MicroRNAs in Normal and Malignant Myelopoiesis

Mir Farshid Alemdehy

Layout and printing: Off Page, Amsterdam, www.offpage.nl

ISBN: 978-94-6182-539-1

The work presented in this thesis was performed at the Department of Hematology at the Erasmus Medical Center in Rotterdam and was financially supported by the Dutch Cancer Society (KWF). Printing of this thesis was financially supported by the Erasmus University Rotterdam and the Dutch Cancer Society (KWF).

MicroRNAs in Normal and Malignant Myelopoiesis

MicroRNAs in normale en maligne myelopoiese

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. H. A. P. Pols

and in accordance with the decision of the Doctorate Board
The public defence shall be held on

Wednesday 11 March 2015 at 13:30

by

Mir Farshid Alemdehy

born in Teheran, Iran

Doctoral Committee

Promotor:
Prof.dr. I. P. Touw

Other members:
Prof.dr. L. H. J. Looijenga
Dr. M. M. von Lindern
Dr. E. A. C. Wiemer

Copromotor:
Dr. S. J. Erkeland

To my love, Setareh,

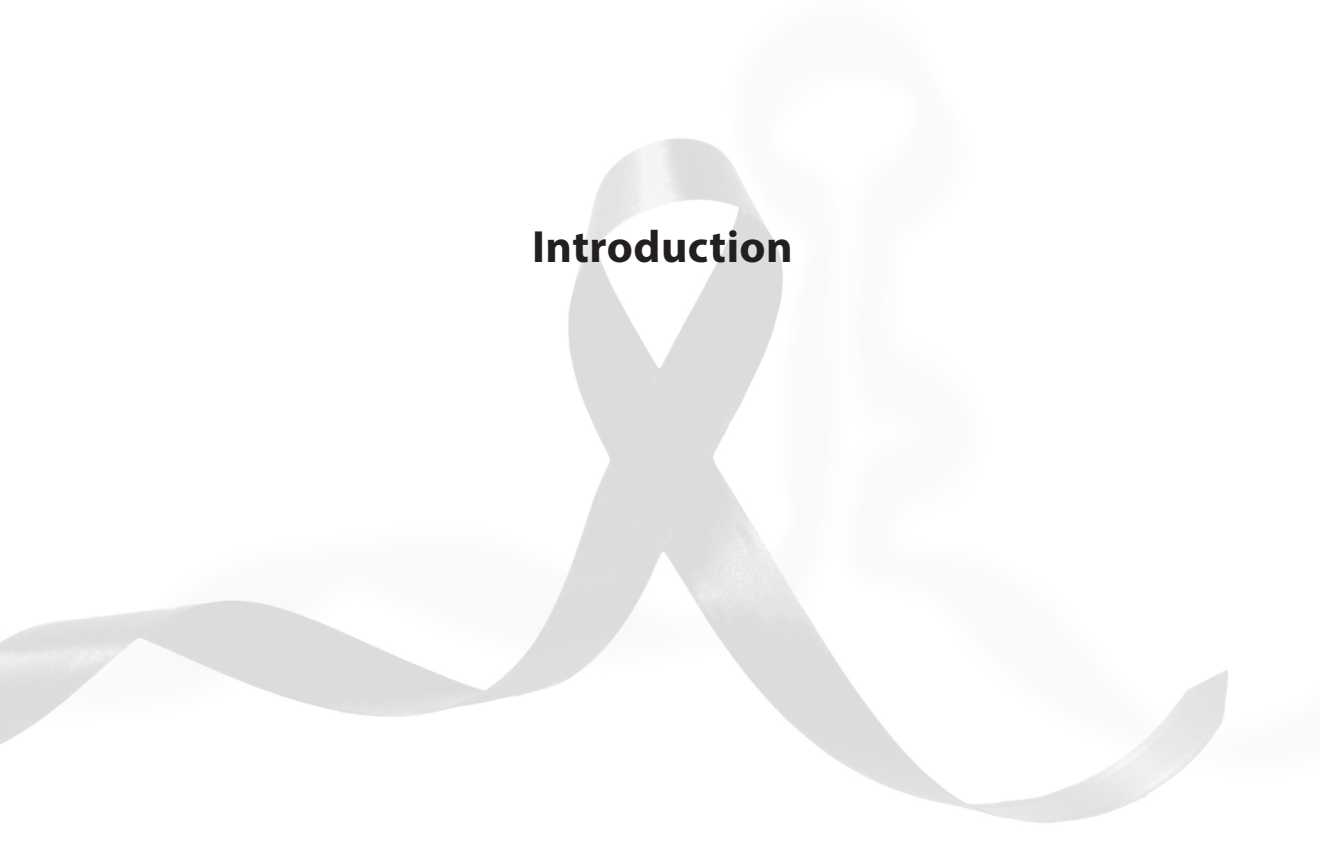
to our Ryan,

and to my parents

CONTENTS

1

Introduction



1. HEMATOPOIESIS

Hematopoiesis is the lifelong continuous process in which hematopoietic stem and progenitor cells (HSPCs) proliferate and differentiate towards mature blood cells. Hematopoiesis is tightly controlled by a network of growth factors and the hematopoietic niche in the bone marrow (BM). This ensures the balanced blood cell production under homeostatic conditions and allows for transient elevation of specific blood cell types production in response to infections or bleeding [1]. In mammalian organisms, long-term hematopoietic stem cells (LT-HSCs) reside in the BM and have self-renewal capacity over the lifespan of the organism [2,3]. The estimated amount of LT-HSCs is approximately 0.007% of all hematopoietic cells in the BM [4]. LT-HSCs give rise to short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs) (Figure 1). These cells have the potential to differentiate into all the different hematopoietic cell types but have less self-renewal capacity [4]. Together, LT-HCSs, ST-HCSs and MPPs constitute 0.05% of mouse BM cells [2]. MPPs differentiate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) (Figure 1). Subsequently, CLPs differentiate into B-cell and T-cell lineages. CMPs first develop into more specified myeloid progenitors, which are megakaryocyte/erythroid progenitors (MEPs) and granulocyte/monocyte progenitors (GMPs). Granulocytes, monocytes and macrophages arise from GMPs, whereas MEPs differentiate towards erythrocytes and thrombocytes (platelets) (Figure 1). The process of differentiation of HSPCs towards mature myeloid cells is referred as myelopoiesis. In adult mammalian organisms, myelopoiesis occurs in the BM.

Disruption of the balance between cell proliferation, differentiation and cell death leads to different hematopoietic disorders, e.g., leukemia, characterized by proliferation of undifferentiated cells, or bone marrow failure (BMF), characterized by impaired hematopoiesis involving one or multiple hematopoietic lineages [5,6]. Proliferation and differentiation of HSPCs are coordinated by gene expression programs driven by endogenous and exogenous factors. MicroRNAs (miRNAs) are a class of non-coding RNAs which function as regulators of gene expression. In the studies described in this thesis the role of miRNAs in normal myelopoiesis and their involvement in acute myeloid leukemia (AML) and Fanconi anemia (FA), the most frequent inherited form of BMF syndromes are investigated. AML, FA and miRNAs will be further introduced in the following sections.

2. ACUTE MYELOID LEUKEMIA

AML is a type of blood cancer characterized by a block in myeloid differentiation and an uncontrolled clonal outgrowth of malignant myeloid progenitor cells, which overgrow normal blood cells and interfere with their functions. Lack of functional blood cells leads to the classical symptoms of AML, i.e., fatigue (due to lack of erythrocytes), infections (due to lack of functional granulocytes and monocytes) and hemorrhage (due to lack of platelets) [7]. AML is mainly a disease of the elderly. The incidence of AML increases with age with 1.7 per 100,000 individuals below the age of 65 compared with 16 per 100,000 individuals over the age of 65 (based on the Surveillance, Epidemiology, and End Results (SEER) cancer statistics review of the National Cancer Institute [8]). The average age at diagnosis is 67 years [8].

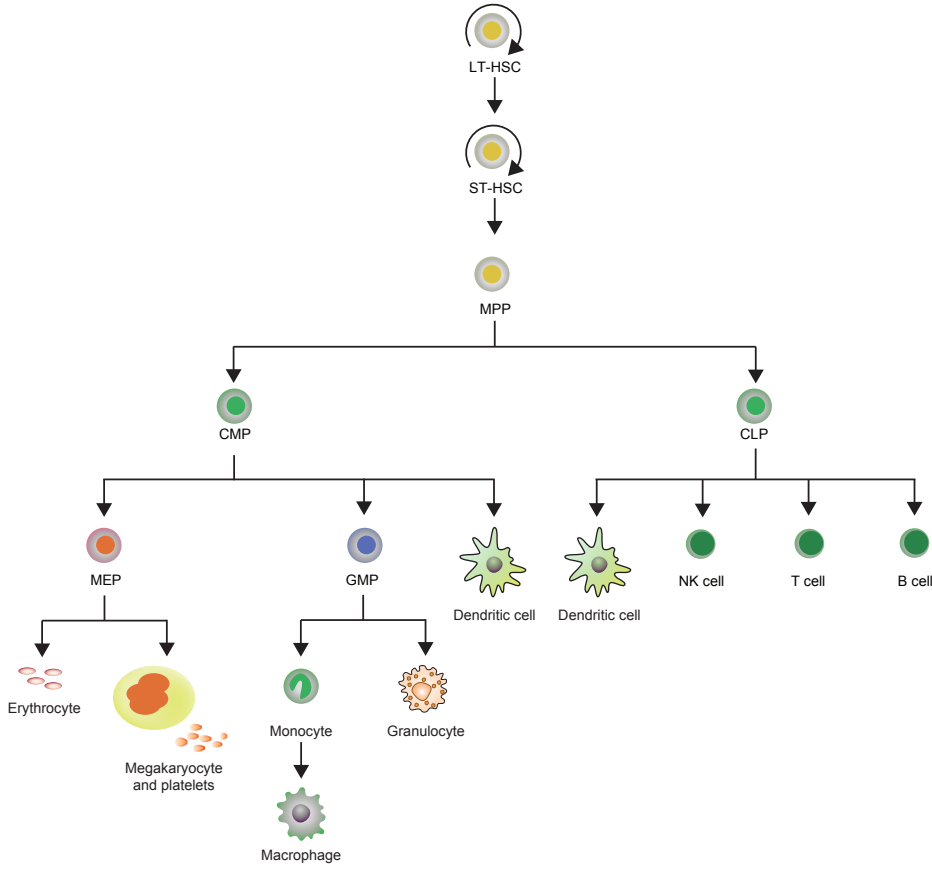


Figure 1. Schematic representation of the hematopoiesis. The hematopoietic stem cells (HSCs) consist of long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs). HSCs differentiate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). Subsequently, CLPs differentiate into B cell and T cell lineages, as well as natural killer cells (NK cells) and dendritic cells. CMPs first develop into more specified myeloid progenitors, which are megakaryocyte/erythroid progenitors (MEPs) and granulocyte/monocyte progenitors (GMPs). Granulocytes, monocytes and macrophages as well as dendritic cells arise from GMPs, whereas MEPs differentiate towards erythrocytes and thrombocytes (platelets). Figure adapted from Reya et al. and Blank et al. [1,2].

AML is a heterogeneous group of diseases characterized by different genetic abnormalities such as chromosomal defects including translocation, deletion or inversion, as well as molecular aberrations such as mutations in the genes encoding important transcription factors. The most recurrent chromosomal aberrations and frequently mutated genes in AML and their prognostic significance are listed in Table 1.

Table 1. Recurrent cytogenetic and genetic abnormalities in adult AML.

Recurrent cytogenetic and genetic aberration	Related fusion gene	Frequency (%)	Prognostic significance
Cytogenetic abnormalities			
Normal karyotype		45	
Complex karyotype*		11	Unfavorable
+8		9	Intermediate
-7/-7q		8	Unfavorable
t(15;17)(q22;q21)	PML-RARA	5-10	Favorable
-5/-5q		7	Unfavorable
t(8;21)(q22;q22)	AML1-ETO	6	Favorable
inv(16)(p13.1q22)	CBFB-MYH11	5	Favorable
-Y		4	Intermediate
11q23	MLL fusions	3	Unfavorable
abn(12p)		3	Intermediate
t(9;11)(p22;q23)	MLLT3-MLL	2	Unfavorable
inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	RPN1-EVI1	2	Unfavorable
+21		2	Intermediate
del(9q)		2	Intermediate
t(9;22)(q34;q11)	BCR-ABL	1	Intermediate/Unfavorable
t(6;9)(p23;q34)	DEK-NUP214	1	Unfavorable
Molecular abnormalities			
ASXL1		11	Unfavorable
CEBPA		10-18	Favorable
c-KIT		17 in CBF leukemia	Unfavorable in t(8;21)
DNMT3A		22	Unfavorable
FLT3-ITD		20-40 (50 in CN)	Unfavorable
FLT3-TKD		11-14	_
IDH1/2		16	Unfavorable
MLL-PTD45		3-5	Unfavorable
NPM1		35 (53 in CN)	Favorable in absence of *FLT3-ITD*
N-Ras/K-Ras		9-14/5	_
PTPN11		2.5	Unfavorable in absence of NPM1 mutation
RUNX1		5-13	Unfavorable
TET2		8-17	Unfavorable

Table 1. Recurrent cytogenetic and genetic abnormalities in adult AML. (*Continued*)

Recurrent cytogenetic and genetic aberration	Related fusion gene	Frequency (%)	Prognostic significance
TP53		<10 (56-78 complex caryotype)	Unfavorable
WT1		10 in CN	Unfavorable combined with *FLT3-ITD*

Cytogenetic abnormalities are ordered based on frequency, moleculare abnormalities are ordered alphabetically, t = translocation, -= loss, + = gain, inv = inversion, abn = abnormality, del = deletion, CN = cytogenetically normal AML, CBF = core binding factor AML, i.e., with inv(16), t(16;16) or t(8;21).
*Complex karyotype has been defined as the presence of 3 or more cytogenetic abnormalities in the absence of t(8;21), inv(16) and t(15;17)
Based on Marcucci et al., 2011 [152]; Mrozek et al., 2004 [153]; Takahashi, 2011 [154]; Walker and Marcucci, 2012 [155]

The treatment of AML patients is performed in two phases. The main objective in the first phase is to induce a remission [7]. The aim of second phase is to prevent relapse by eliminating all undetected remaining leukemic blasts [7]. Remission is defined morphologically when the amount of blasts in the BM decreases to below 5% [7]. Both phases are performed via intensive combinatorial chemotherapy, and depending on the age of the patient, the presence of a suitable stem cell donor and the molecular characteristics of the AML, the second phase may involve allogenic or autologous stem cell transplantation [7]. New insights into the biology of AML are needed to allow introduction of targeted therapies that improve the efficiency of the therapeutic approach. Particularly, AML arising from BMF is associated with a poor prognosis that is probably due to the mechanism of disease progression.

3. FANCONI ANEMIA

FA, the most frequently inherited form of BMF syndromes [6], results from mutations in one of the Fanconi (FANC) genes involved in interstrand cross-link (ICL) DNA damage repair [9-11]. Cells with mutations in FANC genes are hypersensitive to DNA cross-linking agents such as mitomycin C (MMC), and show a dramatic increase in the number of chromosomal aberrations [12-15]. FA is characterized by developmental abnormalities, BMF and an increased susceptibility to develop cancers, including leukemia. Approximately 80% of young FA patients develop BMF of which 16% develop myelodysplastic syndrome (MDS) and AML, with a median age of leukemia onset of 11 years [16-18]. The cumulative incidence of MDS and AML increases to 33% by the age of 40. Typically, the AMLs in older patients are characterized by a poor prognosis [19].

3-1. The FA pathway and ICL repair

Hematopoietic progenitor cells possess a robust proliferative capacity that provides approximately 10^{12} blood cells daily in adult BM [20]. Consequently, the genomic integrity of HSPCs is constantly threatened by replication errors or damages induced by endogenously

formed metabolic products and by-products, such as free radicals, reactive oxygen species (ROS), endogenous alkylating agents and reactive aldehydes [21,22]. Unrepaired DNA damages will ultimately result in accelerated senescence, increased cell death, abnormal cell expansion and an increased risk of cancer [23,24]. To maintain genomic integrity, HSPCs must respond appropriately to genotoxic DNA damages. The DNA repair systems in mammalian cells include nucleotide excision repair (NER), base excision repair (BER), double strand break (DSB) repair, mismatch repair and ICL repair [25]. An ICL consists of a covalent bond between two strands of DNA, leading to a block in DNA replication and transcription, and is an extremely toxic DNA lesion [26]. The FA pathway is involved in swift repair of ICL in higher eukaryotic cells [27]. Eight different FA proteins (FANCA, -B, -C, -E, -F, -G, -L and –M) assemble to form a core protein complex in the nucleus. The FANCL subunit is an E3 ubiquitin ligase [28]. In response to DNA-damage, the FA core complex ubiquitylates two other FA proteins: FANCI and FANCD2 (ID) [29,30] (Figure 2). Monoubiquitylation of (FANC)ID by the FA core complex is required for ICL recognition [12]. Ubiquitylated (FANC)ID recruits FANCP (SLX4) in complex with structure-specific endonuclease complexes including FAN1, MUS81-EME1 and XPF(FANCQ)-ERCC1, to the ICL lesion site [9] (Figure 2). FANCQ-ERCC1 makes incisions

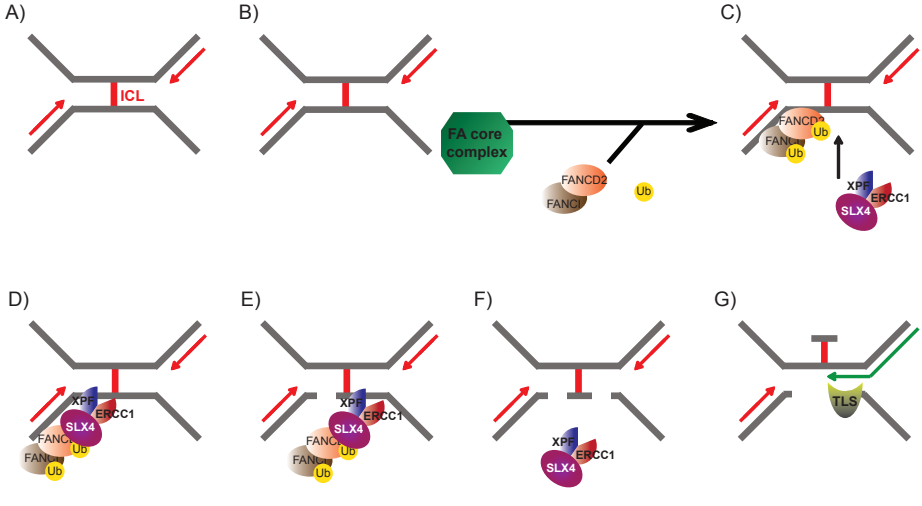


Figure 2. FA pathway and ICL repair. A) An interstrand cross-link (ICL) is a covalent bond between two strands of DNA, blocking DNA replication and transcription. **B)** Eight different FA proteins (FANCA, -B, -C, -E, -F, -G, -L and –M) assemble to form FA core complex in the nucleus. In response to ICL DNA-damage, FA core complex ubiquitylates two other FA proteins: FANCD2 and FANCI (ID). Monoubiquitylation of ID is required for ICL recognition. **C)** Ubiquitylated ID recruits SLX4 in complex with XPF-ERCC1. **D-E-F)** XPF-ERCC1 makes incisions around the ICL site by cutting first 3'- and possibly then 5'- sides, to unhook the cross-linked DNA. **G)** The intact parental strand could then serve as a template for the extension by translesion synthesis (TLS). Figure is adapted from Hodskinson et al. [31].

around the ICL site by cutting first 3' and possibly then 5' sides, to unhook the cross-linked DNA and therefore is an essential endonuclease for ICL repair [31,32] (Figure 2).

3-2. Leukemic transformation in FA

How FA progresses to leukemia is still largely elusive. A genetic reversion of the mutation was reported in an AML cell line from a patient with a biallelic *FANCD1* mutation [33] and it was suggested that this phenomenon may play a general role in FA-related MDS/AML oncogenesis. However, an analysis on 57 FA patients, including 18 FA-MDS and 11 FA-AML patients shows no reversion of the FA mutation, indicating that this does not occur frequently [34]. Further investigation of this group of patients included karyotype analysis of the BM samples and selected oncogene sequencing, which revealed specific patterns of chromosomal abnormalities in FA-MDS and FA-AML [34]. These included gain of 1q (44.8% of FA- MDS/AML) and 3q (containing the *EVI1* gene) (41.4%), and loss of 7/7q (17.2%) and 11q (13.8%). Whereas *RUNX1/AML1* lesions (translocations, deletions, or mutations) are observed in 20.7% of FA patients with MDS or AML, other abnormalities that are frequently found in *de novo* AML, e.g., mutations in *NRAS*, *FLT3-ITD*, *MLL-PTD*, *ERG* amplification, and *ZFP36L2-PRDM16* translocation, were rare [34]. Mutations in *TP53, TET2*, *CBL*, *NPM1*, and *CEBP*A were also rare in FA-AML [34,35]. Abrogation of the DNA damage checkpoints in the cell cycle and resistance to tumor necrosis factor-alpha (TNF-α) have been described as mechanisms favoring the clonal expansion of FA cells [36,37].

4. MICRORNAS

Recent data from high-throughput sequencing platforms have established that in mammalian organisms nearly the entire genome is transcribed [38]. Remarkably, only 2% of the transcripts are translated into proteins [38]. The majority of the transcripts (98%) are so-called non-coding RNAs (ncRNAs) and are roughly categorized into small (< 200 nucleotides (nt)) and large ncRNAs. The small ncRNAs include small nucleolar RNAs, PIWI-interacting RNAs, endogenous small interfering RNAs (siRNAs), and microRNAs (miRNAs). While protein-coding sequences have been extensively investigated in the last decennia, the functions of non-coding RNAs have just recently been addressed [39]. The major features of miRNA functions related to this thesis are introduced below.

MiRNAs are 19-23 nt single-stranded RNA molecules [40]. The first miRNA, *Lin*-4, discovered by Ambros, Ruvkun and colleagues, controls the timing of *Caenorhabditis elegans* (*C. elegans*) larval development [41,42]. Since then, thousands of miRNA encoding genes have been identified in animals, plants and viruses, making them one of the largest gene families [40,43-45]. All 35828 confirmed miRNAs, in up to 223 species, are listed in the miRNA-base database (www.mirbase.org). This database includes 2603 human and 1920 mouse miRNA sequences. Notably, miRNAs are highly conserved between species. For instance, more than half of *C. elegans* miRNAs share sequence homology with miRNAs encoded also in both *Drosophila melanogaster* and human genomes [46]. Furthermore, more than 70% of the miRNAs are

identical between human and mouse [47], indicating that there is a high degree of conservation during animal evolution. Each cell type expresses a unique set of miRNAs at specific levels, suggesting that they are important for cellular identity and functions [45].

4.1- MiRNA biogenesis

MiRNAs are abundantly expressed with levels reaching thousands of molecules per cell, a copy number that is much higher than what is normally found for mRNAs [48]. Most miRNA genes are located in intergenic regions or in antisense orientation in introns of annotated genes, implying that they are expressed from an independent transcription unit [49-51]. About 25-30% of the murine and human miRNA genes are located in introns of other genes in the same orientation of their host genes, suggesting that these miRNAs are co-transcribed and processed from the introns [40,52]. In mammals, 61% of miRNA genes are clustered in the genome and are simultaneously expressed as polycistronic transcripts [52]. Examples of such transcripts are the *miR-17~92* cluster and the *miR-290-295* cluster of miRNAs [53,54].

MiRNAs arise from primary sequences (pri-miRNAs) of various sizes, typically more than 1 kb, which fold into hairpins [40] (Figure 3). Pri-miRNAs contain cap structures and poly(A) tails, and are mainly transcribed by RNA polymerase II (pol II) [55]. However, some miRNAs are transcribed by RNA polymerase III (Pol III), especially those with upstream Alu sequences [56]. Each pri-miRNA hairpin structure contains potentially two mature miRNA sequences, positioned respectively at the 5'- and the 3'-arm of the pri-miRNA sequence. In the first step of processing, pri-miRNAs are cleaved by the microprocessor complex consisting of the highly conserved RNase III endoribonuclease DROSHA and its obligate RNA-binding partner DGCR8 [57]. The product of pri-miRNA cleavage by DROSHA is a ~60-70 nt stem loop intermediate, the so-called precursor miRNA (pre-miRNA) [58]. Importantly, DROSHA cleavage defines the 5' -end of the 5p miRNA and the 3' -end of the 3p miRNA. Next, the pre-miRNAs are exported to the cytoplasm by the karyopherin family member Exportin-5 (EXP5) and its cofactor Ran-GTP [59].

4-2. DICER1

In the cytoplasm, the pre-miRNA is further processed by the enzyme DICER1 [60]. DICER1 is a large (~200 kDa) and evolutionarily conserved member of the RNase III family of endoribonucleases [61]. DICER1 contains three N-terminal helicase domains (HEL1, HEL2i, HEL2), a DUF283 domain, which is presumably involved in binding of double stranded RNA (dsRNA), a "Platform" domain that is tightly associated with the pre-miRNA binding domain PAZ, two tandem RNase III domains a and b, and a C-terminal dsRNA Binding Domain (dsRBD) [62-64]. The RNase III domains of DICER1 cleave pre-miRNAs and double-stranded RNA (dsRNA) substrates into small 5'-phosphorylated and 2 nt 3'-overhangs duplex RNAs of typically 21-23 nt [63,65] (Figure 3).

Genetic studies in plants, zebrafish and mice show that *Dicer1* is essential for normal development [66-68]. For instance, genetic deletion of *Dicer1* in mice depletes the Oct-4-positive pluripotent embryonic stem (ES) cell population at around embryonic day 6-7, and

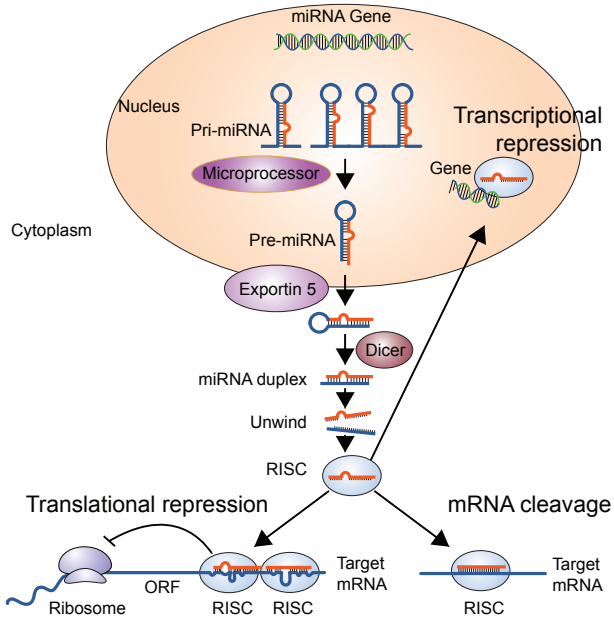


Figure 3. Schematic overview of miRNA biogenesis and activities. Pri-miRNAs are processed by the Microprocessor complex, consisting of the RNase III endoribonuclease DROSHA and its partner DGCR8, exported to the cytoplasm and further cleaved by another endoribonuclease, DICER1. MiRNAs are loaded into RISC and bind to partially complementary sequences of target mRNAs that are predominantly located in the 3' -UTRs. MiRNA-containing RISC may regulate gene expression by transcript destabilization, inhibition of protein translation and transcriptional repression.

results in early embryonic lethality [68]. Because *Dicer1*-null ES cells are incapable to process miRNA hairpins, the lack of miRNAs critical for embryonic development is most likely fully responsible for this lethal phenotype [69-71].

The DICER1 generated double-stranded RNAs become incorporated as single-stranded RNAs in the so-called RNA-induced silencing complex (RISC) of which the AGO proteins (AGO1-4 in mammalian cells) are the main components [72] (Figure 3). Typically, the passenger miRNA sequence, whose 5'-end is less tightly paired, will be degraded [73,74]. However, in some cases both sequences are loaded into RISC [72,75].

4-3. MiRNA activity

The 5'-regions of miRNAs are the most conserved among species. They match conserved elements in the untranslated regions (UTR) of mRNA targets and thereby mediate translational repression and mRNA decay [48,76]. This region of the miRNA (2-8 nt) is called the "seed" sequence. Disruption of this sequence by nucleotide substitutions abrogates miRNA functions [77]. The 3'-end of the miRNA modifies miRNA binding to its target or may

compensate for seed mismatches [78]. Targeting mediated by regions outside the seed sequence has also been reported, but this is uncommon [79,80].

Immunoprecipitation of AGO proteins and identification of co-precipitating mRNAs by RNA sequencing showed that miRNA recognition sites are mainly located in the 3'-UTR of the target genes [81]. After being processed from the primary hairpin structure and RISC loading, the miRNAs pair with the 3'-UTR of mRNAs to control expression of the target mRNA via different mechanisms [82] (Figure 3). MiRNAs may also act at the level of transcriptional regulation [83-87] (Figure 3). These mechanisms are summarized in Table 2.

Individual miRNAs may exert their function by targeting multiple genes. *In silico* analysis showed that functionally related genes are enriched among the targets of some miRNAs [88-90]. For instance, genes involved in growth control are enriched in the predicted targets of *miR-17~92* cluster miRNAs [91]. Among those are several genes of the TGF-β signaling pathway (including *TGF-β receptor II*, *Smad2*, *Smad6*, *Smad7*, and *SARA*), *SOCS* genes (*SOCS1*, *SOCS3*, *SOCS5*, and *SOCS6*), *p130*, and *PTEN* [91].

Table 2. Proposed mechanisms of miRNA action

	Mechanism	Additional comments	Reference
I	Inhibition of translation initiation	Inhibition of Cap-40S ribosomoal subunit association, inhibition of ribosomal 60S and 40S subunits joining	(Pillai et al., 2005) (Chendrimada et al., 2007)
II	Inhibition of translation elongation		(Olsen and Ambros, 1999) (Petersen et al., 2006)
III	Pre-mature termination of translation	Causing ribosomal drop-off	(Petersen et al., 2006) (Wang et al., 2006)
IV	Co-translational protein degradation		(Nottrott et al., 2006) (Petersen et al., 2006)
V	Sequestration of the target mRNA	Sequestration into different structures in the cytoplasm including processing (p)-bodies and stress-granules, where the translational machinery is absent and degradation enzymes are enriched	(Pillai et al., 2005) (Sen and Blau, 2005)
VI	Destabilization of mRNA	Leading to its complete degradation	(Bagga et al., 2005) (Coller and Parker, 2004) (Guo et al., 2010a)
VII	Transcriptional regulation	Chromatin modifications at target promoters, important for cellular senescence and differentiation	(Kim et al., 2008) (Khraiwesh et al., 2010) (Benhamed et al., 2012) (Ketting, 2011) (Zardo et al., 2012)

The expression level of a miRNA is critical for its activity on the target mRNA. Using synthetic miRNA target sites cloned downstream of a reporter gene as miRNA activity sensor, the level of target repression in response to different miRNA expression levels was investigated [92,93]. These studies demonstrated that miRNAs expressed below ~100 copies per cell have little regulatory capacity and only the most abundant miRNAs mediate target suppression [92,93] (Figure 4A). Likewise, when the target mRNAs are expressed below a threshold level, protein production is highly repressed by miRNA activity. However, once the target mRNA levels pass this threshold, miRNAs cannot repress the translation efficiently [94] (Figure 4B). Different mRNA targets having binding sites for the same miRNA may compete for the miRNA (Ebert et al., 2007) (Figure 4C). Also, an mRNA may be targeted by multiple miRNAs. Together, the level of miRNA, the number of miRNAs bound to a target, the level of the target mRNA of interest and the level of competing mRNAs are important parameters determining the miRNA activity on a given mRNA target.

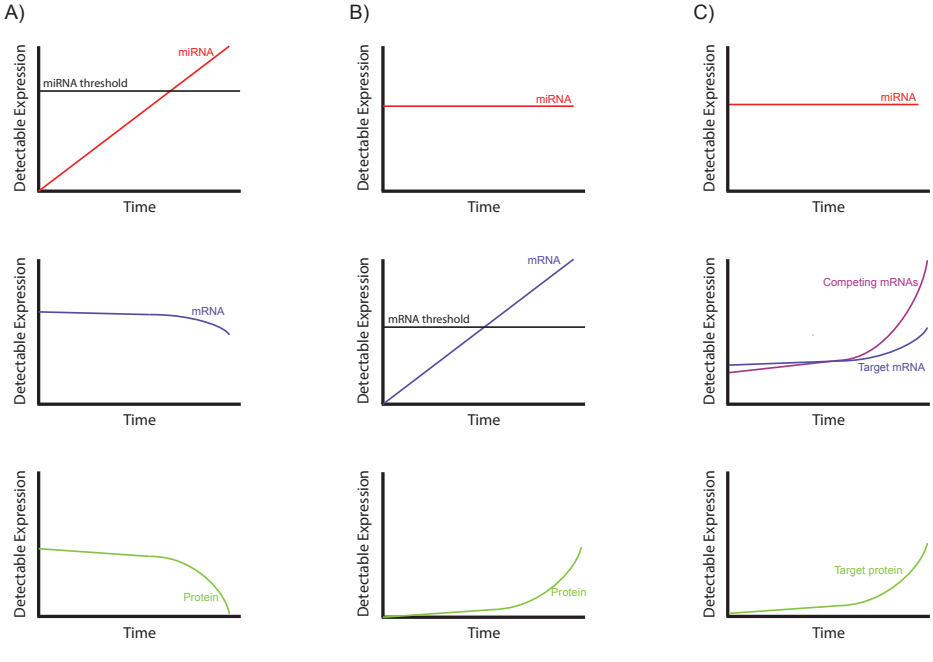


Figure 4. Expression levels of miRNA, target mRNA and target mimics mRNAs affect miRNA activity. The protein output of an mRNA is dependent on the expression level of both miRNA and the mRNA target, as well as the level of the other competing target mRNAs. **A)** MiRNAs expressed below a threshold (~100 molecules per cell) have little regulatory capacity and only the most abundant miRNAs mediate target suppression. **B)** When a particular mRNA target level exceeds the maximum quantity that can be repressed by miRNA, translation of mRNA target is not anymore inhibited by the miRNA. **C)** Expression of a target mRNA is not regulated anymore by a miRNA when the levels of other mRNAs targeted by the same miRNA (competing mRNAs) are increased. Figure is adapted from Leung and Sharp [141].

5. MIRNAS IN HEMATOPOIETIC STEM AND PROGENITOR CELLS

To investigate the role of miRNAs in the development and function of different hematopoietic cell populations, miRNAs have been depleted in different experimental models. As introduced earlier in this chapter, *Dicer1* is essential for normal development and its deletion results in an early embryonic lethality. To bypass this and to enable investigation of miRNA functions in adult tissues in mice, a floxed *Dicer1* allele (*Dicer1*^{fl}) has been generated that allows conditional deletion of *Dicer1* in a cell type- and developmental stage- specific fashion [95]. To address the role of miRNAs in the development and function of hematopoietic cells, different hematopoietic cell specific Cre transgenic strains have been crossed with *Dicer1* floxed mice. For instance, by using an Mx-Cre mouse model, floxed *Dicer1* alleles are efficiently deleted in HSCs by poly I:C injection. *Dicer1* ablation in BM HSCs in this mouse model, results in depletion of all miRNAs [96]. This, results in a rapid induction of apoptosis and consequently causes total disruption of hematopoiesis, mainly due to the lack of *miR-125a*, and thereby de-repression of the proapoptotic gene BAK1 [96].

What is the role of *Dicer1* in myelopoiesis? We addressed this question in **chapter 3** by conditional deletion of *Dicer1* in myeloid progenitors in mice. Furthermore, different hematopoietic cell specific *Dicer1* deletion mouse models are reviewed in **chapter 4**. In addition, we discuss what we have learned from these models about miRNA-controlled pathways in hematopoiesis.

Using computational approaches, cell specific sets of miRNAs and their putative targets that show an inverse pattern of expression have been identified for different subsets of HSCs and myeloid progenitors [97,98]. For instance, 25 miRNAs are expressed at significantly higher level in mouse BM HSCs as compared to more differentiated progenitor populations such as CMP, GMP and MEP [97]. In total, 115 target genes with one or more binding sites for these miRNAs show an inverse pattern of expression with their targeting miRNAs. The network composed of miRNA targets in HSCs is enriched for genes involved in hematological malignancies, cell-to-cell signaling, inflammatory response, hematopoiesis and blood cell function [97].

Other studies focused on the functions of individual miRNAs in HSCs. For example, *miR-142-3p* expressed in HSCs maintains the population of HSCs in the aorta-gonad-mesonephros (AGM) region, one of the regions in embryonic mesoderm in which definitive HSCs are formed [99,100]. Mechanistically, *miR-142a-3p* regulates HSC formation and differentiation through the repression of Interferon regulatory factor-7 (IRF7)-mediated inflammation signaling [99]. Another example is *miR-29a*, which is highly expressed in HSCs and down-regulated in MPPs and in differentiated myeloid cells. *MiR-29* promotes self-renewal and blocks the transition of MPPs towards CMPs [101]. Together, these findings indicate that some miRNAs are critical for cell survival and functions of HSPCs.

6. MIRNA CONTROLLED MYELOID DIFFERENTIATION

Myelopoiesis is mainly driven by transcription factors such as CCAAT enhanced-binding protein alpha (C/EBPα) and Spleen focus forming virus proviral integration oncogene (SPI1, also known as PU.1). These transcription factors activate essential genes for proper expansion and maturation of precursor cells [102]. Several miRNA-encoding genes are activated by these transcription factors.

The transcription factor C/EBPα is critical for granulopoiesis [103,104]. C/EBPα is expressed at very low level in HSCs, but its expression increases in a small fraction of MPPs and is strongly induced during the transition from CMP toward GMP [105-107]. C/EBPα promotes myeloid differentiation of MPPs by activating myeloid-specific genes and suppressing a gene expression program that drives lymphoid differentiation [107]. Deletion of *C/ebpα* in HSCs results in an accumulation of myeloid progenitors because it blocks CMP to GMP transition [104]. C/EBPα regulates multiple miRNAs, including *miR-223*, *miR-27* and *miR-34a*. For instance, C/EBPα directly binds to the promoter of *miR-223* and activates its expression [108]. This finding is consistent with studies reporting a myeloid specific expression of *miR-223* that follows the expression pattern of C/EBPα [109]. The transcription factor Nuclear factor I/A (NFIA) competes with C/EBPα for binding to the *miR-223* promoter [108]. NFIA represses the expression of *miR-223*. Interestingly, NFIA itself is a target of *miR-223* [108]. During the granulocytic differentiation, C/EBPα induces *miR-223* expression, which leads to the repression of NFIA, resulting in a feed-forward loop [108]. Inhibition of NFIA is a critical step in myeloid differentiation [108]. In addition, *miR-223* targets the cell cycle regulator E2F1, whose repression blocks cell cycle progression and contributes to differentiation [110] (Figure 5). Altogether, these data suggest that C/EBPα-mediated *miR-223* expression may play critical roles in granulopoiesis. However, genetic deletion of *miR-223* in mice did not result in a block of granulocytic differentiation [111]. Conversely, *miR-223*-deficient mice have an increased number of circulating neutrophils resulting from an enhanced differentiation and proliferation of the granulocyte progenitor pool [111]. MEF2C, a transcription factor that promotes myeloid progenitor proliferation, is a bona fide target of *miR-223* [111]. Interestingly, genetic ablation of *Mef2c* suppresses progenitor expansion and corrects the neutrophilic phenotype in *miR-223* null mice [111]. This demonstrates that although *miR-223* is dispensable for granulopoiesis, it is essential for normal neutrophil maturation and controls the number of granulocytes [111].

C/EBPα also induces the expression of *miR-27* that targets the transcription factor RUNX1, a potent repressor of G-CSF receptor expression and granulocytic differentiation [112]. Finally, C/EBPα induces the transcription of *miR-34a,* which represses E2F3. The E2F3 repression is a pivotal step in the induction of granulopoiesis [113] (Figure 5). Thus, C/EBPα up-regulated miRNAs control the switch of a progenitor cell state towards a differentiation program that is needed for granulocytic development.

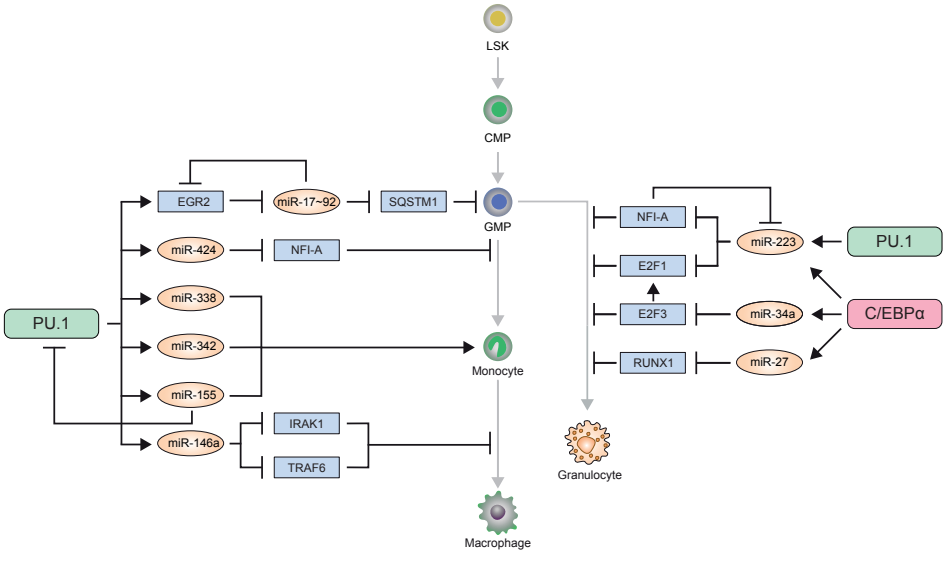


Figure 5. C/EBPα- and PU.1-regulated miRNAs in myelopoiesis. PU.1 regulates transcription of *miR-424*, *miR-146a*, *miR-342*, *miR-338* and *miR-155* in monocyte/macrophage development. PU.1 also induces *EGR2* expression, which in turn causes epigenetic silencing of the *miR-17~92* promoter. Both, C/EBPα and PU.1 enhance the expression of *miR-223*, an important miRNA for the control of progenitor expansion and granulocytic functions. Finally, the C/EBPα controlled transcriptions of *miR-34a* and *miR-27*, repressing E2F family of proteins and RUNX1 respectively, which are together pivotal for the induction of granulopoiesis.

In the studies described in this thesis, we used the *Cebpa-Cre* mice [107]. *Cebpa* promoter-driven CRE recombinase, allowed us to delete our gene of interest specifically in myeloid committed progenitors, among all different populations of HSPCs. In **chapter 3**, we used this model to delete *Dicer1* in myeloid progenitors. In **chapter 4**, we generated myeloid specific conditional *Dicer1; p53* deleted strains, which were used for transplantation experiments to assess the role of *Dicer1* in leukemia development. Finally, in **chapter 5**, to investigate whether enhanced expression of *miR-199a* promotes myeloid leukemia, we overexpressed *miR-199a* in *Cebpa* $^{fl/Cre}$ HSPCs and investigated their leukemogenic potential in transplantation experiments. The Cre enzyme recombines the floxed *Cebpa* allele, resulting in loss of *Cebpa* mainly in myeloid precursors, which leads to a differentiation block at CMP level.

The transcription factor PU.1 is another pivotal regulator of myelopoiesis. PU.1 regulates the expansion of myeloid progenitors, induces myeloid differentiation by repression of

miR-17~92 cluster of miRNAs [114,115], and controls macrophage development, mainly via inducing the transcription of *miR-146a*, *miR-342*, *miR-338* and *miR-155* [114] (Figure 5).

PU.1 controlled miRNAs are summarized in **chapter 2**. Moreover, we discuss how PU.1 and miRNAs interact to regulate myelopoiesis.

Together, regulatory circuits generated by transcription factors and miRNAs allow for a tight control of myeloid development. Aberrant expression of any of these key components may ultimately lead to pathological conditions, including hematopoietic disorders and myeloid malignancies.

7. MIRNAS IN MALIGNANT MYELOPOIESIS

Like in most types of human cancer, miRNAs are aberrantly expressed in AML [38,116,117]. Similar to gene expression profiles, which allow classification of AML [118,119], miRNA-expression signatures also define different subtypes of AML [116,120-123]. Unsupervised miRNA expression analysis reveals distinctive miRNA signatures that correlate with cytogenetic and molecular subtypes of AML, such as the translocation involving chromosomes 8 and 21 (t(8;21)), t(15;17), inversion of chromosome 16 (inv.(16)), and *NPM1* and *CEBPA* mutations [116]. Intriguingly, miRNA expression signatures could predict cytogenetically normal AML with mutations in the genes encoding NPM1, C/EBPα and FLT3-ITD with similar accuracy as mRNA probe set combinations defined by gene expression profiling [116]. As expected from the above described observations, patterns of miRNA expression provide useful information for AML classification and prognosis [116,117,120,121,124,125]. For instance, high *miR-212* expression levels are significantly associated with a prolonged overall- , event-free- and relapse-free survival of AML patients, independent from other known prognostic factors [126]. In a different study, AML patients with high expression of *miR-199a* and *miR-191* were found to have significant shorter overall- and event-free survival [121]. These data suggest an important role for miRNAs in the pathophysiology of AML.

There are strong indications that the deregulated expression of miRNAs plays a role in leukemogenesis. Several aberrantly expressed miRNAs in hematological malignancies show oncogenic activities in experimental models. For example, a subset of AML and MDS patients carrying the translocation t(2;11)(p21;q23) or t(15;17)(q22;q21), or megakaryoblastic leukemia patients with trisomy 21(Down syndrome) show a 6-90-fold increased *miR-125* expression compared with AML cases lacking these translocations [116,127,128]. Enhanced *miR-125* expression in mouse HSPCs caused an enhanced expansion of white blood cells, myeloproliferative disorder, lymphoid leukemia and AML, phenotypes which were largely dependent on the level of *miR-125* overexpression [128-132].

Enhanced *miR-125* expression in HSPCs have been investigated in different studies and, surprisingly, caused different hematopoietic phenotypes, including an enhanced expansion of white blood cells, myeloproliferative disorder, lymphoid leukemia or AML. In **chapter 2** we review these studies and discuss the networks regulated by *miR-125* in normal and malignant myelopoiesis. Furthermore, we discuss the contradictory and confusing observations in relation to the experimental models used.

Global depletion of miRNAs is found in human cancer including different types of leukemia [133]. These data suggest that DICER1 may be a tumor suppressor. Indeed, investigation of the role of *Dicer1* in human cancer development in immune-deficient mice indicated that *Dicer1* is a haploinsufficient tumor suppressor [134]. Deletion of a single copy of *Dicer1* in a genetically engineered mouse model of K-Ras driven lung cancer, led to reduced survival compared with controls [134]. These tumors exhibited impaired miRNA processing but failed to lose the *wild- type* (*wt*) *Dicer1* allele. Consistent with selection against full loss of *Dicer1*, enforced homozygous deletion of *Dicer1*, although tolerated, strongly reduced cell proliferation capacity of the tumor cells [134]. In agreement, heterozygous deletion of *Dicer1*, but not its complete loss, accelerated tumor formation in a mouse model of retinoblastoma [135].

In **chapter 4**, we investigate a role for *Dicer1* in myeloid leukemia by conditional deletion of both *Dicer1* and the tumor suppressor *Trp53* in myeloid-committed progenitors. We show evidence for *Dicer1* dose-dependent tumor suppressor activity in mouse leukemia and discuss the role for DICER1 in AML.

8. MIRNAS IN STRESS CONDITIONS

Following stress conditions and genetic perturbations, cells try to restore homeostasis. The stress response decision is mediated by multiple mechanisms such as rapid clearance of the damaged macromolecules [136], growth arrest [137], reprogrammed gene expression patterns to cope with the new situation [138], or activation of programmed cell death mechanisms when cell damages is excessive [139]. Because miRNAs are potent regulators of gene expression, they contribute significantly to the establishment of the stress responses [140-142].

The functions of miRNAs are modified under stress conditions. Emerging data indicate that stress conditions influence miRNA activities at four different levels:

I) MiRNA expression: Stress conditions can alter the biogenesis of miRNAs by affecting their transcription, their processing by DROSHA and DICER1 or their stability (Figure 6A).

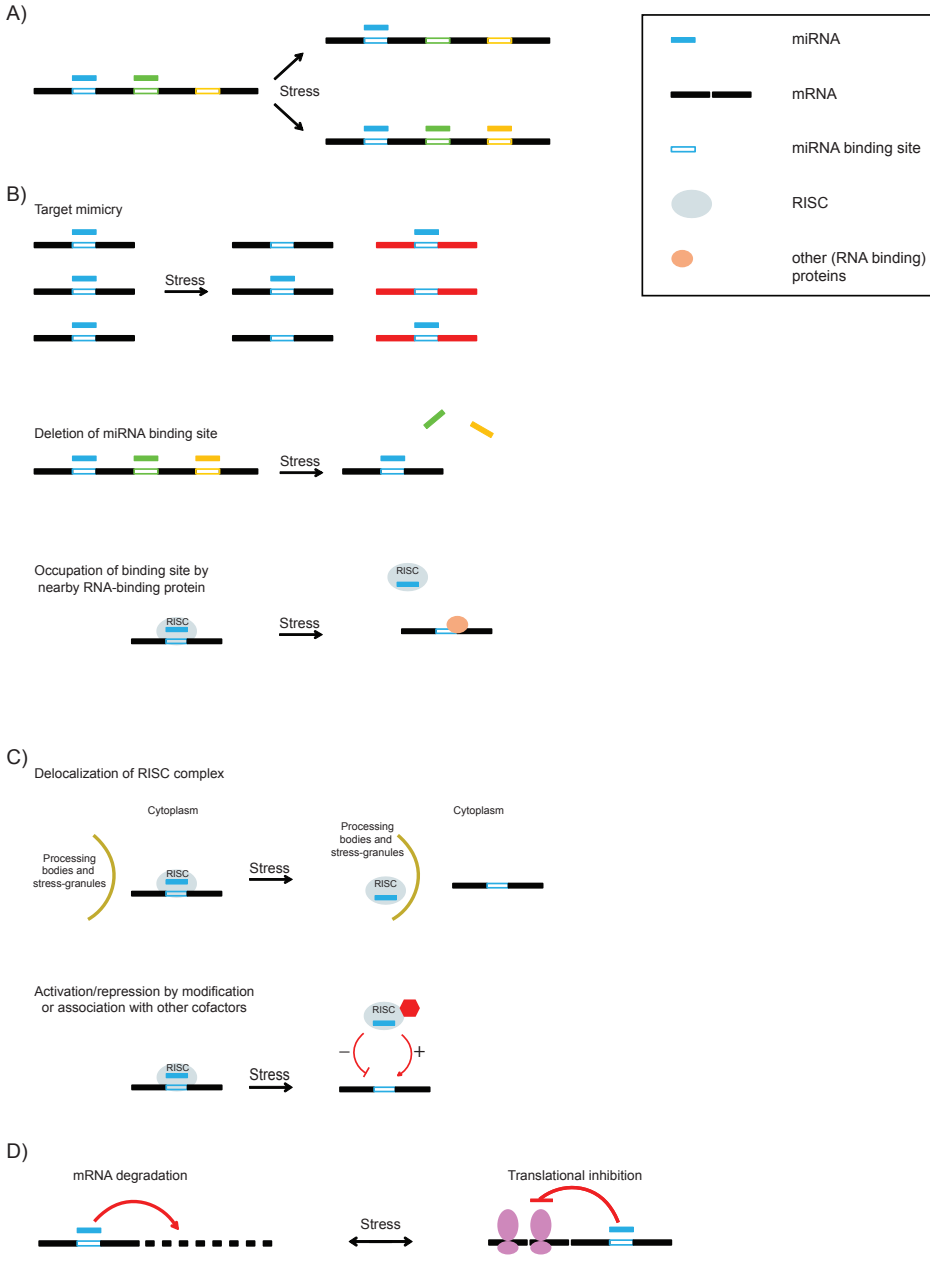


Figure 6. Stress conditions modify miRNA activity. The miRNA function can be modulated at multiple levels: (A) by changing the level of mature miRNAs, (B) modification of the availability of the target mRNA, (C) modification of RISC complex, and (D) a switch in the mode of miRNA action. **A) Modification of expression level of miRNAs:** An mRNA target containing binding sites for three different miRNAs (blue, green and orange) is depicted. In a non-stressed condition, the target is ▶

For instance, p53, induced upon DNA damage, upregulates the expression of several miRNAs at the transcription or the processing levels [143,144].

II) Expression level and accessibility of target mRNA: The modification of the expression levels of mRNA targets and the accessibility of the miRNA binding sites in the 3'-UTR region of a target mRNA, for example via an alternative splicing and polyadenylation, may change the target selection of miRNAs [145] (Figure 6B). Furthermore, RNA Binding Proteins (RBPs) bound nearby miRNA binding sites on the same mRNA target, may hamper the binding of miRNAs and thereby relieve the repression caused by miRNAs (Figure 6B). Intriguingly, AU-rich elements (AREs), which are binding sites for at least of 20 different RBPs, e.g. HuR and HuD, are enriched near miRNA binding sites [146,147], raising the possibility that differential activity of these RBPs under stress condition changes the availability of the miRNA binding site and thereby interact with the activity of miRNAs.

III) RISC stability and localization: The activity of miRNAs could be changed upon stress, via destabilization and relocalization of the RISC complex caused by post-translational modifications of RISC components [148] (Figure 6C). For instance, hydroxylation of AGO2 upon hypoxia increases the stability of RISC [149]. Also, phosphorylation of AGO2 by MAPK-activated protein kinase-2 (MAPKAPK2) in response to the activation of the p38 MAPK pathway, facilitates the localization of miRNA-loaded RISC and the target mRNA into different structures in the cytoplasm including processing (p)-bodies and stress-granules, where the translational machinery is absent and degradation enzymes are enriched [150].

IV) MiRNA mode of action: Stress can alter the mode of action of RISC, from acceleration of mRNA decay to translational inhibition, or vice versa [151] (Figure 6D). The consequences of these two modes of action for mRNA targets are different because the degradation of mRNA is an irreversible process.

▶ repressed by two different miRNAs (blue and green). Upon stress, the expression of one of miRNAs (green) decreases, resulting in a situation where only one site is bound by miRNA and the level of the mRNA target will increase. Alternatively, when the level of another miRNA (orange) increases, three sites are now bound by miRNAs, resulting in a stronger repression of the target. **B) Modification of availability of target mRNA:** Due to competition, the expression of mRNA target (black) increases when the expression of another mRNA with binding sites for the same miRNA (red) increases upon stress. Alternatively, upon stress, cells could express different isoforms of the mRNA targets where miRNA binding sites are added or deleted. Finally, RNA-binding proteins interacting with the 3'-UTR of mRNAs, may inhibit the adjacent interaction of miRNAs and prevent mRNA targets from miRNA-controlled repression. **C) Modification of RISC complex:** A change in the activity of RISC complex upon stress could be caused by differential subcellular localization of the RISC complex, posttranslational modifications of RISC components or direct association with other stress-specific cofactors which hamper or stimulate miRNA activities. **D) Switch in the Mode of miRNA action:** Stress may alter the balance between mRNA decay and inhibition of translation. Figure is adapted from Leung and Sharp [141].

Because miRNAs are potent regulators of gene expression, these precise modifications of miRNA activities determine the specificity and timing of stress responses, which is crucial for regaining homeostasis [140]. However, excessive stress deregulates miRNA expression and activity and hampers the appropriate miRNA-mediated stress response [141,142]. This results in damages in normal physiological processes such as proliferation, differentiation and apoptosis, and thereby contributes to diseases, including cancers [141,142].

Do miRNAs play a role in the response to ICL DNA damage in hematopoietic cells? This question has been addressed in **chapter 5**, where we present evidence for the role of two miRNAs, *miR-139-3p* and *miR-199a-3p*, in ICL-induced BMF and its progression towards leukemia.

REFERENCES

1. Blank U, Karlsson G, Karlsson S. Signaling pathways governing stem-cell fate. *Blood*. 2008;111(2):492-503.
2. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105-111.
3. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. *Blood*. 1993;81(11):2844-2853.
4. Morrison SJ, Wandycz AM, Hemmati HD, Wright DE, Weissman IL. Identification of a lineage of multipotent hematopoietic progenitors. *Development*. 1997;124(10):1929-1939.
5. Bagby GC, Meyers G. Bone marrow failure as a risk factor for clonal evolution: prospects for leukemia prevention. *Hematology Am Soc Hematol Educ Program*. 2007:40-46.
6. Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. *Blood Rev*. 2010;24(3):101-122.
7. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med*. 1999;341(14):1051-1062.
8. SEER. Surveillance Epidemiology and End Results cancer statistic review. Vol. 2014: National Cancer Institute; 2011.
9. Crossan GP, Patel KJ. The Fanconi anaemia pathway orchestrates incisions at sites of crosslinked DNA. *J Pathol*. 2012;226(2):326-337.
10. Bogliolo M, Schuster B, Stoepker C, et al. Mutations in ERCC4, encoding the DNA-repair endonuclease XPF, cause Fanconi anemia. *Am J Hum Genet*. 2013;92(5):800-806.
11. Kashiyama K, Nakazawa Y, Pilz DT, et al. Malfunction of nuclease ERCC1-XPF results in diverse clinical manifestations and causes Cockayne syndrome, xeroderma pigmentosum, and Fanconi anemia. *Am J Hum Genet*. 2013;92(5):807-819.
12. Kim H, D'Andrea AD. Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. *Genes Dev*. 2012;26(13):1393-1408.
13. Otsuki T, Wang J, Demuth I, Digweed M, Liu JM. Assessment of mitomycin C sensitivity in Fanconi anemia complementation group C gene (Fac) knock-out mouse cells. *Int J Hematol*. 1998;67(3):243-248.
14. Prasher JM, Lalai AS, Heijmans-Antonissen C, et al. Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1-/- mice. *EMBO J*. 2005;24(4):861-871.
15. Weeda G, Donker I, de Wit J, et al. Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Curr Biol*. 1997;7(6):427-439.
16. Auerbach AD. Fanconi anemia and its diagnosis. *Mutat Res*. 2009;668(1-2):4-10.
17. Kutler DI, Singh B, Satagopan J, et al. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). *Blood*. 2003;101(4):1249-1256.
18. Rosenberg PS, Greene MH, Alter BP. Cancer incidence in persons with Fanconi anemia. *Blood*. 2003;101(3):822-826.
19. Langmuir PB, Aplenc R, Lange BJ. Acute myeloid leukaemia in children. *Best Pract Res Clin Haematol*. 2001;14(1):77-93.
20. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. *Cell Stem Cell*. 2012;10(2):120-136.
21. Garaycoechea JI, Crossan GP, Langevin F, Daly M, Arends MJ, Patel KJ. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. *Nature*. 2012;489(7417):571-575.
22. Gates KS. An Overview of Chemical Processes That Damage Cellular DNA: Spontaneous Hydrolysis, Alkylation, and Reactions with Radicals. *Chemical Research in Toxicology*. 2009;22(11):1747-1760.
23. Niedernhofer LJ. DNA repair is crucial for maintaining hematopoietic stem cell function. *DNA Repair*. 2008;7(3):523-529.
24. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of

haematopoietic stem cells with age. *Nature*. 2007;447(7145):725-729.

25. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature*. 2001;411(6835):366-374.
26. Deans AJ, West SC. DNA interstrand crosslink repair and cancer. *Nat Rev Cancer*. 2011;11(7):467-480.
27. Kim H, Yang K, Dejsuphong D, D'Andrea AD. Regulation of Rev1 by the Fanconi anemia core complex. *Nat Struct Mol Biol*. 2012;19(2):164-170.
28. Meetei AR, de Winter JP, Medhurst AL, et al. A novel ubiquitin ligase is deficient in Fanconi anemia. *Nat Genet*. 2003;35(2):165-170.
29. Garcia-Higuera I, Taniguchi T, Ganesan S, et al. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell*. 2001;7(2):249-262.
30. Smogorzewska A, Matsuoka S, Vinciguerra P, et al. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell*. 2007;129(2):289-301.
31. Hodskinson MR, Silhan J, Crossan GP, et al. Mouse SLX4 is a tumor suppressor that stimulates the activity of the nuclease XPF-ERCC1 in DNA crosslink repair. *Mol Cell*. 2014;54(3):472-484.
32. Klein Douwel D, Boonen RA, Long DT, et al. XPF-ERCC1 acts in Unhooking DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/SLX4. *Mol Cell*. 2014;54(3):460-471.
33. Ikeda H, Matsushita M, Waisfisz Q, et al. Genetic reversion in an acute myelogenous leukemia cell line from a Fanconi anemia patient with biallelic mutations in BRCA2. *Cancer Res*. 2003;63(10):2688-2694.
34. Quentin S, Cuccuini W, Ceccaldi R, et al. Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions. *Blood*. 2011;117(15):e161-170.
35. Ceccaldi R, Parmar K, Mouly E, et al. Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. *Cell Stem Cell*. 2012;11(1):36-49.
36. Ceccaldi R, Briot D, Larghero J, et al. Spontaneous abrogation of the G2 DNA damage checkpoint has clinical benefits but promotes leukemogenesis in Fanconi anemia patients. *The Journal of Clinical Investigation*. 2011;121(1):184-194.
37. Li J, Sejas DP, Zhang X, et al. TNF-α induces leukemic clonal evolution ex vivo in Fanconi anemia group C murine stem cells. *The Journal of Clinical Investigation*. 2007;117(11):3283-3295.
38. Carninci P, Kasukawa T, Katayama S, et al. The transcriptional landscape of the mammalian genome. *Science*. 2005;309(5740):1559-1563.
39. Cech Thomas R, Steitz Joan A. The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones. *Cell*. 2014;157(1):77-94.
40. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-297.
41. Ambros V. A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. *Cell*. 1989;57(1):49-57.
42. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*. 1993;75(5):855-862.
43. Murchison EP, Hannon GJ. miRNAs on the move: miRNA biogenesis and the RNAi machinery. *Curr Opin Cell Biol*. 2004;16(3):223-229.
44. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research*. 2014;42(D1):D68-D73.
45. Landgraf P, Rusu M, Sheridan R, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*. 2007;129(7):1401-1414.
46. Ibanez-Ventoso C, Vora M, Driscoll M. Sequence relationships among C. elegans,

D. melanogaster and human microRNAs highlight the extensive conservation of microRNAs in biology. *PLoS One*. 2008;3(7):e2818.

47. Meunier J, Lemoine F, Soumillon M, et al. Birth and expression evolution of mammalian microRNA genes. *Genome Research*. 2013;23(1):34-45.
48. Lim LP, Lau NC, Weinstein EG, et al. The microRNAs of Caenorhabditis elegans. *Genes Dev*. 2003;17(8):991-1008.
49. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science*. 2001;294(5543):853-858.
50. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science*. 2001;294(5543):858-862.
51. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. *Science*. 2001;294(5543):862-864.
52. Chiang HR, Schoenfeld LW, Ruby JG, et al. Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. *Genes Dev*. 2010;24(10):992-1009.
53. Concepcion CP, Bonetti C, Ventura A. The microRNA-17-92 family of microRNA clusters in development and disease. *Cancer J*. 2012;18(3):262-267.
54. Houbaviy HB, Dennis L, Jaenisch R, Sharp PA. Characterization of a highly variable eutherian microRNA gene. *RNA*. 2005;11(8):1245-1257.
55. Lee Y, Kim M, Han J, et al. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*. 2004;23(20):4051-4060.
56. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. 2006;13(12):1097-1101.
57. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 2009;10(2):126-139.
58. Lee Y, Ahn C, Han J, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003;425(6956):415-419.
59. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*. 2003;17(24):3011-3016.
60. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*. 2002;21(17):4663-4670.
61. Carmell MA, Hannon GJ. RNase III enzymes and the initiation of gene silencing. *Nat Struct Mol Biol*. 2004;11(3):214-218.
62. Dlakic M. DUF283 domain of Dicer proteins has a double-stranded RNA-binding fold. *Bioinformatics*. 2006;22(22):2711-2714.
63. Lau PW, Guiley KZ, De N, Potter CS, Carragher B, Macrae IJ. The molecular architecture of human Dicer. *Nat Struct Mol Biol*. 2012.
64. Qin H, Chen F, Huan X, Machida S, Song J, Yuan YA. Structure of the Arabidopsis thaliana DCL4 DUF283 domain reveals a noncanonical double-stranded RNA-binding fold for protein-protein interaction. *RNA*. 2010;16(3):474-481.
65. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*. 2001;409(6818):363-366.
66. Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. *Genes Dev*. 2002;16(13):1616-1626.
67. Wienholds E, Koudijs MJ, van Eeden FJ, Cuppen E, Plasterk RH. The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat Genet*. 2003;35(3):217-218.
68. Bernstein E, Kim SY, Carmell MA, et al. Dicer is essential for mouse development. *Nat Genet*. 2003;35(3):215-217.
69. Calabrese JM, Seila AC, Yeo GW, Sharp PA. RNA sequence analysis defines Dicer's role in mouse embryonic stem cells. *Proc Natl Acad Sci U S A*. 2007;104(46):18097-18102.
70. Kanellopoulou C, Muljo SA, Kung AL, et al. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev*. 2005;19(4):489-501.

71. Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. *Proc Natl Acad Sci U S A*. 2005;102(34):12135-12140.

72. Mourelatos Z, Dostie J, Paushkin S, et al. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev*. 2002;16(6):720-728.

73. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. *Cell*. 2003;115(2):209-216.

74. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell*. 2003;115(2):199-208.

75. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. *Science*. 2002;297(5589):2056-2060.

76. Lai EC. Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet*. 2002;30(4):363-364.

77. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev*. 2004;18(5):504-511.

78. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science*. 2004;304(5670):594-596.

79. Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP. Expanding the microRNA targeting code: functional sites with centered pairing. *Mol Cell*. 2010;38(6):789-802.

80. Lal A, Navarro F, Maher CA, et al. miR-24 Inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to "seedless" 3'UTR microRNA recognition elements. *Mol Cell*. 2009;35(5):610-625.

81. Beitzinger M, Peters L, Zhu JY, Kremmer E, Meister G. Identification of Human microRNA Targets From Isolated Argonaute Protein Complexes. *rnabiology*. 2007;4(2):76-84.

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

83. Benhamed M, Herbig U, Ye T, Dejean A, Bischof O. Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. *Nat Cell Biol*. 2012;14(3):266-275.

84. Ketting RF. The many faces of RNAi. *Dev Cell*. 2011;20(2):148-161.

85. Khraiwesh B, Arif MA, Seumel GI, et al. Transcriptional Control of Gene Expression by MicroRNAs. *Cell*. 2010;140(1):111-122.

86. Kim DH, Saetrom P, Snove O, Jr., Rossi JJ. MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A*. 2008;105(42):16230-16235.

87. Zardo G, Ciolfi A, Vian L, et al. Polycombs and microRNA-223 regulate human granulopoiesis by transcriptional control of target gene expression. *Blood*. 2012;119(17):4034-4046.

88. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005;37(5):495-500.

89. Grun D, Wang YL, Langenberger D, Gunsalus KC, Rajewsky N. microRNA target predictions across seven Drosophila species and comparison to mammalian targets. *PLoS Comput Biol*. 2005;1(1):e13.

90. Tsang JS, Ebert MS, van Oudenaarden A. Genome-wide dissection of microRNA functions and cotargeting networks using gene set signatures. *Mol Cell*. 2010;38(1):140-153.

91. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005;120(1):15-20.

92. Brown BD, Gentner B, Cantore A, et al. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol*. 2007;25(12):1457-1467.

93. Mullokandov G, Baccarini A, Ruzo A, et al. High-throughput assessment of microRNA

activity and function using microRNA sensor and decoy libraries. *Nat Methods*. 2012;9(8):840-846.

94. Mukherji S, Ebert MS, Zheng GX, Tsang JS, Sharp PA, van Oudenaarden A. MicroRNAs can generate thresholds in target gene expression. *Nat Genet*. 2011;43(9):854-859.
95. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci U S A*. 2005;102(31):10898-10903.
96. Guo S, Lu J, Schlanger R, et al. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci U S A*. 2010;107(32):14229-14234.
97. Heiser D, Tan YS, Kaplan I, et al. Correlated miR-mRNA Expression Signatures of Mouse Hematopoietic Stem and Progenitor Cell Subsets Predict "Stemness" and "Myeloid" Interaction Networks. *PLoS One*. 2014;9(4):e94852.
98. Raghavachari N, Liu P, Barb JJ, et al. Integrated analysis of miRNA and mRNA during differentiation of human CD34+ cells delineates the regulatory roles of microRNA in hematopoiesis. *Exp Hematol*. 2014;42(1):14-27 e11-12.
99. Lu X, Li X, He Q, et al. miR-142-3p regulates the formation and differentiation of hematopoietic stem cells in vertebrates. *Cell Research*. 2013;23(12):1356-1368.
100. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*. 1996;86(6):897-906.
101. Han YC, Park CY, Bhagat G, et al. microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J Exp Med*. 2010;207(3):475-489.
102. Rosenbauer F, Tenen DG. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol*. 2007;7(2):105-117.
103. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol*. 1998;18(7):4301-4314.
104. Zhang P, Iwasaki-Arai J, Iwasaki H, et al. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity*. 2004;21(6):853-863.
105. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000;404(6774):193-197.
106. Traver D, Miyamoto T, Christensen J, Iwasaki-Arai J, Akashi K, Weissman IL. Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. Vol. 98; 2001.
107. Wolfler A, Danen-van Oorschot AA, Haanstra JR, et al. Lineage-instructive function of C/EBPalpha in multipotent hematopoietic cells and early thymic progenitors. *Blood*. 2010;116(20):4116-4125.
108. Fazi F, Rosa A, Fatica A, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*. 2005;123(5):819-831.
109. Fukao T, Fukuda Y, Kiga K, et al. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell*. 2007;129(3):617-631.
110. Pulikkan JA, Dengler V, Peramangalam PS, et al. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood*. 2010;115(9):1768-1778.
111. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation andgranulocytefunctionbymicroRNA-223. *Nature*. 2008;451(7182):1125-1129.
112. Feng J, Iwama A, Satake M, Kohu K. MicroRNA-27 enhances differentiation of myeloblasts into granulocytes by post-

transcriptionally downregulating Runx1. *Br J Haematol*. 2009;145(3):412-423.

113. PulikkanJA,PeramangalamPS,DenglerV,et al. C/EBPalpha regulated microRNA-34a targets E2F3 during granulopoiesis and is down-regulated in AML with CEBPA mutations. *Blood*. 2010;116(25):5638-5649.
114. GhaniS,RiemkeP,SchonheitJ,etal.Macrophage development from HSCs requires PU.1-coordinated microRNA expression. *Blood*. 2011;118(8):2275-2284.
115. PospisilV,VargovaK,KokavecJ,etal.Epigenetic silencing of the oncogenic miR-17-92 cluster during PU.1-directed macrophage differentiation. *EMBO J*. 2011;30(21):4450-4464.
116. Jongen-LavrencicM,SunSM,DijkstraMK,Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.
117. Marcucci G, Mrozek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. *Blood*. 2011;117(4):1121-1129.
118. BullingerL,DohnerK,BairE,etal.Useofgene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1605-1616.
119. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1617-1628.
120. Garzon R, Garofalo M, Martelli MP, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A*. 2008;105(10):3945-3950.
121. Garzon R, Volinia S, Liu CG, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood*. 2008;111(6):3183-3189.
122. Li Z, Lu J, Sun M, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. *Proc Natl Acad Sci U S A*. 2008;105(40):15535-15540.
123. WangY,LiZ,HeC,etal.MicroRNAsexpression signatures are associated with lineage and survival in acute leukemias. *Blood Cells Mol Dis*. 2010;44(3):191-197.
124. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006;6(11):857-866.
125. RuckerFG,RussAC,CocciardiS,etal.Altered miRNA and gene expression in acute myeloid leukemia with complex karyotype identify networks of prognostic relevance. *Leukemia*. 2012.
126. Sun SM, Rockova V, Bullinger L, et al. The prognostic relevance of miR-212 expression with survival in cytogenetically and molecularly heterogeneous AML. *Leukemia*. 2012.
127. Bousquet M, Quelen C, Rosati R, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation. *J Exp Med*. 2008;205(11):2499-2506.
128. Klusmann JH, Li Z, Bohmer K, et al. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. *Genes Dev*. 2010;24(5):478-490.
129. Bousquet M, Harris MH, Zhou B, Lodish HF. MicroRNA miR-125b causes leukemia. *Proc Natl Acad Sci U S A*. 2010;107(50):21558-21563.
130. GerritsA,WalasekMA,OlthofS,etal.Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. *Blood*. 2012;119(2):377-387.
131. O'Connell RM, Chaudhuri AA, Rao DS, Gibson WS, Balazs AB, Baltimore D. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proc Natl Acad Sci U S A*. 2010;107(32):14235-14240.
132. OoiAG,SahooD,AdornoM,WangY,Weissman IL, Park CY. MicroRNA-125b expands hematopoietic stem cells and enriches for

the lymphoid-balanced and lymphoid-biased subsets. *Proc Natl Acad Sci U S A*. 2010;107(50):21505-21510.

133. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834-838.
134. Kumar MS, Pester RE, Chen CY, et al. Dicer1 functions as a haploinsufficient tumor suppressor. *Genes Dev*. 2009;23(23):2700-2704.
135. Lambertz I, Nittner D, Mestdagh P, et al. Monoallelic but not biallelic loss of Dicer1 promotes tumorigenesis in vivo. *Cell Death Differ*. 2010;17(4):633-641.
136. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. *Mol Cell*. 2010;40(2):280-293.
137. Hoffman B, Liebermann DA. Gadd45 modulation of intrinsic and extrinsic stress responses in myeloid cells. *Journal of Cellular Physiology*. 2009;218(1):26-31.
138. Spriggs KA, Bushell M, Willis AE. Translational regulation of gene expression during conditions of cell stress. *Mol Cell*. 2010;40(2):228-237.
139. Kültz D. MOLECULAR AND EVOLUTIONARY BASIS OF THE CELLULAR STRESS RESPONSE. *Annual Review of Physiology*. 2005;67(1):225-257.
140. Ebert MS, Sharp PA. Roles for microRNAs in conferring robustness to biological processes. *Cell*. 2012;149(3):515-524.
141. Leung AK, Sharp PA. MicroRNA functions in stress responses. *Mol Cell*. 2010;40(2):205-215.
142. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. *Cell*. 2012;148(6):1172-1187.
143. He L, He X, Lim LP, et al. A microRNA component of the p53 tumour suppressor network. *Nature*. 2007;447(7148):1130-1134.
144. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. Modulation of microRNA processing by p53. *Nature*. 2009;460(7254):529-533.
145. Elkon R, Ugalde AP, Agami R. Alternative cleavage and polyadenylation: extent, regulation and function. *Nat Rev Genet*. 2013;14(7):496-506.
146. Jacobsen A, Wen J, Marks DS, Krogh A. Signatures of RNA binding proteins globally coupled to effective microRNA target sites. *Genome Res*. 2010;20(8):1010-1019.
147. Barreau C, Paillard L, Osborne HB. AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Research*. 2005;33(22):7138-7150.
148. Heo I, Kim VN. Regulating the regulators: posttranslational modifications of RNA silencing factors. *Cell*. 2009;139(1):28-31.
149. Qi HH, Ongusaha PP, Myllyharju J, et al. Prolyl 4-hydroxylation regulates Argonaute 2 stability. *Nature*. 2008;455(7211):421-424.
150. Zeng Y, Sankala H, Zhang X, Graves PR. Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. *Biochem J*. 2008;413(3):429-436.
151. Holtz J, Pasquinelli AE. Uncoupling of lin-14 mRNA and protein repression by nutrient deprivation in Caenorhabditis elegans. *RNA*. 2009;15(3):400-405.
152. Marcucci G, Haferlach T, Dohner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. *J Clin Oncol*. 2011;29(5):475-486.
153. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev*. 2004;18(2):115-136.
154. Takahashi S. Current findings for recurring mutations in acute myeloid leukemia. *J Hematol Oncol*. 2011;4:36.
155. Walker A, Marcucci G. Molecular prognostic factors in cytogenetically normal acute myeloid leukemia. *Expert Rev Hematol*. 2012;5(5):547-558.

2

MicroRNAs: Key Players of Normal and Malignant Myelopoiesis

Mir Farshid Alemdehy and Stefan J. Erkeland

Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands

Current Opinion in Hematology. 2012; 19(4):261-7

ABSTRACT

Purpose of the review: Recent data show that microRNAs (miRNAs) play critical roles in the regulation of the developmental process of hematopoietic stem and progenitor cells toward mature myeloid cells. The main focus of the article is the function of some evolutionary conserved miRNAs that are abundantly expressed and tightly regulated during myelopoiesis.

Recent findings: Global miRNA depletion studies in hematopoietic stem cells have shown the importance of miRNA-controlled pathways for hematopoiesis. Recent insights from genetic mouse models and overexpression or deletion of miRNAs in developmental cell intermediates demonstrate strong evidence for evolutionary conserved miRNA-regulated pathways involved in tight control of cellular processes such as proliferation, differentiation and apoptosis at different stages of blood cell development. It is becoming evident that the myeloid transcription factor PU.1 regulates the expression of critical miRNAs including *miR-17~92* and *miR-146a* during myelopoiesis. Furthermore, there is evidence for the contribution of aberrant *miR-125* activities in hematopoietic disorders including myeloid leukemia.

Summary: Despite the large number of articles describing differential miRNA expression during hematopoiesis, miRNA functions and their downstream pathways in myeloid lineage decisions and leukemia are only recently emerging. Here we discuss new findings concerning PU.1-controlled miRNAs and *miR-125*-regulated networks in normal and malignant myelopoiesis.

INTRODUCTION

MicroRNAs (miRNAs) are 19-23 nucleotide RNA molecules and belong to a class of small single-stranded noncoding RNAs. They arise from primary sequences of various sizes (pri-miRNAs) that are further processed by two RNase III endoribonucleases DROSHA and DICER1. MiRNAs are loaded into the RNA-induced silencing complex (RISC) and bind to complementary sequences of target mRNAs that are predominantly located in the 3'-untranslated regions and regulate gene expression by transcript destabilization and inhibition of protein translation. As other transcripts, the expression of miRNAs is regulated at different levels. For instance, the transcription of miRNA genes is controlled by transcription factors, such as E2F, c-MYC, hematopoietic transcription factor spleen focus forming virus proviral integration oncogene (SPI1 or PU.1) and CCAAT/enhancer binding-protein alpha (CEBPα) [1-4]. Recently, multiple factors have been identified that posttranscriptionally regulate miRNA biogenesis, such as Argonaute-2 (AGO-2) [5], arsenic resistance protein-2 (ARS2) [6], monocyte chemoattractant protein 1-induced protein-1 (MCP1P1) [7], and LIN28 and its cofactors Musashi-1 (MSI1) and uridylyltransferase-4 (TUT4) [8,9] .

There is mounting evidence that miRNAs are potent regulators of hematopoiesis [10]. First, miRNA expression profiling studies showed dynamic expression of miRNAs during blood cell development, strongly suggesting that miRNAs play critical roles during this process [11,12] (for review [13]). Second, the importance of miRNAs for normal hematopoiesis has been demonstrated by targeted disruption of miRNA biogenesis in mice. For example, hematopoietic stem cells (HSCs) are impaired by loss of *Dicer1* and these miRNA-depleted HSCs are unable to reconstitute hematopoiesis in mice [14]. In addition, conditional deletion of *Ago-2*, a factor that is critical for miRNA functions, results in impaired differentiation of B-lymphocytes and erythroid cells [15]. Also, hematopoietic lineage-specific conditional deletion of *Dicer1* has revealed the involvement of miRNAs in the survival, maturation and homeostasis of peripheral T-lymphocytes, and antibody diversity and survival of B-lymphocytes [16-18]. Third, several studies demonstrate an interplay of miRNAs and transcription factors, such as the critical regulators of hematopoiesis CEBPα and transcription factor PU.1 [2,4]. Fourth, forced expression of miRNAs such as *miRs-17/20/93/106* promotes expansion of hematopoietic stem and progenitor cells (HSPCs) in mice [19]. Finally, genetic ablation of individual miRNAs such as *miR-223* and *miR-146* in HSCs causes hematopoietic phenotypes [20-22]. Different functions of miRNAs in normal and malignant hematopoiesis have been described previously and have recently been reviewed [23-27]. Here, we discuss new insights concerning a set of miRNAs including PU.1–controlled miRNAs and *miR-125*, of which extensive knowledge has been gained in the past year. These miRNAs are abundantly expressed at different stages of myelopoiesis and are aberrantly expressed in malignant hematopoiesis. Furthermore, we will shed a light on some contradictory and confusing observations in relation to the experimental models used.

PU.1 UPREGULATED MIRNAS

The transcription factor PU.1 is a pivotal regulator of hematopoiesis. PU.1 inhibits GATA1 activity, which shifts the cellular differentiation program from erythroid toward myeloid-lymphoid lineages [28]. In human myeloid leukemia and promyelocytic cell line NB4, *miR-424* has been reported as a direct PU.1 target and regulates tetradecanoylphorbol-13-acetate (TPA)-induced monocytic differentiation by repressing the critical transcription factor nuclear factor I-A (*NFI-A*) and thereby activating differentiation-specific genes such as colony stimulating factor 1 receptor (CSF1R or M-CSFR) [4]. Human *miR-424* is not conserved in mice but belongs to a family of miRNAs that consist of *miR-15/16/195/322/424/497/1907*. Using a mouse hematopoietic PU.1 knockout (K.O.) cell line derived from PU.1 K.O. fetal livers and transduced with tamoxifen-inducible PU.1 expression constructs, Ghani *et al.*, identified a list of 20 PU.1-regulated miRNAs of which 10 miRNAs were downregulated and 10 upregulated [29]. Strikingly, none of the *miR-15/16/195/322/424/497/1907* family members were found to be controlled by PU.1, indicating that this pathway is either not conserved between species or is cell type- and differentiation status-dependent. In mouse progenitors only four miRNAs, namely *miR-146a*, *miR-342*, *miR-338* and *miR-155* are directly controlled by PU.1 [29] (Figure 1). PU.1 permanently regulates the expression of *miR-146a*, *miR-342* and *miR-338* during differentiation, but only initiates *miR-155* expression at the early stage of myeloid development and is not necessary for its maintenance during myelopoiesis [29]. The identification of *miR-342* and the PU.1 binding site in its promoter confirmed the previously published data by De Marchis *et al.* concerning all-trans retinoic acid (ATRA)-mediated differentiation of acute promyelocytic leukemia (APL) cells toward mature granulocytes by De Marchis *et al.* [30]. Notably, this finding indicates that *miR-342* functions are not myeloid-lineage restricted. The expression of *miR-146a*, *miR-342* and *miR-338* steadily increases during myeloid differentiation and is the highest in mature macrophages, whereas *miR-155* transiently increases in cell developmental intermediates but decreases during terminal differentiation [29]. Only *miR-146a* and *miR-155* are upregulated by PU.1 independently from other cooperative factors [29]. However, these data do not exclude a role for additional factors in fine-tuning of miRNA expression in different cell types.

To further assess the functions of *miR-146a* in normal myeloid development, Ghani *et al.* isolated Lineage negative (Lin^-)$Sca\text{-}1^+cKit^+$ (LSK) cells, infected these cells with retroviruses expressing *miR-146a*, transplanted these cells in lethally-irradiated mice and analyzed these mice 6-8 weeks posttransplantation. In contrast to previously published observations at 4 weeks posttransplantation by Starczynowski *et al.* [31], Ghani *et al.* did not observe a transient myeloid expansion [29]. However, this discrepancy can be largely explained by the time point of the phenotypic analysis. Accordingly, Ghani *et al.* found that enforced *miR-146a* expression drives differentiation of HSCs into peritoneal macrophages 8 weeks post-transplantation in mice, an important analysis that was omitted by Starczynowski *et al.* These data largely explain the loss of myeloid cells in the bone marrow, blood and other hematopoietic tissues in both studies.

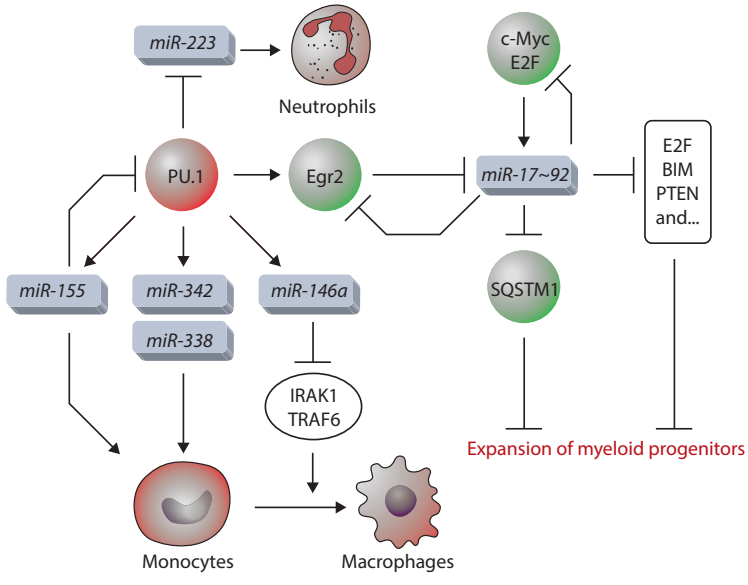


Figure 1. PU.1-regulated miRNAs in myelopoiesis. PU.1 upregulates transcription of *miR-146a*, *miR-342*, *miR-338* and *miR-155*, which control macrophage development. PU.1 also controls the expansion of myeloid progenitors by repression of Egr2-mediated transcription of *miR-17~92* cluster of miRNAs.

In a further analysis of the role of *miR-146a* in myelopoiesis, Ghani *et al.* performed *miR-146a* knockdown studies with antisense oligonucleotides and observed a strong reduction of myelopoiesis in zebrafish, indicating that myelopoiesis requires *miR-146a* activity [29]. Whereas the expression pattern and function of PU.1 and the *miR-146a* seed sequence is highly conserved between zebrafish and mammals, *miR-146a*-deficiency in mice results in massive expansion of myeloid cells and causes myeloid malignancies [20,22]. The discrepancy in experimental outcome between mice and fish demonstrates that evolutionary conservation of molecular networks does not exclude differential functions in species. Notably, many regulatory functions of *miR-146a* in inflammatory responses and hematopoietic disorders have been described in the past year and are previously reviewed [23,24,32].

PU.1 DOWNREGULATED MIRNAS

Fifty percent of the PU.1-controlled miRNAs identified by Ghani *et al.* were transcriptionally downregulated and include several members of the *miR-17~92* cluster such as *miR-20*, *miR-19* and *miR-92*. Although negatively regulated targets of PU.1 are rarely identified, the authors did not further confirm these potentially interesting PU.1 targets that play important roles at the myeloid progenitor stage. We recently identified *miR-17/20/93/106*, all AAAGUGC

seed-containing miRNAs that promote myeloid progenitor expansion by targeting SQSTM1-regulated pathways [19]. Furthermore, Pospidil *et al.* reported that downregulation of *miR-17~92* family of miRNAs is required for PU.1-orchestrated induction of myeloid differentiation [33]. Pospisil *et al.* used the same PU.1 null model as published by Ghani *et al.*, and this study identified all miRNAs encoded by *miR-17~92* and *miR-106b~25* clusters to be indirectly downregulated during PU.1-controlled macrophage development [33]. In their model, PU.1 induces *Egr2* expression that is in turn involved in epigenetic silencing of the *miR-17~92* promoter during macrophage development [33]. In addition, they found that Egr2 itself is a direct target of *miR-17~92*, thereby generating a negative feedback loop of Egr2 in proliferating cells and of *miR-17~92* in differentiating cells (Figure 1) [33]. Interestingly, a correlative analysis of gene and miRNA expression data confirmed a direct association of these factors in human leukemia samples in which low Egr2 expression could largely explain the observed overexpression of *miR-17~92* in clinical acute myeloid leukemia (AML) samples [33]. Together, these recent data demonstrate evidence for a model in which PU.1 controls normal and malignant myelopoiesis at least in part by the regulation of critical miRNAs (Figure 1).

MIR-125 IN MYELOPOIESIS

miR-125 (also known as *Lin-4*) was first identified in *Caenorhabditis elegans* and regulates cell fate progression and differentiation by targeting *Lin-28* and *Lin-14* (for recent review [34]). This miRNA is evolutionary conserved and three *miR-125* family members located at different loci in the human genome exist in mammals (Figure 2A). Several recent publications show differential expression of *miR-125* family members at different stages of myelopoiesis. First, we showed by quantitative PCR that *miR-125b* is highly expressed in human myeloblasts and promyelocytes isolated from the bone marrow (BM) and is strongly downregulated in metamyelocytes and mature neutrophils, whereas *miR-125a* expression remains similar during differentiation [35]. In addition, others showed that in long-term HSCs the endogenous *miR-125a/b* expression is high but decreases rapidly at the progenitor state [14,36-38]. Next, Surdziel *et al.* reported that *miR-125b* expression, but not *miR-125a*, is enhanced (6-fold) during long-term granulocyte colony-stimulating factor (G-CSF) stimulation of the murine myeloid progenitor cell line 32D [39]. Last, Monk *et al.* demonstrated that *miR-125a* is strongly induced in BM-derived monocytes in response to *Candida albicans* and lipopolysaccharide (LPS) [40], whereas in macrophages total *miR-125* levels are downregulated in response to LPS in a protein kinase B AKT-dependent way [41]. Together, these expression data strongly suggest that *miR-125* regulates cellular processes by various mechanisms at different stages of myelopoiesis.

Several recent studies describe cell type and developmental stage specific functions of *miR-125*. For instance, forced expression of *miR-125* in HSCs promotes cell expansion and BM engraftment in mice [14,36-39], eventually resulting in exhaustion of the HSC compartment as recently reported by Gerrits *et al* [38]. In transplantation experiments by Surdziel *et al.*, myeloid

cells of different origin, but not lymphoid cells overexpressing *miR-125b,* exhibit a competitive growth advantage over non-transduced cells. In full agreement, Gerrits *et al.* found that *miR-125* overexpressing HSPCs exhibit an increased myeloid differentiation in vivo [38]. A most intriguing finding was published by Surdziel *et al.*, in which *miR-125b* overexpression in BM-derived HSPCs causes a shift in differentiation of myeloid progenitors toward macrophages in G-CSF-supplemented colony assays [39]. These data indicate that *miR-125b* controls the lineage-decision at the granulocyte-macrophage progenitor stage, however this finding still needs to be confirmed in vivo. The mechanisms behind the observed *miR-125*-induced myeloid lineage decisions remain elusive. The above described results are in full agreement with data from Klusmann *et al.* [42], showing that forced expression of *miR-125b* in HSPCs enhances proliferation and blocks myeloid differentiation of promyelocytes [42]. However, unlike similar effects on proliferation, *miR-125* overexpression does not affect the differentiation capacity of megakaryocytic progenitors and megakaryocytic/erythroid progenitors [42].

MIR-125 TARGETS IN NORMAL HEMATOPOIESIS

The above discussed studies show some evidence for the downstream targets of *miR-125*. In the study of Surdziel *et al.*, *Bak1*, *Stat3* and *Stat3* cofactors *c-Jun* and *Jund* are direct targets [39]. However short hairpin RNAs (shRNAs) against these factors could only partially phenocopy *miR-125* overexpression, suggesting that other additional repressed targets are involved in the observed phenotype [39]. Klusmann *et al.* identified *Dicer1* and the tumor suppressor *St18* as direct targets for *miR-125b* [42]. RNA interference (RNAi)-mediated knockdown of these factors does copy the hyperproliferative phenotype of *miR-125b* overexpression in colony forming units megakaryocytic assays [42], showing the importance of these targets for the phenotype observed. Notably, these targets still remain to be confirmed in vivo. Of the large list of *miR-125* predicted targets, only *Bak1* and *Klf13* are verified by different research groups and could be considered as bona fide targets [14,38]. As antiapoptotic events cannot explain all the observed phenotypes, other *miR-125*-controlled mechanisms remain to be determined to enable full understanding of *miR-125* functions in hematopoiesis. However, their identification is currently one of the main challenges in the field. Recently, it has been shown that the repressing activity of miRNAs on a specific target is dependent on the level of the target transcripts, the level of competing sequences, the abundance of the miRNAs and the activity of RNA binding proteins that shield miRNA binding sequences [43-45]. For these reasons, miRNA-overexpression studies may present confusing results caused by an abnormal potency of *miR-125* to regulate sets of transcripts that reached aberrant targeting thresholds. RISC cross-linking and immunoprecipitation can be helpful to identify endogenous targets [46], but these strategies need large number of cells and the final sequence results are difficult to analyze. For detailed investigation of the cellular mechanisms controlled by endogenous *miR-125* levels during normal myelopoiesis, cell-type and developmental-stage specific *miR-125* family knockout mice are needed. These mouse models will eventually give insights in the molecular pathways controlled by endogenous *miR-125* levels.

MIR-125 IN LEUKEMIA

Several studies show aberrant expression of *miR-125* in hematological disease. For example, miRNA profiling studies show a 6-fold to 90-fold increased-*miR-125* expression in AML and myelodysplastic syndrome (MDS) patients carrying the translocation t(2;11)(p21;q23) compared with healthy controls and other MDS and AML cases [47] and a 20-fold higher expression in AML cases characterized by t(15;17)(q22;q21) compared with the AML cases lacking this translocation [48]. *miR-125b* is also upregulated 26.4-fold in leukemic blasts of children with trisomy 21/Down syndrome with megakaryoblastic leukemia and 18.5-fold in transient leukemia compared with normal $CD34^+$ HSPCs [42].

Multiple recent studies addressed the question whether *miR-125* plays a role in transformation of normal HSPCs toward leukemia. Lin *et al.* reported that *miR-125* is upregulated by the aberrantly expressed homeobox transcription factor CDX2 in myeloid leukemia [49]. In addition, these authors reported that enhanced *mir-125b* levels block progenitor cell differentiation, which is a hallmark of leukemia, through repression of the core binding factor [49]. The study of Klusmann *et al.* also addressed this question. Their data present some evidence that *miR-125*-mediated repression of *Dicer1* results in global downregulation of miRNAs and may explain a functional role of *miR-125* in human megakaryoblastic leukemia. Furthermore, this study suggests that enhanced *miR-125* levels cause expansion of tumorigenic populations of megakaryocytic progenitor cells.

Some other recent reports present strong evidence for a role of *miR-125* in oncogenic transformation in mice, although most published data are contradictory for the observed leukemia phenotypes. For instance, a 1000-fold *miR-125b* overexpression in HSPCs caused an enhanced expansion of white blood cells including myeloid and lymphoid cells and platelets, and a dose-dependent myeloproliferative disorder with progression toward a rapid and lethal myeloid leukemia [36]. However, Ooi *et al.* found that *miR-125b* overexpression in HSCs using a lentivirus that results in an approximately 35-fold overexpression of *miR-125b* causes a significant expansion of exclusively early lymphoid progenitors [37]. Interestingly, a small subset of secondary transplanted mice developed a lymphoproliferative disease that is characterized by splenomegaly, abnormal large thymi and expansion of particularly $CD8^+$ T lymphocytes. In the study of Bousquet *et al.*, *miR-125b* overexpression in fetal liver HSCs increased white blood cell counts and caused macrocytic anemia 16 weeks posttransplantation. In striking contrast with data from Klusmann *et al.* [42], no difference in platelet number was observed [50]. Instead, fifty percent of transplanted mice developed different types of leukemia, such as T cell or B cell acute lymphoblastic leukemia and myeloproliferative neoplasms [50]. These tumors were characterized by particular levels of *miR-125b* overexpression of 500-fold, 1000-fold and up to 1500-fold, respectively [50]. In agreement, Gerrits *et al.* reported that mice reconstituted with HSPCs with a 1500-fold *miR-125a* overexpression, develop myeloproliferative neoplasms [38].

How can we explain the phenotypic discrepancies? It is clear that most cell developmental aberrations and leukemic phenotype variations in the studies mentioned are largely dependent

on the level of *miR-125* overexpression. The higher the level of *miR-125*, the more targets will be considerably repressed by reaching the targeting thresholds, apparently resulting in different types of disease [43]. Also, as suggested by Gerrits *et al.*, the mature forms of *miR-125a* and *miR-125b* share the seed sequence but are different at the 3'-end, presumably influencing the level of repression of a set of targets. In addition, the genes that are deregulated because of viral insertional mutagenesis, which is largely dependent on the characteristics of the viral miRNA-expression system used, collaborate in oncogenic transformation and may play a role in the different leukemia phenotypes observed [51,52]. Therefore, it would be of interest to investigate whether the tumors are clonal or polyclonal. Also, the investigators used a miRNA-expression system that is either retroviral and can only transduce dividing cells such as progenitors, or lentiviral, which also transduces nondividing cells such as dormant HSCs. For that reason, it is essential to investigate whether transformation occurred in HSCs or in specific progenitor fractions, which is very likely to be different in the studies discussed and may explain the observed phenotypic differences.

CRITICAL *MIR-125* TARGETS IN LEUKEMIA

Despite some evidence for *miR-125*-regulated transcripts that are involved in miRNA processing or the regulation of apoptosis such as *BAK1* and *BMF*, the mechanisms behind cellular transformation are still elusive (Figure 2B). The recently published *miR-125*-controlled p53 network is a very attractive candidate [53], however its role in leukemia development is not yet investigated. Strikingly, *miR-125* has recently been identified as a tumor suppressor, for instance by regulating the *ETS-1* proto oncogene in human breast cancer [54] and by targeting the oncogene *LIN28B* in human liver cancer [55]. Furthermore, it remains unclear whether the endogenously coexpressed *miR-99* and *Let-7*, both known to exhibit tumor-suppressing activities [56,57], modulate or even counteract the oncogenic activities of *miR-125*.

CONCLUSION

Recent data show that miRNAs are controlled by critical transcription factors such as PU.1 during myelopoiesis. An important question to resolve is whether miRNAs function through repression of a single or a few targets, or via the cumulative impact of repressing large sets of targets. Some studies show evidence for only a few significant targets making dominant contributions to functions of some miRNAs, for example *miR-150* and *miR-155* [58,59], but this may be different for other miRNAs or may even be cell type and cell developmental stage dependent. Therefore, tissue and developmental stage-specific mouse models, and experimental target identification approaches are needed for the understanding of miRNA functions. Further investigation of miRNA-controlled mechanisms may open new possibilities to develop specific therapies for clinical leukemias.

A)

19q13.14+

miR-125a	UCCCUGAGACCCUUUAACCUGUGA
let-7e	UGAGGUAGGAGGUUGUAUAGUU
miR-99b	CACCCGUAGAACCGACCUUGCG

11q24.1-

miR-125b-1	UCCCUGAGACCCUAACUUGUGA
let-7a-2	UGAGGUAGUAGGUUGUAUAGUU
miR-100	AACCCGUAGAUCCGAACUUGUG

21q21.1+

miR-125b-2	UCCCUGAGACCCUAACUUGUGA
let-7c	UGAGGUAGUAGGUUGUAUGGUU
miR-99a	AACCCGUAGAUCCGAUCUUGUG

B)

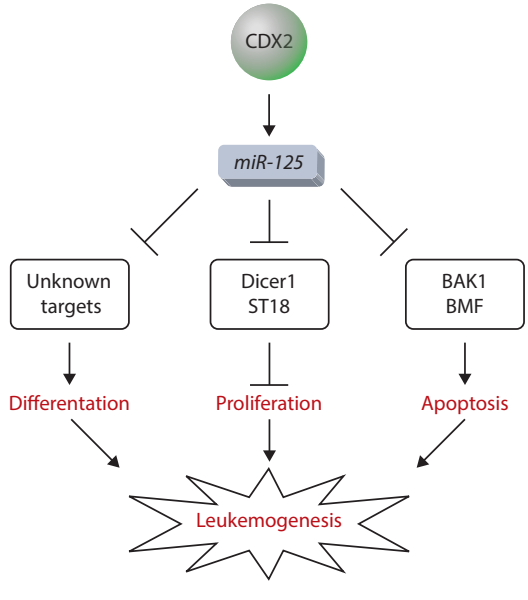


Figure 2. *miR-125* network in leukemia. A) Sequence alignments of miRNAs expressed from three *miR-125* containing clusters: *miR-125a*, *miR-125b-1* and *miR-125b-2*. *MiR-125* family members are cotranscribed with the members of *Let-7* and *miR-99* family of miRNAs. The color code indicates the miRNAs with the same seed sequence. **B)** A schematic model of *miR-125* contribution to leukemogenesis. *MiR-125* family members repress genes that mediate apoptosis, inhibit proliferation, and induce differentiation of hematopoietic stem and progenitor cells.

ACKNOWLEDGEMENTS

We thank Dr. T Cupedo and Dr. M. Buitenhuis for critical reading of the article and E. Simons for assistance with the preparation of the figures. Our work was supported by grants from the Netherlands Organisation for Scientific Research (NWO-VENI) and the Dutch Cancer Society (KWF).

REFERENCES

1. Biyashev D, Qin G. E2F and microRNA regulation of angiogenesis. *Am J Cardiovasc Dis*. 2011;1(2):110-118.
2. Fazi F, Rosa A, Fatica A, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*. 2005;123(5):819-831.
3. Lin CH, Jackson AL, Guo J, Linsley PS, Eisenman RN. Myc-regulated microRNAs attenuate embryonic stem cell differentiation. *EMBO J*. 2009;28(20):3157-3170.
4. Rosa A, Ballarino M, Sorrentino A, et al. The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage differentiation. *Proc Natl Acad Sci U S A. 2007;104(50):19849-19854.*
5. *Diederichs S, Haber DA*. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell*. 2007;131(6):1097-1108.
6. Gruber JJ, Zatechka DS, Sabin LR, et al. Ars2 links the nuclear cap-binding complex to RNA interference and cell proliferation. *Cell*. 2009;138(2):328-339.
7. Suzuki HI, Arase M, Matsuyama H, et al. MCPIP1 Ribonuclease Antagonizes Dicer and Terminates MicroRNA Biogenesis through Precursor MicroRNA Degradation. *Mol Cell*. 2011;44(3):424-436.
8. Heo I, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell*. 2008;32(2):276-284.
9. Heo I, Joo C, Kim YK, et al. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell*. 2009;138(4):696-708.
10. Havelange V, Garzon R. MicroRNAs: emerging key regulators of hematopoiesis. *Am J Hematol*. 2010;85(12):935-942.
11. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004;303(5654):83-86.
12. Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev*. 2007;21(5):578-589.
13. Vasilatou D, Papageorgiou S, Pappa V, Papageorgiou E, Dervenoulas J. The role of microRNAs in normal and malignant hematopoiesis. *Eur J Haematol. 2010;84(1):1-16.*
14. *Guo S, Lu J, Schlanger R, et al*. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci U S A*. 2010;107(32):14229-14234.
15. O'Carroll D, Mecklenbrauker I, Das PP, et al. A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev*. 2007;21(16):1999-2004.
16. Cobb BS, Nesterova TB, Thompson E, et al. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med*. 2005;201(9):1367-1373.
17. Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell*. 2008;132(5):860-874.
18. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med. 2005;202(2):261-269.*
19. *Meenhuis A, van Veelen PA, de Looper H, et al*. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood*. 2011;118(4):916-925.
20. Boldin MP, Taganov KD, Rao DS, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med*. 2011;208(6):1189-1201.
21. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation andgranulocytefunctionbymicroRNA-223. *Nature*. 2008;451(7182):1125-1129.

22. Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D. NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. *Proc Natl Acad Sci U S A*. 2011;108(22):9184-9189.
23. El Gazzar M, McCall CE. MicroRNAs regulatory networks in myeloid lineage development and differentiation: regulators of the regulators. *Immunol Cell Biol*. 2011.
24. O'Connell RM, Zhao JL, Rao DS. MicroRNA function in myeloid biology. *Blood*. 2011;118(11):2960-2969.
25. Turner ML, Schnorfeil FM, Brocker T. MicroRNAs regulate dendritic cell differentiation and function. *J Immunol*. 2011;187(8):3911-3917.
26. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol*. 2010;10(2):111-122.
27. Bissels U, Bosio A, Wagner W. MicroRNAs are shaping the hematopoietic landscape. *Haematologica*. 2011.
28. Zhang P, Zhang X, Iwama A, et al. PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood*. 2000;96(8):2641-2648.
29. Ghani S, Riemke P, Schonheit J, et al. Macrophage development from HSCs requires PU.1-coordinated microRNA expression. *Blood*. 2011;118(8):2275-2284.
30. De Marchis ML, Ballarino M, Salvatori B, Puzzolo MC, Bozzoni I, Fatica A. A new molecular network comprising PU.1, interferon regulatory factor proteins and miR-342 stimulates ATRA-mediated granulocytic differentiation of acute promyelocytic leukemia cells. *Leukemia*. 2009;23(5):856-862.
31. Starczynowski DT, Kuchenbauer F, Wegrzyn J, et al. MicroRNA-146a disrupts hematopoietic differentiation and survival. *Exp Hematol*. 2011;39(2):167-178 e164.
32. Hua Z, Chun W, Fang-Yuan C. MicroRNA-146a and hemopoietic disorders. *Int J Hematol. 2011;94(3):224-229.*
33. *Pospisil V, Vargova K, Kokavec J, et al.* Epigenetic silencing of the oncogenic miR-17-92 cluster during PU.1-directed macrophage differentiation. *EMBO J*. 2011;30(21):4450-4464.
34. Ambros V. MicroRNAs and developmental timing. *Curr Opin Genet Dev*. 2011;21(4):511-517.
35. Sun SM, Dijkstra MK, Bijkerk AC, et al. Transition of highly specific microRNA expression patterns in association with discrete maturation stages of human granulopoiesis. *Br J Haematol*. 2011;155(3):395-398.
36. O'Connell RM, Chaudhuri AA, Rao DS, Gibson WS, Balazs AB, Baltimore D. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proc Natl Acad Sci U S A*. 2010;107(32):14235-14240.
37. Ooi AG, Sahoo D, Adorno M, Wang Y, Weissman IL, Park CY. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc Natl Acad Sci U S A*. 2010;107(50):21505-21510.
38. Gerrits A, Walasek MA, Olthof S, et al. Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. *Blood*. 2011.
39. Surdziel E, Cabanski M, Dallmann I, et al. Enforced expression of miR-125b affects myelopoiesis by targeting multiple signaling pathways. *Blood*. 2011;117(16):4338-4348.
40. Monk CE, Hutvagner G, Arthur JS. Regulation of miRNA transcription in macrophages in response to Candida albicans. *PLoS One*. 2010;5(10):e13669.
41. Androulidaki A, Iliopoulos D, Arranz A, et al. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. *Immunity*. 2009;31(2):220-231.
42. Klusmann JH, Li Z, Bohmer K, et al. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. *Genes Dev*. 2010;24(5):478-490.

43. Mukherji S, Ebert MS, Zheng GX, Tsang JS, Sharp PA, van Oudenaarden A. MicroRNAs can generate thresholds in target gene expression. *Nat Genet*. 2011;43(9):854-859.
44. van Kouwenhove M, Kedde M, Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. *Nat Rev Cancer*. 2011;11(9):644-656.
45. Ebert MS, Sharp PA. Emerging roles for natural microRNA sponges. *Curr Biol. 2010;20(19):R858-861.*
46. *Hafner M, Landthaler M, Burger L, et al.* Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*. 2010;141(1):129-141.
47. Bousquet M, Quelen C, Rosati R, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation. *J Exp Med*. 2008;205(11):2499-2506.
48. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.
49. Lin KY, Zhang XJ, Feng DD, et al. miR-125b, a Target of CDX2, Regulates Cell Differentiation through Repression of the Core Binding Factor in Hematopoietic Malignancies. *J Biol Chem*. 2011;286(44):38253-38263.
50. Bousquet M, Harris MH, Zhou B, Lodish HF. MicroRNA miR-125b causes leukemia. *Proc Natl Acad Sci U S A*. 2010;107(50):21558-21563.
51. Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell*. 2002;110(4):521-529.
52. Wu X, Li Y, Crise B, Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. *Science*. 2003;300(5626):1749-1751.
53. Le MT, Shyh-Chang N, Khaw SL, et al. Conserved regulation of p53 network dosage by microRNA-125b occurs through evolving miRNA-target gene pairs. *PLoS Genet*. 2011;7(9):e1002242.
54. Zhang Y, Yan LX, Wu QN, et al. miR-125b is methylated and functions as a tumor suppressor by regulating the ETS1 proto-oncogene in human invasive breast cancer. *Cancer Res*. 2011;71(10):3552-3562.
55. Liang L, Wong CM, Ying Q, et al. MicroRNA-125b suppressesed human liver cancer cell proliferation and metastasis by directly targeting oncogene LIN28B2. *Hepatology*. 2010;52(5):1731-1740.
56. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME. The role of let-7 in cell differentiation and cancer. *Endocr Relat Cancer*. 2010;17(1):F19-36.
57. Sun D, Lee YS, Malhotra A, et al. miR-99 family of MicroRNAs suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. *Cancer Res*. 2011;71(4):1313-1324.
58. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci U S A*. 2009;106(17):7113-7118.
59. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell. 2007;131(1):146-159.*

3

Dicer1 Deletion in Myeloid-Committed Progenitors Causes Neutrophil Dysplasia and Blocks Macrophage/Dendritic Cell Development in Mice

Mir Farshid Alemdehy, Nicole G.J.A. van Boxtel, Hans W.J. de Looper, Iris J. van den Berge, Mathijs A. Sanders, Tom Cupedo, Ivo P. Touw, and Stefan J. Erkeland

Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands

Blood. 2012; 119(20):4723-30

ABSTRACT

MicroRNAs (miRNAs) have the potential to regulate cellular differentiation programs; however, miRNA deficiency in primary hematopoietic stem cells (HSCs) results in HSC depletion in mice, leaving the question of whether miRNAs play a role in early-lineage decisions unanswered. To address this issue, we deleted *Dicer1*, which encodes an essential RNase III enzyme for miRNA biogenesis, in murine CCAAT/enhancer-binding protein α (C/EBPA)-positive myeloid-committed progenitors *in vivo*. In contrast to the results in HSCs, we found that miRNA depletion affected neither the number of myeloid progenitors nor the percentage of C/EBPA-positive progenitor cells. Analysis of gene-expression profiles from wild-type and *Dicer1*-deficient granulocyte-macrophage progenitors (GMPs) revealed that 20 miRNA families were active in GMPs. Of the derepressed miRNA targets in *Dicer1*-null GMPs, 27% are normally exclusively expressed in HSCs or are specific for multipotent progenitors and erythropoiesis, indicating an altered gene-expression landscape. *Dicer1*-deficient GMPs were defective in myeloid development *in vitro* and exhibited an increased replating capacity, indicating the regained self-renewal potential of these cells. In mice, *Dicer1* deletion blocked monocytic differentiation, depleted macrophages, and caused myeloid dysplasia with morphologic features of Pelger-Huët anomaly. These results provide evidence for a miRNA-controlled switch for a cellular program of self-renewal and expansion toward myeloid differentiation in GMPs.

INTRODUCTION

Hematopoiesis is a tightly regulated process of proliferation and differentiation of hematopoietic stem and progenitor cells (HSPCs) toward mature blood cells. Lineage commitment and differentiation of HSPCs are orchestrated by transcription factors that are expressed at specific developmental stages. For example, CCAAT/Enhancer-Binding Protein-Alpha (C/EBPA) is a master regulatory transcription factor that is not expressed in hematopoietic stem cells (HSCs), but starts to be expressed in a small fraction of multipotent progenitor (MPP) cells and increases steeply during the transition from the common myeloid progenitor (CMP) toward the granulocyte-macrophage progenitor (GMP). C/EBPA drives granulopoiesis by controlling the expression of myeloid specific genes [1,2].

MicroRNAs (miRNAs) belong to a class of small (approximately 22 nt) noncoding RNAs. The RNA-induced silencing complex-bound miRNAs bind to complementary sequences that are predominantly located in the 3'-untranslated regions of target mRNAs and regulate gene expression by transcript destabilization and inhibition of protein translation [3]. Recently, the function of miRNAs in myeloid cells has been investigated using mouse models. For example, *miRs-17/20/93/106* promote progenitor cell expansion by targeting *Sequestosome-1*-regulated pathways [4]. In addition, *miR-223* negatively regulates myeloid progenitor proliferation, and fine-tunes granulocyte differentiation and activity [5]. Also, *miR-146a* inhibits the activity of both myeloid and lymphoid cell lineages and plays key roles in the regulation of inflammation [6].

DICER1 is an evolutionarily conserved member of the RNase III family of endoribonucleases that is critical for processing of specific precursor hairpin sequences, the so-called pre-miRNAs, into miRNAs [7]. Genetic deletion of *Dicer1* in mice results in early embryonic mortality due to depletion of the Oct-4-positive pluripotent embryonic stem cell pool at embryonic day 6-7 (E6-E7) [8]. A floxed *Dicer1* allele (*Dicer1*^{fl}) has been generated that allows conditional deletion of *Dicer1* in a cell type- and developmental stage-specific fashion [9]. Hematopoietic lineage-specific conditional deletion of *Dicer1* has revealed the involvement of miRNAs in the survival, maturation and homeostasis of peripheral T lymphocytes, and in Ab diversity and survival of B lymphocytes [10-12]. In addition, conditional *Dicer1* deletion in osteoprogenitors using mice that have Cre recombinase under the transcriptional control of the osterix promoter (Osx-GFP-Cre) results in myeloid dysplasia and acute myelogenous leukemia with acquired genetic abnormalities but intact *Dicer1* [13].

Mouse primary HSCs are impaired by *Dicer1* loss and are unable to reconstitute hematopoiesis [14]. In addition, conditional deletion of *Ars2*, another gene required for miRNA biogenesis, in HSCs results in BM failure and increased apoptosis of hematopoietic cells in thymus and spleen [15]. Therefore, the overall contribution of miRNAs to myeloid-lineage specification remains elusive. To address this issue, we generated a myeloid specific, *Cebpa-Cre*-driven *Dicer1* deleter mouse strain that also harbors a conditional CRE reporter containing a *loxp*-flanked stop sequence (LSL) and the enhanced yellow fluorescent protein (*Eyfp*) in the ROSA26 locus (*R26-LSL-Eyfp*) [16]. We show that *Cebpa-Cre*-driven *Dicer1* deletion

did not affect the numbers of myeloid-committed progenitors but did play a critical role in the regulation of a developmental program required for normal granulocyte and monocyte/dendritic cell (DC)/macrophages in mice.

METHODS

Mice and reconstitution experiments

To generate *Cebpa-Cre;R26-LSL-Eyfp;Dicer1*$^{wt/fl}$/*Dicer1*$^{fl/fl}$ mice, we crossed mice that contain floxed *Dicer1* alleles (*Dicer1*^{fl} [9]; a kind gift of Dr. P.A. Sharp, David H. Koch Institute for Integrative Cancer Research, Cambridge, MA) with *Cebpa-Cre;R26-LSL-Eyfp* reporter mice [2]. Fetal livers were obtained on E13.5. Routine genotyping of *Dicer1; Cebpa-Cre;R26-LSL-Eyfp* mice was performed by PCR assays of DNA from tail or toe biopsies. Sequences of primers are available on request. All primers were obtained from Biolegio. For transplantation, 6- to 8-week-old recipient mice (C57Bl/6; The Jackson Laboratory) were irradiated (8.5 Gy) and tail-vein injected with fetal liver single-cell suspensions. Typically, cells from each fetal liver were transplanted into 2 recipient mice. Hematopoietic tissues were analyzed 6-10 weeks after transplantation. The percentage of chimerism in hematopoietic tissue was detected by flow cytometric analysis of CD45.1 (recipient) and CD45.2 (*Dicer1*$^{fl/fl}$ and *Dicer1*$^{fl/wt}$ donor) cells in a total of 8 mice. All animal experiments were approved by the Animal Welfare/Ethics Committee of the Erasmus Medical Center.

Cell culture, colony assays and cytospins

DCs were derived from BM cultures in the presence of GM-CSF as described previously [17]. GM-CSF-induced colony formation assays with progenitors from E13.5 fetal livers were performed as described previously [4]. Colonies were counted after 7 days of incubation at 37°C and 5% CO_2 in a humidified atmosphere. For liquid cultures, E13.5 fetal liver cells were isolated and single-cell suspensions were grown in serum free CellGro Stem Cell Growth Medium (Cell Genix) supplemented with 1% penicillin/streptomycin and GM-CSF (10 ng/mL) at a density of $1x10^6$ cells/mL for 7 days. For morphologic analysis of the cells, cytospins were stained with May-Grünwald-Giemsa and examined with a Leica DMLB microscope (100x and 40x objectives) and Leica Application Suite Version 2.7.1 R1 software.

Abs, cell staining, flow cytometry, and cell sorting

To obtain BM cell suspensions, femurs and tibias were crushed in a mortar in PBS with 5% FCS. Cells were passed through a 70-µm nylon sieve, and erythrocytes were lysed. Lineage-positive (Lin^+) cells were determined with Abs against the following lineage markers: CD3ε, CD11b, CD45R/B220, Ly-6G (Gr-1) and Ter119. To recognize HSPC populations, BM cells were stained with Abs against c-Kit, Sca-1, CD34 and FcγRII/III(CD16/32). Myeloid progenitors were defined as $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+CD16/32^{low}$ (CMPs), $Lin^-Sca\text{-}1^{low}c\text{-}Kit^+CD34^+CD16/32^{hi}$ (GMPs) and $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^-CD16/32^{low}$ (megakaryocyte/

erythroid progenitor [MEPs]). For the analysis of differentiated EYFP$^+$ myeloid cells in BM, cells were stained with anti-CD11b and anti-Ly-6G Abs. Peripheral blood obtained by submandibular bleeding was treated with erythrocyte lysis buffer and stained with Abs against CD11b, Ly-6G and Ly-6C for the determination of granulocytes and monocytes. Spleen single-cell suspensions were stained with CD11b and Ly-6G. Macrophages were isolated from the peritoneal cavity with 1.5 mL washes using PBS/5% FCS. For identification of macrophages, the cell suspensions were stained with Abs against F4/80. To identify the *in vitro* expansion and differentiation toward DCs, whole BM cultures were stained with Abs against MHC class II and CD11c. To sort progenitors from BM, Lin$^+$ cells were depleted before staining with the Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set- DM (BD Biosciences) according to the manufacturer's protocol. A forward-side scatter gate excluded cell debris and remaining red blood cells. All sorted populations were more than 95% pure as determined by reanalysis. A full list of Abs used for flow cytometry and suppliers is given in supplemental Table 5.

Gene and miRNA expression profiling

EYFP$^+$ GMPs were sorted into RLT buffer (QIAGEN). RNA was extracted with the RNeasy Micro Kit (QIAGEN). After one step of linear amplification with the RNA MessageAmp II aRNA Kit (Applied Biosystems/AmbionX), aRNA was labeled and hybridized on the Mouse Genome 430 Affymetrix 2.0 Array according to standard protocols. Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). RNA integrity was confirmed on an Agilent 2100 Bioanalyzer (Agilent Technologies) with 6000 nano and pico chips. Microarray data were normalized with the Affymetrix Microarray Suite (MAS Version 5.0). All microarray data are available on the Gene Expression Omnibus under accession number GSE35844. To confirm the expression changes in some target genes, cDNA was produced from 1µg of aRNA using Superscript II (Invitrogen) and quantitative RT-PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN). Primers were obtained from Biolegio. The ΔCt value of *Dicer1*$^{\Delta/\Delta}$ and *Dicer1*$^{wt/\Delta}$ versus *Dicer1* wild-type (*Dicer1*^{wt}) cells was calculated. The fold induction was calculated by the $2^{-\Delta Ct}$ method.

miRNAs were isolated using the RNeasy Plus Mini Kit and RNeasy MinElute Cleanup Kit (QIAGEN) according to manufacturer's protocols. For miRNA profiling, TaqMan Array Rodent MicroRNA A Cards Version 2.0, which enables quantification of 375 mouse miRNAs and 6 controls, were used according to the manufacturer's protocol for Megaplex Pools With Preamplification (Applied Biosystems) using the ABI PRISM 7900HT machine (Applied Biosystems).

Statistics

TargetScan Version 5.2 (http://www.targetscan.org) was used to identify putative miRNA targets. Profiling of mRNA expression was performed in triplicate for each experimental condition (*Dicer1*^{wt}, *Dicer1*$^{wt/\Delta}$ or *Dicer1*$^{\Delta/\Delta}$) and subsequently normalized with MAS5.0.

Probe sets considered indistinguishable from the background signal were omitted from further analyses. Identification of the differentially expressed probe sets was performed using the false discovery rate (FDR)-corrected p values derived by Limma [18]. $p < (0.017)$ was considered statistically significant. We divided the necessary significance level (.05) by the number of pairwise comparisons, in our case 3. The Kolmogorov-Smirnov test was used to infer differences between cumulative distribution functions, and a $p < 0.05$ was considered significant. The Fisher exact test was used to infer enrichment of de-repression for mRNA targets from the identified miRNAs. A FDR-corrected $p < 0.05$ was considered statistically significant. All statistical analyses were performed with R Version 2.12 software (http://www.r-project.org).

RESULTS

Cebpa-Cre-driven deletion of *Dicer1* does not affect the number of HSPCs

In hematopoietic cells, *Cebpa* starts to be expressed at the MPP stage and defines a subpopulation that is instructed to develop toward the myeloid lineage [2], making it a suitable promoter to drive *Dicer1* deletion for studying the role of miRNAs in myelopoiesis. Whereas the *Cebpa-Cre*;*Dicer1*$^{wt/fl}$ mice were viable and born at Mendelian ratios, the *Cebpa-Cre*;*Dicer1*$^{fl/fl}$ mice died rapidly after birth. This phenotype can be largely explained by the fact that the *Cebpa* promoter is highly active during the maturation of the respiratory epithelium in late gestation [19] and deletes *Dicer1*. Lack of *Dicer1* is detrimental to these cells, similar to *Sonic Hedgehog* (*Shh*)-*Cre* conditional *Dicer1* knockout mice [20].

To investigate whether *Dicer1* deletion affects hematopoiesis in the embryo, we isolated fetal livers at E13.5. Cells containing recombined *Dicer1*^{fl} alleles (*Dicer1*$^{\Delta}$) can be identified because they also harbor a conditional CRE reporter *R26-LSL-Eyfp* allele [16]. The E13.5 fetal livers of *Dicer1* mutants and wild type were indistinguishable by eye. In addition, flow cytometric analysis did not show any differences in the fraction of HSCs, hematopoietic progenitors and (Lin$^-$) EYFP$^+$ progenitors in the fetal livers of *Dicer1*$^{fl/fl}$ and *Dicer1*$^{wt/fl}$ mice compared with *Dicer1*^{wt} controls (Figure 1A). To investigate the effects of *Cebpa-Cre*-driven *Dicer1* ablation in hematopoietic cells in adult mice and to bypass early death, E13.5 fetal liver cells were transplanted in lethally irradiated recipients. With this protocol the percentage of chimerism in hematopoietic tissue was at least 92% in the reconstituted mice (data not shown). CRE-dependent deletion of *Dicer1*^{fl} alleles in EYFP$^+$ cells was confirmed by PCR (Figure 1B). Furthermore, more than 95% of total miRNAs were depleted in *Dicer1*$^{\Delta/\Delta}$; EYFP$^+$ cells (supplemental Table 1). The expression level of the remaining miRNAs was less than 10% compared with normal EYFP$^+$ control cells (supplemental Table 1), indicating that *Cebpa-Cre*-mediated deletion of *Dicer1* results in an efficient depletion of miRNAs in myeloid progenitor cells *in vivo*.

Previous studies showed that IFN-responsive promoter-driven *Cre* (*Mx-Cre*)-induced *Dicer1* ablation results in a complete depletion of functional HSCs [14]. We wondered to what extent *Cebpa*-driven *Dicer1* deletion affects the number of myeloid-committed progenitor

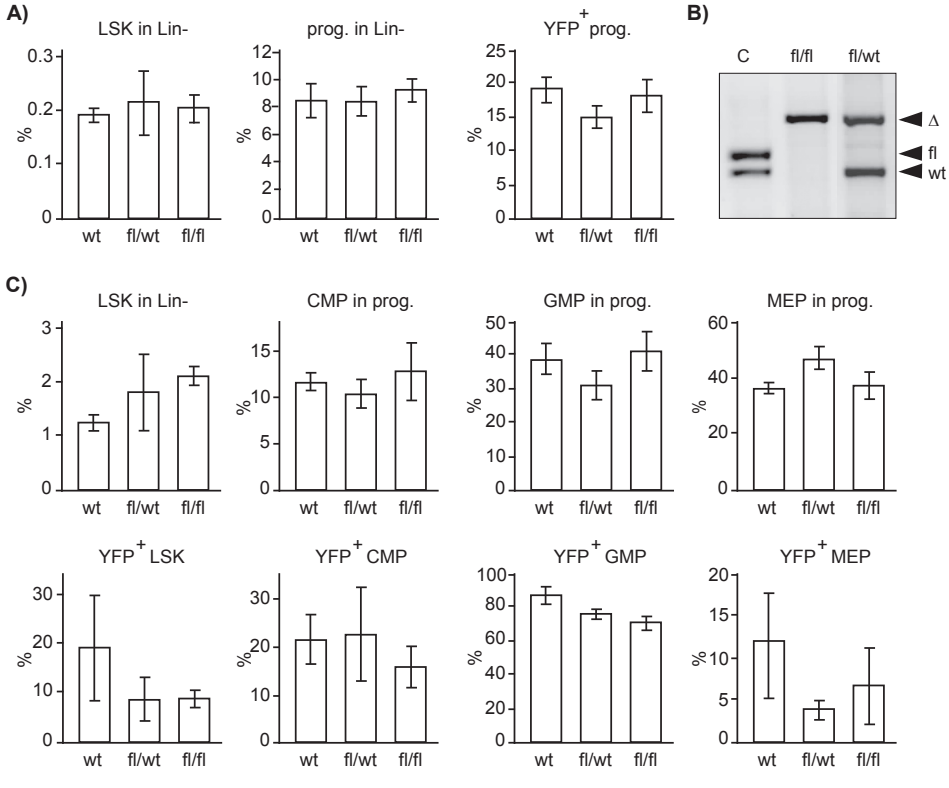


Figure 1. ***Cebpa-Cre*-driven deletion of *Dicer1* does not affect the fraction of myeloid-committed HSPCs in mice. A)** Percentage of LSK cells (Lin-Sca1$^+$C-Kit$^+$), progenitors (Lin-Sca1-C-Kit$^+$) and percentage of EYFP$^+$ progenitor cells of *Dicer1*^{wt} (n=3), *Dicer1*$^{wt/fl}$ (n=4) and *Dicer1*$^{fl/fl}$ cells (n=4) in E13.5 fetal livers. **B)** EYFP$^+$ cells from BM of transplanted mice were sorted by flow cytometry. DNA was isolated and analyzed by PCR. DNA fragments from *Cebpa-Cre; Dicer1*$^{wt/fl}$*;R26-LSL-Eyfp* and *Cebpa-Cre; Dicer1*$^{fl/fl}$*;R26-LSL-Eyfp* are indicated by fl/wt and fl/fl, respectively. Tail DNA of *Dicer1*$^{wt/fl}$ was used as a positive PCR control (c) for the floxed and wild-type alleles. Recombined lox-p sites are indicated by Δ. **C)** Top panel: Percentage of LSK cells (Lin-Sca1$^+$C-Kit$^+$), and CMP, GMP and MEP in the progenitor fraction (Lin-Sca1-C-Kit$^+$) of *Dicer1*^{wt} BM cells (n=5), *Dicer1*$^{wt/fl}$ (n=6) and *Dicer1*$^{fl/fl}$ cells (n=7). Bottom panel: Percentage of EYFP$^+$ cells in indicated fractions.

cells. In contrast to the results published previously for HSCs [14], in the present study, *Dicer1* deletion did not affect the percentage of myeloid-committed EYFP$^+$ cells in the LSK fraction significantly (Figure 1C and supplemental Figure 1). In addition, no significant differences in the fraction of EYFP$^+$ *Dicer1*$^{\Delta/\Delta}$ CMPs, GMPs or MEPs compared with those of *Dicer1*$^{wt/\Delta}$ transplanted mice and *Dicer1*^{wt} mice were observed (Figure 1C). Therefore, unlike the effect of *Dicer1* deletion on HSC maintenance [14], *Cebpa-Cre*-driven *Dicer1* deletion did not affect the numbers of myeloid progenitors in mice.

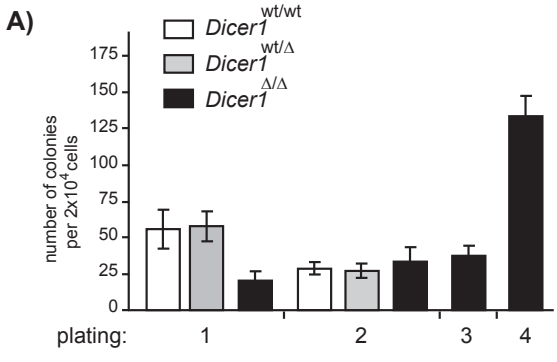
Cebpa-Cre driven deletion of *Dicer1* affects GM-CFU outgrowth, cellular replating capacity and myeloid differentiation

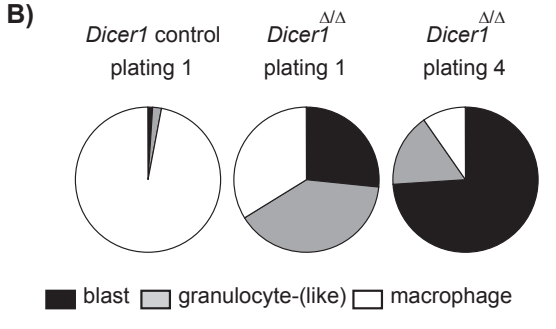
We performed GM-CFU assays to determine whether deletion of *Dicer1* would affect the expansion and differentiation capacity of GMPs. The number of GM-CFUs obtained with *Dicer1*$^{\Delta/\Delta}$ progenitor cells was approximately 50% lower than with *Dicer1*$^{wt/\Delta}$ or *Dicer1*^{wt} cells (Figure 2A). Colony size also decreased as a result of *Dicer1* deletion (data not shown). Morphologic analyses showed a more than 5-fold increase in the number of blast-like cells, a strongly reduced capacity of the *Dicer1*$^{\Delta/\Delta}$ progenitors to differentiate toward macrophages, and the appearance of dysplastic neutrophils (Figure 2B). *Dicer1*$^{\Delta/\Delta}$ progenitor cells gained the ability to form secondary and tertiary colonies after serial replatings, which coincided with a regained self-renewal potential, a blast-like morphology, and a strongly reduced differentiation capacity of the cells (Figure 2A,B). In GM-CSF-containing liquid cultures, Lin$^-$;*Dicer1*$^{\Delta/\Delta}$ progenitors were unable to mature toward macrophages, but instead showed features of dysplastic myeloid cells, including the Pelger-Huët anomaly, which is characterized by neutrophils with a hyposegmented nucleus (~75% of cells) [21] (Figure 2C). Lin$^-$;*Dicer1*$^{\Delta/\Delta}$ BM cells failed to differentiate toward BM-derived DCs (Figure 2D). These results indicate that *Dicer1* is essential for definitive maturation of GMPs toward both the neutrophil and monocyte/macrophage/BM-derived DC lineage *in vitro*.

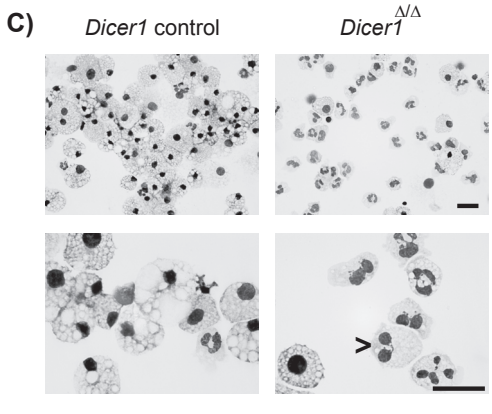
Cebpa-Cre-driven deletion of *Dicer1* causes myeloid dysplasia and monocyte/macrophage depletion *in vivo*

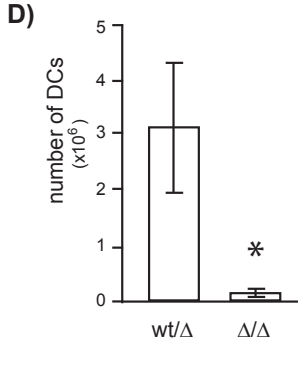
Next, we investigated whether the aberrant myeloid differentiation of GMPs observed *in vitro* also occurs *in vivo*. *Dicer1*$^{\Delta/\Delta}$ BM neutrophils showed increased levels of CD11b (a marker for myeloid cells) and a reduced Ly-6G (a marker for neutrophilic differentiation) expression compared with *Dicer1*$^{wt/\Delta}$ (similar to *Dicer1*^{wt}; Figure 3A). Whereas *Dicer1*$^{wt/\Delta}$ neutrophils appeared normal, *Dicer1*$^{\Delta/\Delta}$ cells showed aberrant nucleus morphologies (Figure 3B) of

Figure 2. Functional analysis of *Dicer1*$^{\Delta/\Delta}$ primary mouse Lin$^-$ BM cells. A) CFU-GM assay and replating of mouse Lin- BM progenitors. Cells were plated in triplicate at densities of 1x10^4 cells per dish in 1 mL methylcellulose medium containing GM-CSF (100 ng/mL). Cells were isolated from dishes, counted and replated under the same conditions. Colonies consisting of more than 50 cells were counted after 7 days of growth. Significance was calculated by comparing *Dicer1*$^{\Delta/\Delta}$ and *Dicer1*$^{wt/\Delta}$ with *Dicer1*^{wt} control using the Mann-Whitney test (asymoptotic significance [2-tailed], * $p < 0.05$. **B)** Average differential cell counts (of at least 100 cells and 3 independent experiments), blast, granulocyte(-like) and macrophages. *Dicer1* control plating 1 (blasts 1%, SD=0, granulocytes 2%, SD=1, macrophages 97%, SD=1). *Dicer1*$^{\Delta/\Delta}$ plating 1 (blast 27%, SD=4.7, granulocyte-like 39%, SD=3.5 and macrophage 34%, SD=8.1). *Dicer1*$^{\Delta/\Delta}$ third and forth replatings (blast 74%, SD =3.6, granulocyte-like 16%, SD=3.2 and macrophage 10%, SD=4.6). **C)** Micrographs show cells isolated from a liquid culture of mouse Lin- BM progenitors with GM-CSF for 7 days. Arrowhead indicates the pince-nez-shaped nucleus, a hallmark for the Pelger-Huët anomaly. Black bar indicates 10 µm. **D)** Number of EYFP$^+$; CD11C$^+$ myeloid DCs per 2x10^6 cells plated in liquid culture after 1 week of expansion (n=3). * $p < 0.05$.


A)
Dicer1 wt/wt
Dicer1 wt/Δ
Dicer1 Δ/Δ
number of colonies per 2x10^4 cells
175
150
125
100
75
50
25
0
plating: 1 2 3 4


B)
Dicer1 control plating 1
Dicer1 Δ/Δ plating 1
Dicer1 Δ/Δ plating 4
blast
granulocyte-(like)
macrophage


C)
Dicer1 control
Dicer1 Δ/Δ


D)
number of DCs (x10^6)
5
4
3
2
1
0
*
wt/Δ
Δ/Δ

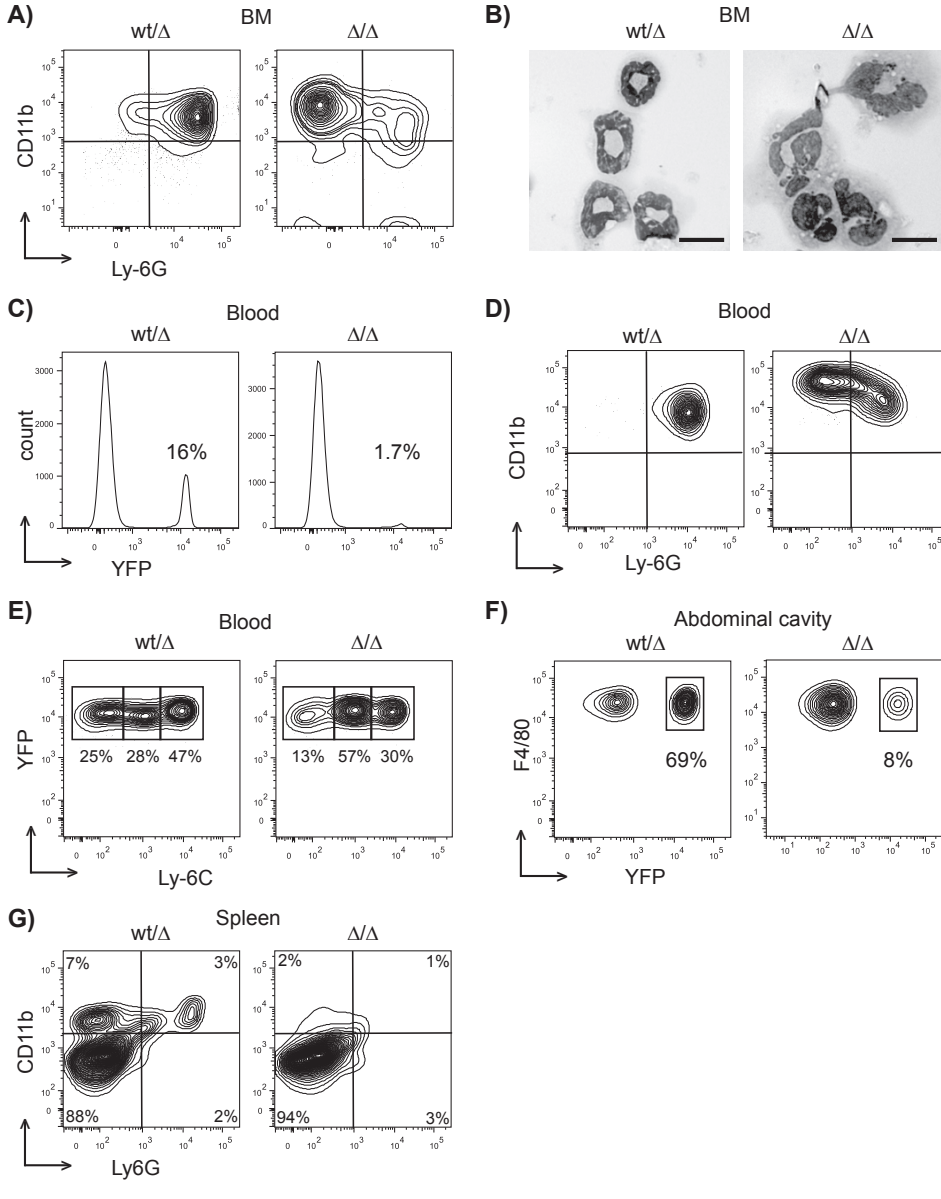


Figure 3. *Cebpa-Cre*-driven deletion of *Dicer1* causes neutrophil dysplasia and monocyte/macrophage depletion in mice. A) FACS analysis of BM neutrophils with Abs against CD11b and Ly-6G for *Dicer1*$^{wt/\Delta}$ (similar to *Dicer1*^{wt}) and *Dicer1*$^{\Delta/\Delta}$ neutrophils. **B)** Micrographs showing morphology of *Dicer1*$^{wt/\Delta}$ (similar to *Dicer1*^{wt}) and *Dicer1*$^{\Delta/\Delta}$ neutrophils. Black bar indicates 10 μm. **C)** Percentage of EYFP^{+} cells in the blood of *Dicer1*$^{wt/\Delta}$ and *Dicer1*$^{\Delta/\Delta}$ mice. **D)** Analysis of peripheral neutrophils with Abs against CD11b and Ly-6G. **E)** Analysis of peripheral EYFP^{+} monocytes (CD11b^{+}, Ly-6G^{-}) with Abs against Ly-6C. **F)** Analysis of EYFP^{+} macrophages from the abdominal cavity with Abs against F4/80 Ag. **G)** FACS analysis of total spleen cells with Abs against CD11b and Ly6G. All analyses were performed on at least 3 independent mice.

which approximately 20% were hyposegmented or bilobed. Furthermore, Ly-6G$^+$ *Dicer1*$^{\Delta/\Delta}$ granulocytes were nearly absent in the peripheral blood and spleen (Figure 3C- D, G), suggesting that the aberrant neutrophils were incapable of emigrating from the BM.

Concerning monocytic/macrophage development, whereas *Dicer1*$^{wt/\Delta}$ monocytes appeared in 3 stages of differentiation: high Ly-6C (immature), intermediate and low Ly-6C (mature) [22], the relative number of mature *Dicer1*$^{\Delta/\Delta}$ monocytes was strongly reduced (Figure 3E). Moreover, we observed a strongly reduced percentage of EYFP$^+$ macrophages in the abdominal cavity of *Dicer1*$^{\Delta/\Delta}$ transplanted mice compared with EYFP$^+$*; Dicer1*$^{wt/\Delta}$ controls (Figure 3F). No CD11b$^+$ cells could be detected in the spleen of *Dicer1*$^{\Delta/\Delta}$ transplanted mice (Figure 3G). *Dicer1*$^{\Delta/\Delta}$ recipients survived for at least 10 months devoid of any sign of myeloproliferative disease or leukemia development.

Twenty miRNA families are active in GMPs and repress expression programs characteristic of HSCs and erythropoiesis

To determine how deletion of *Dicer1*, and the resulting loss of miRNA expression, affects the gene expression landscape of GMPs, we performed gene expression analyses of *Dicer1* mutant and control GMPs. miRNA expression profiling of EYFP$^+$ *Dicer1*^{wt} GMPs identified 104 miRNAs expressed in GMPs (supplemental Table 2). To examine the activities of these miRNAs, we compared the transcriptome of *Dicer1*^{wt}, *Dicer1*$^{wt/\Delta}$ and *Dicer1*$^{\Delta/\Delta}$ GMPs. Transcripts with predicted binding sites for identified miRNAs tended to be up-regulated in *Dicer1*-null GMPs, compared with transcripts without such sites ($p < 2.2x10^{-16}$ by Kolmogorov-Smirnov test; Figure 4A), which is indicative of the activity of the miRNAs in these cells. Transcripts containing putative binding sites for the identified miRNAs had a significant propensity to be up-regulated in the *Dicer1*$^{\Delta/\Delta}$ cells ($p < 2.2x10^{-16}$ by Kolmogorov-Smirnov test), but not in the *Dicer1*$^{wt/\Delta}$ cells (Figure 4B). Deletion of *Dicer1* in GMPs revealed 784 significant differentially expressed probe sets (FDR-corrected $p < 0.05$; Figure 4C). Unsupervised clustering of these probe sets showed that the gene expression pattern of *Dicer1*$^{wt/\Delta}$ GMPs was changed but still appeared very similar to wild-type GMPs (Figure 4C), again indicating that heterozygous deletion of *Dicer1* did not strongly affect the miRNA-regulated targets in GMPs.

Cebpa-Cre-mediated deletion of *Dicer1* in GMPs resulted in 300 significantly up-regulated transcripts (368 probe sets, supplemental Table 3). The up-regulation of some of these genes was confirmed by quantitative RT-PCR (supplemental Figure 2). A set of transcripts that are experimentally confirmed miRNA targets, such as *Bcl2L11* (*Bim*) [11], *K-Ras* and *Hmga2* [23], *Hoxa9* [24], and *Cdkn1a* (*p21*) [25], also appeared to be regulated by *Dicer1* in GMPs (supplemental Table 3). In agreement with data shown in Figure 4B-C, only 11 transcripts (3%) were very sensitive to *Dicer1* deletion because *Dicer1* heterozygosity had a significant effect on their transcript levels (supplemental Table 3). Predicted targets of 20 miRNA families were significantly enriched in the fraction of messages that were up-regulated in *Dicer1*$^{\Delta/\Delta}$ cells compared with the fraction of nonregulated targets (Table 1, FDR-corrected $p < 0.05$ by Fisher exact test). Of the 300 genes that were de-repressed by *Dicer1* depletion, 81 g (>25%) could

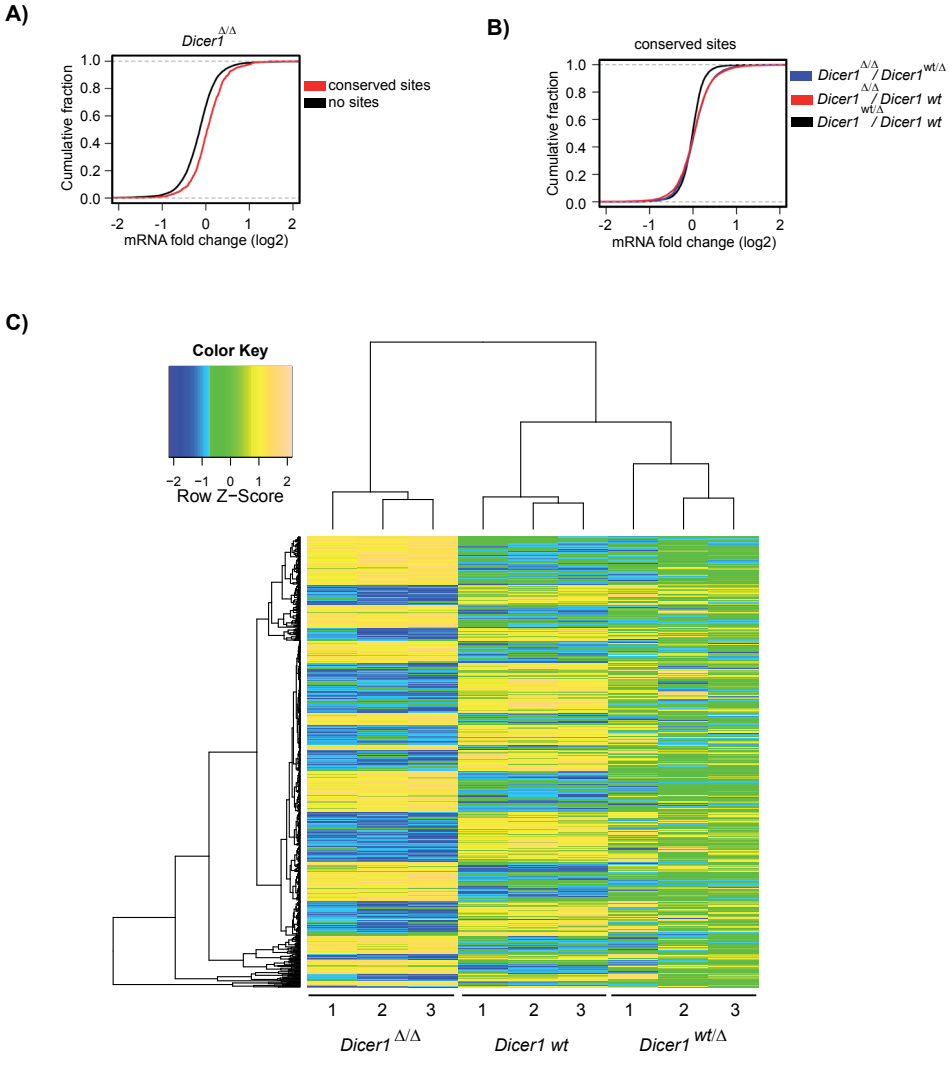


Figure 4. ***Cebpa-Cre*-mediated *Dicer1* deletion affects gene expression in GMPs. A)** Cumulative distribution plot of log fold change of transcripts that contain miRNA-binding sites for identified miRNAs (supplemental Table 2) and messages that do not contain sites compared with wild-type ($p < 2.2x10^{-16}$). **B)** Cumulative distribution plot of log fold change for mRNA that contain miRNA-binding sites for the identified miRNAs (supplemental Table 2) in the indicated populations. The expression ratio of most messages in *Dicer1*$^{\Delta/\Delta}$ cells over *Dicer1*$^{wt/\Delta}$ and *Dicer1*^{wt} are skewed toward higher positive fold changes compared with *Dicer1*$^{wt/\Delta}$ over *Dicer1*^{wt} ($p < 2.2x10^{-16}$). **C)** Significant differentially expressed genes between GMP wild-type and *Dicer1*$^{\Delta/\Delta}$ cells (FDR-corrected $p < 0.05$) were unsupervised clustered with GPLOTS (http://cran.r-project.org/web/packages/gplots/index.html).

Table 1. Active miRNA families in GMP

	Fold enrichment*	P*	No. of transcripts regulated	No. of regulated transcripts linked to lineage-affiliated signatures
let-7b/c/d/e/g/i	1.84	2.452x10-03	55	9 (16.4%)
miR-10a	2.42	1.388x10-02	16	3 (18.8%)
miR-15/16/195	1.65	1.032x10-02	57	8 (14%)
miR-17/20/93/106	2.82	2.787x10-10	83	15 (18.1%)
miR-19a/b	1.93	6.528x10-04	60	15 (25%)
miR-25/92	2.37	1.657x10-05	54	12 (22.2%)
miR-26a/b	1.80	8.623x10-03	45	10 (22.2%)
miR-27a/b	1.79	2.452x10-03	59	12 (20.3%)
miR-30a/b/d/e	1.82	1.073x10-03	69	14 (20.3%)
miR-130b/301a	1.64	2.679x10-02	42	8 (19%)
miR-142-3p	4.02	2.073x10-08	36	10 (27.8%)
miR-181a/c	1.71	9.031x10-03	52	14 (26.9%)
miR-200c	1.68	9.828x10-03	53	9 (17%)
miR-203	1.86	1.388x10-02	33	5 (15.2%)
miR-222	2.30	9.025x10-03	21	5 (23.8%)
miR-223	2.19	2.547x10-02	17	4 (23.5%)
miR-320	2.27	4.134x10-04	42	5 (11.9%)
miR-340-5p	1.82	1.525x10-03	64	7 (10.9%)
miR-494	2.35	4.099x10-03	24	5 (20.8%)
miR-503	2.23	9.025x10-03	23	2 (8.7%)

*The Fold enrichment and FDR-corrected p values of predicted targets in the fraction that are significantly up-regulated in *DicerΔ/Δ* cells compared with the nonregulated targets. Only the significant results ($p < 0.05$) of the miRNAs that are expressed in GMPs are shown. The lineage-affiliated signatures are shown in supplemental Table 4.

be linked to signatures of HSCs, MPPs and early erythropoiesis [26] (supplemental Table 4). These findings imply that certain miRNA families control the switch of a cellular program for self-renewal and expansion toward a granulocyte/monocyte/macrophage differentiation program at the GMP stage.

DISCUSSION

The results of the present study show that *Cebpa-Cre*-driven *Dicer1* deletion in myeloid-committed progenitors, and as a result depletion of miRNAs, disrupts the differentiation program of GMPs that is required for normal myeloid development. This has been demonstrated in 3 ways. First, normal numbers of *Dicer1* null EYFP$^+$ myeloid-committed

progenitors were detected by flow cytometry, indicating that depletion of miRNAs was not detrimental for these cells. Second, gene-expression profiling of *Dicer1*-null GMPs identified an altered gene expression landscape of GMPs, including enhanced expression of a set of genes that is characteristic for HSCs, MPPs and early erythropoiesis. These data imply a disordered differentiation program in *Dicer1*-null GMPs. Finally, flow cytometric analysis of myeloid cells from different hematopoietic tissues in mice showed a developmental block of monocytes, strong reduction of mature macrophages in the abdominal cavity, depletion of myeloid cells in the spleen and the presence of dysplastic neutrophils in the BM.

Recently, Raaijmakers et al. reported that deletion of *Dicer1* specifically in mouse osteoprogenitors disrupts normal hematopoiesis, resulting in a myelodysplastic syndrome and secondary leukemias in mice [13]. In these experiments *Dicer1* was not deleted in HSPCs or in the myelodysplastic cells, indicating that the observed dysplasia was initiated by osteoprogenitor dysfunction [13]. Complementary to those findings, we demonstrate herein a myeloid progenitor cell-intrinsic role for miRNA processing in myelopoiesis, in a system that leaves the BM environment intact. Our results indicate that *Dicer1* may play dual roles in the control of myelopoiesis, both of which are essential for normal myelopoiesis.

Pelger-Huët is characterized by abnormal nuclear shape and chromatin organization in blood granulocytes [21]. A genome-wide linkage scan identified the lamin B receptor (LBR), a member of the sterol reductase family located on the linked genomic region 1q41-43, to be mutated in patients suffering from Pelger-Huët disease [27]. These mutations result in decreased expression of LBR, which is strongly correlated with hyposegmentation of the nucleus in neutrophils [27,28]. In our model, *Dicer1* null neutrophils were hyposegmented, but LBR expression remained unchanged in *Dicer1* null GMPs. This result suggests that other, as-yet-undefined mechanisms may be involved in the observed developmental abnormalities or that downstream LBR pathways are controlled by *Dicer1* and may cause features of Pelger-Huët anomaly.

In contrast to our results in GMPs, recently published data show that *Dicer1* deletion in hematopoietic cells from different origins causes cell death due to derepression of mRNAs coding for proteins involved in the induction of apoptosis. For example, *Dicer1* ablation in HSCs depletes functional HSCs, induces rapid apoptosis in HSPCs and results in total disruption of hematopoiesis [14]. These deleterious effects of miRNA depletion in HSCs can be largely circumvented by the reintroduction of a single miRNA, *miR-125a*, which targets the proapoptotic protein BAK1 [14]. In addition, *Dicer1* ablation in early B-cell progenitors induces apoptosis at the pre-B cell state because of de-repression of the *miR-17~92* proapoptotic target *Bcl2L11* (also known as *Bim*) as the consequence of *Dicer1* loss [11]. Strikingly, although *Bcl2L11* is approximately 1.8 fold up-regulated in *Dicer1* null GMPs (supplemental Table 3), this did not induce an increase in apoptosis. In T cells, *Dicer1* is also essential for cell viability [10,12,29], suggesting that *Dicer1*-dependent RNAs in HSCs and cells from lymphoid origin regulate cell survival, which is, according to our data, different in myeloid-committed progenitors. In addition to the regulation of apoptosis, *Dicer1* plays

a pivotal role in the regulation of activation, migration, lineage choice and differentiation of T cells [10,12,29]. To our knowledge, this is the first report describing an exclusive role for *Dicer1* in the developmental switch of myeloid-committed progenitors toward mature neutrophils, macrophages and myeloid DCs.

A limitation of the *Dicer1* deletion models is the global depletion of miRNAs that presumably results in disruption of many cellular pathways simultaneously, which hampers the analysis of individual miRNA functions. Our data showed that *Cebpa-Cre*-driven *Dicer1* ablation in GMPs depleted 20 active miRNA families simultaneously, resulting in de-repression of at least 300 potential miRNA targets in GMPs. Because some miRNAs regulate expression by translational inhibition without affecting mRNA stability to a detectable level, the determined level of miRNA activity is most likely an underestimation. In addition, transcripts can be regulated by multiple miRNAs, and the action of miRNAs is dependent on both miRNA and target gene levels, which complicates the functional analysis of single miRNAs in this model [30]. Despite the limitations of our model, a set of miRNA target genes in GMPs is normally exclusively expressed in HSCs and when derepressed due to *Dicer1* deletion in GMPs might explain at least in part some of the phenotypic features of the *Dicer1* null GMPs. For example, *HOXA9*, a confirmed target of *miR-126* [24], is up-regulated in *Dicer1* null GMPs and forced expression in myeloid progenitors blocks differentiation and results in enhanced replating capacity [31]. In addition, the *Let-7* target *HMGA2* [23] is strongly up-regulated by *Dicer1* depletion in GMPs, and overexpression or truncation of *HMGA2* has been found in patients with myelodysplastic syndromes [32]. In addition, the robust de-repression of the HSC-specific genes *HMGA2* (8-fold) and *HOXA9* (3-fold), instead of the moderate regulation shown for most miRNA targets, suggests a miRNA-driven switch-like transition from stem cell fate toward differentiation as shown for miRNAs *lin-4* and *let-7* targeting the genes *lin-14* and *lin-41*, respectively, in Caenorhabditis elegans [33]. Therefore, our data suggest that the inability to silence stem cell genes in myeloid-committed progenitors interferes with the switch of stemness toward a myeloid differentiation program. Although these findings suggest that some miRNAs function in myeloid progenitors, an extensive miRNA add-back screen in *Dicer1* null cells is needed to identify their specific role in the control of myeloid development and this is one of the challenges in the field.

In conclusion, this study has demonstrated that *Dicer1* ablation by *C/ebpa-Cre* does not affect the numbers of HSCs, CMPs, and GMPs, but results in defective GMPs, which are unable to mature toward monocytes, macrophages, and myeloid DCs, and leads instead to neutrophil dysplasia. We have identified a set of 20 highly active miRNA families in GMPs and provided evidence that *Dicer1* controls a gene expression program that is normally active in HSCs and MPPs, and counteracts the expression of messages that are linked to early erythropoiesis. Our data uncover a *Dicer1*-controlled differentiation program in GMPs that is required for normal myelopoiesis.

ACKNOWLEDGEMENTS

The authors thank Dr. P. Leenen for providing Abs, and Prof.dr.P.A. Sharp for the *Dicer1* floxed mice; N. Papazian, Dr. K. van Lom, P. van Strien, A. Prins and Dr. E. Rombouts for technical assistance; Prof.dr. H. Delwel, M. Huston, Dr. F. Cornelissen for critical reading of the manuscript; and E. Simons for assistance with the figure preparation. This work was supported by grants from the Netherlands Organisation for Scientific Research (NWO-VENI) and the Dutch Cancer Society (KWF).

AUTHORSHIP

M.F.A. and S.J.E., designed and performed the research, analyzed the data, and wrote the manuscript. N.v.B., H. d. L. and I.J.vd.B. performed the cellular and *in vivo* research, M.A.S., performed the bioinformatic analysis, and T.C. and I.P.T., designed the research and discussed the data. The authors declare no competing financial interests.

REFERENCES

1. Tsukada J, Yoshida Y, Kominato Y, Auron PE. The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine*. 2011;54(1):6-19.
2. Wolfler A, Danen-van Oorschot AA, Haanstra JR, et al. Lineage-instructive function of C/EBPalpha in multipotent hematopoietic cells and early thymic progenitors. *Blood*. 2010;116(20):4116-4125.
3. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*. 2000;404(6775):293-296.
4. Meenhuis A, van Veelen PA, de Looper H, et al. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood*. 2011;118(4):916-925.
5. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation andgranulocytefunctionbymicroRNA-223. *Nature*. 2008;451(7182):1125-1129.
6. Boldin MP, Taganov KD, Rao DS, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med*. 2011;208(6):1189-1201.
7. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*. 2001;409(6818):363-366.
8. Bernstein E, Kim SY, Carmell MA, et al. Dicer is essential for mouse development. *Nat Genet*. 2003;35(3):215-217.
9. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(31):10898-10903.
10. Cobb BS, Nesterova TB, Thompson E, et al. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *The Journal of Experimental Medicine*. 2005;201(9):1367-1373.
11. Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell*. 2008;132(5):860-874.
12. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med*. 2005;202(2):261-269.
13. Raaijmakers MH, Mukherjee S, Guo S, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 2010;464(7290):852-857.
14. Guo S, Lu J, Schlanger R, et al. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci U S A*. 2010;107(32):14229-14234.
15. Gruber JJ, Zatechka DS, Sabin LR, et al. Ars2 links the nuclear cap-binding complex to RNA interference and cell proliferation. *Cell*. 2009;138(2):328-339.
16. Srinivas S, Watanabe T, Lin CS, et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol*. 2001;1:4.
17. Stockinger B, Hausmann B. Functional recognition of in vivo processed self antigen. *Int Immunol*. 1994;6(2):247-254.
18. Smyth G. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*. 2004;3:article 3.
19. Martis PC, Whitsett JA, Xu Y, Perl AK, Wan H, Ikegami M. C/EBPalpha is required for lung maturation at birth. *Development*. 2006;133(6):1155-1164.
20. Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X. Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci U S A*. 2006;103(7):2208-2213.
21. Cunningham JM, Patnaik MM, Hammerschmidt DE, Vercellotti GM.

Historical perspective and clinical implications of the Pelger-Huet cell. *Am J Hematol*. 2009;84(2):116-119.

22. Sunderkotter C, Nikolic T, Dillon MJ, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004;172(7):4410-4417.
23. Kumar MS, Erkeland SJ, Pester RE, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci U S A*. 2008;105(10):3903-3908.
24. Shen WF, Hu YL, Uttarwar L, Passegue E, Largman C. MicroRNA-126 regulates HOXA9 by binding to the homeobox. *Mol Cell Biol*. 2008;28(14):4609-4619.
25. Ivanovska I, Ball AS, Diaz RL, et al. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. *Mol Cell Biol*. 2008;28(7):2167-2174.
26. Ng SY, Yoshida T, Zhang J, Georgopoulos K. Genome-widelineage-specifictranscriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. *Immunity*. 2009;30(4):493-507.
27. Hoffmann K, Dreger CK, Olins AL, et al. Mutations in the gene encoding the lamin B receptor produce an altered nuclear morphology in granulocytes (Pelger-Huet anomaly). *Nat Genet*. 2002;31(4):410-414.
28. Gravemann S, Schnipper N, Meyer H, et al. Dosage effect of zero to three functional LBR-genes in vivo and in vitro. *Nucleus*. 2010;1(2):179-189.
29. Zhang N, Bevan MJ. Dicer controls $CD8^+$ T-cell activation, migration, and survival. *Proc Natl Acad Sci U S A*. 2010;107(50):21629-21634.
30. Mukherji S, Ebert MS, Zheng GX, Tsang JS, Sharp PA, van Oudenaarden A. MicroRNAs can generate thresholds in target gene expression. *Nat Genet*. 2011;43(9):854-859.
31. Bach C, Buhl S, Mueller D, Garcia-Cuellar MP, Maethner E, Slany RK. Leukemogenic transformation by HOXA cluster genes. *Blood*. 2010;115(14):2910-2918.
32. Odero MD, Grand FH, Iqbal S, et al. Disruption and aberrant expression of HMGA2 as a consequence of diverse chromosomal translocations in myeloid malignancies. *Leukemia*. 2005;19(2):245-252.
33. Bagga S, Bracht J, Hunter S, et al. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*. 2005;122(4):553-563.

SUPPLEMENTARY MATERIALS

Suppl. Table 1. List of miRNAs expressed in *EYFP*$^{+}$;*CD11b*$^{+}$ control cells

	miRNA	Expression after *Dicer1* deletion		miRNA	Expression after *Dicer1* deletion
1	mmu-let-7f	not detectable	69	mmu-miR-15b	not detectable
2	mmu-miR-124	not detectable	70	mmu-miR-195	not detectable
3	mmu-miR-188-3p	not detectable	71	mmu-miR-20a	not detectable
4	mmu-miR-339-3p	not detectable	72	mmu-miR-125b-5p	not detectable
5	mmu-miR-500	not detectable	73	mmu-miR-136	not detectable
6	mmu-miR-677	not detectable	74	mmu-miR-484	not detectable
7	mmu-miR-582-5p	not detectable	75	mmu-miR-17	not detectable
8	mmu-miR-547	not detectable	76	mmu-miR-126-5p	not detectable
9	mmu-miR-376b	not detectable	77	mmu-miR-148b	not detectable
10	mmu-miR-197	not detectable	78	mmu-miR-7a	not detectable
11	mmu-miR-139-5p	not detectable	79	mmu-miR-100	not detectable
12	mmu-miR-30c	not detectable	80	mmu-miR-509-3p	not detectable
13	mmu-let-7g	not detectable	81	mmu-miR-135b	not detectable
14	mmu-miR-140	not detectable	82	mmu-miR-222	not detectable
15	mmu-miR-106b	not detectable	83	mmu-miR-30a	not detectable
16	mmu-miR-25	not detectable	84	mmu-miR-208	not detectable
17	mmu-miR-744	not detectable	85	mmu-miR-450b-5p	not detectable
18	mmu-miR-103	not detectable	86	mmu-miR-107	not detectable
19	mmu-miR-322	not detectable	87	mmu-miR-98	not detectable
20	mmu-miR-21	not detectable	88	mmu-miR-30d	not detectable
21	mmu-miR-146b	not detectable	89	mmu-let-7i	not detectable
22	mmu-miR-340-5p	not detectable	90	mmu-miR-101a	not detectable
23	mmu-miR-18a	not detectable	91	mmu-miR-873	not detectable
24	mmu-miR-301a	not detectable	92	mmu-miR-381	not detectable
25	mmu-miR-142-5p	not detectable	93	mmu-miR-672	not detectable
26	mmu-miR-340-3p	not detectable	94	mmu-miR-181a	not detectable
27	mmu-miR-221	not detectable	95	mmu-miR-330	not detectable
28	mmu-miR-532-5p	not detectable	96	mmu-miR-99a	not detectable
29	mmu-miR-148a	not detectable	97	mmu-miR-296-5p	not detectable
30	mmu-miR-301b	not detectable	98	mmu-miR-465b-5p	not detectable
31	mmu-miR-425	not detectable	99	mmu-miR-342-3p	not detectable
32	mmu-let-7e	not detectable	100	mmu-miR-324-5p	not detectable
33	mmu-miR-29c	not detectable	101	mmu-miR-145	not detectable

Suppl. Table 1. List of miRNAs expressed in *EYFP*$^{+}$*;CD11b*$^{+}$ control cells (*Continued*)

	miRNA	Expression after *Dicer1* deletion		miRNA	Expression after *Dicer1* deletion
34	mmu-miR-15a	not detectable	102	mmu-miR-127	not detectable
35	mmu-miR-200c	not detectable	103	mmu-miR-574-3p	not detectable
36	mmu-miR-93	not detectable	104	mmu-miR-152	not detectable
37	mmu-miR-194	not detectable	105	mmu-miR-671-3p	not detectable
38	mmu-let-7d	not detectable	106	mmu-miR-328	not detectable
39	mmu-let-7c	not detectable	107	mmu-miR-544	not detectable
40	mmu-miR-339-5p	not detectable	108	mmu-miR-362-3p	not detectable
41	mmu-miR-130b	not detectable	109	mmu-miR-147	not detectable
42	mmu-miR-350	not detectable	110	mmu-miR-28	not detectable
43	mmu-miR-27b	not detectable	111	mmu-miR-186	not detectable
44	mmu-miR-10a	not detectable	112	mmu-miR-193b	not detectable
45	mmu-miR-532-3p	not detectable	113	mmu-miR-150	not detectable
46	mmu-miR-320	not detectable	114	mmu-let-7b	not detectable
47	mmu-miR-652	not detectable	115	mmu-miR-30e	not detectable
48	mmu-miR-331-3p	not detectable	116	mmu-miR-685	not detectable
49	mmu-miR-467a	not detectable	117	mmu-miR-192	not detectable
50	mmu-miR-20b	not detectable	118	mmu-miR-34b-3p	not detectable
51	mmu-miR-203	not detectable	119	mmu-miR-146a	not detectable
52	mmu-let-7a	not detectable	120	mmu-miR-126-3p	not detectable
53	mmu-miR-467c	not detectable	121	mmu-miR-667	not detectable
54	mmu-miR-365	not detectable	122	mmu-miR-546	not detectable
55	mmu-miR-423-5p	not detectable	123	mmu-miR-155	not detectable
56	mmu-miR-135a	not detectable	124	mmu-miR-361	not detectable
57	mmu-miR-30b	not detectable	125	mmu-miR-411	not detectable
58	mmu-miR-19a	not detectable	126	mmu-miR-410	not detectable
59	mmu-miR-872	not detectable	127	mmu-miR-188-5p	not detectable
60	mmu-miR-26b	not detectable	128	mmu-miR-331-5p	not detectable
61	mmu-miR-142-3p	not detectable	129	mmu-miR-223	0.07% of WT
62	mmu-miR-29a	not detectable	130	mmu-miR-16	0.29% of WT
63	mmu-miR-450a-5p	not detectable	131	mmu-miR-24	0.32% of WT
64	mmu-miR-467b	not detectable	132	mmu-miR-19b	0.62% of WT
65	mmu-miR-345-5p	not detectable	133	mmu-miR-191	0.77% of WT
66	mmu-miR-185	not detectable	134	mmu-miR-106a	2.06% of WT
67	mmu-miR-26a	not detectable	135	mmu-miR-92a	2.23% of WT
68	mmu-miR-27a	not detectable	136	mmu-miR-494	6.92% of WT

Suppl. Table 2. List of miRNAs that are expressed in GMPs

	miRNA	family	Ct		miRNA	family	Ct
1	mmu-let-7e	let-7	24,7	53	mmu-miR-194	mir-194	27,8
2	mmu-let-7g	let-7	25,8	54	mmu-miR-196b	mir-196	24,2
3	mmu-let-7i	let-7	27,2	55	mmu-miR-199a-3p	mir-199	30,8
4	mmu-let-7d	let-7	27,8	56	mmu-miR-203	mir-203	27,4
5	mmu-let-7b	let-7	29,8	57	mmu-miR-222	mir-221	20,9
6	mmu-let-7c	let-7	27,5	58	mmu-miR-223	mir-223	19,6
7	mmu-miR-10a	mir-10	30,4	59	mmu-miR-24	mir-24	20,4
8	mmu-miR-101a	mir-101	27,1	60	mmu-miR-25	mir-25	30,4
9	mmu-miR-103	mir-103	27,5	61	mmu-miR-92a	mir-25	21,8
10	mmu-miR-124	mir-124	30,1	62	mmu-miR-26b	mir-26	29,9
11	mmu-miR-125b-5p	mir-125	25,8	63	mmu-miR-26a	mir-26	27,2
12	mmu-miR-125a-5p	mir-125	25,8	64	mmu-miR-27a	mir-27	29,9
13	mmu-miR-126-5p	mir-126-5p	27,3	65	mmu-miR-27b	mir-27	30,5
14	mmu-miR-126-3p	mir-126-3p	23,2	66	mmu-miR-28	mir-28	28,4
15	mmu-miR-130b	mir-130	27,9	67	mmu-miR-29c	mir-29	24,8
16	mmu-miR-301a	mir-130	30,1	68	mmu-miR-29a	mir-29	25,3
17	mmu-miR-133b	mir-133	30,6	69	mmu-miR-30b	mir-30	23,2
18	mmu-miR-133a	mir-133	24,7	70	mmu-miR-30d	mir-30	23,8
19	mmu-miR-138	mir-138	29,8	71	mmu-miR-30e	mir-30	24,8
20	mmu-miR-139-3p	mir-139-3p	27,3	72	mmu-miR-30a	mir-30	28,6
21	mmu-miR-139-5p	mir-139-5p	23,6	73	mmu-miR-30c	mir-30	23,8
22	mmu-miR-140	mir-140	26,4	74	mmu-miR-301b	mir-301b	28,8
23	mmu-miR-142-3p	mir-142	26,2	75	mmu-miR-31	mir-31	29,5
24	mmu-miR-146a	mir-146	23,1	76	mmu-miR-320	mir-320	23,2
25	mmu-miR-146b	mir-146	19,7	77	mmu-miR-324-5p	mir-324-5p	31,8
26	mmu-miR-148a	mir-148	28,1	78	mmu-miR-328	mir-328	26,3
27	mmu-miR-152	mir-148	30,5	79	mmu-miR-331-3p	mir-331-3p	26,3
28	mmu-miR-148b	mir-148	30,7	80	mmu-miR-339-3p	mir-339-3p	24,6
29	mmu-miR-195	mir-15	25,5	81	mmu-miR-340-3p	mir-340-3p	29,1
30	mmu-miR-15b	mir-15	25,7	82	mmu-miR-340-5p	mir-340-5p	27,8
31	mmu-miR-16	mir-15	19,4	83	mmu-miR-342-3p	mir-342-3p	23,2
32	mmu-miR-150	mir-150	20,3	84	mmu-miR-365	mir-365	31,9
33	mmu-miR-494	mir-154	30,7	85	mmu-miR-423-5p	mir-423-5p	31,8
34	mmu-miR-155	mir-155	23,3	86	mmu-miR-425	mir-425	25,2
35	mmu-miR-106a	mir-17	20,2	87	mmu-miR-467a	mir-467	29,5
36	mmu-miR-17	mir-17	20,9	88	mmu-miR-467b	mir-467	28,9

Suppl. Table 2. List of miRNAs that are expressed in GMPs (*Continued*)

	miRNA	family	Ct		miRNA	family	Ct
37	mmu-miR-20a	mir-17	23,8	89	mmu-miR-484	mir-484	19,9
38	mmu-miR-93	mir-17	25,3	90	mmu-miR-486	mir-486	27,3
39	mmu-miR-106b	mir-17	27,5	91	mmu-miR-503	mir-503	28,0
40	mmu-miR-18a	mir-17	28,2	92	mmu-miR-532-5p	mir-532-5p	30,0
41	mmu-miR-20b	mir-17	24,7	93	mmu-miR-547	mir-547	31,4
42	mmu-miR-181c	mir-181	31,9	94	mmu-miR-574-3p	mir-574-3p	24,2
43	mmu-miR-181a	mir-181	28,2	95	mmu-miR-652	mir-652	31,0
44	mmu-miR-184	mir-184	26,5	96	mmu-miR-672	mir-672	31,0
45	mmu-miR-186	mir-186	22,9	97	mmu-miR-676	mir-676	31,3
46	mmu-miR-188-5p	mir-188	28,8	98	mmu-miR-680	mir-680	29,6
47	mmu-miR-532-3p	mir-188	28,5	99	mmu-miR-682	mir-682	26,3
48	mmu-miR-19a	mir-19	23,7	100	mmu-miR-685	mir-685	26,8
49	mmu-miR-19b	mir-19	21,8	101	mmu-miR-744	mir-744	26,0
50	mmu-miR-191	mir-191	15,5	102	mmu-miR-200c	mir-8	25,7
51	mmu-miR-192	mir-192	28,5	103	mmu-miR-872	mir-872	29,4
52	mmu-miR-193b	mir-193	28,6	104	mmu-miR-100	mir-99	31,6
	MammU6 (loading cont.)		**14,2**		**ath-miR-159a (neg. cont.)**		**40,0**

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion

	probes	Gene Symbol	Fold induction ΔΔ/wt
1	1438254_at	1110007A13Rik	1,228
2	1434613_at	1810013L24Rik	1,498
3	1425193_at	2010106G01Rik	1,301
4	1452159_at	2310001A20Rik	1,365
5	1452607_at	2610030H06Rik	1,341
6	1455352_at	2610101N10Rik	1,409
7	1423472_at	2-Sep	1,523
8	1427978_at	4732418C07Rik	1,504
9	1428236_at	Acbd5	1,797
10	1423883_at	Acsl1	1,346
11	1428585_at	Actn1	1,647
12	1416094_at	Adam9	2,394
13	1454918_at	Agps	1,828
14	1435879_at	Akt3	1,822
15	1448104_at	Aldh6a1	1,835
16	1422573_at	Ampd3	1,875
17	1429193_at	Ankib1	1,681
18	1433543_at	Anln	1,464
19	1427077_a_at	Ap2b1	1,617
20	1427442_a_at	App	1,580
21	1434039_at	Appbp2	1,398
22	1451251_at	Appbp2	1,492
23	1455166_at	Arl5b	1,274
24	1434949_at	Armc8	1,660
25	1455113_at	Armc8	2,054
26	1450685_at	Arpp19	1,833
27	1416735_at	Asah1	1,522
28	1450072_at	Ash1l	1,874
29	1426015_s_at	Asph	3,941
30	1427250_at	Atp2a2	1,231
31	1437688_x_at	Atp6ap2	1,233
32	1423662_at	Atp6ap2	1,301
33	**1438925_x_at**	**Atp6v0c**	1,285
34	1436921_at	Atp7a	2,450
35	1455507_s_at	Atxn1l	1,540
36	1423228_at	B4galt6	2,163

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
37	1434352_at	B630005N14Rik	1,509
38	1440831_at	Bach1	1,636
39	1435240_at	Baz2b	1,289
40	1424025_at	BC013529	1,659
41	1424026_s_at	BC013529	1,855
42	1424663_at	BC017647	1,499
43	1417077_at	Bcap29	1,397
44	1456005_a_at	Bcl2l11	1,788
45	1417493_at	Bmi1	1,238
46	**1458370_at**	**Bmp2k**	2,531
47	1437419_at	Bmp2k	1,751
48	1422490_at	Bnip2	1,286
49	1454874_at	Btbd7	1,450
50	1451222_at	Btf3l4	1,343
51	1451202_at	C330007P06Rik	1,487
52	**1417461_at**	**Cap1**	2,826
53	1451980_at	Casd1	1,376
54	1435972_at	Cast	1,325
55	1417327_at	Cav2	6,869
56	1420827_a_at	Ccng1	1,766
57	1424420_at	Ccpg1	1,395
58	1437670_x_at	Cd151	1,416
59	1416440_at	Cd164	1,327
60	1420907_at	Cd2ap	1,723
61	1417740_at	Cdc37l1	1,485
62	1434082_at	Cdk17	1,504
63	1424638_at	Cdkn1a	3,539
64	1434045_at	Cdkn1b	1,357
65	1427630_x_at	Ceacam1	1,967
66	1425538_x_at	Ceacam1	2,059
67	1426407_at	Celf1	1,276
68	1427413_a_at	Celf1	1,467
69	1418066_at	Cfl2	1,770
70	1418067_at	Cfl2	3,370
71	1448026_at	Chd7	1,707

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
72	1438606_a_at	Clic4	1,501
73	1450484_a_at	Cmpk2	1,725
74	1426682_at	Cnot6	1,666
75	1423641_s_at	Cnot7	1,404
76	1434547_at	Cpd	1,767
77	1420618_at	Cpeb4	1,737
78	1452857_at	Crebzf	1,433
79	1448248_at	Crk	1,799
80	1450966_at	Crot	1,381
81	1448592_at	Crtap	1,674
82	1448128_at	Ctsa	1,238
83	1433908_a_at	Cttn	7,351
84	1423917_a_at	Cttn	5,069
85	1417454_at	Cul4b	1,556
86	1422186_s_at	Cyb5r3	1,702
87	1423043_s_at	Ddx3x	1,326
88	1448438_at	Derl2	1,308
89	1454654_at	Dirc2	1,130
90	1417182_at	Dnaja2	2,070
91	1417191_at	Dnajb9	1,497
92	1428086_at	Dnm1l	1,341
93	1424782_at	Dram2	1,541
94	1459854_s_at	Dynlt3	1,458
95	1424065_at	Edem1	1,245
96	1434331_at	Eif2c1	1,266
97	1423220_at	Eif4e	1,240
98	1415856_at	Emb	1,405
99	1435264_at	Emilin2	1,753
100	1424800_at	Enah	2,196
101	1435223_at	Erlin2	1,713
102	1433702_at	Ermp1	1,271
103	1449324_at	Ero1l	1,789
104	1433514_at	Etnk1	1,460
105	1417513_at	Evi5	1,905
106	1417512_at	Evi5	2,167

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
107	1433572_a_at	Fam120a	1,286
108	1424683_at	Fam134b	1,658
109	1455429_at	Fam160b1	1,351
110	1417953_at	Fam3c	1,653
111	1448904_at	Fam3c	1,764
112	1431337_a_at	Fam45a	1,449
113	1436842_at	Fam63b	1,588
114	1429503_at	Fam69a	1,470
115	1435315_s_at	Far1	1,381
116	1451558_at	Fbxw7	1,267
117	1435801_at	Fktn	1,924
118	1443863_at	Fndc3a	1,904
119	1426903_at	Fndc3a	2,807
120	1433833_at	Fndc3b	1,972
121	1434002_at	Foxn3	1,631
122	1427177_at	Fyco1	2,466
123	1455915_at	Galnt4	1,648
124	1452232_at	Galnt7	1,601
125	1426908_at	Galnt7	1,645
126	1425156_at	Gbp6	1,882
127	1435749_at	Gda	1,816
128	1435748_at	Gda	2,058
129	1456581_x_at	Gdi2	1,169
130	1417679_at	Gfi1	1,518
131	1428715_at	Gfpt1	1,759
132	1428681_at	Gm608	1,212
133	1429559_at	Gnaq	1,507
134	1428940_at	Gnaq	1,640
135	1455729_at	Gnaq	1,869
136	1428939_s_at	Gnaq	1,883
137	1428938_at	Gnaq	2,091
138	1455089_at	Gng12	1,664
139	1426524_at	Gnpda2	1,480
140	1433546_at	Gns	1,852
141	1433488_x_at	Gns	2,377

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
142	1415698_at	Golm1	2,231
143	1428323_at	Gpd2	1,627
144	1437171_x_at	Gsn	1,763
145	1456312_x_at	Gsn	1,866
146	1436991_x_at	Gsn	1,907
147	1434876_at	Gxylt1	1,473
148	1420376_a_at	H3f3b	1,247
149	1460367_at	Hbp1	1,321
150	1434478_at	Heca	1,385
151	1427418_a_at	Hif1a	1,686
152	1428433_at	Hipk2	1,559
153	1450780_s_at	Hmga2	6,915
154	1422851_at	Hmga2	18,200
155	1450781_at	Hmga2	14,215
156	1433443_a_at	Hmgcs1	1,599
157	1433445_x_at	Hmgcs1	1,603
158	1452712_at	Hnrnpa3	1,156
159	1456698_s_at	Hnrpdl	1,199
160	1455626_at	Hoxa9	4,052
161	1415889_a_at	Hsp90b1	1,396
162	1452982_at	Igf1r	1,814
163	**1433732_x_at**	**Igf2bp3**	14,093
164	**1433731_at**	**Igf2bp3**	15,548
165	**1422611_s_at**	**Igf2bp3**	14,381
166	**1422610_s_at**	**Igf2bp3**	28,606
167	1437289_at	Impad1	2,180
168	1437290_at	Impad1	2,316
169	1436069_at	Ing5	1,222
170	1434446_at	Insr	1,290
171	1419041_at	Itfg1	1,194
172	1452784_at	Itgav	2,514
173	1435023_at	Itsn2	1,650
174	1434037_s_at	Kat2b	2,213
175	1455816_a_at	Kctd3	1,281
176	1418629_a_at	Khdrbs1	1,118

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
177	1418431_at	Kif5b	1,384
178	1418430_at	Kif5b	1,500
179	1426530_a_at	Klhl5	1,299
180	1449505_at	Kpna1	1,556
181	1460260_s_at	Kpna1	1,741
182	1419548_at	Kpna1	1,871
183	1451979_at	Kras	1,373
184	1434000_at	Kras	1,554
185	1435739_at	Lats1	1,793
186	1443779_s_at	Lcor	3,658
187	1455121_at	Lcor	4,612
188	1455260_at	Lcorl	1,685
189	1434129_s_at	Lhfpl2	1,745
190	1435326_at	Lpgat1	1,699
191	1440167_s_at	Lpp	2,452
192	1435461_at	Magi3	1,726
193	1439830_at	Map3k5	1,614
194	1426245_s_at	Mapre2	1,356
195	1451989_a_at	Mapre2	1,509
196	**1456028_x_at**	**Marcks**	3,748
197	1456700_x_at	Marcks	2,607
198	1415971_at	Marcks	2,674
199	1415973_at	Marcks	2,529
200	1427040_at	Mdfic	1,712
201	1423610_at	Metap2	1,210
202	1434120_a_at	Metap2	1,223
203	1424463_at	Mfsd6	2,692
204	1424464_s_at	Mfsd6	3,280
205	1435641_at	Mgat4a	2,023
206	1434179_at	Mll3	1,288
207	1423488_at	Mmd	1,276
208	1453032_at	Mobkl3	1,430
209	1423168_at	Mobkl3	1,884
210	1424124_at	Mospd2	1,421
211	1418514_at	Mtf2	1,215

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
212	1452608_at	Mycbp	1,587
213	1419648_at	Myo1c	2,003
214	1419649_s_at	Myo1c	3,015
215	1436051_at	Myo5a	1,885
216	1428453_at	Naa30	1,396
217	1428410_at	Naa50	1,322
218	1417624_at	Nab1	1,651
219	1454952_s_at	Ncapd3	1,100
220	1438452_at	Nebl	5,724
221	1416543_at	Nfe2l2	1,404
222	1454919_at	Nmt2	2,282
223	1420487_at	Nol7	1,293
224	1456599_at	Nxt2	2,670
225	1419534_at	Olr1	3,714
226	1422792_at	Pafah1b2	1,426
227	**1451740_at**	**Paip1**	3,327
228	**1425521_at**	**Paip1**	19,532
229	1428423_at	Pcgf3	1,779
230	1419047_at	Pcnx	1,581
231	1448527_at	Pdcd10	1,115
232	1448528_at	Pdcd10	1,422
233	1433694_at	Pde3b	1,895
234	1423423_at	Pdia3	1,414
235	1456478_at	Pgm2l1	1,404
236	1452841_at	Pgm2l1	1,491
237	1437067_at	Phtf2	1,866
238	1416489_at	Pi4k2b	1,553
239	1435458_at	Pim1	2,208
240	1437295_at	Pkn2	2,931
241	1438677_at	Pkp4	1,303
242	1417288_at	Plekha2	1,613
243	1420840_at	Plekha3	1,345
244	1415901_at	Plod3	1,730
245	1429019_s_at	Pon2	1,518
246	1450686_at	Pon2	1,610

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
247	1425537_at	Ppm1a	1,852
248	1417367_at	Ppp2ca	1,125
249	1428265_at	Ppp2r1b	1,117
250	1452056_s_at	Ppp3ca	1,319
251	1428473_at	Ppp3cb	1,303
252	1425550_a_at	Prkar1a	1,233
253	1419700_a_at	Prom1	2,229
254	1455724_at	Prrg1	1,957
255	**1449342_at**	**Ptplb**	1,544
256	1419642_at	Purb	1,995
257	1428254_at	Purb	2,451
258	1427992_a_at	Rab12	1,989
259	1419246_s_at	Rab14	1,330
260	1418622_at	Rab2a	1,594
261	1419945_s_at	Rab2a	1,727
262	1434062_at	Rabgap1l	1,699
263	1426476_at	Rasa1	1,412
264	1418703_at	Rbms1	1,654
265	1422449_s_at	Rcn2	1,438
266	1428342_at	Rcor3	1,268
267	1450784_at	Reck	3,960
268	1433976_at	Reep3	1,389
269	1452359_at	Rell1	1,285
270	1427243_at	Rell1	1,692
271	1415747_s_at	Riok3	1,141
272	1422650_a_at	Riok3	1,482
273	1454064_a_at	Rnf138	1,144
274	1419369_at	Rnf138	1,382
275	1433655_at	Rnf141	1,352
276	1427898_at	Rnf6	1,384
277	1452767_at	Rrbp1	1,125
278	1436058_at	Rsad2	2,345
279	1428219_at	Rybp	1,377
280	1454704_at	Scarb2	1,476
281	1416267_at	Scoc	2,429

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
282	1450941_at	Sdcbp	1,610
283	**1439882_at**	**Sec23ip**	3,353
284	1433934_at	Sec24a	1,738
285	1424925_at	Sec63	1,512
286	1448108_at	Serinc1	1,676
287	1435437_at	Setd7	1,469
288	1428663_at	Sgms2	1,893
289	1437503_a_at	Shisa5	1,441
290	1423149_at	Skp1a	1,243
291	1436000_a_at	Skp2	1,317
292	1437033_a_at	Skp2	1,483
293	1452286_at	Slain2	1,291
294	1417902_at	Slc19a2	2,045
295	1441315_s_at	Slc19a2	2,087
296	1452717_at	Slc25a24	1,801
297	1419657_a_at	Slc25a36	1,249
298	1417061_at	Slc40a1	3,155
299	1438673_at	Slc4a7	1,451
300	1438116_x_at	Slc9a3r1	1,182
301	1422486_a_at	Smad4	1,372
302	1422487_at	Smad4	1,380
303	1435251_at	Snx13	2,261
304	1436015_s_at	Stk4	1,229
305	1421664_a_at	Styx	1,402
306	1429711_at	Styx	1,550
307	1424603_at	Sumf1	1,333
308	1426666_a_at	Sun1	1,666
309	1454961_at	Synj1	1,360
310	1426357_at	Taok1	1,646
311	1455432_at	Taok1	2,225
312	1430133_at	Tbc1d8b	2,080
313	1436226_at	Tceb1	1,272
314	**1452213_at**	**Tex2**	1,526
315	1420895_at	Tgfbr1	2,059
316	1426397_at	Tgfbr2	2,462

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
317	1424574_at	Tmed5	1,572
318	1428854_at	Tmed8	1,848
319	**1415741_at**	**Tmem165**	1,422
320	1452813_a_at	Tmem188	1,222
321	1451652_a_at	Tmem188	1,248
322	1451458_at	Tmem2	2,288
323	1424711_at	Tmem2	2,372
324	1452942_at	Tmem65	1,800
325	**1424454_at**	**Tmem87a**	4,146
326	1453120_at	Tmx4	1,513
327	1425036_a_at	Tnrc6a	1,567
328	1455128_x_at	Tnrc6a	1,693
329	1434898_at	Tnrc6a	1,723
330	1439244_a_at	Tnrc6a	1,739
331	1455141_at	Tnrc6a	2,223
332	1434899_s_at	Tnrc6a	2,047
333	1455333_at	Tns3	1,429
334	1435526_at	Tor1aip2	1,915
335	1434768_at	Tpp1	1,512
336	1427407_s_at	Trip11	1,681
337	1416926_at	Trp53inp1	2,448
338	1420875_at	Twf1	1,922
339	1428945_at	Uba6	1,609
340	1417609_at	Ube2a	1,555
341	**1416943_at**	**Ube2e1**	1,222
342	1426461_at	Ugp2	1,339
343	1435325_at	Usp46	1,569
344	1452011_a_at	Uxs1	1,731
345	1437708_x_at	Vamp3	1,803
346	1456245_x_at	Vamp3	1,906
347	1415990_at	Vdac2	1,179
348	1438118_x_at	Vim	1,267
349	1456292_a_at	Vim	1,399
350	**1451720_at**	**Vps39**	1,981
351	1449095_at	Vps54	2,113

Suppl.Table 3. Up-regulated and not regulated expressed probe sets. Probesets shown in bold are significantly derepressed also by *Dicer1* heterozygous deletion (*Continued*)

	probes	Gene Symbol	Fold induction ΔΔ/wt
352	1418479_at	Vps54	2,079
353	1451495_at	Wac	1,358
354	1452193_a_at	Wasl	2,276
355	1434076_at	Wdr37	1,466
356	1427098_at	Wwp1	1,863
357	1452299_at	Wwp1	1,966
358	1455111_at	Yipf6	1,694
359	1426842_at	Ythdf3	1,349
360	1426841_at	Ythdf3	1,602
361	1428779_at	Zbtb41	2,474
362	1439089_at	Zbtb41	2,133
363	1449947_s_at	Zfhx3	2,934
364	1433623_at	Zfp367	2,225
365	1460589_at	Zfp597	2,233
366	1429615_at	Zfp91	1,339
367	1424670_s_at	Zfyve21	1,360
368	1452970_at	Zmym2	1,216

Suppl.Table 4. Upregulated genes in Dicer null GMPs linked to lineage-affiliated signatures as published by Samuel Yao-Ming Ng, et al, Immunity 30, 493-507, april 17, 2009.

Gene.Symbol	sign. group	Definition
Cav2	1 stem	Contains self-renewing genes expressed in
Bach1	1	HSCs (LT- + ST-)
Hmga2	2 s-mpp	No significant expression of lineage
Hoxa9	2	specific genes expressed in
2210010L05Rik	2	HSC/MPP and LMPP
Atbf1	2	
Tgfbr2	2	
Trp53inp1	2	
Rsad2	2	
Actn1	2	
Scotin	2	
Cd151	2	
Cttn	3 s-ery	Eerythroid lineage specific genes primed in HSC.
Slc40a1	3	1st wave of erythroid lineage specific expression program in
Ampd3	3	HSC/MPP and MEP.
2810423A18Rik	3	
Tyki	3	
Ches1	3	
Rcor3	3	
Hmga2	4 s-myly	Lymphoid and myeloid lineage specific genes primed in HSC.
Reck	4	First wave of lymphoid- and myeloid-lineage -specific
Marcks	4	expression program in HSC/MPP, LMPP, GMP and ProB.
Cdkn1a	4	
Fndc3a	4	
Marcks	4	
Itgav	4	
Atp7a	4	
Snx13	4	
Ceacam1	4	
Fndc3b	4	
Myo5a	4	
Gnaq	4	
Mdfic	4	
AW112037	4	
1810015C04Rik	4	
LOC675366	4	

Suppl.Table 4. Upregulated genes in Dicer null GMPs linked to lineage-affiliated signatures as published by Samuel Yao-Ming Ng, et al, Immunity 30, 493-507, april 17, 2009. (*Continued*)

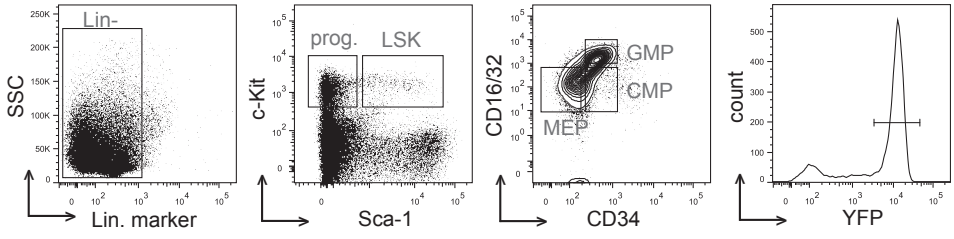
Gene.Symbol	sign. group	Definition
Plekha2	4	
Hmgcs1	4	
Pcnx	4	
Asah1	4	
Gfi1	4	
Tns3	4	
Emb	4	
Vim	4	
Reep3	4	
Smad4	4	
Ugp2	4	
Pkp4	4	
Klhl5	4	
Baz2b	4	
Vim	4	
Slc9a3r1	4	
Kpna1	6 diff	No expression with lineage specific genes.
D6Wsu176e	6	Demarcating a progenitor-restricted state in GMP, MEP and
Kif5b	6	ProB.
Tmem165	6	
Pdia3	6	
Hsp90b1	6	
Sumf1	6	
Nat13	6	
Atp6ap2	6	
Metap2	6	
Itfg1	6	
Khdrbs1	6	
Asph	7 d-ery	Erythroid progenitor-specific.
Purb	7	2rd wave of erythroid lineage specific expression program
Scoc	7	in MEP.
Golph2	7	
Wwp1	7	
Ccng1	7	
Emilin2	7	
Lhfpl2	7	

Suppl.Table 4. Upregulated genes in Dicer null GMPs linked to lineage-affiliated signatures as published by Samuel Yao-Ming Ng, et al, Immunity 30, 493-507, april 17, 2009. (*Continued*)

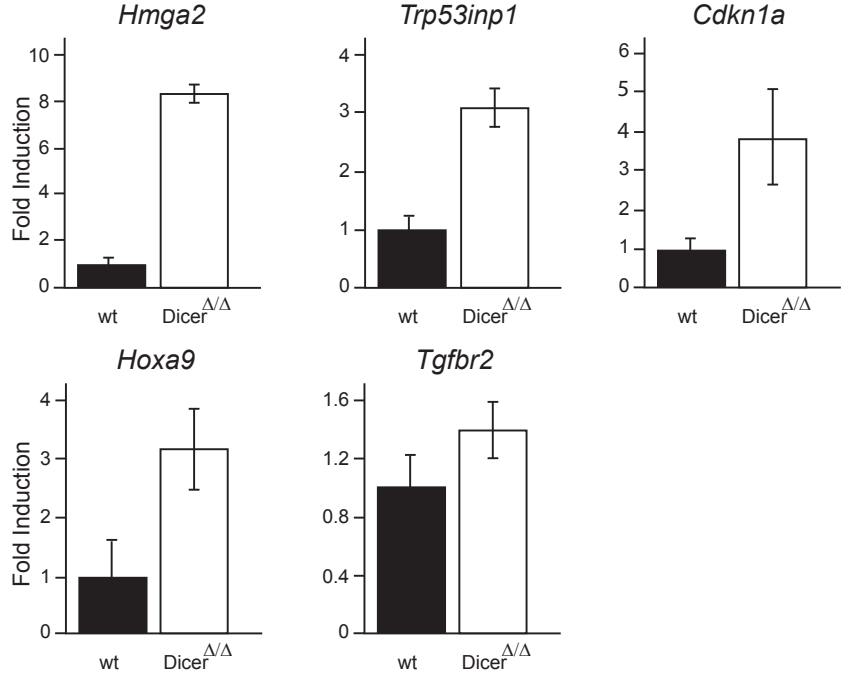
Gene.Symbol	sign. group	Definition
Yipf6	7	
Unc84a	7	
Pon2	7	
Usp46	7	
Pon2	7	
Tpp1	7	
Nfe2l2	7	
Pgm2l1	7	
Acsl1	7	
Gm608	7	
Ppp2r1b	7	
5133401H06Rik	8 d-my	Myeloid progenitor-specific.
Plod3	8	3rd wave of myeloid lineage program in GMP.
Gng12	8	
Tex2	8	
Edem1	8	
Dirc2	8	

Suppl.Table 5. List of antibodies

Antibody	Conjugate	Clone identifier	Company
CD3e	Biotin	145-2C11	BectonDickinson (BD) Bioscience
Ter119	Biotin	Ter119	BD Bioscience
Gr-1	Biotin, APC	RB6-8C5	BD Bioscience
CD45R/B220	Biotin	RA3-6B2	BD Bioscience
CD11b	Biotin, PE	M1/70	BD Bioscience
c-Kit	APC	2B8	BD Bioscience
Sca-1	PE-Cy7	D7	eBioscience
CD16/32	PE	2.4G2	BD Bioscience
CD34	Pacific blue	RAM34	eBioscience
streptavidine	APC-Cy7		BD Bioscience
MHC class II	Alexa Fluor 700	M5/114.15.2	eBioscience
CD11c	PE-Cy7	HL3	BD Bioscience
F4/80	PE-Cy5	200 BM8	eBioscience
Ly6C	Alexa Fluor 700		BD Bioscience



Suppl. Fig. 1. Analysis of the percentage of YFP-positive cells in the progenitor (MEPs, CMPs and GMPs) and the LSK fraction of the bone marrow of transplanted mice.



Suppl. Fig. 2. Confirmation of some transcript upregulation in *Dicer1* null cells by QPCR.

4

Stop the Dicing in Hematopoiesis; What have we Learned?

Mir Farshid Alemdehy and Stefan J. Erkeland

Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands

Cell Cycle. 2012; 11(15):2799-807

ABSTRACT

MicroRNAs (miRNAs) belong to an abundant class of highly conserved small (22nt) non-coding RNAs. MiRNA profiling studies indicate that their expression is highly cell-type dependent. DICER1 is an essential RNase III endoribonuclease for miRNA processing. Hematopoietic cell-type and developmental stage specific *Dicer1* deletion models show that miRNAs are essential regulators of cellular survival, differentiation and function. For instance, miRNA-deficiency in hematopoietic stem cells and progenitors of different origins results in decreased cell survival, dramatic developmental aberrations or dysfunctions in mice. We recently found that homozygous *Dicer1* deletion in myeloid-committed progenitors results in an aberrant expression of stem cell genes and induces a regained self-renewal capacity. Moreover, *Dicer1* deletion causes a block in macrophage development and myeloid dysplasia, a cellular condition that may be considered as a preleukemic state. However, *Dicer1* null cells do not develop leukemia in mice, indicating that depletion of miRNAs is not enough for tumorigenesis. Surprisingly, we found that heterozygous *Dicer1* deletion in myeloid-committed progenitors, but not *Dicer1* knockout, collaborates with *p53* deletion in leukemic progression and results in various types of leukemia. Our data indicate that *Dicer1* is a haploinsufficient tumorsuppressor in hematopoietic neoplasms which is consistent with the observed downregulation of miRNA expression in human leukemia samples. Here, we review the various hematopoietic specific *Dicer1* deletion mouse models and the phenotypes observed within the different hematopoietic lineages and cell developmental stages. Finally, we discuss the role for DICER1 in mouse and human malignant hematopoiesis.

INTRODUCTION

DICER1 is an evolutionarily conserved member of the RNase III family of endoribonucleases. The gene encoding DICER1 is located on human chromosome 14q32 and mouse chromosome 12E. DICER1 is a complex protein and contains three N-terminal helicase domains (HEL1, HEL2i, HEL2), a DUF283 domain which is presumably involved in binding of double stranded RNA (dsRNA), a Platform domain, the pre-miRNA binding domain PAZ, RNase IIIa, RNase IIIb and a C-terminal double-stranded RNA binding domain (dsRBD) [1-3]. The RNase III domains of DICER1 cleave double-stranded RNA (dsRNA) substrates and specific precursor hairpin sequences, including so-called pre-miRNAs, into small 5'-phosphorylated RNAs of typically 21-23 nucleotides called miRNA [4]. Deep sequencing of 5'-phosphorylated short RNAs in ES cells showed that the miRNA is the only class of short RNAs to be fully DICER1 dependent [5]. However, the premature *miR-451* is the single well-conserved miRNA-containing sequence known to bypass DICER1 processing and is matured by an Argonaute-2 (Ago-2)-dependent mechanism [6-9]. The DICER1-generated short RNAs bind to Argonaute proteins in the so-called RNA-induced silencing complex (RISC). This complex induces degradation or inhibits translation of homologs target mRNAs. Moreover RISC triggers gene silencing via chromatin modifications at target promoters under specific conditions such as cellular senescence [10,11].

Genetic studies in plants, zebrafish and mice show that *Dicer1* is essential for normal development [12-14]. For instance, genetic deletion of *Dicer1* in mice results in early embryonic mortality due to depletion of the Oct-4-positive pluripotent embryonic stem cell pool at embryonic day (E) 6-E7 [14]. *Dicer1* null ES cells are incapable of processing miRNA hairpins or dsRNAs [5,15,16]. However, *Dicer1* is dispensable for the small interfering (siRNA)-mediated gene silencing response [16]. Although a role for *Dicer1* in centromeric silencing has been suggested, deep sequencing of small RNAs in *Dicer1* null and *Dicer1* wild type ES cells indicates that the production of miRNAs is the sole catalytic function of DICER1 in these cells [5]. To bypass embryonic lethality and to enable investigation of *Dicer1* functions in adult tissues in mice, a floxed *Dicer1* allele (*Dicer1*^{fl}) has been generated that allows conditional deletion of *Dicer1* in a cell type and developmental stage-specific fashion [17]. To address the overall role of miRNAs in the development and function of hematopoietic cells, different hematopoietic cell stage and lineage-specific conditional *Dicer1* deletion strains have been used. First, we will review the phenotypic consequences of *Dicer1* deletion at different stages of hematopoiesis and cell types. Second, we discuss what we have learned from these models about miRNA-controlled pathways in hematopoiesis. Finally, we show evidence for *Dicer1* haploinsufficient tumorsuppressor activity in mouse leukemia and discuss the role for *DICER1* in human AML.

THE ROLE OF *DICER1* IN T- LYMPHOCYTE DEVELOPMENT

In one of the first studies addressing the role of *Dicer1* in hematopoiesis *in vivo*, floxed *Dicer1* alleles were deleted by CRE in lymphocyte-specific protein tyrosine kinase (Lck) positive

cells. In this model, *Cre* is active at the double negative (DN) $CD4^-CD8^-$ T cell developmental stage and results in *Dicer1* null $CD44^-CD25^-$ (DN4), $CD4^+CD8^+$ and $CD4^+CD8^-$, $CD4^-CD8^-$ cells (Figure 1A) [18]. *Dicer1* seems to be essential for the generation and survival of αβ T-cells. However, in the surviving T-cells *Dicer1* is dispensable for $CD4^+$ and $CD8^+$-single positive lineage commitment [18]. These results strongly suggest that *Dicer1* deletion does not affect normal T-cell lineage-specific gene expression programs. In these cells, the transcriptional repression of centromeric satellite repeats and features of facultative heterochromatin are maintained in the absence of *Dicer1* [18], suggesting that survival of immature T-cells is regulated directly by a miRNA-controlled mechanism.

The *CD4-Cre* transgenic mouse model enables investigation of the consequences of *Dicer1* deletion at a later stage of T cell development (Figure 1B). These mice show four major phenotypes: (1) *Dicer1* is required for basic cellular processes such as proliferation and survival, as also proposed by Cobb *et al.* and therefore *Dicer1* deficiency results in decreased number of T-cells [18,19]. (2) *Dicer1* deletion appears to favor T-cell lineage production from $CD4^+CD8^+$ double positive stage toward $CD4^+$ single positive peripheral T-cells over $CD8^+$ single positive cells. However, this phenotype was less obvious from thymic T-cell lineage analysis. This discrepancy may be explained by the fact that *CD4-Cre*-driven deletion of *Dicer1* does not result in complete depletion of all miRNAs, presumably due to high miRNA stability and limited cell divisions of a small fraction of $CD4^+$ T-cells, which may be different for *Dicer1* null $CD8^+$ T-cells. (3) *Dicer1* null $CD4^+$ T-cells produce increased levels of IFN-γ, a pro-Th1 cytokine, indicating that *Dicer1* controls Th1-lineage commitment [19]. (4) *CD4-Cre*; $Dicer1^{fl/fl}$ mice show a more than 2-fold decreased proportion of $Foxp3^+$ regulatory T cells (Treg) [20]. Interestingly, these mice developed a splenomegaly and their lymph nodes were severely enlarged at the age of 3 to 4 months. Moreover, organs such as colon, lung and liver were affected by immune pathology caused by an overactive immune system, which is less severe as compared with *Foxp3* knockout mice lacking functional Tregs [20]. However, this phenotype suggests that *Dicer1*-deficient Tregs are functionally aberrant as well.

Two studies revealed the role of *Dicer1* more specifically in the function of mature Tregs, using a *Foxp3-Cre* knock-in mouse (Figure 1C) [21,22]. Under steady state conditions, Foxp3-controlled deletion of *Dicer1* has minimal effects on Treg cell development, cellular proliferation and survival in the peripheral compartments [22]. However, a diminished fitness of *Dicer1*-deficient Treg cells in the periphery was observed in a competitive experiment in mice [21]. Under inflammatory conditions, the immune-repressive capacity of the mutant Treg cells is markedly reduced and results in rapid fatal autoimmunity and complete failure of immune suppression activity [21,22]. Moreover, *Dicer1* deletion in Treg cells leads to the progression of fatal lymphoproliferative autoimmune syndrome with an early onset, which is indistinguishably comparable to T-cell-specific Foxp3-deficiency [21]. The expression of putative suppressor effector molecules including CTLA4, IL-10, EBV-induced gene 3 (Ebi-3), and granzyme B was decreased by still unidentified miRNA-controlled mechanisms [21]. Tregs express a specific set of miRNAs including

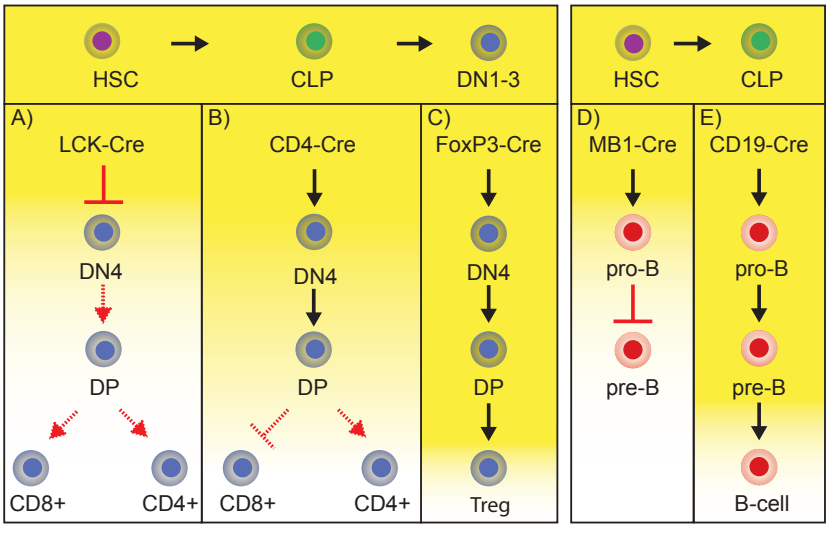


Figure 1. Schematic overview of the phenotypic characteristics of different CRE-mediated *Dicer1*-deletion models in lymphopoiesis. A) HSCs develop via different progenitors toward mature $CD4^+$ or $CD8^+$ single positive cells. The effects of *LCK-Cre*-mediated *Dicer1* deletion are depicted. The apparent level of *Dicer1* expression is indicated by the yellow background color (yellow: normal endogenous levels; white: no Dicer1 expression). HSC: hematopoietic stem cells, CLP: common lymphoid progenitor, DN1-3: double negative stage 1 to 3 ($CD4^-CD8^-$), DN4: double negative stage 4, DP: double positive $CD4^+CD8^+$ cells. Phenotypic characteristics are indicated by the red arrows and lines. Dashed lines indicate less cells than in wild-type situation. **B)** See also A. The effects of *CD4-Cre*-mediated *Dicer1* deletion are depicted. Phenotypic characteristics are indicated by the red arrow and lines. **C)** See also A. The effects of *FoxP3-Cre*-mediated *Dicer1* deletion results in normal numbers of regulatoty T-cells (Tregs) but these cells are functionally aberrant. **D)** HSCs develop via indicated progenitors toward mature B-cells. The effects of *MB1-Cre*-mediated *Dicer1* deletion are indicated by the red lines and arrow and result in developmental block from the pro-B-cell to the pre-B-cell stage. Pro-B: earliest stage of progenitor B-cell development, pre-B-cell, precursor stage of B-cell development. **E)** See also D. *CD19-Cre*-mediated deletion of *Dicer1* results in mature B-cells which are functionally aberrant.

miR-223, *miR-155* and *miR-146*, which is distinct from naïve $CD4^+$ T-cells [20]. Therefore, the expression of these miRNAs may be under direct or indirect control of the transcription factor Foxp3 [20]. Identification of the targets that are controlled by these miRNAs in Tregs may provide new insights about the molecular pathways involved in the activity of these cells.

The role of miRNAs in invariant Natural Killer T (iNKT) cells was studied in a mouse strain by *Tie2-Cre*-mediated disruption of *Dicer1* [23]. The Tie2 kinase is specifically expressed in hematopoietic progenitors and endothelial cells [24]. Similar to the immune phenotypes in *CD4-Cre;Dicer1*$^{fl/fl}$ and *Lck-Cre;Dicer1*$^{fl/fl}$, these mice show reduced numbers of iNKT

cells in the thymus, spleen, and liver. Moreover, *Dicer1* deletion results in developmental abnormalities of iNKT cells [23,25]. In addition, *Dicer1*-deficient peripheral iNKT cell numbers are decreased and displayed profound defects in α-GalCer, phorbol myristate acetate (PMA) and ionomycin-induced cellular activation and production of cytokines such as IL-4 and IFN-γ [23]. Together, these data indicate that *Dicer1* controls survival at the early T-cell developmental stage. At the later stage, *Dicer1* is critical for the balance of Th1/Th2 lineage production and controls functions such as immune-repression and specific cellular activity.

DICER1 FUNCTION DURING B CELL DEVELOPMENT

Ablation of *Dicer1* in early B cell progenitors, mediated by the *Mb1-Cre* allele, which is expressed at the earliest stage of B-cell development, blocks B-cell development almost completely at the pro-B-cell ($B220^{low}$, c-kit^+ $CD25^-$) to pre-B-cell ($B220^{int}$, c-kit^-, $CD25^+$) transition (Figure 1D) [26]. This block in B-cell development is caused by a strong induction of apoptosis and results in total depletion of B cells in the BM and the peripheral lymphoid organs in mice [26]. Gene expression profiling of Abelson virus (v-Abl)-transformed *Dicer1* null pro-B-cells revealed that *miR-142-3p* and different members of the *miR-17~92* family of miRNA such as *miR-17*, *miR-19*, *miR-20* and *miR-92* are the most-active at the pro-B-cell stage [26]. Derepression of the proapoptotic protein BIM, a confirmed target of *miR-17~92*, was shown to be mainly responsible for the failure of the cells to respond to survival signals [26]. In full agreement, Ventura A and colleagues have demonstrated that deletion of the *miR-17~92* in mouse hematopoietic stem cells (HSCs) leads to a cell development arrest at the pro-B to pre-B transition that is highly reminiscent of what has been observed in the *Dicer1*-deficient mice [27].

The role of miRNAs in terminal B cell differentiation is addressed by the analysis of *CD19-Cre* driven *Dicer1*-deletion mouse model (Figure 1E) [28]. In contrast to early *MB1-Cre* driven *Dicer1* deletion, depletion of *Dicer1* with *CD19-Cre* in immature $B220^+$ IgM^+ cells does not induce cell death and allowed analysis of the role for *Dicer1* in mature B cells in peripheral tissues [28]. In the absence of *Dicer1*, transitional and marginal zone B cells are overrepresented and the generation of follicular B cells is impaired [28]. The *miR-185* is abundantly expressed in follicular B-cells and controls the expression of B cell antigen receptor (BCR) signaling effector Bruton tyrosine kinase (BtK) in activated B cells [28]. *Dicer1*-deficient B cells produce high titers of autoreactive antibodies and as a result cause autoimmune disease in aged female mice [28]. However, the miRNAs that control autoreactivity are still unidentified.

To investigate the role for *Dicer1* in antigen-activated, but not naive B cells, an activation-induced cytadine deaminase *(Aicda)-Cre*-mediated *Dicer1* deletion mouse model has been generated [29]. This mouse model showed that *Dicer1* is required for the production of antigen-specific high-affinity antibodies during a T-cell dependent immune response [29]. Also, the formation of germinal center B cells is drastically impaired in *Dicer1*-deficient mice [29]. These mutant mice fail to generate memory B and long-lived plasma cells after immunization with a T cell-dependent antigen. This study provides evidence for *Dicer1*-controlled cell proliferation

of activated germinal center B-cells by strong repression of cell cycle inhibitory genes, such as *Cdkn1c* (*p57*^{Kip2}), *Cdkn2b* (*p16*^{INK4a}), *Cdk1a* (*p21*^{Cip1}) and *Cdkn1b* (*p27*^{Kip}) [29]. Furthermore, *Dicer1* deletion in B-cells leads to massive induction of apoptosis due to derepression of the proapoptotic protein BIM1 as described for early stages of B-cell development [29]. Together, these data show that *Dicer1* controls survival of B-cells at different stages of B-cell development, regulates cellular proliferation and is critical for proper B- and plasma cell functions.

THE ROLE FOR *DICER1* IN NK CELL FUNCTION

Bezman *et al.* induced ablation of conditional *Dicer1* alleles with a tamoxifen–inducible Cre recombinase (human estrogen receptor *(ER*^{T2}*)-Cre*), and studied the effects of miRNA depletion in NK cells [30]. This non-specific model revealed a role for *Dicer1* in the maintenance of survival and function of NK cells [30]. They found that in response to a viral infection with mouse cytomegalovirus (MCMV), the expansion of NK cells, but not the IFN-γ production, is *Dicer1* dependent, suggesting that survival but not activity of NK cells is affected by *Dicer1* deficiency [30]. Similarly, *HCD2-Cre;Dicer1*$^{fl/fl}$ mice, which enable a lymphocyte-restricted *Dicer1*-deletion at the early stage of NK cells development also showed reduced NK-cell maturation and survival [30,31]. However, *Dicer1* null NK cells showed enhanced degranulation and IFN-γ production in response to cytokines such as IL-15 and IL-12, tumor target cells, activating NK cell receptor ligation, as well as during acute MCMV infection *in vivo* [31]. The *miR-15/16* family of miRNAs is potentially contributing to IFN-γ suppression and may control dampening of NK cell functions [31].

DICER1 DELETION IN MYELOID-COMMITTED PROGENITORS REVEALED AN UNEXPECTED FUNCTION IN HEMATOPOIESIS

The consequences of *Dicer1* deletion in hematopoietic stem and progenitor cells (HSPCs) was first studied by breeding *Dicer1*$^{fl/fl}$ with *Mx-Cre* mice [32]. These mice express the Cre-recombinase in response to interferons and are highly efficient in recombination of floxed alleles in the hematopoietic system *in vivo* via peritoneal injection of polyI:polyC (pIpC) [32]. *Dicer1* ablation in these mice depletes functional HSCs, induces rapid apoptosis in HSPCs and consequently causes total disruption of hematopoiesis [32]. In addition, miRNA-depleted HSCs are unable to reconstitute hematopoiesis in mice [32]. In full agreement, *Dicer1*$^{fl/fl}$ HSCs containing the *VAVi-Cre* transgene that is highly active in HSCs and efficient in deletion of floxed alleleles [33], are incapable to reconstitute lethally-irradiated recipient mice (Erkeland SJ, *et al.*, unpublished data). Together, these data show that *Dicer1* is essential for HSCs survival. Interestingly, *miR-125a*, controls the expansion of HSCs *in vivo* through targeting the proapototic gene *Bak1*. Whether *miR-125a* as a single miRNA can rescue *Dicer1*-null HSC survival and functions remains elusive but it is more likely that multiple miRNAs are critical at this stage.

To address the question whether miRNAs play a role in early myeloid-lineage decisions, we deleted *Dicer1* in CCAAT/enhancer-binding protein alpha (C/EBPA)-positive myeloid-committed progenitors *in vivo* (Figure 2) [34]. In striking contrast to the results in HSCs and early lymphoid progenitors, we recently found that miRNA depletion does not affect the number of myeloid-committed progenitor cells in mice [34]. However, isolated *Dicer1*-deficient granulocyte-macrophage progenitors (GMPs) were defective in myeloid development and exhibited an increased self-renewal potential [34]. In mice, *Dicer1* deletion by *C/ebpa-Cre* blocked monocytic differentiation, depleted macrophages and myelo-dendritic cells and caused myeloid dysplasia with morphological features of Pelger-Huet anomaly [34] (Figure 2). Strikingly, monocytes express low levels of proteins involved in miRNA processing and functions such as DROSHA, AGO1 and AGO2, compared with the levels found in T-cells, and are deficient for DICER1, unless the cells are forced to differentiate toward macrophages [35,36]. The presence of some miRNAs in the monocytic and *Dicer1*-deficient cell line U937 suggests that some miRNAs can be generated by proteins other than DICER1, such as PIWIL4 [35] but this hypothesis still needs proper validation. However, the fact that *Dicer1*-null monocytes are blocked in their differentiation *in vivo* indicates that *Dicer1* is essential at this stage and its function cannot be bypassed by other miRNA processing mechanisms.

MiRNA profiling of wild type GMPs showed that 104 miRNAs are abundantly expressed at this stage, of which at least 20 miRNA families are potentially active by reducing their target mRNA abundance [34]. Interestingly, of the derepressed miRNA targets in *Dicer1*-null GMPs, 27% are normally exclusively expressed in HSCs or are specific for multi-potent progenitors and erythropoiesis [34]. Unlike the results from HSCs and lymphoid progenitors showing functions of *Dicer1* mainly in survival pathways, these results provide evidence for a miRNA-controlled switch of a hematopoietic stem cell program of self-renewal and expansion toward myeloid differentiation (Figure 2) [34].

THE ROLE FOR *DICER1* IN LEUKEMIA

Human cancer including different types of leukemia is characterized by a global reduction in miRNA expression [37]. The first experimental evidence for a role of global downregulation of miRNAs in cellular transformation and tumorigenesis has been presented in a *K-Ras*-induced mouse model for lung cancer [38]. Further studies of the role for *Dicer1* in human cancer development in immune-deficient mice, strongly suggested that *Dicer1* is a haploinsufficient tumorsuppressor [39]. In this model, homozygous deletion of *Dicer1* is tolerated by the tumor cells, however lack of miRNAs abrogates tumor outgrowth due to strongly reduced cell proliferation capacity of the DICER1-null cells [39]. In agreement, heterozygous deletion of *Dicer1*, but not *Dicer1* knockout, accelerated tumor formation on a retinoblastoma-sensitized background [40]. In mouse B-cells, *Dicer1* is required for Myc-induced B-cell lymphomagenesis and survival of B-cell lymphomas [41]. However, in this model *Dicer1* is not a haploinsufficient tumor suppressor, as heterozygous deletion of *Dicer1* does not affect lymphoma latency and

overall survival [41]. This discrepancy may indicate that the tumorsuppressing activity of DICER1 is cell type dependent.

We asked whether *Dicer1* deletion enhances myeloid leukemia development in mice. In hematopoietic cells, *C/ebpa* starts to be expressed in early myeloid-committed progenitors, making it a suitable promoter to drive *Dicer1* deletion for studying the role of miRNA

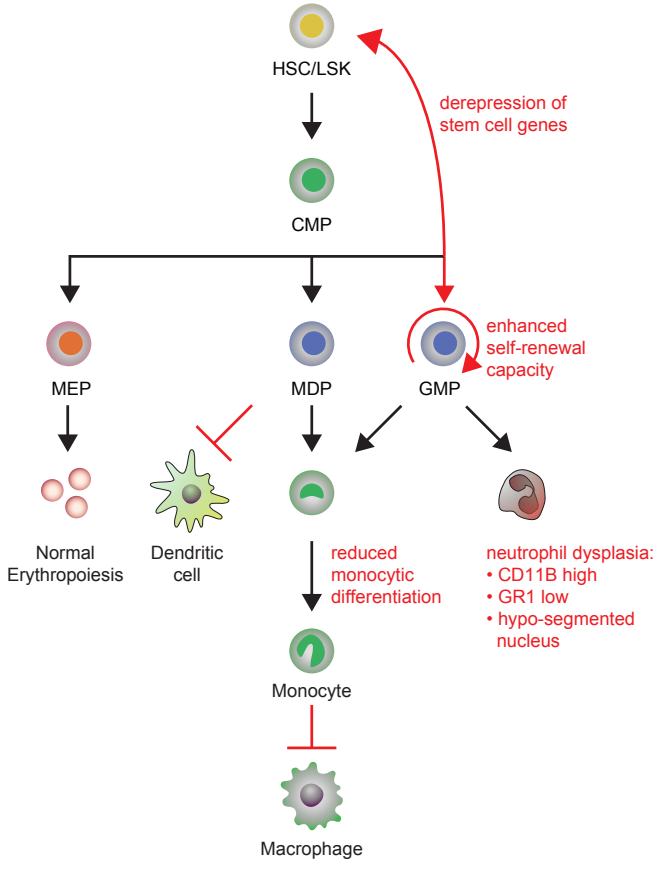
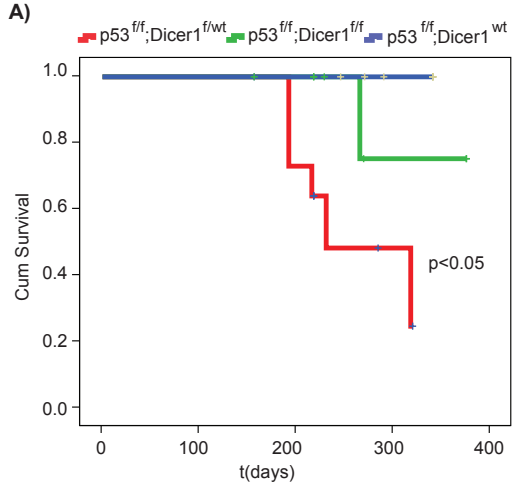
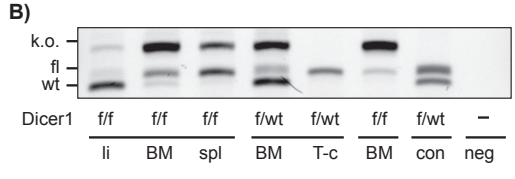


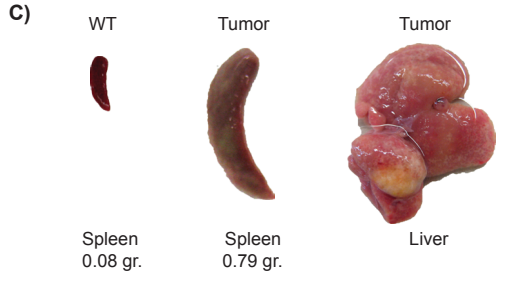
Figure 2. Schematic overview of the results of *C/ebpa-Cre*-mediated deletion of *Dicer1* in myeloid-committed progenitors. Phenotypic characteristics are indicated in red. In short, deletion of *Dicer1* results in derepression of stem cell genes in myeloid progenitors and an enhanced self-renewal capacity. Furthermore, MDPs and GMPs are blocked in macrophage and dendritic cell development. In addition, *Dicer1* deletion results in neutrophil dysplasia with cells that are characteristic for Pelger-Huet anomaly. HSC/LSK: hematopoietic stem cells/Lin^-;$ScaI^+$; Kit^+, CMP: common myeloid progenitor, GMP: granulocyte-macrophage progenitor, MEP: megakaryocytic-erythroid progenitor, MDP: macrophage-dendritic cell progenitor, CD11B: pan marker for myeloid cells, GR-1: marker for mature granulocytes.

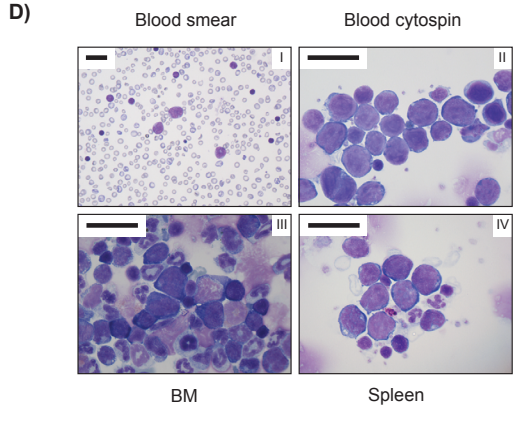
depletion in myeloid leukemias [34,42]. To circumvent prenatal lethality, we transplanted fetal liver cells from mutant and control embryos into lethally-irradiated recipient mice. While heterozygous deletion of *Dicer1* in myeloid-committed progenitors does not affect myeloid development, homozygous *Dicer1* deletion results in block of macrophage/dendritic cell development and myeloid-dysplasia, a cellular condition that may be considered as a preleukemic state [34] (Figure 2). However, mice transplanted with either heterozygous floxed *Dicer1* or homozygous floxed *Dicer1* cells survived devoid of any signs of myeloproliferative disease or leukemia development within a year of observation, indicating that loss of *Dicer1* in myeloid-committed progenitors is not sufficient to initiate short-term leukemogenesis in mice [34] (Figure 3A). To further investigate whether depletion of miRNAs accelerates myeloid leukemia development in a tumor susceptible model, we crossed *Dicer1* ^{fl} alleles with *P53*$^{fl/fl}$ mice and transplanted fetal liver cells from double mutants and control embryos into lethally-irradiated recipient mice. *C/ebpa-cre* driven deletion of *p53* and hemizygous deletion of *Dicer1* in mice caused development of various types of leukemias in half of the reconstituted mice with a latency of approximately 6 months (Figure 3). Only one out of eight *Dicer1*$^{fl/fl}$*;p53*$^{fl/fl}$ recipient mice developed a leukemia with a latency of 9 months (Figure 3A). However, PCR analysis on genomic DNA isolated from the *Dicer1*$^{fl/fl}$ tumor cells in liver and spleen showed that the *Dicer1* floxed alleles were incompletely recombined (Figure 3B). These results are in full agreement with data published by Kumar *et al.*, and strongly suggest that only reduced levels of *Dicer1*, but not bialleleic loss of *Dicer1*, may play a functional role in leukemia development [39,40]. However, the fact that total depletion of miRNAs does not affect the viability of myeloid progenitors in mice, may suggest that no negative selection due to reduced survival or proliferation by lack of miRNAs occurs in these cells. Together, these data provide evidence for a model in which reduced level of miRNAs is an oncogenic event in the development of leukemia but that activity of at least some miRNA species is essential for oncogenic transformation (Figure 4). This is in full agreement with experimental data showing tumor suppressing and oncogenic activities of investigated miRNAs such as *miR-17~92* and *miR-125* [43]. Moreover, miRNA expression profiling data of human cancer and AML samples are consistent with this hypothesis, as a small subset of miRNAs, including e.g. *miR-9*, *miR-125* and *miR-17~92* are highly expressed whereas most other miRNAs are downregulated [44].

Figure 3. Leukemia developed from *C/ebpa-Cre; p53*$^{f/f}$*; Dicer1* $^{f/wt}$ HSCs. A) Cumulative survival of mice transplanted with HSCs from fetal livers of *C/ebpa-Cre; p53*$^{f/f}$*; Dicer1* ^{wt} (n=8), *C/ebpa-Cre; p53*$^{f/f}$*; Dicer1* $^{f/wt}$ (n=12) and *C/ebpa-Cre; p53*$^{f/f}$*; Dicer1* $^{f/f}$ (n=8) embryos. Significance: p<0.05 (log-rank Mantel-Cox test). **B)** PCR on genomic DNA extracted from tumor cells. K.O.: knockout allele, fl: floxed allele, wt: wild type allele, li: liver, BM: bone marrow, spl: spleen, con: control DNA heterozygous floxed *Dicer1*, neg: loading control. **C)** Example of tumor infiltration in liver and spleen of leukemic mice transplanted with *C/ebpa-Cre; p53* $^{f/f}$*; Dicer1* $^{f/wt}$ HSCs. **D)** Micrographs showing morphology of tumor cells in blood, bone marrow and Spleen. Bar indicates 10 μm.


A)
p53 f/f;Dicer1 f/wt
p53 f/f;Dicer1 f/f
p53 f/f;Dicer1 wt
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
p<0.05
0
100
200
300
400
t(days)


B)
k.o.
fl
wt
Dicer1
f/f
f/f
f/f
f/wt
f/wt
f/f
f/wt
–
li
BM
spl
BM
T-c
BM
con
neg


C)
WT
Tumor
Tumor
Spleen
0.08 gr.
Spleen
0.79 gr.
Liver


D)
Blood smear
Blood cytospin
I
II
III
IV
BM
Spleen

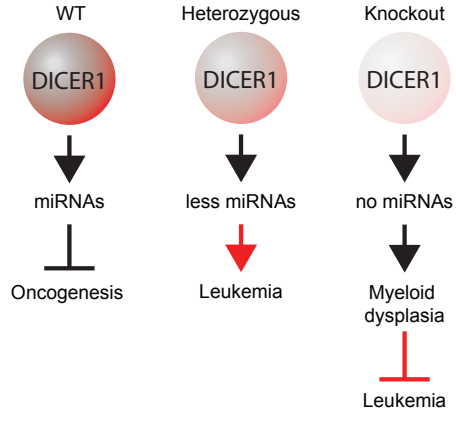


Figure 4. Model for the role of *Dicer1* in leukemia development. *Dicer1* knockout and as a result total loss of miRNA biogenesis, lead to myeloid dysplasia but not leukemia in a p53 knockout background. In contrast, heterozygous loss of *Dicer1* conserves the expression of a set of miRNAs needed for normal differentiation. Furthermore, our model suggests that at least some miRNA activity is needed for oncogenic transformation.

DICER1 MUTATIONS IN HUMAN LEUKEMIA

To date, the mechanism behind the reduced miRNA expression in subsets of human myeloid leukemia samples still remains elusive. One possibility is that the widespread silencing of miRNAs is the result of a defect in miRNA biogenesis caused by mutations in the gene encoding DICER1. For instance, data from Cancer Genome Project at the Wellcome Trust Sanger Institute (**www.sanger.ac.uk/cosmic**) show that somatic *DICER1* mutations occur in different human tumors including lung carcinoma, malignant melanoma and ovarian cancer [45]. Recently, Hill *et al.*, found *DICER1* mutations in familial pleuro-pilmonary blastoma [46]. In addition, a recent study in human non-epithelial ovarian cancers revealed mutations in the codons encoding metal-binding sites within the RNase IIIb catalytic centers of *DICER1* in 30 of 102 (29%) of the tumors [47]. These authors also detected mutations in 1 out of 14 non-seminomatous testicular germ-cell tumors, in 2 of 5 embryonal rhabdomyosarcomas, and in 1 of 266 epithelial ovarian and endometrial carcinomas [47]. The RNase III domains of DICER1 are essential for miRNA maturation, and introduced mutations in the RNase IIIa and in RNase IIIb abrogate *in vitro* processing of the 3p and 5p miRNAs respectively [47,48]. In agreement, transient expression experiments of mutant human *DICER1* constructs in murine *Dicer1*-null mesenchymal stem cells showed that inactivation of the RNase IIIb domain by mutation of D1709, results in complete loss of particularly 5p-derived mature miRNAs, including the tumor-suppressive *Let-7* family of miRNAs [49]. This mutation is found in subsets of nonepithelial ovarian cancers. Indeed, the identified *Dicer1* hot spot mutations in cancer result in reduced RNase IIIb activity but retain the RNase IIIa activity, strongly

suggesting a positive selection for the mutations that reduces *Let7*-tumorsuppressing activity in cancer development [47].

In a first attempt to gain more functional insight into the mechanisms behind the reduced miRNA expression in AML, a panel of 45 AML samples, characterized by activation of the oncogene EVI-1 due to t(3;3)(q21;q26) or inv(3)(q21q26) and poor prognosis, and five AML cell lines including U937, MOLM1, MUTZ3, KASUMI-3 and F36P were sequenced. In this panel of high risk AML samples, no mutation in *Dicer1* coding sequences and untranslated regions were identified (unpublished data, Erkeland S.J., Valk P., Delwel H., Sanders M.A., Groschel S. and Hoogenboezem R., 2012). Despite the limited set of data, this result suggests that other mechanisms are involved in deregulation of miRNA expression in human AML.

DIFFERENT MECHANISMS OF *DICER1* ACTIVITY REDUCTION IN HUMAN LEUKEMIA

The expression of miRNAs may be deregulated by different mechanisms in human cancer [50]. For instance, the activity of DICER1 may be reduced as *DICER1* is frequently deleted in various human cancers [39]. In addition, low expression of *DICER1* independently predicted poor outcomes in ovarian cancer patients [51]. In chronic lymphocytic leukemia (CLL), low expression of *DICER1* has been correlated with increased aggressiveness of the disease, shorter overall survival as well as reduced treatment free survival [52]. Notably, no such correlation between *DICER1* transcript levels and disease outcome were found in human AML [53]. However, there is evidence for regulation of *DICER1* expression by miRNAs such as *miR-15a* and *miR-16* in a cohort of del(13q14) in CLL [52], *miR-9* in Hodgkin lymphoma [54], *miR-125* in human megakaryoblastic leukemia [55] and *miR-106a* in the undifferentiated primary monocytes [35]. Interestingly, *miR-9, miR-125* and *miR-106a* are frequently aberrantly expressed at high levels in human AML [44,56] (and review [43]) and may control *DICER1* translation, leaving mRNA levels intact. Thus, aberrant miRNA biogenesis in human AML may occur via direct miRNA-controlled feedback mechanisms on translation of DICER1 transcripts, but this hypothesis still needs proper experimental confirmation.

Reduction of miRNA expression may be controlled by other mechanisms as well. This hypothesis is supported by recently described mutations in the TAR RNA-binding protein 2 (TARBP2), a critical protein for processing of miRNAs, in sporadic and hereditary carcinomas, and the inactivating mutations in *Exportin-5*, which results in trap of pre-miRNAs in the nucleus in human cancer cells [57-59]. Other possible mechanisms behind aberrant miRNA expression are single nucleotide polymorphisms (SNPs) that influence processing of miRNAs [60] or RNA editing of miRNA precursors that blocks cleavage by DICER1 [61,62]. Sequencing of factors involved in the biogenesis of miRNAs or a better understanding of miRNA expression regulation by e.g. transcription factors, epigenetic events or miRNA stability are needed to unravel the mechanisms behind the reduced miRNA activity in human AML.

CONCLUSION

Recent data show that *Dicer1* is an essential factor at different stages of normal hematopoiesis. A limitation of the *Dicer1*-deletion models is the global depletion of miRNAs that presumably results in disruption of many cellular pathways simultaneously, which hampers the identification of the functions of individual miRNAs. Although some studies show evidence for only a few miRNAs making dominant contributions, such as *miR-17~92* in B-cell development, this may be different for other cell types or even be developmental stage dependent. Overall, *Dicer1* mainly controls survival and expansion at the early stages of lymphoid development and controls cellular activities at the terminal maturation stage. The function of *Dicer1* is different in myelopoiesis at the earliest developmental stage as *Dicer1* is not essential for cell viability, but instead controls essential steps in switching from the stem cell stage toward myeloid lineage development. Although the functions of some miRNAs such as *miR-17/20/93/106* and *miR-223*, are well-described in immature and mature myeloid cells respectively [56,63], the miRNA-controlled pathways that are involved at different stages of myelopoiesis are still largely elusive. Therefore, tissue and developmental stage-specific miRNA-add-back in the *Dicer1*-deficient models, and experimental target identification approaches may be of help for the understanding of the miRNA activities in hematopoiesis.

METHODS

Mice and reconstitution experiments

To generate the different mouse lines of interest, we first crossed *C/ebpa-Cre;R26-LSL-Eyfp;Dicer1*$^{wt/fl}$ mice [34] with mice that contain floxed *p53* conditional alleles (Jackson Laboratories). Finally, *C/ebpa-Cre;R26-LSL-Eyfp;Dicer1*$^{wt/fl}$/*Dicer1*$^{fl/fl}$;*p53*$^{fl/fl}$ mice were obtained from breeding *C/ebpa-Cre;Dicer1*$^{wt/fl}$; *p53*$^{fl/wt}$ mice with *R26-LSL-Eyfp;Dicer1*$^{fl/fl}$;*p53*$^{fl/fl}$ mice. Fetal livers were obtained on embryonic day (E) 13.5. Genotyping of *Dicer1; p53; C/ebpa-Cre;R26-LSL-Eyfp* embryos was performed by PCR assays of DNA from tail or foot biopsies. Sequences of primers are available upon request. All primers were obtained from Biolegio BV. For transplantation, 8-week-old recipient mice C57Bl/6, (Jackson Laboratories) were irradiated (8.5 Gy) and tail-vein injected with fetal liver single-cell suspensions. Typically, cells from each fetal liver were transplanted into two recipient mice. Tumorigenicity was subsequently monitored by daily examination of the transplanted mice. Mice were euthanized when moribund. All animal experiments were approved by the Animal Welfare/Ethics Committee of the Erasmus Medical Center.

Antibodies, cell staining, flow cytometry and cytospins

Peripheral blood was obtained by heart puncture at the moment of euthanasia. Bone marrow cell suspensions were prepared as described previously [34]. Tumor samples were prepared as single-cell suspension for cytospins or FACS analysis. For morphological analysis of the cells, cytospins were stained with May-Grünwald-Giemsa and examined with a Leica DMLB microscope (100x and 40x objectives) and Leica Application Suite software Version 2.7.1 R1.

Statistics

Kaplan-Meier survival curves were plotted using SPSS software (SPSS, PASW, 17.0.2), and log-rank Mantel-Cox test was used to determine statistical significance.

ACKNOWLEDGEMENTS

We thank Dr. T Cupedo and Dr. M. Buitenhuis for critical reading of the manuscript and E. Simons for assistance with the preparation of the figures. We also thank Dr. K. van Lom for the microscopic analysis of leukemias. Our work was supported by grants from the Netherlands Organisation for Scientific Research (NWO-VENI) and the Dutch Cancer Society (KWF).

REFERENCES

1. Dlakic M. DUF283 domain of Dicer proteins has a double-stranded RNA-binding fold. *Bioinformatics*. 2006;22(22):2711-2714.
2. Qin H, Chen F, Huan X, Machida S, Song J, Yuan YA. Structure of the Arabidopsis thaliana DCL4 DUF283 domain reveals a noncanonical double-stranded RNA-binding fold for protein-protein interaction. *RNA*. 2010;16(3):474-481.
3. Lau PW, Guiley KZ, De N, Potter CS, Carragher B, Macrae IJ. The molecular architecture of human Dicer. *Nat Struct Mol Biol*. 2012.
4. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*. 2001;409(6818):363-366.
5. Calabrese JM, Seila AC, Yeo GW, Sharp PA. RNA sequence analysis defines Dicer's role in mouse embryonic stem cells. *Proc Natl Acad Sci U S A*. 2007;104(46):18097-18102.
6. Cheloufi S, Dos Santos CO, Chong MM, Hannon GJ. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature*. 2010;465(7298):584-589.
7. Cifuentes D, Xue H, Taylor DW, et al. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science*. 2010;328(5986):1694-1698.
8. Yang JS, Maurin T, Robine N, et al. Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. *Proc Natl Acad Sci U S A. 2010;107(34):15163-15168.*
9. *Yang JS, Lai EC.* Dicer-independent, Ago2-mediated microRNA biogenesis in vertebrates. *Cell Cycle*. 2010;9(22):4455-4460.
10. Ketting RF. The many faces of RNAi. *Dev Cell*. 2011;20(2):148-161.
11. Benhamed M, Herbig U, Ye T, Dejean A, Bischof O. Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. *Nat Cell Biol. 2012;14(3):266-275.*
12. *Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. Genes Dev. 2002;16(13):1616-1626.*
13. *Wienholds E, Koudijs MJ, van Eeden FJ, Cuppen E, Plasterk RH.* The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat Genet*. 2003;35(3):217-218.
14. Bernstein E, Kim SY, Carmell MA, et al. Dicer is essential for mouse development. *Nat Genet. 2003;35(3):215-217.*
15. *Kanellopoulou C, Muljo SA, Kung AL, et al.* Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev*. 2005;19(4):489-501.
16. Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. *Proc Natl Acad Sci U S A*. 2005;102(34):12135-12140.
17. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci U S A*. 2005;102(31):10898-10903.
18. Cobb BS, Nesterova TB, Thompson E, et al. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med*. 2005;201(9):1367-1373.
19. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med*. 2005;202(2):261-269.
20. Cobb BS, Hertweck A, Smith J, et al. A role for Dicer in immune regulation. *J Exp Med*. 2006;203(11):2519-2527.
21. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J Exp Med. 2008;205(9):1993-2004.*
22. *Zhou X, Jeker LT, Fife BT, et al.* Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med*. 2008;205(9):1983-1991.

23. Zhou L, Seo KH, He HZ, et al. Tie2cre-induced inactivation of the miRNA-processing enzyme Dicer disrupts invariant NKT cell development. *Proc Natl Acad Sci U S A*. 2009;106(25):10266-10271.
24. Batard P, Sansilvestri P, Scheinecker C, et al. The Tie receptor tyrosine kinase is expressed by human hematopoietic progenitor cells and by a subset of megakaryocytic cells. *Blood*. 1996;87(6):2212-2220.
25. Fedeli M, Napolitano A, Wong MP, et al. Dicer-dependent microRNA pathway controls invariant NKT cell development. *J Immunol*. 2009;183(4):2506-2512.
26. Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell*. 2008;132(5):860-874.
27. Ventura A, Young AG, Winslow MM, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*. 2008;132(5):875-886.
28. Belver L, de Yebenes VG, Ramiro AR. MicroRNAs prevent the generation of autoreactive antibodies. *Immunity*. 2010;33(5):713-722.
29. Xu S, Guo K, Zeng Q, Huo J, Lam KP. The RNase III enzyme Dicer is essential for germinal center B-cell formation. *Blood. 2012;119(3):767-776.*
30. *Bezman NA, Cedars E, Steiner DF, Blelloch R, Hesslein DG, Lanier LL.* Distinct requirements of microRNAs in NK cell activation, survival, and function. *J Immunol*. 2010;185(7):3835-3846.
31. Sullivan RP, Leong JW, Schneider SE, et al. MicroRNA-deficient NK cells exhibit decreased survival but enhanced function. *J Immunol. 2012;188(7):3019-3030.*
32. *Guo S, Lu J, Schlanger R, et al.* MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci U S A. 2010;107(32):14229-14234.*
33. *de Boer J, Williams A, Skavdis G, et al.* Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Eur J Immunol. 2003;33(2):314-325.*
34. *Alemdehy MF, van Boxtel NG, de Looper HW, et al.* Dicer1 deletion in myeloid-committed progenitors causes neutrophil dysplasia and blocks macrophage/dendritic cell development in mice. *Blood*. 2012.
35. Coley W, Van Duyne R, Carpio L, et al. Absence of DICER in monocytes and its regulation by HIV-1. *J Biol Chem. 2010;285(42):31930-31943.*
36. *Klase Z, Kale P, Winograd R, et al.* HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. *BMC Mol Biol. 2007;8:63.*
37. *Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435(7043):834-838.*
38. *Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007;39(5):673-677.*
39. *Kumar MS, Pester RE, Chen CY, et al. Dicer1 functions as a haploinsufficient tumor suppressor. Genes Dev. 2009;23(23):2700-2704.*
40. *Lambertz I, Nittner D, Mestdagh P, et al.* Monoallelic but not biallelic loss of Dicer1 promotes tumorigenesis in vivo. *Cell Death Differ*. 2010;17(4):633-641.
41. Arrate MP, Vincent T, Odvody J, Kar R, Jones SN, Eischen CM. MicroRNA biogenesis is required for Myc-induced B-cell lymphoma development and survival. *Cancer Res. 2010;70(14):6083-6092.*
42. *Wolfler A, Danen-van Oorschot AA, Haanstra JR, et al.* Lineage-instructive function of C/EBPalpha in multipotent hematopoietic cells and early thymic progenitors. *Blood*. 2010;116(20):4116-4125.
43. Alemdehy MF, Erkeland SJ. MicroRNAs: key players of normal and malignant myelopoiesis. *Curr Opin Hematol*. 2012.
44. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.

45. Forbes SA, Bhamra G, Bamford S, et al. The Catalogue of Somatic Mutations in Cancer (COSMIC). *Curr Protoc Hum Genet*. 2008;Chapter 10:Unit 10 11.

46. Hill DA, Ivanovich J, Priest JR, et al. DICER1 mutations in familial pleuropulmonary blastoma. *Science*. 2009;325(5943):965.

47. Heravi-Moussavi A, Anglesio MS, Cheng SW, et al. Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. *N Engl J Med*. 2012;366(3):234-242.

48. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. *Cell*. 2004;118(1):57-68.

49. Gurtan AM, Lu V, Bhutkar A, Sharp PA. In vivo structure-function analysis of human Dicer reveals directional processing of precursor miRNAs. *RNA*. 2012.

50. Deng S, Calin GA, Croce CM, Coukos G, Zhang L. Mechanisms of microRNA deregulation in human cancer. *Cell Cycle*. 2008;7(17):2643-2646.

51. Merritt WM, Lin YG, Han LY, et al. Dicer, Drosha, and outcomes in patients with ovarian cancer. *N Engl J Med*. 2008;359(25):2641-2650.

52. Zhu DX, Fan L, Lu RN, et al. Downregulated Dicer expression predicts poor prognosis in chronic lymphocytic leukemia. *Cancer Sci*. 2012;103(5):875-881.

53. Martin MG, Payton JE, Link DC. Dicer and outcomes in patients with acute myeloid leukemia (AML). *Leuk Res*. 2009;33(8):e127.

54. Leucci E, Zriwil A, Gregersen LH, et al. Inhibition of miR-9 de-represses HuR and DICER1 and impairs Hodgkin lymphoma tumour outgrowth in vivo. *Oncogene*. 2012.

55. Klusmann JH, Li Z, Bohmer K, et al. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. *Genes Dev. 2010;24(5):478-490.*

56. *Meenhuis A, van Veelen PA, de Looper H, et al.* MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood*. 2011;118(4):916-925.

57. Melo SA, Ropero S, Moutinho C, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet*. 2009;41(3):365-370.

58. Melo SA, Moutinho C, Ropero S, et al. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell*. 2010;18(4):303-315.

59. Melo SA, Esteller M. A precursor microRNA in a cancer cell nucleus: get me out of here! *Cell Cycle*. 2011;10(6):922-925.

60. Sun G, Yan J, Noltner K, et al. SNPs in human miRNA genes affect biogenesis and function. *RNA*. 2009;15(9):1640-1651.

61. Kawahara Y, Zinshteyn B, Chendrimada TP, Shiekhattar R, Nishikura K. RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. *EMBO Rep*. 2007;8(8):763-769.

62. Heale BS, Keegan LP, O'Connell MA. ADARs have effects beyond RNA editing. *Cell Cycle*. 2009;8(24):4011-4012.

63. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation andgranulocytefunctionbymicroRNA-223. *Nature. 2008;451(7182):1125-1129.*

5

Interstrand Cross-link Induced *miR-139-3p* and *miR-199a-3p* have Opposite Roles in Hematopoietic Cell Expansion and Leukemic Transformation

Mir Farshid Alemdehy[1], Jurgen R. Haanstra[1], Hans W. J. de Looper[1], Paulina M.H. van Strien[1], Judith Verhagen-Oldenampsen[1], Yvette Caljouw[1], Mathijs A. Sanders[1], Remco Hoogenboezem[1], Arnoud H. de Ru[2], George M.C. Janssen[2], Stephanie E. Smetsers[3], Marc B. Bierings[3,4], Marieke von Lindern[1], Peter A. van Veelen[2], Ivo P. Touw[1] and Stefan J. Erkeland[1]

[1] Department of Hematology, Erasmus University Medical Center Cancer Institute, Rotterdam, the Netherlands; [2] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands; [3] Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, the Netherlands; [4] Dutch Childhood Oncology Group, the Hague, the Netherlands

Submitted

ABSTRACT

Interstrand cross-links (ICLs) are toxic DNA lesions that cause severe genomic damage during replication. In the hematopoietic system, this results in a progressive bone marrow failure (BMF) and predisposes to acute myeloid leukemia (AML). The molecular mechanisms responsible for these defects are largely unknown. Using *Ercc1*-deficient mice, we show that *Trp53* is responsible for ICL-induced BMF and that loss of *Trp53* is leukemogenic in this model. In addition, *Ercc1*-deficient myeloid progenitors expressed elevated levels of *miR-139-3p* and *miR-199a-3p* with age. These microRNAs exert opposite effects on hematopoiesis. Ectopic expression of *miR-139-3p* strongly inhibited proliferation of myeloid progenitors, whereas inhibition of *miR-139-3p* activity restored defective proliferation of *Ercc1*-deficient progenitors. Conversely, inhibition of *miR-199a-3p* functions aggravated the myeloid proliferation defect in the *Ercc1*-deficient model, whereas its enforced expression enhanced proliferation of progenitors. Importantly, *miR-199a-3p* caused AML in a pre-leukemic mouse model, supporting its role as an onco-miR. Target genes include *HuR* for *miR-139-3p* and *PRDX6*, *RUNX1* and *SUZ12* for *miR-199a-3p.* The latter genes have previously been implicated as tumor suppressors in de novo and secondary AML. These findings show that, in addition to TRP53-controlled mechanisms, *miR-139-3p* and *miR-199a-3p* are involved in the defective hematopoietic function of ICL-repair deficient myeloid progenitors.

INTRODUCTION

Hematopoiesis is tightly controlled by both cell-intrinsic and -extrinsic cues. This ensures the life-long balanced blood cell production from hematopoietic stem cells (HSCs) under homeostatic conditions and allows the transient expansion of specific blood cell types in response to infections or blood loss [1]. Bone marrow failure (BMF) syndromes are characterized by impaired hematopoiesis, leading to single- or multi-lineage cytopenia [2]. Although the underlying causes of BMF syndromes are heterogeneous, they share the elevated risk to progress towards leukemia [3]. Ineffective repair of DNA damage is one of the major causes of BMF and leukemic transformation [4]. Several mouse models have shown that defective DNA repair reduces the function but not the absolute number of long-term hematopoietic stem cells (LT-HSCs) with age. For instance, in aged mice deficient in nucleotide excision repair (NER) the numbers of common myeloid progenitors (CMPs) are significantly decreased, whereas the numbers LT-HSCs remain unaltered [5].

The DNA repair protein ERCC1 is active in both NER and interstrand cross-link (ICL) repair [6]. ERCC1 acts in a complex with XPF as a structure specific endonuclease. Mice lacking ERCC1 show accelerated aging and reduced hematopoietic reserves within 3 weeks of age [7]. The observation that XPA knockout mice, which are exclusively NER-deficient, did not show the accelerated bone marrow (BM) exhaustion suggested that defective ICL repair was predominantly responsible for this phenotype. Indeed, *Ercc1*-deficient hematopoietic progenitor cells showed the hypersensitivity to the DNA cross-linking agent mitomycin C (MMC) that is characteristic of ICL-repair deficiency [7]. Additional studies showed that proliferative myeloid progenitors, i.e., CMPs, granulocyte monocyte progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs), but not the non-cycling LT-HSCs or short-term (ST)-HSCs were severely reduced with progressive loss of ERCC1 activity [8].

The underlying DNA-damage response (DDR) pathways responsible for the depletion of ICL-deficient hematopoietic progenitors are still largely unknown. In Fanconi anemia (FA), the best characterized clinical condition of ICL-induced BMF, it has been shown that the inflammatory cytokines TNF-α and IFN-γ contribute to the BMF and are produced at increased levels in FA mouse models and in patients [9-11]. However, an elevated production of these and other inflammatory cytokines in FA patients was not observed in another study [12]. Here, we used *Ercc1*-deficient mice to identify additional mechanisms that contribute to ICL-induced BMF and leukemic transformation. We show that deletion of *Trp53* but not *Cdkn2a* alleviates the loss of lineage negative (lin-) Sca1+ c-kit+ (LSK) BM cells in *Ercc1*-deficient mice and identified TRP53 as a major gatekeeper preventing leukemic transformation of *Ercc1*-deficient BM cells, corroborating studies in clinical FA [13]. In addition, we identified two miRNAs, *miR-139-3p* and *miR-199a-3p*, that were expressed at enhanced levels in CMPs from *Ercc1*-deficient mice compared to control littermates which exert strikingly opposite effects on the proliferation of myeloid progenitors. These miRNAs were also expressed at an elevated level in CD34+ BM cells from FA patients.

Importantly, *miR-199a-3p* caused AML in a preleukemic mouse transplantation model, establishing its role as an onco-miR. These findings uncover novel mechanisms of BMF and leukemogenesis involving miRNAs in an ICL-defective mouse model, which may bear relevance for FA.

MATERIALS AND METHODS

Mice

The *Cdkn2a* $^{+/-}$ [14], *Trp53* $^{+/-}$ [15], *Ercc1* $^{+/*292}$ and *Ercc1* $^{+/-}$ mice have been previously described [16]. *Ercc1* mice were generated in a F1 mixed background of C57BL/6 and FVB/n. To generate *Cebpa* $^{cre/fl}$ mice, *Cebpa* $^{fl/fl}$ [17] were crossed with *Cebpa-cre* mice [18].

For transplantation experiments with *Ercc1*-deficient cells, 12 to 14 week old recipient F1 mice (FVB/n x C57BL/6) were irradiated (9 Gy) and transplanted by tail vein injection with $1x10^7$ total BM cells and $1x10^5$ spleen cells. For other transplantation experiments, 6-8 week old C57BL/6 recipient mice (The Jackson Laboratory) were irradiated (8.5 Gy) and tail-vein injected with retrovirally-transduced HSPCs ($1\text{-}5x10^5$ cells/mouse). Leukemia cells isolated from BM of primary recipients were injected into irradiated (5 Gy) 6-8-week old C57BL/6 mice. All animal experiments were approved by the animal Welfare/Ethics Committee of the Erasmus MC.

Retroviral infection and colony assays

MSCV-BC-miRNA vectors and virus particles were generated as described previously [19]. The 32D cells and BM-derived HSPCs were infected with MSCV-BC-miRNA virus using RetroNectin (Takara Bio Inc) according to manufacturer's instructions. Colony assays were performed as described [18]. In brief, 10.000 MSCV-transduced cells (based on EGFP expression determined by flow cytometry) or 50.000 total BM cells, per mL per 35 mm dish were plated in triplicate in methyl cellulose medium (Methocult M3234, StemCell Technologies SARL), containing human G-CSF (0.1 µg/ml), or mouse GM-CSF (0.1 µg/ml), or human Epo (4 mU/ml) plus transferrin (0.3 mM), hemin (0.2 mM) and mouse SCF (0.1 µg/ml), and puromycin (1.5 µg/mL, only for transduced cells). Colonies containing 50 cells or more were scored on day 7 of culture. For miRNA inhibitory experiments HSPCs were transfected with fluorescent tagged miRCURY LNA inhibitors (Exiqon) with DharmaFECT1 (Thermo Scientific). The transfection efficiency (100%) was confirmed with flow cytometry. The colony assays were performed as described above.

Patient samples

BM HSPCs were obtained from patients as described previously [20]. All FA patients provided written informed consent in accordance with the Declaration of Helsinki.

See Supplemental Materials and Methods for quantitative proteomics, whole exome sequencing and data analysis, Luciferase reporter assays, Luminex experiments, antibodies, cell staining flow-cytometry and cytospins, gene- and miRNA profiling qPCR and statistics.

RESULTS

Exhaustion of *Ercc1*$^{-/*292}$ HSPCs is caused by TRP53- rather than CDKN2A-dependent mechanisms

Ercc1 knock-out mice are severely runted, weigh only about 20% compared to their normal littermates and die around 3 weeks of age [16,21]. A premature stop codon at position 292 of mouse *Ercc1* (*Ercc1*^{*292}), causes a C-terminal deletion of 7 amino acids of ERCC1, which impairs dimerization with XPF [16]. The life span of *Ercc1*$^{-/*292}$ mice is approximately 22 weeks, and their hematopoietic phenotype is comparable to that of *Ercc1* knockout mice [8]. The BM of *Ercc1*$^{-/*292}$ mice contains decreased numbers of myeloid progenitors that are strongly hampered in their ability to proliferate in colony assays [8]. Both TRP53 and CDKN2A have been shown to regulate the DDR in HSPCs [13,22]. We investigated how disruption of these individual loci affects *Ercc1*$^{-/*292}$ LSK and hematopoietic progenitor maintenance. At 3 weeks of age, *Ercc1*$^{-/*292}$ BM contained less than 50% of LSKs compared with *wild type* (*wt*) (Figure 1A). Furthermore, the *in vitro* colony forming capacity of *Ercc1*$^{-/*292}$ myeloid and erythroid progenitor cells was markedly reduced (Figure 1B-D). Deletion of *Cdkn2a* did not affect this phenotype (Figure 1A-D). In contrast, homozygous deletion of *Trp53* in *Ercc1*$^{-/*292}$ mice restored the fraction of LSK cells and granulocyte-macrophage colony forming units (CFU-GM) (Figure 1E-H). These results, showing that TRP53-driven, rather than CDKN2A-dependent DDR mechanisms cause ICL-induced loss of HSPCs, corroborate studies in clinical FA [13]. Under conditions of G-CSF and SCF/EPO stimulation, a partial rescue of CFU-G or BFU-E colony growth was seen (Figure 1G, 1H).

Affected pathways in *Ercc1*$^{-/*292}$ HSPCs

To investigate how *Ercc1*-deficiency affects BM HSPCs at the molecular level, we performed gene expression profiling (GEP) and proteomics. Ingenuity Downstream Effect Analysis (IDEA) showed decreased activity of networks required for hematopoietic development and leukocyte differentiation in *Ercc1*$^{-/*292}$ LSK BM cells at week 20 to 22 of age compared to control cells (Suppl. Figure 1A, 1B, Suppl. Table 1). Differentially expressed DDR genes included *P53 and DNA damage regulated 1* (*Pdrg1*), *Cyclin-dependent kinase inhibitor 1a* (*Cdkn1a, p21*) and *Xiap-associated factor 1* (*Xaf1*), all TRP53-induced genes (Suppl. Table 1).

LSK cells are enriched for hematopoietic stem cells (HSCs) that are mostly non-cycling. Because of the higher proliferation rate of committed hematopoietic progenitors, the consequences of defective ICL-repair are expected to be more severe in these cells. We therefore also analyzed lineage depleted BM cells that contain HSCs and progenitor cells. GEP identified 887 differentially expressed transcripts in *Ercc1*$^{-/*292}$ lin- cells relative to controls (Supplemental Figure 1C, Supplemental Table 2). IDEA showed that pathways involved in cell death, the regulation of reactive oxygen species (ROS), cell cycle control, hematopoiesis and DNA repair are more prominently deregulated in *Ercc1*$^{-/*292}$ lin- cells compared to LSKs (Supplemental Figure 1B versus 1D). Expression of TRP53 target genes, e.g. *p21* and *Gadd45b*, was increased in *Ercc1*$^{-/*292}$ lin- cells, indicating an activated DDR (Supplemental Table 2).

Quantitative proteome analysis on *Ercc1*$^{-/*292}$ and control HSPCs showed that 457 proteins were significantly differentially expressed (Supplemental Table 3). Notably, a high correlation between the changes in mRNA and protein level was observed (Supplemental Figure 1E). IDEA on these protein data showed that the main deregulated biological functions identified by proteomics are similar to those detected by gene expression profiling, i.e. pathways involved in cell death and regulation of ROS (Supplemental Figure 1F).

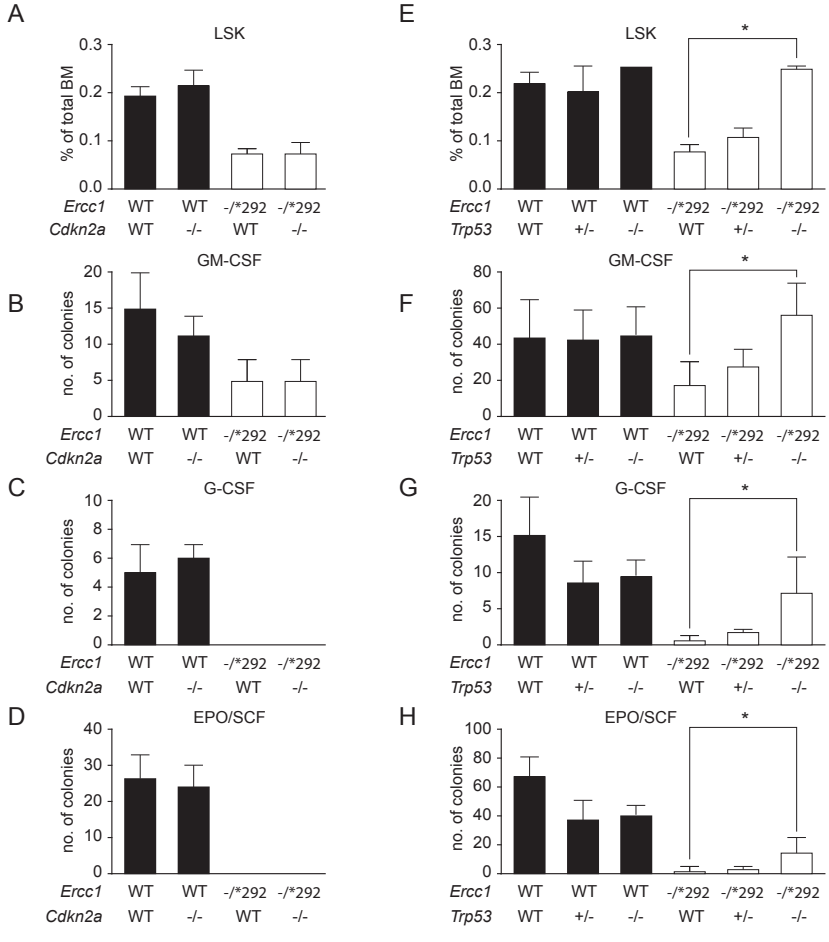


Figure 1. Loss of *Trp53*, but not of *Cdkn2a*, restores HSPC content in *Ercc1*$^{-/*292}$ mice. A, E) LSK frequencies in lin- fractions of indicated mouse genotypes at 3 weeks of age are shown. **B-D** and **F-H)** Colony-forming units (CFU) per 5x10^4 unfractionated BM cells in the presence of indicated growth factors relative to the *wt* controls are shown. All bars represent the mean and standard deviations of n≥3 mice. The significance was calculated by the student t-tests (asymptotic significance [2-tailed]) (* $p<0.05$).

MiRNA expression in *Ercc1*$^{-/*292}$ HSPCs

Because miRNAs play key roles in cellular stress responses, including the regulation of TRP53-dependent pathways [23,24], we interrogated which miRNAs are differentially expressed in *Ercc1*$^{-/*292}$ versus *Ercc1 wt* LSK and lin- cells. From a panel of 365 miRNAs tested by Taqman qPCR in *Ercc1*$^{-/*292}$ LSKs isolated from 20 week old mice, none was significantly altered in its expression relative to control LSKs. In contrast, in lin- *Ercc1*$^{-/*292}$ cells, expression of 4 miRNAs, *miR-139-3p*, *miR-199a-3p*, *miR-34a-5p* and *miR-342-5p* was significantly elevated relative to *wt* lin- controls (2 to 4 Fold, $p<0.05$) (Figure 2A). This result suggested that expression of these 4 miRNAs is induced in cycling *Ercc1*$^{-/*292}$ progenitors enriched in the lin- fraction, rather than in the mostly non-cycling LSK fraction. To further specify which myeloid progenitor subsets express these miRNAs, we analyzed FACS-purified CMPs, GMPs and MEPs from 20 weeks old mice. In *Ercc1*$^{-/*292}$ CMPs, the levels of *miR-34a-5p*, *miR-139-3p* and *miR-199a-3p* were elevated relative to controls, whereas *miR-342-5p* was not expressed (Figure 2B). In *Ercc1*$^{-/*292}$ GMPs, *miR-139-3p* was not expressed and only the level of *miR-199a-3p* was significantly elevated (Figure 2B). In *Ercc1*$^{-/*292}$ MEPs only the expression of *miR-342-5p* was significantly induced (Figure 2B).

At week 3 of age, the levels of *miR-34a-5p* and *miR-342-5p* were already significantly elevated in *Ercc1*$^{-/*292}$ lin- cells (Figure 2C). Crossings of *Ercc1*$^{-/*292}$ mice with *Trp53*$^{-/-}$ mice revealed that the expression of *miR-34a-5p* and *miR-342-5p* was fully TRP53 dependent (Figure 2C). In contrast, the expression of *miR-139-3p* and *miR-199a-3p* is only induced at week 20, when DNA damage due to unrepaired ICLs accumulates (Figure 2A, C). In support of this, the ICL-inducing agent mitomycin C (MMC) induced the expression of *miR-199a-3p* and *miR-139-3p* in normal lin- cells (Figure 2D).

Opposite roles of *miR-139* and *miR-199a* in myeloid progenitor exhaustion and expansion

To determine their role in myeloid cell survival, proliferation and differentiation, we ectopically expressed the miRNAs from barcoded retroviral vectors (MSCV) in the murine cell line 32D expressing human CSF3R (32D-CSF3R) [19,25]. In IL-3-containing expansion medium, *miR-139* expressing 32D-CSF3R cells died rapidly, whereas no alteration in cell survival and proliferation was seen in cells expressing *miR-34a*, *miR-199a* or *miR-342* (Figure 3A). When these cells were mixed in a 1:1 ratio with MSCV-EV transduced control cells and transferred to G-CSF-containing differentiation medium, *miR-139* again inhibited proliferation (Figure 3B), whereas *miR-34a* and *miR-342* had little or no effect (Figure 3B). In contrast, *miR-199a* expressing 32D-CSF3R cells had gained a competitive growth advantage in this setting (Figure 3B), suggesting that *miR-199a* inhibits differentiation and/or enhances proliferation of myeloid progenitors in response to G-CSF. Similar results were obtained in primary CFU-GM colony cultures. Whereas ectopic expression of *miR-34a* and *miR-342* did not alter, and *miR-139* completely abrogated colony outgrowth, the CFU-GM colony formation capacity of *miR-199a* expressing BM cells was 1.5 fold ($p<0.05$) increased (Figure 3C).

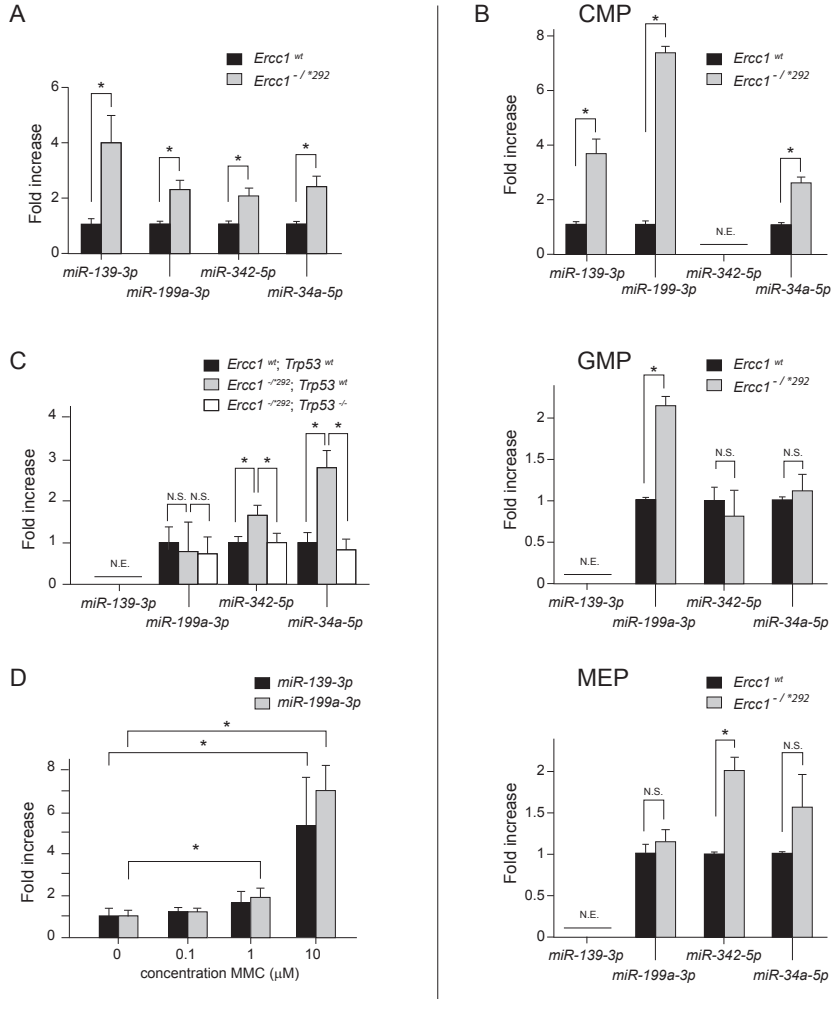


Figure 2. Differentially expressed miRNAs in *Ercc1*$^{-/*292}$ cells. A) The expression of miRNAs and U6 in lin- BM cells was determined by q-PCR in quadruplicate. The expression of indicated miRNAs relative to U6 and normalized to *wt* controls is depicted. The error bars represent the standard deviations (SD) of 5 mice. **B)** The expression of indicated miRNAs relative to U6 and normalized to *wt* is depicted for different hematopoietic progenitor subpopulations isolated from the BM of 20 weeks old mice. The error bars represent SD of three measurements. **C)** The expression of indicated miRNAs relative to U6 in lin- BM cells of three week old *Ercc1*$^{-/*292}$*;Trp53 wt* mice and *Ercc1*$^{-/*292}$*;Trp53*$^{-/-}$ and relative to *wt* controls is shown. All bars represent the mean and SD of n≥3 mice **D)** The expression of *miR-139-3p* and *miR-199a-3p* relative to U6 in *wt* lin- cells and normalized to the control condition without MMC, is depicted for the indicated MMC concentrations. The error bars represent SD of three measurements. In all panels, the significance was calculated with the Mann-Whitney U test (asymptotic significance [2-tailed]) (* $p<0.05$). N.E.: not expressed. N.S.: not significant.

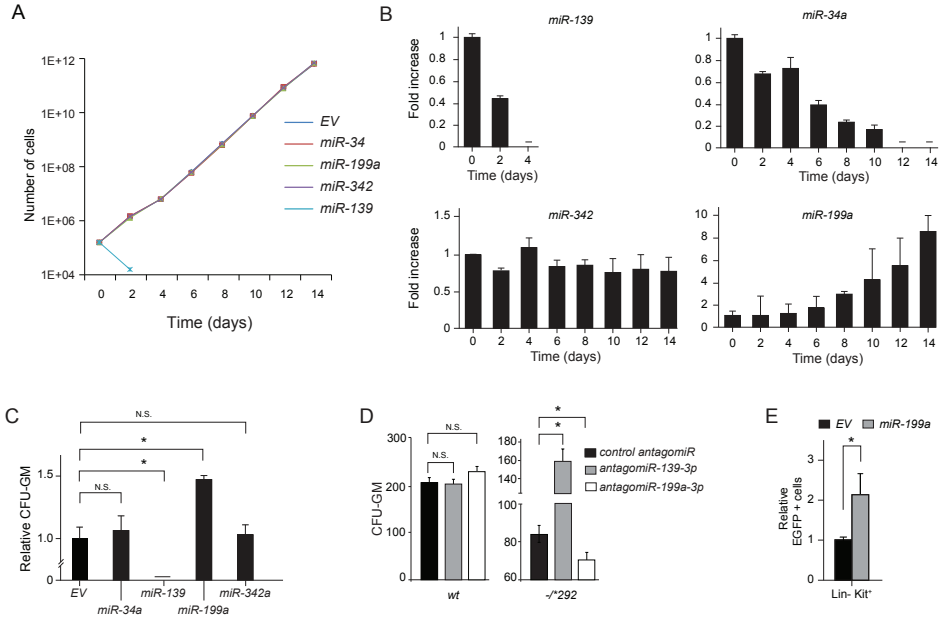


Figure 3. Expression of *miR-139* and *miR-199a* alters the balance of HSPC loss and expansion. **A)** Murine 32D cells were infected with MSCV-BC vectors containing either, *miR-139*, *miR-199a*, *miR-34a*, *miR-342* or no miRNA (EV) as control. 32D cells expressing miRNAs were expanded in IL-3 containing medium and the number of cells at indicated time points is plotted. **B)** Equal numbers of 32D cells expressing indicated miRNAs were mixed with control EV-expressing 32D cells and switched to G-CSF-containing medium. Cell samples were taken at indicated time points and genomic DNA was isolated. The abundance of the different the barcodes relative to the EV barcode signal and normalized to day 0 is depicted. Representative data of three independent experiments are shown. The error bars represent SD of three measurements. **C)** Colony assays were performed with $1x10^4$ lin- cells transduced with different miRNA expressing viruses. The number of CFU-GMs consisting of more than 50 cells after 7 days of growth relative to EV control is depicted and is a representative result of 2 independent experiments. Data represent the mean and SD of 3 plates counted. **D)** Same as C. The number of CFU-GM of $1x10^4$ mouse *Ercc1*$^{-/*292}$ and *wt* lin- cells transfected with LNA antagomiR against *miR-139-3p* and *miR-199a-3p* are shown. Data represent the mean and SD of 3 plates counted. **E)** Lin- cells transduced with MSCV-*miR-199a* or MSCV-EV and mixed with untransduced *wt* lin- cells in a 1:1 ratio were transplanted in irradiated recipient mice (n=8 per group). The change in lin-Kit+ (LK, progenitors) fraction in the BM 10 weeks post-transplantation and relative to the EV control is shown (* $p<0.05$). The error bars represent SD of n=8 mice. In panels C, D and E, the significance was calculated with the student t-test [2-tailed] (* $p<0.05$).

Next, we used LNA antagomiRs to explore how inhibition of *miR-139-3p* and *miR-199a-3p* affects the outgrowth of *Ercc1*$^{-/*292}$ CFU-GM. Treatment of *Ercc1*$^{-/*292}$ lin- cells with *miR-199a-3p* LNA antagomiR reduced the number of CFU-GM by ~20% (Figure 3D). Conversely, *miR-139-3p* LNA antagomiR rescued the colony forming capacity of *Ercc1*$^{-/*292}$ progenitors. Both antagomiRs had no effect on CFU-GM colony formation from normal BM (Figure 3D).

Finally, we assessed the effects of *miR-199a* expression on HSPC expansion *in vivo*, by transplanting MSCV-*miR-199a* and MSCV-EV infected lin- cells mixed with non-infected lin- cells in mice. Myeloid progenitor cells expressing *miR-199a* significantly increased in numbers over non-transduced controls at 10 weeks post-transplantation, whereas cells transduced with MSCV-EV were maintained at a similar frequency compared to input (Figure 3E). In summary, these data show that ectopic expression of *miR-139-3p* inhibits and *miR-199a-3p* enhances the outgrowth of normal myeloid progenitors, both *in vitro* and *in vivo* and that antagonizing their endogenous expression in *Ercc1*-deficient cells restores (anti-*miR-139-3p*) or further aggravates (anti-*miR-199a-3p*) the ICL-induced phenotype.

Identification of *miR-139-3p* and *miR-199a-3p* targets

Combined transcriptome and proteome analysis (Supplementary Figure 1) revealed that in *Ercc1*$^{-/*292}$ lin- cells, 7 genes were down-regulated that contain predicted and evolutionary well-conserved binding sites for either *miR-139-3p* or *miR-199a-3p* in their 3'-UTRs (TargetScan, www.targetscan.org) (Supplemental Table 2 and 3). The RNA-binding protein HuR (also known as Elavl1*)*, was the only identified target of *miR-139-3p* in *Ercc1*$^{-/*292}$ HSPCs, whereas 6 genes, *Prdx6*, *Suz12*, *Pon2*, *Fubp1*, *Calu*, and *Runx1*, contain *miR-199a-3p* recognition sites. To test whether *miR-139-3p* and *miR-199a-3p* directly control the expression of these genes by binding to the predicted miRNA binding sites, we cloned these 3'-UTR regions downstream of a luciferase reporter (Figure 4A). Ectopic expression of *miR-139* and *miR-199a* caused a 30% to 65% reduction of luciferase activity of all target 3'-UTR fragments tested (Figure 4B). Mutation of the predicted miRNA binding sites abolished the inhibition of luciferase activity, confirming the predicted target sites as major determinants for *miR-139-3p* and *miR-199a-3p*-mediated regulation (Figure 4B).

MiR-139-3p, *miR-199a-3p* and their targets are deregulated in human ICL-repair deficient BM cells

Because defective ICL repair is a major hallmark of FA [26], we assessed the expression of *miR-139-3p* and *miR-199a-3p* in CD34+ BM progenitor cells from FA patients (Supplemental Table 4). Similar to the *Ercc1*$^{-/*292}$ lin- cells, *miR-139-3p* expression was increased in CD34+ cells from FA patient BM compared to healthy individuals (median=14.5 fold ($p<0.05$)) (Figure 4C). Expression of *miR-199a-3p* was significantly higher in FA patients with a severe BMF (median=15.7 fold ($p<0.05$)) than in patients with mild BMF (Figure 4C, supplemental Table 4). This is suggestive of a correlation between the severity of ICL-induced damage and the expression of *miR-199a-3p* in FA-BM.

RNA-seq analysis on BM CD34+ cells of a FA patient with progressive BMF showed that the identified targets of *miR-139-3p* and *miR-199a-3p* were downregulated in this patient compared with normal CD34+ cells (Supplemental Figure 2). Conversely, expression of *GADD45B* and *CDKN1A*, two TP53-controlled DDR genes was significantly increased in FA BM CD34+ cells (Supplemental Figure 2). To corroborate these results in a larger group of patients, we analyzed the FA Transcriptome Consortium (FTC) database (Gene Expression Omnibus, 2009, publicly available as GSE16334), containing the transcriptome data of low-density mononuclear BM cells from 21 FA patients and 11 healthy donors [27]. In this data set, expression of the target genes of *miR-139-3p* and *miR-199a-3p* was again significantly lower in FA patients relative to healthy controls (Figure 4D), whereas the DDR-induced genes *GADD45B* and *CDKN1A* were upregulated (Figure 4D). These results establish that *miR-139-3p*, *miR-199a-3p* and their targets are also deregulated in ICL-repair deficient BM cells from FA patients.

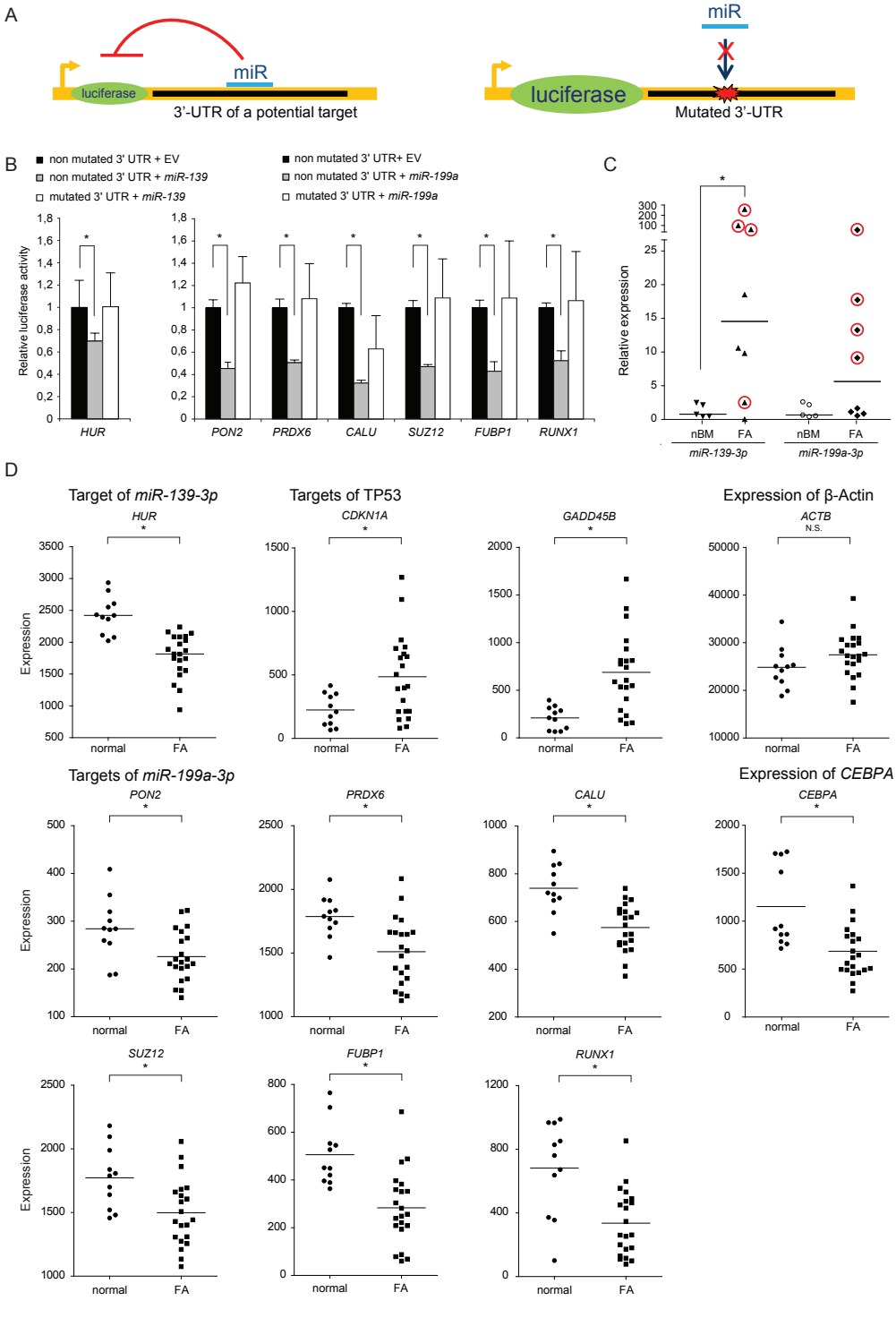
Loss of *Trp53* uncovers the leukemogenic nature of *Ercc1* deficiency

Having initially established that genetic deletion of *Trp53* rescued *Ercc1*$^{-/*292}$ HSPCs from exhaustion, we next investigated whether the loss of one *Trp53* allele promotes the leukemic transformation of *Ercc1*-deficient HSPCs. From 51 mice transplanted with *Ercc1*$^{-/*292}$;*Trp53*$^{+/-}$ HSPCs, 41 died with an average latency of 28 weeks, while all control transplanted mice survived without symptoms (Figure 5A). Of the 31 mice that could be fully analyzed all had developed leukemia, characterized by enlarged liver, spleen or thymus. The leukemia cells had a blast-like morphology and with few exceptions expressed T-cell receptor alpha/beta rearrangements, and CD4 and CD8, indicative of T-cell leukemia (data not shown).

To identify somatic mutations and insertion/deletions (indels), 17 leukemia samples and germ line control DNAs isolated from the brain of 6 donor mice were subjected to whole exome sequencing (WES). Because the chromosomal damage caused by ICLs is repaired by error-prone non-homologous end-joining rather than homologous recombination, leukemia genomes from patients deficient in ICL-repair frequently contain gross genetic aberrations [28]. We developed a new algorithm (Sanders M.A., et al., 2014, manuscript in preparation), allowing for the identification of these abnormalities based on WES data sets. Sporadic patterns of copy number variations (CNVs) were seen, such as gains on chromosomes 4, 5, 11 and 15 and losses on chromosomes 3, 6-8, 13, 16 and 19 (Figure 5B). However, the copy number neutral loss of heterozygosity (CNN-LOH) of chromosome 11 caused complete loss of *Trp53* in all leukemia samples analyzed (Figure 5B, 5C). Expression of *miR-199a-3p* was markedly increased (3.14 fold, $p<0.05$) in *Ercc1*$^{-/*292}$ leukemia cells, whereas *miR-139-3p* was not detectable (Figure 5D).

MiR-199a contributes to the development of AML

MiR-199a-3p was expressed at elevated levels in the *Ercc1*$^{-/*292}$ leukemia samples (Figure 5D). Because the leukemias arising in this model are mostly of T-cell origin, we sought to investigate the consequences of enhanced *miR-199a-3p* in a mouse model prone to develop


A
miR
luciferase
3'-UTR of a potential target
miR
luciferase
Mutated 3'-UTR
B
non mutated 3' UTR + EV
non mutated 3' UTR + miR-139
mutated 3' UTR + miR-139
non mutated 3' UTR+ EV
non mutated 3' UTR + miR-199a
mutated 3' UTR + miR-199a
Relative luciferase activity
HUR
PON2
PRDX6
CALU
SUZ12
FUBP1
RUNX1
C
Relative expression
nBM
FA
miR-139-3p
miR-199a-3p
D
Target of miR-139-3p
HUR
Targets of TP53
CDKN1A
GADD45B
Expression of β-Actin
ACTB
N.S.
Targets of miR-199a-3p
PON2
PRDX6
CALU
Expression of CEBPA
CEBPA
SUZ12
FUBP1
RUNX1
Expression
normal
FA

AML. CEBPA-deficient mice are defective in myeloid differentiation without displaying overt leukemia [29]. Because upregulation of *miR-199a-3p* in *Ercc1*$^{-/*292}$ mice occurs mainly in CMPs and because CEBPA expression is significantly decreased in FA patients (Figure 4D, 40% downregulated, p<0.00080) [27], we reasoned that a *Cebpa* conditional knockout mouse model is especially suitable to investigate the leukemogenic potential of *miR-199a*. We used the *Cebpa*$^{Cre/fl}$ mouse model in which the expression of Cre-recombinase is driven by the *Cebpa* promoter [18]. The Cre enzyme recombines the floxed *Cebpa* allele, resulting in loss of *Cebpa* mainly in myeloid precursors which leads to a differentiation block at CMP level. Fetal liver *Cebpa*$^{Cre/fl}$ HSPCs were transduced with MSCV-EGFP control virus, MSCV-EGFP-*miR-199a,* or -*miR-106*, a non-oncogenic miRNA that induces expansion of HSPCs [19,30], and transplanted in irradiated recipients (Figure 6A). All mice transplanted with *miR-199a* transduced HSPCs (n=5) developed AML within 60-200 days, whereas no malignancies occurred in mice transplanted with control cells (EV, n=9 or *miR-106*, n=4) (Figure 6B). Transplantation of the leukemic cells in secondary recipients caused leukemia with a shortened latency (60-150 days) and more than 95% blasts in the BM (Figure 6B, Supplemental Table 5). The complete loss of *Cebpa* expression in EGFP-positive leukemia cells was confirmed by *Cebpa* locus specific PCR and *Cebpa* expression analysis of leukemia samples (data not shown). Leukemic mice had splenomegaly, severe anemia, high percentages (70-90 %) of blast cells in the BM (Figure 6C, Supplemental Table 5) and leukemic infiltration in peripheral blood, liver and spleen (Figure 6D, 6E). All leukemia samples expressed c-Kit and/or Sca1, indicative of their immature HSPC-like state and lacked markers for terminal

Figure 4. *MiR-139-3p*, *miR-199a-3p* and their targets are aberrantly expressed in FA. A) A schematic overview of the luciferase reporter assay. The 3'-UTR region of a potential target containing the predicted miRNA binding site is cloned downstream of a luciferase reporter. Ectopic expression of the miRNA reduces the luciferase activity. Mutation of the predicted miRNA binding site abolishes the miRNA-dependent inhibition of luciferase activity. **B)** Luciferase reporter plasmids containing the 3'-UTR sequences of indicated targets of *miR-139-3p* or *miR-199a-3p* with a *wt* or mutated miRNA binding site were generated. The luciferase activities in the miRNA expressing cells and relative to the EV control are shown. Error bars represent the SD of 3 experiments. The significance was calculated with the student t-test [2-tailed]) (* p<0.05). **C)** The expression of *miR-139-3p* and *miR-199a-3p* normalized to U6 in BM CD34+ cells isolated from healthy individuals (normal (n) BM) and FA patients (FA), and relative to the average of normal BM samples are plotted. The patients with clinical progressive BMF are indicated with a red circle. The bars show the median relative expression of each group. The significance was calculated with the Mann-Whitney U test (asymptotic significance [2-tailed]) (* p<0.05). **D)** The transcript expression of indicated *miR-139-3p* or *miR-199a-3p* targets, TP53 targets i.e., *CDKN1A* and *GADD45B*, *CEBPA*, and the negative control *ACTB* in low-density mononuclear BM cells from 21 FA patients (FA) and 11 healthy volunteers (normal) is shown. Data are taken from FA Transcriptome Consortium (FTC) database (Gene Expression Omnibus, 2009 and publicly available as GSE16334). The bars indicate the mean relative expression of each group. The significance was calculated with the non-paired student t.test (asymptotic significance [2-tailed]) *p<0.05). N.S.: not significant.

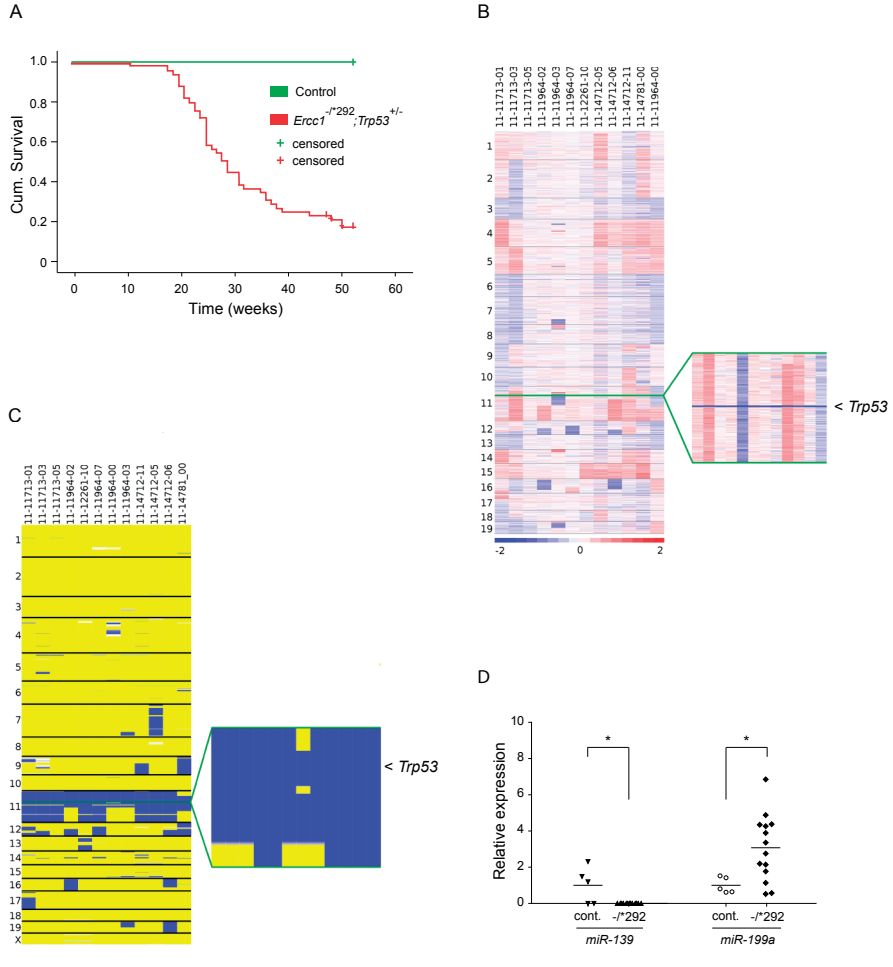


Figure 5. Development of leukemia in *Ercc1*$^{-/*292}$*Trp53*$^{+/-}$ transplanted mice. A) Kaplan-Meier plot of overall survival (*Ercc1*$^{-/*292}$ *Trp53*$^{+/-}$ (n=51); control group contained *Ercc1*$^{+/-}$*Trp53*$^{+/-}$ and *Ercc1*$^{+/*292}$*Trp53*$^{+/-}$ (n=17)). Live animals at the end of the experiment appear as censored (+ symbols). **B)** Heat map of copy number variation (CNV) estimation showing consistent patterns of CNVs. The CNVs are displayed for each leukemia sample (indicated by a 9-digit number, e.g. 11-11713-01) along the genome. The autosomal chromosomes are given on the left side. Blue: loss of genetic material, red: gain of genetic material, and white: retention of genetic material. **C)** The loss of heterozygosity (LOH) along the genome of isolated leukemias is shown. In all leukemias, chromosome 11 harbors partial or complete LOH. *Trp53* is located within regions affected by LOH, leading to homozygous deletions. Blue: LOH; yellow: retention of heterozygosity; white: no informative SNPs. **D)** The expression of ICL-induced miRNAs normalized to U6 in cells isolated from *Ercc1*$^{-/*292}$ leukemias relative to the average of *wt* lin- controls is shown. Bars show the mean relative expression of each group. The significance was calculated with the Mann-Whitney U test (asymptotic significance [2-tailed]) (* $p<0.05$).

myelo-monocytic differentiation (CD11b, GR1), T cells (CD4, CD8) or erythroid cells (TER-119) (Figure 6F). These data establish that *miR-199a* is an onco-miR that drives AML in differentiation defective preleukemic HSPCs.

DISCUSSION

In this study, we used *Ercc1*-deficient mice as a model to identify mechanisms involved in ICL-driven BMF and leukemic progression. There is convincing evidence that the endonuclease complex ERCC1-XPF participates in the FA ICL-repair pathway [31]. *XPF* (*ERCC4*) is mutated in rare cases of FA and has been renamed *FANQ* [32]. FANCQ-ERCC1 is recruited to ICL lesions where it functions as the essential endonuclease required for repair [26,33,34]. Similar to the observations in FA patients [13], we found that *Trp53* is crucially involved in the exhaustion of HSPCs in *Ercc1*$^{-/*292}$ mice. In contrast, *Cdkn2a* expression did not restore the colony-forming capacity of *Ercc1*$^{-/*292}$ HSPCs. The *Cdkn2a* locus encodes the cyclin-dependent kinase inhibitor p16INK4a [35]. While being a major effector of BMF caused by the loss of ataxia telangiectasia mutated (ATM) protein [22], p16INK4a thus appears dispensable for ICL-induced DDR in HSPCs, which is mainly controlled by ATM related (ATR) protein [36]. *Cdkn2a* also encodes p19^{ARF}, which sequesters the TRP53 degradation protein MDM2. Hence, the lack of effects of *Cdkn2a* deletion on myeloid colony formation of *Ercc1*$^{-/*292}$ HSPCs also argues against a dominant role of p19^{ARF}/MDM2-controlled TRP53 stability in HSPCs.

Loss of *Trp53* was a common genetic abnormality in the *Ercc1*$^{-/*292}$ leukemia genomes. This is in line with studies showing that deletion of *Trp53* cooperates with the loss of *Fancc* or *Fancd2* in tumorigenesis [37,38]. Loss of *TP53* functions also occurs frequently in AML secondary to myeloproliferative neoplasms or MDS [39]. In contrast, *TP53* mutations appear to be rare in ICL-repair deficient MDS/AML [40], although a systematic analysis of TP53 activity that, e.g., includes epigenetic silencing of critical TP53 target genes in these patients remains to be done.

Two miRNAs, *miR-139-3p* and *miR-199a-3p* that play opposite roles in *Ercc1*$^{-/*292}$ CMPs were also found to be expressed in CD34+ cells from FA patients, suggesting that they may be involved in the hematopoietic defects seen in FA. We identified *HuR* as a major target of *miR-139-3p*, and blocking its activity by antagomirs partly restored myeloid colony-formation from *Ercc1*$^{-/*292}$ BM cells. HuR has a broad pro-survival function in hematopoietic progenitors by controlling the expression of *Bcl-2*, *Bcl-xl, Survivin, Caspase-9*, *Noxa* and *Puma* [41,42]. HuR levels increase progressively in the transition from the chronic phase to the blast crisis of chronic myeloid leukemia [43] and *HuR* is overexpressed in subsets of AML [44]. Thus, one of the likely mechanisms by which *miR-139-3p* inhibits myeloid progenitor outgrowth of ICL-repair deficient BM cells is through inhibition of the pro-survival activities of HuR.

Expression of *miR-199a-3p* in *Cebpa*-deficient HSPCs gave rise to a transplantable AML in mice, establishing its role as an onco-miR. The leukemogenic activity of *miR-199a-3p* is suggestive of a tumor suppressive function of its major target gene(s). PON2 and PRDX6 protect cells from oxidative stress [45,46] and their repression may contribute to the excessive

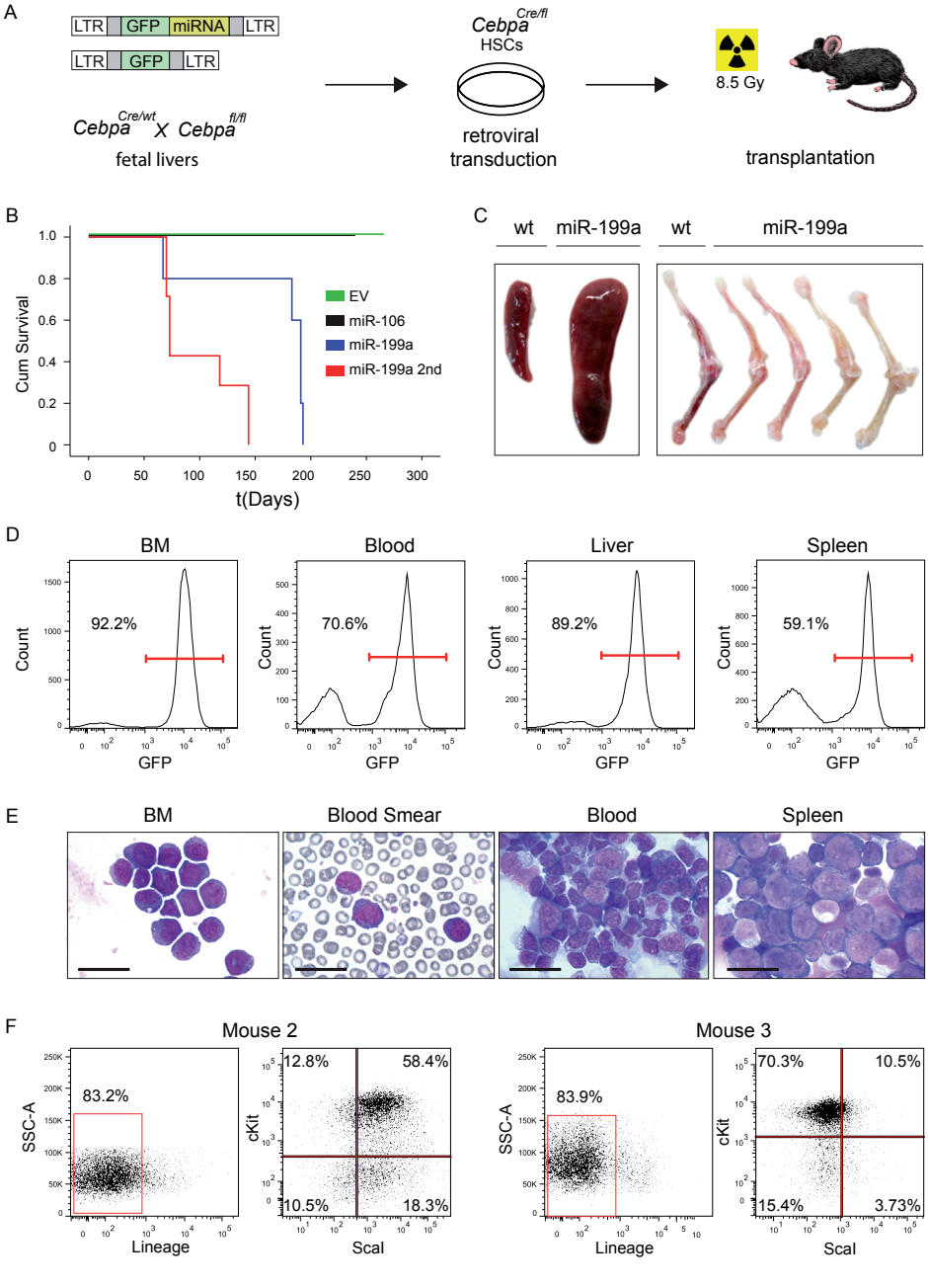


Figure 6. Forced expression of *miR-199a* drives leukemogenesis in mice. A) Schematic overview of the HSC transplantation experiment. HSPCs were isolated from *Cebpa*$^{Cre/fl}$ fetal livers and infected with MSCV-GFP-miRNA or with MSCV-GFP-EV control viruses. Recipient mice were lethally-irradiated (8.5 Gy) and transplanted with transduced cells by tail-vein injection. **B)** Cumulative survival of mice transplanted with lin- cells expressing GFP with, *miR-199a* (n=5) ($p<0.0005$ compared to EV n=9), ▶

ROS levels in ICL-deficient progenitors [47]. SUZ12 is a component of the polycomb repressor complex 2 (PRC2) involved in the silencing of multiple genes, including *HOX* genes [48]. Indeed, expression of the PRC2 target *HOXA11* was increased in FA samples, whereas expression of *HOXA5*, which is not controlled by PRC2 [49], was not changed (Supplemental Figure 3). *MiR-199a*-mediated repression of *Suz12* may at least partly explain the increased expansion of progenitors without impairing their differentiation capacity, in line with what has been reported in a heterozygous *Suz12* (*Suz12*$^{Plt8/wt}$) model [50]. Additionally, *miR-199a-3p*-induced down-regulation of *Runx1,* a transcription factor critical for normal hematopoiesis [51], may contribute to leukemogenesis. Intriguingly, Runx1 has been shown to protect hematopoietic stem and progenitor cells from oncogenic insults via a fail-safe mechanism involving BMI-1 that neutralizes oncogenic RAS signaling [52]. Other targets of *miR-199a-3p* that have been implicated in leukemogenesis, i.e., mTOR and CD44 [53-55] were not found in our study. We cannot exclude that these targets were missed for technical reasons, e.g., the limited sensitivity of the GEP microarray and proteomics strategy used in our study. Finally, it is of interest that *miR-199a-3p* has been shown to compete with pluripotency factors in the reprogramming of mouse embryonic fibroblasts (MEFs) to induced pluripotent stem cells [56]. A difference between this and our study is that *miR-199a-3p* processing in MEFs depended on TRP53, contrary to what was observed in *Ercc1*-deficient leukemia cells (Figure 2C). This discrepancy probably reflects the complex control of miRNA activities depending on cell type, expression levels of targets and type of cellular stress [23,57].

In conclusion, we have presented evidence that ICL-stress causes a loss of HSPCs through the combinatorial action of both *Trp53* and *miR-139-3p*. Elevated expression of *miR-199a-3p* appears insufficient to compensate for the exhaustion of HSPCs at the stage of BMF, but when expression of *Trp53* and *miR-139-3p* is low or absent, *miR-199a-3p* acts as an onco-miR that promotes the development of AML. These findings shed new light on the molecular pathogenesis of ICL-repair deficient BMFS and transformation to leukemia and may open therapeutic avenues to reduce stem cell exhaustion and leukemic transformation, e.g., by selective use of antagomiRs.

miR-106 (n=4) (a non-oncogenic miRNA that promotes myeloid progenitor expansion (Meenhuis et al., 2011; Mu et al., 2009)), GFP only, or secondary recipients of *miR-199a* leukemia cells (n=7) (*miR-199* compared to EV p<0.0005, and secondary transplants compared to primary tumors p<0.03). The statistical significance was calculated with the log-rank Mantel-Cox test. **C)** Typical examples of splenomegaly and femurs and tibiae isolated from *miR-199a*-transplanted mice with leukemia. The pale bones indicate a severe anemia in the leukemic mice. **D)** Typical examples of FACS plots showing the percentage of GFP-expressing AML cells in the BM, blood, liver and spleen of the leukemic mice. **E)** Micrographs showing the morphology of leukemic blasts in the different hematological organs (BM, blood (blood smear), blood (erythrocyte lysis, cytospins) and spleen). Black bar indicates 10 µm. **F)** Flow cytometric analysis of GFP-positive BM cells. GFP-positive AML cells from mouse 2 are an example of a stem cell-like phenotype (c-Kit, Sca1 double positive), whereas the AML cells from mouse 3 have a progenitor-like (c-Kit high, Sca1 low) phenotype.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Cancer Society (KWF kankerbestrijding). PMHvS is supported by a ZonMw E-rare grant. SES was supported by a KIKA Children Cancer Free grant. We gratefully acknowledge the personnel of the animal facility of Erasmus MC (EDC) for mouse care, Dr. E. Rombouts, P. van Geel and N. Spierenburg-Papazian for assistance with flow cytometry and cell sorting, Dr. R. Beekman Dr. E. Bindels for experimental assistance and E. Simons for assistance with the preparation of figures. Prof. Dr. Björn Schumacher, Cologne Excellence Cluster for Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Germany is gratefully acknowledged for providing *Trp53*- and *Cdkn2a*-deficient mouse models. We thank Prof. Dr. D. Tenen for the floxed *Cebpa* mice. Some results are based upon data generated by The Cancer Genome Atlas managed by the NCI and NHGRI (http://cancergenome.nih.gov).

AUTHOR CONTRIBUTIONS

MFA and JRH performed experiments and analyzed data; HWJdL, PMHvS, JV-O, YC performed experiments; RH and MS processed and analyzed next generation sequencing data; AHdR, GMCJ and PAvV, designed and performed proteomics and analyzed proteomics data; SES and MBB isolated and investigated BM samples from FA patients; MvL, IPT and SJE supervised the project. MFA, JRH, IPT and SJE designed the experiments and wrote the manuscript.

REFERENCES

1. Blank U, Karlsson G, Karlsson S. Signaling pathways governing stem-cell fate. *Blood*. 2008;111(2):492-503.
2. Dokal I, Vulliamy T. Inherited aplastic anaemias/bone marrow failure syndromes. *Blood Rev*. 2008;22(3):141-153.
3. Parikh S, Bessler M. Recent insights into inherited bone marrow failure syndromes. *Curr Opin Pediatr*. 2012;24(1):23-32.
4. Bakker ST, Passegue E. Resilient and resourceful: genome maintenance strategies in hematopoietic stem cells. *Exp Hematol*. 2013;41(11):915-923.
5. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature*. 2007;447(7145):725-729.
6. Rahn JJ, Adair GM, Nairn RS. Multiple roles of ERCC1-XPF in mammalian interstrand crosslink repair. *Environ Mol Mutagen*. 2010;51(6):567-581.
7. Prasher JM, Lalai AS, Heijmans-Antonissen C, et al. Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1-/- mice. *EMBO J*. 2005;24(4):861-871.
8. Verhagen-Oldenampsen JH, Haanstra JR, van Strien PM, Valkhof M, Touw IP, von Lindern M. Loss of ercc1 results in a time- and dose-dependent reduction of proliferating early hematopoietic progenitors. *Anemia*. 2012;2012:783068.
9. Dufour C, Corcione A, Svahn J, et al. TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis in vitro. *Blood*. 2003;102(6):2053-2059.
10. Garaycoechea JI, Patel KJ. Why does the bone marrow fail in Fanconi anemia? *Blood*. 2014;123(1):26-34.
11. Garbati MR, Hays LE, Keeble W, Yates JE, Rathbun RK, Bagby GC. FANCA and FANCC modulate TLR and p38 MAPK-dependent expression of IL-1beta in macrophages. *Blood*. 2013;122(18):3197-3205.
12. Matsui K, Giri N, Alter BP, Pinto LA. Cytokine production by bone marrow mononuclear cells in inherited bone marrow failure syndromes. *Br J Haematol*. 2013;163(1):81-92.
13. Ceccaldi R, Parmar K, Mouly E, et al. Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. *Cell Stem Cell*. 2012;11(1):36-49.
14. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. *Cell*. 1996;85(1):27-37.
15. Clarke AR, Purdie CA, Harrison DJ, et al. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*. 1993;362(6423):849-852.
16. Weeda G, Donker I, de Wit J, et al. Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Curr Biol*. 1997;7(6):427-439.
17. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A*. 1997;94(2):569-574.
18. Wolfler A, Danen-van Oorschot AA, Haanstra JR, et al. Lineage-instructive function of C/EBPalpha in multipotent hematopoietic cells and early thymic progenitors. *Blood*. 2010;116(20):4116-4125.
19. Meenhuis A, van Veelen PA, de Looper H, et al. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood*. 2011;118(4):916-925.
20. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.

21. McWhir J, Selfridge J, Harrison DJ, Squires S, Melton DW. Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nat Genet*. 1993;5(3):217-224.
22. Ito K, Hirao A, Arai F, et al. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature*. 2004;431(7011):997-1002.
23. Leung AK, Sharp PA. MicroRNA functions in stress responses. *Mol Cell*. 2010;40(2):205-215.
24. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. *Cell*. 2012;148(6):1172-1187.
25. de Koning JP, Soede-Bobok AA, Ward AC, et al. STAT3-mediated differentiation and survival and of myeloid cells in response to granulocyte colony-stimulating factor: role for the cyclin-dependent kinase inhibitor p27(Kip1). *Oncogene*. 2000;19(29):3290-3298.
26. Crossan GP, Patel KJ. The Fanconi anaemia pathway orchestrates incisions at sites of crosslinked DNA. *J Pathol*. 2012;226(2):326-337.
27. Vanderwerf SM, Svahn J, Olson S, et al. TLR8-dependent TNF-(alpha) overexpression in Fanconi anemia group C cells. *Blood*. 2009;114(26):5290-5298.
28. Deans AJ, West SC. DNA interstrand crosslink repair and cancer. *Nat Rev Cancer*. 2011;11(7):467-480.
29. Zhang P, Iwasaki-Arai J, Iwasaki H, et al. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity*. 2004;21(6):853-863.
30. Mu P, Han YC, Betel D, et al. Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas. *Genes Dev*. 2009;23(24):2806-2811.
31. Bhagwat N, Olsen AL, Wang AT, et al. XPF-ERCC1 participates in the Fanconi anemia pathway of cross-link repair. *Mol Cell Biol*. 2009;29(24):6427-6437.
32. Bogliolo M, Schuster B, Stoepker C, et al. Mutations in ERCC4, encoding the DNA-repair endonuclease XPF, cause Fanconi anemia. *Am J Hum Genet*. 2013;92(5):800-806.
33. Hodskinson MR, Silhan J, Crossan GP, et al. Mouse SLX4 Is a Tumor Suppressor that Stimulates the Activity of the Nuclease XPF-ERCC1 in DNA Crosslink Repair. *Mol Cell*. 2014.
34. Klein Douwel D, Boonen RA, Long DT, et al. XPF-ERCC1 Acts in Unhooking DNA Interstrand Crosslinks in Cooperation with FANCD2 and FANCP/SLX4. *Mol Cell*. 2014.
35. Weber JD, Taylor LJ, Roussel MF, Sherr CJ, Bar-Sagi D. Nucleolar Arf sequesters Mdm2 and activates p53. *Nat Cell Biol*. 1999;1(1):20-26.
36. Ceccaldi R, Briot D, Larghero J, et al. Spontaneous abrogation of the G(2) DNA damage checkpoint has clinical benefits but promotes leukemogenesis in Fanconi anemia patients. *J Clin Invest*. 2011;121(1):184-194.
37. Freie B, Li X, Ciccone SL, et al. Fanconi anemia type C and p53 cooperate in apoptosis and tumorigenesis. *Blood*. 2003;102(12):4146-4152.
38. Houghtaling S, Granville L, Akkari Y, et al. Heterozygosity for p53 ($Trp53^{+/-}$) accelerates epithelial tumor formation in fanconi anemia complementation group D2 (Fancd2) knockout mice. *Cancer Res*. 2005;65(1):85-91.
39. Milosevic JD, Puda A, Malcovati L, et al. Clinical significance of genetic aberrations in secondary acute myeloid leukemia. *Am J Hematol*. 2012;87(11):1010-1016.
40. Quentin S, Cuccuini W, Ceccaldi R, et al. Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions. *Blood*. 2011;117(15):e161-170.
41. Abdelmohsen K, Lal A, Kim HH, Gorospe M. Posttranscriptional orchestration of an anti-apoptotic program by HuR. *Cell Cycle*. 2007;6(11):1288-1292.

42. Ghosh M, Aguila HL, Michaud J, et al. Essential role of the RNA-binding protein HuR in progenitor cell survival in mice. *J Clin Invest*. 2009;119(12):3530-3543.

43. Radich JP, Dai H, Mao M, et al. Gene expression changes associated with progression and response in chronic myeloid leukemia. *Proc Natl Acad Sci U S A*. 2006;103(8):2794-2799.

44. Topisirovic I, Siddiqui N, Orolicki S, et al. Stability of eukaryotic translation initiation factor 4E mRNA is regulated by HuR, and this activity is dysregulated in cancer. *Mol Cell Biol*. 2009;29(5):1152-1162.

45. Fisher AB. Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A(2) activities. *Antioxid Redox Signal*. 2011;15(3):831-844.

46. Ng CJ, Wadleigh DJ, Gangopadhyay A, et al. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *J Biol Chem*. 2001;276(48):44444-44449.

47. Du W, Adam Z, Rani R, Zhang X, Pang Q. Oxidative stress in Fanconi anemia hematopoiesis and disease progression. *Antioxid Redox Signal*. 2008;10(11):1909-1921.

48. Cao R, Zhang Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell*. 2004;15(1):57-67.

49. Abdel-Wahab O, Adli M, LaFave LM, et al. ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. *Cancer Cell*. 2012;22(2):180-193.

50. Majewski IJ, Blewitt ME, de Graaf CA, et al. Polycomb repressive complex 2 (PRC2) restricts hematopoietic stem cell activity. *PLoS Biol*. 2008;6(4):e93.

51. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*. 1996;84(2):321-330.

52. Motoda L, Osato M, Yamashita N, et al. Runx1 protects hematopoietic stem/progenitor cells from oncogenic insult. *Stem Cells*. 2007;25(12):2976-2986.

53. Fornari F, Milazzo M, Chieco P, et al. MiR-199a-3p regulates mTOR and c-Met to influence the doxorubicin sensitivity of human hepatocarcinoma cells. *Cancer Res*. 2010;70(12):5184-5193.

54. Henry JC, Park JK, Jiang J, et al. miR-199a-3p targets CD44 and reduces proliferation of CD44 positive hepatocellular carcinoma cell lines. *Biochem Biophys Res Commun*. 2010;403(1):120-125.

55. Wu D, Huang HJ, He CN, Wang KY. MicroRNA-199a-3p regulates endometrial cancer cell proliferation by targeting mammalian target of rapamycin (mTOR). *Int J Gynecol Cancer*. 2013;23(7):1191-1197.

56. Wang J, He Q, Han C, et al. p53-facilitated miR-199a-3p regulates somatic cell reprogramming. *Stem Cells*. 2012;30(7):1405-1413.

57. Ebert MS, Sharp PA. Roles for microRNAs in conferring robustness to biological processes. *Cell*. 2012;149(3):515-524.

SUPPLEMENTARY MATERIALS AND METHODS

Quantitative proteomics

Sample preparation: HSPCs (experiment 1: 3 x 10^6 cells, experiment 2: 5 x 10^6 cells, experiment 3: 5 x 10^6 cells) were washed with PBS and lysed in ST lysis buffer (5% SDS, 100 mM Tris-Cl pH 7.6). DTT (0.5M) was added to a concentration of 100 mM. The sample was incubated for 4 min at 90°C. Samples were processed using filter-aided sample preparation on 30 kDa Microcon filters as previously described [1].

Reductive amination: Peptide concentration was determined using the bicinchoninic acid protein assay (Pierce). Twenty-five µg peptide was labeled by reductive amination essentially as described [2]. Peptides from *Ercc1* $^{-/*292}$ and *Ercc1* control cells were sequentially labeled on the same C18 extraction cartridge (HLB 1cc Oasis, Waters Corporation) with either of the dimethyl isotopologues. Briefly, peptides from *Ercc1* $^{-/*292}$ were diluted in 1 ml 0.1% (vol/vol) formic acid, applied to the column, washed twice with 0.1% (vol/vol) formic acid and labeled with five 0.5 ml aliquots of labeling mixture 'light', applied in 2-minute intervals. The column was washed twice with 0.1% (vol/vol) formic acid followed by application of peptides from *Ercc1*control cells and its labeling with the 'medium' dimethyl isotopologue. Finally the differentially labeled peptides were eluted from the column with 400 µl 80% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid and freeze-dried. 'Light' dimethyl labeling mixture consists of formaldehyde (37% (wt/vol) in H_2O, Sigma Aldrich) and sodium cyanoborohydride (Fluka), 'medium' labeling mixture consists of formaldehyde-D2 (20% (wt/vol) in D_2O, Isotec) and sodium cyanoborohydride.

Strong cation exchange chromatography (SCX): SCX separations were performed on a home-made SCX column (320 µm ID, 15 cm, polysulfoethyl A 3 um, Poly LC). Peptides were applied to the column and washed for 10 min with solvent A (water, 35% acetonitrile, 0.1% TFA) at 4 µl/min. The column was developed with a linear gradient to reach 100% solvent B (250 mM KCl, 35% acetonitrile, 0.1% TFA) in 15 min, followed by 100 % solvent C (500 mM KCl, 35% acetonitrile, 0.1% TFA) in the next 15 min. After 5 min at 100 % solvent C, the column was conditioned with solvent A. About twenty 4 µl-fractions were collected in vials prefilled with 100 µl 3% acetonitrile, 0.1% formic acid.

On-line nanoHPLC-tandem mass spectrometry: SCX fractions were analyzed by nano-flow liquid chromatography using an Agilent 1100 HPLC binary system (Agilent Technologies), using the setup as described [3] and coupled on-line to a 7-tesla LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific). The end of the nano-column was drawn to a tip (internal diameter ~5 µm), from which the eluent was sprayed into the mass spectrometer. Peptides were trapped at 5 µl/min on a 1 cm column (100-µm ID; ReproSil-Pur C18-AQ, 3 µm), and eluted to a 15 cm column (50-µm ID; ReproSil-Pur C18-AQ, 3 µm) at 150 nl/min in a 120 minutes gradient

from 0 to 50% acetonitrile in 0.1% formic acid. All chromatographic columns were prepared in-house. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 3,000,000. The five most intense ions were then isolated for collision induced dissociation in the linear ion trap at a target value of 10,000, and placed on the dynamic exclusion list for 45 seconds. Nano-HPLC MS/MS experiments of the biological replicates were run in duplicate.

Data analysis: Raw files were processed using two different software packages, Proteome Discoverer (v1.2.0.208) (Thermo Fisher Scientific) and MaxQuant (version 1.2.2.5) (www.maxquant.org), for relative quantitation. In Proteome Discoverer, peak lists were generated using default settings, and searched with the MASCOT search engine v2.2.04 (Matrix Science) against the IPImouse387 database (trypsin, precursor tolerance 10 ppm, fragment tolerance 0.5 Da), with static modification carbamidomethyl (Cys), and dynamic modifications oxidation (Met), light (d0)-dimethylation (Lys and N-terminus) and heavy (d4)-dimethylation (Lys and N-terminus).

MaxQuant was used with the default settings against the same database with the same parameters as for the mascot search. The only difference was the precursor tolerance, which was 20 ppm in MaxQuant. In each experiment, the proteins with a PEP value > 0.05 when analyzed by MaxqQuant, were discarded.

Protein fold changes between *Ercc1* $^{-/*292}$ HSPCs and *Ercc1 control* HSPCs were calculated with PD and Maxquant based on at least 2 counts per protein. The protein fold changes were discarded in case of a relative variability of > 30% between different counts in each experiment. In addition, protein fold changes from PD and Maxquant were compared and if the results from different programs were contradictory i.e., showing upregulation in one program and downregulation in the other, proteins were discarded. With this procedure we identified 2000 (experiment 1), 1685 (experiment 2), and 1310 (experiment 3) proteins. In total, we identified 457 proteins to be up- or down- regulated in at least 2 out of 3 experiments. The final fold change shows the average of different experiments analyzed by either Proteome Discoverer or MaxQuant software packages.

To correlate the protein names-output from the proteomics data with the expression data-based gene names on the MMU array we developed a software package that scans the NCBI, Ensembl and Swiss- Prot databases. In this way 97% of the MaxQuant-identified proteins could be linked to the expression data. Normalization and mapping were performed by the use of BrainArray annotation. Associations to predicted targets of related miRNA's were inferred using TargetScan.

Whole exome sequencing (WES)

Sequencing libraries from the leukemias were prepared using the Sure-select target enrichment mouse exome capture (G7550B, Agilent Technologies) according to manufacturer's protocol (Version 2.3, January 2011; for Illumina sequencing kit). In short,

3 μg genomic DNA was sheared to fragments of approximately 170 base pairs using the Covaris S-series Single Tube Sample Preparation System, Model S2 (Covaris). Fragment sizes were determined on a Bioanalyzer (Agilent Technologies). Adapter ligated libraries were prepared according to the manufacturer's protocol using the Paired-End Genomic DNA Sample Prep Kit (Illumina). Of the adaptor-ligated library ~6 pM was sequenced (101 bp; paired-end) along with 1% Phix control with a v2 flow cell on a Hiseq2000 (Illumina). In the second cohort exome capture was performed using the NimbleGen SeqCap EZ exome library MM9 according to manufacturer's protocol (NimbleGen SeqCap EZ Library SR User's Guide v3.0, November 2011). Adapter ligation and amplifications were done with Illumina Truseq DNA sample Preparation v2 (protocol August 2011). Mouse Cot-1 DNA (Life technologies) was used during hybridization. For sequencing a v3 flow cell was used. Brain DNA from the 6 donor *Ercc1*$^{-/*292}$ donor mice, used for BM transplantation was sequenced to filter out germ-line single nucleotide polymorphisms (SNP's) and indels from the leukemia genomes.

WES data analysis: Paired-end sequence alignment was done using the Burrows and Wheeler aligner (BWA) [4]. The copy number variation (CNV) profiles were determined by an in-house constructed package called CNVsvd (M. Sanders et al, 2014, submitted). In short, the algorithm extracts the fragment count information from aligned paired-end reads. Subsequently, it determines the variance components from the count profiles of the control samples which are used to filter the noise from the count profiles of the tumor samples. Finally, the count distribution of the control samples is used to estimate the copy number from the count information of the tumor samples.

Luciferase reporter assays

The wild-type 3'UTR fragments were cloned behind the Firefly Luciferase gene of the pGL3-promoter vector (Promega). Mutations were introduced with Quick change site-directed mutagenesis kit (Agilent Technologies). HEK293 cells were transfected with pGL3-3'UTR and Renilla control vectors with Fugene6 transfection reagent (Roche). For dual luciferase reporter assays, cell were lysed and analyzed according to the manufacturer's instruction (Promega) with a Victor multi-label counter.

Primers for 3'UTR cloning:	
PRDX6 hsa fw XbaI	AATCTAGATGTGAGCCAGAGGATGTCAGC
PRDX6 hsa rv ApaI	AAGGGCCCAATGGGTGACAGGAGAGGACG
PRDX6 mmu fw XbaI	AATCTAGAAGCCGTGGGTCCTTGC
PRDX6 mmu rv ApaI	AAGGGCCCTCTCCCTATGTCAGTGCTCTCC
SUZ12 mmu fw XbaI	AATCTAGATAGGATGGTAAAGCAAGAGAAACAG
SUZ12 mmu rv ApaI	AAGGGCCCTCTGATGGTGGTGGAATGAAAT
RUNX1 mmu fw XbaI	AATCTAGAGAAGACGCAGCCCATCCT
RUNX1 mmu rv ApaI	AAGGGCCCTCCAGACATCTTCAACGCAAT
PON2 mmu fw XbaI	AATCTAGAATCCAGCGAGAAAGAAGAAAGC
PON2 mmu rv ApaI	AAGGGCCCGCTGAACACTGGAAGGCTGA
FUBP1 mmu fw XbaI	AATCTAGATATGGATGCAGACGACTTGATG
FUBP1 mmu rv ApaI	AAGGGCCCGAAATAGCCAAATACAACAGAAAAG
CALU mmu fw XbaI	AATCTAGAGACAGAGGAACCTACATTTCTTCA
CALU mmu rv ApaI	AAGGGCCCTCAACAGGCAAGAGAAAGCATT
HuR 3'UTR_f1 XbaI	AATCTAGAtactctgggatgcaaccgac
HuR 3'UTR_r1 ApaI	AAGGGCCCTGAGTGAGCAGGAGGTGGCA
HuR 3'UTR_f2 XbaI	AATCTAGAgatgacgggcttgtagcacc
HuR 3'UTR_r2 ApaI	AAGGGCCCGCCAGCAACTAGAACCTTGATG

Primers used for mutagenesis:	**MRE1**
PRDX6 hsa fw XbaI	GGTTTTTAGGTTGCTATATCACTGGCTTATTAAATGAAAATGGC
PRDX6 hsa rv ApaI	GCCATTTTCATTTAATAAGCCAGTGATATAGCAACCTAAAAACC
PRDX6 mmu fw XbaI	ATGTAGATCGCTCGCTATAATAATGGGTCATTAAATGGAAATG
PRDX6 mmu rv ApaI	CATTTCCATTTAATGACCCATTATTATAGCGAGCGATCTACAG
SUZ12 mmu fw XbaI	CAACAGAAAGTGGTTTCATTAATGGCACGGATAGCTTTTTATTC
SUZ12 mmu rv ApaI	GAATAAAAAGCTATCCGTGCCATTAATGAAACCACTTTCTGTTG
RUNX1 mmu fw XbaI	GCTTTGGGTCATTTTTTAATTAATGTATTTCCACAAAGAAATCCC
RUNX1 mmu rv ApaI	GGGATTTCTTTGTGGAAATACATTAATTAAAAAATGACCCAAAGC
PON2 mmu fw XbaI	CCT GCT GGG CTT GTG CAG GAA TAA TGA TAG ATA ACA CTT G
PON2 mmu rv ApaI	CAA GTG TTA TCT ATC ATT ATT CCT GCA CAA GCC CAG CAG G
FUBP1 mmu fw XbaI	CCC CCC TTT TTT TTT ATT TTG AAA ATG TAC AAA ATA ACT ATC AAT AAT GAT AGG AGG TTA ATA TTT CTG
FUBP1 mmu rv ApaI	CAG AAA TAT TAA CCT CCT ATC ATT ATT GAT AGT TAT TTT GTA CAT TTT CAA AAT AAA AAA AAA GGG GGG

Primers used for mutagenesis:	MRE1
CALU mmu fw XbaI	CTC TGG TTT CAC ATA AAA TTG CGC TGC AGA GAC TGT TAT TAC AAA CTT TTT AA
CALU mmu rv ApaI	TTA AAA AGT TTG TAA TAA CAG TCT CTG CAG CGC AAT TTT ATG TGA AAC CAG AG
HuR 3'UTR_f1 XbaI	CTA ACA CGC GTT TCA TTC AAT GTA TAC ACA GAC TGG GTA GCA AAA AAA
HuR 3'UTR_r1 ApaI	TTT TTT TGC TAC CCA GTC TGT GTA TAC ATT GAA TGA AAC GCG TGT TAG
HuR 3'UTR_f2 XbaI	AGG AAC ATT CTC ATT GTA TGT GGT AGC CGC TGT TTG AAC AGC
HuR 3'UTR_r2 ApaI	GCT GTT CAA ACA GCG GCT ACC ACA TAC AAT GAG AAT GTT CCT
	MRE2
PRDX6 hsa fw XbaI	CATTCATACATCAGCACTCTACTAGTTCTGTTTGAAATATGTT
PRDX6 hsa rv ApaI	AACATATTTCAAACAGAACTAGTAGAGTGCTGATGTATGAATG
PRDX6 mmu fw XbaI	CTGTCACAGTGCCCAATGACTGGCTCTCTTTG
PRDX6 mmu rv ApaI	CAAAGAGAGCCAGTCATTGGGCACTGTGACAG

Luminex experiments

The 32D cells expressing human colony-stimulating factor 3 (CSF3)-receptor were expanded as described [5]. GFP-positive MSCV-BC-miRNA 32D cell populations were sorted with FACSAria II cell sorter (Becton-Dickinson Biosciences). MiRNA expressing cell populations were mixed in a 1:1 ratio, further expanded in RPMI medium supplemented with FCS and IL-3 (1:1000, supernatant) or switched to CSF3-containing medium. Luminex experiments were performed as described previously [6].

Antibodies, cell staining, flowcytometry and cytospins

Peripheral blood was obtained by heart puncture at the moment of euthanasia. BM samples were prepared as described previously [7]. For morphological analysis of tumor cells, cytospins were stained with May-Grünwald-Giemsa and examined with a Leica DMLB microscope (40X objective) and Leica Application Suite Version 2.7.1 R1 Software. For flow cytometric analyses, cells were stained with combinations of antibodies as described previously [7]. Cell populations were determined with BD™ LSR II Flow Cytometer System (Becton-Dickinson Biosciences). Data was analyzed with FlowJo (Tree Star, Ashland, OR). For live cell gating, cells were stained with 7-AAD (Invitrogen). HSPC subpopulations were gated as described [8].

Gene and miRNA expression profiling and qPCR

LSK fractions of the BM of 3 *Ercc1* $^{-/*292}$ and 3 littermate-matched *Ercc1*-proficient (*Ercc1* $^{+/*292}$ or *Ercc1* $^{+/-}$) control mice were sorted on a FACSAria II instrument (Becton-Dickinson). BM HSPCs were isolated as previously described [7]. HSPCs were lysed in Trizol and total

RNA was isolated with Trizol reagent according to manufacturer's protocol (Invitrogen). Quality control of total RNA was done with RNA 6000 Nano assay on the Aligent 2100 Bioanalyzer (Aligent). Concentrations and purity of the RNA samples were determined with a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). Gene- and miRNA-expression arrays, using Mouse Genome 430 Affymetrix 2.0 Array and Taqman Array Rodent or Human MicroRNA A Cards Version 2.0 (Applied Biosystems) respectively, were performed as previously described [7]. Single miRNA expression assays, using Taqman single real-time miRNA and U6 qPCR reactions (Applied Biosystems), were performed as previously described [7].

Statistics

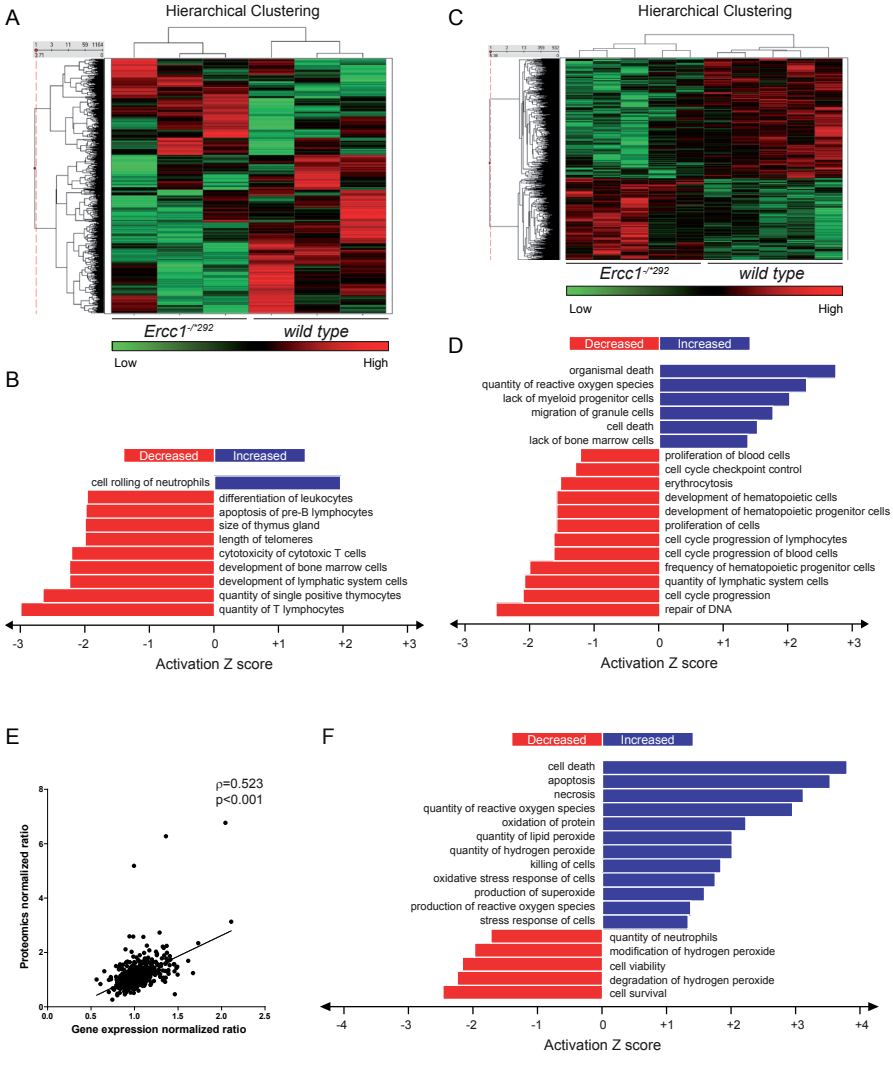
Gene expression profiling: Profiling of mRNA expression was performed in multiplicate (3x in LSKs and 5x in HSPCs) for each experimental condition (*Ercc1*$^{-/*292}$ or *Ercc1*^{wt}). The data were subsequently normalized with MAS5.0. Probe sets considered indistinguishable from the background signal were omitted from further analyses. Identification of the differentially expressed probe sets was performed using the FDR corrected p-values. A p-value < 0.05 was considered statistically significant.

MiRNA profiling: The expression of miRNAs determined by Taqman Array Rodent or Human MicroRNA A Cards Version 2.0 (Applied Biosystems) was normalized by the average expression value of 4 U6 snRNA probesets available on the cards. QPCR analysis on single miRNAs and U6 expression levels were determined in triplicate. Differentially expressed miRNAs were determined using the Mann-Whitney U test (asymptotic significance [2-tailed]). A p-value < 0.05 was considered statistically significant. The fold induction was calculated by the $2^{-\Delta\Delta Ct}$ method.

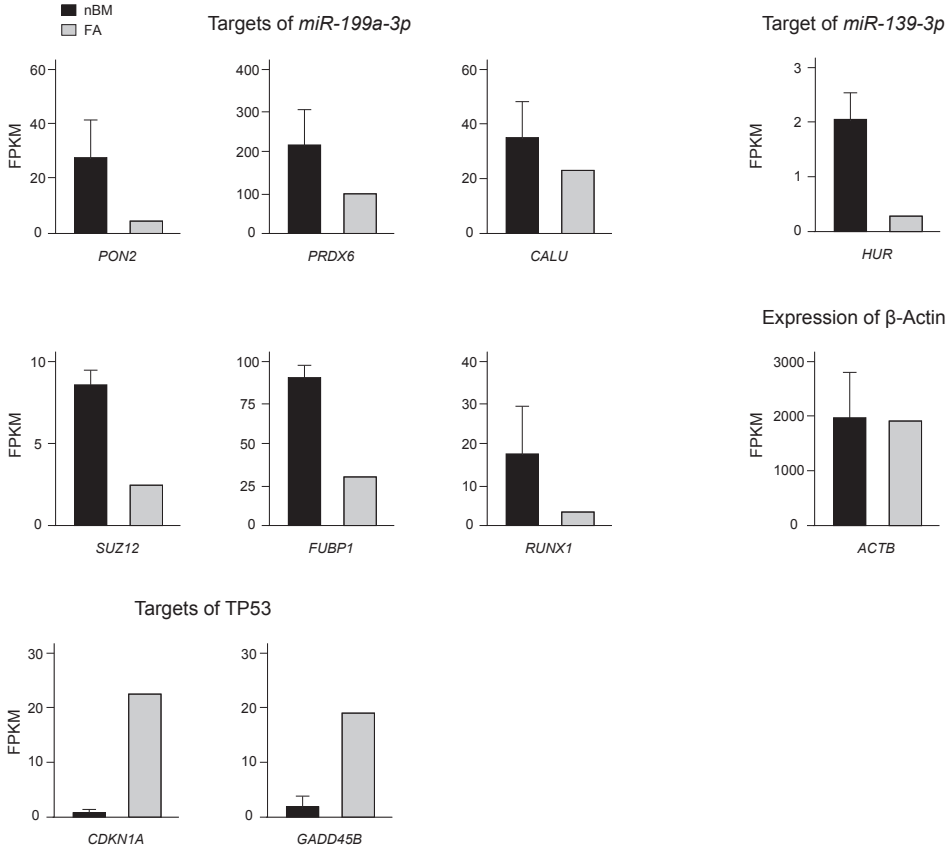
REFERENCES

1. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009;6(5):359-362.
2. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc*. 2009;4(4):484-494.
3. Meiring MS, Litthauer D, Harsfalvi J, van Wyk V, Badenhorst PN, Kotze HF. In vitro effect of a thrombin inhibition peptide selected by phage display technology. *Thromb Res*. 2002;107(6):365-371.
4. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14):1754-1760.
5. de Koning JP, Soede-Bobok AA, Ward AC, et al. STAT3-mediated differentiation and survival and of myeloid cells in response to granulocyte colony-stimulating factor: role for the cyclin-dependent kinase inhibitor p27(Kip1). *Oncogene*. 2000;19(29):3290-3298.
6. Meenhuis A, van Veelen PA, de Looper H, et al. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood*. 2011;118(4):916-925.
7. Alemdehy MF, van Boxtel NG, de Looper HW, et al. Dicer1 deletion in myeloid-committed progenitors causes neutrophil dysplasia and blocks macrophage/dendritic cell development in mice. *Blood*. 2012;119(20):4723-4730.
8. Wolfler A, Danen-van Oorschot AA, Haanstra JR, et al. Lineage-instructive function of C/EBPalpha in multipotent hematopoietic cells and early thymic progenitors. *Blood*. 2010;116(20):4116-4125.

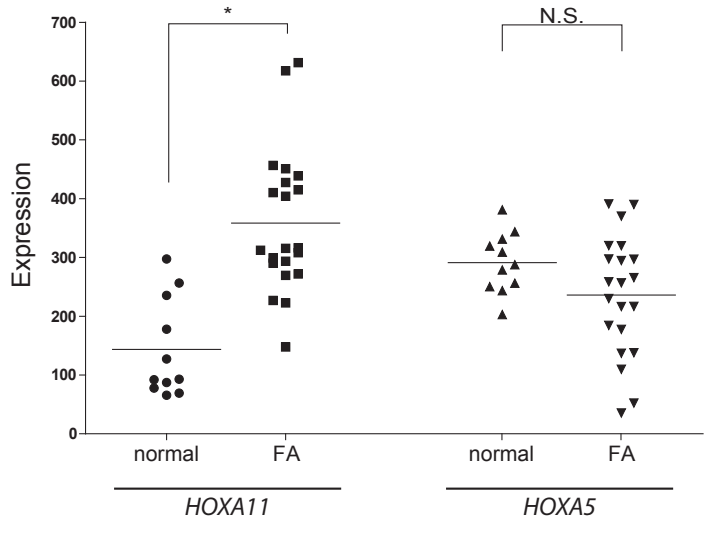
SUPPLEMENTAL FIGURES AND TABLES



Supplemental Figure 1. Gene expression analysis of *Ercc1*-proficient and -deficient HSPCs. A) Unsupervised clustering of differentially expressed genes (FDR-corrected p<0.05) in LSK cells from 20-22 week old *Ercc1*$^{-/*292}$ mice and *wt* littermates. The scale bar shows the normalized expression z-scores and indicates an increased (red) or decreased (green) expression relative to the row mean (black). **B)** Altered biological functions of differentially expressed genes in *Ercc1*$^{-/*292}$ LSK cells identified by IDEA. **C)** Unsupervised clustering of significant differentially expressed genes (FDR-corrected p<0.05) in *Ercc1*$^{-/*292}$ and *wt* Lin- BM cells. **D)** Altered biological functions of differentially expressed genes in *Ercc1*$^{-/*292}$ Lin- BM cells identified by IDEA. **E)** Example of the correlation of the ratio's determined by gene expression profiling and proteomics. Line shows the Pearson correlation. **F)** Altered biological functions of differentially expressed proteins in *Ercc1*$^{-/*292}$ Lin- BM cells identified by IDEA.



Supplemental Figure 2. Identified targets of *miR-139-3p* and *miR-199a-3p* are transcriptionally deregulated in FA. RNA from CD34+ cells isolated from the BM cells of a FA patient with progressive BMF (patient nr. 5 in Suppl.Table 4) is sequenced by Illumina. The number of bases mapped to the genome for FA and controls are in the same range (data not shown). The expression of *HUR* (identified target of *miR-139-3p*), *PON2*, *PRDX6*, *CALU*, *SUZ12*, *FUBP1* and *RUNX1* (identified targets of *miR-199a-3p*), and also *CDKN1A* and *GADD45B* (targets of TP53) is compared in CD34+ BM cells obtained from FA patient (grey bars) with CD34+ BM cells from 3 healthy volunteers (black bars).The expression of *Beta-ACTIN* is shown as a negative control.



Supplemental Figure 3. Expression of the PRC2 target *HOXA11* is increased in the BM of FA patients. Transcript expression of *HOXA11* and *HOXA5* in low-density mononuclear BM cells from 21 FA patients (FA) and 11 healthy volunteers (normal). Data are taken from FA Transcriptome Consortium (FTC) database (Gene Expression Omnibus, 2009 and publicly available as GSE16334). Lines show the mean of each group. Significance was calculated by comparing the expression level in healthy controls with the level in FA patients with the non-paired student t.test (asymptotic significance [2-tailed]) * $p < 0.05$).

Supplemental Tables 1-3 are accessible online via:
http://hema13.erasmusmc.nl/Farshid/Table%20S1.xlsx
http://hema13.erasmusmc.nl/Farshid/Table%20S2.xlsx
http://hema13.erasmusmc.nl/Farshid/Table%20S3.xlsx

Supplemental Table 4. Clinical characteristics of FA patients

case	affected FA gene	mutation I	mutation II	effect I	effect II
1	*FANC-C*	c.67delG	c.67delG	p.Asp23Ilefs*23	p.Asp23Ilefs*23
2	*FANC-A*	c.2852G>A	c.3624T>C[(1)]	p.Arg951Gln	p.Ser1208Ser[(2)]
3	*FANC-A*	c.487delC	c.2851C>T	p.Arg163Valfs*29	p.Arg951Trp
4	*FANC-A*	exon31-35del[(3)]	c.2851C>T	-	p.Arg951Trp
5	*FANC-A*	c.2852G>A	c.3788_3790del	p.Arg951Gln	p.Phe1263del
6	*FANC-A*	c.2852G>A	c.3788_3790del	p.Arg951Gln	p.Phe1263del
7	*FANC-C*	c.67delG	c.67delG	p.Asp23Ilefs*23	p.Asp23Ilefs*23
8	*FANC-C*	c.67delG	c.553C>T	p.Asp23Ilefs*23	p.R185*

Description of mutations based on cDNA sequence. GenBank reference sequences FANCA: NM_000135; FANCC: NM_000136. [1]synonymous; [2]aberrant splicing, Hum Mutat. 2008 Jan;29(1):159-66; [3]exons absent by MLPA, breakpoints unknown, effect: probably non sense mediated decay; [4]MDS morphologically not excluded; [5]material not representative, mixed with blood. Hb = haemoglobin; PLT = platelet count; WBC = white blood cell count. Patients 5 and 6 are siblings. §Received allogeneic stem cell transplantation.

Supplemental Table 5. Analysis of blood, BM and spleen derived from leukemic mice transplanted with CEBPA$^{fl/Cre}$; *miR-199a* **cells**

Characteristic	Normal level	Primary leukemia* Median (range)	Secondary leukemia# Median (range)
Survival (Days)		191 (67 - 193)	73 (70 - 144)
WBC (x10^9/l)	3 - 15	2.8 (2.8 - 9.2)	44.3 (2.9 - 57.5)
RBC (x10^{12}/l)	5 - 12	4.87 (0.87 - 5.61)	3.27 (1.91 - 5.1)
HGB (mmol/l)	6.9 - 11.2	3.2 (1.4 - 6.2)	4.9 (2.5 - 6.9)
HCT (l/l)	0.360 - 0.520	0.153 (0.06 - 0.329)	0.249 (0.115 - 0.356)
Blast in BM (%)	1%	79.8 (70.7 - 98.0)	97 (95 - 100)
Spleen weight (g)	0.09 - 0.17	0.35 (0.21 - 0.41)	0.68 (0.48 - 1.4)

* n=5, # n=7
WBC: white blood cells, RBC: red blood cells, HGB: hemoglobin, HCT: hematocrit, BM: bone marrow.

	Blood	Blood	Blood			
	anemia Hb (mmol/L)	**trombopenia PLT (x10⁹/L)**	**leucopenia WBC (x10⁹/L)**	**Bone marrow morphology**	**cytogenetics**	**conclusion**
	mild 7.3	yes 111	no 5.8	mild dysplasia, no MDS	no abnormalities	mild BMF
	mild 7.7	yes 67	yes 2.9	mild dysplasia, no MDS	no abnormalities	mild BMF
	mild 7.2	yes 43	no 5.3	mild dysplasia, no MDS	no abnormalities	mild BMF
	mild 7.1	yes 62	no 6.8	mild dysplasia, no MDS	no abnormalities	mild BMF
	yes 6.1	yes 30	yes 3.0	dysplasia[4]	no abnormalities	progressive BMF
	mild 7.3	yes 133	yes 3.0	mild dysplasia[4]	no abnormalities	progressive BMF
	mild 8.3	yes 21	yes 2.5	not available[5]	no abnormalities	Severe BMF§
	not available	not available	not available	not available	not available	Severe BMF§

6

Summary and General Discussion

1. SUMMARY

This thesis contains two main experimental parts. In the first part we asked whether *Dicer1*, the gene encoding a crucial RNase III enzyme for microRNA (miRNA) maturation, is essential for normal myelopoiesis. In Chapter 3, we addressed this question by deletion of *Dicer1* in murine C/EBPα-positive myeloid-committed progenitors. In striking contrast to the results in hematopoietic stem cells (HSCs), miRNA depletion in myeloid-committed progenitor did not significantly affect the number of multipotent progenitors (MPPs), common myeloid progenitors (CMPs), megakaryocyte/erythroid progenitors (MEPs) and granulocyte/monocyte progenitors (GMPs) in mice. However, *Dicer1*-deficient GMPs were defective in myeloid development and exhibit an increased self-renewal potential *in vitro*. In mice, *Dicer1* deletion in myeloid committed progenitor cells blocked monocytic differentiation, depleted macrophages and myelo-dendritic cells (DCs), and caused myeloid dysplasia with morphological features of Pelger-Huet anomaly, a benign hematopoietic disorder leading to defect of terminal neutrophil differentiation [1]. MiRNA profiling of wild type GMPs showed that 104 miRNAs are abundantly expressed at this stage. In *Dicer1*–null GMPs, predicted targeted transcripts of at least 20 miRNA families were significantly higher expressed, suggesting that these miRNAs are active in GMPs. Interestingly, of the de-repressed miRNA targets in *Dicer1*-null GMPs 27% are normally exclusively expressed in HSCs or are specific for MPPs and erythropoiesis. Thus, unlike the results from HSCs and other hematopoietic cell fractions (reviewed in Chapter 4) showing functions of *Dicer1* mainly in survival pathways, these results provide evidence for a miRNA-controlled switch of a stem cell program of self-renewal and expansion, towards myeloid differentiation.

The depletion of miRNAs caused by deletion of *Dicer1* in C/EBPα positive myeloid progenitor cells blocked monocytic/macrophage differentiation and resulted in production of dysplastic myeloid cells, a condition that may be considered as a preleukemic state. However *Dicer1*-null myeloid progenitors did not develop leukemia in mice after 1 year of monitoring, indicating that total depletion of miRNAs does not lead to leukemia. In agreement, in Chapter 4, we showed that homozygous *Dicer1* deletion did not collaborate with loss of *Trp53* in leukemic progression. In striking contrast, mice transplanted with heterozygous *Dicer1* and *Trp53*-null alleles developed frank leukemias. Thus, we provided evidence for a model in which *Dicer1* heterozygosity, causing reduced levels of miRNAs, is an oncogenic event in the leukemia development, but expression of at least some miRNAs is needed for leukemogenesis.

In the second experimental part of this thesis, we addressed the question whether deregulation of miRNA expression upon interstrand cross-link (ICL) DNA damage could be involved in bone marrow failure (BMF) and leukemia. In Chapter 5, we showed that 2 miRNAs, *miR-139-3p* and *miR-199a-3p*, play a key role in BMF and leukemic transformation. Hematopoietic progenitors from FA patients and from mice deficient for ERCC1, an endonuclease functioning downstream of FA pathway and essential for ICL repair, expressed elevated levels of *miR-139-3p* and *miR-199a-3p* during BMF. *MiR-139-3p* induced cell death

and its expression declined during leukemic progression, whereas *miR-199a-3p* expression remained high and induced acute myeloid leukemia (AML) in mice. Inactivation of *miR-139-3p* expressed in *Ercc1*-deficient hematopoietic stem and progenitor cells (HSPCs) restored their reduced proliferative capacity, whereas inhibition of *miR-199a-3p* further aggravated the proliferation defect of *Ercc1*-deficient HSPCs. Combined transcriptome and proteome analyses to identify targets revealed that *miR-139-3p* controls the expression of the RNA binding protein (RBP) HUR, whereas *miR-199a-3p* inhibits the expression of *SUZ12* and *RUNX1*, genes that are involved in human AML. These findings uncover a major involvement of *miR-139-3p* and *miR-199a-3p* in the pathogenesis of ICL-induced BMF and leukemic transformation.

2. GENERAL DISCUSSION

2-1. MiRNAs involved in normal myeloid development

In Chapter 3, we showed that miRNAs are required for normal myelopoiesis. Disruption of their biogenesis by deletion of *Dicer1* in myeloid progenitors blocks monocyte differentiation and leads to neutrophil dysplasia. The consequences of *Dicer1* deletion at different stages of hematopoiesis and the role of *Dicer1* in leukemia development are discussed in detail in Chapter 4. A limitation of the *Dicer1*-deletion model is that it disrupts the biogenesis of virtually all miRNAs, which prohibits investigation of the role of individual miRNAs. Strategies to unravel the contribution of single miRNAs to myelopoiesis are discussed in sections 2-6-1 and 2-6-2.

2-2. ICL-induced upregulation of *miR-139-3p* and *miR-199a-3p*

In Chapter 5, we showed that expression of *miR-139-3p* and *miR-199a-3p* is enhanced upon ICL DNA damage in HSPCs. *MiR-199* is a vertebrate specific miRNA family containing 3 paralogs known as *miR-199a-1*, *miR-199a-2* and *miR-199b*, localized on human chromosomes 19, 1 and 9, and mouse chromosomes 9, 1 and 2 respectively [2]. All *miR-199* genes are located on the opposite strand in introns of *Dynamin* (*Dnm*) genes and are expressed independently of *Dnm* transcription, indicating that they are controlled by independent promoters [2]. Within *Dnm3*, *miR-199a* is coexpressed with *miR-214* as a common primary 6-kb transcript in mouse, human and zebrafish [3]. *MiR-139* is an intronic miRNA, located on the sense strand of the *PDE2A* gene on chromosome 11 in human and chromosome 7 in mouse (miRBase, www.mirbase.org), within a highly conserved sequence. The molecular mechanism driving *miR-139-3p* and *miR-199a-3p* expression upon ICL DNA damage is still elusive. The elevated levels of *miR-139-3p* and *miR-199a-3p* could be the consequence of transcriptional upregulation, modified pri-miRNA processing, increased stability of the mature miRNA or combinations of these. In order to investigate the possibility of the transcriptional upregulation, the expression of host genes as well as co-expressed miRNAs were analyzed. *Dnm2*, *Dnm3* or *Pde2a* were not differentially expressed in *Ercc1*$^{-/*292}$ HSPCs compared to controls (Chapter 5, supplementary Table 2). *MiR-199a-5p* was not expressed

in HSPCs of *Ercc1* deficient or proficient mice. *MiR-214* and *miR-139-5p* were expressed in HSPCs but their expression levels were not increased upon ICL stress. These data suggest that the expression of *miR-199a-3p* and *miR-139-3p* is not induced at the transcriptional level. TP53 has been reported to bind to endonuclease DROSHA and to facilitate the processing of primary miRNAs to precursor miRNA [4,5]. Although we have shown that *miR-199a-3p* and *miR-139-3p* are not direct targets of Trp53 (Chapter 5), our data do not rule out that these miRNAs are upregulated as a result of Trp53-induced post-transcriptional regulation. Finally, the upregulation of *miR-199a-3p* and *miR-139-3p* could be the consequence of miRNA modifications that may influence the stability of the miRNA. For instance, adenylation or 2'-O-methylation of the 3'-end of miRNA have been reported to increase miRNA stability [6,7]. Several enzymes such as GLD2, TUT4 and XRN2 are implicated in these modifications [8]. These enzymes were not differentially expressed between HSPCs of *Ercc1*-deficient and proficient mice (Chapter 5, supplementary Tables 2 and 3), but their activity may be different upon ICL damage. Therefore, post-transcriptional modification may still be involved in the upregulation of *miR-199a-3p* and *miR-139-3p*.

2-3. Target of *miR-139-3p*

Using a combination of gene expression arrays and proteomics, we identified *Elavl1* (HuR) as a target of *miR-139-3p* in HSPCs upon ICL-induced stress (Chapter 5). HuR is a member of the embryonic lethal abnormal vision Drosophila-like ELAV family of RBP [9]. HuR elicits a broad anti-apoptotic function by regulating the expression of many target RNAs [10]. Relevant for our study is that HuR controls the expression of genes involved in DNA damage response, including the tumor suppressor *p53*, *cyclins A*, *B1* and *D1*, proto-oncogenes *c-Fos* and *c-Myc*, cyclin-dependent kinases inhibitors *p21* and *p27*, and anti-apoptotic *Bcl-2* [11]. Reduction of HuR levels decreases cell expansion and survival of hematopoietic progenitors by downregulation of survival factors, such as BCL-2, BCL-XL and Survivin, whereas apoptotic inducers, such as CASPASE-9, and the TP53 targets NOXA and PUMA are upregulated [12]. Thus, repression of HuR may well explain all the observed effect of *miR-139-3p* on cell growth.

HuR also influences miRNAs activities, in both positive and negative ways. HuR may interact with mRNA targets of miRNAs. Binding of HuR near a miRNA recognition sequence may interfere or stimulate the miRNA binding. For example, HuR binding to AU-rich elements on 3'-UTR of *CAT1* mRNA, blocks the complementary site for *miR-122* and thereby relieves the mRNA from repression [13]. However, HuR may also stimulate miRNA activity by recruiting miRNAs to the 3'-UTRs of target genes. For instance, HuR promotes the interaction between *Let-7* and the *c-Myc* 3'-UTR, and thereby represses the expression of *c-Myc* [14].

2-4. Targets of *miR-199a-3p*

We identified *Suz12* as a target of the ICL-induced *miR-199a-3p*. SUZ12 is a critical component of the Polycomb Repressive Complex 2 (PRC2), which is responsible for di- and tri-methylation of Histone 3 at lysine 27 (H3K27) [15]. SUZ12 is required for histone methyltransferase activity

and gene silencing functions of PRC2 [16]. PRC2 consists of multiple proteins, including the methyltransferase enhancer of Zeste 2 (EZH2) and the Embryonic Ectoderm Development protein (EED), and interacts with Addition of Sex Combs-Like 1 (ASXL1). Each component of the PRC2 complex is essential for mammalian development [17-19]. Furthermore, members of the PRC2 complex are frequently mutated in cancers including hematological neoplasms [20]. Inactivating *EZH2* mutations are found in approximately 12% of myelodysplastic syndrome (MDS)/ myeloproliferative neoplasm (MPN) patients [21]. Also, mutations and deletions of *SUZ12* have been identified at a low frequency in myeloid neoplasms [22].

In our experiments, EZH2 levels were also decreased in *miR-199a-3p* overexpressing human AML cell line (HL-60) and primary mouse leukemia cells (Figure 1A and 1B), which is consistent with previous reports showing that SUZ12 stabilizes other PRC2 components [18]. Moreover, it has been reported that a heterozygous mutation in *Suz12* (*Suz12*$^{Plt8/wt}$) enhances expansion of HSPCs without impairing their differentiation capacity [23]. Thus, the repression of *Suz12* by *miR-199a-3p* may in part explain the enhanced expansion of HSPCs.

Peroxiredoxin-6 (*PRDX6*) and Paraoxonase-2 (*PON2*) are two other targets of *miR-199a-3p* that were identified in our study. PRDX6 is a member of the thiol-specific antioxidant protein family [24]. This protein is a dual function enzyme with peroxidase and phospholipase activities [24]. PRDX6 is involved in redox regulation of the cell by reduction of hydrogen peroxide and short chain organic, fatty acid and phospholipid hydroperoxides [24]. *PON2* encodes a member of the paraoxonase gene family, which includes three known paraoxonases located adjacent to each other on the long arm of chromosome 7. PON2 is ubiquitously expressed in human tissues, is membrane-bound, acts as a cellular antioxidant protecting cells from oxidative stress and prevents the cell-mediated oxidation of LDL [25]. Moreover, the

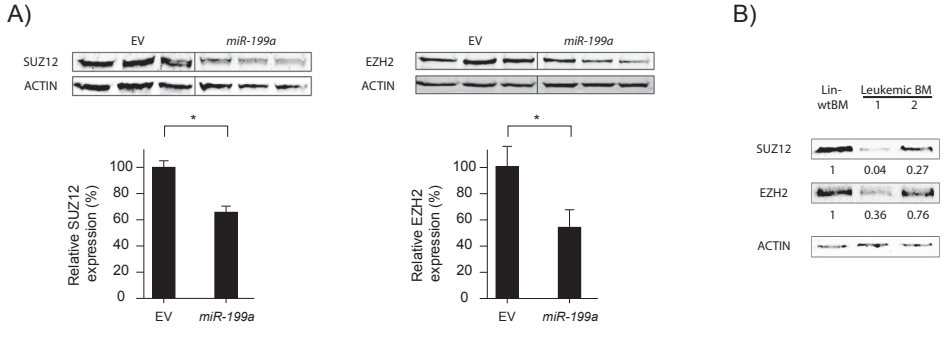


Figure 1. *MiR-199a* represses endogenous SUZ12 and EZH2 expression in human HL60 and mouse *Cebpa*-null leukemic cells. A) Protein levels of SUZ12 and EZH2 decreased significantly upon introduction of *miR-199a* in HL-60 human AML cell lines. The normalized quantifications are shown in bar plots. Significance were calculated with student t-test [2-tailed]) (* $p<0.05$). **B)** Protein levels of SUZ12 and EZH2 in the lineage negative BM cells of leukemic mice, transplanted with *miR-199a* expressing *Cebpa*-null cells, or in the lin- BM of wt control mice are shown (the mouse model is explained in Chapter 5).

overexpression of PON2 decreased the oxidative stress in the cells which were treated with hydrogen peroxide or oxidized phospholipids [26]. Thus, PRDX6 and PON2 play important roles in antioxidant stress responses. Oxidative stress, defined as an imbalance between the production and elimination of reactive oxygen species (ROS) is considered as an important pathogenic factor in BMF and leukemia progression in FA patients [27]. Therefore, the repression of PRDX6 and PON2 by *miR-199a-3p* may play a crucial role in the incapacity of FA-HSPCs to cope with oxidative stress and may contribute to the onset of BMF and its progression towards leukemia.

2-5. Expression and prognostic significance of *miR-139-3p* and *miR-199a-3p* in human AML

Having established that *miR-199a-3p* is an onco-miR in mouse myeloid leukemia and that *miR-139-3p* expression is lost in *Ercc1*$^{-/*292}$*; Trp53*-deficient leukemias (Chapter 5), we asked whether the combined absence of *miR-139-3p* and enhanced expression of *miR-199a-3p* is a more common feature of AML. Two unique cell lines, SB1690CB and FA-AML1, both characterized by bi-allelic *BRCA2/FANCD1* mutations, have previously been established from FA/AML patients [28,29]. Similar to mouse *Ercc1*$^{-/*292}$ leukemia, *miR-139-3p* was not detectable in these cell lines, whereas *miR-199a-3p* expression was elevated compared with normal BM CD34+ cells (Figure 2A).

We analyzed the expression of *miR-199a-3p* and *miR-139-3p* in 177 clinical AML samples randomly picked from our biobank. Expression of *miR-139-3p* was undetectable in 175 and very low in the remaining 2 samples, whereas the U6 loading control was detected at high levels in all samples (Ct=20.6 +/- 1.1) (Table 1). Analysis of RNA-seq data of 275 AML samples from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/) confirmed that *miR-139-3p* is not or very lowly expressed in AML (data not shown). *MiR-199a-3p* expression was variable in our biobank samples (Table 1, Figure 2B) and in the TCGA cases (data not shown). No significant correlation was seen between *miR-199a-3p* expression and the most frequent cytogenetic markers (t(8;21), t(15;17), inv(16) and 11q23) and molecular abnormalities. We then divided the 177 AML cases from our biobank in two groups based on the expression level of *miR-199a-3p*, i.e., above and below the median expression. AML cases with high *miR-199a-3p* expression were significantly enriched among AML patients lacking mutations in *NPM1*, *DNMT3A* and *IDH2* (Table 1). Kaplan-Meier survival analysis showed that AML patients with high *miR-199a-3p* expression had a significantly shorter overall survival ($p < 0.05$) than patients with low or no *miR-199a-3p* expression (Figure 2C).

Next, we performed a multivariate survival analysis with the Cox proportional hazard model considering the following variables: favorable karyotype (i.e. t(15;17), inv(16), t(8;21)), *FLT3-ITD*, *FLT3-TKD*, *NPM1* mutation, *CEBPA* double mutants, *IDH1* mutation, *IDH2* mutation, *DNMT3A* mutation, age (continuous), white blood cell count (continuous), abnormalities involving *MLL* and *miR-199a-3p* (dichotomized on the median expression level). Variables were included based on a step-wise manner using the Akaike Information Criterion (AIC) [30]. We found that high *miR-199a-3p* expression is an independent marker for

adverse overall survival (p=0.022, hazard ratio (HR)=1.66) (Table 2). Increased expression of *miR-199a* has been associated with poor prognosis in AML [31]. Our study showed that high *miR-199a-3p* expression, but not *miR-199a-5p*, has prognostic significance in AML.

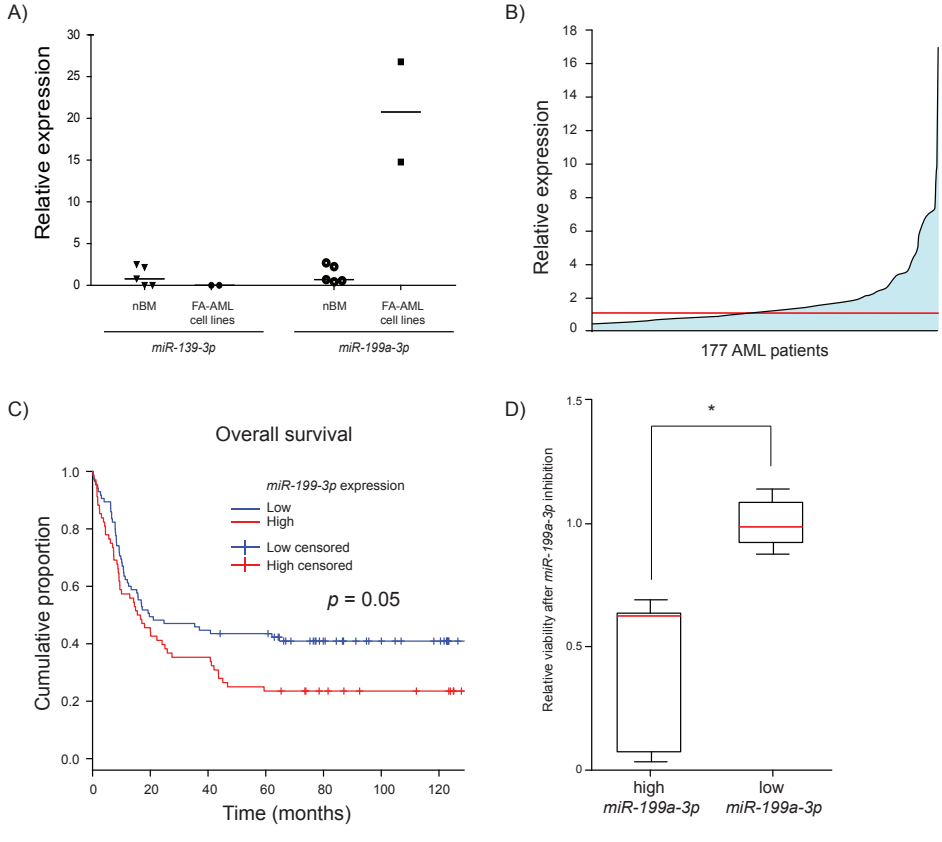


Figure 2. Expression and prognostic significance of *miR-199a-3p* in human AML. A) Expression of *miR-139-3p* and *miR-199a-3p* normalized to *snU6* in BM CD34+ cells isolated from healthy individuals (nBM) and FA-AML cell lines SB1690CB and FA-AML1 relative to the average of nBM samples are shown. Bars show the median of each group. **B)** *MiR-199a-3p* expression in AML samples relative to *U6* (n=177) are plotted. The relative expression levels are normalized to the median level as indicated by the red line. **C)** Kaplan-Meier curves of overall survival of AML patients with high (red) or low (blue) expression of *miR-199a-3p*. The log-rank test was used to calculate the significance of the difference between survival curves. **D)** The viability of BM cells obtained from AML patients with high (n=7 samples) and low (n=5 samples) *miR-199a-3p* expression, after *miR-199a-3p* inhibition with antagomiRs and relative to the viability value of scrambled control antagomiR treated cells is shown. Cell viability was assessed using the CellTiter-Glo luminescent cell viability assay (Promega), 24 hours post transfection of LNA antagomiRs, according to manufacturer's protocol. The transfection efficiency of at least 50% was confirmed by flow cytometry. The red line indicates the median values of each group.

Table 1. Clinical, cytogenetic and molecular characteristics of the cohort of newly-diagnosed AML

	Low *miR-199a-3p*-expressing patients*		High *miR-199a-3p*-expressing patients*		*P*
Sex (N = 177), no. (%)					0.0881†
Male	46	(50%)	47	(50%)	
Female	43	(51%)	41	(49%)	
Age (N = 177), y					0.8223‡
Median (range)	49	(15-72)	48	(17-77)	
Transplantation status					0.3338§
Allogeneic transplantation	31		22		0.1895†
Autologous transplantation	9		12		0.4952†
none	49		54		0.4471†
FAB classification					0.0204§
M0	3		1		-
M1	22		14		0.1910†
M2	13		31		0.0017†
M3	0		4		0.0590†
M4	17		16		1†
M5	26		15		0.0743†
M6	1		0		-
Secondary AML (RAEB-t)	5		5		1†
Not determined	2		2		-
Cytogenetic abnormalities					
t(15;17)	0	(0%)	4	(4.5%)	0.0590†
t(8;21)	2	(2.2%)	6	(6.8%)	0.1684†
Inv.(16)	3	(3.4%)	9	(10.2%)	0.0805†
11q23	3	(3.4%)	8	(9.1%)	0.1322†
3q	4	(4.5%)	3	(3.4%)	1†
7q	5	(5.6%)	6	(6.8%)	0.7664†
t(6;9)	0	(0%)	2	(2.3%)	0.2458†
t(9;22)	3	(3.4%)	1	(1.1%)	0.6207†
t(9;11)	1	(1.1%)	3	(3.4%)	0.6203†
+8	4	(4.5%)	9	(10.2%)	0.1620†
-5/-5q/-7/-7q	6	(6.7%)	5	(5.7%)	1†
Complex	2	(2.2%)	5	(5.7%)	0.2778†
Normal karyotype	51	(57.3%)	32	(36.4%)	0.0067†
Other	16	(18.0%)	16	(18.0%)	1†

Table 1. Clinical, cytogenetic and molecular characteristics of the cohort of newly-diagnosed AML (*Continued*)

	Low *miR-199a-3p*-expressing patients*		**High *miR-199a-3p*-expressing patients***		***P***
Other molecular abnormalities					
FLT3-ITD	28	(31.5%)	24	(27.3%)	0.6212†
FLT3-TKD	10	(11.2%)	8	(9.1%)	0.8945†
NPM1	40	(44.9%)	22	(25%)	0.0072†
DNMT3A	29	(32.6%)	15	(17.0%)	0.0230†
CEBPA_double	4	(4.5%)	7	(8.0%)	0.3708†
CEBPA_silenced	5	(5.6%)	1	(1.1%)	0.2108†
IDH1	6	(6.7%)	8	(9.1%)	0.5909†
IDH2	15	(16.9%)	3	(3.4%)	0.0049†
NRAS	10	(11.2%)	10	(11.2%)	1†
KRAS	1	(1.1%)	0	(0%)	-
EVI1 overexpression	10	(11.2%)	11	(12.5%)	0.8204†
ASXL1	2	(2.2%)	6	(6.8%)	0.1685†

FAB, French-American-British.*Cutoff used for high and low *miR-199a-3p* expression was the median of all AML. †*P* values are based on Fisher exact tests; ‡*P* value is based on Mann-Whitney *U* tests; §*P* values are based on χ^2 tests.

Finally, we investigated the functional significance of enhanced *miR-199a-3p* levels in AML cells (Table 3). Inhibition of *miR-199a-3p* by *antagomiR-199a-3p* significantly decreased the viability of AML blasts with high *miR-199a-3p* expression, but had no effect on AML samples with low *miR-199a-3p* levels (Figure 2D). These results indicate that *miR-199a-3p*, when aberrantly expressed, is critical for survival of AML blasts.

2-6. Future directions

2-6-1. MiRNA add-back

In Chapter 3, *in silico* analyses of gene and miRNA expression profiles suggest that a set of 20 miRNAs is highly active in GMPs. Different approaches could be applied to investigate the contribution of each single miRNA to myelopoiesis. Adding-back of miRNAs in *Dicer1*-null progenitor cells is one of them. To investigate the function of individual miRNAs in *Dicer1*-null cells, pre-miRNAs need to bypass the *Dicer1*-dependent processing. For this, we can employ the alternative processing mechanism of *miR-451*. *MiR-451* is the only miRNA reported to be *Dicer1*-independent and is processed by Ago-2 instead [32,33]. The structure of the pre-*miR-451* hairpin differs from other miRNAs. The mature *miR-451* sequence is extended to the loop of the hairpin. Furthermore, the length of the hairpin stem is shorter compared with other pre-miRNAs. These differences lead to a non-canonical processing mechanism of *miR-451* [32,33]. Testing structural mimics of *miR-451*, showed that for the

Table 2. High *miR-199a-3p* expression is an independent prognostic factor for overall survival in AML based on the multivariate Cox regression hazard model.

Marker	Hazard ratio	Lower bound 95% CI	Upper bound 95% CI	P-value
NPM1	0.253944	0.15408	0.4185	7.60E-08
CEBPA_DM	0.176413	0.06223	0.5001	0.0011
FLT3_ITD	1.648418	1.05903	2.5658	0.02683
Age*	1.016535	1.00257	1.0307	0.02018
Wbc*	1.008561	1.00445	1.0127	4.40E-05
favo	0.412988	0.21487	0.7938	0.00799
abn11q23	0.456555	0.19724	1.0568	0.06711
miR-199a-3p	1.66494	1.07484	2.579	0.02242
IDH2	1.625292	0.85445	3.0915	0.13874

For the construction of the multivariate Cox proportional hazard model the following variables were considered: favorable karyotype (i.e. t(15;17), inv(16), t(8;21)), FLT3-ITD, FLT3-TKD, NPM1 mutation, CEBPA double mutants, IDH1 mutation, IDH2 mutation, DNMT3A mutation, age, white blood cell count (wbc), abnormalities involving MLL , *miR-199a-3p* (dichotomized on the median expression level).
* Continuous variable

Table 3. Characteristics of patients in viability test.

	Low *miR-199a-3p**	High *miR-199a-3p**
Sex		
Male	2	2
Female	3	5
Age		
Median (range)	43 (38-54)	54 (31-68)
WBC (x10 ^{9} /L)		
Median (range)	84 (5,8-263,4)	21,5 (2-37,1)
Transplantation status	.	.
No transplantation	3	2
Autologous transplantation	0	1
Allogenic transplantation	2	4
FAB classification	.	.
M0	0	0
M1	2	2
M2	0	3
M3	0	0
M4	1	1
M5	1	1
M6	0	0

Table 3. Characteristics of patients in viability test. (*Continued*)

	Low *miR-199a-3p**	High *miR-199a-3p**
Secondary AML (RAEB-t)	1	0
Other/unknown	0	0
Cytogenetic abnormalities		
t(15;17)	.	.
t(8;21)	.	.
inv(16)	.	1
11q23	1	.
3q	.	.
7q	.	.
t(6;9)	.	.
t(9;22)	.	.
t(9;11)	1	.
ł8	.	2
-5/-5q/-7/-7q	.	.
complex	.	1
Normal karyotype	3	3
other	1	1
Other molecular aberrations		
FLT3ITD	3	1
FLT3TKD835	.	2
NPM1 mutation	1	4
NRAS	.	1
KRAS	.	.
CEBPA_silenced		.
CEBPA_SM	1	
CEBPA_DM	.	.
EVI1_overexpression	1	.
ASXL1	.	.
IDH1	.	2
IDH2	.	.
DNMT3A_mut	.	2

WBC indicates white blood cell count; and FAB, French-American-British.
*Cutoff used for high and low miR-199a-3p expression was the median of all AML.

alternative Ago2-dependent miRNA biogenesis pathway, the structure, but not the sequence of *miR-451* is required [32,33]. In other words, by replacing the mature sequence of *miR-451* in the *miR-451* precursor structure with sequences encoding other miRNAs, it is possible to generate miRNAs in a *Dicer1*-independent manner. We used this approach to produce *Let-7g* miRNA in *Dicer1*-null cells (Figure 3). *Let-7g* was one of the miRNAs identified to

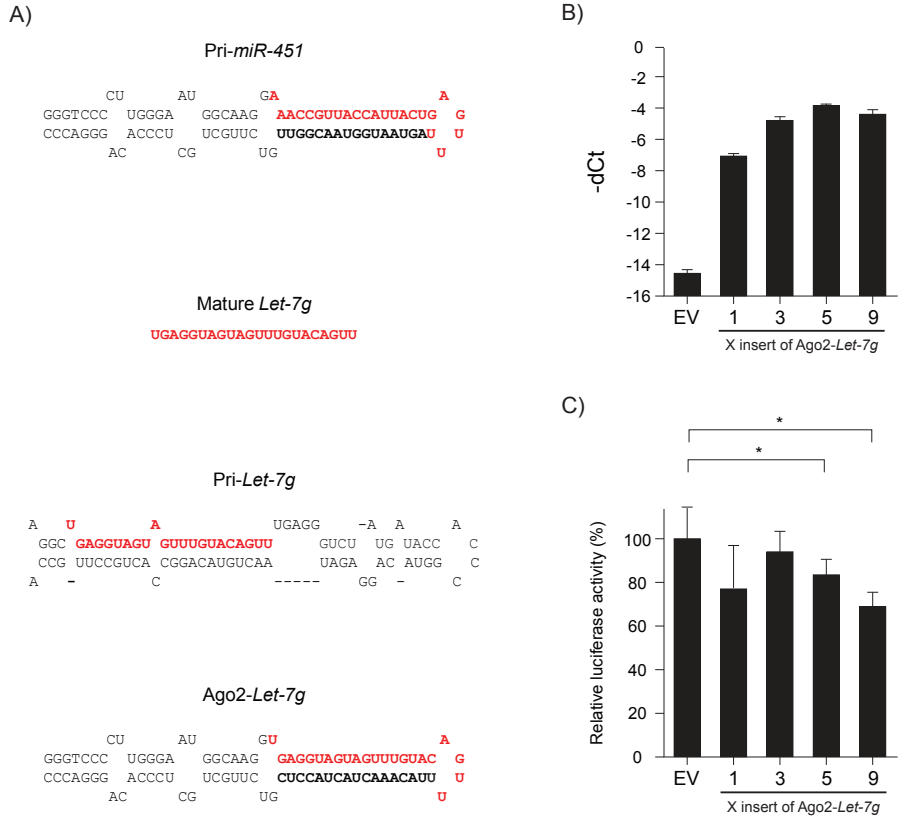


Figure 3. Production of *Dicer1*-independent *Let-7g*. **A)** Sequence and secondary hairpin structure of Pri-*miR-451*, Pri-*Let-7g* and designed Ago2-*Let-7g* hairpin, and mature *Let-7g* sequence. The sequence of the mature miRNA is depicted in red. The modified nucleotides of Pri-*miR-451* in order to make Ago2-*Let-7g*, without changing the hairpin structure, are shown in bold. **B)** Relative expression levels of *Let-7g* mature transcripts in *Dicer1* knockout murine mesenchymal stem cells (MSCs) after transduction of the retroviral expression vector (MSCV-BC-miRNA) containing 1, 3, 5 or 9 inserts of Ago2-*Let-7g*. Expression is presented as –dCt values measured by quantitative RT–PCR using *snU6* RNA as loading control. The error bars represent standard deviations (SD) of n=3 experiments. **C)** Luciferase reporter plasmids containing 3 binding sites for *Let-7g* behind the luciferase coding sequence were generated. The luciferase activity values of the cells co-expressing luciferase reporter plasmid and MSCV-BC containing 1, 3, 5 or 9 inserts of Ago2-*Let-7g* relative to the EV control are shown. Error bars represent the SD of n=5 experiments. Data were analyzed by student t-test [2-tailed]) (* $p<0.05$).

be active during myelopoiesis. Interestingly, HMGA2 and HOXA9, both known targets of *Let-7g* involved in the stemness of HSC, were de-repressed and upregulated in *Dicer1*-null GMPs and may explain the enhanced self-renewal capacity of *Dicer1*-null progenitors. We replaced the sequence of *miR-451* by *Let-7g* and mimicked the pre-*miR-451* structure and length (Ago2-*Let-7g*) (Figure 3A) and cloned the Ago2-*Let-7g* sequence in an expression vector (MSCV-BC) [34]. MiRNA q-PCR showed that mature *Let-7g* miRNA is produced in *Dicer1* knockout murine mesenchymal stem cells (MSCs) from this vector (Figure 3B). Also, Ago2-*Let-7g* was active in luciferase reporter assay (Figure 3C). Thus, these results show that this expression system can be applied to add back single miRNAs in *Dicer1*-null myeloid progenitor cells.

2-6-2. Inactivation of single miRNAs

Another approach to address the involvement of single miRNAs in myelopoiesis is to inactivate single miRNAs, or miRNA family, in myeloid progenitors. Gene-targeting methods enable us to delete miRNA encoding regions in the genome and investigate the consequences on cell behavior. The so-called clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system has been shown to facilitate RNA-guided site-specific (sgRNAs) DNA cleavage in human and mouse cell lines, mouse ES cells and even in one-cell embryos with high efficiency [35,36]. The double strand breaks generated will then be repaired by error prone non-homologues end joining or homology-directed repair (HDR) pathways, resulting in mutant cells carrying deletions or defined alterations at the cut sites [37]. To show the feasibility of this technology to delete miRNA encoding region in the genome of hematopoietic cells, we deleted the coding region of *miR-139*, in the murine myeloid progenitor cell line 32D (Figure 4). To reduce off-target activity, we used a combination of a new mutant of Cas9, the so-called Cas9 nickase (Cas9n), with paired guide RNAs to introduce targeted double strand breaks (Figure 4A) [37]. With this approach we were able to knockout a 1 kb fragment containing the full *miR-139* coding sequences in 32D cell line as confirmed by Sanger sequencing (Figure 4B and 4C). These results indicate that we are able to generate miRNA knockout cells by CRISPR and that the sgRNA combination can be used to generate *miR-139* knockout mouse embryos. The recently generated Cas9 knock in mouse allows wide range application of CRISPR technology for genome editing *in vivo* by tissue specific delivery of sgRNAs [38]. In this way, by expressing a library of sgRNAs against a set of miRNA encoding loci in HSPCs of Cas9 knock in mice, and subsequently transplantation of these cells in recipient mice, we can investigate the functions of single miRNAs in myelopoiesis.

2-6-3. Identification of miRNA targets

MiRNA target identification and elucidation of critical downstream pathways are still major challenges in the field. MiRNAs are predicted to control many target mRNAs, most of which are only moderately repressed. Slight repression of multiple genes that act in the same network may have significant effects together on cellular processes. Detection of these slight expression differences needs accurate experimental approaches followed by

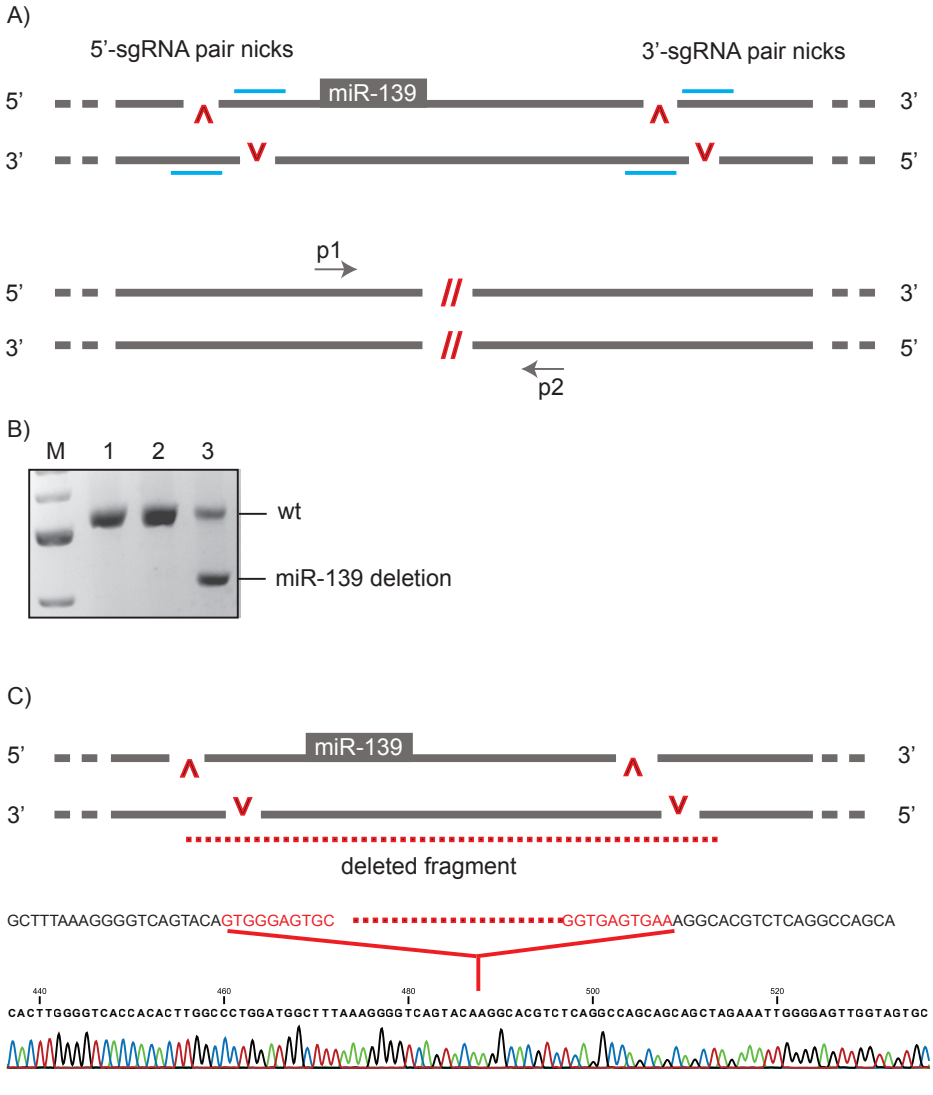
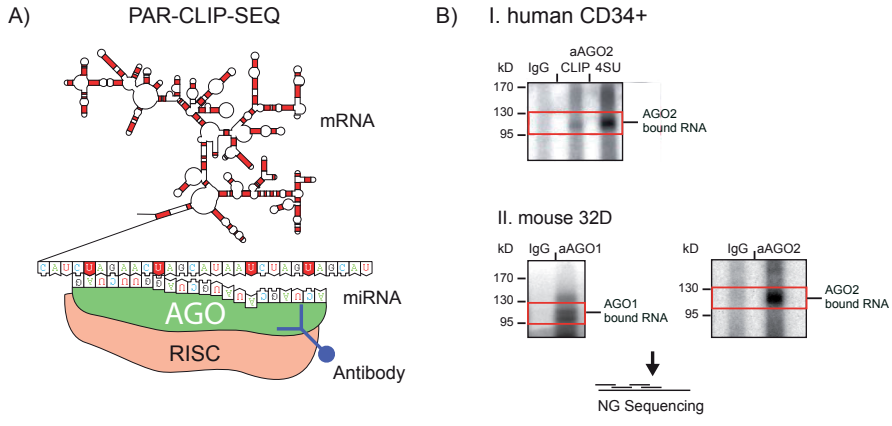


Figure 4. Double nicking with CAS9n facilitates genomic *miR-139* deletion. A) Schematic overview of CAS9n-mediated targeting of *miR-139*. sgRNAs are depicted in blue. The nicking sites are depicted in red. Genomic deletions will be determined by PCR. **B)** 32D cells were transfected with plasmids expressing the *miR-139*-locus specific sgRNAs and CAS9n by electroporation. Genomic DNA from the transfected 32D cell subpopulations was used as a template for PCR primer 1 and primer 2 (A). Samples 1 and 2 are control populations showing only a large wt band. Sample 3 contains *miR-139* knockout cells as determined by the lower band. **C)** The *miR-139* deletion band was isolated out of the gel, cloned in TOPO-PCR cloning vector. The expected genomic deletion was confirmed by Sanger sequencing.



C)

3'UTR *EDEM3* — # reads — T->C

miR-10a-3p

```
CTTATAATTTTAGAGGGGGGTAGAATTTAGTAAATATT
..........TAGAGGGGGGTAGAATTC..........      3      1
..........CAGAGGGGGGTAGAATTT..........     17      1
..........TAGAGGGGGGTAGAATTT..........      9      0
...........AGAGGGGGGTAGAATTT..........     10      0
............GAGGGGGGTAGAATTT..........      2      0
..........TAGAGGGGGGTAGA..............      9      0
```

3'UTR *SEMA7A* — # reads — T->C

miR-27a-3p

```
TGTTATAACTTAGGCTAAACTGTGAAAATTCCGCACCCC
.....TAACTTAGGCTAAACTGTGAAAATCCCG......      4      1
......AACTTAGGCTAAACTGTGAAAATCCCG......      8      1
..............CTAAACTGTGAAAACTCCG......     10      1
......AACTTAGGCTAAACTGTGAAAATTCC.......      6      0
```

3'UTR *AHNAK* — # reads — T->C

miR-30a-e-5p

```
TGCATTGACTGCTGAGAGCTCCTGTTTACTAAGCAAGCT
.......ACTGCTGAGAGCCCTGTTTACTAAG......      8      1
.......ACTGCTGAGAGCTCCTGTTTACCAAG......     10      1
```

Figure 5. Photoactivatable ribonucleoside-enhanced cross-linking immunoprecipitation followed by deep sequencing (PAR-CLIP-SEQ). A) Cells will be expanded overnight in expansion medium containing photo-activatable nucleoside 4-thioridine (4SU). 4SU-labeled transcripts (miRNA and target RNA) will be UV-cross-linked to RNA-binding proteins. RNA in immunoprecipitated complexes will be radiolabeled and loaded on gel. **B)** Examples of audiographs of indicated samples and antibodies are shown. Bands were cut from the gel and RNA was isolated and prepared for Illumina RNA deep sequencing. **C)** Examples of identified miRNAs and their targets. The extracted sequence reads were mapped to the human genome and publicly available RNA databases. In red are depicted the T to C transitions caused by 4SU-UV cross-linking. The red line indicates the binding motifs of identified miRNAs.

high sensitivity profiling of the transcriptome or proteome. More direct approaches for miRNA target identification have previously been described. For instance, photoactivatable ribonucleoside-enhanced cross-linking immunoprecipitation followed by deep sequencing (PAR-CLIP-SEQ), allows determination of direct miRNA-bound mRNA targets [39]. In this method, using specific antibodies against AGO proteins, the main components of RISC complex, the cross-linked miRNA and their target sequences will be co-immunoprecipitated and subsequently identified by RNA sequencing [39] (Figure 5).

We applied this method on human CD34+ and murine 32D cells. Cells were labeled with photoactivatable nucleoside 4-thioridine (4SU) overnight in HSPC expansion medium, lyzed and specific antibodies against AGO1 and AGO2, the most abundantly expressed member of AGO proteins in HSPCs, were used (Figure 5). We also used an optimized cDNA library preparing protocol (also called iCLIP) [40] followed by Illumina small RNA deep-sequencing. The extracted sequence reads were mapped to the genome and publicly available RNA databases. The miRNA binding sites (seed matches) on the target mRNAs were determined by bioinformatic analyses. This bioinformatic pipeline allows for identification and quantification of AGO-bound miRNAs, their direct RNA targets and the sites of interaction (Figure 5). In this way, transcripts that are relevant for pathways involved in hematopoiesis, and hematopoietic malignancies can be selected for further investigation.

In Chapter 5, we applied a combination of proteomics and gene expression arrays to identify the targets of *miR-199a-3p* and *miR-139-3p* upon ICL DNA damage. The gene regulation by miRNAs is mediated by translational inhibiton or by promoting mRNA degradation, but the relative contributions of translational inhibition and mRNA degradation on the outcome are still largely unknown. Destabilization of target mRNAs is the predominant reason for reduced protein output [41]. However, transcriptome profiling is not an appropriate method to identify the minority of targets which are only hampered at translation level, but these genes may still be highly relevant. Furthermore, since the final effect of a miRNA on its target gene is a change in protein expression, proteomic approaches are still relevant tools for identification of few targets that are mainly blocked at the translation level. Recent proteomics technologies such as labeling of amino acids with stable isotopes are appropriate methods to identify the change of protein levels upon inhibition or induction of miRNAs [42,43]. Nevertheless, by using this approach, the direct and indirect targets of miRNAs cannot be distinguished. Another pitfall of proteomic approaches is the low sensitivity. This is due to the limited capacity to detect low-abundance peptides. Together, considering the pitfalls of each approach, a combination of PAR-CLIP, proteomics and RNA sequencing protocols could give essential insights into the direct targets regulated by miRNAs.

The presence and activity of the AGO protein as well as other RNA interfering factors such as DICER1 in the cell nucleus, demonstrate that miRNAs may be active in the nucleus [44]. Consistently, certain mature miRNAs have been reported to re-enter the nucleus after the final step of maturation in the cytoplasm [45]. Some recent publications show that miRNAs target nuclear non-coding (nc) RNAs including other miRNA primary transcripts, as well as long ncRNAs [46]. For instance, nuclear located *miR-709* is reported to target pri-*miR-15a/16-1*

and suppress its maturation to pre-*miR-15a* and pre-miR-*16* [47]. Also a circular ncRNA, the antisense transcript of CDR1 is cleaved by nucleus-enriched *miR-671* in an AGO2-dependent manner, which results in the decrease of corresponding CDR1 sense transcript [48]. MiRNAs are also reported to regulate gene expression in hematopoietic cells via transcriptional gene silencing [49,50]. To identify genomic targets of miRNAs, chromatin immunoprecipitation (ChIP) with specific antibodies against AGO protein has been performed. Including antibodies against histone markers of active and silenced chromatin state will unravel the effect of the miRNA on the epigenetic status of the miRNA bound genomic loci [49,50].

2-6-4. Translational relevance and potential of miRNAs in therapeutic settings

Aberrant expression levels of different miRNAs are associated with different stage of onset, progression and outcome of human cancers, including AML [51,52]. These findings suggest that miRNA expression profiles are potential indicators for diagnosis or prognosis. More importantly, identification of miRNA-controlled pathways and studying their roles in AML pathogenesis may lead to recognition of novel intervention points for AML therapy. For instance, functional identification of oncogenic miRNAs in AML may allow therapeutic targeting by using inhibitory small RNAs against these miRNAs or their downstream targets [53]. One recent example of an advanced miRNA therapeutic in human disease is the locked nucleic acid (LNA)-modified anti-*miR-122* inhibitor sequence for treatment of patients with Hepatitis-C virus (HCV) infection [54]. In fact, subcutaneous delivery of the LNA *miR-122* inhibitor effectively suppressed HCV replication in monkeys without evidence of toxicity [55]. The subcutaneous delivery of the *miR-122* LNA inhibitor was then tested in a clinical phase-II study on 36 patients with chronic HCV infection [56]. The results of this study showed an LNA inhibitor dose-dependent reduction in HCV RNA levels without any dose-limiting adverse events [56]. Another recent study show therapeutic potential of *miR-155* inhibition using 8-mer LNA oligonucleotides complementary to the miRNA seed region in the treatment of B-cell leukemia in mice [57]. These results highlight the potential of miRNA inhibitors for therapeutic targeting in hematological malignancies. In Chapter 5, we showed that deregulated expression of *miR-199a-3p*, which is observed at BMF stage of FA, plays a major role in the malignant transformation of BM HSPCs. FA-AML cannot be treated with standard protocols because FA patients do not tolerate high dosages of chemotherapeutic agents [58]. We showed that transfection with antago*miR-199a-3p* did not affect expansion and differentiation of wt HSPCs, suggesting low toxicity of this agent. Thus, the inhibition of the oncogenic *miR-199a-3p* by antagomiRs may be a new opportunity to prevent the transition of BMF towards leukemia.

On the other hand, the fact that global downregulation of miRNAs is observed in many human cancers [59] and that heterozygous deletion of *Dicer1* contributes to leukemogenesis (Chapter 4), indicates that it may be beneficial to deliver tumor-suppressing miRNAs. For instance, recent data showed suppression of non-small cell lung tumor development by re-introduction of *Let-7* in tumor cells [60-62]. Recently, lipid-based nano-particles were used for systemic delivery of tumor-suppressing miRNA expressing vectors or miRNA mimics to

cancer cells and showed efficient elimination of cancer cells in a dose-dependent manner and without detectable toxicity in mouse models for ovarian, pancreatic and lung cancers [63-65]. This liposome technology is now being used to deliver synthetic double strand mimics of *miR-34a* in a phase-I trial in patients with primary liver cancer or metastatic cancer with liver involvement [66]. This suggests that this approach may be successful for AML treatment as well. For example, delivery of *Let-7c* promotes granulocytic differentiation in AML cell lines as well as in primary AML blasts in culture, and could be an interesting approach [67]. The loss of *miR-139-3p* seems to be a common event in the onset of malignant transformation. Therefore, delivering of this miRNA could be of benefit in preventing the outgrowth of leukemic cells.

The increasing knowledge about the functions of miRNAs may open doors for new drug development. Small interfering RNAs may be used to target aberrant factors that control miRNA activities in AML. Up to date, more than 50 RNA-based therapeutics have reached clinical testing and have demonstrated promising results in the treatment of viral infections, genetic disorders and cancers including leukemia [68]. Therefore, *in vivo* experimental models for AML should be generated to enable testing and further developing of small-RNA based therapeutic protocols, which may ultimately lead to a better treatment of this disease.

REFERENCES

1. Cunningham JM, Patnaik MM, Hammerschmidt DE, Vercellotti GM. Historical perspective and clinical implications of the Pelger-Huet cell. *American Journal of Hematology*. 2009;84(2):116-119.
2. Desvignes T, Contreras A, Postlethwait JH. Evolution of the miR199-214 cluster and vertebrate skeletal development. *RNA Biol*. 2014;11(4):281-294.
3. Loebel DA, Tsoi B, Wong N, Tam PP. A conserved noncoding intronic transcript at the mouse Dnm3 locus. *Genomics*. 2005;85(6):782-789.
4. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. Modulation of microRNA processing by p53. *Nature*. 2009;460(7254):529-533.
5. Wang J, He Q, Han C, et al. p53-facilitated miR-199a-3p regulates somatic cell reprogramming. *Stem Cells*. 2012;30(7):1405-1413.
6. Katoh T, Sakaguchi Y, Miyauchi K, Suzuki T, Kashiwabara S, Baba T. Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev*. 2009;23(4):433-438.
7. Li J, Yang Z, Yu B, Liu J, Chen X. Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in Arabidopsis. *Curr Biol*. 2005;15(16):1501-1507.
8. Kai ZS, Pasquinelli AE. MicroRNA assassins: factors that regulate the disappearance of miRNAs. *Nat Struct Mol Biol*. 2010;17(1):5-10.
9. Abdelmohsen K, Pullmann R, Jr., Lal A, et al. Phosphorylation of HuR by Chk2 regulates SIRT1 expression. *Mol Cell*. 2007;25(4):543-557.
10. Abdelmohsen K, Lal A, Kim HH, Gorospe M. Posttranscriptional orchestration of an anti-apoptotic program by HuR. *Cell Cycle*. 2007;6(11):1288-1292.
11. Kim HH, Abdelmohsen K, Gorospe M. Regulation of HuR by DNA Damage Response Kinases. *J Nucleic Acids*. 2010;2010.
12. Ghosh M, Aguila HL, Michaud J, et al. Essential role of the RNA-binding protein HuR in progenitor cell survival in mice. *J Clin Invest*. 2009;119(12):3530-3543.
13. Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell*. 2006;125(6):1111-1124.
14. Kim HH, Kuwano Y, Srikantan S, Lee EK, Martindale JL, Gorospe M. HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev*. 2009;23(15):1743-1748.
15. Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature*. 2011;469(7330):343-349.
16. Cao R, Zhang Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell*. 2004;15(1):57-67.
17. O'Carroll D, Erhardt S, Pagani M, Barton SC, Surani MA, Jenuwein T. The polycomb-group gene Ezh2 is required for early mouse development. *Mol Cell Biol*. 2001;21(13):4330-4336.
18. Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E, Helin K. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *EMBO J*. 2004;23(20):4061-4071.
19. Shumacher A, Faust C, Magnuson T. Positional cloning of a global regulator of anterior-posterior patterning in mice. *Nature*. 1996;383(6597):250-253.
20. Radulovic V, de Haan G, Klauke K. Polycomb-group proteins in hematopoietic stem cell regulation and hematopoietic neoplasms. *Leukemia*. 2012.
21. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet*. 2010;42(8):722-726.
22. Brecqueville M, Cervera N, Adelaide J, et al. Mutations and deletions of the SUZ12

polycomb gene in myeloproliferative neoplasms. *Blood Cancer J*. 2011;1(8):e33.

23. Majewski IJ, Blewitt ME, de Graaf CA, et al. Polycomb repressive complex 2 (PRC2) restricts hematopoietic stem cell activity. *PLoS Biol*. 2008;6(4):e93.
24. Fisher AB. Peroxiredoxin 6: A Bifunctional Enzyme with Glutathione Peroxidase and Phospholipase A2 Activities. *Antioxidants & Redox Signaling*. 2010;15(3):831-844.
25. Rajkovic MG, Rumora L, Barisic K. The paraoxonase 1, 2 and 3 in humans. *Biochem Med (Zagreb)*. 2011;21(2):122-130.
26. Ng CJ, Wadleigh DJ, Gangopadhyay A, et al. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *J Biol Chem*. 2001;276(48):44444-44449.
27. Du W, Adam Z, Rani R, Zhang X, Pang Q. Oxidative stress in Fanconi anemia hematopoiesis and disease progression. *Antioxid Redox Signal*. 2008;10(11):1909-1921.
28. Ikeda H, Matsushita M, Waisfisz Q, et al. Genetic reversion in an acute myelogenous leukemia cell line from a Fanconi anemia patient with biallelic mutations in BRCA2. *Cancer Res*. 2003;63(10):2688-2694.
29. Meyer S, Fergusson WD, Whetton AD, et al. Amplification and translocation of 3q26 with overexpression of EVI1 in Fanconi anemia-derived childhood acute myeloid leukemia with biallelic FANCD1/BRCA2 disruption. *Genes Chromosomes Cancer*. 2007;46(4):359-372.
30. Akaike H. A new look at the statistical model identification. *Automatic Control, IEEE Transactions on*. 1974;19(6):716-723.
31. Garzon R, Volinia S, Liu CG, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood*. 2008;111(6):3183-3189.
32. Cheloufi S, Dos Santos CO, Chong MM, Hannon GJ. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature*. 2010;465(7298):584-589.
33. Cifuentes D, Xue H, Taylor DW, et al. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science*. 2010;328(5986):1694-1698.
34. Meenhuis A, van Veelen PA, de Looper H, et al. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood*. 2011;118(4):916-925.
35. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013;8(11):2281-2308.
36. Wang H, Yang H, Shivalila CS, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;153(4):910-918.
37. Ran FA, Hsu PD, Lin CY, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell*. 2013;154(6):1380-1389.
38. Platt Randall J, Chen S, Zhou Y, et al. CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. *Cell*. 2014;159(2):440-455.
39. Hafner M, Landthaler M, Burger L, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*. 2010;141(1):129-141.
40. Sugimoto Y, Konig J, Hussain S, et al. Analysis of CLIP and iCLIP methods for nucleotide-resolution studies of protein-RNA interactions. *Genome Biol*. 2012;13(8):R67.
41. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*. 2010;466(7308):835-840.
42. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc*. 2009;4(4):484-494.
43. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009;6(5):359-362.

44. Gagnon Keith T, Li L, Chu Y, Janowski Bethany A, Corey David R. RNAi Factors Are Present and Active in Human Cell Nuclei. *Cell Reports*. 2014;6(1):211-221.
45. Hwang HW, Wentzel EA, Mendell JT. A hexanucleotide element directs microRNA nuclear import. *Science*. 2007;315(5808):97-100.
46. Chen X, Liang H, Zhang C-Y, Zen K. miRNA regulates noncoding RNA: a noncanonical function model. *Trends in Biochemical Sciences*. 2012;37(11):457-459.
47. Tang R, Li L, Zhu D, et al. Mouse miRNA-709 directly regulates miRNA-15a/16-1 biogenesis at the posttranscriptional level in the nucleus: evidence for a microRNA hierarchy system. *Cell Res*. 2012;22(3):504-515.
48. Hansen TB, Wiklund ED, Bramsen JB, et al. miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. *EMBO J*. 2011;30(21):4414-4422.
49. Zardo G, Ciolfi A, Vian L, et al. Polycombs and microRNA-223 regulate human granulopoiesis by transcriptional control of target gene expression. *Blood*. 2012;119(17):4034-4046.
50. Benhamed M, Herbig U, Ye T, Dejean A, Bischof O. Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. *Nat Cell Biol*. 2012;14(3):266-275.
51. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.
52. Nana-Sinkam P, Croce CM. MicroRNAs in diagnosis and prognosis in cancer: what does the future hold? *Pharmacogenomics*. 2010;11(5):667-669.
53. Stenvang J, Silahtaroglu AN, Lindow M, Elmen J, Kauppinen S. The utility of LNA in microRNA-based cancer diagnostics and therapeutics. *Semin Cancer Biol*. 2008;18(2):89-102.
54. Elmen J, Lindow M, Silahtaroglu A, et al. Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res*. 2008;36(4):1153-1162.
55. Elmen J, Lindow M, Schutz S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature*. 2008;452(7189):896-899.
56. Janssen HLA, Reesink HW, Lawitz EJ, et al. Treatment of HCV Infection by Targeting MicroRNA. *New England Journal of Medicine*. 2013;368(18):1685-1694.
57. Zhang Y, Roccaro AM, Rombaoa C, et al. LNA-mediated anti-miR-155 silencing in low-grade B-cell lymphomas. *Blood*. 2012;120(8):1678-1686.
58. Kee Y, D'Andrea AD. Molecular pathogenesis and clinical management of Fanconi anemia. *J Clin Invest*. 2012;122(11):3799-3806.
59. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834-838.
60. Kumar MS, Erkeland SJ, Pester RE, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci U S A*. 2008;105(10):3903-3908.
61. Esquela-Kerscher A, Trang P, Wiggins JF, et al. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle*. 2008;7(6):759-764.
62. Trang P, Medina PP, Wiggins JF, et al. Regression of murine lung tumors by the let-7 microRNA. *Oncogene*. 2010;29(11):1580-1587.
63. Goldberg MS, Xing D, Ren Y, Orsulic S, Bhatia SN, Sharp PA. Nanoparticle-mediated delivery of siRNA targeting Parp1 extends survival of mice bearing tumors derived from Brca1-deficient ovarian cancer cells. *Proc Natl Acad Sci U S A*. 2011;108(2):745-750.
64. Pramanik D, Campbell NR, Karikari C, et al. Restitution of tumor suppressor

microRNAs using a systemic nanovector inhibits pancreatic cancer growth in mice. *Mol Cancer Ther*. 2011;10(8):1470-1480.

65. Trang P, Wiggins JF, Daige CL, et al. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther*. 2011;19(6):1116-1122.
66. Bouchie A. First microRNA mimic enters clinic. *Nat Biotech*. 2013;31(7):577-577.
67. Pelosi A, Careccia S, Lulli V, et al. miRNA let-7c promotes granulocytic differentiation in acute myeloid leukemia. *Oncogene*. 2012.
68. Burnett JC, Rossi JJ. RNA-based therapeutics: current progress and future prospects. *Chem Biol*. 2012;19(1):60-71.

Addendum

LIST OF ABBREVIATIONS

4SU	4-thioridine
Ab	antibody
AGM	aorta-gonad-mesonephros
AIC	Akaike information criterion
AML	acute myeloid leukemia
ARE	AU-rich element
BER	base excision repair
BM	bone marrow
BMF	bone marrow failure
CFU	colony-forming unit
CFU-GM	granulocyte-macrophage colony-forming unit
ChIP	chromatin immunoprecipitation
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNN-LOH	copy number-neutral loss of heterozygosity
CNV	copy number variation
CRISPR	clustered regularly interspaced short palindromic repeats
DC	dendritic cell
DDR	DNA-damage response
DN	double-negative
DSB	double strand break
dsRNA	double-stranded RNA
EGFP	enhanced green fluorescent protein
ES cell	embryonic stem cell
EV	empty vector
EYFP	enhanced yellow fluorescent protein
FA	Fanconi anemia

FDR	false discovery rate
FTC	Fanconi anemia transcriptome consortium
G-CSF	granulocyte colony-stimulating factor
GEP	gene expression profiling
GMP	granulocyte monocyte progenitor
HCV	hepatitis-C virus
HDR	homology-directed repair
HSC	hematopoietic stem cell
ICL	interstrand cross-link
IDEA	Ingenuity downstream effect analysis
LNA	locked nucleic acid
LPS	lipopolysaccharide
LSK	lineage negative (Lin-) Sca1+ c-Kit+
LSL	loxp-flanked stop sequence
LT-HSC	long-term hematopoietic stem cell
MCMV	mouse cytomegalovirus
MDS	myelodysplatic syndrome
MEF	mouse embryonic fibroblast
MEP	megakaryocyte-erythroid progenitor
miRNA	microRNA
MMC	mitomycin C
MPN	myeloproliferative neoplasm
MPP	multipotent progenitor
MSC	mesenchymal stem cell
NER	nucleotide excision repair
NK cells	natural killer cells
nt	nucleotide
PAR-CLIP	photoactivatable ribonucleoside-enhanced cross-linking immunoprecipitation
pIpC	polyI:polyC

PMA	phorbol myristate acetate
PRC	polycomb repressive complex
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
RBD	RNA binding domain
RBP	RNA binding protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	reactive oxygen species
SD	standard deviation
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
ST-HSC	short-term HSC
TCGA	the cancer genome atlas
Treg	regulatory T cell
UTR	untranslated region
WES	whole exome sequencing
WT	wild type

NEDERLANDSE SAMENVATTING

Hematopoëse (bloedcelvorming) is een continu proces waarin hematopoëtische stam- en voorlopercellen vermenigvuldigen en differentiëren naar volwassen bloedcellen. Hematopoëse wordt sterk gecontroleerd door een netwerk van groeifactoren en de hematopoëtische niche in het beenmerg. Verstoring van de balans tussen proliferatie, differentiatie en celdood kan leiden tot verschillende hematopoëtische aandoeningen zoals beenmerg falen, gekarakteriseerd door verstoorde bloedaanmaak, of leukemie, gekarakteriseerd door ongecontroleerde expansie van ongedifferentieerde bloedcellen.

MicroRNAs (miRNAs) behoren tot een klasse van niet-coderende RNAs die de expressie van genen reguleren. Dit proefschrift bevat twee experimentele gedeelten. In het eerste deel hebben we de vraag gesteld of *Dicer1*, het gen wat codeert voor het cruciale RNAse III enzym voor het verwerken van langere voorlopersequenties naar miRNAs, essentieel is voor normale myeloïde bloedcelvorming. In hoofdstuk 3 hebben we dit onderzocht door het gen wat codeert voor DICER1 specifiek in myeloïde voorlopercellen te verwijderen. In tegenstelling tot de resultaten in hematopoëtische stamcellen (HSCs) had het verwijderen van het *Dicer1* gen in myeloïde voorlopercellen geen significant effect op het aantal multipotente voorlopercellen (MPPs), algemene myeloïde voorlopercellen (CMPs), megakaryocyte/erythroïde voorlopercellen (MEPs) en granulocyte/monocyte voorlopercellen (GMPs) in de muis. Echter, Dicer1 knockout GMPs waren niet in staat om uit te rijpen tot volwassen myeloïde cellen en hadden een verhoogde zelfvernieuwingscapaciteit. Verwijdering van *Dicer1* in myeloïde voorlopercellen verhinderde de differentiatie van monocyten, macrofagen en dendritische cellen en veroorzaakte myeloïde dysplasie met morfologische kenmerken van de Pelger Huët afwijking (een goedaardige hematopoëtische aandoening wat een defect in neutrofiele differentiatie veroorzaakt). We hebben gevonden dat 104 miRNAs hoog tot expressie komen in normale GMPs. In Dicer-knockout GMPs kwamen voorspelde targets van tenminste 20 miRNA families significant hoger tot expressie, wat sterk suggereert dat deze miRNAs actief zijn in GMPs. Interessant is dat 27% van deze miRNA-gereguleerde transcripten normaal alleen in HSCs tot expressie komen of specifiek zijn voor MPPs en rode bloedcellen. Dus, hoewel de resultaten in HSCs en andere bloedcellen fracties voornamelijk functies van Dicer1 in overlevingsmechanismen laten zien (beschreven in Hoofdstuk 4), hebben wij bewijs verkregen voor een miRNA-gecontroleerde omschakeling van een stamcelprogramma van zelfvernieuwing en celdeling naar myeloïde differentiatie.

In hoofdstuk 4, laten we zien dat totale verlies van *Dicer1* niet samenwerkt met het verlies van het tumor remmende gen P53 in leukemie-ontwikkeling. In plaats daarvan ontwikkelde de muizen die getransplanteerd waren met heterozygote *Dicer1* deletie en P53 nul allelen ernstige leukemie. Deze experimenten lieten zien dat verminderde concentratie van miRNAs een oncogene gebeurtenis is en dat tenminste sommige miRNAs nodig zijn voor het leukemie proces.

In het tweede gedeelte van dit proefschrift hebben we de vraag gesteld of de deregulatie van miRNA expressie als gevolg van DNA-koppelingsschade een rol speelt in beenmerg

falen en leukemie. In hoofdstuk 5 hebben we de activiteiten van 2 miRNAs, *miR-139-3p* en *miR-199a-3p* beschreven. Beiden miRNAs kwamen verhoogd tot expressie in Fanconi anemie. *MiR-139-3p* induceerde celdood en de expressie van *miR-139-3p* was verlaagd in leukemie, terwijl de expressie van *miR-199a-3p* verhoogd bleef en acute leukemie veroorzaakte in de muis. Remming van *miR-139-3p* expressie in *Ercc1*-knockout voorlopercellen herstelde de verminderde proliferatiecapaciteit, terwijl de remming van *miR-199a-3p* het proliferatieve defect van *Ercc1*-knockoutcellen verergerde. Gecombineerde genexpressie- en eiwitanalyse voor de identificatie van targets, onthulde dat *miR-139-3p* de expressie van het RNA-bindend eiwit HUR reguleert, en dat *miR-199a-3p* de expressie van de AML genen *SUZ12* en *RUNX1* remt. Deze bevindingen laten zie dat *miR-139-3p* en *miR-199a-3p* een belangrijke rol spelen in het ziekteverloop van Fanconi anemie en de transformatie naar leukemie.

WORDS OF THANKS

…and that is how another season of my life comes to its end. The realization of this season was not possible without the help and support of many people and it gives me great pleasure in acknowledging them.

I wish to thank, first and foremost, my supervisor: Dear Stefan, many thanks for letting me be a member of your team, for your supervision and for your patience. Your guidance made my life in science enjoyable. Out of science, our wonderful trips, football chats and exciting ski lessons also gave me so many nice memories. You were not only an exceptional scientific mentor, but also an excellent person I am really proud of having as a friend. I would also like here to thank Sabine, who always warmly welcomed me, and wish all the best for Lars and Koen.

Dear Ivo, I would like to thank you for your continuous support through all these years. Your sense of criticism is always a great example of scientific way of thinking for me. I am thankful for your aspiring guidance, invaluably constructive criticism and friendly advice during my time at hematology department.

I would like to express my gratitude to the members of my thesis reading committee, Dr. Marieke von Lindern, Prof. Leendert Looijenga and Dr. Erik Wiemer for careful reading of my thesis and sharing their critical and illuminating inputs. Dear Marieke, I am also sincerely grateful for sharing with me your truthful and illuminating views on a number of issues related to my manuscript. Furthermore, my deepest appreciation to Prof. Ruud Delwel, Prof. Gerard de Haan, Prof. Sjaak Philipsen and Dr. Marc Bierings, the members of my large thesis committee, for their time and attention.

Dear Hans, we both started our carrier at Erasmus MC together. I enjoyed your company in the lab and it was always a pleasure for me to work with you. It was great that we could grow together in Stefan's lab. I wish you all the best in your career in science as well as in sport. Dear Paulette, your technical expertise always helped me when I was blocked in an experiment. I really appreciate your help and support. Dear Hans and Paulette, also thanks for being with me all along the promotion day as my "paranimfen". Nicole and Yvette, thanks for all your patience in showing me how to work with smaller organisms, for all your helps and for giving grateful technical support, which made the lab work more fun, and of course efficient for me. Nahuel and Jana, thank you for helping me to bring my projects a few steps further. I wish you lots of luck during your PhD period, and further in your professional and personal life.

My warm thanks belong also to all my fellow PhD students, postdocs, research analysts, technicians and group leaders in hematology department, for offering me pleasant friendly moments, for constructive scientific and non scientific discussions, technical help and mental support and for a lot of good memories we shared during last five years. I wish you all good luck.

Special thanks to all the people in 13th floor of Erasmus MC that provided me technical and administrative support over the last years. Dear Ans, Leenke, Annelies, Egied, Jan,

Thomasia and Natasja, I have caused quite some work for you. Thank you for all your assistance during these years.

I was so lucky to have great office mates during my PhD training, and I am thankful to all of them. Elnaz, who helped me find my first steps in Erasmus MC and in Hematology department, and Lucila and Athina, with whom I shared the office for the majority of my time at 13th floor. It is great to see your adorable, little family now, and wish you lots of luck in your career. I also shared the office with Kean and Piotr for the last few months of my stay at the department. Thank you for making the office such a pleasant place and I wish you all the best for your future.

During my PhD-training, I was a member of Molecular Medicine postgraduate school (MolMed) and I learned a lot from the courses, seminars and workshops offered and organized by MolMed. Representing the whole team, I would like to thank Frank and Joris for these exciting opportunities and valuable trainings.

The Iranian community of the Erasmus MC, thank you good friends for your personal support. You made me feel at home. It was great that we got to know each other here and hopefully despite the distance, our friendships will go on.

The work presented in this thesis would have been impossible without many collaborators. Therefore, I would like to express my warm thanks to Dr. Peter van Veelen, Dr. George Janssen, and the rest of van Veelen group at LUMC, as well as to Dr. Marc Bierings and to Stephanie from Wilhelmina Children's Hospital (UMCU).

From September 2014 I have been working at NKI in Amsterdam and already, just after a few months, I really feel knowing you guys for a long time and enjoy working with you. Heinz, thanks for your comprehension during the busy period of time I experienced, and for your personal and scientific support. Muhammad, Paul, Mark, Alessandra and Bas, we will hopefully go for a couple of years of fun in producing good quality science together, as always said by Heinz.

Maman and Baba, there is no word to explain my feeling about you. Though Confucius said "While one's family are alive, one should not travel to distant places", you are always supportive and encourage me to follow my dreams. I always feel lucky to have you as parents. Thank you for all your patience, for the lovely environment you made for us at home and for all love you gave me.

مامان و بابای مهربانم، ممنون برای همه ی مهری که به من داشته اید و دارید، که همیشه به آن محتاجم و همیشه قدردان شما خواهم بود.

Mrs. and Mr. Moghadasi, because of you, I never felt being far from home. I always was welcomed warmly in the family, which is now a part of my new big family. Thanks a lot. Farzaneh, Ali, Farnaz, Anoushiravan, Maryam and Ali, thanks for being, on top of family members, best friends for me and Setareh.

Every journey starts with preparation steps. My preparation for PhD period was done in Belgium. Without the support of my aunts, uncles and cousins, it was impossible to be

in the way I am following today. I would like to express the deepest appreciation to all of them, as well as to all my good friends and colleagues in Belgium.

And finally, I would like to thank my love, Setareh. Dear Setareh, I thank you for your support, your care and your smile, which not only help me go through all the difficulties, but also make all my moments so precious. I feel the luckiest to share my life with you. During last days of preparation of this book, an exceptional tiny happiness came to our life. Little Ryan, you are an unlimited source of joy and motivation for your parents. Our best of love to you.

Writing these words made me recall lots of nice memories for being together and working with a lot of amazing people in Erasmus MC, hematology department or Erkeland lab. I did not show my appreciations in more detail by naming them all one by one, but they will always be in my mind and heart. Thank you all!

Farshid Alemdehy
March 2015

CURRICULUM VITAE

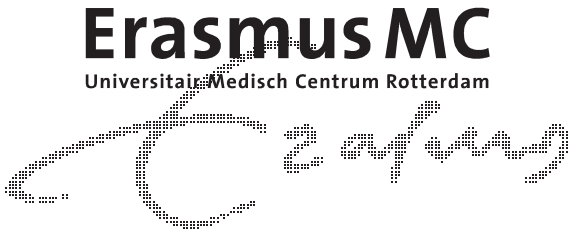
Farshid Alemdehy was born in Tehran, Iran, on 28 July 1976. After receiving his high school diploma from the National Organization for Development of Exceptional Talents (NODET, Tehran, Iran), he studied microbiology at University of Tehran and gained his Bachelor of Science degree in 1998 (Tehran, Iran). He then moved to Belgium and studied bioengineering in biotechnology. After completing a research project on immunomodulatory properties of a recombinant lactic acid bacterium against the dust mite allergy, performed in the Institute of Molecular Biology and Medicine (IBMM, Gosselies, Belgium), he gained the degree of Master of Science *with distinction*, from Université Libre de Bruxelles (ULB, Brussels, Belgium). He worked for a short period in CARAH (Applied Agronomic Research Centre of province of Hainaut, Ath, Belgium) as a research assistant. He then moved to the Netherlands in 2010 and started his PhD in the group of Dr. Stefan Erkeland in Hematology Department, Erasmus University Medical Center, in Rotterdam. During his PhD training, he studied the role of microRNAs in normal and malignant myelopoiesis. In September 2014 he started working on a post-doctoral project on the non-coding role of stable immunoglobulin transcripts in establishing allelic exclusion and B cell development, in the group of Dr. Heinz Jacobs in the Netherlands Cancer Institute in Amsterdam, the Netherlands.

AWARDS

- **American Society of Hematology Abstract Achievement Award** (2012), 53rd ASH annual meeting
- **Best Poster Award** (2013), 17th Molecular Medicine Day
- **Lady TATA Memorial Trust International Award for Research in Leukaemia** (2014)

LIST OF PUBLICATIONS

- **Alemdehy, M. F.**, van Boxtel, N. G., de Looper, H. W., van den Berge, I. J., Sanders, M. A., Cupedo, T., Touw, I. P., and Erkeland, S. J. (2012). Dicer1 deletion in myeloid-committed progenitors causes neutrophil dysplasia and blocks macrophage/dendritic cell development in mice. *Blood 119*, 4723-4730.
- **Alemdehy, M. F.**, and Erkeland, S. J. (2012). MicroRNAs: key players of normal and malignant myelopoiesis. *Current opinion in hematology 19*, 261-267.
- **Alemdehy, M. F.**, and Erkeland, S. J. (2012). Stop the dicing in hematopoiesis: What have we learned? *Cell Cycle 11*, 2799-2807.
- **Alemdehy, M. F.**, Haanstra, J. R., de Looper H. W., van Strien P. M. H., Verhagen-Oldenampsen J., Caljouw Y., Sanders M. A., Hoogenboezem R., de Ru A. H., Janssen G. M. C., Smetsers S. E., Bierings M. B., von Lindern M., van Veelen P. A., Touw I. P. and Erkeland S. J. Interstrand cross-link induced miR-139-3p and miR-199a-3p have opposite roles in hematopoietic cell expansion and leukemic transformation. *Submitted*
- **Alemdehy M. F.**, Erkeland S. J. MicroRNAs in normal and malignant myelopoiesis, A chapter of the book :" *MicroRNA in Regenerative Medicine*", Elsevier, In press.



PHD PORTFOLIO SUMMARY

Summary of PhD training and teaching activities

Name PhD student: Mir Farshid Alemdehy
Erasmus MC Department: Hematology
Research School: Molecular Medicine (MolMed)

PhD period: Mai 2010-August 2014
Promotor: Prof.Dr. Ivo P. Touw
Supervisor: Dr. Stefan J. Erkeland

1.PhD training		
	Year	**ECTS**
General academic/research skills		
Research Management for PhDs/Postdocs	2010	1
Laboratory animal science	2011	3
Basic introduction course on SPSS	2011	0.6
Photoshop & Illustrator CS5 workshop	2011	0.3
English biomedical writing and communication	2011	4
Indesign CS5 workshop	2012	0.15
In-depth courses and workshops		
workshop on Basic data analysis on gene expresison arrays (BAGE)	2010	1.2
Scientific workshop on AML molecular	2011	1
Basic and translational oncology	2013	1.8
Basic course on R	2013	0.7
Analysis of microarray and RNA seq data	2013	1.6
Molecular aspects of hematological disorders	2014	1
Scientific meetings department of hematology		
Erasmus hematology lecture	2010-2014	1.3
Workdiscussion	2010-2014	4

1.PhD training		
	Year	**ECTS**
AIO/Postdoc meeting	2010-2014	1.6
Journal club	2010-2014	2
Seminars and conferences		
Dutch Hematology Congress	2011-2014	1.8
Molecular Medicine Day	2011-2014	1.2
53rd ASH annual meeting	2011	1
Keystone Symposia: MicroRNAs and Non-Coding RNAs and Cancer	2011	1
Keystone Symposia: Non-Coding RNAs in development and Cancer	2013	1
Presentations		
Workdiscussion (department of hematology, 9X)	2010-2014	4.5
Journal club presentation (department of hematology, 4X)	2011-2014	2
AIO/Postdoc meeting (department of hematology, 3X)	2010-2014	1.5
5th Dutch Hematology Congress (oral)	2011	1
15th Molecular Medicine Day (oral)	2011	1
Keystone Symposia: MicroRNAs and Non-Coding RNAs and Cancer (poster)	2011	1
53rd ASH annual meeting (oral)	2011	1
Keystone Symposia: Non-Coding RNAs in development and Cancer (poster)	2013	1
17th Molecular Medicine Day (poster)	2013	1
8th Dutch Hematology Congress (oral)	2014	1
Molecular aspects of hematological disorders (oral)	2014	1

2.Teaching activities		
Supervising Master's thesis	2013-2014	3

Other		
Organizing Hematology PhD lunch with invited speakers	2011-2012	0.2

Total	50.45