

Long noncoding RNAs during normal and malignant hematopoiesis

Juan R. Alvarez-Dominguez · Wenqian Hu ·
Austin A. Gromatzky · Harvey F. Lodish

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Abstract Long noncoding RNAs (lncRNAs) are increasingly recognized to contribute to cellular development via diverse mechanisms during both health and disease. Here, we highlight recent progress on the study of lncRNAs that function in the development of blood cells. We emphasize lncRNAs that regulate blood cell fates through epigenetic control of gene expression, an emerging theme among functional lncRNAs. Many of these noncoding genes and their targets become dysregulated during malignant hematopoiesis, directly implicating lncRNAs in blood cancers such as leukemia. In a few cases, dysregulation of an lncRNA alone leads to malignant hematopoiesis in a mouse model. Thus, lncRNAs may be not only useful as markers for the diagnosis and prognosis of cancers of the blood, but also as potential targets for novel therapies.

Keywords Hematopoiesis · Long noncoding RNAs (lncRNAs) · Leukemia

Introduction

Mammalian genomes comprise thousands of protein-coding and noncoding genes. The latter can produce small structural

and regulatory RNAs (including rRNAs, tRNAs, snoRNAs and microRNAs) as well as long noncoding RNAs (lncRNAs), which are >200 nt yet do not function through an encoded protein product. lncRNAs mirror mRNAs in their biogenesis and regulation: most are Pol II transcripts that undergo splicing, capping and polyadenylation and are regulated by specific transcription factors (TFs) and chromatin modifiers [1, 2]. However, unlike mRNAs they are mainly found in the nucleus [2, 3] and rarely engage translating ribosomes in the cytoplasm [4, 5]. lncRNAs have been implicated in a variety of processes: they can recruit and regulate the activity of TFs and chromatin modifiers, organize chromosomal domains, and modulate mRNA splicing, translation, and degradation [6–8]. Such regulatory capacities are enabled by the versatility with which RNA molecules can interact with DNA, with other RNAs and with protein complexes [9, 10]. Crucial to this versatility in lncRNAs are specific features of primary sequence, secondary structure, and genomic positioning that experience different levels of evolutionary constraint [2, 11, 12].

Many lncRNAs are differentially expressed across tissues, developmental stages and physiological states [2, 13–15]. Some are required for life [16–20], and some are not but fulfill specialized functions in response to specific developmental or physiological cues [21–24]. Thus, lncRNAs are increasingly recognized as important players during the development and functioning of mammalian cells and tissues. In this review, we summarize recent progress on the study of lncRNAs with roles during normal and malignant hematopoiesis. We highlight lncRNAs that regulate cell fate and differentiation decisions via epigenetic mechanisms, and discuss the clinical relevance of lncRNAs as diagnostic and prognostic biomarkers of hematopoietic malignancies and their potential as therapeutic targets.

J. R. Alvarez-Dominguez · W. Hu · A. A. Gromatzky ·
H. F. Lodish
Whitehead Institute for Biomedical Research, Cambridge,
MA 02142, USA

J. R. Alvarez-Dominguez · H. F. Lodish
Department of Biology, Massachusetts Institute of Technology,
Cambridge, MA 02142, USA

A. A. Gromatzky · H. F. Lodish (✉)
Department of Biological Engineering, Massachusetts Institute of
Technology, Cambridge, MA 02142, USA
e-mail: lodish@wi.mit.edu

lncRNAs during normal hematopoiesis

Hematopoiesis is a highly coordinated process comprising a hierarchy of well-defined cell differentiation states, making it an ideal system to identify lncRNAs that regulate cell fate. All hematopoietic effector cells—erythrocytes, myelocytes, and lymphocytes—derive from hematopoietic stem cells (HSCs) through a cascade of cell lineage specification, proliferation, and differentiation events. An analysis of the hematopoietic lineage expression of lncRNAs identified in the fetal liver of E14.5 mice (Fig. 1) reveals many with exquisite lineage specificity, suggesting that distinct collections of lncRNAs modulate the development

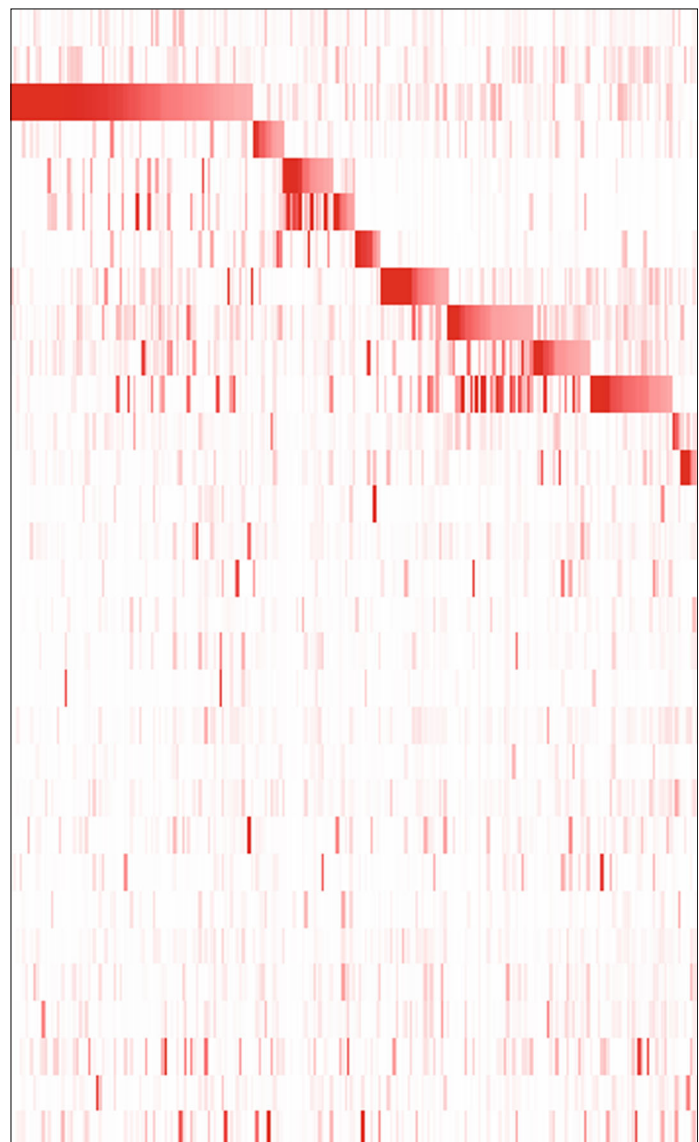
or functioning of distinct blood cell types. Indeed, recent studies have described several lncRNAs associated with development or function of specific types of blood cells (Table 1; Fig. 2). In the next sections, we will discuss selected examples of functional lncRNAs that illustrate recent advances in the field.

lncRNAs in hematopoietic stem cells

Self-renewal of hematopoietic stem cells (HSCs) is in part sustained by developmental and environmental signals from their niche, which act through developmentally conserved signaling pathways to promote proliferation while

Fig. 1 Tissue specificity of mouse fetal liver lncRNAs. Relative abundance of lncRNAs (rows) expressed in the E14.5 mouse fetal liver across 30 primary cell and tissue types (columns) from the mouse ENCODE consortium. Color intensity represents the fractional gene-level expression across all tissues examined. Hematopoietic tissues are highlighted in red. Adapted from [44]

Placenta
ESC
Bone_marrow
MEP
Erythroblast_1
Erythroblast_2
Megakaryocyte
Macrophage
B-cell_CD19+
T-cell_naive
B-cell_CD43-
Spleen
Thymus
Adrenal
Bladder
Brown_adipose
Colon
Kidney
Liver
Lung
Heart
Mammary_gland
MEF
Skeletal_muscle
Stomach
Subcutaneous_fat
Whole_brain
Cerebellum
Cerebrum
Ovary
Testis



Fractional expression level across tissues
0 0.1 0.2 0.3 0.4 0.5

Table 1 Examples of lncRNAs with roles in normal hematopoiesis

Name	Cell type	Loss of function phenotype	Functional assays	Reference
H19	HSC	Increased activation and proliferation; impaired repopulation ability	Mouse knockout models; transplantation assays	[29]
EGO	Eosinophils	Impaired expression of eosinophil differentiation genes	siRNA knockdown	[40]
HOTAIRM1	Myeloid progenitors	Inhibition of the nearby HoxA1 and HoxA4 genes during granulocytic differentiation	siRNA and shRNA knockdown	[41]
LincRNA-EPS	Erythroblasts	Compromised differentiation; elevated apoptosis	shRNA knockdown; ectopic expression	[42]
DLEU2; eLincRNA-EC1,3; lincRNA-EC2,4,5,8,9; aLincRNA-EC1,2,3,7	Erythroblasts	Impaired erythrocyte maturation	shRNA knockdown	[43]
NeST	T cells	Sensitivity to viral and bacterial pathogenesis in mice	Ectopic expression	[47, 48]
lincRNA-Cox2	Dendritic cells; macrophages	Impaired gene regulation upon IFN stimulation	siRNA knockdown; overexpression	[52]
LincR-Ccr2-5' AS	T cells	Downregulation of the nearby Ccr1, Ccr2, Ccr3, and Ccr5 genes	shRNA knockdown	[53]

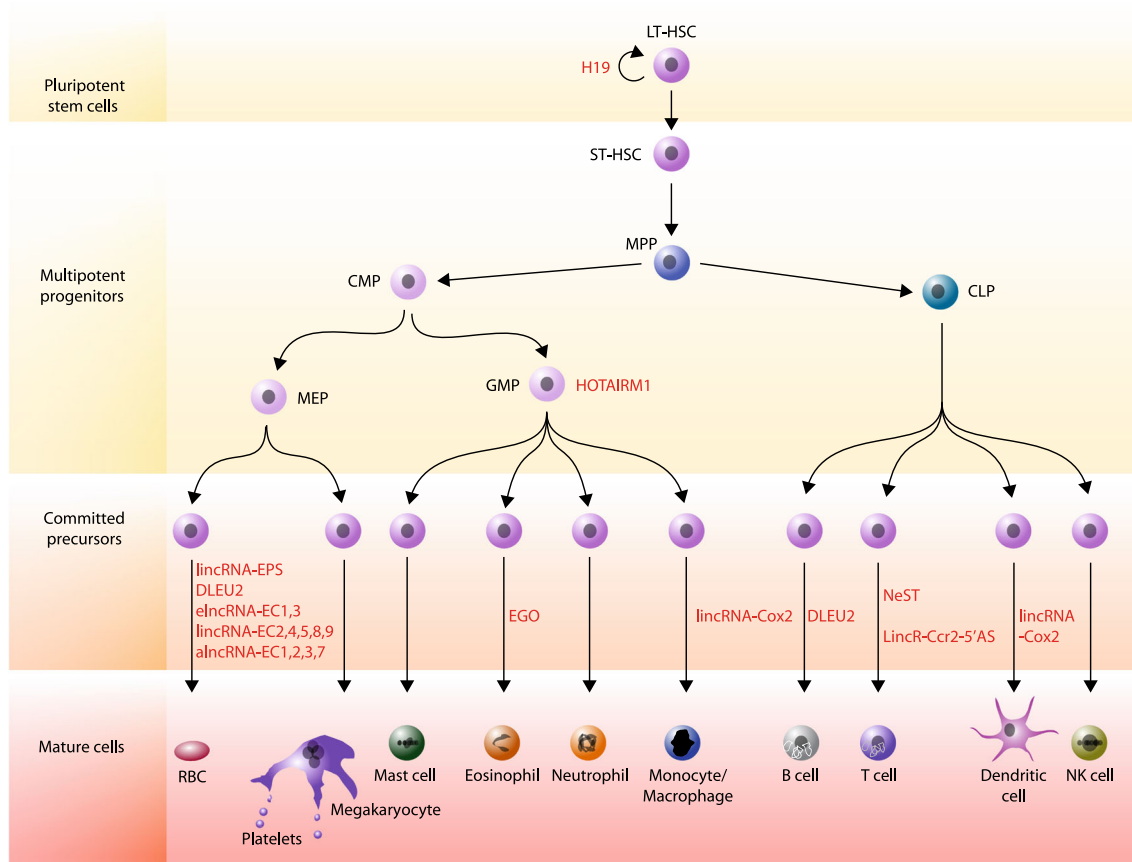


Fig. 2 Involvement of lncRNAs in blood cell development. lncRNAs required for blood cell development, as determined through loss-of-function studies, are depicted next to the stage of hematopoietic development affected by their inhibition. LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic cell; MPP,

multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP; granulocyte/monocyte progenitor; RBC, red blood cell; NK cell, natural killer cell

suppressing apoptosis and differentiation [25, 26]. During hematopoiesis, this self-renewing capability decreases as cells transition from multipotent stem cells to lineage-committed progenitors. The fact that lncRNAs have the capacity to modulate self-renewal of embryonic stem cells [27–29] suggests that they too may act in the circuitry controlling the HSC state.

Li and colleagues recently described the first example of lncRNA-mediated maintenance of adult HSC quiescence [30]. The H19 gene was in fact the first lncRNA to be identified, enriched in the embryonic fetal liver but downregulated after birth [31, 32]. The H19 transcript is a Pol II product that undergoes capping and polyadenylation, can be found in both nucleus and cytoplasm, and contains only small ORFs that do not template protein synthesis *in vivo*. During embryogenesis, H19 is transcribed from the maternal allele and regulates growth via imprinting *in cis* of Igf2, as well as by encoding miR-675, which downregulates the receptor of the Igf2 ligand expressed from the paternal allele (Igf1r) [33–35]. H19 remains active in long-term HSCs and is gradually downregulated in short-term HSCs and multipotent progenitors. Deletion of H19 from the maternal allele results in increased HSC activation and proliferation and impairs repopulating ability. As expected, this effect is mediated by de-repression of maternal Igf2 expression and by increased Igf1r translation, resulting in increased signaling through the Igf1r. Overexpression of H19-derived mir-675 restores proper levels of Igf1r protein, and concomitant deletion of the Igf1r locus partially rescues the H19 knockout phenotype. Thus, H19 promotes HSC quiescence by regulating the Igf2-Igf1r pathway at the transcriptional and posttranscriptional levels.

Interestingly, several other imprinted lncRNAs are upregulated in HSCs or in other adult stem cells relative to their differentiated progeny [30, 36]. These include Air, which acts *in cis* to silence the paternal allele of Igf2 via transcriptional interference and also Slc22a3 and Slc22a2 via recruitment of the G9a histone methyltransferase [37–39], as well as the small RNA hosts from the Dlk1-Dio3 imprinted region Rian and Gtl2, thought to act in stem cell maintenance [28, 40].

lncRNAs in the myeloid and erythroid lineages

The first lncRNA with a role in hematopoiesis to be described was EGO, a conserved gene transcribed antisense to ITPR1 that modulates the development of eosinophils [41]. EGO is normally expressed in human CD34 + HSCs and becomes upregulated during their differentiation into eosinophils. The EGO transcript is non-coding, as it does not associate with ribosomes. Knockdown of EGO by siRNAs in cultured CD34 + progenitors impaired the expression of genes critical for

eosinophil development, including major basic protein and eosinophil-derived neurotoxin. Thus, EGO can contribute to eosinophilopoiesis by enhancing the expression of genes needed for this process.

lncRNAs are also implicated in the regulation of myelopoiesis, the formation of granulocytes and monocytes. Zhang et al. [42] identified HOTAIRM1, a gene transcribed antisense to the HOXA1/2 intergenic region that is strongly upregulated during retinoic acid-induced granulocytic differentiation of myeloid progenitors. The HOTAIRM1 transcript is ~500 nt and does not associate with ribosomes. Its expression is coordinated along the body plan with that of HoxA1 and HoxA2, suggesting that it may be involved in keeping these genes active. Indeed, knockdown of HOTAIRM1 inhibits HoxA1 and HoxA4 activation during granulocytic differentiation in the NB4 acute promyelocytic leukemia cell line, resulting in impaired expression of myeloid differentiation markers such as CD11b and CD18. This effect may be mediated through the interaction of HOTAIRM1 with various chromatin modifiers [28], suggesting that this lncRNA modulates myelopoiesis by regulating the epigenetic state of neighboring genes at the HoxA locus.

Our group has extensively profiled the lncRNA transcriptome of the erythroid lineage, uncovering numerous erythroid-specific lncRNAs that become dramatically induced during terminal differentiation of mouse fetal liver red blood cells *in vivo* [43, 44]. Many of these RNAs—including intergenic, antisense, and enhancer transcripts—are targets of erythroid-important transcription factors GATA1, TAL1 or KLF1 and are needed for proper red cell development. We characterized 13 such lncRNAs and showed that they are nuclear-localized and exhibit complex developmental expression patterns. Depleting any of them severely impaired erythrocyte maturation, inhibiting cell size reduction and subsequent enucleation. One antisense lncRNA, alncRNA-EC7, is transcribed from an enhancer and is specifically needed for activation of the neighboring gene encoding BAND3. One intergenic lncRNA, lincRNA-EPS, produces a ~2.5 kb capped and polyadenylated transcript that is alternatively spliced and resides in the nucleus. Depleting lincRNA-EPS by shRNAs severely compromised terminal differentiation of erythroid progenitors and resulted in elevated apoptosis. Conversely, ectopic expression of lincRNA-EPS protected erythroid progenitors from apoptosis triggered by erythropoietin starvation. These effects persisted after disruption of putative short ORFs within the transcript, and rather are mediated by a conserved 500 bp 3' terminal domain that is sufficient for anti-apoptotic activity. Functional studies indicated that lincRNA-EPS acts by repressing a number of pro-apoptotic proteins, most prominently the caspase activating adaptor protein Pycard. Thus, lincRNA-EPS acts to

promote red blood cell maturation by downregulating proapoptotic pathways. These examples show that lncRNAs can modulate the development of myeloid and erythroid cells through their capacity to regulate gene expression.

lncRNAs in lymphoid lineages

Roles for lncRNAs in lymphoid cells were first proposed by early observations of lymphoid-specific lncRNAs that are dynamically regulated during T cell differentiation and activation [45–47]. Recent studies have now provided evidence for the importance of several lncRNAs in immune cell function. The lncRNA NeST (Tmevpg1) modulates the ability of mice to respond to viral and bacterial infections [48, 49]. The NeST locus was identified through a forward genetic screen as a susceptibility locus for sensitivity to pathogenesis of Thelie's virus in mice [50–52]. The NeST locus encodes an lncRNA specifically expressed by the T_H1 subset of helper T cells. Endogenous or ectopic expression of NeST regulates the degree of inflammation induced by infecting pathogens, such as Thelie's virus or Salmonella. Mechanistically, NeST regulates expression of the cytokine IFN- γ , critical for innate and adaptive immunity, by specifically interacting with WDR5, a component of the H3K4 methyltransferase complex, in CD8⁺ T cells. Thus, NeST acts in immune effector cells to regulate the outcome of viral or bacterial infections by epigenetically activating expression of the IFN- γ locus via direct interactions with chromatin modifiers.

Fitzgerald and colleagues have characterized lincRNA-Cox2, which becomes dramatically upregulated by Toll-like receptor (TLR) signaling in mouse bone marrow-derived dendritic cells and macrophages [1, 53]. The lincRNA-Cox2 locus encodes several RNA isoforms that do not associate with ribosomes. Upon silencing them with shRNAs, Ccl5, Ccr1, and several IFN-stimulated genes were particularly upregulated. Overexpression of lincRNA-Cox2 in turn resulted in severe attenuation of Ccl5 and overexpression of TLR-induced interleukin 6. lincRNA-Cox2 is found in both nucleus and cytoplasm and for inhibitory activity interacts with heterogeneous nuclear ribonucleoproteins A/B and A2/B2. Thus, lincRNA-Cox2 also acts during inflammatory signaling by modulating the

expression of several immune response genes via interactions with regulatory complexes.

In another recent study, Hu et al. globally profiled lncRNA gene expression during the differentiation of naïve CD8⁺ T cells into various helper T cell subsets, and characterized LincR-Ccr2-5'AS, a T_H2-specific gene activated by the transcription factor GATA3 [54]. LincR-Ccr2-5'AS is located between the genes encoding the chemokine receptors Ccr3 and Ccr2 and is coregulated with them. Depleting LincR-Ccr2-5'AS with shRNAs in T_H2 cells downregulated expression of the nearby Ccr1, Ccr2, Ccr3 and Ccr5 genes, without affecting their chromatin architecture, and impaired the ability of the cells to migrate to the lungs in vivo. The global gene expression response of T_H2 cells depleted for LincR-Ccr2-5'AS significantly overlaps with that following GATA3 depletion, suggesting a functional link between the two during T cell differentiation and immunological function. These examples document critical roles for lncRNAs in modulating immune responses by associating with regulatory complexes to specify immune gene expression programs.

lncRNAs during malignant hematopoiesis

Defects in the proper coordination of hematopoiesis can result in the uncontrolled proliferation of blood cells at different stages of development, resulting in cancer. Because of their roles in regulating blood cell fates, including differentiation, proliferation and survival, lncRNAs are likely suspects in the pathogenesis of hematopoietic malignancies. Thus far, a handful of lncRNAs have been conclusively linked to cancers of the blood (Table 2; Fig. 3). We discuss these in the next sections, with an emphasis on their mechanism of action.

lncRNAs and leukemia

B cell chronic lymphocytic leukemia (CLL), the most common adult B cell-derived malignancy, has been linked to deletion of 13q14, which is observed at >50 % frequency in both CLL and CD5⁺ monoclonal B cell lymphocytosis (MBL), and at lower frequencies in CD5⁻ B

Table 2 Examples of lncRNAs with roles in malignant hematopoiesis

Name	Cell type	Loss of function molecular phenotype	Loss of function malignant phenotype	Functional assays	Reference
DLEU2	B cells	Dysregulated cell cycle progression and apoptosis	MBL, CLL, DLBCL	Mouse knockout models; ectopic expression	[58]
Xist	HSC	Compromised X inactivation; impaired maturation; loss of long-term HSCs	Mixed MPN/MDS	Mouse knockout models; transplantation assays	[72]

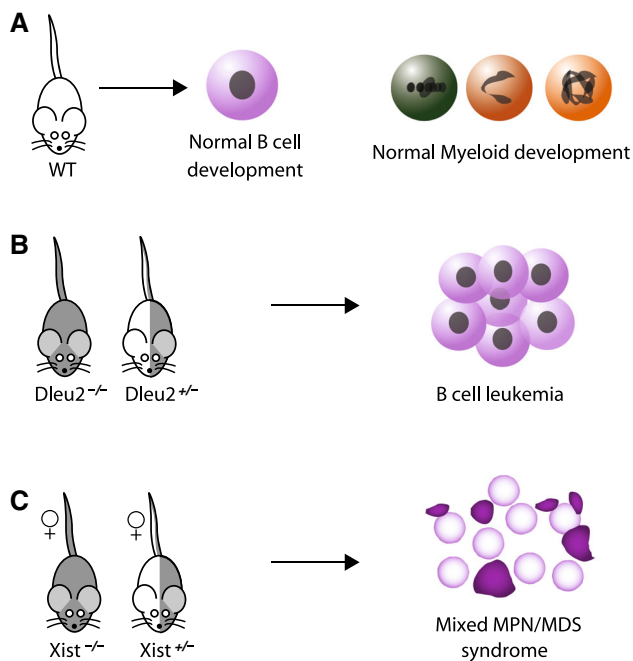


Fig. 3 Mouse knockout models link lncRNAs to blood cancer pathogenesis. Proper expression of *Dleu2* and *Xist* is required for normal hematopoiesis. **a** Wild-type mice develop B cells and myeloid cells normally. **b** *Dleu2*^{-/-} and *Dleu2*^{+/-} mice fail to properly regulate cell cycle progression and apoptosis of B cells, developing a B cell chronic lymphocytic leukemia reminiscent of human CLL that significantly reduces lifespan. **c** *Xist*^{-/-} and *Xist*^{+/-} female mice fail to maintain proper X dosage, developing a deficiency in HSC maturation that leads to a lethal mixed myeloproliferative neoplasm and myelodysplastic (MPN/MDS) syndrome

cell-derived malignancies and T cell lymphomas [55–58]. The minimal deleted region (MDR) within 13q14 consists of a ~110 kb stretch comprising the *DLEU2* lncRNA, which hosts microRNAs 15a and 16-1 and is spliced into a mature RNA that can be found in the nucleus and cytoplasm [44]. Mice deleted for the entire MDR or only for miR-15a/16-1 in all cells (or only in B cells) displayed MBL, developed CLL at moderate penetrance, and in a fraction of cases progressed into diffuse large B cell lymphoma, thus recapitulating the spectrum of human CLL phenotypes [59]. Re-expression of miR-15a/16-1 in a human CLL cell line reduced proliferation and downregulated multiple genes involved in the G0/G1-S phase transition but did not affect expression of antiapoptotic ones such as *BCL2* or NF- κ B, which are normally dysregulated in CLL. Moreover, disease penetrance in *MDR*^{-/-} mice was 42 % as opposed to 26 % in miR-15a/16-1^{-/-} mice, which translated in shortened lifespan in the former but not in the latter. These findings indicated that loss of *DLEU2* leads to CLL at least in part through the absence of the cell cycle inhibitory microRNAs 15a and 16-1, but additional roles of *DLEU2* that contribute to a more aggressive disease course remained unexplained.

Indeed, recent studies have now linked *DLEU2* to the *in cis* repression of protein-coding neighboring genes that positively regulate NF- κ B activity, consistent with the NF- κ B downregulation and resistance to apoptosis seen in *DLEU2*-deficient CLL cells [44, 60, 61]. Thus, *DLEU2* acts as a tumor suppressor by regulating both cell cycle progression and the NF- κ B signaling pathway in B cells, although the precise mechanisms for the latter role remain elusive. This lncRNA is therefore not only a useful prognostic marker in CLL, but also a potential target for novel CLL therapies.

Interestingly, other lncRNAs are dysregulated in other types of leukemia. *MEG3* lncRNA is commonly downregulated in acute myeloid leukemia (AML) and hypermethylation of its promoter is a marker of poor prognosis [62, 63]. Indeed, *MEG3* has been implicated in regulation of the Rb and p16^{INK4a} pathway and thus of cell proliferation in a variety of cancers [64, 65]. Similarly, the lncRNA *ANRIL* is upregulated in acute lymphoblastic leukemia (ALL) and modulates cell proliferation via regulation of the Rb and p16^{INK4a} pathway in multiple cancers [66, 67]. Although no clear role in the etiology of AML or ALL has emerged for *MEG3* and *ANRIL*, it is likely that they contribute to the disease course via their known effects on cell cycle regulation. Thus, dysregulation of these or of other lncRNAs in specific blood cancer subtypes may present at the very least useful markers of disease progression.

lncRNAs in myeloproliferation and myelodysplasia

Perhaps the most well-understood lncRNA to date, *Xist*, has recently been found to act as a potent suppressor of hematological cancer *in vivo*. The *Xist* locus is transcribed into multiple polyadenylated, nuclear transcripts that can reach ~18–19 kb in length in mouse and human and are required for X chromosome inactivation during embryogenesis [68]. *Xist* is expressed from a region within the future inactive X chromosome called the X-inactivation center (*Xic*). *Xist* recruits the chromatin repressive complex PRC2 to its transcription site through a structured RNA domain, and the two co-migrate to spatially proximal gene-dense regions of active chromatin within the X chromosome, leading to their PRC2-mediated silencing [69–72]. These regions are repositioned into a growing heterochromatin compartment, effectively bringing new sites into close contact with the *Xic* for further proximity transfer of the *Xist*-PRC2 complex. By this mechanism, *Xist* spreads over a ~150 Mb scale to silence most genes in the inactive X chromosome. Attesting to its importance during development, paternally inherited loss of *Xist* is lethal due to lack of X-inactivation in extra-embryonic tissues [16].

X chromosome dosage has long been associated with cancer, since selective loss of the inactive X and duplication of the active one is frequently seen in breast and ovarian malignancies. However, it was not until a recent report by Lee and colleagues that regulation by the Xist lncRNA was causally linked to blood cancer [73]. Conditional deletion of Xist in HSCs after the occurrence of X-inactivation was lethal for both Xist^{-/-} and Xist^{-/+} females (at 100 % penetrance) but not for their male counterparts. Deceased mutant females exhibited massive splenomegaly and extramedullary hematopoiesis, associated with hyperproliferation of all hematopoietic lineages, with myeloid cells outproliferating those of the other lineages. Bone marrow dysfunction was also observed, including myelofibrosis, myeloproliferation and myelodysplasia, leading to chronic myelomonocytic leukemia and erythroleukemia and thus recapitulating human myeloproliferative neoplasm and myelodysplastic syndrome (MPN/MDS). Wild-type mice transplanted with Xist^{-/-} bone marrow developed MPN/MDS, whereas Xist^{-/-} mice transplanted with wild-type bone marrow did not, indicating a cell autonomous HSC defect. Indeed, Xist^{-/-} mice displayed impaired HSC maturation and loss of long-term HSCs. Molecularly, these effects were mediated by widespread X reactivation leading to genome-wide changes including upregulation or downregulation of oncogenes or tumor suppressors implicated in MPN and MDS, respectively. Thus, the Xist lncRNA is required for both establishment and long-term maintenance of proper X dosage *in vivo*, and its loss of function leads to blood cancer.

Interestingly, several other lncRNAs modulate Xist expression and thus X chromosome dosage. These include Tsix, which silences Xist *in cis* via recruitment of the DNMT3A methyltransferase to its promoter, and Jpx, which acts to upregulate Xist *in trans* at the Xic. Importantly, loss of Tsix or of Jpx is female-lethal [17, 19]. Since X chromosome dosage is so frequently associated with human cancer, the lncRNAs that regulate it present a unique opportunity for the study of functional lncRNAs in the context of hematological cancer.

Conclusions and future directions

The number of human lncRNAs described has exploded over the past 10 years, owing to the rapid development of high throughput transcriptome profiling techniques and their application to a variety of developmental and pathological conditions. Interestingly, many of these genes are highly cell/tissue-specific. Functional characterization of lncRNAs has revealed and likely will continue to reveal roles in various physiological processes, including hematopoiesis. Clearly, lncRNAs are a group of novel regulatory

genes that contribute to animal development and pathogenesis. Here, we discuss from our own perspectives some of the outstanding challenges and opportunities for the study of lncRNAs in the hematology field.

Investigating the molecular mechanisms by which lncRNAs regulate gene expression

lncRNAs regulate gene expression during hematopoiesis via multiple mechanisms. One common theme for nuclear-localized lncRNAs is to recruit protein partners, such as chromatin modifying complexes, to target regions in the genome to modulate gene expression. Thus, it is important to identify both the protein partners of lncRNAs and the target genomic regions with which they associate. To this end, an expanding toolbox of biochemical approaches for RNA purification coupled with high throughput sequencing or with mass spectrometry is rapidly becoming available. These include CHART [74], ChIRP-Seq [75], RIA-Seq [76] and RAP [71], which share the same principle of using biotinylated antisense probes that hybridize to a target RNA to purify it together with any associated proteins, RNAs or genomic DNA segments from cross-linked cell lysates. In addition to these biochemical approaches, simple cell biology approaches, such as direct visualization of lncRNAs *in situ* by single-molecule RNA-FISH can provide important insights into their molecular mechanisms. For example, RNA-FISH can evidence localization to functional subcellular compartments, such as paraspeckles or the nucleolus [38, 77–79], or even to specific chromosomal regions [80, 81], and can also be used to examine multimerization potential and co-localization with RNA or protein partners [82–84]. Thus, when used in combination, these biochemical and cell biology approaches can greatly facilitate the exploration of lncRNA mechanisms during hematopoietic cell differentiation.

Integrating lncRNAs into regulatory networks in control of hematopoiesis

Hematopoiesis is regulated at multiple levels by both intracellular factors and cytokines to ensure the proper generation of multiple cell lineages under both normal and pathophysiological conditions [85, 86]. As more and more functions for lncRNAs in the hematopoietic system are being described, it is increasingly clear that lncRNAs are important components of these regulatory circuitries. Thus, it is of great interest to determine just how these noncoding transcripts fit into known regulatory networks in control of hematopoiesis.

The expression of many lncRNAs that modulate differentiation into a specific cell type is indeed controlled by the key TFs needed for differentiation of that cell type. For

example, during erythroid terminal differentiation, the induction of many lncRNAs that are required for proper red cell maturation is mediated by the master erythroid TFs GATA1 and TAL1 [44]. Since the genomic binding sites of many TFs with roles during hematopoiesis have been already assayed by CHIP-seq studies, it is now feasible to intersect these binding sites with active lncRNA loci. This should help integrate lncRNAs into the transcriptional networks established by these TFs, and can additionally help researchers prioritize which lncRNA candidates to choose for functional studies. Interestingly, some lncRNAs may physically bind TFs [29], suggesting that lncRNAs might also regulate TF activity. Further progress in identifying the global binding sites of key TFs during hematopoiesis, as well as the protein interactome of lncRNAs, should be of great help in deciphering potential lncRNA and TF regulatory networks.

MicroRNAs also control the differentiation of many blood cell lineages [87, 88]. Interestingly, certain lncRNAs and microRNAs can regulate each other [89–91]. However, functional validation of lncRNA targets of microRNAs in the hematopoietic system remains largely unexplored. Identifying miRNA and lncRNA genes with complementary expression patterns during blood cell differentiation may thus generate candidate lncRNA-microRNA pairs to be tested for inference of lncRNA-microRNA regulatory relationships. These studies should not only lead to the identification of ncRNA regulatory networks in the hematopoietic system, but may also enhance our understanding of how these RNA genes contribute to its development.

Testing lncRNA functions in vivo

The effects of inhibiting some lncRNAs *in vitro* are relatively small, usually only a ~2 fold change in the expression of target loci, placing them closer to the effects of miRNAs than of TFs. This may be in part due to limitations in achieving efficient depletion of lncRNAs by current si/shRNA approaches, or it may indicate that many lncRNAs, like microRNAs, mainly act to fine tune target gene expression. In addition, any effects of lncRNA perturbation observed *in vitro* are not guaranteed to elicit phenotypic changes *in vivo*. Thus, complete deletion of lncRNA loci is required to properly assess the relative contribution of lncRNAs to target gene expression and to establish roles for lncRNAs during hematopoiesis *in vivo*.

Only a few lncRNA knockout mouse models have been generated to date to address these questions. Encouragingly, in both *Xist*^{-/-} and *Xist*^{-/+} female mice, compromised maintenance of X-inactivation causes a critical deficiency in HSC maturation that leads to a highly aggressive myeloproliferative neoplasm and myelodysplastic syndrome with full penetrance [73]. Similarly,

Dleu2^{-/-} and *Dleu2*^{-/+} mice fail to properly regulate cell cycle progression and apoptosis of B cells, developing a chronic lymphocytic leukemia reminiscent of human CLL with moderate penetrance [59]. Thus, proper expression of the *Xist* and *Dleu2* lncRNAs is clearly required for normal hematopoiesis, and dysregulation of these genes leads to lethal blood cancer phenotypes.

Despite the examples of *Xist* and *Dleu2*, whether other lncRNAs, particularly those that are lineage specific and affect blood cell differentiation *in vitro*, contribute to hematopoiesis *in vivo* remains largely unanswered. Examining the phenotypic effects of animal models deleted for these RNAs will require careful evaluation of the specific tissue, developmental stage and environmental condition in which the gene is active. Valuable lessons have been learned in this respect through recent studies. For example, despite the fact that *Xist* is continuously expressed throughout female life, it had been widely believed to be indispensable for maintenance of X chromosome inactivation, since deleting *Xist* post-inactivation does not cause immediate reactivation in fibroblasts, in somatic cell hybrids or in ES models of autosomal silencing by an autosomal *Xist* transgene [92–95]. However, deleting *Xist* post-inactivation in the blood compartment *in vivo* clearly leads to X reactivation and potent hematologic cancer. Analogously, the lncRNA MALAT1, first identified as a marker for metastasis development of lung adenocarcinoma [96], is dispensable for life and development [97–99], but targeted deletion of MALAT1 in human lung tumor cells indeed impaired tumor metastasis in a mouse xenograft model [21]. These examples highlight the need for studying lncRNAs under informative physiological conditions.

The strategy for deleting an lncRNA gene is also a crucial factor for elucidating its function *in vivo*. This is exemplified by the case of the lncRNA HOTAIR, transcribed from the *HoxC* gene cluster and originally found to act *in trans* to epigenetically repress the *HoxD* cluster in human cells with anterior and distal positional identities [100]. Deleting a large chromosomal region including eight *HoxC* genes, two microRNAs, HOTAIR and several other lncRNAs in a mouse model did not elicit obvious developmental defects and only resulted in modest de-repression of *HoxD* genes. However, targeted deletion of only the HOTAIR locus indeed affected *HoxD* gene expression in forming anterior and distal skeletal structures, leading to homeotic transformations of the spine and malformation of metacarpal-carpal bones. Thus, deletions that specifically target lncRNAs may be necessary for uncovering their specific roles *in vivo*, especially in the case of lncRNAs that overlap other loci. Recently developed techniques for efficient targeted genome engineering, such as the CRISPR-Cas9 system, should aid researchers in this

respect. Such targeted deletion studies *in vivo* will be necessary to uncover the true functions of the many uncharacterized lncRNAs that are active during normal and malignant hematopoiesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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