PhD-FSTC-2013-12
The Faculty of Sciences, Technology and Communication

Medizinische Fakultät (Bereich klinische Medizin)
der Universität des Saarlandes

DISSERTATION

Defense held on 07/05/2013 in Luxembourg
to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG EN BIOLOGIE

and

DOKTOR DER NATURWISSENSCHAFTEN der medizinischen Fakultät der Universität des Saarlandes

by

Martina SCHMITT

Born on October 18th 1983 in Völklingen (Germany)

Regulation of microRNA Expression by STAT1 Transcription Factors – Relevance for Melanoma Development

Dissertation defense committee

Dr Paul. Wilmes, Chairman
Luxembourg Centre for Systems Biomedicine, University of Luxembourg

Prof. Dr. Klaus Römer, Vice Chairman
José Carreras Research Center, University of Saarland Medical School, Homburg, Germany

Prof. Dr. Iris Behrmann, Dissertation Supervisor
Life Science Research Unit, University of Luxembourg

Prof. Dr. Friedrich Grässer, Dissertation Supervisor
Institute of Virology, University of Saarland Medical School, Homburg, Germany

Prof. Dr. Gerhard Müller-Newen
Institute of Biochemistry and Molecular Biology, University Hospital Aachen, Germany

Prof. Dr. Anja Bosserhoff
Institute of Pathology, University of Regensburg, Germany

To my family

I hereby declare that this dissertation has been written only by the undersigned and that no sources have been used in preparation of this thesis other than those indicated here.

Luxembourg, 18.02.13

Martina Schmitt

The results on pages 58-60 (Fig. 19, Fig. 20, Fig. 21) were obtained in the master thesis project of Susanne Reinsbach and are partially published in (Reinsbach *et al.* 2012). I participated in supervision of the student as well as in the planning and performing the shown experiments.

This PhD project led to two first authorship publications 'miRNA-29: A microRNA Family with Tumor-Suppressing and Immune-Modulating Properties' (review article) and 'Interferon-γ-induced activation of Signal Transducer and Activator of Transcription 1 (STAT1) up-regulates the tumor suppressing microRNA-29 family in melanoma cells' (original research article) which can be found in chapter 11 (Publications). The corresponding results provided the basics for this manuscript. Sentences from my thesis manuscript, which likewise appear in those first authorship publications are marked with [1] (review article) or [2] (original research article). Furthermore, I contributed to three more publications (see chapter 11), of which two are briefly mentioned in the thesis and also attached in chapter 11.

Table of Contents

List of Figures

List of tables

ABBREVIATIONS

3’/5’-UTR	3’/5’-untranslated region
ABCDE	Asymmetry, Border, Color, Diameter, Evolving
Ago	Argonaute protein
AJCC	American Joint Committee on Cancer
ALM	acranal lentiginous melanoma
Amp	ampicillin
ANOVA	analysis of variance
AP-1	activator protein 1
APC	antigen-presenting cell
APS	ammonium persulfate
ARF	alternative reading frame
ATCC	American Type Culture Collection
Bcl-2	B-cell CLL/lymphoma 2
BCLL	B-cell chronic lymphocytic leukemia
Bim	Bcl-like 11
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CBP	CREB-binding protein
CD	cluster of differentiation
cdc42	cell division control protein 42 homolog
CDK	cyclin-dependent kinase
CDKN	cyclin-dependent kinase inhibitor
CEBP	CCAAT-enhancer-binding protein
ChIP	chromatin immunoprecipitation
COL	collagen
Ct	cycle threshold
CTLA	Cytotoxic T-Lymphocyte Antigen 4
CycloA	Peptidylprolyl isomerase A (cyclophilin A)
Da	Dalton
DEPC	Diethylpyrocarbonate
DGCR	DiGeorge syndrome critical region
DMEM	Dulbecco´s Modified Eagle´s Medium
DMSO	Dimethylsulfoxide
DNA	desoxyribonucleic acid
DNMT	DNA (cytosine-5-)-methyltransferase
dNTPs	desoxyribonucleotides
DPE	downstream promoter element
DSMZ	Deutsche Sammlung von Mikoorganismen und Zellkulturen
DTT	Dithiothreitol
E2F1/3	E2F transcription factor 1/3
ECL	enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eIF4	eukaryotic initiation factor4
ENCODE	ENCyclopedia of DNA Elements
Eomes	Eomesodermin (T-box brain protein 2 (TBR2))
ERK	extracellular regulated kinase

ESTDAB	European Searchable Tumor Line Database
FCS	fetal calf serum
FDA	food and drug administration
FFPE	formalin-fixed paraffin-embedded tissue
G418	Geneticin
GAS	Interferon-Gamma Activated Sequence
gp130	glycoprotein 130
GTP	guanosine triphosphate
H	histone
H3K4Me3	trimethylation of histone H3 lysine 4
HAT	histone acetyl transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hg19	human genome version 19
HIF	hypoxia inducible factor
HIV	human immunodeficiency virus
HMGA2	high mobility group AT-hook 2
HPRT	hypoxanthine phosphoribosyltransferase 1
HRP	horseradish peroxidase
IFN	interferon
IFNAR	interferon-alpha receptor
IFNGR	interferon-gamma receptor
Ig	immunoglobulin
IL	interleukin
Imp8	importin 8
INR	initiator element
IRF	interferon regulated factor
ISGF3	interferon-stimulated gamma factor 3
ISRE	interferon-stimulated response element
Jak	Janus kinase
JII	Jak inhibitor 1
JNK	Jun N-terminal kinase
K	lysine residue
kDa	kilodalton
LAR	luciferase assay reagent
LATS2	large tumor suppressor, homolog 2
LB	Luria Bertani
LMM	lentigo maligna melanoma
LNA	locked nucleic acid
maf	v-maf musculoaponeurotic fibrosarcoma oncogene homolog
MAGE	melanoma-associated antigen
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
MCS	multiple cloning site
MDM	mouse double minute
Me	methylated
MEK	dual specificity mitogen-activated protein kinase kinase
MHC	major histocompatibility complex

MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
miRNA	microRNA
miRNA*	microRNA star
MITF	Microphthalmia-associated transcription factor
MMP	matrix metalloprotease
NC	negative control
NEB	New England Biolabs
Nef	negative regulatory factor
NF	normalisation factor
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEM	normal human epidermal melanocytes
NK	natural killer cell
NLS	nuclear localisation signal
NM	nodular melanoma
N-RAS	neuroblastoma RAS viral (v-ras) oncogene homolog
OD	optical density
PABPC1	polyadenylate-binding protein 1
PACT	protein activator of PKR
P-bodies	processing bodies
PDCD4	Programmed cell death protein 4
PDGFR	platelet-derived growth factor receptor
PI3K	phosphoinositide 3-kinase
PI3KR	phosphoinositide-3-kinase regulatory subunit
PIAS	protein inhibitors of activated STATs
piRNA	Piwi-interacting RNA
PitX3	Pituitary homeobox 3
PKR	protein kinase R
pre-miRNA	precursor-miRNA
pri-miRNA	primary miRNA
PTEN	Phosphatase and tensin homolog
PTP	protein tyrosine phosphatase
qPCR	quantitative polymerase chain reaction
Raf	RAF proto-oncogene serine/threonine-protein kinase
Ras	rat sarcoma virus
Rb	Retinoblastoma
Reck	Reversion-inducing-cysteine-rich protein with kazal motifs
REL	relative expression
RGP	radial growth phase
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RNA	ribonucleic acid
RNU1A, 5A	RNA, U1A/5A small nuclear
rpm	rotations per minute
RWTH	Rheinisch-Westfälische Technische Hochschule
SCARNA17	small Cajal body-specific RNA 17
SD	standard deviation
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH	src homology
SHP-2	SH2 domain-containing phosphatase
siRNA	small interfering RNA
SMAD3	mothers against decapentaplegic homolog 3
SOCS	suppressor of cytokine signalling
SP-1	specificity protein 1
SSM	superficial spreading melanoma
STAT	signal transducer and activator of transcription
TBP	TATA binding protein
Tcl-1	T-cell leukemia/lymphoma 1
TE	tris-EDTA
TEMED	tetramethylethylendiamine
TFIIB	transcription factor 2B
Th1	T helper type 1
TNF	tumor necrosis factor
TPM1	Tropomyosin alpha-1 chain
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TRPB	TAR RNA binding protein
TRPM	melastatin
Tyk	non-receptor protein tyrosine kinase
UCSC	University of California Santa Cruz
UV	ultraviolet
VGP	vertical growth phase
WHO	World Health Organization
WT-1	Wilms tumor 1

Abstract

[2]The type-II-cytokine IFN-γ (interferon gamma) is not only a pivotal player in innate immune responses but also assumes functions in controlling tumor cell growth by orchestrating cellular responses against neoplastic cells. It predominantly triggers cellular responses through the Janus Kinase (Jak)/ Signal Transducer and Activator of Transcription 1 (STAT1) pathway leading to STAT1 binding to the promoter region of target genes. As key regulators of mRNA and protein expression levels, microRNAs (small non-coding RNAs) take part in fine-tuning complex biological processes such as cell proliferation, neoplastic transformation, apoptosis, immune surveillance and differentiation. [1]MiR-29, one of the most interesting miRNA families in humans to date, consists of three mature members miR-29a, miR-29b and miR-29c, which are encoded in two genetic clusters. In this PhD thesis, the miR-29 primary cluster pri-29a~b-1 was shown to be IFN-γ-induced and STAT1-dependently up-regulated in melanoma cell lines. [2]Furthermore, expression levels of mature miR-29a and miR-29b were elevated in cell lines and in primary melanoma patient samples while the pri-29b-2~c cluster was almost undetectable in cell lines. Moreover, tumor-suppressing properties of miR-29 family members have been detected: inhibition of melanoma cell proliferation could be induced by miR-29a, which down-regulated CDK6 (cyclin-dependent kinase 6), an important player in cell cycle G1/S transition. Also, knockdown of CDK6 resulted in reduced proliferation of melanoma cells, suggesting that miR-29-mediated growth inhibitory effects may be brought about by CDK6-downregulation.

These findings identify the pri-29a~b-1 cluster as a novel IFN-γ-regulated gene. Furthermore, a potential novel signaling pathway was identified: IFN-γ → Jaks → P-STAT1 → miR-29 → CDK6, which opens up new connections between miRNAs, interferon signaling and malignant melanoma, possibly clearing the way to novel concepts for new treatment options in the future.

Zusammenfassung

Das Typ-II-Zytokin IFN-γ ist nicht nur ein zentraler Akteur in der angeborenen Immunantwort, sondern besitzt auch wichtige Funktionen in der Wachstumskontrolle von Tumorzellen, indem es zelluläre Antworten gegen neoplastische Zellen vermittelt. Es wirkt hauptsächlich über den Janus Kinase (Jak)/ *Signal Transducer and Activator of Transcription 1* (STAT1) Signalweg, welcher zur Bindung von STAT1 in der Promoterregion von Targetgenen führt. MikroRNAs (kleine, nicht-codierende RNAs) sind bedeutsame Regulatoren von mRNA- und Proteinexpressionsspiegeln und tragen zur Feinjustierung komplexer biologischer Prozesse bei, wie z.B. Zellproliferation, neoplastische Transformation, Apoptose, Immunkontrolle und Differenzierung. miR-29, momentan eine der interessantesten der bisher bekannten miRNA-Familien, besteht im Menschen aus den drei „reifen" Familienmitgliedern, miR-29a, miR-29b und miR-29c, welche in zwei genetischen Clustern codiert sind. In dieser Doktorarbeit wurde gezeigt, dass das miR-29 Primärtranskript pri-29a~b-1 in Melanomzellen IFN-γ-induziert und STAT1-abhängig hochreguliert ist. Desweiteren waren die Expressionsspiegel der reifen miRNAs miR-29a und miR-29b in Zelllinien und primären Melanompatientenproben erhöht, während das pri-29b-2~b Transkript in Zelllinien fast nicht detektierbar war. Darüber hinaus wurden Tumor-supprimierende Eigenschaften von miR-29-Familienmitgliedern ausfindig gemacht: eine Inhibierung der Proliferation von Melanomzellen konnte durch miR-29a induziert werden, welche CDK6 (*cyclin-dependent kinase 6*) herunterregulierte, einen wichtigen Akteur im G1/S-Übergang des Zellzyklus. Auch „Knock-down" von CDK6 führte zu verminderter Proliferation von Melanomzellen, was darauf hindeutet, dass die durch miR-29a vermittelte Wachstumsinhibition durch Verminderung der CDK6-Expression bewerkstelligt werden könnte.

Diese Ergebnisse beschreiben das pri-29a~b-1-Cluster als neues, IFN-γ-reguliertes Gen. Außerdem wurde ein neuer Signalweg entdeckt, IFN-γ → Jaks → P-STAT1 → miR-29 → CDK6, welcher neue Verbindungen zwischen miRNAs, Interferon Signaling und malignen Melanomen aufzeigt und möglicherweise den Weg für neuartige Behandlungskonzepte ebnet.

1 Introduction

This PhD project addresses the fundamental question of if and how miRNA expression can be regulated by the cytokine IFN-γ and STAT1 transcription factors and furthermore considers their potential relevance for melanoma development. Accordingly, the introduction focuses on three major topics: miRNAs, Interferon/Jak/STAT signaling and melanoma. The first part of the introduction provides an overview on miRNAs, their biogenesis, regulation, mode of action and role in cancer as well as the miR-29 family, which became of special interest while the project was in progress. The second part introduces the canonical Jak-STAT signaling pathway and its main players. The last part of the introduction presents a brief overview on melanoma, an aggressive type of skin cancer, which was the main biological system investigated in this PhD project.

1.1 MicroRNAs

In the past decade, small non-coding microRNAs (miRNAs) have been identified as important novel players in post-transcriptional gene regulation and ever since, their expression patterns and cellular functions have been investigated in cancer and other diseases (Winter *et al.* 2009; Krol *et al.* 2010b). MicroRNAs (miRNAs) are a class of small non-coding RNA molecules (~22 nucleotides), whose main function is the negative regulation of gene expression at a post-transcriptional level. They are conserved in plants and animals (Ambros 2003), but not present in bacteria (Tjaden *et al.* 2006). In human cells, miRNAs are expressed in all cell types and are involved in the control of fundamental cellular processes such as differentiation, apoptosis, proliferation, cell death and others (Esau *et al.* 2004; Hwang and Mendell 2006; Jovanovic and Hengartner 2006). MiRNAs were initially discovered in 1993, when Victor Ambros, Rosalind Lee and Rhonda Feinbaum were studying the larval development in the nematode *C. elegans* and discovered the miRNA lin-4, which they showed to inhibit the lin-14 mRNA (Lee *et al.* 1993). However at that stage, lin-4 was only described as a short RNA product from the lin-4 gene. The term 'miRNA' was only introduced following their characterization as a separate class of biologically relevant molecules around the year 2000 (Pasquinelli *et al.* 2000; Lagos-Quintana *et al.* 2001; Lau *et al.* 2001; Lee and Ambros 2001). The rapidly growing interest in various aspects of miRNA biology is reflected by the constantly increasing number of publications since their first discovery. For the current version 19 of miRBase, entries for newly identified miRNAs have risen to 26,264 representing 193 different species (Kozomara and Griffiths-Jones 2011). Over the past decade, many diseases including almost all types of cancer have been connected to aberrant expression of miRNAs (Esquela-Kerscher and Slack 2006; Chang and Mendell 2007; Iorio and Croce 2012b). [1]Like protein-coding genes, miRNAs can either act as tumor

suppressors when down-regulating potentially oncogenic targets or they can exert oncogenic functions when tumor-suppressive target mRNAs are down-regulated (Carleton *et al.* 2007). Apart from their role in regulation of mRNA expression and involvement in cancer, miRNAs were postulated to confer robustness to biological processes by compensating for alterations of cellular transcription levels (Ebert and Sharp 2012).

Biogenesis of miRNAs

The canonical miRNA biogenesis pathway (Winter *et al.* 2009; Krol *et al.* 2010a; Treiber *et al.* 2012) is a multi-step process and an overview is shown in Fig. 1. miRNAs are transcribed in the nucleus mainly by RNA-polymerases II, but the involvement of RNA-polymerases III has also been reported for some cases (Faller and Guo 2008). The polymerases create a primary transcript, which is several thousand nucleotides long and contains a 5'-methylguanosine-cap as well as a 3'-poly(A)-tail like mRNAs. This primary (pri-) miRNA is then processed into a 70-80 nucleotide-long precursor form (pre-miRNA) by the RNase III enzyme Drosha and the dsRNA-binding protein DGCR8 (DiGeorge syndrome critical region 8). This pre-miRNA exerts a typical hairpin structure with a 2 nucleotide 3'-overhang derived from Drosha-processing (Graves and Zeng 2012). Its nuclear export is subsequently mediated by Exportin 5 which, supported by Ran-GTP, delivers the pre-miRNA to the cytoplasm (Davis-Dusenbery and Hata 2011). The following step, i.e. cleaving of the precursor molecule into its bioactive, mature form, is performed by the RNase enzyme Dicer, one of the most important proteins within the miRNA biogenesis pathway (Ma *et al.* 2011b). Together with its interacting partners TRBP (TAR RNA-binding protein), PACT (protein activator of PKR) and Ago2 (Argonaute 2) (Koscianska *et al.* 2011), it is often referred to as RISC (RNA-induced silencing complex) loading complex (RLC) (Winter *et al.* 2009). The Dicer-derived mature miRNA duplex is 21-23 nucleotides long. It is quickly separated by helicase into two single strands whose further distribution depends on thermodynamic stability and which are subsequently named differently: The 'major' strand (also known as 'guide' strand) is generally incorporated in the RISC and is found in a higher concentration in the cell in comparison to the 'minor' strand (also known as 'passenger' strand or miRNA star (*) sequences). However, recently evidence was provided that also star (*) sequences, which are now labeled as '-3p' in contrast to the '-5p'-suffix for the 'major' strands, are not always degraded but can exhibit biological functions and thus target an own set of mRNAs (Fig. 1) (Czech and Hannon 2011). The RISC, sometimes referred to as 'miRISC' when loaded with a miRNA, is in charge of directing the incorporated, single-stranded miRNA to its target mRNA (Winter *et al.* 2009). The miRNA subsequently represses expression of mRNAs and their encoded proteins in the cytosol, mostly by binding to their 3'-UTR but binding to the 5'-UTR (Orom *et al.* 2008) or the coding region (Fang and Rajewsky 2011) has also been reported.

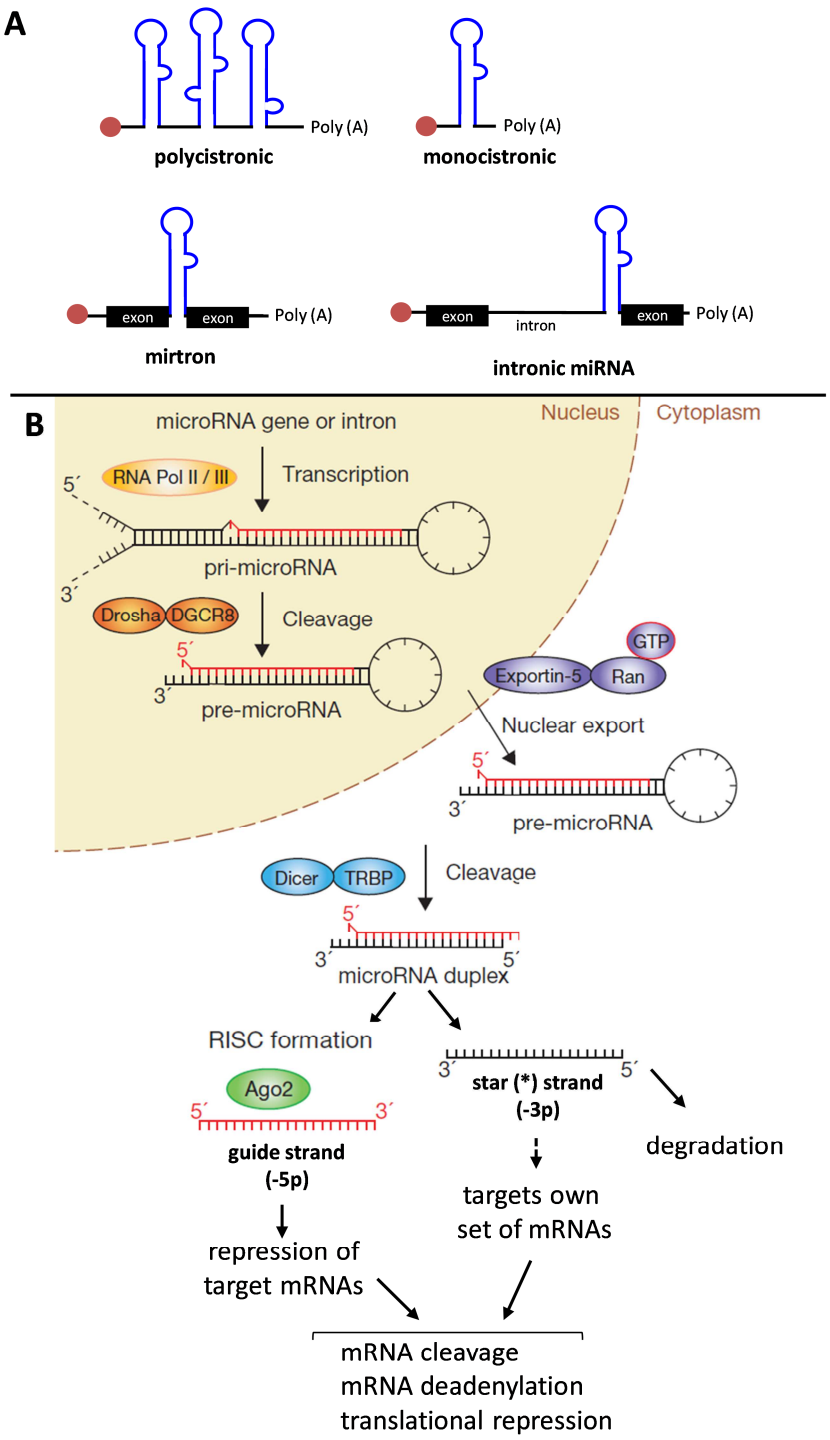


Fig. 1 Schematic overview: genomic organization and biogenesis of human miRNAs ((Winter *et al.* 2009; Meister 2011), adapted). (A) miRNAs can be encoded in polycistronic clusters or as monocistronic genes. Most miRNA genes are encoded within introns of protein coding genes; some miRNAs, which are processed by the splicosome, form entire introns. (B) Pri-miRNAs are transcribed in the nucleus mainly by RNA-polymerase II. After first processing of the primary transcript to the pre-miRNA by Drosha and DGCR8, the precursor molecule is exported to the cytoplasm by Exportin 5. Cleavage by Dicer leads to the ~22 nucleotide long mature miRNA duplex. The major strand gets incorporated into the RISC complex, where it can act on target mRNAs while the other strand is degraded or targets an own set of mRNAs. Ago2 - Argonaute protein 2; DGCR8 - DiGeorge syndrome critical region; RISC – RNA-induced silencing complex; TRBP – TAR RNA binding protein.

The seed region determins, which mRNA can potentially be targeted by a given miRNA. It comprises nucleotides 2-7, which generally exhibit a perfect complementarity to the corresponding binding site of the mRNA-3'-UTR (Fabian *et al.* 2010). The seed sequence also influences target gene recognition and the efficiency of the subsequent repressing procedure. Interaction with the 6 nucleotides of positions 2-7, ('6-mer') results in the weakest possible binding to the target UTR and consequently leads to a weak repression. The repression is enhanced, if an adenosine is present at position 1 ('7-mer-A1'-site, Fig. 2) and even more so, if nucleotides 2-8 exibit a perfect match ('7mer-m8'-site) or if eight nucleotides perfectly pair with the target mRNA as shown in Fig. 2 (Bartel 2009; Schnall-Levin *et al.* 2011). However, so-called atypical sites with mismatches can also occur (Bartel 2009).

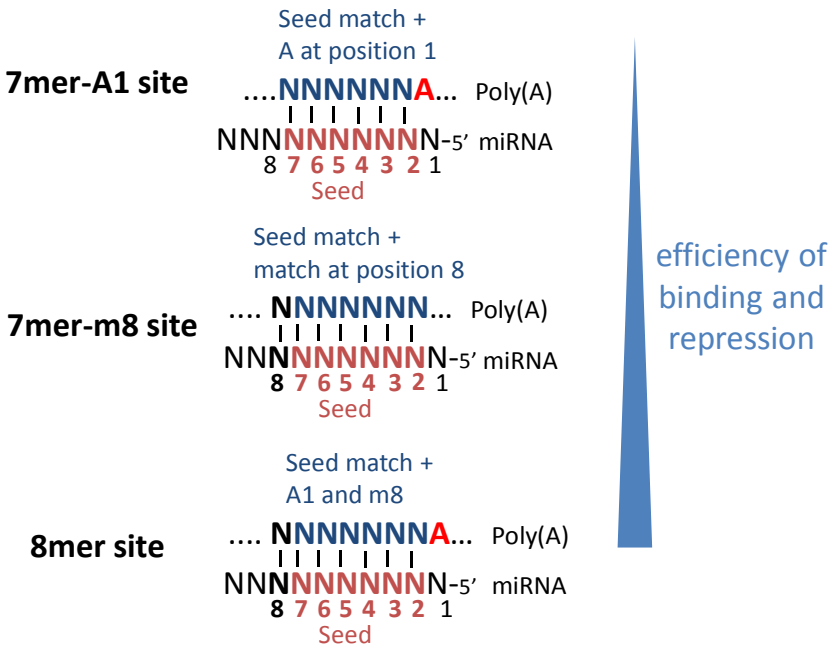


Fig. 2: Canonical miRNA target sites
Different possibilities of target gene recognition and binding by miRNAs as described in the text. The binding efficiency increases together with the efficiency of target gene repression from the 7mer-A1 pairing to the 8mer site. Adapted from (Bartel 2009).

Mode of action

MiRNAs and target mRNAs interact while the mature miRNA strand is incorporated in the RISC complex. The two main mechanisms which had been described initially for miRNA-mediated gene silencing were mRNA degradation and inhibition of translation of the respective mRNA (Filipowicz *et al.* 2008). It was furthermore claimed that the detailed mode of action depends on the complementarity between miRNA and target mRNA sequence with full complementarity leading to mRNA degradation while incomplete complementarity induces prevention of

translation (Lim *et al.* 2005; Brodersen and Voinnet 2009). In principle, protein translation can be inhibited at multiple steps, for example during initiation, assembly of the ribosomal subunits as well as during elongation and termination of translation (Chekulaeva and Filipowicz 2009). Additional modes of action have been proposed in recent years. Very recently, Morozova *et al.* published an overview of miRNA-mediated mechanisms based on literature analysis and on a mathematical model, suggesting that the various reported mechanisms coexist in the cell, as shown in Fig. 3 (Morozova *et al.* 2012). These mechanisms include: cap inhibition, 60 S joining inhibition, inhibition of elongation, ribosome drop-off, co-translational protein degradation, sequestration in P-bodies (processing bodies), mRNA decay, mRNA cleavage and transcriptional inhibition. Most of them have been confirmed experimentally, however, some remain controversial (Morozova *et al.* 2012; Zinovyev *et al.* 2012).

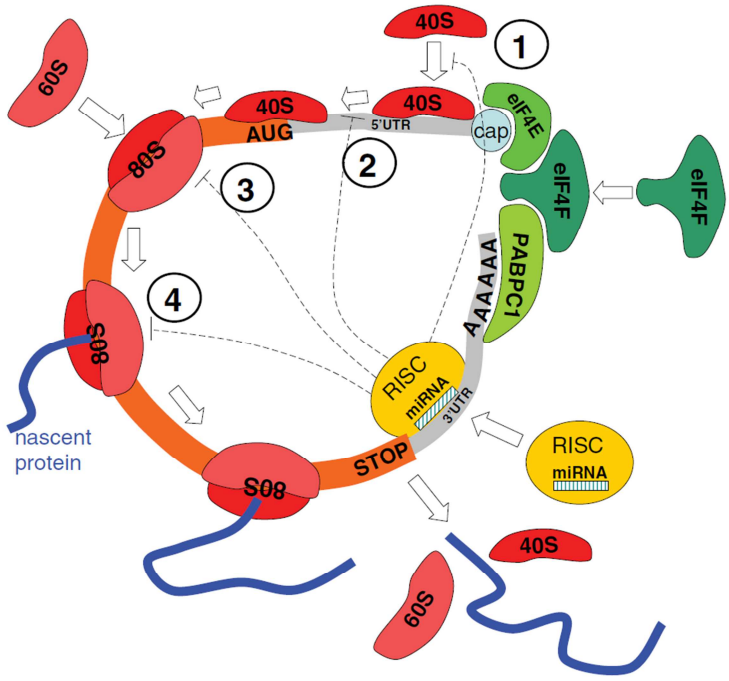


Fig. 3: Possible miRNA modes of action on protein translation
From (Zinovyev *et al.* 2012). Translation can be inhibited at several steps: (1) initiation of translation can be prohibited by preventing the assembly of the initiation complex (for example by action on eIF4 or 40S), (2) searching for the start codon can be prevented, (3) ribosome assembly can be affected, (4) the translation process can be inhibited. Other mechanisms include for example transport to P-bodies, ribosome drop-off, co-translational protein degradation and others (not shown here).
40S, 60S: ribosomal subunits; 80S: assembled ribosome, RISC: RNA-induced silencing complex; eIF4 - eukaryotic translation initiation factor 4; PABPC1 - Polyadenylate-binding protein 1; cap – mRNA cap structure.

In terms of target gene repression, *in silico*, *in vitro* and *in vivo* studies show that a single miRNA can down-regulate hundreds of genes (Krek *et al.* 2005; Lim *et al.* 2005). On the other

hand, one gene can be targeted by several miRNAs (Wu *et al.* 2010). According to recent publications, miRNA functions are not solely limited to actions in the cytoplasm, but can also perform tasks in the nucleus (Huang and Li 2012).

Genomic organization and regulation of miRNA expression

miRNAs can be organized in genetic clusters with several members being encoded close to each other or within a certain distance between genomic locations of single miRNAs (Fig. 1). Cluster members are often generated within a transcriptional unit from a common primary transcript, but can also be regulated separately. For miRNA families, precursor sequences of the different members are positioned in different genomic locations, but all members share the same conserved seed region. miRNA family members have the same name which is followed by a letter to differentiate between family members from different genomic locations: for example hsa-miR-23a is encoded on chromosome 19 whereas its 'sister' miRNA hsa-miR-23b is transcribed from chromosome 9.

MiRNAs exhibit a relatively low rate of evolutionary changes and thus can be used as phylogenetic markers (Wheeler *et al.* 2009). Their genes can be located in intergenic regions or within introns or exons of protein-coding genes (Kim and Nam 2006) and entire pri-miRNAs can also be spliced like normal mRNAs (miRtrons) (Kim 2005) (Fig. 1). In contrast to the transcriptional start sites (TSSs) of protein-coding genes, identification of miRNA TSSs and assignment of promoter regions remains difficult and conventional methods for TSS identification are often not suitable, mainly due to the quick processing and rapid turnover times of primary miRNA transcripts (Krol *et al.* 2010a). Thus, only a few precise miRNA promoters have been experimentally characterised so far (Chien *et al.* 2011).

Transcriptional regulation is thought to be the main mechanism for regulation of miRNA expression (Bartel 2004), but other controlling processes including epigenetic silencing (Bueno *et al.* 2008) and different processing and turnover times have also been described (Krol *et al.* 2010b). Depending on the surrounding cellular context or tissue, differential expression of proteins of the miRNA processing machinery will affect miRNA amounts (Lu *et al.* 2005b). Furthermore, expression levels of certain miRNAs can be drastically altered if the primary transcript is encoded at genomically instable sites, which are often deleted or multiplied in cancer (Calin *et al.* 2004). Binding of transcription factors to the promoter region of a gene normally leads to activation of targets, however, transcription can also be repressed. Identifying the transcription factors, which regulate miRNAs or their respective host genes (in the case of intragenic miRNAs) is crucial for understanding regulation and different miRNA expression patterns in a healthy versus a diseased cellular context.

As miRNAs are predicted to control approximately 50 % of human genes (Friedman *et al.* 2009) and are often de-regulated in cancer and other diseases, deciphering cellular networks of their

own regulation is of obvious importance. Different mechanisms are implicated in the regulation of miRNAs, which can act at several cellular stages as was recently reviewed by Krol and colleagues (Krol *et al.* 2010b). For example the protein machinery involved in miRNA processing can be influenced by other proteins: Drosha and Dicer efficiencies are largely dependent on their binding partners DGCR8 and TRBP, respectively (Han *et al.* 2009; Melo *et al.* 2009). Furthermore, miRNA stability can be influenced by modifications at the 3'-end, as exemplified by the RNA-binding protein LIN-28 which can repress the maturation of the let-7 miRNA (Viswanathan *et al.* 2009). Furthermore, RNA-binding proteins partake in the regulation of miRNAs by direct interaction with the RISC-complex (Galgano *et al.* 2008) and miRNAs can also be epigenetically silenced (Bueno *et al.* 2008; Yan *et al.* 2011).

Like mRNAs, most miRNAs are transcribed by RNA-polymerase II. Likewise, transcription factors can regulate miRNA expression in the same way as they control expression levels of protein-coding genes. Among others, miRNA-regulation by c-myc (O'Donnell *et al.* 2005), p53 (Jin *et al.* 2011) and HIF (hypoxia-inducible factor) (Kulshreshtha *et al.* 2007) has been demonstrated. Hence, transcription factors provide the opportunity to fine-tune miRNA expression levels also in a tissue-specific manner. Additionally, transcription factor-miRNA interactions can be part of a regulatory network including feedback loops: Transcription factors can guard the expression of a certain miRNA and *vice versa*, a transcription factor can be negatively regulated by a miRNA either via direct interaction or via a secondary regulator. The feedback loop involving miR-133b and the transcription factor PitX3 (Pituitary homeobox 3) in neurons is a well-described example with miR-133b-mediated repression of PitX3 and PitX3-dependent transcriptional regulation of miR-133b (Kim *et al.* 2007). More loops involving miRNA-transcription factor-interaction will be discussed below. A major focus of this PhD thesis was the investigation of transcriptional regulation of miRNA expression and therefore a brief introduction of eukaryotic transcription is provided in chapter 2.3.

miRNAs in cancer

miRNAs have been implicated in the regulation of processes that promote cancer growth or conversely, in processes that might prevent cancers from developing. Since their discovery, deregulation of miRNA expression has been connected to a plethora of malignancies and other diseases. Like protein-coding genes, miRNAs can be classified as being either 'oncogenic' or 'tumor-suppressive'. A very recent review lists the 'key microRNAs involved in cancer', whose oncogenic or tumor-suppressive functions have been extensively studied in several cancer types (Lujambio and Lowe 2012). Table 1 shows an overview of the most prominent cancer microRNAs.

Table 1: Selection of most prominent cancer microRNAs
(green) Tumor suppressor miRNAs and (red) oncogenic miRNAs, ↑ ≙ up-regulated; ↓ ≙ down-regulated. Adapted from (Garzon *et al.* 2009a; Iorio and Croce 2012a).

miRNA	Expression in patients	Selection of confirmed targets	Experimental data
miR-15a/16-1	↓ in CLL	Bcl-2, WT-1	Induce apoptosis and decrease tumorigenicity
Let-7 (a-d)	↓ in lung and breast cancer	Ras, c-myc, HMGA2	Induce apoptosis
miR-29	↓in CLL, AML, lung and breast cancers, cholangiocarcinoma and others	TCL-1, MCL-1, CDK6, DNMT3s	Induce apoptosis and decrease tumorigenicity
miR-34a-c	↓in pancreatic, colon and breast cancers	CDK4, CDK6, cyclinE2, E2F3	Induce apoptosis
miR-155	↑ in CLL, DLBCL, AML, BL, lung and breast cancers	c-maf	Induces lymphoproliferation, pre-B lymphoma/leukemia in mice
miR-17~92 cluster	↑ in lymphomas, breast, lung, colon, stomach and pancreatic cancers	E2F1, Bim, PTEN	Cooperates with c.myc to induce lymphoma in mice, transgenic miR-17-92 develop lymphoproliferative disorders
miR-21	↑ in breast, colon, pancreas, lung, prostate, liver and stomach cancer, AML, CLL and glioblastoma	PTEN, PDCD4, TPM1	Stimulates invasion and metastasis in different tumour types
miR-372/373	↑ in testicular tumors	LATS2	Promote tumorigenesis in cooperation with RAS

One of the first 'oncomirs' described was miR-21, which exhibits elevated levels in many human cancers and which down-regulates tumor suppressor genes such as those encoding PTEN (phosphatase and tensin homolog) and RECK (reversion-inducing-cysteine-rich protein with kazal motifs) (Meng *et al.* 2007a; Gabriely *et al.* 2008; Pan *et al.* 2010). Another well-known example is oncogenic miR-155, which was shown to repress genes responsible for repair of DNA damage (Costinean *et al.* 2006; Tili *et al.* 2011). On the other hand, the most prominent representatives of miRNAs exhibiting predominantly tumor-suppressive properties include miR-34, miR-15~16, members of the let-7 family as well as the miR-29 family (Calin *et al.* 2002; Garzon *et al.* 2009b; Hermeking 2009; Buechner *et al.* 2011; Zhang *et al.* 2011b). The miR-29 family or its single members, which will be described in more detail below, have been shown to be down-regulated in glioblastoma (Cortez *et al.* 2010), lung cancer (Yanaihara *et al.* 2006), prostate cancer (Porkka *et al.* 2007), colon cancer (Cummins *et al.* 2006), chronic lymphocytic leukemia (Calin *et al.* 2005) and many other malignancies. Apart from those miRNAs which can clearly be attributed to a distinct class, miRNAs with 'dual' functions as oncogenic or tumor-suppressive, depending on the cellular context, have been reported. Examples are miR-125b, the miR-181 family and miR-220 (Fabbri *et al.* 2007b; Visone *et al.* 2007; Nam *et al.* 2008). Reliable and detailed knowledge of these miRNA functions is crucial to pave their way to the

clinic: The allocation to the 'oncomir' or 'tumor-suppressive' group should be determined before levels of miRNAs are manipulated in clinical treatments.

miRNAs as biomarkers and therapeutical agents

[1]Since it has been demonstrated that miRNAs often have tissue- and disease-specific expression patterns, the possibility to use miRNAs as biomarkers for early diagnosis of malignancies and other diseases has been studied extensively in recent years. They exhibit an extreme high stability in formalin-fixed tissues, plasma and serum samples (Mitchell *et al.* 2008) and are present in most solid tissues (Lu *et al.* 2005a; Liang *et al.* 2007). Apart from blood, where they are currently being examined and evaluated as 'secreted or circulating' miRNAs, the small RNA molecules have been detected in many body fluids such as tears, breast milk, urine, bronchial lavage and others (Weber *et al.* 2010). Thus, as soon as there are robust and standardized methods for extraction, quantification and analysis of these secreted miRNAs, they are likely to become promising biomarkers, offering a non-invasive approach for the diagnosis of diseases, which alter miRNA expression profiles. The respective studies, which have been initiated for many different cancer types mostly rely on a panel of different miRNAs instead of single molecules (Chen *et al.* 2012).

As described above, a cancer cell can emerge following the over-expression of classic oncogenes and so-called 'oncomirs' (such as the miR-17-92 family, miR-21, -155, miR-34a etc.), which down-regulate tumor-suppressors that normally control cell proliferation (Krutovskikh and Herceg 2010). On the other hand, miRNAs that function as tumor-suppressors by targeting cellular oncoproteins (such as let-7 family members, miR-15a, -16, -29, etc.) are frequently down-regulated in cancer tissues (Henry *et al.* 2011). Therapeutics opting to replace the diminished tumor-suppressor miRNAs are currently being investigated and seem promising, as miRNAs exhibit high stability as well as high specificity for their target mRNAs (Henry *et al.* 2011; Kasinski and Slack 2011). [1]Furthermore, miRNAs and their target genes represent interesting pharmaceutical targets as part of a general or personalized therapy in the future. However, several problems need to be adressed, such as efficient delivery and potential side-effects. The miRNA treatment with the biggest potential for clinical application is currently a LNA (locked nucleic acid)-inhibitor of liver-specific miR-122 termed 'Miraversen', which is in clinical trials as an application against hepatitis C virus (HCV) infections, which can lead to the development of hepatocellular carcinoma (HCC). Remarkably, miR-122 inhibition was shown to reduce HCV viremia in monkeys (Lanford *et al.* 2010; Hildebrandt-Eriksen *et al.* 2012). [1]Otherwise, clinical applications are still rare and before miRNAs will routinely be incorporated in clinical therapeutic interventions, more detailed information on their precise biological functions within different cellular contexts will need to be collected.

The miR-29 family

[1]The miR-29 family is among the earliest ones discovered (Lagos-Quintana *et al.* 2001) and is highly conserved among species (Kozomara and Griffiths-Jones 2011). An overview on the genomic organization and the mature sequences of the miR-29 family is shown in Fig. 4. [1]In humans, it is encoded by two clusters, miR-29a~29b-1 (chromosome 7q32.3) and miR-29b-2~29c (chr 1q32.2). miR-29a and miR-29b-1 (GenBank accession number EU154353 as well as miR-29c and miR-29b-2 (EU 154351 and EU154352) are co-transcribed by RNA-polymerase II as a polycistronic primary transcript from the minus strand (Chang *et al.* 2008; Mott *et al.* 2010), only encoded 649bp and 504bp apart from each other, respectively. [1]With identical seed sequences, miR-29 family members share most of their predicted targets. [1]MiR-29b-1/b-2 have the same mature sequence and miR-29a and 29c mature sequences only differ by one nucleotide (Fig. 4).

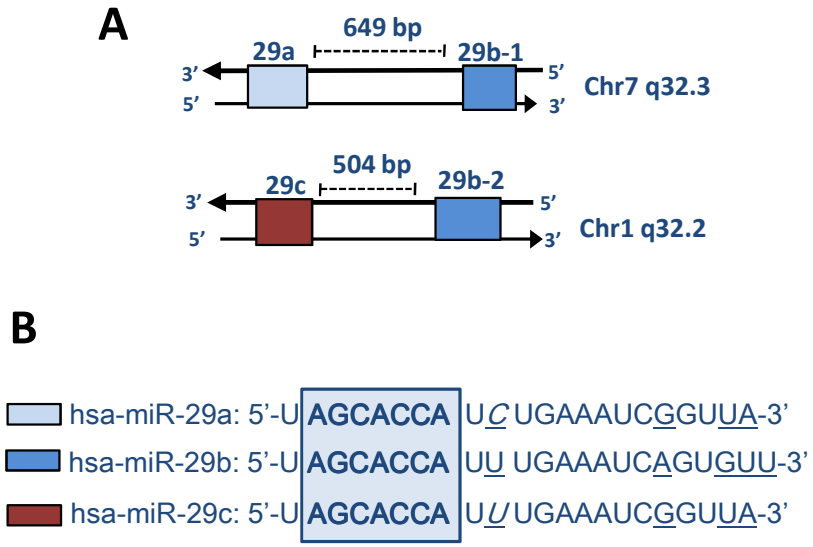


Fig. 4: miR-29 family: genomic organization and mature sequences.
(A) [2]The miR-29 family is transcribed from the respective antisense strand from two genetic clusters of chromosomes 7 (pri-29a~b-1) and 1 (pri-29b-2~c). (B) The three mature forms miR-29a/29b/29c share the same seed region (blue box). [2]Differences between the mature sequences are underlined; the one nucleotide difference between miR-29a and miR-29c is shown in italics.

[1]All miR-29 family members are ubiquitously expressed in healthy tissues (www.microrna.org, (Betel *et al.* 2008)). [1]Both clusters, miR-29a~29b-1 and miR-29b-2~29c, are intergenic with no protein-coding genes in close proximity. [1]Only a non-coding RNA (LOC646329), which has not been further characterized yet, shares part of the sequence with miR-29a~29b-1 (www.genome.ucsc.edu) (Kent *et al.* 2002). The human miR-29 family represents a very important miRNA family whose members are increasingly recognized as tumor suppressors in a variety of malignancies. [1]Since their sequence was added to miRBase in 2001 (Lagos-Quintana

et al. 2001), the number of publications on one or more members of the human miR-29 family rose quickly to more than 100. In recent overview articles on miRNAs in cancer, the miR-29 family resided among the topmost cancer-associated miRNAs (Spizzo *et al.* 2009; Lujambio and Lowe 2012). Members of the miR-29 family have been shown to be implicated in many divergent cellular processes like extracellular matrix homeostasis (Villarreal *et al.* 2011), collagen expression (van Rooij *et al.* 2008), insulin signaling (Pandey *et al.* 2010), aging (Ugalde *et al.* 2011) and others. The number of confirmed targets for one or more family members is constantly rising, including many different protein classes ranging from transcription factors (Steiner *et al.* 2011; Ugalde *et al.* 2011), viral proteins (Ahluwalia *et al.* 2008) to growth factors (Hand *et al.* 2012), structural cell components (van Rooij *et al.* 2008) and others. [1]Noteworthy, miR-29 members have also been connected to diseases other than cancer, for example myocardial infarction (van Rooij *et al.* 2008), diabetes (Pandey *et al.* 2010) and atherosclerosis (Chen *et al.* 2011). The implication of the miR-29 family in diseases like myocardial infarction and renal injury has recently been reviewed as well as its tumor-suppressing functions and its role in the immune system (Kriegel *et al.* 2012; Liston *et al.* 2012; Schmitt *et al.* 2012a).

1.2 Signal Transduction

Signal transduction in cellular communication

A task which the human body solves with a fascinating accuracy is the allocation of an efficient communication system for intercellular correspondence. Already in the early steps of embryonal development, the few cells which will grow up to organs and constitute the complex organism later on, exchange information to ensure proper division, differentiation and positioning. After formation of organs and the organism as such, cell communication remains crucial for everyday survival. Signal transduction in general represents a fundamental basis for the regulation and maintenance of the body functions. It provides the basic elements for the communication of cells with each other, enables them to take up extracellular information in form of signals and process them to the inside of the cell where subsequent tasks like gene regulation can be fulfilled (Eckardstein 2009).

The impact of signal transduction in cancer and other diseases is obvious - false regulation of the extracellular signal or entire signal transduction pathways leads to wrong transmission of the information from the extracellular environment to the inside and thus can cause imbalances in the homeostasis of the cell. Therefore, it is important to decipher the functions of single components, which take part in signal transduction processes in order to understand those malfunctions and to develop therapeutical treatment options. One of the most evolutionary conserved and simultaneously very prominent signal cascade in eukaryotic cells is the Jak-STAT signaling pathway, which will be of major interest for this PhD project.

Interferons as inducers of the Jak-STAT pathway

Cytokines are key glycoproteins involved in cellular signaling, which regulate, amongst other fundamental processes, growth and differentiation of cells. They are subdivided into five main groups, i.e. interleukins, interferons, tumor necrosis factors, colony-stimulating factors and chemokines (Eckardstein 2009). The respective cytokine receptors can also be divided into five groups according to structural features of their extracellular domains. These are type-I- and type-II-cytokine receptors, TNF (tumor necrosis factor) receptors, receptors of the Ig (immunoglobulin) superfamily and seven-transmembrane receptors (Eckardstein 2009).

Interferons comprise a cytokine family with important functions in immune responses and they signal via type-II-cytokine receptors. They were originally described as agents that 'interfere with viral replication' by Isaacs and Lindenmann in 1957 and are classified into two groups type I ('viral interferons': IFN-α with subtypes, IFN-β, IFN-ω and IFN-τ) and type II ('immune interferon': only IFN-γ) interferons (Borden *et al.* 2007). Apart from their specific anti-viral activities, common actions of interferons are the regulation of cell growth, differentiation and apoptosis as well as the activation of immune cells (Samuel 2001; Santos and Costa-Pereira

2011). Because of their above mentioned properties in infection and other cellular processes, interferons have first been introduced as therapeutical agents already in 1986 (Pestka 2007). Today, IFN-α is still used for treatment against melanoma, (hairy cell) leukemia, chronic hepatitis B and C and other diseases, whereas multiple sclerosis patients receive IFN-β treatment and IFN-γ is given in chronic granulomatous disease and malignant osteopetrosis (Pestka 2007). The probably best known example for the use of interferons as medication is a pegylated interferon α 2 which is used in combination with ribavirin for treatment of hepatitis C and has been FDA (food and drug administration)-approved in 2002. However, interferon-treated patients regularly suffer from severe side effects such as flu-like symptoms (headache, fever, fatigue etc.), depression and others (Dusheiko 1997).

IFN-γ has long been recognized for its crucial role in defense against viral and bacterial infections as well as in tumor control (Dunn *et al.* 2006; Schreiber *et al.* 2011). It can be produced by APCs (antigen-presenting cells, such as dendritic cells, monocytes and macrophages) and NK (natural killer)-cells and primarily signals through the Jak/STAT pathway via binding to the IFNGR1 (IFN-γ-receptor 1, associates Jak1) and IFNGR2 (associates Jak2). Examples for IFN-γ-regulated genes are STAT1, IRF-1 and SOCS1 (Fig. 6). IFN-α and IFN-β signal via IFNAR1/2 (IFN-α-receptor 1) (Fig. 6).

The Jak-STAT signaling pathway

Conserved among eukaryotes, STAT pathways are also present in slime molds, worms, flies and vertebrates, but not in fungi and plants (Darnell 1997; Aaronson and Horvath 2002). The Jak-STAT pathway was discovered 20 years ago (Stark and Darnell 2012) and can be activated by cytokines, but also by growth factors and hormones (Subramaniam *et al.* 2001; Eckardstein 2009). Main components of the pathway are the respective receptor, Janus kinases and STAT transcription factors. STAT transcription factors can also become activated independently of the Jak-STAT pathway, for example by receptors with intrinsic tyrosine kinase activity such as the EGF (epidermal growth factor) and PDGF (platelet-derived growth factor) receptor (Levy and Darnell 2002). This PhD project focuses on the induction of the Jak-STAT pathway by interferons, following the path: IFN-γ → IFNGR1/2 → Jak1/2 → P-STAT1 → STAT1 target genes.

Main players of the Jak-STAT pathway: Janus kinases and the STAT family of transcription factors

The Janus kinase family consists of the four cytoplasmic, receptor-associated tyrosine kinases Jak1, Jak2, Jak3 and Tyk2. Jak1, Jak2 and Tyk2 show ubiquitous expression, whereas Jak3 is only expressed in hematopoietic cells (Cornejo *et al.* 2009). Janus kinases are named after the two-faced roman god Janus, which stands for the past and the future. Similar to the two faces

of the god Janus, Jaks have two kinase domains, i.e. a functional kinase domain and a so-called pseudokinase domain, which is catalytically inactive (Saharinen and Silvennoinen 2002).
In total seven STAT genes (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) have been identified in mammals. The common feature of the mammalian STAT protein family are their conserved domains (Levy and Darnell 2002; Reich and Liu 2006), some of which are shown in Fig. 5 for STAT1. The sizes of STAT proteins range from 750 to 850 amino acids (Kisseleva *et al.* 2002).

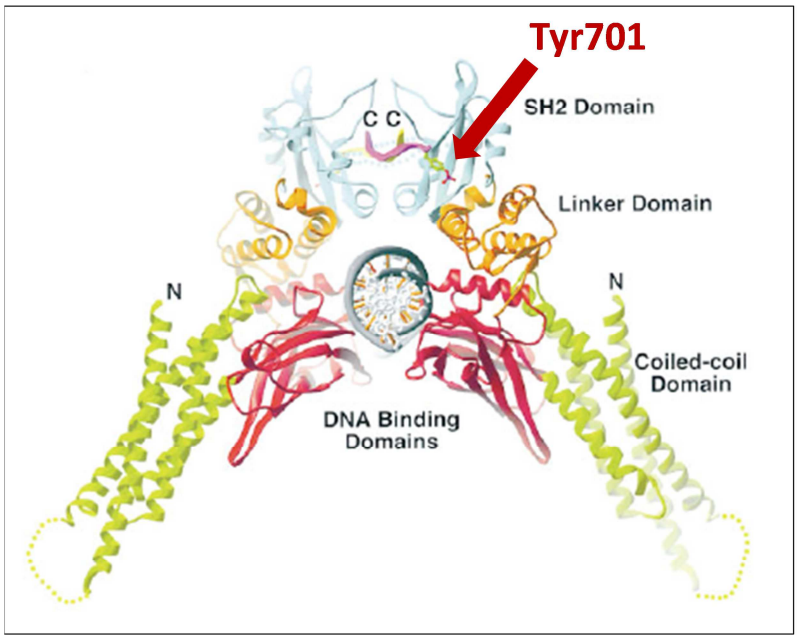


Fig. 5: STAT1 crystal structure and DNA binding motif
Crystal structure of the STAT1 dimer bound to DNA with the following protein domains; green: coiled-coil domain; red: DNA-binding domain; orange: linker domain; cyan: SH2-domain. The phosphotyrosine residue Tyr701, which is critical for activation is indicated by the red arrow, the DNA backbone is grey. (taken from (Chen *et al.* 1998))

The N-terminal and the coiled-coil domain are important for protein-protein-interaction, as for example the interplay of STAT1 with PIAS (protein inhibitor of activated STAT) (Shuai 2000). The DNA binding domain provides the possibility for dimerized STATs to interact with promoter regions of their respective target genes. Another important region, which is crucial for the dimerization of STAT proteins is the SH2 protein domain (Src homology 2). This motif is structurally conserved, present in many signaling molecules and generally recognizes phosphotyrosine residues. Importantly, phosphorylation of a single tyrosine residue (pY 701, Fig. 5) only within the C-terminus is required for STAT-activation (Santos and Costa-Pereira 2011). Briefly, STATs can dimerize by interaction between the SH2 domain of each STAT protein with the phosphotyrosine of the other STAT monomer. In contrast to the other domains, the sequence of the C-terminal transactivation domain is quite variable and therefore mostly

responsible for the formation of the seven different STAT proteins. STAT1 represents a central player of interferon signaling and consists of the two isoforms alpha (p91) and beta (p84), which are produced by alternative splicing of the STAT1 gene with p91 being the designated canonical form (UniprotConsortium 2011). The importance of STAT1 proteins as transducers of interferon signaling is emphasized by studies, which observed effects after impairment of STAT1 function: STAT1 knockout mice exhibited an increased susceptibility to viral and bacterial infections (Meraz *et al.* 1996; Schroder *et al.* 2004). Diminished immune reaction to mycobacterial attacks were observed in humans with STAT1 germline mutations (Dupuis *et al.* 2001; Boisson-Dupuis *et al.* 2012). Apart from dimerization with other STAT factors, STAT1 can also associate with other proteins and transcription factors, as for example with p48 (also known as IRF-9 within the ISGF3 complex (Fig. 6) (Boisson-Dupuis *et al.* 2012). STATs can function in cooperation with other transcription factors such as c-Jun and SP-1 (Shuai 2000; Ginsberg *et al.* 2007). Another well-described interaction of STATs is the association with the co-activators p300 and CBP (CREB-binding protein), which improve the interaction with the basal transcription factors (Wojciak *et al.* 2009).

The Jak-STAT pathway: Events following IFN-γ stimulation

Binding of IFN-γ to its receptor leads to oligomerization of two IFNGR1/IFNGR2 complexes, which are pre-assembled with Jaks (Samuel 2001; Saha *et al.* 2010). Subsequently, Jak1 and Jak2 get trans-phosphorylated and activated. Subsequently, they phosphorylate the tyrosine residues Y440 of the IFN-γ-receptor chains, which thereby create docking sites for the SH2 domains of STAT1. The attracted STAT1 is then phosphorylated at the tyrosine residue Y701 (Fig. 5). Additionally to tyrosine Y701, also the STAT1 serine residue S727 can be phosphorylated, which is required for maximal transcriptional activity (Sun *et al.* 2005). In addition to Tyr701 and Ser727 phosphorylation, acetylation, methylation and sumoylation as additional posttranslational modifications have been reported for STAT1 (Boisson-Dupuis *et al.* 2012). After activation, the resulting STAT1-STAT1 homodimers dissociate from the receptor and translocate to the nucleus. Nuclear import is supported by importins (Jerke *et al.* 2009), mediated by nuclear localization signal (NLS) (Fagerlund *et al.* 2002). There the STAT1-STAT1 homodimer binds to GAS-elements in promoter regions of target genes, which carry the consensus sequence $TTCN_{(2\text{-}4)}GAA$ (Decker *et al.* 1997). However, also unphosphorylated STAT1 is able to drive gene expression (Cheon *et al.* 2011).

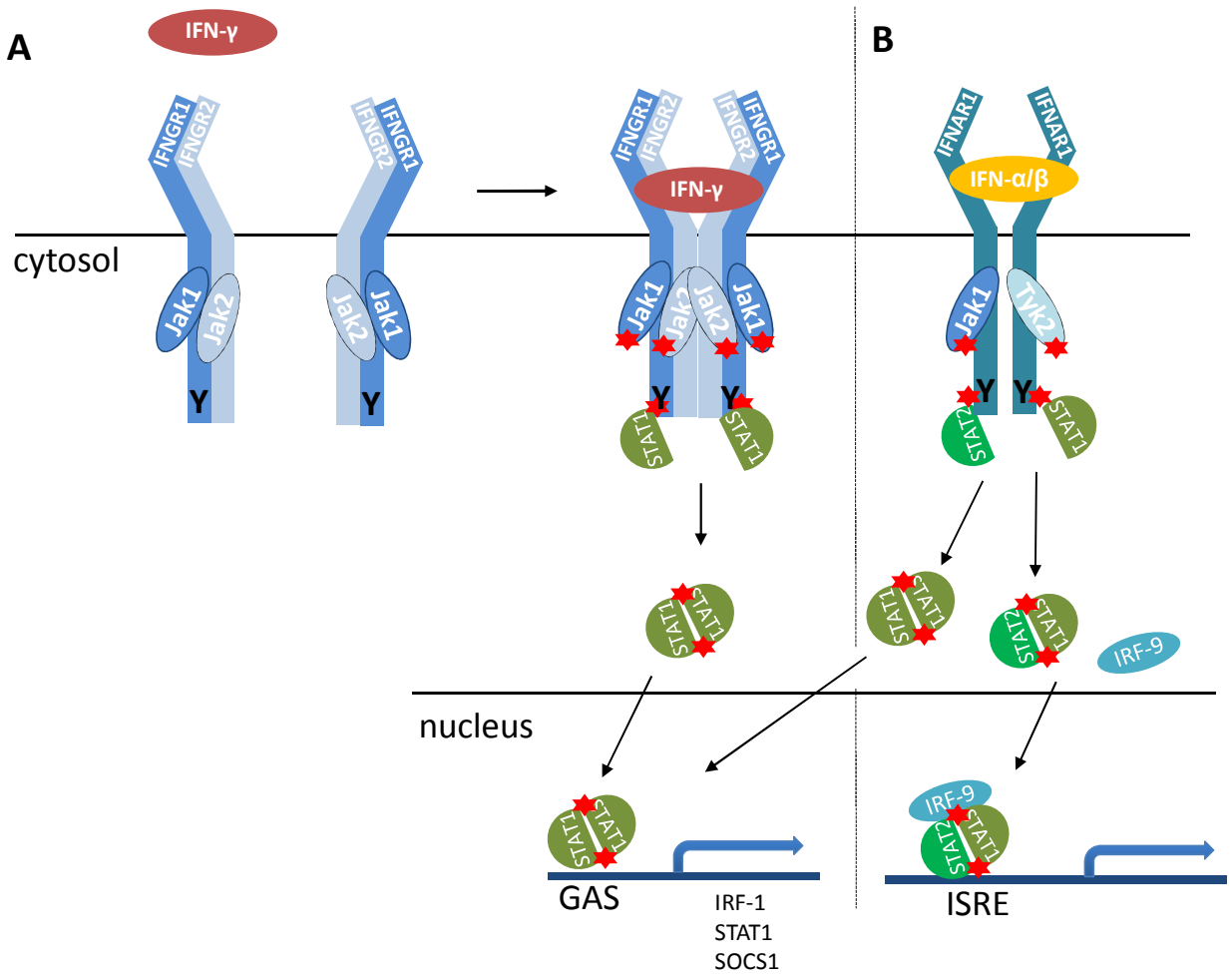


Fig. 6: Signal transduction processes following IFN-γ and IFN-α/β stimulation
(A) IFN-γ stimulation of the IFN-γ receptor induces a conformational change in the receptor chains, resulting in phosphorylation of Jak1/Jak2 and subsequent phosphorylation of the tyrosine residues Y440 in the IFNGR1 chains. These form docking sites for latent STAT1 proteins, which get recruited to the cytosolic receptor chains, become phosphorylated, dimerize and translocate to the nucleus. There they bind to regulatory promoter elements, i.e. GAS elements (gamma activated sequences). Known STAT1 target genes are for example STAT1, IRF-1 and SOCS1. (B) IFN-α/β signaling is accomplished via the IFNAR, which leads to the production of STAT1-STAT2-IRF-9 trimers. They bind to ISREs (IFN stimulated response elements) in promoter regions of target genes and initiate their transcription. Alternatively, STAT1-STAT1 dimers can also be formed upon IFN-α/β stimulation of the IFNAR, as indicated by the arrow.

For IFN-α and IFN-β, signal transduction follows a similar way via IFNAR1/2, which are associated with Tyk2/Jak1, respectively, the subsequent formation of STAT1/STAT1 dimers or STAT1/STAT2/IRF-9 heterotrimers, which are also known as ISGF3 (interferon-stimulated gamma factor 3) complexes. Following IFN-α/β stimulation, they bind to ISRE (IFN stimulated response elements) in promoter regions of the respective genes, represented by the consensus sequence AGTTTN$_3$TTTCC (Kessler *et al.* 1988).

Negative regulation of the Jak-STAT pathway

Several negative regulators can partake in the inactivation of the Jak-STAT pathway, such as SOCS (suppressor of cytokine signaling) and PIAS (protein inhibitor of activated STAT) proteins. SOCS proteins are rapidly up-regulated with enhanced STAT activation, thus providing a typical loop of feedback inhibition. They bind to phosphorylated Janus kinases and the respective receptors to block the pathway; for example binding of phosphotyrosines in a receptor chain by SOCS proteins prevents the subsequent binding of STAT factors (Rawlings *et al.* 2004). PIAS proteins are constitutively expressed E3-SUMO protein ligases which directly interact with phosphorylated STAT dimers and inhibit the DNA recognition process (Wormald and Hilton 2004). Another group of negative regulators are PTPs (protein tyrosine phosphatases), as for example the tyrosine phosphatase SHP-1 (Src homology region 2 domain-containing phosphatase-1), which can dephosphorylate Jaks or their receptors (Rawlings *et al.* 2004). Several phosphatases such as SHP-2 which can act as negative regulators of STAT proteins have been described (Shuai and Liu 2003; Xu and Qu 2008).

Concerning negative regulation of the Jak-STAT-pathway following IFN-γ-stimulation in particular, several specific mechanisms have been reported. For example, the IFN-γ/IFNGR complex can be internalized and subjected to degradation in the endosomal pathway, making it unavailable for further transduction of the signal, possibly to hamper overstimulation (Claudinon *et al.* 2007; Trinchieri 2010). However, receptors are not always degraded but can be recycled and send back to the surface (Claudinon *et al.* 2007). STAT1 can be sumoylated by PIAS1, leading to inhibition of its activity (Ungureanu *et al.* 2005). Additionally, SOCS1 provides specific feedback inhibition of IFN-γ-signaling, which itself induces SOCS1 (Fig. 6).

Connecting the Jak-STAT pathway to miRNAs

STAT factors have been first described to regulate transcription of miRNA genes a few years ago (Löffler *et al.* 2007; Meng *et al.* 2007b) and the importance of miRNA-STAT factor interactions has very recently been summarized in a review (Kohanbash and Okada 2012). However, only few connections have been described in detail. One of the first relationships between cytokine-induced Jak/STAT signaling and miRNAs has been established by Löffler *et al.*, who showed that IL-6 increased the expression of oncogenic miR-21 via STAT3 activation in myeloma cells (Löffler *et al.* 2007), which has been subsequently confirmed by other groups in different cell lines (Iliopoulos *et al.* 2010; Yang *et al.* 2010; Kohanbash and Okada 2012). Also, miR-181b, miR-17-92 and miR-199a-5p have been shown to be STAT3-induced (Brock *et al.* 2009; Iliopoulos *et al.* 2010; Haghikia *et al.* 2011). A positive feedback loop between STAT1 and miR-155 has recently been discovered in epithelial cells, where STAT1 induced up-regulation of miR-155, which in turn negatively regulated SOCS1 (Kutty *et al.* 2010).

1.3 Regulation of gene expression and eukaroytic transcription

Several different ways to control the regulation of gene expression are known to date including transcriptional and posttranscriptional regulation, epigenetic mechanisms including structural or chemical modifications of DNA, posttranscriptional regulation by miRNAs and regulation of translation. The regulation by miRNAs has been briefly explained above (2.1 MicroRNAs – mode of action) and the transcriptional regulation of gene expression as well as additional epigenetic mechanisms will be addressed below.

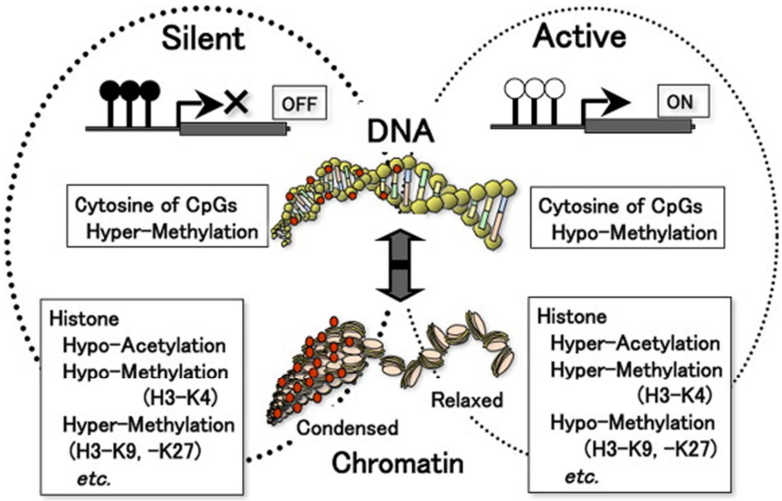


Fig. 7: Epigenetic changes in mammalian cells: DNA methylation and different chromatin structures
taken from (Ohgane *et al.* 2008)
(upper part) Methylation of CpG islands within the DNA leads to silencing of corresponding genes (methylation of CpG islands is indicated by black circles (left) in contrast to white circles (right), which represent hypo-methylated CpG islands). (lower part) Chromatin structure (condensed – in closed conformation, transcriptionally inactive in contrast to relaxed – in more open conformation, at transcriptionally active sites) is accommodated by histone modifications, i.e. acetylation and methylation. H3 – histone 3; K – lysine residus.

To fit into the nucleus and to prevent the DNA from damage, it is generally tightly packed around histone proteins into nucleosomes (Fig. 7). For the initiation of transcription, the compact chromatin ('heterochromatin') has to be re-arranged to a more open comformation ('euchromatin') in order to provide enough space for the polymerase. This is accomplished by several well-described modifications at specific histones, which become enzymatically modified and include methylation and acetylation. In contrast to DNA-methylation, which occurs at CpG islands and epigenetically silences genes, the methylation status of distinct histone residues can be correlated with the chromatin status: for example H3K4me3 (tri-methylation of histone H3 at lysine 4) is often found close to the transcription start site (TSS) of active promoters (Benevolenskaya 2007), whereas H3K9me3 (tri-methylation of lysine 9 within histone 3) is a

marker for inactive genes (Rosenfeld *et al.* 2009) (Fig. 7). Also acetylation of lysines, as mediated by histone acetyltransferases (HATs) influences chromatin structure; as for methylation, acetylation of histones is associated with a less condensed chromatin structure whereas hypo-acetylated chromatin is tightly packed (Fig. 7). Only in the open conformation, chromatin is accessible for the RNA-polymerase and the transcription process can be initiated.

As the first step in gene expression, eukaryotic transcription enables cells to elicit a response following exposure to extracellular signals via diverse signaling processes (Lee and Young 2000; Maston *et al.* 2006). To start the transcription process, assembly of the initiation machinery including the positioning of the RNA polymerase at the respective DNA sequence is required. For protein-coding genes and the majority of miRNA genes, transcription is performed by RNA-polymerase II and can be subdivided into initiation, elongation and termination, with the majority of regulation happening at the initiation step. Initiation of transcription takes place at the promoter region of DNA, which is located upstream of the transcription start site. The so-called 'core promoter' region mostly contains the minimum number of elements which are necessary for transcription like a TATA box, an initiator element (INR), a downstream promoter element (DPE) and a TFIIB (transcription factor 2B) recognition element. Furthermore, the TSS and a binding site for the RNA-polymerase are located here. Besides this region, other regulatory elements such as silencers and enhancers, which provide recognition sequences for the specific transcription factors are part of the genomic regulatory region. In contrast to the 'core promoter' elements, they can be localized up to many thousands of base pairs away from the transcription start site. The transcription process starts with the assembly of the 'pre-initiation' complex. Briefly, TBP (TATA-binding protein), a subunit of TFIID, binds to the TATA box and ensures subsequent gathering of the remaining general transcription factors (TFII-A, B, E, F, H) to the promoter region, where they lead RNA polymerase II to the transcription start site. Afterwards, RNA polymerase II creates a complementary RNA copy of the DNA during the elongation step which is polyadenylated at the termination step and transcription of the respective gene is accomplished. Further fine-tuning and activation or silencing of gene-expression requires the activity of additional transcription factors (Maston *et al.* 2006; Spitz and Furlong 2012).

Transcription factors

Eukaryotic control of gene expression is much more complex than the mechanisms that exist in prokaryotes, which can be mainly attributed to the existence of transcription factors and their influence on RNA polymerase II activity. Controlling the transcription rate is crucial for the fine-tuning of gene expression. Transcription factors are composed of a DNA-binding domain and a transactivating (or transrepressing-) domain as well as further domains responsible for the interaction with other proteins, as shown in Fig. 5 for STAT1. Their function is to recognize and

bind to specific, 6-10 nucleotide long DNA sequences (enhancer or silencer elements of target genes) and to interact with RNA-polymerase II to influence transcriptional activity either by blocking (repressors) or enhancing (activators) the action of the polymerase (Maston *et al.* 2006). The respective regulatory elements can be located hundreds or thousands of basepairs upstream and downstream of the promoter region. Several public databases allow for the scanning of user-provided DNA-sequences for conserved transcription factor binding sites, as for example 'Genomatix', 'Transfac' and 'Jaspar' (Matys *et al.* 2003; Vlieghe *et al.* 2006); the latter two were used within this PhD project.

Currently, ChIP (chromatin immunoprecipitation) provides the method of choice to verify transcription factor binding sites within promoter regions of target genes. Briefly, the chromatin including the DNA-bound transcription factors is fragmented by sonication and subsequently immune-precipitated with an antibody for the analyzed transcription factor. After purification, the respective DNA-sequence can be amplified by PCR or detected by microarray (then known as ChIP-chip) or deep sequencing (ChIP-Seq, Fig. 8) (Collas 2010). ChIP-Seq is a commonly used high-throughput method which allows for determination of genome-wide binding sites for a given transcription factor. A comprehensive study including ChIP-Seq data for STAT1 has been performed by Robertson *et al.* in Hela cells (Robertson *et al.* 2007).

Apart from the ChIP analysis for binding sites in the promoter regions of target genes for the transcription factor of interest, other components of the transcription process can be investigated: For example ChIP-Seq can also be performed with an antibody, which recognizes the characteristic histone signatures (e.g. H3K4Me3) as explained above (Fig. 7) to search for transcriptionally active promoters (Young *et al.* 2011; Ntziachristos *et al.* 2012).

In addition to transcription factors, co-activators and co-repressors mediate the interaction between general and specific transcription factors and influence transcription efficiency (Spitz and Furlong 2012). For example the co-activators CBP (CREB binding protein)/p300, which have been shown to interact with STAT1, exhibit histone acetyltransferase activity, enabling them to promote transcription by acetylation of histones (Bandyopadhyay *et al.* 2002) (Fig. 7). Along with the combinatorial control mediated by transcription factors and their tissue-specific distribution, these above mentioned factors and processes contribute to the specificity of transcription, which is a major reason for the general complexity of eukaryotic organisms.

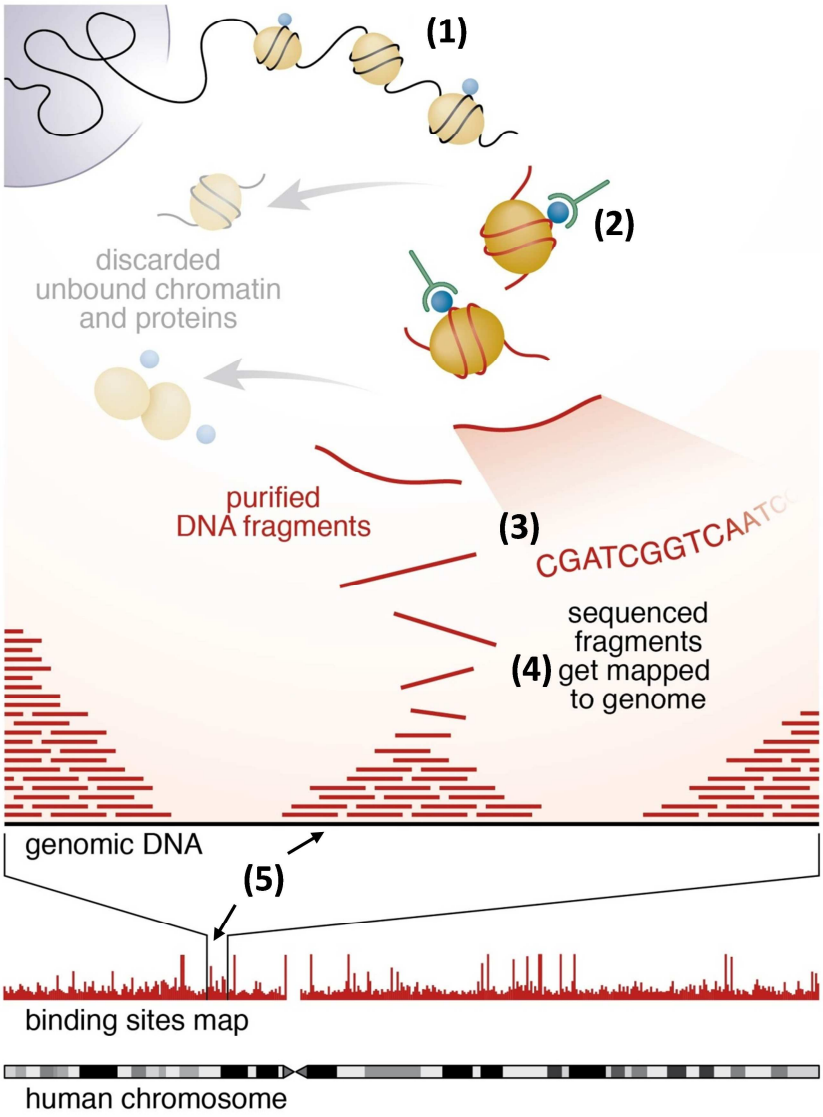


Fig. 8: ChIP-Seq (scheme)
Taken from http://www.bnl.gov/newsroom/news.php?a=11351
(from top to bottom) Initial steps include (1) cross-linking of transcription factors to DNA, (2) fragmentation of DNA and binding of the specific antibody of choice followed by precipitation of the fragments which are bound by this antibody. (3) Those DNA fragments are subjected to high-throughput sequencing and (4) the respective sequences can be mapped to the genome. (5) Note that the number of enriched sequences corresponds to the height of the peak, which is obtained as a read-out of ChIP-Seq data.

1.4 Melanoma

Composition of the skin and types of skin cancer

The skin represents the largest and most versatile organ of the body. Apart from protection against infection, injury and temperature loss, one of its most important functions is to protect against UV radiation. The outermost part of the skin is divided into epidermis, dermis and adjacent subcutaneous tissue (Fig. 9).

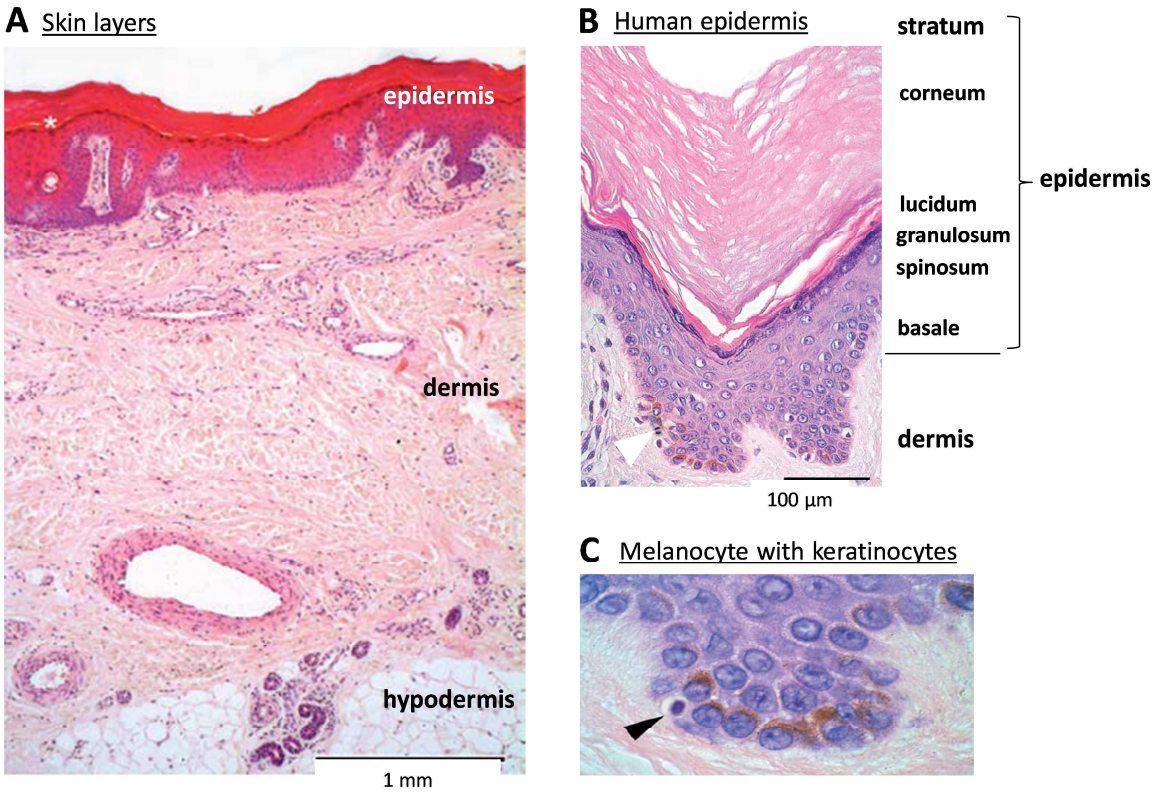


Fig. 9 Layers of the skin
(Pictures from (Junqueira *et al.* 2005), chapter 17 'The skin'). (A) The outer layer of the skin is divided into epidermis, dermis and the adjacent subcutaneous tissue (hypodermis), 'thick' skin is shown in this picture. (B) Magnified view on the human epidermis, which is composed of the different layers (from outside to inside) *stratum corneum*, *lucidum*, *granulosum*, *spinosum* and *basale* and separated from the dermis by a basement membrane. Melanocytes as the melanin-producing cells of the skin are generally located in the *stratum basale* in the deepest part of the epidermis, just above the basement membrane. (C) Enlarged picture of the *stratum basale* with keratinocytes and a melanocyte. The arrow indicates a melanocyte which is surrounded by keratinocytes and melanin (brownish structure which covers the nuclei of the keratinocytes), which is secreted by melanocytes and is taken up by the cells in their vicinity.

Keratinocytes represent the main components of the epidermis (> 90 % of the cells). Moreover, the epidermal layer contains squamous cells, basal cells and melanocytes which are all prone to skin cancer development. Named according to the cell type they develop from, basal and squamous cell carcinoma (often referred to as 'non-melanoma skin cancer') as well as melanoma represent the three most common types of skin cancer (AmericanCancerSociety

2012). For all of them, the number of cases has increased over the past couple of years (Garbe and Blum 2001; Thompson *et al.* 2005). Basal cell carcinoma is one of the most common skin tumors. It derives from the lowest layer of the epidermis (*stratum basale*, Fig. 9). Survival rates are extremely good as the tumor rarely metastasizes. Nevertheless, it grows invasively and can - if not treated - lead to a massive destruction of the surrounding tissue. Squamous cell cancer is less common, but metastasizes with a higher frequency. It develops from keratinocytes of the *stratum spinosum* (Fig. 9). Other types of skin cancer - though occurring much less frequently - are for example Merkel cell carcinoma, Kaposi's sarcoma and others. While the mortality rates of basal and squamous cell carcinoma are relatively low (Stulberg *et al.* 2004), (metastatic) melanoma is less common, but a far more dangerous and aggressive type of skin cancer, leading to 5-year survival rates of < 5% (Miller and Mihm 2006). Melanoma contribute to the vast majority of deaths caused by skin cancer, although they only contribute to less than 5% of all skin cancer cases (AmericanCancerSociety 2012). The incidence of melanoma and non melanoma skin cancers has been increasing during the last couple of years (WHO, www.who.int) and it is estimated to rise even further. Fig. 10 illustrates a comparison of international age-standardized incidences of melanoma together with the respective mortality rates. Australia shows by far the highest incidence of melanoma: In 2008, incidence rates were 60.5 cases/100.000 men and 39.3 cases/100.000 women (http://www.aihw.gov.au).

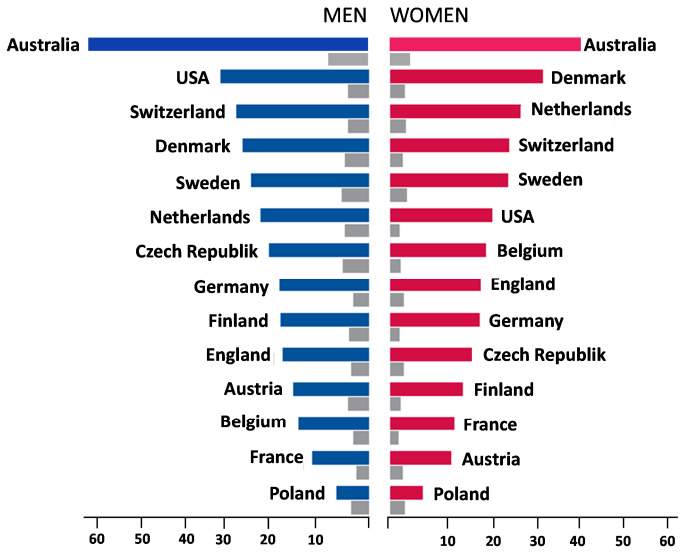


Fig. 10: Melanoma: international comparison of age-standardized incidences
(blue: men, red: women) and mortality rates (grey), calculated per 100.000 inhabitants (data from 2007-2008 or the last year which was available, graph adapted from Robert-Koch-Institut Germany; (www.rki.de));. Data from Australia are from 2008 (age-standardized incidences) and 2007 (mortality rates) and obtained from the 'Australian Cancer Incidence and Mortality books', ACIM (http://www.aihw.gov.au).

Melanocytes and their normal function: production of melanin

Melanocytes are the pigment-producing cells of the skin and located at the bottom layer of the epidermis (Fig. 9). Together with their derivative pigment melanin, their function is the protection of DNA from UV-radiation-induced DNA damages (Hu 2008). Melanogenesis is induced as soon as a part of the skin is exposed to sun. Briefly, tyrosinase catalyzes the conversion of tyrosine to 3,4-dihydrophenlyalanine (dopa) and to dopachinone, which is then subsequently converted to melanin. It accumulates in melanosome vesicles, which migrate to the tips of the melanocytes, where they get released and taken up by the surrounding keratinocytes (Park *et al.* 2009a). Every melanocyte has contact to approximately 30 keratinocytes. Skin pigmentation in general is determined by the specific type of melanin pigment that is predominant: eumelanin is rather brownish, while pheomelanin causes the reddish color of red hair and freckles. Differences in pigmentation are inherited; for example red haired people, who usually exhibit fair skin have more pheomelanin than eumelanin (Mitra *et al.* 2012). Also the size, number and density of melanosomes plays a role in skin pigmentation. Benign accumulations of melanocytes are referred to as 'melanocytic nevi' or 'nevi' (Markovic *et al.* 2007) and commonly known as 'moles'. Melanoma can develop from pre-existing moles or they can derive from a new location on normal skin spontaneously.

Melanoma – origin and development

The WHO classifies melanoma in four different subtypes, i.e. superficial spreading melanoma (SSM), acral lentiginious melanoma (ALM), lentigo maligna melanoma (LMM), nodular melanoma (NM). They differ in several features as the age at which they mostly occur or in their location on the body. For example, LMM are most common in elderly people and develop from pigmented areas of the skin. NM occur in sun-exposed areas of the skin and develop quickly. SSM is the most common type of melanoma whereas acral lentiginous melanoma are quite rare and develop in palms, nails etc. (www.skincancer.org).

The mechanical progression of healthy epidermal tissue to metastatic melanoma is a multi-step process and a known set of mutations accompanies the single steps towards malignancy (Miller and Mihm 2006) (Fig. 11). In normal skin or in a benign nevus, melanocytes proliferate slowly. The next step towards malignancy is a dysplastic nevus, which shows atypical growth behavior. Such spots can potentially be identified by the 'ABCDE'-rule (which will be explained below, Fig. 12), as they show suspicious asymmetry or borders, a large diameter or multiple colors. The following radial growth phase represents the first malignant stage of melanoma development and progression. Still, cells show low invasiveness and proliferation. Proliferation increases dramatically during the vertical growth phase, thus cells are able to penetrate the basement membrane, infiltrate the dermis and invade the connective tissue. In the last and metastatic

state, melanoma cells can spread over the lymphatic system as well as the blood and metastasize.

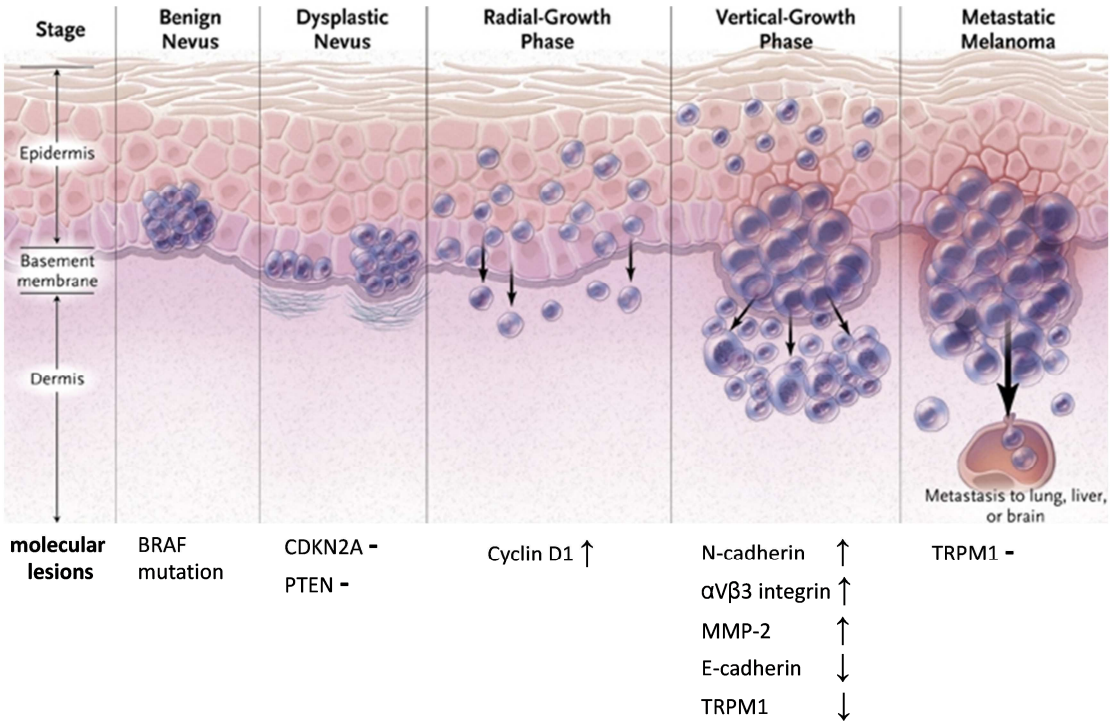


Fig. 11: Histological progression of melanocyte transformation to melanoma is a multi-step process

(Picture from (Miller and Mihm 2006), modified).

A benign nevus shows normal growth behavior, although somatic BRAF mutations can already occur at this early stage. Loss of CDKN2A/PTEN is observed for dysplastic nevi, while the radial growth phase is characterized by increased Cyclin D1 expression and the tumor cells acquire the ability to penetrate the basement membrane. During the vertical growth phase, increased expression of N-Cadherin, αVβ3 integrin and MMP-2 are observed while E-cadherin expression is lost and TRPM1 is reduced, which is absent in the following, metastatic phase. In this last phase of melanocyte transformation, melanoma cells are able to metastasize to other organs. CDKN2A – cyclin-dependent kinase inhibitor 2A, PTEN-phosphatase and tensin homolog, MMP – matrix-metalloprotease, TRPM - Melastatin.

Genetic characteristics of melanoma

Many cellular pathways have been described to be de-regulated in melanoma, which include the MITF (microphtalmia-associated transcription factor) pathway, the PI3K (phosphoinositide-3 kinase)-Akt pathway, the RAS (rat sarcoma virus)-RAF-MEK (dual specificity mitogen-activated protein kinase kinase)-ERK (extracellular regulated kinase) pathway as well as the $p16^{INK4A}$-CDK4 (cyclin-depent kinase 4) -RB (Retinoblastoma) pathway. Table 2 provides an overview of the most important genes in melanoma and melanoma development. A selection of affected pathways and their functions will be explained in more detail below.

Table 2: Genes and cellular pathways important in melanoma
(adapted from (Miller and Mihm 2006)) TF = transcription factor; TS = tumor suppressor; Onc = oncogene; VGP = vertical growth phase; RGP = radial growth phase; expr. = expression; oncogenes are marked in red, whereas tumor suppressors are shown in green.

Pathway	Gene/Protein		Normal function	Changes in melanoma
MAPK RAS	N-RAS BRAF	neuroblastoma RAS viral (v-ras) oncogene homolog/ v-raf murine sarcoma viral oncogene homolog B1	Oncogenes	Sporadic activating mutation (N-RAS: G13R, BRAF: V600E)
	MEK	Mitogen-activated protein kinase – extracellular-regulated kinase	Signal Transduction	Up-regulated in RGP & VGP
	ERK1/2 (MAPK)	Extracellular-regulated kinase 1 / 2 (mitogen-activated kinase)	Signal Transduction	Activity enhanced
INK4A CDK Rb	CDKN2A/ INK4A	Cyclin-dependent kinase inhibitor 2A / inhibitor of kinase 4A	TS, negative regulator of cell proliferation	Germline mutations, sporadic deletions, promoter inactivation
	CDK4	Cyclin-dependent kinase 4	Promoter of proliferation	Familial germline mutations
	CCND1	Cyclin D1	Promoter of proliferation	Sporadic amplification
	Rb	Retinoblastoma	TS, negative regulator of cell proliferation	Phosphorylation → G1/S transition
ARF p53	ARF	Alternate reading frame (CDKN2A)	TS, degrades MDM2	Germline mutations, sporadic deletions, promoter inactivation etc.
	p53	Tumor protein p53	TS, induces apoptosis and suppresses proliferation after DNA-damage	Expr. usually present in melanoma
	MDM2	Mouse double minute 2	Targets p53 for ubiquitination & destruction	Up-regulated in presence of ARF mutation
PTEN Akt	PTEN	Phosphatase and Tensin homolog	TS, counteracts PI3K activity	Sporadic deletion
	PI3K	Phosphatidylinositol 3 kinase	Signaling molecule for many growth factors	Active, when PTEN is mutated
	Akt (PKB)	Protein kinase B	Onc, activated by PI3K, leads to increased cell survival	Sometimes amplified
MSH MITF	MC1R	Melanocortin receptor 1	Receptor for α-MSH	affects hair and skin color
	MITF	Micropthalmia-associated transcription factor	TF	Expr. decreased
	TYR	Tyrosinase	Pigment synthesis	
	MLANA	Melan-A	Melanoma antigen recognized by T-cells 1	
	TRPM1	Melastatin 1	?	
	BCL-2		Cell survival	Variable up-regulation (in various phases of melanoma)
Cell Adhesion molecules	β-Catenin		Adherens junction protein; also affects gene expression	Sporadic mutations
	E-cadherin		Cell adhesion molecule	Reduced expr. in VGP
	N-cadherin		Cell adhesion molecule	Aberrant expr. in VGP
	αVβ3 integrin		Dimer that forms cell adhesion molecule	

A transcription factor of particular importance for melanoma development is MITF (microphthalmia-associated transcription factor). It is responsible for melanocyte development and controls their proliferation, survival and differentiation (Tsao *et al.* 2012). Amplified in 10-20 % of melanoma, it has widely been described as oncogene, but has also been attributed tumor-suppressive functions (Bell and Levy 2011).

The CDKN2A locus

The CDKN2A (cyclin-dependent kinase 2A) gene is a well-known melanoma susceptibility locus (Hussussian *et al.* 1994) (Table 2), which encodes for two proteins, $p16^{INK4A}$ and $p14^{ARF}$. $p16^{INK4A}$ is deleted in about 50% of melanoma (Bennett 2008) while $p14^{ARF}$ is affected less often (Zuo *et al.* 1996; Soufir *et al.* 1998; Tang *et al.* 1999). Both act as tumor suppressors in the cell cycle by controlling G_1 → S transition via the phosphorylation status of Rb1. $p14^{ARF}$ positively regulates p53 via MDM2 (murine double minute) and thus controls induction of $p21^{WAF1/CIP1}$, which controls CDK2/Cyclin E–mediated phosphorylation of Rb1. $p16^{INK4A}$ inhibits the activation of CDK4 and CDK6, which are normally complexed with Cyclin D1 and $p16^{INK4A}$ can also phosphorylate Rb1. In an unphosphorylated form, Rb1 binds to the transcription factor E2F, thereby hindering it from expression of genes necessary for G_1 → S transition. Genetic defects in the CDKN2A locus concerning either of the two proteins result in uncontrolled cell proliferation as will be further discussed below (Fig. 42).

The MAP-kinase pathway in melanoma

The Ras-Raf-MEK-ERK-signaling cascade plays a prominent role in most cancer types. It is normally activated by growth factors via receptor tyrosine kinases, leading to cell proliferation and survival. Initial activation of Ras is a trigger for subsequent phosphorylation of single proteins along the Raf → MEK (MAPK/ERK kinase) → ERK path with each phosphorylation acting as an activation of the subsequent protein in the pathway. Consequently, constitutive activation of only one of the members can lead to uncontrolled proliferation and cancer. Members of the MAP-kinase pathway are mutated in many cancer types. NRAS (member of the Ras family together with KRAS and HRAS) is mutated in ~15-30 % of melanomas (Rosenfeld *et al.* 2008) while BRAF is mutated in ~60 % (Davies *et al.* 2002), mostly showing the $BRAF^{V600E}$ mutation. An inhibitor is available for clinical treatment of individuals with $BRAF^{V600E}$ mutations, which will be further discussed below. The importance of this kinase for melanoma development is emphasized by the fact that BRAF-mutated melanoma metastasize with a higher percentage than not mutated ones (Broekaert *et al.* 2010) and therefore show a more aggressive clinical progression (Long *et al.* 2011). As NRAS and BRAF mutations are mostly mutually exclusive, the majority of melanomas exhibit at least one of them (Glud and Gniadecki 2012).

The PI3K-Akt pathway in melanoma

Upon stimulation by growth factors and hormones, the family of PI3K mediates the phosphorylation of the inositol ring on phosphoinositides, converting PIP_2 (Phosphatidylinositol 4,5-bisphosphate) to PIP_3 (Phosphatidylinositol (3,4,5)-triphosphate). After phosphorylation, the second messenger PIP_3 is required for phosphorylation of signaling proteins containing pleckstrin-homology domains, like the serine-threonine kinase Akt (Proteinkinase B), which subsequently activates other proteins that act on cellular growth, survival and the cell cycle (Cantley 2002). The tumor suppressor and phosphatase PTEN counteracts the $PIP_2 \rightarrow PIP_3$ phosphorylation mediated by PI3K. All of the proteins mentioned above are prone to genetic changes in melanoma (Table 2): Akt3 (member of the Akt family of serine/threonine protein kinases together with Akt1 and Akt2) activity is increased in around 70 % of sporadic melanoma (Sharma *et al.* 2009) and loss of PTEN occurs frequently (Wu *et al.* 2003) while mutations within PI3K genes are rare (Davies 2012). Apart from genetic changes in the human skin, several external risk factors can influence melanoma development.

Environmental and genetic risk factors

The impact of sun exposure on melanoma development has been assessed in many studies and a firm connection has been established. Sun-derived UV radiation is thought to be the cause for around 86 % of melanomas (Parkin *et al.* 2010), as well as for the majority of mutations in melanoma (Pleasance *et al.* 2009). Already one blistering sunburn increases the general risk to develop melanoma (Lew *et al.* 1983), while the risk doubles after more than five sunburns at any age (Pfahlberg *et al.* 2001). Sunburns which have been acquired during childhood increase the risk even more. Of course, the geographical location of an individual has an important impact on daily sun-exposure and is enhanced in the southern hemisphere. Also UV-light from so-called 'non-solar' resources as tanning beds and sunlamps augment the melanoma burden. Interestingly, UV-tanning devices were listed in 'group 1' of the most dangerous cancer-causing substances by the International Agency for Research on Cancer (partner organization of the WHO), which also includes plutonium and cigarettes (El Ghissassi *et al.* 2009). Users of indoor tanning devices are 74 % more likely to develop melanoma than subjects who have never used them (Lazovich *et al.* 2010). Also the risk for squamous cell carcinoma (x 2.5) and basal cell carcinoma (x 1.5) is increased (Karagas *et al.* 2002).

Apart from environmental factors, the genetic background of an individual also influences the development of this cancer. For example the number of nevi on the body correlates directly with the risk for melanoma as nevi have the potential to progress to a malignant state (Markovic *et al.* 2007). Immunosuppressed individuals or people suffering from *Xeroderma pigmentosum* exhibit a higher risk, which is also the case for individuals with a familial history of melanoma

(Cho *et al.* 2005a; Cho *et al.* 2005b) often showing germline mutations in the respective susceptibility genes (as listed in Table 2).

Melanoma detection and current treatment options

Once a melanoma has developed, early discovery is crucial. A commonly established way of melanoma self-control is the 'ABCDE'-rule (Fig. 12). Here, features of existing moles or spots on the body get evaluated for their Asymmetry, Border irregularity, Color variation, Diameter and if they are Evolving (any itching, bleeding or crusting).

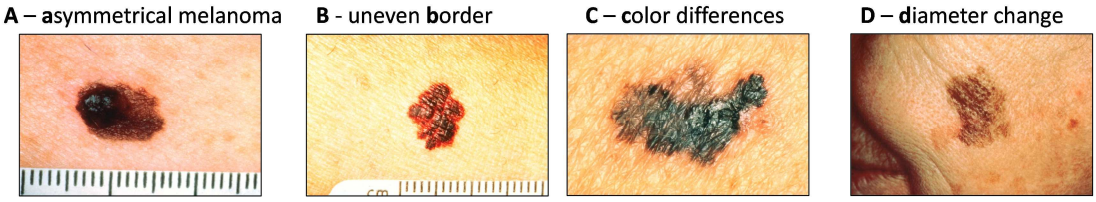


Fig. 12: Images for illustration of the 'ABCD' rule
As described in the text. Taken from NCI Visuals online (http://visualsonline.cancer.gov/)

The 'ugly duckling' sign is a rather recent innovation. This analysis aims at identification of moles or spots that look totally different from the spots in the vicinity. Most affected sites for melanoma development are arms and legs for women and the back for men (Boyle *et al.* 1995; Tsai *et al.* 2005). Suspicious moles, which show abnormal growth behavior, color or even lesions should directly be presented to a dermatologist.

The most obvious and elementary treatment option of malignant melanoma is physical excision of the primary tumour, which – like for most types of skin cancer - can be seen on the surface of the skin. If the tumor has not reached the radial growth phase yet and thus did not invade the basement membrane (compare Fig. 1, then referred to as 'melanoma-*in-situ* '), the chance for formation of metastasis is extremely low (Bene *et al.* 2008). Nevertheless, except excision at early stages, no curative therapies exist and metastatic melanoma exhibit a severe resistance to available therapies. The 5-year survival rate for melanoma patients heavily depends on the time of detection: if detected before the tumor has penetrated the basement membrane, chances to survive are 98 %. However, this quickly drops to 62 %, when lymph nodes are affected and further to 15% when the tumor has metastasized (AmericanCancerSociety 2012). For clinical reports, staging of tumors is performed according to the American Joint Committee on Cancer (AJCC) (Balch and Soong 2008). Here, the vertical tumor thickness (divided into 5 stages according to Breslow (Breslow 1970), the mitotic rate, ulceration and the lymph node status are analyzed (Glud and Gniadecki 2012). Additionally, the 'Clark' classification, which characterizes the invasion level of the tumor (stages 0 to IV) has been widely used (www.skincancer.org). As mentioned above, when detected early, melanoma can be efficiently treated by surgical

resection. However, all therapeutics which are used to treat metastasized malignant melanoma to date merely increase survival by several months. Consequently, current treatment options are rather palliative and aim to minimize reccurence. Often, therapies against melanoma include administration of high-dose IFN-α-2b as an adjuvant treatment, which has, however, severe side effects such as fever, fatigue and weight loss. Around 50% of melanoma patients exhibit V600E mutations in the cellular kinase BRAF (Ascierto *et al.* 2012). In 2011, a targeted kinase inhibitor therapy was licensed. The BRAF-inhibitor Zelboraf® (also known as Vemurafenib, Roche) has been FDA-approved for treatment of late-stage melanoma patients who carry the V600E mutation and this treatment increases life expectancy by several months (Sala *et al.* 2008; Flaherty *et al.* 2010). Nevertheless, none of the available treatment options (kinase inhibitors, chemotherapy) can entirely extinguish the malignancy (Chapman *et al.* 2011). Ipilimumab (Yervoy®, Bristol-Myers Squibb) which is also used for treatment of malignant melanoma, is a monoclonal antibody targeting CTLA-4 (cytotoxic T-lymphocyte antigen 4) to induce an antitumor immune response (Scheier *et al.* 2011). More drugs are currently under investigation in clinical trials, for example the monoclonal antibody Bevacizumab (Avastin®, Roche), which inhibits angiogenesis and has already been approved for the treatment of other cancer types. However, the most promising approach to date in immunotherapy against melanoma uses T-cells, which have been genetically modified to express receptors directed against tumor antigens, such as MAGE A3 and –A12 (melanoma antigen encoding gene) (Zhu *et al.* 2012).

Melanoma and miRNAs

As they are involved in virtually all cellular processes, clearly miRNAs are supposed to be also be involved in melanoma development and progression, as reviewed extensively during the last couple of years (Mueller and Bosserhoff 2009; Howell *et al.* 2010; Glud and Gniadecki 2012; Völler *et al.* 2013). This is supported by the fact that miRNAs have been shown to play decisive roles already in normal skin development and homeostasis (Botchkareva 2012; Schneider 2012): Knockouts of the key miRNA processing enzymes DGCR8 (Yi *et al.* 2009) and Dicer (Bernstein *et al.* 2003) led to death of mice in early embryogenesis. Furthermore, individual miRNAs could be attributed important functions in skin development, as for example miR-203, which can promote epidermal differentiation (Yi *et al.* 2008). However, concerning malignant melanoma, relatively few studies on the function of miRNAs have been published so far in comparison to other cancer types. The majority of studies on miRNAs in melanoma established miRNA expression profiles in different healthy and diseased skin stages, i.e. healthy nevi, primary and malignant melanoma. Pilot studies in this area investigated microarray expression profiles in healthy melanocytes versus melanoma cell lines and reported distinct miRNA signatures (Zhang *et al.* 2006; Gaur *et al.* 2007; Mueller and Bosserhoff 2009). Along this line,

we have previously analyzed the miRNome of a set of melanoma cell lines and FFPE patient samples (Philippidou *et al.* 2010; Schmitz Ulf *et al.* 2013) and other groups have investigated blood samples of melanoma patients for screenings to identify potential biomarkers (Leidinger *et al.* 2010). Latest screening studies added previously unknown miRNAs (as miR-4291, miR-4317 and miR-4324) to the miRNAs de-regulated in melanoma as the corresponding microarray experiments were based on the current version 19 in contrast to earlier studies (Sand *et al.* 2012). Apart from those evaluations which concerned the whole miRNome, individual miRNAs have been subsequently connected with proteins involved in melanoma growth and behaviour.

The first link between a single miRNA and melanoma tumorigenesis was the discovery that the transcription factor MITF (*microphthalmia-associated transcription factor*) was regulated by miR-137 (Bemis *et al.* 2008) and also by miR-182 (Segura *et al.* 2009). MITF mediates melanocyte proliferation, survival and differentiation and primary as well as metastatic melanomas exhibit alterations in the connected pathway (Cronin *et al.* 2009) (Table 2). Also other well-known cancer-associated miRNAs, as for example the let-7 family, have been connected to melanoma: let-7a down-regulated integrin β3 (Muller and Bosserhoff 2008), which contributes to the invasive potential of melanoma cells and let-7b has been confirmed to target the cell cycle regulator Cyclin D1 (Table 2) (Schultz *et al.* 2008). Other examples for 'key' cancer-associated miRNAs with melanoma involvement are miR-34a, which is silenced in melanoma (Lodygin *et al.* 2008), miR-155, which is over-expressed and negatively regulates melanoma growth as well as the oncogenic miR-17-92 cluster, which is also over-expressed (Levati *et al.* 2009). Although only a small selection of melanoma-connected miRNAs was addressed here, it is certain that they play a pivotal role in general skin functions and melanoma development. This PhD project aims to decipher further miRNA-melanoma cellular networks, with the focus on STAT1-regulated miRNAs.

2 Objectives

The main focus of this PhD project was the investigation of miRNAs that could be induced by IFN-γ and that are regulated STAT1-dependently. By elucidating STAT1 regulation of miRNA expression and their respective target genes, an important objective was to identify new regulatory pathways for the growth control of melanoma and later, other cancer cells. Following this path, the aim was to identify and describe a complete regulatory line of events starting with cytokine stimulations of different melanoma cells → STAT induction → identification of STAT-regulated miRNAs → identification and analysis of miRNA target genes → functional role of target genes in cancer. Apart from this major objective, another important issue was expression profiling of melanoma to find potential biomarkers. The main objectives of this PhD project are briefly summarized below:

- Identification of differentially regulated miRNAs in untreated versus IFN-γ-stimulated cell lines (microarray): list potentially STAT1-regulated miRNAs
- Validation of candidate STAT1-regulated miRNAs
- *In silico* analysis of promoter regions of identified miRNAs
- Subsequent identification of the role of STAT1-regulated miRNAs in defined cellular processes and their implication in melanoma development
- *In silico* identification of respective miRNA target genes
- Experimental verification of potential target genes, which are likely to play a role in melanoma development and/or progression or exhibit other interesting cancer-related properties
- Analysis of the functional relevance of miRNA-regulated target genes for the growth characteristics of melanoma cells: proliferation, migration/invasion, apoptosis.
- Analysis of global and selected miRNA expression patterns in melanoma cell lines and patient samples

3 Materials

Tables with the suppliers for all laboratory equipment as well as solution and buffer recipes can be found in the Appendix in (Table 8-Table 11).

Cell lines

Table 3: Human cell lines used in this study
DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen (german collection of microorganism and cell cultures), RWTH = Rheinisch-Westfälische Technische Hochschule Aachen

Cell line	description	Medium	Company/ obtained from
A375	melanoma, metastatic	RPMI + 10 % FCS	ATCC
A375 (wt) **A375 (F)**	A375 stable transfectants	RPMI + 10 % FCS + 400 µg/ml G418	Dr. Marcin Kortylewski (Kortylewski *et al.* 1999)
FM-55M1	melanoma late stage (metastatic)	RPMI + 10 % FCS	ESTDAB, Tübingen, Germany
FM-55P	melanoma, early stage (primary)	RPMI + 10 % FCS	ESTDAB, Tübingen, Germany
G361	melanoma	RPMI + 10 % FCS	RWTH Aachen, Germany
IGR-37	melanoma, late stage	RPMI + 10 % FCS	DSMZ, Braunschweig, Germany
IGR-39	melanoma, early stage	RPMI + 10 % FCS	DSMZ, Braunschweig, Germany
MelIm **MelJuso**	melanoma	RPMI + 10 % FCS	Prof. Bosserhoff, Regensburg, Germany
MeWo	melanoma	RPMI + 10 % FCS	Prof. Schadendorf, Essen, Germany
SK-Mel30	melanoma	RPMI + 10 % FCS	Prof. Böhm, Münster, Germany
NHEM-M2	normal human epidermal melanocytes	Melanocyte medium M2	PromoCell
HaCat	keratinocytes	DMEM + 10 % FCS	Prof. Fusenig, Heidelberg, Germany
Hek293T	human embr. kidney cells	DMEM + 10 % FCS	RWTH Aachen, Germany
Jurkat	Leukemia T cells	RPMI + 10 % FCS	RWTH Aachen, Germany
MT4	T cells	RPMI + 10 % FCS	Dr. Devaux, Luxembourg

FFPE (formalin-fixed paraffin-embedded) patient material

Skin tissue samples from patients with either benign nevi or melanoma were collected at the Dermatology Department of the University Hospital of Freiburg (Germany) under supervision of

Prof. Dorothee Nashan and histopathologically examined to confirm clinical diagnoses. Upon excision, tissues were fixed in FFPE according to standard dermatohistopathologic techniques. In this PhD project, RNAs of 5 healthy skin samples, 4 benign nevi, 12 primary and 14 metastatic melanoma samples were analyzed by qRT-PCR. [2]The primary and metastatic samples were collected from different parts of the body from a total number of 5 melanoma patients. The study was approved by the ethical review board of EK Freiburg (reference 196/09) as well as by Luxembourg's ethics comission, and written informed consent was obtained from healthy controls and live patients.

Bacteria

E. coli DH5 α (Invitrogen) were used for cloning experiments and were cultivated in LB medium.

Vectors

pmirGLO Dual Luciferase miRNA target expression vector

(Promega, #E1330)

pmirGLO was used for cloning of the luciferase constructs. The vector itself is designed for quantitative evaluation of microRNA activity and the corresponding mRNA regulation. miRNA 3´-UTRs containing miRNA target sites or isolated target sites can be inserted 3´of the firefly luciferase gene (luc2), which represents the primary reporter gene. Thus, reduced firefly luciferase expression indicates the binding of endogenous or introduced miRNAs to the cloned miRNA target sequence. Furthermore, the vector contains a second luciferase gene, encoding the Renilla luciferase (hRluc-neo), which acts as an internal control reporter and is used for normalization purposes. The 3'-UTRs and the single binding sites of potential miR-29 target genes were inserted into the multiple cloning site (MCS) by standard cloning procedures as will be described below.

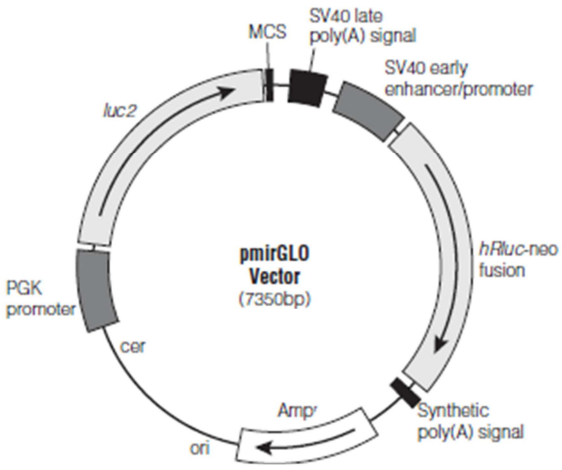


Fig. 13: pmirGLO vector (Promega)
3'UTRs of CDK6 and PI3KR1 or isolated miR-29 binding sites were cloned into the multiple cloning site (MCS), which is located downstream of the firefly luciferase reporter gene (luc2) and upstream of the renilla luciferase gene (hRluc-neo fusion). The vector furthermore contains an ampicillin resistance (Amp) for selection of bacteria that contain the plasmid.

Antibodies

Table 4 : Antibodies

Primary antibodies	Dilution	species	company
Actin (C4)	1:4000	rabbit	Millipore
CDK6	1:500	mouse	Santa Cruz
FIN13	1:3000	mouse	BD
IRF-1 (C-20)	1:1000	rabbit	Santa Cruz
P (Y701)-STAT1	1:1000	mouse	BD
p85α (PI3K)	1:1000	mouse	Upstate
STAT1	1:1000	mouse	Santa Cruz
Tubulin	1:4000	mouse	Santa Cruz
Secondary antibodies	**Dilution**		**company**
HRP-labeled (for ECL detection)	1:5000		Cell Signaling Technology
fluorophor-coupled (for quantification in Licor)	1:10.000		Licor Biosciences

All primary antibodies were diluted in TBS-N + 0.01-0,1 % NaN_3. Secondary antibodies for ECL-detection were diluted in TBS-N and secondary antibodies for quantification in a mixture of Licor Blocking Buffer (1/5) and PBS + 0.1 % Triton (4/5).

miR-mimics/inhibitors and siRNA

MiR-29a and miR-29b mimics and inhibitors and their respective negative controls (NC-mimic and NC-inhibitor) were obtained from Qiagen, whereas 'ON-TARGET' siRNA for CDK6 and the corresponding negative control were purchased from Dharmacon.

Oligonucleotides

Primers for amplification of mature miRNAs were purchased from Qiagen. All other oligonucleotides were self-designed in our laboratory, obtained from Eurogentec and are listed in Table 6 and Table 5.

Software

For text files and analysis of data Microsoft Office 2010™ was used, for further statistical analyses and graphs we used Graph Pad Prism5. MicroRNA target gene prediction was performed by *in silico* analysis of the 3´-UTR of potential miR-29 target genes using a combination of the open source databases TargetScan ((Friedman *et al.* 2009), http://www.targetscan.org/), DIANA-microT v3.0 ((Maragkakis *et al.* 2009), http://diana.cslab.ece.ntua.gr/micro-CDS) and microRNA.org ((Betel *et al.* 2008), http://www.microrna.org). Quantification of Western blots was performed with the analysis software V3.0 provided by Licor Biosciences whereas ECL-detected Western blot bands were detected with the program provided by ChemiStar (Intas) or the ImageLab™ software (Bio-Rad) and further processed with Adobe Photoshop CS3. The NCBI homepage (http://www.ncbi.nlm.nih.gov/) and Netprimer (http://www.premierbiosoft.com/netprimer/) were used for analysis of primer sequences and the Excel applet GeNorm (Vandesompele *et al.* 2002) was used for qPCR normalization. The UCSC-tracks which have been used for the *in silico* analysis will be explained below.

Table 5: Primer sequences I

Cloning primers and oligonucleotides used for the construction of luciferase constructs.

	amplicon		sequence	amplicon size (bp)
cloning primers for luciferase constructs	CDK6 3'UTR	F	5'-TTTGCTAGCTGTATTAGTGTTTCTGCATTGCC-3'	1607
		R	5'-TTTCTCGAGTTGGACAGTGATATTTCAACACC-3'	
	PI3KR1 3'UTR	F	5'-TTTGCTAGCACGTTCCTAAGCTGGAGTGCTT-3'	1552
		R	5'-TTTCTCGAGCAGTCCAGAGCAGTGACAGTATGA-3'	
oligonucleotides for luciferase constructs	29a FC	F	5'-TCGACTAACCGATTTCAGATGGTGCTAT-3'	
		R	5'-CTAGATAGCACCATCTGAAATCGGTTAG-3'	
	CDK6-BS1	F	5'-TCGACATGGAGAGCACCATGTGGACAAG-3'	
		R	5'-CTAGACTTGTCCACATGGTGCTCTCCATG-3'	
	CDK6-BS2	F	5'-TCGACACTCAAAGCACCAAAACAGAGCATTCTG-3'	
		R	5'-CTAGACAGAATGCTCTGTTTTGGTGCTTTGAGTG-3'	
	CDK6-BS3	F	5'-TCGACTCATTCTAGCACCCAGTAAGACATCCAG-3'	
		R	5'-CTAGACTGGATGTCTTACTGGGTGCTAGAATGAG-3'	

Table 6: Primer sequences II

Primers used for qRT-PCR of primary and precursor miRNAs, reference genes and mRNAs of target genes.

	amplicon		sequence	amplicon size (bp)
primary miRNAs	pri-29a~b-1	F	5'-GGGCTTTCTGGAACCAATCC-3'	68
		R	5'-ACAATGCGATATCCTGTACAATTACAT-3'	
	pri-29b-2~c	F	5'-AAGAGCAAAATACACTCTTGAGTT-3'	63
		R	5'-AACCCCCTTCTCTACTGTCAC-3'	
precursor miRNAs	pre-29a	F	5'-ATGACTGATTTCTTTTGGTGTTCA-3'	64
		R	5'-ATAACCGATTTCAGATGGTGCTA-3'	
	pre-29b-1	F	5'-CTTCAGGAAGCTGGTTTCATAT-3'	64
		R	5'-TGATTTCAAATGGTGCTAGACA-3'	
	pre-29b-2	F	5'-CTGGTTTCACATGGTGGCTTA-3'	62
		R	5'-CACTGATTTCAAATGGTGCTAGATA-3'	
	pre-29c	F	5'-GGCTGACCGATTTCTCCTGG-3'	76
		R	5'-TCCCCCTACATCATAACCGATTT-3'	
reference genes	HPRT1	F	5'-TGGACAGGACTGAACGTCTT-3'	77
		R	5'-GAGCACACAGAGGGCTACAA-3'	
	β-Actin	F	5'-TGACCCAGATCATGTTTGAGA-3'	108
		R	5'-AGTCCATCACGATGCCAGT-3'	
	CycloA	F	5'-CAGACAAGGTCCCAAAGACA-3'	139
		R	5'-CCATTATGGCGTGTGAAGTC-3'	
	TBP	F	5'-ACCCAGCAGCATCACTGTT-3'	127
		R	5'-CGCTGGAACTCGTCTCACTA-3'	
Potential miR-29 target genes	DNMT3A	F	5'-TATTGATGAGCGCACAAGAGAGC-3'	111
		R	5'-GGGTGTTCCAGGGTAACATTGAG-3'	
	DNMT3B	F	5'-GGCAAGTTCTCCGAGGTCTCTG-3'	113
		R	5'-TGGTACATGGCTTTTCGATAGGA-3'	
	Bcl-2	F	5'-TGTGGAGAGCGTCAACCG-3'	113
		R	5'-CCCAGCCTCCGTTATCCT-3'	
	PTEN	F	5'-TAAGGACCAGAGACAAAAAGG-3'	143
		R	5'-CATTGGAATAGTTTCAAACATCA-3'	
	Akt3	F	5'-AAAGGGAAGAATGGACAGAA-3'	143
		R	5'-TATGATGGGTTGTAGAGGCA-3'	
	cdc42	F	5'-CGTGACCTGAAGGCTGTCAA-3'	129
		R	5'-ACACACCTGCGGCTCTTCTT-3'	
	Mcl-1	F	5'-AGTATCACAGACGTTCTCGTAAGG-3'	106
		R	5'-GCCACCTTCTAGGTCCTCTACA-3'	
	Dicer1	F	5'-GAAAATATCAGGTTGAACTGCTTG-3'	116
		R	5'-GATAGGACAGCTCTTTAGTGAGTAGTAC-3'	
	CDK6	F	5'-CCAGCAGCGGACAAATAA-3'	92
		R	5'-CCACAGCGTGACGACCA-3'	
	PI3KR1	F	5'-ATACCCGCACATCCCAGG-3'	118
		R	5'-TGTATTCTTTGCTGTACCGCTC-3'	

4 Methods

4.1 Cell culture

All cells were grown in a humidified atmosphere with 5 % CO_2 in T75 CELLSTAR™ cell culture flasks in a Hera cell 100 incubator in 15 ml of the respective medium. All cell lines were routinely tested to be mycoplasma-negative by PCR, taken in to culture (from frozen aliquots) the week before the experiment and split after reaching 70-90 % confluence. Splitting was performed by detaching the cells with 200 mg/l Trypsin/EDTA (Versene) and subsequent division in new culture flasks with fresh medium. For storing, the content of a 10 cm-cell culture dish was pelleted for 5 min at 1400 rpm (Eppendorf centrifuge 5702); the pellet was dissolved in the respective medium containing 10 % DMSO. Cells were stored in CRYO.S™-tubes at -80 °C for short term storage and in liquid nitrogen for longtime storage. Before seeding, cells were quantified with a Bürker cell counting chamber.

4.2 Stimulations with cytokines

Cytokine stimulation: time-course experiments

For stimulation of melanoma cell lines and HEK 293T cells, 10^5 cells per well were seeded in 6-well-plates in at least triplicates for each treatment. For Jurkat and MT4 T-cells, 10^5 cells per well were seeded in 12-well plates. Cells were either left untreated or stimulated with a final concentration of 50 ng/ml human IFN-γ, IFN-α, IFN-β or IL-27 for different periods of time and harvested all together at the end of the experiment, as indicated in Fig. 14.

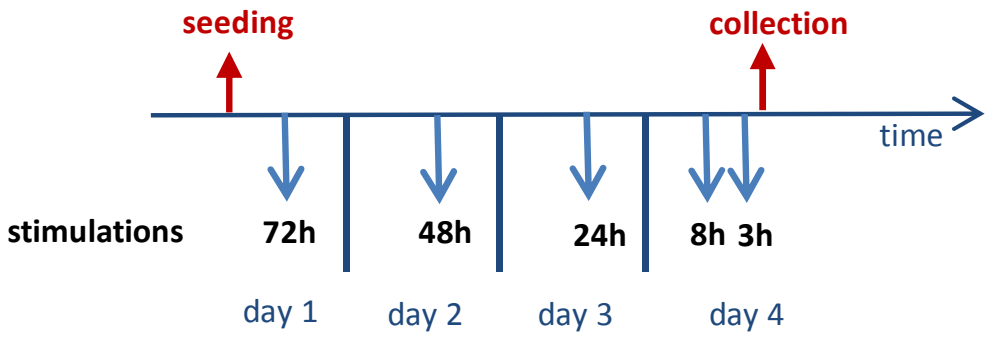


Fig. 14: Experimental setup of cytokine stimulation time-course experiments
Cells were seeded at day 1 of the experiment and the stimulations at the different time points were performed as indicated above. For subsequent Western Blot analysis or RNA-extraction, cells were all harvested together at the end of the experiment.

Pre-treatment with Janus kinase inhibitor I (JII)

Experiments with Jak-inhibitor 1 included a pre-treatment step with 5 µM of the inhibitor, which was added one hour before commencing cytokine stimulation. For all experiments, cells were collected all together at the end of the time course for further experimental analyses. Concentrations and length of treatment with inhibitor had previously been optimised in our laboratory (Kreis *et al.* 2007; Reinsbach *et al.* 2012).

4.3 RNA extraction

Isolation of total RNA from cell lines

For all experiments except cloning of the 3'-UTRs and microarray analyses, total RNA of cell lines was extracted using TRIsure (Guanidium thiocyanate-phenol-chloroform extraction). Briefly, for adherent cells, the medium was aspirated and cells were washed once at RT with 1 x PBS. Cells were lysed directly in the culture dish by addition of 1 ml TRIsure per well. Suspension cells were washed with PBS, trypsinized and pelleted. The pellet was dissolved in 1 ml of TRIsure. For both, the lysates were transferred to 1.5 ml Safe-Lock Eppendorf microcentrifuge tubes. Phase separation was carried out by adding 200 µl chloroform to each sample followed by a centrifugation step for 15 min at 4 °C at 13,200 rpm (Eppendorf centrifuge 5415D). The RNA accumulates in the (upper) aqueous phase, while DNA and proteins are discarded with the interphase and the (lower) organic phase. The upper aqueous phase was carefully collected and the RNA was precipitated after addition of 500 µl isopropanol and centrifugation for 10 min at 4 °C at 13,200 rpm (Eppendorf centrifuge 5415D). Afterwards, the RNA pellets were washed three times with 1 ml EtOH (75 %), dissolved in DEPC-H_2O and stored at -20°C until further use or at -80 °C for long-term storage. Quantity and purity of RNA samples were assessed by determining the ratios of absorbance at 260 nm/280 nm and 260 nm/230 nm using a NanoDrop ND-2000c spectrophotometer.

Isolation of total RNA from cell lines for subsequent microarray analyses

For LC Sciences and Affymetrix miRNA microarrays, RNA was extracted with the miRNeasy kit according to the manufacturer's protocol with an additional on-column DNase I digestion.

DNase I treatment

To degrade any DNA contamination in total RNA samples, DNAse I treatment was performed before the reverse transcription. Briefly, 2 µg of RNA were mixed with 2 µl of 10 x DNase I-buffer, 0.5 µl DNase I (2 U/µl) and the respective amount of DEPC-H_2O (up to a total volume of 20 µl) on ice. The mixture was incubated at 37 °C for 10 min and then placed on ice. 0.5 µl of 0.02 M EDTA was added to each sample (final concentration of 0.5 mM) to protect the RNA during the following heat-inactivating step. After vortexing, the DNase was heat-inactivated at

75 °C for 10 min. The DNase-treated RNA was re-quantified in Nanodrop to ensure RNA amounts between 50-90 ng/µl.

Extraction of total RNA from FFPE patient material

For FFPE-samples, five scalpel-scraped slices of FFPE tissue were pooled and RNA was extracted using the RT_2-FFPE extraction kit. Quantity and purity of RNA samples were assessed using a NanoDrop ND-2000c spectrophotometer.

4.4 Reverse transcription, quantitative Realtime PCR and Analysis

Reverse Transcription to obtain cDNA for subsequent cloning

Total RNA from A375 cells was extracted with the Nucleospin RNA extraction kit according to the manufacturer´s instructions. cDNA synthesis was performed with the Thermoscript RT-PCR system. Briefly, 500 ng RNA was incubated with 1 µl dNTPs (10 mM), 0.5 µl oligo dT (50 µM) and H_20 (up to 10 µl) for 5 min at 65 °C and then placed on ice. After addition of 2 µl 5x cDNA buffer, 0.5 µl 0.1 M DTT, 0.5 µl RNaseOut, 1 µl of H_20 and 0.5 µl of thermoscript polymerase the mixture was incubated for 1h at 50 °C and for 5 min at 85 °C. The resulting cDNA served as input for PCR amplifications as described below.

Reverse Transcription for subsequent miRNA and mRNA expression analysis

For FFPE samples and cell lines, 250 ng of total RNA was reversely transcribed using the miScript System according to the manufacturer's instructions and as depicted schematically in Fig. 15. The system allows for simultaneous reverse transcription of mRNA and miRNAs. The reverse transcription mix includes a poly(A) polymerase to allow the addition of Poly(A) tails to miRNAs, which are not polyadenylated in nature. In the first step of the reaction, miRNAs get polyadenylated whereas the reverse transcription is performed in the second step. Additionally, a universal tag is added to the 3'-end of miRNAs and mRNAs, which is needed for later amplification during the real-time PCR. For reverse transcription, briefly, 250 ng of total RNA were diluted in DEPC-H_2O to a total volume of 7.5 µl and mixed with 2 µl miScript RT Buffer and 0.5 µl reverse transcriptase mix. Incubation times were 37 °C for 60 min followed by 95 °C for 5 min.

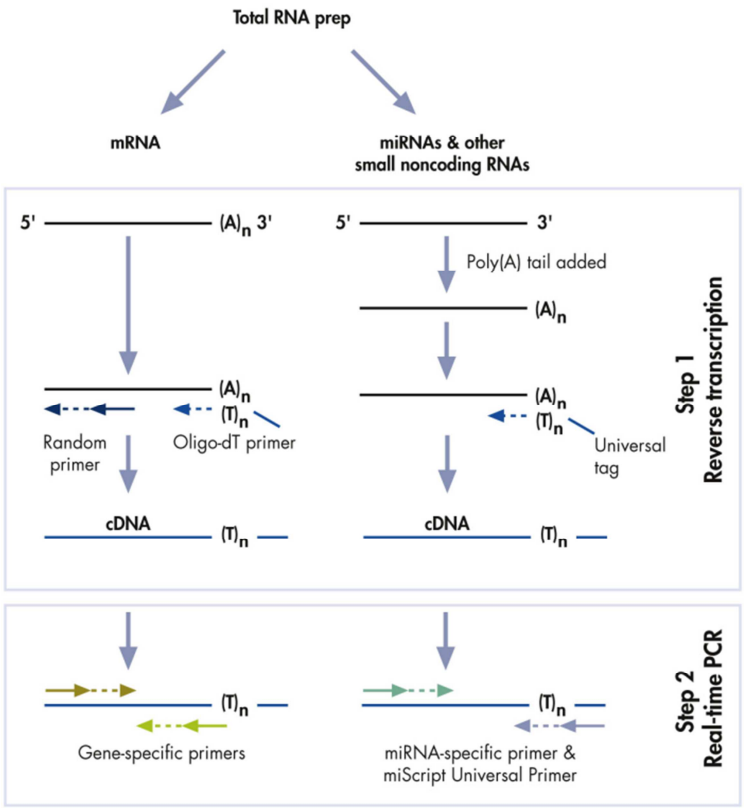


Fig. 15: miScript system – schematic overview
(from miScript handbook, Qiagen) Polyadenylation, reverse transcription and real-time amplification of miRNAs as described in the text.

Realtime-qPCR

Realtime polymerase chain reaction or quantitative real time polymerase chain reaction (qPCR) allows for amplification and simultaneous quantification of specific DNA fragments. Apart from Northern Blotting, microarray analysis or sequencing, it provides a standard method for detection and quantification of miRNAs and mRNAs (VanGuilder *et al.* 2008). It offers the opportunity to calculate absolute miRNA amounts, when standard concentrations are used in the same assay. However, mostly relative miRNA or mRNA expression levels are assessed and are determined with respect to a panel of so-called 'house-keeping' or reference genes, which show stable expression levels even after treatment (Bustin *et al.* 2009).

For the quantification of mature miRNAs, real-time PCR was carried out on a CFX96 or CFX384 detection system using 5 ng RNA input, 2 x iQ SYBR Green Supermix and 10 x miRNA-specific primer assay (Qiagen). To detect mRNAs and primary/precursor miRNAs, 25 ng or 125 ng RNA input, 2 x iQ SYBR Supermix and 5 pmol gene-specific primer pairs (Table 6) were used. Thermal cycling conditions for all reactions were 95 °C for 3 min, 39 x (95 °C for 15 s, 60 °C for 30 s), 95 °C for 1 min, 60 °C for 1 min, followed by a melting-curve analysis: 60 °C to 95 °C, increment 0.5 °C for 20 s.

Analysis of realtime-qPCR results

If not stated otherwise, expression analysis was performed using the GeNorm VBA applet for Microsoft Excel, version 3.5 (Vandesompele *et al.* 2002; Mestdagh *et al.* 2009). The add-in calculates a normalization factor (NF) which is based on the raw quantity of the house keeping genes (HKG), calculated from their respective cycle thresholds (C_t's) with

$$\text{raw HKG quantity} = 2^{(\text{Ct min} - \text{Ct sample})}$$

and

$$\text{NF} = \text{geometric mean of raw HKG quantity}$$

wherein C_t min = minimum C_t of the respective house keeping gene for all samples. The housekeeping genes TBP (TATA-binding protein), HPRT (Hypoxanthine phosphoribosyltransferase 1), CycloA (Peptidylpropyl isomerase A) and β-Actin (see Table 6 for sequences) were used for normalization of mRNA amplification and primary / precursor miRNAs and RNU1A, RNU5A (RNA, U1A/5A, small nuclear) and SCARNA17 (small Cajal body-specific RNA 17) (all from Qiagen) for mature miRNAs. After determination of the NF, the gene of interest (GOI) is normalized by dividing the raw gene of interest quantity by the normalization factor:

$$\text{raw GOI quantity} = 2^{(\text{Ct min} - \text{Ct sample})}$$

and

$$\text{normalized GOI} = \frac{\text{raw GOI quantity}}{\text{NF}}$$

For stimulation experiments, the normalized GOIs were then divided by the mean of the untreated control and the values of the single time points were subsequently averaged to obtain the relative expression (REL). Apart from normalization, the Genorm applet calculates a gene expression stability measure M for the reference genes, allowing for further quality control of the experiment and for elimination of genes with the most unstable expression. For our studies, analysis was performed based on the Genorm-calculated normalization factor of three reference genes with an M-value below 1.5 (in most cases < 0.5).

4.5 miRNA microarrays

LC Sciences

Total RNA from the NHEM, IGR39 and IGR37 lines was subjected to genome-wide microRNA expression profiling (miRBase, version 11.0) using the µParaflo® microarray technology as described on the web site of LC Sciences (Houston, TX, USA) and by us (Philippidou *et al.* 2010). Briefly, total RNA (2-5 µg) was size-fractionated with a YM-100 Microcon filter (Millipore) to isolate small RNAs (<300 nt). These were subsequently 3'-poly(A) tailed and fused to nucleotide tags for later Cy3 and Cy5 dye labeling. Hybridisation of RNAs with the probes (which are complementary to target miRNAs (miRBase)) was carried out overnight on a µParaflo microfluidic chip. The respective hybridization images were collected by a laser scanner (GenePix 4000B, Molecular Device) and subsequently processed with the Array-Pro image analysis software (Media Cybernetics). Data analysis was performed by LC Sciences.

Affymetrix

Duplicate total RNA samples from A375 melanoma cells (stimulated for different periods of time with IFN-γ or including the pre-treatment with JII at the 72 h time point) were analyzed as published previously by members of our group (Reinsbach *et al.* 2012). Affymetrix GeneChip miRNA 2.0 Arrays (based on miRBase version 15, Affymetrix Datasheet P/N EXP00180) were performed at the microarray facility of the CRP Santé (Luxembourg), which included use of the FlashTag Biotin HSR RNA labelling kit (Genisphere, USA) according to the manufacturer's instructions.

4.6 Transfection of cells

Mimic/inhibitor experiments

10^5 cells/well were seeded in 6-well plates in antibiotic-free medium and transfected after 24 h with 50 nM of each miR-29a and miR-29b mimics, with 150 nM miR-29a inhibitor or with corresponding amounts of negative controls (NC-mimic or NC-inhibitor) using the DharmafectDuo transfection reagent according to the supplied protocol; 1 ml of medium was added 24 h after transfection. Efficient transfection was confirmed by qRT-PCR (Fig. 29). For miR-29 target gene expression, RNA and protein lysates were collected 24 h, 48 h and 72 h after transfection and subsequently analyzed by RT-qPCR and Western blot.

Combination experiment with miR-29 inhibitor and IFN-γ

2 x 10^3 cells/well were seeded in 96-well plates in antibiotic-free medium and transfected 24 h with 150 nM of miR-29a inhibitor or the same amount of negative control (NC-inhibitor) or were left untreated. For the combination experiment and for the samples with IFN-γ treatment only, stimulation (50 ng/ml IFN-γ) was performed 8h after transfection.

CDK6 siRNA transfection

5 x 10^4 cells were transfected with 75 nM ON-TARGET siRNA or siRNA negative control (si-NC) 24 h after seeding in 6-well plates using the HiPerfect transfection reagent according to the manufacturer's instructions in a total volume of 1 ml antibiotic-free medium and 1 ml medium was added 24 h after transfection. CDK6 mRNA and protein levels were assessed after 24 h, 48 h and 72 h to confirm efficient down-regulation (Fig. 38).

4.7 Reporter gene assays

Cloning of 3'-UTRs and single miR-29a binding sites

For **PCR amplification of 3'-UTRs**, a ~1600 bp part of the 3'-UTR of two predicted miR-29 target genes was amplified from A375 cDNA using primer pairs (Table 5) with incorporated XhoI (forward) or NheI (reverse) restriction sites. The CDK6 construct (1600 bp) contained three putative miR-29 binding sites, whereas the PI3KR1 construct (1567 bp) contained one binding site (Fig. 37). Briefly, 2 µl of cDNA were subjected to PCR using the Phusion DNA polymerase in a total reaction volume of 50 µl with 10 µl buffer, 1 µl dNTPs, 1 µl of each primer (forward/reverse, 100 µM), 0.2 µl polymerase, 2.5 µl DMSO and 32.3 µl H_20. Cycling conditions (for 35 cycles) were 98 °C for 30 s (initial denaturation), 98 °C for 10 s (denaturation), 65°C for 20 s (annealing), 72 °C for 15 s (extension) and 72 °C for 10 min (final extension). Purification of PCR products and all following purifications of plasmid DNA as well as separation of DNA after restriction digests were performed by cutting bands of appropriate size from a 1 % agarose gel under UV-light. Subsequent purification of the DNA from agarose gels was performed with the innuPREP DOUBLE pure kit according to the manufacturer's instructions.

For vector preparation for subsequent **cloning**, 2 µg of pmirGLO DNA were digested in a total volume of 30 µl with 3 µl NEB2 buffer, 3 µl BSA, 0.3 µl XhoI, 0.3 µl NheI and H_20 (up to 30 µl). 30 µl of purified PCR products (amplified 3´-UTRs) were digested with 5 µl NEB2 buffer, 5 µl BSA, 0.3 µl XhoI, 0.3 µl NheI and 9.4 µl H_20. Both digestions were performed for 1.5 h at 37 °C, followed by a heat-inactivating step at 65 °C for 20 min. For subsequent cloning, linearized vector DNA was dephosphorylated to avoid re-ligation of the vector: after digestion, plasmid DNA was incubated for 1 h at 37 °C with 0.4 µl CIP upon addition of 2 µl NEB2 buffer and 18 µl H_20 in a total volume of 50 µl. Linearized plasmid DNA and inserts were purified on a 1 % agarose gel.

For **ligation**, 100 ng of digested vector DNA was incubated with inserts in two different molar ratios (vector to insert - 1:3 or 1:7) along with 2 µl of 10x ligase buffer, 0.5 µl T4 DNA ligase and H_20 ad 20 µl overnight at 16 °C. Bacteria were transformed with ligated plasmids (as will be described below).

For **cloning of single miR-29 binding sites** (CDK6, BS 1-3 and 29a full complementary site,

FC), oligo annealing was performed. Oligonucleotides (obtained from Eurogentec, Table 5) were diluted to a concentration of 10 µM. 2 µl of each forward/reverse oligo were incubated with 46 µl of oligo annealing buffer for 2 min at 100 °C and then left on the heatblock to cool down to room temperature. For ligation, oligos were diluted 1:10 to obtain a concentration of 4 ng/ µl per oligo. 4 ng of oligo and 50 ng of linearized vector were ligated with 2 µl buffer and 0.5 µl of T4 DNA ligase at 16 °C overnight. 2 µg of pmirGLO plasmid were digested in a total volume of 30 µl with 3 µl NEB2 buffer, 3 µl BSA, 0.3 µl XhoI, 0.3 µl XbaI and 21 µl of H_2O for 1.5 h at 37 °C and oligos were ligated into open vectors as described above. Purification was performed on a 1 % agarose gel as described before.

Chemically competent *E. coli* cells were prepared for subsequent transformation of plasmids. For the starter culture, 2 ml LB medium was inoculated with a single colony of *E. coli* DH5 α and incubated overnight at 37 °C with 250 rpm (Certomat MOII, Sartorius). 20 ml of pre-warmed LB were inoculated with the starter culture at 37 °C, 250 rpm (Certomat MOII, Sartorius) until the OD_{600} reached approximately 0.4. The culture was centrifuged at 4300 rpm (Heraeus Megafuge 1.0R) for 15 min at 4 °C and the pellet was then resuspended in 2 ml ice-cold TSS buffer. 20 % glycerol was added and the cell suspension was dispensed in 300 µl aliquots to be stored at -20 °C until further use.

For **transformation** of competent cells, an aliquot of competent bacteria was thawed on ice. 150 µl of competent DH5 α bacteria were incubated with 100 ng of the ligated plasmid DNA for 30 min on ice. The cells were subjected to heat shock at 42 °C for 90 s and placed on ice for 2 min. 1 ml of LB-medium was added to the mixture, followed by pre-culturing at 37 °C with shaking at 250 rpm for 1 h. The cells were then centrifuged at 10.000 rpm for 1 min and plated on LB agar supplemented with ampicillin to a final concentration of 50 µg/ml. The plate was incubated overnight at 37°C.

To verify correct insertion of the constructs (**mini preparation**), 2 ml LB medium supplemented with ampicillin (final concentration of 50 µg/ml) was inoculated with a single bacterial colony and incubated overnight at 37 °C with shaking (250 rpm). The cells were harvested by centrifugation at 13.000 rpm. For the following steps, plasmid DNA was purified using the 5prime Miniprep kit according to the instruction manual provided. Instead of the supplied elution buffer, 10 mM Tris (pH 8) was used.

For **analytical restriction digest** after small scale plasmid isolation, plasmid DNA was incubated with the restriction enzymes XhoI and NheI for 1.5 h at 37 °C to confirm the presence of the correct ligation of 3'-UTR inserts in the pmirGLO vector. For oligo cloning, control digest was performed with XhoI and HindIII. For both, correct sizes were monitored on 1 % agarose gels. **DNA sequencing** was performed to verify correct ligation of fragments into the pmirGLO vector, constructs were sequenced by GATC (Konstanz, Germany) and DNA

sequences were verified using the GATC viewer, which is provided on the GATC homepage (http://www.gatc-biotech.com/en/index.html).

For subsequent **preparative isolation of plasmid DNA** (**midi preparation**), 100 ml LB medium supplemented with ampicillin (final concentration of 50 µg/ml) were inoculated with a single bacterial colony and incubated overnight at 37 °C with shaking (250 rpm, Certomat MOII, Sartorius). The cells were harvested by centrifugation at 4300 rpm (Heraeus Megafuge 1.0R). Plasmid DNA was purified using the Xtra Midi Plus kit according to the instruction manual provided.

Luciferase reporter gene assays

For luciferase assay, the dual luciferase reporter assay system was used according to the manufacturer´s instructions. The sequence parts of CDK6 and PI3KR1- 3'UTRs containing miR-29 binding sites, CDK6 miR-29a single binding sites and the miR-29a full complementary sequence were cloned into the pmirGLO Dual Luciferase miRNA target expression vector downstream of the luciferase gene (see Table 5 for primer sequences and oligonucleotides). A375 cells were seeded at a density of 5 x 10^4 cells/well in 24-well plates 24 h prior to transfection. Cells were transiently co-transfected with 500 ng plasmid and 50 nM miR-29a mimic or negative control (NC-mimic) for 48 h and 72 h. Samples were lysed with 1x Passive Lysis Buffer for 15 min at room temperature. Lysates were transferred in 96-well plates and frozen at -20°C until the measurement for both time points was performed together.

For measurement, 10 µl of lysate were transferred into a LIA luminescence 96-well plate. Measurement of firefly activity was performed after addition of 50 µl of LARII (luciferase assay reagent II) reagent per well and measurement of renilla activity was monitored after subsequent addition of 50 µl of Stop & Glo reagent per well. The firefly/renilla activity ratios of mimic-transfected samples were calculated and normalized to the respective ratios of the negative control-transfected samples for each construct and each time point. [2]Significance was assessed by one-way ANOVA followed by a Bonferroni Post-Hoc test.

4.8 Western blots and Licor quantification of protein levels

Preparation of protein extracts from cell lines

Adherent cells were washed 1x with PBS, followed by direct lysis on ice with Laemmli buffer. Generally 500 µl were used for 1 well from a 6-well plate, if confluent. Alternatively, suspension cells were pelleted in Eppendorf reaction tubes, the pellet was lysed in Triton lysis buffer for 10 min on ice and Laemmli buffer was added to the solution.

SDS page and Western blotting

Extracts containing equal amounts of protein were boiled for 5 min at 95 °C and 10-20 µl of each sample per lane were separated by 8-12 % SDS-PAGEs at 20-30 mA/gel. A protein standard ladder (Precision Plus Protein™ Standard) was loaded to confirm the sizes of proteins investigated. The proteins were then transferred to a nitrocellulose membrane (for Licor quantification, see below) or PVDF membrane (the latter had been activated in methanol for 1 minute before blotting, for ECL (enhanced chemoluminescence)-detection) in a semi-dry Western blotting chamber for 1 h at 48 V per blot. The membrane was blocked for 30 min at room temperature with blocking buffer. After three washes (5 min each) in TBS-N buffer, the membrane was probed with 10 ml of the respective first antibodies (Table 4) overnight at 4 °C with gently shaking. After three more TBS-N washing steps, it was subsequently incubated with the HRP (horseradish peroxidase)-labeled (for ECL detection) or fluorophor-coupled (for Licor quantification) secondary antibody of the respective species (Table 4) for 1h at RT with shaking. For ECL-detection, membranes were washed three times for 5 min in TBS-N and placed in ECL substrate reagent supplemented with H_2O_2 for 1 min. Membranes were exposed in the Molecular Imager ChemiDocTM XRS+ system or the ChemiStar system for various times and chemiluminescent signals were detected. Before re-probing, PVDF membranes were stripped for 30 min at 70 ^{o}C in stripping buffer, washed in H_2O, blocked for 30 min and incubated with the next antibody. Equal loading was confirmed by actin, tubulin or FIN13 loading controls for PVDF membranes.

Licor quantification (Li-cor Biosciences)

The Li-cor Odyssey Infrared Imaging System (Li-cor Biosciences) enables infrared fluorescence detection of protein signals by two independent infrared channels (700 nm and 800 nm). This technology allows for a wide dynamic range and thus accurate quantification of protein bands from membranes (www.licor.com). This is achieved by using fluorophor-coupled secondary antibodies instead of peroxidase-coupled ones as routinely used for ECL detection. For quantification of CDK6 and p85α protein levels, signal intensities of the different fluorophor channels 700 nm and 800 nm were assessed with the Odyssey imaging system at a resolution of 84 µm and analyzed with the provided software. CDK6 and p85α signals were normalized to the respective tubulin loading controls.

4.9: Functional assays: Incucyte experiments

The Incucyte machine is an automated live-cell imaging system which allows label-free and kinetic analysis of cell growth rates and morphology. It is positioned inside a standard cell culture incubator and equipped with a camera, which can e.g. photograph cells in phase-contrast or detect fluorescence signals to provide raw data for subsequent quantification of

cellular growth rates or other analyses. After seeding or treatment of the cells, imaging can take place inside the machine under normal cell-culture conditions without removing the cells to RT in between (www.essenbioscience.com).

Apoptosis assay

2 x 10^3 cells/well were seeded in 96-well plates and transfected 24 h after seeding with the respective amounts of mimic, inhibitor or negative controls as described above (5.6). After transfection, cells were additionally incubated with 5 µM of NucView 488 Caspase detection reagent. The NucView reagent combines a caspase-3 substrate and a non-functional dye, which becomes functional and emits fluorescence upon enzymatic cleavage of the substrate. Thus, caspase activity in living cells can be followed up in real-time. 50 µM Etoposide was used as positive control for apoptosis and fluorescence was monitored by Incucyte over 72 h. For subsequent analysis, object counting thresholds were set to recognize fluorescent cells over background fluorescence of the medium and the number of fluorescent cells per well was calculated by the Incucyte software.

Real-time proliferation assays

All proliferation assays were performed by real-time monitoring in the Incucyte live-cell imaging system, which photographed cells in phase contrast every 3 h, taking 9 (for 96-well plate) to 25 (for 6-well plate) pictures per well. For monitoring the growth of untreated melanoma cell lines, 25 x 10^3 cells/well of eight untreated melanoma cell lines were seeded in 12-well plates and harvested after 96 h. To monitor basal miRNA levels in these cell lines, RNA was extracted and miR-29 species were amplified by qRT-PCR as described below.

4.10 *In silico* analysis of the miR-29 promoter region

For the TSS (transcription start site) overview, all published TSSs on both miR-29 clusters, which were available before May 2012 were collected and converted to genome version 19 (hg19) with the UCSC (University of California Santa Cruz) Liftover utility for coordinate conversion (http://genome.ucsc.edu/cgi-bin/hgLiftOver). The subsequent *in silico* analysis on the promoter region was performed based on the UCSC Genome Browser (Kent *et al.* 2002) using the UCSC tracks described below, which are described in more detail on the UCSC homepage (http://genome.ucsc.edu/). The analysis included several characteristic features of promoter regions, which are explained below. The aim of this analysis was to overlay these characteristic features (conservation score, histone mark) with predicted and experimentally verified STAT1 binding sites in order to identify candidate STAT1 transcription factor binding sites which could be responsible for miR-29 regulation.

Vertebrate Basewise Conservation by PhyloP

(Siepel *et al.* 2005; Pollard *et al.* 2009) (found under 'comparative genomics')

Evolutionary conservation of promoter regions, especially transcription factor binding sites is correlated with a higher possibility of actual usage of the site for transcription factor binding. The UCSC track shows a multiple alignment of the genomes of 46 vertebrate species and the corresponding evolutionary conservation. In the plots, conserved sites are represented in blue, with positive scores; sites shown in red and assigned to negative scores are considered to be fast-evolving.

H3K4Me3 Mark

(trimethylation of histone H3 lysine 4) (Ram *et al.* 2011)

(found under 'regulation' → 'integrated regulation' from ENCODE tracks)

The H3K4Me3 signature is often found near active promoters because it changes the chromatin to a more open conformation, thus making it more accessible for transcription, as explained in the introduction (Fig. 7). The track shows the levels of enrichment of H3K4Me3 as investigated by ChIP-seq in K562 cells.

Transcription Factor Binding Sites determined by ChIP-seq

from ENCODE/Stanford/Yale/USC/Harvard (track 'ENCODE transcription factor binding' → SYDH found under 'regulation')

The track shows predicted transcription factor binding sites as determined by ChIP-seq experiments which were performed in IFN-γ-treated K562 leukemia cells and in HeLaS3 cells (for HeLaS3 data: (Robertson *et al.* 2007)(both experiments: Snyder laboratory, Stanford; IFN-γ treatment for 30 min). Both ChIP experiments were carried out with an STAT1 antibody (sc-345). The bar (Fig. 26) represents the enrichment for binding of the transcription factor

together with the site having the greatest evidence of transcription factor binding (peak, here represented by a red line). According to UCSC, both measures are based on processed data (normalized data from pooled replicates).

Predicted transcription factor binding sites based on ChIP-seq data

Transcription factor ChIP-seq from ENCODE (found under 'regulation' → integrated regulation from ENCODE tracks): This track also shows predicted transcription factor binding sites, in which the peaks from the ChIP-seq data were analyzed by a pipeline developed from Anshul Kundaje (for further details see UCSC (http://genome.ucsc.edu/)), which combined data from a panel of different cell lines.

Putative pri-29a~b-1 transcription start sites

Putative transcription start sites of the pri-29a~b-1 cluster were collected from literature (derived from *in vitro* experiments as well as computational predictions), converted to the human genome version hg19 and allocated to the pri-29a~b-1 promoter as shown in (Schmitt *et al.* 2012a) and references therein.

Potential STAT1 binding sites

(predicted by Jaspar and Transfac)

For own investigation of the pri-29a~b-1 promoter sequence for potential STAT1 binding sites, we used the open-source website 'Jaspar' (Vlieghe *et al.* 2006) as well as the commercial program 'Transfac' (Matys *et al.* 2003) which both predict transcription factor binding sites in a given DNA sequence.

GAS elements

The sequence search for GAS elements in the promoter region was performed for the consensus sequences TT(C/A)CNNNAA(A/G) (Pagliaccetti *et al.* 2008) and TT(C/A)NNN(G/T)AA (Smith *et al.* 2012).

5 Results

5.1 IFN-γ-induced miRNAs

Identification of differentially regulated miRNAs in melanoma cell lines following cytokine treatment

The main focus of this thesis was the identification and characterization of IFN-γ-induced miRNAs. To this end, there was a previous report showing that IL-6 induced STAT3-dependent up-regulation of miR-21 in multiple myeloma cells (Löffler *et al.* 2007). Here we turned our interest to IFN-γ, which is known to play pivotal roles in immune regulation (Schroder *et al.* 2004; Borden *et al.* 2007) and regulation of growth behavior as was previously demonstrated for melanoma cells (Kortylewski *et al.* 2004). However, no STAT1-dependent miRNAs had been reported yet, and thus all 837 human miRNAs listed in the former miRBase version 11.0 were potential candidates. To investigate basal expression levels of miRNAs and their possible transcriptional regulations by STAT1 factors, IGR39 (representing early stage, primary melanoma) and IGR37 (late stage, metastatic melanoma from the same patient as IGR39) melanoma cell lines as well as NHEM (normal human epidermal melanocytes) were treated with 50 ng/ml IFN-γ for different periods of time (3 h and 72 h) and total RNA of the cells was subsequently analyzed by miRNA microarray (LC Sciences, miRBase version 11.0). This initial miRNA microarray experiment served as a starting point for this PhD project to provide important information to the following questions: Firstly, it offered an overview on basal miRNA expression levels of the three (untreated) cell lines as well as on differentially expressed miRNAs within the untreated cell lines, which represented different melanoma stages (Fig. 16). The latter could be subsequently classified in up- or down-regulated miRNAs according to melanoma progression stages (NHEM → IGR39 → IGR37) (Philippidou *et al.* 2010). In this study, we compared these array results on untreated melanoma cell lines with miRNA expression profiles of 88 cancer-related miRNAs by PCR-array which was performed on melanoma patient samples. Interestinlgy, miR-200c was consistently down-regulated with tumour progression in all samples (melanoma cell lines and patient samples). miR-146a and miR-155 levels were increased in patient samples, but decreased in cell lines with melanoma progression whereas miR-205 and miR-23b exhibited reduced expression levels in patient samples. Subsequent Ingenuity pathway analysis determined a de-regulated gene network around MITF, the key transcription factor in melanoma development (Philippidou *et al.* 2010; Schmitz Ulf *et al.* 2013).

Secondly and of special importance for this PhD project, the microarray provided the fundamental information necessary for consecutive selection of potential STAT1-regulated miRNAs. All three cell lines exhibit a clearly distinct miRNA expression pattern after IFN-γ-

stimulation, with IGR39 showing the highest number of regulations (Fig. 16). Furthermore, miRNAs which showed differential regulation between the three treatments (untreated, 3 h and 72 h IFN-γ) could be allocated into several groups according to their distinct differential expression pattern (Fig. 16). The expression level of several miRNAs was already altered after a short time interval (3 h) and remained in this state even after 72 h (D_1, U_1, Fig. 16). Most miRNAs were either up-regulated or down-regulated over time with most regulations occurring solely at the late time point (72 h, D_2 and U_2, Fig. 16). Interestingly, especially in NHEM cells, differential expression levels over time partly exhibited 'curve'-like shapes, f.e. a clear up- or down-regulation at the early time point (3 h) and returning to basal levels after 72 h of IFN-γ stimulation (C_1, C_2, Fig. 16). In addition to this clustering analysis, array results were analyzed for differential miRNA expression levels after IFN-γ-stimulation in comparison to the respective untreated control at both time points to identify a 'working list' of potentially STAT1-regulated miRNAs. This analysis led to further selection of a panel of seven microRNAs, which were differentially regulated in the cell lines analyzed and which were of interest for other biological reasons. Those included miR-21, miR-23a, miR-29a, miR-146a, miR-211, miR-1246 and miR-1826 (Fig. 16). miR-211 was excluded from further experiments because it was found to be undetectable in most melanomas (data not shown) and as it was the focus of another study from our group where the functional role of this miRNA in melanoma was investigated (Margue *et al.* submitted). Interestingly, miR-1246 showed the strongest up-regulation after IFN-γ-stimulation on the microarrays and has been reported to exhibit a high basal expression level on different array platforms (Zhang *et al.* 2011a; Piepoli *et al.* 2012), however, these findings could not be confirmed by qRT-PCR. This could be due to unspecific hybridization to the miR-1246 probes or because current commercially available primers are not suitable for qPCR amplification of this miRNA.

Fig. 16: Differentially expressed miRNAs on clustered heatmaps based on LC Sciences miRNA microarrays

Heatmaps illustrate significantly differentially expressed miRNAs (p-value < 0.01, ANOVA) between the different treatments (untreated, 3 h or 72 h stimulation with 50 ng/ml IFN-γ, respectively). Single miRNAs showing differential expression are represented in the vertical area, whereas the three treatments are aligned horizontally. Note that every miRNA is spotted four times on the array, resulting in four rectangles per cell line and treatment. Colors represent Z-score values as indicated at the bottom. According to their differential regulation patterns, miRNAs can be divided into several groups: down-regulated (D) and up-regulated (U) miRNAs as well as miRNAs regulated in a 'curve' (C) (with down-/up-regulation at the time points D_1 = early (3 h) and late (72 h), D_2 = late, U_1 = early and late, U_2 = late whereas the curve-regulation can be divided into C_1 = down-regulation followed by return to basal level and C_2 = up-regulation followed by return to basal levels). miRNAs with a high differential expression over time which were selected for further analysis are marked in green.

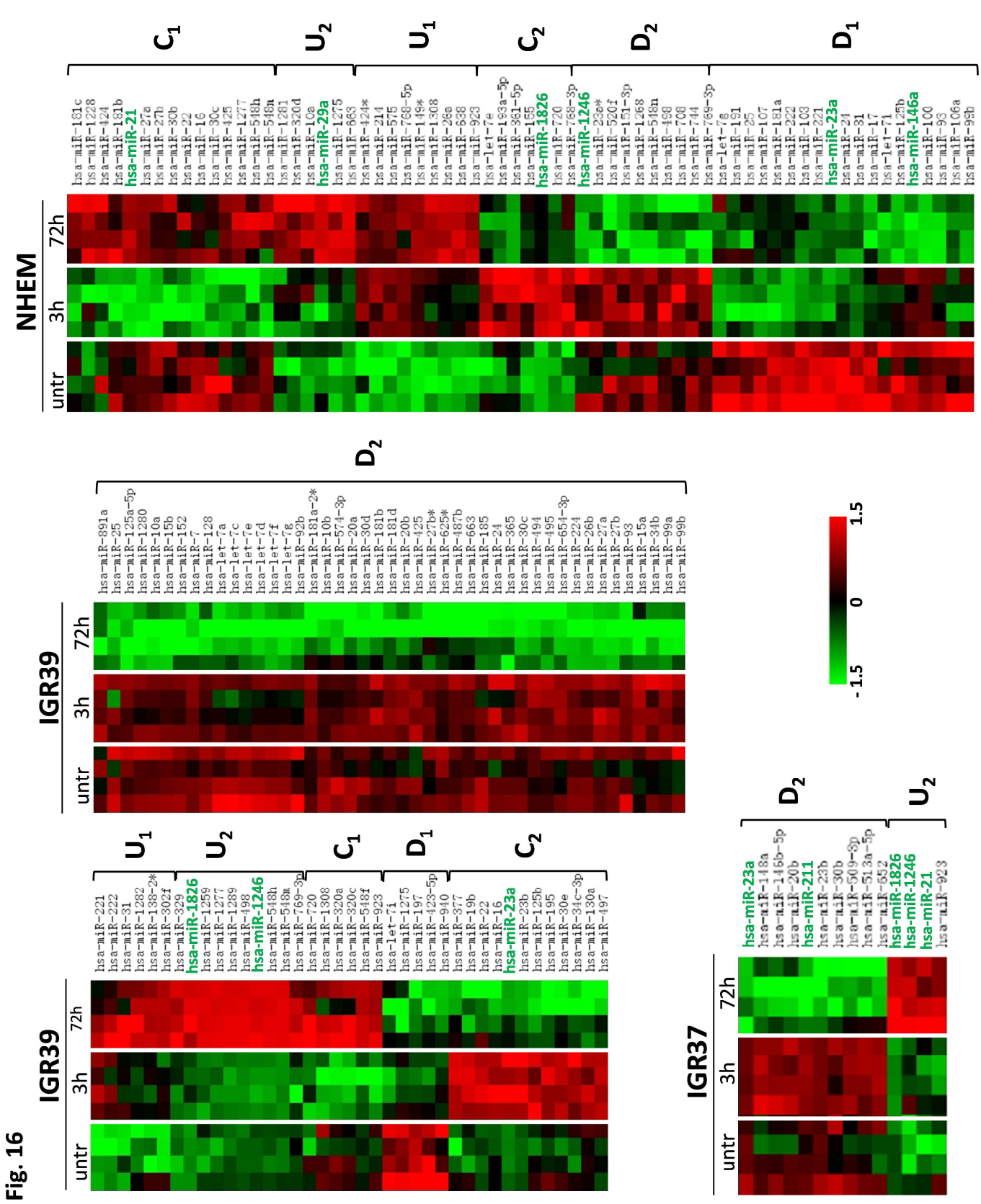

NHEM
untr
3h
72h
C1
U2
U1
C2
D2
D1
hsa-miR-21
hsa-miR-29a
hsa-miR-1826
hsa-miR-1246
hsa-miR-23a
hsa-miR-146a
IGR39
IGR37
1.5
0
-1.5

Fig. 16

Otherwise, differential expression of miRNAs in IFN-γ-stimulated samples in comparison to the untreated control was confirmed by qPCR for all miRNAs tested and different regulation patterns were detected in the different cell lines (Fig. 17). Efficient IFN-γ-stimulation was confirmed by induction of P-STAT1 and up-regulation of the STAT1 target gene STAT1 itself by parallel Western blot analysis (Fig. 17). Surprisingly, different regulation patterns for the same RNA samples were detected, when miRNA expression was normalized either to the housekeeping gene RNU6B or SCARNA17. For example in IGR37 cells, most of the analyzed miRNAs (miR-21, -23a, -29a, -146a, -1826) were found to be up-regulated, when referred to RNU6B, but were down-regulated, when SCARNA17 was used for normalization (Fig. 17). miR-21 was not induced in A375 and MeWo cells when normalized to SCARNA17, but was up-regulated when calibrated to RNU6B. Both genes are commonly used reference RNAs for qPCR amplification of miRNAs. Nevertheless, miR-29a and miR-1826 showed the most robust regulation of all miRNAs tested in the investigated cell lines and were thus selected for further analyses. Because the miR-1826 sequence was later on identified to be a fragment of 5.8S rRNA (ribosomal RNA), it was removed from miRBase and thus from our list of human miRNAs of interest. Subsequently, the focus was set on the miR-29 family with its mature members miR-29a, -29b and -29c, which is one of the most important miRNA families to date, implicated in the regulation of various cellular processes (Kriegel *et al.* 2012; Schmitt *et al.* 2012a). For control purposes, miR-100 (slightly down-regulated after IFN-γ stimulation) and miR-25 (stable levels) were selected. To circumvent the above described normalization problem and to be able to reliably identify the small expression changes typical for miRNAs (1.5 – 5 fold), the Genorm normalization method (for details see methods section) has been used for all following experiments, which uses a minimum number of three housekeeping genes (in our case RNU1A, RNU5A and SCARNA17), thus allowing for more accurate normalization of miRNA expression changes according to the MIQE-guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) (Bustin *et al.* 2009).

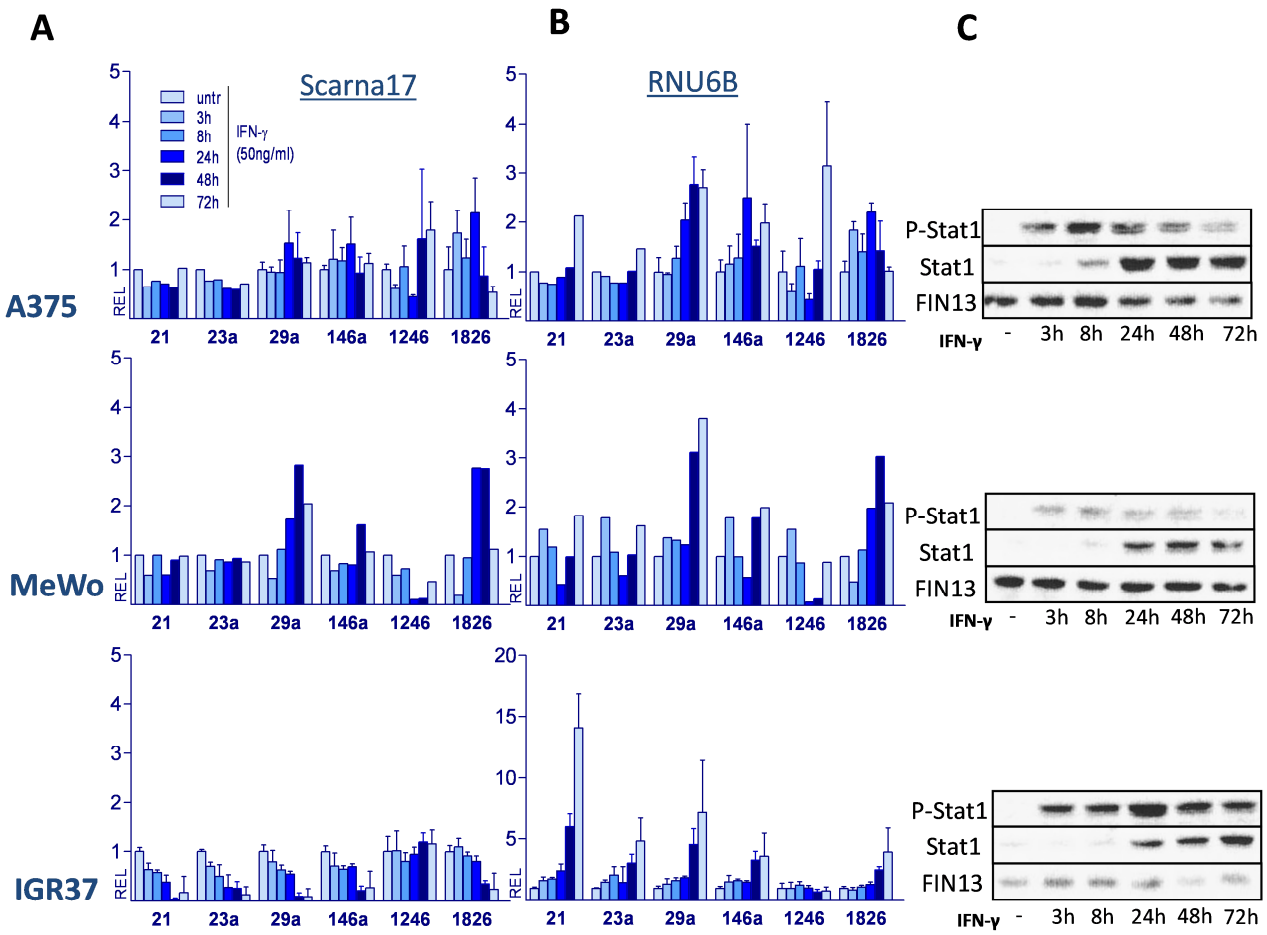


Fig. 17: IFN-γ stimulation of three in melanoma cell lines: qPCR amplification of candidate miRNAs as selected from the LC Sciences microarray experiment
(A,B) The miRNAs miR-21, -23a, -29a, -146a, -1246 and -1826 were amplified from A375, MeWo and IGR37 melanoma cell lines, which had been left untreated or stimulated with IFN-γ for 3 h, 8 h, 24 h, 48 h or 72 h. Graphs show miRNA relative expression (REL) normalized to the house keeping genes SCARNA17 (A) or RNU6B (B) and to the untreated control. Bars ≙ mean with SD of technical triplicates; only single experiments are shown for other experiments. (C) Efficient STAT1-activation was confirmed by Western blot analysis.

To gain a comprehensive view on the regulation of the miR-29 family, the expression of all miR-29 species following IFN-γ-stimulation was addressed in detail: kinetics of both primary clusters pri-29a~b-1 and pri-29b-2~c, the different precursor molecules pre-29a, pre-29b-1, pre-29b-2 and pre-29c as well as the mature miRNAs miR-29a and miR-29b were analyzed. Nevertheless, it must be noted that precursor primers can also amplify primary miRNAs and up-regulation of miR-29 precursors may partially reflect the pri-29a~b-1 signal. Expression of mature miR-29c, which is derived from the pri-29b-2~c cluster was not analyzed as it only bears one nucleotide difference to miR-29a (Fig. 4). [2]Therefore, specific and correct qPCR amplification of miR-29c expression was not possible with the SYBR system (data not shown).

The pri-29a~b-1 cluster and mature miR-29a/29b are regulated by IFN-γ in melanoma cell lines

To accurately assess the regulation of the miR-29 family by IFN-γ-induced STAT1, the melanoma cell lines MeWo and A375, as well as stably transfected A375 derivates were used for further stimulation experiments with IFN-γ. [2]A375-STAT1(F) represent STAT1-dominant negative cells harboring a phenylalanine replacement of tyrosine residue 701 crucial for STAT1 phosphorylation and dimerization (Kortylewski *et al.* 2004)(Fig. 5). [2]Thus, transcription of STAT1 target genes is abolished despite IFN-γ stimulation. [2]The corresponding positive control cells A375-STAT1(wt) express the STAT1 wild-type construct instead (Kortylewski *et al.* 2004). [2]Stimulation of A375, MeWo and A375-STAT1(wt) cell lines with 50 ng/ml of IFN-γ induced a prominent STAT1 phosphorylation, which decreased after 48 h of IFN-γ treatment, whereas the STAT1-dominant negative cells A375-STAT1(F) only exhibited a delayed and weak P-STAT1 signal after IFN-γ stimulation (Fig. 18), see also (Kortylewski *et al.* 1999)). [2]Functional activity of the P-STAT1 transcription factor was confirmed by up-regulation of the STAT1 target genes IRF-1 and STAT1 itself, which showed induced expression after 3 h and 8 h, respectively. [2]Following stimulation, changes in miRNA expression levels were assessed by qRT-PCR. A375, A375-STAT1(wt) and MeWo cell lines showed a strong and significant up-regulation (>5 fold) of the pri-29a~b-1 cluster, starting 24 h after IFN-γ stimulation, while expression of the pri-29b-2~c cluster was not altered (Fig. 18, upper panel). [2]Accordingly, miRNA precursors pre-29a and pre-29b-1 were also augmented whereas pre-29b-2 and pre-29c levels remained unaffected (Fig. 18, middle panel). [2]Subsequently, significant up-regulation of both mature miR-29a and miR-29b following IFN-γ stimulation was confirmed (Fig. 18, lower panel). The two control amplifications of miR-100 (slightly down-regulated) and miR-25, which remained stable over time following IFN-γ stimulation confirmed the initial microarray-based expression profiles (Fig. 18, lower panel). [2]Except for minor expression changes of the Pri/Pre-miR-29 species after 72h of IFN-γ treatment, no up-regulation was detected in the A375-STAT1(F) dominant negative control cells, clearly suggesting that STAT1 activity is required for the IFN-γ-induced regulation of miR-29.

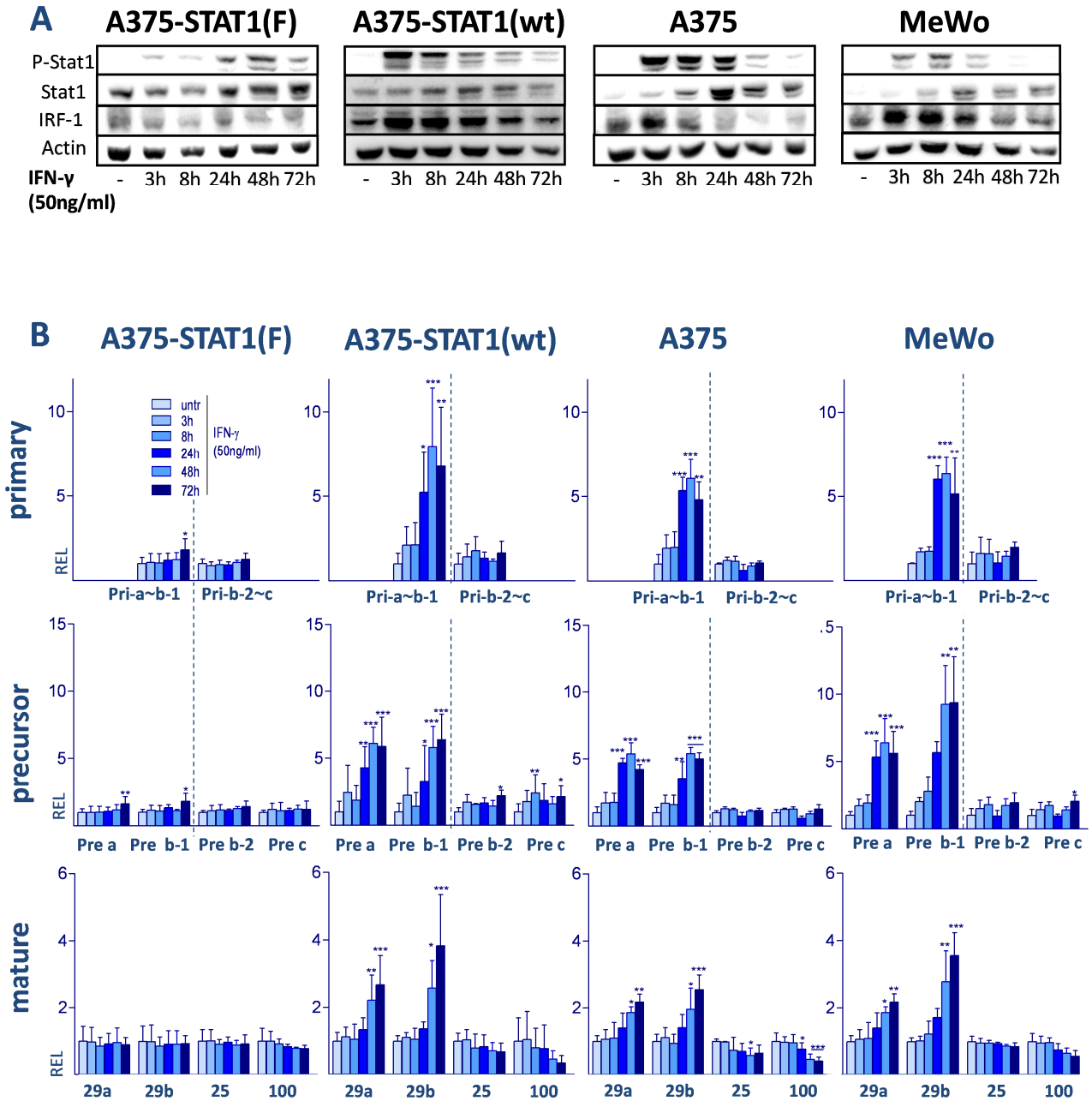


Fig. 18: [2] Expression profiles of miR-29 clusters in melanoma cells

[2]A375-STAT1(F), A375-STAT1(wt), A375 and MeWo melanoma cells were stimulated with IFN-γ for different time points. [2](A) Western Blot analysis (representative blots of biological triplicates) confirms activation of P-STAT1 and induction of STAT1 and IRF-1 after IFN-γ stimulation while dominant negative A375-STAT1(F) cells show minor effects. [2](B) Time course study of miRNA-expression after IFN-γ-stimulation. [2]Graphs show relative expression (REL) from quantitative qRT-PCR data for the pri-29a~b-1 and the pri-29b-2~c clusters, the precursors pre-29a/29b-1/29b-2/29c and mature miR-29a/29b/25/100. [2]Fold expression was calculated relative to the untreated control. Bars = mean with SD for biological triplicates. [2]Statistical significance was tested with one-way ANOVA, followed by a Dunnett Post-Hoc test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Detailed time course microarray experiment confirms IFN-γ-induced regulation of miR-29

In the course of a master thesis project in our group (Reinsbach *et al.* 2012), a more detailed time course miRNA microarray experiment was performed using IFN-γ-stimulated A375 cells (including untreated, 30 min, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h time points). In parallel and as a negative control, cells had been pre-treated with Jak inhibitor 1 (JI1), which specifically inhibits Janus tyrosine kinases and subsequently prevented miR-29 up-regulation after IFN-γ stimulation. Optimal concentrations of inhibitor had previously been established in our laboratory. As shown in Fig. 19, the STAT1 phosphorylation for cells pretreated with JI1 was almost undetectable.

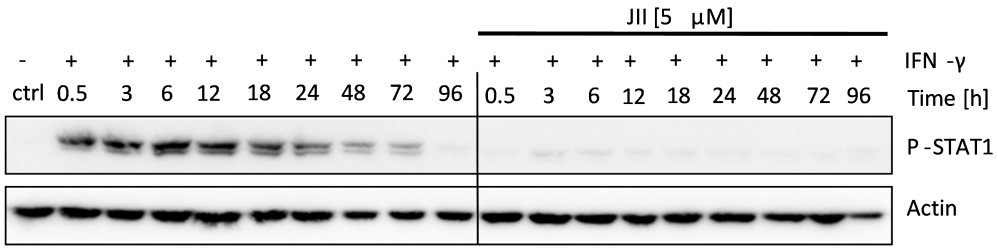


Fig. 19: STAT1-activation after IFN-γ stimulation and abrogation of P-STAT1 signals following JI1-pretreatment
Detailed IFN-γ kinetics in A375 cells (50 ng/ml IFN-γ or pre-treated with 5 µM JII followed by IFN-γ stimulation). Activation of P-STAT1 (left) and Jak-inhibition (right) were confirmed prior to microarray analysis of the corresponding RNA samples (Reinsbach *et al.* 2012).

Following the confirmation of successful IFN-γ-stimulation, duplicate RNA samples of A375 cells from all time points (untr, 0.5 to 96 h) and from the 72 h JI1-pre-treated cells were subjected to miRNA-microarray (Affymetrix) (Fig. 20) and subsequent qRT-PCR validation of selected miRNAs (data not shown). Up-regulation of mature miR-29a and miR-29b after IFN-γ-stimulation, as well as no modulation of miR-25 and slight down-regulation of miR-100 over time was confirmed (Fig. 20). No expression change in comparison to untreated cells was observed after JI1-pre-treatment and subsequent IFN-γ stimulation for any of the miRNAs, indicating that the transcriptional induction of miRNAs following IFN-γ-stimulation is Jak-dependent and STAT-mediated. Altogether, these data substantiate the time-dependent up-regulation of the expression of pri-29a~b-1 cluster as well as of the mature miRNAs miR-29a and -29b in melanoma cells, which is triggered by IFN-γ-induced STAT1 signaling.

Interestingly, also miRNA star sequences showed differential regulation. The miRNA star (miR*)-sequences, as named on the microarray (and as explained before, introduction Fig. 3), represent the miRNA, which arises from the 3´-arm of the hairpin and is conventionally

considered as "minor" product. In this case, miR-29a* and miR-29c* were not induced whereas miR-29b-1* and miR-25* was up-regulated after IFN-γ-stimulation.

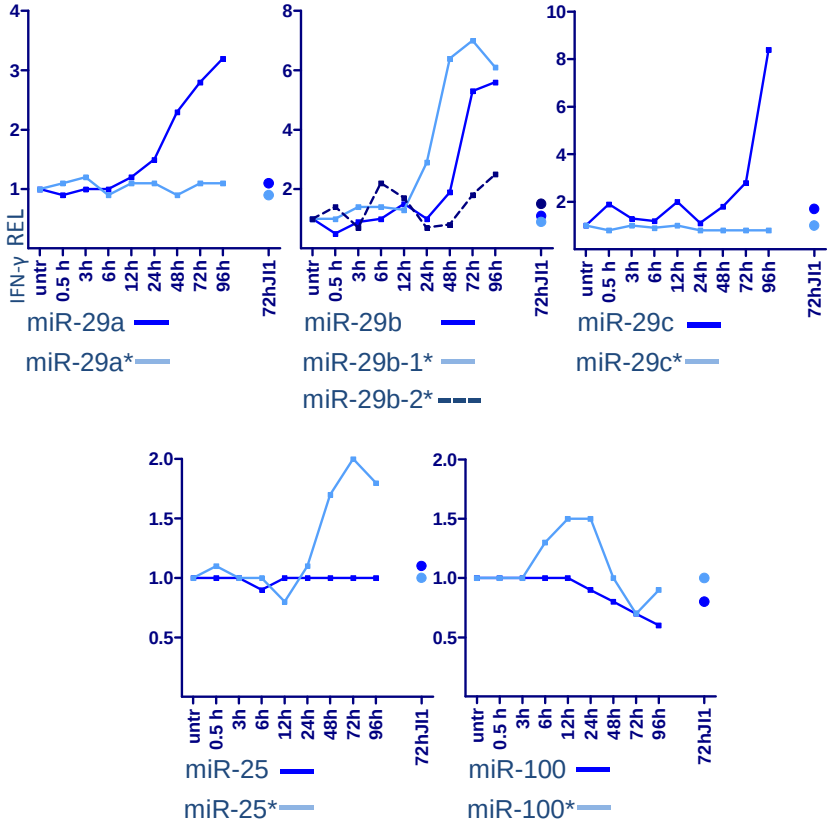


Fig. 20: miRNA and miRNA* expression profiles in A375 cells
Corresponding data were derived from a more detailed IFN-γ time course miRNA microarray experiment including cells treated with JI1 (72h JI1, IFN-γ stimulation for 72 h after pre-treatment with JI1, blue dots). [2]Depicted are log2-values of the mean of duplicate microarray experiments.

miRNAs from the miR-23a/27a/24-2 clusters show strand-specific IFN-γ-induction

Other IFN-γ-induced miRNAs from the detailed miRNA microarray experiment included also several miRNA star sequences which showed regulation patterns different to their partner duplex strands (Fig. 21). For example, miR-23a* and miR-27a* were up-regulated, whereas miR-23a and miR-27a did not exhibit differential expression following IFN-γ. For the second miR-23/27/24 cluster, all miR-23/27 species were down-regulated (Fig. 21). Interestingly, both clusters are composed of three miRNAs from different families each (miR-23/miR-27/miR-24). This leads to the suggestion that the cellular functions of miR-23a*/miR-23b* and miR-27a*/miR-27b* could complement each other (Reinsbach *et al.* 2012).

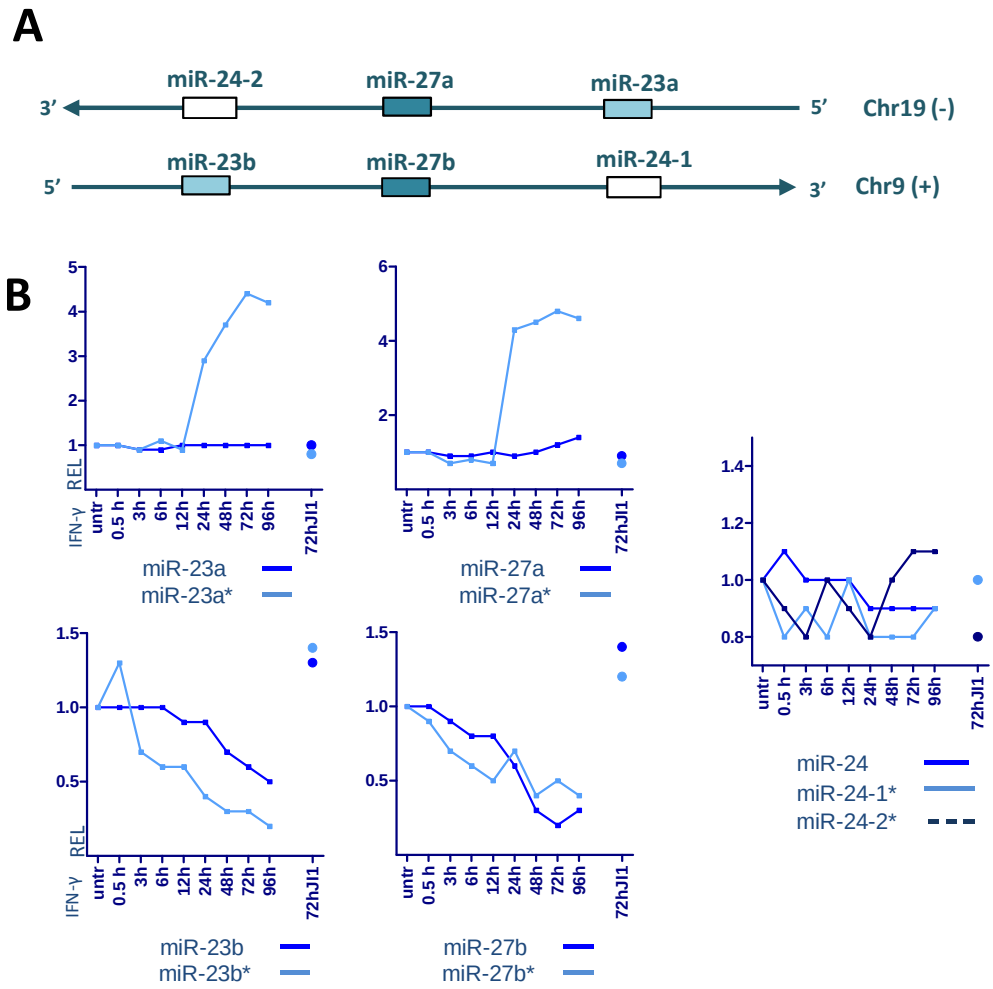


Fig. 21: Strand-specific IFN-γ induction the miR-23a/27a/24-2 cluster and down-regulation of the miR-23b/27b/24-1 cluster

(A) Genomic organization of both clusters miR-23a/27a/24-2 (chr 19, -) and miR-23b/27b/24-1 (chr 9, +) (B) miRNA and miRNA* expression profiles in A375 cells derived from the detailed IFN-γ time course miRNA microarray experiment including cells treated with JI1 (72hJI1, IFN-γ stimulation for 72h after pre-treatment with JI1, blue dots). [2]Depicted are log2-values of the mean of duplicate microarray experiments.

Analysis of miR-29 regulation in other biological systems

To investigate whether the observed regulation patterns are specific for melanoma cells or if they can be reproduced in other cell types, we performed the same stimulation experiments in HEK293T kidney cells and Jurkat T-cells and also used the type-I interferons IFN-α and IFN-β in preliminary stimulation experiments (Fig. 22). Up-regulation of mature miR-29a/b after IFN-γ-stimulation and unchanged miR-25 levels were also observed in these two other cell lines (Fig. 22), albeit miR-29a up-regulation was slightly weaker than in melanoma cells. Interestingly, also IFN-α and IFN-β-stimulation led to enhanced miR-29a and miR-29a expression in Jurkat and HEK cells, proving that also type-I-interferons can induce these miRNAs. However, effects were

stronger in Jurkat cells than in HEK cells and miR-29a/b induction after IFN-β stimulation was higher in comparison to IFN-α stimulation in Jurkat cells (Fig. 22).

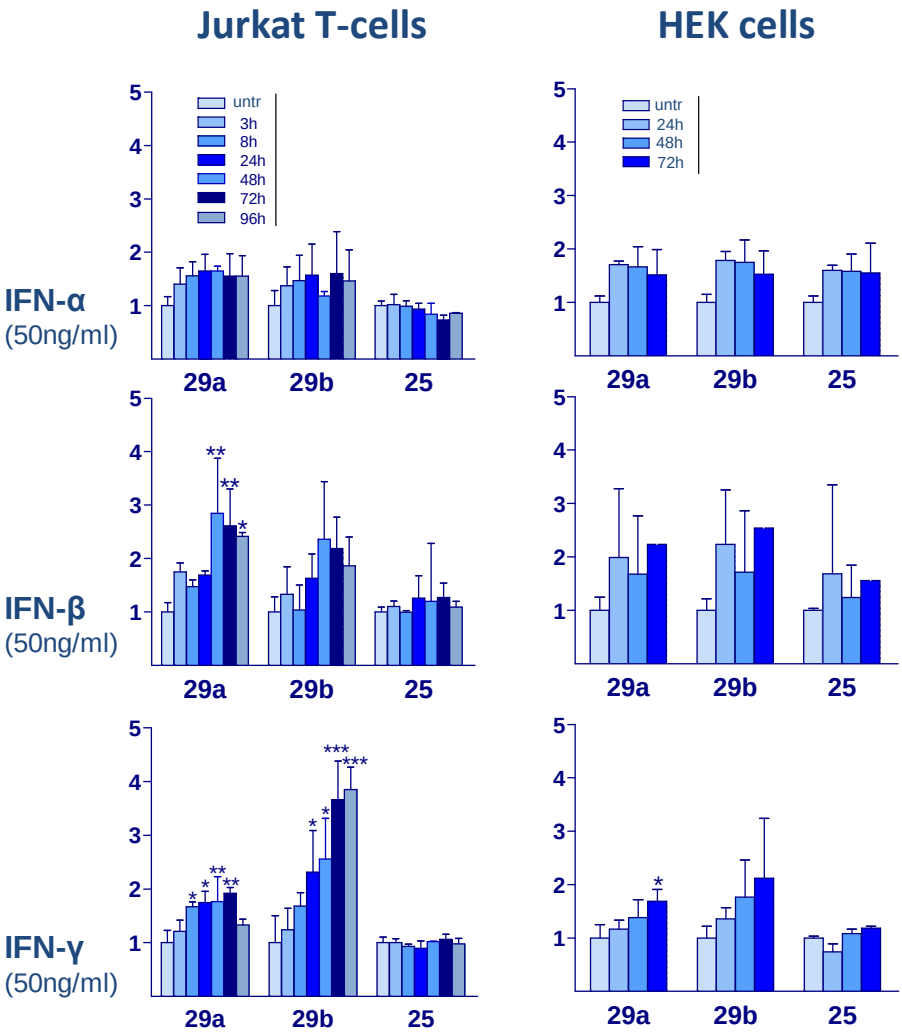


Fig. 22: Analysis of miR-29 regulation patterns in HEK293T and Jurkat cells
Up-regulation of mature miR-29a/29b after IFN-α, IFN-β and IFN-γ stimulation in Jurkat T cells and HEK293T kidney cells. miR-25 levels remained unchanged in Jurkat cells and were slightly affected after IFN-α and IFN-β stimulation of HEK293T cells. Graphs show relative expression (REL). Bars = mean with SD for biological triplicates (IFN-γ) or technical triplicates (IFN-α, IFN-β). Statistical significance was tested with one-way ANOVA, followed by a Dunnett Post-Hoc test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

In addition to our findings for melanoma cells (Fig. 18) and previously published data on the role of miR-29 in various other cancer types (Sengupta *et al.* 2008; Wang *et al.* 2008; Zhao *et al.* 2010) miR-29 family members were also shown to be up-regulated in T-cells (Fig. 22).
When analyzing potential target genes of miR-29 with publicly available databases and literature searches (see below, Fig. 33) we found that miR-29a/29b had also been reported to target the HIV (human immunodeficiency virus) -protein nef (negative regulatory factor) (Hariharan *et al.*

2005). Nef has a positive influence on HIV infectivity and replication by down-regulating cell surface molecules like CD4 (cluster of differentiation 4) and MHC (major histocompatibility complex) I and II (Kirchhoff *et al.* 2008). Following this interesting connection, we found in a report that miR-29a modulates HIV-production and infectivity (Nathans *et al.* 2009). With this information at hand we initiated initial experiments to investigate a potential connection between IFN-γ/miR-29a/nef in T-cells, using MT4 T-cells, which can be infected by HIV. MiR-29a/b were up-regulated after stimulation of MT4 T-cells with IFN-α-, IFN-β- and IFN-γ. Follow-up studies are currently being planned to investigate whether IFN-induced up-regulation of miR-29a/b is sufficient to reduce nef expression in HIV-infected T-cells and to possibly decipher a new role for interferons and miRNAs during HIV infection.

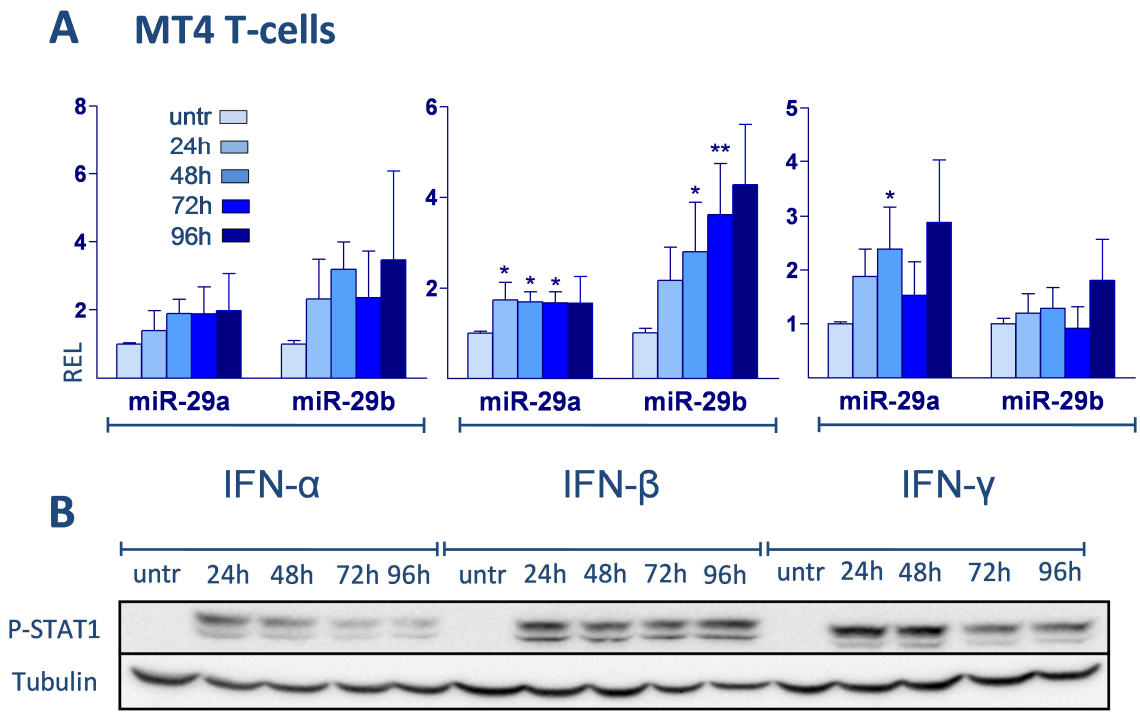


Fig. 23: Initial experiments on miR-29 in MT4 cells
(A) Mature miR-29a/29b up-regulation after IFN-α-, IFN-β- and IFN-γ-stimulation (50 ng/ml) in MT4 T cells. Graphs show relative expression (REL). Bars ≙ mean with SD for biological triplicates. Statistical significance was tested with one-way ANOVA, followed by a Dunnett Post-Hoc test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. (B) Efficient phosphorylation of P-STAT1 after stimulation (IFN-α-, IFN-β- and IFN-γ, 50 ng/ml) was confirmed by Western blotting.

IL-27 induces a P-STAT1 response in melanoma cell lines and up-regulates miR-29

For all following experiments, we focused again on melanoma cells. Members of our group had shown that although it belongs to the IL-6-type cytokine receptor family, IL-27 (interleukin 27) assumes IFN-γ-like functions in hepatoma cells and induces a STAT1 response (Schoenherr *et*

al. 2010). We tested whether IL-27 could also activate STAT1 in melanoma cells leading to up-regulation of miR-29 as it was confirmed that IL-27 exhibits anti-proliferative activities on melanoma cell lines (Yoshimoto *et al.* 2008). The required receptors for IL-27 signal transduction are WSX-1 and gp130 (glycoprotein 130), which are known to be expressed on MeWo cells (Pflanz *et al.* 2004). Indeed, P-STAT1 activity as well as miR-29a/b up-regulation was confirmed in A375 and MeWo cells (Fig. 24). Also phosphorylation of STAT3 following IL-27 stimulation could be detected in the early time points (3 h – 24 h). Interestingly, also the untreated samples showed a P-STAT3 signal, which can be explained by constitutive activity of STAT3 in cell culture experiments, which include wells with a high cell density (Kreis *et al.* 2007).

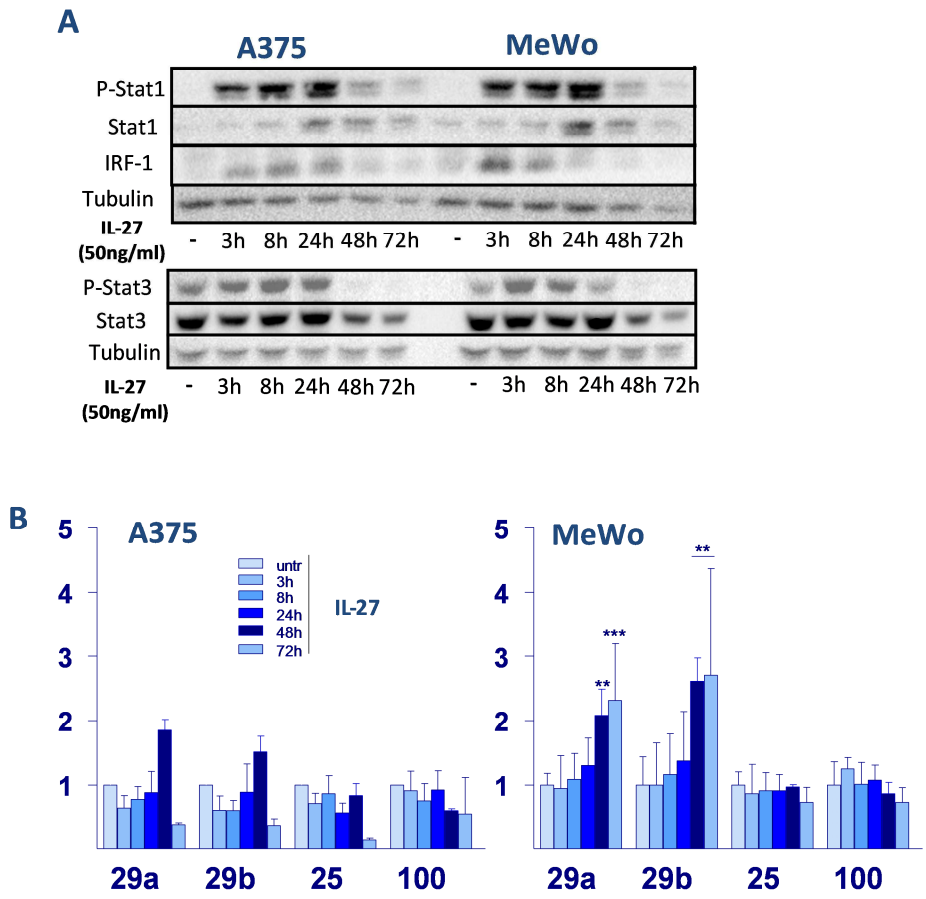


Fig. 24: IL-27 induces a STAT1-response in A375 and MeWo melanoma cell lines
Time course study of miRNA-expression after IL-27-stimulation (50 ng/ml) in A375 and MeWo melanoma cells. (A) Western Blot analysis confirms activation of P-STAT1 and induction of STAT1 and IRF-1 after IL-27 stimulation as well as activation of P-STAT3. (B) qRT-PCR: graphs show relative expression (REL) from quantitative qRT-PCR data for the mature miRNAs miR-29a, miR-29b, miR-25 and miR-100. Fold expression was calculated relative to the untreated control. Bars ≙ mean with SD for technical (A375) or biological (MeWo) triplicates. Statistical significance (MeWo) was tested with one-way ANOVA, followed by a Dunnett Post-Hoc test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

5.1 *In silico* analysis of the miR-29 promoter regions

As miR-29 family members could be induced by STAT1, we next analyzed their respective promoter regions more closely. Both clusters of the miR-29 family are intergenic and currently there is no common agreement on their transcription start sites (TSSs). To taper the region which could be responsible for miR-29 regulation, a detailed literature search was performed on all published TSS coordinates for both clusters (Saini *et al.* 2007; Chang *et al.* 2008; Marson *et al.* 2008; Saini *et al.* 2008; Corcoran *et al.* 2009; Mott *et al.* 2010; Chien *et al.* 2011), which were then converted into the human genome version 19 (hg19) as described in more detail in our recent review (Schmitt *et al.* 2012a). So far, 14 different TSSs have been annotated for the miR-29 family (Fig. 25). TSS analysis revealed that the genomic locations of the published transcription start sites were distributed over a large distance.

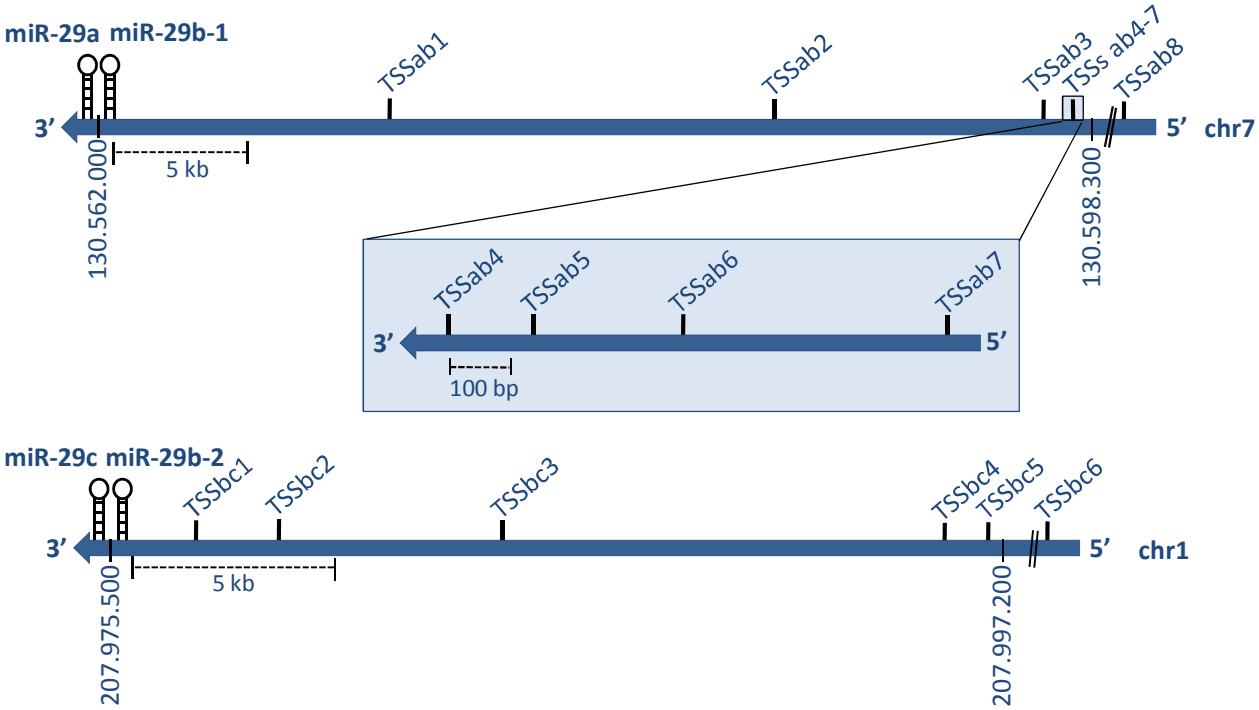


Fig. 25: Promoter organization and potential transcription start sites for miR-29 clusters
[1]Potential TSSs for both clusters miR-29a~29b-1 (TSSab1-8) and miR-29a~29b-2 (TSSbc1-6) from literature, as described in (Schmitt *et al.* 2012a). [1]Coordinates have been converted to genome version hg19/GRCh37 and are as follows: TSSab1 chr7:130.572.487; TSSab2 chr7:130.586.832; TSSab3 chr7:130.596.983; TSSab4 chr7:130.597.889; TSSab5 chr7:130.598.020; TSSab6 chr7:130.598.638; TSSab7 chr7:130.598.268; TSSab8 chr7:130.800.298; TSSbc1 chr1:207.977.425; TSSbc2 chr1:207.979.479; TSSbc3 chr1:207.985.009; TSSbc4 chr1:207.996.050; TSSbc5 chr1:207.997.156;TSSbc6 chr1:207.037.276. The magnified insert (TSSab4-7) indicates a culmination of described TSSs and thus a putative promoter region of the miR-29a~29b-1 cluster.

However, four of the TSSs for the miR-29a~29b-1 cluster accumulated in a 700 bp-region, which could be its most important regulatory region, while no refinement was possible for the

miR-29c~29b-2 cluster (Fig. 25). Genomic coordinates for those four transcription start sites were obtained by experimental (TSSab4 and TSSab5) (Chang *et al.* 2008; Mott *et al.* 2010) and computational (TSSab6 and TSSab7) analyses (www.switchgeargenomics.com).

In silico analysis of the pri-29a~b-1 promoter region identifies a putative regulatory region containing two STAT1 binding sites

After the region for the approximate location of the pri-29a~b-1 promoter had been narrowed down and to further confirm the STAT1-mediated regulation of this cluster, a comprehensive *in silico* examination of the region chr7:130,560,000-130,610,000 (hg19) was conducted, spanning the sequence 40.000 bp upstream and 10.000 downstream of the putative pri-29a~b-1 transcription start site as described in Schmitt *et al.* (Schmitt *et al.* 2012a); an illustration is shown in Fig. 25. This analysis included the UCSC genome browser features 'H3K4Me3 mark', 'vertebrate conservation' and also incorporated data from a ChIP-Seq experiment which had been performed on IFN-γ treated Hela cells within the ENCODE project (ENCyclopedia of DNA Elements) (Kent *et al.* 2002) (all features are further described in the methods section). This initial analysis identified a region (chr7:130,596,800-130,599,000; hg19) with high vertebrate conservation, a H3K4Me3-mark as marker for open chromatin and a prominent ChIP-Seq peak and also contained five of the putative transcription start sites described in literature (Fig. 26 A). We thus concentrated on the sequence mentioned above for the following analyses.

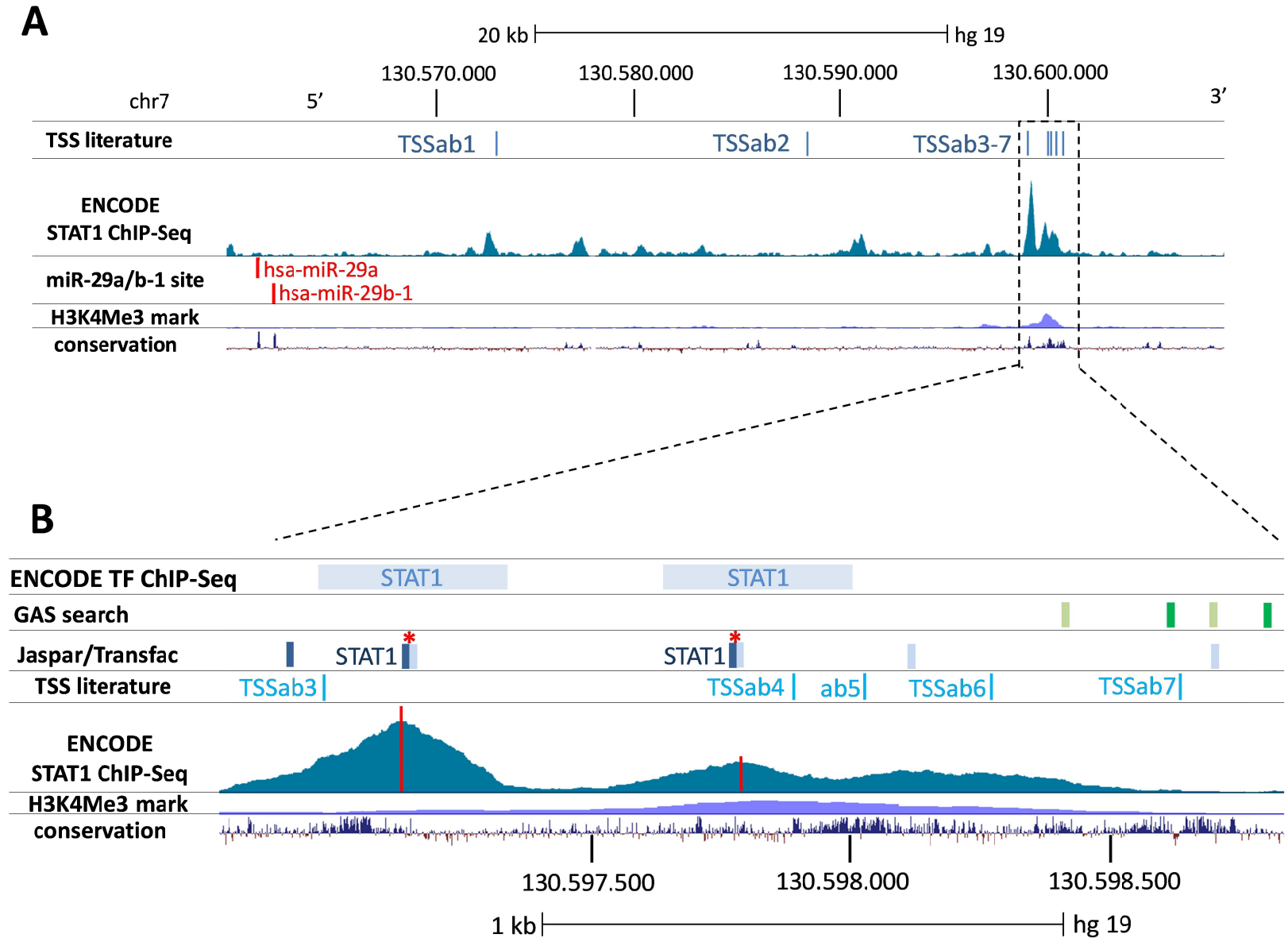
By combination of several sequence searches and publicly available *in vitro* data and *in silico* analyses, we identified two STAT1 bindings sites (chr7:130597141-130597155 and chr7:130597778-130597792) within a conserved region of chromosome 7 in the promoter region of the pri-29a~b-1 cluster. The binding sites which could be responsible for pri-29a~b-1 regulation have been predicted by two different algorithms ('Jaspar' and 'Transfac') (Fig. 26 B) and overlapped with ChIP-Seq-based data as well as ENCODE-predictions for STAT1 binding sites. Furthermore, they were located in close proximity of a H3K4Me3-mark (marker for open chromatin) and are located close to putative transcription start sites found in literature. Interestingly, transcription of the pri-29a~b-1 from the region described above would lead to a very long primary construct of ~36 kb.

Fig. 26: *In silico* analysis of a putative STAT1-regulatory region within the pri-29a~b-1 promoter

(A) Initial analysis on chr7:130,560,000-130,610,000 (hg19) included (from top to bottom) putative TSSs from literature (Fig. 25 and (Schmitt *et al.* 2012a)). ENCODE STAT1-ChIP-seq peaks from a ChIP experiment performed on IFN-γ-stimulated Hela cells, position of mature hsa-miR-29a and hsa-miR-29b-1 sequences, H3K4Me3 peaks and vertebrate conservation scores (blue / positive scores: conserved sites; red / negative scores: fast-evolving sites) were all obtained from UCSC (Kent *et al.* 2002).

(B) Detailed analysis on chr7:130,596,800-130,599,000 (hg19), magnified insert from (A). From top to bottom: ENCODE TF-ChIP-seq: STAT1 binding sites as predicted by the UCSC track ENCODE (Hudson and Snyder 2006; Euskirchen *et al.* 2007) transcription factor binding; GAS-search: GAS elements from computational screen for the consensus sequences TT(C/A)CNNNAA(A/G) (light green) (Pagliaccetti *et al.* 2008) and from screen for consensus sequence TT(C/A)NNN(G/T)AA (green) which had been used by Smith *et al.* (Smith *et al.* 2012). Jaspar/Transfac: putative STAT1 binding sites predicted by Jaspar (dark blue) or Transfac (light blue); the red star indicates the sites found by both of the programs. TSS literature: potential transcription start sites collected from literature, as summarized in (Schmitt *et al.* 2012a) and corresponding to TSS labelling from Fig. 25: TSSab3: (Chien *et al.* 2011) miRStart; TSSab4: (Mott *et al.* 2010); (TSS)ab5: (Chang *et al.* 2008); TSSab6 & TSSab7: UCSC: 'SwitchGear'. ENCODE STAT1 ChIP-seq and H3K4Me3 mark and vertebrate conservation: magnified insert from A), the red line indicates the middle of the STAT1-ChIP-seq-peak.

Fig. 26


A
20 kb
hg 19
130.570.000
130.580.000
130.590.000
130.600.000
chr7
5'
3'
TSS literature
TSSab1
TSSab2
TSSab3-7
ENCODE
STAT1 ChIP-Seq
miR-29a/b-1 site
hsa-miR-29a
hsa-miR-29b-1
H3K4Me3 mark
conservation
B
ENCODE TF ChIP-Seq
STAT1
STAT1
GAS search
Jaspar/Transfac
STAT1
STAT1
TSS literature
TSSab3
TSSab4
ab5
TSSab6
TSSab7
ENCODE
STAT1 ChIP-Seq
H3K4Me3 mark
conservation
130.597.500
130.598.000
130.598.500
1 kb
hg 19

5.3 Functional characterization of miRNA-29 in melanoma

The miR-29b-2~c cluster is undetectable in melanoma cell lines, melanocytes and keratinocytes

[2]As both miR-29 primary clusters as well as the mature miR-29a/29b showed different basal expression levels in stimulation experiments and are known to be differentially expressed in several other types of cancer (Pekarsky *et al.* 2006; Stamatopoulos *et al.* 2009), we next analyzed the miR-29 basal expression profiles in a panel of melanoma cell lines, primary human melanocytes (NHEM-M2) and HaCaT keratinocytes (Fig. 27).

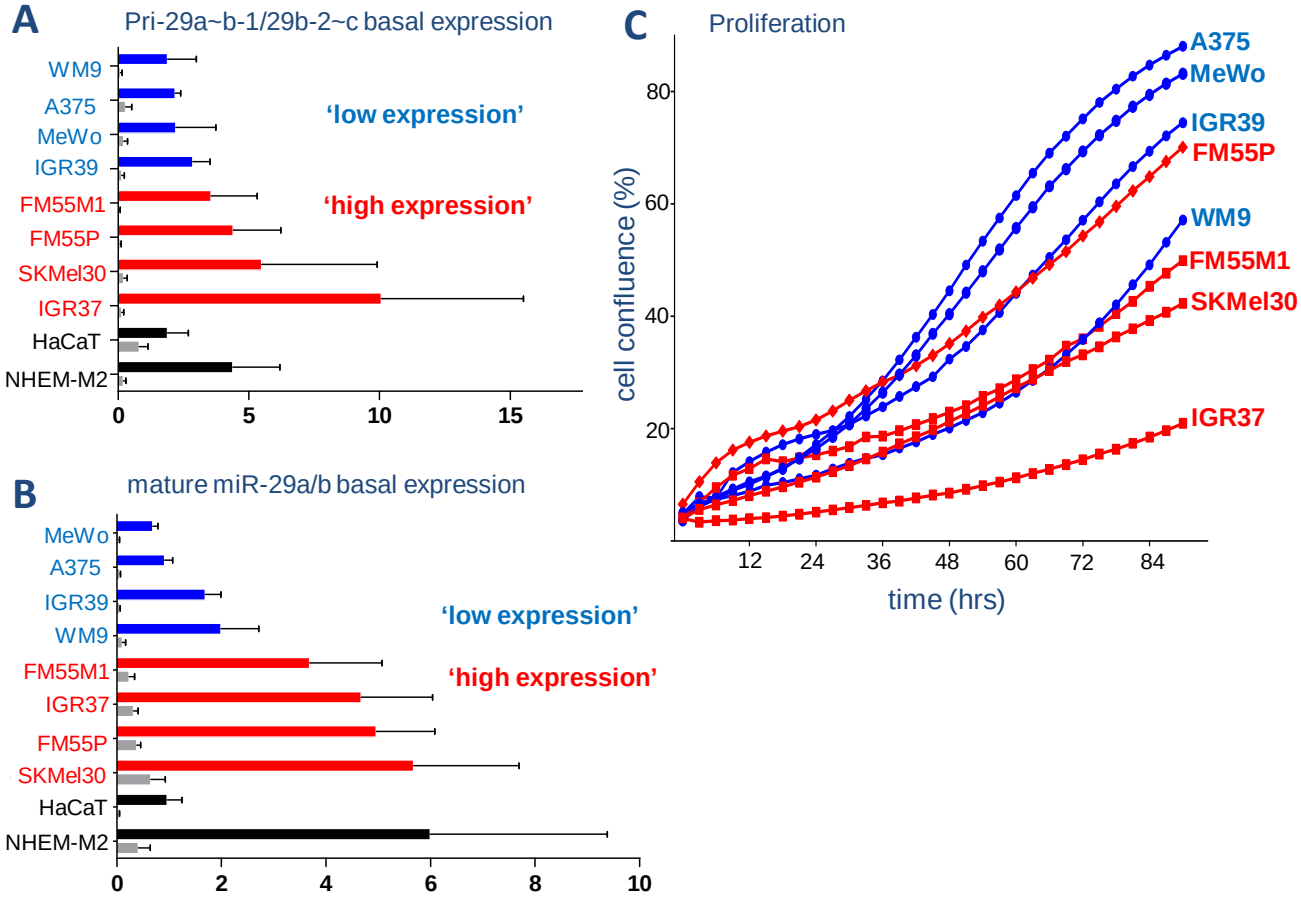


Fig. 27: [2]Analysis of miR-29 basal expression levels and proliferation of untreated melanoma cell lines

[2]Comparison of basal expression levels of (A) primary miRNA clusters pri-29a~b-1 (blue/red/black bars) and pri-29b-2~c (grey bars) and (B) mature miR-29a (blue/red/black bars) and miR-29b (grey bars) in NHEM-M2, eight melanoma cell lines and HaCaT keratinocytes. Bars = mean of = $2^{(dCt)}$ x10^2 with SD of biological triplicates (dct = ct target – ct GEOMEAN of 3 reference genes). (C) [2]Mean growth curves of untreated melanoma cell lines over 4 days (biological quadruplicates). [2]Melanoma cell lines with 'low expression' of pri-29a~b-1 and miR-29a show faster proliferation whereas cells with a relatively 'high expression' proliferate slower.

[2]Pri-29a~b-1 was strongly expressed whereas pri-29b-2~c was almost undetectable in all cell lines analyzed (Fig. 27). This is in accordance with previous studies reporting down-regulation of the pri-29b-2~c cluster in rhabdomyosarcoma (Wang *et al.* 2008) and B-cell lymphoma (Chang *et al.* 2008). Mature miR-29a consistently showed higher basal expression levels than miR-29b in all cell lines examined (Fig. 27). To exclude the possibility that the differences in expression levels mentioned above were caused by different primer efficiencies (e.g. miR-29a versus miR-29b primer and pri-29a~b-1 primer versus pri-29b-2~c), we tested the primer efficiencies of all miR-29 primer and confirmed them to be between 80% and 120% (data not shown).

MiR-29a/29b expression levels inversely correlate with growth behavior of melanoma cell lines

The classification of miR-29 as tumor-suppressor miRNA has been widely accepted and the possibility to use synthetic miR-29 as therapeutic agent in treatments of cancer seems to become increasingly realistic. [2]Properties counteracting the development and spreading of cancer cells that have been observed *in vitro* and *in vivo* after miR-29 overexpression include reduced invasion and proliferation and induction of apoptosis (Xiong *et al.* 2010; Fang *et al.* 2011). [2]These findings prompted us to analyze a potential correlation of basal miR-29 expression levels with cell growth. Proliferation of untreated melanoma cell lines was monitored over time (Fig. 27) in order to correlate it with miR-29a and pri-29a~b-1 basal expression levels obtained from cells harvested 96 h after seeding. NHEM-M2 primary melanocytes were not included in this experiment as they hardly proliferate. [2]According to their expression levels, melanoma cell lines could be grouped in miR-29a and pri-29a~b-1 'low-expression' lines (A375, MeWo, IGR39, WM9) and 'high expression' cell lines (FM55P, FM55M1, SK-Mel30, IGR37) (Fig. 27). [2]Generally, cell lines with lower miR-29a showed an increased proliferation rate compared to lines with higher basal miR-29a levels (Fig. 27). Furthermore, the inverse correlation between pri-29a~b-1/miR-29a expression and the proliferation rate of melanoma cell lines might suggest a potential involvement of miR-29 in anti-proliferative effects in melanoma cells.

MiR-29a/b are responsible for growth inhibition of melanoma cells

To follow up these initial findings on proliferation (Fig. 27), we applied miR-29a/29b mimics to A375 cells, which exhibit a relatively low miR-29a/29b basal expression and, *vice versa*, we applied a miR-29a inhibitor to FM55P cells, which have a high basal miR-29a/29b expression (Fig. 27). [2]Proliferation assays with mimics and inhibitors and the corresponding amounts of scrambled controls, NC (negative control)-mimic and NC-inhibitor, corroborated that miR-29 indeed inhibited growth of melanoma cells: transfection of miR-29a/29b mimics caused a remarkable reduction of proliferation as compared to NC-mimic-transfected A375 cells.

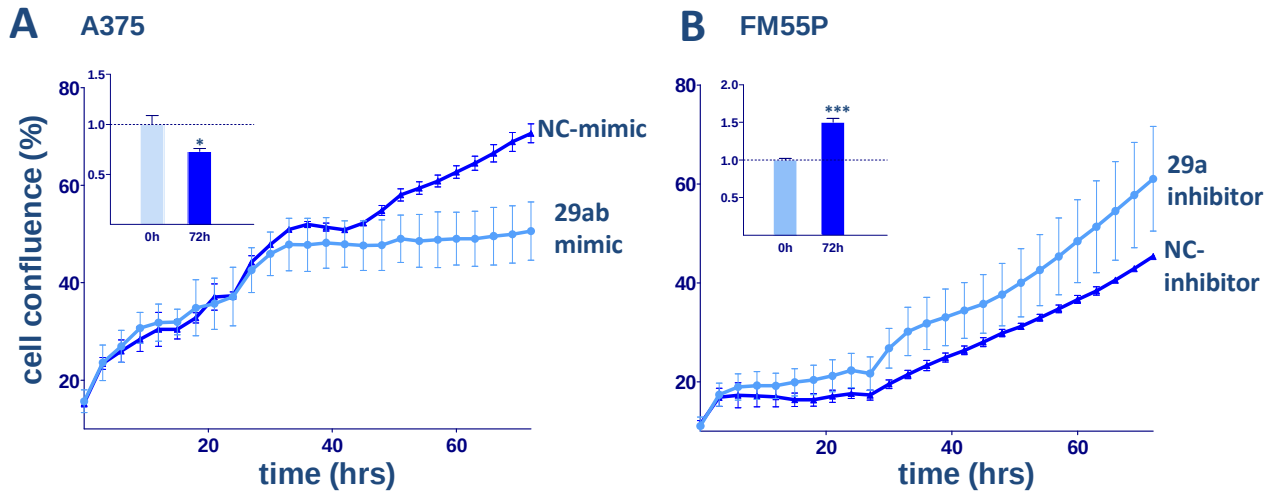


Fig. 28: [2] Proliferation assay of miR-29a/b-mimic-transfected and miR-29a-inhibitor-transfected melanoma cells confirms the growth-inhibitory role of miR-29

[2]Proliferation assay of (dark blue) mimic/inhibitor- and (light blue) NC (negative control)-mimic/NC-inhibitor-transfected cells over 72h in (A) A375 and (B) FM55P cells; representative graphs of four independent experiments. [2]Error bars depict SDs of technical triplicates. [2]The inserted graphs (upper left corners) show the mean confluence of 4 biological replicates at 0 h and 72 h time points of the proliferation assay. [2]Depicted are ratios of confluence of miR-29a/b-mimic / NC-mimic treated cells (A) and miR-29a-inhibitor / NC-inhibitor treated cells (B). Bars ≙ mean with SEM. Significance was assessed by a two-tailed t-test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

In turn, FM55P cells, in which miR-29a was inhibited, proliferated faster than NC-inhibitor-transfected control cells (Fig. 28). [2]To confirm efficient transfection, miR-29a/29b tracking by qPCR was performed in parallel to the proliferation experiments (Fig. 29). [2]Application of miR-29b inhibitor was not possible as it turned out that the inhibitor itself was amplified by miR-29b primers in qRT-PCR, thus inhibition of miR-29b could not be properly monitored and controlled. [2]However, as shown in Fig. 27, miR-29b was generally expressed to a much lower degree than miR-29a, so that inhibition of miR-29a was considered to be more important regarding cellular effects. Furthermore, initial experiments on combinatorial miR-29a/29b inhibition revealed no additional or synergistic effects on potential target genes (see below) compared to miR-29a inhibition alone (data not shown).

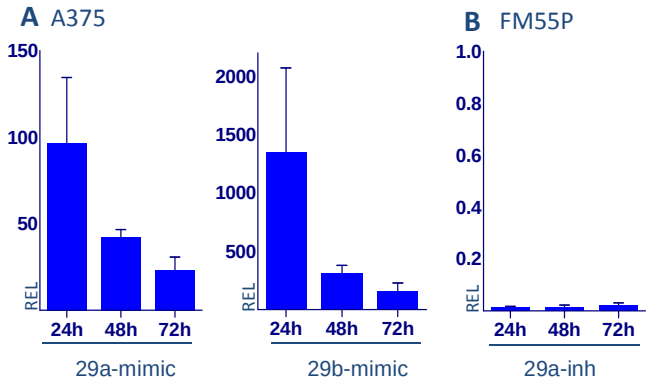


Fig. 29: qRT-PCR tracking experiments to confirm efficient mimic/inhibitor transfection
miR-29a/29b mimics were tracked in A375 cells (A) and miR-29a suppression after inhibitor transfection was quantified in FM55P cells (B) by qRT-PCR. [2]Expression levels of miR-29a/29b were assessed 24h, 48h and 72h after mimic/inhibitor transfection; bars show means of biological triplicates with SD, relative to negative control (NC)-mimic/NC-inhibitor controls. [2]Note that miR-29b transfection and/or amplification was more efficient than for miR-29a.

In the course of the miR-29-mimic/inhibitor-experiments in melanoma cell lines we analyzed their impact on proliferation by measuring growth rates following IFN-γ stimulation. Dose-dependent inhibition of proliferation by IFN-γ-treatment alone of up to 40 % was detected for A375 and MeWo cells (Fig. 30), and this was in line with previous findings for melanoma and other cancer cells (Garbe and Krasagakis 1993; Knüpfer *et al.* 2001; Kortylewski *et al.* 2004; Saha *et al.* 2010).

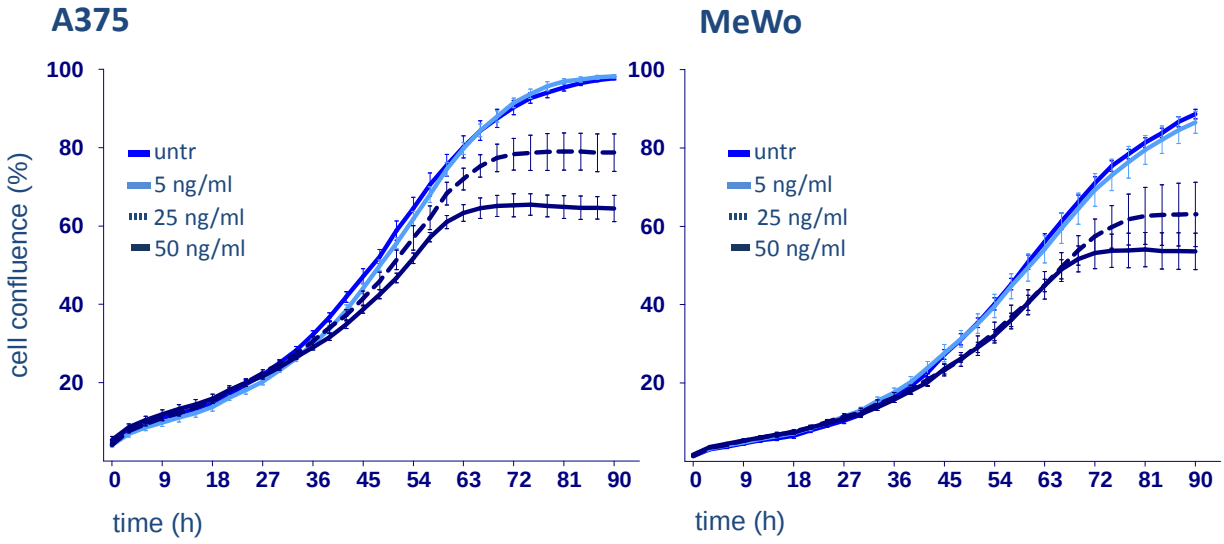


Fig. 30: Proliferation assay following IFN-γ stimulation
A375 (left) and MeWo (right) melanoma cell lines were left untreated or stimulated with 5, 25 or 50 ng/ml IFN-γ and growth was monitored over 90 h. Error bars show SEM of technical triplicates. IFN-γ dose-dependently inhibited melanoma proliferation in both cell lines.

Both miR-29a/b and IFN-γ inhibited melanoma growth (Fig. 28, Fig. 30) and inhibition of miR-29a led to enhanced proliferation with respect to the control treatment (Fig. 28). These findings raised the question whether in course of a combined IFN-γ/miR-29a inhibitor treatment, inhibition of miR-29a would be able to compensate for the inhibition of proliferation mediated by IFN-γ (Fig. 31). Inhibitor/IFN-γ double treated cells indeed proliferated faster than NCI/IFN-γ treated ones (Fig. 31). However, growth differences were not very prominent and not consistently reproducible, probably due to the harsh effects of the double treatment on the cells.

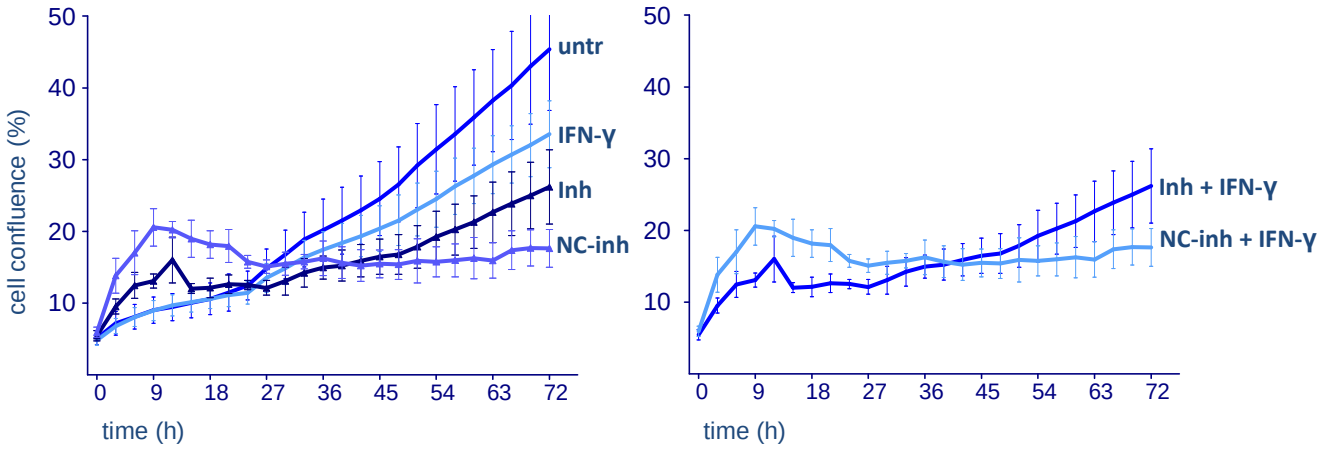


Fig. 31: Proliferation assay: combination experiment with miR-29 inhibitor and IFN-γ in FM55P-cells

FM55P melanoma cells were left untreated, stimulated with 50 ng/ml IFN-γ or transfected with miR-29a-inhibitor (Inh), the respective negative control (NC-inhibitor) or a combination of either of them with 50 ng/ml IFN-γ. Left: as shown above, IFN-γ inhibited proliferation and mir-29a inhibitor transfection (Inh) led to enhanced proliferation in comparison to the scrambled control (NCI). Right: Following combined treatment, the proliferation rate of inhibitor/IFN-γ treated cells was higher than for NCI/IFN-γ treated cells. Error bars show SD of technical triplicates.

Manipulation of miR-29a/b expression levels has no significant impact on apoptosis

To expand the findings on miR-29-mediated cellular effects, apoptosis was monitored after transfection of miR-29a/b mimics and inhibition of miR-29a in A375 and FM55P melanoma cell lines by a caspase-3 based Incucyte assay (Fig. 32). Apoptosis rates for miR-29a/b mimic-transfected cells were slightly enhanced in comparison to the control treatment and, *vice versa*, less apoptosis was observed when miR-29a was inhibited. It has been described previously that miR-29 induces apoptosis (Mott *et al.* 2007; Wang *et al.* 2011b). Here, effects were not very prominent and a reliable difference in apoptosis levels of miR-29a/b-mimic or miR-29a-inhibitor-transfected cells in comparison to the respective negative controls could not be confirmed.

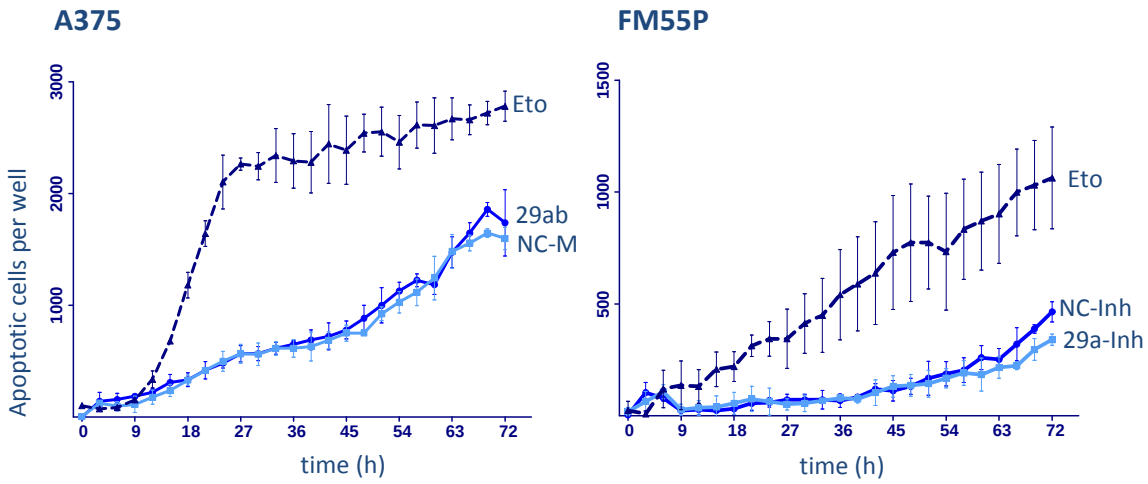


Fig. 32: Apoptosis assay of miR-29a/b-transfected A375 cells and miR-29a-inhibitor-transfected FM55P cells

Caspase-3 activity was monitored in Incucyte over 72h for miR-29a/b-transfected cells (29ab) in comparison to NC-mimic (NC-M)-transfected A375 cells (left) and for miR-29a-inhibitor (29a-Inh) in comparison to NC-inhibitor (NC-Inh)-transfected FM55P-cells (right). Graphs show the number of apoptotic cells per well (fluorescent cells with a fluorescence intensity above the background) as counted by the Incucyte software. Graphs show a representative replicate out of biological triplicates for each cell line, error bars show SD of technical triplicates. Etoposide (Eto) was used as positive control for apoptosis.

5.4 The quest for miR-29a/b target genes

MiR-29 is predicted to regulate more than 1000 human genes (TargetScanHuman 6.1). To further elucidate the role of the miR-29 family in melanoma, a combination of several algorithms (TargetScanHuman 6.1, Diana-microT v3.0, and miRanda) was consulted in order to compile a list of potentially interesting target genes, which carry predicted miR-29 target sites and could play a role in melanoma development and progression. As the three mature miR-29 species share their seed sequence and the remaining mature sequences are very similar, the lists of target genes for miR-29a, -29b and -29c can be considered identical. Venn diagram analysis of the three target gene lists identified 97 targets as commonly predicted by the three programs (Fig. 33).

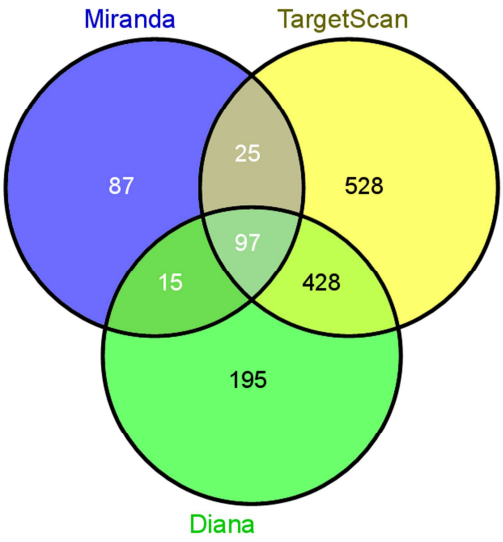


Fig. 33: Venn diagram of potential miR-29 target genes as predicted by Diana, miRanda and TargetScan
The three programs were requested to search for miR-29a target genes. Venn diagram analysis (http://bioinfogp.cnb.csic.es/tools/venny/index.html) identified 97 target genes, which had been predicted by all three of the programs.

To shorten this list of potential miR-29 target genes, the candidates were screened for their potential relevance in melanoma development and the list was expanded by several more candidates from literature analysis. Furthermore, this initial selection was cross-checked with data from a previous mRNA microarray experiment, which had been performed on a panel of five untreated melanoma cell lines (Master Thesis Project, Stefanie Schmitz). Expression analysis revealed that most of the potential target genes were either not expressed in the majority of cell lines or only showed a very low basal expression (data not shown). Based on this information, ten potential target genes were chosen for further tests (Fig. 33). The selection of potential miR-29 target genes is shown in Table 7.

Table 7: Selection of potential miR-29 target genes selected for further analysis
(descriptions taken from http://www.ncbi.nlm.nih.gov/gene)

Gene	Name	description
Akt3	v-akt murine thymoma viral oncogene homolog 3	Member of the Akt kinase family, which regulate cell signaling in response to insulin and growth factors
Bcl-2	B-cell CLL/lymphoma 2	Integral outer mitochondrial membrane protein that blocks apoptotic death of several cells (f.e. lymphocytes)
Cdc42	Cell division control protein 42 homolog	Small GTPase of the Rho-subfamily, involved in cell morphology, migration etc.
CDK6	cyclin-dependent kinase 6	Important player in cell cycle; phosphorylates RB, which subsequently leads to G1/S transition
Dicer1		Endoribonuclease, involved in miRNA processing
DNMT3A DNMT3B	DNA (cytosine-5-)-methyltransferase A/B	Catalyzes transfer of methyl group to DNA ➔ involved in gene silencing
Mcl-1	myeloid cell leukemia sequence 1	Member of Bcl-2 family; 2 isoforms: one enhanced cell survival, while the other one promotes apoptosis
PI3KR1	Phosphoinositide-3-kinase regulatory subunit	Subunit of PI3K, which is a signaling molecule for growth factors and hydroxylates phosphatidylinositols; involved in cell growth. proliferation etc.
PTEN	Phosphatase and tensin homolog	Tumor suppressor; counteracts PI3K; negatively regulates the Akt-pathway

To further reduce and verify this target gene selection, expression levels of the potential target genes were evaluated by RT-qPCR 24h after transfection with NC-mimic as this represents the approximate control for the subsequent miR-29a/b-mimic-transfections (Fig. 34).

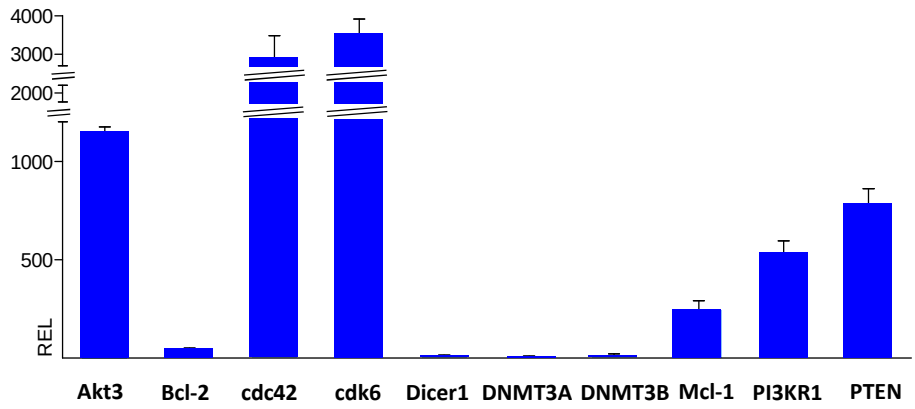


Fig. 34: Expression of potential target genes in A375 cells after NC-mimic-transfection
Basal expression level of target genes in A375 cells 24h after transfection of 100 nM NC-(negative control) mimic. Graphs show relative expression level with REL = $2^{(Ct\ target\ -\ Ct\ TBP)}$ x 10^2 with SD of technical triplicates. Akt3, cdc42, CDK6, Mcl-1, PI3KR1 and PTEN show a high or moderate basal expression while Bcl-2, Dicer1 and DNMT3A/B only exhibit a low expression in A375 cells.

Indeed, some of the target genes (DNMTA/B, Bcl-2, Dicer1) had very low basal expression levels whereas others (CDK6, cdc42, Akt3) were comparatively stronlgy expressed. Subsequently, the miR-29a/b mimic was applied to A375 cells to examine whether tentative target genes could be down-regulated (Fig. 35).

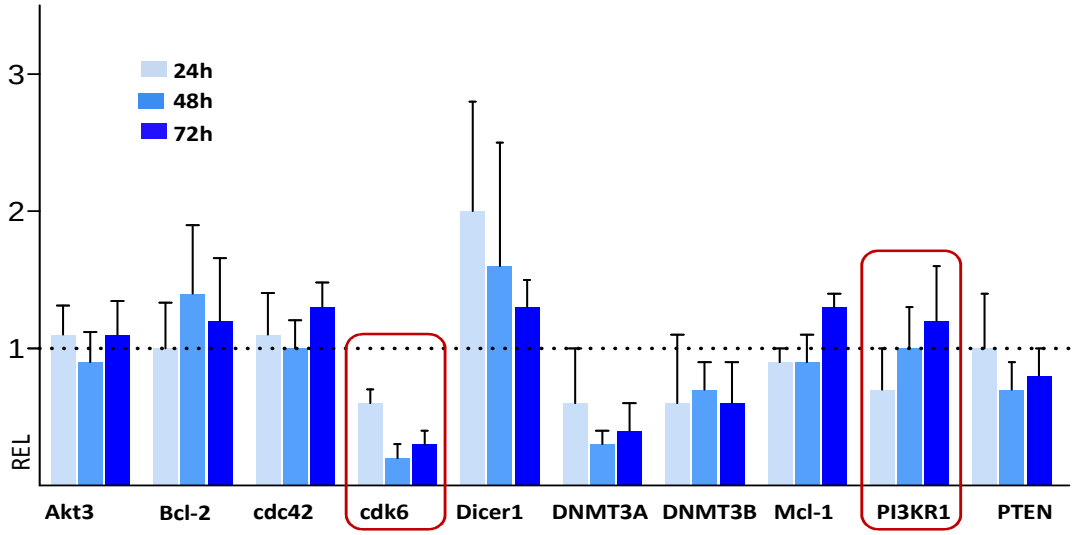


Fig. 35: Expression levels of target genes in A375 cells following miR-29a/b mimic transfection

mRNA levels were assessed 24h, 48h and 72h after transfection of 50nM each miR-29a and miR-29b mimic and were normalized to NC-mimic controls (REL-values were obtained by dividing target gene mRNA levels after mimic treatment by target gene mRNA levels of the corresponding NC-mimic-treated cells). Bars show mean of technical replicates with the propagated error.

mRNA levels of Akt3, Bcl-2, cdc42 remained unchanged after miR-29a/b mimic treatment, whereas a modest down-regulation was observed for PI3KR1 and PTEN (Fig. 35). Interestingly, Dicer1 and Mcl-1 were up-regulated, while a clear down-regulation was monitored for CDK6, DNMT3A and DNMT3B. The PI3K regulatory subunit (gene: PI3KR1; protein: PI3K/p85α) and CDK6 were selected for further analyses. Bcl-2, Dicer1 and DNMT3A/B showed a very low basal expression level in A375 cells (Fig. 34), hence a miR-29-mediated down-regulation would unlikely be of particular importance in the cellular context and therefore they were not considered any further. CDK6 had a high basal expression level, which was clearly reduced after mimic-transfection, while PI3KR1 exhibited a moderate basal expression and only a minor reaction to the treatment (Fig. 34, Fig. 35). Both genes play important roles in cell cycle control, cellular signaling and proliferation and had already been confirmed as miR-29 targets in several cancers (Garzon *et al.* 2009b; Park *et al.* 2009b; Zhao *et al.* 2010; Li *et al.* 2011), however not for melanoma.

MiR-29a/29b down-regulate CDK6, but not PI3K

[2]To assess the effect of miR-29 on CDK6 and PI3K expression in melanoma, mRNA and protein levels were examined after miR-29 mimic or inhibitor treatments by qRT-PCR and quantitative immunoblotting, respectively (Fig. 36).

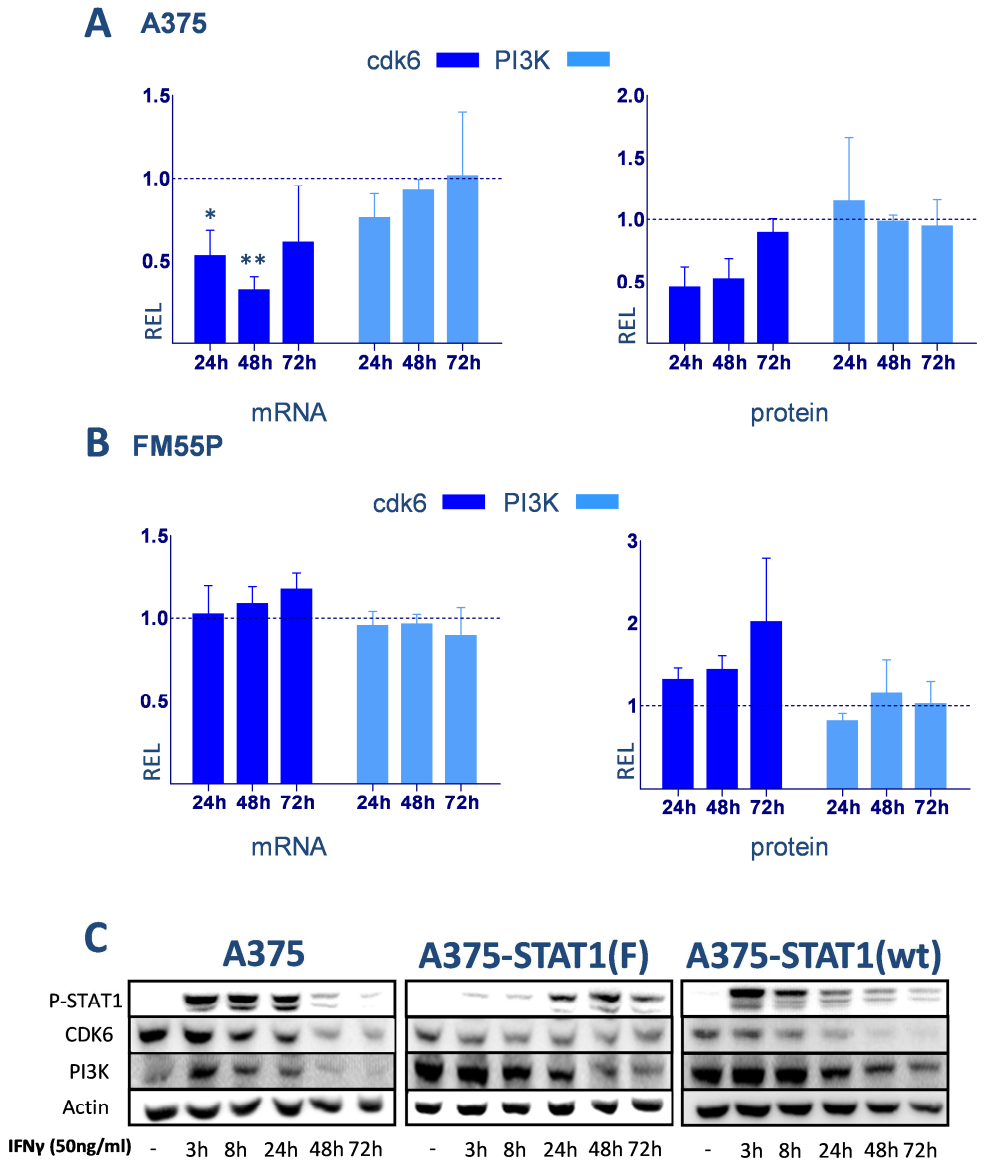


Fig. 36: Effects of miR-29 on potential target genes CDK6 and PI3K
(A,B) [2]relative mRNA and protein expression levels (REL) of miR-29 target genes CDK6 (dark blue) and PI3K (light blue), assessed 24 h, 48 h and 72 h after mimic/inhibitor transfection compared to NC-mimic/NC-inhibitor controls (REL-values were obtained by dividing target gene mRNA/protein levels after mimic/inhibitor treatment by target gene mRNA/protein levels of the corresponding NC-mimic/NC-inhibitor treated cells); bars show means of biological triplicates with SD. Statistical significance was assessed by one-way ANOVA followed by a Bonferroni Post-Hoc test. (C) Down-regulation of miR-29 target proteins CDK6 and PI3K is observed after IFN-γ stimulation of melanoma cells.

[2]Combined transfection of miR-29a/29b reduced CDK6 mRNA and protein levels in A375 cells as compared to scrambled controls whereas PI3K levels were not affected (Fig. 36A). [2]In agreement with that, knockdown of miR-29a in FM55P cells resulted in a slight up-regulation of CDK6 levels while PI3K remained unchanged (Fig. 36B). These data indicate that miR-29 is involved in down-regulation of CDK6 protein while PI3K does not seem to be a specific target in melanoma cells. Upon analysis of protein levels, CDK6 was found to be down-regulated in response to miR-29 induction after IFN-γ stimulation in A375 cells and A375-STAT1(wt) but not in A375-STAT1(F) cells, suggesting STAT1 dependency (Fig. 36C). In contrast, PI3K levels were reduced in all three cell lines, hinting at STAT1-independent effects.

CDK6 is a direct target of miR-29a

To determine whether a direct interaction between target genes and miR-29 exist, we performed luciferase assays with reporter constructs containing a part of the CDK6 3´-UTR, its three single miR-29 binding sites as predicted by TargetScan (www.targetscan.org), a part of the PI3KR1-3´-UTR or the miR-29a full complementary sequence as a positive control (Fig. 37). Luciferase activity dropped by ~60 % for both time points, 48 h and 72 h, when the CDK6 3´-UTR construct was co-transfected with miR-29a mimic in A375 melanoma cells. [2]The corresponding single binding sites contributed to this suppression with 38 % (BS1), 34 % (BS2) and 35 % (BS3), though only 72 h after transfection (Fig. 37). [2]This suggests that all three miR-29 binding sites partake in the suppression of CDK6. [2]Surprisingly, the PI3KR1 construct was also significantly suppressed by the miR-29a mimic in luciferase assays (Fig. 37) while only marginal effects had been observed on mRNA and protein level (Fig. 36). [2]Taken together, these findings indicate that both CDK6 and PI3KR1 3´-UTRs are directly targeted by miR-29 in melanoma cells; however, only CDK6 suppression seems to be relevant in a cellular context. Because of these overall inconsistent results for PI3KR1, we continued our experiments only with CDK6.

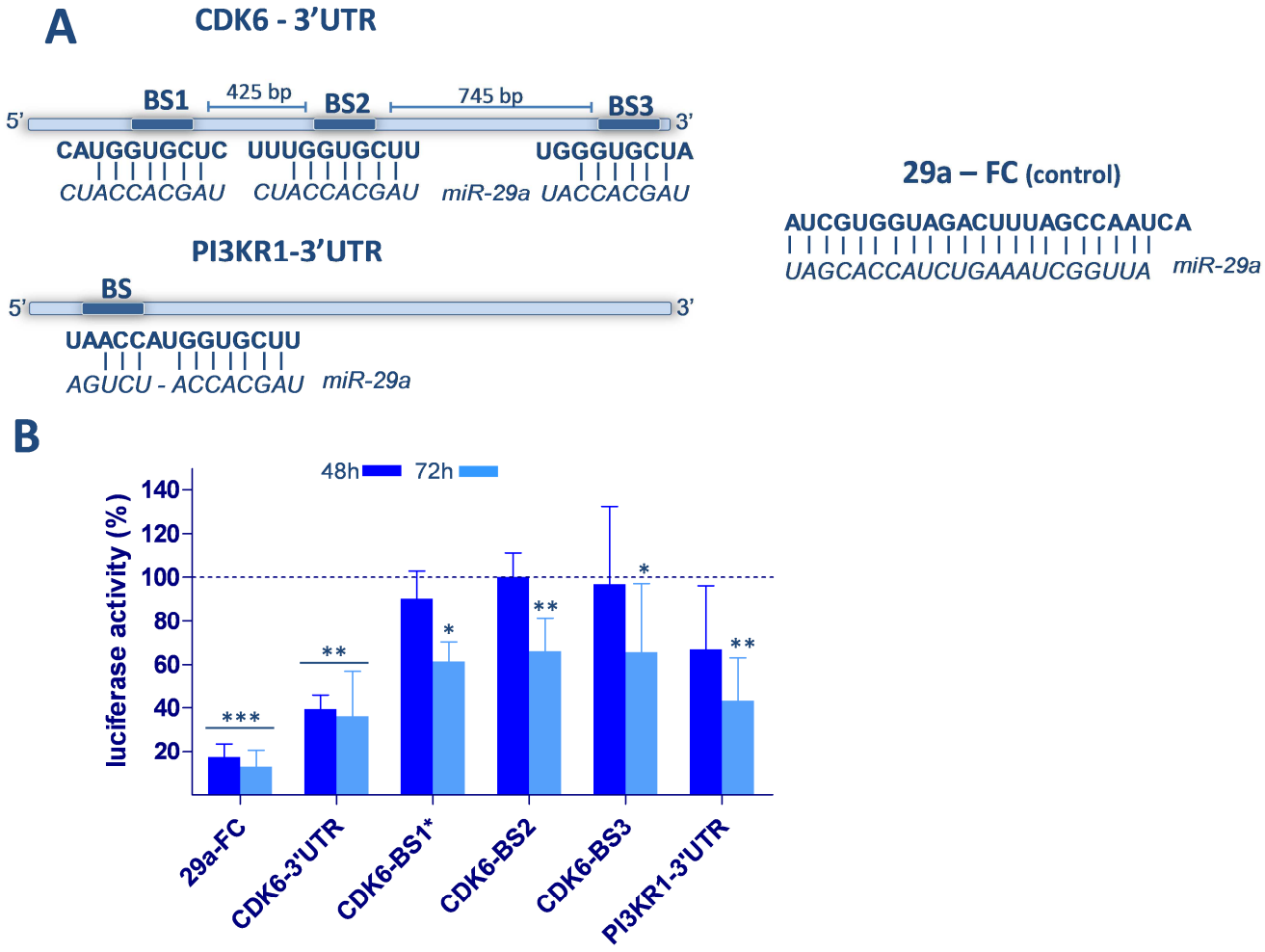


Fig. 37: Luciferase assay experiments for 3'-UTR sequences of predicted miR-29 target genes CDK6 and PI3KR1

(A) Schematic overview of CDK6 and PI3KR1 luciferase constructs with positions of conserved miR-29a binding sites predicted by TargetScan (bold) in the CDK6-3'UTR (BS1-3) and in the PI3KR1-3'UTR (BS) and corresponding miR-29a sequences (italics). (B) Luciferase activity in A375 cells transfected with constructs containing the positive control miR-29a full complementary sequence (29a-FC), parts of CDK6- or PI3K1-3'UTRs or CDK6 single binding sites (BS1-BS3) and miR-29a mimic or the same amount of negative control for 48 h and 72 h. Relative luciferase activity (as calculated by the ratio of firefly and renilla activities) of miR-29a-transfected samples was normalized to NC-mimic-transfected control samples (with luciferase activity of miR-29a-transfected samples divided by NC-mimic-transfected samples = 1 set to 100%). Bars show the mean of biological triplicates with SD, relative to NC-mimic controls for each construct. Statistical significance was tested with one-way ANOVA, followed by a Bonferroni Post-Hoc test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

The direct miR-29 target CDK6 regulates the growth behavior in melanoma cells

[2]To further explore the relevance of reduced CDK6 levels for the cell, we used siRNA against CDK6 and assessed proliferation over 72h in A375 and FM55P cells (Fig. 39). Reduction of CDK6 mRNA and protein levels led to a clearly diminished proliferation in both cell lines. Efficient knockdown of CDK6 was confirmed by qRT-PCR and Western blotting (Fig. 38).

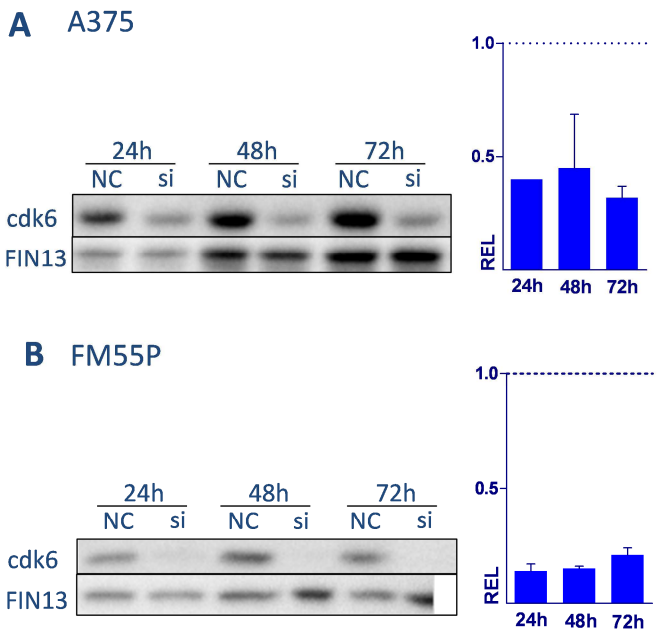


Fig. 38: Tracking of CDK6 mRNA and protein levels after si-RNA transfection
[2]To confirm efficient knock-down by CDK6-siRNA, CDK6 expression was tracked in (A) A375 and (B) FM55P cells by Western blot (left) and qRT-PCR (right), which both confirm efficient knockdown of CDK6. For qRT-PCR, expression levels of CDK6 were assessed 24h, 48h and 72h after siRNA transfection; bars show means of biological triplicates with SD relative to negative control.

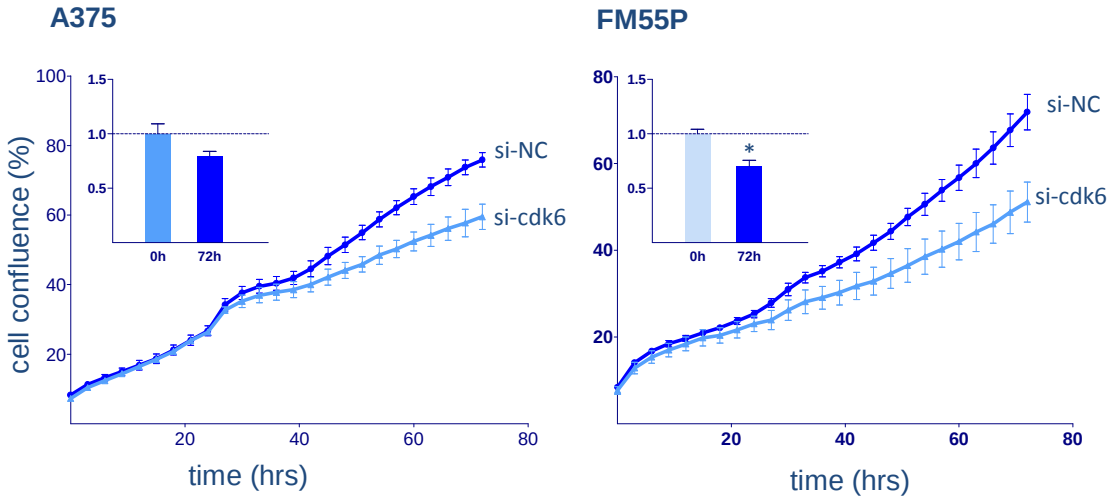


Fig. 39: Effects of siRNA-mediated knockdown of CDK6 on proliferation of melanoma cells
A375 (left) and FM55P (right) cells transfected with CDK6 siRNA (si-cdk6, light blue) show reduced proliferation in comparison to cells transfected with siRNA negative control (si-NC, blue). Results were reproduced in at least two biological replicates. [2]The inserted bar diagrams (upper left corners) show the mean confluence of at least 3 biological replicates at 0h and 72h time points of the proliferation assay. Bars show ratios of confluence of si-CDK6 / si-NC; with SEM. Significance was assessed by a two-tailed t-test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

MiR-29a and miR-29b are up-regulated in primary melanoma patient samples

[2]MiR-29a/29b expression profiles were analyzed in FFPE melanoma patient samples from normal skin, nevi, primary and metastatic melanoma by qRT-PCR (Fig. 40). [2]Nevi represent the most appropriate control samples as they contain predominantly melanocytes while normal skin samples are mostly composed of keratinocytes. [2]In comparison to healthy skin and nevi, both miR-29a and miR-29b showed an up-regulation in primary melanoma samples whereas in metastatic tumors, expression levels were only slightly enhanced compared to healthy controls. [2]Closer sub-classification of the patient samples revealed, however, that only two of five patients demonstrated the enhanced miR-29a/29b expression, indicating that expression levels are heterogeneous and will have to be assessed in larger patient cohorts.

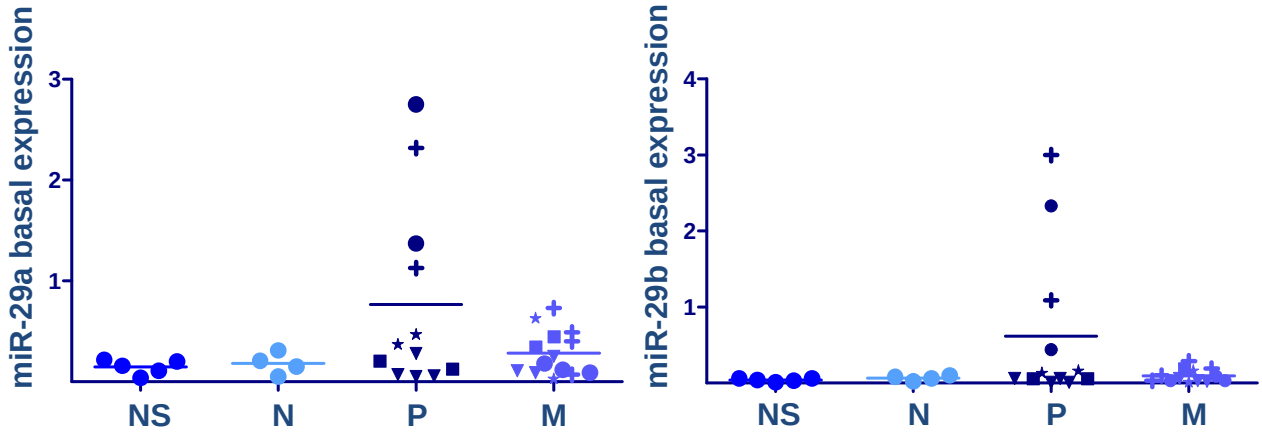


Fig. 40: [2]miR-29 expression in FFPE melanoma patient samples
[2]Analysis of miR-29a (left) and miR-29b (right) basal expression of individual FFPE-patient samples from NS = normal skin, N = nevi, P = primary melanoma and M = metastatic melanoma of a total number of five patients. [2]All graphs show $2^{-\Delta ct}$ with $\Delta ct = (ct_{miR\text{-}29a/29b} - ct_{RNU5A})$. [2]Primary and metastatic tumor samples were sorted according to patients P1-5: P1-circles, P2-rectangles, P3-crosses, P4-triangles, P5-asterisks.

6 Discussion

Regulation of miRNA expression. The broad impact of miRNAs on the initiation, development and maintenance of cancer and other diseases but also their possible use as therapeutic compounds or targets has become increasingly evident over the past decade. Substantial experimental efforts have been undertaken to reveal miRNA sequences, their modes of actions and their roles in cellular homeostasis and malignancies. Along this line it became soon obvious that it is of particular importance to figure out how miRNAs themselves are being regulated in order to further understand miRNA-cancer-pathways and to be able to efficiently manipulate the abundance of these small non-coding RNA molecules. Generally, expression levels of miRNAs can be regulated transcriptionally, by epigenetic silencing, differential biosynthesis or different turnover times (Bartel 2004; Bueno *et al.* 2008; Krol *et al.* 2010b). Moreover, miRNAs have also been shown to be regulated by extracellular stimuli, e.g. cytokines like interferons (Pedersen *et al.* 2007).

Cytokines play a pivotal role in the immune system by communicating cellular responses to inflammation and infections (O'Shea and Murray 2008). Nearly all cell types can come into contact with cytokines such as IFN-γ, which is mainly secreted by activated T and NK cells and which represents the most important Th1 (T helper type 1)– cytokine in innate and adaptive immunity to infection (Schroder *et al.* 2004; Saha *et al.* 2010). Generally, cytokines can activate transcription factors, subsequently leading to transcription of target genes including miRNAs. IFN-γ primarily triggers the JAK/STAT pathway resulting in a STAT1-mediated transcriptional activation of target genes. The importance of interactions between miRNAs and STAT transcription factors has very recently been summarized in a review (Kohanbash and Okada 2012). Classical and well-described examples for miRNAs with connection to STATs are miR-21 (Löffler *et al.* 2007) and miR-155 (Kutty *et al.* 2010), which both are regulated by STAT factors, while miR-29 represents a miRNA family which had not been associated to STATs before.

Connecting the miR-29 family to Jak-STAT signaling. In this PhD project, the miR-29a~29b-1 primary cluster as well as mature miR-29a/b were found to be specifically and dose-dependently up-regulated after IFN-γ stimulation of melanoma, HEK and T-cells (Fig. 17, Fig. 19, Fig. 22). IFN-γ stimulation of a control cell line expressing dominant-negative STAT1 (A375-STAT1(F)) did not cause an up-regulation of miR-29, providing strong evidence that STAT1 is indeed mediating IFN-γ-induced effects on miR-29 expression levels (Schmitt *et al.* 2012b) (Fig. 17). This finding was further strengthened by an experiment with a specific Jak inhibitor which abrogated STAT1 activation (Reinsbach *et al.* 2012).

The miR-29 family is one of the first ones described, is highly conserved among species and the increasing number of publications on single family members emphasizes their important role in many cellular processes of cancer biology. Along with miR-34, miR-15~16 and members of the let-7 family, miR-29 is currently the most prominent representative of miRNAs exhibiting predominantly tumor-suppressive properties (Calin *et al.* 2002; Garzon *et al.* 2009b; Hermeking 2009; Buechner *et al.* 2011; Zhang *et al.* 2011b) and as reviewed in the course of this PhD project (Schmitt *et al.* 2012a).

In general, human miRNAs, especially the majority of tumor suppressor miRNAs, are located in fragile regions of the genome and often show loss of heterozygosity in cancer (Calin *et al.* 2004). However, miR-29 genes are encoded in chromosomal locations, which do not experience frequent genomic rearrangements (Douglass *et al.* 1985; Barr 2001). [1]This suggests that transcriptional regulatory mechanisms are responsible for modulation of miR-29 expression levels rather than deletions, translocations or mutations. In line with our findings about the STAT1-dependent up-regulation of the miR-29a~29b-1 cluster, the contribution of other transcription factors to control miR-29 expression has been demonstrated. Transcriptional up-regulation of miR-29a~29b-1 was mediated by the transcription factor CEBPα (CCAAT/enhancer-binding protein alpha) (Eyholzer *et al.* 2010). In addition, miR-29a and miR-29c were up-regulated by canonical Wnt-signaling and SMAD3 (mothers against decapentaplegic homolog 3) induced miR-29b expression (Kapinas *et al.* 2009; Villarreal *et al.* 2011). On the contrary, the miR-29a~29b-1 cluster was found to be negatively regulated by c-myc, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and hedgehog signaling (with involvement of Gli as the responsible transcription factor) in different cell lines (Chang *et al.* 2008; Mott *et al.* 2010; Ma *et al.* 2011a). Taken together, it is likely that the transcriptional regulation of the miR-29 clusters is mediated by a combination of various transcription factors. Furthermore, STAT1 collaborating transcription factors like SP-1 (specificity protein 1), AP-1 (activator protein 1) or CEBP (CCAAT/enhancer-binding protein) presumably also play a role in the regulation process (Zhou *et al.* 1998; Wang *et al.* 2010). In this context it is noteworthy that several miRNAs in addition to miR-29 were IFN-γ-induced in our experiments and could play a role for the cellular context. For example, miR-155 was among the miRNAs, which were up-regulated upon IFN-γ stimulation (Reinsbach *et al.* 2012). Interestingly, SOCS1 has been shown to be directly targeted by miR-let7a/b and miR-155 in hepatic stellate and human hepatoma cells, respectively (Su *et al.* 2011; Meng *et al.* 2012). Thus, IFN-γ-induced miR-155 expression might contribute to IFN-γ signaling by prolonging the STAT1 signal due to inhibition of SOCS1.

Within this PhD project, STAT1-dependent up-regulation of the miR-29a~29b-1 cluster was reported, while miR-29b-2~29c was not IFN-γ-induced (Fig. 20). The results on miR-29a~29b-1

were further corroborated by *in silico* analyses: A combination of computational and experimental evidence identified two STAT1 binding sites as likely candidates for miR-29a~29b-1-regulation inside a putative miR-29a~29b-1 promoter region containing several potential transcription start sites (Fig. 26). However, although several types of transcriptional regulation on the miR-29a~29b-1 cluster have been experimentally elucidated, disagreement about the corresponding transcription start sites continues to exist (Fig. 25). A considerable number of studies has contributed to the investigation of the transcription start sites of both miR-29 clusters by computational and experimental methods (Saini *et al.* 2007; Chang *et al.* 2008; Marson *et al.* 2008; Saini *et al.* 2008; Corcoran *et al.* 2009; Mott *et al.* 2010; Chien *et al.* 2011) (www.switchgeargenomics.com). For the miR-29b-2~29c cluster, current data do not allow for a clear assignment of a TSS. However, for miR-29a~29b-1, the position of the most likely TSS could here be narrowed down to a 700 bp region around four predicted and partly experimentally validated TSSs which most likely contains the 'real' transcription start sites (Schmitt *et al.* 2012a) (Fig. 25). Possible reasons for the discrepancies in the reported TSSs could be the existence of multiple TSSs and/or differences due to the diverse experimental and computational methods that have been applied. [1]Another source of variation may result from the use of different genome versions and GenBank assemblies causing variation of coordinates within the published data.

MiR-29 family members exhibit deviating expression levels in different cellular contexts. Although both transcriptional activation and repression have been reported for miR-29a~29b-1 regulation, the vast majority of publications report a down-regulation of miR-29 or its single members in cancer (Calin *et al.* 2005; Yanaihara *et al.* 2006; Eyholzer *et al.* 2010). These reduced expression levels could be confirmed within this project by screening a panel of melanoma cell lines for different miR-29 species and family members, which revealed almost no expression of the pri-29b-2~c cluster and a much higher basal miR-29a than miR-29b level (Fig. 27). In tumor cells, reduced miR-29 expression is frequently observed and diminished expression of miRNAs in general is often associated with enhanced oncogenesis (Henry *et al.* 2011; Nguyen *et al.* 2011). The difference in pri-29a~b-1 and pri-29b-2~c expression levels, which has been detected here, is consistent with the results in other types of cancer, in which the pri-29b-2~c cluster was mostly down-regulated (Pekarsky *et al.* 2006; Wang *et al.* 2008; Stamatopoulos *et al.* 2009). Also the revative expression levels of human mature forms of miR-29a, miR-29b and miR-29c are divergent in different tissues (Sempere *et al.* 2004).

Apart from differential regulation, miRNA stability could influence these expression differences: it can range from several hours (Bail *et al.* 2010) to weeks (van Rooij *et al.* 2007). For example Xiong *et al.* observed that transfected miR-29b duplexes were only stable for 4-7 days in HepG2

cells and were not detectable any more four weeks after transfection in nude mice (Xiong *et al.* 2010). An even more rapid decay (8-12 hours) for miR-29 family members has been reported by others (Hwang *et al.* 2007; Zhang *et al.* 2011c). In addition to those findings, another explanation for miR-29b regulation has been suggested by Hwang *et al.* who found miR-29a highly expressed throughout the cell cycle, while miR-29b only exhibited high levels during mitosis (Hwang *et al.* 2007). Considering the large amount of time which cells spend for the whole cell cycle in contrast to mitosis, this could be a possible explanation for the enhanced miR-29a/miR-29b ratio found by us and others.

The tumor-suppressing properties of the miR-29 family. The fact that miR-29 family members are often not expressed in cancer cells could be crucial for cancer cell growth: miR-29 down-regulates important genes such as CDC42, TCL-1 and MCL-1, which normally confer tumor-suppressing traits. In this context, anti-proliferating as well as anti-invasive and pro-apoptotic effects have been observed after miR-29 introduction into a variety of cancer cells (Fig. 41) (Xiong *et al.* 2010; Fang *et al.* 2011). Fig. 41 summarizes reported findings of miR-29 functions in different cancer types.

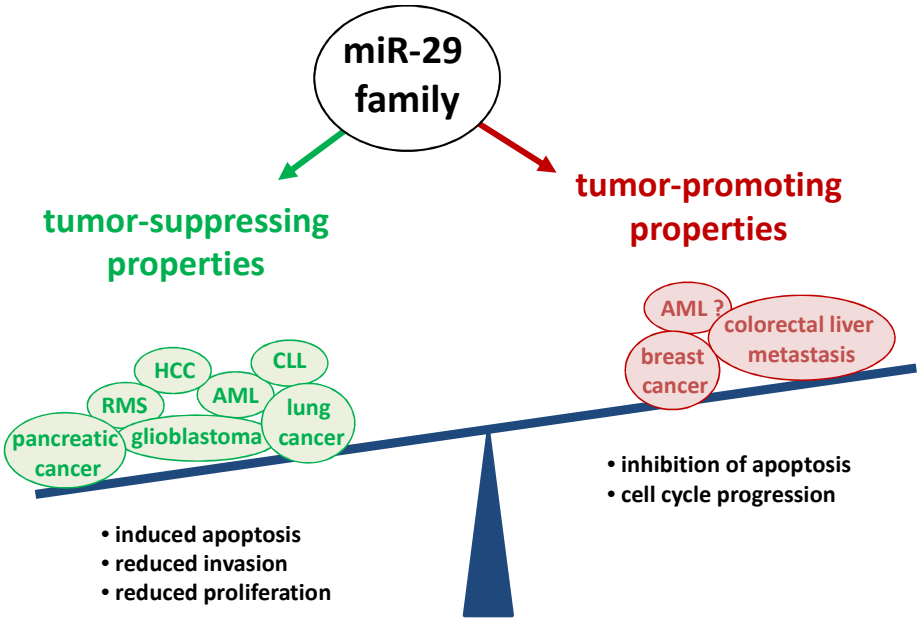


Fig. 41: Types of cancer in which miR-29 family members have been shown to exert tumor suppressive or oncogenic properties
Tumor-suppressing properties have been demonstrated (among others) for RMS (rhabdomyosarcoma) (Wang *et al.* 2008), HCC (hepatocellular carcinoma) (Xiong *et al.* 2010; Fang *et al.* 2011), AML (acute myeloid leukemia) (Eyholzer *et al.* 2010), CLL (chronic lymphocytic leukemia) (Santanam *et al.* 2010), glioblastoma (Cortez *et al.* 2010) and lung and pancreatic cancer (Muniyappa *et al.* 2009) whereas tumor-promoting properties have been observed for AML (Han *et al.* 2010), colorectal liver metastasis (Wang and Gu 2011) and breast cancer (Gebeshuber *et al.* 2009; Wang *et al.* 2011a).

Along this line, an inverse correlation between miR-29a/b expression and the proliferation rate of various melanoma cell lines has been observed in this project, which was corroborated in miR-29 mimic or -inhibitor transfected cells, whereas the pro-apoptotic effects could not be confirmed reliably (Fig. 27, Fig. 28, Fig. 32). These anti-proliferative effects of miR-29a/b on melanoma cell lines furthermore agree with current literature on miR-29 properties. As mentioned above, the tumor-suppressor functions which have been demonstrated for miR-29 family members in different cell lines mostly include induction of apoptosis as well as a reduction of proliferation and invasion (Muniyappa *et al.* 2009; Cortez *et al.* 2010). Importantly, *in vivo* experiments in mice further confirmed these findings: re-expression of otherwise silenced miR-29 inhibited tumor growth of rhabdomyosarcoma (Wang *et al.* 2008) and reduced tumor formation by HCC (hepatocellular carcinoma) cells (Xiong *et al.* 2010) along with a decreased invasive potential, angiogenesis and metastasis in HCC-transplanted nude mice (Fang *et al.* 2011).

In contrast to the majority of studies highlighting tumor-suppressive properties (Fig. 41), up-regulation of miR-29b in highly metastatic breast cancer cells in comparison to low-metastatic types together with enhanced migration and invasion as well as increased resistance to apoptosis has been observed (Wang *et al.* 2011a). Also, elevated levels of miR-29a in patients with invasive breast carcinomas in comparison to benign samples from patients with non-invasive hyperplasia have been reported (Gebeshuber *et al.* 2009). Interestingly and in line with these augmented levels of miR-29 in breast cancer and AML (Han *et al.* 2010), we have made similar observations in melanoma patient samples: miR-29a and 29b were up-regulated in primary melanoma relative to healthy nevi (Philippidou *et al.* 2010). Similarly, analysis of primary and metastatic melanoma patient samples during this PhD project revealed increased miR-29a/29b expression in some primary tumor samples in comparison to normal skin, nevi and metastatic tissue while all metastatic lesions had low levels of these miRNAs (Schmitt *et al.* 2012b) (Fig. 40). Possibly, IFN-γ, which is produced by macrophages and/or T cells and NK-cells as a start of a signaling cascade and first line of defense against the developing tumors, induces miR-29 expression via STAT1, which in turn could act as tumor-suppressing miRNA counteracting manifestation of the cancer at early stages. The fact that miR-29a/29b were only up-regulated in two out of five primary melanoma patients is striking and requires further evaluation in a larger panel of patient samples including early neoplasia and advanced metastatic stages. Surprisingly, a recent study on cutaneous melanoma reported unchanged miR-29a and-29b expression levels while miR-29c was down-regulated in metastatic melanoma in comparison to primary tumor samples. However, no healthy control tissue or nevi were included in this study, hampering the interpretation of the results (Nguyen *et al.* 2011). Apart from the noteworthy exceptions in AML and breast cancer as mentioned above and in the report

on primary melanoma by Nguyen *et al.*, miR-29 family members have consistently and predominantly been reported to be down-regulated and to assume tumor-suppressive properties in cancers.

CDK6 and other miR-29 target genes. The importance of miR-29 regulation has been addressed above, however the role of miR-29 family members in cancer is mainly determined by their respective target genes. As for all miRNAs, the identification and analysis of mir-29 target genes in various tissues and malignancies is of obvious importance. Down-regulation of several important cellular proteins by miR-29 family members has been reported, among others the DNA-methyltransferases DNMT3A and B (Fabbri *et al.* 2007a; Garzon *et al.* 2009b; Hand *et al.* 2012). [1]DNMTs are involved in DNA-methylation of CpG islands causing epigenetic silencing of the corresponding genes. [1]Interestingly, DNMT3 is frequently up-regulated in various malignancies such as hepatoma, lung, prostate, colorectal and breast cancer (Eads *et al.* 1999; Patra *et al.* 2002; Girault *et al.* 2003; Saito *et al.* 2003; Kim *et al.* 2006), which emphasizes the importance of this link between DNMTs and reduced miR-29 expression.

[1]Other miR-29 target genes include extracellular matrix proteins such as collagens, de-regulation of which can favor cancer growth and progression (Sengupta *et al.* 2008; van Rooij *et al.* 2008; Maurer *et al.* 2010). Also the matrix-metalloprotease MMP-2 (Liu *et al.* 2010; Fang *et al.* 2011), elastin and fibrillin 1 (van Rooij *et al.* 2008), laminin γ1 (Sengupta *et al.* 2008) and integrin beta 1 (Liu *et al.* 2010) have been confirmed as miR-29 target genes, which all have recently been discussed in a review by Kriegel *et al.* and summarized by us (Kriegel *et al.* 2012; Schmitt *et al.* 2012a). An overview on miR-29 target genes is shown in the Appendix.

In this PhD thesis, CDK6 was for the first time confirmed as a direct miR-29 target in melanoma cells (Fig. 36, Fig. 37) (Schmitt *et al.* 2012b). It was furthermore demonstrated that knockdown of CDK6 expression resulted in reduced proliferation (Fig. 39) as it had been shown for other types of cancer before (Ismail *et al.* 2011; Whiteway *et al.* 2012). This suggests, that the inhibition of proliferation which was observed in miR-29a/b-mimic-transfected cells may largely be mediated via CDK6. CDK6 plays a pivotal role in control of G1/S cell cycle transition (Fig. 42) (Grossel and Hinds 2006) and loss thereof is a common event in neoplastic growth (Musat *et al.* 2004). The special relevance of CDK6 activity for melanoma growth is emphasized by the fact that the gene which encodes tumor suppressor $p16^{INK4A}$ (an inhibitor of CDK6 and CDK4), which is transcribed from the CDKN2A melanoma susceptibility locus, is deleted in about 50% of melanoma patients (Hussussian *et al.* 1994; Bennett 2008) (Table 2, Fig. 42). This supports the tumor-suppressing functions of miR-29a/b and attributes obvious novel functions to miR-29 as CDK6 inhibitor in melanoma. It moreover suggests the hypothesis that enhanced miR-29a/b levels, which were up-regulated in some primary melanoma patient samples could take over the

tumor-suppressing properties of $p16^{INK4A}$ by inhibiting CDK6, even if the locus is deleted, subsequently preventing uncontrolled cell cycle progression (Fig. 43).

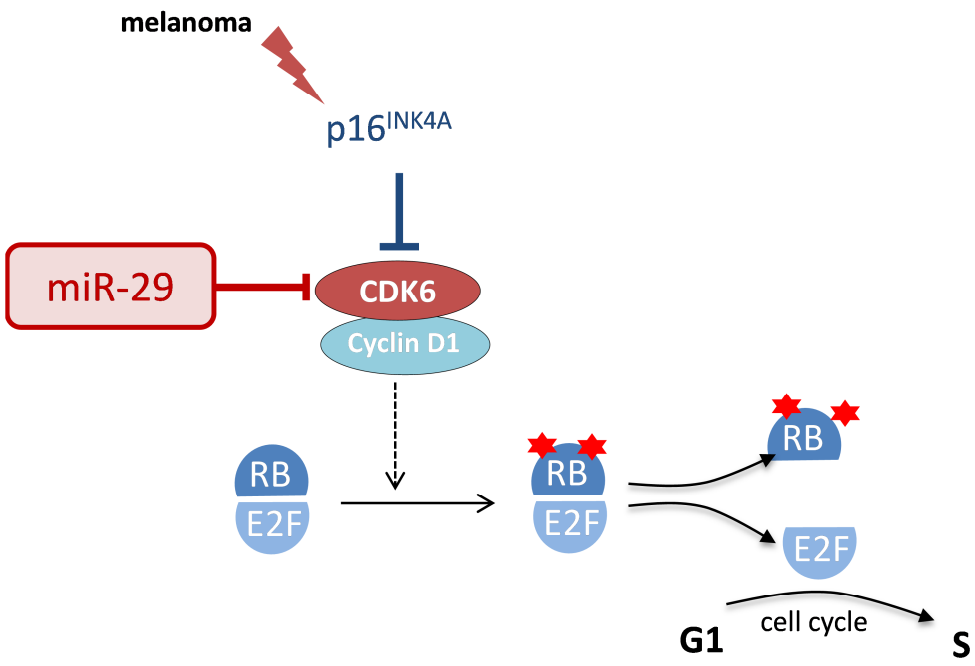


Fig. 42: Interplay of miR-29 and CDK6
The $p16^{INK4A}$ gene is encoded within the CDKN2A melanoma susceptibility locus and shows often mutations or deletions in melanoma (indicated by the red bolt). The $p16^{INK4A}$ protein acts as negative regulator of cell cycle progression by inhibiting the activation of CDK6, which is complexed with Cyclin D1 during G1/S-phase of the cell cycle and can phosphorylate RB. If unphosphorylated, RB binds to the transcription factor E2F, thereby hindering it from expression of genes necessary for G1 → S cell cycle transition. The latter is accomplished as soon as Rb and E2F dissociate after phosphorylation of Rb via Cyclin D1/CDK4/CDK6. miR-29 inhibits CDK6 in melanoma cells and could therefore act as a tumor suppressor. For reasons of clarity, this illustration only focuses on miR-29/ $p16^{INK4A}$ /CDK6/RB/E2F and does not show the CDKN2A melanoma susceptibility locus as well as $p14^{ARF}$, p53, CDK4 and other participating proteins.

Noteworthy, CDK6 has also been demonstrated to be a direct miR-29 target in mantle cell lymphoma (Zhao *et al.* 2010), acute myeloid leukemia (Garzon *et al.* 2009b) and cervical cancer (Li *et al.* 2011). Interestingly, the anti-proliferative effects of IFN-γ in many cancers (Garbe and Krasagakis 1993; Kortylewski *et al.* 2004; Mori *et al.* 2008) which have also been confirmed here (Fig. 30) can be explained by an attenuated G1/S transition and may in part be explained by a G1 arrest involving down-regulation of G1/S cyclins (Cyclins A and E) and CDK2/4 (Kortylewski *et al.* 2004). Here we add CDK6 as another cyclin-dependent kinase which is involved in IFN-γ-mediated anti-proliferative effects. These anti-proliferative activities of IFN-γ and miR-29 as well as a STAT1-dependent induction of miR-29 expression following IFN-γ stimulation have been demonstrated in melanoma cells within this PhD project (Fig. 18, Fig. 20,

Fig. 27, Fig. 28, Fig. 30). A series of proliferation assays including combined IFN-γ/miR-29a inhibitor treatment indicated that the growth-inhibitory effects of miR-29a could be partially responsible for IFN-γ-mediated inhibition of proliferation in melanoma cells which, however, could not be confirmed reliably (Fig. 31). This was probably due to too much stress for the cells, which were suffering from the double treatment (IFN-γ stimulation plus miR-29 inhibitor transfection). Concerning a connection between IFN-γ and melanoma, at a first glance it seems questionable how IFN-γ as a cytokine can come into direct contact with melanocytes located in the outer layer of the skin. However, this has been recently addressed in an elegant study by Zaidi *et al.* who have shown that IFN-γ-producing macrophages are recruited to the UV-exposed skin, where they stimulate proliferation and migration of melanocytes as well as expression of genes implicated in immunoevasion and survival. Furthermore, analysis of 27 human melanoma samples confirmed that 19 contained IFN-γ-expressing macrophages (Zaidi *et al.* 2011). A scheme summarizing the key events is shown in Fig. 43.

miR-29 and its importance for interferon signaling. Other reports have previously connected individual miRNAs to interferon signaling: for example, miR-155 was shown to inhibit IFN-γ-signaling in $CD4^+$ T-cells (Banerjee *et al.* 2010) and miR-29a suppressed interferon-α-receptor expression in the thymic epithelium (Papadopoulou *et al.* 2012), implying that IFN signaling in general may actively partake in the regulation of miRNA expression levels in cells.
Moreover, the role of cytokines as inducers of miRNA expression has recently been proposed in several studies and examples for cytokine-induced miRNA up- or down-regulation range from pro-inflammatory signaling molecules like TNF-α and IL1-β (Roggli *et al.* 2010; Ruan *et al.* 2012) to different types of interferons, which are of special interest as they are central players in tumor-immune system interactions (Dunn *et al.* 2006; Diamond *et al.* 2011). Pedersen *et al.* analyzed changes of miRNA expression levels in human hepatoma cells after IFN-β stimulation, and interestingly found two miRNAs to respond already within 30 min to the cytokine (Pedersen *et al.* 2007), while others exhibited a retarded response as it was mostly observed in this project. We have shown here that also IL-27 as well as IFN-α/β induced miR-29.
[2]The concept of 'cancer immunosurveillance', defined as the immunological protection of a host against the development of cancer, has evoked much interest during the last decade: Mediated by the host's immune system, it is triggered by immune recognition of stress ligands or antigens expressed on transformed cells.
IFN-γ has long been recognized for its crucial role in defense against viral and bacterial infections as well as in tumor control (Dunn *et al.* 2006; Schreiber *et al.* 2011) and this PhD project provides evidence for a novel IFN-γ - miR-29 connection via STAT1, which has also been confirmed in T-cells (Fig. 23). These findings can be embedded in the context of already known

connections between interferons and miRNAs and the important regulatory role for miR-29 in the adaptive immune system which has only been unraveled during the past two years, as was recently reviewed (Liston *et al.* 2012; Schmitt *et al.* 2012a). In accordance with our findings in melanoma and T-cells, Smith et al. showed that IFN-γ-induced STAT1 leads to enhanced miR-29a~b-1 expression in murine and human T-cells with a maximal peak of miR-29a/b expression at 48-72h. Moreover, IFN-γ itself (Ma *et al.* 2011a; Smith *et al.* 2012) as well as the transcription factors T-bet (Steiner *et al.* 2011; Smith *et al.* 2012) and Eomes (Eomesodermin = T-box brain protein 2) (Steiner *et al.* 2011), known inducers of the IFN-γ-gene, have recently been confirmed to be direct miR-29 targets, thus suggesting a negative regulation of IFN-γ expression by miR-29. These findings indicate that a regulatory loop could exist with IFN-γ inducing miR-29 expression via STAT1 and miR-29 subsequently repressing IFN-γ directly as well as indirectly by down-regulating the IFN-γ-inducing transcription factors T-bet and Eomes (Fig. 43).

In addition to the newly discovered properties in melanoma, the herein described IFN-γ/miR-29 regulatory loop also reveals new perspectives for the understanding of immunological host defense: interestingly, miR-29a/29b have both been reported to target the HIV-protein nef (negative regulatory factor) (Hariharan *et al.* 2005), which down-regulates cell surface molecules like CD4 and MHCI and II (Kirchhoff *et al.* 2008), thereby enhancing HIV infectivity and replication. These findings imply another potential regulatory line of events: IFN-γ, secreted rapidly in response to HIV infection (Twigg *et al.* 1999), triggers transcriptional activation of miR-29 that in turn down-regulates nef and thus may participate in host control of early HIV infection. This connection has been explored in preliminary experiments and IFN-γ-induced up-regulation of miR-29a and miR-29b could be confirmed in T-cells, which can be infected by HIV-1 (data not shown). Together with these findings, the overall results obtained in this PhD thesis can be described as a regulatory circuit: IFN-γ, which is e.g. secreted by macrophages following diverse assaults such as infections or UV light induces a STAT1-dependent up-regulation of miR-29, which in turn can down-regulate IFN-γ directly or indirectly via T-bet and Eomes (Fig. 43).

This study extends the current knowledge on the miRNA family miR-29, adding a novel regulatory loop involving IFN-γ-mediated Jak/STAT signaling in melanoma cells. The newly discovered signaling pathway of IFN-γ → P-STAT1 → miR-29a/b → down-regulation of CDK6 → inhibition of tumor growth points at new connections between the immune system, miRNAs, cell cycle control and potentially tumorigenesis, which could lead to novel concepts for future therapeutic approaches. Currently, melanoma patient samples are characterized for the well-known mutations in BRAF, NRAS and KIT genes (Berger *et al.* 2012) or newly discovered genetic changes in MITF (Yokoyama *et al.* 2012). To further tailor personalized and targeted therapy options in the future, evaluation of selected miRNA expression levels could be an add-

on to the routine clinical analysis. Optimal would surely be the use as biomarkers from blood as a non-invasive approach. For miR-29 however, this could lead to difficulties because at least in cell lines and tissue samples, it exhibits expression changes in most cancer types, as reported above.

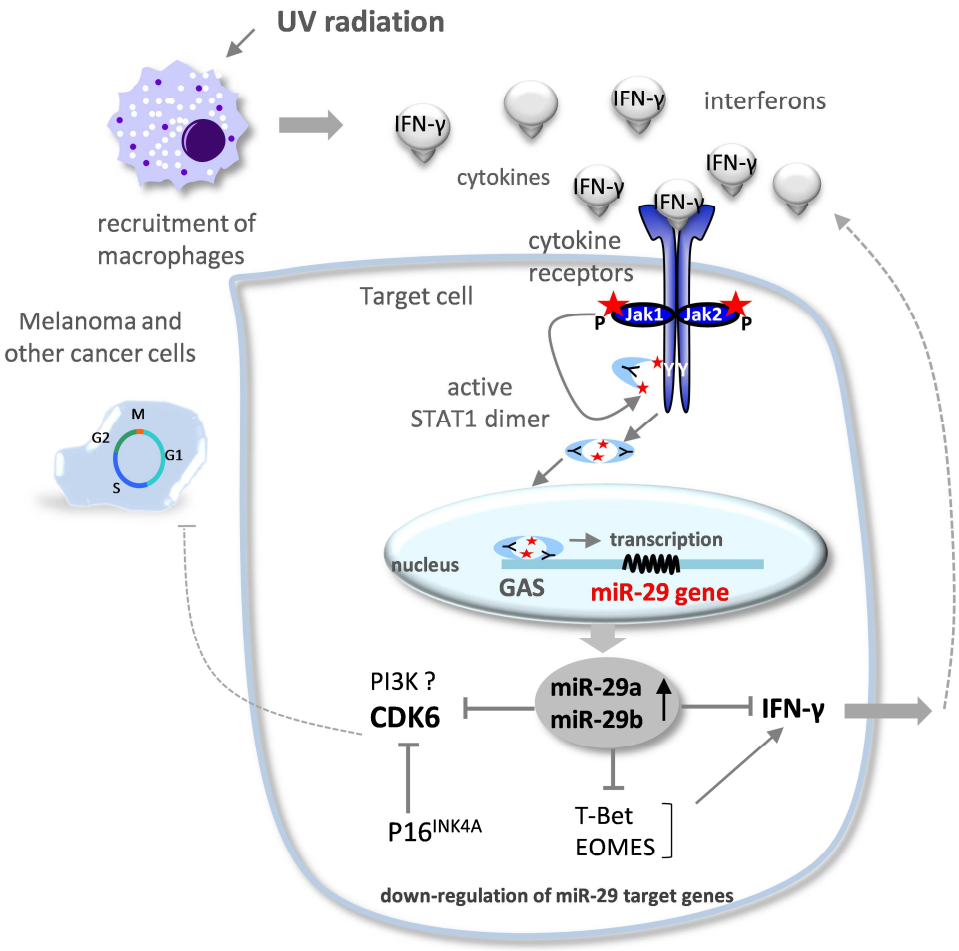


Fig. 43: Involvement of the miR-29 family in multiple cellular processes
[2]UV-radiation triggers the recruitment of macrophages to the skin, which secrete cytokines like interferon gamma (IFN-γ). [2]By binding to its receptors, IFN-γ signals through the Jak/STAT pathway triggering subsequent activation of STAT1, which then binds to GAS-elements in the promoter region of target genes and initiates their transcription. [2]IFN-γ-induced, STAT1-dependent up-regulation of miR-29 causes a down-regulation of CDK6, a novel miR-29 target gene in melanoma, which plays a crucial role in G1/S-cell cycle transition and thus growth control of cancer cells. The genetic locus of the cell cycle inhibitor $p16^{INK4A}$ shows often deletions or mutations in melanoma and its function (inhibition of CDK6) might be compensated by miR-29a/b. [2]IFN-γ-activating transcription factors T-bet and Eomes and IFN-γ itself are also targeted by miR-29.

Furthermore, in a pilot biomarker study on melanoma samples, miR-29 had not been detected among the de-regulated miRNAs (Leidinger *et al.* 2010). Concerning therapeutic treatment approaches, miR-29 application in form of miR-29-mimicking oligonucleotides seems to have an effect on tumor growth *in vivo* in some cancer types. For example Fabbri *et al.* validated the

down-regulation of DNMTs 3A and 3B by all miR-29 family members and demonstrated reduced tumor sizes in mice engrafted with lung cancer cells that had been transfected with single miR-29 family members (Fabbri *et al.* 2007a). The same group reported similar findings for AML and suggested the use of synthetic miR-29b as therapeutic agent (Garzon *et al.* 2009b).

This PhD project has deciphered a previously unknown regulatory pathway around the miR-29 family with importance for melanoma development. With its often tumor-suppressing functions, the miR-29 family lends itself to be explored further in future cancer therapy. However, before this becomes a feasible treatment option, we need to acquire a more complete systems-biological view of the complex interactions of the miR-29 family, including the regulation of miR-29 gene expression with respect to transcription factors, complete characterization of melanoma-specific target genes, and the interplay of miR-29 with other miRNAs.

7 Perspectives

To further validate the STAT1 regulation of the pri-29a/b1 cluster, the potential STAT1 binding sites should be experimentally verified by ChIP analysis in melanoma cells. For the investigation of the cellular consequences of miR-29 up- or down-regulation, we examined proliferation and apoptosis in miR-29-mimic or inhibitor-transfected melanoma cell lines. To further follow up on this functional role of miR-29 in cancer-related processes, more functional assays (such as migration and invasion assays and cell cycle analysis) could be performed. Knowing the precise mechanisms and functional role of the miR-29 family members would be a pre-requisite to evaluate potential therapeutic approaches involving this miRNA family.

Apart from the miR-29 family, on which the main focus was set after the first year of the PhD project, many other interesting miRNAs turned out to be potentially STAT1-regulated. For example miRNAs from the miR-23/27/24 clusters (chromosome 9 and 19) seem interesting because of their distinct regulation patterns and could be investigated further.

Although this PhD project focused on IFN-γ as a well-known inducer of a prominent STAT1-response, we also tested IFN-α and IFN-β for stimulation in several initial setup experiments for their ability to induce STAT1-dependent miRNAs. Follow-up studies could be performed especially using IFN-α as IFN-α 2B is still used for treatment of melanoma patients, but not much is known about its regulatory role on miRNAs. It would be interesting to further investigate whether different STAT factors induce specific sets of miRNAs or whether there is redundancy between the various STATs.

Moreover, we also examined the miR-29a/b expression in FFPE patient material. Unfortunately, only mature miRNAs can be amplified from the total RNA of these samples whereas amplification of mRNAs or primary miRNA molecules is generally not possible. Thus, it would be important to analyze the miR-29a/b level as well as the expression of the primary and precursor molecules of the miR-29 tumor-suppressor family in a larger number of patients, ideally from 'fresh-frozen' nevi, primary and metastatic samples. CDK6-expression could also be addressed. In this context, our group recently started a project in which we examine melanoma tumour material together with the corresponding blood samples from the same patients. The aim of the project is to investigate a melanoma-connected miRNA signature in blood and it would be interesting to find out if members of the miR-29 family belong to those circulating miRNAs. Finally, the results obtained during the course of this PhD thesis around the newly discovered pathway along IFN-γ → P-STAT1 → miR-29 → CDK6 should also be investigated in other types of cancer.

8 Literature

Aaronson, D.S. and Horvath, C.M. 2002. A road map for those who don't know JAK-STAT. *Science* **296**(5573): 1653-1655.

Ahluwalia, J.K., Khan, S.Z., Soni, K., Rawat, P., Gupta, A., Hariharan, M., Scaria, V., Lalwani, M., Pillai, B., Mitra, D. *et al.* 2008. Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology* **5**: 117.

Ambros, V. 2003. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* **113**(6): 673-676.

AmericanCancerSociety. 2012. Cancer Facts & Figures 2012, www.cancer.org, accessed December 2012.

Anastasiadou, E., Boccellato, F., Vincenti, S., Rosato, P., Bozzoni, I., Frati, L., Faggioni, A., Presutti, C., and Trivedi, P. 2010. Epstein-Barr virus encoded LMP1 downregulates TCL1 oncogene through miR-29b. *Oncogene* **29**(9): 1316-1328.

Ascierto, P.A., Kirkwood, J.M., Grob, J.J., Simeone, E., Grimaldi, A.M., Maio, M., Palmieri, G., Testori, A., Marincola, F.M., and Mozzillo, N. 2012. The role of BRAF V600 mutation in melanoma. *J Transl Med* **10**(1): 85.

Bail, S., Swerdel, M., Liu, H., Jiao, X., Goff, L.A., Hart, R.P., and Kiledjian, M. 2010. Differential regulation of microRNA stability. *RNA* **16**(5): 1032-1039.

Balch, C.M. and Soong, S.J. 2008. Predicting outcomes in metastatic melanoma. *J Clin Oncol* **26**(2): 168-169.

Bandyopadhyay, D., Okan, N.A., Bales, E., Nascimento, L., Cole, P.A., and Medrano, E.E. 2002. Down-regulation of p300/CBP histone acetyltransferase activates a senescence checkpoint in human melanocytes. *Cancer Res* **62**(21): 6231-6239.

Banerjee, A., Schambach, F., DeJong, C.S., Hammond, S.M., and Reiner, S.L. 2010. Micro-RNA-155 inhibits IFN-gamma signaling in CD4+ T cells. *Eur J Immunol* **40**(1): 225-231.

Barr, F.G. 2001. Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma. *Oncogene* **20**(40): 5736-5746.

Bartel, D. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**(2): 215-233.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**(2): 281-297.

Bell, R.E. and Levy, C. 2011. The three M's: melanoma, microphthalmia-associated transcription factor and microRNA. *Pigment Cell Melanoma Res* **24**(6): 1088-1106.

Bemis, L.T., Chen, R., Amato, C.M., Classen, E.H., Robinson, S.E., Coffey, D.G., Erickson, P.F., Shellman, Y.G., and Robinson, W.A. 2008. MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. *Cancer Res* **68**(5): 1362-1368.

Bene, N.I., Healy, C., and Coldiron, B.M. 2008. Mohs micrographic surgery is accurate 95.1% of the time for melanoma in situ: a prospective study of 167 cases. *Dermatol Surg* **34**(5): 660-664.

Benevolenskaya, E.V. 2007. Histone H3K4 demethylases are essential in development and differentiation. *Biochem Cell Biol* **85**(4): 435-443.

Bennett, D.C. 2008. How to make a melanoma: what do we know of the primary clonal events? *Pigment Cell Melanoma Res* **21**(1): 27-38.

Berger, M.F., Hodis, E., Heffernan, T.P., Deribe, Y.L., Lawrence, M.S., Protopopov, A., Ivanova, E., Watson, I.R., Nickerson, E., Ghosh, P. *et al.* 2012. Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature* **485**(7399): 502-506.

Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V., and Hannon, G.J. 2003. Dicer is essential for mouse development. *Nat Genet* **35**(3): 215-217.

Betel, D., Wilson, M., Gabow, A., Marks, D.S., and Sander, C. 2008. The microRNA.org resource: targets and expression. *Nucleic Acids Res* **36**(Database issue): D149-153.

Boisson-Dupuis, S., Kong, X.F., Okada, S., Cypowyj, S., Puel, A., Abel, L., and Casanova, J.L. 2012. Inborn errors of human STAT1: allelic heterogeneity governs the diversity of immunological and infectious phenotypes. *Curr Opin Immunol* **24**(4): 364-378.

Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R., and Stark, G.R. 2007. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* **6**(12): 975-990.

Botchkareva, N.V. 2012. MicroRNA/mRNA regulatory networks in the control of skin development and regeneration. *Cell Cycle* **11**(3): 468-474.

Boyle, P., Maisonneuve, P., and Dore, J.F. 1995. Epidemiology of malignant melanoma. *Br Med Bull* **51**(3): 523-547.

Breslow, A. 1970. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg* **172**(5): 902-908.

Brock, M., Trenkmann, M., Gay, R.E., Michel, B.A., Gay, S., Fischler, M., Ulrich, S., Speich, R., and Huber, L.C. 2009. Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. *Circ Res* **104**(10): 1184-1191.

Brodersen, P. and Voinnet, O. 2009. Revisiting the principles of microRNA target recognition and mode of action. *Nat Rev Mol Cell Biol* **10**(2): 141-148.

Broekaert, S.M., Roy, R., Okamoto, I., van den Oord, J., Bauer, J., Garbe, C., Barnhill, R.L., Busam, K.J., Cochran, A.J., Cook, M.G. *et al.* 2010. Genetic and morphologic features for melanoma classification. *Pigment Cell Melanoma Res* **23**(6): 763-770.

Buechner, J., Tomte, E., Haug, B.H., Henriksen, J.R., Lokke, C., Flaegstad, T., and Einvik, C. 2011. Tumour-suppressor microRNAs let-7 and mir-101 target the proto-oncogene MYCN and inhibit cell proliferation in MYCN-amplified neuroblastoma. *Br J Cancer* **105**(2): 296-303.

Bueno, M.J., Perez de Castro, I., Gomez de Cedron, M., Santos, J., Calin, G.A., Cigudosa, J.C., Croce, C.M., Fernandez-Piqueras, J., and Malumbres, M. 2008. Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. *Cancer Cell* **13**(6): 496-506.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. *et al.* 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**(4): 611-622.

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K. *et al.* 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* **99**(24): 15524-15529.

Calin, G.A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S.E., Iorio, M.V., Visone, R., Sever, N.I., Fabbri, M. *et al.* 2005. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med* **353**(17): 1793-1801.

Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M. *et al.* 2004. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A* **101**(9): 2999-3004.

Cantley, L.C. 2002. The phosphoinositide 3-kinase pathway. *Science* **296**(5573): 1655-1657.

Carleton, M., Cleary, M.A., and Linsley, P.S. 2007. MicroRNAs and cell cycle regulation. *Cell Cycle* **6**(17): 2127-2132.

Chang, T.C. and Mendell, J.T. 2007. microRNAs in vertebrate physiology and human disease. *Annu Rev Genomics Hum Genet* **8**: 215-239.

Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., and Mendell, J.T. 2008. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* **40**(1): 43-50.

Chapman, P.B., Hauschild, A., Robert, C., Haanen, J.B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M. *et al.* 2011. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**(26): 2507-2516.

Chekulaeva, M. and Filipowicz, W. 2009. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol* **21**(3): 452-460.

Chen, T., Li, Z., Tu, J., Zhu, W., Ge, J., Zheng, X., Yang, L., Pan, X., Yan, H., and Zhu, J. 2011. MicroRNA-29a regulates pro-inflammatory cytokine secretion and scavenger receptor expression by targeting LPL in oxLDL-stimulated dendritic cells. *FEBS Lett* **585**(4): 657-663.

Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, J.E., Jr., and Kuriyan, J. 1998. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. *Cell* **93**(5): 827-839.

Chen, Z.H., Zhang, G.L., Li, H.R., Luo, J.D., Li, Z.X., Chen, G.M., and Yang, J. 2012. A panel of five circulating microRNAs as potential biomarkers for prostate cancer. *Prostate* **72**(13): 1443-1452.

Cheon, H., Yang, J., and Stark, G.R. 2011. The functions of signal transducers and activators of transcriptions 1 and 3 as cytokine-inducible proteins. *J Interferon Cytokine Res* **31**(1): 33-40.

Chien, C.H., Sun, Y.M., Chang, W.C., Chiang-Hsieh, P.Y., Lee, T.Y., Tsai, W.C., Horng, J.T., Tsou, A.P., and Huang, H.D. 2011. Identifying transcriptional start sites of human microRNAs based on high-throughput sequencing data. *Nucleic Acids Res* **39**(21): 9345-9356.

Cho, E., Rosner, B.A., and Colditz, G.A. 2005a. Risk factors for melanoma by body site. *Cancer Epidemiol Biomarkers Prev* **14**(5): 1241-1244.

Cho, E., Rosner, B.A., Feskanich, D., and Colditz, G.A. 2005b. Risk factors and individual probabilities of melanoma for whites. *J Clin Oncol* **23**(12): 2669-2675.

Claudinon, J., Monier, M.N., and Lamaze, C. 2007. Interfering with interferon receptor sorting and trafficking: impact on signaling. *Biochimie* **89**(6-7): 735-743.

Collas, P. 2010. The current state of chromatin immunoprecipitation. *Mol Biotechnol* **45**(1): 87-100.

Corcoran, D.L., Pandit, K.V., Gordon, B., Bhattacharjee, A., Kaminski, N., and Benos, P.V. 2009. Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. *PLoS One* **4**(4): e5279.

Cornejo, M.G., Boggon, T.J., and Mercher, T. 2009. JAK3: a two-faced player in hematological disorders. *Int J Biochem Cell Biol* **41**(12): 2376-2379.

Cortez, M.A., Nicoloso, M.S., Shimizu, M., Rossi, S., Gopisetty, G., Molina, J.R., Carlotti, C., Jr., Tirapelli, D., Neder, L., Brassesco, M.S. *et al.* 2010. miR-29b and miR-125a regulate podoplanin and suppress invasion in glioblastoma. *Genes Chromosomes Cancer* **49**(11): 981-990.

Costinean, S., Zanesi, N., Pekarsky, Y., Tili, E., Volinia, S., Heerema, N., and Croce, C.M. 2006. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci U S A* **103**(18): 7024-7029.

Cronin, J.C., Wunderlich, J., Loftus, S.K., Prickett, T.D., Wei, X., Ridd, K., Vemula, S., Burrell, A.S., Agrawal, N.S., Lin, J.C. *et al.* 2009. Frequent mutations in the MITF pathway in melanoma. *Pigment Cell Melanoma Res* **22**(4): 435-444.

Cui, Y., Su, W.Y., Xing, J., Wang, Y.C., Wang, P., Chen, X.Y., Shen, Z.Y., Cao, H., Lu, Y.Y., and Fang, J.Y. 2011. MiR-29a inhibits cell proliferation and induces cell cycle arrest through the downregulation of p42.3 in human gastric cancer. *PLoS One* **6**(10): e25872.

Cummins, J.M., He, Y., Leary, R.J., Pagliarini, R., Diaz, L.A., Jr., Sjoblom, T., Barad, O., Bentwich, Z., Szafranska, A.E., Labourier, E. *et al.* 2006. The colorectal microRNAome. *Proc Natl Acad Sci U S A* **103**(10): 3687-3692.

Czech, B. and Hannon, G.J. 2011. Small RNA sorting: matchmaking for Argonautes. *Nat Rev Genet* **12**(1): 19-31.

Darnell, J.E., Jr. 1997. STATs and gene regulation. *Science* **277**(5332): 1630-1635.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W. *et al.* 2002. Mutations of the BRAF gene in human cancer. *Nature* **417**(6892): 949-954.

Davies, M.A. 2012. The role of the PI3K-AKT pathway in melanoma. *Cancer J* **18**(2): 142-147.

Davis-Dusenbery, B.N. and Hata, A. 2011. MicroRNA in Cancer: The Involvement of Aberrant MicroRNA Biogenesis Regulatory Pathways. *Genes Cancer* **1**(11): 1100-1114.

Decker, T., Kovarik, P., and Meinke, A. 1997. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. *J Interferon Cytokine Res* **17**(3): 121-134.

Diamond, M.S., Kinder, M., Matsushita, H., Mashayekhi, M., Dunn, G.P., Archambault, J.M., Lee, H., Arthur, C.D., White, J.M., Kalinke, U. *et al.* 2011. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med* **208**(10): 1989-2003.

Douglass, E.C., Green, A.A., Hayes, F.A., Etcubanas, E., Horowitz, M., and Wilimas, J.A. 1985. Chromosome 1 abnormalities: a common feature of pediatric solid tumors. *J Natl Cancer Inst* **75**(1): 51-54.

Dunn, G.P., Koebel, C.M., and Schreiber, R.D. 2006. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* **6**(11): 836-848.

Dupuis, S., Dargemont, C., Fieschi, C., Thomassin, N., Rosenzweig, S., Harris, J., Holland, S.M., Schreiber, R.D., and Casanova, J.L. 2001. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. *Science* **293**(5528): 300-303.

Dusheiko, G. 1997. Side effects of alpha interferon in chronic hepatitis C. *Hepatology* **26**(3 Suppl 1): 112S-121S.

Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Danenberg, P.V., and Laird, P.W. 1999. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res* **59**(10): 2302-2306.

Ebert, M.S. and Sharp, P.A. 2012. Roles for microRNAs in conferring robustness to biological processes. *Cell* **149**(3): 515-524.

Eckardstein, S.G.v., ed. 2009. *Biochemie und Molekularbiologie des Menschen*. Elsevier.

El Ghissassi, F., Baan, R., Straif, K., Grosse, Y., Secretan, B., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Freeman, C., Galichet, L. *et al.* 2009. A review of human carcinogens--part D: radiation. *Lancet Oncol* **10**(8): 751-752.

Esau, C., Kang, X., Peralta, E., Hanson, E., Marcusson, E.G., Ravichandran, L.V., Sun, Y., Koo, S., Perera, R.J., Jain, R. *et al.* 2004. MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem* **279**(50): 52361-52365.

Esquela-Kerscher, A. and Slack, F.J. 2006. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* **6**(4): 259-269.

Euskirchen, G.M., Rozowsky, J.S., Wei, C.L., Lee, W.H., Zhang, Z.D., Hartman, S., Emanuelsson, O., Stolc, V., Weissman, S., Gerstein, M.B. *et al.* 2007. Mapping of transcription factor binding regions in mammalian cells by ChIP: comparison of array- and sequencing-based technologies. *Genome Res* **17**(6): 898-909.

Eyholzer, M., Schmid, S., Wilkens, L., Mueller, B.U., and Pabst, T. 2010. The tumour-suppressive miR-29a/b1 cluster is regulated by CEBPA and blocked in human AML. *Br J Cancer* **103**(2): 275-284.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Alder, H., Costinean, S., Fernandez-Cymering, C. *et al.* 2007a. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A* **104**(40): 15805-15810.

Fabbri, M., Ivan, M., Cimmino, A., Negrini, M., and Calin, G.A. 2007b. Regulatory mechanisms of microRNAs involvement in cancer. *Expert Opin Biol Ther* **7**(7): 1009-1019.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. 2010. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* **79**: 351-379.

Fagerlund, R., Melen, K., Kinnunen, L., and Julkunen, I. 2002. Arginine/lysine-rich nuclear localization signals mediate interactions between dimeric STATs and importin alpha 5. *J Biol Chem* **277**(33): 30072-30078.

Faller, M. and Guo, F. 2008. MicroRNA biogenesis: there's more than one way to skin a cat. *Biochim Biophys Acta* **1779**(11): 663-667.

Fang, J.H., Zhou, H.C., Zeng, C., Yang, J., Liu, Y., Huang, X., Zhang, J.P., Guan, X.Y., and Zhuang, S.M. 2011. MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. *Hepatology* **54**(5): 1729-1740.

Fang, Z. and Rajewsky, N. 2011. The impact of miRNA target sites in coding sequences and in 3'UTRs. *PLoS One* **6**(3): e18067.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**(2): 102-114.

Flaherty, K.T., Puzanov, I., Kim, K.B., Ribas, A., McArthur, G.A., Sosman, J.A., O'Dwyer, P.J., Lee, R.J., Grippo, J.F., Nolop, K. *et al.* 2010. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* **363**(9): 809-819.

Friedman, R.C., Farh, K.K., Burge, C.B., and Bartel, D.P. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19**(1): 92-105.

Gabriely, G., Wurdinger, T., Kesari, S., Esau, C.C., Burchard, J., Linsley, P.S., and Krichevsky, A.M. 2008. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. *Mol Cell Biol* **28**(17): 5369-5380.

Galgano, A., Forrer, M., Jaskiewicz, L., Kanitz, A., Zavolan, M., and Gerber, A.P. 2008. Comparative analysis of mRNA targets for human PUF-family proteins suggests extensive interaction with the miRNA regulatory system. *PLoS One* **3**(9): e3164.

Garbe, C. and Blum, A. 2001. Epidemiology of cutaneous melanoma in Germany and worldwide. *Skin Pharmacol Appl Skin Physiol* **14**(5): 280-290.

Garbe, C. and Krasagakis, K. 1993. Effects of interferons and cytokines on melanoma cells. *J Invest Dermatol* **100**(2 Suppl): 239S-244S.

Garzon, R., Calin, G.A., and Croce, C.M. 2009a. MicroRNAs in Cancer. *Annu Rev Med* **60**: 167-179.

Garzon, R., Liu, S., Fabbri, M., Liu, Z., Heaphy, C.E., Callegari, E., Schwind, S., Pang, J., Yu, J., Muthusamy, N. *et al.* 2009b. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood* **113**(25): 6411-6418.

Gaur, A., Jewell, D.A., Liang, Y., Ridzon, D., Moore, J.H., Chen, C., Ambros, V.R., and Israel, M.A. 2007. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res* **67**(6): 2456-2468.

Gebeshuber, C.A., Zatloukal, K., and Martinez, J. 2009. miR-29a suppresses tristetraprolin, which is a regulator of epithelial polarity and metastasis. *EMBO Rep* **10**(4): 400-405.

Ginsberg, M., Czeko, E., Muller, P., Ren, Z., Chen, X., and Darnell, J.E., Jr. 2007. Amino acid residues required for physical and cooperative transcriptional interaction of STAT3 and AP-1 proteins c-Jun and c-Fos. *Mol Cell Biol* **27**(18): 6300-6308.

Girault, I., Tozlu, S., Lidereau, R., and Bieche, I. 2003. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. *Clin Cancer Res* **9**(12): 4415-4422.

Glud, M. and Gniadecki, R. 2012. MicroRNAs in the pathogenesis of malignant melanoma. *J Eur Acad Dermatol Venereol*.

Graves, P. and Zeng, Y. 2012. Biogenesis of mammalian microRNAs: a global view. *Genomics Proteomics Bioinformatics* **10**(5): 239-245.

Grossel, M.J. and Hinds, P.W. 2006. From cell cycle to differentiation: an expanding role for cdk6. *Cell Cycle* **5**(3): 266-270.

Haan, C. and Behrmann, I. 2007. A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. *J Immunol Methods* **318**(1-2): 11-19.

Haghikia, A., Missol-Kolka, E., Tsikas, D., Venturini, L., Brundiers, S., Castoldi, M., Muckenthaler, M.U., Eder, M., Stapel, B., Thum, T. *et al.* 2011. Signal transducer and activator of transcription 3-mediated regulation of miR-199a-5p links cardiomyocyte and endothelial cell function in the heart: a key role for ubiquitin-conjugating enzymes. *Eur Heart J* **32**(10): 1287-1297.

Han, J., Pedersen, J.S., Kwon, S.C., Belair, C.D., Kim, Y.K., Yeom, K.H., Yang, W.Y., Haussler, D., Blelloch, R., and Kim, V.N. 2009. Posttranscriptional crossregulation between Drosha and DGCR8. *Cell* **136**(1): 75-84.

Han, Y.C., Park, C.Y., Bhagat, G., Zhang, J., Wang, Y., Fan, J.B., Liu, M., Zou, Y., Weissman, I.L., and Gu, H. 2010. microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J Exp Med* **207**(3): 475-489.

Hand, N.J., Horner, A.M., Master, Z.R., Boateng, L.A., Leguen, C., Uvaydova, M., and Friedman, J.R. 2012. MicroRNA Profiling Identifies miR-29 as a Regulator of Disease-associated Pathways in Experimental Biliary Atresia. *J Pediatr Gastroenterol Nutr* **54**(2): 186-192.

Hariharan, M., Scaria, V., Pillai, B., and Brahmachari, S.K. 2005. Targets for human encoded microRNAs in HIV genes. *Biochem Biophys Res Commun* **337**(4): 1214-1218.

Henry, J.C., Azevedo-Pouly, A.C., and Schmittgen, T.D. 2011. MicroRNA replacement therapy for cancer. *Pharm Res* **28**(12): 3030-3042.

Hermeking, H. 2009. The miR-34 family in cancer and apoptosis. *Cell Death Differ* **17**(2): 193-199.

Hildebrandt-Eriksen, E.S., Aarup, V., Persson, R., Hansen, H.F., Munk, M.E., and Orum, H. 2012. A locked nucleic acid oligonucleotide targeting microRNA 122 is well-tolerated in cynomolgus monkeys. *Nucleic Acid Ther* **22**(3): 152-161.

Howell, P.M., Jr., Li, X., Riker, A.I., and Xi, Y. 2010. MicroRNA in Melanoma. *Ochsner J* **10**(2): 83-92.

Hu, D.N. 2008. Methodology for evaluation of melanin content and production of pigment cells in vitro. *Photochem Photobiol* **84**(3): 645-649.

Huang, V. and Li, L.C. 2012. miRNA goes nuclear. *RNA Biol* **9**(3): 269-273.

Hudson, M.E. and Snyder, M. 2006. High-throughput methods of regulatory element discovery. *Biotechniques* **41**(6): 673, 675, 677 passim.

Hussussian, C.J., Struewing, J.P., Goldstein, A.M., Higgins, P.A., Ally, D.S., Sheahan, M.D., Clark, W.H., Jr., Tucker, M.A., and Dracopoli, N.C. 1994. Germline p16 mutations in familial melanoma. *Nat Genet* **8**(1): 15-21.

Hwang, H.W. and Mendell, J.T. 2006. MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br J Cancer* **94**(6): 776-780.

Hwang, H.W., Wentzel, E.A., and Mendell, J.T. 2007. A hexanucleotide element directs microRNA nuclear import. *Science* **315**(5808): 97-100.

Iliopoulos, D., Jaeger, S.A., Hirsch, H.A., Bulyk, M.L., and Struhl, K. 2010. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol Cell* **39**(4): 493-506.

Iorio, M. and Croce, C. 2012a. microRNA involvement in human cancer. *Carcinogenesis* **33**(6): 1126-1133.

Iorio, M.V. and Croce, C.M. 2012b. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med* **4**(3): 143-159.

Ismail, A., Bandla, S., Reveiller, M., Toia, L., Zhou, Z., Gooding, W.E., Kalatskaya, I., Stein, L., D'Souza, M., Litle, V.R. *et al.* 2011. Early G(1) cyclin-dependent kinases as prognostic markers and potential therapeutic targets in esophageal adenocarcinoma. *Clin Cancer Res* **17**(13): 4513-4522.

Jerke, U., Tkachuk, S., Kiyan, J., Stepanova, V., Kusch, A., Hinz, M., Dietz, R., Haller, H., Fuhrman, B., and Dumler, I. 2009. Stat1 nuclear translocation by nucleolin upon monocyte differentiation. *PLoS One* **4**(12): e8302.

Jin, L., Hu, W.L., Jiang, C.C., Wang, J.X., Han, C.C., Chu, P., Zhang, L.J., Thorne, R.F., Wilmott, J., Scolyer, R.A. *et al.* 2011. MicroRNA-149*, a p53-responsive microRNA, functions as an oncogenic regulator in human melanoma. *Proc Natl Acad Sci U S A* **108**(38): 15840-15845.

Jovanovic, M. and Hengartner, M.O. 2006. miRNAs and apoptosis: RNAs to die for. *Oncogene* **25**(46): 6176-6187.

Junqueira, Carneiro, and Gratzl. 2005. *Histologie*. Springer.

Kapinas, K., Kessler, C.B., and Delany, A.M. 2009. miR-29 suppression of osteonectin in osteoblasts: regulation during differentiation and by canonical Wnt signaling. *J Cell Biochem* **108**(1): 216-224.

Karagas, M.R., Stannard, V.A., Mott, L.A., Slattery, M.J., Spencer, S.K., and Weinstock, M.A. 2002. Use of tanning devices and risk of basal cell and squamous cell skin cancers. *J Natl Cancer Inst* **94**(3): 224-226.

Kasinski, A.L. and Slack, F.J. 2011. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev Cancer* **11**(12): 849-864.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., and Haussler, D. 2002. The human genome browser at UCSC. *Genome Res* **12**(6): 996-1006.

Kessler, D.S., Levy, D.E., and Darnell, J.E., Jr. 1988. Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. *Proc Natl Acad Sci U S A* **85**(22): 8521-8525.

Kim, H., Kwon, Y.M., Kim, J.S., Han, J., Shim, Y.M., Park, J., and Kim, D.H. 2006. Elevated mRNA levels of DNA methyltransferase-1 as an independent prognostic factor in primary nonsmall cell lung cancer. *Cancer* **107**(5): 1042-1049.

Kim, J., Inoue, K., Ishii, J., Vanti, W.B., Voronov, S.V., Murchison, E., Hannon, G., and Abeliovich, A. 2007. A MicroRNA feedback circuit in midbrain dopamine neurons. *Science* **317**(5842): 1220-1224.

Kim, V.N. 2005. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* **6**(5): 376-385.

Kim, V.N. and Nam, J.W. 2006. Genomics of microRNA. *Trends Genet* **22**(3): 165-173.

Kirchhoff, F., Schindler, M., Specht, A., Arhel, N., and Munch, J. 2008. Role of Nef in primate lentiviral immunopathogenesis. *Cell Mol Life Sci* **65**(17): 2621-2636.

Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C.W. 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* **285**(1-2): 1-24.

Knüpfer, M.M., Knüpfer, H., Jendrossek, V., Van Gool, S., Wolff, J.E., and Keller, E. 2001. Interferon-gamma inhibits growth and migration of A172 human glioblastoma cells. *Anticancer Res* **21**(6A): 3989-3994.

Kohanbash, G. and Okada, H. 2012. MicroRNAs and STAT interplay. *Semin Cancer Biol* **22**(1): 70-75.

Kole, A.J., Swahari, V., Hammond, S.M., and Deshmukh, M. 2011. miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis. *Genes Dev* **25**(2): 125-130.

Kong, G., Zhang, J., Zhang, S., Shan, C., Ye, L., and Zhang, X. 2011. Upregulated microRNA-29a by hepatitis B virus X protein enhances hepatoma cell migration by targeting PTEN in cell culture model. *PLoS One* **6**(5): e19518.

Kortylewski, M., Heinrich, P.C., Mackiewicz, A., Schniertshauer, U., Klingmuller, U., Nakajima, K., Hirano, T., Horn, F., and Behrmann, I. 1999. Interleukin-6 and oncostatin M-induced growth inhibition of human A375 melanoma cells is STAT-dependent and involves upregulation of the cyclin-dependent kinase inhibitor p27/Kip1. *Oncogene* **18**(25): 3742-3753.

Kortylewski, M., Komyod, W., Kauffmann, M.E., Bosserhoff, A., Heinrich, P.C., and Behrmann, I. 2004. Interferon-gamma-mediated growth regulation of melanoma cells: involvement of STAT1-dependent and STAT1-independent signals. *J Invest Dermatol* **122**(2): 414-422.

Koscianska, E., Starega-Roslan, J., and Krzyzosiak, W.J. 2011. The role of Dicer protein partners in the processing of microRNA precursors. *PLoS One* **6**(12): e28548.

Kozomara, A. and Griffiths-Jones, S. 2011. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* **39**(Database issue): D152-157.

Kreis, S., Munz, G.A., Haan, S., Heinrich, P.C., and Behrmann, I. 2007. Cell density dependent increase of constitutive signal transducers and activators of transcription 3 activity in melanoma cells is mediated by Janus kinases. *Mol Cancer Res* **5**(12): 1331-1341.

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M. *et al.* 2005. Combinatorial microRNA target predictions. *Nat Genet* **37**(5): 495-500.

Kriegel, A.J., Liu, Y., Fang, Y., Ding, X., and Liang, M. 2012. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiol Genomics* **44**(4): 237-244.

Krol, J., Busskamp, V., Markiewicz, I., Stadler, M.B., Ribi, S., Richter, J., Duebel, J., Bicker, S., Fehling, H.J., Schubeler, D. *et al.* 2010a. Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. *Cell* **141**(4): 618-631.

Krol, J., Loedige, I., and Filipowicz, W. 2010b. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* **11**(9): 597-610.

Krutovskikh, V.A. and Herceg, Z. 2010. Oncogenic microRNAs (OncomiRs) as a new class of cancer biomarkers. *Bioessays* **32**(10): 894-904.

Kulshreshtha, R., Ferracin, M., Wojcik, S.E., Garzon, R., Alder, H., Agosto-Perez, F.J., Davuluri, R., Liu, C.G., Croce, C.M., Negrini, M. *et al.* 2007. A microRNA signature of hypoxia. *Mol Cell Biol* **27**(5): 1859-1867.

Kutty, R.K., Nagineni, C.N., Samuel, W., Vijayasarathy, C., Hooks, J.J., and Redmond, T.M. 2010. Inflammatory cytokines regulate microRNA-155 expression in human retinal pigment epithelial cells by activating JAK/STAT pathway. *Biochem Biophys Res Commun* **402**(2): 390-395.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. 2001. Identification of novel genes coding for small expressed RNAs. *Science* **294**(5543): 853-858.

Lanford, R.E., Hildebrandt-Eriksen, E.S., Petri, A., Persson, R., Lindow, M., Munk, M.E., Kauppinen, S., and Orum, H. 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* **327**(5962): 198-201.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. 2001. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **294**(5543): 858-862.

Lazovich, D., Vogel, R.I., Berwick, M., Weinstock, M.A., Anderson, K.E., and Warshaw, E.M. 2010. Indoor tanning and risk of melanoma: a case-control study in a highly exposed population. *Cancer Epidemiol Biomarkers Prev* **19**(6): 1557-1568.

Lee, R.C. and Ambros, V. 2001. An extensive class of small RNAs in Caenorhabditis elegans. *Science* **294**(5543): 862-864.

Lee, R.C., Feinbaum, R.L., and Ambros, V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**(5): 843-854.

Lee, T.I. and Young, R.A. 2000. Transcription of eukaryotic protein-coding genes. *Annu Rev Genet* **34**: 77-137.

Leidinger, P., Keller, A., Borries, A., Reichrath, J., Rass, K., Jager, S.U., Lenhof, H.P., and Meese, E. 2010. High-throughput miRNA profiling of human melanoma blood samples. *BMC Cancer* **10**: 262.

Levati, L., Alvino, E., Pagani, E., Arcelli, D., Caporaso, P., Bondanza, S., Di Leva, G., Ferracin, M., Volinia, S., Bonmassar, E. *et al.* 2009. Altered expression of selected microRNAs in melanoma: antiproliferative and proapoptotic activity of miRNA-155. *Int J Oncol* **35**(2): 393-400.

Levy, D.E. and Darnell, J.E., Jr. 2002. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* **3**(9): 651-662.

Lew, R.A., Sober, A.J., Cook, N., Marvell, R., and Fitzpatrick, T.B. 1983. Sun exposure habits in patients with cutaneous melanoma: a case control study. *J Dermatol Surg Oncol* **9**(12): 981-986.

Li, Y., Wang, F., Xu, J., Ye, F., Shen, Y., Zhou, J., Lu, W., Wan, X., Ma, D., and Xie, X. 2011. Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29. *J Pathol* **224**(4): 484-495.

Liang, Y., Ridzon, D., Wong, L., and Chen, C. 2007. Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* **8**: 166.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**(7027): 769-773.

Liston, A., Papadopoulou, A.S., Danso-Abeam, D., and Dooley, J. 2012. MicroRNA-29 in the adaptive immune system: setting the threshold. *Cell Mol Life Sci*.

Liu, Y., Taylor, N.E., Lu, L., Usa, K., Cowley, A.W., Jr., Ferreri, N.R., Yeo, N.C., and Liang, M. 2010. Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes. *Hypertension* **55**(4): 974-982.

Lodygin, D., Tarasov, V., Epanchintsev, A., Berking, C., Knyazeva, T., Korner, H., Knyazev, P., Diebold, J., and Hermeking, H. 2008. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle* **7**(16): 2591-2600.

Löffler, D., Brocke-Heidrich, K., Pfeifer, G., Stocsits, C., Hackermuller, J., Kretzschmar, A.K., Burger, R., Gramatzki, M., Blumert, C., Bauer, K. *et al.* 2007. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* **110**(4): 1330-1333.

Long, G.V., Menzies, A.M., Nagrial, A.M., Haydu, L.E., Hamilton, A.L., Mann, G.J., Hughes, T.M., Thompson, J.F., Scolyer, R.A., and Kefford, R.F. 2011. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* **29**(10): 1239-1246.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A. *et al.* 2005a. MicroRNA expression profiles classify human cancers. *Nature* **435**(7043): 834-838.

Lu, J., Qian, J., Chen, F., Tang, X., Li, C., and Cardoso, W.V. 2005b. Differential expression of components of the microRNA machinery during mouse organogenesis. *Biochem Biophys Res Commun* **334**(2): 319-323.

Lujambio, A. and Lowe, S.W. 2012. The microcosmos of cancer. *Nature* **482**(7385): 347-355.

Ma, F., Xu, S., Liu, X., Zhang, Q., Xu, X., Liu, M., Hua, M., Li, N., Yao, H., and Cao, X. 2011a. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. *Nat Immunol* **12**(9): 861-869.

Ma, Z., Swede, H., Cassarino, D., Fleming, E., Fire, A., and Dadras, S.S. 2011b. Up-regulated Dicer expression in patients with cutaneous melanoma. *PLoS One* **6**(6): e20494.

Maragkakis, M., Alexiou, P., Papadopoulos, G.L., Reczko, M., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Simossis, V.A. *et al.* 2009. Accurate microRNA target prediction correlates with protein repression levels. *BMC bioinformatics* **10**: 295.

Margue, C., Philippidou, D., Reinsbach, S., Schmitt, M., and Kreis, S. submitted. New target genes of MITF-induced microRNA-211 contribute to melanoma cell invasion. *Pigment Cell & Melanoma Research*.

Markovic, S.N., Erickson, L.A., Rao, R.D., Weenig, R.H., Pockaj, B.A., Bardia, A., Vachon, C.M., Schild, S.E., McWilliams, R.R., Hand, J.L. *et al.* 2007. Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. *Mayo Clin Proc* **82**(3): 364-380.

Marson, A., Levine, S.S., Cole, M.F., Frampton, G.M., Brambrink, T., Johnstone, S., Guenther, M.G., Johnston, W.K., Wernig, M., Newman, J. *et al.* 2008. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* **134**(3): 521-533.

Maston, G.A., Evans, S.K., and Green, M.R. 2006. Transcriptional regulatory elements in the human genome. *Annu Rev Genomics Hum Genet* **7**: 29-59.

Matys, V., Fricke, E., Geffers, R., Gossling, E., Haubrock, M., Hehl, R., Hornischer, K., Karas, D., Kel, A.E., Kel-Margoulis, O.V. *et al.* 2003. TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res* **31**(1): 374-378.

Maurer, B., Stanczyk, J., Jungel, A., Akhmetshina, A., Trenkmann, M., Brock, M., Kowal-Bielecka, O., Gay, R.E., Michel, B.A., Distler, J.H. *et al.* 2010. MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. *Arthritis Rheum* **62**(6): 1733-1743.

Meister, G., ed. 2011. *RNA Biology*. WILEY-VCH.

Melo, S.A., Ropero, S., Moutinho, C., Aaltonen, L.A., Yamamoto, H., Calin, G.A., Rossi, S., Fernandez, A.F., Carneiro, F., Oliveira, C. *et al.* 2009. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet* **41**(3): 365-370.

Meng, F., Glaser, S.S., Francis, H., DeMorrow, S., Han, Y., Passarini, J.D., Stokes, A., Cleary, J.P., Liu, X., Venter, J. *et al.* 2012. Functional analysis of microRNAs in human hepatocellular cancer stem cells. *J Cell Mol Med* **16**(1): 160-173.

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T., and Patel, T. 2007a. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* **133**(2): 647-658.

Meng, F., Henson, R., Wehbe-Janek, H., Smith, H., Ueno, Y., and Patel, T. 2007b. The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes. *J Biol Chem* **282**(11): 8256-8264.

Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D. *et al.* 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84**(3): 431-442.

Mestdagh, P., Van Vlierberghe, P., De Weer, A., Muth, D., Westermann, F., Speleman, F., and Vandesompele, J. 2009. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* **10**(6): R64.

Miller, A.J. and Mihm, M.C., Jr. 2006. Melanoma. *N Engl J Med* **355**(1): 51-65.

Mitchell, P.S., Parkin, R.K., Kroh, E.M., Fritz, B.R., Wyman, S.K., Pogosova-Agadjanyan, E.L., Peterson, A., Noteboom, J., O'Briant, K.C., Allen, A. *et al.* 2008. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* **105**(30): 10513-10518.

Mitra, D., Luo, X., Morgan, A., Wang, J., Hoang, M.P., Lo, J., Guerrero, C.R., Lennerz, J.K., Mihm, M.C., Wargo, J.A. *et al.* 2012. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. *Nature* **491**(7424): 449-453.

Mori, S., Sawada, T., Okada, T., and Kubota, K. 2008. Anti-proliferative effect of interferon-gamma is enhanced by iron chelation in colon cancer cell lines in vitro. *Hepatogastroenterology* **55**(85): 1274-1279.

Morozova, N., Zinovyev, A., Nonne, N., Pritchard, L.L., Gorban, A.N., and Harel-Bellan, A. 2012. Kinetic signatures of microRNA modes of action. *RNA* **18**(9): 1635-1655.

Mott, J.L., Kobayashi, S., Bronk, S.F., and Gores, G.J. 2007. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* **26**(42): 6133-6140.

Mott, J.L., Kurita, S., Cazanave, S.C., Bronk, S.F., Werneburg, N.W., and Fernandez-Zapico, M.E. 2010. Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. *J Cell Biochem* **110**(5): 1155-1164.

Mueller, D.W. and Bosserhoff, A.K. 2009. Role of miRNAs in the progression of malignant melanoma. *Br J Cancer* **101**(4): 551-556.

Muller, D.W. and Bosserhoff, A.K. 2008. Integrin beta 3 expression is regulated by let-7a miRNA in malignant melanoma. *Oncogene* **27**(52): 6698-6706.

Muniyappa, M.K., Dowling, P., Henry, M., Meleady, P., Doolan, P., Gammell, P., Clynes, M., and Barron, N. 2009. MiRNA-29a regulates the expression of numerous proteins and reduces the invasiveness and proliferation of human carcinoma cell lines. *Eur J Cancer* **45**(17): 3104-3118.

Musat, M., Vax, V.V., Borboli, N., Gueorguiev, M., Bonner, S., Korbonits, M., and Grossman, A.B. 2004. Cell cycle dysregulation in pituitary oncogenesis. *Front Horm Res* **32**: 34-62.

Nam, E.J., Yoon, H., Kim, S.W., Kim, H., Kim, Y.T., Kim, J.H., Kim, J.W., and Kim, S. 2008. MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* **14**(9): 2690-2695.

Nathans, R., Chu, C.Y., Serquina, A.K., Lu, C.C., Cao, H., and Rana, T.M. 2009. Cellular microRNA and P bodies modulate host-HIV-1 interactions. *Mol Cell* **34**(6): 696-709.

Nguyen, T., Kuo, C., Nicholl, M.B., Sim, M.S., Turner, R.R., Morton, D.L., and Hoon, D.S. 2011. Downregulation of microRNA-29c is associated with hypermethylation of tumor-related genes and disease outcome in cutaneous melanoma. *Epigenetics* **6**(3): 388-394.

Ntziachristos, P., Tsirigos, A., Van Vlierberghe, P., Nedjic, J., Trimarchi, T., Flaherty, M.S., Ferres-Marco, D., da Ros, V., Tang, Z., Siegle, J. *et al.* 2012. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. *Nat Med* **18**(2): 298-301.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**(7043): 839-843.

O'Shea, J.J. and Murray, P.J. 2008. Cytokine signaling modules in inflammatory responses. *Immunity* **28**(4): 477-487.

Ohgane, J., Yagi, S., and Shiota, K. 2008. Epigenetics: the DNA methylation profile of tissue-dependent and differentially methylated regions in cells. *Placenta* **29 Suppl A**: S29-35.

Orom, U.A., Nielsen, F.C., and Lund, A.H. 2008. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell* **30**(4): 460-471.

Pagliaccetti, N.E., Eduardo, R., Kleinstein, S.H., Mu, X.J., Bandi, P., and Robek, M.D. 2008. Interleukin-29 functions cooperatively with interferon to induce antiviral gene expression and inhibit hepatitis C virus replication. *J Biol Chem* **283**(44): 30079-30089.

Pan, X., Wang, Z.X., and Wang, R. 2010. MicroRNA-21: a novel therapeutic target in human cancer. *Cancer Biol Ther* **10**(12): 1224-1232.

Pandey, A.K., Verma, G., Vig, S., Srivastava, S., Srivastava, A.K., and Datta, M. 2010. miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. *Mol Cell Endocrinol* **332**(1-2): 125-133.

Papadopoulou, A.S., Dooley, J., Linterman, M.A., Pierson, W., Ucar, O., Kyewski, B., Zuklys, S., Hollander, G.A., Matthys, P., Gray, D.H. *et al.* 2012. The thymic epithelial microRNA network elevates the threshold for infection-associated thymic involution via miR-29a mediated suppression of the IFN-alpha receptor. *Nat Immunol* **13**(2): 181-187.

Park, H.Y., Kosmadaki, M., Yaar, M., and Gilchrest, B.A. 2009a. Cellular mechanisms regulating human melanogenesis. *Cell Mol Life Sci* **66**(9): 1493-1506.

Park, S.Y., Lee, J.H., Ha, M., Nam, J.W., and Kim, V.N. 2009b. miR-29 miRNAs activate p53 by targeting p85 alpha and CDC42. *Nat Struct Mol Biol* **16**(1): 23-29.

Parkin, D.M., Mesher, D., and Sasieni, P. 2010. 13. Cancers attributable to solar (ultraviolet) radiation exposure in the UK in 2010. *Br J Cancer* **105 Suppl 2**: S66-69.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Muller, P. *et al.* 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408**(6808): 86-89.

Patra, S.K., Patra, A., Zhao, H., and Dahiya, R. 2002. DNA methyltransferase and demethylase in human prostate cancer. *Mol Carcinog* **33**(3): 163-171.

Pedersen, I.M., Cheng, G., Wieland, S., Volinia, S., Croce, C.M., Chisari, F.V., and David, M. 2007. Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* **449**(7164): 919-922.

Pekarsky, Y., Santanam, U., Cimmino, A., Palamarchuk, A., Efanov, A., Maximov, V., Volinia, S., Alder, H., Liu, C.G., Rassenti, L. *et al.* 2006. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res* **66**(24): 11590-11593.

Pestka, S. 2007. The interferons: 50 years after their discovery, there is much more to learn. *J Biol Chem* **282**(28): 20047-20051.

Pfahlberg, A., Kolmel, K.F., and Gefeller, O. 2001. Timing of excessive ultraviolet radiation and melanoma: epidemiology does not support the existence of a critical period of high susceptibility to solar ultraviolet radiation- induced melanoma. *Br J Dermatol* **144**(3): 471-475.

Pflanz, S., Hibbert, L., Mattson, J., Rosales, R., Vaisberg, E., Bazan, J.F., Phillips, J.H., McClanahan, T.K., de Waal Malefyt, R., and Kastelein, R.A. 2004. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *J Immunol* **172**(4): 2225-2231.

Philippidou, D., Schmitt, M., Moser, D., Margue, C., Nazarov, P.V., Muller, A., Vallar, L., Nashan, D., Behrmann, I., and Kreis, S. 2010. Signatures of microRNAs and selected microRNA target genes in human melanoma. *Cancer Res* **70**(10): 4163-4173.

Piepoli, A., Tavano, F., Copetti, M., Mazza, T., Palumbo, O., Panza, A., di Mola, F.F., Pazienza, V., Mazzoccoli, G., Biscaglia, G. *et al.* 2012. Mirna expression profiles identify drivers in colorectal and pancreatic cancers. *PLoS One* **7**(3): e33663.

Pleasance, E.D., Cheetham, R.K., Stephens, P.J., McBride, D.J., Humphray, S.J., Greenman, C.D., Varela, I., Lin, M.L., Ordonez, G.R., Bignell, G.R. *et al.* 2009. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* **463**(7278): 191-196.

Pollard, K.S., Hubisz, M.J., Rosenbloom, K.R., and Siepel, A. 2009. Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Res* **20**(1): 110-121.

Porkka, K.P., Pfeiffer, M.J., Waltering, K.K., Vessella, R.L., Tammela, T.L., and Visakorpi, T. 2007. MicroRNA expression profiling in prostate cancer. *Cancer Res* **67**(13): 6130-6135.

Ram, O., Goren, A., Amit, I., Shoresh, N., Yosef, N., Ernst, J., Kellis, M., Gymrek, M., Issner, R., Coyne, M. *et al.* 2011. Combinatorial patterning of chromatin regulators uncovered by genome-wide location analysis in human cells. *Cell* **147**(7): 1628-1639.

Rawlings, J.S., Rosler, K.M., and Harrison, D.A. 2004. The JAK/STAT signaling pathway. *J Cell Sci* **117**(Pt 8): 1281-1283.

Reich, N.C. and Liu, L. 2006. Tracking STAT nuclear traffic. *Nat Rev Immunol* **6**(8): 602-612.

Reinsbach, S., Nazarov, P.V., Philippidou, D., Schmitt, M., Wienecke-Baldacchino, A., Muller, A., Vallar, L., Behrmann, I., and Kreis, S. 2012. Dynamic regulation of microRNA expression following Interferon-gamma-induced gene transcription. *RNA Biol* **9**(7): 978-989.

Robertson, G., Hirst, M., Bainbridge, M., Bilenky, M., Zhao, Y., Zeng, T., Euskirchen, G., Bernier, B., Varhol, R., Delaney, A. *et al.* 2007. Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nat Methods* **4**(8): 651-657.

Roggli, E., Britan, A., Gattesco, S., Lin-Marq, N., Abderrahmani, A., Meda, P., and Regazzi, R. 2010. Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. *Diabetes* **59**(4): 978-986.

Rosenfeld, J.A., Wang, Z., Schones, D.E., Zhao, K., DeSalle, R., and Zhang, M.Q. 2009. Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics* **10**: 143.

Rosenfeld, N., Aharonov, R., Meiri, E., Rosenwald, S., Spector, Y., Zepeniuk, M., Benjamin, H., Shabes, N., Tabak, S., Levy, A. *et al.* 2008. MicroRNAs accurately identify cancer tissue origin. *Nat Biotechnol* **26**(4): 462-469.

Ruan, W., Xu, J.M., Li, S.B., Yuan, L.Q., and Dai, R.P. 2012. Effects of down-regulation of microRNA-23a on TNF-alpha-induced endothelial cell apoptosis through caspase-dependent pathways. *Cardiovasc Res* **93**(4): 623-632.

Saha, B., Jyothi Prasanna, S., Chandrasekar, B., and Nandi, D. 2010. Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine* **50**(1): 1-14.

Saharinen, P. and Silvennoinen, O. 2002. The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. *J Biol Chem* **277**(49): 47954-47963.

Saini, H.K., Enright, A.J., and Griffiths-Jones, S. 2008. Annotation of mammalian primary microRNAs. *BMC Genomics* **9**: 564.

Saini, H.K., Griffiths-Jones, S., and Enright, A.J. 2007. Genomic analysis of human microRNA transcripts. *Proc Natl Acad Sci U S A* **104**(45): 17719-17724.

Saito, Y., Kanai, Y., Nakagawa, T., Sakamoto, M., Saito, H., Ishii, H., and Hirohashi, S. 2003. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. *Int J Cancer* **105**(4): 527-532.

Sala, E., Mologni, L., Truffa, S., Gaetano, C., Bollag, G.E., and Gambacorti-Passerini, C. 2008. BRAF silencing by short hairpin RNA or chemical blockade by PLX4032 leads to different responses in melanoma and thyroid carcinoma cells. *Mol Cancer Res* **6**(5): 751-759.

Samuel, C.E. 2001. Antiviral actions of interferons. *Clin Microbiol Rev* **14**(4): 778-809, table of contents.

Sand, M., Skrygan, M., Sand, D., Georgas, D., Gambichler, T., Hahn, S.A., Altmeyer, P., and Bechara, F.G. 2012. Comparative microarray analysis of microRNA expression profiles in primary cutaneous malignant melanoma, cutaneous malignant melanoma metastases, and benign melanocytic nevi. *Cell Tissue Res* **351**(1): 85-98.

Santanam, U., Zanesi, N., Efanov, A., Costinean, S., Palamarchuk, A., Hagan, J.P., Volinia, S., Alder, H., Rassenti, L., Kipps, T. *et al.* 2010. Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. *Proc Natl Acad Sci U S A* **107**(27): 12210-12215.

Santos, C.I. and Costa-Pereira, A.P. 2011. Signal transducers and activators of transcription-from cytokine signalling to cancer biology. *Biochim Biophys Acta* **1816**(1): 38-49.

Scheier, B., Amaria, R., Lewis, K., and Gonzalez, R. 2011. Novel therapies in melanoma. *Immunotherapy* **3**(12): 1461-1469.

Schmitt, M.J., Margue, C., Behrmann, I., and Kreis, S. 2012a. miRNA-29: A microRNA Family with Tumor-Suppressing and Immune-Modulating Properties. *Curr Mol Med*.

Schmitt, M.J., Philippidou, D., Reinsbach, S.E., Margue, C., Wienecke-Baldacchino, A., Nashan, D., Behrmann, I., and Kreis, S. 2012b. Interferon-gamma-induced activation of Signal Transducer and Activator of Transcription 1 (STAT1) up-regulates the tumor suppressing microRNA-29 family in melanoma cells. *Cell Commun Signal* **10**(1): 41.

Schmitz Ulf, Wolkenhauer Olaf, and Julio, V., ed. 2013. *MicroRNAs in Melanoma Biology*. Springer.

Schnall-Levin, M., Rissland, O.S., Johnston, W.K., Perrimon, N., Bartel, D.P., and Berger, B. 2011. Unusually effective microRNA targeting within repeat-rich coding regions of mammalian mRNAs. *Genome Res* **21**(9): 1395-1403.

Schneider, M.R. 2012. MicroRNAs as novel players in skin development, homeostasis and disease. *Br J Dermatol* **166**(1): 22-28.

Schoenherr, C., Weiskirchen, R., and Haan, S. 2010. Interleukin-27 acts on hepatic stellate cells and induces signal transducer and activator of transcription 1-dependent responses. *Cell Commun Signal* **8**: 19.

Schreiber, R.D., Old, L.J., and Smyth, M.J. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* **331**(6024): 1565-1570.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**(2): 163-189.

Schultz, J., Lorenz, P., Gross, G., Ibrahim, S., and Kunz, M. 2008. MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. *Cell Res* **18**(5): 549-557.

Segura, M.F., Hanniford, D., Menendez, S., Reavie, L., Zou, X., Alvarez-Diaz, S., Zakrzewski, J., Blochin, E., Rose, A., Bogunovic, D. *et al.* 2009. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. *Proc Natl Acad Sci U S A* **106**(6): 1814-1819.

Sempere, L.F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E., and Ambros, V. 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol* **5**(3): R13.

Sengupta, S., den Boon, J.A., Chen, I.H., Newton, M.A., Stanhope, S.A., Cheng, Y.J., Chen, C.J., Hildesheim, A., Sugden, B., and Ahlquist, P. 2008. MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins. *Proc Natl Acad Sci U S A* **105**(15): 5874-5878.

Sharma, A., Sharma, A.K., Madhunapantula, S.V., Desai, D., Huh, S.J., Mosca, P., Amin, S., and Robertson, G.P. 2009. Targeting Akt3 signaling in malignant melanoma using isoselenocyanates. *Clin Cancer Res* **15**(5): 1674-1685.

Shuai, K. 2000. Modulation of STAT signaling by STAT-interacting proteins. *Oncogene* **19**(21): 2638-2644.

Shuai, K. and Liu, B. 2003. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* **3**(11): 900-911.

Siepel, A., Bejerano, G., Pedersen, J.S., Hinrichs, A.S., Hou, M., Rosenbloom, K., Clawson, H., Spieth, J., Hillier, L.W., Richards, S. *et al.* 2005. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Res* **15**(8): 1034-1050.

Smith, K.M., Guerau-de-Arellano, M., Costinean, S., Williams, J.L., Bottoni, A., Mavrikis Cox, G., Satoskar, A.R., Croce, C.M., Racke, M.K., Lovett-Racke, A.E. *et al.* 2012. miR-29ab1 Deficiency Identifies a Negative Feedback Loop Controlling Th1 Bias That Is Dysregulated in Multiple Sclerosis. *J Immunol* **189**(4): 1567-1576.

Soufir, N., Avril, M.F., Chompret, A., Demenais, F., Bombled, J., Spatz, A., Stoppa-Lyonnet, D., Benard, J., and Bressac-de Paillerets, B. 1998. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. *Hum Mol Genet* **7**(2): 209-216.

Spitz, F. and Furlong, E.E. 2012. Transcription factors: from enhancer binding to developmental control. *Nat Rev Genet* **13**(9): 613-626.

Spizzo, R., Nicoloso, M.S., Croce, C.M., and Calin, G.A. 2009. SnapShot: MicroRNAs in Cancer. *Cell* **137**(3): 586-586 e581.

Stamatopoulos, B., Meuleman, N., Haibe-Kains, B., Saussoy, P., Van Den Neste, E., Michaux, L., Heimann, P., Martiat, P., Bron, D., and Lagneaux, L. 2009. microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. *Blood* **113**(21): 5237-5245.

Stark, G.R. and Darnell, J.E., Jr. 2012. The JAK-STAT pathway at twenty. *Immunity* **36**(4): 503-514.

Steiner, D.F., Thomas, M.F., Hu, J.K., Yang, Z., Babiarz, J.E., Allen, C.D., Matloubian, M., Blelloch, R., and Ansel, K.M. 2011. MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells. *Immunity* **35**(2): 169-181.

Stulberg, D.L., Crandell, B., and Fawcett, R.S. 2004. Diagnosis and treatment of basal cell and squamous cell carcinomas. *Am Fam Physician* **70**(8): 1481-1488.

Su, C., Hou, Z., Zhang, C., Tian, Z., and Zhang, J. 2011. Ectopic expression of microRNA-155 enhances innate antiviral immunity against HBV infection in human hepatoma cells. *Virol J* **8**: 354.

Subramaniam, P.S., Torres, B.A., and Johnson, H.M. 2001. So many ligands, so few transcription factors: a new paradigm for signaling through the STAT transcription factors. *Cytokine* **15**(4): 175-187.

Sun, W., Xu, W., Snyder, M., He, W., Ho, H., Ivashkiv, L.B., and Zhang, J.J. 2005. The conserved Leu-724 residue is required for both serine phosphorylation and co-activator recruitment for Stat1-mediated transcription activation in response to interferon-gamma. *J Biol Chem* **280**(51): 41844-41851.

Tang, L., Li, G., Tron, V.A., Trotter, M.J., and Ho, V.C. 1999. Expression of cell cycle regulators in human cutaneous malignant melanoma. *Melanoma Res* **9**(2): 148-154.

Thompson, J.F., Scolyer, R.A., and Kefford, R.F. 2005. Cutaneous melanoma. *Lancet* **365**(9460): 687-701.

Tili, E., Michaille, J.J., Wernicke, D., Alder, H., Costinean, S., Volinia, S., and Croce, C.M. 2011. Mutator activity induced by microRNA-155 (miR-155) links inflammation and cancer. *Proc Natl Acad Sci U S A* **108**(12): 4908-4913.

Tjaden, B., Goodwin, S.S., Opdyke, J.A., Guillier, M., Fu, D.X., Gottesman, S., and Storz, G. 2006. Target prediction for small, noncoding RNAs in bacteria. *Nucleic Acids Res* **34**(9): 2791-2802.

Treiber, T., Treiber, N., and Meister, G. 2012. Regulation of microRNA biogenesis and function. *Thromb Haemost* **107**(4): 605-610.

Trinchieri, G. 2010. Type I interferon: friend or foe? *J Exp Med* **207**(10): 2053-2063.

Tsai, T., Vu, C., and Henson, D.E. 2005. Cutaneous, ocular and visceral melanoma in African Americans and Caucasians. *Melanoma Res* **15**(3): 213-217.

Tsao, H., Chin, L., Garraway, L.A., and Fisher, D.E. 2012. Melanoma: from mutations to medicine. *Genes Dev* **26**(11): 1131-1155.

Twigg, H.L., 3rd, Spain, B.A., Soliman, D.M., Knox, K., Sidner, R.A., Schnizlein-Bick, C., Wilkes, D.S., and Iwamoto, G.K. 1999. Production of interferon-gamma by lung lymphocytes in HIV-infected individuals. *Am J Physiol* **276**(2 Pt 1): L256-262.

Ugalde, A.P., Ramsay, A.J., de la Rosa, J., Varela, I., Marino, G., Cadinanos, J., Lu, J., Freije, J.M., and Lopez-Otin, C. 2011. Aging and chronic DNA damage response activate a regulatory pathway involving miR-29 and p53. *EMBO J* **30**(11): 2219-2232.

Ungureanu, D., Vanhatupa, S., Gronholm, J., Palvimo, J.J., and Silvennoinen, O. 2005. SUMO-1 conjugation selectively modulates STAT1-mediated gene responses. *Blood* **106**(1): 224-226.

UniprotConsortium. 2011. Reorganizing the protein space at the Universal Protein Resource (UniProt). *Nucleic Acids Res* **40**(Database issue): D71-75.

van Rooij, E., Sutherland, L.B., Qi, X., Richardson, J.A., Hill, J., and Olson, E.N. 2007. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316**(5824): 575-579.

van Rooij, E., Sutherland, L.B., Thatcher, J.E., DiMaio, J.M., Naseem, R.H., Marshall, W.S., Hill, J.A., and Olson, E.N. 2008. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci U S A* **105**(35): 13027-13032.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**(7): RESEARCH0034.

VanGuilder, H.D., Vrana, K.E., and Freeman, W.M. 2008. Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* **44**(5): 619-626.

Verrier, J.D., Lau, P., Hudson, L., Murashov, A.K., Renne, R., and Notterpek, L. 2009. Peripheral myelin protein 22 is regulated post-transcriptionally by miRNA-29a. *Glia* **57**(12): 1265-1279.

Villarreal, G., Jr., Oh, D.J., Kang, M.H., and Rhee, D.J. 2011. Coordinated Regulation of Extracellular Matrix Synthesis by the MicroRNA-29 Family in the Trabecular Meshwork. *Invest Ophthalmol Vis Sci*.

Visone, R., Pallante, P., Vecchione, A., Cirombella, R., Ferracin, M., Ferraro, A., Volinia, S., Coluzzi, S., Leone, V., Borbone, E. *et al.* 2007. Specific microRNAs are downregulated in human thyroid anaplastic carcinomas. *Oncogene* **26**(54): 7590-7595.

Viswanathan, S.R., Powers, J.T., Einhorn, W., Hoshida, Y., Ng, T.L., Toffanin, S., O'Sullivan, M., Lu, J., Phillips, L.A., Lockhart, V.L. *et al.* 2009. Lin28 promotes transformation and is associated with advanced human malignancies. *Nat Genet* **41**(7): 843-848.

Vlieghe, D., Sandelin, A., De Bleser, P.J., Vleminckx, K., Wasserman, W.W., van Roy, F., and Lenhard, B. 2006. A new generation of JASPAR, the open-access repository for transcription factor binding site profiles. *Nucleic Acids Res* **34**(Database issue): D95-97.

Völler, D., Ott, C., and Bosserhoff, A. 2013. MicroRNAs in malignant melanoma. *Clin Biochem*.

Wang, C., Bian, Z., Wei, D., and Zhang, J.G. 2011a. miR-29b regulates migration of human breast cancer cells. *Mol Cell Biochem* **352**(1-2): 197-207.

Wang, C.M., Wang, Y., Fan, C.G., Xu, F.F., Sun, W.S., Liu, Y.G., and Jia, J.H. 2011b. miR-29c targets TNFAIP3, inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma. *Biochem Biophys Res Commun* **411**(3): 586-592.

Wang, G., Wang, Y., Teng, M., Zhang, D., Li, L., and Liu, Y. 2010. Signal transducers and activators of transcription-1 (STAT1) regulates microRNA transcription in interferon gamma-stimulated HeLa cells. *PLoS One* **5**(7): e11794.

Wang, H., Garzon, R., Sun, H., Ladner, K.J., Singh, R., Dahlman, J., Cheng, A., Hall, B.M., Qualman, S.J., Chandler, D.S. *et al.* 2008. NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. *Cancer Cell* **14**(5): 369-381.

Wang, L.G. and Gu, J. 2011. Serum microRNA-29a is a promising novel marker for early detection of colorectal liver metastasis. *Cancer Epidemiol* **36**(1): e61-67.

Weber, J.A., Baxter, D.H., Zhang, S., Huang, D.Y., Huang, K.H., Lee, M.J., Galas, D.J., and Wang, K. 2010. The microRNA spectrum in 12 body fluids. *Clin Chem* **56**(11): 1733-1741.

Wheeler, B.M., Heimberg, A.M., Moy, V.N., Sperling, E.A., Holstein, T.W., Heber, S., and Peterson, K.J. 2009. The deep evolution of metazoan microRNAs. *Evol Dev* **11**(1): 50-68.

Whiteway, S.L., Harris, P.S., Venkataraman, S., Alimova, I., Birks, D.K., Donson, A.M., Foreman, N.K., and Vibhakar, R. 2012. Inhibition of cyclin-dependent kinase 6 suppresses cell proliferation and enhances radiation sensitivity in medulloblastoma cells. *J Neurooncol*.

Winter, J., Jung, S., Keller, S., Gregory, R.I., and Diederichs, S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* **11**(3): 228-234.

Wojciak, J.M., Martinez-Yamout, M.A., Dyson, H.J., and Wright, P.E. 2009. Structural basis for recruitment of CBP/p300 coactivators by STAT1 and STAT2 transactivation domains. *EMBO J* **28**(7): 948-958.

Wormald, S. and Hilton, D.J. 2004. Inhibitors of cytokine signal transduction. *J Biol Chem* **279**(2): 821-824.

Wu, H., Goel, V., and Haluska, F.G. 2003. PTEN signaling pathways in melanoma. *Oncogene* **22**(20): 3113-3122.

Wu, S., Huang, S., Ding, J., Zhao, Y., Liang, L., Liu, T., Zhan, R., and He, X. 2010. Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3' untranslated region. *Oncogene* **29**(15): 2302-2308.

Xiong, Y., Fang, J.H., Yun, J.P., Yang, J., Zhang, Y., Jia, W.H., and Zhuang, S.M. 2010. Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. *Hepatology* **51**(3): 836-845.

Xu, D. and Qu, C.K. 2008. Protein tyrosine phosphatases in the JAK/STAT pathway. *Front Biosci* **13**: 4925-4932.

Yan, H., Choi, A.J., Lee, B.H., and Ting, A.H. 2011. Identification and functional analysis of epigenetically silenced microRNAs in colorectal cancer cells. *PLoS One* **6**(6): e20628.

Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumamoto, K., Yi, M., Stephens, R.M., Okamoto, A., Yokota, J., Tanaka, T. *et al.* 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* **9**(3): 189-198.

Yang, C.H., Yue, J., Fan, M., and Pfeffer, L.M. 2010. IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis. *Cancer Res* **70**(20): 8108-8116.

Ye, Y., Hu, Z., Lin, Y., Zhang, C., and Perez-Polo, J.R. 2010. Downregulation of microRNA-29 by antisense inhibitors and a PPAR-gamma agonist protects against myocardial ischaemia-reperfusion injury. *Cardiovasc Res* **87**(3): 535-544.

Yi, R., Pasolli, H.A., Landthaler, M., Hafner, M., Ojo, T., Sheridan, R., Sander, C., O'Carroll, D., Stoffel, M., Tuschl, T. *et al.* 2009. DGCR8-dependent microRNA biogenesis is essential for skin development. *Proc Natl Acad Sci U S A* **106**(2): 498-502.

Yi, R., Poy, M.N., Stoffel, M., and Fuchs, E. 2008. A skin microRNA promotes differentiation by repressing 'stemness'. *Nature* **452**(7184): 225-229.

Yokoyama, S., Woods, S.L., Boyle, G.M., Aoude, L.G., MacGregor, S., Zismann, V., Gartside, M., Cust, A.E., Haq, R., Harland, M. *et al.* 2012. A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. *Nature* **480**(7375): 99-103.

Yoshimoto, T., Morishima, N., Mizoguchi, I., Shimizu, M., Nagai, H., Oniki, S., Oka, M., Nishigori, C., and Mizuguchi, J. 2008. Antiproliferative activity of IL-27 on melanoma. *J Immunol* **180**(10): 6527-6535.

Young, M.D., Willson, T.A., Wakefield, M.J., Trounson, E., Hilton, D.J., Blewitt, M.E., Oshlack, A., and Majewski, I.J. 2011. ChIP-seq analysis reveals distinct H3K27me3 profiles that correlate with transcriptional activity. *Nucleic Acids Res* **39**(17): 7415-7427.

Zaidi, M.R., Davis, S., Noonan, F.P., Graff-Cherry, C., Hawley, T.S., Walker, R.L., Feigenbaum, L., Fuchs, E., Lyakh, L., Young, H.A. *et al.* 2011. Interferon-gamma links ultraviolet radiation to melanomagenesis in mice. *Nature* **469**(7331): 548-553.

Zhang, L., Huang, J., Yang, N., Greshock, J., Megraw, M.S., Giannakakis, A., Liang, S., Naylor, T.L., Barchetti, A., Ward, M.R. *et al.* 2006. microRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci U S A* **103**(24): 9136-9141.

Zhang, Y., Liao, J.M., Zeng, S.X., and Lu, H. 2011a. p53 downregulates Down syndrome-associated DYRK1A through miR-1246. *EMBO Rep* **12**(8): 811-817.

Zhang, Y.K., Wang, H., Leng, Y., Li, Z.L., Yang, Y.F., Xiao, F.J., Li, Q.F., Chen, X.Q., and Wang, L.S. 2011b. Overexpression of microRNA-29b induces apoptosis of multiple myeloma cells through down regulating Mcl-1. *Biochem Biophys Res Commun* **414**(1): 233-239.

Zhang, Z., Zou, J., Wang, G.K., Zhang, J.T., Huang, S., Qin, Y.W., and Jing, Q. 2011c. Uracils at nucleotide position 9-11 are required for the rapid turnover of miR-29 family. *Nucleic Acids Res* **39**(10): 4387-4395.

Zhao, J.J., Lin, J., Lwin, T., Yang, H., Guo, J., Kong, W., Dessureault, S., Moscinski, L.C., Rezania, D., Dalton, W.S. *et al.* 2010. microRNA expression profile and identification of miR-29 as a prognostic

marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood* **115**(13): 2630-2639.

Zhou, Z.H., Chaturvedi, P., Han, Y.L., Aras, S., Li, Y.S., Kolattukudy, P.E., Ping, D., Boss, J.M., and Ransohoff, R.M. 1998. IFN-gamma induction of the human monocyte chemoattractant protein (hMCP)-1 gene in astrocytoma cells: functional interaction between an IFN-gamma-activated site and a GC-rich element. *J Immunol* **160**(8): 3908-3916.

Zhu, S., Van den Eynde, B.J., Coulie, P.G., Li, Y.F., El-Gamil, M., Rosenberg, S.A., and Robbins, P.F. 2012. Characterization of T-cell receptors directed against HLA-A*01-restricted and C*07-restricted epitopes of MAGE-A3 and MAGE-A12. *J Immunother* **35**(9): 680-688.

Zinovyev, A., Morozova, N., Nonne, N., Barillot, E., Harel-Bellan, A., and Gorban, A.N. 2012. Dynamical modeling of microRNA action on the protein translation process. *BMC Syst Biol* **4**: 13.

Zuo, L., Weger, J., Yang, Q., Goldstein, A.M., Tucker, M.A., Walker, G.J., Hayward, N., and Dracopoli, N.C. 1996. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat Genet* **12**(1): 97-99.

9 Appendix

Suppliers

Table 8: Machines / laboratory equipment

Machines / laboratory equipment	Supplier
µ clear 96 well plates	Greiner
-80°C freezer	New Brunswick Scientific
Autoclave 5075 ELV	Systec
Bürker Cell Counting Chamber	Marienfeld Superior
Cell culture hood	Faster BHA48
Cell culture incubator Hera cell 100	Heraeus
Centrifuge Megafuge 1.0R	Heraeus
Centrifuges 5702 and 5415D	Eppendorf
Certomat MOII (shaker)	Sartorius
CFX96 and CFX384 Real-time System C1000 Thermal Cycler	Bio-Rad
Electrophoresis power supply	Amersham Biosciences
Incucyte	Essen Bioscience
Lia (luminescence) white 96 well plates	Greiner
LI-COR Odyssey® Infrared Imaging System	LI-COR Biosciences
Luminescence Reader Fluostar Optima	BMG labtech
Molecular Imager ChemiDocTM XRS+	Bio-Rad
Nano Drop 2000c Spectrophotometer	Thermo Scientific
Nunclon 96 well plates	Thermo Scientific
SDS PAGE chamber	Biometra
Semi-dry blotting chamber TransBlot SD Cell	Bio-Rad
Thermocycler Gene Amp PCR System 9700	Applied Biosystems
Thermomixer compact	Eppendorf

Table 9: Chemicals, solutions, ladders and enzymes.
Chemicals not mentioned in the table were obtained from well-established companies.

Chemicals, solutions, ladders and enzymes	Supplier
1kb Plus DNA ladder	Invitrogen
Oligo annealing buffer	Promega
Acrylamide	Applichem
Agarose	FisherScientific
Ampicillin	Carl Roth
APS	Sigma-Aldrich
BSA (blocking buffer)	Applichem
BSA (restriction digestion)	New England Biolabs
CIP (alcaline phosphatase, calf intestinal)	New England Biolabs
DEPC	Sigma-Aldrich
DNase I	New England Biolabs
dNTP´s	Invitrogen
Ethanol	VWR
Etoposide	Sigma-Aldrich
FCS (fetal calf serum)	PAA
G418 (Geneticin)	Gibco
Glutamine	Lonza BioWhittaker
H_20_2	Sigma-Aldrich
IL-27 (human)	R & D Systems
Interferons (human, α,β,γ)	Peprotech
iQ SYBR green supermix	Bio-Rad
Isopropanol	VWR
Jak Inhibitor I	Calbiochem
NucView 488 Caspase	Biotium
Penicillin/Streptomycin	Lonza BioWhittaker
Phusion DNA polymerase	Finnzymes
Precision Plus Protein™ Standard	Bio-Rad
Restriction enzymes	New England Biolabs
SDS	Carl Roth
SYBR® Safe DNA gel stain	Invitrogen
T4 DNA ligase	New England Biolabs
TEMED	GE Healthcare
TRIsure	Bioline USA
Trypan blue stain	Lonza BioWhittaker
Trypsine	Lonza BioWhittaker

Table 10: Commercial kits, transfection reagents, buffers, media, membranes, consumables

Kits	Supplier
Dual-luciferase reporter assay system	Promega
Fast Plasmid Mini extraction kit	5 prime
innuPrep DOUBLE pure kit	Analytical Jena
miRNeasy kit	Qiagen
miScript Reverse Transcription Kit	Qiagen
Nucleobond Xtra Midi Plus	Macherey Nagel
Nucleospin RNA extraction kit	Macherey-Nagel
RT_2-FFPE extraction kit	SA Bioscience
Thermoscript RT-PCR system	Invitrogen
Transfection reagents, buffers and media	
1x passive lysis buffer	Promega
Buffers for restriction digestion	New England Biolabs
Dharmafect Duo	Dharmacon
DMEM	Lonza BioWhittaker
HiPerfect	Qiagen
RPMI	Lonza BioWhittaker
Melanocyte medium M2	PromoCell
Membranes and consumables	
(Whatman) chromatography paper	GE Healthcare
1.5 ml safe-lock reaction tubes	Eppendorf
Cell culture dishes	Greiner
CRYO.S™-tubes	Greiner
nitrocellulose membrane	GE Healthcare
PCR Sealers Microseal 'B' Film	Bio-Rad
PVDF membrane	Carl Roth

Buffers

Table 11: Buffer Recipes

Blocking buffer	10 % BSA in TBS-N 0.01 % NaN_3
Laemmli buffer	20 % glycerol 10 % ß-mercaptoethanol 4 % SDS 0.125 M Tris-HCl, pH 6.8 0.002 % bromophenol blue
DNase I buffer (New England Biolabs)	10mM Tris-HCl 2.5mM $MgCl_2$ 10mM $CaCl_2$
Stripping buffer	60 mM Tris/HCl pH 6,7 2 % SDS 100 mM β-mercaptoethanol
1x PBS	NaCl 8 g/L KCl 0.2 g/L KH_2PO_4 0.24 g/L Na_2HPO_4 14.4 g/L
TSS solution	1 g tryptone 0.5 g yeast extract 0.5 g NaCl 10 g PEG3350 5 ml DMSO 5 ml 1 M $MgCl_2$
5x DNA loading buffer	5 ml 10 x TBE 3 ml glycerol 2 ml H_2O 3 µg bromophenol blue
ECL solution	20 ml ECL solution (stock) 6.6 µl 30 % H_2O_2 H_2O_2 to be added freshly
ECL stock	The ECL solution pCA was used as described before (Haan and Behrmann 2007) 100 mM Tris/HCl pH 8.8 2.5 mM luminol 0.2 mM p-coumaric acid
LB medium	10 g tryptone (1 %) 5 g yeast extract (0.5 %) 10 g NaCl (1 %) H_2O up to 1000 ml to be autoclaved prior to use
LB agar (bacteria plates)	10 g tryptone (1 %) 5 g yeast extract (0.5 %) 10 g NaCl (1 %) up to 1000 ml H_2O

	add 15 g agar-agar (1.5 %) to be autoclaved prior to use
10x PBS, 2 l	160 g NaCl 4 g KCl 4.8 g KH_2PO_4 28.8 g Na_2HPO_4 H_2O up to 2 l
10x SDS running buffer, 2 l	60 g Tris (0.25 M) 288 g glycine (1.92 M) In 1.5 l H_2O add 50 ml 20 % SDS H_2O up to 2 l
4x separating gel buffer, 1 l (1.5 M Tris/HCl, pH 8.8)	181.7 g Tris add 800 ml H_2O add 20 ml 20 % SDS H_2O up to 1 l
4x stacking gel buffer, 500 ml (1 M Tris/HCl, pH 6.8)	250 ml 1 M Tris /HCl pH 6.3 200 ml H_2O 10 ml 20 % SDS H_2O up to 500 ml
Stacking gel (3 %)	3 ml 4x stacking gel buffer 7.2 ml H_2O 1.8 ml 30 % acrylamide 100 µl 20 % APS 20 µl TEMED
Separating gel (10 %)	3.5 ml 4x separating gel buffer 5.8 ml H_2O 4.7 ml 30 % acrylamide 105 µl 20 % APS 20 µl TEMED
10x TBE buffer, 2 l	216 g Tris 110 g boric acid 14.6 g EDTA H_2O up to 2 l
10x TBS-N, 2 l	200 ml 1 M Tris/HCl 7.4 540 ml 5 M NaCl 20 ml NP-40 (IGEPAL A-630) H_2O up to 2 l

Confirmed direct targets of the miR-29 family

Table 12: [1]Confirmed direct targets of the miR-29 family

(as extracted from literature search in April 2012); L = luciferase assay, mRNA = mRNA level; iv = *in vivo* (mice), WB = western blot; **b** = mainly b

Target		L	mRNA	WB	29 a/b/c	Ref	description
DNMT3A	DNA-methyltransferases	√	√	√	29b	(Garzon *et al.* 2009b)	Methyltransferases
			√ **iv**		29a	(Hand *et al.* 2012)	
		√	√	√	29abc	(Fabbri *et al.* 2007a)	
DNMT3B		√	√	√	29b	(Garzon *et al.* 2009b)	
			√ **iv**		29a	(Hand *et al.* 2012)	
		√	√	√	29abc	(Fabbri *et al.* 2007a)	
Bim	Bcl-2-like 11	√			29b	(Kole *et al.* 2011)	Members of Bcl-2 family and others involved in apoptosis
Puma	p53 up-regulated modulator of apoptosis	√			29b	(Kole *et al.* 2011)	
Bmf	Bcl-2-modifying factor	√			29b	(Kole *et al.* 2011)	
Bcl-2	B-cell lymphoma 2	√	√	√	29abc	(Xiong *et al.* 2010)	
Mcl-1	Myeloid cell leukemia sequence 1	√	no effect	√	29b	(Mott *et al.* 2007)	
		√	√	√	29abc	(Xiong *et al.* 2010)	
				√ **iv**	29ac	(Ye *et al.* 2010)	
Hrk	Activator of apoptosis harakiri	√			29b	(Kole *et al.* 2011)	
N-Bak	Bcl-2 homologous antagonist	√			29b	(Kole *et al.* 2011)	
Adamts18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	√			29abc	(Ugalde *et al.* 2011)	Extracellular matrix proteins
COL15A1	Collagens	√	√		29c	(Sengupta *et al.* 2008)	
COL1A1		√			29c		
		√	√		29b	(Liu *et al.* 2010)	
COL1A1		√	√ & **iv**		29**b**	(van Rooij *et al.* 2008)	
COL1A2		√			29c	(Sengupta *et al.* 2008)	
		√	√ & **iv**		29**b**	(van Rooij *et al.* 2008)	
COL3A1		√	√		29c	(Sengupta *et al.* 2008)	
		√	√	√	29abc	(Maurer *et al.* 2010)	
		√	√		29b	(Liu *et al.* 2010)	
		√	√ & **iv**		29**b**	(van Rooij *et al.* 2008)	
COL4A1		√	√		29c	(Sengupta *et al.* 2008)	
COL4A2		√			29c		
COL4A1		√	√		29b	(Liu *et al.* 2010)	
COL5A1		√	√		29b		
COL5A2		√	√		29b		
COL5A3		√	√		29b		
COL7A1		√	√		29b	(Liu *et al.* 2010)	
COL8A1		√	√		29b		
Eln1	Elastin	√			29b	(van Rooij *et al.* 2008)	
Fbn1	Fibrillin1	√			29b		
Itgb1	Integrin β1	√	√		29b	(Liu *et al.* 2010)	
Laminin γ1		√	√		29c	(Sengupta *et al.* 2008)	
MMP-2	Matrix metalloprotease	√		√	29b	(Fang *et al.* 2011)	
		√	√		29b	(Liu *et al.* 2010)	
Osteonectin/ SPARC	secreted protein, acidic, cysteine-rich	√	√		29ac	(Kapinas *et al.* 2009)	
T-bet	T-box transcription factor TBX21	√	√		29b	(Steiner *et al.* 2011)	
Eomes	Eomesodermin = T-box	√	√		29b	(Steiner *et al.* 2011)	

	brain protein 2						Transcription factors
HBP1	HMG-box transcription factor 1	√			29abc	(Ugalde *et al.* 2011)	
Mycn	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived	√			29abc	(Ugalde *et al.* 2011)	
YY1	Yin-yang 1	√		√ **iv**	29abc	(Wang *et al.* 2008)	
			√	√	29ab	(Li *et al.* 2011)	
cdc42	Cell division control protein 42 homolog	√		√	29abc	(Park *et al.* 2009b)	Cell cycle proteins
cdk6	Cyclin-dependent kinase 6	√	√	√	29abc	(Zhao *et al.* 2010)	
			√	√	29ab	(Li *et al.* 2011)	
Dusp2	Dual specificity protein phosphatase 2	√			29abc	(Ugalde *et al.* 2011)	Phosphatases
Ppm1d	Protein phosphatase 1D	√			29abc	(Ugalde *et al.* 2011)	
PTEN	Phosphatase and tensin homolog			√	29b	(Wang *et al.* 2011a)	
		√	√	√	29a	(Kong *et al.* 2011)	
FUSIP1	FUS-interacting serine-arginine-rich protein 1	√	√		29c	(Sengupta *et al.* 2008)	RNA splicing
Ifi30	Gamma-interferon-inducible lysosomal thiol reductase	√			29abc	(Ugalde *et al.* 2011)	Thiol reductase
IFNAR1	Interferon alpha receptor 1	√			29a	(Papadopoulou *et al.* 2012)	Cytokine signaling
IFN-γ	Interferon γ	√			29abc	(Ma *et al.* 2011a)	
Igf1	Insulin-like growth factor 1	√	√ **iv**		29a	(Hand *et al.* 2012)	
Il1RAP	Interleukin-1 receptor accessory protein	√	√ **iv**		29a	(Hand *et al.* 2012)	
LPL	lipoproteinlipase	√	√	√	29a	(Chen *et al.* 2011)	Lipase
Narf	Nuclear prelamin A recognition factor	√			29abc	(Ugalde *et al.* 2011)	Nuclear protein
nef	Negative regulatory factor	√		√	29ab	(Ahluwalia *et al.* 2008)	Virulence factor (HIV)
		√			29a	(Nathans *et al.* 2009)	
p85 α	Phosphatidylinositol 3-kinase, regulatory subunit α	√		√	29abc	(Park *et al.* 2009b)	Kinase
				√	29a	(Pandey *et al.* 2010)	
PDPN	Podoplanin	√	√	√	29b	(Cortez *et al.* 2010)	Membrane glycoprotein
PMP22	Peripheral myelin protein 22	√	√	√	29abc	(Verrier *et al.* 2009)	Major component of myelin
Tcl-1	T-cell leukemia 1		√	√	29c	(Stamatopoulos *et al.* 2009)	Co-activator of Akt
		√		√	29b	(Pekarsky *et al.* 2006)	
				√	29b	(Anastasiadou *et al.* 2010)	
TDG	G/T mismatch-specific thymine DNA glycosylase	√	√		29c	(Sengupta *et al.* 2008)	Glycosylase
TTP	Tristetraprolin	√		√	29a	(Gebeshuber *et al.* 2009)	mRNA degradation
p42.3		√	√	√	29a	(Cui *et al.* 2011)	unknown

10 Acknowledgements

First, I would like to thank my supervisor Dr. Stephanie Kreis for creating this interesting project I have been working on and giving me the opportunity to join the young and newly established group of the 'micro-people'. She always provided great help and support for technical, project-related and other emergencies and always had time to give feedback and to answer questions.
I would like to thank Prof. Iris Behrmann for accepting me as a PhD student in the Signal Transduction Group and for offering the possibility to participate in this project. Her scientific memory is amazing (although she says that this is not true ☺) and she always manages it to have a couple of minutes for an important meeting although her schedule is already full.
From both of you I learned from scratch how to carefully plan experiments with all crucial controls, how to interpret them and not to believe everything I see or read, even from peer-reviewed articles. I was always amazed by your ideas and suggestions and your ability to proof-read quicker than your shadow, when necassary.

Prof. Friedrich Grässer I would like to thank for his external supervision; he did not hesitate to accept me as a cotutelle PhD student although he is always quite busy and had to travel far for the CET-meetings. I am grateful for his suggestions and critical comments on the project and the opportunity for Stephanie and me to keep a link to Homburg.

Prof. Anja Bosserhoff, Prof. Müller-Newen and Dr. Paul Wilmes – I would like to thank for their agreement to critically read my manuscript and their immediate agreement to come to Luxembourg University as members of the thesis defense comittee.

Demetra Philippidou and Catherine Rolvering are clearly to be stressed here for their outstanding technical assistance – all members of the lab would be completely lost without them (maybe not everyone emphasizes that, but it is true).

Christiane Margue as another force from the miRNA team I would like to thank for her participation in the review and the paper, including proof-reading, discussions and valuable advices.

Furthermore, I would like to thank Demetra Philippidou and Dr. Elisabeth Letellier for their encouragement and support during our pregnancy – it was not always easy, but in the end, we managed and hey – all three boys are almost a year by now and all healthy and fine ☺.

Another gratitude goes to my fellow PhD students Karoline Gäbler, Susanne Reinsbach, Elisabeth John and Andreas Zimmer, as well as our office-member Dr. Mirko Moreno for their continuous support and motivation. It was always nice how we sticked together and managed everything in the end – I hope you keep up the nice atmosphere in the office and teach it to all future office-mates.

Dr. Anke Wienecke-Baldaccino helped us a lot with computational analyses, also on short-notice, and without her help we would still search for the STAT1 binding sites by eye (probably forever).

Dr. Lasse Sinkkonen I would like to thank for being a member of my CET committee during the first two years and for his valuable advices on microRNA biology and experiments.

Concerning all other – former and present - members of the signal transduction group and the Life Science Research Unit - all of them somehow participated to this thesis, in terms of answers to a countless number of questions, ideas, suggestions, mental support or others – thank you !!

Ein ganz großes Dankeschön geht natürlich an die Familien Schmitt und Biehler, deren tatkräftige Unterstützung wesentlich zum Gelingen dieser Doktorarbeit beigetragen hat. Ich freue mich besonders, dass auch meine beiden Großmütter bald die gedruckte Version der Arbeit anschauen können, denn auch sie waren in Gedanken immer dabei - Oma Friedel mach Dir keine Sorgen – es ist alles korrekt zitiert.

Last but not least, this thesis would not have been possible without the Fonds National de la Recherche Luxembourg, which offered the generous funding for my PhD position, including the great opportunity to participate in international conferences and summer schools and to interact with fellow researchers.

11 Curriculum vitae

Martina Schmitt (married Biehler)

Am Seffersbach 23
D-66663 Merzig
Germany

18.10.1983
German
married, 1 child (*26.06.12)

Education

2009 - current	**PhD thesis**, University of Luxembourg 'Regulation of microRNA expression by STAT transcription factors: relevance for melanoma development' Signal Transduction Group, LSRU, FSTC (Supervision: Dr. Stephanie Kreis/Prof. Iris Behrmann) in cooperation with Prof. Friedrich Grässer, University Medical Center, Homburg
2008	**Diploma thesis**, Institute for Virology, University Medical Center, Homburg 'Characterization of the interaction between the Human Endogenous Retrovirus K (HERV-K (HML-2)) protein Rec and the Testis Zinc Finger Protein (TZFP)'
2003 – 2008	Studies in **Biology**, Saarland University (Human- and Molecular Biology) to obtain the title 'Diplom-Biologin'
1994 - 2003	Marie-Luise-Kaschnitz-Gymnasium, Völklingen

11 Publications

[1]miRNA-29: A microRNA Family with Tumor-Suppressing and Immune-Modulating Properties

Schmitt MJ, Margue C, Behrmann I, Kreis S.

Curr Mol Med. 2012 Aug 31 PMID:22934851

[2]Interferon-γ-induced activation of Signal Transducer and Activator of Transcription 1 (STAT1) up-regulates the tumor suppressing microRNA-29 family in melanoma cells

Schmitt MJ, Philippidou D, Reinsbach SE, Margue C, Wienecke-Baldacchino A, Nashan D, Behrmann I, Kreis S.

Cell Commun Signal. 2012 Dec 17;10(1):41 PMID:23245396

Signatures of microRNAs and selected microRNA target genes in human melanoma

Philippidou D, Schmitt M, Moser D, Margue C, Nazarov PV, Muller A, Vallar L, Nashan D, Behrmann I, Kreis S.

Cancer Res. 2010 May 15;70(10):4163-73 PMID: 20442294

Dynamic regulation of microRNA expression following Interferon-gamma-induced gene transcription

Reinsbach S, Nazarov PV, Philippidou D, Schmitt M, Wienecke-Baldacchino A, Muller A, Vallar L, Behrmann I, Kreis S.

RNA Biol. 2012 Jul 1;9(7) PMID: 22767256

New target genes of MITF-induced microRNA-211 contribute to melanoma cell invasion

Margue C, Philippidou D; Reinsbach SE; Schmitt MJ; Behrmann I; Kreis S

submitted to Pigment Cell & Melanoma Research

MiRNA-29: a microRNA Family with Tumor-Suppressing and Immune-Modulating Properties

Martina J. Schmitt, Christiane Margue, Iris Behrmann and Stephanie Kreis*

Signal Transduction Laboratory, Life Sciences Research Unit, University of Luxembourg,

L-1511 Luxembourg

*To whom correspondence should be addressed:

Tel: +352 4666446884; Fax: +352 4666446435, Email: Stephanie.Kreis@uni.lu

Key words biomarker; immune response; miR-29 family; oncogenic; therapeutic target; tumor suppressor

Abstract

MicroRNAs (miRNAs) are ubiquitously expressed small, non-coding RNAs that negatively regulate gene expression at a post-transcriptional level. So far, over 1000 miRNAs have been identified in human cells and their diverse functions in normal cell homeostasis and many different diseases have been thoroughly investigated during the past decade. MiR-29, one of the most interesting miRNA families in humans to date, consists of three mature members miR-29a, miR-29b and miR-29c, which are encoded in two genetic clusters. Members of this family have been shown to be silenced or down-regulated in many different types of cancer and have subsequently been attributed predominantly tumor-suppressing properties, albeit exceptions have been described where miR-29s have tumor-promoting functions. MiR-29 targets expression of diverse proteins like collagens, transcription factors, methyltransferases and others, which may partake in abnormal migration, invasion or proliferation of cells and may favor development of cancer. Furthermore, members of the miR-29 family can be activated by interferon signaling, which suggests a role in the immune system and in host-pathogen interactions, especially in response to viral infections. In this review, we summarize current knowledge on the genomic organization and regulation of the miR-29 family and we provide an overview of its implication in cancer suppression and promotion as well as in host immune responses. The numerous remarkable properties of these miRNAs and their often altered expression patterns might make the miR-29 family promising biomarkers and therapeutic targets for various diseases in future.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules (~22 nt), whose main function is the negative regulation of gene expression at a post-transcriptional level. MiRNAs are expressed in all cell types and are involved in the control of fundamental cellular processes such as differentiation, apoptosis, proliferation, cell death and others [1-3]. In recent years, many diseases including almost all types of cancer have been connected to aberrant expression of miRNAs [4, 5]. The rapidly growing interest in various aspects of miRNA biology is reflected by the constantly increasing number of publications since their first discovery in 1999. For the current version 18 of miRBase, entries for newly identified miRNAs have risen to 18226 representing 168 different species [6]. The biogenesis of miRNAs has been extensively and expertly reviewed elsewhere [7-10].

The present review focuses on the human miR-29, a very important miRNA family whose members are increasingly recognized as tumor suppressors in a variety of malignancies. Since their sequence was added to miRBase in 2001 [11], the number of publications on one or more members of the human miR-29 family rose quickly to approximately 100. Members of the miR-29 family have been shown to be implicated in many divergent cellular processes like extracellular matrix homeostasis [12], collagen expression [13], insulin signaling [14], ageing [15] and others. The number of confirmed targets for one or more family members (Table 1) is constantly rising, including many different protein classes ranging from transcription factors [15, 16], viral proteins [17] to growth factors [18], structural cell components [13] and others. Noteworthy, miR-29 members have been connected to diseases other than cancer, for example myocardial infarction [13], diabetes [14] and atherosclerosis [19]. The implication of the miR-29 family in diseases like myocardial infarction and renal injury has recently been reviewed [20]. The topic of the present review, however, is the involvement of miR-29 in malignancies: in a recent overview article on miRNAs in cancer, the miR-29 family resided among the topmost cancer-associated miRNAs [21]. Here, we will focus on the role of miR-29 in cancer and discuss the transcriptional regulation of this miRNA family, it`s tumor suppressing functions, it`s potential as a therapeutic target in cancer therapy as well as it`s exceptional tumor promoting properties. Furthermore, we will summarize several interesting findings, which attribute immune-modulating functions to miR-29 family members and discuss their potential role in immune responses to viral infections.

The miR-29 family: genomic organization and transcriptional regulation of expression

The miR-29 family is among the earliest ones discovered [11] and is highly conserved among species [6]. In humans, it is encoded by two clusters, miR-29a~29b-1 (chromosome 7q32.3) and miR-29b-2~29c (chr 1q32.2) (Fig. 1A). MiR-29a and miR-29b-1 (GenBank accession number EU154353) as well as miR-29c and miR-29b-2 (EU 154351 and EU154352) are co-transcribed by RNA-polymerase II as a polycistronic primary transcript from the minus strand [22, 23], only encoded 649bp and 504bp apart from each other, respectively. With identical seed sequences, miR-29 family members share most of their predicted targets. MiR-29b-1/b-2 both have the same mature sequence and miR-29a and 29c mature sequences only differ by one nucleotide (Fig. 1A). All family members are ubiquitously expressed in healthy tissue (microRNA.org). Chang et al. were the first to experimentally characterize the primary transcripts of both miR-29 clusters in B-cells. They found that the miR-29a~29b-1cluster is produced from the last intronic region of the primary transcript in contrast to the miR-29b-2~29c cluster, which is processed from the last exon of its corresponding transcript [22] (Fig. 1B). Both clusters, miR-29a~29b-1 and miR-29b-2~29c are intergenic with no protein-coding genes in close proximity. Currently, there is no common agreement on the TSSs of miR-29 family members.

Both clusters of the miR-29 family are intergenic and currently there is no common agreement on their TSSs. So far, 14 different TSSs have been annotated for the miR-29 family, which are shown (numbered consecutively) in Figure 1C. In large-scale computational analyses focusing on multiple genomic features, Saini et al. predicted many boundaries of intergenic miRNA transcripts, among others the miR-29b-2~29c clusters' 5' and 3'ends (TSSbc1, Fig.

1C)[24] and in a different report the ends of miR-29a~29b-1 (TSSab8, Fig. 1C) and miR-29b-2~29c (TSSbc2, Fig. 1C)[25]. In a study on embryonic stem cells, Marson et al. collected coordinates of H3K4me3-enriched loci from several cell lines and predicted different TSSs for the miR-29 clusters miR-29a~29b-1 (TSSab2, Fig. 1C) and miR-29b-2~29c (TSSbc6, Fig. 1C)[26]. Corcoran and colleagues annotated the TSS of the miR-29a~29b-1 cluster (TSSab1) based on a Pol II ChIP-chip of A549 lung epithelial cells using an own 'Core Promoter Prediction Program' [27]. A study by Chang et al. provided the first RACE-experiments for the miR-29 family, which annotated the 5'- and 3'-ends of both clusters in a human Burkitt lymphoma cell line (TSSab5 and TSSbc4, Fig. 1C)[22]. Mott et al. confirmed the miR-29a~29b-1 TSS by RACE in H69 non-malignant cholangiocytes (TSSab4, Fig. 1C), albeit with a 131 bp downstream shift to the one described by Chang (TSSab5) [23]. Finally, even more TSS predictions can be extracted from the miRStart website [28] (TSSab3 and TSSbc5, Fig. 1C) or from the UCSC genome browser feature 'SwitchGear genomics' (www.switchgeargenomics.com) (TSSab6 & TSSab7 for miR29a~29b-1 and TSSbc3 for miR29b-2~29c, Fig. 1C).

Transcriptional regulation is thought to be the main mechanism for regulation of miRNA expression [7], but other controlling processes including epigenetic silencing [29] and different turnover or processing times [10] have also been described. Identifying the transcription factors, which regulate miRNAs or their respective host genes, in the case of intragenic miRNAs, is crucial for understanding regulation and different miRNA expression patterns in a healthy versus diseased cellular context. In a recent review by Kriegel et al. [20], known transcription factor binding sites in the promoter region of the miR-29 family have been summarized. The vast majority of publications report a down-regulation of miR-29 in cancer [30-32], which will be discussed in more detail below (see also Table 2). To some extent, decreased miR-29 levels can be explained by transcriptional repression and an overview of the current knowledge about miR-29 regulation is given in Figure 2.

Although most recent publications report a clear down-regulation of the miR-29 family members in cancer, especially with progression, some studies have shown opposite effects with miR-29 being transcriptionally up-regulated. The transcription factors CEBPA (CCAAT/enhancer-binding protein alpha), p53, SMAD3 (mothers against decapentaplegic homolog 3) and canonical Wnt-signaling have all been shown to activate the expression of miR-29 family members in various cell types [12, 15, 32-35] (Fig. 2A). In this context, we have recently observed a profound and robust up-regulation of miR-29 by the STAT1 (signal transducer and activator of transcription) transcription factor induced by interferon γ (IFN-γ) signaling in melanoma and T-cells (dashed box, Fig. 2A). These results will be further discussed below. Taken together, depending on the cellular context, several transcription factors directly up-regulate or repress the expression of the miR-29 family members and these data are crucial for further deciphering miR-29 regulatory networks (Fig. 2A).

Involvement of the miR-29 family in cancer

The predominantly tumor-suppressing properties of miR-29 in solid tissue cancers. Since their discovery, miRNAs have been implicated in malignancies and many studies on miRNA deregulation in all types of cancer have been carried out. Like protein-coding genes, miRNAs can be classified as being either 'oncogenic' or 'tumor-suppressive'. Mir-21, one of the first known 'oncomirs', has elevated expression levels in many human cancers [36] while the miR-29 family or its single members have been shown to be down-regulated in glioblastoma [37], lung cancer [30], prostate cancer [38], colon cancer [39], chronic lymphocytic leukemia [31] and many other malignancies (Table 2).

Tumor-suppressor functions have clearly been demonstrated in glioblastoma cells, where the introduction of miR-29b-mimicking RNA-duplexes was found to induce apoptosis and reduction of proliferation and invasion [37]. In a study involving a RMS (rhabdomyosarcoma) xenograft model, it was observed that re-expression of otherwise silenced miR-29 inhibited tumor growth in mice [40]. Nude mice showed reduced tumor formation by HCC (hepatocellular carcinoma) cells after transfection with miR-29b [41] and the same group later described that miR-

29b inhibited the invasive potential of HCC cells, as well as angiogenesis and metastasis in HCC-transplanted nude mice [42]. Muniyappa et al. focused on a more general approach and compared miR-29a expression levels in various cancer cell lines with divergent invasive capacity [43]. A higher miR-29a expression was correlated with a lower invasiveness of NSCLC (non-small-cell lung carcinoma) cell lines and over-expression of miR-29a resulted in anti-invasive and anti-proliferative effects in lung and pancreatic cancer cells [43].

In contrast to the majority of studies highlighting tumor-suppressive properties, opposing expressing patterns and roles seem to exist in breast cancer and primary melanoma. Wang et al. reported an up-regulation of miR-29b in highly metastatic breast cancer cells in comparison to low-metastatic ones and showed that miR-29b promotes migration and invasion and increases resistance to apoptosis. However, healthy control tissue or primary tumor material for determining basal expression levels were not analyzed [44]. Similar to this study, Gebeshuber and colleagues recently reported enhanced levels of miR-29a in patients with invasive breast carcinomas in comparison to benign samples from patients with non-invasive hyperplasia [45]. MiR-29a transfection into mice had no effect on cell proliferation but resulted in enhanced lung metastasis. It remains to be clarified why Wang et al. only detected miR-29b while Gebeshuber et al. exclusively reported an up-regulation of miR-29a with neither group seeing differential regulation of the other miRNA in the same tissue. Nevertheless, both studies provide evidence that miR-29 may indeed have tumor-promoting traits in breast cancer and to our knowledge a down-regulation of miR-29 in breast cancer has not been reported so far. Interestingly and in line with elevated levels of miR-29 in breast cancer, we have recently made similar observations in melanoma patient samples: both miR-29a and 29b were up-regulated, however only in primary melanoma relative to healthy nevi while expression levels in metastatic melanoma were decreased even below the levels scored in nevi [46]. Surprisingly, a recent study on cutaneous melanoma reported unchanged miR-29a and-29b expression levels while miR-29c was down-regulated in metastatic melanoma in comparison to primary tumor samples [47] but again no healthy control tissue or nevi were included. Apart from these noteworthy exceptions in primary melanoma and breast cancer, miR-29 family members have consistently and predominantly been reported to be down-regulated and to assume tumor-suppressive properties in solid tissue cancers.

Properties of the miR-29 family in hematological malignancies. In acute myeloid leukemia (AML), most studies have demonstrated a down-regulation of miR-29 in patient material or malignant cell lines. Eyholzer et al. reported a suppression of miR-29b expression in AML patient-derived bone marrow samples and showed that the whole miR-29a~29b-1 cluster was blocked in the disease [32]. They reasoned that this was due to the disruption of CEBPA-expression in AML and could further prove that the CEBPA transcription factor indeed mediates miR-29b expression. In contrast to this, a recent study suggested oncogenic properties of miR-29a in AML. Purified leukemia stem cell (LSC)-blast populations of AML patients had up-regulated levels of miR-29a and over-expression of miR-29a led to the acquisition of the self-renewal capacity of myeloid progenitors and subsequently to the development of a myeloproliferative disorder, which progressed to AML in a mouse model [48]. A possible explanation for the contradictory results on miR-29 in AML could be the different clinical sample and cell types that were used: patient-derived bone marrow [32] or PBMCs [49], versus purified human LCSs [48].

In addition to acute leukemia, miR-29 has been implicated in chronic leukemia, mainly CLL. Again, most publications describe a down-regulation of miR-29 members in CLL patients: miR-29a, -b and –c levels were reduced in lymphocytes of CLL-patients and miR-29c was also down-regulated in PBMCs [31, 50-52]. In this context, reports by Santanam and colleagues emphasized how crucial adequately controlled samples are, especially when comparing miRNA expression levels. Interestingly, they reported that miR-29 decreased when expression levels from aggressive CLL samples were normalized to samples from indolent CLL [52] while an up-regulation of miR-29 was detected when indolent and aggressive CLL were compared to normal CD19+ lymphocytes [53]. Furthermore, the group hypothesized that miR-29 can contribute to the pathogenesis of CLL. This was demonstrated in miR-29a~b-1 –transgenic mice, which developed a CLL phenotype in comparison to wild-type mice indicating an oncogenic potential for miR-29 in chronic leukemia after over-expression [53].

Taken together, a substantial body of evidence on the role of miR-29 and its family members in human malignancies has been collected over recent years, spanning many different solid tumors as well as several types of leukemia. Like other miRNAs, miR-29 appears to have an important role in development, progression and control of cancer and many of the cited studies also addressed the potential of miR-29 in disease-associated prognosis and therapy. Most studies corroborate that miR-29 mainly exerts tumor-suppressive properties, which are subsequently lost when it is silenced in cancer and the cellular homeostasis is changed depending on the functional role of the target genes in the respective type of cancer (Tables 1 & 2). Potentially opposing roles and up-regulated expression levels have been reported for AML, primary melanoma and breast cancer. Nevertheless, differential expression patterns of miR-29 family members in many cancer types offer some potential for the development of targeted therapy once the downstream target genes have been clearly identified.

Selected miR-29 target genes and their potential involvement in cancer suppression. Beyond assessing the expression levels of the miR-29s in different cancer types, it is crucial to identify their target genes in order to decipher cancer-associated cellular pathways and networks that might be regulated by miR-29. Table 1 summarizes experimentally confirmed miR-29 targets with potential functional roles in various aspects of cancer biology.

Several groups have independently established that miR-29 family members target and thus down-regulate the methyltransferases DNMT3A and DNMT3B (DNA-methyltransferases) [18, 54, 55] (Table 1). DNMTs are involved in DNA-methylation of CpG islands causing epigenetic silencing of the corresponding genes. Hyper-methylation is a normal regulatory mechanism, but can be fatal if tumor suppressor genes are silenced, which has been shown for $p16^{INK4A}$ (CDKN2A, cyclin-dependent kinase inhibitor 2A) in lung carcinoma cells [56]. Interestingly, DNMT3 is frequently up-regulated in various malignancies such as hepatoma, lung, prostate, colorectal and breast cancer [57-61]. An inverse correlation was shown for miR-29c and DNMT expression levels in cutaneous melanoma with high expression of miR-29c and low expression of DNMTs in primary melanoma and the reverse situation in metastasis [47]. Fabbri et al. validated the down-regulation of DNMTs 3A and 3B by all miR-29 family members [55] and demonstrated reduced tumor sizes in mice, engrafted with lung cancer cells that had been transfected with single miR-29 family members. These important findings provide first indications towards the potential miR-29s might have in therapeutic treatment approaches of cancer.

Another important group of miR-29 targets with a role in cancer progression is the Bcl-2 family (B-cell lymphoma 2), which encodes genes involved in the regulation of apoptosis [62]. Interestingly, the pro-apoptotic members Bim (Bcl-2 like 11), Puma (p53 up-regulated modulator of apoptosis) and Hrk (activator of apoptosis harakiri) as well as the anti-apoptotic n-BAK (Bcl-2 homologous antagonist) and Bmf (Bcl-2-modifiying factor) can all be targeted by miR-29 [63]. Among the most prominent members, Bcl-2 itself and Mcl-1 (myeloid cell leukemia sequence 1), both anti-apoptotic, have been experimentally validated to be direct miR-29 targets in multiple cell types [41, 64, 65]. All these studies suggest that decreased expression of miR-29 leads to an up-regulation of Bcl-2 and Mcl-1 and consequently to protection of cells from apoptosis, which in turn promotes tumorigenesis.

Other miR-29 target genes include extracellular matrix proteins, de-regulation of which can favor cancer growth and progression. Confirmed miR-29 target genes include collagens [13, 66, 67], the matrix-metalloprotease MMP-2 [42, 68], elastin and fibrillin 1 [13], laminin γ1 [66] and integrin beta 1 [68], which have recently been summarized in a review [20]. Although many targets with distinct functions in numerous cellular processes and different cells types, tissues and diseases have been put forward, no 'universal' miR-29 target gene seems to exist, which is de-regulated in most cancers (Table1). Depending on the cellular context, not all targets are similarly found to be down-regulated after mimic treatment. For example, down-regulation of the methyltransferases DNMT3A and DNMT3B was shown in AML, lung cancer and liver tissue [18, 54, 55], but could not be confirmed in nasopharyngeal carcinomas [69]. Nevertheless, cell-type specific target genes may offer the possibility for a more precise and targeted therapeutic intervention by miRNAs with potentially less side effects.

MiR-29: a player in immunological host defense?

Apart from direct regulation affecting biogenesis or processing speed mediated by genetic factors and epigenetic silencing, miRNAs have also been shown to be indirectly regulated by cytokines like interferons [70]. Cytokine signalling plays a pivotal role in the immune system by communicating cellular responses to inflammation and infections [71]. Nearly all cell types can come into contact with cytokines such as IFN-γ, which is mainly secreted by activated T and NK cells and represents the most important Th1 (T helper type 1) –cytokine in innate and adaptive immunity to infection [72]. Generally, cytokines activate transcription factors, which subsequently lead to transcription of target genes including those encoding miRNAs. IFN-γ primarily triggers the JAK/STAT pathway resulting in a STAT-mediated transcriptional activation of target genes. The importance of interactions between miRNAs and STAT transcription factors has very recently been summarised in a review by Kohanbash and Okada [73]. In this context, we have found that miR-29a/b was specifically and dose-dependently up-regulated after IFN-γ stimulation of melanoma and T-cells. Using a STAT1 dominant-negative control cell line as well as specific Jak inhibitors, we have established that this activation is mediated by STAT1 (manuscript in preparation). Along that line, we have also investigated the dynamic regulation of miRNA expression profiles following IFN-γ-induced gene transcription and observed temporal changes in levels of about 10% of all miRNAs including miR-29a and -29b in response to STAT1-activation [74].

Other reports have previously connected individual miRNAs to interferon signaling: for example, miR-155 was shown to inhibit IFN-γ-signaling in $CD4^+$ T-cells [75] and miR-29a suppressed interferon-α-receptor expression in the thymic epithelium [76], implying that IFN signaling in general may actively partake in the regulation of miRNA expression levels in cells. Recently, two excellent publications provided further insights into the implication of the miR-29 family in immune responses. Ma et al. [77] showed that miR-29a and -29b were down-regulated in IFN-γ-secreting T-cells. Furthermore, they observed a down-regulation of miR-29a and -29b and an up-regulation of IFN-γ in activated NK cells and T-cells following infection of mice with *Listeria monocytogenes* or *Mycobacterium bovis*. By luciferase reporter gene assays, IFN-γ was confirmed to be a direct target of all miR-29 family members and this suggests an immunosuppressive role for miR-29 in bacterial infections with the specific down-regulation of IFN-γ by miR-29.These findings were confirmed by an experiment in mice transgenically expressing a 'sponge' target to compete with endogenous miR-29 targets [16]. These mice showed increased Th1-responses and better resistance to mycobacteria. Interestingly, Steiner et al. proved that the transcription factors T-bet and Eomes (Eomesodermin = T-box brain protein 2), both of which are known inducers of IFN-γ production in helper T-cells, were directly targeted by miR-29a and -29b, thus facilitating an immune response to intracellular pathogens [16]. Both publications suggest that miR-29 negatively regulates IFN-γ production. As mentioned above, we generally see a clear induction of miR-29 expression following IFN-γ stimulation and have shown this regulation to be STAT1-dependent. These findings indicate that a regulatory loop exists with IFN-γ inducing miR-29 expression via STAT1 and miR-29 subsequently repressing IFN-γ directly as well as indirectly by down-regulating the IFN-γ-inducing transcription factors T-bet and Eomes (Figure 2B).

Apart from immune responses to bacterial infection, the miR-29 family has also been implicated in immune reactions to viral infections. Cellular miRNAs can down-regulate viral protein-coding genes and partake in host-pathogen interactions, supporting antiviral mechanisms of the host defense. For example, interferon-β rapidly elevates the expression of several cellular miRNAs in Hepatitis C-infected cells and in turn, some human miRNAs like miR-196 and miR-448 down-regulate viral genomic RNA and viral replication is controlled and reduced [70]. For EBV (Epstein-Barr virus) it was reported that EBV-encoded LMP1 (latent membrane protein 1) suppresses the Tcl-1 (T-cell leukemia 1) oncogene through miR-29b [78] (Fig. 2B). More interestingly, several studies have connected the miR-29 family to HIV. Hariharan et al. reported that miR-29a/29b targets the HIV-nef protein (negative regulatory factor) [79]. Nef is a membrane-associated accessory protein able to down-regulate cell-surface molecules like CD4, MHCI and II, which enhances virion infectivity and stimulates viral replication [80]. By miR-29-induced reduction of nef levels, overall protein expression and HIV-1 replication was slowed down [17][patent

US 2007/0087335 A1; Brahmachari et al.]. Nathans et al. confirmed the ability of miR-29a to bind to the HIV-1 3'UTR and reported a high conservation of the otherwise variable nef sequence, which is targeted by the miRNA seed region. They found that miR-29a is highly abundant in HIV-infected T-lymphocytes and HIV-1 infection induced elevated miR-29a expression in HEK 293T cells. Mimicking miR-29a efficiently suppressed HIV-production and infectivity, confirming miR-29a as a cellular repressor of HIV-1 mRNA expression. Also, miR-29a enhanced HIV-1 mRNA interactions with P bodies and the RISC (RNA-induced silencing complex) proteins resulting in an accumulation of viral mRNA at P bodies for translational suppression and thereby reducing the infectivity of HIV [81]. It is noteworthy that in contrast to the reports where induced expression of miR-29 after HIV-infection and a potential antiviral role of the miR-29 family have been demonstrated, two other publications came to different conclusions, both reporting a down-regulation of miR-29 in HIV-infected PBMCs [82, 83] and patients [83]. Nevertheless, accumulating evidence suggests that miR-29 could be useful for the control of HIV infection at early stages [17, 81], however, the high variability and quick mutation rates of the HIV-genome might hamper therapeutic interventions with chemical or miRNA-based drugs.

Potential for cancer diagnosis and therapy

Mir-29: potential predictive biomarkers. Several studies have already described the potential of miR-29 family members as biomarkers for a variety of malignancies and assessment of miR-29 expression in serum or in various types of cancer-derived tissues could contribute to predictions about disease onset and prognosis. In chronic leukemia, expression of miR-29 family members discriminated between CLL samples with good and bad prognosis: in a patient group with unmutated IgV_H (rearranged immunoglobulin heavy-chain variable region gene) and high expression of ZAP-70 (70kDa zeta-associated protein), both of which are known predictors of poor prognosis in CLL, miR-29a, -29b-2 and -29c had low expression levels and were included in a signature group of 13 miRNAs as potential prognostic markers for CLL [31]. In agreement with that, Stamatopulous and colleagues showed that miR-29c levels significantly decreased in subjects with poor prognosis. Furthermore, miR-29c levels could predict individual treatment-free survival and overall survival in a cohort of 110 CLL patients with long-term follow-up [50] and likewise, low expression of miR-29c and miR-29b was associated with disease progression in some CLL cases [51]. Similarly, Wang et al. reported a down-regulation of miR-29a in PBMCs of AML patients compared to healthy individuals [84].

MiR-29 was also suggested as a prognostic marker for hepatocellular carcinoma as it was frequently down-regulated in HCC (hepatocellular carcinoma), which correlated with a worse prognosis for those patients [41]. A decreased expression level of the miR-29 family was furthermore associated with a shorter survival of patients with MCL (mantle cell lymphoma) [85]. In colorectal cancer (CRC), serum miR-29a was found to be significantly higher expressed in patients suffering from advanced CRC or colorectal liver metastasis than in CRC patients without metastasis [86] and very recently, Weissmann-Brenner et al. have shown that high expression of miR-29a was associated with longer disease-free survival of stage II CRC patients [87]. Likewise, miR-29a was significantly over-expressed in serum from ovarian cancer patients [88]. For a variety of cancers, the miR-29 family holds promise to become a prognostic and predictive biomarker, however, detection protocols, reference tissues or blood samples, and appropriate controls need to be standardized in order to ensure reliable and correct measurements of miRNA levels. This is even more important for detecting and distinguishing members of the miR-29 family, whose expression changes may potentially be small and who share substantial sequence homology.

Mir-29 as therapeutic tools or targets. Beside their potential function as non-invasive biomarkers and prognostic factors for disease outcome, a few research groups have begun to delineate miR-29's capacity as therapeutic agents, for example in leukemia treatment. A common issue in the development of leukemia is aberrant DNA hyper-methylation, which silences tumor suppressor genes involved in hematopoiesis [89, 90]. As described above, studies

by Garzon and Fabbri showed that miR-29 family members target DNA methyltransferases and thus induce global hypo-methylation in lung cancer and AML, leading to a reduction of tumor growth in nude mice [54, 55]. The same group also described a positive correlation between high levels of miR-29b and improved clinical responses to Decitabine, a DNMT-inhibitor used for treatment of AML patients [91]. Both Decitabine and miR-29 have hypo-methylating effects. Thus, the authors suggested that miR-29b levels might be useful in predicting potential Decitabine responses in AML patients. Furthermore, synthetic miR-29b could be used as a therapeutic DNA hypo-methylation agent for leukemic blasts because over-expression of miR-29b has been shown to reduce global DNA-methylation, thus inducing apoptosis [54]. Since miR-29 family members mainly exhibit tumor-suppressive functions and are often down-regulated in human cancers, re-introduction of synthetic miR-29 at specific tumor sites could become a possible treatment option. Vice versa, anti-miR-29 oligonucleotides could be applied in rare cases, where one or more miR-29 family members are over-expressed in human cancers (Table 2), similar to applications of other antagomirs, which are currently under investigation [92]. However, several issues need to be assessed before miR-29 or other miRNAs can be used in clinical applications, the most important of which are efficient delivery of miRNAs to target tissues, their potential side effects and their stability[93, 94] .

In this context, it has been shown that miRNA stability can range from several hours [95] to weeks [96]. Xiong et al. observed that transfected miR-29b duplexes were only stable for 4-7 days in HepG2 cells [41] and were not detectable any more four weeks after transfection in nude mice. In HeLa cells, synthetic miR-29b was already degraded after several hours [97]. Using artificial RNA-duplexes in the same cell line, Zhang et al. also found that miR-29 family members decay very rapidly, after 8-12 hours [98]. The question of half-lives for endogenous as well as synthetic miRNAs in the cells is important and will influence if and how applicable certain groups of miRNAs are for future use in clinical interventions.

Summary and Conclusions

The increasing number of publications on miR-29 family members emphasizes their important role in many cellular processes of cancer biology. The family is one of the first ones described and is highly conserved among species. Several studies have contributed to identifying the transcription start sites and managed to narrow down a possible region, at least for the miR-29a~29b-1 cluster. MiR-29 targets and down-regulates a diverse set of mRNAs with often survival-promoting properties and by doing so, miR-29 can reduce tumor growth and proliferation. In concordance with this, the vast majority of studies reports a down-regulation of miR-29 in different cancers and clearly assigns tumor-suppressive functions to this microRNA family. Future studies will have to establish the reasons for some seemingly tissue-specific exceptions, where miR-29 has tumor-promoting properties. The advances in clinical applications of antagomirs and mimics (miR replacement therapy) certainly hold potential for future treatment of diseases where miR-29 and others miRNAs are deregulated. However, the use of miRNAs as drugs is still in its infancy and questions about delivery, stability and potential side effects remain to be solved before a safe and effective treatment options will become available.

Several groups have begun to decipher the role of miR-29 in immune responses to viruses and bacteria and have described opposing immune-modulating properties against intra- or extracellular pathogens. More studies including a wider range of different pathogens will be necessary to specify the functional role of miR-29 in immune responses to infectious agents.

Another very interesting connection has been established between the miR-29 family, IFN-induced Jak/STAT signaling and as such immune responses to pathogens or potentially to cancer. Interferons are key coordinators of interactions between tumors and the immune system. Tumor-infiltrating immune cells can secrete cytokines that could trigger transcriptional up-regulation of miR-29 family members, which in turn down-regulate potentially oncogenic mRNAs. The concept of “immunoediting” has been introduced to explain observable anti-tumor responses that are mainly mediated by interferons as well as opposing effects where the immune system appears to promote cancer growth [99, 100]. STAT transcription factors, which are activated by cytokine signaling, have long

been recognized as important mediators of the crosstalk between immune and cancer cells [101, 102]. Considering our and other recent findings [103], we argue that miRNAs, and especially miR-29 could assume important functions in coordinating interferon-induced responses to suppress transformation into cancer cells: IFN-γ can specifically up-regulate miR-29, which in turn may suppress target genes involved in tumorigenesis. In this context, it will be interesting to investigate whether some cell types that have generally low levels of miR-29 may have acquired resistance to interferons, which would normally drive transcription of these miR genes. MicroRNAs in general and miR-29 in particular might be part of the sought after intercellular targets that are mediating the interferon-induced immune response to cancer.

Many recent studies corroborate on the potential use of the miR-29 family as predictive biomarkers for early diagnosis of malignancies and other diseases, either alone or in a panel of validated disease-specific miRNAs. However, expression levels have to be assessed more accurately in larger patient cohorts paying attention to appropriate controls, standardized techniques and amplification protocols, before miRNAs can routinely be used as diagnostic tools [104, 105]. Some progress has also been made towards the use of synthetic miRNAs as therapeutic agents or tools [106]. With its often tumor-suppressing functions, the miR-29 family lends itself to be explored further in future cancer therapy. Once important issues of miR delivery and stability are solved, replacement therapy with synthetic miR-29 in cancers where expression levels are low or absent can be envisaged. However, before this becomes a feasible treatment option, we need to acquire a more complete systems-biological view of the complex interactions of the miR-29 family, including characterization of disease-specific target genes, transcription factors, and their interplay with other miRNAs.

Acknowledgements

M.S. is supported by an AFR-grant by the Fonds National de la Recherche Luxembourg (TR-PHD BFR08-77). The work was supported by funding from the University of Luxembourg (F1R-LSC-PUL-09MIRN) and the Fondation Cancer (Luxembourg).

References

[1] Jovanovic M, Hengartner MO. miRNAs and apoptosis: RNAs to die for. Oncogene. 2006 Oct 9;25(46):6176-87.
[2] Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, et al. MicroRNA-143 regulates adipocyte differentiation. J Biol Chem. 2004 Dec 10;279(50):52361-5.
[3] Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer. 2006 Mar 27;94(6):776-80.
[4] Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006 Apr;6(4):259-69.
[5] Chang TC, Mendell JT. microRNAs in vertebrate physiology and human disease. Annu Rev Genomics Hum Genet. 2007;8:215-39.
[6] Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 2011 Jan;39(Database issue):D152-7.
[7] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004 Jan 23;116(2):281-97.
[8] Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 2004 Oct 13;23(20):4051-60.
[9] Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol. 2005 May;6(5):376-85.
[10] Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010 Sep;11(9):597-610.
[11] Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science. 2001 Oct 26;294(5543):853-8.
[12] Villarreal G, Jr., Oh DJ, Kang MH, Rhee DJ. Coordinated Regulation of Extracellular Matrix Synthesis by the MicroRNA-29 Family in the Trabecular Meshwork. Invest Ophthalmol Vis Sci. 2011 May 27.
[13] van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A. 2008 Sep 2;105(35):13027-32.
[14] Pandey AK, Verma G, Vig S, Srivastava S, Srivastava AK, Datta M. miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. Mol Cell Endocrinol. 2010 Jan 30;332(1-2):125-33.
[15] Ugalde AP, Ramsay AJ, de la Rosa J, Varela I, Marino G, Cadinanos J, et al. Aging and chronic DNA damage response activate a regulatory pathway involving miR-29 and p53. EMBO J. 2011 Jun 1;30(11):2219-32.
[16] Steiner DF, Thomas MF, Hu JK, Yang Z, Babiarz JE, Allen CD, et al. MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells. Immunity. 2011 Aug 26;35(2):169-81.
[17] Ahluwalia JK, Khan SZ, Soni K, Rawat P, Gupta A, Hariharan M, et al. Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. Retrovirology. 2008;5:117.
[18] Hand NJ, Horner AM, Master ZR, Boateng LA, Leguen C, Uvaydova M, et al. MicroRNA Profiling Identifies miR-29 as a Regulator of Disease-associated Pathways in Experimental Biliary Atresia. J Pediatr Gastroenterol Nutr. 2012 Feb;54(2):186-92.
[19] Chen T, Li Z, Tu J, Zhu W, Ge J, Zheng X, et al. MicroRNA-29a regulates pro-inflammatory cytokine secretion and scavenger receptor expression by targeting LPL in oxLDL-stimulated dendritic cells. FEBS Lett. 2011 Feb 18;585(4):657-63.
[20] Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 Family: Genomics, Cell Biology, and Relevance to Renal and Cardiovascular Injury. Physiol Genomics. 2012 Jan 3.
[21] Spizzo R, Nicoloso MS, Croce CM, Calin GA. SnapShot: MicroRNAs in Cancer. Cell. 2009 May 1;137(3):586- e1.
[22] Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet. 2008 Jan;40(1):43-50.
[23] Mott JL, Kurita S, Cazanave SC, Bronk SF, Werneburg NW, Fernandez-Zapico ME. Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. J Cell Biochem. 2010 Aug 1;110(5):1155-64.
[24] Saini HK, Griffiths-Jones S, Enright AJ. Genomic analysis of human microRNA transcripts. Proc Natl Acad Sci U S A. 2007 Nov 6;104(45):17719-24.
[25] Saini HK, Enright AJ, Griffiths-Jones S. Annotation of mammalian primary microRNAs. BMC Genomics. 2008;9:564.

[26] Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell. 2008 Aug 8;134(3):521-33.
[27] Corcoran DL, Pandit KV, Gordon B, Bhattacharjee A, Kaminski N, Benos PV. Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. PLoS One. 2009;4(4):e5279.
[28] Chien CH, Sun YM, Chang WC, Chiang-Hsieh PY, Lee TY, Tsai WC, et al. Identifying transcriptional start sites of human microRNAs based on high-throughput sequencing data. Nucleic Acids Res. 2011 Nov;39(21):9345-56.
[29] Bueno MJ, Perez de Castro I, Gomez de Cedron M, Santos J, Calin GA, Cigudosa JC, et al. Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. Cancer Cell. 2008 Jun;13(6):496-506.
[30] Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006 Mar;9(3):189-98.
[31] Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 2005 Oct 27;353(17):1793-801.
[32] Eyholzer M, Schmid S, Wilkens L, Mueller BU, Pabst T. The tumour-suppressive miR-29a/b1 cluster is regulated by CEBPA and blocked in human AML. Br J Cancer. 2010 Jul 13;103(2):275-84.
[33] Tarasov V, Jung P, Verdoodt B, Lodygin D, Epanchintsev A, Menssen A, et al. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle. 2007 Jul 1;6(13):1586-93.
[34] Park SY, Lee JH, Ha M, Nam JW, Kim VN. miR-29 miRNAs activate p53 by targeting p85 alpha and CDC42. Nat Struct Mol Biol. 2009 Jan;16(1):23-9.
[35] Kapinas K, Kessler CB, Delany AM. miR-29 suppression of osteonectin in osteoblasts: regulation during differentiation and by canonical Wnt signaling. J Cell Biochem. 2009 Sep 1;108(1):216-24.
[36] Pan X, Wang ZX, Wang R. MicroRNA-21: a novel therapeutic target in human cancer. Cancer Biol Ther. 2010 Jan 12;10(12):1224-32.
[37] Cortez MA, Nicoloso MS, Shimizu M, Rossi S, Gopisetty G, Molina JR, et al. miR-29b and miR-125a regulate podoplanin and suppress invasion in glioblastoma. Genes Chromosomes Cancer. 2010 Nov;49(11):981-90.
[38] Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T. MicroRNA expression profiling in prostate cancer. Cancer Res. 2007 Jul 1;67(13):6130-5.
[39] Cummins JM, He Y, Leary RJ, Pagliarini R, Diaz LA, Jr., Sjoblom T, et al. The colorectal microRNAome. Proc Natl Acad Sci U S A. 2006 Mar 7;103(10):3687-92.
[40] Wang H, Garzon R, Sun H, Ladner KJ, Singh R, Dahlman J, et al. NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. Cancer Cell. 2008 Nov 4;14(5):369-81.
[41] Xiong Y, Fang JH, Yun JP, Yang J, Zhang Y, Jia WH, et al. Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. Hepatology. 2010 Mar;51(3):836-45.
[42] Fang JH, Zhou HC, Zeng C, Yang J, Liu Y, Huang X, et al. MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. Hepatology. 2011 Nov;54(5):1729-40.
[43] Muniyappa MK, Dowling P, Henry M, Meleady P, Doolan P, Gammell P, et al. MiRNA-29a regulates the expression of numerous proteins and reduces the invasiveness and proliferation of human carcinoma cell lines. Eur J Cancer. 2009 Nov;45(17):3104-18.
[44] Wang C, Bian Z, Wei D, Zhang JG. miR-29b regulates migration of human breast cancer cells. Mol Cell Biochem. 2011 Jun;352(1-2):197-207.
[45] Gebeshuber CA, Zatloukal K, Martinez J. miR-29a suppresses tristetraprolin, which is a regulator of epithelial polarity and metastasis. EMBO Rep. 2009 Apr;10(4):400-5.
[46] Philippidou D, Schmitt M, Moser D, Margue C, Nazarov PV, Muller A, et al. Signatures of microRNAs and selected microRNA target genes in human melanoma. Cancer Res. 2010 May 15;70(10):4163-73.
[47] Nguyen T, Kuo C, Nicholl MB, Sim MS, Turner RR, Morton DL, et al. Downregulation of microRNA-29c is associated with hypermethylation of tumor-related genes and disease outcome in cutaneous melanoma. Epigenetics. 2011 Mar;6(3):388-94.
[48] Han YC, Park CY, Bhagat G, Zhang J, Wang Y, Fan JB, et al. microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. J Exp Med. 2010 Mar 15;207(3):475-89.
[49] Wang F, Wang XS, Yang GH, Zhai PF, Xiao Z, Xia LY, et al. miR-29a and miR-142-3p downregulation and diagnostic implication in human acute myeloid leukemia. Mol Biol Rep. 2011 Mar;39(3):2713-22.

[50] Stamatopoulos B, Meuleman N, Haibe-Kains B, Saussoy P, Van Den Neste E, Michaux L, et al. microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. Blood. 2009 May 21;113(21):5237-45.
[51] Visone R, Rassenti LZ, Veronese A, Taccioli C, Costinean S, Aguda BD, et al. Karyotype-specific microRNA signature in chronic lymphocytic leukemia. Blood. 2009 Oct 29;114(18):3872-9.
[52] Pekarsky Y, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, et al. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. Cancer Res. 2006 Dec 15;66(24):11590-3.
[53] Santanam U, Zanesi N, Efanov A, Costinean S, Palamarchuk A, Hagan JP, et al. Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. Proc Natl Acad Sci U S A. 2010 Jul 6;107(27):12210-5.
[54] Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood. 2009 Jun 18;113(25):6411-8.
[55] Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A. 2007 Oct 2;104(40):15805-10.
[56] Ulivi P, Zoli W, Calistri D, Fabbri F, Tesei A, Rosetti M, et al. p16INK4A and CDH13 hypermethylation in tumor and serum of non-small cell lung cancer patients. J Cell Physiol. 2006 Mar;206(3):611-5.
[57] Girault I, Tozlu S, Lidereau R, Bieche I. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res. 2003 Oct 1;9(12):4415-22.
[58] Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H, et al. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer. 2003 Jul 1;105(4):527-32.
[59] Patra SK, Patra A, Zhao H, Dahiya R. DNA methyltransferase and demethylase in human prostate cancer. Mol Carcinog. 2002 Mar;33(3):163-71.
[60] Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. Cancer Res. 1999 May 15;59(10):2302-6.
[61] Kim H, Kwon YM, Kim JS, Han J, Shim YM, Park J, et al. Elevated mRNA levels of DNA methyltransferase-1 as an independent prognostic factor in primary nonsmall cell lung cancer. Cancer. 2006 Sep 1;107(5):1042-9.
[62] Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. 2008 Jan;9(1):47-59.
[63] Kole AJ, Swahari V, Hammond SM, Deshmukh M. miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis. Genes Dev. 2011 Jan 15;25(2):125-30.
[64] Mott JL, Kobayashi S, Bronk SF, Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene. 2007 Sep 13;26(42):6133-40.
[65] Ye Y, Hu Z, Lin Y, Zhang C, Perez-Polo JR. Downregulation of microRNA-29 by antisense inhibitors and a PPAR-gamma agonist protects against myocardial ischaemia-reperfusion injury. Cardiovasc Res. 2010 Aug 1;87(3):535-44.
[66] Sengupta S, den Boon JA, Chen IH, Newton MA, Stanhope SA, Cheng YJ, et al. MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins. Proc Natl Acad Sci U S A. 2008 Apr 15;105(15):5874-8.
[67] Maurer B, Stanczyk J, Jungel A, Akhmetshina A, Trenkmann M, Brock M, et al. MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. Arthritis Rheum. 2010 Jun;62(6):1733-43.
[68] Liu Y, Taylor NE, Lu L, Usa K, Cowley AW, Jr., Ferreri NR, et al. Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes. Hypertension. 2010 Apr;55(4):974-82.
[69] Sengupta S, den Boon JA, Chen IH, Newton MA, Dahl DB, Chen M, et al. Genome-wide expression profiling reveals EBV-associated inhibition of MHC class I expression in nasopharyngeal carcinoma. Cancer Res. 2006 Aug 15;66(16):7999-8006.
[70] Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, et al. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature. 2007 Oct 18;449(7164):919-22.
[71] O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. Immunity. 2008 Apr;28(4):477-87.
[72] Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004 Feb;75(2):163-89.
[73] Kohanbash G, Okada H. MicroRNAs and STAT interplay. Semin Cancer Biol. 2012 Feb;22(1):70-5.

[74] Reinsbach S, Nazarov PV, Philippidou D, Schmitt M, Wienecke-Baldacchino A, Muller A, et al. Dynamic regulation of microRNA expression following Interferon-gamma-induced gene transcription. RNA Biol. Jul 1;9(7).
[75] Banerjee A, Schambach F, DeJong CS, Hammond SM, Reiner SL. Micro-RNA-155 inhibits IFN-gamma signaling in CD4+ T cells. Eur J Immunol. 2010 Jan;40(1):225-31.
[76] Papadopoulou AS, Dooley J, Linterman MA, Pierson W, Ucar O, Kyewski B, et al. The thymic epithelial microRNA network elevates the threshold for infection-associated thymic involution via miR-29a mediated suppression of the IFN-alpha receptor. Nat Immunol. 2012;13(2):181-7.
[77] Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, et al. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. Nat Immunol. 2011 Sep;12(9):861-9.
[78] Anastasiadou E, Boccellato F, Vincenti S, Rosato P, Bozzoni I, Frati L, et al. Epstein-Barr virus encoded LMP1 downregulates TCL1 oncogene through miR-29b. Oncogene. 2010 Mar 4;29(9):1316-28.
[79] Hariharan M, Scaria V, Pillai B, Brahmachari SK. Targets for human encoded microRNAs in HIV genes. Biochem Biophys Res Commun. 2005 Dec 2;337(4):1214-8.
[80] Kirchhoff F, Schindler M, Specht A, Arhel N, Munch J. Role of Nef in primate lentiviral immunopathogenesis. Cell Mol Life Sci. 2008 Sep;65(17):2621-36.
[81] Nathans R, Chu CY, Serquina AK, Lu CC, Cao H, Rana TM. Cellular microRNA and P bodies modulate host-HIV-1 interactions. Mol Cell. 2009 Jun 26;34(6):696-709.
[82] Houzet L, Yeung ML, de Lame V, Desai D, Smith SM, Jeang KT. MicroRNA profile changes in human immunodeficiency virus type 1 (HIV-1) seropositive individuals. Retrovirology. 2008;5:118.
[83] Sun G, Li H, Wu X, Covarrubias M, Scherer L, Meinking K, et al. Interplay between HIV-1 infection and host microRNAs. Nucleic Acids Res. 2011 Nov 10.
[84] Wang F, Wang XS, Yang GH, Zhai PF, Xiao Z, Xia LY, et al. miR-29a and miR-142-3p downregulation and diagnostic implication in human acute myeloid leukemia. Mol Biol Rep. 2011 Jun 16.
[85] Zhao JJ, Lin J, Lwin T, Yang H, Guo J, Kong W, et al. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. Blood. 2010 Apr 1;115(13):2630-9.
[86] Wang LG, Gu J. Serum microRNA-29a is a promising novel marker for early detection of colorectal liver metastasis. Cancer Epidemiol. 2011 Feb;36(1):e61-7.
[87] Weissmann-Brenner A, Kushnir M, Lithwick Yanai G, Aharonov R, Gibori H, Purim O, et al. Tumor microRNA-29a expression and the risk of recurrence in stage II colon cancer. Int J Oncol. 2012 Mar 16.
[88] Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, Cohn DE. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. Gynecol Oncol. 2009 Jan;112(1):55-9.
[89] Toyota M, Kopecky KJ, Toyota MO, Jair KW, Willman CL, Issa JP. Methylation profiling in acute myeloid leukemia. Blood. 2001 May 1;97(9):2823-9.
[90] Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, et al. Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood. 2001 Mar 1;97(5):1172-9.
[91] Blum W, Garzon R, Klisovic RB, Schwind S, Walker A, Geyer S, et al. Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. Proc Natl Acad Sci U S A. 2010 Apr 20;107(16):7473-8.
[92] Poltronieri P, D'Urso PI, Mezzolla V, D'Urso OF. Potential of anti-cancer therapy based on anti-miR-155 oligonucleotides in glioma and brain tumours. Chem Biol Drug Des. Jul 26.
[93] Seth S, Johns R, Templin MV. Delivery and biodistribution of siRNA for cancer therapy: challenges and future prospects. Ther Deliv. Feb;3(2):245-61.
[94] Osman A. MicroRNAs in health and disease--basic science and clinical applications. Clin Lab.58(5-6):393-402.
[95] Bail S, Swerdel M, Liu H, Jiao X, Goff LA, Hart RP, et al. Differential regulation of microRNA stability. RNA. 2010 May;16(5):1032-9.
[96] van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007 Apr 27;316(5824):575-9.
[97] Hwang HW, Wentzel EA, Mendell JT. A hexanucleotide element directs microRNA nuclear import. Science. 2007 Jan 5;315(5808):97-100.
[98] Zhang Z, Zou J, Wang GK, Zhang JT, Huang S, Qin YW, et al. Uracils at nucleotide position 9-11 are required for the rapid turnover of miR-29 family. Nucleic Acids Res. 2011 May;39(10):4387-95.

[99] Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol. 2006 Nov;6(11):836-48.
[100] Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011 Mar 25;331(6024):1565-70.
[101] Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol. 2007 Jan;7(1):41-51.
[102] Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009 Nov;9(11):798-809.
[103] Smith KM, Guerau-de-Arellano M, Costinean S, Williams JL, Bottoni A, Mavrikis Cox G, et al. miR-29ab1 Deficiency Identifies a Negative Feedback Loop Controlling Th1 Bias That Is Dysregulated in Multiple Sclerosis. J Immunol. 2012 Aug 15;189(4):1567-76.
[104] Mizuarai S, Irie H, Kotani H. Gene expression-based pharmacodynamic biomarkers: the beginning of a new era in biomarker-driven anti-tumor drug development. Curr Mol Med. 2010 Aug;10(6):596-607.
[105] Vlassov VV, Laktionov PP, Rykova EY. Circulating nucleic acids as a potential source for cancer biomarkers. Curr Mol Med. 2010 Mar;10(2):142-65.
[106] Rossbach M. Small non-coding RNAs as novel therapeutics. Curr Mol Med. 2010 Jun;10(4):361-8.
[107] Li Y, Wang F, Xu J, Ye F, Shen Y, Zhou J, et al. Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29. J Pathol. 2011 Aug;224(4):484-95.
[108] Kong G, Zhang J, Zhang S, Shan C, Ye L, Zhang X. Upregulated microRNA-29a by hepatitis B virus X protein enhances hepatoma cell migration by targeting PTEN in cell culture model. PLoS One. 2011;6(5):e19518.
[109] Verrier JD, Lau P, Hudson L, Murashov AK, Renne R, Notterpek L. Peripheral myelin protein 22 is regulated post-transcriptionally by miRNA-29a. Glia. 2009 Sep;57(12):1265-79.
[110] Cui Y, Su WY, Xing J, Wang YC, Wang P, Chen XY, et al. MiR-29a inhibits cell proliferation and induces cell cycle arrest through the downregulation of p42.3 in human gastric cancer. PLoS One. 2011;6(10):e25872.
[111] Su H, Yang JR, Xu T, Huang J, Xu L, Yuan Y, et al. MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer Res. 2009 Feb 1;69(3):1135-42.
[112] Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer. 2010 Jul 1;127(1):118-26.

Table 1: Confirmed direct targets of the miR-29 family

Target		L	mRNA	WB	29 a/b/c	Ref	description
	DNA-methyltransferases	√	√	√	29b	[54]	**Methyltransferases**
			√ **iv**		29a	[18]	
DNMT3A		√	√	√	29abc	[55]	
		√	√	√	29b	[54]	
			√ **iv**		29a	[18]	
DNMT3B		√	√	√	29abc	[55]	
Bim	Bcl-2-like 11	√			29b	[63]	**Members of Bcl-2 family and others involved in apoptosis**
Puma	p53 up-regulated modulator of apoptosis	√			29b	[63]	
Bmf	Bcl-2-modifying factor	√			29b	[63]	
Bcl-2	B-cell lymphoma 2	√	√	√	29abc	[41]	
	Myeloid cell leukemia sequence 1	√	no effect	√	29b	[64]	
		√	√	√	29abc	[41]	
Mcl-1				√ **iv**	29ac	[65]	
Hrk	Activator of apoptosis harakiri	√			29b	[63]	
N-Bak	Bcl-2 homologous antagonist	√			29b	[63]	
Adamts18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	√			29abc	[15]	**Extracellular matrix proteins**
COL15A1	Collagens	√	√		29c	[66]	
		√			29c		
COL1A1		√	√		29b	[68]	
COL1A1		√	√ & **iv**		29**b**	[13]	
COL1A2		√			29c	[66]	
		√	√ & **iv**		29**b**	[13]	
		√	√		29c	[66]	
		√	√	√	29abc	[67]	
		√	√		29b	[68]	
COL3A1		√	√ & **iv**		29**b**	[13]	
COL4A1		√	√		29c	[66]	
COL4A2		√			29c		
COL4A1		√	√		29b	[68]	
COL5A1		√	√		29b		
COL5A2		√	√		29b		
COL5A3		√	√		29b		
COL7A1		√	√		29b	[68]	
COL8A1		√	√		29b		
Eln1	Elastin	√			29b	[13]	
Fbn1	Fibrillin1	√			29b		
Itgb1	Integrin β1	√	√		29b	[68]	
Laminin γ1		√	√		29c	[66]	
	Matrix metalloprotease	√		√	29b	[42]	
MMP-2		√	√		29b	[68]	

Osteonectin/ SPARC	secreted protein, acidic, cysteine-rich	√	√		29ac	[35]	
T-bet	T-box transcription factor TBX21	√	√		29b	[16]	**Transcription factors**
Eomes	Eomesodermin = T-box brain protein 2	√	√		29b	[16]	
HBP1	HMG-box transcription factor 1	√			29abc	[15]	
Mycn	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived	√			29abc	[15]	
	Yin-yang 1	√		√ **iv**	29abc	[40]	
YY1			√	√	29ab	[107]	
cdc42	Cell division control protein 42 homolog	√		√	29abc	[34]	**Cell cycle proteins**
	Cyclin-dependent kinase 6	√	√	√	29abc	[85]	
cdk6			√	√	29ab	[107]	
Dusp2	Dual specificity protein phosphatase 2	√			29abc	[15]	**Phosphatases**
Ppm1d	Protein phosphatase 1D	√			29abc	[15]	
	Phosphatase and tensin homolog			√	29b	[44]	
PTEN		√	√	√	29a	[108]	
FUSIP1	FUS-interacting serine-arginine-rich protein 1	√	√		29c	[66]	**RNA splicing**
Ifi30	Gamma-interferon-inducible lysosomal thiol reductase	√			29abc	[15]	**Thiol reductase**
Ifnar1	Interferon alpha receptor 1	√			29a	[76]	**Cytokine signaling**
IFN-γ	Interferon γ	√			29abc	[77]	
Igf1	Insulin-like growth factor 1	√	√ **iv**		29a	[18]	
Il1RAP	Interleukin-1 receptor accessory protein	√	√ **iv**		29a	[18]	
LPL	lipoproteinlipase	√	√	√	29a	[19]	**Lipase**
Narf	Nuclear prelamin A recognition factor	√			29abc	[15]	**Nuclear protein**
	Negative regulatory factor	√		√	29ab	[17]	**Virulence factor (HIV)**
nef		√			29a	[81]	
	Phosphatidylinositol 3-kinase, regulatory subunit α	√		√	29abc	[34]	**Kinase**
p85 α				√	29a	[14]	
PDPN	Podoplanin	√	√	√	29b	[37]	**Membrane glycoprotein**
PMP22	Peripheral myelin protein 22	√	√	√	29abc	[109]	**Major component of myelin**
	T-cell leukemia 1		√	√	29c	[50]	**Co-activator of Akt**
		√		√	29b	[52]	
Tcl-1				√	29b	[78]	
TDG	G/T mismatch-specific thymine DNA glycosylase	√	√		29c	[66]	**Glycosylase**
TTP	Tristetraprolin	√		√	29a	[45]	**mRNA degradation**
p42.3		√	√	√	29a	[110]	**unknown**

L = luciferase assay, mRNA = mRNA level; iv = *in vivo*/mice, WB = western blot; **b** = mainly b

Table 2: Summary of studies reporting an up- or down-regulation of one or more miR-29 family members in cancer

Down-regulation of miR-29 family members in cancer ↓			
type of cancer	**sample types**	**miRNA**	**ref**
acute myeloid leukemia (AML)	patient bone marrow	29b	[32]
	PBMCs	29a	[84]
chronic lymphocytic leukemia (CLL)	patient lymphocytes	29abc	[31]
		29bc	[51]
		29bc	[52]
	PBMCs	29c	[50]
malignant cholangiocytes	cell line	29b	[64]
colorectal cancer	tissue	29	[39]
glioblastoma	tissue	29b	[37]
rhabdomyosarcoma	cell lines & tissue	29b	[40]
hepatocellular carcinoma	tissue	29	[111]
lung cancer	cell line	29a	[43]
	tissue	29b	[30]
mantle cell lymphoma	tissue	29abc	[85]
nasopharyngeal carcinoma	tissue	29c	[66]
invasive pancreatic cancer	cell line	29a	[43]
prostate cancer	tissue	29ab	[38]
Burkitt lymphoma	cell line	29	[22]
Up-regulation of miR-29 family members in cancer ↑			
type of cancer	**sample types**	**miRNA**	**ref**
AML	patient LSC/ non-LSC-blasts	29a	[48]
CLL	tissue	29ab	[53]
colorectal liver metastasis colorectal cancer	serum	29a	[86]
	plasma		[112]
breast	tissue	29a	[45]
ovarian cancer	serum	29a	[88]
primary melanoma	tissue	29ab	[46]

Figure Legends

Figure 1:

A) Scheme of miR-29 genomic organization: miR-29a~29b-1 and miR-29a~29b-2 are encoded as a cluster on Chr7 q32.3 and Chr1 q32.2, respectively (left panel). Mature sequences of the miR-29 family members are shown on the right. Note identical mature sequences for miR-29b-1 and miR-29b-2. Seed regions are highlighted by a box and nucleotides differing between members are underlined, the single nucleotide difference between miR-29a and miR-29c is shown in italics (right panel). B) Human primary transcripts of the miR-29 genes. Exons (E1-4/5) are shown as white boxes, introns as connecting lines; hairpin structures illustrate the coding position of precursor sequences. A scale is given below. C) Promoter organization and potential transcription start sites for both clusters miR-29a~29b-1 (TSSab1-8) and miR-29a~29b-2 (TSSbc1-6) from literature, as mentioned in the text. Coordinates have all been converted to genome version hg19/GRCh37 and are as follows: TSSab1 chr7:130.572.487; TSSab2 chr7:130.586.832; TSSab3 chr7:130.596.983; TSSab4 chr7:130.597.889; TSSab5 chr7:130.598.020; TSSab6 chr7:130.598.638; TSSab7 chr7:130.598.268; TSSab8 chr7:130.800.298; TSSbc1 chr1:207.977.425; TSSbc2 chr1:207.979.479; TSSbc3 chr1:207.985.009; TSSbc4 chr1:207.996.050; TSSbc5 chr1:207.997.156; TSSbc6 chr1:207.037.276.

Figure 2:

A) Overview of miR-29 family members and their regulation: transcriptional activators and repressors of the miR-29a~29b-1 and miR-29a~29b-2 clusters are shown that actively influence miR-29 expression levels. The dashed box indicates our recent findings on miR-29 regulation by STAT1. (Note that SMAD3 activates miR-29b only and canonical Wnt-signaling induced miR-29a and 29c only. B) Immune-modulatory effects of miR-29 and interaction with pathogens (as described in “MiR-29: a player in immunological host defense?”).

Figure 1

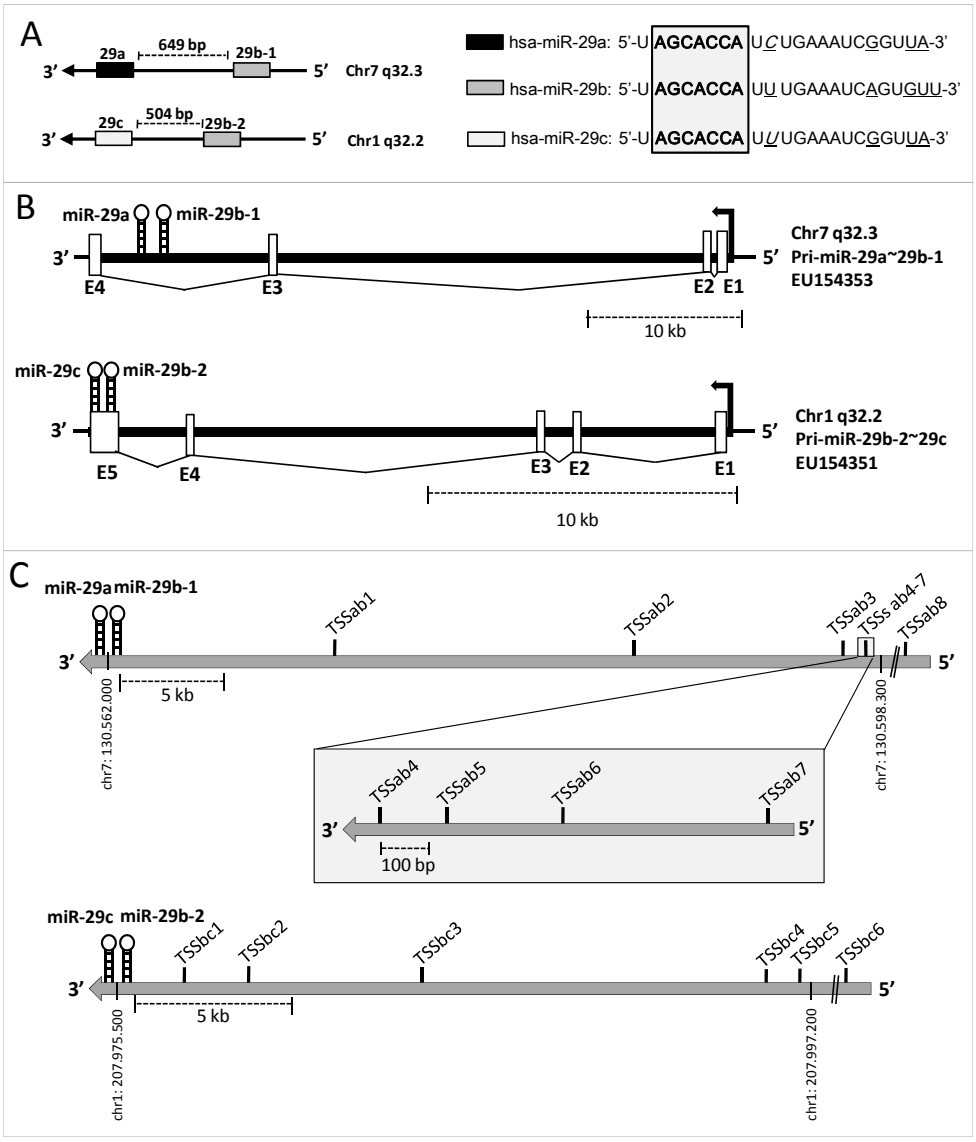
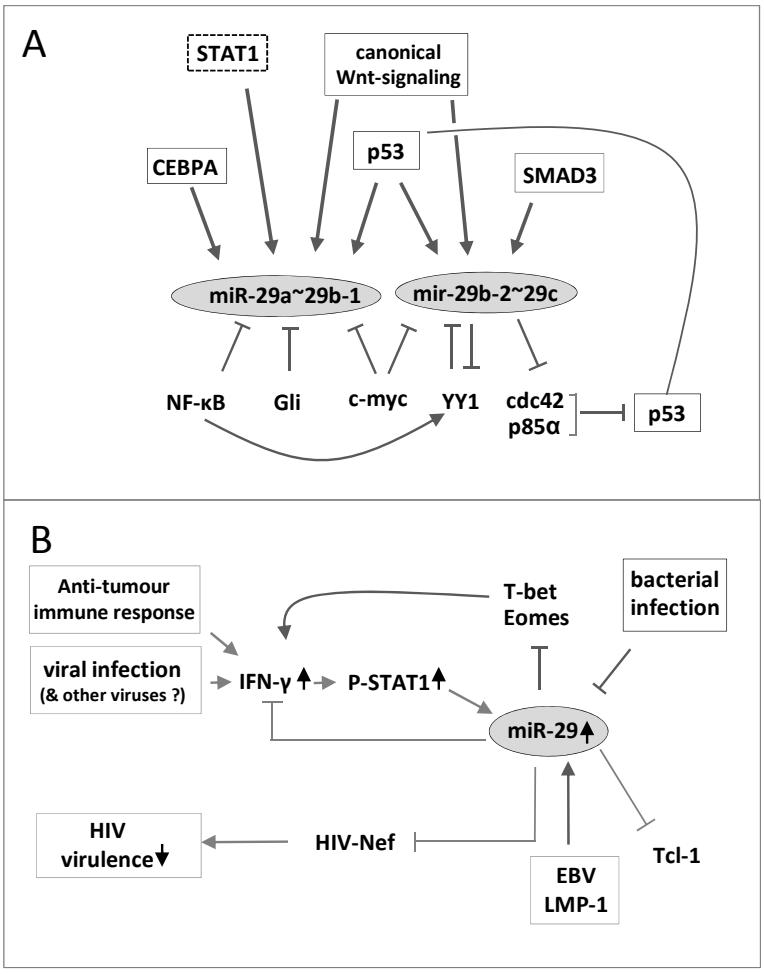

A
29a
649 bp
29b-1
3'
5'
Chr7 q32.3
29c
504 bp
29b-2
Chr1 q32.2
hsa-miR-29a: 5'-U AGCACCA UUGAAAUCGGUUA-3'
hsa-miR-29b: 5'-U AGCACCA UUUGAAAUCAGUGUU-3'
hsa-miR-29c: 5'-U AGCACCA UUUGAAAUCGGUUA-3'
B
miR-29a
miR-29b-1
E4
E3
E2
E1
10 kb
Chr7 q32.3
Pri-miR-29a~29b-1
EU154353
miR-29c
miR-29b-2
E5
E4
E3
E2
E1
10 kb
Chr1 q32.2
Pri-miR-29b-2~29c
EU154351
C
miR-29a miR-29b-1
TSSab1
TSSab2
TSSab3
TSSs ab4-7
TSSab8
5 kb
chr7: 130.562.000
chr7: 130.598.300
TSSab4
TSSab5
TSSab6
TSSab7
100 bp
miR-29c miR-29b-2
TSSbc1
TSSbc2
TSSbc3
TSSbc4
TSSbc5
TSSbc6
5 kb
chr1: 207.975.500
chr1: 207.997.200

Figure 2


A
STAT1
canonical Wnt-signaling
CEBPA
p53
SMAD3
miR-29a~29b-1
mir-29b-2~29c
NF-κB
Gli
c-myc
YY1
cdc42 p85α
p53
B
Anti-tumour immune response
viral infection (& other viruses ?)
IFN-γ
P-STAT1
T-bet Eomes
bacterial infection
miR-29
HIV virulence
HIV-Nef
Tcl-1
EBV LMP-1

Schmitt et al. Cell Communication and Signaling 2012, 10:41
http://www.biosignaling.com/content/10/1/41

CELL COMMUNICATION & SIGNALING

RESEARCH Open Access

Interferon-γ-induced activation of Signal Transducer and Activator of Transcription 1 (STAT1) up-regulates the tumor suppressing microRNA-29 family in melanoma cells

Martina J Schmitt[1], Demetra Philippidou[1], Susanne E Reinsbach[1], Christiane Margue[1], Anke Wienecke-Baldacchino[2], Dorothee Nashan[3], Iris Behrmann[1] and Stephanie Kreis[1*]

Abstract

Background: The type-II-cytokine IFN-γ is a pivotal player in innate immune responses but also assumes functions in controlling tumor cell growth by orchestrating cellular responses against neoplastic cells. The role of IFN-γ in melanoma is not fully understood: it is a well-known growth inhibitor of melanoma cells *in vitro*. On the other hand, IFN-γ may also facilitate melanoma progression. While interferon-regulated genes encoding proteins have been intensively studied since decades, the contribution of miRNAs to effects mediated by interferons is an emerging area of research.
We recently described a distinct and dynamic regulation of a whole panel of microRNAs (miRNAs) after IFN-γ-stimulation. The aim of this study was to analyze the transcriptional regulation of miR-29 family members in detail, identify potential interesting target genes and thus further elucidate a potential signaling pathway IFN-γ → Jak→ P-STAT1 → miR-29 → miR-29 target genes and its implication for melanoma growth.

Results: Here we show that IFN-γ induces STAT1-dependently a profound up-regulation of the miR-29 primary cluster pri-29a~b-1 in melanoma cell lines. Furthermore, expression levels of pri-29a~b-1 and mature miR-29a and miR-29b were elevated while the pri-29b-2~c cluster was almost undetectable. We observed an inverse correlation between miR-29a/b expression and the proliferation rate of various melanoma cell lines. This finding could be corroborated in cells transfected with either miR-29 mimics or inhibitors. The IFN-γ-induced G1-arrest of melanoma cells involves down-regulation of CDK6, which we proved to be a direct target of miR-29 in these cells. Compared to nevi and normal skin, and metastatic melanoma samples, miR-29a and miR-29b levels were found strikingly elevated in certain patient samples derived from primary melanoma.

Conclusions: Our findings reveal that the miR-29a/b1 cluster is to be included in the group of IFN- and STAT-regulated genes. The up-regulated miR-29 family members may act as effectors of cytokine signalling in melanoma and other cancer cells as well as in the immune system.

Keywords: IFN-γ, Jak/STAT pathway, STAT1, Signaling, Melanoma, miR-29, Tumor-suppressor

* Correspondence: Stephanie.Kreis@uni.lu
[1]Signal Transduction Laboratory, University of Luxembourg, 162A Avenue de la Faïencerie, Luxembourg L-1511, Luxembourg
Full list of author information is available at the end of the article

Background

In the past decade, small non-coding microRNAs (miRNAs) have been identified as new and important players in post-transcriptional gene regulation and ever since, their expression patterns and cellular functions have been investigated in cancer and other diseases [1,2]. MiRNA biogenesis can be differentially regulated [3], but generally starts with the generation of a primary (pri-) miRNA transcript (several thousand nucleotides long), which is subsequently processed into a 70–80 nucleotide precursor form (pre-miRNA), which, following nuclear export, is then cleaved into the ~22 nucleotide mature miRNA. One strand of the mature duplex is incorporated in the RISC (RNA-induced silencing complex), where it recognizes, binds to and represses mRNA target sequences [1]. MiRNAs are involved in many fundamental cellular processes as they are estimated to control >50% of all protein-coding genes in mammals [4]. Consequently, they have been implicated in the regulation of processes that promote cancer growth or conversely, in processes that might prevent cancers from developing. For instance, a cancer cell can emerge following the over-expression of so-called "oncomirs" (such as the miR-17-92 family, miR-21, -155, etc.) which down-regulate tumor-suppressors that control cell proliferation. On the other hand, miRNAs that function as tumor-suppressors by targeting cellular oncoproteins (such as let-7 family members, miR-15a, -16, -29, etc.) are frequently down-regulated in cancer tissues [5]. Therapeutics opting to replace the diminished tumor-suppressor miRNAs are currently being investigated and seem promising, as miRNAs exhibit high stability as well as high specificity for their target mRNAs [5,6].

A disease where patients are in urgent need of more effective treatments is advanced melanoma, the most aggressive form of skin cancer. Metastatic melanoma exhibit a severe resistance to therapy leading to 5-year survival rates of below 5% [7]. Around 50% of patients exhibit V600E mutations in the cellular kinase BRAF [8]. Recently, the BRAF-inhibitor Zelboraf® has been approved for treatment of late-stage malignant melanoma patients with V600E mutations, increasing life expectancy by several months [9,10]. Nevertheless, except excision at early stages, no curative therapies exist. Routinely, therapies against melanoma include IFN-α as an adjuvant treatment. Interferons are cytokines and constitute a major part of the innate immune response, but they are also recognized for their anti-proliferative properties. We and others have shown that the type-II-cytokine IFN-γ mediates growth inhibition of cancer cells by activating the transcription factor STAT1 [11,12]. After IFN-γ stimulation, STAT1 forms homodimers, which bind to GAS (IFN-γ-activated sequences) elements in the promoter regions of target genes. Very recently, we have found several miRNAs to be dynamically regulated following stimulation with IFN-γ [13]. One of the first connections between cytokine-induced Jak/STAT signaling and miRNAs has been established by Löffler *et al.*, who showed that IL-6 increased the expression of oncogenic miR-21 via STAT3 activation in myeloma cells [14]. The signaling cascades involving IL-6 or IFN-α/β/STAT3/miR-21 and others have been confirmed in several types of cancer and diseases [15-17].

In the current study, we have focused on the biochemical analysis of individual miRNAs regulated by IFN-γ which we have recently identified in a detailed-time course microarray experiment [13], and further concentrated on the interesting miRNA family miR-29 with its three mature members, miR-29a, -29b and -29c. It is transcribed into two primary transcripts, pri-29a~b-1 and pri-29b-2~c, from chromosomes 7 and 1, respectively. MiR-29 family members target the expression of proteins such as methyltransferases, extracellular matrix proteins and transcription factors [18-20], which are potentially involved in triggering enhanced invasion, migration or proliferation of cells. They are silenced or down-regulated in many types of cancer and have consequently been assigned tumor-suppressing properties, although in some cases also oncogenic roles have been reported [21,22]. Here, we demonstrate a specific and profound IFN-γ-induced, STAT1-dependent up-regulation of miR-29a and -29b in melanoma cells and importantly, also increased expression in primary melanoma patient samples (but not in metastatic tumors) whereas the second cluster pri-29b-2~c was consistently undetectable. Moreover, we provide evidence for the tumor-suppressing properties of miR-29 family members: inhibition of melanoma cell proliferation could be mediated by miR-29a, which down-regulated *CDK6* (cyclin-dependent kinase 6), an important player in cell cycle G1/S transition. Our findings identify the pri-29a~b-1cluster as a novel IFN-γ-regulated gene and open up new connections between miRNAs, interferon signaling and malignant melanoma, which could lead to novel concepts for potential treatment options in the future.

Results

To investigate possible transcriptional regulations of miRNAs by STAT transcription factors, several melanoma cell lines were treated with IFN-γ for different time intervals and were subsequently analyzed by miRNA microarray as previously described [13]. The top 10 IFN-γ-induced miRNAs from a microarray experiment, which showed highest differential expression compared to untreated cells, and detailed time-course expression profiles thereof are depicted in Figure 1A and Additional file 1: Figure S1. For further analysis, we focused on the miR-29 family, as its mature members miR-29a and

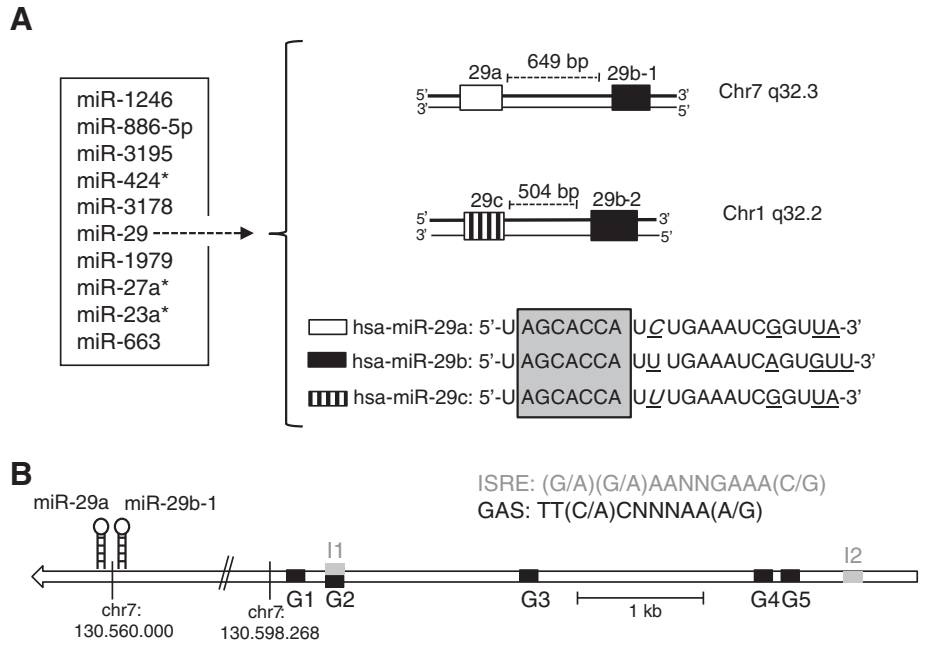


Figure 1 Top 10 IFN-γ-up-regulated miRNAs. (**A**) Ten miRNAs with highest positive fold changes (as determined by previous microarray experiments [13]) include miR-29 family members (left). Detailed time course profiles are shown in Additional file 1: Figure S1. The miR-29 family is transcribed from the respective antisense strand from two genetic clusters of chromosomes 7 (pri-29a~b-1) and 1 (pri-29b-2~c) (right). The three mature forms miR-29a/29b/29c share the same seed region (grey box). Differences between the mature sequences are underlined; a nucleotide difference between miR-29a and miR-29c is shown in italics. (**B**) The presumed pri-29a~b-1 promoter region [22-24] contains five GAS-elements G1-5 (TT(C/A)CNNNAA(A/G)) and two ISRE-elements I1-2 (((G/A)(G/A)AANNGAAA(C/G)) (GRCh37/hg19).

miR-29b showed the most robust regulations across all tested melanoma cell lines and because of its interesting properties regarding tumor biology. To identify the presence of potential IFN-response elements, we performed *in silico* screening of the promoter region 5 kb upstream of a putative transcription start of pri-29a~b-1 [22-24] and found five GAS-elements (TT(C/A)CNNNAA(A/G)) and two ISRE (interferon stimulated response element)-elements ((G/A)(G/A)AANNGAAA(C/G)) (Figure 1B). For control purposes, we selected miR-100, which was slightly down-regulated after IFN-γ stimulation and miR-25, whose levels were not induced in the microarray experiments. Other regulated candidates included several miRNA star sequences ("miR*" which here represents the miR strand, which arises from the 3'-arm of the hairpin, while the 5'-arm would be the guide or parent strand and is conventionally considered as "minor" product) which are currently being further assessed in our laboratory (Figure 1, Additional file 1: Figure S1).

The pri-29a~b-1 cluster and mature miR-29a/29b are regulated by IFN-γ

For stimulation experiments with IFN-γ, melanoma cell lines MeWo and A375, as well as stably transfected A375 derivates were used. A375-STAT1(F) represent STAT1-dominant negative cells harboring a phenylalanine replacement of tyrosine residue 701 crucial for STAT1 phosphorylation and dimerization [12]. Thus, transcription of STAT1 target genes is abolished despite IFN-γ stimulation. The corresponding control cells A375-STAT1(wt) express the STAT1 wild-type construct instead [12]. To accurately assess the regulation of the miR-29 family by IFN-γ-induced STAT1, we performed time course experiments (Figure 2). Stimulation of A375, MeWo and A375-STAT1(wt) cell lines with 50 ng/ml of IFN-γ induced a prominent STAT1 phosphorylation, which decreased after 48h of IFN-γ treatment, whereas the STAT1-dominant negative cells A375-STAT1(F) only exhibited a delayed and weak P-STAT1 signal after IFN-γ stimulation (Figure 2A, see also [25]). Functional activity of the P-STAT1 transcription factor was confirmed by up-regulation of the STAT1 target genes IRF-1 and STAT1 itself, which showed induced expression after 3h and 8h, respectively.

Following stimulation, changes in miRNA expression levels were assessed by qRT-PCR (Figure 2B). A375, A375-STAT1(wt) and MeWo cell lines showed a strong and significant up-regulation (>5 fold) of the pri-29a~b-1 cluster, starting 24h after IFN-γ stimulation, while expression of the pri-29b-2~c cluster was not altered (Figure 2B, upper panel). Accordingly, miRNA precursors pre-29a and pre-29b-1 were also augmented whereas

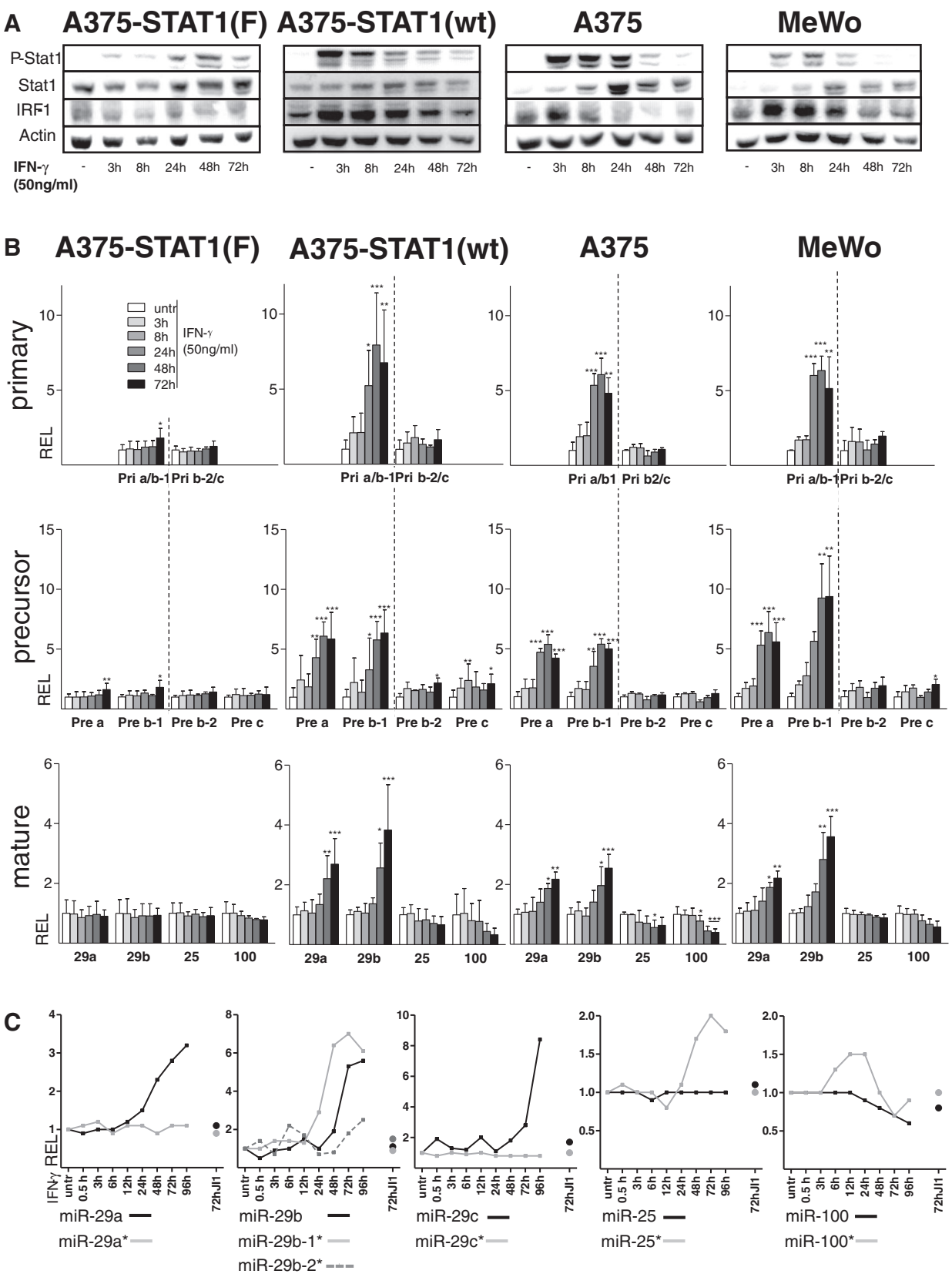


Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Expression profiles of miR-29 clusters in melanoma cells. A375-STAT1(F), A375-STAT1(wt), A375 and MeWo melanoma cells were stimulated with IFN-γ for different time points. (**A**) Western Blot analysis (representative blots of biological triplicates) confirms activation of P-STAT1 and induction of STAT1 and IRF-1 after IFN-γ stimulation while dominant negative A375-STAT1(F) cells show minor effects. (**B**) Time course study of miRNA-expression after IFNγ-stimulation. Graphs show relative expression (REL) from quantitative qRT-PCR data for the pri-29a~b-1 and the pri-29b-2~c clusters, the precursors pre-29a/29b-1/29b-2/29c and mature miR-29a/29b/25/100. Fold expression was calculated relative to the untreated control and SDs are shown for biological triplicates. Statistical significance was tested with one-way ANOVA, followed by a Dunnett Post-Hoc test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. (**C**) MiRNA and miRNA* expression profiles in A375 cells derived from a more detailed IFN-γ time course miRNA microarray experiment including cells treated with JI1 (IFN-γ stimulation for 72h after pre-treatment with JI1, black and grey dots). Depicted are log2-values of the mean of duplicate microarray experiments.

pre-29b-2 and pre-29c levels remained unaffected (Figure 2B, middle panel). Subsequently, significant up-regulation of both mature miR-29a and miR-29b following IFN-γ stimulation was confirmed (Figure 2B, lower panel). The two control amplifications of miR-100 (slightly down-regulated) and miR-25, which remained stable over time following IFN-γ stimulation confirmed the initial microarray-based expression profiles (Figure 2B, lower panel). Similar regulation patterns were also found in Jurkat and MT4 T-cells and in HEK293T kidney cells (for mature miR-29a, miR-29b, and miR-25, Additional file 2: Figure S2). Except for minor expression changes of the Pri/Pre-miR-29 species after 72h of IFN-γ treatment, no up-regulation was detected in the A375-STAT1(F) dominant negative control cells, clearly suggesting that STAT1 activity is required for the IFN-γ-induced regulation of miR-29 family members.

Figure 2C shows expression results of a detailed time course microarray experiment using IFN-γ-stimulated A375 cells. In parallel and as described before, cells had been pre-treated with Jak inhibitor 1 (JI1), which specifically inhibits Janus tyrosine kinases and subsequently prevented miR-29 up-regulation after IFN-γ stimulation [13].

Altogether, these data substantiate for the first time a time-dependent up-regulation of the expression of pri-29a~b-1 cluster as well as of the mature miRNAs miR-29a and -29b in melanoma cells, which is triggered by IFN-γ-induced STAT1 signaling.

The miR-29b-2~c cluster is undetectable in melanoma cell lines, melanocytes and keratinocytes

As both miR-29 primary clusters as well as the mature miR-29a/29b showed different basal expression levels in stimulation experiments and are known to be differentially expressed in several types of cancer [26,27], we next analyzed the miR-29 basal expression profiles in a panel of melanoma cell lines, primary human melanocytes (NHEM-M2) and HaCaT keratinocytes (Figure 3A and B). Pri-29a~b-1 was strongly expressed whereas pri-29b-2~c was almost undetectable in all cell lines analyzed (Figure 3A). This is in accordance with previous studies reporting down-regulation of the pri-29b-2~c cluster in rhabdomyosarcoma [28] and B-cell lymphoma [23]. Also, mature miR-29a consistently showed higher basal expression levels than miR-29b in all cell lines examined (Figure 3B).

MiR-29a/29b expression levels inversely correlate with growth behavior of melanoma cell lines

The classification of miR-29 as tumor-suppressor miRNA has been widely accepted and the possibility to use synthetic miR-29 as therapeutic agent in treatments of cancer seems to become increasingly realistic. Properties counteracting the development and spreading of cancer cells that have been observed *in vitro* and *in vivo* after miR-29 overexpression include reduced invasion and proliferation and induction of apoptosis [29,30]. These findings prompted us to analyze a potential correlation of basal miR-29 expression levels with cell growth. Proliferation of untreated melanoma cell lines was monitored over time (Figure 3C) in order to correlate the growth rate with miR-29a and pri-29a~b-1 basal expression levels obtained from cells harvested 96h after seeding. Melanoma cell lines were grouped in miR-29a and pri-29a~b-1 'low-expression' (A375, MeWo, IGR39, WM9) and 'high expression' cell lines (FM55P, FM55M1, SK-Mel30, IGR37) (Figure 3A and B). Generally, cell lines with lower miR-29a showed an increased proliferation rate compared to lines with higher basal miR-29a levels (Figure 3C). Furthermore, the inverse correlation between pri-29a~b-1/miR-29a expression and the proliferation rate of melanoma cell lines might suggest a potential involvement of miR-29 in anti-proliferative effects on melanoma cells. To follow up these findings, we applied miR-29a/29b mimics to A375 cells, which exhibit a relatively low miR-29a/29b basal expression and, *vice versa*, we applied a miR-29a inhibitor to FM55P cells, which have a high basal miR-29a/29b expression (Figure 3B). Proliferation assays with mimics and inhibitors and the corresponding amounts of scrambled controls, NC (negative control)-mimic and NC-inhibitor, corroborated that miR-29 indeed inhibited growth of melanoma cells: transfection of miR-29a/29b mimics caused a remarkable reduction of proliferation as compared to NC-mimic-transfected A375 cells (Figure 3D). In turn, FM55P cells,

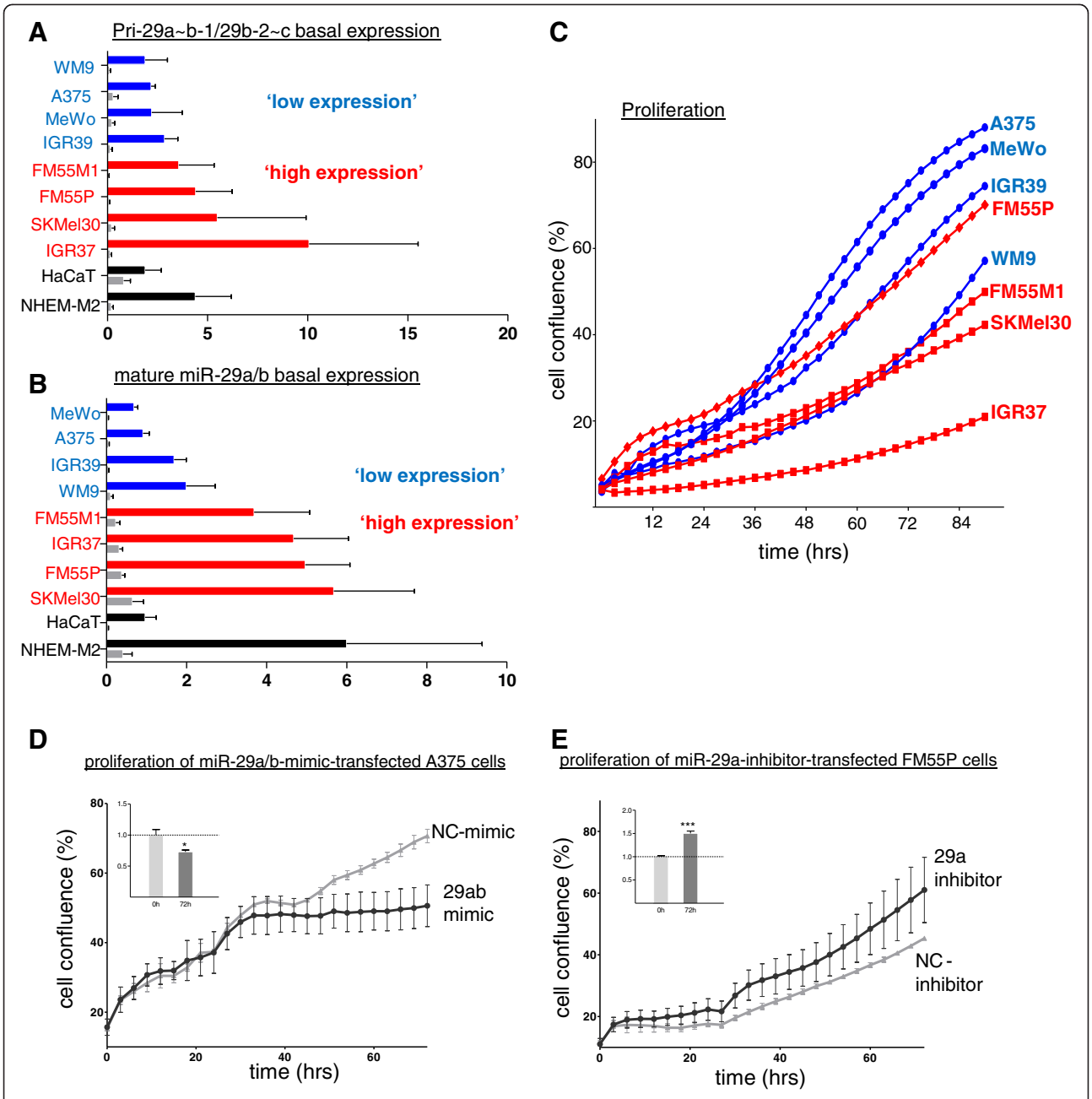


Figure 3 Inverse correlation of miR-29a expression levels and melanoma proliferation. Comparison of basal expression levels of (**A**) primary miRNA clusters pri-29a~b-1 (blue/red/black bars) and pri-29b-2~c (grey bars) and (**B**) mature miR-29a (blue/red/black bars) and miR-29b (grey bars) in NHEM-M2, eight melanoma cell lines and HaCaT keratinocytes. Graphs show $2^{-\Delta ct}$ x10^2 with SD of biological triplicates. (**C**) Mean growth curves of untreated melanoma cell lines over 4 days (biological quadruplicates). Melanoma cell lines with 'low expression' of pri-29a~b-1 and miR-29a show faster proliferation whereas cells with a relatively 'high expression' proliferate slower. (D,E) Proliferation assay of (black) mimic/inhibitor- and (grey) NC-mimic/NC-inhibitor-transfected cells over 72h in (**D**) A375 and (**E**) FM55P cells; representative graphs of four independent experiments. Error bars depict SDs of technical triplicates. The inserted graphs (upper left corners) show the mean confluence of 4 biological replicates at 0h and 72h time points of the proliferation assay. Depicted are ratios of confluence of 29ab-mimic/NC-mimic treated cells (D) and 29a-inhibitor/NC-inhibitor treated cells (E). Error bars show SEM. Significance was assessed by a two-tailed *t*-test with * p<0.05, ** p<0.01 and *** p<0.001.

in which miR-29a was inhibited, proliferated faster than NC-inhibitor-transfected control cells (Figure 3E).

MiR-29a/29b down-regulate *CDK6*, but not *PI3K*

MiR-29 is predicted to regulate more than 1000 human genes (TargetScanHuman 6.1). We have used a combination of several algorithms (TargetScanHuman 6.1, Diana-microT v3.0, microRNA.org) to compile a list of potentially interesting genes, which carry predicted miR-29 target sites. After detailed expression analysis of potential candidates in melanoma cells and initial screening for their response to miR-29 mimic and inhibitor treatment (data not shown), we concentrated on the *PI3K* regulatory subunit (gene: *PI3KR1*; protein: PI3K/p85α) and *CDK6*, which play important roles in cell cycle control, cellular signaling and thus, proliferation. Both have already been confirmed as miR-29 targets in several cancers [31-34].

To assess the effect of miR-29 on *CDK6* and *PI3K* expression in melanoma, mRNA and protein levels were examined after miR-29 mimic or inhibitor treatments by qRT-PCR and quantitative immunoblotting, respectively (Figure 4A and B). Combined transfection of miR-29a/29b reduced *CDK6* mRNA and protein levels in A375 cells as compared to scrambled controls whereas *PI3K* levels were not affected (Figure 4A). In agreement with that, knockdown of miR-29a in FM55P cells resulted in a slight up-regulation of *CDK6* levels while *PI3K* levels remained unchanged (Figure 4B). These data indicate that miR-29 is involved in down-regulation of *CDK6* protein while *PI3K* was not specifically targeted in melanoma cells. *CDK6* was also down-regulated in response to miR-29 induction after IFN-γ stimulation in A375 cells and A375-STAT1(wt) but not in A375-STAT1(F) cells, suggesting STAT1 dependency (Figure 4C). In contrast, PI3K levels were reduced in all three cell lines, hinting at STAT1-independent effects. To further prove regulation of *CDK6* by miR-29, we performed luciferase assays with reporter constructs containing part of the *CDK6* 3'-UTR, its three single miR-29 binding sites as predicted by TargetScan (www.targetscan.org), part of the *PI3KR1*-3'-UTR or the miR-29a full complementary sequence as a positive control (Figure 4D). Luciferase activity, as compared to the respective negative control, dropped by ~60 % for both time points in A375 melanoma cells when the *CDK6* 3'-UTR construct was co-transfected with miR-29a mimic. The corresponding single binding sites contributed to this suppression significantly with 38% (BS1), 34% (BS2) and 35% (BS3) (Figure 4E). This suggests that all three miR-29 binding sites partake in the suppression of *CDK6*. Surprisingly, the *PI3KR1* construct was also significantly suppressed by the miR-29a mimic in luciferase assays (Figure 4E) while only marginal effects had been observed on mRNA and protein level (Figure 4A,B). Taken together, these findings indicate that both *CDK6* and *PI3KR1* 3'-UTRs are directly targeted by miR-29 in melanoma cells; however, only *CDK6* suppression seems to be important in a cellular context. To further explore the relevance of reduced *CDK6* levels in the cell, we used siRNA against *CDK6* and assessed proliferation over 72h in A375 (Figure 4F) and FM55P cells (Figure 4G). Reduction of CDK6 mRNA and protein level (Additional file 3: Figure S3) led to a clearly diminished proliferation in both cell lines.

MiR-29a and miR-29b are up-regulated in primary melanoma patient samples

Finally, we investigated miR-29a/29b expression profiles in FFPE melanoma patient samples from normal skin, nevi, primary and metastatic melanoma by qRT-PCR (Figure 5). Nevi represent the most appropriate control samples as they contain predominantly melanocytes while normal skin samples are mostly composed of keratinocytes. In comparison to healthy skin and nevi, both miR-29a and miR-29b showed an up-regulation in primary melanoma samples whereas in metastatic tumors, expression levels were only slightly enhanced compared to healthy controls. Closer sub-classification of the patient samples revealed, however, that only two of five patients demonstrated the enhanced miR-29a/29b expression, indicating that expression levels are heterogeneous and will have to be assessed in larger patient cohorts.

Discussion

Generally, expression levels of miRNAs can be regulated transcriptionally, by epigenetic silencing or different turnover times [1,35,36]. The role of cytokines as inducers of miRNA expression has recently been proposed in several studies and examples for cytokine-induced miRNA up- or down-regulation include pro-inflammatory signaling molecules like TNF-α and IL1-β [37,38]. Interferons are central players in tumor-immune interactions [39,40]. In this context, the theory of 'cancer immunosurveillance', defined as the immunological protection of the host against development of cancer, has evoked much interest during the last decade: mediated by the host's immune system, it is triggered by immune recognition of stress ligands or antigens expressed on transformed cells. IFN-γ has long been recognized for its crucial role in defense against viral and bacterial infections as well as in tumor control [40,41]. It primarily signals through the Jak/STAT pathway and activated STAT1 homodimers bind to GAS-elements in promoter regions of target genes, while IFN-α/β signal additionally through ISRE–elements. In our study, we have identified several GAS-elements in the proposed pri-29a~b-1 promoter region. IFN-γ stimulation of a control cell line expressing

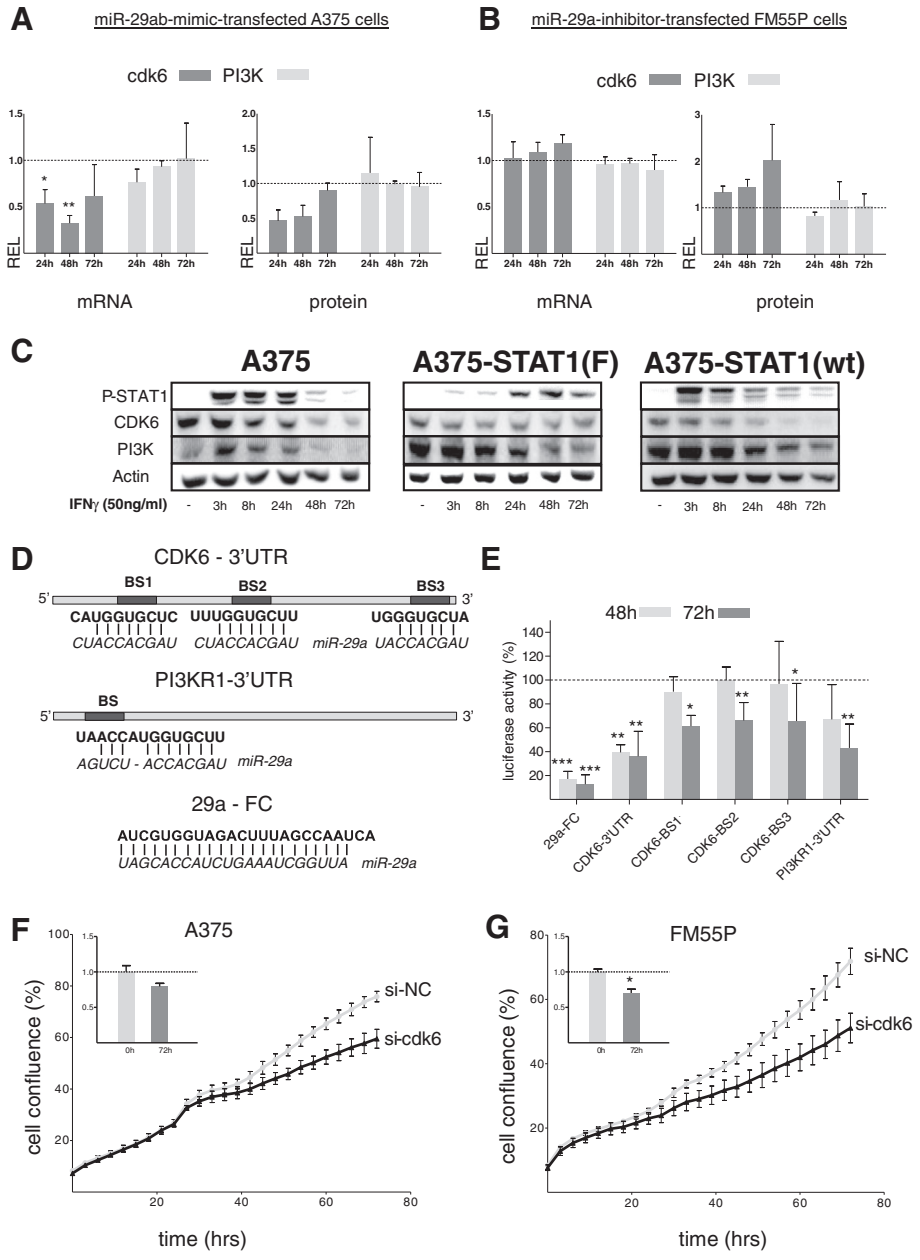


Figure 4 Effects of miR-29 on target genes CDK6 and PI3K. (**A**,**B**) relative mRNA and protein expression levels (REL) of miR-29 target genes CDK6 (dark grey) and PI3K (light grey), assessed 24h, 48h and 72h after mimic/inhibitor transfection compared to NC-mimic/NC-inhibitor controls; graphs show means of biological triplicates ± SD. (**C**) Down-regulation of miR-29 target proteins CDK6 and PI3K is observed after IFN-γ stimulation of melanoma cells. (**D**) Schematic overview of CDK6 and PI3KR1 luciferase constructs with positions of conserved miR-29a binding sites predicted by TargetScan (bold) in the CDK6-3'UTR (BS1-3) and PI3KR1-3'UTR (BS) and corresponding miR-29a sequences (italics). (**E**) Luciferase activity in A375 cells transfected with constructs containing the positive control miR-29a full complementary sequence (29a-FC), parts of CDK6- or PI3K1-3'UTRs or CDK6 single binding sites (BS1-BS3) and miR-29a mimic or NC for 48h and 72h. Relative luciferase activity of miR-29a-transfected samples was normalized to NC-mimic-transfected control samples (luciferase activity of NC-mimic transfected samples was set to 100%). Bars show means of biological triplicates ± SD for each construct. (**F**) A375 and (**G**) FM55P cells transfected with CDK6 siRNA (black) show reduced proliferation compared to cells transfected with siRNA NC (grey). Results were reproduced at least in biological duplicates. Inserted bar diagrams show the mean confluence of at least biological triplicates at 0h and 72h. Shown are confluence ratios of si-CDK6/si-NC ± SEM. Significance was assessed by one-way ANOVA followed by a Bonferroni Post-Hoc test (A,B,E) or by a two-tailed t-test (F,G). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

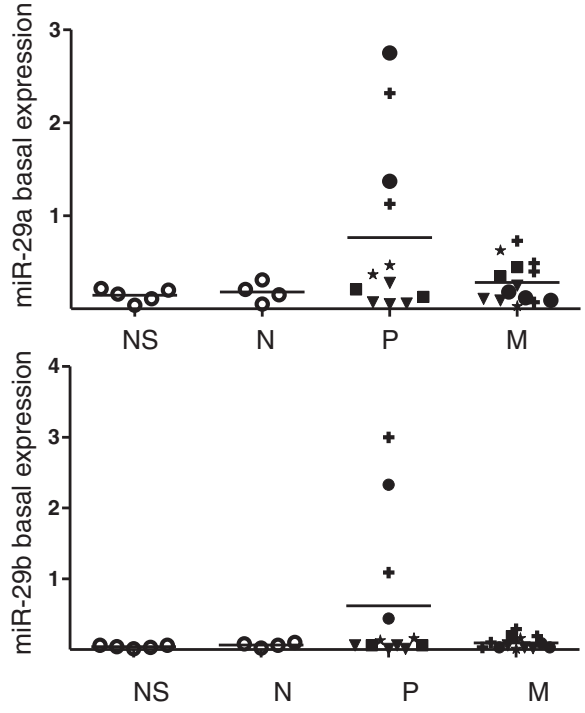


Figure 5 miR-29 expression in patient samples. Analysis of miR-29a (upper panel) and miR-29b (lower panel) basal expression of individual FFPE-patient samples from NS = normal skin, N = nevi, P = primary melanoma and M = metastatic melanoma. All graphs show $2^{-\Delta ct}$ with Δct= (ct (miR-29a/29b)– ct (RNU5A)). Primary and metastatic tumor samples were sorted according to patients (P1-5: P1-circles; P2-rectangles; P3-crosses; P4-triangles; P5-asterisks).

dominant-negative STAT1 (A375-STAT1(F)) did not cause an up-regulation of miR-29, providing strong evidence that STAT1 is indeed mediating IFN-γ-induced effects on miR-29 expression levels.

IFN-γ has anti-proliferative effects on cancer cells including melanoma [11,12] and we show here that miR-29a, which is induced by IFN-γ exhibited the same effects. Overall effects on growth were relatively small, but robust and reproducible, considering that we only manipulated levels of one miRNA and only used relatively small amounts of mimics/inhibitors (50 nM/150 nM) to be as close as possible to physiological relevance. However, IFN-γ may also facilitate melanoma progression: Zaidi *et al.* have shown that IFN-γ-producing macrophages are recruited to the UV-exposed skin and can stimulate proliferation and migration of melanocytes as well as induce expression of genes implicated in immunoevasion and survival. When added to transplanted melanoma, these skin-recruited macrophages enhanced the growth and survival of melanoma. All these effects were IFN-γ-dependent as demonstrated by antibody blocking of IFN-γ [42].

In our study, analysis of primary and metastatic melanoma patient samples revealed increased miR-29a/29b expression in some primary tumor samples in comparison to normal skin, nevi and metastatic tissue while all metastatic lesions had low levels of these miRNAs. Possibly, IFN-γ, which can be produced by macrophages, T cells and NK cells induces miR-29 expression via STAT1. miR-29a/29b were only up-regulated in two out of five primary melanoma patients. In this respect, it is interesting to note that IFN-γ producing macrophages have been observed in 70 % of melanoma samples [42]. A further evaluation of a larger panel of patient samples including early neoplasia and advanced metastatic stages is needed where a special focus will be placed on immune cell infiltration, interferon concentration and an interferon-responsive gene signature.

miR-29 has very recently been linked to interferon biology: it directly targets IFN-γ [43,44], the transcription factors Tbet and Eomes crucial for IFN-γ expression [19,44], and the receptor IFNAR1 [45], thereby drastically affecting immune regulation such as T cell polarization and thymic function.

While this manuscript was in preparation, IFN-γ involvement in the regulation of miR-29 expression was also reported by a group studying T cell activation and polarization in autoimmune diseases [44]. We here confirmed IFN-γ-induced miR-29 up-regulation in T cells (Jurkat and MT4, Additional file 2: Figure S2) and have also observed this effect in human embryonic kidney cells implying a regulatory mechanism of broader relevance. Interestingly, also type I interferons led to an up-regulation of miR-29 (Additional file 2: Figure S2).

Screening of a panel of melanoma cell lines for different miR-29 species and family members revealed that the pri-29b-2~c cluster was almost not expressed and that miR-29a exhibited a much higher basal expression level than miR-29b. In tumor cells, reduced miR-29 expression is frequently observed and diminished expression of miRNAs in general is often associated with enhanced oncogenesis [5,46]. The difference in pri-29a~b-1 and pri-29b-2~c expression levels, which we w?>have detected, is consistent with other types of cancer, in which the pri-29b-2~c cluster was mostly down-regulated [26-28]. The fact that miR-29 family members are often not expressed in cancer cells could be crucial for cancer control: miR-29 down-regulates important genes such as *CDC42*, *TCL-1* and *MCL-1*, which normally confer tumor-suppressing traits. In this context, anti-proliferating as well as anti-invasive and pro-apoptotic effects have been observed after miR-29 re-introduction in a variety of cancer cells [29,30]. In line with this, we show anti-proliferative effects of miR-29 and confirm for the first time *CDK6* as a direct miR-29 target in melanoma cells. This suggests that miR-29-mediated down-regulation of

CDK6 is involved in decreasing proliferation rates of miR-29a/b-mimic-transfected melanoma cells. SiRNA-mediated knockdown of *CDK6* resulted in reduced proliferation of melanoma cells similar to what has been shown for other cancer types [47,48]. *CDK6* plays a pivotal role in control of G1/S cell cycle transition [49] and loss thereof is a common event in neoplastic growth [50]. Noteworthy, *CDK6* has also been shown to be a direct miR-29 target in mantle cell lymphoma [31], acute myeloid leukemia [34] and cervical cancer [32].

The numerous anti-proliferative effects of IFN-γ in many cancers may in part be explained by a G1 arrest involving down-regulation of *G1/S cyclins* (cyclins A and E) and *CDK2/4* [12]. Accordingly, we find that IFN-γ as well as miR-29 exhibit anti-proliferative activities in melanoma cells involving down-regulation of cell cycle control players such as *CDK6*. The relevance of *CDK6* activity for melanoma growth is further emphasized by the fact that the tumor suppressor $p16^{INK4A}$ (an inhibitor for CDK6 and 4) is deleted in about 50% of melanoma patients [51,52]. Here, we describe for the first time that *CDK6* is a direct target of miR-29 involved in regulating growth behavior of melanoma cells.

Conclusion

Our study extends the current knowledge on the miRNA family miR-29, adding a novel regulatory loop of IFN-γ-mediated Jak/STAT signaling in melanoma cells. Figure 6 summarizes the proposed regulatory circuit involving IFN-γ and miR29: IFN-γ, which is e.g. secreted by macrophages following diverse assaults such as infections or UV light induces a STAT1-dependent up-regulation of miR-29, which in turn can down-regulate IFN-γ expression directly and indirectly via T-bet and Eomes. Down-regulation of cell cycle regulators like CDK6 contributes to IFN-γ-mediated growth arrest.

We report that the pri-29 b-2~c cluster is almost undetectable in melanoma, which might markedly reduce the ability of the miR-29 family to exhibit its tumor-suppressing properties in these cancer cells. The fact that miR-29a and miR-29b had enhanced expression levels in some primary melanoma patients but not in metastatic tumor samples is in line with many studies showing down-regulation or low levels of miR-29 in various advanced cancers [21,22,31,53]. We hypothesize that the reduced miR-29 expression in cancer cells could be a consequence of diminished IFN-γ signaling in those cells, which might already have escaped immune surveillance [41]. In regard to the proposed regulatory circuit, our study may open new connections between the immune system, miRNAs and growth control and thus, tumorigenesis.

Methods

Cell lines and patient samples

Melanoma cell lines A375 (American Type Culture Collection, ATCC), A375-STAT1(F) and A375-STAT1(wt) [25], FM55P and FM55M1 (European Searchable Tumor Line Database and Cell Bank, ESTDAB), IGR39 and IGR37 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ), MeWo (Dr. D. Schadendorf, Essen, Germany) and SK-Mel30 (Dr. M. Böhm, Münster, Germany) as well as the T cell lines Jurkat and MT4 (Dr. C. Devaux, Luxembourg) were maintained in RPMI 1640 supplemented with 10% FCS (PAA), 50 μg/ml penicillin, 100 μg/ml streptomycin and 0.5 mmol/l L-glutamine. The stably transfected A375 cell clones A375-STAT1(F) and A375-STAT1(wt) were grown under selective pressure with 400 μg/ml Geneticin (G418, Gibco). HaCaT keratinocytes (Dr. N. Fusenig, Heidelberg, Germany) and HEK293T were grown in DMEM supplemented with 10% FCS, 50 μg/ml penicillin, 100 μg/ml streptomycin and 2.5% HEPES. NHEM-M2 (normal epidermal human melanocytes, PromoCell) were cultured in melanocyte growth medium M2 (PromoCell) and harvested after reaching ~50% confluence in a 10 cm^2 cell culture dish. All cells were maintained in a humidified atmosphere with 5% CO_2 and were routinely tested to be mycoplasma-negative by PCR. Reagents and media were purchased from Lonza unless specified otherwise.

Ethical approval for use of the patient FFPE (formalin-fixed paraffin-embedded) and healthy control samples was obtained by the Ethical review board, Freiburg, Germany (Reference 196/09). Collection, histopathological analysis, fixation and RNA extraction were performed as described before [54]. In total, RNAs of 5 healthy skin samples, 4 benign nevi, 12 primary and 14 metastatic melanoma samples were analyzed by qRT-PCR. The primary and metastatic samples were collected from different parts of the body from a total number of 5 melanoma patients. Basal miR-expression levels were calculated as $2^{-\Delta ct}$ with Δct= (ct (miR-29a/29b) – ct (RNU5A)) (Figure 5).

IFN-γ stimulation, RNA extraction, and miRNA microarray analysis

For IFN-γ time course stimulation experiments, $100x10^3$ cells/well were seeded in 6-well plates (Greiner). Cells were either left untreated or stimulated with 50 ng/ml of IFN-γ (PeproTech) for the time periods indicated. 5 μM Jak inhibitor 1 (JI1, Calbiochem) pre-treatment was included (72h JI1-time point) in the detailed time course miRNA microarray experiment one hour before IFN-γ-stimulation. Samples for RNA extraction and protein lysates were collected altogether at the end of the treatment for further analyses by qRT-PCR and Western Blotting, respectively. Total RNA was extracted using

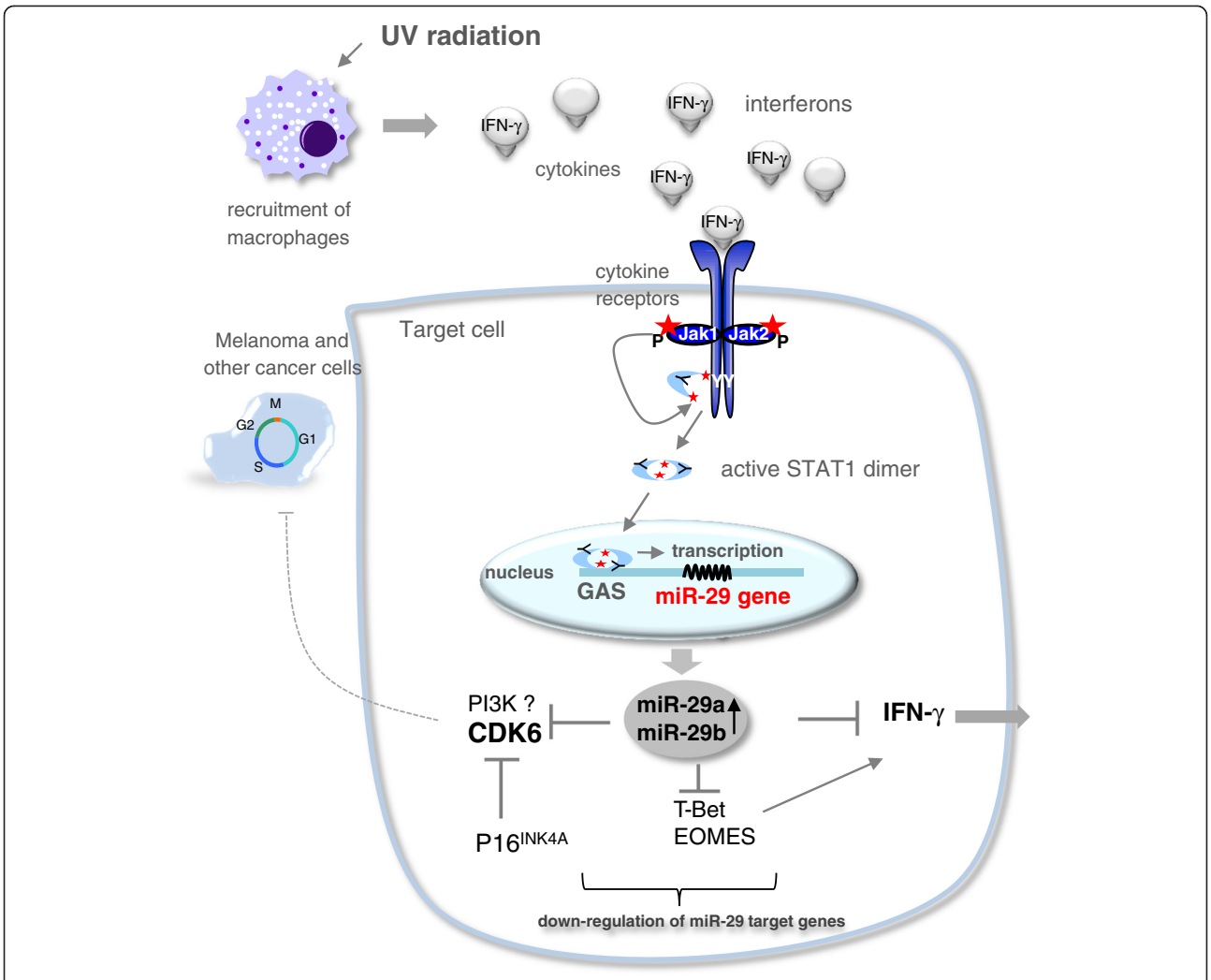


Figure 6 The miR-29 family is involved in multiple cellular processes. UV-radiation triggers the recruitment of macrophages to the skin, which secrete cytokines like interferon gamma (IFN-γ). By binding to its receptors, IFN-γ signals through the Jak/STAT pathway triggering subsequent activation of STAT1, which then binds to GAS-elements in the promoter region of target genes and initiates their transcription. IFN-γ-induced, STAT1-dependent up-regulation of miR-29 causes a down-regulation of CDK6, a novel miR-29 target gene in melanoma, which plays a crucial role in cell cycle G1/S-transition and thus growth control of cancer cells. The cell cycle inhibitor $p16^{INK4A}$ is often deleted in melanoma and its function (inhibition of *CDK6*) might be compensated by miR-29a/b. IFN-γ-activating transcription factors T-bet and Eomes and IFN-γ itself are also targeted by miR-29.

TRIsure (Bioline USA, Inc.) and subsequently treated with DNaseI (New England Biolabs) as described before [54]. Quantity and purity of RNA samples were assessed using a NanoDrop ND-2000 spectrophotometer. Global miRNA expression levels were profiled on Affymetrix GeneCHip miRNA 2.0 Arrays as described before [13].

Relative quantification of primary, precursor and mature miRNAs and mRNAs

For FFPE samples and cell lines, 250 ng of total RNA was reversely transcribed using the miScript Reverse Transcription kit (Qiagen) according to the supplied protocol. Real-time PCR was carried out on a CFX detection system (Bio-Rad). For quantification of mature miRNAs, 5 ng RNA input, 2x iQ SYBR Green Supermix (Bio-Rad) and 10x miRNA-specific primer assay (Qiagen) were used. To detect mRNAs, miRNA primary clusters and precursors, 2x iQ SYBR Supermix and 5 pmol gene-specific primers (for sequences see Additional file 4: Table S1) were used together with 50 ng (mRNA detection) or 125 ng (primary/precursor miRNAs) RNA input. PCR conditions for all qRT-PCR reactions were 95°C-3 min; 39x (95°C-15s; 60°C-30s); 95°C-1 min; 60°C-1 min, followed by a melt curve analysis (60°C to 95°C, increment 0.5°C for 20s) to confirm specificity of the PCR primers. If not stated otherwise, Ct-values for mRNA and miRNA species were normalized to at least three housekeeping genes: TBP (TATA binding protein),

HPRT1 (Hypoxanthine phosphoribosyltransferase 1), CycloA (cyclophilin A) and β-Actin for mRNAs and primary/precursor miRNAs; RNU1A, RNU5A (RNA, U1A/5A small nuclear) and SCARNA17 (small Cajal body-specific RNA 17) for mature miRNAs. Based on the geometric mean of the three reference genes, a normalization factor was calculated for each sample using geNorm, a VBA applet for Microsoft Excel [55]. The relative amount of each target in each sample was then corrected by dividing its amount by the corresponding normalization factor. Fold changes were calculated by dividing the normalized relative amount of treated samples with the normalized relative amount of the untreated sample that served as a control. Statistical significance was tested with one-way ANOVA, followed by a Dunnett Post-Hoc test. Except for the FFPE patient samples, all experiments were performed at least in biological triplicates. P values of <0.05 (*), <0.01 (**) and <0.001 (***) were considered significant.

Western blot analysis

Cell lysis, SDS-PAGE, ECL detection, stripping and reprobing was performed as previously described [56,57] using the following antibodies: Actin (C4, Milipore), Tubulin, IRF-1, STAT1 and CDK6 (Santa Cruz), P-STAT1 (Cell Signaling), p85α (PI3K) (Upstate) and the corresponding HRP-labeled (ECL detection, Cell Signaling Technology) or fluorophor-coupled (quantification, Li-cor Biosciences) secondary antibodies. For quantification of proteins, signal intensities were assessed with a Li-cor Odyssey Infrared Imaging System (Li-cor Biosciences) and analyzed with the provided software. CDK6 and p85α signals were normalized to the respective Tubulin loading controls.

Real-time proliferation assays

25 $x10^3$ cells/well of eight untreated melanoma cell lines were seeded in 12-well plates and harvested after 96h of real-time monitoring in the IncuCyte live-cell imaging system (Essen Bioscience), which photographed cells in phase contrast every 3h. RNA was extracted and miR-29 species were amplified by qRT-PCR as described before [54] and above.

miRNA mimic/inhibitor transfection

100 $x10^3$ cells/well were seeded in 6-well plates and transfected after 24h with 50 nM of each miR-29a and miR-29b mimics or with 150 nM miR-29a inhibitor or corresponding amounts of negative controls (Qiagen) using the DharmafectDuo transfection reagent (Dharmacon) according to the supplied protocol; efficient transfection was confirmed by qRT-PCR (Additional file 3: Figure S3). For miR-29 target gene expression, RNA and protein lysates were collected 24h, 48h and 72h after transfection and subsequently analyzed by RT-qPCR and western blot. Proliferation was monitored by the IncuCyte cell-imaging system as described above.

CDK6 siRNA transfection

50$x10^3$ cells were transfected with 75nM ON-TARGET siRNA or siRNA negative control (si-NC) 24h after seeding in 6-well plates using the HiPerfect transfection reagent according to the manufacturer's instructions (Qiagen). Proliferation was monitored in the IncuCyte as described above. CDK6 mRNA and protein levels were assessed after 24h, 48h and 72h to confirm efficient down-regulation (Additional file 3: Figure S3C,D).

Luciferase reporter gene assays

The parts of CDK6 and PI3KR1 (Phosphatidylinositol 3-kinase) 3'UTRs containing miR-29 binding sites, CDK6 miR-29a single binding sites and the miR-29a full complementary sequence were cloned into the pmirGLO Dual Luciferase miRNA target expression vector (Promega) downstream of the luciferase gene (see Additional file 4: Table S1 for primer sequences and oligonucleotides). A375 cells were seeded at a density of 50 $x10^3$ cells/well in 24-well plates one day before transfection. Cells were transiently co-transfected with 500 ng plasmid and 50 nM miR-29a mimic or negative control for 48h and 72h. Samples were lysed with 1x Passive Lysis Buffer (Promega) and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly was divided by Renilla activity and normalized to the negative control for each construct. Significance was assessed by one-way ANOVA followed by a Bonferroni Post-Hoc test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Additional files

Additional file 1: Figure S1. Schmitt_et_al_2012_Contains a graphical representation of array results: Top 10 up-regulated miRNAs (as listed in Figure 1A) after IFN-γ stimulation for the indicated time periods and 72h JI1.

Additional file 2: Figure S2. Schmitt_et_al_2012_Contains bar diagrams of qRT-PCR results: MiR-29a/29b up-regulation after IFN-γ-stimulation and unchanged miR-25 levels in A) HEK293T kidney and B) Jurkat T cells. C) MiR-29a/29b up-regulation after IFN-α-, IFN-β- and IFN-γ-stimulation (50 ng/ml) in MT4 T cells.

Additional file 3: Figure S3. Schmitt_et_al_2012_Contains bar diagrams of qRT-PCR results an western blots: Tracking of miR-29a/29b mimics in A375 cells (A) and miR-29a suppression after inhibitor transfection in FM55P cells (B); and knock-down of CDK6 mRNA (C) and protein levels (D) in both cell lines.

Additional file 4: Table S1. within Schmitt_et_al_2012_Contains primer sequences. Additional Figure legends: Schmitt_et_al_2012_ Contains additional Figure legends. Powerpoint documents.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
The study was carried out in collaboration with all authors. MS, DP, SR and CM performed the laboratory experiments and analyzed the results. AWB performed the *in silico* analysis of the miR-29 promoter region and provided bioinformatic support. MS, DP and SR drafted the manuscript. DN provided the primary melanoma patient samples and scientific background about the disease. IB and SK developed the experimental design of the study, interpreted results and participated in writing and critical revision of the manuscript. All authors read and approved the manuscript.

Acknowledgments
M. and SR are supported by an AFR fellowship of the Fonds National de la Recherche, Luxembourg (MS: TR-PHD BFR08-077, SR: 4019604). The study was supported by an internal research grant from the University of Luxembourg (F1R-LSC-PUL-09MIRN) and by a research grant from the Fondation Cancer (Luxembourg). We thank Dr. Carole Devaux and Gilles Iserentant (CRP-Santé, Luxembourg) for providing MT4 cells and Dr. Lasse Sinkkonen for providing pri-29a~b-1 primer sequences.

Author details
[1]Signal Transduction Laboratory, University of Luxembourg, 162A Avenue de la Faïencerie, Luxembourg L-1511, Luxembourg. [2]Life Sciences Research Unit, University of Luxembourg, 162A Avenue de la Faïencerie, Luxembourg L-1511, Luxembourg. [3]Hautklinik, Klinikum Dortmund GmbH, Beurhausstraße 40, Dortmund 44137, Germany.

Received: 19 October 2012 Accepted: 27 November 2012
Published: 17 December 2012

References

1. Krol J, Loedige I, Filipowicz W: **The widespread regulation of microRNA biogenesis, function and decay.** *Nat Rev Genet* 2010, **11:**597–610.
2. Winter J, Jung S, Keller S, Gregory RI, Diederichs S: **Many roads to maturity: microRNA biogenesis pathways and their regulation.** *Nat Cell Biol* 2009, **11:**228–234.
3. Davis BN, Hata A: **Regulation of MicroRNA Biogenesis: A miRiad of mechanisms.** *Cell Commun Signal* 2009, **7:**18.
4. Friedman RC, Farh KK, Burge CB, Bartel DP: **Most mammalian mRNAs are conserved targets of microRNAs.** *Genome Res* 2009, **19:**92–105.
5. Henry JC, Azevedo-Pouly AC, Schmittgen TD: **MicroRNA replacement therapy for cancer.** *Pharm Res* 2011, **28:**3030–3042.
6. Kasinski AL, Slack FJ: **Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy.** *Nat Rev Cancer* 2011, **11:**849–864.
7. Miller AJ, Mihm MC Jr: **Melanoma.** *N Engl J Med* 2006, **355:**51–65.
8. Ascierto PA, Kirkwood JM, Grob JJ, Simeone E, Grimaldi AM, Maio M, Palmieri G, Testori A, Marincola FM, Mozzillo N: **The role of BRAF V600 mutation in melanoma.** *J Transl Med* 2012, **10:**85.
9. Sala E, Mologni L, Truffa S, Gaetano C, Bollag GE, Gambacorti-Passerini C: **BRAF silencing by short hairpin RNA or chemical blockade by PLX4032 leads to different responses in melanoma and thyroid carcinoma cells.** *Mol Cancer Res* 2008, **6:**751–759.
10. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'Dwyer PJ, Lee RJ, Grippo JF, Nolop K, Chapman PB: **Inhibition of mutated, activated BRAF in metastatic melanoma.** *N Engl J Med* 2010, **363:**809–819.
11. Garbe C, Krasagakis K: **Effects of interferons and cytokines on melanoma cells.** *J Invest Dermatol* 1993, **100:**239S–244S.
12. Kortylewski M, Komyod W, Kauffmann ME, Bosserhoff A, Heinrich PC, Behrmann I: **Interferon-gamma-mediated growth regulation of melanoma cells: involvement of STAT1-dependent and STAT1-independent signals.** *J Invest Dermatol* 2004, **122:**414–422.
13. Reinsbach S, Nazarov P, Philippidou D, Schmitt M, Wienecke-Baldacchino A, Muller A, Vallar L, Behrmann I, Kreis S: **Dynamic regulation of microRNA expression following Interferon-γ-induced gene transcription.** *RNA Biol* 2012, **9:**978–989.
14. Loffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermuller J, Kretzschmar AK, Burger R, Gramatzki M, Blumert C, Bauer K, *et al*: **Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer.** *Blood* 2007, **110:**1330–1333.
15. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K: **STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer.** *Mol Cell* 2010, **39:**493–506.
16. Yang CH, Yue J, Fan M, Pfeffer LM: **IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis.** *Cancer Res* 2010, **70:**8108–8116.
17. Kohanbash G, Okada H: **MicroRNAs and STAT interplay.** *Semin Cancer Biol* 2012, **22:**70–75.
18. Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, *et al*: **MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B.** *Proc Natl Acad Sci USA* 2007, **104:**15805–15810.
19. Steiner DF, Thomas MF, Hu JK, Yang Z, Babiarz JE, Allen CD, Matloubian M, Blelloch R, Ansel KM: **MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells.** *Immunity* 2011, **35:**169–181.
20. Sengupta S, den Boon JA, Chen IH, Newton MA, Stanhope SA, Cheng YJ, Chen CJ, Hildesheim A, Sugden B, Ahlquist P: **MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins.** *Proc Natl Acad Sci USA* 2008, **105:**5874–5878.
21. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M: **The miR-29 Family: Genomics, Cell Biology, and Relevance to Renal and Cardiovascular Injury.** *Physiol Genomics* 2012, **44:**237–244.
22. Schmitt M, Margue C, Behrmann I, Kreis S: **MiRNA-29: a microRNA Family with Tumor-Suppressing and Immune-Modulating Properties.** *Curr Mol Med* 2012, in press.
23. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, Dang CV, Thomas-Tikhonenko A, Mendell JT: **Widespread microRNA repression by Myc contributes to tumorigenesis.** *Nat Genet* 2008, **40:**43–50.
24. Mott JL, Kurita S, Cazanave SC, Bronk SF, Werneburg NW, Fernandez-Zapico ME: **Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB.** *J Cell Biochem* 2010, **110:**1155–1164.
25. Kortylewski M, Heinrich PC, Mackiewicz A, Schniertshauer U, Klingmuller U, Nakajima K, Hirano T, Horn F, Behrmann I: **Interleukin-6 and oncostatin M-induced growth inhibition of human A375 melanoma cells is STAT-dependent and involves upregulation of the cyclin-dependent kinase inhibitor p27/Kip1.** *Oncogene* 1999, **18:**3742–3753.
26. Stamatopoulos B, Meuleman N, Haibe-Kains B, Saussoy P, Van Den Neste E, Michaux L, Heimann P, Martiat P, Bron D, Lagneaux L: **microRNA-29c and microRNA-223 down-regulation has *in vivo* significance in chronic lymphocytic leukemia and improves disease risk stratification.** *Blood* 2009, **113:**5237–5245.
27. Pekarsky Y, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, Volinia S, Alder H, Liu CG, Rassenti L, *et al*: **Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181.** *Cancer Res* 2006, **66:**11590–11593.
28. Wang H, Garzon R, Sun H, Ladner KJ, Singh R, Dahlman J, Cheng A, Hall BM, Qualman SJ, Chandler DS, *et al*: **NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma.** *Cancer Cell* 2008, **14:**369–381.
29. Fang JH, Zhou HC, Zeng C, Yang J, Liu Y, Huang X, Zhang JP, Guan XY, Zhuang SM: **MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression.** *Hepatology* 2011, **54:**1729–1740.
30. Xiong Y, Fang JH, Yun JP, Yang J, Zhang Y, Jia WH, Zhuang SM: **Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma.** *Hepatology* 2010, **51:**836–845.
31. Zhao JJ, Lin J, Lwin T, Yang H, Guo J, Kong W, Dessureault S, Moscinski LC, Rezania D, Dalton WS, *et al*: **microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma.** *Blood* 2010, **115:**2630–2639.
32. Li Y, Wang F, Xu J, Ye F, Shen Y, Zhou J, Lu W, Wan X, Ma D, Xie X: **Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29.** *J Pathol* 2011, **224:**484–495.
33. Park SY, Lee JH, Ha M, Nam JW, Kim VN: **miR-29 miRNAs activate p53 by targeting p85 alpha and CDC42.** *Nat Struct Mol Biol* 2009, **16:**23–29.

34. Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N, *et al*: **MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1.** *Blood* 2009, **113:**6411–6418.
35. Bartel DP: **MicroRNAs: genomics, biogenesis, mechanism, and function.** *Cell* 2004, **116:**281–297.
36. Bueno MJ, Perez de Castro I, Gomez de Cedron M, Santos J, Calin GA, Cigudosa JC, Croce CM, Fernandez-Piqueras J, Malumbres M: **Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression.** *Cancer Cell* 2008, **13:**496–506.
37. Roggli E, Britan A, Gattesco S, Lin-Marq N, Abderrahmani A, Meda P, Regazzi R: **Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells.** *Diabetes* 2010, **59:**978–986.
38. Ruan W, Xu JM, Li SB, Yuan LQ, Dai RP: **Effects of down-regulation of microRNA-23a on TNF-alpha-induced endothelial cell apoptosis through caspase-dependent pathways.** *Cardiovasc Res* 2012, **93:**623–632.
39. Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, Lee H, Arthur CD, White JM, Kalinke U, *et al*: **Type I interferon is selectively required by dendritic cells for immune rejection of tumors.** *J Exp Med* 2011, **208:**1989–2003.
40. Dunn GP, Koebel CM, Schreiber RD: **Interferons, immunity and cancer immunoediting.** *Nat Rev Immunol* 2006, **6:**836–848.
41. Schreiber RD, Old LJ, Smyth MJ: **Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion.** *Science* 2011, **331:**1565–1570.
42. Zaidi MR, Davis S, Noonan FP, Graff-Cherry C, Hawley TS, Walker RL, Feigenbaum L, Fuchs E, Lyakh L, Young HA, *et al*: **Interferon-gamma links ultraviolet radiation to melanomagenesis in mice.** *Nature* 2011, **469:**548–553.
43. Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, Hua M, Li N, Yao H, Cao X: **The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma.** *Nat Immunol* 2011, **12:**861–869.
44. Smith KM, Guerau-de-Arellano M, Costinean S, Williams JL, Bottoni A, Mavrikis Cox G, Satoskar AR, Croce CM, Racke MK, Lovett-Racke AE, Whitacre CC: **miR-29ab1 Deficiency Identifies a Negative Feedback Loop Controlling Th1 Bias That Is Dysregulated in Multiple Sclerosis.** *J Immunol* 2012, **189:**1567–1576.
45. Papadopoulou AS, Dooley J, Linterman MA, Pierson W, Ucar O, Kyewski B, Zuklys S, Hollander GA, Matthys P, Gray DH, *et al*: **The thymic epithelial microRNA network elevates the threshold for infection-associated thymic involution via miR-29a mediated suppression of the IFN-alpha receptor.** *Nat Immunol* 2012, **13:**181–187.
46. Nguyen T, Kuo C, Nicholl MB, Sim MS, Turner RR, Morton DL, Hoon DS: **Downregulation of microRNA-29c is associated with hypermethylation of tumor-related genes and disease outcome in cutaneous melanoma.** *Epigenetics* 2011, **6:**388–394.
47. Ismail A, Bandla S, Reveiller M, Toia L, Zhou Z, Gooding WE, Kalatskaya I, Stein L, D'Souza M, Litle VR, *et al*: **Early G(1) cyclin-dependent kinases as prognostic markers and potential therapeutic targets in esophageal adenocarcinoma.** *Clin Cancer Res* 2011, **17:**4513–4522.
48. Whiteway SL, Harris PS, Venkataraman S, Alimova I, Birks DK, Donson AM, Foreman NK, Vibhakar R: **Inhibition of cyclin-dependent kinase 6 suppresses cell proliferation and enhances radiation sensitivity in medulloblastoma cells.** *J Neurooncol* 2012, in press.
49. Grossel MJ, Hinds PW: **From cell cycle to differentiation: an expanding role for cdk6.** *Cell Cycle* 2006, **5:**266–270.
50. Musat M, Vax VV, Borboli N, Gueorguiev M, Bonner S, Korbonits M, Grossman AB: **Cell cycle dysregulation in pituitary oncogenesis.** *Front Horm Res* 2004, **32:**34–62.
51. Bennett DC: **How to make a melanoma: what do we know of the primary clonal events?** *Pigment Cell Melanoma Res* 2008, **21:**27–38.
52. Hussussian CJ, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, Clark WH Jr, Tucker MA, Dracopoli NC: **Germline p16 mutations in familial melanoma.** *Nat Genet* 1994, **8:**15–21.
53. Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T: **MicroRNA expression profiling in prostate cancer.** *Cancer Res* 2007, **67:**6130–6135.
54. Philippidou D, Schmitt M, Moser D, Margue C, Nazarov PV, Muller A, Vallar L, Nashan D, Behrmann I, Kreis S: **Signatures of microRNAs and selected microRNA target genes in human melanoma.** *Cancer Res* 2010, **70:**4163–4173.
55. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** *Genome Biol* 2002, **3**. research0034.1-0034.11-0031-0034.0011.
56. Kreis S, Philippidou D, Margue C, Behrmann I: **IL-24: a classic cytokine and/or a potential cure for cancer?** *J Cell Mol Med* 2008, **12:**2505–2510.
57. Haan C, Behrmann I: **A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background.** *J Immunol Methods* 2007, **318:**11–19.

doi:10.1186/1478-811X-10-41
Cite this article as: Schmitt *et al.*: Interferon-γ-induced activation of Signal Transducer and Activator of Transcription 1 (STAT1) up-regulates the tumor suppressing microRNA-29 family in melanoma cells. *Cell Communication and Signaling* 2012 **10**:41.

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

Tumor and Stem Cell Biology

Cancer Research

Signatures of MicroRNAs and Selected MicroRNA Target Genes in Human Melanoma

Demetra Philippidou[1], Martina Schmitt[1], Dirk Moser[1], Christiane Margue[1], Petr V. Nazarov[2], Arnaud Muller[2], Laurent Vallar[2], Dorothee Nashan[3], Iris Behrmann[1], and Stephanie Kreis[1]

Abstract

Small noncoding microRNAs (miRNA) regulate the expression of target mRNAs by repressing their translation or orchestrating their sequence-specific degradation. In this study, we investigated miRNA and miRNA target gene expression patterns in melanoma to identify candidate biomarkers for early and progressive disease. Because data presently available on miRNA expression in melanoma are inconsistent thus far, we applied several different miRNA detection and profiling techniques on a panel of 10 cell lines and 20 patient samples representing nevi and primary or metastatic melanoma. Expression of selected miRNAs was inconsistent when comparing cell line–derived and patient-derived data. Moreover, as expected, some discrepancies were also detected when miRNA microarray data were correlated with qPCR-measured expression levels. Nevertheless, we identified miRNA-200c to be consistently downregulated in melanocytes, melanoma cell lines, and patient samples, whereas miRNA-205 and miRNA-23b were markedly reduced only in patient samples. In contrast, miR-146a and miR-155 were upregulated in all analyzed patients but none of the cell lines. Whole-genome microarrays were performed for analysis of selected melanoma cell lines to identify potential transcriptionally regulated miRNA target genes. Using Ingenuity pathway analysis, we identified a deregulated gene network centered around microphthalmia-associated transcription factor, a transcription factor known to play a key role in melanoma development. Our findings define miRNAs and miRNA target genes that offer candidate biomarkers in human melanoma. *Cancer Res; 70(10); 4163–73. ©2010 AACR.*

Introduction

MicroRNAs (miRNA) are noncoding ~22 nucleotide short RNAs that typically downregulate expression of their target genes. Nucleotides 2 to 8, the so-called "seed" region of miRNAs, bind to completely or partially complementary regions in the 3′ untranslated region (UTR) of target genes, which are generally present in multiple copies to amplify the regulatory effects of the miRNA (1, 2). To date, 721 human miRNAs have been identified (3), which are thought to regulate at least 30% of human genes. A recent in-depth analysis of human 3′ UTR sequences indicated, however, that even >60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs (4).

miRNAs influence most fundamental biological processes by ultimately altering the expression levels of proteins either through interference with mRNA translation or by reducing the stability of the mRNA in the cytoplasm (5). Downregulated target gene mRNAs can be detected by whole-genome microarray technology, keeping in mind that translationally repressed miRNA targets would be missed by such an approach (6).

Given the tremendous regulatory potential of miRNAs and their often tissue-specific and disease-specific expression patterns (7–9), there is increasing evidence that miRNA expression profiles could be indicative of disease risks and burdens, and as such, miRNAs are being assessed as possible biomarkers to aid diagnosis and prediction of different types and stages of cancers, including melanoma (10–12). Melanoma arises from melanocytes, which are pigmented cells present in the basal layer of the epidermis (13). The global incidence of melanoma continues to rise faster than any other malignancy, and despite considerable research efforts, no efficient therapy is available to date. Once melanoma has metastasized, the median 5-year survival rate is <5% (14).

Relatively few miRNA expression profiling analyses have thus far included or focused on melanoma samples, and the available data sets show little agreement with regards to expression patterns of individual miRNAs or the entire miRNome. Using real-time PCR on 241 individual miRNAs, Gaur and colleagues (15) have identified a set of 15 miRNAs that distinguished melanomas from other solid cancers whereas others (16) have described four melanoma-characteristic miRNAs detected by microarray analysis with only miR-335

Authors' Affiliations: [1]Life Sciences Research Unit, University of Luxembourg; [2]CRP Santé/Microarray Center, Luxembourg, Luxembourg and [3]Department of Dermatology, University Clinic of Freiburg, Freiburg, Germany

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Stephanie Kreis, Life Sciences Research Unit, University of Luxembourg, 162A Avenue de la Faiencerie, L-1511 Luxembourg, Luxembourg. Phone: 352-4666446884; Fax: 011-00352-4666446435; E-mail: stephanie.kreis@uni.lu.

doi: 10.1158/0008-5472.CAN-09-4512

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

common to both studies. Mueller and colleagues (17) have recently described a more detailed analysis of miRNA expression in melanocytes versus seven different melanoma cell lines based on miRNA microarray analysis. In primary melanoma cell lines, a set of 49 miRNAs was found to be strongly upregulated whereas 14 were downregulated; however, most of these had not been shown before to be substantially regulated in melanoma development. miRNA expression in melanocytic nevi derived from formalin-fixed paraffin-embedded (FFPE) samples has recently been investigated by Glud and colleagues, who, among others, have shown that FFPE samples are useful sources for miRNA profiling (18, 19).

Owing to the many factors that differed between these studies (real-time PCR versus miRNA microarray approaches; different microarray platforms and mirBase versions; different sample types and melanoma cell lines) it is not surprising that largely different miRNA sets were considered to be characteristic for melanoma or melanoma progression. However, thus far no miRNAs have been identified that would allow for reproducible and specific distinction between early-stage or late-stage melanoma samples from healthy melanocytes or benign nevi in cell lines and patients.

To evaluate the discrepancies and/or concordances in miRNA expression patterns that might result from different technical approaches on different biological samples, we established miRNA expression profiles in primary human melanocytes [normal human epidermal melanocyte (NHEM)] versus melanoma cell lines by miRNA microarray, real-time miRNA reverse transcription–PCR arrays (RT^2 Profiler), followed by individual quantitative real-time PCR (qPCR) validations. To then correlate cell line–derived expression patterns with melanoma patients, we next analyzed FFPE patient samples including benign nevi, primary melanoma, and metastatic melanoma samples. Cross-correlation of all miRNA expression data revealed that only miRNA-200c was commonly downregulated in different sample types robustly detectable by the various technical approaches. Other miRNAs were only found to be significantly deregulated in either cell lines or patients or could not be reproduced using different detection techniques.

Finally, whole-genome arrays were used on melanoma cell lines and melanocytes to identify possible target genes of some of the differentially regulated miRNAs. We focused on genes commonly deregulated in melanoma and inversely correlated their expression patterns with miRNAs predicted to target such genes, identifying several possible miRNA/target gene pairs likely to play a role in melanoma development. Ingenuity network analysis identified with a high statistical confidence deregulated pathway around the transcription factors microphthalmia-associated transcription factor (MITF), SRY-related HMG box (SOX10), and T-box transcription factor 2.

Materials and Methods

Cell lines and cell culture. In total, nine melanoma cell lines were analyzed: Wm9 (Dr. M. Böhm, Münster, Germany), FM55-M1 (European Searchable Tumor Line Database and Cell Bank), IGR39 and IGR37 (Deutsche Sammlung von Mikroorganismen und Zellkulturen), A375 (American Type Culture Collection), 1102 (Dr. M. Kortylewski, City of Hope, California), MeWo (Dr. Schadendorf, Essen, Germany), and MelIm and MelJuso (Dr. A. Bosserhoff, Regensburg, Germany). All cells were maintained in RPMI 1640 supplemented with 10% FCS, 50 μg/mL penicillin, 100 μg/mL streptomycin, and 0.5 mmol/L L-glutamine. NHEMs from lightly pigmented adult skin (PromoCell) were maintained in serum-free and phorbol 12-myristate 13-acetate–free MGM-M2 medium and were used at passage number 5 or 6. MCF-7 breast cancer cells (Dr. G. Vetter, Luxembourg) were grown in DMEM supplemented with 10% FCS, 50 μg/mL penicillin, and 100 μg/mL streptomycin. All cells were grown in a humidified atmosphere with 5% CO_2 supply and were routinely PCR tested to be *Mycoplasma* negative.

Patient samples. Skin tissue samples from patients with either benign nevi or melanoma were collected at the Dermatology Department of University Hospital of Freiburg (Germany) and histopathologically examined to confirm clinical diagnoses. Upon excision, tissues were fixed in FFPE according to standard dermatohistopathologic techniques. In total, 3 pools of benign nevi (RNAs of two different donors each) and 17 primary and subcutaneous melanoma metastasis patient samples were analyzed (age and gender information is included in Supplementary Table S1). Additionally, four breast cancer FFPE samples from two patients were included in this study. The study was approved by the ethical review board of EK Freiburg (reference 196/09), and written informed consent was obtained from healthy controls and live patients.

Total RNA extraction and quality control. Total RNA of cell lines was extracted using TRIsure (Bioline USA, Inc.) and treated with DNaseI (New England Biolabs) following each manufacturer's instructions. For miRNA and whole-genome microarray analyses, total RNA was extracted using the miRNeasy kit (Qiagen) according to the manufacturer's protocol with additional on-column DNaseI digestion. In FFPE samples, for total RNA extraction, five scalpel-scraped slices of FFPE tissue were pooled and processed using the RT^2-FFPE RNA Extraction kit (SABiosciences) according to the supplied protocol. Quantity and purity of RNA samples were assessed using a NanoDrop ND-100 Spectrophotometer. For FFPE tissues, in particular, when RNA quantity or quality was insufficient, samples were further processed by standard ethanol precipitation and resuspended in appropriate volumes of DEPC-H_2O to achieve total RNA concentrations of >350 ng/μL and absorbance ratios of > 1.8 (260/280) and > 1.7 (260/230).

miRNA and whole-genome microarrays. Total RNA from NHEM, IGR39, and IGR37 cells was subjected to (*a*) genome-wide miRNA expression profiling (miRBase, version 11.0) using the μParaflo microarray technology (LCSciences) and (b) whole-genome expression profiling using GeneChip Human Gene 1.0 ST arrays (Affymetrix). Gene network analyses were performed with the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems). Detailed methodologies

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

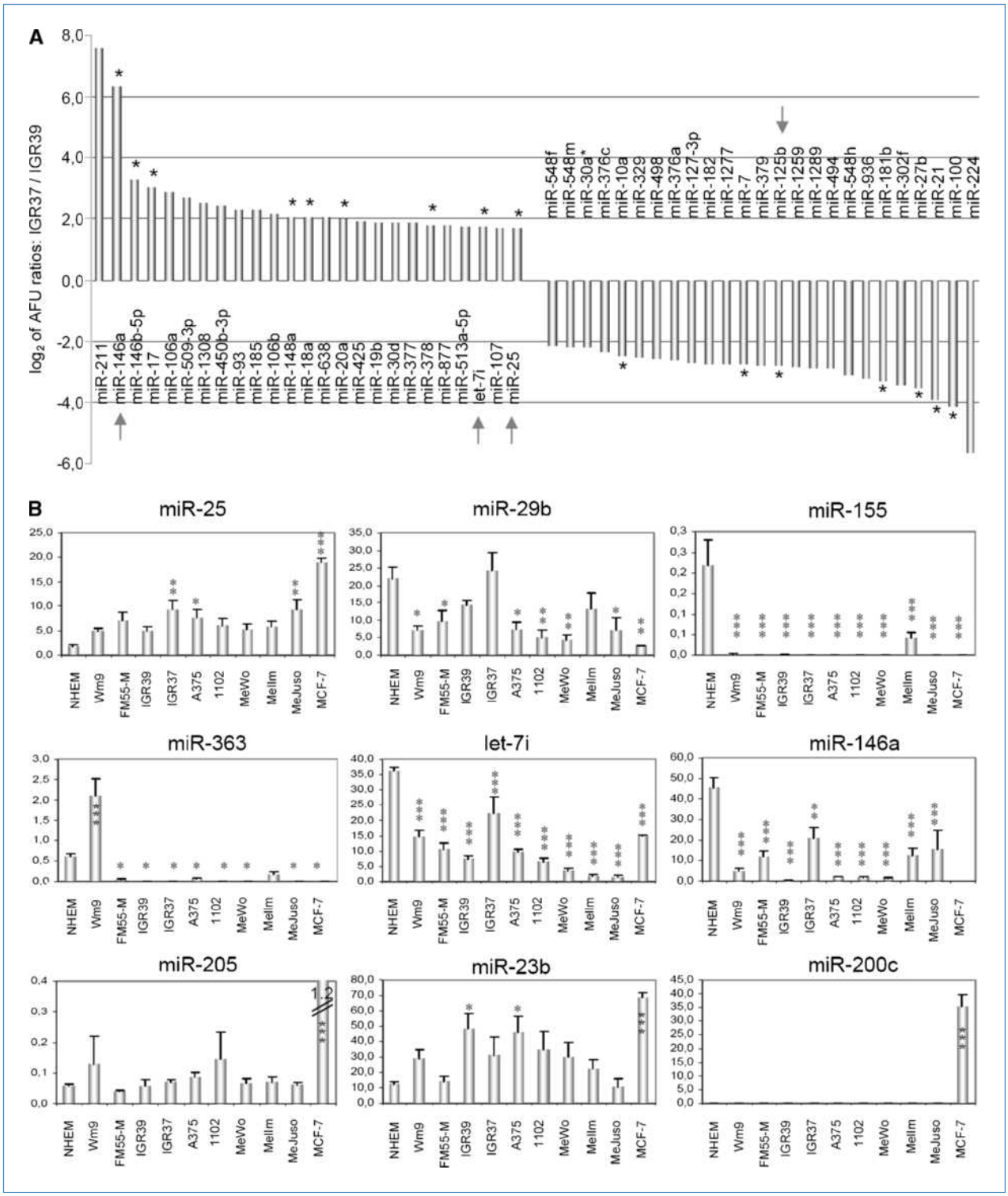


Figure 1. A, top 25 gradually upregulated and downregulated miRNAs in metastatic (IGR37) versus primary (IGR39) melanoma cell lines established from the same patient. AFUs were derived from at least duplicate miRNA microarrays (LCSciences). AFUs of >500 were selected and sorted, and $\log_2$ values of AFU ratios were plotted. miRNAs marked with an arrow were further validated; the ones marked with an asterisk were confirmed by RT^2 Profiler results. B, relative expression of selected miRNAs in primary human melanocytes (NHEM, passage no. 5), a panel of nine melanoma cell lines, and MCF-7 breast cancer cells (relative to SCARNA17 expression). Results are depicted as mean of biological triplicates ± SEM. Statistical analysis was performed using a one-way ANOVA test, comparing results for each cell line to NHEM cells. ***, $P < 0.001$; **, $P = 0.001$–0.01; *, $P = 0.01$–0.05; no star, not significant at $P > 0.05$.

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

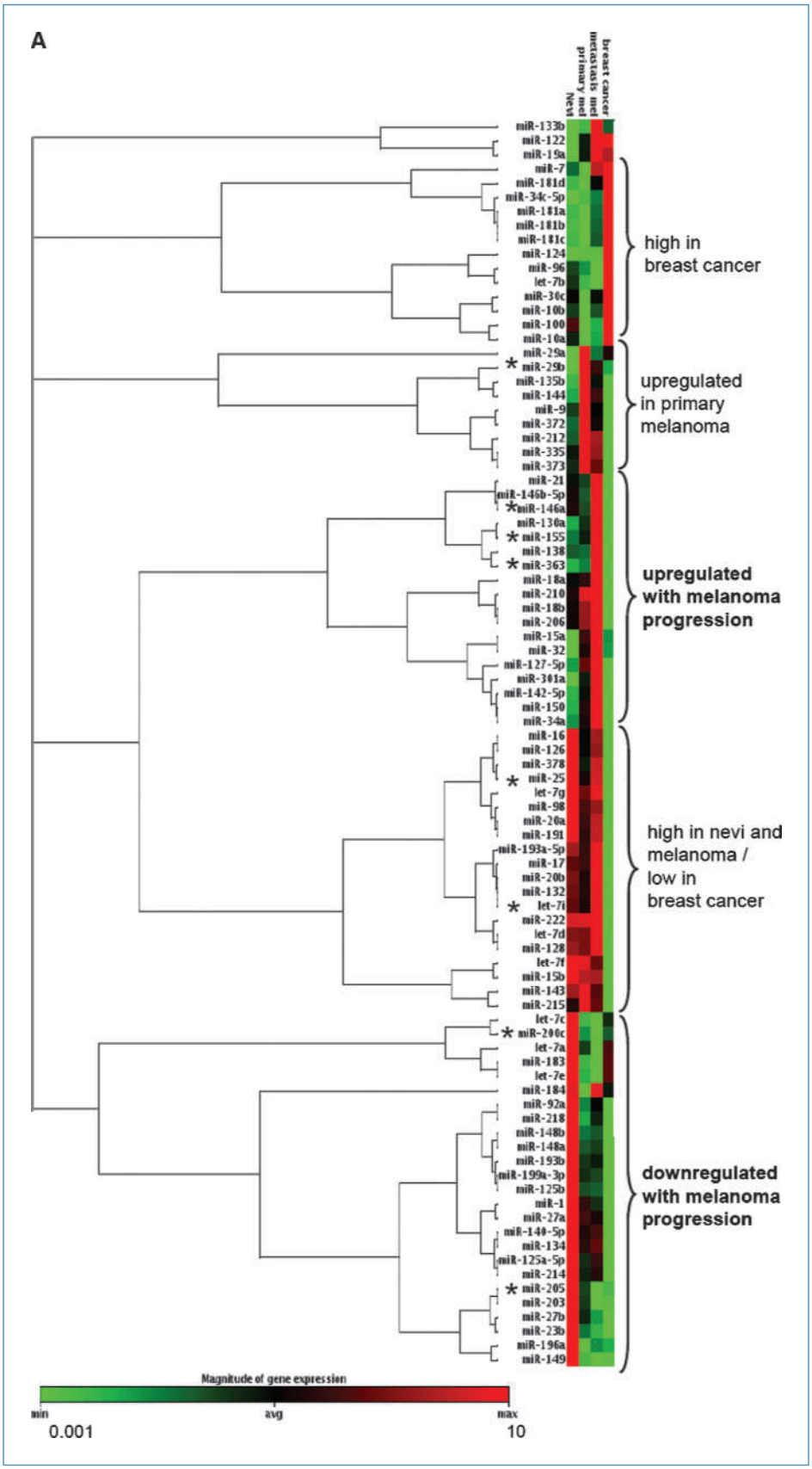


Figure 2. A, miRNA expression profiling in melanoma patients (RT^2 Profiler). Clustergram generated from average miRNA C_t values of individual FFPE patient samples from nevi (3), primary melanoma (7), metastatic melanoma (10), and breast cancer samples (4). For each patient group, average C_t values were normalized to a panel of four different housekeeping genes (Supplementary Table S1). The range of $2^{-\Delta C_t}$ values is given below the color scale. miRNAs that were further validated by qPCR are marked by an asterisk.

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

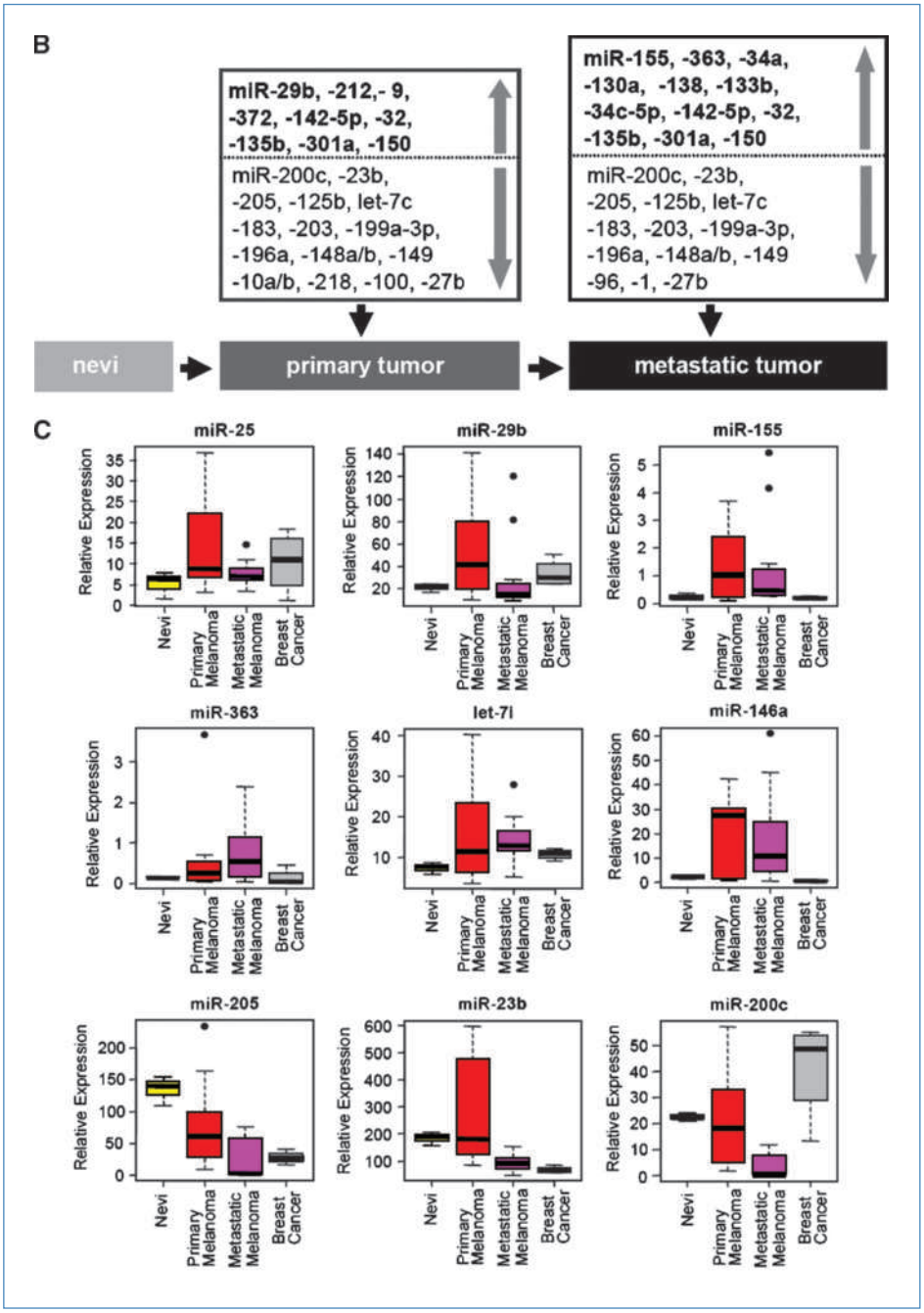


Figure 2. ***Continued.*** B, miRNA signatures for stages of melanoma progression. miRNAs of >2.0-fold upregulated (bold) or downregulated relative to nevi are shown for each patient group. C, box plots showing triplicate qPCR validations for nine selected miRNAs of individually analyzed patient samples (as in A).

and bioinformatic analyses are described in Supplementary Materials and Methods.

Real-time PCR-based miRNA expression profiling. FFPE patient samples were analyzed for the presence and differential expression of a panel of 88 cancer-related miRNAs using cancer RT^2 miRNA PCR arrays (called RT^2 Profiler; SABiosciences) according to the manufacturer's instructions. Data analysis was performed with the web-based software package for the miRNA PCR array system (20).

miRNA and gene expression validation by real-time qPCR. Briefly, 250 and 100 ng of total RNA from cell lines and FFPE tissues, respectively, were reverse transcribed in a 10-μL reaction volume with the miScript System (Qiagen) following the manufacturer's instructions. To quantify mature miRNAs, real-time qPCR was carried out on a CFX96 Detection System (Bio-Rad) using 5 ng RNA input, 2× iQ SYBR Green Supermix (Bio-Rad), and 10× miRNA-specific primer assay (Qiagen). For the detection of cKIT and MITF,

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

50 ng RNA input, 2× iQ SYBR Green Supermix, and 5 pmol gene-specific primer pairs were used. Thermal cycling conditions for all assays were 95°C for 3 minutes, 40 cycles at 95°C for 10 seconds, and 60°C for 30 seconds, followed by melting curve analyses. RNA input was normalized to endogenous controls: SCARNA17 for miRNAs and TATA-binding protein for protein encoding genes. The $2^{-\Delta C_t}$ method was used to calculate the fold relationships in miRNA or gene expression among the tested samples (21).

miRNA target gene prediction. Three publicly available databases, TargetScanHuman 5.1 (4), DIANA-microT v3.0 (22), and MicroCosm Targets Version 5 (3), were used for miRNA target gene predictions. Predicted target genes in combination with miRNA and whole-genome microarray data were used to visualize possible biological miRNA/mRNA processes correlating to melanoma development and/or progression.

Results

miRNA expression profiling of primary human melanocytes (NHEM) and melanoma cell lines. As a first step, we analyzed the miRNome of two melanoma cell lines from a single patient representing primary (IGR39) and metastatic tumors (IGR37) and compared them to NHEM using LCSciences microarray data and RT^2 Profiler PCR arrays, followed by individual qPCR validations for selected miRNAs (Fig. 1, Supplementary Table S1). Figure 1A depicts the top 25 miRNAs upregulated and downregulated with tumor progression as detected by miRNA microarray. Sixteen miRNAs (asterisk-marked) were also present on RT^2 Profiler assays. Of those, 12 had matching expression ratios (except for miR-10a, miR-7, miR-181b, and miR-21) following tumor progression in cell lines (Supplementary Table S1) and melanoma patients (see Fig. 2A). This suggests that cell lines give some indication of relevant expression patterns, but individual results may change in patient-derived samples. Complete array-based expression profiles of all miRNAs (miRBase version 11.0) served as a first base for selection of miRNAs to be investigated further. Comparison of microarray results with data from the RT^2 Profiler revealed that 66 of the 88 common miRNAs (75%) showed comparable results, with both of the assays being clearly positive [C_t < 30 or arbitrary fluorescence unit (AFU) > 500] or negative.

The expression levels of several miRNAs were further validated by qPCR, extending the cell line panel to nine different melanoma cell lines, NHEMs, and MCF-7 breast cancer cells (Fig. 1B). qPCR results for NHEM, IGR39, and IGR37 correlated with microarray-measured expression levels for all tested miRNAs except let-7i, wherein levels were lower in NHEM compared with IGR39 and IGR37. Figure 1B further illustrates that some miRNAs have considerably different expression levels in individual cell lines, suggesting that profound variations would also be apparent when analyzing the entire miRNomes of such cell lines. Nevertheless, a few robustly regulated miRNAs seemed to be useful candidates to distinguish between NHEMs and melanoma cell lines in general: miR-155 and miR-146a with decreased expression levels in all melanoma lines tested and miR-25 and miR-23b (as well as miR-23a; data not shown), which were consistently upregulated.

miRNA expression profiling of FFPE melanoma patient samples. Due to the small amplicon size of miRNAs, FFPE samples represent useful sources to analyze miRNA expression even when some degree of RNA degradation is apparent (18). In total, 24 FFPE samples were analyzed by RT^2 Profiler arrays (Fig. 2A) followed by qPCR for selected miRNAs (Fig. 2B). The heat map (Fig. 2A) depicts expression values for 88 cancer-relevant miRNAs of four donor groups relative to four different house-keeping genes (Supplementary Table S1). Nevi (consisting of pools of RNAs extracted from five healthy volunteers) showed very similar C_t values across 85% of analyzed miRNAs. The primary melanoma group represents mean expression values from seven individual primary tumors, whereas the metastatic melanoma group consisted of 10 different patient samples. The breast cancer group (four samples from two patients) was included to allow for identification of miRNAs that were distinct between the two cancer types. miRNA-16, miRNA-27a, miRNA-125b, 199a-3p, 199a-21, and 199a-205 were highly expressed in melanoma having greater RNA levels than the four housekeeping genes (U6, SNORD44, SNORD46, and SNORD47), whereas the breast cancer group was characterized by highest expression of miRNA-16, miRNA-100, miRNA-21, and let-7b (Supplementary Table S1). Figure 2B shows that each melanoma stage bore a distinct miRNA signature. Considerable interpatient variability (>2.0 C_t) among the different melanoma patient groups was detected for <10% of the analyzed 88 cancer-related miRNAs (Supplementary Table S1).

qPCR validation on duplicate RNAs extracted from individual FFPE samples revealed a generally good correlation when compared with expression patterns achieved with averaged values for the different patient groups. Figure 2C shows box plots for nine selected miRNAs. Most striking were the levels of miR-200c, miR-205, and miR-23b, which were strongly downregulated in melanoma patients when compared with nevi. miR-200c is further interesting, as it allows for discrimination between melanoma and breast cancer samples.

miR-363, miR-146a, and miR-155 were clearly upregulated in all melanoma patients but not in breast cancer samples. Interestingly, the latter two were consistently low or undetectable in cell lines. In this context, it has recently been suggested that miR-155 was a negative regulator of melanoma proliferation, as its expression was downregulated or lost in the majority of melanoma cell lines (23). Here, we also monitored an almost complete loss of miR-155 in our panel of cell lines (Fig. 1B); however, miR-155 was clearly detectable in all of the 17 patient-derived samples, indicating that the loss of miR-155 expression may be a tissue culture–related phenomenon. Taken together, miR-155, miR-205, miR-146a, and miR-23b emerged as useful markers to distinguish between melanoma cell lines and patient samples relative to human melanocytes and nevi, respectively.

Correlation of expression levels of selected miRNAs with their predicted target genes. Whole-genome cDNA arrays

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

were performed on melanoma cell lines and melanocytes to obtain a pattern of transcriptionally regulated candidate genes that could serve as possible targets of some of the identified differentially regulated miRNAs. Scatter plots with highlighted genes known to be important for melanoma development and/or progression are shown in Fig. 3. Interestingly, melanoma-associated antigen C2, a predicted (although nonconserved) target of miR-200a, miR-200b, and miR-200c family, was strongly expressed in most melanoma cell lines but not in MCF-7 breast cancer cells, whereas inversely miR-200c expression was undetectable in all tested melanomas (Figs. 1B and 2A; Supplementary Fig. S1). Expression of the receptor tyrosine kinase cKIT, a confirmed target of miR-221/miR-222 (24), was augmented in IGR37, which showed correspondingly low levels of miR-221. An inversely and perfectly correlated expression pattern of miR-221 and cKIT was also confirmed for all nine melanoma cell lines but interestingly not for the MCF-7 cell line (Supplementary Fig. S1). miR-23a and miR-23b, upregulated in melanoma cell lines, are predicted to target many genes, of which several were found to be reciprocally downregulated in this data set [MITF (Supplementary Fig. S1), TYR (tyrosinase), TRPM1 (transient receptor potential cation channel 1), MLANA (Melan-A), and others]. All of these genes have previously been implicated in melanoma development (25–27). We further validated and confirmed the array-based expression levels for four additional genes [CDKN1B (p27), CDK6, E-cadherin, and N-cadherin] for NHEM, IGR39, and IGR37 (Supplementary Fig. S2).

Using differentially expressed gene lists of NHEM and IGR39, Ingenuity analysis identified 33 pathways with statistical scores of >10 (score = –log (*P* value) from Fisher's exact test analysis). Network no. 3 "skin development and function" (score = 37) was selected based on statistical and biological relevance for the study (Fig. 4A). We then prompted Ingenuity to include miRNAs that were predicted to target the strongly regulated and melanoma-relevant genes. The resulting network shows some of the known key players in melanoma development and progression with *MITF* regulating the expression of several genes known to be involved in pigmentation and skin development, such as TYR, tyrosinase-related protein 1, SILV (melanocyte protein Pmel 17), and TRPM1, whose expression is inversely correlated with melanoma metastasis (25).

To evaluate the robustness of the interactions predicted by the software, we analyzed gene levels of MITF and its tentative targeting miRNA-23a and miRNA-23b, as well as four genes that have previously been shown to be regulated by MITF (Fig. 4B; refs. 26, 28, 29). In IGR39 cells, wherein miR-23a/miR-23b expression was highest, MITF levels were almost undetectable. Correspondingly, the expression patterns of three of the four tested and potentially MITF-regulated genes (miR-146a, let-7I, and cKIT) matched the expression trends of the transcription factor, indicating that they could indeed be regulated by MITF. miRNA-363, which is also regulated by MITF (28), was however an exception as such that IGR37 had reproducibly undetectable levels of miR-363.

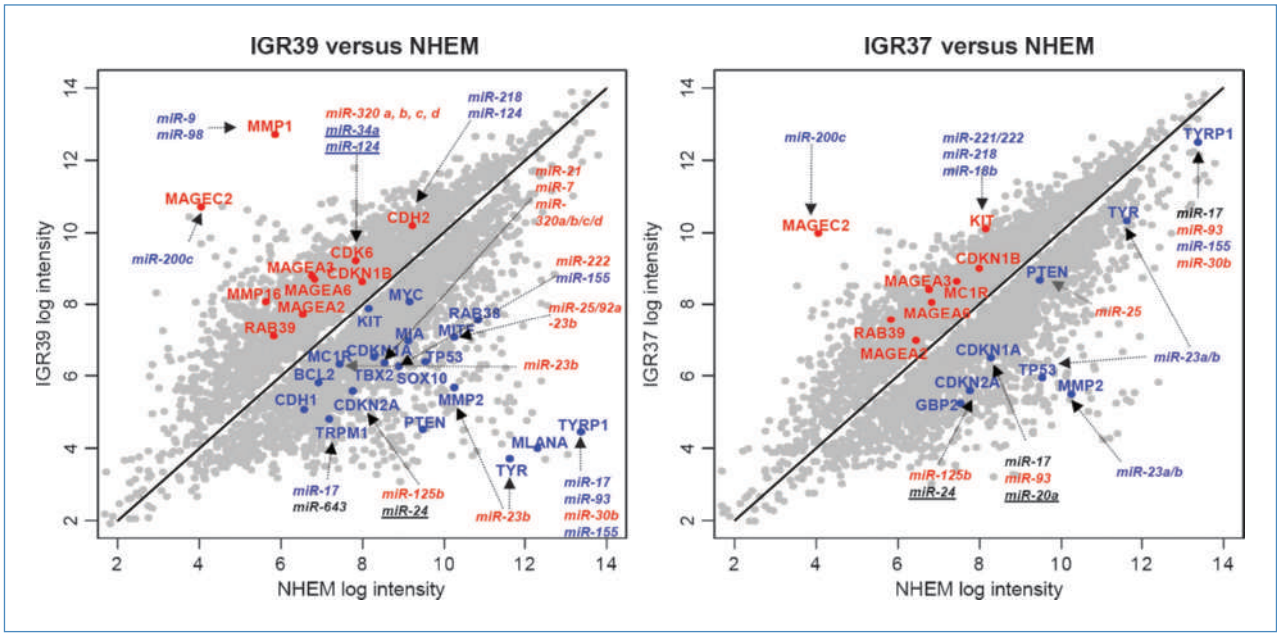


Figure 3. Mapping of potential miRNA targets in gene expression profiles. Scatter graphs were generated by plotting normalized log expression data obtained with IGR39 or IGR37 cells against those found with NHEM control cells. Each gray dot in the graphs corresponds to the expression value of a particular gene found to be significantly differentially expressed (FDR < 0.05). Selected genes previously implicated in melanoma are highlighted in red or blue (upregulated or downregulated in melanoma versus melanocytes, respectively). miRNAs computationally predicted to target some of the highlighted genes are depicted in italics: red or blue (upregulated or downregulated in the respective melanoma cell line versus melanocytes, respectively), or black (unchanged expression). Underlined are miRNAs that have been experimentally confirmed to regulate the respective target genes. For some genes, no targeting miRNA was predicted by Targetscan or MicroCosm Targets.

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

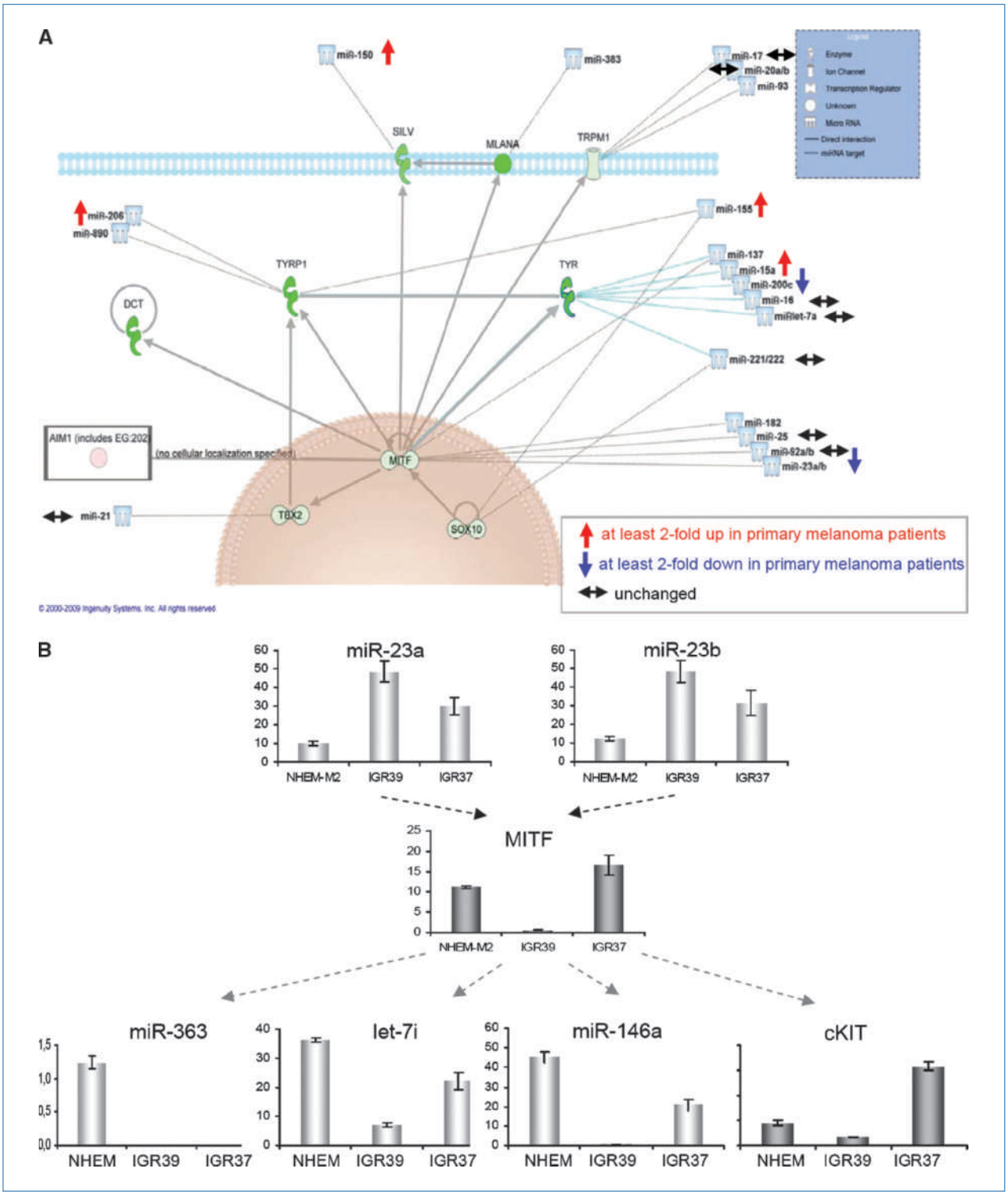


Figure 4. A, miRNA target map illustrating parts of the "skin development and function" network, which IPA identified to be highly significant in our experimental data set (IGR39 versus NHEM). Central to this network is MITF and its predicted interactions with pigmentation-relevant and melanoma-relevant proteins together with their subcellular localization. The node color intensity indicates the expression level of genes: red, upregulated; green, downregulated in the comparison IGR39 versus NHEM. IPA-predicted miRNAs and their relative expression levels in our primary melanoma patients are also shown (red, up; blue, down). B, relative expression levels of MITF of biological triplicates of NHEM, IGR39, and IGR37. Inverse expression levels for miR-23a and miR-23b, which are predicted to target MITF, are shown above. Expression patterns for miRNA-363, miRNA-146a, let-7i, and cKIT, suggested to be regulated by MITF, are shown in the bottom, all of which (except miR-363) show MITF-concordant expression trends.

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

Taken together, computational pathway analysis proved to be a useful tool to select key-regulated genes. Inversely correlated expression patterns of such selected genes and their predicted miRNAs in an extended number of biological samples can point to regulatory relationships between miRNAs and their tentative target genes.

Discussion

Recent years have seen a tremendous number of miRNA profiling studies aiming at identifying unique miRNA signatures for many diseases, for different stages thereof, or for prediction of disease risks (9, 11). These efforts have been paralleled by continuous discovery of new miRNAs and by rapidly changing developments and improvements in miRNA detection techniques. Given the different technical approaches applied to miRNA profiling (various microarray chemistries and real-time PCR quantifications) together with the plethora of different biological samples that are being profiled, it is not surprising that there is often little correlation between data sets coming from different laboratories. Here, we have addressed this question by comparing miRNA expression patterns in well-characterized melanoma cell lines with patient-derived samples of different melanoma stages by applying microarray and qPCR profiling techniques.

A key issue for comparability or lack thereof of different expression data sets is the selected reference line or tissue, whose basal miRNA expression levels will determine the calculated fold expressions in the tested samples. We have observed that primary human melanocytes (NHEM), which are commonly used as calibrators for melanoma studies, considerably change their absolute levels of several important miRNAs with increased passage number (data not shown). Therefore, great care should be taken in choosing negative controls for comparative and consecutive studies. In this context, we noted very little agreement between our miRNA expression patterns of NHEM melanocytes and melanoma cell lines with a recent study by Mueller and colleagues (17), who have used Agilent miRNA microarrays. Of the 76 listed miRNAs associated with melanoma development and progression, we only identified six to be correspondingly regulated in our data set (miR-27b, miR-92b, miR-10a, miR-182, miR-26b, and miR-379), with the latter four being only weakly expressed. This indicates that the use of different profiling platforms together with the negative control cells and likely even their passage number may result in strikingly different conclusions drawn from the data. On the other hand, our miRNA expression profiles of FFPE-extracted nevi was in good agreement with results recently reported by Glud and colleagues, who used Invitrogen arrays to profile miRNA expression in 15 nevi (18). Taking into account the different array platforms and miRBase versions that were used, we found a remarkable 81% (68 of 84) of miRNAs to be similarly expressed in both studies. Although FFPE samples represent useful sources for miRNA profiling, it is important to keep in mind that variable handling conditions as well as lengths and conditions of storage may directly influence the degree of RNA degradation, which in turn could affect expression levels of some miRNAs.

Another source for intrinsic data variability stems from the various biological sources that are used in miRNA profiling studies. Several miRNAs, such as miR-155, miR-146a, miR-23b, and miR-205, showed divergent expression patterns in cell lines and patient samples, indicating tissue culture–induced adaption processes that likely lead to the loss or upregulation of certain miRNAs, which are not present in patient material. Nonetheless, we identified miR-25 to be upregulated in our samples, and this was in line with a recent observation made in melanoma (30). miR-200c was reproducibly downregulated in patients relative to nevi and was absent in normal human melanocytes and cell lines. The loss or reduced expression of miR-200c in melanoma cell lines has also been reported previously (15). The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) is clearly emerging as a key regulator of differentiation in various cell types, and a marked downregulation with tumor progression has been noted for several cancers (31–33). The loss of E-cadherin expression in our melanoma cell lines is consistent with absent miR-200c expression, which releases repression of the transcription factor ZEB1, which in turn suppresses E-cadherin gene transcription (34). In doing so, the miR-200 family becomes an important regulator of epithelial-to-mesenchymal transition (EMT), a key process for initializing metastasis, and might therefore represent one of the most promising miRNA candidates for therapeutic intervention (35). Interestingly, downregulation of miR-205 has also been implicated in the EMT processes (33). Reduced amounts of miR-205 with tumor progression were detected in all of our melanoma patients, although melanoma cell lines showed slightly increased expression of this miRNA.

Olson and colleagues showed that miR-25 was upregulated 2.6-fold in hyperproliferative stages of pancreatic tumors whereas miR-146a was downregulated 8.4-fold in metastatic lesions, a pattern that we have also observed in melanoma patient samples (36). It is noteworthy that miRNA-15b has recently been suggested to be a prognostic marker for melanoma progression (37). Although melanomas have indeed high levels of miR-15b, we also detected these elevated levels in nevi, questioning the usefulness of this miRNA as a biomarker for melanoma.

Taken together, miRNA-205, miRNA-200, and let-7 family (-125b, -146a, -155, -21, -25, -23a, -29b) have repeatedly been shown to become deregulated with tumor progression in general and in melanomas in particular (this study and refs. 12, 38). Although this is by no means an exclusive list, it certainly contains some of the most promising miRNAs for therapeutic intervention or to be further evaluated for their biomarker potential in larger patient cohorts.

Having identified several miRNAs that could be of value for the understanding of melanoma development, we set out to find potential target genes by analyzing mRNA expression patterns in primary melanocytes (NHEM) and two melanoma cell lines. As expected, many genes previously linked to melanoma development were found to be deregulated,

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

and several were differentially regulated when comparing IGR39 cells (primary melanoma) with the cell line derived from a metastatic tumor of the same patient (IGR37; Fig. 3). For example, cKIT was slightly downregulated in IGR39 whereas its levels were augmented with disease progression in IGR37. It has been shown that miR-221/miR-222 downregulates cKIT and p27 in melanomas, thus favoring a malignant phenotype (30), and another study suggested that melanoma subgroups exist, which overexpress mutated and active cKIT (39). qPCR for miR-221 and cKIT confirmed this by a perfect inversely correlated expression pattern on all our melanoma cell lines (Supplementary Fig. S1).

Inverse correlation of miRNA expression with their tentative target genes is a useful approach to reduce the generally extensive number of computationally predicted target genes for further analysis. In combination with examination of biological pathways targeted by the deregulated miRNAs, Ingenuity analysis indicated that the regulatory network around MITF was most prominent in melanoma cell lines (Fig. 4A). MITF is critically involved in regulation of melanocyte growth, maturation, apoptosis, and pigmentation (40, 41). Over 20% of metastatic melanomas have been found to carry genetic alterations in the MITF pathway (MITF or SOX10 mutations; ref. 42), and amplification of MITF has been linked to poor patient survival (26). By nucleosome mapping and chromatin structure analysis, Ozsolak and colleagues have identified several miRNA promoters that were occupied by MITF (28). Of those, miR-146a, miR-221, and miR-363 were experimentally confirmed to be regulated by MITF. qPCR analysis of candidate miRNA-23a and miRNA-23b levels predicted to target MITF, as well as of cKIT and miRNAs, which are transcriptionally regulated by MITF, supported the central part of the predicted pathway (Fig. 4B).

Although altered expression of the miRNome has been well documented in many cancers, it remains to be shown how single and/or groups of miRNAs or the whole of the miRNome drives or allows for neoplastic transformation. A key prerequisite to these analyses is, however, an accurate and standardized quantification of the miRNA expression levels in a sufficient number of clinical samples and matching cell lines, as effects seen in one cell line or patient might be quite different from those found in another, in which other potential target genes and also other modulators of miRNA activity may be present. This is even more so as minute changes in miRNA levels may have profound consequences for the cell (2). Accurate and extensive measurements of miRNA levels together with improved computational target gene predictions and pathway analyses will surely be necessary before miRNAs make their way into the clinic as robust biomarkers and/or as therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

University of Luxembourg grant F1R-LSC-PUL-09MIRN and Fonds National de la Recherche Luxembourg AFR fellowship grant TR-PHD BFR08-077 (M. Schmitt).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/14/2009; revised 02/08/2010; accepted 02/25/2010; published OnlineFirst 05/04/2010.

References

1. Grimson A, Farh KK, Johnston WK, et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 2007;27:91–105.
2. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215–33.
3. Griffiths-Jones S, Saini HK, van Dongen S, et al. miRBase: tools for microRNA genomics. Nucleic Acids Res 2008;36:D154–8. Available from: http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5.
4. Friedman RC, Farh KK, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19:92–105. Available from: http://www.targetscan.org.
5. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9:102–14.
6. Grosshans H, Filipowicz W. Proteomics joins the search for microRNA targets. Cell 2008;134:560–2.
7. Bargaje R, Hariharan M, Scaria V, et al. Consensus miRNA expression profiles derived from interplatform normalization of microarray data. RNA 2010;16:16–25.
8. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–66.
9. Saunders MA, Lim LP. (micro)Genomic medicine: microRNAs as therapeutics and biomarkers. RNA Biol 2009;6:324–8.
10. Yi R, Fuchs E. MicroRNA-mediated control in the skin. Cell Death Differ 2010;17:229–35.
11. Bartels CL, Tsongalis GJ. MicroRNAs: novel biomarkers for human cancer. Clin Chem 2009;55:623–31.
12. Sand M, Gambichler T, Sand D, et al. MicroRNAs and the skin: tiny players in the body's largest organ. J Dermatol Sci 2009;53:169–75.
13. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature 2007;445:851–7.
14. Miller AJ, Mihm MC, Jr. Melanoma. N Engl J Med 2006;355:51–65.
15. Gaur A, Jewell DA, Liang Y, et al. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res 2007;67:2456–68.
16. Blower PE, Verducci JS, Lin S, et al. MicroRNA expression profiles for the NCI-60 cancer cell panel. Mol Cancer Ther 2007;6:1483–91.
17. Mueller DW, Rehli M, Bosserhoff AK. miRNA expression profiling in melanocytes and melanoma cell lines reveals miRNAs associated with formation and progression of malignant melanoma. J Invest Dermatol 2009;129:1740–51.
18. Glud M, Klausen M, Gniadecki R, et al. MicroRNA expression in melanocytic nevi: the usefulness of formalin-fixed, paraffin-embedded material for miRNA microarray profiling. J Invest Dermatol 2009;129:1219–24.
19. Ma Z, Lui WO, Fire A, et al. Profiling and discovery of novel miRNAs from formalin-fixed, paraffin-embedded melanoma and nodal specimens. J Mol Diagn 2009;11:420–9.
20. http://www.sabiosciences.com/pcr/arrayanalysis.php
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Δ Δ C(T)) method. Methods 2001;25:402–8.
22. Maragkakis M, Alexiou P, Papadopoulos GL, et al. Accurate microRNA target prediction correlates with protein repression levels. BMC

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

Bioinformatics 2009;10:295. Available from: http://diana.cslab.ece.ntua.gr.
23. Levati L, Alvino E, Pagani E, et al. Altered expression of selected microRNAs in melanoma: antiproliferative and proapoptotic activity of miRNA-155. Int J Oncol 2009;35:393–400.
24. Felicetti F, Errico MC, Bottero L, et al. The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. Cancer Res 2008;68:2745–54.
25. Miller AJ, Du J, Rowan S, et al. Transcriptional regulation of the melanoma prognostic marker melastatin (TRPM1) by MITF in melanocytes and melanoma. Cancer Res 2004;64:509–16.
26. Hoek KS, Schlegel NC, Eichhoff OM, et al. Novel MITF targets identified using a two-step DNA microarray strategy. Pigment Cell Melanoma Res 2008;21:665–76.
27. Ugurel S, Utikal J, Becker JC. Tumor biomarkers in melanoma. Cancer Control 2009;16:219–24.
28. Ozsolak F, Poling LL, Wang Z, et al. Chromatin structure analyses identify miRNA promoters. Genes Dev 2008;22:3172–83.
29. Hou L, Pavan WJ. Transcriptional and signaling regulation in neural crest stem cell-derived melanocyte development: do all roads lead to Mitf? Cell Res 2008;18:1163–76.
30. Igoucheva O, Alexeev V. MicroRNA-dependent regulation of cKit in cutaneous melanoma. Biochem Biophys Res Commun 2009;379:790–4.
31. Peter ME. Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression. Cell Cycle 2009;8:843–52.
32. Maillot G, Lacroix-Triki M, Pierredon S, et al. Widespread estrogen-dependent repression of micrornas involved in breast tumor cell growth. Cancer Res 2009;69:8332–40.
33. Gregory PA, Bracken CP, Bert AG, et al. MicroRNAs as regulators of epithelial-mesenchymal transition. Cell Cycle 2008;7:3112–8.
34. Hurteau GJ, Carlson JA, Spivack SD, et al. Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. Cancer Res 2007;67:7972–6.
35. Bracken CP, Gregory PA, Khew-Goodall Y, et al. The role of microRNAs in metastasis and epithelial-mesenchymal transition. Cell Mol Life Sci 2009;66:1682–99.
36. Olson P, Lu J, Zhang H, et al. MicroRNA dynamics in the stages of tumorigenesis correlate with hallmark capabilities of cancer. Genes Dev 2009;23:2152–65.
37. Satzger I, Mattern A, Kuettler U, et al. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. Int J Cancer 2010;126:2553–62.
38. Baffa R, Fassan M, Volinia S, et al. MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. J Pathol 2009;219:214–21.
39. Garrido MC, Bastian BC. KIT as a therapeutic target in melanoma. J Invest Dermatol 2010;130:20–7.
40. Levy C, Khaled M, Fisher DE. MITF: master regulator of melanocyte development and melanoma oncogene. Trends Mol Med 2006;12:406–14.
41. Wellbrock C, Rana S, Paterson H, et al. Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. PLoS One 2008;3:e2734.
42. Cronin JC, Wunderlich J, Loftus SK, et al. Frequent mutations in the MITF pathway in melanoma. Pigment Cell Melanoma Res 2009;22:435–44.

Published OnlineFirst May 4, 2010; DOI:10.1158/0008-5472.CAN-09-4512

Cancer Research

Correction: Online Publication Dates for *Cancer Research* April 15, 2010 Articles

The following articles in the April 15, 2010 issue of *Cancer Research* were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:

Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, Feral C, Papayannopoulou T, Lowy A, Blair SL, Cheresh D, Ginsberg M, Varner JA. Integrin $\alpha4\beta1$ signaling is required for lymphangiogenesis and tumor metastasis. Cancer Res 2010;70:3042–51. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3761.

Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, Martin F, Apetoh L, Rébé C, Ghiringhelli F. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res 2010;70:3052–61. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3690.

Nagasaka T, Rhees J, Kloor M, Gebert J, Naomoto Y, Boland CR, Goel A. Somatic hypermethylation of *MSH2* is a frequent event in Lynch syndrome colorectal cancers. Cancer Res 2010;70:3098–108. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3290.

He X, Ota T, Liu P, Su C, Chien J, Shridhar V. Downregulation of HtrA1 promotes resistance to anoikis and peritoneal dissemination of ovarian cancer cells. Cancer Res 2010;70:3109–18. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3557.

Fiorentino M, Judson G, Penney K, Flavin R, Stark J, Fiore C, Fall K, Martin N, Ma J, Sinnott J, Giovannucci E, Stampfer M, Sesso HD, Kantoff PW, Finn S, Loda M, Mucci L. Immunohistochemical expression of BRCA1 and lethal prostate cancer. Cancer Res 2010;70:3136–9. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4100.

Veronese A, Lupini L, Consiglio J, Visone R, Ferracin M, Fornari F, Zanesi N, Alder H, D'Elia G, Gramantieri L, Bolondi L, Lanza G, Querzoli P, Angioni A, Croce CM, Negrini M. Oncogenic role of *miR-483-3p* at the *IGF2/483* locus. Cancer Res 2010;70:3140–9. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4456.

Lu W, Zhang G, Zhang R, Flores LG II, Huang Q, Gelovani JG, Li C. Tumor site–specific silencing of *NF-κB p65* by targeted hollow gold nanosphere–mediated photothermal transfection. Cancer Res 2010;70:3177–88. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3379.

Geng H, Rademacher BL, Pittsenbarger J, Huang C-Y, Harvey CT, Lafortune MC, Myrthue A, Garzotto M, Nelson PS, Beer TM, Qian DZ. ID1 enhances docetaxel cytotoxicity in prostate cancer cells through inhibition of p21. Cancer Res 2010;70:3239–48. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3186.

Yoo BK, Chen D, Su Z-z, Gredler R, Yoo J, Shah K, Fisher PB, Sarkar D. Molecular mechanism of chemoresistance by astrocyte elevated gene-1. Cancer Res 2010;70:3249–58. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4009.

Lu ZH, Shvartsman MB, Lee AY, Shao JM, Murray MM, Kladney RD, Fan D, Krajewski S, Chiang GG, Mills GB, Arbeit JM. Mammalian target of rapamycin activator RHEB is frequently overexpressed in human carcinomas and is critical and sufficient for skin epithelial carcinogenesis. Cancer Res 2010;70:3287–98. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3467.

Hattermann K, Held-Feindt J, Lucius R, Müerköster SS, Penfold MET, Schall TJ, Mentlein R. The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates antiapoptotic effects. Cancer Res 2010;70:3299–308. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3642.

Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung H-J, Evans CP, Zhou Q, Gao AC. Aberrant activation of the androgen receptor by NF-κB2/p52 in prostate cancer cells. Cancer Res 2010;70:3309–19. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3703.

Acu ID, Liu T, Suino-Powell K, Mooney SM, D'Assoro AB, Rowland N, Muotri AR, Correa RG, Niu Y, Kumar R, Salisbury JL. Coordination of centrosome homeostasis and DNA repair is intact in MCF-7 and disrupted in MDA-MB 231 breast cancer cells. Cancer Res 2010;70:3320–8. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3800.

McFarlane C, Kelvin AA, de la Vega M, Govender U, Scott CJ, Burrows JF, Johnston JA. The deubiquitinating enzyme USP17 is highly expressed in tumor biopsies, is cell cycle regulated, and is required for G_1-S progression. Cancer Res 2010;70:3329–39. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4152.

Dudka AA, Sweet SMM, Heath JK. Signal transducers and activators of transcription-3 binding to the fibroblast growth factor receptor is activated by receptor amplification. Cancer Res 2010;70:3391–401. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3033.

Cho SY, Xu M, Roboz J, Lu M, Mascarenhas J, Hoffman R. The effect of CXCL12 processing on $CD34^+$ cell migration in myeloproliferative neoplasms. Cancer Res 2010;70:3402–10. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3977.

Published OnlineFirst 05/11/2010.

doi: 10.1158/0008-5472.CAN-10-1347

RNA Biology 9:7, 978-989; July 2012; © 2012 Landes Bioscience

Dynamic regulation of microRNA expression following interferon-γ-induced gene transcription

Susanne E. Reinsbach,[1] Petr V. Nazarov,[2] Demetra Philippidou,[1] Martina Schmitt,[1] Anke Wienecke-Baldacchino,[3] Arnaud Muller,[2] Laurent Vallar,[2] Iris Behrmann[1] and Stephanie Kreis[1,*]

[1]Signal Transduction Laboratory; Life Sciences Research Unit; University of Luxembourg; Luxembourg; [2]Microarray Center; Centre de Recherche Public de la Santé; Luxembourg; [3]Life Sciences Research Unit; University of Luxembourg; Luxembourg

Keywords: microRNA, dynamic expression, transcriptional regulation, time-series microarrays

MicroRNAs are major players in post-transcriptional gene regulation. Even small changes in miRNA levels may have profound consequences for the expression levels of target genes. Hence, miRNAs themselves need to be tightly, albeit dynamically, regulated. Here, we investigated the dynamic behavior of miRNAs over a wide time range following stimulation of melanoma cells with interferonγ (IFNγ), which activates the transcription factor STAT1. By applying several bioinformatic and statistical software tools for visualization and identification of differentially expressed miRNAs derived from time-series microarray experiments, 8.9% of 1105 miRNAs appeared to be directly or indirectly regulated by STAT1. Focusing on distinct dynamic expression patterns, we found that the majority of robust miRNA expression changes occurred in the intermediate time range (24–48 h). Three miRNAs (miR-27a, miR-30a and miR-34a) had a delayed regulation occurring at 72 h while none showed significant expression changes at early time points between 30 min and 6 h. Expression patterns of individual miRNAs were altered gradually over time or abruptly increased or decreased between two time points. Furthermore, we observed coordinated dynamic transcription of most miRNA clusters while few were found to be regulated independently of their genetic cluster. Most interestingly, several "star" or passenger strand sequences were specifically regulated over time while their "guide" strands were not.

Introduction

MicroRNAs (miRNAs), a class of small non-coding RNAs (~22 nt), were initially discovered in *Caenorhabditis elegans* almost two decades ago[1] and have attracted much attention as major regulators of gene expression since.[2] The biogenesis of miRNAs from miRNA genes has been extensively studied in recent years and is reviewed in references 2 and 3. Most miRNAs are encoded in intergenic regions, but some are also hosted within introns of pre-mRNAs.[4,5] Approximately 50% of mammalian miRNA loci are located in close proximity to those of other miRNAs. So-called miRNA clusters are usually composed of two or three miRNAs, which may share similarity in sequence but which are not necessarily identical.[6] Accumulating evidence suggests that most clustered miRNAs are transcribed from single but complex transcription units, even though there might be exceptional cases in which clustered miRNAs are transcribed individually from separate gene promoters.[7] Although the precise localization of promoter regions of the majority of autonomously expressed miRNAs has not been fully mapped, it has been shown that these regions are highly similar to those of protein-coding genes.[8] In general, intergenic miRNAs are believed to be transcribed by their own promoters, whereas intronic miRNAs are coordinately transcribed with their host gene.[9]

In recent years, miRNAs have emerged as key post-transcriptional regulators of gene expression as they are predicted to directly control the expression of at least 50% of all protein-coding genes in mammals.[2] A single miRNA may control the expression levels of several hundred mRNA targets and on the other hand, a given mRNA can be regulated by several miRNAs.[10,11] Due to the fact that deregulation of miRNAs is often implicated in human diseases, precise control of miRNA amounts is crucial in order to maintain normal cellular functions. An initial point of regulation in miRNA biogenesis is transcription of miRNA genes, which is a tightly controlled multi-step process. Levels of gene expression are often controlled by auto-regulatory feedback loops in which miRNAs participate together with transcription factors (TFs).[2,3,12] However, only few examples of TF-mediated miRNA expression have been described so far: the oncogenic TF c-myc and the hypoxia-induced factor (HIF) have been shown to regulate the expression of individual or clustered miRNAs positively or negatively in a tissue-specific manner.[13-15] Also, STATs (signal transducer and activator of transcription) do not only induce protein-encoding target genes (like SOCS1, IRF1 and many others) but also appear to drive transcription of miRNA genes.[16-18] The few known interactions include a positive feedback loop between STAT1 and miR-155, where STAT1 upregulates miR-155, which in turn downregulates

*Correspondence to: Stephanie Kreis; Email: Stephanie.Kreis@uni.lu
Submitted: 02/10/12; Revised: 04/04/12; Accepted: 04/24/12
http://dx.doi.org/10.4161/rna.20494

SOCS1, a negative inhibitor of JAK/STAT signaling.[19] Further, STAT3-mediated regulation of miR-21, miR-181b, miR-17–92 and miR-199a-5p has also been described in references 16 and 20–22. Inactive STATs become activated by extracellular signals, mainly through cytokines and growth factors that bind to cell surface receptors.[23,24] STAT1 responds to type I and type II interferons and has been implicated in host anti-tumor responses.[25,26] In this context, Yang and colleagues have recently shown that type I interferon-induced upregulation of miR-21 was STAT3-dependent and resulted in inhibition of IFN-induced apoptosis.[27] Here, we used IFNγ-induced activation of STAT1 to trigger miRNA transcription in a melanoma cell model. Although some TF-mediated miRNA activations have been documented, the dynamic nature of this transcriptional regulation has, to our knowledge, not been investigated much so far.

Microarray assays have become a widely used technology to study gene regulations by measuring global expression patterns of genes or miRNAs. The comparison of steady-state expression levels of healthy vs. diseased states or treated vs. untreated samples has become a routine tool in many laboratories in recent years.[28] In a static experiment, the samples are obtained irrespective of time, providing only a "snapshot" of gene expression. However, gene expression is generally not considered a static process resulting in a steady-state level of transcripts, but is highly variable over time depending on cell types, environmental influences, cellular physiology, phenotypes and circadian rhythms and can as such be considered a "dynamic information processing system."[29-31] The aim of this study was the investigation of dynamic and global miRNA expression patterns following activation of miRNA gene transcription in a detailed time-course experiment. In the experimental setup, the transcription factor STAT1 was activated by IFNγ stimulation of melanoma cells in order to identify STAT1-regulated miRNAs over time. We chose the melanoma cell system and the JAK/STAT signaling cascade as these experimental systems are well characterized in our laboratory[32-34] and have previously been established to be suitable for specific activation of subsets of miRNAs.[18,35] For analysis (clustering, visualization and identification of differentially expressed miRNAs) of time-series microarray data sets, we used and compared different biostatistical approaches in order to gain insights into global regulation of the miRNome as well as into the behavior of individual miRNAs after transcriptional activation.

Results

In a detailed time-course, A375 cells were stimulated with IFNγ and, in parallel, pre-treatment with JAK Inhibitor I (JII) was performed. Selected RNA samples were subjected to microarray analysis, followed by identification of differentially expressed miRNAs using different statistical methods. Temporal expression changes of selected miRNAs were verified by RT-qPCR (**Fig. S1**).

Stimulation of A375 cells resulting in subsequent activation of STAT1 was confirmed by western blot analysis of protein lysates (**Fig. 1**). Strong STAT1 phosphorylation was already detectable after 15 min of IFNγ treatment and remained elevated for 12 h while P-STAT1 signals began to gradually wane after 18 h. Functional activity of the TF was assured by analyzing two target genes of STAT1. In line with previous reports in reference 36, STAT1 itself and IRF-1 were both upregulated by STAT1, albeit with different kinetics. Furthermore, different concentrations of the JAK-specific kinase inhibitor JII were tested in order to define a minimum concentration to prevent activation of STAT1 (**Fig. 1B**). Lower concentrations (200 nM and 1 μM) were not sufficient to fully suppress STAT1 activation. Pre-incubation with 5 μM JII was adequate to almost completely inhibit STAT phosphorylation (right) and this concentration was used in the time-course experiments. **Figure 1C** shows STAT1 activation status of samples that were subjected to microarray and/or RT-qPCR analyses.

Analysis of time-series microarrays. In total, 20 microarrays (two samples for every time point including the untreated and the JII pre-treated samples at 72 h) were included in the study. Quality control scatter plots illustrated good reproducibility of the expression data (**Fig. S3A and S3B**). Reproducibility of array experiments was evaluated by calculating coefficients of determinations (r^2) between expression data (**Fig. S4**). Overall, the coefficient of determination between duplicate RNA samples was high (mean r^2 ~ 0.967) and was enhanced even more (mean r^2 ~ 0.989) after filtering out lowly expressed miRNAs. This indicates that most variability between arrays was detected in the very low expression range close to the detection limit. Mean r^2 values for biological replicates were significantly higher than r^2 for non-replicate samples: 0.989 vs. 0.890, respectively (p-value is 9.45×10^{-8}), indicating a good reproducibility for the biological replicate samples considered in the study.

To obtain a global view of the time evolution of the miRNome, all miRNAs were analyzed by GEDI (**Fig. 2**). Each sample exhibits a characteristic color pattern, reflecting the transcriptional behavior over time. Although IFNγ treatment has shifted global miRNA expression patterns already after 30 min, overall expression changes were small (note the color bar: maximum change range 0.7, with all values normalized to the mean of the untreated control). Candidates that were upregulated between 0.5 h and 12 h (upper right corners of self organizing maps (SOMs)-based mosaics) included miRNAs present in the complete data set only, which were removed after threshold filtering for downstream analyses (see below). The majority of robust expression changes were scored after 24 h of IFNγ treatment and after 48 h a clear separation between up and downregulated miRNAs became evident.

After filtering out miRNAs whose maximum $\log_2$ expression values never exceeded 7.0 in all of the 9 different time points, 158 miRNAs remained, which were then analyzed by unsupervised hierarchical clustering (**Fig. 3A**). The filtering threshold was chosen because we had previously established that miRNAs associated with hybridization signal intensities below $\log_2$ of 7.0 using Affymetrix technology were not reliably detectable by RT-PCR (data not shown). The heatmap illustrates that the majority of differentially expressed miRNAs were either upregulated (~50 miRNAs) or downregulated (~80 miRNAs) at around 24 h of IFNγ treatment. Several miRNAs appeared to

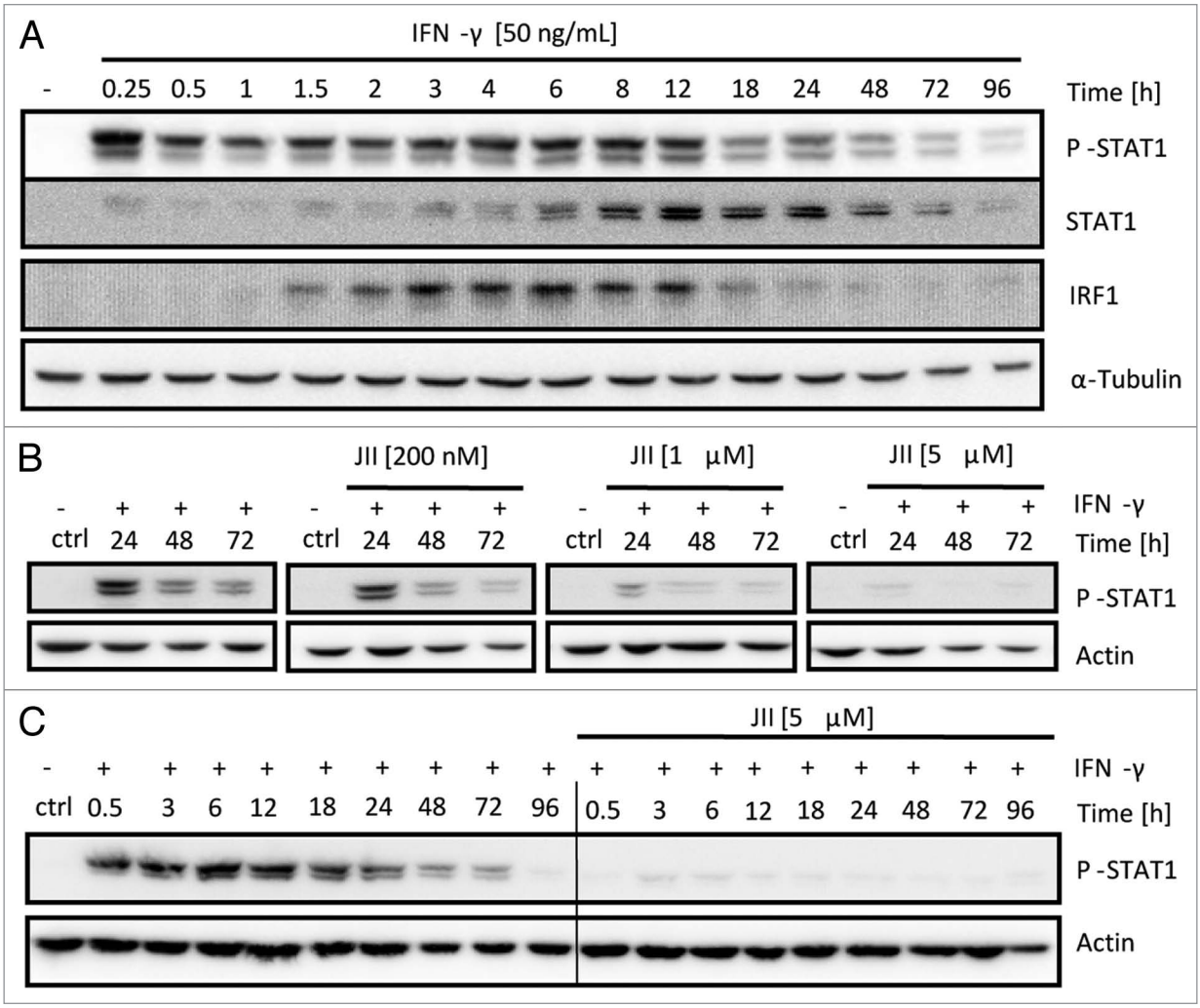


Figure 1. STAT1 activation by IFNγ. (A) A375 cells were treated with human IFNγ (50 ng/mL) up to 96 h. Proteins were detected with antibodies against pSTAT1, STAT1 and IRF1. Tubulin or actin served as loading controls. (B) Titration of JAK-Inhibitor I (JII). A375 cells were pre-treated with different JII concentrations followed by IFNγ (50 ng/mL) to monitor the inhibition of the Janus kinases. (C) All samples selected for microarray and/or RT-qPCR analysis were monitored for STAT1 phosphorylation and the lack thereof following JII treatment.

be upregulated very rapidly after IFNγ stimulation (30 min–6 h) however, these changes were not significant, rather small with maximum fold changes up to 1.5 relative to the untreated control and could also be unspecific fluctuations in expression changes. On the other hand, a clearly delayed upregulation, which only occurred after 48 h was detected for miR-34a, miR-30a and miR-27a (**Fig. 4**).

Next, we subjected the filtered miRNA data set to soft clustering by *Mfuzz* (**Fig. 3B**). The upper part represents time evolution of individual miRNA expressions that were altered upon treatment, and in the lower panel the averaged expression profiles of all miRNAs in the respective cluster are displayed. Cluster 2 and 3 were comprised of miRNAs with high membership values (colored in red) representing very similar expression profiles. Three major expression profiles were found with either very small randomly fluctuating changes (cluster 1) or pronounced up or downregulated miRNAs after 24 h of IFNγ treatment (cluster 2 and 3, respectively). Although the averaged profile in the first cluster had no significant regulation, the sharp increase in between the two untreated control samples was likely due to the slightly higher variation of those duplicate data sets. Similarly, the larger positive and negative peaks for the 6 h time point also reflect higher variability among those duplicate samples.

Taken together, different clustering approaches revealed that during the analyzed time course reproducible dynamic changes in miRNA expression levels occurred. In samples from short periods of IFNγ stimulations (less than 12 h), small expression changes were detected for five individual miRNAs (**Fig. 3A**). However, after 24 h, levels of most of the expressed miRNAs were profoundly altered indicating that IFNγ-induced JAK/STAT signaling takes approximately one day to result in measurable transcriptional expression changes of the miRNome.

Identification of groups of differentially expressed miRNAs over time. In order to elucidate the transcriptional responses to IFNγ-treatment in more detail, three statistical programs were used that were specifically designed to analyze replicated,

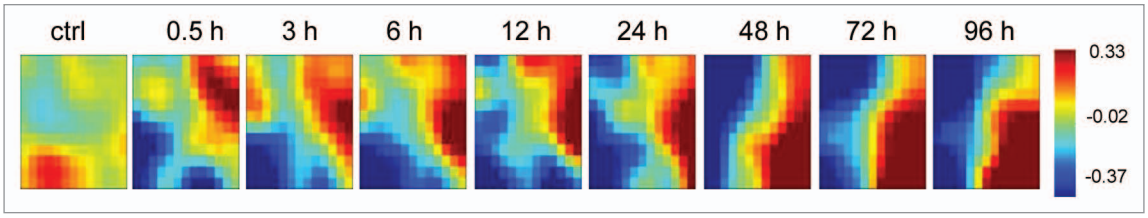


Figure 2. GEDI maps (mosaics) representing progression of expression profiles after IFNγ stimulation of A375 cells for the indicated time periods. Log_2-transformed expression levels were used for analysis of each miRNA relative to the untreated sample (ctrl), which served as a control. On the right, a SOM map color bar indicates log_2 values: highest expression levels (red) and lowest expression levels (blue). GEDI maps are displayed for all 1,105 miRNAs simultaneously for each treatment time point. The expression profiles of 18 arrays (duplicates) corresponding to 9 time points were visualized as 9 GEDI maps, each consisting of a 13-by-12 mosaic, representing 156 mini clusters.

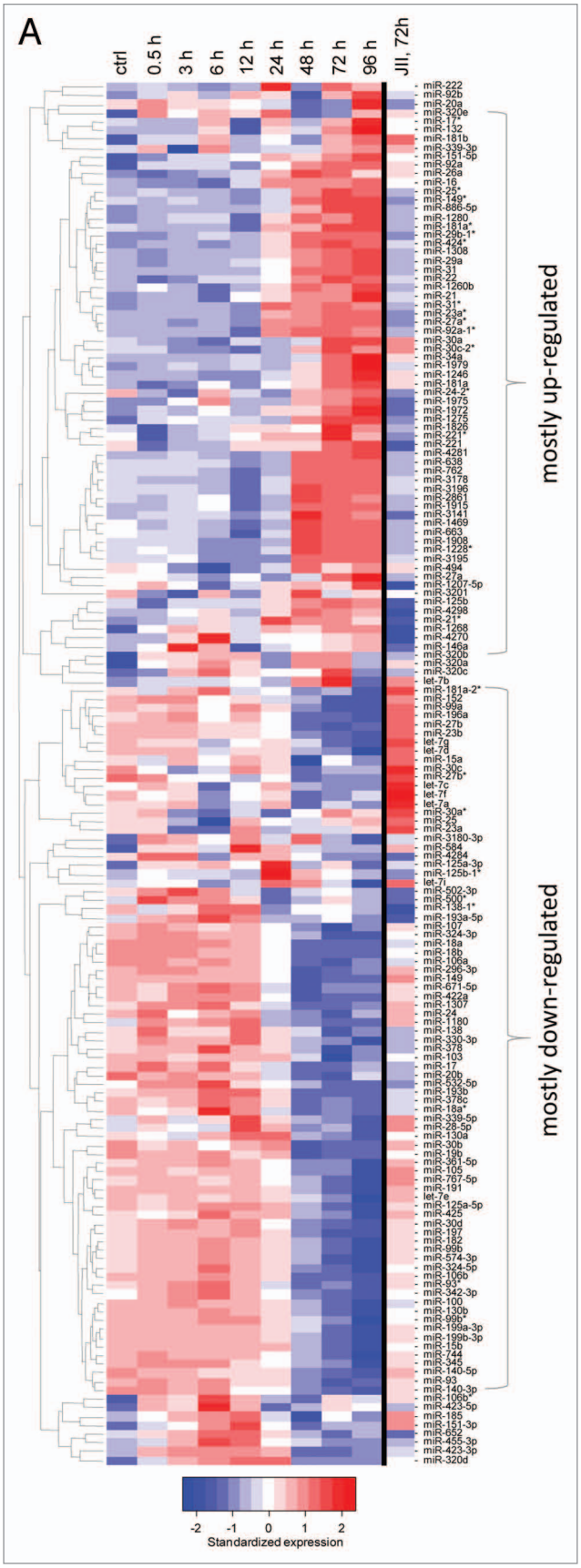
time-series microarray data. The individual programs are described in more detail in Material and Methods and in the Supplements. In the current study, these programs were applied in order to generate a robust set of differentially expressed miRNAs over time, which were jointly identified by the different algorithms. For all calculations only expressed miRNAs (after the filtering step) served as input. The resulting 158 miRNAs (14.3% of all human and mature miRNAs on the chip) were used for further statistical analysis. **Figure 5A** summarizes the results of all three programs in a Venn diagram with a total of 98 miRNAs being differentially expressed. EDGE returned a list of 95 differentially expressed miRNAs (60%), while the *betr* function identified 69 out of 158 miRNAs (~43%) with a probability of 1 to be differentially expressed. The *timecourse* package ranked all 158 miRNAs according to the Hotelling T^2 statistics (see **Sup. Materials and Methods**). By setting the cut-off threshold to a FDR of 0.05, we identified 23 miRNAs (~15% of expressed miRNAs), which had also been detected by the other two programs (listed in **Table S2**). The heatmap (**Fig. 5B**) visualizes expression levels of those 23 commonly identified miRNAs over time.

Bioinformatic analysis revealed distinct dynamic activation patterns for groups of seemingly co-regulated miRNAs. In contrast to a recent study where several miRNAs were found to be strongly upregulated after 30 min of IFNβ treatment,[37] we found several miRNAs reacting slightly to our transcriptional stimulus in the early time periods, albeit none of these expression changes were significant (**Figs. 3A and 4**). The majority of strong miRNA alterations occurred in the intermediate (around 24 h) time ranges. In total, we identified 98 differentially expressed miRNAs, whose levels were either increased or attenuated in response to IFNγ (**Fig. 5A**). The three statistical programs used here commonly identified 23 differentially and significantly regulated miRNAs (all amplitudes of regulations > 2.3-fold). Of those, seven were downregulated at around 24 h while 16 were upregulated following STAT1 activation (**Fig. 5B**). The heatmap visualizes expression changes over time and further illustrates that the 24 h time point appears to be a transitional phase where most regulatory events are beginning to manifest.

Dynamic expression changes of individual miRNAs. In order to visualize time evolution of expression changes, we built smooth regression models (**Fig. 4**) by the logistic function described in **Supplemental Materials and Methods**. Analyzing the models of expression evolution for 158 filtered miRNAs, we identified four distinct dynamic patterns: (1) gradual upregulations, the most frequent type of activation model; (2) abrupt upregulations, where expression was sharply amplified between two time points; (3) delayed upregulations at around 72 h, which are possibly due to secondary effects and (4) gradual downregulations, which might be caused by indirect effects following STAT1 activation that result in suppression of these miRNAs. In addition, we observed very isolated events with an expression peak at one time point followed by a rapid return to baseline levels (miR-146b and miR-367). Parallel duplicate treatments with JII (at 72 h) abrogated JAK/STAT signaling and therefore counteracted IFNγ-specific transcriptional regulation of all selected miRNAs (depicted by crosses, **Fig. 4**). This modeling approach documents the different dynamic courses of individual miRNA regulations, highlighting the importance to consider the parameters "time" and "dynamics of expression" when analyzing miRNA expression patterns.

Validation of individual miRNA expression patterns. For the validation of microarray results by RT-qPCR, 14 miRNAs, some of which had already been described to play a role in cancer, were chosen to represent up, down and non-regulated miRNAs as well as "star"-sequences and genetically clustered miRNAs. The majority of clustered miRNAs share a genome location in a region smaller than 10 kb, together with two or more other miRNAs. **Table S3** lists those miRNAs and the corresponding program(s) by which they were identified, their direction of regulation as well as information derived from miRBase (version 17).[38] **Figure 6A** illustrates that microarray-measured expression levels over time were confirmed for both strands of the selected miRNAs and irrespective of type, direction or time of regulation. In qPCRs, JII-treated control samples were analyzed for all time points (hatched bars), clearly demonstrating that regulations of miRNAs were averted when JAK signaling was interrupted, indicating that observed modulations of miRNA levels were indeed caused by cytokine-induced activation of the JAK/STAT pathway. Individual miRNAs differed considerably with regard to their temporal induction patterns. For example miR-21, a known target of STAT3,[16] was upregulated 48 h after IFNγ stimulation and expression remained high up to 96 h. The same expression pattern, however, with a stronger upregulation (fold change of 8.7 in microarray analysis and up to 4 fold change in RT-qPCR) was found for miR-424* while miR-149 was downregulated after 24 h in response to IFNγ. MiR-25 remained unaffected by IFNγ

Figure 3. (A) Expression evolution of 158 miRNAs presented as a heat-map. Expression values were standardized (transformed to z-scores) and miRNAs were re-ordered by unsupervised hierarchical clustering using Euclidean distance. Colors for each miRNA represent z-scores and derive from "inter-miRNA-standardisations" with red corresponding to high standardised expression levels and blue representing low expression levels.



stimulation (**Table S1**) and therefore served as a non-regulated control miRNA in qPCR validations (**Fig. 6A**).

To examine whether clustered miRNAs are co-regulated, we compared profiles of members of several genetic clusters. For example, the miR-23 and miR-27 families are coordinately regulated with miR-23a~27a of the first cluster being upregulated while members of the second cluster (miR-23b- and -27b) were downregulated (**Fig. 6B**). Other clusters like miR-29a~29b1 and miR-29b2~29c also showed a very obvious co-regulation (data not shown), which was to be expected given that clustered miRNAs are generally (but not always) transcribed together. Adversely, some genetically clustered miRNAs like miR-92a~18a and miR-93~25 appeared to be regulated independently showing completely divergent expression patterns over time (data not shown).

Apart from the different dynamic regulation patterns, biostatistical analysis revealed another interesting and rather unexpected finding: passenger strands (so called "star" sequences, labeled with "*") were often and specifically regulated. Surprisingly, four "star" sequences (miR-424*, miR-29b-1*, miR-27a* and miR-23a*) were among the top 10 miRNAs displaying maximum fold changes > 4 in qPCR analyses and even higher values in microarray results. Examples of "star" strand regulations are given in **Figure 6B** with miR-23a* and miR-27a* being clearly increased between 12–24 h of IFNγ stimulation, while expression levels of the respective guide strands were largely unaffected. In contrast, both strands of miR-27b and miR-23a were similarly downregulated over time.

In summary, detailed data analyses of this miRNA time course revealed several new findings: (1) in the melanoma cell model used here, 14.3% (158 of 1105 human and mature miRNAs) were detected to be expressed and of those 62% (98 of 158) were differentially expressed following IFNγ stimulation, which activated the STAT1 TF; (2) 23 differentially expressed miRNAs were commonly identified by three different statistical programs, which included gradual as well as abrupt up and downregulation events over time. (3) Most regulatory events occurred around 24 h after IFNγ stimulation with only three miRNAs showing a delayed reaction at 72 h. (4) Inhibition of JAK/STAT signaling (by JII treatment) abrogated most responses implying a specific JAK-dependent and likely STAT-mediated transcriptional induction. (5) Most genetically clustered miRNAs were transcriptionally co-regulated. (6) Finally, several "star" strands were dynamically and strongly regulated while their guide strands remained unaffected.

Discussion

MicroRNAs are known to influence fundamental cellular activities in health and disease through their ability to post-transcriptionally

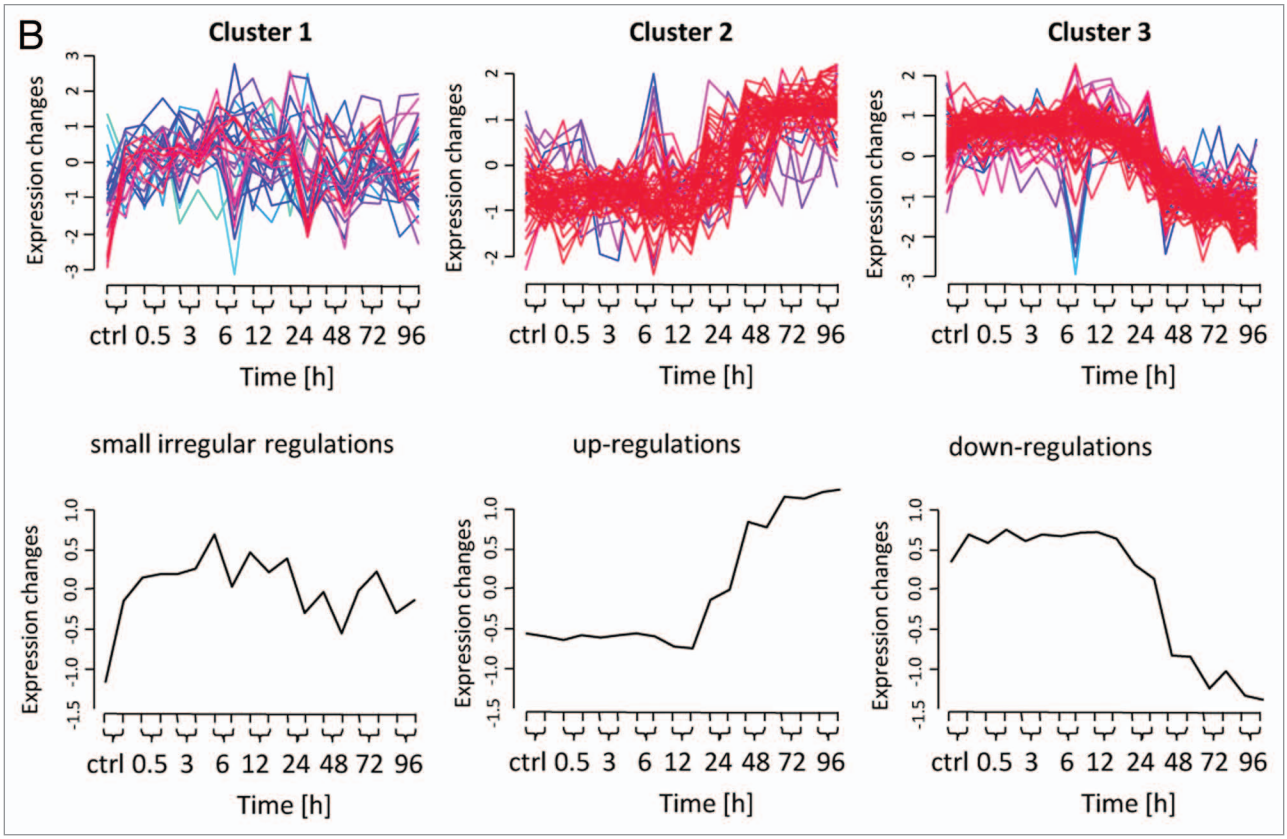


Figure 3. (B) Soft clusters of miRNA expression data using *Mfuzz*. Temporal expression patterns obtained during the time-course experiment of A375 cells treated with IFNγ for the indicated time points. Overall expression patterns (upper part) and average expression patterns (lower part) were displayed. Profiles of three major clusters were found: small irregular regulations (cluster 1), up and downregulation of miRNAs over time (cluster 2 and 3, respectively). Light and dark blue colored lines correspond to miRNAs with low membership; red colored lines represent miRNAs with high membership values. On the x-axis arrays for the replicated time points were placed next to each other, the y-axis represents standardised expression values.

regulate gene expression of most genes. However, not much is known about the transcriptional activation and regulation of miRNA genes themselves. Transcription is a dynamic and well controlled process allowing the cell to respond flexibly to environmental stimuli and internal signals. Transcription factors (TFs), among other gene transcriptional regulators, mediate such responses in order to quickly adapt to changes by influencing the expression of relevant genes including those encoding miRNAs. In order to better understand the principles of these complex interactions and to investigate the dynamics of TF-mediated miRNA regulation, we chose the JAK/STAT pathway as a model system in A375 malignant melanoma cells, both well-established experimental systems in our laboratory, which furthermore, can be controlled at several steps.[32,34,39] Cytokine signaling in general is of major importance for the mammalian immune system as it mediates cellular response to infections, inflammatory processes and transformation of normal into malignant cells.[40] Almost all cell types can come into contact with surrounding cytokines, e.g., interferonγ, which is mainly secreted by activated T and NK cells.[41] Interferons predominantly signal through the JAK/STAT pathway culminating in STAT-mediated transcriptional activation of target genes. Interestingly, IFNγ-mediated STAT1 activation has previously been shown to induce growth inhibition of melanoma cells.[42] Here, we asked if and which miRNA genes could be specifically targeted by IFNγ-induced STAT activation and which dynamic patterns these potential activation processes would follow.

Thus far, only a small number of studies have addressed interactions between miRNAs and STAT factors and even fewer have investigated dynamic issues of miRNA regulation. Very recently, IFNα-induced upregulation of miR-21 has been demonstrated to be an early event (after 2 h of IFNα treatment), which was directly co-mediated by STAT3 and NFκB.[27] Löffler and colleagues investigated the role of STAT3 in the regulation of miR-21 by quantifying primary and mature miR-21 by RT-qPCRs. They showed that the induction of miR-21 was controlled by IL-6 and required STAT3 in myeloma cells. However, in contrast to the rapid induction of the primary miR-21 already after 1 h, the mature miR-21 levels increased slowly (up to 5 d),[16] which is in line with our findings of miR-21 remaining upregulated for up to 96 h. At the same time, Pedersen et al. analyzed kinetic induction of miRNAs following IFNβ stimulation. They performed a short time-series experiment with 5 time points after stimulation of a human hepatoma cell line and primary hepatocytes for 0.5 h, 2 h, 8 h, 24 h and 48 h. IFNβ rapidly and strongly modulated the expression of few miRNAs (among them miR-1 and miR-196)

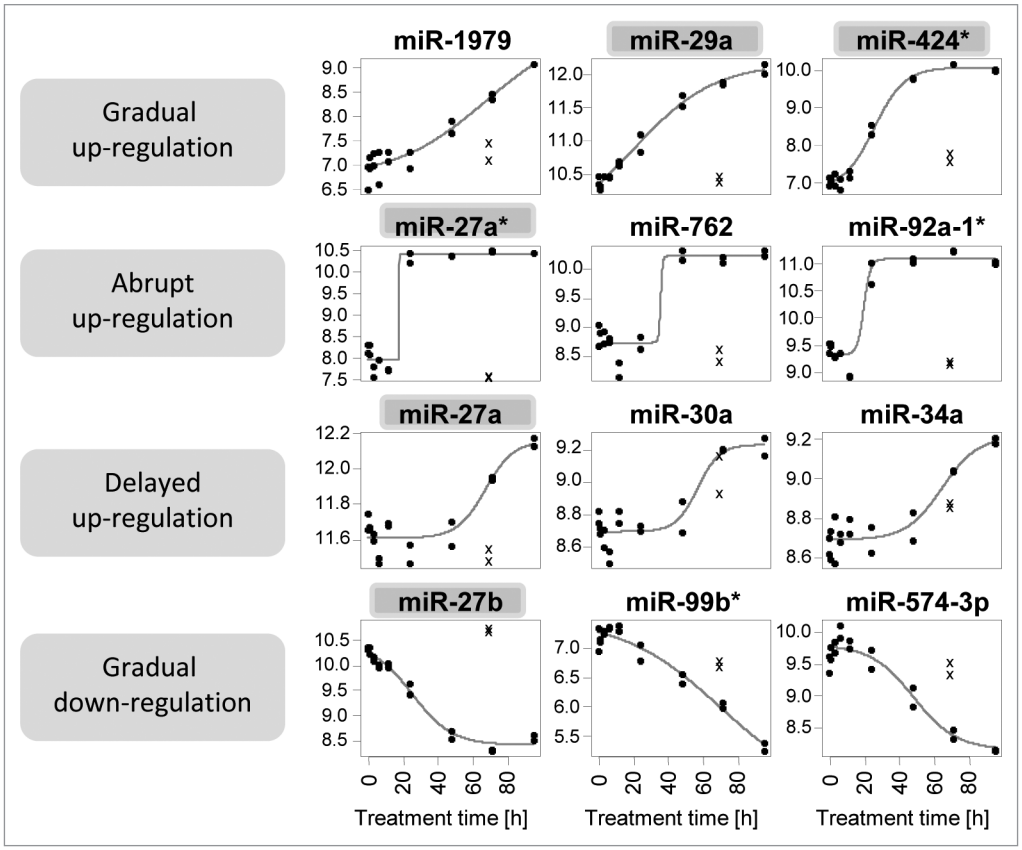


Figure 4. Time evolution of $\log_2$ expression values for selected miRNAs exemplifying the four different dynamic expression patterns. Individual microarray-based expression results are illustrated by black dots in a natural timescale (hours). A smooth model was built using regression with a parameterised logistic function as detailed in **Supplemental Materials and Methods.** Crosses represent JII-control experiments at 72 h. Expression profiles of miRNAs highlighted with gray boxes have been validated by RT-qPCR.

within 30 min.[37] In the melanoma cell line used here, we did not observe any statistically significant regulations during early time periods (30 min–6 h). Expression level alterations for those few "early responders" were weak and not sufficiently reproducible between duplicate samples. Therefore, in our experimental setup, earliest and robust expression changes started to emerge well after 12 h.

Another interesting interaction between a miRNA (miR-155) and STAT factors (STAT1) in form of a positive feedback loop has recently been described by Kutty et al.[19] who demonstrated that miR-155 expression levels in human retinal pigment epithelial (HRPE) cells gradually increased over a period of 24 h after exposure to inflammatory signals (cytokine mixture TNFα, IL-1β and IFNγ). Recently, Wang and colleagues identified 83 active promoter regions of miRNA genes in IFNγ-treated HeLa cells, 41 of which contained STAT1 binding sites.[43] In their in silico study, they used a bioinformatic approach to analyze high-throughput functional genomic data. ChIP-Seq experiments provided TF as well as RNA Pol II binding sites, which were used to construct potential feedback loops containing regulatory cascades of TFs and miRNAs. They found that 51.4% of STAT1-targeted miRNAs contained AP-1 binding sites suggesting that such interactions could also be involved in the regulation of miRNA transcription. In this context, TFs other than STAT1, but also activated by IFNγ (e.g., via the MAPK pathway) could participate in the direct or indirect regulatory events we have observed here. Repressors of miRNA transcription, which are regulated by STAT1 or other IFNγ-regulated TFs could explain the delayed responses and downregulation events we have scored and might indicate that most of the late regulatory events are rather indirect effects of STAT1 activation (**Figs. 5 and 6**). Even though we and others have previously shown that JII specifically inhibits JAK/STAT signaling in melanoma cells and human retinal pigment epithelial cells,[19,34,44] it cannot be entirely ruled out that JAK-independent factors also play a role in IFNγ-induced miRNA transcription.

To investigate the presence of potential STAT binding sites, we performed an overlay of a publicly available ChIP-Seq data set of IFNγ-stimulated HeLa cells[45] with Jaspar-generated profiles of promoter regions of the 23 commonly identified miRNAs. All 23 miRNAs turned out to have 2 to 14 STAT binding sites within 50 kb upstream of their genomic location, while 15 miRNAs had 1–3 STAT binding sites within 10 kb (data not shown). Taken together, the presence of STAT binding sites in their promoter regions, the dynamic regulation of these miRNAs as well as the abrogation of their transcriptional activation by JII are highly suggestive of a direct regulation of a subset of miRNAs by STAT TFs. The importance of these complex, but so far scarcely

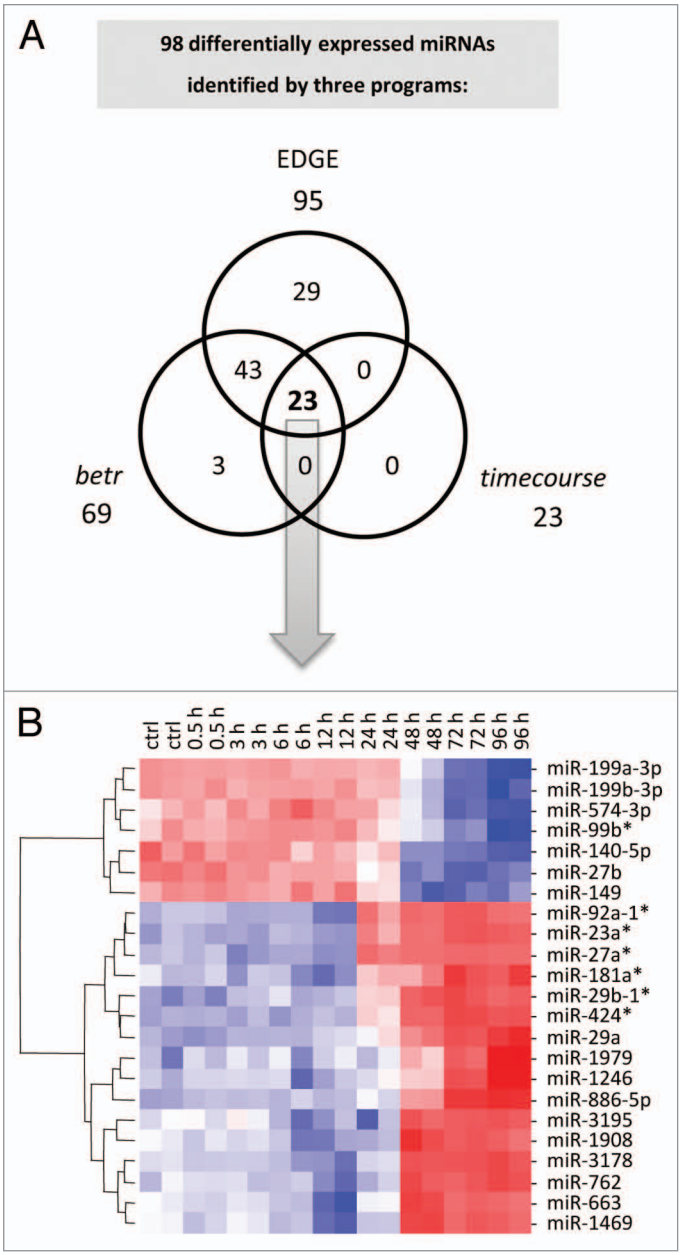


Figure 5. (A) Venn diagram displaying the overlay of results from three biostatistical programs. MiRNAs from each program are listed in **Table S2**. (B) Heatmap displaying the expression of 23 miRNAs identified by all three statistical tools.

described interactions between STAT factors and miRNAs has been highlighted in a recent review in reference 18.

It is generally assumed that genetically clustered miRNAs are coordinately transcribed.[7] In this context, the TF c-Myc has been shown to directly regulate the miR-17 cluster, which comprises a region encoding six miRNAs.[15] Here, we have found genetically clustered miRNAs to be regulated together as well as independently of one another. For instance, miR-23b and miR-27b were both downregulated during the time-course experiment while miR-24-1, the third member of this cluster (**Fig. 6B; Table S3**), was not detected in A375 cells. Recently, miRNA clusters have been classified into two types: homo-clusters (composed of miRNAs from a single family) and hetero-clusters (miRNAs from multiple families).[46] Usually members of the same miRNA family target a similar set of mRNAs because they share the same seed region,[47] which predominantly determines the targets of miRNAs.[48] Accordingly, we have identified a co-regulated homo-cluster (miR-29a~29b1*, data not shown) and two hetero-clusters (miR-23a~27a and miR-23b~27b), which had reverse expression patterns: miR-23a~27a was upregulated, while miR-23b~27b was downregulated (**Figs. 5 and 6**) suggesting overlapping regulatory roles of these important miRNA clusters, which are often augmented in multiple types of cancers.[49]

Surprisingly, almost 10% of the 98 differentially expressed miRNAs were passenger strands (*). The co-accumulation of both "sister" strands of miRNA pairs does not necessarily imply that both are functional, however, functional roles of miRs* are being increasingly recognized and described. Tsang and Kwok showed that miR-18* may function as a potential tumor suppressive miRNA by targeting K-Ras.[50] In contrast to let-7, which targets both K-Ras and H-Ras, miR-18* only targets K-Ras mRNA. This specificity might be important for targeting the specific isoforms of Ras and therefore it was suggested that miR-18* could serve as therapeutic agent in future cancer therapy. Recently, it was demonstrated that well-conserved vertebrate miR* species such as miR-19* have an impact on regulatory networks and should be taken into account when studying functional roles of miRNAs and their contribution to disease.[51] In this context, "*"-designations have been removed in the latest version of miRBase (v18) and only 5'- and 3'-designations are used to describe the two opposite miRNA strands indicating equally important roles the two strands may have. Remarkably, among the 10 most upregulated miRNAs we noted four passenger strands (miR-424*, miR-29b1*, miR-27a* and miR-23a*). A possible tissue-specificity of passenger vs. guide strand sequences as well as the regulatory function of the miRs*-sequences will have to be investigated further in order to establish differential, similar or possibly synergistic roles of both miRNA strands.

Another aspect of this study was the grouping of miRNAs according to their temporal expression patterns. We applied several unsupervised learning techniques (GEDI, *Mfuzz*, heatmaps) and a modeling approach to reveal hidden structures in large expression data sets. Using only miRNAs which were detectable in our melanoma cells (filtered data set), most robust expression changes (either gradual or abruptly between two time points) became evident at around 24 h after stimulation with IFNγ. Later time intervals (48 h and later) produced a distinct separation in either up or downregulated miRNAs with only three examples showing a delayed activation type (72 h). Early expression changes (30 min–6 h) detectable in our melanoma cell line were few and negligible. Whether some of these rather small increases in miRNA levels may still have functional consequences for their respective target genes and as such for the cellular responses to IFN-induced signaling, remains to be investigated. In this context, Perry and colleagues[52] have shown that two miRNAs (miR-146a and miR-146b) were induced 3 h after IL-1β treatment in A549 human alveolar lung epithelial

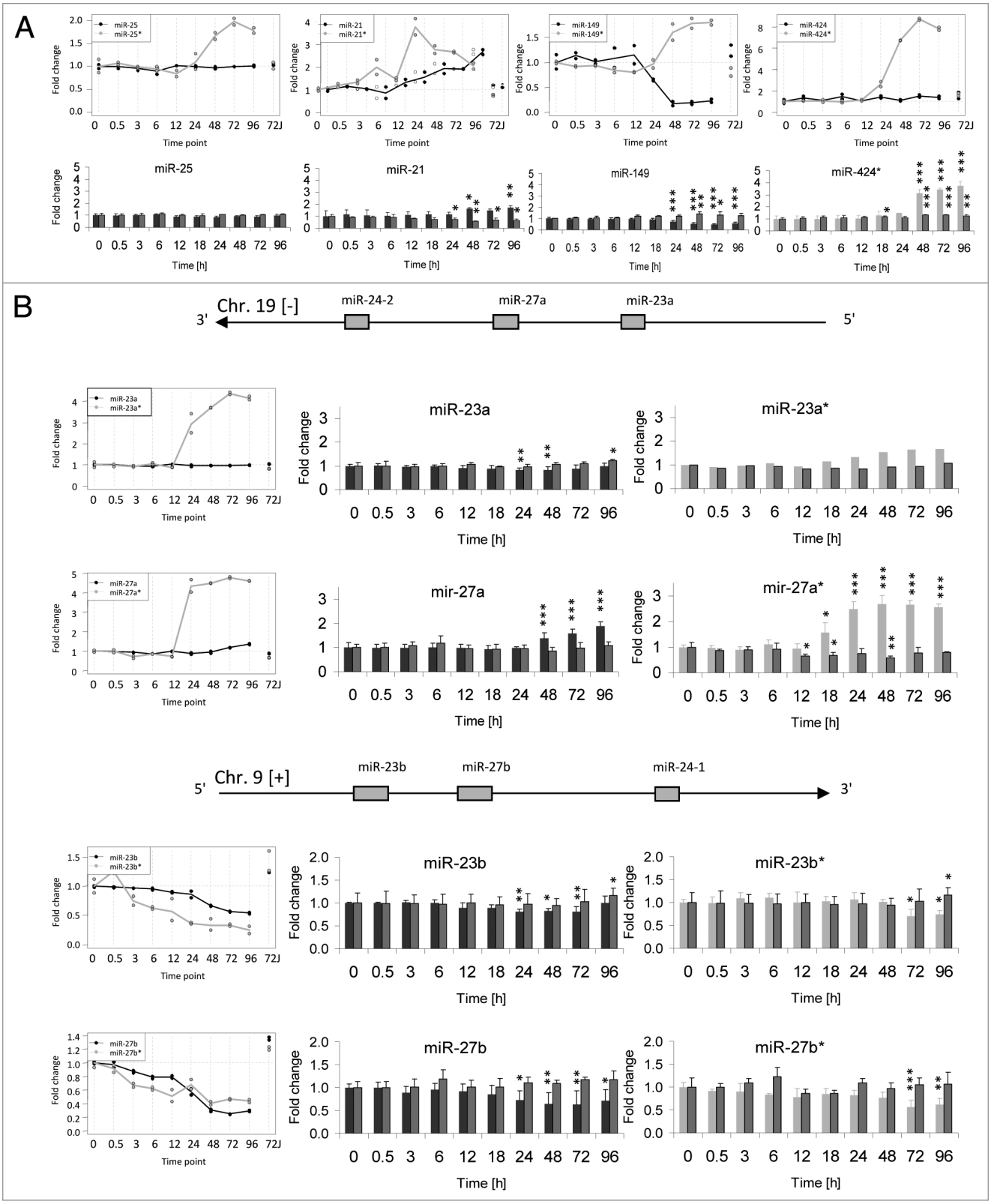


Figure 6. For figure legend, see page 987.

Figure 6. (See opposite page) Quantitative RT-PCR validation of selected miRNAs. Fold changes were calculated for each miRNA relative to the untreated control. Results are depicted as mean of biological triplicates plus standard deviation. Grey bars and lines illustrate "*" sequences while black bars and lines show guide strands. (A) Microarray-measured expression levels of miRNAs exemplifying no regulation over time (miR-25), upregulation of "star" strands only (miR-424*), of both strands (miR-21) and gradual downregulation (miR-149) are shown in the top graphs. Duplicate microarrays are plotted by two circles for each time point. Expression values for the JII-control measurements at 72 h (72J) are shown on the far right of the graphs. In the graphs below, qPCR validations are depicted with hatched bars showing JII-treated controls. (B) Co-regulations of genetically clustered miRNAs. All RT-qPCR expression levels were normalized to reference genes RNU1A, RNU5A and Scarna17. Statistical significance was determined by one-way ANOVA followed by a Dunnett's Multi Comparison test with ***p < 0.001, **p: 0.001–0.01, *p: 0.01–0.05.

cells with their expression levels remaining elevated until the end of the time-course at 24 h. In contrast, we found miR-146b to have a strong expression peak at 24 h but then it immediately returned to baseline levels (data not shown). Although the regulation of miRNA stability may play an important role in the control of gene expression, very little is known about the decay and the half-lives of individual miRNAs. Further, it remains to be shown whether distinct dynamic miRNA expression behaviors are specific to certain cell types and transcriptional stimuli.

The current study aimed at investigating dynamic expression changes of miRNAs following transcriptional activation. Detailed microarray time-course experiments were performed to globally analyze miRNA expression patterns. A computational analysis pipeline consisting of several tools for efficient biostatistical investigation of dynamic data sets has been generated. Examination of the microarray-based results revealed distinct temporal groups of miRNA activation with most expression changes taking place at around 24 h. Individual miRNAs were found to follow highly diverse dynamic expression profiles emphasizing the importance of the parameter "time" when studying miRNAs. Furthermore, we observed that several miRNA passenger strands (miR*) were also specifically regulated and that genetically clustered miRNAs were often but not always co-regulated. Future elucidation of transcriptional regulation of miRNAs over time following diverse cellular or environmental stimuli will be necessary to obtain a more complete systems-biological view of complex regulatory networks in cells.

Materials and Methods

Cell culture. The human malignant melanoma cell line, A375 (ATCC, CRL-1619™), was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FCS, PAA Laboratories), 50 μg/mL penicillin, 100 μg/mL streptomycin and 0.5 mmol/L L-glutamine (all from Lonza) at 37°C in a humidified atmosphere with 5% CO_2. Cells were tested routinely to be *Mycoplasma* negative. For all experiments described below, 50,000 cells/well were seeded in 6-well plates.

Time-course experiments. To investigate a possible dynamic regulation of miRNAs, time-series experiments were performed as summarized in **Figure S1**. During the course of the experiment, biological triplicates of A375 cells were stimulated with human interferon gamma (IFNγ, PeproTech Inc., final concentration of 50 ng/mL) for the indicated periods of time or were left untreated (ctrl). In parallel, a second time-course experiment was performed including a pre-treatment step with 5 μM of Janus kinase inhibitor I (JII; Calbiochem), added one hour before commencing IFNγ stimulation. Cells were collected all together at the end of the course for further experimental analyses. Prior to this, optimal concentrations of JII, a potent JAK inhibitor, which prevents activation/phosphorylation of STAT1, were determined (**Fig. 1**).

RNA extraction and quality control. Two methods for RNA extraction were used according to the manufacturer's instructions for different downstream applications: (1) miRNeasy Mini kit (Qiagen) with an additional on-column DNase I digestion for microarrays and (2) TRIsure (Bioline) for downstream RT-qPCR validations. All RNA extractions were performed on three biological replicates each consisting of three technical replicates for each of the stimulation time points. RNA quality and purity were assessed by a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and with an Agilent 2100 Bioanalyser (Agilent Technologies).

Real-time qPCR. A total of 14 miRNAs were selected for real-time quantitative PCR (RT-qPCR, Bio-Rad CFX96 system, Bio-Rad Laboratories) in order to validate the microarray results. Reverse transcription (RT) of triplicate RNAs from IFNγ and JII-treated cells was performed for all time points using the miScript Reverse Transcription kit and miScript Primer assays (both from Qiagen) as described in reference 32. For miRNA expression analysis, three endogenous reference controls were included: RNU1A, RNU5A and SCARNA17, which are small non-coding RNA species expressed in the A375 cell line and which had previously been tested not to be differentially regulated by IFNγ stimulation or JII pre-treatment (data not shown). Based on the geometric mean of the three reference genes, a normalization factor was calculated for each sample using geNorm, a VBA applet for Microsoft Excel.[53] The relative amount of each miRNA in each sample was then corrected by dividing its amount with the corresponding normalization factor. Finally, the fold change of each miRNA for each time point after IFNγ stimulation with or without JII pre-treatment, was calculated by dividing its normalized relative amount by the normalized relative amount of the untreated sample that served as control. All RT-qPCRs were performed in technical triplicates on each of the biological replicates.

Western blot. Western blots were performed as described before in reference 34, to detect phosphorylation of STAT1 and upregulation of STAT1 target genes using the following primary antibodies: anti-(pY701)-STAT1 (dilution 1:1,000, BD Transduction Laboratories), anti-STAT1 (dilution 1:1,000, BD Transduction Laboratories), anti-IRF1 (dilution 1:1,000, Santa Cruz), anti-α-tubulin (dilution 1:4,000, Santa Cruz) and anti-actin (dilution 1:4,000, Millipore).

miRNA microarray expression profiling. The global expression patterns of miRNAs after IFNγ stimulation were

analyzed using the Affymetrix GeneChip miRNA 2.0 Arrays and the FlashTag Biotin HSR RNA labeling kit (Genisphere, USA) according to the manufacturer's instructions. The Affymetrix chip was designed based on the miRBase version 15 to target 1,105 human precursor and mature RNAs as well as 32 scaRNAs and 2302 snoRNAs (Affymetrix Datasheet P/N EXP00180). Duplicate RNAs of samples were analyzed, which were selected to represent different periods of IFNγ stimulations and the 72 h time point samples that were pre-treated with JII. Microarray data are available at ArrayExpress (ebi.ac.uk/arrayexpress) under accession number E-MEXP-3544.

Analysis of time-series-derived data. The workflow of biostatistical analyses is outlined in **Figure S2**.

(A) Data pre-processing: Microarray data were pre-processed, quality was controlled and a filtering step was performed. Preprocessing of microarray data was performed with Partek Genomics Suite (version 6.5) using the robust multi-chip analysis (RMA) algorithm, which performs background adjustment, quantile normalization and probe summarization as described before in reference 54. Since low expression estimates are unreliable, a filtering step was included to remove all miRNAs, for which maximum expression over all arrays did not reach a threshold of 7.0 in a $\log_2$ scale. We have previously established that microarray-measured $\log_2$ values < 7.0 are generally not detectable by RT-qPCR (data not shown).

(B) Visualization and clustering: Microarray data were visualized before and after the filtering step. Gene Expression Dynamics Inspector (GEDI) analysis was performed to obtain an overall picture of all miRNAs before filtering[55] whereas the *Mfuzz* package[56] was applied to cluster and visualize dynamic expression patterns of the remaining miRNAs after filtering. Hierarchical cluster analysis and expression visualization in form of heatmaps were performed using the *heatmap.2* function of the R package *gplots*.

(C) To identify differentially expressed miRNAs statistical analyses were performed using three different approaches. The Extraction of Differential Gene Expression (EDGE) measures statistical significance of genome-wide studies based on the concept of false discovery rate (FDR).[57] The Bayesian Estimator of Temporal Regulation (BETR) algorithm[58] calculates the probability of differential expression for each miRNA in the microarray time-course data set. Finally, the *timecourse* package[59] uses a multivariate empirical Bayes (MB) approach to rank differentially expressed miRNAs according to the Hotelling T^2 statistics. Additional and more detailed information on bioinformatics and statistical analyses is provided in the **Supplemental Materials and Methods**.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

We would like to thank Nathalie Nicot for expert generation of high quality microarray data and Dr Christiane Margue for critically reading the manuscript.

Funding

This work was supported by project grants from the University of Luxembourg (F1R-LSC-PUL-09MIRN) and the Fondation Cancer (Luxembourg).

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/rnabiology/article/20494/

References

1. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 2004; 23:4051-60; PMID:15372072; http://dx.doi.org/10.1038/sj.emboj.7600385.
2. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010; 11:597-610; PMID:20661255.
3. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 2009; 11:228-34; PMID:19255566; http://dx.doi.org/10.1038/ncb0309-228.
4. Altuvia Y, Landgraf P, Lithwick G, Elefant N, Pfeffer S, Aravin A, et al. Clustering and conservation patterns of human microRNAs. Nucleic Acids Res 2005; 33:2697-706; PMID:15891114; http://dx.doi.org/10.1093/nar/gki567.
5. He C, Li Z, Chen P, Huang H, Hurst LD, Chen J. Young intragenic miRNAs are less coexpressed with host genes than old ones: implications of miRNA-host gene coevolution. Nucleic Acids Res 2012; PMID:22238379; http://dx.doi.org/10.1093/nar/gkr1312.
6. Guo L, Lu Z. Global expression analysis of miRNA gene cluster and family based on isomiRs from deep sequencing data. Comput Biol Chem 2010; 34:165-71; PMID:20619743; http://dx.doi.org/10.1016/j.compbiolchem.2010.06.001.
7. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009; 10:126-39; PMID:19165215; http://dx.doi.org/10.1038/nrm2632.
8. Ozsolak F, Poling LL, Wang Z, Liu H, Liu XS, Roeder RG, et al. Chromatin structure analyses identify miRNA promoters. Genes Dev 2008; 22:3172-83; PMID:19056895; http://dx.doi.org/10.1101/gad.1706508.
9. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 2005; 11:241-7; PMID:15701730; http://dx.doi.org/10.1261/rna.7240905.
10. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008; 455:64-71; PMID:18668037; http://dx.doi.org/10.1038/nature07242.
11. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet 2004; 5:396-400; PMID:15143321; http://dx.doi.org/10.1038/nrg1328.
12. Wang J, Lu M, Qiu C, Cui Q. TransmiR: a transcription factor-microRNA regulation database. Nucleic Acids Res 2010; 38:119-22; PMID:19786497; http://dx.doi.org/10.1093/nar/gkp803.
13. Dews M, Homayouni A, Yu D, Murphy D, Sevignani C, Wentzel E, et al. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat Genet 2006; 38:1060-5; PMID:16878133; http://dx.doi.org/10.1038/ng1855.
14. Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, et al. A microRNA signature of hypoxia. Mol Cell Biol 2007; 27:1859-67; PMID:17194750; http://dx.doi.org/10.1128/MCB.01395-06.
15. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005; 435:839-43; PMID:15944709; http://dx.doi.org/10.1038/nature03677.
16. Löffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermüller J, Kretzschmar AK, et al. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. Blood 2007; 110:1330-3; PMID:17496199; http://dx.doi.org/10.1182/blood-2007-03-081133.
17. Meng F, Henson R, Wehbe-Janek H, Smith H, Ueno Y, Patel T. The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes. J Biol Chem 2007; 282:8256-64; PMID:17220301; http://dx.doi.org/10.1074/jbc.M607712200.
18. Kohanbash G, Okada H. MicroRNAs and STAT interplay. Semin Cancer Biol 2012; 22:70-5; PMID:22210182; http://dx.doi.org/10.1016/j.semcancer.2011.12.010.

19. Kutty RK, Nagineni CN, Samuel W, Vijayasarathy C, Hooks JJ, Redmond TM. Inflammatory cytokines regulate microRNA-155 expression in human retinal pigment epithelial cells by activating JAK/STAT pathway. Biochem Biophys Res Commun 2010; 402:390-5; PMID:20950585; http://dx.doi.org/10.1016/j.bbrc.2010.10.042.
20. Brock M, Trenkmann M, Gay RE, Michel BA, Gay S, Fischler M, et al. Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. Circ Res 2009; 104:1184-91; PMID:19390056; http://dx.doi.org/10.1161/CIRCRESAHA.109.197491.
21. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. Mol Cell 2010; 39:493-506; PMID:20797623; http://dx.doi.org/10.1016/j.molcel.2010.07.023.
22. Haghikia A, Missol-Kolka E, Tsikas D, Venturini L, Brundiers S, Castoldi M, et al. Signal transducer and activator of transcription 3-mediated regulation of miR-199a-5p links cardiomyocyte and endothelial cell function in the heart: a key role for ubiquitin-conjugating enzymes. Eur Heart J 2011; 32:1287-97; PMID:20965886; http://dx.doi.org/10.1093/eurheartj/ehq369.
23. Brivanlou AH, Darnell JE Jr. Signal transduction and the control of gene expression. Science 2002; 295:813-8; PMID:11823631; http://dx.doi.org/10.1126/science.1066355.
24. Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002; 3:651-62; PMID:12209125; http://dx.doi.org/10.1038/nrm909.
25. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 2005; 5:375-86; PMID:15864272; http://dx.doi.org/10.1038/nri1604.
26. Lesinski GB, Anghelina M, Zimmerer J, Bakalakos T, Badgwell B, Parihar R, et al. The antitumor effects of IFNalpha are abrogated in a STAT1-deficient mouse. J Clin Invest 2003; 112:170-80; PMID:12865406.
27. Yang CH, Yue J, Fan M, Pfeffer LM. IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis. Cancer Res 2010; 70:8108-16; PMID:20813833; http://dx.doi.org/10.1158/0008-5472.CAN-10-2579.
28. Wang X, Wu M, Li Z, Chan C. Short time-series microarray analysis: methods and challenges. BMC Syst Biol 2008; 2:58; PMID:18605994; http://dx.doi.org/10.1186/1752-0509-2-58.
29. Turner M. Is transcription the dominant force during dynamic changes in gene expression? Adv Exp Med Biol 2011; 780:1-13; PMID:21842360; http://dx.doi.org/10.1007/978-1-4419-5632-3_1.
30. Lowrey PL, Takahashi JS. Genetics of circadian rhythms in Mammalian model organisms. Adv Genet 2011; 74:175-230; PMID:21924978; http://dx.doi.org/10.1016/B978-0-12-387690-4.00006-4.
31. Dougherty ER, Shmulevich I, Bittner ML. Genomic signal processing: the salient issues. EURASIP J Appl Signal Process 2004; 2004:146-53.
32. Philippidou D, Schmitt M, Moser D, Margue C, Nazarov PV, Muller A, et al. Signatures of microRNAs and selected microRNA target genes in human melanoma. Cancer Res 2010; 70:4163-73; PMID:20442294; http://dx.doi.org/10.1158/0008-5472.CAN-09-4512.
33. Haan C, Kreis S, Margue C, Behrmann I. Jaks and cytokine receptors—an intimate relationship. Biochem Pharmacol 2006; 72:1538-46; PMID:16750817; http://dx.doi.org/10.1016/j.bcp.2006.04.013.
34. Kreis S, Munz GA, Haan S, Heinrich PC, Behrmann I. Cell density dependent increase of constitutive signal transducers and activators of transcription 3 activity in melanoma cells is mediated by Janus kinases. Mol Cancer Res 2007; 5:1331-41; PMID:18171991; http://dx.doi.org/10.1158/1541-7786.MCR-07-0317.
35. Mueller DW, Bosserhoff AK. The evolving concept of 'melano-miRs'-microRNAs in melanomagenesis. Pigment Cell Melanoma Res 2010; 23:620-6; PMID:20557479; http://dx.doi.org/10.1111/j.1755-148X.2010.00734.x.
36. Morris AC, Beresford GW, Mooney MR, Boss JM. Kinetics of a gamma interferon response: expression and assembly of CIITA promoter IV and inhibition by methylation. Mol Cell Biol 2002; 22:4781-91; PMID:12052885; http://dx.doi.org/10.1128/MCB.22.13.4781-91.2002.
37. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, et al. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature 2007; 449:919-22; PMID:17943132; http://dx.doi.org/10.1038/nature06205.
38. Griffiths-Jones S. The microRNA Registry. Nucleic Acids Res 2004; 32:109-11; PMID:14681370; http://dx.doi.org/10.1093/nar/gkh023.
39. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 2003; 374:1-20; PMID:12773095; http://dx.doi.org/10.1042/BJ20030407.
40. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. Immunity 2008; 28:477-87; PMID:18400190; http://dx.doi.org/10.1016/j.immuni.2008.03.002.
41. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferongamma: an overview of signals, mechanisms and functions. J Leukoc Biol 2004; 75:163-89; PMID:14525967; http://dx.doi.org/10.1189/jlb.0603252.
42. Kortylewski M, Komyod W, Kauffmann ME, Bosserhoff A, Heinrich PC, Behrmann I. Interferongamma-mediated growth regulation of melanoma cells: involvement of STAT1-dependent and STAT1-independent signals. J Invest Dermatol 2004; 122:414-22; PMID:15009724; http://dx.doi.org/10.1046/j.0022-202X.2004.22237.x.
43. Wang G, Wang Y, Teng M, Zhang D, Li L, Liu Y. Signal transducers and activators of transcription-1 (STAT1) regulates microRNA transcription in interferon gamma-stimulated HeLa cells. PLoS One 2010; 5:11794; PMID:20668688; http://dx.doi.org/10.1371/journal.pone.0011794.
44. Thompson JE, Cubbon RM, Cummings RT, Wicker LS, Frankshun R, Cunningham BR, et al. Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor. Bioorg Med Chem Lett 2002; 12:1219-23; PMID:11934592; http://dx.doi.org/10.1016/S0960-894X(02)00106-3.
45. Robertson G, Hirst M, Bainbridge M, Bilenky M, Zhao Y, Zeng T, et al. Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. Nat Methods 2007; 4:651-7; PMID:17558387; http://dx.doi.org/10.1038/nmeth1068.
46. Wang J, Haubrock M, Cao KM, Hua X, Zhang CY, Wingender E, et al. Regulatory coordination of clustered microRNAs based on microRNA-transcription factor regulatory network. BMC Syst Biol 2011; 5:199; PMID:22176772; http://dx.doi.org/10.1186/1752-0509-5-199.
47. Griffiths-Jones S. miRBase: the microRNA sequence database. Methods Mol Biol 2006; 342:129-38; PMID:16957372.
48. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 2003; 115:787-98; PMID:14697198; http://dx.doi.org/10.1016/S0092-8674(03)01018-3.
49. Huang S, He X, Ding J, Liang L, Zhao Y, Zhang Z, et al. Upregulation of miR-23a approximately 27a approximately 24 decreases transforming growth factorbeta-induced tumor-suppressive activities in human hepatocellular carcinoma cells. Int J Cancer 2008; 123:972-8; PMID:18508316; http://dx.doi.org/10.1002/ijc.23580.
50. Tsang WP, Kwok TT. The miR-18a* microRNA functions as a potential tumor suppressor by targeting on K-Ras. Carcinogenesis 2009; 30:953-9; PMID:19372139; http://dx.doi.org/10.1093/carcin/bgp094.
51. Yang JS, Phillips MD, Betel D, Mu P, Ventura A, Siepel AC, et al. Widespread regulatory activity of vertebrate microRNA* species. RNA 2011; 17:312-26; PMID:21177881; http://dx.doi.org/10.1261/rna.2537911.
52. Perry MM, Williams AE, Tsitsiou E, Larner-Svensson HM, Lindsay MA. Divergent intracellular pathways regulate interleukin-1beta-induced miR-146a and miR-146b expression and chemokine release in human alveolar epithelial cells. FEBS Lett 2009; 583:3349-55; PMID:19786024; http://dx.doi.org/10.1016/j.febslet.2009.09.038.
53. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3:34; PMID:12184808; http://dx.doi.org/10.1186/gb-2002-3-7-research0034.
54. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization and summaries of high density oligonucleotide array probe level data. Biostatistics 2003; 4:249-64; PMID:12925520; http://dx.doi.org/10.1093/biostatistics/4.2.249.
55. Eichler GS, Huang S, Ingber DE. Gene Expression Dynamics Inspector (GEDI): for integrative analysis of expression profiles. Bioinformatics 2003; 19:2321-2; PMID:14630665; http://dx.doi.org/10.1093/bioinformatics/btg307.
56. Futschik ME, Carlisle B. Noise-robust soft clustering of gene expression time-course data. J Bioinform Comput Biol 2005; 3:965-88; PMID:16078370; http://dx.doi.org/10.1142/S0219720005001375.
57. StoreyJD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci USA 2003; 100:9440-5; PMID:12883005; http://dx.doi.org/10.1073/pnas.1530509100.
58. Aryee MJ, Gutiérrez-Pabello JA, Kramnik I, Maiti T, Quackenbush J. An improved empirical bayes approach to estimating differential gene expression in microarray time-course data: BETR (Bayesian Estimation of Temporal Regulation). BMC Bioinformatics 2009; 10:409; PMID:20003283; http://dx.doi.org/10.1186/1471-2105-10-409.
59. Tai YC, Speed TP. A multivariate empirical Bayes statistic for replicated microarray time course data. Ann Stat 2006; 34; http://dx.doi.org/10.1214/009053606000000759.