The AS-RBM15 lncRNA enhances RBM15 protein translation during megakaryocyte differentiation

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Abstract

Antisense RNAs regulate the transcription and translation of the corresponding sense genes. Here, we report that an antisense RNA, AS-RBM15, is transcribed in the opposite direction within exon 1 of *RBM15*. *RBM15* is a regulator of megakaryocyte (MK) differentiation and is also involved in a chromosome translocation t(1;22) in acute megakaryocytic leukemia. MK terminal differentiation is enhanced by up-regulation of AS-RBM15 expression and attenuated by AS-RBM15 knockdown. At the molecular level, AS-RBM15 enhances RBM15 protein translation in a CAP-dependent manner. The region of the antisense AS-RBM15 RNA, which overlaps with the 5'UTR of *RBM15*, is sufficient for the up-regulation of RBM15 protein translation. In addition, we find that transcription of both *RBM15* and AS-RBM15 is activated by the transcription factor RUNX1 and repressed by RUNX1-ETO, a leukemic fusion protein. Therefore, AS-RBM15 is a regulator of megakaryocyte differentiation and may play a regulatory role in leukemogenesis.

Keywords antisense RNA; hematopoiesis; RBM15

Subject Categories Development & Differentiation; Protein Biosynthesis & Quality Control; RNA Biology

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Introduction

Long non-coding RNAs (lncRNAs) by definition are RNA molecules longer than 200 nucleotides without protein coding potential. The total number of non-coding RNA genes are greater than the number of protein-coding genes in the human genome and are involved in various aspects of biological functions including cell fate decisions [1]. lncRNAs play vital roles in self-renewal of hematopoietic stem cells and malignant hematopoiesis [2–4]. Inhibition of a long intergenic non-coding RNA (lincRNA-EPS) blocks erythroid differentiation by promoting apoptosis in erythroid cells [5]. LincRNA HOTAIRM1, which is transcribed between the *HOXA1* and *HOXA2*

genes in the *HOXA* cluster, controls myeloid cell development by facilitating the retinoic acid-mediated transcriptional activation of *HOXA1* and *HOXA4* [6]. lncRNA *NRON* is associated with cytoplasmic RNA–protein scaffold complex and controls the export of NFAT and hence the production of NFAT-dependent cytokines in T-cell differentiation [7]. Many lineage-specific lncRNA genes were identified in mouse erythroblasts, megakaryocytes, and megakaryocyte–erythroid progenitors by using RNA-seq analysis [8,9].

Based on chromosomal locations, lncRNAs are further divided into several groups: antisense, intronic, bidirectional, intergenic, and overlapping lncRNAs [10]. Transcription of antisense RNA is initiated in the opposite direction either within the gene body of its sense counterpart or at the end of its sense transcript. Termination of antisense RNA transcription can occur inside of the sense gene or somewhere upstream of the sense gene transcription start site. Genome-wide analysis shows that sense–antisense (S/AS) pairs account for more than 20% of human genes. Relative to sense genes, antisense genes are transcribed head-to-head, tail-to-tail, and fully overlapping [11,12]. The biological functions as well as the functioning mechanisms of antisense RNAs are very diverse [11,13–15]. Antisense p15 overlaps completely with the sense p15 transcript for silencing the p15 gene epigenetically [16]. c-Myc [17], Pu.1, p53, WT1, and RBM15 antisense RNAs do not overlap with their sense genes completely. The p53 gene locus has an antisense RNA gene (*Wrap53*). Exon 1 of *Wrap53*, which overlaps with p53 exon 1, is responsible for up-regulation of p53 mRNA as well as p53 protein [18]. In addition, *Wrap53* RNA has been shown to bind to CTCF to modulate the transcription of both *Wrap53* and *p53* [19]. An antisense RNA is transcribed from the transcription factor *PU.1* locus [20]. In that case, the antisense RNA of *PU.1* attenuates *PU.1* protein translation by serving as a decoy to compete away the translational machinery from *PU.1* mRNA. The whole process resembles the antisense RNA from bacteria *SymE* [21]. WT1, another transcription factor related to multiple organ development including blood, has a lncRNA (*WT1-AS*) transcribed from the *WT1* intron 1 in the opposite direction [22]. Expression of AS-*WT1* exon 1 elevates *WT1* protein level [23]. AS-*Uchl1*, an antisense RNA transcribed from the *Uchl1* gene locus in brain, enhances the CAP-independent protein translation of *Uchl1* mRNA via their

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overlapping region and a SINEB2 repeat [24]. Antisense RNA ZEB2-AS1 controls alternative splicing of ZEB2 pre-mRNA, which introduces an IRES element to promote ZEB2 mRNA protein translation [25]. Here, we describe a new long antisense RNA, AS-RBM15, which is transcribed from the *RBM15* gene locus.

RBM15, an RNA binding protein, promotes megakaryocyte (MK) terminal differentiation [26]. RBM15 belongs to the SPEN (split end) family of evolutionarily conserved proteins involved in cell fate decisions [27]. Previously, we demonstrated that RBM15 regulates alternative RNA splicing of a few key transcription factors including GATA1 and RUNX1 in MK differentiation [28]. RBM15 is chromosome translocated t(1;22) in infant acute megakaryocytic leukemia [29]. The translocation generates a fusion protein, RBM15-MKL1, which leads to haploid deficiency of RBM15 expression. Given that RBM15 is required for MK differentiation, low expression of RBM15 in RBM15-MKL1-initiated leukemia may be a causal factor.

In this report, we characterize a human antisense lncRNA (AS-RBM15) transcribed head-to-head with RBM15. AS-RBM15 augments CAP-dependent RBM15 protein translation via the overlapping regions and promotes MK terminal differentiation. lncRNA genes, like protein-coding genes, are regulated by transcription factors. RUNX1 (aka AML1) is a master transcription factor for hematopoiesis, especially for megakaryopoiesis [30–33]. We discovered that RUNX1 controls transcription of the lncRNA gene *AS-RBM15* as well as *RBM15*. Given that both *RUNX1* and *RBM15* are involved in chromosome translocations, down-regulation of AS-RBM15 may contribute to the genesis of both AML1-ETO and RBM15-MKL1 initiated leukemia.

Results

AS-RBM15 is an antisense lncRNA of *RBM15*

RBM15 is located on human chromosome 1 (1p13.3). A long non-coding RNA gene (ENSG00000227963), which we refer to here as *AS-RBM15*, is transcribed in the opposite direction at +666 bp downstream of the *RBM15* transcription start site. According to the ENSEMBL annotation, *AS-RBM15* consists of 4 exons. Exon 1 (175 nucleotides) of *AS-RBM15* is found within the 5'UTR of *RBM15* mRNA (Fig 1A, also in Fig EV1A). We cloned the full-length AS-RBM15 (2.6 kb) from MEG-01 cells. The putative open reading frame (ORF) in AS-RBM15 exon 4 (from 1,508 nt to 1,804 nt) has no matched protein domains in the PFAM database [34]. PhyloCSF, used to distinguish protein-coding RNAs from non-coding RNAs [35], also scored negative for the protein coding potential of *AS-RBM15* (Fig EV1B). CPAT, another protein coding potential analysis program [36], rated AS-RBM15 ORF 0.029 lower than the cutoff score (0.364) for a protein-coding transcript (Fig EV1C). Based on these algorithms, we conclude that *AS-RBM15* encodes a long non-coding RNA.

Given that the repeat element SINEB2 in lncRNA *AS-Uchl1* is a functional element for protein translation [24], we analyzed the AS-RBM15 sequence using RepeatMasker finder (<http://www.repeat-masker.org>). We found five repeat elements in exon 4 and one AluSx element in exon 2 (from nucleotide 170 to 294) of AS-RBM15 (Fig EV1D).

RBM15 protein is ubiquitously expressed in many tissues but is higher in blood lineages [37]. We analyzed the expression levels of

AS-RBM15 and RBM15 using RNA-seq data from blood lineages directly sorted from human cord blood cells [38]. AS-RBM15 RNA levels are about ten-fold less than RBM15 mRNA levels in different blood lineages. While RBM15 mRNA levels fluctuate within a twofold range, AS-RBM15 RNA levels decrease as hematopoietic stem cells differentiate into megakaryocyte–erythrocyte progenitor cells (MEP) and then increase as MEP cells give rise to MK progenitor cells (Fig 1B). MEG-01 cell line was established from acute megakaryocytic leukemia cells with MK maturation potentials upon PMA stimulation [39]. We also found that AS-RBM15 expression was up-regulated in both MEG-01 cells (Fig 1C) and human CD34⁺ cells (Fig 1D) grown in pro-MK differentiation culture systems over a time course. These results indicate that AS-RBM15 may promote MK differentiation.

AS-RBM15 promotes MK terminal differentiation

Human cord blood CD34⁺ cells were transduced with a lentivirus co-expressing AS-RBM15 and GFP. The GFP-positive cells were sorted for CFU-MK assays. The colonies of MK origin were counted by acetylcholinesterase assays [40]. We found that cells transduced with lentivirus expressing AS-RBM15 produced significantly less colonies than the control cells transduced with an empty lentiviral vector (Fig 2A), indicating that AS-RBM15 blocks the proliferation of megakaryocyte progenitor cells. Consistently, knockdown of AS-RBM15 by two different shRNAs attenuated MK maturation as measured by CD42⁺CD41⁺ cell population in liquid culture assays (Fig 2B). The role of AS-RBM15 in MK terminal differentiation was further confirmed by experiments with MEG-01 cells (Fig EV2).

In order to understand the molecular functions of AS-RBM15, we made truncations to dissect functional regions. Interestingly, when we overexpressed two truncations (Fig 2C) in cord blood CD34⁺ cells for MK differentiation analysis, the $\Delta 3$ truncation, which overlaps with the 5' end of RBM15 mRNA, enhanced MK differentiation (Fig 2D). Given that the $\Delta 3$ did not contain the putative ORF, we further excluded the role of a putative protein encoded by AS-RBM15 in MK maturation. The $\Delta 3$ truncation contains an AluSx repeat (from nucleotide 170 to 294 in exon 2). To further assess the function of the AluSx repeat in MK differentiation, we made a truncation (exon 1) that only expresses the first 175 nucleotides of AS-RBM15, which is the size of exon 1 and completely overlaps with the 5'UTR of RBM15 mRNA. Ectopic expression of only exon 1 still promoted MK terminal differentiation, as the percentage of CD41⁺CD42⁺ cells was significantly higher than that of the control group (Fig 2E). Taken together, we found that the RNA element for MK differentiation is exon 1 of *AS-RBM15*, and the repeat element in the antisense RNA is dispensable for MK differentiation.

AS-RBM15 up-regulates RBM15 protein

Given that antisense RNA molecules have been shown to regulate the expression of their sense counterparts at the transcriptional or translational level, we tested how AS-RBM15 regulates RBM15 expression. We overexpressed AS-RBM15 in HEK 293T cells and leukemic cell lines (HEL, MEG-01, and K562). Overexpression of AS-RBM15 did not affect the mRNA levels of RBM15 in these cell lines (Fig 3A right panel) but increased the protein levels (Fig 3A left panel). Increasingly higher levels of RBM15 protein was detected in 293T cells transfected with increasingly higher concentration of

Figure 1. Expression of RBM15 and AS-RBM15 in hematopoietic cells.

A Genomic organization of the human *RBM15* locus. Exons are represented by black boxes. Arrows indicate transcriptional direction. 5'UTR and 3'UTR are represented by open boxes.

B Expression levels of RBM15 and AS-RBM15 in different blood lineages by meta-analysis of RNA-seq data [38]: HSC (hematopoietic stem cells), MPP (multipotent progenitor cells), CMP (common myeloid progenitor cells), CLP (common lymphoid progenitor cells), GMP (granulocyte–macrophage progenitor cells), MEP (megakaryocyte–erythrocyte progenitor cells), MK (megakaryocyte), and EB (erythroid body). Relative expression levels were calculated as the average numbers of sequence reads ($n = 3$, mean $\pm$ SD).

C Relative expression levels of AS-RBM15 (left) and RBM15 (right) in MEG-01 cells treated with PMA were measured by real-time PCR assays ($n = 4$, mean $\pm$ SD).

D Relative expression levels of AS-RBM15 (left) and RBM15 (right) in CD34⁺ cells grown in pro-MK medium were measured by real-time PCR. GAPDH was used as an internal control ($n = 3$, mean $\pm$ SD). See also Fig EV1.

Data information: *P*-values were calculated using one-way ANOVA (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

plasmids expressing AS-RBM15 (Fig EV3A). In contrast, knockdown of AS-RBM15 in MEG-01 cells reduced the protein level but not the mRNA level of RBM15 (Fig 3B). Moreover, only $\Delta 3$ truncation and exon 1 truncation but not $\Delta 5$ truncation elevated RBM15 protein levels, which is consistent with AS-RBM15's role in MK differentiation (Fig 3C). A reporter with the 5'UTR of RBM15 mRNA ligated to firefly luciferase and followed by an IRES element controlling Renilla

luciferase as an internal control was used to test the effect of AS-RBM15 on protein translation (Fig 3 D). Both the full-length and exon 1 AS-RBM15 generated higher luciferase counts which means higher efficiency of protein translation, while $\Delta 5$ AS-RBM15 cannot stimulate the protein translation via 5'UTR. As a negative control, we demonstrated that AS-RBM15 did not enhance the translation of luciferase mRNA without RBM15 5'UTR.

Figure 2. AS-RBM15 enhances the megakaryocyte terminal differentiation of human umbilical CD34⁺ cells (see also Fig EV2).

- A** CFU-MK assays with CD34⁺ cells expressing AS-RBM15. MK colonies staining for acetylcholinesterase activity were counted (right). *P*-value was calculated using Student's *t*-test (*n* = 3, mean ± SD).
- B** The number of CD41⁺CD42⁺ mature MK cells was determined by FACS analysis. CD34⁺ cells expressing sh-AS-RBM15 were grown in pro-MK medium containing thrombopoietin and stem cell factor (TPO/SCF) for 5 days. The CD41⁺CD42⁺ cell percentages were analyzed using one-way ANOVA, ***P* < 0.01 (*n* = 4, mean ± SD).
- C** Schematic diagram of AS-RBM15 truncations, FL (full-length), Δ5 (nt 463–2611), Δ3 (nt 1–471), and exon 1 (nt 1–175). Exon 2 (blue) contains repeat element (SINE/Alu).
- D** FACS analysis of CD34⁺ cells grown in pro-MK medium and expressing full-length AS-RBM15 or Δ5 or Δ3. The CD41⁺CD42⁺ cell percentages were analyzed using one-way ANOVA, ***P* < 0.01 (*n* = 4, mean ± SD).
- E** The percentage of CD41⁺CD42⁺ cells in CD34⁺ cells expressing AS-RBM15 exon 1 were determined by FACS analysis after these cells were grown in pro-MK medium for 5 days. *P*-value was calculated by Student's *t*-test, ***P* < 0.01 (*n* = 3, mean ± SD).

AS-RBM15 promotes protein translation of RBM15 mRNA

Each actively translated mRNA is associated with polyribosomes. Both the density of ribosomes on a single mRNA molecule and the

number of mRNA molecules associated with polyribosome (poly-some) determine overall protein translation efficiency. We performed polysome fractionation assays and found that neither overexpression nor knockdown of AS-RBM15 changes the global

Figure 3. AS-RBM15 up-regulates RBM15 protein level.

A RBM15 protein levels in leukemia cell lines (MEG-01, K562, HEL) were assessed by Western blotting (WB; left) ($n = 3$). The mRNA levels of RBM15 in cells overexpressing AS-RBM15 were measured by real-time PCR (right, $n = 3$, mean $\pm$ SD). GAPDH was used as an internal control for WB and PCR.

B Protein levels (left) and mRNA levels (right) of RBM15 in MEG-01 cells expressing two different shRNAs against AS-RBM15, respectively, were measured using WBs and real-time PCR assays, respectively ($n = 3$, mean $\pm$ SD). *** $P < 0.001$; NS: not significant with Student's t -test.

C Protein levels (left) and mRNA levels (right) of RBM15 in MEG-01 cells expressing truncated AS-RBM15 RNAs (the schematic as shown on Fig 2C) were measured using WB and real-time PCR, respectively ($n = 3$, mean $\pm$ SD).

D The role of AS-RBM15 in protein translation was assessed by reporter assays. The reporter under control of the CMV promoter contains the 5'UTR of RBM15 mRNA ligated to firefly luciferase followed by an IRES element controlling Renilla luciferase as an internal control. The reporter plasmid was co-transfected with plasmids expressing full-length, exon 1 and $\Delta 5$ AS-RBM15, respectively. The vector reporter without 5'UTR is a negative control ($n = 3$, mean $\pm$ SD). P -values were calculated by one-way ANOVA (* $P < 0.05$, ** $P < 0.01$).

Source data are available online for this figure.

Figure 4.

Figure 4. AS-RBM15 enhances RBM15 protein translation.

- A Cytoplasmic extracts from MEG-01 cell lines stably expressing AS-RBM15, exon 1, or shRNA-02 against AS-RBM15 were fractionated through sucrose gradients (15–60%) ($n = 4$). Global polyribosome profiles of these cell lines were generated by UV absorbance at 254 nm (A_{254}). The arrow indicates increasing polyribosome weights. Low molecular weight, LMW; high molecular weight, HMW.
- B The distributions of RBM15 mRNA (top), GAPDH mRNA (middle), and AS-RBM15 (bottom) were measured by real-time PCR and represented as percentages of total RNA in the fractions ($n = 3$).
- C MS2 pull-down assays: Full-length (FL) AS-RBM15 and exon 1 AS-RBM15 were fused to MS2 RNA as baits. Plasmids expressing MS2-binding protein (HA-tagged) and fusion baits were co-transfected into 293T cells. HA antibody was used to immunoprecipitate HA-MS2-binding protein as shown by a WB on the bottom panel. RBM15 mRNA and MS2 fusions were assessed by real-time PCR ($n = 3$, mean $\pm$ SD). Fold enrichment was calculated as 2 to the power of $[(C_{I,R}-C_{b,R})-(C_{I,MS2}-C_{b,MS2})]$. I: input, that is, the RNA in solution before immunoprecipitation, R: RBM15 mRNA, b: bead-bound mRNA, MS2: RNA levels of MS2 fusions, C: number of cycles in real-time PCR. One-way ANOVA for P -value: $*P < 0.05$.
- D WB analysis of the endogenous RBM15 protein levels in 293T cells overexpressing two different doses of AS-RBM15-MS2 and MS2 ($n = 3$).
- E RBM15 mRNA interacts with AS-RBM15 via RNA–RNA interaction. AS-RBM15-MS2-associated RNAs were purified by phenol extraction and measured by reverse transcription and PCR for detection of RBM15, AS-RBM15, and MS2 under denaturing (heat) and non-denaturing conditions ($n = 3$).
- F RNase A protection assays for detecting endogenous association of AS-RBM15 and RBM15 mRNA ($n = 4$). RNA was extracted from MEG-01 cells with phenol and digested with RNase A. cDNA was synthesized after RNase A treatment and detected using AS-RBM15 exon-specific primers as shown in the schematic diagram on top. RT: reverse transcriptase.
- G MEG-01 cells expressing AS-RBM15 or vector control were treated with DMSO or rapamycin for 45 min. Cells were harvested for WB using antibodies against GAPDH and RBM15 ($n = 2$).
- H The RBM15 5'UTR reporter remains sensitive to rapamycin treatment with or without overexpression of antisense AS-RBM15 ($n = 4$, mean $\pm$ SD). P -values were calculated by one-way ANOVA ($**P < 0.01$ and $***P < 0.001$).
- Source data are available online for this figure.

polysome profiles (Fig 4A), indicating AS-RBM15 does not have a global impact on protein translation. However, the peak polysome fraction containing RBM15 mRNA was shifted to higher molecular weight fractions when the full-length AS-RBM15 and the exon 1 truncation were overexpressed, indicating that the full-length AS-RBM15 and the exon 1 truncation enhanced the RBM15 protein translation (Fig 4B, top panel). Conversely, when AS-RBM15 was knocked down by shRNA, the peak polysome fraction containing RBM15 mRNA was shifted to lower molecular weight fractions (Fig 4B, top panel), indicating that the translation rate of RBM15 mRNA was reduced. In addition, the $\Delta 5$ truncation did not change the polysome profile (Fig EV4). Neither overexpression nor knock-down of AS-RBM15 changed the polysomes profile of GAPDH mRNA (Fig 4B, middle panel). In aggregate, we conclude that AS-RBM15 specifically enhances the translation efficiency of RBM15 mRNA via exon 1.

Strikingly, we found that AS-RBM15 co-migrated with RBM15 mRNA in sucrose gradients indicating that AS-RBM15 may be incorporated into translational machinery associated with RBM15 mRNA (Fig 4B, bottom panel). We fused AS-RBM15 and its truncations to MS2 RNA, so that the AS-RBM15 RNA and its truncations can be pulled down by HA-tagged MS2-binding protein as described [41]. We found that RBM15 mRNA was associated with AS-RBM15-MS2 RNA, $\Delta 3$ -MS2 RNA, and exon 1-MS2 RNA in HA immunoprecipitation assays (Fig 4C), while $\Delta 5$ -MS2 RNA showed a modest interaction with RBM15 mRNA. As a control, we found that the MS2 fusions still enhanced RBM15 protein translation (Fig 4D). The data implies that the endogenous AS-RBM15 RNA may interact with RBM15 mRNA via the overlapping region for enhancing protein translation. To further validate that AS-RBM15 binds to RBM15 mRNA, we used proteinase K to digest proteins in extracts of 293T cells expressing AS-RBM15-MS2. The purified RNA was denatured by heating to 94°C for 5 min and then loaded onto an affinity column with immobilized HA antibody bound to HA-MS2BP protein. We detected the association of RBM15 mRNA to AS-RBM15 only under no-heating condition, which suggests that AS-RBM15 binds to RBM15 mRNA (Fig 4E). To demonstrate whether the

endogenous association between RBM15 mRNA and exon 1 AS-RBM15 can be formed in leukemia cells, we treated phenol-extracted RNA with RNase A that digests single-stranded RNA but not RNA duplex or protein–RNA complex [42]. PCR was performed with cDNA synthesized from the RNase A-treated endogenous RNA. We only detected RNA from exon 1 (Fig 4F). In aggregate, we conclude that endogenous AS-RBM15 enhances RBM15 protein translation via association with RBM15 mRNA.

Rapamycin treatment for a short time inhibits CAP-dependent protein translation [43]. We treated MEG-01 cells expressing AS-RBM15 with rapamycin for 45 min. Given that rapamycin treatment reduced RBM15 protein level (Fig 4G), AS-RBM15 did not direct the RBM15 mRNA to translate protein in a CAP-independent manner

Figure 5. Up-regulation of AS-RBM15 RNA level in the cytoplasm is a response to MK differentiation signal.

The subcellular distribution of RBM15 and AS-RBM15 in CD34⁺ cells grown in TPO-containing medium for 5 days. Cytoplasmic and nuclear RNA levels of AS-RBM15 and RBM15 were normalized to GAPDH (control for cytoplasmic fraction) and MALAT1 (control for nuclear fraction), respectively, using the $\Delta\Delta C_t$ method ($n = 4$, mean $\pm$ SD).

similar to another antisense RNA AS-Uchl1 [24]. To further prove that RBM15 5'UTR-mediated protein translation is CAP-dependent, we measured the luciferase counts expressed from the reporters with or without the treatment of rapamycin. Again, rapamycin inhibits RBM15 5'UTR function (Fig 4H).

Cytoplasmic AS-RBM15 is up-regulated during MK differentiation

Given that AS-RBM15 regulates protein translation, we expected that AS-RBM15 would mainly reside in the cytoplasm. We fractionated MEG-01 cells into cytoplasmic and nuclear fractions to examine

Figure 6. RUNX1 and RUNX1-ETO regulate the transcription of RBM15 and AS-RBM15.

- A RUNX1 expression levels in human CD34⁺ cells grown in pro-MK medium in a time course were measured by real-time PCR ($n = 4$, mean $\pm$ SD).
- B CD34⁺ cells were grown in pro-MK medium for 5 days and stained with antibodies against CD41 and CD42 for flow cytometric analysis. The CD41⁺CD42⁺ cell percentages were analyzed using one-way ANOVA, $*P < 0.05$ ($n = 4$, mean $\pm$ SD).
- C The mRNA expression levels of RUNX1, RBM15, and AS-RBM15 in RUNX1 knockdown MEG-01 cells were measured by real-time PCR ($n = 4$, mean $\pm$ SD).
- D Chromatin immunoprecipitation using anti-RUNX1 antibody. Immunoprecipitated DNA fragments were measured by real-time PCR using HEL cells expressing shRUNX1 and control ($n = 4$, means $\pm$ SD).
- E CD34⁺ human cord blood cells were transduced with MIG retroviruses co-expressing A/E (AML1-ETO, aka RUNX1-ETO) and GFP. Around 5 days after transduction, the GFP⁺CD11b⁺ cells were sorted for real-time PCR ($n = 4$). See also Fig EV6 for RUNX1-ETO binding profile on *RBM15* locus.

Data information: Data are presented as mean $\pm$ SD. P -values were determined using a Student's t -test (E), one-way ANOVA (A, C), and two-way ANOVA (D) ($*P < 0.05$, $**P < 0.01$, and $***P < 0.0001$).

the distribution of AS-RBM15. Strikingly, most of the AS-RBM15 RNA actually was inside the nucleus (Fig EV5). However, when we treated MEG-01 cells with PMA to induce MK differentiation, we found the substantial increase of AS-RBM15 RNA in the cytoplasmic fraction (Fig EV5). Next, we took CD34⁺ cord blood cells grown in TPO-containing medium for subcellular fractionation. Within 5 days, a larger proportion of AS-RBM15 was identified in the cytoplasm as the cells differentiated into MK cells (Fig 5). Thus, pro-MK differentiation signals regulate the AS-RBM15 RNA level in the cytoplasm.

RUNX1 activates the transcription of RBM15 and AS-RBM15

Long non-coding RNA genes are regulated by master transcription factors for hematopoiesis [4]. Available RUNX1 ChIP-seq data showed that RUNX1 binds to several regions both upstream and downstream of the RBM15 transcriptional start site (Fig EV6) [44–47]. We found a gradual increase of RUNX1 expression in CD34⁺ cells grown in pro-MK differentiation medium (Fig 6A), while RUNX1 knockdown in CD34⁺ cells attenuated MK terminal differentiation as measured by CD42⁺CD41⁺ cell populations (Fig 6B). In addition, the RNA levels of both RBM15 and AS-RBM15 were significantly decreased (Fig 6C) in RUNX1 knockdown cells. To investigate whether RUNX1 directly regulates RBM15 and AS-RBM15 transcription, we performed chromatin immunoprecipitation assays to show that RUNX1 binds to several regions of the RBM15 promoter (Fig 6D). The results are consistent with published RUNX1 ChIP-seq data (Fig EV6A–C). Taken together, these results show that RUNX1 regulates the transcription of RBM15 and AS-RBM15.

Chromosome translocation t(8;21) produces the RUNX1 (aka AML1) and ETO1 fusion which is found in over 20% of acute myelogenous leukemia (AML) patients. Meta-analysis indicates that RUNX1-ETO binds to the RBM15 promoter (Fig EV6D) [47]. We overexpressed RUNX1-ETO in cord blood CD34⁺ cells and found that RUNX1-ETO repressed the transcription of both RBM15 and AS-RBM15 (Fig 6E). Thus, we conclude that RBM15 and AS-RBM15 are both regulated by RUNX1 and RUNX1-ETO.

Discussion

In this report, we demonstrate that the antisense RNA AS-RBM15 promotes protein translation of the sense RBM15 mRNA via the overlapping region of the sense/antisense (S/AS) pair as summarized in Fig 7. Genes with S/AS pairs are prevalent in mammalian genomes. An antisense RNA of AS-Uchl1 can enhance protein translation of its sense mRNA counterpart via both the overlapping region and an SINEB2 element [24]. Furthermore, AS-Uchl1 dictates the protein translation of Uchl1 mRNA in a CAP-independent manner as probed with rapamycin treatment. Here, we offer an example that AS-RBM15-enhanced translation only requires the overlapping region but is still sensitive to rapamycin treatment (Fig 4G and H), which implies that even in the presence of AS-RBM15, RBM15 protein translation requires a 5'CAP of RBM15 mRNA for the formation of translational initiation complex. Of note, the overlapping region between AS-RBM15 and RBM15 is deep inside the 5'UTR of RBM15 mRNA (Fig 1A), thus scanning 5'CAP of RBM15 mRNA may still be required to initiate protein translation. Further investigation to

Figure 7. AS-RBM15 controls RBM15 protein translation in megakaryocyte differentiation.

- A** Exon 1 of AS-RBM15 antisense RNA is used to enhance RBM15 protein translation via incorporation into the RBM15 mRNA-containing polysome via putative RNA interaction. Given that RBM15 protein translation in the presence of AS-RBM15 is rapamycin-resistant, the 5'CAP of RBM15 mRNA is needed for scanning of the protein translation initiation complex consisting of eIFs (eukaryotic initiation factors). The repeat elements such as AluSx may play no roles in protein translation.
- B** During megakaryocyte terminal differentiation, AS-RBM15, activated by RUNX1 at transcriptional level, elevates RBM15 protein via translation. Thus, AS-RBM15 increases the dosage range of RBM15 during MK terminal differentiation.

identify proteins associated with S/AS pair will offer definitive mechanistic details for antisense RNA-mediated protein translation. AS-RBM15 is primarily a nuclear RNA. When stimulated for differentiation, the amount of AS-RBM15 in the cytoplasm increases, while the amount of RBM15 mRNA does not increase in the cytoplasm. Thus, AS-RBM15-mediated enhancement of protein translation is a response to signals for cellular differentiation.

RBM15 was initially identified as a leukemic chromosome translocation that produces a fusion protein RBM15-MKL1 involved in pediatric acute megakaryocytic leukemia [29,48]. Later RBM15 was found to be essential for stress responses in hematopoietic stem cells and for megakaryocyte differentiation [26,49]. Like RBM15, RUNX1 is required for the self-renewal of hematopoietic stem cells and for megakaryocyte differentiation [30,31]. Therefore, RUNX1 is epistatic to RBM15 in hematopoiesis. Meta-analysis of transcription factors binding to the RBM15 promoter reveals that RUNX1 binds to the RBM15 promoter (Fig EV6) [44]. We further demonstrated that RUNX1-ETO (aka AML1-ETO), a well-studied fusion oncogene, represses the expression of RBM15 and AS-RBM15. Thus, RBM15-mediated regulation may be a common denominator for leukemia initiated by AML1-ETO and RBM15-MKL1.

We also investigated whether the mouse *Rbm15* locus contains a long antisense RNA. After analyzing the CAGE database in detail [50], we found that antisense RNA is transcribed from the mouse locus. At the primary sequence level, the AS-Rbm15 RNA is very different from human AS-RBM15. However, the mouse AS-Rbm15 does overlap with the 5'UTR of mouse RBM15 mRNA (data not shown). We expect that the function of AS-Rbm15 to promote Rbm15 translation is evolutionarily conserved.

In summary, we reveal a new protein translation mechanism mediated by an antisense RNA. Many antisense RNAs may apply the same strategy used by AS-RBM15 to regulate protein translation of the sense counterpart. The sense and antisense RNA pair works as a regulatory unit to fine-tune megakaryocyte differentiation by widening the RBM15 protein dosage range.

Materials and Methods

Cell culture

MEG-01 cells (ATCC CRL-2021) were cultured in RPMI 1640 (GIBCO, NY) medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were treated with phorbol myristate acetate (10 nM PMA) to induce megakaryocyte differentiation. Subcellular fractionation was performed as described previously [51]. RNA was extracted from these fractions using TRIzol (Invitrogen, CA).

Human cord blood CD34⁺ cells purchased from Cincinnati Children's Hospital were cultured in IMDM (Invitrogen, NY) supplemented with 20% BIT 9500 (Stem Cell Technologies), 100 ng/ml human recombinant stem cell factor (SCF), 100 ng/ml human recombinant FLT3, 50 ng/ml hIL-6, and 20 ng/ml hTPO. All cytokines were purchased from Peprotech, NJ. To induce megakaryocyte differentiation, cells were cultured in IMDM supplemented with 20% BIT 9500, 50 ng/ml TPO, and 2 ng/ml SCF.

Megakaryocyte colony formation assays

Human cord blood CD34⁺ cells were infected with lentiviruses expressing AS-RBM15 and GFP. The GFP-positive cells were sorted. Five thousand GFP-positive cells were plated into one 6-cm dish of MegaCult medium containing thrombopoietin, IL-3, and IL-6 (Stem-Cell Technology, Vancouver, Canada). The colonies formed by megakaryocyte progenitor cells were verified by acetylcholinesterase staining [40]. Briefly, colonies were air-dried before staining. The acetylcholinesterase substrate solution was made by dissolving 10 mg of acetylthiocholine iodide in 15 ml of 0.1 M dibasic sodium phosphate (pH 6.0). Before staining, 1 ml of 0.1 M sodium citrate, 2 ml of 30 mM copper sulfate, and 2 ml of 5 mM potassium ferricyanide were added to produce the complete staining solution. The air-dried cells or colonies were incubated in staining solution for 5 h at ambient temperature. The staining solution was replaced with 95% ethanol, and the colonies were fixed for 10 min. Hematoxylin solution was used to counterstain for 30 s. Dishes were rinsed with running tap water, incubated with saturated Li₂CO₃ for 30 s, and washed under the running tap water again. MK cells or colonies (acetylcholinesterase-positive) were visualized under a light microscope.

Retrovirus production and transduction

AS-RBM15 and its truncations were cloned into pBGJR lentiviral vectors that use the EF1 α promoter to express inserts [52]. Primers for cloning AS-RBM15: TTAATCGGTCCGGGACAAAAAGTCTGCCCTGT and GCGCTCGACTTTTGACTTTGTACCGATAG. The shRNAs against AS-RBM15 (shAS-RBM15#1: GCCCTTCAAGTAGCTGGG

ATT; shAS-RBM15#2: GCCTCCCAGGAGCTGGGATTA) were cloned into the inducible lentiviral vector Tet-pLKO-Puro [53]. To make lentiviruses, lentiviral vectors were co-transfected with envelope vector pMD2.G and packaging vector psPAX2 (Addgene) into HEK293T cells. Viruses were harvested and concentrated with PEG6000 (Sigma, St. Louis, MO). Briefly, one volume of 50% PEG6000 was mixed with four volumes of viral supernatant by rotating for 5 h at 4°C. The virus was concentrated by centrifugation at 1,800 g for 20 min. The pellet was resuspended in cell culture medium. Cells were transduced with concentrated viruses at an m.o.i. 100 in the presence of polybrene (8 μ g/ml) for 48 h and then sorted using a BD FACSARIA III cell sorter (for pBGJR lentiviruses co-expressing GFP) or selected with puromycin (1 μ g/ml) (Tet-pLKO-Puro lentiviruses). MIG retrovirus expressing AML1/ETO (A/E) was reported [54].

Chromatin immunoprecipitation (ChIP)

We performed RUNX1 chromatin immunoprecipitation as we described [55]. Briefly, ten million cells at exponential phase were cross-linked with 1% formaldehyde for 10 min at 37°C. Cells were washed twice with ice-cold PBS and then resuspended in 1 ml of lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40). Cell nuclei were collected by centrifugation at 400 g for 5 min at 4°C, resuspended in RIPA buffer (1 $\times$ PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and then sonicated with Bioruptor (Diagenode, Denville NJ) for 25 min at 4°C. Supernatant was used for immunoprecipitation with protein A agarose beads (Roche, Indianapolis) and rabbit anti-RUNX1 antibody. After rotating overnight at 4°C, beads were washed four times with RIPA buffer and once with TE buffer (10 mM Tris-Cl pH 7.9, 1 mM EDTA). Immunoprecipitated complexes were eluted by adding 250 μ l of ChIP elution buffer (1% SDS, 0.1 M NaHCO₃) and 10 μ l of 5 M NaCl and then incubated at 65°C overnight for reverse cross-linking. The eluted DNA was used for real-time PCR to detect the RUNX1-bound DNA.

Western blotting

Cells (10⁷ cells/ml) were lysed in SDS-PAGE sample buffer and sonicated. Western blotting antibodies are rabbit polyclonal RBM15 antibody [28], mouse anti-human RBM15 monoclonal antibody (#66059-1-1g, Proteintech, Chicago), and mouse anti-human GAPDH monoclonal antibody (#MA5-15738, Thermo Scientific, Waltham). Proteins were detected with the Immobilon Western Chemiluminescent horseradish peroxidase substrate detection kit (Millipore) and documented using Bio-Rad Chem-Doc instrument.

Flow cytometric analysis (FACS)

Cultured cells were harvested, washed with ice-cold PBS, and then passed through strainers to collect a single-cell population. Cells were resuspended in FACS buffer (2% FBS in PBS) and stained with phycoerythrin (PE)-conjugated anti-CD41 and allophycocyanin (APC)-conjugated anti-CD42b antibodies (BD Biosciences, San Jose) for 30 min at 4°C. Cells were washed once with FACS buffer and then analyzed using a FACS Fortessa flow cytometer (BD Biosciences), and FlowJo software (TreeStar Inc).

Polyploidy assay

Cells were harvested, washed with cold PBS, and fixed with 70% ethanol on ice for 1 h. Fixed cells were washed with cold PBS twice and then resuspended in a propidium iodide staining solution: 0.1% v/v Triton X-100 (Sigma) in PBS. Cells were treated with RNase A (final concentration 0.2–0.5 mg/ml) for 30 min at 37°C. Just prior to cytometric analysis, propidium iodide was added to cells at a final concentration of 10 µg/ml.

Real-time PCR

Total RNA was prepared using a Direct-zol miniprep kit (Zymo Research, Irvine). cDNA was generated with random hexamer priming using the Verso cDNA synthesis Kit (Thermo Scientific). Real-time PCR assays were performed with Absolute Blue qPCR SYBR Green Mix (Thermo Scientific) using a ViiA 7 system (Applied Biosystems). Primers are listed in Table 1. GAPDH was used as an internal control for normalization. Relative expression levels were calculated by the $\Delta\Delta C_t$ method and presented as mean $\pm$ standard deviation from at least 3 independent experiments.

MS2 pull-down assay

AS-RBM15 and its truncations were fused to MS2 RNA. AS-RBM15-MS2 was co-transfected with HA-MS2-binding protein into HEK293T cells. Cells were collected 36 h after transfection, washed twice with

Table 2. Primers for specific exons of AS-RBM15

Primer	Sequence
Exon 1 Forward	GGACAAAAAGTCTGCCCTGT
Exon 1 Reverse	CGTCTCTCGGATATCGCTG
Exon 2–3 Forward	GAATTGCTCTGTACGCAAG
Exon 2–3 Reverse	CAGCTTCTCCCCATTACAT
Exon 4.1 Forward	TGGACACAGACCCCTACCA
Exon 4.1 Reverse	GGAATGTATTGGCTCGCTGT
Exon 4.2 Forward	CTCCTGGAATGGCTCATTGT
Exon 4.2 Reverse	AGCAGCATCCCTAGACCTGA

PBS, and lysed in H lysis buffer (20 mM HEPES pH 7.9, 300 mM NaCl, 1 mM MgCl₂, 1% NP-40, 10 mM NaF, 0.2 mM NaVO₄, 10 mM β -glycerol phosphate, 5% glycerol, 1 mM DTT, and proteinase inhibitors) for 30 min on ice. Cells were spun at 15,000 g for 15 min at 4°C. Mouse monoclonal anti-HA antibody (Thermo Scientific) was used to pull down HA-MS2-binding proteins that recognize MS2 RNA fusions. After overnight incubation at 4°C, beads were washed five times with HA wash buffer (20 mM Tris pH 7.9, 300 mM KCl, 0.2% Triton X-100, 1 mM EDTA, 1 mM DTT, and 0.2 mM PMSF). RNA was extracted using a Direct-zol miniprep kit (Zymo Research, Irvine) for real-time PCR assays. Fold enrichment was calculated using this formula: $2^{\frac{[(C_{L,R}-C_{b,R})-(C_{L,MS2}-C_{b,MS2})]}{I}}$. I: input, that is, the RNA in solution before immunoprecipitation, R: mRNA of RBM15, b: bead-bound mRNA, MS2: RNA levels of MS2 fusions, C: number of cycles in real-time PCR.

To detect RNA–RNA interaction, the whole-cell extract was digested with proteinase K and RNA was extracted with phenol–chloroform. The purified RNA was denatured by heating to 95°C for 5 min and chilled immediately on ice. An affinity column with HA-MS2BP-bound resins was used to bind MS2-AS-RBM15 in H lysis buffer for 1 h at room temperature. The MS2-AS-RBM15-associated RNA will be lost under heat. Moreover, to detect endogenous RNA–RNA interaction *in vivo*, we performed an RNase A protection assay. Briefly, MEG-01 cell extract was prepared with H lysis buffer and 2 µl of proteinase K (20 mg/ml) was added to 250 µl of cell extract and incubated 30 min at 37°C. RNA was extracted with phenol–chloroform, and 1 µg of RNA was resuspended in 100 µl of RNase-free water and digested with or without 1 µl of RNase A (10 µg/µl) at 37°C for 45 min. RNA was then purified with TRIzol to remove RNase A and subjected to cDNA synthesis. AS-RBM15 exon-specific primers were used to detect the RNA duplex. See Table 2 for primer information.

Polyribosome profile assay

Cells grown at exponential phase were treated with cycloheximide (100 µg/ml) for 30 min. Cytoplasmic extract was prepared in hypotonic buffer (10 mM HEPES pH 7.9 (KOH), 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.1 mM PMSF, proteinase inhibitor, and Thermo Scientific RiboLock RNase inhibitor) on ice for 5 min. The cell lysate was spun at 1,300 g for 15 min at 4°C. Supernatant was collected and precleared by centrifugation at 15,000 g for 10 min. Cytosolic extract was loaded on top of a 15–60% linear sucrose gradient in

Table 1. Real-time PCR primer list

Primer	Sequence
RBM15 promoter-3843F	ACC GAA AAT CCT CCT CAC CT
RBM15 promoter-3843R	CAC TGC ACC CAA CCA AGT TA
RBM15 promoter-2619F	ATT TTG GGG GCA ATA ACA CA
RBM15 promoter-2619R	GAC TGG AAA TCT GCC TGG AG
RBM15 promoter-2058F	GTC ACT CTG GTG CCT GGT TT
RBM15 promoter-2058R	AAA ATC TGG ATG CTG GAG GA
RBM15 promoter-1502F	CAC ACC TCT TCA GGG CAA TC
RBM15 promoter-1502R	AAA CTC GAA GGA AGA AGC TCA A
RBM15 promoter-1182F	CAG CTT CAC ATT CCT CAC GA
RBM15 promoter-1182R	GAT CCG GTC CTC AAC CCT AT
RBM15 promoter-853F	AGC GAC GTA AAA TGG TGT CC
RBM15 promoter-853R	TGT TAA GTT CCG TGG GGT GT
RBM15 promoter-570F	CAC CTT CAG TCA GCC TCC TC
RBM15 promoter-570R	CTC TTT CGA CCC CCT TTT TC
human RBM15 forward	TCC CAC CTT GTG AGT TCT CC
human RBM15 reverse	GTC AGC GCC AAG TTT TCT CT
human RUNX1 forward	AAC CCT CAG CCT CAG AGT CA
human RUNX1 reverse	CCA CAT TCT GCC TTC CTC AT
AS-RBM15 Forward	TGGACACAGACCCCTACCA
AS-RBM15 reverse	GGAATGTATTGGCTCGCTGT
human GAPDH forward	GAGTCAACGGATTTGGTCGT
human GAPDH reverse	GACAAGCTTCCCGTTCTCAG

buffer (50 mM Tris-Ac pH 7.6, 50 mM NH₄Cl, 12 mM MgCl₂, 1 mM DTT, heparin 1 mg/ml) and spun at 221,632 g for 3 h at 4°C in an SW41Ti rotor. Twenty fractions were collected, and the polyribosome global profiles were monitored by light absorption at 254 nm (A₂₅₄). RNA was purified from all fractions for detecting RBM15 mRNA, AS-RBM15, and GAPDH mRNA levels by real-time PCR.

Bioinformatic analysis

All of the reads from RNA-seq data available from the Blueprint Consortium Web site (www.blueprint-epigenome.eu/) and the European Genome-phenome Archive with accession no. EGAD00001000745 were mapped to the human reference genome (GRCh37/hg19) using TopHat (v2.0.13) [56]. The mean insert size and its standard deviation were calculated using Picard (v1.126) (<http://broadinstitute.github.io/picard/>). The alignment was guided using a Gene Transfer File (GTF version GRCh37.70), and count tables were generated using HT-seq (v0.6.0) [57]. The DESeq (v3.0) package was used for differential expression (DE) analysis. The statistical analyses were performed in R (v3.1.1) (<http://www.r-project.org/>) [58]. All of the BigWig files were generated using Bedtools (v2.17.0) and bedGraphToBigWig tool (v4) [59].

Expanded View for this article is available online.

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Author contributions

N-TT and HS contributed to acquisition of data, analysis and interpretation of data, and article draft; AK-J contributed to analysis and interpretation of data; SL and LZ contributed to acquisition of data; DZ, JCM, and TMT contributed reagents; JCM contributed to acquisition and analysis of data; KMP and YC contributed reagents and revised the article; XZ contributed to conception and design, analysis and interpretation of data, article draft, and revision.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Fatica A, Bozzoni I (2014) Long non-coding RNAs: new players in cell differentiation and development. *Nat Rev Genet* 15: 7–21
- Paralkar VR, Weiss MJ (2013) Long noncoding RNAs in biology and hematopoiesis. *Blood* 121: 4842–4846
- Wei P, Han B, Chen Y (2013) Role of long non-coding RNAs in normal and malignant hematopoiesis. *Sci China Life Sci* 56: 867–875
- Luo M, Jeong M, Sun D, Park HJ, Rodríguez BA, Xia Z, Yang L, Zhang X, Sheng K, Darlington GJ et al (2015) Long non-coding RNAs control hematopoietic stem cell function. *Cell Stem Cell* 16: 426–438
- Hu W, Yuan B, Flygare J, Lodish HF (2011) Long noncoding RNA-mediated anti-apoptotic activity in murine erythroid terminal differentiation. *Genes Dev* 25: 2573–2578
- Zhang X, Lian Z, Padden C, Gerstein MB, Rozowsky J, Snyder M, Gingeras TR, Kapranov P, Weissman SM, Newburger PE (2009) A myelopoiesis-associated regulatory intergenic noncoding RNA transcript within the human HOXA cluster. *Blood* 113: 2526–2534
- Sharma S, Findlay GM, Bandukwala HS, Oberdoerffer S, Baust B, Li Z, Schmidt V, Hogan PG, Sacks DB, Rao A (2011) Dephosphorylation of the nuclear factor of activated T cells (NFAT) transcription factor is regulated by an RNA-protein scaffold complex. *Proc Natl Acad Sci USA* 108: 11381–11386
- Alvarez-Dominguez JR, Hu W, Yuan B, Shi J, Park SS, Gromatzky AA, van Oudenaarden A, Lodish HF (2014) Global discovery of erythroid long noncoding RNAs reveals novel regulators of red cell maturation. *Blood* 123: 570–581
- Paralkar VR, Mishra T, Luan J, Yao Y, Kossenkova AV, Anderson SM, Dunagin M, Pimkin M, Gore M, Sun D et al (2014) Lineage and species-specific long noncoding RNAs during erythro-megakaryocytic development. *Blood* 123: 1927–1937
- Mattick JS, Rinn JL (2015) Discovery and annotation of long noncoding RNAs. *Nat Struct Mol Biol* 22: 5–7
- Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J et al (2005) Antisense transcription in the mammalian transcriptome. *Science* 309: 1564–1566
- Chen J, Sun M, Hurst LD, Carmichael GG, Rowley JD (2005) Genome-wide analysis of coordinate expression and evolution of human cis-encoded sense-antisense transcripts. *Trends Genet* 21: 326–329
- Pelechano V, Steinmetz LM (2013) Gene regulation by antisense transcription. *Nat Rev Genet* 14: 880–893
- Lehner B, Williams G, Campbell RD, Sanderson CM (2002) Antisense transcripts in the human genome. *Trends Genet* 18: 63–65
- Csörba T, Questa JI, Sun Q, Dean C (2014) Antisense COOLAIR mediates the coordinated switching of chromatin states at FLC during vernalization. *Proc Natl Acad Sci USA* 111: 16160–16165
- Yu W, Gius D, Onyango P, Muldoon-Jacobs K, Karp J, Feinberg AP, Cui H (2008) Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA. *Nature* 451: 202–206
- Celano P, Berchtold CM, Kizer DL, Weeraratna A, Nelkin BD, Baylin SB, Casero RA Jr (1992) Characterization of an endogenous RNA transcript with homology to the antisense strand of the human c-myc gene. *J Biol Chem* 267: 15092–15096
- Mahmoudi S, Henriksson S, Corcoran M, Mendez-Vidal C, Wiman KG, Farnebo M (2009) Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage. *Mol Cell* 33: 462–471
- Saldana-Meyer R, Gonzalez-Buendia E, Guerrero I, Narendran V, Bonasio R, Recillas-Targa F, Reinberg D (2014) CTCF regulates the human p53 gene through direct interaction with its natural antisense transcript, Wrap53. *Genes Dev* 28: 723–734
- Ebralidze AK, Guibal FC, Steidl U, Zhang P, Lee S, Bartholdy B, Jorda MA, Petkova V, Rosenbauer F, Huang G et al (2008) PU.1 expression is modulated by the balance of functional sense and antisense RNAs regulated by a shared cis-regulatory element. *Genes Dev* 22: 2085–2092
- Kawano M, Aravind L, Storz G (2007) An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Mol Microbiol* 64: 738–754

22. Dallosso AR, Hancock AL, Malik S, Salpekar A, King-Underwood L, Pritchard-Jones K, Peters J, Moorwood K, Ward A, Malik KT *et al* (2007) Alternately spliced WT1 antisense transcripts interact with WT1 sense RNA and show epigenetic and splicing defects in cancer. *RNA* 13: 2287–2299
23. Moorwood K, Charles AK, Salpekar A, Wallace JL, Brown KW, Malik K (1998) Antisense WT1 transcription parallels sense mRNA and protein expression in fetal kidney and can elevate protein levels in vitro. *J Pathol* 185: 352–359
24. Carrieri C, Cimatti L, Biagioli M, Beugnet A, Zucchelli S, Fedele S, Pesce E, Ferrer I, Collavin L, Santoro C *et al* (2012) Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature* 491: 454–457
25. Beltran M, Puig I, Pena C, Garcia JM, Alvarez AB, Pena R, Bonilla F, de Herreros AG (2008) A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. *Genes Dev* 22: 756–769
26. Niu C, Zhang J, Breslin P, Onciu M, Ma Z, Morris SW (2009) c-Myc is a target of RNA-binding motif protein 15 in the regulation of adult hematopoietic stem cell and megakaryocyte development. *Blood* 114: 2087–2096
27. Su H, Liu Y, Zhao X (2015) SPEN family RNA binding proteins are novel tumor suppressors coupling transcriptional regulation with RNA processing. *Cancer Trans Med* 1: 21–25
28. Zhang L, Tran NT, Su H, Wang R, Lu Y, Tang H, Aoyagi S, Guo A, Khodadadi-Jamayran A, Zhou D *et al* (2015) Cross-talk between PRMT1-mediated methylation and ubiquitylation on RBM15 controls RNA splicing. *elife* 4: e07938
29. Mercher T, Coniat MB, Monni R, Mauchauffe M, Nguyen Khac F, Gressin L, Mugneret F, Leblanc T, Dastugue N, Berger R *et al* (2001) Involvement of a human gene related to the Drosophila spen gene in the recurrent t(1;22) translocation of acute megakaryocytic leukemia. *Proc Natl Acad Sci USA* 98: 5776–5779
30. Ichikawa M, Asai T, Saito T, Seo S, Yamazaki I, Yamagata T, Mitani K, Chiba S, Ogawa S, Kurokawa M *et al* (2004) AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nat Med* 10: 299–304
31. Gowney JD, Shigematsu H, Li Z, Lee BH, Adelsperger J, Rowan R, Curley DP, Kutok JL, Akashi K, Williams IR *et al* (2005) Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. *Blood* 106: 494–504
32. Pencovich N, Jaschek R, Tanay A, Groner Y (2011) Dynamic combinatorial interactions of RUNX1 and cooperating partners regulates megakaryocytic differentiation in cell line models. *Blood* 117: e1–e14
33. Elagib KE, Racke FK, Mogass M, Khetawat R, Delehanty LL, Goldfarb AN (2003) RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. *Blood* 101: 4333–4341
34. Finn RD, Bateman A, Clements J, Coghill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J *et al* (2014) Pfam: the protein families database. *Nucleic Acids Res* 42: D222–D230
35. Lin MF, Jungreis I, Kellis M (2011) PhyloCSF: a comparative genomics method to distinguish protein coding and non-coding regions. *Bioinformatics* 27: i275–i282
36. Wang L, Park HJ, Dasari S, Wang S, Kocher JP, Li W (2013) CPAT: Coding-Potential Assessment Tool using an alignment-free logistic regression model. *Nucleic Acids Res* 41: e74
37. Kim MS, Pinto SM, Getnet D, Nirujogi RS, Manda SS, Chaerkady R, Madugundu AK, Kelkar DS, Isserlin R, Jain S *et al* (2014) A draft map of the human proteome. *Nature* 509: 575–581
38. Chen L, Kostadima M, Martens JH, Canu G, Garcia SP, Turro E, Downes K, Macaulay IC, Bielczyk-Maczynska E, Coe S *et al* (2014) Transcriptional diversity during lineage commitment of human blood progenitors. *Science* 345: 1251033
39. Ogura M, Morishima Y, Okumura M, Hotta T, Takamoto S, Ohno R, Hirabayashi N, Nagura H, Saito H (1988) Functional and morphological differentiation induction of a human megakaryoblastic leukemia cell line (MEG-01s) by phorbol diesters. *Blood* 72: 49–60
40. Lev-Lehman E, Deutsch V, Eldor A, Soreq H (1997) Immature human megakaryocytes produce nuclear-associated acetylcholinesterase. *Blood* 89: 3644–3653
41. Yoon JH, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S, Huarte M, Zhan M, Becker KG, Gorospe M (2012) LincRNA-p21 suppresses target mRNA translation. *Mol Cell* 47: 648–655
42. Podlowski S, Bramlage P, Baumann G, Morano I, Luther HP (2002) Cardiac troponin I sense-antisense RNA duplexes in the myocardium. *J Cell Biochem* 85: 198–207
43. Beretta L, Gingras AC, Svitkin YV, Hall MN, Sonenberg N (1996) Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J* 15: 658–664
44. Tijssen MR, Cvejic A, Joshi A, Hannah RL, Ferreira R, Forrai A, Bellissimo DC, Oram SH, Smethurst PA, Wilson NK *et al* (2011) Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators. *Dev Cell* 20: 597–609
45. Wilkinson AC, Ballabio E, Geng H, North P, Tapia M, Kerry J, Biswas D, Roeder RG, Allis CD, Melnick A *et al* (2013) RUNX1 is a key target in t(4;11) leukemias that contributes to gene activation through an AF4-MLL complex interaction. *Cell Rep* 3: 116–127
46. Mandoli A, Singh AA, Jansen PW, Wierenga AT, Riahi H, Franci G, Prange K, Saeed S, Vellenga E, Vermeulen M *et al* (2014) CBFB-MYH11/RUNX1 together with a compendium of hematopoietic regulators, chromatin modifiers and basal transcription factors occupies self-renewal genes in inv(16) acute myeloid leukemia. *Leukemia* 28: 770–778
47. Sun XJ, Wang Z, Wang L, Jiang Y, Kost N, Soong TD, Chen WY, Tang Z, Nakadai T, Elemento O *et al* (2013) A stable transcription factor complex nucleated by oligomeric AML1-ETO controls leukaemogenesis. *Nature* 500: 93–97
48. Ma Z, Morris SW, Valentine V, Li M, Herbrick JA, Cui X, Bouman D, Li Y, Mehta PK, Nizetic D *et al* (2001) Fusion of two novel genes, RBM15 and MKL1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia. *Nat Genet* 28: 220–221
49. Xiao N, Jani K, Morgan K, Okabe R, Cullen DE, Jesneck JL, Raffel GD (2012) Hematopoietic stem cells lacking Ott1 display aspects associated with aging and are unable to maintain quiescence during proliferative stress. *Blood* 119: 4898–4907
50. Kawaji H, Kasukawa T, Fukuda S, Katayama S, Kai C, Kawai J, Carninci P, Hayashizaki Y (2006) CAGE Basic/Analysis Databases: the CAGE resource for comprehensive promoter analysis. *Nucleic Acids Res* 34: D632–D636
51. Kalakonda N, Fischle W, Boccuni P, Gurvich N, Hoya-Arias R, Zhao X, Miyata Y, Macgrogan D, Zhang J, Sims JK *et al* (2008) Histone H4 lysine 20 monomethylation promotes transcriptional repression by L3MBTL1. *Oncogene* 27: 4293–4304
52. Vu LP, Perna F, Wang L, Voza F, Figueroa ME, Tempst P, Erdjument-Bromage H, Gao R, Chen S, Paietta E *et al* (2013) PRMT4 blocks myeloid

- differentiation by assembling a methyl-RUNX1-dependent repressor complex. *Cell Rep* 5: 1625–1638
53. Wiederschain D, Wee S, Chen L, Loo A, Yang G, Huang A, Chen Y, Caponigro G, Yao YM, Lengauer C *et al* (2009) Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle* 8: 498–504
54. Mulloy JC, Cammenga J, MacKenzie KL, Berguido FJ, Moore MA, Nimer SD (2002) The AML1-ETO fusion protein promotes the expansion of human hematopoietic stem cells. *Blood* 99: 15–23
55. Zhao X, Jankovic V, Gural A, Huang G, Pardanani A, Menendez S, Zhang J, Dunne R, Xiao A, Erdjument-Bromage H *et al* (2008) Methylation of RUNX1 by PRMT1 abrogates SIN3A binding and potentiates its transcriptional activity. *Genes Dev* 22: 640–653
56. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 14: R36
57. Anders S, Pyl PT, Huber W (2015) HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31: 166–169
58. Anders S, Huber W (2010) Differential expression analysis for sequence count data. *Genome Biol* 11: R106
59. Quinlan AR, Hall IM (2010) BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26: 841–842