

From the Department of Microbiology, Tumor and Cell Biology  
Karolinska Institutet, Stockholm, Sweden

# **FUNCTIONAL STUDIES OF microRNAs IN DEVELOPMENT AND CANCER**

---

Jakob Lovén



Stockholm 2010

**All previously published papers were reproduced with permission from the publisher.**

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

© Jakob Lovén, 2010  
ISBN 978-91-7457-031-1

## **TO MY LOVING FAMILY**

“Eventually, everything connects.”  
– Charles Eames





# ABSTRACT

---

**MicroRNAs (miRNAs) COMPRISE** a large family of small (~23 nucleotide in length), endogenous RNAs that regulate gene expression at the posttranscriptional level. Functional studies have indicated that miRNAs participate in the regulation of nearly all cellular processes investigated so far, including differentiation, apoptosis, and proliferation. Further, the deregulation of miRNA expression greatly contributes to human diseases, and is associated with many human pathologies, such as cancer.

The studies in this thesis have focused on miRNA expression and regulation in various forms of malignancies. Specifically, we wanted to provide mechanistic insights into the role of miRNAs in tumorigenesis. In parallel, we hoped to discover new therapeutic targets that could be exploited clinically to treat childhood and adult cancer. In the work presented, we describe the functional consequences of miRNA perturbations in three distinct neoplasias: (1) chronic lymphocytic leukemia (CLL), the second most common type of blood cancer in adults; (2) neuroblastoma (NB), an embryonal malignancy of the sympathetic nervous system that is derived from primordial neural crest cells and occurs almost exclusively in infants and young children; and, (3) basal cell carcinoma (BCC), a basal cell-derived malignancy of the epidermis, which ranks as the most commonly diagnosed human cancer among fair-skinned individuals.

Our CLL studies revealed that the *DLEU2* transcript functions as a regulatory host gene for the miRNAs *miR-15a* and *miR-16-1*. These miRNAs were shown to target the G<sub>1</sub> cyclins D<sub>1</sub> and E<sub>1</sub> for translational repression, resulting in a prominent cell cycle arrest. Further, ectopic expression of *DLEU2* inhibited the colony-forming capacity of tumor cell lines, suggesting a tumor-suppressive function for *miR-15a* and *miR-16-1*. We also demonstrate that *DLEU2* is transcriptionally regulated by the oncoprotein c-MYC, providing a novel mechanism by which MYC can regulate the G<sub>1</sub> cyclins in a posttranscriptional manner. Functional loss of *DLEU2* may thus constitute an important step in CLL tumorigenesis and various c-MYC-dependent cancers.

In our analysis of *MYCN*-amplified neuroblastoma (NB), we investigated the molecular consequences and functional outcome of abnormal miRNA regulation and discovered that miR-17~92 cluster-derived miRNAs potentiate the tumorigenic behavior of this childhood cancer. Importantly, we could show that miR-18a and miR-19a target and repress the expression of estrogen receptor- $\alpha$  (ESR $\alpha$ ), a ligand-inducible transcription factor implicated in neuronal differentiation. We propose that ESR $\alpha$  represents a previously undescribed *MYCN* target in NB and demonstrate a unique oncogenic circuitry in which the repression of ESR $\alpha$  through *MYCN*-regulated miRNAs may play a fundamental role in NB tumorigenesis.

Finally, based on our genome-wide miRNA expression analysis of a non-melanoma skin cancer, we found that the skin-specific miRNA, miR-203, is preferentially lost in BCC. Functional analyses demonstrated that the inappropriate activation of the Hedgehog and MAPK pathways in BCCs may contribute to cancer progression via severely reduced expression of miR-203, which dramatically facilitates the misexpression of genes involved in the regulation of cell proliferation and cell cycle, including c-JUN and c-MYC. In this respect, miR-203 constitutes a 'gatekeeper' miRNA controlling keratinocyte proliferation. The molecular reconstitution of miR-203 could therefore serve as a novel therapeutic strategy in the treatment of BCC tumors.



# LIST OF PUBLICATIONS

---

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

- I. Mikael Lerner, Masako Harada, **Jakob Lovén**, Juan Castro, Zadie Davis, David Oscier, Marie Henriksson, Olle Sangfelt, Dan Grandér, and Martin M. Corcoran. (2009)  
DLEU2, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1. *Experimental Cell Research*, **315**, 2941–2952.
- II. **Jakob Lovén**, Nikolay Zinin, Therese Wahlström, Inga Müller, Petter Brodin, Erik Fredlund, Ulf Ribacke, Andor Pivarsci, Sven Pählman, and Marie Henriksson. (2010)  
MYCN-regulated microRNAs repress estrogen receptor- $\alpha$  (ESR1) expression and neuronal differentiation in human neuroblastoma. *Proceeding of the National Academy of Science (PNAS)*, **107**, 1553–1558.
- III. Eniko Sonkoly\*, **Jakob Lovén**\*, Tianling Wei, Ning Xu, Florian Meisgen, Petter Brodin, Viljar Jaks, Maria Kasper, Takashi Shimokawa, Masako Harada, Johann Heilborn, Mari-Anne Hedblad, Andreas Hippe, Dan Grandér, Bernhard Homey, Peter Zaphiropoulos, Marie Arsenian-Henriksson, Mona Stähle, Andor Pivarsci. (2010)  
miR-203 functions as a bona fide tumor suppressor microRNA in basal cell carcinoma.  
*Manuscript*.  
\*Equal contribution

# CONTENTS

---

<b>1. INTRODUCTION</b>	<b>1</b>
<b>THE microRNAs</b>	<b>2</b>
<b>miRNA Biogenesis</b>	<b>3</b>
In the Nucleus	4
In the Cytoplasm	5
<b>The Biological Functions of miRNAs</b>	<b>7</b>
miRNAs, Transcription Factors, & Regulatory Networks	9
Variability, Robustness, and Stochasticity	10
Network Buffering and Genetic Canalisation	14
Pleiotropism in Function	15
<b>CANCER</b>	<b>16</b>
<b>Adult Cancer</b>	<b>17</b>
<b>Childhood Cancer</b>	<b>18</b>
<b>Tumor Suppressor Genes &amp; Oncogenes</b>	<b>19</b>
The MYC Oncoprotein	20
<b>miRNAs in Cancer</b>	<b>21</b>
Tumor Suppressor miRNAs	21
Oncogenic miRNAs	23
<b>2. AIMS</b>	<b>26</b>
<b>3. RESULTS &amp; DISCUSSION</b>	<b>27</b>
Paper I	27
Paper II	29
Paper III	30
<b>4. CONCLUDING REMARKS</b>	<b>36</b>
<b>5. ACKNOWLEDGEMENTS</b>	<b>37</b>
<b>6. REFERENCES</b>	<b>40</b>
<b>7. PUBLICATIONS &amp; MANUSCRIPTS</b>	<b>55</b>



# LIST OF ABBREVIATIONS

---

3'UTR	3' untranslated region
5'UTR	5' untranslated region
AGO	argonaute
AKT	v-akt murine thymoma viral oncogene homolog
ATXN1	spinocerebellar ataxia type 1 protein
BARD1	BRCA1 associated RING domain 1
BCC	basal cell carcinoma
BCL2	B-cell CLL/lymphoma 2
BCNS	basal cell nevus syndrome
bHLH	basic-helix-loop-helix
<i>C.elegans</i>	<i>Caenorhabditis elegans</i>
CCND2	cyclin D2
ChIP	chromatin immunoprecipitation
CLL	chronic lymphocytic leukemia
CNS	central nervous system
<i>D.melanogaster</i>	<i>Drosophila melanogaster</i>
DGRC8	DiGeorge syndrome critical region
DLEU2	deleted in lymphocytic leukemia 2
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EcR	ecdysone receptor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
endo-siRNA	endogenous small interfering RNA
ESR1	estrogen receptor- $\alpha$
EXP5	exportin-5
FFL	feedforward loop
GLI	glioma-associated oncogene family zinc finger 1
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HES	hairy e(spl)
HH	hedgehog
HOX	homeobox

IFE	interfollicular epidermis
IHC	immunohistochemistry
JNK	Jun N-terminal kinase
<i>lin</i>	lineage abnormal
LNA	locked nucleic acid
MAPK	mitogen-activated protein kinase
MAX	MYC-associated protein X
MDR	minimal deleted region
mESC	mouse embryonic stem cells
miRISC	miRNA-programmed RISC
miRNA	microRNA
MIZ1	MYC-interacting zinc finger protein 1
MNT	MAX-interacting protein
MXD	MAX dimerization protein
MYC	myelocytomatosis
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
ncRNA	noncoding RNA
NGF	nerve growth factor
PACT	protein activator of PKR
piRNA	Piwi-interacting RNA
pre-miRNA	miRNA precursor
pri-miRNA	primary miRNA
PTCH1	patched homologue 1
PTEN	phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
RB	retinoblastoma
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNA Pol	RNA polymerase
RNAi	RNA interference
ROBO2	roundabout, axon guidance receptor, homolog 2
ROS	reactive oxygen species
SA	sympatho adrenal
SHH	sonic hedgehog
SMO	smoothened
snoRNA	small nucleolar RNA
SNS	sympathetic nervous system
SOP	sense organ precursor
stRNA	short temporal RNA
TAR	trans-activation responsive
TF	transcription factor
TFBS	transcription factor binding site
TRBP	TAR RNA binding protein
TRKA	tyrosine kinase receptor A
tRNA	transfer RNA
TSG	tumor suppressor gene
TSS	transcriptional start site



# 1. INTRODUCTION

---

IN THE LAST decade, the biology of ribonucleic acid (RNA) has witnessed a transformation unmatched by any other area in medical research. The discovery that RNA molecules act as versatile regulators of eukaryotic gene expression has reshaped our understanding of gene regulation and function. At present, any transcript, regardless of coding potential, may have an intrinsic function as an RNA. Whether long or short, noncoding RNAs (ncRNAs) have been found to regulate some of the most important levels of genome function, including chromatin structure, transcription, RNA processing, RNA stability and translation.

In 1986, Walter Gilbert proposed the RNA world hypothesis, a theory about the origin of life based on the view that the most critical event is the emergence of a self-replicating molecule, a molecule that can both copy itself and mutate and, hence, evolve to more efficient copying (Gilbert, 1986). RNA is such a molecule. During the very early stages of life on Earth, proteins were not yet engaged in biochemical reactions and RNA carried out both the information storage task of genetic information and the full range of catalytic roles necessary in a very primitive self-replicating system.

Despite this functional versatility, RNA was long considered a docile molecular entity, largely operating as an inert intermediary between gene and protein. However, the finding that most of the genomes of complex organisms are transcribed in a regulated fashion along with the discovery of several classes of regulatory, noncoding RNAs (including microRNAs) has challenged this assumption (Mercer et al., 2009). Recent advances have revealed unexpected diversity of function for various ncRNAs, suggesting that RNA has continued to evolve and expand alongside DNA and protein.

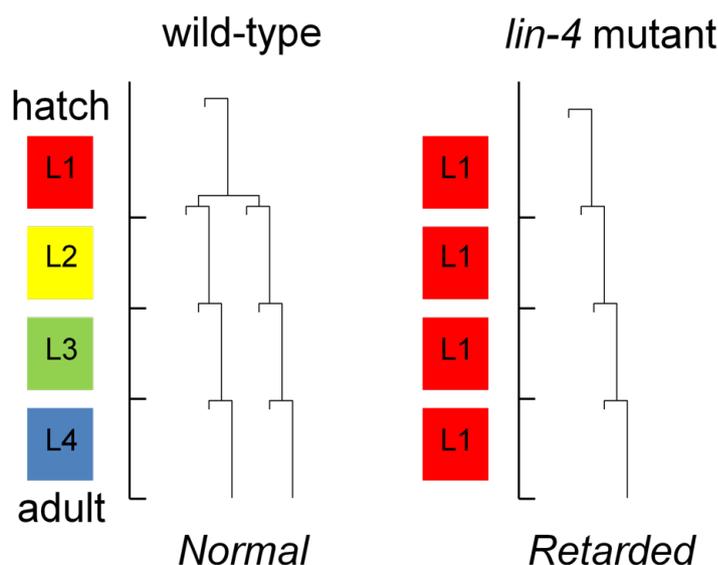
The work presented in this thesis focuses on a particular class of small noncoding RNAs termed microRNAs, or miRNAs, and their functional role in developmental pathways and cancer etiology.

# THE microRNAs

---

**IN BIOLOGY, HETEROCHRONY** refers to changes, over evolutionary time, in the rate or timing of developmental events (Moss, 2007). Differences in the relative timing of developmental events have long been believed to be a major force in the evolution of morphology. Early attempts to identify heterochronic phenotypes in *Caenorhabditis elegans* (*C. elegans*) revealed a collection of genes that, when mutated, execute stage-specific cell fates at inappropriately early or late time-points (Chalfie et al., 1981). These so-called ‘heterochronic’ genes were identified among a larger group of lineage-abnormal (*lin*) mutants, and revealed a developmental timing mechanism that functions independently of other types of developmental regulation, including growth, induction and differentiation (Moss, 2007).

Among the first heterochronic mutants to be described was *lin-4*. Mutations in *lin-4* caused the first larval stage (L1) to reiterate at later developmental stages (*figure 1*) (Chalfie et al., 1981). The converse phenotype was later observed for the *lin-14* mutant, which arose spontaneously in a culture of *lin-4* mutant animals. Omission of the L1 cell fates and premature development into the L2 stage are observed in worms that are deficient for *lin-14* (Ambros and Horvitz, 1984). Following extensive mutagenic analyses, it was realised that *lin-4* encodes a pair of small untranslated RNAs measuring 61 and 22 nucleotides (nt) in length (Lee et al., 1993). Surprisingly, these *lin-4* RNAs had antisense complementarity to multiple sites of the 3'-untranslated region (3'UTR) of the *lin-14* gene (Lee et al., 1993). *lin-14*, on the other hand, encodes a nuclear protein, downregulation of which at the end of L1 initiates the developmental progression into the second larval stage (L2) (Lee et al., 1993; Ruvkun and Giusto, 1989). In addition to a functional *lin-4* gene, the negative regulation of the LIN-14 protein requires an intact 3'UTR (Lee et al., 1993; Wightman et al., 1991).



*Figure 1.* The heterochronic phenotype of the *lin-4* mutant in *C. elegans*. Worms with mutations in *lin-4* do not develop normally and cell divisions characteristic of larval stage 1 (L1) reiterate throughout development.

Together, these findings led to the characterisation of a novel regulatory pathway in which *lin-4* RNAs negatively regulate *lin-14* translation by binding to its 3'UTR. The subsequent discovery that another temporally regulated 21 nt small RNA in *C. elegans*, *let-7*, was readily detected among phylogenetically distinct bilaterians indicated a more general role for small RNAs in developmental regulation (Pasquinelli et al., 2000). It was also established that several additional small RNAs with structural characteristics resembling *lin-4* and *let-7* were detectable throughout metazoan development (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). However, unlike the *lin-4* and *let-7* RNAs, which had been classified as short temporal RNAs (stRNAs) due to their common roles in controlling developmental timing, many of the newly cloned small RNAs did not display heterochronic features. Thus, the annotation stRNA was abandoned and replaced with the term microRNA (miRNA), to denote all small RNAs with similar features but unknown functions (Bartel, 2004).

Since their original discovery, miRNAs have been detected in all metazoans surveyed so far for their presence, including poriferans and cnidarians, and are even present in other distantly related eukaryotic lineages, such as plants and algae (Grimson et al., 2008). Hence, miRNAs constitute one of the most abundant classes of gene-regulatory molecules in the animal and plant branches of eukaryota.

## microRNA Biogenesis

**miRNAs ARE BROADLY** defined as small (~23 nt in length), endogenous RNAs that play important gene-regulatory roles by pairing to the mRNAs of protein-coding genes to direct their posttranscriptional repression (Bartel, 2009). MiRNAs belong to a broad class of small RNAs, including endogenous small interfering RNAs (endo-siRNAs) (Reinhart and Bartel, 2002) and the Piwi-interacting RNAs (piRNAs) (Aravin et al., 2007). Together, these RNAs act as sequence-specific guides in RNA silencing pathways. Processed from distinctive hairpin-shaped transcripts, miRNAs act as guide molecules in posttranscriptional processes that involve base-pairing with cognate messenger RNA (mRNA) targets, usually in the 3' untranslated region (3'UTR).

Binding of a miRNA to the target mRNA typically leads to translational repression and/or mRNA destabilisation, although other types of regulation, including translational activation and heterochromatin formation, have been described (Filipowicz et al., 2008; Kim et al., 2008; Orom et al., 2008). At present, the number of confidently identified miRNAs has surpassed 110 in *C. elegans*, 140 in the fly *Drosophila melanogaster* and 400 in humans, corresponding to ~ 1–2% of the number of protein-coding genes in these species (Bartel, 2009). Initial efforts based on computational methods estimated that more than one third of human genes appear to be conserved miRNA targets (Lewis et al., 2005), while recent estimates suggest that more than 60% of all human protein-coding genes are under selective pressure to maintain pairing to miRNAs (Friedman et al., 2009).

With regard to their genomic organisation, a sizable portion (~40%) of miRNAs are present within introns of pre-mRNAs, providing a convenient mechanism for the coordinated expression of intron-embedded miRNAs and protein-encoding mRNAs (Bartel, 2004). Furthermore, most mammalian miRNA genes are clustered in the genome, allowing them to be transcribed simultaneously as polycistronic transcription units (Altuvia et al., 2005; Bartel, 2004). Intronic incorporation and genomic miRNA aggregation allows for miRNAs to employ existing functional promoter elements, eliminating the need for *de novo* assembly of transcriptional features, such as promoter-enhancer sequences, transcriptional start sites (TSS), and transcription factor binding sites (TFBS) (Saini et al., 2007; Zhou et al., 2007). Although a majority of miRNA genes are transcribed by RNA polymerase II (Pol II) (Cai et al., 2004; Lee et al., 2004), a subset of miRNAs are processed by RNA polymerase III (Pol III), including the dense cluster of human miRNAs interspersed among repetitive *Alu* elements on chromosome 19 (Borchert et al., 2006). Both RNA polymerases are regulated differently and recognize specific promoter and terminator elements, facilitating a wide variety of regulatory options, thus allowing miRNA genes to be elaborately expressed in particular contexts and cell types.

## In the Nucleus

**MATURATION OF miRNAs** involves several sequential processing steps. In animals, canonical miRNA genes are transcribed by Pol II into primary miRNAs (pri-miRNAs) that are typically several kilobases (kb) long, 5'-capped, polyadenylated, and contain local stem-loop structures (Kim, 2005; Kim et al., 2009). These stem-loop structures serve as substrates for nuclear cleavage of the pri-miRNA by the Microprocessor complex, which contains the RNase III endonuclease Drosha and the cofactor DiGeorge syndrome critical region gene 8 (DGCR8) in humans (or Pasha in *D. melanogaster* and *C. elegans*) (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Lee et al., 2003). The two RNase domains of Drosha cleave the 5' and 3' arms of the pri-miRNA hairpin ~11 bp away from the single-stranded RNA-double-stranded RNA (ssRNA-dsRNA) junction with the aid of DGCR8, which stably interacts with the ssRNA segments and ~33 bp of the stem (Han et al., 2004; Han et al., 2006). This nuclear reaction liberates a ~60-70 nt stem-loop intermediate, known as the miRNA precursor (pre-miRNA) (Lee et al., 2002).

Drosha-mediated cleavage of the pri-miRNA occurs co-transcriptionally and precedes splicing of the protein-encoding or non-coding host RNA containing the miRNAs. Splicing is not influenced by Drosha-mediated cleavage, thereby ensuring both miRNA biogenesis and protein synthesis from a single primary transcript (Kim and Kim, 2007; Morlando et al., 2008). However, the inclusion of miRNA-resembling stem-loop structures in exonic regions of mRNAs may affect transcript stability. Drosha negatively regulates its co-factor DGCR8 via two highly conserved hairpin structures in its 5'UTR and in the coding sequence near the start codon, which results in DGCR8 mRNA destabilisation following Drosha-mediated cleavage (Han et al., 2009).

Additional observations support a functional role for miRNA-5'UTR/exonic interactions in conveying target-associated repression, implying that putative miRNA-resembling stem-loop structures in non-3'UTR regions of mRNAs may serve as RNA control elements influencing mRNA stability (Duursma et al., 2008; Lee et al., 2009; Lytle et al., 2007). The participation of other, yet-to-be-described nucleases in this mode of mRNA metabolism are also thought to exist (Karginov et al., 2010).

In addition to canonical processing pathways, a subset of miRNAs mature following the completion of splicing. These miRNAs, termed mirtrons, derive from short introns and their biogenesis does not require Drosha processing (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Such non-canonical small RNAs are produced when debranching of lariat-shaped introns results in the appropriate formation of a hairpin structure resembling a pre-miR. Additional 5'- or 3'-end exonucleolytic trimming of mirtron tails is occasionally required before these RNAs are suitable as substrates for nuclear export. Deletion of *DGCR8* in mouse embryonic stem cells (mESC) has also revealed Microprocessor-independent, Dicer-dependent generation of small non-mirtronic RNAs from other non-coding RNA loci, such as the isoleucine transfer RNA (tRNA) gene, while Dicer-dependent processing of the small nucleolar RNA (snoRNA) *ACA45* generates small RNAs with miRNA-like function (Babiarz et al., 2008; Ender et al., 2008). Evidently, multiple non-canonical pathways contribute to a substantial fraction of pre-miR abundance in mammalian cells through Drosha-independent pathways.

## In the Cytoplasm

**AFTER THE COMPLETION** of nuclear processing, pre-miRNAs are exported into to the cytoplasm by Exportin-5, (EXP5), a nuclear transport receptor, in complex with Ran-GTP (Lund et al., 2004). Originally assumed to be a minor export factor of tRNAs, EXP5 predominantly mediates nuclear export of both short-hairpin RNAs (shRNA) and pre-miRs (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2005). EXP5 binds cooperatively to double-stranded RNA (dsRNA) independently of sequence or loop structure (Gwizdek et al., 2003; Zeng and Cullen, 2004), thus ensuring the export of only correctly processed small RNAs with a characteristic stem-motif of 14-16 bp and short 3' overhangs (Lund and Dahlberg, 2006).

Once in the cytoplasm, pre-miRs are cleaved near the terminal loop by the RNase III endonuclease Dicer, releasing ~22 nt miRNA duplexes with ~2 nt 3' overhangs at either end. This cleavage is essential for miRNA processing and has been described in many organisms, including *C. elegans*, *D. melanogaster*, and mammals (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). In humans, Dicer interacts with the dsRNA-binding domain proteins TAR RNA-binding protein (TRBP) and protein activator of PKR (PACT) and the core component Argonaute proteins 1-4 (Ago 1-4) (Gregory et al., 2005; Lee et al., 2006). Together, these proteins contribute to miRNA-programmed RNA-induced silencing complex (miRISC) assembly by forming a

RISC-loading complex (RLC). Although TBRP and PACT are not essential for Dicer-mediated cleavage of pre-miRNAs, RLC formation is facilitated in their presence (Chendrimada et al., 2005; Lee et al., 2006).

After Dicer-mediated cleavage, Dicer and its interactors TRBP and PACT dissociate from the miRNA duplex. The two miRNA strands are then separated and one of the strands associates with an Ago protein within RISC, where it acts as a guide to repress target messages. Strand selection is determined by inherent features of the miRNA duplex, including thermodynamic asymmetry/stability (Khvorova et al., 2003; Schwarz et al., 2003). Typically, the strand whose 5' end is less stably base-paired will be more frequently chosen as the functional guide strand. The other strand (the passenger strand or miRNA\*) is presumed to be excluded from miRISC incorporation and subsequently degraded (Carthew and Sontheimer, 2009). However, recent deep-sequencing efforts have revealed that a large number of miRNA\* strands can be appreciably detected in Ago complexes, and are in large part functional. Differential sorting of miRNA duplex strands correlates with specific mismatches at positions 9 and 10 of the mature miRNA and the 5' nucleotide identity of the strand (Czech et al., 2009; Okamura et al., 2009; Okamura et al., 2008). In addition, relative levels of the miRNA/miRNA\* strands vary widely across tissues (Hu et al., 2009; Landgraf et al., 2007). Together, these findings suggest that miRNA precursors can be bifunctional, with individual strands adopting different fates within small RNA pathways (*figure 2*).

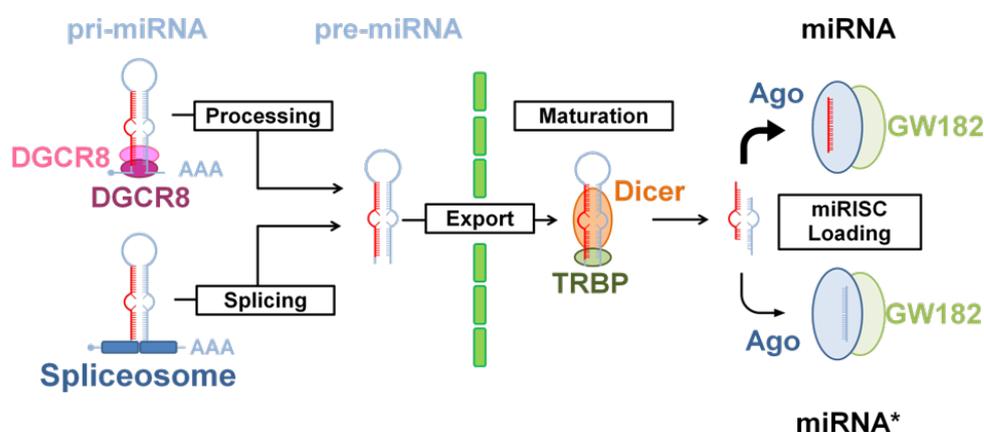


Figure 2. miRNA biogenesis and assembly into miRISC

Once a single strand has been selected, the miRNA acts as an adaptor for miRISC to specifically recognise and regulate particular mRNAs. With the exception of a few aforementioned examples, miRNA-binding sites in metazoan mRNAs lie in the 3'UTR and are present in multiple copies. Most miRNAs base pair imperfectly with their targets, following a set of rules determined by experimental and bioinformatic analyses (Brennecke et al., 2005b; Doench and Sharp, 2004; Grimson et al., 2007; Lewis et al., 2005; Nielsen et al., 2007). The most stringent requirement of miRNAs base-pairing to their targets is a contiguous and perfectly matched Watson-Crick interaction of miRNA nucleotides 2-7, representing the seed region. Other molecular determinants, such as

pairing to the 3' region of the miRNA (3'-supplementary and 3'-compensatory pairing) and UTR accessibility, are known to enhance binding specificity and affinity (Bartel, 2009). Recently, the miRNA targeting code expanded to include functional sites with centered pairing, to describe target sites that lack both perfect seed pairing and 3'-compensatory sites and instead have 11-12 contiguous Watson-Crick pairs to the center of the miRNA (Shin et al., 2010).

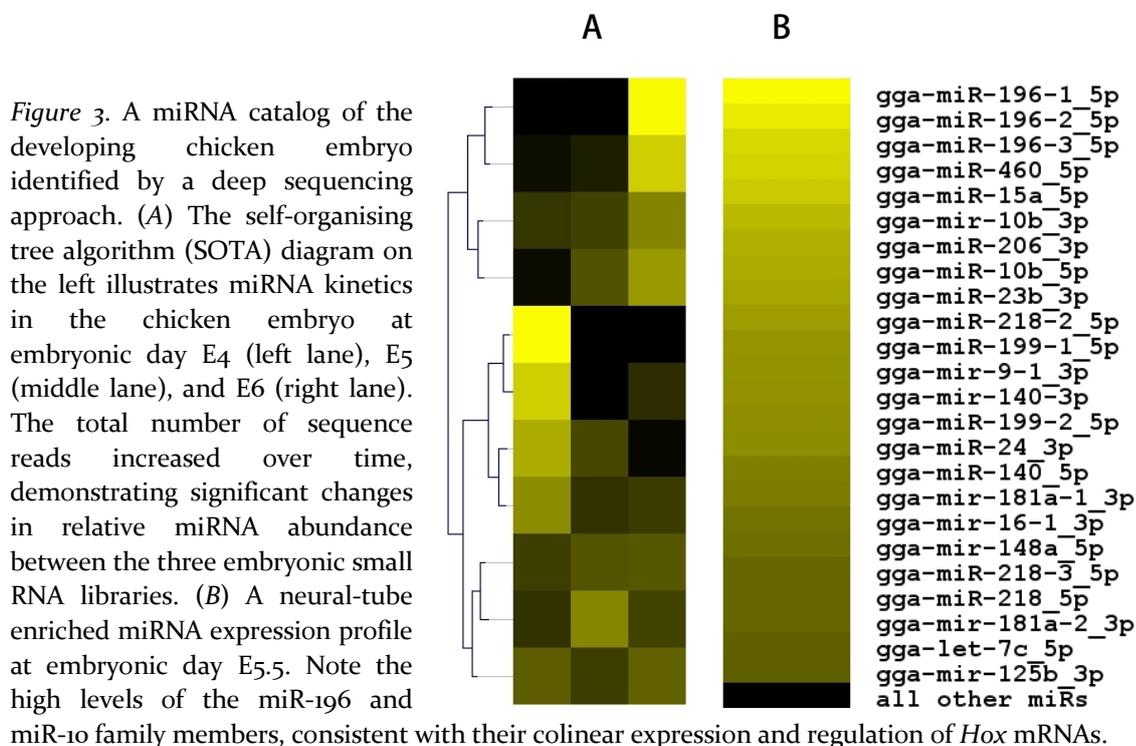
The degree of miRNA-mRNA complementarity will generally dictate the outcome of the miRNA-mRNA interaction. Perfect complementarity allows Ago-catalysed cleavage of the mRNA strand, although very few examples of miRNA-dependent cleavage have been observed in mammals (Meister et al., 2004; Yekta et al., 2004). More commonly, metazoan miRNAs direct translational repression, mRNA destabilisation, or a combination of both (Filipowicz et al., 2008). The mechanisms by which miRISC regulates translation are not entirely clear; i.e. whether repression occurs at the translational initiation or postinitiation step of mRNA maturation. Several models have been proposed, including repression of initiation at the 7-methylguanosine (m<sup>7</sup>G) cap stage, miRISC-dependent obstruction of translationally competent ribosomes at the AUG start codon, or premature ribosome dissociation from mRNAs at the postinitiation stage (i.e. the elongation phase) (Carthew and Sontheimer, 2009). The mechanisms associated with mRNA destabilisation, however, are proposed to operate through deadenylation, decapping, and exonucleolytic digestion of the miRNA-bound mRNA, resulting in a significant reduction in mRNA abundance (Bagga et al., 2005; Behm-Ansmant et al., 2006; Giraldez et al., 2006). This reaction requires Ago, GW182, and the cellular decapping and deadenylation machinery (Behm-Ansmant et al., 2006). Although the molecular principles underlying the preferred mode of posttranscriptional repression remain elusive, it has been suggested that the number, type and position of mismatches in the miRNA/mRNA duplex play an important role in triggering degradation or translational arrest (Aleman et al., 2007). Suffice to say, miRNAs affect protein production at many different levels with distinct biological outcomes.

## The Biological Functions of miRNAs

**SINCE THE DISCOVERY** that *lin-4* and *let-7* play critical roles in the timing of larval development in *C. elegans*, miRNAs have been implicated in a bewildering array of biological settings, and the importance of miRNAs in select developmental processes in model organisms is indisputable. In particular, miRNAs appear to partake in pathways associated with cell fate determination and differentiation, thereby contributing to the specification of many cell types. Global attenuation of miRNA biogenesis through genetic manipulation of *Dicer* has revealed several clues to miRNA function in developmental contexts. *Dicer* loss of function results in profound phenotypic defects in both zebrafish and mice, emphasising the importance of the miRNA pathway in vertebrate development. However, by in large, early stages of development progress normally in *Dicer*<sup>-/-</sup>embryos. For example, zebrafish embryos lacking *Dicer* develop

without profound defects until maternal stores of *Dicer* are depleted, approximately eight days post-fertilisation (Wienholds et al., 2003). Even *Dicer*-deficient zebrafish embryos lacking maternal *Dicer* (*MZdicer* mutants) exhibit only mild phenotypic perturbations within the first 24 hours of development (Giraldez et al., 2005), suggesting that miRNAs are not essential for cell fate determination and early patterning, but provide critical functions at subsequent steps in embryonic development. Like zebrafish *MZdicer* mutants, mouse embryos lacking *Dicer* do not display gross morphological defects before the onset of gastrulation (~embryonic day 7.5), again pointing to an important role for miRNAs in later stages of development (Bernstein et al., 2003).

This notion is further supported by the temporal and spatial expression patterns of conserved vertebrate miRNAs in zebrafish embryos, as well as other model systems, including mouse and chicken (figure 3) (Ason et al., 2006; Darnell et al., 2006; Wienholds et al., 2005). For example, *miR-196* and *miR-10* genes of various vertebrates reside in the homeobox (*HOX*) gene clusters and function at later stages of vertebrate development. Like the *HOX* genes, *miR-196* and *miR-10* are colinearly expressed in a spatial and temporal manner along the anterior-posterior body axis during development and preferentially target *HOX* mRNAs (Yekta et al., 2008). In mouse embryos, both miRNA families have highest expression in the neural tube and lower expression levels in the trunk mesoderm, with ill-defined anterior limits and broad posterior expression through the tail (Mansfield et al., 2004). The observed expression domains of these miRNA families are in agreement with the patterns that are expected on the basis of their locations within the *HOX* clusters.



Although the expression patterns among orthologous miRNAs may vary between species, these studies demonstrate that many miRNAs are expressed with precise tissue specificity late (rather than early) in development, presumably due to their participation in tissue-specific functions and lineage-promoting effects. Indeed, recent studies have shown that the unique modalities by which specific miRNAs exert their widespread function in various developmental settings depend on both the timing and pattern of their expression, as well as the repertoire of co-expressed targets.

## **miRNAs, Transcription Factors, and Regulatory Networks**

**GIVEN THAT MORE** than half of mammalian mRNAs are under selective pressure to maintain base-pairing interactions with miRNAs (Friedman et al., 2009), most biological processes are likely to be influenced by miRNA function. With such a broad impact on gene regulation, miRNAs have rapidly emerged as one of the most abundant gene regulatory factors in multicellular genomes. In this respect, miRNAs share many regulatory characteristics with transcription factors (TF), which suggests that miRNAs and TFs share a common regulatory logic (Hobert, 2004).

Transcription factors exert their effects by directly (or indirectly) binding DNA at specific genomic loci to control the transcription of nearby genes. In doing so, transcription factors can influence cell fate decisions, functioning as key switches by regulating gene expression programs on a genome-wide level. A single transcription factor can thus, through positively or negatively regulating transcription of numerous genes, execute entire cellular or tissue-level programs. To this end, gene repression has surfaced as an important theme in shaping cell-specific gene regulatory programs, especially in developmental contexts (Bilodeau et al., 2009; Boyer et al., 2006; Hobert, 2004).

However, like miRNAs, TFs rarely operate single-handedly; rather, sets of combinatorially expressed TFs and miRNAs act together to precisely delineate individual cell types and fates (Hobert, 2008; Marson et al., 2008). Cooperativity therefore provides a mechanistic basis for describing the overall output of combinatorial expression patterns of TFs and miRNAs. These combinatorial codes are in turn controlled by more upstream events, such as signaling cascades, which play an integral part in regulatory transactions in cells (Weake and Workman, 2010). Developmental processes can therefore be considered as a succession of hierarchically acting regulatory states (Davidson et al., 2002). Consequently, transcriptional regulatory programs have been placed into well-defined regulatory networks that are characterised by small sets of recurring network motifs that endow the system with specific properties such as signal amplification, dampening, and oscillation (Alon, 2007). As trans-acting factors, TFs and miRNAs frequently associate in small-scale gene regulatory networks with defined topology (Martinez et al., 2008; Tsang et al., 2007). Similar to the network motifs shared by transcription factors, miRNA network motifs are typically composed of feedforward and feedback loops (Herranz and Cohen, 2010). Thus, miRNA regulatory events

interface with known TF and signaling networks that control cell fate and differentiation, modulating their activity through positive and negative feedback loops to reinforce cellular decisions.

## Variability, Robustness, and Stochastic Gene Expression

**BIOLOGICAL SYSTEMS ARE** continuously challenged by an environment that is variable, yet developmental and physiological processes are remarkably stable, resulting in stereotyped outcomes (Hornstein and Shomron, 2006). Such variability, also referred to as 'noise', has multiple sources, including variations in the activity of individual genes, cell-to-cell variations in metabolic activity, or fluctuating levels of an external signal, and must be controlled in living systems in order to maintain phenotypic stability. Gene regulatory networks, involving feedforward and feedback loops, are particularly suited to control the effects of noise, by buffering its impact on gene expression (Raser and O'Shea, 2005).

The ways in which cells and organisms use noise and deal with can greatly influence cellular behavior and phenotypic consequences. By in large, developmental programs are deterministic; however, mechanisms have evolved to buffer stochastic fluctuations, thereby conferring robustness to gene regulatory networks (Hornstein and Shomron, 2006). One form of noise management, termed canalisation, has been selected over the course of evolution to impart developmental pathways with high phenotypic reproducibility. However, some biological processes utilise noise to trigger stochastic developmental decisions (Losick and Desplan, 2008). As miRNAs are frequently embedded in network motifs, their function is often related to the nature and topology of the network to which they belong, suggesting that they confer useful regulatory possibilities to facilitate network decision-making and biological outcomes (e.g. homeostasis, differentiation, and/or lineage specification). In some contexts, miRNAs act as binary switches to help repress target protein output to inconsequential levels while in other settings miRNAs act as rheostats to dampen (tune) protein output to more optimal levels (Bartel, 2009). The ways in which network motifs and miRNAs manage the impact of noise therefore differs from one biological process/circuit to another.

**CLASSICAL SWITCH INTERACTIONS** shaped the initial paradigm of miRNA targeting, whereby miRNA induction turns off expression of a pre-existing target. In network language, these interactions are examples of feedback (positive or negative) motifs (*figure 4*). Initial examples of switch interactions include *lin-4* targeting *lin-14* and *lin-28*, and *let-7* targeting of *lin-41* (Bartel, 2009; Reinhart et al., 2000). The recent characterisation of *D. melanogaster miR-14* has revealed a different type of regulatory switch that acts by limiting stochastic expression of its target, the ecdysone receptor (EcR). In *Drosophila*, the steroid hormone Ecdysone and its receptor EcR play a key role

in control of developmental timing. Pulses of Ecdysone followed by EcR activation trigger a complex hierarchy of gene expression programs that control the physiological and morphological changes involved in metamorphosis (Thummel, 1996, 2001). An important feature of this process is its all-or-none character, which depends on a positive feedback loop involving transcriptional autoregulation of EcR (Karim and Thummel, 1992; Koelle et al., 1991). Random transcription (noise) is a stochastic event that often occurs in bursts (Raj and van Oudenaarden, 2008). Random transcriptional fluctuations of EcR need to be limited to avoid a self-amplifying response. miR-14 acts directly to reduce EcR levels, and reciprocally, EcR negatively regulates *miR-14*, thereby keeping the circuit in balance. Upon hormonal induction, EcR will negatively regulate *de novo* transcription of *miR-14* and residual miR-14 levels will eventually subside, permitting the cell to distinguish between sustained input of hormone-induced EcR activation and transcriptional fluctuations in EcR levels (Varghese and Cohen, 2007). Thus, while miR-14 prevents inappropriate transitions to occur during development due to noise, it constitutes a developmental switch that allows major transformations in gene expression programs when a certain threshold level is reached.

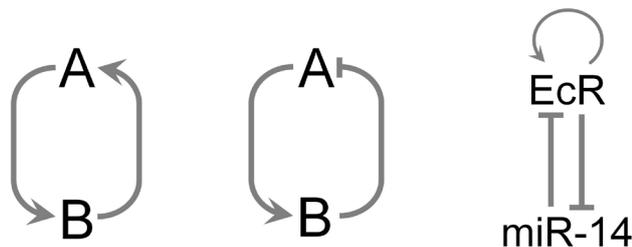


Figure 4. Examples of simple positive and negative feedback loops. *miR-14* acts in a negative feedback loop modulating EcR activity.

**FAILSAFE INTERACTIONS REFER** to switch interactions in which a miRNA is already present when the target is first expressed. For example, sense organ specification in the *D. melanogaster* peripheral nervous system is a stochastic event modulated by *miR-9a*, which inhibits the sporadic production of additional neuronal precursor cells (Li et al., 2006). Sense organ primordia are initially defined as small groups of cells that express a set of proneural basic helix-loop-helix (bHLH) transcription factors of the Hairy-E(spl) (HES) family. Definition of the proneural cluster at this level is deterministic, based on programmed control of gene expression, but fluctuations in the level of the transcription factor Senseless determines which cell adopts the sense organ

precursor (SOP) fate (Herranz and Cohen, 2010). The stochastic selection of a SOP cell leads to an increase in proneural gene expression, which feeds back to increase *Senseless* expression. *Senseless* also increases expression of the Notch ligand *Delta*, which in turn leads to increased Notch signaling activity in the adjacent cells. Notch activity represses proneural gene expression in these cells, and reinforces the advantage of the *Senseless*- and *Delta*-expressing cell toward becoming the SOP (Jafar-Nejad et al., 2003). As positive feedback loops are inherently labile, additional (failsafe) mechanisms are needed to ensure that random fluctuation in transcription factor levels does not trigger positive feedback inappropriately.

In this biological context, *miR-9a* sets a threshold that *Senseless* transcription must overcome in order to activate the *Senseless*-proneural feedback loop (Li et al., 2006). Importantly, *miR-9a* expression is under proneural gene control and *miR-9a* levels are at first uniform in the proneural cluster. Following stochastic selection of the SOP, *Senseless* activity increases proneural gene expression with concurrent reduction of *miR-9a* expression. However, *miR-9a* levels remain high in the surrounding cells, where proneural gene activity is kept low through Notch activity. The opposing regulation of *Senseless* and *miR-9a* is an integral element of the switch. The transcriptional feedback system is triggered by random fluctuations in gene expression, and *miR-9a* helps to reinforce selection of the SOP cell, thereby bestowing the noise-dependent switch with a failsafe, functionally redundant layer of control (Herranz and Cohen, 2010).

**FEEDFORWARD REGULATION REPRESENTS** the second type of network motifs. Feedforward loops (FFLs) have been described in a multitude of gene systems, from bacteria and yeast to mammals (Alon, 2007). Feedforward motifs consist of three components: an upstream regulator (TF) and two targets (e.g. a miRNA and a target gene). Assuming that each of the three regulatory interactions can be either positive or negative, there are eight possible structural types of FFL (Alon, 2007). Further, depending on the nature of the relationships between the components, FFLs can be classified as being either coherent or incoherent (Mangan and Alon, 2003). In a coherent FFL, the miRNA is induced, directly or indirectly, by a TF that represses the target, i.e. the posttranscriptional repression by the miRNA is synergistic with transcriptional inhibition of the same target. In this regard, the coherent FFL can be considered failsafe, as it enhances the fidelity of a genetic program by ensuring that aberrant transcripts do not give rise to consequential amounts of protein. Reciprocally, the TF may activate target transcription and repress miRNA production, thereby removing miRNA-mediated constraints on target gene expression. The impact of coherent FFLs has recently been described in both developmental and oncogenic contexts. For example, miR-7 has been implicated in two coherent feedforward motifs involved in the specification of photoreceptor cell in the *Drosophila* eye (Li et al., 2009). Likewise, *let-7* belongs to a feedforward motif underlying oncogenic transformation in response to inflammatory signals (Iliopoulos et al., 2009).

Incoherent FFLs are less intuitive and have different properties than coherent FFLs, but are just as common as coherent types (Tsang et al., 2007). Incoherent FFLs are particularly suited as triggers for oscillatory systems, enabling more customised expression in different cell types as well as more uniform expression within each cell type. In this scenario, the miRNA is important in fine-tuning the expression level of the target gene. One well-studied example of an incoherent FFL involves c-MYC, *miR-17~92* cluster-derived miRNAs and cell cycle progression (O'Donnell et al., 2005). c-MYC transcriptionally induces the expression of *E2F1*, which in turn regulates an array of cell cycle genes. This is accompanied by the concomitant, c-MYC-dependent induction of *miR-17* and *miR-20a*, which repress *E2F1*. The overall outcome of these interactions results in the precise pulse of c-MYC-induced *E2F1* expression needed for optimal cell cycle progression (figure 5).

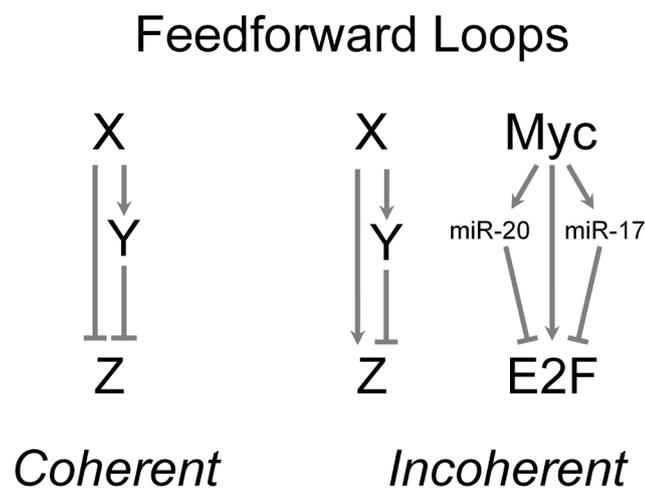


Figure 5. Feedforward motifs where X regulates Z directly and indirectly through regulation of Y. Coherent motifs have the direct and indirect paths from X acting on the target Z in the same direction. Incoherent motifs have the opposite outcomes for the two paths. miR-20 and miR-17 act in an incoherent feedforward motif. c-MYC activates E2F directly while repressing its expression posttranscriptionally via miR-20 and miR-17. This opposing activity sets a precise E2F expression level, and thus prompts cell cycle progression.

### Network Buffering and Genetic Canalisation

**ALTHOUGH miRNAs AND TFs** are interconnected in shared regulatory motifs and these regulatory events appear quite similar, their individual importance as regulatory factors is different. Thorough genetic analyses in unicellular and multicellular organisms has firmly demonstrated the importance of TFs in controlling development and homeostasis (Davidson et al., 2002; Kamath et al., 2003). Likewise, the genetic elimination of *Dicer* can produce striking phenotypes, especially defects in later developmental events (Levy et al., 2010; Zehir et al., 2010). However, many miRNAs, even the most highly conserved, can be eliminated individually without causing obvious phenotypes (Miska et al., 2007). In fact, the deletion of 95 *C. elegans* miRNA loci revealed that less than 10% of miRNA knockouts results in clear developmental or

morphological defects (Miska et al., 2007). In contrast, recent RNA interference (RNAi) loss-of-function analysis show that a substantially higher proportion of genes required for viability in *C. elegans* are enriched for TFs, associated with developmental processes, and cause easily discernable phenotypes (Fraser et al., 2000; Kamath et al., 2003). This implies that many miRNAs may be functionally redundant, yet it is difficult to reconcile their extreme conservation with redundancy. Moreover, gene-knockout phenotypes in flies and vertebrates can be very subtle, and are commonly masked by the considerable derepression of only a few conserved targets (Baek et al., 2008; Nakahara et al., 2005; Selbach et al., 2008).

Large-scale proteomic analysis following miR-223 disruption *in vivo* indicated that very few messages in a biologically accurate setting were repressed by more than 50% (Baek et al., 2008). Instead, miR-223 had more modest effects on its endogenous targets (including conserved targets), with individual sites reducing protein output by ~30% (Bartel, 2009). Several reasons may account for these observations. First, the nature of specific miRNA:target interactions may play an important role. Messages with multiple conserved sites and particularly favorable sites are more likely to represent highly responsive miRNA:target interactions, resulting in substantial target repression. This has been illustrated by numerous examples, including the *lin-4:lin-14* interaction, the *let-7:Hmg2* interaction and the *miR-18:ESR1* interaction (Lee et al., 1993; Loven et al.; Mayr et al., 2007; Wightman et al., 1993). However, these types of interactions represent only a minority of preferentially conserved targets, and modest repression appears to be the more common regulatory outcome of a miRNA:target interaction. With more than 90% of conserved miRNA:target interactions involving only a single site to the miRNA, most of these targets would be expected to be downregulated by less than 50% (Bartel, 2009).

In addition, since most messages with a conserved site to one miRNA have at least one other conserved site to an unrelated miRNA, disruption of multiple miRNA:target interaction sites might be necessary before the derepression generated has overt consequences (Bartel, 2009). Mild phenotypes may also be the result of the vast functional diversity of many miRNA targets. Often, ~2-fold protein fluctuations can be tolerated, as evidenced by the rarity of haploinsufficient phenotypes, even when the targets themselves are regulatory proteins (e.g. TFs). This phenomenon, known as network buffering, may constitute an additional function for miRNAs.

Many regulatory interactions, including many miRNA:target interactions, fall within complex regulatory networks with bifurcating pathways and feedback control that enable accurate response despite a defective node in the network. With this ability to buffer the effects of losing a node, such networks must be perturbed elsewhere before the lost miRNA interaction has discernable phenotypic consequences (Bartel, 2009). In line with their role as genetic buffers, mounting evidence suggests that miRNAs may serve as canalising genes, i.e. that miRNA regulatory networks evolved under natural selection in order to stabilise phenotypes and decrease the variability of specific traits (Hornstein and Shomron, 2006). For example, the muscle-specific miRNA *miR-1* is conserved in sequence and expression pattern throughout the bilaterian lineage. On this

basis, *miR-1* was presumed to play an important role muscle patterning or differentiation. However, the knockout of *miR-1* in *D. melanogaster* resulted in functional muscles that develop normally (Sokol and Ambros, 2005). Only at the onset of rapid larval growth phase does the phenotypic muscle crisis emerge, suggesting that *miR-1* confers robustness to the identity and physiology of muscle cells (Brennecke et al., 2005a). In this setting, *miR-1* is dispensable for muscle differentiation but upon growth stress conditions the lack of *miR-1*-mediated target interactions causes discernable phenotypic consequences and decanalisation of the network (Hornstein and Shomron, 2006).

### **miRNAs Operate As Pleiotropic RNAs with Diverse Function**

**WITH SUCH SEEMINGLY** diverse modes of regulation and action, it is difficult to assign a generic role to miRNA function. However, a few basic assertions can be made based on the hitherto mentioned examples: (1) miRNAs participate in regulatory networks driven by TF and signaling events that control cell fate and differentiation. miRNAs modulate these events through positive and negative feedback loops to facilitate cellular decisions and reinforce biological outcomes; (2) miRNAs can act as binary switches to help repress target protein output to inconsequential levels or as rheostats to dampen (tune) protein output to more optimal levels. This, in turn, depends on the nature and topology of the network to which the miRNA belongs, and the nature of specific miRNA:target interactions in a particular cell type; and (3) miRNAs are essential for the normal development of animals. However, since miRNAs tend to have highly tissue-specific expression patterns during later stages of development, miRNAs are most likely controlling particular aspects of terminal differentiation programs of individual cell types, i.e. miRNAs do not govern early cell/tissue fate establishment but are critical in later differentiation steps and in the maintenance of tissue identity and integrity.

It is noteworthy to mention that while many miRNAs participate in tissue development and identity, several vertebrate miRNAs partake in cell-autonomous processes associated with cellular physiology. For example, the liver-specific *miR-122* plays an important role in cholesterol biosynthesis and fatty acid metabolism (Esau et al., 2006; Krutzfeldt et al., 2005). Murine *miR-375* is specifically expressed in pancreatic islet  $\beta$ -cells and regulates glucose-stimulated insulin secretion and exocytosis through its interactions with *Myotrophin*. Restricted predominantly to keratinocytes, *miR-203* functions as a switch between proliferative and terminally differentiating compartments in vertebrate skin (Yi et al., 2008), while the miR-23 family members *miR-23a* and *miR-23b* play a more general role in controlling glutamine metabolism reactive oxygen species (ROS) homeostasis (Gao et al., 2009). Given the high number of miRNA genes identified so far, their regulation and diverse expression patterns along with the overwhelming abundance of putative miRNA:target interactions, it is not surprising that miRNAs have been implicated in a broad spectrum of diseases, including cancer.

# CANCER

---

**THE TERM CANCER** encompasses more than 100 distinct diseases with diverse risk factors and epidemiology which originate from most of the cell types and organs of the human body and which are characterized by relatively unrestrained proliferation of cells that can invade beyond normal tissue boundaries and metastasize to distant organs (Stratton et al., 2009). With such an extensive range of origins and features, it is difficult to pinpoint a single attribute common to all cancers. Nonetheless, cancer can be broadly described as a genetic, disease-causing accumulation of mutations which arises through a multistep, evolutionary process occurring among somatic cell populations within the microenvironments provided by the tissues of a multicellular organism (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 1993). In this respect, malignancy can be considered both a penalty and a potent selection force in the evolution of multicellularity. On one hand, normal physiology requires large, continuously renewing cellular populations, while on the other hand every cell division risks the initiation of evolutionary events leading to a fatal malignancy.

Analogous to Darwinian evolution of species, cancer development resembles evolution on the somatic level, characterised by the continuous acquisition of heritable genetic variation in individual cells by random mutation and natural selection acting on the resultant phenotypic diversity (Stratton et al., 2009). In fact, carcinogenesis can be considered as two evolutionary competing processes: (1) potential cancer-forming cells evolving with adaptations to overcome the tumor suppression mechanisms of the host organism; and (2) the organism evolving strategies to reduce the probability of death from cancer prior to and during reproductive maturity (Gatenby et al., 2010). Constraining the emergence of cancer has thus become an evolutionary imperative in metazoans, especially in large, long-lived organisms with tissues that continuously regenerate (Lowe et al., 2004).

Cell growth, cell division, and the programmed elimination of cells (apoptosis) are tightly controlled processes which act to benefit multicellular organisms in response to environmental cues. The precise regulation of these processes is a requisite to the proper functioning of the organism. Occasionally, a single cell acquires a set of sufficiently advantageous mutations that allows it to proliferate autonomously, invade tissues and metastasize. However, no single mutation is sufficient to cause cancer; rather cancer results from various genetic and epigenetic alterations (i.e. heritable changes in gene expression that are not accompanied by changes in DNA sequence) that eventually confer cells with an ability to escape normal regulation. Most (if not all) malignant tumors acquire the same set of functional capabilities, commonly referred to as the hallmarks of cancer. These include (1) self-sufficiency in growth signals; (2) insensitivity to antigrowth signals; (3) evasion of apoptosis; (4) limitless replicative potential; (5) sustained angiogenesis; and (6) tissue invasion and metastasis (Hanahan and Weinberg,

2000). Once endowed with these properties, a neoplastic cell rapidly gains a proliferative advantage and eventually outcompetes its surroundings in order to thrive and grow (Nowell, 1976).

## **Adult Cancer**

**WHATEVER THE ROUTES** or mechanistic strategies needed to reach malignancy may be, the progressive conversion of normal cells into cancer cells is fundamentally a function of three aspects: inheritance, the environment, and changes over time (evolution). First, with respect to inheritance, most attributes of tumor development can be explained by genetic alterations of primary DNA sequence. While some mutations are inherited through the germline, others appear *de novo* in somatic cells and are restricted to the neoplastic lineage. Additionally, epigenetic information, encoded as binomial patterns of DNA methylation, also participates in cancer progression (Feinberg and Tycko, 2004; Jones and Baylin, 2007). As a heritable disease, cancer constitutes the propagation of genetic and epigenetic information, either inherited or transmitted, on a cellular and multicellular level. Second, epidemiologic data has demonstrated a clear association between cancer development and environmental factors like smoking, diet, and radiation (Vineis and Berwick, 2006). Numerous studies have substantiated the causal relationship between environmental mutagens and the initiation of cancer (Soto and Sonnenschein, 2010). Finally, cancer is, by in large, an age-related disease that develops over long periods of time. Cancer incidence is known to increase dramatically with age (Vogelstein and Kinzler, 1993) and virtually all individuals over the age of 50 carry some kind of *in situ* carcinoma (Folkman and Kalluri, 2004). Genes and mutations thus evolve in dynamic interaction with the surrounding environment, and cancer development is best described as an evolutionary process within the organism (Gatenby et al., 2010). Accordingly, cancers in adults result from a multistep process and often progress over many years or decades.

## **Childhood Cancer**

**THIS IS IN** contrast to tumors that develop during childhood (0-15 years of age), which set them apart from adult (solid) tumors. Childhood tumors, including neuroblastomas, Wilm's tumor, retinoblastomas, lymphomas, and CNS malignancies, are fundamentally developmental disorders; the nature of the progenitor cells from which these tumors arise allows these cancers to develop with fewer defects in cell regulatory processes than adult cancers. Much of the cellular behavior (e.g. spontaneous regression) which typically characterises pediatric cancers derives from their developmental nature. Further, processes which typify adult cancers (proliferation, survival, self-renewal, and migration), are all aspects of normal developmental programs. Essentially, cancers that arise during prenatal and postnatal development are driven by normal growth and

differentiation-promoting mechanisms associated with organogenesis, tissue growth, and maturation (Scotting et al., 2005).

The relationship between childhood cancer development and the onset of organogenesis is supported by the fact that the age-specific pattern of these tumors often coincides with the periods of maximum growth of the related normal tissue. Neuroblastoma, Wilms' tumor, and hepatoblastoma occur in children younger than four years of age, whereas bone tumors, germ-cell tumors, and Hodgkin's disease occur predominantly in adolescents (Altekruse et al., 2009). The immature tissue environment represents an additional feature intimately linked to the onset and maintenance of perinatal cancers. In developing tissues, the progeny of most dividing cells are primarily more dividing cells, providing a favorable context for errors in DNA replication and a microenvironment in which proliferation is favored over differentiation (Scotting et al., 2005). Perturbations in the microenvironment can therefore accelerate uncontrolled cell growth and survival of immature cells that retain the ability to undergo high levels of proliferation and migration.

Likewise, the changing environment of the growing host can partially explain the biological and clinical heterogeneity observed in childhood cancers, but not in adult cancers, such as spontaneous regression. It should also be noted that many childhood tumors, with the exception of high-risk neuroblastoma, do not necessarily follow the dogmatic route of transformation during tumor progression, such as chromosomal instability, aneuploidy, or amplification of genetic damage. For example, infant teratomas generally show no signs of gross genetic damage and childhood cancer cells generally have fewer cytogenic defects than adult tumors (Oosterhuis and Looijenga, 2005). With fewer aberrations, childhood malignancies offer more therapeutic avenues and respond more readily to biologically targeted interventions (e.g. retinoic acid treatment for neuroblastoma and acute myeloid leukemia). In summary, childhood tumors represent a unique category of neoplasms that deviate from adult-onset cancers, both in their cell biology and their tissue microenvironment.

## **Tumor Suppressor Genes and Oncogenes**

**THE THREE FEATURES** of cancer development mentioned in the earlier section (inheritance, environment, and time) have been consolidated in a linear model, which has long served as a standard paradigm of carcinogenesis (Arends, 2000; Fearon and Vogelstein, 1990). Central to this model are the two categories of genes that are affected by mutational events during tumor initiation and progression: oncogenes and tumor suppressor genes (TSG) (Vogelstein and Kinzler, 1993).

Oncogenes are classified as genes that have acquired gain-of-function mutations (or exhibit abnormal expression levels) and are capable of promoting cancer. Under normal circumstances, oncogenes are referred to as proto-oncogenes and function to regulate cell growth and differentiation. Proto-oncogenes are frequently involved in signal transduction pathways, and play important roles in the execution of mitogenic signals.

Upon inappropriate activation however, a proto-oncogene (or its product) may, in the presence of a selective environment, promote cell autonomy and thereby support transformation and tumorigenesis. Examples of proto-oncogenes include *c-MYC*, *GLI*, and *RAS*.

Tumor suppressor genes, on the other hand, represent gene products that can function to counteract the onset of malignancy. Since the cancer-preventive effects of TSGs usually require the presence of only a single functional gene, prototypic tumor suppressor genes are recessive, requiring “two-hit” inactivation of both alleles, a concept formulated by Alfred Knudson in 1971 following studies of inherited and sporadic cases of retinoblastoma (Knudson, 1971; Sherr, 2004). TSGs are commonly inactivated (via deletions, mutations, or epigenetically) in human tumors and participate in a multitude of critical cellular processes, including apoptosis, senescence, and DNA repair. Accordingly, the involvement of TSGs in cell cycle checkpoint control, mitogenic signaling pathways, protein turnover, DNA damage, hypoxia, and other stress responses reflects the broad spectrum of cell-autonomous processes that can be deregulated in cancer cells (Sherr, 2004). Bona fide TSGs include *p53*, *PTEN*, and *RB*, all of which are frequently deregulated in a vast majority of cancers (Harris and Hollstein, 1993; Li et al., 1997).

Since its formulation, the ‘standard model’ has embodied the efforts of cancer research in its pursuit to describe the multifaceted nature of cancer development. These efforts have primarily focused on identifying the genetic and/or epigenetic alterations of protein coding elements in the genome. With the realisation that noncoding RNAs, notably miRNAs, play functional roles in developmental and cancer-associated processes, cancer should now be regarded as a complex genetic disease involving countless abnormalities in both coding and non-coding genes.

## The MYC Oncoprotein

**COMPARABLE TO THE** *p53* gene and its protein product (which are mutated in more than 50% of all human cancers), the prevalence of MYC deregulation in human cancers is staggering (Levine et al., 2004). No category of tumor, whether it be adult or pediatric, solid or hematological, has managed to entirely elude MYC, and the broad spectrum of neoplasms associated with MYC perturbations reflects its central role in the onset and progression of tumorigenesis (Nesbit et al., 1999).

Originally identified as the cellular homologue of the avian acute leukemic (MC29) viral transforming sequence, *c-myc* was the first cellular oncogene shown to be activated through retroviral promoter insertion (Hayward et al., 1981). Unlike the other proto-oncogenes discovered during this pioneering period of cancer research (e.g. *RAS*), MYC activation was not the result of mutations in its coding sequence. Instead, novel mechanisms, including insertional mutagenesis, chromosomal translocations, and gene amplification, were found to deregulate MYC (and other oncogenes), providing new

paradigms for the genetic basis of cancer. The function of MYC, in both normal and pathological settings, has been intensively examined ever since, and the *MYC* gene family ranks among the most exhaustively studied groups of genes in biology (Eilers and Eisenman, 2008).

Briefly, the *MYC* family of proto-oncogenes encodes a number of transcription factors (c-MYC, MYCN, and L-MYC) that heterodimerise with the cofactor MYC-associated protein X (MAX), bind DNA, and activate a substantial portion of coding and non-coding elements in the genome (Cawley et al., 2004; Fernandez et al., 2003). Functionally, Myc-activated genes serve to stimulate cell growth by initiating ribosome biogenesis, protein synthesis, metabolism, and cell cycle progression. Simultaneously, genes involved in cell-cycle arrest, cell adhesion, and cell-cell communication are inhibited. The manner in which gene expression is restricted upon MYC activation is less clear but at least one mechanism has been reported to involve MYC:MAX-dependent displacement of co-factor recruitment to genes bound by the zinc finger transcriptional activator Miz-1 (Herold et al., 2002; Staller et al., 2001). Furthermore, MYC:MAX complexes are counteracted by the MXD family proteins, which antagonise MYC function by sequestering accessible MAX and, as heterodimers (MNT:MAX or MAD:MAX), promote repression at genomic loci otherwise activated by MYC (Eilers and Eisenman, 2008).

Taken together, MYC possesses the capacity to both activate (directly) genes that stimulate growth and deactivate (directly and/or indirectly) genes that abrogate cell cycle inhibition. Thus, by responding to both external and internal cues, MYC proteins modulate and influence a myriad of cellular processes, including proliferation, growth, apoptosis, metabolism, and differentiation, all of which are frequently deregulated in MYC-dependent cancers (Albihn et al., 2010).

## miRNAs In Cancer

**THE EARLIEST COMPREHENSIVE** evidence connecting miRNAs to cancer came from the observation that miRNA genes are greatly enriched in genomic loci known to undergo chromosomal rearrangements, deletion, and amplification (Calin et al., 2004). More direct proof was attained when high-throughput technologies enabled the detection of miRNA expression on a genome-wide level, which revealed that abnormal expression of miRNAs was a common feature of all tumors investigated (Lu et al., 2005; Volinia et al., 2006). Interestingly, these studies reported a global downregulation of miRNA expression in cancer cells, with the exception of a few, highly overexpressed miRNA family members (e.g. members of the miR-17~92 family). These data were consistent with the hypothesis that in mammals, as in *C. elegans*, miRNAs can function to prevent cell division and drive terminal differentiation.

Indeed, global miRNA expression patterns reflect the state of cellular differentiation in tumors, highlighting the potential of miRNA profiling in cancer diagnosis. An

extension of this hypothesis suggested that differential expression levels of certain miRNAs might play a causal role in the generation or maintenance of tumors (Lu et al., 2005). The belief that miRNAs dysfunction can be a causative event in cancer pathogenesis has very recently been addressed in transgenic mouse models (Costinean et al., 2006; Dorsett et al., 2008; Klein et al., 2010; Medina et al., 2010). These studies clearly demonstrate that, similar to prototypic protein-coding oncogenes and TSGs, the misexpression of select miRNAs (even the misexpression of a single miRNA) is sufficient to promote and maintain tumor growth and survival *in vivo*. Below, I have highlighted examples of miRNAs with tumor-suppressive activity and miRNAs with oncogenic properties, paying special attention to the cancer forms which I have focused on during my PhD studies.

## Tumor Suppressor miRNAs

**THE FIRST miRNAs** demonstrated to have altered expression in tumor cells and causatively linked to cancer were miR-15a and miR-16-1 (Calin et al., 2002b). These miRNAs are clustered on chromosome 13 within the larger consensus minimal deleted region (MDR) 13q14, a region that is deleted in more than half of B-cell chronic lymphocyte leukemias (CLL) (Dohner et al., 2000). CLL represents the most common B cell-derived malignancy in the adult population, accounting for ~30% of all leukemias (Redaelli et al., 2004). An insidious disease with a chronic course, CLL is characterized by the clonal expansion of proliferating, neoplastic CD5<sup>+</sup> B lymphocytes (Chiorazzi and Ferrarini, 2003).

The course of the disease is variable and while some patients with CLL have a normal life span (indolent CLL), others present with more aggressive disease and die within five years after diagnosis (Rozman and Montserrat, 1995). In cases of disease progression, overexpression of *c-MYC*, deletions of the *Rb* gene, and mutations of the *p53* have been reported (Rozman and Montserrat, 1995). Nevertheless, the genetic cause of CLL remains unknown and its pathogenesis is obscure. On the one hand, CLL is a morphologically homogenous tumor, while on the other, it represents a lymphoma with high clinical heterogeneity. However, there are a few molecular and clinical features which unify CLLs. These include the presence or absence of somatically mutated immunoglobulin variable region (IgV) genes (i.e. pre-germinal versus post-germinal center leukemia), a set of different recurring chromosomal alterations, mainly deletions (including 13q14), and the accumulation of malignant CD5<sup>+</sup> B lymphocytes. In view of these characteristics, CLL might result from a multistep process, beginning with an antigen-driven polyclonal expansion of CD5<sup>+</sup> B lymphocytes that, in a mutationally conducive setting, would eventually transform into monoclonal proliferation.

The accumulation of malignant cells in CLL has long been considered a consequence of an inherent apoptosis defect, rather than excessive proliferation, since CLL cells frequently overexpress the antiapoptotic protein Bcl-2. In fact, it was suggested that loss of function of miR-15a and miR-16-1 promotes enhanced expression of Bcl-2 and thus

abnormal survival of CLL cells (Cimmino et al., 2005). Lately, these concepts have been challenged. For example, CLLs express significantly shorter telomeres than aged-matched B cells as a result of more frequent cell divisions (Damle et al., 2004). Furthermore, by measuring CLL cell kinetics *in vivo*, the production rate of leukemic cells in patients has been estimated to fall in the range of  $10^9 - 10^{12}$  per day (Messmer et al., 2004), reflecting the high proliferative capacity of CLL cells. Importantly, our careful analyses of miR-15a/16-1 function (see Paper I) strongly suggest a role in cell cycle progression, a finding that was recently corroborated *in vivo* using a *miR-15a/16-1* transgenic mouse model (Klein et al., 2010). Other studies have reached the same conclusion in *miR-15a/16-1*-deficient cancers, including non-small cell lung cancer and prostate cancer (Bandi et al., 2009; Bonci et al., 2008). A more thorough analysis of Paper I and its implications are provided in the Results and Discussion section.

**miR-203**, A miRNA preferentially expressed in the suprabasal layer of skin, represents another *bona-fide* tumor suppressor miRNA. Early insights into its function revealed that miR-203 acts as a switch between proliferation and differentiation during embryonic skin development (Yi et al., 2008). By altering the embryonic, spatiotemporal expression pattern of miR-203 *in vivo*, it was found that premature expression of miR-203 promoted epidermal differentiation by restricting the proliferative potential of targeted basal stem cells and inducing early cell cycle exit. When miR-203 was inhibited chemically using antagomiR technology (Krutzfeldt et al., 2005) in neonatal mice, antagomiR-203-treated dorsal skin showed clear elevations in epidermal proliferation and atypical expansion of *p63* expression, an essential regulator of stem cell maintenance in epithelial tissue (Yi et al., 2008).

These findings point to an antiproliferative function for miR-203 in the skin and, as evidenced by its tumor-suppressing effects in select hematopoietic malignancies, suggests that loss of miR-203 expression and/or function may promote tumorigenesis (Bueno et al., 2008). As miR-203 is preferentially expressed in the skin, it is highly probable that significant disruption of miR-203 levels in the epidermis may contribute to various types of skin disorders, such as skin cancer. Paper III in this thesis demonstrates that severely reduced expression of miR-203 is a typical feature of basal cell carcinoma (BCC), resulting in a deregulation of critical genes in the Hedgehog (Hh) pathway and subsequent hyperproliferation of transformed keratinocytes.

BCCs, the most common malignancy among persons of European ancestry, are keratinocyte tumors and appear as slow-growing, elevated lesions on sun-exposed skin (Epstein, 2008). Exposure to ultraviolet radiation is generally accepted as the major cause of BCCs, although a rare, familial variant of BCCs frequently occurs in basal cell nevus syndrome (BCNS) patients (commonly referred to as Gorlin syndrome) (Rubin et al., 2005). Despite the high incidence of BCCs among Caucasians, these tumors rarely metastasize; nevertheless, they can cause significant tissue destruction by tissue invasion.

Central to both sporadic and familial BCCs is the inappropriate activation of the Hh signaling pathway, originally identified as a determinant of segment polarity in *D. melanogaster* (Nusslein-Volhard and Wieschaus, 1980). Deregulation of the HH pathway is also common to other tumors, such as medulloblastoma and rhabdomyosarcoma (Taipale and Beachy, 2001). Briefly, secreted Sonic hedgehog (SHH) protein binds the TSG patched homologue 1 (PTCH1), relieving its suppression of smoothened (SMO). SMO signaling culminates in the activation of the Gli family of transcription factors (GLI1, GLI2, and GLI3), which execute SHH-induced signals. As the constituents of the HH pathway are intimately associated with proliferative responses in target cells, proteins belonging to the HH network are frequently misexpressed in BCCs (including loss-of-function mutations of *PTCH1* and gain-of-function mutations of *SMO*). Our work presented in Paper III of this thesis establishes that miR-203 plays an intricate role in fine-tuning key components of the HH network. By assuring the precise protein output of downstream effectors (e.g. *c-MYC* and *c-JUN*) of Hh signaling and other mitogenic pathways, miR-203 constitutes a 'gatekeeper' miRNA controlling keratinocyte proliferation.

## Oncogenic miRNAs

**IN 2005, TWO** seminal papers published in Nature provided the first demonstration that miRNAs are functionally integrated into oncogenic pathways and participate in cancer development (He et al., 2005; O'Donnell et al., 2005). Analyses of a genomic locus (13q31.3) frequently amplified in various lymphomas and solid tumors revealed that *C13orf25*, the only gene found to be upregulated within this amplicon, was a non-coding miRNA polycistron encoding six miRNAs: miR-17, miR-18a, mi-19a, miR-19b-1, miR-20a, and miR-92a. Collectively referred to as the miR-17~92 cluster, enforced expression of these miRNAs hematopoietic stem cells were found to dramatically accelerate the onset of B cell lymphoma in the Eμ-*myc* transgenic mouse model, providing direct evidence that the miR-17~92 cluster has oncogenic activity *in vivo* (He et al., 2005). In the same issue of Nature, O'Donnell et al. reported that the miR-17~92 cluster is transcriptionally activated by the transcription factor *c-MYC* (O'Donnell et al., 2005). Since then, expression profiling studies have revealed a widespread overexpression of miR-17~92-derived miRNAs in several tumor subtypes, including cancers of the breast, colon, brain, prostate, lung, and nervous system (Volinia et al., 2006). The second paper included in this thesis (Paper II) demonstrates the molecular outcome of abnormal miR-17~92 expression in *MYCN* amplified neuroblastoma (NB), an embryonal tumor derived from the sympathetic nervous system (SNS).

NB is an embryonal tumor derived from the immature cells of the developing SNS and represents the most common cancer of infancy that occurs during the first year of life (Altekruse et al., 2009). NB tumors account for more than 7% of malignancies in patients younger than 15 years and approximately 15% of all pediatric oncology deaths (Maris et al., 2007). The clinical hallmark of neuroblastoma is heterogeneity, with the

likelihood of tumor progression varying widely according to anatomic stage and age at diagnosis. Although great improvements in disease outcome have been observed in certain well-defined subsets of patients, the outcome for children with high-risk NB remains poor, with long-term survival still less than 40% (Maris et al., 2007). In general, children diagnosed before 1 year of age and/or with localized disease are curable with surgery and little or no adjuvant therapy. Intriguingly, some of these tumors undergo spontaneous regression or differentiate into benign ganglioneuromas (Matthay, 1999), referred to as stage 4S (S=special) disease. This striking clinical phenotype, which occurs in about 5% of cases, was first described by D'Angio and colleagues, who observed that infants with small, localised primary tumors accompanied by metastases to liver, skin, or bone marrow frequently experience spontaneously regression (D'Angio et al., 1971). In contrast, older children often have extensive hematogenous metastases at diagnosis, and the majority die from disease progression despite intensive multimodal therapy.

The clinical diversity observed in patients correlates closely with several molecular biologic features of neuroblastoma. Activating, germ-line mutations in the anaplastic lymphoma kinase (ALK) oncogene were recently discovered in hereditary NB, and occasionally occur in sporadic NBs (Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). The genetic events underlying sporadic neuroblastoma are less clear, although enrichments of single-nucleotide-polymorphisms (SNP) within the non-coding RNA *FLJ22536* and the DNA-repair ligase *BARD1* have been detected in patients with progressing disease (Capasso et al., 2009; Maris et al., 2008). The biological variables that define advanced stage disease include 1p36 deletion (with subsequent loss of the p53-regulated tumor-suppressor *miR-34a*), allelic loss of 11q, 17q22-qter gain. In addition, amplification of the *MYCN* locus, present in ~20 to 30% of all cases, represents the most important genetic aberration, and is strongly related to poor clinical diagnosis (Maris et al., 2007).

Originally cloned in 1983 by identifying an amplified DNA sequence with partial homology to the *c-MYC* gene, *MYCN* amplification is strongly associated with rapid disease progression and low event-free survival (Brodeur, 2003; Schwab et al., 1983). From a pathway perspective, poor outcome neuroblastomas (including all *MYCN*-amplified cases) exhibit elevated signaling through the MYC transcriptional network (*c-MYC*, *MYCN*, and *L-MYC* target genes), along with low expression of lineage marker genes relating to late neuronal differentiation. Additionally, these gene expression traits were not only present in high-risk tumors, but also in patients with tumors initially diagnosed as low or intermediate risk that ultimately had an adverse outcome (Fredlund et al., 2008). In agreement with these findings, NB tumors with high MYC pathway activity also display specific miRNA gene signatures, including elevated expression levels of miRNAs belonging to the miR-17~92 cluster (Mestdagh et al., 2010).

Our findings in Paper II suggest that specific expression changes in miR-17~92 cluster-derived miRNAs may promote tumorigenic behavior of *MYCN*-amplified NB, the consequences of which are discussed in further detail in the following sections.



## 2. AIMS

---

**THE OVERALL PURPOSE** of this thesis was to explore the role of miRNAs in cancer in order to gain a more complete molecular understanding of the mechanisms underlying select childhood and adult malignancies. More specifically, this thesis aimed to:

- Characterise the regulation and function of the *miR-15a/miR-16-1* host transcript *DLEU2* and examine its role in tumor development, with particular emphasis on CLL (Paper I)
- Investigate the molecular consequences and functional outcome of abnormal miRNA transcription in *MYCN*-amplified NB (Paper II)
- Identify and elucidate the mechanism(s) by which abnormal miRNA expression in the epidermis may contribute to carcinogenesis of the skin and BCC tumor formation (Paper III)

## 3. RESULTS AND DISCUSSION

---

### PAPER I.

#### ***DLEU2*, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1**

---

**IN THEIR EFFORTS** to clone a tumor suppressor gene at the 13q14 locus, Calin and coworkers were the first to show a causal link between miRNAs and cancer when they discovered that *miR-15a* and *miR-16-1* reside in region commonly deleted in CLL. Specifically, they claimed that the 13q14 MDR in CLL includes *miR-15a* and *miR-16-1* and occurs in 70% of all CLLs (Calin et al., 2002a). This is in contrast to a recent expression profiling study which demonstrated that only 11% (6 of 56 samples) of all CLL cases analysed had exceedingly low expression of miR-15a/miR-16-1 (Fulci et al., 2007). Furthermore, only three of these six patients showed biallelic loss of 13q14 (Fulci et al., 2007), suggesting that alternative mechanisms contribute to miR-15a/miR-16-1 loss-of-function in CLL. Germline mutations affecting pri-miR-15a/16-1 processing have been detected, but represented only a trivial fraction of patients analysed (2 of 75 CLL cases) and must therefore be considered a rare event (Calin et al., 2005). A similar finding has been reported in New Zealand Black (NZB) mice that spontaneously develop a lymphoproliferative disorder highly reminiscent of CLL. These mice carry a syntenic point mutation in the 3' flanking region of miR-16-1, resulting in decreased expression of miR-16 (Raveche et al., 2007). While biallelic loss and germline mutations negatively impact miR-15a/16-1 expression levels, these genetic perturbations do not account for all cases of *miR-15a* and *miR-16-1* dysregulation in CLL. Moreover, breakpoint analyses of the 13q14 MDR in CLL consistently demonstrate that the *miR-15a* and *miR-16-1* genes are frequently retained following deletion (Lerner et al., 2009; Liu et al., 1997). Instead, the refined 13q14 MDR involves the deletion of portions of *DLEU1* and *DLEU2*, both of which are non-coding transcripts oriented in a sense/antisense fashion. Given that *DLEU2*, in contrast to *DLEU1*, is evolutionarily conserved (as are *miR-15a* and *miR-16-1* positioned

intronic in *DLEU2*), it seems more plausible that *DLEU2* represents the candidate tumor suppressor targeted by deletions and/or deregulation in CLL. In Paper I, we set out to investigate the regulation and function of the *miR-15a/miR-16-1* host transcript *DLEU2* and examine its role in CLL tumorigenesis.

As mentioned previously, most mammalian miRNA genes are clustered in the genome, allowing them to be transcribed simultaneously as polycistronic transcription units (Altuvia et al., 2005; Bartel, 2004). In addition, intronic incorporation and genomic miRNA aggregation allows for miRNAs to employ existing functional promoter elements. To determine if *DLEU2* functions as a bicistronic host gene for miR-15a and miR-16-1, we assessed if the maturation of *DLEU2* RNA was Drosha-dependent using RNAi. Knockdown of Drosha caused a significant accumulation of partially spliced *DLEU2*, yet did not affect the maturation of fully spliced *DLEU2*, suggesting that *DLEU2* transcripts are normally processed by Drosha in order to liberate functional miRNAs.

To further assess the regulatory features of *DLEU2*, we decided to investigate the role of both *DLEU2* promoters and additionally assess the relevance of putative regulatory units just upstream of miR-15a/miR-16-1. As a recent report had demonstrated that MYC transcriptionally represses a substantial portion of miRNAs, including miR-15a and miR-16-1 (Chang et al., 2008), we decided to test if reduction of miR-15a/miR-16-1 expression was associated with concomitant *DLEU2* downregulation. Using a doxycycline-regulated model system in which MYC expression can be turned off (Schuhmacher et al., 1999), we found that both miR-15a/miR-16-1 and *DLEU2* expression levels increased in the absence of Myc. Further, our chromatin immunoprecipitation (ChIP) assays implied that reduced expression of *DLEU2* is the result of direct Myc-mediated transcriptional binding of the two alternative *DLEU2* promoters. In contrast, no binding was detected to any of the loci downstream of the first two *DLEU2* exons, including the most conserved region immediately upstream of *miR-15-a/miR-16-1*. These findings conclude that *DLEU2* functions as a host gene for *miR-15-a/miR-16*.

Next, we wanted to analyse the function of miR-15-a/miR-16-1. In order to assess the tumor-suppressive effects of *DLEU2*, we performed colony formation assays in osteosarcoma (U2OS) and breast adenocarcinoma (MCF-7) cells. To this end, we cloned the *miR-15-a/miR-16*-containing *DLEU2* transcript into a cytomegalovirus promoter (CMV)-driven expression vector. As expected, transfection of the *DLEU2* plasmid resulted in the rapid upregulation of miR-15a and miR-16-1 in several cell lines, demonstrating that proper miRNA processing occurs after vector expression. We also generated a deletion mutant, *DLEU2-ΔMIR* that lacks miR-15a and miR-16-1. Ectopic expression of *DLEU2* led to an ~80% decrease in colony number as compared to both mock- and *DLEU2-ΔMIR*-transfected cells. In parallel, DNA histogram analysis performed on HEK293 and U2OS cells transfected with *DLEU2* showed a clear G<sub>1</sub> arrest, suggesting an antiproliferative as opposed to an antiapoptotic effect. G<sub>1</sub> arrest in *DLEU2* expressing cells was accompanied by hypophosphorylation of the Rb protein, further indicating that cell cycle entry was abrogated.

Although it was initially reported that miR-15-a and miR-16-1 target the anti-apoptotic gene *bcl-2* (Cimmino et al., 2005), others have reported that miR-15-a/miR-16-1 function to inhibit cell cycle progression (Huang et al., 2007; Linsley et al., 2007), a notion that was recently confirmed *in vivo* using a *miR-15a/16-1* transgenic mouse model (Klein et al., 2010). Using the PicTar and miRBase algorithms, we searched for putative miR-15-a/miR-16-1 targets and identified Cyclin D1 and Cyclin E1 as high confidence predictions. Transfection of *DLEU2* into several different cell lines resulted in prominent downregulation of Cyclin D1 and Cyclin E1 on protein level. RT-PCR and cyclohexamide chase analysis established that the *DLEU2*-mediated repression of the G<sub>1</sub>-related cyclin proteins was not the result of a decreased mRNA levels or increased cyclin protein turnover. *DLEU2*-ΔMIR-transfected cells did not show protein abundance alterations of Cyclin D1 and Cyclin E1. Importantly, luciferase reporter assays established that the *DLEU2*-mediated repression of Cyclin D1 and Cyclin E1 was dependent on the 3'UTRs of these targets.

Taken together, this study delineated a functional role of the *miR-15a/miR-16-1* host transcript *DLEU2*. The identification of *DLEU2* as a regulatory host gene of these miRNAs elucidates how CLL deletions with *miR-15a/16-1* retention may still result in their functional loss. The refined 13q14 MDR suggest that that loss of the promoters (and perhaps the first exons of *DLEU2*) result in the functional loss of these miRNAs. This notion is supported by the absence of miR-16-1 expression in CLL case 4 that retains *miR-15a/miR-16-1* at the genomic level while deleting both alternative *DLEU2* promoters (Figure 1). We also show that *DLEU2* is negatively regulated by MYC, providing yet another mechanism by which *DLEU2* may be deregulated in CLL and other cancers, some of which are Myc-dependent (Bonci et al., 2008). These data also demonstrate how activation of MYC can lead to the induction of multiple G<sub>1</sub> cyclins in a posttranscriptional manner. The exact molecular features relating to miR-15a and miR-16-1 interactions during cell cycle dynamics remain to be addressed. Their constitutive expression across the cell cycle phases (unpublished data) suggests that miR-15a and miR-16-1 may act as an additional regulatory layer to ensure both the precise expression of G<sub>1</sub> cyclins levels during G<sub>0</sub>/G<sub>1</sub>-S transition and to reduce potentially harmful leaky expression of these cyclins in later cell cycle phases.

## PAPER II.

### MYCN-regulated microRNAs repress estrogen receptor-alpha (ESR1) expression and neuronal differentiation in human neuroblastoma

---

**NEUROBLASTOMA HAS LONG** been considered a clinical enigma (Brodeur, 2003), owing to the incredibly diverse and often dramatic clinical behavior of NB tumors. NB accounts for disproportionate morbidity and mortality among childhood cancers while conversely representing the cancer with the highest incidence of spontaneous regression and complete tumor involution (Maris, 2010). While low risk and intermediate risk patients have a very high chance of survival, the outcome for children with high-risk NB remains poor, with long-term survival still less than 40% (Maris et al., 2007). A common feature of high-risk NB is *MYCN* amplification, which occurs in ~20% of primary NB tumors and is strongly correlated with advanced stage disease and treatment failure (Maris et al., 2007). While 1p36 deletions have been shown to precede *MYCN* amplification, the mechanisms underlying *MYCN*-mediated NB progression are poorly understood. In Paper II, we wanted to gain further insights into the molecular processes associated *MYCN*-amplified NB, with specific emphasis on deregulated miRNA expression patterns and downstream targets. This, we hoped, would advance our understanding of *MYCN*-amplified NB and uncover novel pathways that could serve as clinical alternatives in NB disease management.

To begin, we employed a genome-wide miRNA expression array in order to identify *MYCN*-regulated miRNAs, using Tet<sub>2</sub>1N NB cells with doxycycline-inducible *MYCN* expression. Our analyses revealed consistent ~2-fold overexpression of several *MYC*-associated miRNAs previously identified, including miRNAs from the oncogenic miR-17~92 cluster (e.g. miR-17, miR-18a, and miR-19a) and its paralogs on chromosome 7 and chromosome X (He et al., 2005; O'Donnell et al., 2005). We also noticed a robust decrease of miRNA expression in *MYCN* expressing cells, many of which exert tumor-suppressive functions in various cancers (Croce, 2009). Further, our chromatin immunoprecipitation (ChIP) assays confirmed that *MYCN*:MAX associated with canonical E-box sequences upstream of all polycistronic miRNA units assayed, resulting in the transcriptional activation of miR-17~92 cluster-derived miRNAs and its paralogs.

Next, we wanted to analyse the function of a subset of the miRNAs identified in our screen. It had previously been reported that overexpression of the miR-17~92 cluster strongly augments NB tumorigenesis both *in vitro* and *in vivo* (Fontana et al., 2008). While the oncogenic contribution of miR-17 and miR-20a in relation to NB was described in this paper, the biological significance of other miRNAs residing in the miR-17~92 polycistron (including miR-18a and miR-19a) was not addressed. We therefore decided to interrogate miR-18a and miR-19a function in *MYCN*-amplified NB cells using miRNA-specific inhibitors followed by fluorescence-activated cell sorting (FACS)

analysis. These initial experiments conclusively demonstrated that miR-18a and miR-19a affect cell cycle progression. To further extend this finding, we employed lentiviral constructs encoding specific antisense miRNA sequences designed to target miR-18a and miR-19a function. Long-term inhibition of miR-18a resulted in a dramatic phenotype, characterised by both morphological and biochemical differentiation, while the miR-19a knockdown was more difficult to assess. Nevertheless, our results suggested that miR-18a (and to a lesser degree miR-19a) provide *MYCN*-amplified cells with a proliferative advantage by deregulating messages linked to cell cycle progression and differentiation.

Using the PicTar and Targetscan algorithms, we identified several genes downstream miR-18a and miR-19a that are involved in neural and cancer-associated processes (such as *ROBO2*, *ATXN1*, *CCND2*, and *ESR1*). A literature survey provided the first evidence that estrogen receptor- $\alpha$  (*ESR1*) could potentially account for the phenotype we observed in our miRNA knockdown experiments. In 1993, Ma and colleagues described the direct involvement of activated *ESR1* in a series of morphological and biochemical changes that lead the transfected cells toward a differentiated state (Ma et al., 1993). Unintentionally, the authors used the *MYCN*-amplified cell line SK-N-BE, which, by virtue of its *MYCN* amplification, expresses high levels of miR-18a and miR-19a, potentially leading to the aberrant repression of endogenous *ESR1*. Importantly, upon ligand-dependent activation, ectopic *ESR1* in the genetically engineered NB cell line SK-N-E3 resulted in growth arrest (Ma et al., 1993). This led us to hypothesise that *MYCN*-regulated miRNAs might disrupt estrogen signaling sensitivity in cells derived from SNS precursors through deregulation of *ESR1*, thereby preventing the normal induction of neuroblast differentiation.

To experimentally assess this idea, we first used luciferase reporter assays to demonstrate that miR-18a and miR-19a repress *ESR1* expression through miRNA-binding elements in its 3'UTR. Western blot and quantitative PCR (qPCR) analyses of MCF-7 cells (which express high levels of endogenous *ESR1*) transfected with miR-18a and miR-19a mimic oligonucleotides confirmed that these miRNAs also target endogenous *ESR1*. Next, to reaffirm Ma's original work, we used lentiviral constructs expressing *ESR1* cDNA and transduced SK-N-BE(2) cells. Upon lentiviral reconstitution of *ESR1*, we observed cell cycle arrest and morphological changes indicative of differentiation.

In addition, we were able to demonstrate *ESR1* expression in human fetal sympathetic ganglia, suggesting that *ESR1* plays an important role during the development of the sympathetic nervous system. Finally, our Kaplan-Meier survival analyses of 251 NB patients indicate that *ESR1*-positive NBs are associated with a favorable disease outcome.

In summary, we have uncovered a novel mechanism by which miRNA-mediated deregulation of *ESR1* expression potentiates tumorigenesis in human neuroblastoma. On a far more fundamental level of neurobiology, the demonstration of *ESR1* expression in human fetal sympathetic ganglia suggests that *ESR1* may act as a key constituent in the specification/diversification of the neural crest-derived sympatho-adrenal (SA) lineage.

The importance of estrogen and its receptors during neural development is recapitulated in embryonic neuronal stem cells, which undergo differentiation in response to estradiol exposure (Brannvall et al., 2002). Analyses of *ESR1* expression during peripheral nervous system development in chicken embryos show *ESR1*-immunoreactivity in the dorsal root ganglia (DRG) at cervical, thoracic and lumbosacral levels, as well as in the sympathetic ganglia and primary spinal motoneurons (Cui and Goldstein, 2000). Interestingly, it has been shown that *ESR1* potentiates mRNA expression of the tyrosine kinase receptor *TrkA* using a cancer cell line derived from a pheochromocytoma (PC12 cells) of the rat adrenal medulla (Sohrabji et al., 1994a; Sohrabji et al., 1994b). *TrkA* is the high affinity catalytic receptor for the neurotrophin (NGF) and mediates the multiple effects of NGF, which includes neuronal differentiation. Both undifferentiated (naïve to NGF) and NGF-exposed, differentiated PC12 cells (which resemble the sympathetic neuron phenotype) express *ESR1* mRNA. While naive cells express very low levels of *ESR1* mRNA, NGF elicits a significant increase in *ESR1* mRNA. As in sensory neurons, NGF-mediated increases in *ESR1* expression resulted a ~3-fold upregulation of *trkA* mRNA (Toran-Allerand, 1996).

The cooperative interaction between *ESR1*, the neurotrophins and their receptors could be of great significance to the onset and progression of high-risk, *MYCN*-amplified NB. High levels of *TrkA* expression are correlated with younger age, lower stage, and absence of *MYCN* amplification. Furthermore, *TrkA* expression is highly correlated with favorable disease outcome (Brodeur, 2003). Thus, *ESR1*-positive NBs may represent a potential subgroup of tumors amenable to estrogen treatment, providing a means to force these tumors into spontaneous regression. Likewise, inhibition of miR-18a may represent a promising therapeutic opportunity for *MYCN*-amplified NB patients.

## PAPER III.

### miR-203 functions as a bona fide tumor suppressor microRNA in basal cell carcinoma

---

**BASAL CELL CARCINOMA** (BCC) is the most common type of malignant cancer in fair-skinned individuals. Common to both sporadic and familial BCCs is the inappropriate activation of the Hedgehog (Hh) signaling pathway, which plays a central role in the pathogenesis of BCC tumors, as well as medulloblastoma (MB) (Wicking et al., 1999). In sporadic BCCs, 10% show *SMO* gain-of-function mutations while ~70% of BCC tumors exhibit inactivating mutations in at least one allele of *PTCH1* (Epstein, 2008). Several murine models support the concept that aberrant Hh pathway activation is sufficient to drive development of BCCs or BCC-like tumors, including mice with constitutive or conditional overexpression of *GLI1* or of *GLI2*, activating *SMO* mutations, or *PTCH1* haploinsufficiency (Aszterbaum et al., 1999; Grachtchouk et al.,

2000; Nilsson et al., 2000; Xie et al., 1998). To date, all investigations on BCC tumor onset and development have focused on the mutations and/or expression of protein-coding genes, and although several advances have been made, a comprehensive molecular description detailing BCC pathogenesis is still lacking. To this end, we set out to investigate the role of miRNA deregulation in BCC tumors.

To explore the potential involvement of miRNAs in basal cell carcinoma, we performed the first comprehensive, genome-wide analysis of miRNA expression in human healthy skin and BCCs. Unsupervised hierarchical clustering based on miRNA expression clearly separated BCC tumor samples from healthy skin, and most miRNAs with significant differential expression were suppressed in BCC. These findings suggest that the altered expression of miRNA has a role in the pathogenesis of BCC and enforces the observation that global downregulation of miRNA expression is a common feature of solid tumors (Lu et al., 2005). Of all miRNAs assayed, miR-203 showed the greatest decrease (~5-fold) in expression in BCC when compared to healthy skin, a finding which was further validated in a larger set of healthy and BCC samples. Expression analyses using *in situ* hybridization with specific locked nucleic acid (LNA) probes demonstrated that miR-203 was preferentially expressed in the suprabasal layers of healthy skin, almost in a gradient-like fashion, while BCC tumors consistently lacked miR-203 expression.

Next, we wanted to explore the potential association between miR-203 downregulation and the activation of the Hedgehog pathway. Quantitative real-time PCR results showed that both *PTCH1* and *GLI1* were significantly overexpressed ( $p < 0.001$ ) in BCC tumors as compared with healthy skin, in accordance with previously published data. Further, correlation analysis showed a significant negative correlation between miR-203 expression and *GLI1*, as well as between miR-203 and *PTCH1*, suggesting that a loss-of-function of miR-203 may be associated with aberrant Hedgehog signaling in BCC.

Our next aim was to elucidate the potential mechanisms and/or pathways accounting for miR-203 suppression in BCC. Previous signal transduction analyses indicated that miR-203 is regulated by the PKC/AP-1 pathway and suppressed by growth factors, such as KGF and EGF in keratinocytes (Sonkoly et al., 2010). In BCC, EGFR has been shown to synergize with Hedgehog/GLI in oncogenic transformation via activation of the MEK/ERK/JUN pathway (Schnidar et al., 2009). Therefore, we set out to further explore the role of the EGFR signaling pathway in the regulation of miR-203. By measuring miR-203 expression in primary human keratinocytes treated with inhibitors of EGFR, MEK1/2, JNK, or Akt in combination with EGF or DMSO alone, we were able to determine that the EGFR-MAPK signaling pathway represses miR-203 expression, indicating that this pathway may negatively regulate miR-203 levels in BCC.

A bioinformatic search for putative miR-203 targets identified several genes in the MEK/ERK/JUN pathway, including c-JUN, as well as the Hh family member *PTCH1*. Interestingly, the c-MYC 3'UTR also harbors a miR-203 binding site, albeit poorly conserved. 3'UTR-based luciferase reporter assays subsequently demonstrated that all

genes identified were indeed regulated by miR-203. Moreover, cell-cycle analysis of primary keratinocytes transfected with miR-203 mimic oligonucleotides revealed a clear obstruction in the G<sub>1</sub> to S-phase transition of the cell cycle. Immunohistochemical (IHC) analyses of these targets in BCC tumors confirmed substantial increases of protein expression when compared to healthy skin. Furthermore, IHC analyses in skin collected from the transgenic K5-TreGli mouse (which develop skin tumors closely resembling human BCCs) corroborated our findings in human tumors: BCC tumors consistently exhibit severely reduced expression of miR-203, which dramatically influences the expression of genes involved in the regulation of cell proliferation and cell cycle, including key constituents of the Hh pathway (figure 6).

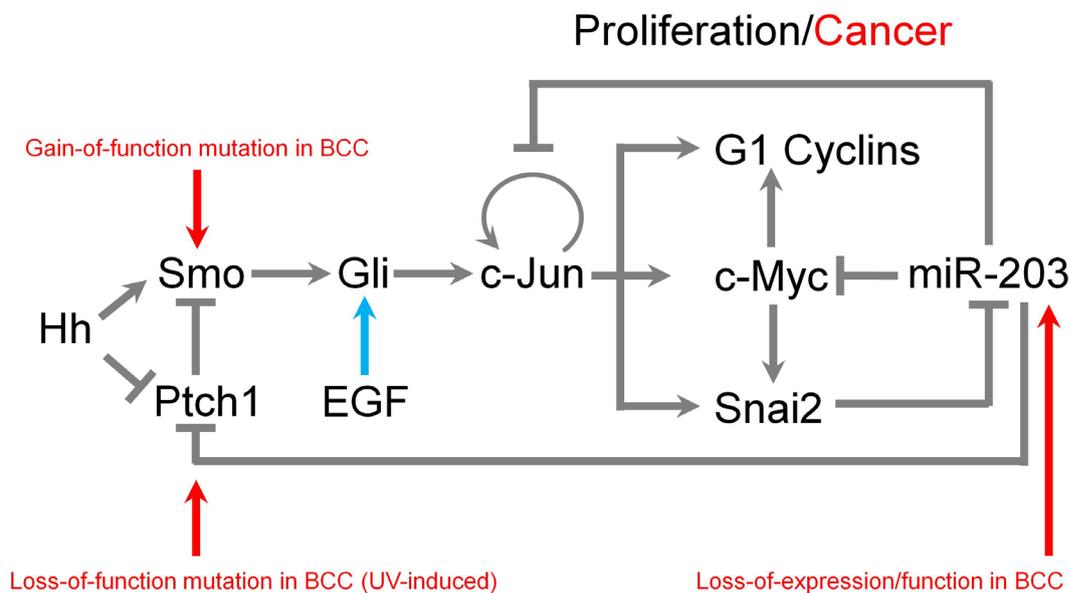


Figure 6. A proposed autoregulatory loop involving the Hedgehog (Hh) and MAPK pathways, the proto-oncogenes *c-JUN* and *c-MYC*, and the *bona fide* tumor suppressor miR-203. See text for details.

In physiological settings, Hh signaling is required for the proliferation of hair follicle epithelium during development (Chiang et al., 1999; St-Jacques et al., 1998) and postnatal hair cycles, which include a growth phase (anagen), regression phase (catagen), and resting phase (telogen) (Wang et al., 2000). While Hh signaling activity in follicle epithelium is restricted to periods of active growth and is limited by transient levels of Sonic hedgehog (SHH), this pathway is constitutively active in BCCs. Recently, it was demonstrated that BCCs arise from long-lived progenitor cells residing in the interfollicular epidermis (IFE), a stem-cell containing compartment that can give rise to all epidermal lineages in response to appropriate stimuli (Owens and Watt, 2003;

Youssef et al., 2010). Importantly, like other cell lineages of the mammalian epidermis, IFE stem cells respond to Hh signaling, which results in *de novo* hair follicle formation (Silva-Vargas et al., 2005). As BCC tumors have several characteristics in common with immature hair follicles (i.e. stem-cell properties), including similar histology, ultrastructure, and gene expression patterns, it has been proposed that BCC tumors (and other tumors) reflect aberrant organogenesis caused by defects in developmental signaling pathways, such as Hh (Hutchin et al., 2005; Millar, 2002). In this respect, the gradient-like expression pattern of miR-203 in the suprabasal layers of the epidermis may serve as a molecular barrier to limit proliferative cues in replenishing skin. The loss of miR-203 in BCC may thus facilitate the inappropriate activation of Hh signaling, causing the reiteration of molecular programs normally regulating skin and hair development and ultimately leading to tumors which phenotypically resemble immature hair follicles.

In conclusion, our study represents the first analysis of miRNA expression and function in a non-melanoma skin cancer. The loss of miR-203 in BCC suggests that this miRNA could be used as a biomarker of the disease and may represent a potential therapeutic target for the treatment of BCC, especially for the rare cases of metastatic, locally advanced BCCs where surgical treatment options are limited. Further investigation will be needed to demonstrate whether the molecular reconstitution of miR-203 may serve as a novel therapeutic strategy in the treatment of BCC tumors.

## 4. CONCLUDING REMARKS

---

**CONSIDERING THE FUNDAMENTAL** role of miRNAs in organismal development, cellular differentiation, metabolism, viral infection, and oncogenesis, the future of miRNA-based therapeutics holds great potential. In 2008, Elmén et al. demonstrated potent antagonism of miR-122 by the simple delivery of a unconjugated high-affinity LNA-antimiR oligonucleotide in mice and non-human primates (Elmen et al., 2008), providing a new molecular intervention strategy to target hepatitis C virus (HCV) in humans (Jopling et al., 2005). Moreover, the systemic administration of miR-26a in a mouse model of hepatocellular carcinoma (HCC) using adeno-associated virus (AAV) led to induction of tumor-specific apoptosis and dramatic protection from disease progression without measurable toxicity (Kota et al., 2009). Very recently, the first mechanistic evidence of RNAi in humans from an administered siRNA was demonstrated, verifying that RNAi can be used as a gene-specific therapeutic in human solid tumors (Davis et al., 2010). Together, these studies offer reassuring evidence that RNAi-based technologies are feasible in clinical settings and represent toxilogically sound approaches, with sparingly few side-effects reported so far (Bonetta, 2009). To conclude, the work provided herein has identified new miRNA targets that may be exploited therapeutically to treat childhood and adult malignancies. While delivery and specificity remain a challenge for many diseases, especially for cancer, these issues will hopefully be resolved at the same rapid pace as the field of RNAi research has developed in the last few years. The future looks bright.

## 5. ACKNOWLEDGEMENTS

---

**THE WORK PRESENTED** in this thesis was supported by the Ph.D. program for new doctoral students (KID-funding) at Karolinska Institutet (KI). I am also grateful to the Cancer Research Institute (USA) for financing the first year of my studies at MTC, KI.

On a more personal level, this thesis would not have been realised without the unyielding support of my family, friends, and colleagues. I am very thankful to have such loving, amusing, and intellectual people in my life. Without you, I wouldn't amount to much. I therefore want to express my deepest appreciation to all the kind and generous people who have inspired, encouraged, and helped me for 30 odd years. In particular, I want to thank the following:

My loving wife **Stella** and my beautiful son **Gustaf**. You are both all that matter. As I type these words, I realise how much has happened since I decided to commence my Ph.D. studies. **Stella**, this thesis is for us, our growing family and for our future together. Thank you for being such a good listener, for all your valuable advice, for your affection and endurance, for everything I could ever ask for. I love you from the bottom of my heart; I am very fortunate to be the person you wanted to share the rest of your life with. I look forward to every minute we have together. **Gustaf**, you are just as inquisitive as your father was at your age. Never stop interrogating the world around you, it's full of surprises. You give so many people so much happiness and warmth, including me. I love you; don't forget to be nice to your sister.

**Mamma** och **Pappa**, tack för allt stöd och all kärlek ni har givit mig. Jag kunde inte ha önskat mig finare föräldrar eller en härligare uppväxt. En bättre start i livet kan man inte få. Älskar er och saknar er hela tiden.

**Sofia**, my incredible sister. No one can *ever* accuse us for not working hard! You M.D., me Ph.D., who would have guessed? You were the best thing that happened when I was 3½ years old. I am very lucky to have you and I treasure our relationship dearly. Come and visit us soon.

**Marie Henriksson**, my supervisor. Over the years, you have helped me develop and mature, both on a scientific and personal level. Thank you for your patience, guidance, encouragement, and for believing in me so that I could explore my ideas freely and cultivate my passion for research and RNAi.

**Georg Klein**, professor emeritus. During my Ph.D. studies, I had the pleasure to interact with Georg on a regular basis at MTC. He has been, and continues to be, a true inspiration for so many scientists, myself included. Georg, thank you for your kind support, your sharp intellect, and your exquisite questions during our meetings.

**Members of the Henriksson Lab** – **Inga** and **Hanna**, keep up the good work. When the going gets tough, the tough get going. It's tough all the time. **Nikolay**, I wish you all the best with your Ph.D. studies. Just remember that you are going to have to write one of these. Either way, your work ethic is every PI's dream! **Therese**, your journey is soon over. Feel free to copy/paste anything you might need from this thesis. To the female post-doc trio **Anna**, **Ulrica**, **Margarita**, thanks for your advice and support along the way and for proof-reading all this bla bla bla. **Ulrica** again, don't forget to frequently remind Marie that estrogen receptor-alpha is the new Myc. **Hovanness** and **Melanie**, good luck with your scientific endeavors.

**Others at MTC** – **Petter Brodin**, grämt att vara klar! Tack för all din hjälp med mitt, hoppas all min hjälp med ditt kommer betala av sig fett snart. Kom över till Boston så gör vi något knasigt projekt ihop, får gärna vara ytterst komplicerat, både experimentellt och konceptuellt. **Emelie Flaberg**, sjövid i klädseln, behärskad vid mikroskopet, en oslagbar kombination! Tack för alla pratstunder, för att inte nämna den delikata fruktkorgen som damp ner i knät förra veckan, pluspoäng! **Speranta Puiaç**, you are an amazing person and great friend. I'm glad we had the chance to get to know one another. Good luck with everything! **Eduard Hejll**, hang in there buddy! **L-G Larsson**, thanks for taking the time to discuss my erratic ideas on Myc function from time to time. I'll let you know if any of these ideas ever holds up experimentally. **Adnane Anhour**, there lives a drama queen in every masculine man, even in men from Morocco. Thank you also to all the friendly members of L-G Larsson's group. **Hamid**, you really have to get that ChIP going soon!

Collaborators, near and far, who made the work easier and so much more interesting – **Igor Adameyko**, **Francoise Lallemand**, **Michael Andäng** (MBB, KI), it's been such a pleasure to work with you all. If I hadn't settled for cancer research, chances are that I would have been a pretty decent neurobiologist by now. Keep in touch. **Martin Corcoran**, **Mikael Lerner**, **Masako Harada**, and **Per Johnsson** (CCK, KI), the true miRNA pioneers at KI. **Martin** is the only person I know who can build a functioning apparatus from laboratory leftovers, true MacGyver style. **Mikael**, your hair doesn't

need more wax, ever. Thanks for an excellent collaboration and great discussions. **Per**, you taught me well! Enjoy your time in the Morris Lab. **Andor Pivarcsi** and **Enkiö Sonkoly (CMM, KI)**, the other branch of the miRNA family. You have been amazing to work alongside. I will miss our discussions about parenting intertwined with miRNA biology. I hope our BBC project gets the credit it deserves! **Florian Meisgen** and **Tianling Wei (CMM, KI)**, you are both in safe hands, thanks for all your help. I also want to extend my sincere gratitude to **Sven Pålman (LU)**, who appeared at just the right moment during my Ph.D. studies and helped us resolve some difficult questions we were facing in our project. **Erik Fredlund (LU)** was also instrumental in making our PNAS paper an exceptional piece of work, thanks. I also want to thank **Markus Hafner** from the **Tuschl Lab (Rockefeller University, NY)** for helping us with the deep-sequencing data and for being so forthcoming and helpful in every way possible.

Finally, I want to thank my all my friends who have stood by me in times of joy and in times of sadness. My life is bright and colorful thanks to all of you.

## 6. REFERENCES

---

---

- Albihn, A., Johnsen, J.I., and Henriksson, M.A. (2010). MYC in oncogenesis and as a target for cancer therapies. *Adv Cancer Res* 107, 163-224.
- Altekruse SF, Kosary CL, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlander N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK (eds). SEER Cancer Statistics Review, 1975-2007, National Cancer Institute. Bethesda, MD, [http://seer.cancer.gov/csr/1975\\_2007/](http://seer.cancer.gov/csr/1975_2007/), based on November 2009 SEER data submission, posted to the SEER web site, 2010.
- Aleman, L.M., Doench, J., and Sharp, P.A. (2007). Comparison of siRNA-induced off-target RNA and protein effects. *Rna* 13, 385-395.
- Alon, U. (2007). Network motifs: theory and experimental approaches. *Nat Rev Genet* 8, 450-461.
- Altuvia, Y., Landgraf, P., Lithwick, G., Elefant, N., Pfeffer, S., Aravin, A., Brownstein, M.J., Tuschl, T., and Margalit, H. (2005). Clustering and conservation patterns of human microRNAs. *Nucleic Acids Res* 33, 2697- 2706.
- Ambros, V., and Horvitz, H.R. (1984). Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science* 226, 409-416.
- Aravin, A.A., Hannon, G.J., and Brennecke, J. (2007). The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* 318, 761-764.
- Arends, J.W. (2000). Molecular interactions in the Vogelstein model of colorectal carcinoma. *J Pathol* 190, 412-416.
- Ason, B., Darnell, D.K., Wittbrodt, B., Berezikov, E., Kloosterman, W.P., Wittbrodt, J., Antin, P.B., and Plasterk, R.H. (2006). Differences in vertebrate microRNA expression. *Proc Natl Acad Sci U S A* 103, 14385-14389.
- Aszterbaum, M., Epstein, J., Oro, A., Douglas, V., LeBoit, P.E., Scott, M.P., and Epstein, E.H., Jr. (1999). Ultraviolet and ionizing radiation enhance the growth of BCCs and trichoblastomas in patched heterozygous knockout mice. *Nat Med* 5, 1285-1291.
- Babiarz, J.E., Ruby, J.G., Wang, Y., Bartel, D.P., and Blelloch, R. (2008). Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev* 22, 2773-2785.
- Baek, D., Villen, J., Shin, C., Camargo, F.D., Gygi, S.P., and Bartel, D.P. (2008). The impact of microRNAs on protein output. *Nature* 455, 64-71.

- Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A.E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 122, 553-563.
- Bandi, N., Zbinden, S., Gugger, M., Arnold, M., Kocher, V., Hasan, L., Kappeler, A., Brunner, T., and Vassella, E. (2009). miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. *Cancer Res* 69, 5553-5559.
- Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281-297.
- Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215-233.
- Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* 20, 1885-1898.
- Berezikov, E., Chung, W.J., Willis, J., Cuppen, E., and Lai, E.C. (2007). Mammalian mirtron genes. *Mol Cell* 28, 328-336.
- Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363-366.
- 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, 215-217.
- Bilodeau, S., Kagey, M.H., Frampton, G.M., Rahl, P.B., and Young, R.A. (2009). SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. *Genes Dev* 23, 2484-2489.
- Bohnsack, M.T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *Rna* 10, 185-191.
- Bonci, D., Coppola, V., Musumeci, M., Addario, A., Giuffrida, R., Memeo, L., D'Urso, L., Pagliuca, A., Biffoni, M., Labbaye, C., *et al.* (2008). The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nat Med* 14, 1271-1277.
- Bonetta, L. (2009). RNA-based therapeutics: ready for delivery? *Cell* 136, 581-584.
- Borchert, G.M., Lanier, W., and Davidson, B.L. (2006). RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* 13, 1097-1101.
- Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., *et al.* (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441, 349-353.
- Brannvall, K., Korhonen, L., and Lindholm, D. (2002). Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. *Mol Cell Neurosci* 21, 512-520.
- Brennecke, J., Stark, A., and Cohen, S.M. (2005a). Not miR-ly muscular: microRNAs and muscle development. *Genes Dev* 19, 2261-2264.
- Brennecke, J., Stark, A., Russell, R.B., and Cohen, S.M. (2005b). Principles of microRNA-target recognition. *PLoS Biol* 3, e85.

- Brodeur, G.M. (2003). Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 3, 203-216.
- 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, 496-506.
- Cai, X., Hagedorn, C.H., and Cullen, B.R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *Rna* 10, 1957-1966.
- Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., *et al.* (2002a). 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, 15524-15529.
- Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., *et al.* (2002b). Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America* 99, 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, 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, 2999-3004.
- Capasso, M., Devoto, M., Hou, C., Asgharzadeh, S., Glessner, J.T., Attiyeh, E.F., Mosse, Y.P., Kim, C., Diskin, S.J., Cole, K.A., *et al.* (2009). Common variations in BARD1 influence susceptibility to high-risk neuroblastoma. *Nat Genet* 41, 718-723.
- Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell* 136, 642-655.
- Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A.J., *et al.* (2004). Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell* 116, 499-509.
- Chalfie, M., Horvitz, H.R., and Sulston, J.E. (1981). Mutations that lead to reiterations in the cell lineages of *C. elegans*. *Cell* 24, 59-69.
- 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, 43-50.
- Chen, Y., Takita, J., Choi, Y.L., Kato, M., Ohira, M., Sanada, M., Wang, L., Soda, M., Kikuchi, A., Igarashi, T., *et al.* (2008). Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 455, 971-974.
- Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 436, 740-744.

- Chiang, C., Swan, R.Z., Grachtchouk, M., Bolinger, M., Litingtung, Y., Robertson, E.K., Cooper, M.K., Gaffield, W., Westphal, H., Beachy, P.A., *et al.* (1999). Essential role for Sonic hedgehog during hair follicle morphogenesis. *Dev Biol* 205, 1-9.
- Chiorazzi, N., and Ferrarini, M. (2003). B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. *Annu Rev Immunol* 21, 841-894.
- Cimmino, A., Calin, G.A., Fabbri, M., Iorio, M.V., Ferracin, M., Shimizu, M., Wojcik, S.E., Aqeilan, R.I., Zupo, S., Dono, M., *et al.* (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A* 102, 13944-13949.
- 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, 7024-7029.
- Croce, C.M. (2009). Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 10, 704-714.
- Cui, S., and Goldstein, R.S. (2000). Expression of estrogen receptors in the dorsal root ganglia of the chick embryo. *Brain Res* 882, 236-240.
- Czech, B., Zhou, R., Erlich, Y., Brennecke, J., Binari, R., Villalta, C., Gordon, A., Perrimon, N., and Hannon, G.J. (2009). Hierarchical rules for Argonaute loading in *Drosophila*. *Mol Cell* 36, 445-456.
- D'Angio, G.J., Evans, A.E., and Koop, C.E. (1971). Special pattern of widespread neuroblastoma with a favourable prognosis. *Lancet* 1, 1046-1049.
- Damle, R.N., Batliwalla, F.M., Ghiotto, F., Valetto, A., Albesiano, E., Sison, C., Allen, S.L., Kolitz, J., Vinciguerra, V.P., Kudalkar, P., *et al.* (2004). Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations. *Blood* 103, 375-382.
- Darnell, D.K., Kaur, S., Stanislaw, S., Konieczka, J.H., Yatskievych, T.A., and Antin, P.B. (2006). MicroRNA expression during chick embryo development. *Dev Dyn* 235, 3156-3165.
- Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., *et al.* (2002). A genomic regulatory network for development. *Science* 295, 1669-1678.
- Davis, M.E., Zuckerman, J.E., Choi, C.H., Seligson, D., Tolcher, A., Alabi, C.A., Yen, Y., Heidel, J.D., and Ribas, A. (2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* 464, 1067-1070.
- Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F., and Hannon, G.J. (2004). Processing of primary microRNAs by the Microprocessor complex. *Nature* 432, 231-235.
- Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. *Genes Dev* 18, 504-511.
- Dohner, H., Stilgenbauer, S., Benner, A., Leupolt, E., Krober, A., Bullinger, L., Dohner, K., Bentz, M., and Lichter, P. (2000). Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 343, 1910-1916.
- Dorsett, Y., McBride, K.M., Jankovic, M., Gazumyan, A., Thai, T.H., Robbiani, D.F., Di Virgilio, M., San-Martin, B.R., Heidkamp, G., Schwickert, T.A., *et al.* (2008).

- MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity* 28, 630-638.
- Duursma, A.M., Kedde, M., Schrier, M., le Sage, C., and Agami, R. (2008). miR-148 targets human DNMT3b protein coding region. *Rna* 14, 872-877.
- Eilers, M., and Eisenman, R.N. (2008). Myc's broad reach. *Genes Dev* 22, 2755-2766.
- Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjarn, M., Hansen, H.F., Berger, U., *et al.* (2008). LNA-mediated microRNA silencing in non-human primates. *Nature* 452, 896-899.
- Ender, C., Krek, A., Friedlander, M.R., Beitzinger, M., Weinmann, L., Chen, W., Pfeffer, S., Rajewsky, N., and Meister, G. (2008). A human snoRNA with microRNA-like functions. *Mol Cell* 32, 519-528.
- Epstein, E.H. (2008). Basal cell carcinomas: attack of the hedgehog. *Nat Rev Cancer* 8, 743-754.
- Esau, C., Davis, S., Murray, S.F., Yu, X.X., Pandey, S.K., Pear, M., Watts, L., Booten, S.L., Graham, M., McKay, R., *et al.* (2006). miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 3, 87-98.
- Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* 61, 759-767.
- Feinberg, A.P., and Tycko, B. (2004). The history of cancer epigenetics. *Nat Rev Cancer* 4, 143-153.
- Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. *Genes Dev* 17, 1115-1129.
- 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, 102-114.
- Folkman, J., and Kalluri, R. (2004). Cancer without disease. *Nature* 427, 787.
- Fontana, L., Fiori, M.E., Albini, S., Cifaldi, L., Giovinnazzi, S., Forloni, M., Boldrini, R., Donfrancesco, A., Federici, V., Giacomini, P., *et al.* (2008). Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS One* 3, e2236.
- Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* 408, 325-330.
- Fredlund, E., Ringner, M., Maris, J.M., and Pahlman, S. (2008). High Myc pathway activity and low stage of neuronal differentiation associate with poor outcome in neuroblastoma. *Proc Natl Acad Sci U S A* 105, 14094-14099.
- 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, 92-105.
- Fulci, V., Chiaretti, S., Goldoni, M., Azzalin, G., Carucci, N., Tavoraro, S., Castellano, L., Magrelli, A., Citarella, F., Messina, M., *et al.* (2007). Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. *Blood* 109, 4944-4951.
- Gao, P., Tchernyshyov, I., Chang, T.C., Lee, Y.S., Kita, K., Ochi, T., Zeller, K.I., De Marzo, A.M., Van Eyk, J.E., Mendell, J.T., *et al.* (2009). c-Myc suppression of miR-23a/b

- enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 458, 762-765.
- Gatenby, R.A., Gillies, R.J., and Brown, J.S. (2010). The evolutionary dynamics of cancer prevention. *Nature Reviews Cancer* 10, 526-527.
- George, R.E., Sanda, T., Hanna, M., Frohling, S., Luther, W., 2nd, Zhang, J., Ahn, Y., Zhou, W., London, W.B., McGrady, P., *et al.* (2008). Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 455, 975-978.
- Gilbert, W. (1986). Origin of Life - the Rna World. *Nature* 319, 618-618.
- Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., Hammond, S.M., Bartel, D.P., and Schier, A.F. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. *Science* 308, 833-838.
- Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* 312, 75-79.
- Grachtchouk, M., Mo, R., Yu, S., Zhang, X., Sasaki, H., Hui, C.C., and Dlugosz, A.A. (2000). Basal cell carcinomas in mice overexpressing Gliz in skin. *Nat Genet* 24, 216-217.
- Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123, 631-640.
- Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature* 432, 235-240.
- Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* 27, 91-105.
- Grimson, A., Srivastava, M., Fahey, B., Woodcroft, B.J., Chiang, H.R., King, N., Degnan, B.M., Rokhsar, D.S., and Bartel, D.P. (2008). Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature* 455, 1193-1197.
- Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* 106, 23-34.
- Gwizdek, C., Ossareh-Nazari, B., Brownawell, A.M., Doglio, A., Bertrand, E., Macara, I.G., and Dargemont, C. (2003). Exportin-5 mediates nuclear export of minihelix-containing RNAs. *J Biol Chem* 278, 5505-5508.
- Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H., and Kim, V.N. (2004). The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* 18, 3016-3027.
- Han, J., Lee, Y., Yeom, K.H., Nam, J.W., Heo, I., Rhee, J.K., Sohn, S.Y., Cho, Y., Zhang, B.T., and Kim, V.N. (2006). Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 125, 887-901.
- Han, J., Pedersen, J.S., Kwon, S.C., Belair, C.D., Kim, Y.K., Yeom, K.H., Yang, W.Y., Haussler, D., Belloch, R., and Kim, V.N. (2009). Posttranscriptional crossregulation between Drosha and DGCR8. *Cell* 136, 75-84.
- Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100, 57-70.

- Harris, C.C., and Hollstein, M. (1993). Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* 329, 1318-1327.
- Hayward, W.S., Neel, B.G., and Astrin, S.M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature* 290, 475-480.
- He, L., Thomson, J.M., Hemann, M.T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J., *et al.* (2005). A microRNA polycistron as a potential human oncogene. *Nature* 435, 828-833.
- Herold, S., Wanzel, M., Beuger, V., Frohme, C., Beul, D., Hillukkala, T., Syvaaja, J., Saluz, H.P., Haenel, F., and Eilers, M. (2002). Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell* 10, 509-521.
- Herranz, H., and Cohen, S.M. (2010). MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems. *Genes & Development* 24, 1339-1344.
- Hobert, O. (2004). Common logic of transcription factor and microRNA action. *Trends Biochem Sci* 29, 462-468.
- Hobert, O. (2008). Gene regulation by transcription factors and microRNAs. *Science* 319, 1785-1786.
- Hornstein, E., and Shomron, N. (2006). Canalization of development by microRNAs. *Nat Genet* 38 *Suppl*, S20-24.
- Hu, H.Y., Yan, Z., Xu, Y., Hu, H., Menzel, C., Zhou, Y.H., Chen, W., and Khaitovich, P. (2009). Sequence features associated with microRNA strand selection in humans and flies. *BMC Genomics* 10, 413.
- Huang, J.C., Babak, T., Corson, T.W., Chua, G., Khan, S., Gallie, B.L., Hughes, T.R., Blencowe, B.J., Frey, B.J., and Morris, Q.D. (2007). Using expression profiling data to identify human microRNA targets. *Nat Methods* 4, 1045-1049.
- Hutchin, M.E., Kariapper, M.S., Grachtchouk, M., Wang, A., Wei, L., Cummings, D., Liu, J., Michael, L.E., Glick, A., and Dlugosz, A.A. (2005). Sustained Hedgehog signaling is required for basal cell carcinoma proliferation and survival: conditional skin tumorigenesis recapitulates the hair growth cycle. *Genes Dev* 19, 214-223.
- Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293, 834-838.
- Iliopoulos, D., Hirsch, H.A., and Struhl, K. (2009). An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell* 139, 693-706.
- Jafar-Nejad, H., Acar, M., Nolo, R., Lacin, H., Pan, H., Parkhurst, S.M., and Bellen, H.J. (2003). Senseless acts as a binary switch during sensory organ precursor selection. *Genes Dev* 17, 2966-2978.
- Janoueix-Lerosey, I., Lequin, D., Brugieres, L., Ribeiro, A., de Pontual, L., Combaret, V., Raynal, V., Puisieux, A., Schleiermacher, G., Pierron, G., *et al.* (2008). Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 455, 967-970.
- Jones, P.A., and Baylin, S.B. (2007). The epigenomics of cancer. *Cell* 128, 683-692.

- Jopling, C.L., Yi, M., Lancaster, A.M., Lemon, S.M., and Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 309, 1577-1581.
- Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., *et al.* (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421, 231-237.
- Karginov, F.V., Cheloufi, S., Chong, M.M., Stark, A., Smith, A.D., and Hannon, G.J. (2010). Diverse endonucleolytic cleavage sites in the mammalian transcriptome depend upon microRNAs, Drosha, and additional nucleases. *Mol Cell* 38, 781-788.
- Karim, F.D., and Thummel, C.S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *Embo J* 11, 4083-4093.
- Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev* 15, 2654-2659.
- Khvorova, A., Reynolds, A., and Jayasena, S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115, 209-216.
- Kim, D.H., Saetrom, P., Snove, O., Jr., and Rossi, J.J. (2008). MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A* 105, 16230-16235.
- Kim, V.N. (2005). MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 6, 376-385.
- Kim, V.N., Han, J., and Siomi, M.C. (2009). Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 10, 126-139.
- Kim, Y.K., and Kim, V.N. (2007). Processing of intronic microRNAs. *Embo J* 26, 775-783.
- Klein, U., Lia, M., Crespo, M., Siegel, R., Shen, Q., Mo, T., Ambesi-Impimbato, A., Califano, A., Migliazza, A., Bhagat, G., *et al.* (2010). The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell* 17, 28-40.
- Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68, 820-823.
- Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59-77.
- Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.W., Chang, T.C., Vivekanandan, P., Torbenson, M., Clark, K.R., *et al.* (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 137, 1005-1017.
- Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K.G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 438, 685-689.
- Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science* 294, 853-858.
- Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A.O., Landthaler, M., *et al.* (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 129, 1401-1414.

- 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, 858-862.
- Lee, I., Ajay, S.S., Yook, J.I., Kim, H.S., Hong, S.H., Kim, N.H., Dhanasekaran, S.M., Chinnaiyan, A.M., and Athey, B.D. (2009). New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. *Genome Res* 19, 1175-1183.
- Lee, R.C., and Ambros, V. (2001). An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* 294, 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, 843-854.
- Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., *et al.* (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415-419.
- Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R., and Kim, V.N. (2006). The role of PACT in the RNA silencing pathway. *Embo J* 25, 522-532.
- Lee, Y., Jeon, K., Lee, J.T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. *Embo J* 21, 4663-4670.
- Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. *Embo J* 23, 4051-4060.
- Lerner, M., Harada, M., Loven, J., Castro, J., Davis, Z., Oscier, D., Henriksson, M., Sangfelt, O., Grander, D., and Corcoran, M.M. (2009). DLEU2, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1. *Exp Cell Res* 315, 2941-2952.
- Levine, A.J., Finlay, C.A., and Hinds, P.W. (2004). P53 is a tumor suppressor gene. *Cell* 116, S67-69, 61 p following S69.
- Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15-20.
- Levy, C., Khaled, M., Robinson, K.C., Veguilla, R.A., Chen, P.H., Yokoyama, S., Makino, E., Lu, J., Larue, L., Beermann, F., *et al.* (2010). Lineage-Specific Transcriptional Regulation of DICER by MITF in Melanocytes. *Cell* 141, 994-1005.
- Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliareis, C., Rodgers, L., McCombie, R., *et al.* (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275, 1943-1947.
- Li, X., Cassidy, J.J., Reinke, C.A., Fischboeck, S., and Carthew, R.W. (2009). A microRNA imparts robustness against environmental fluctuation during development. *Cell* 137, 273-282.
- Li, Y., Wang, F., Lee, J.A., and Gao, F.B. (2006). MicroRNA-9a ensures the precise specification of sensory organ precursors in *Drosophila*. *Genes Dev* 20, 2793-2805.
- Linsley, P.S., Schelter, J., Burchard, J., Kibukawa, M., Martin, M.M., Bartz, S.R., Johnson, J.M., Cummins, J.M., Raymond, C.K., Dai, H., *et al.* (2007). Transcripts targeted

- by the microRNA-16 family cooperatively regulate cell cycle progression. *Mol Cell Biol* 27, 2240-2252.
- Liu, Y., Corcoran, M., Rasool, O., Ivanova, G., Ibbotson, R., Grander, D., Iyengar, A., Baranova, A., Kashuba, V., Merup, M., *et al.* (1997). Cloning of two candidate tumor suppressor genes within a 10 kb region on chromosome 13q14, frequently deleted in chronic lymphocytic leukemia. *Oncogene* 15, 2463-2473.
- Losick, R., and Desplan, C. (2008). Stochasticity and cell fate. *Science* 320, 65-68.
- Lowe, S.W., Cepero, E., and Evan, G. (2004). Intrinsic tumour suppression. *Nature* 432, 307-315.
- Loven, J., Zinin, N., Wahlstrom, T., Muller, I., Brodin, P., Fredlund, E., Ribacke, U., Pivarcsi, A., Pahlman, S., and Henriksson, M. MYCN-regulated microRNAs repress estrogen receptor-alpha (ESR1) expression and neuronal differentiation in human neuroblastoma. *Proc Natl Acad Sci U S A* 107, 1553-1558.
- 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.* (2005). MicroRNA expression profiles classify human cancers. *Nature* 435, 834-838.
- Lund, E., and Dahlberg, J.E. (2006). Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. *Cold Spring Harb Symp Quant Biol* 71, 59-66.
- Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. *Science* 303, 95-98.
- Lytle, J.R., Yario, T.A., and Steitz, J.A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A* 104, 9667-9672.
- Ma, Z.Q., Bondiolotti, G.P., Olasmaa, M., Violani, E., Patrone, C., Picotti, G.B., and Maggi, A. (1993). Estrogen modulation of catecholamine synthesis and monoamine oxidase A activity in the human neuroblastoma cell line SK-ER3. *J Steroid Biochem Mol Biol* 47, 207-211.
- Mangan, S., and Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proc Natl Acad Sci U S A* 100, 11980-11985.
- Mansfield, J.H., Harfe, B.D., Nissen, R., Obenaus, J., Srineel, J., Chaudhuri, A., Farzan-Kashani, R., Zuker, M., Pasquinelli, A.E., Ruvkun, G., *et al.* (2004). MicroRNA-responsive 'sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. *Nat Genet* 36, 1079-1083.
- Maris, J.M. (2010). Recent advances in neuroblastoma. *N Engl J Med* 362, 2202-2211.
- Maris, J.M., Hogarty, M.D., Bagatell, R., and Cohn, S.L. (2007). Neuroblastoma. *Lancet* 369, 2106-2120.
- Maris, J.M., Mosse, Y.P., Bradfield, J.P., Hou, C., Monni, S., Scott, R.H., Asgharzadeh, S., Attiyeh, E.F., Diskin, S.J., Laudenslager, M., *et al.* (2008). Chromosome 6p22 locus associated with clinically aggressive neuroblastoma. *N Engl J Med* 358, 2585-2593.
- 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, 521-533.
- Martinez, N.J., Ow, M.C., Barrasa, M.I., Hammell, M., Sequerra, R., Doucette-Stamm, L., Roth, F.P., Ambros, V.R., and Walhout, A.J. (2008). A *C. elegans* genome-scale

- microRNA network contains composite feedback motifs with high flux capacity. *Genes Dev* 22, 2535-2549.
- Matthay, K.K. (1999). Intensification of therapy using hematopoietic stem-cell support for high-risk neuroblastoma. *Pediatr Transplant* 3 *Suppl 1*, 72-77.
- Mayr, C., Hemann, M.T., and Bartel, D.P. (2007). Disrupting the pairing between let-7 and Hmgaz enhances oncogenic transformation. *Science* 315, 1576-1579.
- Medina, P.P., Nolde, M., and Slack, F.J. (2010). OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature*.
- Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 15, 185-197.
- Mercer, T.R., Dinger, M.E., and Mattick, J.S. (2009). Long non-coding RNAs: insights into functions. *Nature Reviews Genetics* 10, 155-159.
- Messmer, B.T., Albesiano, E., Messmer, D., and Chiorazzi, N. (2004). The pattern and distribution of immunoglobulin VH gene mutations in chronic lymphocytic leukemia B cells are consistent with the canonical somatic hypermutation process. *Blood* 103, 3490-3495.
- Mestdagh, P., Fredlund, E., Pattyn, F., Schulte, J.H., Muth, D., Vermeulen, J., Kumps, C., Schlierf, S., De Preter, K., Van Roy, N., *et al.* (2010). MYCN/c-MYC-induced microRNAs repress coding gene networks associated with poor outcome in MYCN/c-MYC-activated tumors. *Oncogene* 29, 1394-1404.
- Millar, S.E. (2002). Molecular mechanisms regulating hair follicle development. *J Invest Dermatol* 118, 216-225.
- Miska, E.A., Alvarez-Saavedra, E., Abbott, A.L., Lau, N.C., Hellman, A.B., McGonagle, S.M., Bartel, D.P., Ambros, V.R., and Horvitz, H.R. (2007). Most *Caenorhabditis elegans* microRNAs are individually not essential for development or viability. *PLoS Genet* 3, e215.
- Morlando, M., Ballarino, M., Gromak, N., Pagano, F., Bozzoni, I., and Proudfoot, N.J. (2008). Primary microRNA transcripts are processed co-transcriptionally. *Nat Struct Mol Biol* 15, 902-909.
- Moss, E.G. (2007). Heterochronic genes and the nature of developmental time. *Curr Biol* 17, R425-434.
- Mosse, Y.P., Laudenslager, M., Longo, L., Cole, K.A., Wood, A., Attiyeh, E.F., Laquaglia, M.J., Sennett, R., Lynch, J.E., Perri, P., *et al.* (2008). Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 455, 930-935.
- Nakahara, K., Kim, K., Sciulli, C., Dowd, S.R., Minden, J.S., and Carthew, R.W. (2005). Targets of microRNA regulation in the *Drosophila* oocyte proteome. *Proc Natl Acad Sci U S A* 102, 12023-12028.
- Nesbit, C.E., Tersak, J.M., and Prochownik, E.V. (1999). MYC oncogenes and human neoplastic disease. *Oncogene* 18, 3004-3016.
- Nielsen, C.B., Shomron, N., Sandberg, R., Hornstein, E., Kitzman, J., and Burge, C.B. (2007). Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. *Rna* 13, 1894-1910.
- Nilsson, M., Unden, A.B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P.G., and Toftgard, R. (2000). Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. *Proc Natl Acad Sci U S A* 97, 3438-3443.

- Nowell, P.C. (1976). The clonal evolution of tumor cell populations. *Science* 194, 23-28.
- Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795-801.
- 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, 839-843.
- Okamura, K., Hagen, J.W., Duan, H., Tyler, D.M., and Lai, E.C. (2007). The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*. *Cell* 130, 89-100.
- Okamura, K., Liu, N., and Lai, E.C. (2009). Distinct mechanisms for microRNA strand selection by *Drosophila* Argonautes. *Mol Cell* 36, 431-444.
- Okamura, K., Phillips, M.D., Tyler, D.M., Duan, H., Chou, Y.T., and Lai, E.C. (2008). The regulatory activity of microRNA\* species has substantial influence on microRNA and 3' UTR evolution. *Nat Struct Mol Biol* 15, 354-363.
- Oosterhuis, J.W., and Looijenga, L.H. (2005). Testicular germ-cell tumours in a broader perspective. *Nat Rev Cancer* 5, 210-222.
- 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, 460-471.
- Owens, D.M., and Watt, F.M. (2003). Contribution of stem cells and differentiated cells to epidermal tumours. *Nat Rev Cancer* 3, 444-451.
- 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, 86-89.
- Raj, A., and van Oudenaarden, A. (2008). Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell* 135, 216-226.
- Raser, J.M., and O'Shea, E.K. (2005). Noise in gene expression: origins, consequences, and control. *Science* 309, 2010-2013.
- Raveche, E.S., Salerno, E., Scaglione, B.J., Manohar, V., Abbasi, F., Lin, Y.C., Fredrickson, T., Landgraf, P., Ramachandra, S., Huppi, K., *et al.* (2007). Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. *Blood* 109, 5079-5086.
- Redaelli, A., Laskin, B.L., Stephens, J.M., Botteman, M.F., and Pashos, C.L. (2004). The clinical and epidemiological burden of chronic lymphocytic leukaemia. *Eur J Cancer Care (Engl)* 13, 279-287.
- Reinhart, B.J., and Bartel, D.P. (2002). Small RNAs correspond to centromere heterochromatic repeats. *Science* 297, 1831.
- Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403, 901-906.
- Rozman, C., and Montserrat, E. (1995). Chronic lymphocytic leukemia. *N Engl J Med* 333, 1052-1057.
- Rubin, A.I., Chen, E.H., and Ratner, D. (2005). Basal-cell carcinoma. *N Engl J Med* 353, 2262-2269.
- Ruby, J.G., Jan, C.H., and Bartel, D.P. (2007). Intronic microRNA precursors that bypass Drosha processing. *Nature* 448, 83-86.

- Ruvkun, G., and Giusto, J. (1989). The *Caenorhabditis elegans* heterochronic gene *lin-14* encodes a nuclear protein that forms a temporal developmental switch. *Nature* 338, 313-319.
- 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, 17719-17724.
- Schnidar, H., Eberl, M., Klingler, S., Mangelberger, D., Kasper, M., Hauser-Kronberger, C., Regl, G., Kroismayr, R., Moriggl, R., Sibilias, M., *et al.* (2009). Epidermal growth factor receptor signaling synergizes with Hedgehog/GLI in oncogenic transformation via activation of the MEK/ERK/JUN pathway. *Cancer Res* 69, 1284-1292.
- Schuhmacher, M., Staeger, M.S., Pajic, A., Polack, A., Weidle, U.H., Bornkamm, G.W., Eick, D., and Kohlhuber, F. (1999). Control of cell growth by c-Myc in the absence of cell division. *Curr Biol* 9, 1255-1258.
- Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. (1983). Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 305, 245-248.
- Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115, 199-208.
- Scotting, P.J., Walker, D.A., and Perilongo, G. (2005). Childhood solid tumours: a developmental disorder. *Nat Rev Cancer* 5, 481-488.
- Selbach, M., Schwanhaussner, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. *Nature* 455, 58-63.
- Sherr, C.J. (2004). Principles of tumor suppression. *Cell* 116, 235-246.
- Shin, C., Nam, J.W., Farh, K.K., Chiang, H.R., Shkumatava, A., and Bartel, D.P. (2010). Expanding the microRNA targeting code: functional sites with centered pairing. *Mol Cell* 38, 789-802.
- Silva-Vargas, V., Lo Celso, C., Giangreco, A., Ofstad, T., Prowse, D.M., Braun, K.M., and Watt, F.M. (2005). Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells. *Dev Cell* 9, 121-131.
- Sohrabji, F., Greene, L.A., Miranda, R.C., and Toran-Allerand, C.D. (1994a). Reciprocal regulation of estrogen and NGF receptors by their ligands in PC12 cells. *J Neurobiol* 25, 974-988.
- Sohrabji, F., Miranda, R.C., and Toran-Allerand, C.D. (1994b). Estrogen differentially regulates estrogen and nerve growth factor receptor mRNAs in adult sensory neurons. *J Neurosci* 14, 459-471.
- Sokol, N.S., and Ambros, V. (2005). Mesodermally expressed *Drosophila* microRNA-1 is regulated by Twist and is required in muscles during larval growth. *Genes Dev* 19, 2343-2354.
- Sonkoly, E., Wei, T., Pavez-Lorie, E., Suzuki, H., Kato, M., Torma, H., Stahle, M., and Pivarcsi, A. (2010). Protein kinase C-dependent upregulation of miR-203 induces the differentiation of human keratinocytes. *J Invest Dermatol* 130, 124-134.

- Soto, A.M., and Sonnenschein, C. (2010). Environmental causes of cancer: endocrine disruptors as carcinogens. *Nature Reviews Endocrinology* 6, 364-371.
- St-Jacques, B., Dassule, H.R., Karavanova, I., Botchkarev, V.A., Li, J., Danielian, P.S., McMahon, J.A., Lewis, P.M., Paus, R., and McMahon, A.P. (1998). Sonic hedgehog signaling is essential for hair development. *Curr Biol* 8, 1058-1068.
- Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F., *et al.* (2001). Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* 3, 392-399.
- Stratton, M.R., Campbell, P.J., and Futreal, P.A. (2009). The cancer genome. *Nature* 458, 719-724.
- Taipale, J., and Beachy, P.A. (2001). The Hedgehog and Wnt signalling pathways in cancer. *Nature* 411, 349-354.
- Thummel, C.S. (1996). Flies on steroids--Drosophila metamorphosis and the mechanisms of steroid hormone action. *Trends Genet* 12, 306-310.
- Thummel, C.S. (2001). Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. *Dev Cell* 1, 453-465.
- Toran-Allerand, C.D. (1996). Mechanisms of estrogen action during neural development: mediation by interactions with the neurotrophins and their receptors? *J Steroid Biochem Mol Biol* 56, 169-178.
- Tsang, J., Zhu, J., and van Oudenaarden, A. (2007). MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. *Mol Cell* 26, 753-767.
- Wang, L.C., Liu, Z.Y., Gambardella, L., Delacour, A., Shapiro, R., Yang, J., Sizing, I., Rayhorn, P., Garber, E.A., Benjamin, C.D., *et al.* (2000). Regular articles: conditional disruption of hedgehog signaling pathway defines its critical role in hair development and regeneration. *J Invest Dermatol* 114, 901-908.
- Varghese, J., and Cohen, S.M. (2007). microRNA miR-14 acts to modulate a positive autoregulatory loop controlling steroid hormone signaling in *Drosophila*. *Genes Dev* 21, 2277-2282.
- Weake, V.M., and Workman, J.L. (2010). Inducible gene expression: diverse regulatory mechanisms. *Nature Reviews Genetics* 11, 426-437.
- Wicking, C., Smyth, I., and Bale, A. (1999). The hedgehog signalling pathway in tumorigenesis and development. *Oncogene* 18, 7844-7851.
- Wienholds, E., Kloosterman, W.P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H.R., Kauppinen, S., and Plasterk, R.H. (2005). MicroRNA expression in zebrafish embryonic development. *Science* 309, 310-311.
- Wienholds, E., Koudijs, M.J., van Eeden, F.J., Cuppen, E., and Plasterk, R.H. (2003). The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat Genet* 35, 217-218.
- Wightman, B., Burglin, T.R., Gatto, J., Arasu, P., and Ruvkun, G. (1991). Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev* 5, 1813-1824.
- Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* 75, 855-862.

- Vineis, P., and Berwick, M. (2006). The population dynamics of cancer: a Darwinian perspective. *Int J Epidemiol* 35, 1151-1159.
- Vogelstein, B., and Kinzler, K.W. (1993). The multistep nature of cancer. *Trends Genet* 9, 138-141.
- Volinia, S., Calin, G.A., Liu, C.G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M., *et al.* (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A* 103, 2257-2261.
- Xie, J., Murone, M., Luoh, S.M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J.M., Lam, C.W., Hynes, M., Goddard, A., *et al.* (1998). Activating Smoothed mutations in sporadic basal-cell carcinoma. *Nature* 391, 90-92.
- Yekta, S., Shih, I.H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 304, 594-596.
- Yekta, S., Tabin, C.J., and Bartel, D.P. (2008). MicroRNAs in the Hox network: an apparent link to posterior prevalence. *Nat Rev Genet* 9, 789-796.
- Yi, R., Doehle, B.P., Qin, Y., Macara, I.G., and Cullen, B.R. (2005). Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *Rna* 11, 220-226.
- Yi, R., Poy, M.N., Stoffel, M., and Fuchs, E. (2008). A skin microRNA promotes differentiation by repressing 'stemness'. *Nature* 452, 225-229.
- Youssef, K.K., Van Keymeulen, A., Lapouge, G., Beck, B., Michaux, C., Achouri, Y., Sotiropoulou, P.A., and Blanpain, C. (2010). Identification of the cell lineage at the origin of basal cell carcinoma. *Nat Cell Biol* 12, 299-305.
- Zehir, A., Hua, L.L., Maska, E.L., Morikawa, Y., and Cserjesi, P. (2010). Dicer is required for survival of differentiating neural crest cells. *Developmental Biology* 340, 459-467.
- Zeng, Y., and Cullen, B.R. (2004). Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. *Nucleic Acids Res* 32, 4776-4785.
- Zhou, X., Ruan, J., Wang, G., and Zhang, W. (2007). Characterization and identification of microRNA core promoters in four model species. *PLoS Comput Biol* 3, e37.

## **PUBLICATIONS & MANUSCRIPTS**

---