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Alternative translation initiation generates the N-terminal truncated form of RUNX1 that retains hematopoietic activity

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Transcription factor RUNX1 plays a crucial role in hematopoiesis and its activity is tightly regulated at both the transcriptional and posttranslational levels. However, translational control of RUNX1 expression has not been fully understood. In this study, we demonstrated that RUNX1b mRNA is translated from two alternative initiation sites, Met-1 and Met-25, giving full-length RUNX1b and a shorter protein lacking the first 24 amino acids (RUNX1ΔN24). Presence/absence of strong Kozak consensus sequences around Met-1 determines which initiation site is mainly used in RUNX1b cDNA. Selective disruption of either Met-1 or Met-25 abrogates expression of the corresponding protein while facilitating the production of another protein. The RUNX1b cDNA containing 65 bp natural promoter sequences mainly produces full-length RUNX1b in human cord blood cells, but disruption of Met-1 in this cDNA also induced translation from Met-25. Consistent with these data, disruption of endogenous RUNX1b around Met-1 using CRISPR/Cas9 induced selective expression of RUNX1ΔN24 in several leukemia cell lines. RUNX1ΔN24 protein is more stable than full-length RUNX1b and retains hematopoietic activity. We also found that FLAG-tagged full-length RUNX1 showed altered activity, indicating the influence of N-terminal FLAG-tag on RUNX1 function. The alternative translation initiation of RUNX1b may participate in fine tuning RUNX1 activity. © 2019 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

The mammalian Runx transcription factors RUNX1, RUNX2, and RUNX3 are key regulators of lineage-specific gene expression in diverse developmental processes [1,2]. The RUNX proteins contain a conserved 128-amino acid Runt domain responsible for sequence-specific DNA binding. The Runt domain is also required to form a heterodimeric complex with a partner protein, CBFB. RUNX1 is a master regulator of hematopoiesis, and disruption of RUNX1 function either through mutations or generation of fusion genes leads to the development of hematopoietic diseases [3–6]. RUNX1 function is under the tight control of multiple mechanisms, including transcriptional regulation, translational control, and posttranslational modifications [7–9]. Transcription of RUNX1 is controlled by two distantly located promoter regions, distal P1 and proximal P2, which produce the RUNX1c and RUNX1b protein isoforms, respectively [10]. P1 and P2 transcripts are structurally and functionally different in their 5′ untranslated region (5′-UTR) [11,12]. The P1 5′-UTR is relatively short, lacks GC-rich elements, and mediates efficient cap-dependent translation of RUNX1c. In contrast, the P2 5′-UTR is long, possesses GC-rich regions, contains an internal ribosome entry site (IRES), and mediates IRES-dependent translation of RUNX1b.

Eukaryotic protein translation initiation is known to occur primarily at AUG codons encompassed within the conserved Kozak consensus sequence RccAUGG, where R is a purine (A or G) three bases upstream of AUG and the AUG is followed by another G [13]. Alternative translation initiation occurs when the ribosome skips the first translation initiation site and moves down to the next AUG codon. Such a mechanism is known to contribute to the diversity of protein products and functions. RUNX1b has relatively "weak" Kozak consensus around Met-1 (i.e., the first AUG is followed by "C" instead of "G"), which raises the possibility that RUNX1b may have the downstream translation initiation sites.

Through analyses using two RUNX1b cDNAs with identical coding region sequences, we found that

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RUNX1b is translated from a downstream AUG, yielding a RUNX1 protein lacking the first 24 amino acids (RUNX1ΔN24) when the cDNA does not have "strong" Kozak consensus around the first AUG. RUNX1ΔN24 is more stable than full-length RUNX1b and retains hematopoietic activity. Importantly, FLAG-tagged full-length RUNX1 and RUNX1ΔN24 showed different results in a cell growth assay using MLL-AF9-expressing cord blood (CB) cells, indicating the influence of N-terminal Flag tag on RUNX1 function. The alternative translation initiation will increase the molecular and functional complexity of RUNX1, which may be important in fine tuning gene expression levels in the hematopoietic system.

Methods

Plasmids and viral transduction

RUNX1b-(A) (a gift from Dr. T. Kitamura) and RUNX1b-(B) (a gift from Dr. M. Kurokawa) were inserted into the retroviral vector pMYs-IRES-EGFP (pMYs-IG) [14] or the lentiviral vector LeGO-iV2 [15]. Both are fused to FLAG tag at the N-terminus. RUNX1b containing natural 65 bp 5′-UTR sequences was provided by Dr. M. Kurokawa and inserted into pMYs-IG. Point mutations were introduced using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Retroviral and lentiviral transduction were performed by transfecting RUNX1 plasmids along with gag, pol, and env-expressing plasmids into 293T cells, as described previously [16,17].

Cell culture

Human umbilical CB cells were obtained from Translational Trials Development and Support Laboratory at Cincinnati Children's Hospital Medical Center according to an institutional review board-approved protocol. Informed consent was obtained in accordance with the Declaration of Helsinki. $CD34^+$ cells were separated using the EasySep CD34 selection kit (StemCell Technologies, Vancouver, BC, Canada). Human CB cells and MLL-AF9-expressing CB cells [18] were cultured in Iscove's modified Dulbecco's medium containing 20% BIT9500 (StemCell Technologies) and 10 ng/mL stem cell factor, megakaryocyte growth and development factor (thrombopoietin), Flt3 ligand, interleukin-3 (IL-3), and IL-6, as described previously [16,17]. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). K562 cells, Molm13 cells, and Jurkat cells were cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% FBS. TF-1 cells were cultured in RPMI medium containing 10% FBS and 1 ng/mL IL-3.

Transfection and Western blotting

293T cells were transfected with plasmids by the calcium phosphate method. Cells were directly lysed in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blots were probed with the following antibodies: α-RUNX1 (4336, Cell Signaling Technology), α-FLAG (F1804, Sigma-Aldrich), α-β-actin (A5316, Sigma-Aldrich), α-tubulin (T0198, Sigma-Aldrich). Band intensity was measured using LabWorks Version 4.5 software (UVP, LLC).

Flow cytometry

Green fluorescent protein (GFP) expression in cells was analyzed by a FACSCanto I (BD Biosciences). Sorting of GFP^+ cells was performed using a FACSAria (BD Biosciences).

RUNX1 *depletion using CRISPR/Cas9*

To generate single-guide RNA (sgRNA) expression vectors targeting *RUNX1*, annealed oligonucleotides were cloned into the pLKO5.sgRNA.EFS.tRFP vector, which was obtained from Addgene (plasmid #52823). The expression vector for Cas9 (lentiCas9-Blast #52962) was also obtained from Addgene. K562, Molm13, Jurkat, and TF-1 cells were infected with the lentiviruses for 24 hours and selected for stable expression of Cas9 using blasticidin (10 μg/mL) and for stable expression of sgRNAs using puromycin (1 μg/mL) in RPMI medium supplemented with 10% FBS (together with 1 ng/mL IL-3 for TF-1 cells). Sequences for the nontargeting (NT) control and sgRNAs targeting *RUNX1* are provided as follows:

NT: 5′-CGCTTCCGCGGCCCGTTCAA 3′

sgRUNX1-(1): 5′-GGCTCGTGCTGGCATCTACG 3′

sgRUNX1-(2): 5′-CTGATCGTAGGACCACGGTG 3′

Results

RUNX1b mRNA is translated from two alternative initiation sites

We used two human RUNX1b cDNAs in the expression vectors, designated RUNX1b-(A) and RUNX1b-(B) herein, for experiments. They both are fused with a FLAG epitope tag at the N-terminus, have identical sequence in the coding region, and have been widely used in the scientific community. To our surprise, transfection of RUNX1b-(A) or RUNX1b-(B) into 293T cells resulted in dominant expression of either a 53 kDa or a 51 kDa protein, respectively, irrespective of the expression vectors that they are inserted in (Figure 1A). Furthermore, only RUNX1b-(B), but not RUNX1b-(A), showed a growth-promoting effect in MLL-AF9-expressing CB cells in which an optimal level of RUNX1 is required for their efficient growth [19] (Figure 1B). Therefore, despite their identical coding region sequences, these two RUNX1b constructs produce distinct proteins with different functions.

Sequence analyses of the constructs revealed that RUNX1b-(A) has Kozak consensus sequence around the first ATG, whereas RUNX1b-(B) does not. Another difference is that RUNX1b-(B) contains ~500 bp 3′-UTR/PolyA sequences after a stop codon, whereas RUNX1b-(A) only includes coding region sequences (Figure 2A). To

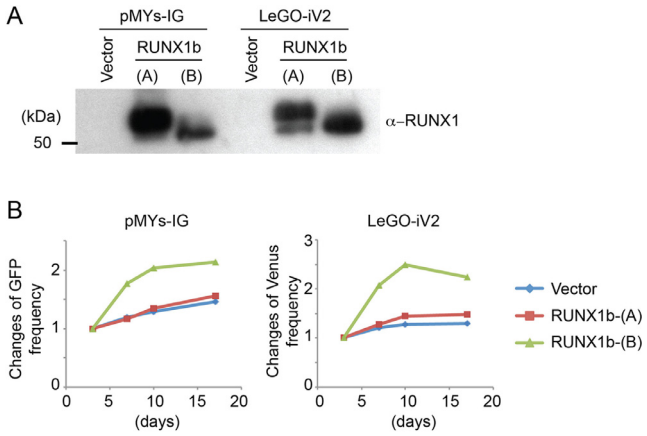

Figure 1. Two RUNX1b constructs with identical coding region sequences produce distinct proteins with different functions. **(A)** Western blot of protein produced in transfected 293T cells. RUNX1b-(A) and RUNX1b-(B) mainly produced either a 53 kDa or a 51 kDa protein, respectively, irrespective of the expression vectors (pMYs-IRES-EGFP and LeGO-iV2) in which they are inserted. Blot was probed with α-RUNX1 antibody. **(B)** Changes in frequency of GFP$^+$ cells (vector- or RUNX1b-transduced cells) in cultures of MLL-AF9 cells. RUNX1b-(B), but not RUNX1b-(A), showed a growth-promoting effect.

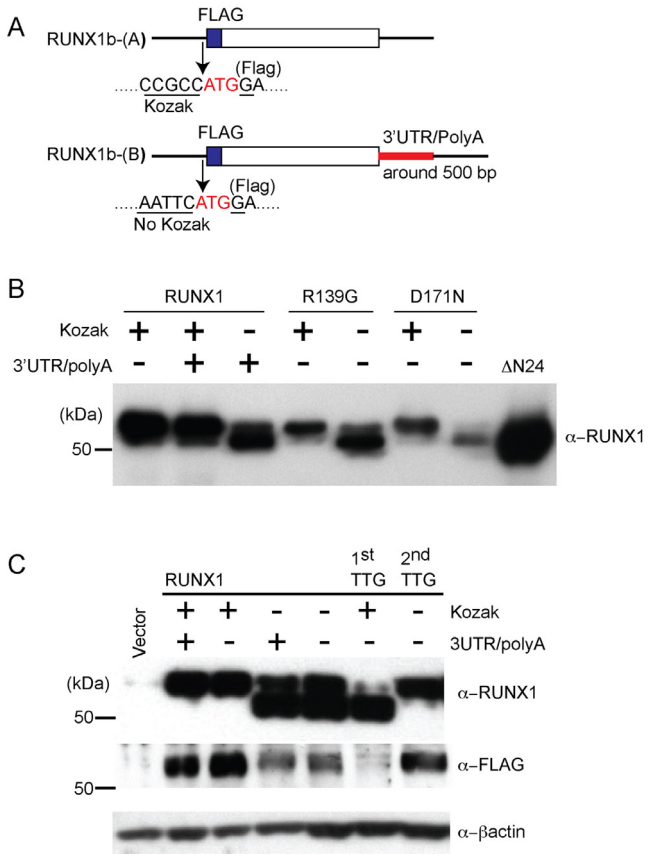

Figure 2. Alternative translation initiation produces full-length RUNX1 and RUNX1Δ24 proteins from RUNX1b mRNA. **(A)** Scheme of the two expression plasmids of RUNX1b. RUNX1b-(A) has a Kozak consensus sequence around the first ATG and only includes coding region sequences. RUNX1b-(B) does not have a Kozak sequence and contains 3′-UTR and polyA tail sequences after the stop codon. **(B)** Western blot of protein produced in 293T cells transfected with various RUNX1 plasmids with or without Kozak/3′-UTR sequences. RUNX1 plasmids with Kozak sequences mainly produced 53 kDa protein, whereas those without a Kozak sequence mainly produced a 51 kDa protein. The RUNX1 mutant lacking the first 24 amino acids (RUNX1ΔN24) appeared to have the same size as the 51 kDa protein. The blot was probed with α-RUNX1 antibody. R139G and D171N are RUNX1 mutants found in leukemia patients. **(C)** Western blot of protein produced in human CB cells transduced with various RUNX1 plasmids including those with a point mutation at the first or second ATG. The blot was probed with α-RUNX1 and α-FLAG antibody. Both the 53 kDa and 51 kDa proteins were detected by α-RUNX1 antibody depending on the presence of Kozak sequences, whereas only the 53 kDa protein was detected by α-FLAG antibody. Selective disruption of each ATG abrogated expression of the corresponding protein while facilitating production of another protein.

investigate a cause for the different protein migration, we then transfected a set of RUNX1b or its disease-related mutants (R139G and D171N) with or without Kozak/3′-UTR sequences into 293T cells and assessed their protein expression. Clearly, the constructs with a Kozak sequence mainly produced a 53 kDa protein, whereas those without a Kozak sequence preferentially produced 51 kDa protein. The presence or absence of 3′-UTR/PolyA sequences did not affect the protein migration. In addition, a RUNX1 mutant lacking first 24 amino acids (RUNX1ΔN24) appeared to have the same size as the 51 kDa protein (Figure 2B). Given that the Kozak sequence is important for efficient translation from the first AUG, these results indicate that the inframe AUG at 25 amino acids downstream serves as an internal translation initiation site to produce the 51 kDa protein. This AUG is evolutionally conserved among RUNX factors and internal mRNA translation from the corresponding AUG was reported in RUNX1 and RUNX3 proteins [20–22].

To determine whether the alternative translation occurs in primary human hematopoietic cells, we next transduced a set of RUNX1 constructs, including those with a point mutation at either the first (+1) or the second (+73) ATG site into human CB CD34$^+$ cells. Consistent with the above results, RUNX1 with or without Kozak sequence mainly produced a 53 kDa or a 51 kDa protein, respectively. Mutating each ATG (+1 or +73ATG) to TTG abrogated expression of the upper or lower protein, respectively, while facilitating production of another protein. Blocking the translation from the second AUG (Met-25) recovered efficient translation from Met-1 even in the RUNX1b cDNA lacking Kozak sequences, suggesting the competitive regulation of these alternative translation initiations. Furthermore, the α-FLAG antibody detected only the 53 kDa protein but not the 51 kDa protein (Figure 2C). Because FLAG is tagged at the N-terminus, it further confirms that the 51 kDa protein is translated from a downstream internal AUG. Therefore, RUNX1b is translated from both the first AUG (Met-1) and the

second AUG (Met-25) in a competitive manner, yielding full-length RUNX1b and RUNX1ΔN24 proteins. RUNX1b-(A), which has a Kozak sequence around the first AUG, mainly produces full-length RUNX1b, whereas RUNX1b-(B), which does not have a Kozak sequence, mainly produces RUNX1ΔN24 in cells.

Full-length RUNX1b is a major protein translated from the RUNX1b mRNA with natural promoter sequences

Because both RUNX1b-(A) and RUNX1b-(B) have artificial 5′-UTR sequences and the N-terminal FLAG tag, we next investigated which RUNX1b protein (full-length or ΔN24) is mainly translated from RUNX1b mRNA with 65 bp sequences that exist naturally in the 5′-UTR of RUNX1b (RUNX1b with natural sequences: RUNX1b-NS; Figure 3A). Transduction of RUNX1b-NS into CB cells revealed that full-length RUNX1b is a major protein produced from this cDNA. Consistent with earlier results, RUNX1b-NS with an ATG-to-TTG mutation at Met-1 (RUNX1b-NS-1st TTG) produced RUNX1ΔN24 (Figure 3B). Furthermore, it appears that full-length RUNX1b is endogenously expressed in CB cells according to its protein size (Figure 3B, long exposure). However, whether it is, in fact, the full-length RUNX1b protein is not clear given the existence of multiple RUNX1 transcripts (RUNX1b and RUNX1c). Therefore, RUNX1b cDNA with natural 5′-UTR sequences mainly generates full-length RUNX1b protein in human CB cells.

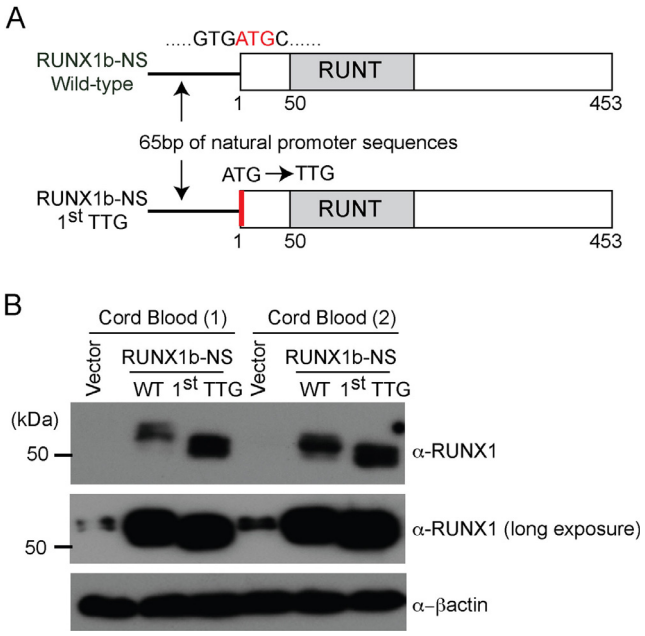

Figure 3. Full-length RUNX1 is a major protein translated from RUNX1b mRNA with natural promoter sequences. **(A)** Scheme of RUNX1b constructs with 65 bp sequences that exist naturally in the 5′-UTR of RUNX1b (RUNX1b-NS). The RUNX1b-NS-1st TTG has an ATG-to-TTG mutation at Met-1. Numbers indicate the positions of amino acid residues from the N terminus. **(B)** Western blot of protein produced in two independent human CB cells transduced with RUNX1b-NS or RUNX1b-NS-1st TTG. Blot was probed with α-RUNX1. RUNX1b-NS mainly produced a 53 kDa protein (full-length RUNX1b), whereas RUNX1b-NS-1st TTG produced a 51 kDa protein (RUNX1ΔN24). Note that the protein level of RUNX1ΔN24 produced by RUNX1b-NS-1st TTG was higher than that of full-length RUNX1b produced by RUNX1b-NS. Endogenous RUNX1 that was detected after long exposure appears to be full-length RUNX1b.

RUNX1ΔN24 protein is more stable than full-length RUNX1b protein

Interestingly, the protein level of RUNX1ΔN24 produced by RUNX1b-NS-1st TTG was consistently higher than that of full-length RUNX1b produced by RUNX1b-NS in CB cells (Figure 4B). To compare the stability of each protein, we cultured NIH3T3 cells transduced with RUNX1b-NS or RUNX1b-NS-1st TTG in the presence of the protein synthesis inhibitor cycloheximide (CHX). Total cell extracts isolated 0, 4, 8, or 16 hours after the addition of CHX were subjected to immunoblotting for RUNX1. The steady-state level of RUNX1ΔN24 protein was higher than full-length RUNX1b in NIH3T3 cells. Furthermore, CHX addition showed a slower rate of RUNX1ΔN24 degradation (Figures 5A and 5B).

It was shown that RUNX1 is ubiquitinated at multiple lysine residues, including Lys-24, and is continuously subjected to proteolytic degradation mediated by the

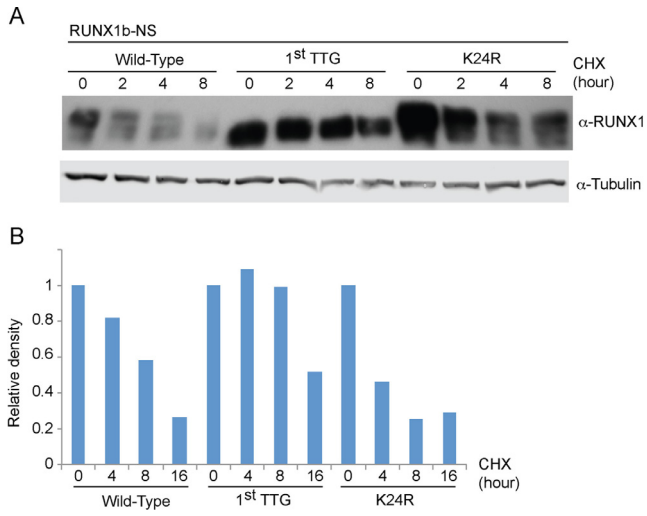

Figure 4. RUNX1ΔN24 is a long-lived protein partly due to the lack of a ubiquitination site, Lys-24. **(A)** NIH3T3 cells were transduced with wild-type RUNX1b-NS, RUNX1b-NS-1st TTG, or RUNX1b-NS-K24R. The cells were treated with 50 μg/mL CHX for the indicated times and cell extracts were analyzed with α-RUNX1 antibody. **(B)** Band intensities of RUNX1b produced in RUNX1b-NS- or RUNX1b-NS-TTG-expressing NIH3T3 cells were quantified with LabWorks. The intensity of RUNX1 without CHX treatment was set to one.

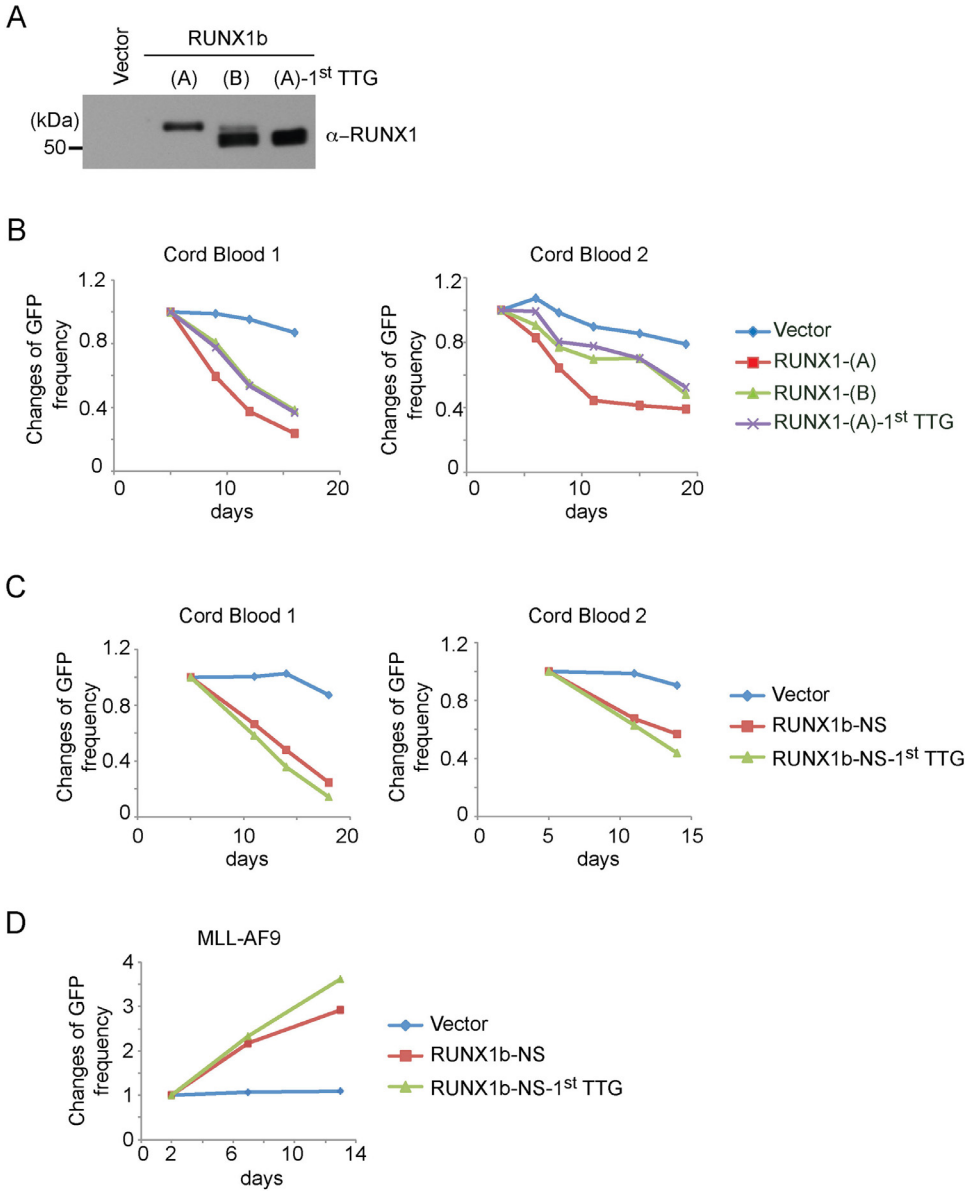

Figure 5. RUNX1ΔN24 retains hematopoietic activity. **(A)** Western blot of protein produced in human CB cells transduced with RUNX1b-(A), RUNX1b-(B), or RUNX1b-(A) with an ATG-to-TTG mutation at Met-1, RUNX1b-(A)-1st TTG. Blot was probed with α-RUNX1. RUNX1b-(A) mainly produced 53 kDa protein (FLAG-tagged full-length RUNX1b), whereas the other two constructs produced a 51 kDa protein (RUNX1ΔN24). **(B)** Human CB $CD34^+$ cells transduced with the RUNX1b constructs (coexpressing GFP) were cultured with cytokines inducing myelopoiesis. Changes of GFP frequency in culture were monitored every 3 or 4 days. RUNX1b-(A) showed stronger growth inhibitory effects than the other two RUNX1b constructs. Results are normalized to the frequency of GFP^+ cells at day 3 or 5, set to one. Two independent experiments using different CB cells were performed. **(C,D)** Human CB $CD34^+$ cells or MLL-AF9-expressing CB cells were transduced with the untagged RUNX1b constructs containing a 65 bp natural sequence at the promoter region (RUNX1b-NS and RUNX1b-NS-1st TTG). Cells were cultured with cytokines that induce myelopoiesis. Changes of GFP frequency in culture were monitored every 3 or 4 days. Both the untagged RUNX1b constructs showed a similar growth-inhibitory effect in CB cells **(C)** or showed a similar growth-promoting effect in MLL-AF9 cells **(D)**, indicating that RUNX1ΔN24 has similar function to the untagged full-length RUNX1b.

ubiquitin-proteasome pathway [23,24]. To determine whether the lack of Lys-24 explains the increased stability of RUNX1ΔN24, we introduced a lysine to arginine mutation at Lys-24 in RUNX1b-NS (RUNX1b-NS-K24R) and transduced it into NIH3T3 cells. The expression level of RUNX1b-NS-K24R protein was higher than that of wild-type RUNX1b. However, turnover of K24R protein was similar to that of wild-type RUNX1b in the presence of CHX (Figures 4A and 4B). Therefore, it appears that lack of Lys-24 contributes to the increased protein level of RUNX1ΔN24 in steady state, but does not fully account for the longer half-life of RUNX1ΔN24 protein.

RUNX1ΔN24 retains similar hematopoietic activity to full-length RUNX1

We previously showed that forced expression of RUNX1 inhibits the growth of CB cells by inducing myeloid differentiation [19]. This assay provides efficient approach to quantify the activity of RUNX1 isoforms and mutants in hematopoietic cells. Using this experimental system, we compared the function of RUNX1b-(A), RUNX1b-(B), and RUNX1b-(A) with an ATG-to-TTG mutation at Met-1 [RUNX1b-(A)-1st TTG]. Consistent with the earlier results, RUNX1b-(A) mainly produced full-length RUNX1 protein, whereas RUNX1b-(B) and RUNX1b-(A)-1st TTG produced RUNX1ΔN24 in CB cells (Figure 5A). Functionally, all of the RUNX1 constructs inhibited the growth of CB cells, as evidenced by the loss of GFP-expressing cells in culture. RUNX1b-(A) showed a more potent growth-inhibitory effect than the other two constructs, indicating the stronger activity of FLAG-tagged full-length RUNX1 compared with RUNX1ΔN24 (Figure 5B).

To examine the potential influence of the N-terminal FLAG tag on RUNX1 function, we next performed similar experiments using the untagged RUNX1b constructs RUNX1b-NS and RUNX1b-NS-1st TTG. To our surprise, both RUNX1b constructs showed a similar ability to inhibit the growth of CB cells in this experimental condition (Figure 5C). We also transduced these untagged RUNX1b into MLL-AF9-expressing CB cells to assess their biological function. In contrast to the results shown in Figure 1B, both constructs showed a similar growth-promoting effect in MLL-AF9 cells (Figure 5D). These results indicate that the N-terminal FLAG tag is responsible for the functional difference between RUNX1b-(A) and RUNX1b-(B) observed in earlier experiments (Figure 1B). In addition, the data suggest that RUNX1ΔN24 retains normal function.

Disruption of endogenous RUNX1b around Met-1 induced selective expression of RUNX1ΔN24 in leukemia cell lines

Several previous reports have revealed the growth-promoting role of endogenous RUNX1 in myeloid and lymphoid leukemia cells [19,25,26]. To assess the physiologic role of full-length RUNX1b and RUNX1ΔN24, we next induced depletion of *RUNX1* gene using the CRISPR/Cas9 system in four leukemia cell lines: K562, Molm13, TF-1, and Jurkat cells. We transduced Cas9 together with an NT sgRNA and two independent *RUNX1*-targeting sgRNAs into the cells. sgRUNX1-(1) recognizes only full-length RUNX1b, but not RUNX1ΔN24 and another RUNX1 isoform, RUNX1c, because the PAM sequence for this sgRNA does not exist in RUNX1ΔN24 and RUNX1c. sgRUNX1-(2) recognizes all long isoforms of RUNX1 (Figure 6A). sgRUNX1-(1) induced disruption of full-length RUNX1b around Met-1, which resulted in the selective expression of RUNX1ΔN24 in these cells. sgRUNX1-(2) depleted all long RUNX1 isoforms, which induced aberrant expression of multiple (probably nonfunctional) RUNX1 proteins. Expression levels of RUNX1ΔN24 was relatively low in most sgRUNX1-(1)-transduced cells probably due to the disruption of promoter sequences by CRISPR/Cas9 (Figure 6B). Both sgRNAs showed a growth-inhibitory effect (Figure 6C), indicating that RUNX1b plays a critical role in supporting the efficient growth of these leukemia cells. TF-1 and Jurkat cells were more dependent on RUNX1 activity for their growth, as evidenced by the rapid decrease of tRFP^{+} (sgRUNX1-transduced) cells in culture. Importantly, sgRUNX1-(1) showed a more potent inhibitory effect than sgRUNX1-(2) in K562 and Molm13 cells, suggesting that RUNX1ΔN24 has some compensatory functions to promote the growth of these cells. RUNX1ΔN24 did not support the growth of TF-1 and Jurkat cells, probably because the remaining RUNX1ΔN24 expression was not sufficient for these RUNX1-dependent cell lines. Therefore, disruption of RUNX1b around Met-1 induced selective expression of RUNX1ΔN24, which partially compensates for the loss of full-length RUNX1b in K562 and Molm13 cells.

Discussion

Alternative translation initiation contributes to protein diversity by expressing multiple protein isoforms from a single transcript. Here, we demonstrated that RUNX1b mRNA could be translated from internal AUG (Met-25) to produce the RUNX1Δ24 protein when the translation from Met-1 is perturbed. RUNX1ΔN24 is more stable than full-length RUNX1b, partly due to the lack of a lysine residue (Lys-24). Given that RUNX1ΔN24 is less active at upregulating GM-CSF and Iα1 promoters [27], it appears that the enhanced protein stability of RUNX1ΔN24 compensates for the reduced transcriptional activity to retain hematopoietic activity.

Physiological significance of the alternative translation initiation for RUNX1 remains elusive. Several lines of evidence suggest that RUNX1ΔN24 possesses the function to promote hematopoiesis. RUNX1b-(B), which mainly produces RUNX1ΔN24, can rescue the defective hematopoiesis of *Runx1*-deficient murine para-aortic splanchnopleural cells in vitro [28]. Another report showed that the N-terminal region of RUNX1 was dispensable for in vitro megakaryocytopoiesis from mouse embryonic stem cells [27]. Furthermore, a very recent study showed that RUNX1ΔN24 alone is sufficient for early hematopoiesis using mutant mice with defective RUNX1b translation from the first AUG [22]. In this study, we also demonstrated that RUNX1ΔN24 has similar activity to inhibit the growth of

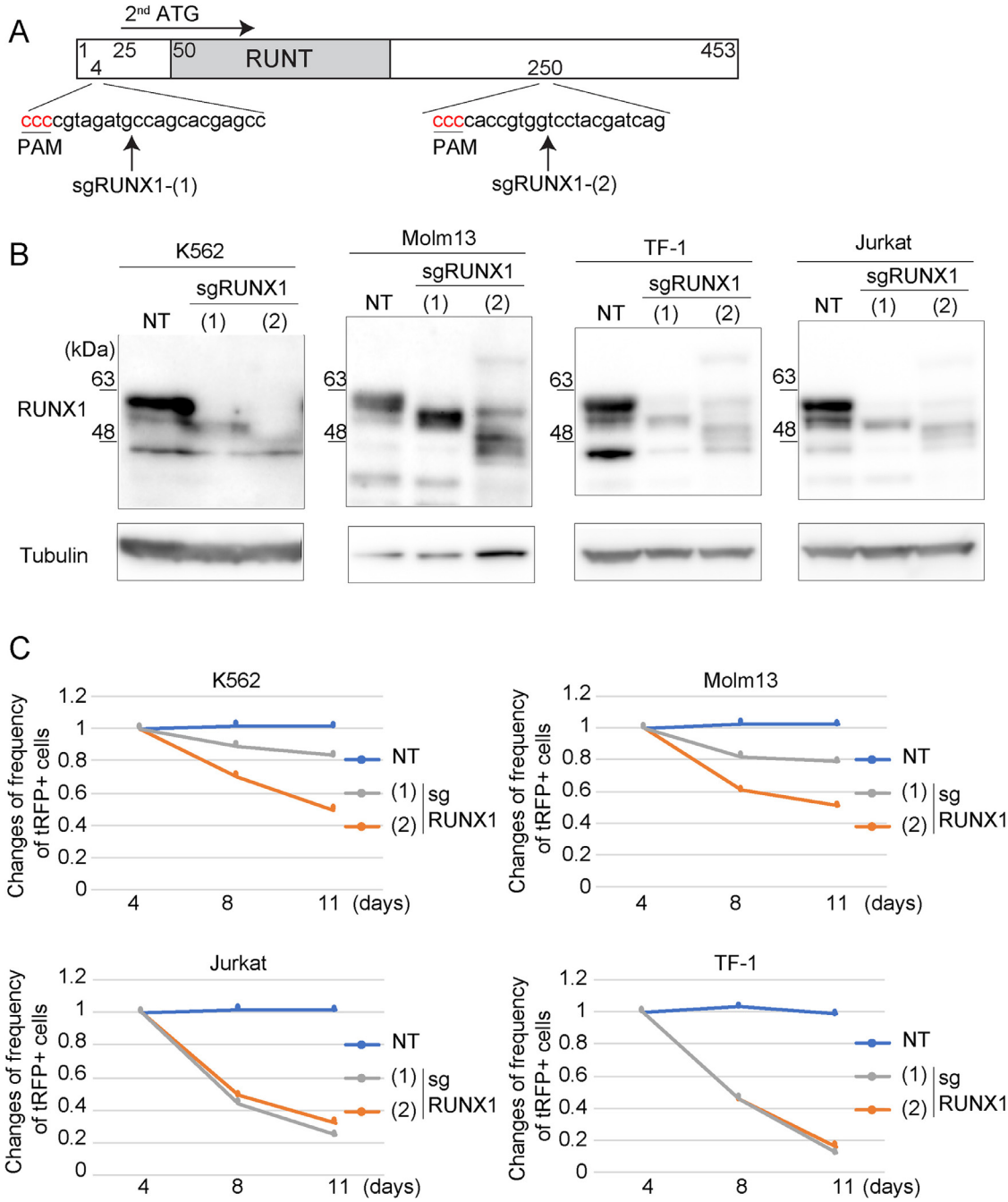

Figure 6. Disruption of endogenous RUNX1b around Met-1 induced selective expression of RUNX1ΔN24 in leukemia cell lines. **(A)** Schematic representation of RUNX1 and sgRNA-targeting regions. **(B)** Levels of RUNX1 protein in leukemia cell lines (K562, Molm13, TF-1, and Jurkat) transduced with NT or *RUNX1*-targeting sgRNAs. Transduction of sgRUNX1-(1) resulted in expression of RUNX1ΔN24 in all leukemia cell lines, whereas that of sgRUNX1-(2) led to expression of many aberrant (probably nonfunctional) proteins. **(C)** Four leukemia cell lines were transduced with NT or *RUNX1*-targeting sgRNAs (coexpress tRFP). The cells were cultured in vitro to monitor the changes of tRFP frequency.

CB cells. Interestingly, we found that disruption of either Met-1 or Met-25 promotes translation from another AUG, indicating that total level of RUNX1b protein is tightly controlled by the competitive regulation of these alternative translation initiations.

The regulatory mechanism of the alternative translation initiation also merits further studies. A recent report showed that translation of a Runx3 transcript is restricted because of lack of an efficient Kozak sequence in T cells, which promotes the alternative translational initiation from internal AUG. Introduction of an optimal Kozak sequence into the endogenous locus by genome editing enhanced the translation efficiency of RUNX3 from Met-1 [21]. Similar

mechanisms to regulate RUNX1b translation should exist in hematopoietic cells. It was also reported that knock-in mice expressing Runx1b deleted for the IRES element died in utero with defective hematopoiesis and angiogenesis, indicating the essential role of IRES-mediated translational regulation of RUNX1b in vivo [29]. The IRES element may control the delicate balance of the alternative translation initiation of RUNX1b. Our study revealed that disruption of full-length RUNX1b around Met-1 induces selective expression of RUNX1ΔN24 in leukemia cells, which has some compensatory function to support their growth. At present, it is technically challenging to determine which endogenous RUNX1 protein is mainly expressed in cells by the standard immunoblotting method because there are multiple RUNX1 transcripts and migration of each protein is affected by posttranslational modifications including phosphorylation. In the future, ribosomeprofiling [30] combined with CRISPR/Cas9-mediated genome editing will reveal the exact expression pattern and specific function of individual RUNX1 isoforms in normal and malignant hematopoietic cells.

The use of alternative translation initiation codons in a single mRNA has been shown for several hematopoietic transcriptional factors, including GATA1 and CEBPA [31,32]. The *GATA1* and *CEBPA* genes are targets for mutations that selectively disrupt expression of full-length protein in leukemia patients and the remaining N-terminal-truncated forms have significantly reduced function. In contrast to these genes, mutations selectively inhibiting full-length RUNX1 expression are rare in hematopoietic diseases. Given that RUNX1ΔN24 retains similar hematopoietic activity to full-length RUNX1, the translation initiation from Met-25 may act as a fail-safe mechanism to maintain normal hematopoiesis when the production of full-length RUNX1 protein is inhibited by genetic mutations. Although it is rare, a frame-shift mutation at Phe-13 was previously reported in myelodysplastic syndrome patients [5]. It will be interesting to determine whether the RUNX1ΔN24 protein is expressed in malignant cells with such mutations.

Experimentally, we should pay attention to which RUNX1 protein, full-length or RUNX1ΔN24, is expressed from each expression plasmid. The presence or absence of Kozak consensus sequences around Met-1 determines which initiation site, Met-1 or Met-25, is mainly used. This is particularly important when the plasmid contains an N-terminal tag because the N-terminal tag can alter RUNX1 function.

In summary, we identified Met-25 of RUNX1b as an internal translation initiator, adding to the complexity of RUNX1 expression and function. Given the distinct biochemical properties of RUNX1ΔN24, namely the enhanced protein stability and the attenuated transcriptional activity, RUNX1ΔN24 may play a specific role in maintaining the hematopoietic system.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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