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

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LIN28B is overexpressed in specific subtypes of paediatric leukaemia and regulates lncRNA *H19*

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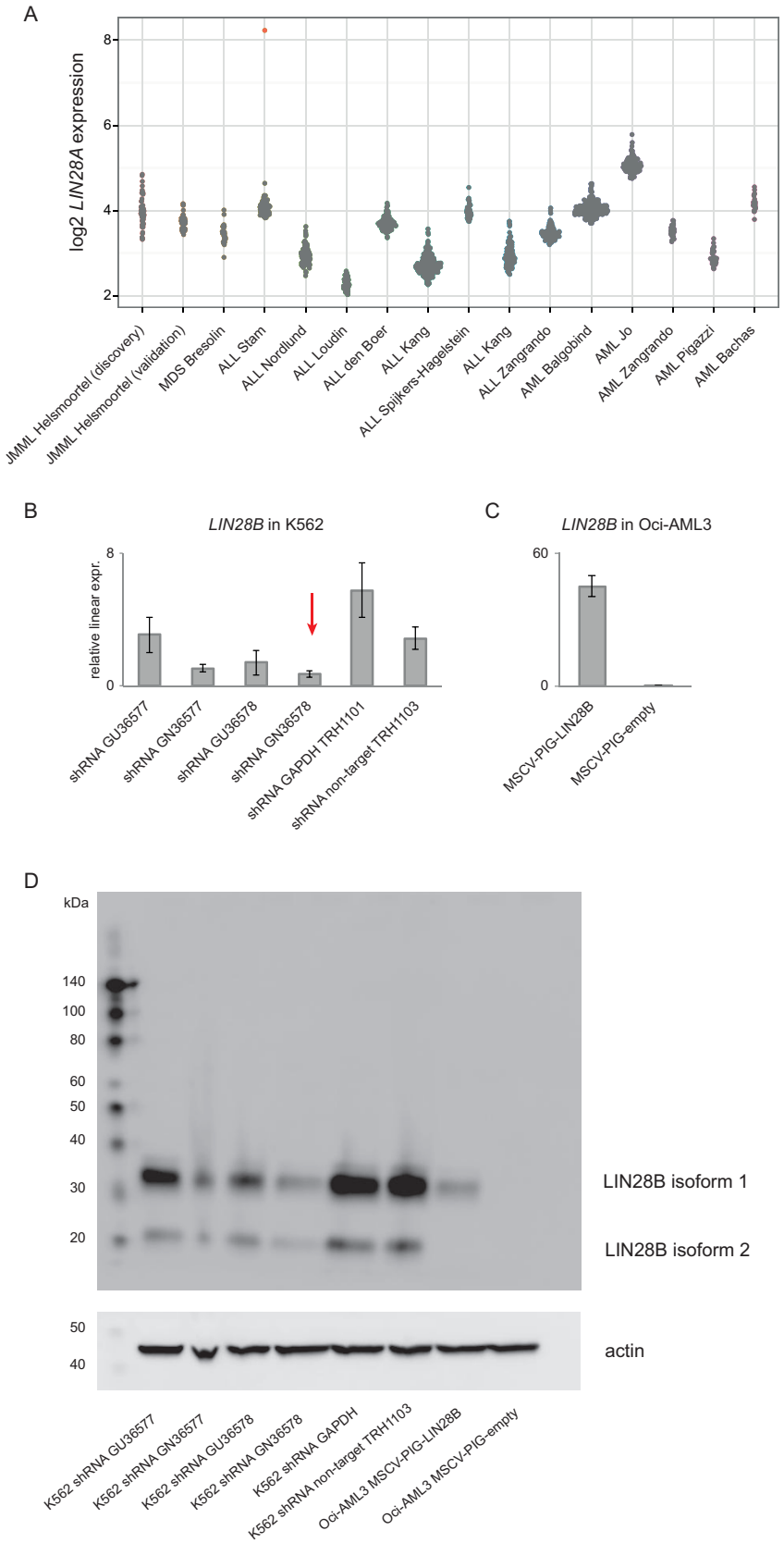
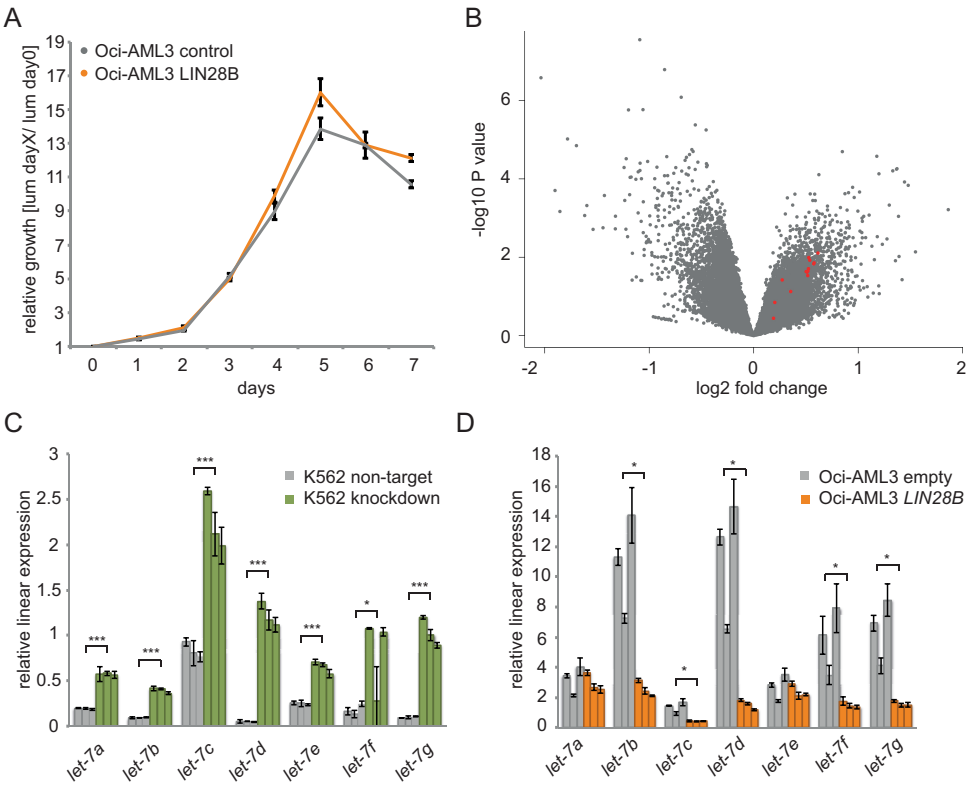
Supplemental figures and legends

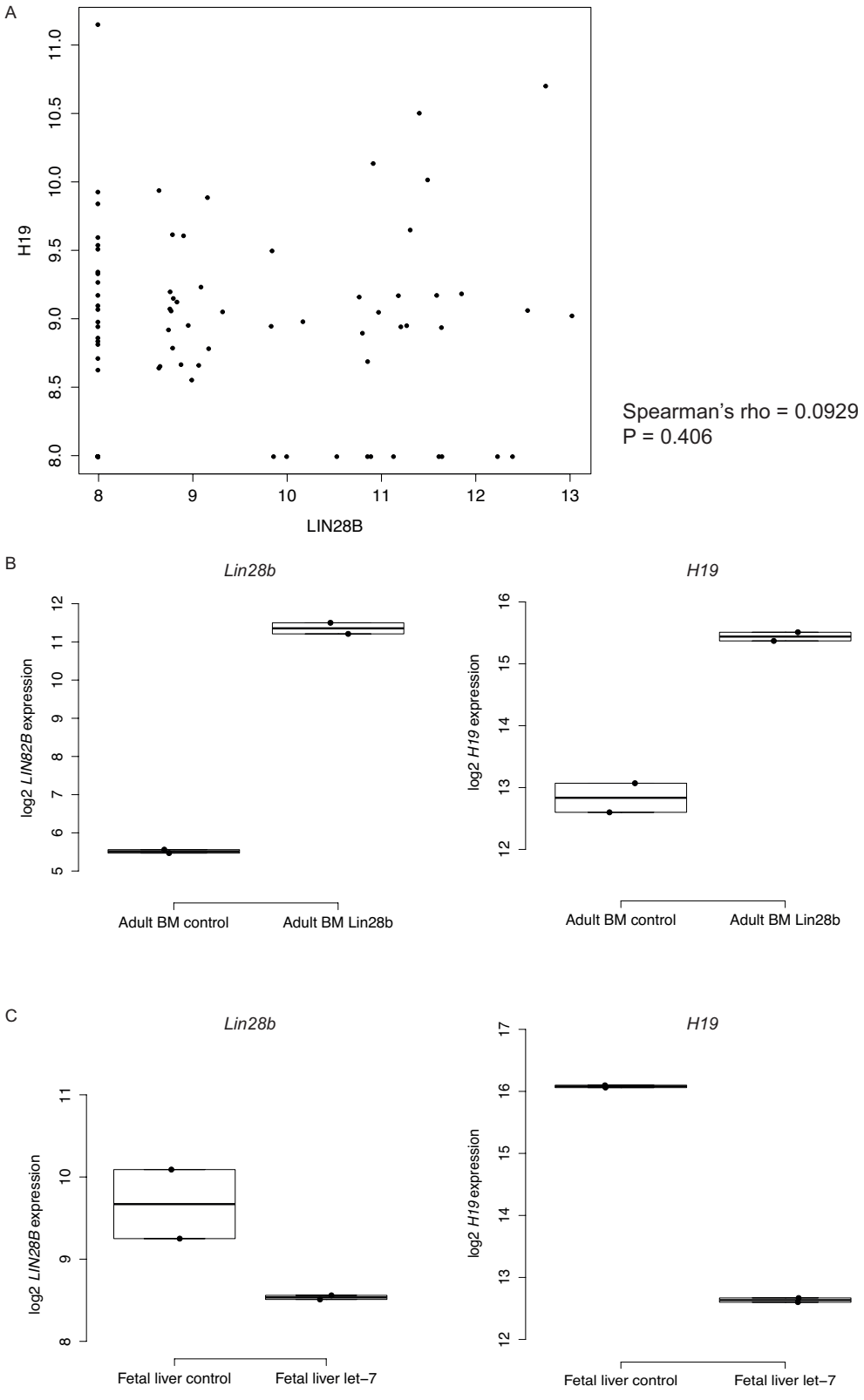
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure S3



Supplemental Figure 4

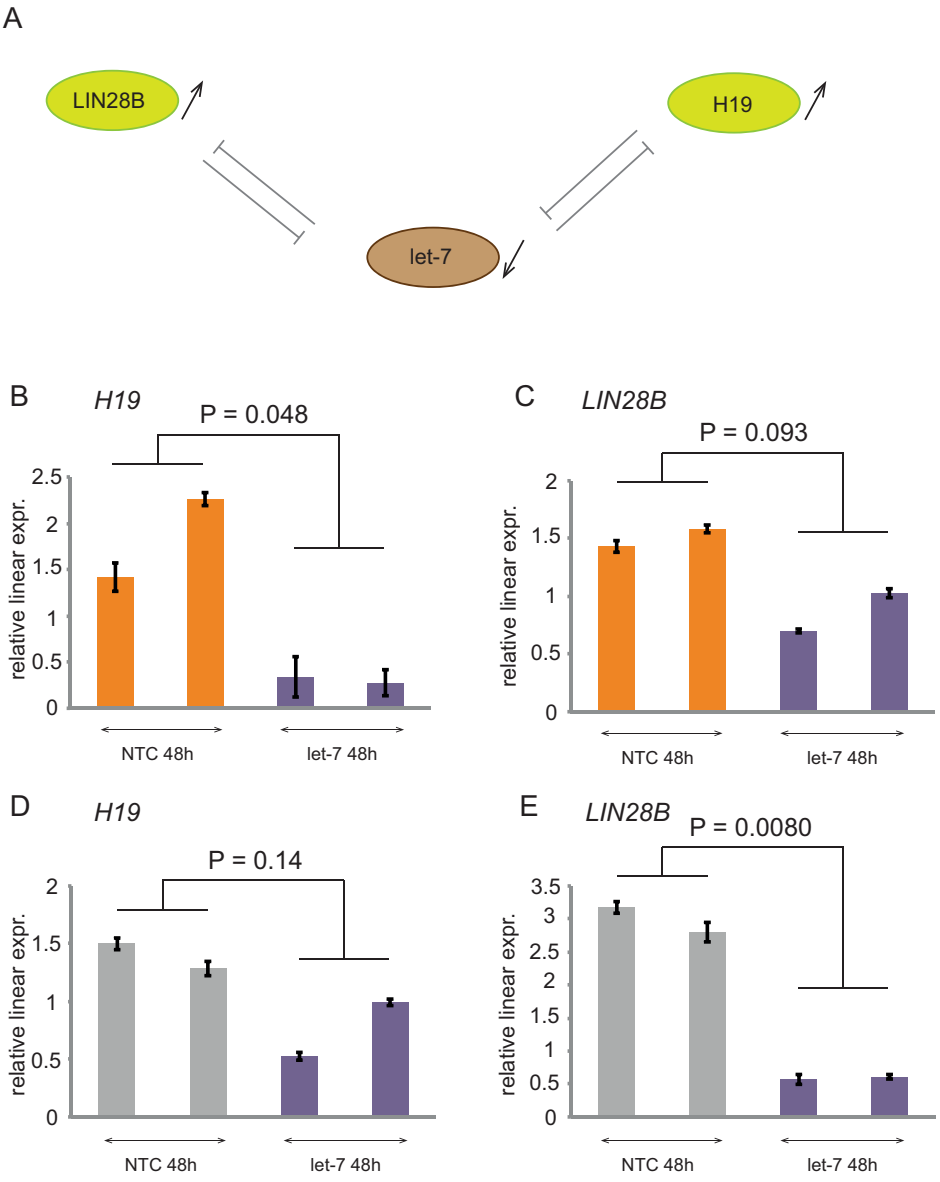


Figure S1. (A) Log2 *LIN28A* expression values in 14 publicly available paediatric leukaemia datasets, visualized using SinaPlot. Each dot is a patient and the red dot represents a value above the microarray average. (B) RT-qPCR *LIN28B* expression of retrovirally transduced K562 cells. (C) RT-qPCR *LIN28B* expression of retrovirally transduced Oci-AML3 cells. (D) Western blot measuring LIN28B protein levels relative to actin in the stable K562 and Oci-AML3 cell lines.

Figure S2. (A) Relative growth compared to the day of cell seeding of control (grey) and *LIN28B* overexpressing Oci-AML3 cells (orange). Lum = luminescence. (B) Volcano plot representing differentially expressed genes between JMML patients with high and low *LIN28B* expression. Dots on the right (log2 fold change > 0) are probes overexpressed in patients with

high *LIN28B* levels. Red dots represent the 15 different *H19* probes. The *LIN28B* probe was omitted for scalability reasons. (C) Multiplex RT-qPCR results showing the expression level of *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f* and *let-7g* in K562 control (grey) and *LIN28B* knockdown cells (green) in triplicates isolated at three different time points. (D) Multiplex RT-qPCR results showing the expression level of *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f* and *let-7g* in Oci-AML3 control (grey) and *LIN28B* overexpression cells (orange) in triplicates isolated at three different time points.

Figure S3. (A) Correlation analysis between *LIN28B* and *H19* in 82 publicly available leukaemia/lymphoma cell lines. (B) Microarray results of *Lin28b* and *H19* expression after *Lin28b* introduction in murine adult bone marrow cells according to experiment GSE65536 in the GEO database. (C) Microarray results of *Lin28b* and *H19* expression after *let-7* overexpression in murine foetal liver cells according to experiment GSE65536 in the GEO database.

Figure S4. (A) Schematic representation of the interaction between *LIN28B*, *let-7* and *H19*. (B) RT-qPCR expression of *H19* and (C) *LIN28B* in FACS-sorted Oci-AML3-LIN28B cells electroporated with a negative control (orange) or a cocktail of *let-7* miRNAs (purple). (D) qRT-PCR expression of *H19* and (E) *LIN28B* in FACS-sorted K562-control cells electroporated with a negative control (grey) or a cocktail of *let-7* miRNAs (purple). Biological qRT-PCR replicates underwent the same electroporation but independent growth, RNA isolation and cDNA synthesis. All experiments were replicated using the puromycin-selected cells and yielded the same results; the graphs presented are representative.

Supplemental tables

Supplemental Table T1. Overlapping probes

Probe	Gene name	K562 Fold Change	Oci-AML3 Fold Change	JMML Fold Change
A_33_P3401902	ANKRD20A2	3.784	0.580	0.709
CUST_11022_PI427704219	lnc-C11orf89-2:14 (H19)	2.339	1.949	0.760
CUST_5212_PI427704219	lnc-C11orf89-2:6 (H19)	1.530	2.756	0.756
CUST_16121_PI427704219	lnc-C11orf89-2:4 (H19)	1.882	2.227	0.724
CUST_13030_PI427704219	lnc-C11orf89-2:9 (H19)	1.467	2.380	0.834
CUST_1184_PI427704219	lnc-C11orf89-2:5 (H19)	1.561	2.225	0.773
CUST_21457_PI427704219	lnc-C11orf89-2:8 (H19)	1.468	2.097	0.748
CUST_5_PI427704219	lnc-C11orf89-2:12 (H19)	1.483	2.051	0.746
CUST_11168_PI427704219	lnc-C11orf89-2:3 (H19)	1.482	1.825	0.887
CUST_18927_PI427704219	lnc-C11orf89-2:7 (H19)	1.518	1.503	0.828
CUST_4470_PI427704219	lnc-C11orf89-2:2 (H19)	1.515	1.461	0.736
A_33_P3298159	PTGDS	0.920	1.603	0.896
CUST_10036_PI427704219	lnc-DLK1-9:3	1.258	0.760	1.073
A_24_P329795	C10orf10	0.482	1.086	1.513
A_33_P3396214	KREMEN2	0.777	1.044	1.182
A_23_P329890	TMEM136	0.578	1.222	0.932
A_23_P380614	ATP9A	0.576	1.024	1.060
A_33_P3415633	TMEM136	0.219	1.711	0.632
CUST_21386_PI427704219	lnc-TMEM206-5:1	1.072	0.991	0.420
A_23_P25706	CLMN	0.368	0.653	1.436
A_24_P112032	KCNK17	0.463	0.435	1.177
A_23_P200685	MOSC2	0.326	0.661	1.081
CUST_19628_PI427704219	lnc-AC005481.5.1-1:4	0.840	0.758	0.452
A_23_P386320	MFI2	0.225	0.839	0.557
A_24_P318897	SNX21	0.582	0.428	0.549
A_33_P3272921	ARID3A	0.332	0.581	0.619
A_23_P360874	LRWD1	0.414	0.367	0.737
CUST_4552_PI427704219	lnc-ZC3H12D-6:1	0.638	0.492	0.369
A_24_P410605	ROR1	0.480	0.276	0.738
CUST_3307_PI427704219	lnc-FRMD4A-1:4	0.188	0.460	0.841
A_23_P50276	ANGPTL6	0.340	0.280	0.853
A_24_P182620	CELSR2	0.576	0.417	0.461
CUST_393_PI427704219	lnc-FRMD4A-1:2	0.266	0.415	0.739
CUST_10068_PI427704219	lnc-TSPY10-2:1	0.489	0.487	0.358
CUST_10166_PI427704219	lnc-LMOD1-3:1	0.393	0.514	0.264
CUST_7371_PI427704219	lnc-BTBD10-3:1	0.411	0.137	0.325
A_23_P349406	RIMKLA	0.291	0.178	0.372

Supplemental Table T2. Genotyping mice

Lin28b

95°C 5 min
39 CYCLES 95°C 10s, 59.5°C 15s, 72°C 10s
72°C 5 min
10°C hold

Wild type band	Forward primer	CATGTCTTTAATCTACCTCGATGG
(299 bp)	Reverse primer	CTCTTCCCTCGTGATCTGCAACTCC
Mutant band	Forward primer	GTGACATTGACATCCACTTTGC
(461 bp)	Reverse primer	CCCAAGGCACACAAAAAACC

Vav

94°C 3 min
35 CYCLES 94°C 30s, 51.7°C 60s, 72°C 60s
72°C 2 min
10°C hold

Mutant band	Forward primer	AGATGCCAGGACATCAGGAACCTG
(236 bp)	Reverse primer	ATCAGCCACACCAGACACAGAGATC

Supplemental Table T3. Primer sequences

Human primer sequences

LIN28B	o Forward: 5’-TCTTCCAAAGGCCTTGAGTC-3’ o Reverse: 5’- GCACTTCTTTGGCTGAGGAG-3’
H19 (Fig 1)	o Forward: 5’-GCACCTTGGACATCTGGAGT-3' o Reverse: 5’-TTCTTTCCAGCCCTAGCTCA-3'
H19 (Fig S4)	o Forward: 5’-CCCACAACATGAAAGAAATGGTGC-3' o Reverse: 5’-CACCTTCGAGAGCCGATTCC-3'
TBP	o Forward: 5’-CACGAACCACGGCACTGATT-3’ o Reverse: 5’-TTTTCTTGCTGCCAGTCTGGAC-3’
HMBS	o Forward: 5’-GGCAATGCGGCTGCAA-3’ o Reverse: 5’-GGGTACCCACGCGAATCAC-3’
SDC4	o Forward: 5'-CAGGGTCTGGGAGCCAAGT-3' o Reverse: 5'-GCACAGTGCTGGACATTGACA-3'
SDHA	o Forward: 5’-TGGGAACAAGAGGGCATCTG-3’ o Reverse: 5’-CCACCACTGCATCAAATTCATG-3’
HPRT1	o Forward: 5’-TGACACTGGCAAAACAATGCA-3’ o Reverse: 5’-GGTCCTTTTCACCAGCAAGCT-3’
YHWAZ	o Forward: 5'-ACTTTTGGTACATTGTGGCTTCAA-3' o Reverse: 5'-CCGCCAGGACAAACCAGTAT-3'
RPL13A	o Forward: 5'-CCTGGAGGAGAAGAGGAAAGAGA-3' o Reverse: 5'-TTGAGGACCTCTGTGTATTTGTCAA-3'
UBC	o Forward: 5’-ATTTGGGTCGCGGTTCTTG-3’ o Reverse: 5’-TGCCTTGACATTCTCGATGGT-3’

Mouse primer sequences

mGapdh	o Forward: 5’-CCCCAATGTGTCCGTCGTG-3’ o Reverse: 5’-GCCTGCTTCACCACCTTCT-3’
mTbp	o Forward: 5'-CCCCACAACTCTTCCATTCT-3' o Reverse: 5'-GCAGGAGTGATAGGGGTCAT-3'
mG6pdh	o Forward: 5'-ATGCAGAACCACCTCCT-3’ o Reverse: 5’-TTCAACACTTTGACCTTCTCA-3’
mUbc	o Forward: 5'-GCAGATCTTTGTGAAGACCC-3' o Forward: 5'-GAAGGTACGTCTGTCTTCCT-3'
mLin28b 3	o Forward: 5’-GAGTCCAGGATGATTCCAAGA-3’ o Reverse: 5’-TGCTCTGACAGTAATGGCACTT-3’
mH19	o Forward: 5’-AATGGTGCTACCCAGCTCAT -3' o Reverse: 5’-TCAGAACGAGACGGACTTAAAGAA-3'

Supplemental methods

Generating stable cell lines

K562 cells were retrovirally transduced with four Transomic Platinum Select MLP Retroviral shRNAs against *LIN28B* (RLGH-GU36577, RLGH-GN36577, RLGH-GU36578 and RLGH-GN36578), a negative control (shRNA-miR non-targeting control TRH1103) and a positive control (shRNA against *GAPDH* TRH1101). Oci-AML3 cells were retrovirally transduced with MSCV-PIG-LIN28B and MSCV-PIG-empty vectors (gifts from Joshua Mendel lab). Cells were selected using puromycin, a fraction of the cells were harvested and immediately frozen at -80°C (in Trizol for RNA isolation and as a pellet for Western blot). *LIN28B* knockdown or overexpression was analyzed by RT-qPCR (see below). Total protein isolation was performed with RIPA-lysis buffer, supplemented with protease inhibitors and SDS-PAGE was performed according to standard protocols. For immunoblotting, the rabbit antibody against LIN28B (Cell Signaling, #4196S) was used in a 1:3000 dilution in milk and the mouse antibody against actin (Sigma, A2228) in a 1:5000 dilution.

The best knockdown in K562 was achieved with shRNA GN36578. For subsequent analyses, RNA was isolated at three different time points from the GN36578 (K562 *LIN28B* knockdown) and the negative control TRH1103 cell line (K562 control). The same was done for the Oci-AML3 MSCV-PIG-LIN28B (Oci-AML3 *LIN28B*) and MSCV-PIG-empty cell line (Oci-AML3 control). For all experiments involving *let-7* electroporation (see below), transduced Oci-AML3 and K562 cells were FACS-sorted (GFP+) instead of selected by puromycin.

Growth assays

K562 control, K562 *LIN28B* knockdown, Oci-AML3 control and Oci-AML3-*LIN28B* cells were seeded in triplicate in eight 96-well plates at a density of 10.000 cells. The number of viable cells was measured every 24 hours for eight consecutive days using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The same experiment with 5.000 and 7.500 cells yields similar results.

Let-7 electroporation

10μL of a 20μM stock cocktail of four let-7 mimics (*let-7a* (AM17100/PM10050), *let-7b* (AM17100/PM11050), *let-7e* (AM17100/PM12304), *let-7g* (AM17100/PM11758),

Life Technologies) or a negative control pre-miR (AM17110, Life Technologies) was electroporated in 500μL Oci-AML3-LIN28B or K562-TRH1103 cells at a density of 50 million cells per mL RPMI. Subsequently, two replicates of 100μL were seeded in 1.9 mL RPMI + 15% foetal calf serum and harvested after 48h. RNA isolation and RT-qPCR were then conducted as described below.

Multiplex PCR of *let-7* family in Oci-AML3 and K562 cells

A primerpool of 11 stem-loop RT primers (U6 snRNA, RNU24, RNU48, RNU6B, let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, Life Technologies) was concentrated 2.75 times by vacuum centrifugation. Sample RNA was diluted to 10 ng/μL and cDNA generated using the TaqMan MicroRNA Reverse Transcription Kit in a total volume of 20μL (0.4μL dNTPs, 4μL Multiscribe reverse transciptase, 2μL RT-buffer (10X), 0.25μL RNase inhibitor, 8.35μL water, 4μL stem-loop RT primerpool and 1μL RNA). Cycling conditions were 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and 4°C for 5 min. RT-qPCR was performed with 4μL Taqman Universal Master Mix II, no UNG (Life Technologies), 0.2μL TaqMan probe and primers, 0.8μL water and 3μL cDNA (5 times diluted) and read with a LightCycler 480 (Roche). Data were analysed with qBasePLUS software according to the ΔΔCt-method (Biogazelle)[1]. Cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, and finally 40°C for 30 sec.

***Lin28b* overexpressing ESC and embryoid bodies**

Murine Lin28b (Transomic) was cloned in a Topo vector and was shuttled by Gateway® cloning into a recombination-mediated cassette exchange (RMCE)-compatible cre-excised pRMCE-DV1 vector[2]. G4 ROSALUC ESCs were cotransfected with a cre-excised pRMCE-DV1-Lin28b vector and a FlpE-expressing plasmid (pCAGGS-FlpE-IRES-puromycin-pA)[3] in a 1:1 ratio using Lipofectamin 2000 reagent (Invitrogen). The FlpE-mediated cassette exchange inserted *Lin28b* into the ROSA26 locus and, in addition, restored neomycin-resistance. After 7 to 10 days G418 selection (200 mg/ml), individual G418-resistant RMCE-targeted ES colonies were picked, expanded and validated. Two independent clones were selected for further analysis. The procedure for forming embryoid bodies (EBs) was similar to that described before[4]. In brief, ES cells were feeder-depleted and passaged once on gelatinized plates. For random differentiation into EBs, ES cells were plated in different dilutions unto bacterial grade Petri-dishes in differentiation medium (IMDM medium (Invitrogen) supplemented with 15% FBS (Hyclone), 5% PFMHII

(Invitrogen), 2 mM L-glutamine (Invitro- gen), 0.4 mM MTG (Sigma), 50 µg/mL ascorbic acid (Sigma) and penicillin (100 U/mL)-streptomycin (100 µg/mL) (Invitrogen). EBs were allowed to form in these dishes for ten days.

Vav-Lin28b mice

LSL-*Lin28b* mice with a loxP-flanked transcriptional termination site upstream of the *Lin28b* gene in the ROSA26 locus were a gift from the Schulte lab[5]. The animals were crossed with Vav-iCre[6] obtained from The Jackson Laboratory mice to achieve stable ectopic Lin28b expression in the hematopoietic system. One $Vav^{+/+}$ $Lin28b^{+/+}$ from the first litter was sacrificed after 26 weeks. Four adult mice from the second litter were sacrificed 12 weeks after birth (one $Vav^{+/+}$ $Lin28b^{tg/+}$ and three $Vav^{tg/+}$ $Lin28b^{tg/+}$) and bone marrow was isolated from the tibia and femur. Crushed cells were treated with red cell lysis buffer. Mouse tails were genotyped using the KAPA Taq HotStart PCR kit (Kapa Biosystems). Primers and PCR protocol are listed in Supplemental Table T2. The ethical committee of Ghent University approved the animal experiments (ECD 13/23) and all experiments were performed in accordance with the guidelines and regulations of this approval.

RT-qPCR for LIN28B and H19 in *in vitro* and *in vivo* samples

RNA was isolated with the miRNeasy mini kit (Qiagen), the concentration was measured using NanoDrop (Thermo Scientific) and 500 ng cDNA was synthesized with iScript (Bio-Rad, #1708891) and diluted to 2.5 ng/µL. RT-qPCR was conducted using 2.5 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.25 µL forward primer, 0.25 µL reverse primer and 2µL (5ng) cDNA per well, always in two technical replicates. For experiments with human cell lines, *LIN28B, H19* and five reference genes (*TBP*, *HMBS*, *SDHA, HPRT1* and *UBC*) were included, except for the validation of LIN28B overexpression/ knockdown in the stable cell lines, where six reference genes were used (*TBP*, *HMBS*, *SDC4*, *YHWAZ*, *RPL13A* and *UBC*). Plates were read with a LightCycler 480 (Roche). Data were analysed and p-values were calculated with qBasePLUS software (Biogazelle)[1]. One-sided t-tests were used given that the direction of the observed effect was known. The most stable reference genes according to GeNorm were used for normalization and calculation of expression levels relative to the reference genes (i.e. relative expression). The results are presented in a linear scale (i.e. relative linear expression). In the experiments with mouse cells, mouse primers for *Lin28b, H19* and four reference genes (*Gapdh*, *Tbp*, *G6pdh* and *Ubc*) were included. All primer sequences are listed

in Supplemental Table T3.

Flow cytometric analyses

Cells from DIV10 embryoid bodies after 14 days on methylcellulose were stained and detected with FCER1a-biotin/streptavidin (eBioscience), CD3-FITC (eBioscience), CD19-FITC (BD Pharmingen), NK1.1-FITC (eBioscience), CD115-PerCpEfl (eBioscience), Ly6C-PacBlue (eBioscience), life/dead AmCyan (eBioscience), CD45-Qdot (Molecular probes), CD11b-APC (BD Pharmingen), Ly6G-AF700 (BD Pharmingen), MHCII-APC AF7 (Biolegend), CD170-PE (BD Pharmingen), CD11c-PE-TR (Invitrogen) and Ter119-PE-C5 (eBioscience). Of note, CD3, CD19 and NK1.1 (FITC) data could not be interpreted because of the presence of GFP in the *Lin28b* construct. Data were analyzed with an LSRII (BD Biosciences), FACSDiva software (BD Biosciences) and FlowJo vX.0.7.

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