LIN28B is over-expressed in specific subtypes of pediatric leukemia and regulates lncRNA *H19*

LIN28B is an RNA-binding protein with an oncofetal expression pattern. High *LIN28B* expression is crucial during human embryogenesis and is down-regulated in most tissues after birth.[1] However, reactivation during oncogenesis is common in a plethora of adult cancers, including leukemia, and was also detected in solid pediatric tumors such as neuroblastoma, Wilms tumor, pediatric hepatoblastoma and hepatocellular carcinoma, germ cell tumors and rhabdoid tumors.[2] More recently, we identified high *LIN28B* expression in a large subgroup of

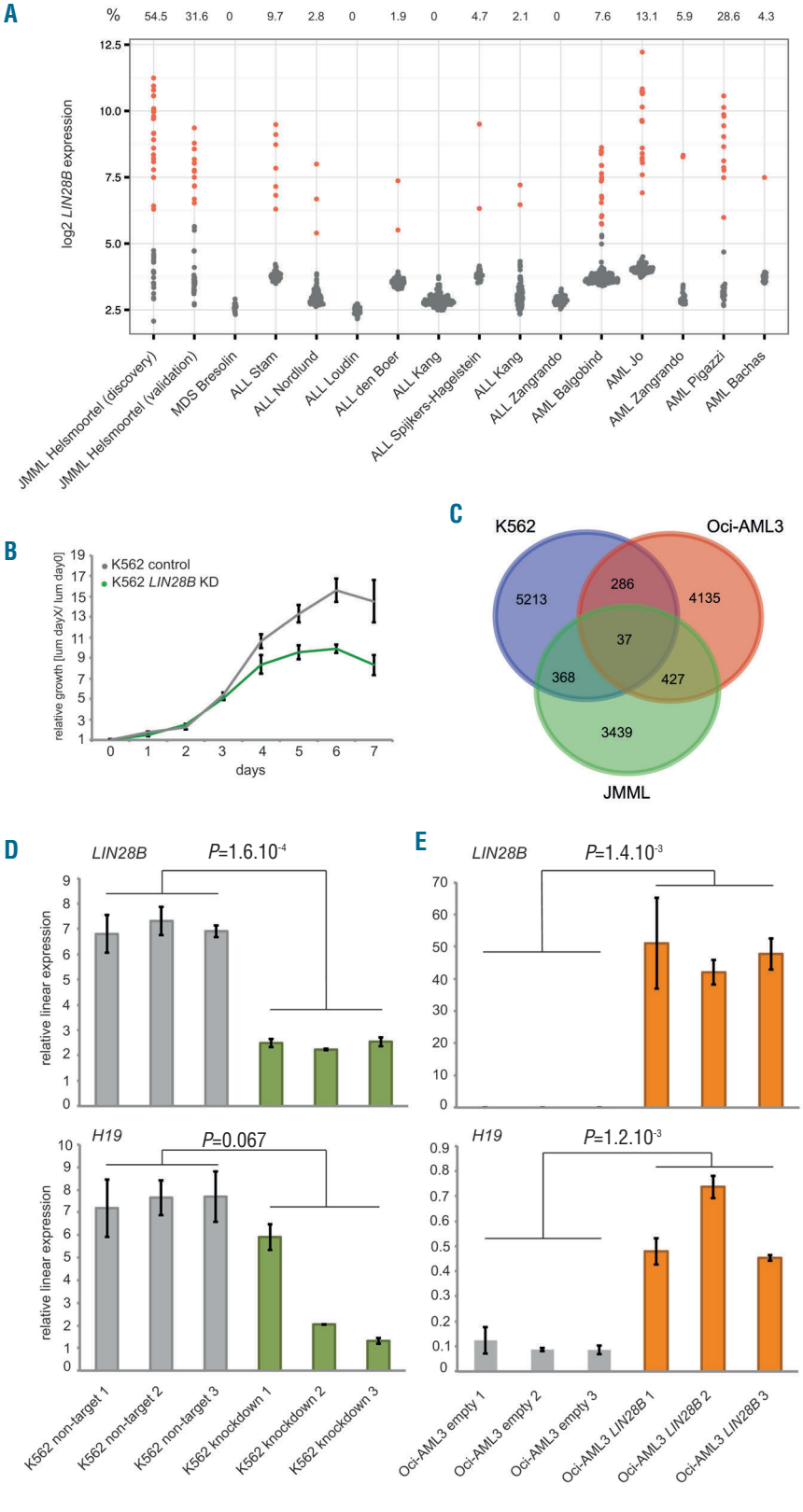

Figure 1. (A) **Log2 *LIN28B* expression values in 14 publicly available pediatric leukemia datasets,[3-16] visualized using SinaPlot.** Each dot is a patient and red dots represent values above the microarray average. The numbers on top represent the percentage of patients with *LIN28B* expression higher than the average of the study. (B) Relative growth compared to the day of cell seeding of control (gray) and *LIN28B* knockdown K562 cells (green). Lum: luminescence. (C) Differential expressed genes between K562 *LIN28B* knockdown and control cells, Oci-AML3 *LIN28B* over-expressing and control cells, and juvenile myelomonocytic leukemia (JMML) patients with high and low *LIN28B* expression. Venn diagram was generated using http://bioinformatics.psb.ugent.be/webtools/Venn/ (D) RT-qPCR expression of *LIN28B* and *H19* in K562 control (gray) and *LIN28B* knockdown cells (green). (E) RT-qPCR expression of *LIN28B* and *H19* in Oci-AML3 control (gray) and *LIN28B* over-expressing cells (orange).

juvenile myelomonocytic leukemia (JMML), an aggressive childhood hematopoietic neoplasm.[3]

To further evaluate putative oncogenic activity of *LIN28B* in a broad panel of pediatric hematologic malignancies, we conducted a meta-analysis of *LIN28B* expression in different types of childhood leukemia using publicly available Affymetrix Human Genome U133 Plus 2.0 microarrays. For this, we investigated gene expression profiles from 1361 childhood leukemia patients in 14 independent studies: GSE71454,[3] GSE29326,[4] GSE19475,[5] GSE47051,[6] GSE20910,[7] GSE13351,[8] GSE11877,[9] GSE32962,[10] GSE68720,[11] GSE14062,[12] GSE17855,[13] GSE35784,[14] GSE19577[15] and GSE52891.[16] All available Affymetrix data were renormalized using RMA in R Bioconductor and, given the absence of *LIN28B* expression after birth, *LIN28B* was considered over-expressed if it exceeded the average expression of the array (Figure 1A, visualized using SinaPlot[17]). In contrast to JMML, *LIN28B* is not over-expressed in the evaluated pediatric myelodysplastic syndrome (MDS) cohort and, in general, *LIN28B* is more frequently over-expressed in pediatric acute myeloid leukemia (AML, 10.7%) compared to acute lymphoblastic leukemia samples (ALL, 2%). In a similar meta-analysis for *LIN28A*, only one patient with *LIN28A* overexpression was detected in the whole cohort, suggesting distinct functions for both human *LIN28* homologs (*Online Supplementary Figure S1A*).

Despite the fact that increasing evidence points to the importance of *LIN28B* in malignant hematopoiesis, including the observation that *LIN28B* is able to reprogram adult hematopoietic progenitors to mediate fetal-like lymphopoiesis,[18] the role of *LIN28B* in leukemic development is far from understood. Therefore, we altered *LIN28B* expression in normal and malignant hematopoietic cells. First, we retrovirally modulated *LIN28B* expression in two human myeloid leukemia cell lines with different *LIN28B* levels (*Online Supplementary Methods*). Stable shRNA mediated knockdown was achieved in K562, a cell line with very high intrinsic *LIN28B* levels that originates from a chronic myeloid leukemia patient in blast crisis (*Online Supplementary Figure S1B and D*). In addition, we stably over-expressed

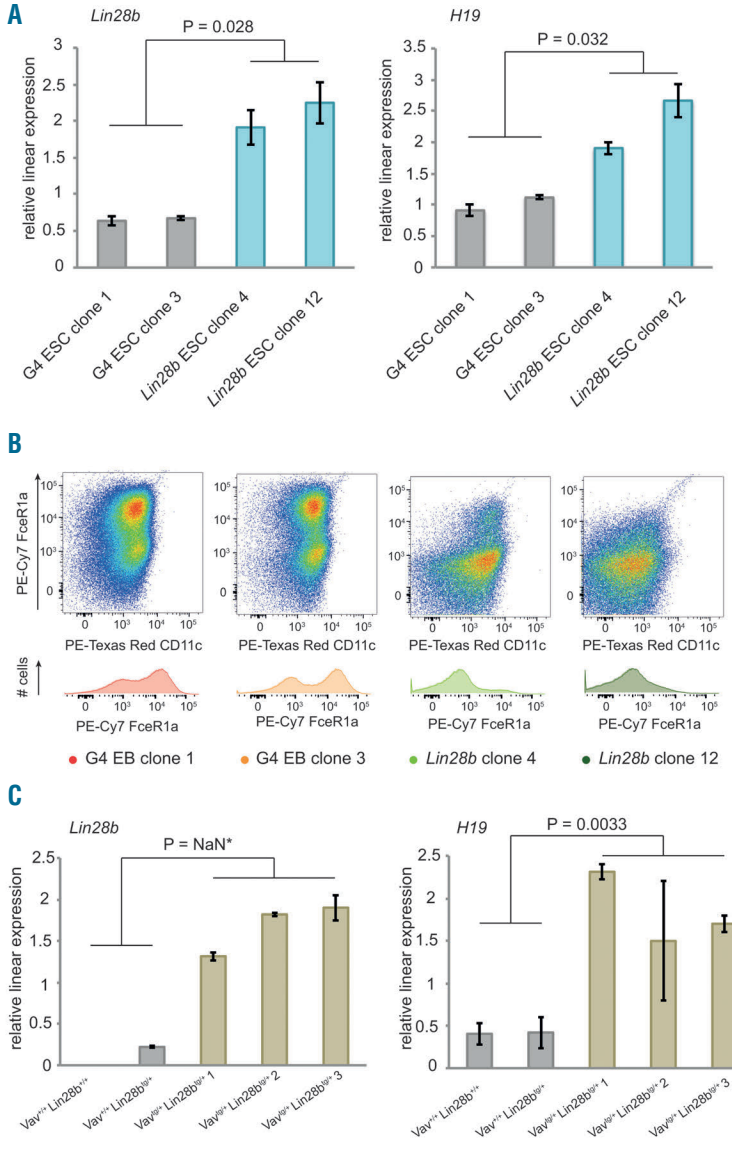

Figure 2. (A) ***Lin28b*** and ***H19*** **expression in G4 embryonic stem cells (ESCs) (gray) and *Lin28b* ESCs (blue).** (B) FACS analysis of G4 and *Lin28b* DIV10 embryoid bodies that were grown on methylcellulose plates for 14 days. (Top) CD11c is plotted *versus* FceR1a in CD45$^+$ cells. The bottom graph represents the distribution of the FceR1a receptor. (C) RT-qPCR results showing *Lin28b* and *H19* expression in Vav-Lin28b mice. Mice without *Lin28b* expression (Vav$^{+/+}$Lin28b$^{+/+}$ and Vav$^{+/+}$Lin28b$^{tg/+}$) are indicated in gray; mice with *Lin28b* expression (Vav$^{tg/+}$ Lin28b$^{tg/+}$) in brown. Tg: transgene. **P*-value for *Lin28b* could not be calculated in qBasePlus due to complete absence of gene expression in the left mouse.

LIN28B in the acute myeloid leukemia cell line Oci-AML3, which lacks endogenous *LIN28B* expression (*Online Supplementary Figure S1C and D*). In line with previous reports,[19] loss of *LIN28B* decreased leukemic cell growth in K562 cells (Figure 1B and *Online Supplementary Methods*). However, growth rates were comparable between Oci-AML3 cells with and without *LIN28B* expression (*Online Supplementary Figure S2A*).

To study the transcriptional consequences of *LIN28B* modulation in the context of human leukemia, we profiled both stable cell line models and their respective controls using Agilent gene expression microarrays containing probes for 27958 mRNA transcripts and 23042 long non-coding RNAs (GSE79981).[20] In addition, expression profiles from 44 JMML patients were previously generated using the same platform (GSE71449). Differential gene expression (GenePattern;[21] raw *P*-values<0.05) revealed 5904 differentially expressed probes between the *LIN28B* knockdown K562 cells and control cells, 4885 probes between the *LIN28B* over-expressing Oci-AML3 cells and control cells, and 4271 probes between *LIN28B*-high and *LIN28B*-low JMML patients. Interestingly, the intersection of these three analyses contained 37 probes, all of which correlated with *LIN28B* expression (Figure 1C). However, and most notably, a large proportion of probes within this intersection hybridized to lncRNA *H19* (*Online Supplementary Table S1 and Figure S2B*). *H19* regulation by *LIN28B* was validated by RT-qPCR in both cell lines, with the exception of one outlier (Figure 1D and E and *Online Supplementary Methods*). In addition, we confirmed the previously established reciprocal relationship between *LIN28B* and miRNA *let-7* expression in these *in vitro* models (*Online Supplementary Figure S2C and D and Online Supplementary Methods*). We did not observe a correlation between *LIN28B* and *H19* expression in 82 publicly available leukemia and lymphoma cell line data (Spearman's rho=0.0929, *P*=0.406) (*Online Supplementary Figure S3A*).[22]

Although four potential *let-7* binding sites have been shown previously identified in the human *H19* sequence[23] and *H19* has been shown to be a target of *let-7* in muscle cells,[24] there has been no report of a direct regulation of *H19* by *LIN28B*. Remarkably, *H19* was described as the uttermost differentially expressed gene between fetal and adult murine hematopoietic stem cells, followed by *Lin28b*.[25] In addition, *H19* is known as an oncofetal gene with high expression before birth and has a critical role in tumor growth and metastasis.[26] Recently, Zhou *et al*. evaluated the transcriptional effects of *Lin28b* expression in adult mouse bone marrow as well as *let-7* activation in murine fetal liver hematopoietic cells.[27] Notably, reanalysis of the data revealed strong *H19* activation upon reintroduction of *Lin28b* in the adult bone marrow (*Online Supplementary Figure S3B*). In contrast, *let-7* induction in the fetal liver triggered a profound decrease in *H19* and *Lin28b* expression (*Online Supplementary Figure S3C*).

Given these observations, we wondered whether the regulation of *H19* by *LIN28B* in hematopoietic cells could be driven, at least in part, by the inhibitory effect of LIN28B protein on the *let-7* family of microRNAs (*Online Supplementary Figure S4A*). To this end, we introduced a *let-7* mimic cocktail in fluorescence activated cell sorted (FACS) stable retrovirally transduced *LIN28B*-over-expressing Oci-AML3 cells and evaluated *LIN28B* and *H19* expression after 48 h (*Online Supplementary Methods*). Given that the ectopically expressed *LIN28B* has no 3'UTR, we hypothesized that every effect of *let-7* on *H19* should be *LIN28B*-independent and not the result of diminished direct binding of *LIN28B* to *H19*. As expected, *let-7* mimics induced a significant reduction of *H19* expression (*Online Supplementary Figure S4B*), whereas *LIN28B* levels were not significantly affected. Nevertheless, there was a trend towards lower *LIN28B* expression in *let-7* electroporated cells, suggesting the presence of putative *let-7* binding sites in the protein-coding region of *LIN28B* (*Online Supplementary Figure S4C*). In contrast, introducing a *let-7* mimic cocktail in the empty control K562 cell line significantly down-regulated *LIN28B* (with 3'UTR), but not *H19* (*Online Supplementary Figure S4D and E*). All experiments were replicated with the puromycin-selected cells and yielded the same results (*data not shown*). These results indicate that investigating the role of *let-7* is hampered by the double negative feedback interaction between *LIN28B* and *H19* and that other regulatory mechanisms, such as direct binding of *LIN28B* to *H19*, could be involved in this complex regulatory network, a notion that warrants further investigation. In the light of our previous study, we analyzed the expression of 768 miRNAs, including the *H19*-encoded *miR-675*, in 21 JMML patients using an RT-qPCR-based platform.[3] Although half of the patients showed enhanced *LIN28B* and *H19* levels, *miR-675* was not expressed in any of the samples.

To further evaluate the association between *LIN28B* and *H19* in non-malignant cells, we investigated two replicates of G4 ROSALUC mouse embryonic stem cells (G4 ESCs) and generated two independent ESC clones with ectopic ROSA26 driven *Lin28b* expression (R26-*Lin28b* ESCs) (*Online Supplementary Methods*).[28] After G418 selection, RT-qPCR showed significant upregulation of murine *H19* in *Lin28b* ESCs as compared to G4 ESC controls (Figure 2A). These observations extend the regulation of *H19* by *Lin28b* to a murine, non-malignant model system. Subsequently, the four ESC clones described above were differentiated into embryoid bodies for ten days and seeded on methylcellulose plates for fourteen days. All colonies were harvested and subjected to a 12-color FACS-analysis. We measured 13 different cell surface markers associated with distinct hematopoietic populations (CD45, CD3, CD19, NK1.1, CD115, Ly6C, CD11b, Ly6G, MHCII, CD170, CD11c, Ter119 and FceR1a) (*Online Supplementary Methods*). *Lin28b* overexpression clearly affected FceR1a receptor levels, with declining FceR1a upon *Lin28b* activation (Figure 2B). Interestingly, these observations are in line with a recent report from Wang *et al*. in which bone marrow-derived mast cell cultures and common myeloid progenitors from induced iLIN28B mice were characterized by reduced levels of *Fcer1a*.[29]

Finally, we used a previously established conditional ROSA26 *Lin28b* knock-in mouse model[30] to provide independent confirmation of the putative association between *Lin28b* and *H19* in murine hematopoietic cells. Enforced *Lin28b* expression in the hematopoietic compartment of adult mice using Vav-iCre resulted in increased levels of murine *H19* when compared to littermate controls, thereby confirming the regulation of *H19* by *Lin28b* (Figure 2C).

In conclusion, we show that lncRNA *H19*, one of the first discovered long non-coding RNAs, is regulated by *LIN28B*. *H19* has been shown to alter DNA methylation genome wide and associates with EZH2, a member of the Polycomb Respressive Complex 2 (PRC2), which was recently identified to be mutated in JMML. *LIN28B*-driven deregulated *H19* expression might provide yet another mechanism driving aberrant methylation in JMML and other leukemias. The discovery of *LIN28B* as a regulator

of *H19*, known to be involved in hematopoiesis, embryonic development and oncogenesis, can help to clarify the function of this intriguing lncRNA.

Hetty H. Helsmoortel,[1,2] Barbara De Moerloose,[1] Tim Pieters,[2-4] Farzaneh Ghazavi,[1,2] Silvia Bresolin,[5] Hélène Cavé,[6] Andrica de Vries,[7] Valerie de Haas,[8] Christian Flotho,[9] Veerle Labarque,[10] Charlotte Niemeyer,[9] Pascale De Paepe,[11] Nadine Van Roy,[2] Jan Stary,[12] Marry M. van den Heuvel-Eibrink,[8,13] Yves Benoit,[1] Johannes Schulte,[14-18] Steven Goossens,[3,4] Geert Berx,[3,4] Jody J. Haigh,[19] Frank Speleman,[2] Pieter Van Vlierberghe,[2] and Tim Lammens[1*]*

[1]Department of Paediatric Haematology-Oncology and Stem Cell Transplantation, Ghent University Hospital, Belgium; [2]Center for Medical Genetics, Ghent University, Belgium; [3]Unit for Molecular and Cellular Oncology, VIB Inflammation Research Center, Ghent University, Belgium; [4]Department of Biomedical Molecular Biology, Ghent University, Belgium; [5]Department of Women and Child Health, University of Padova, Italy; [6]Department of Genetics, University Hospital of Robert Debré and Paris-Diderot University, Paris, France; [7]Department of Paediatric Oncology/Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands; [8]Dutch Childhood Oncology Group (DCOG), the Hague, the Netherlands; [9]Department of Paediatrics and Adolescent Medicine, Division of Paediatric Haematology and Oncology, University of Freiburg, Germany; [10]Pediatric Haemato-Oncology, University Hospitals Leuven, Belgium; [11]Pathological Anatomy, AZ Sint Jan Bruges, Belgium; [12]Department of Paediatric Haematology/Oncology, Charles University and University Hospital Motol, Prague, Czech Republic; [13]Princess Maxima Center for Paediatric Oncology, Utrecht, the Netherlands; [14]Division of Neuroblastoma Genomics, German Cancer Research Center (DKFZ), Heidelberg, Germany; [15]Department of Paediatric Oncology and Haematology, University Children's Hospital Essen, Essen, Germany; [16]German Consortium for Translational Cancer Research (DKTK), Essen, Germany; [17]Translational Neuro-Oncology, West German Cancer Center (WTZ), University Hospital Essen, University Duisburg-Essen, Germany; [18]Centre for Medical Biotechnology, University Duisburg-Essen, Germany; and [19]Mammalian Functional Genetics Laboratory, Division of Blood Cancers, Australian Centre for Blood Diseases, Monash University, Melbourne, Victoria, Australia.

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The online version of this letter has a Supplementary Appendix.

** PVV and TL contributed equally to this work*

Correspondence: Tim.Lammens@UGent.be

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