Definitive hematopoiesis requires Runx1 C-terminal-mediated subnuclear targeting and transactivation

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Runx1 is a key hematopoietic transcription factor required for definitive hematopoiesis and is a frequent target of leukemia-related chromosomal translocations. The resulting fusion proteins, while retaining DNA binding activity, display loss of subnuclear targeting and associated transactivation functions encoded by the C-terminus of the protein. To define the precise contribution of the Runx1 C-terminus in development and leukemia, we created a knock-in mouse with a C-terminal truncation by introducing a single nucleic acid substitution in the native Runx1 locus. This mutation (Runx1^{Q307X}) models genetic lesions observed in patients with leukemia and myeloproliferative disorders. The Runx1^{Q307X} homozygous mouse exhibits embryonic lethality at E12.5 due to central nervous system hemorrhages and a complete lack of hematopoietic stem cell function. While able to bind DNA, Runx1^{Q307X} is unable to activate target genes, resulting in deregulation of various hematopoietic markers. Thus, we demonstrate that the subnuclear targeting and transcriptional regulatory activities of the Runx1 C-terminus are critical for hematopoietic development. We propose that compromising the C-terminal functions of Runx1 is a common mechanism for the pathological consequences of a variety of somatic mutations and Runx1-related leukemic fusion proteins observed in human patients.

INTRODUCTION

Runx1 is required for the emergence (1,2) and maintenance (3) of hematopoietic stem cells (HSCs) and definitive hematopoiesis. Clinically relevant are the observations that Runx1 point mutations and translocations are associated with acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML), as well as myeloid dysplastic syndromes (MDS), refractory anemia with excess blasts (RAEB) and familial platelet disorder which also have predisposition toward AML (4–9). Somatic mutations observed in patients cluster either in the DNA-binding Runt Homology Domain (RHD) or within the C-terminus (10). Recently, Runx1 mutations in the C-terminus of CMML patients have been shown to predict transformation to

AML (4). The domains disrupted by the C-terminal mutations are critical for protein–protein interactions (11–13) and for appropriate subnuclear targeting of Runx1 (14–17). Consequently, cells exhibit deregulation of Runx1 target genes (15). Loss of subnuclear targeting enhances proliferation of myeloid progenitor cells concomitant with a differentiation block, analogous to a transformed phenotype (15,18). This phenotype is similar to that caused by various chromosomal translocations that retain the RHD of Runx1, but replace the C-terminus with segments of other proteins, generating leukemic fusion proteins such as AML1/ETO, Runx1/Evi1 and Runx1/MDS. Together these results strongly suggest a role of the Runx1 C-terminus and associated functions in the biology of hematopoiesis and leukemogenesis.

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Patient Category	Mutation	Reference
Sporadic MDS, followed by AML	Ser291fsTer300	Harada et al., 2004
Sporadic MDS, followed by AML	Arg292fsTer574	Harada et al., 2004
Sporadic MDS, RAEB in transformation	Thr296fsTer305	Harada et al., 2004
Sporadic MDS, RAEB	Thr296fsTer338	Harada et al., 2004
Sporadic MDS, RAEB	Ala364fsTer570	Harada et al., 2004
Secondary MDS, RAEB in transformation	Leu378fsTer573	Harada et al., 2004
CMML	Ser268fsX578	Kuo et al., 2009
CMML	Arg293X	Kuo et al., 2009
CMML	Gly324fsX565	Kuo et al., 2009
CMML	Pro332fsX573	Kuo et al., 2009
CMML	Met341fsX569	Kuo et al., 2009
CMML	Phe369fsX572	Kuo et al., 2009
Mouse Models	Mutation	Reference
Runx1 ^{tm1Dow}	Original RHD knockout	Okuda et al., 1996
Runx1 ^{tm4Spe}	Patient RHD mutation: T149A	Matheny et al., 2007
Runx1 ^{tm5Spe}	Patient RHD mutation: R174Q	Matheny et al., 2007
Runx1 ^{tm6Spe}	Patient RHD mutation: L148F	Matheny et al., 2007
Runx1 ^{tm7Spe}	Patient RHD mutation: R177X	Matheny et al., 2007
Runx1 ^{tm8Spe}	Patient RHD mutation: T161A	Matheny et al., 2007
Runx1 ^{LacZ}	Replace exons 7 and 8 with LacZ	Wang, et al., 1996
Runx1 ^{delta446}	Delete final 5 amino acids VWRPY	Nishimura et al., 2004
Runx1 ^{Q307X}	Stop codon in exon 8	This study

Figure 1. Runx1 patient mutations and mouse models. Diagram of Runx1 with regions of interest highlighted. Runx1^{Q307X} was designed to model several human mutations observed in MDS, AML, RAEB and CMML. Also illustrated are the first RHD knock out mouse, existing mouse models bearing patient mutations in the RHD and previous models with mutations in the Runx1 C-terminus. Runx1-LacZ is broken to show the full length of the chimeric fusion protein.

To directly examine the biological relevance of the Runx1 C-terminus in a mouse model *in vivo*, we introduced a premature translational stop codon after amino acid 307, mimicking Runx1 mutations identified in human MDS/AML and CMML patients (Fig. 1) and observed to cause MDS/AML in mouse bone marrow transfer models (4,10,19,20). The truncated Runx1, Runx1^{Q307X}, lacks the transactivation domain and nuclear matrix targeting signal (NMTS), but retains sequences for the entire endogenous Runx1 mRNA, in contrast to a previous mouse model that replaced a portion of the mRNA with bacterial LacZ sequences (21). In this study, Runx1^{Q307X} homozygous mice die at embryonic day 12.5 (E12.5) from central nervous system hemorrhages and a lack of HSC function. This phenotype is similar to that observed with a DNA-binding knockout (1). We also show deregulation of genes important for hematopoiesis and HSC function in E12.5

Runx1^{Q307X} homozygous mice. Our results demonstrate that the transactivation and subnuclear targeting domains lost in Runx1^{Q307X} are essential for Runx1 function during development.

RESULTS

Loss of Runx1 C-terminal domains causes aberrant subnuclear targeting

The region lost in Runx1^{Q307X} includes the NMTS, a domain required for subnuclear targeting of Runx1 to transcriptionally active sites. In contrast, previous mouse models have focused on the Runt-homology DNA-binding domain or substituted the C-terminus (Fig. 1). To examine subnuclear targeting of the mutated protein, nuclear matrix-intermediate filament

Figure 2. *In vitro* characterization of Runx1^{Q307X} and generation of Runx1^{Q307X} mice. (A) Western blot showing expression of wild-type Runx1 or Runx1^{Q307X} in transfected HeLa cells. (B) Whole cell (upper panels) or NMIF preparations (lower panels) of HeLa cells transfected with wild-type Runx1 or Runx1^{Q307X}. Anti-Runx1 immunofluorescence images are shown with phase contrast insets. Scale bars are 100 μ m. The signal for Runx1^{Q307X} cells in NMIF preparations decreased by 4.5-fold compared with wild-type Runx1, based on observation of at least 200 cells per sample with representative cells shown. (C) Luciferase expression from GM-CSF promoter construct in the presence of empty vector, wild-type or Runx1^{Q307X} in HeLa and K562 cells. (D) EMSA showing DNA binding activity of Runx1 and Runx1^{Q307X} from transduced HeLa cell nuclear extracts: lane 1, free probe; lane 2, empty vector; lane 3, wild-type Runx1; lane 4, Runx1^{Q307X}. (E) Targeting strategy to replace a portion of exon 8 with exon 8 containing the point mutation to cause a stop codon after amino acid 307 (CAG to TAG). Probes A and B and restriction enzyme cut sites are shown. The Neomycin resistance gene (Neo) was removed by using embryonic stem cells containing the sperm-specific PC3Cre, excising Neo during generation of founder mice. Southern blot analysis of genomic DNA from tails of E12.5 embryos was performed using restriction enzymes and probes shown.

(NMIF) preparations of HeLa cells transduced with wild-type Runx1 or Runx1^{Q307X} were examined by *in situ* immunofluorescence microscopy. Expression of the truncated Runx1^{Q307X} (denoted ΔC in figures) was confirmed by western blot (Fig. 2A). In whole cell preparations, Runx1^{Q307X} retained the characteristic punctate nuclear foci observed with wild-type Runx1 (Fig. 2B). However, the Runx1^{Q307X} signal was significantly reduced in NMIF preparations, indicating that this mutant has lost the ability to interact with the nuclear matrix (Fig. 2B). Thus, our results are consistent with previous observations that the C-terminally encoded NMTS of Runx1 is required for subnuclear targeting. We performed luciferase reporter assays to determine whether Runx1^{Q307X} is able to activate target promoters. The GM-CSF promoter was activated in the presence of wild-type Runx1, but not Runx1^{Q307X}, in both HeLa and K562 cells (Fig. 2C). Runx1^{Q307X} forms stable protein-DNA complexes in electrophoretic mobility shift assay (EMSA) (Fig. 2D). Thus, while Runx1^{Q307X} is capable of binding to DNA, this truncated protein is unable to associate with the nuclear matrix and fails to activate target gene promoters.

Runx1 translocations observed in human leukemia patients often lose C-terminal domains critical for subnuclear targeting and protein-protein interactions. Previous mouse models examined complete loss-of-function Runx1 mutations by

ablating DNA binding. Other mutants created hypomorphic deletions or fusion proteins produced from chimeric mRNAs lacking endogenous 3'-UTR sequences that are required for fidelity of expression. We investigated the biological function of the Runx1 C-terminus by introducing the Runx1^{Q307X} point mutation into the endogenous Runx1 locus by homologous recombination (Fig. 2E).

Runx1 C-terminal subnuclear targeting and associated transactivation are required during development

Embryos homozygous for the truncated Runx1^{Q307X} died at E12.5 from central nervous system hemorrhage, similar to the Runx1 RHD mutant knockout mice (1). The presence of the Runx1^{Q307X} mutation in the transcripts was confirmed by sequencing; furthermore, there was no evidence of an alternatively spliced transcript (data not shown). We observed near Mendelian ratios of wild-type, heterozygous and homozygous mutant pups at E11.5 and E12.5 (Table 1). However, no live homozygous Runx1^{Q307X} pups were observed at E13.5 (Table 1). Gross examination of homozygous embryos consistently showed multiple areas of hemorrhage and a pale fetal liver (Fig. 3A). The severity of the phenotype ranged from a few small areas of hemorrhage in the spinal cord to large regions of hemorrhage throughout the spinal cord, isthmus

Table 1. Genotyping of embryos from Runx1^{Q307X} heterozygous intercrosses.

Age	Embryos	Litters	+/+	+/ Δ C	Δ C/ Δ C
E11.5	37	4	9	21	7
E12.5	97	12	27	48	22
Total	134	16	36	69	29
Ratio			1.00	1.92	0.81
E13.5	19 ^a	5	9	10	0

Genotyping was performed by Southern blot as described in Figure 2 or by PCR using the following primers, forward primer ACT CTG GCA GTC TAG GAA GCC and reverse primer AGG CGC CGT AGT ATA GAT GGT A.

^aAt E13.5, only live embryos were counted.

and ventral metencephalon (Supplementary Material, Fig. S2). Areas of hemorrhage were examined in more detail with whole mount hematoxylin and eosin staining (Fig. 3B and C). The sinusoids of Runx1^{Q307X} homozygous embryo fetal livers were devoid of hematopoietic precursor cells that were present in wild-type embryos (Fig. 3C middle and lower panels).

Consistent with the absence of hematopoietic progenitor cells by histological analysis, fetal liver cells from Runx1^{Q307X} homozygous embryos were unable to form colonies in a methocellulose colony forming unit assay (Fig. 4A) despite robust activity of fetal liver cells from their wild-type and heterozygous littermates. Runx1 is not required for erythropoiesis and primitive erythrocytes are abundant in the peripheral blood of both wild-type and mutant embryos (Fig. 4B). Thus, the C-terminal domains of Runx1 are required for *in vivo* HSC function during development.

Aberrant subnuclear targeting and lost protein–protein interactions of Runx1^{Q307X} cause deregulation of Runx1 targets

Runx1 is a key regulator of hematopoiesis and controls many hematopoietic target genes. Considering the severity of the phenotype of the Runx1^{Q307X} homozygous mice, we performed RT–PCR analysis to assess the effects of Runx1^{Q307X} on Runx1 target genes and markers of hematopoiesis (Fig. 5). Expression of hematopoietic transcription factors (Fig. 5A) and phenotypic markers (Fig. 5B) was clearly decreased in the Runx1^{Q307X} homozygous mice, with a gene dosage-dependent reduction generally seen in heterozygous littermates. Runx1 expression was not substantially altered, suggesting that the point mutation does not change the stability of the transcript (e.g. nonsense-mediated mRNA decay). Markers for cell survival and proliferation were comparable for the three genotypes (Fig. 5C). Genes important for HSC function (Fig. 5D) were dramatically downregulated in the Runx1^{Q307X} homozygous mice, confirming the hematopoietic specificity of the mutation. Signaling proteins that are indirect targets were also affected in Runx1^{Q307X} mice (Fig. 5E). Interestingly, VEGF was upregulated in the Runx1^{Q307X} homozygous mice, which may contribute to the observed hemorrhages by increasing vascular permeability. Consistent with mRNA expression data, a drop in protein levels of PU.1 and YAP was observed (Supplementary Material, Fig. S3). These results indicate that the loss of key domains

responsible for protein–protein interactions and subnuclear targeting in Runx1^{Q307X} caused widespread gene deregulation.

DISCUSSION

Human leukemias often involve Runx1 translocations that result in a fusion protein retaining the DNA-binding domain of Runx1, but losing the C-terminus encompassing subnuclear targeting and transactivation domains. In this study, we have generated a knock-in mouse model that contains a point mutation causing a premature stop codon within exon 8. This mutation truncates the protein after amino acid 307, but maintains the integrity of the endogenous Runx1 mRNA by retaining the extensive 3'-untranslated region and potential regulation by miRNAs (22,23). In many human leukemias, a naturally occurring truncated protein isoform, AML1a [Runx1/p27 (24)], is upregulated, and there is evidence that overexpression of AML1a can act as an initiating leukemogenic mutation by inhibiting Runx1 function (25,26). Taken together, these findings suggest that Runx1 C-terminal domains play important mechanistic roles in leukemogenesis and that the loss of subnuclear targeting and transactivation may contribute to the etiology of human leukemia.

Our mouse model with a point mutation in exon 8 complements previous mouse models with mutations in exon 4, which encodes a part of the Runt-homology DNA-binding domain. These exon 4 mouse mutations mimic genetic lesions found in leukemia patients (27; Fig. 1) and have defined the phenotypic consequences of complete loss of function due to inhibition of DNA binding. Recent evidence has shown the importance of the Runx1 C-terminus, which includes subnuclear targeting and transactivation domains, during embryoid body formation with mouse embryonic stem (ES) cells (28). Hematopoietic differentiation was lost in embryoid bodies made with Runx1 null ES cells, but rescued by targeted insertion of cDNAs for full-length Runx1 or Runx1 C-terminal truncations that retained transactivation domains. Recent *in vitro* studies used a retroviral rescue of Runx1 null cells to assess which domains of Runx1 were important for hematopoietic colony formation (29). These studies revealed that the C-terminal transactivation domain is required for colony formation, but did not further characterize the rescued cells or address the effects of mutations *in vivo*.

The Runx1^{Q307X} mutation we have characterized here provides a mouse model for several C-terminal mutations observed in human patients with MDS, AML, RAEB and CMML (Fig. 1) (4,10). A previous mouse model involving the loss of the Runx1 C-terminus replaced exons 7 and 8 with the bacterial LacZ gene to create a fusion protein (21). This model allowed the tracking of Runx1 expression and supported the concept that Runx1 is required during hematopoietic development. However, the genetic strategy produced a larger Runx1 C-terminal deletion and generated a large Runx1-LacZ fusion protein, as well as eliminated 3'-UTR sequences that are now known to support regulation by miRNAs (e.g. miR-27a) (22). Runx1, Runx2 and Runx3 all have homologous subnuclear targeting signals (14,22,30). The Runx1^{Q307X} mouse contains a precise mutation that

Figure 3. Histology of $Runx1^{Q307X}$ at E12.5. (A) Gross image of wild-type (left panel) and $Runx1^{Q307X}$ (right panel) E12.5 embryos. White arrowheads note sites of hemorrhage in the $Runx1^{Q307X}$ embryo. Scale bars are 1 mm. (B) Whole mount hematoxylin and eosin staining of embryos in A. Scale bars are 1 mm. (C) Magnification of heads of E12.5, noting hemorrhages with black arrows (upper panels, scale bars are 500 μ m). Magnification of fetal livers of E12.5 embryos (middle panels, scale bars are 250 μ m) and magnification showing liver sinusoids (lower panels, scale bars are 100 μ m) with arrow noting the hematopoietic precursor cells in the wild-type sinusoids that are absent from $Runx1^{Q307X}$.

Figure 4. Lack of hematopoietic progenitor cell function in $Runx1^{Q307X}$. (A) Colony forming unit assays using fetal liver cells from wild-type, heterozygous or $Runx1^{Q307X}$ homozygous E12.5 embryos. (B) Nucleated erythroid progenitors are morphologically normal in peripheral blood smears of wild-type and $Runx1^{Q307X}$ E12.5 embryos. Scale bars are 10 μ m.

removes only the subnuclear targeting and transactivation domains, thus compromising the function of Runx1 as a scaffolding protein attached to the nuclear matrix, DNA and other proteins within the complicated three-dimensional architecture of the nucleus (31,32). The resulting aberrant subnuclear localization of $Runx1^{Q307X}$ is predicted to trigger a cascade of deregulation.

In this study, we show that $Runx1^{Q307X}$ is no longer associated with the nuclear matrix due to the loss of domains critical

for interaction with the nuclear architecture. The region lost in $Runx1^{Q307X}$ corresponds with the mapped interaction domains for many Runx co-factors, including p300 (12), YAP (33,34), MOZ (35), Groucho/TLE1 (36–38), SUV39H1 (13), Smad 3 (39,40), HDAC 1 and HDAC 3 (11,13). $Runx1^{Q307X}$ binds DNA but without the transactivation domains or subnuclear targeting it obstructs regulation rather than bringing components together. Removal of the C-terminal domains of Runx1 responsible for subnuclear targeting and protein–protein interactions causes the same embryonic lethal phenotype as a DNA-binding knockout. Homozygous $Runx1^{Q307X}$ embryos die at E12.5 from lack of definitive hematopoiesis and central nervous system hemorrhages. Hence, $Runx1^{Q307X}$ is not a hypomorphic mutation but rather results in a phenocopy of RHD mutations. These findings complement the observations with an analogous mutation in the Runx2 gene, which was also a phenocopy of a null mutation (41). $Runx1^{Q307X}$ contrasts with a mouse mutant lacking the C-terminal VWRPY motif, which has a mild hypomorphic phenotype with defects in thymocyte development (42). Our results with the $Runx1^{Q307X}$ mouse establish that the subnuclear targeting and transactivation functions of Runx1 are essential for hematopoietic development and are consistent

Figure 5. Runx1^{Q307X} causes deregulation of hematopoietic genes. Relative expression of hematopoietic transcription factors (A), phenotypic markers (B), proliferation and cell survival markers (C), genes required for HSC maintenance (D), and signaling molecules (E) by RT-PCR in wild-type, heterozygous and homozygous Runx1^{Q307X} E12.5 mice ($N=3$, 2 and 4, respectively). All RT-PCR results were normalized to GAPDH expression. See Materials and Methods for specific primers.

with the observation that human genetic lesions perturbing these functions contribute to leukemogenesis.

MATERIALS AND METHODS

Immunofluorescence microscopy

HeLa cervical carcinoma cells were grown on cover slips coated with 0.05% gelatin and transfected with Eugene 6 (Roche Diagnostics, Indianapolis, IN, USA) following manufacturer's instructions. After 24 h, cells were fixed using formaldehyde (3.7%), and permeabilized with 0.5% Triton

X-100 for whole-cell preparations. NMIF preparations were obtained as described (43). Runx1 protein was detected by the AML1(RHD) antibody (Oncogene Science, Cambridge, MA, USA; 1:200 dilution) followed by fluorochrome-conjugated Alexa Fluor 488 secondary antibody (Invitrogen Molecular Probes, Eugene, OR, USA; 1:800 dilution). Cells were mounted in Prolong Gold antifade mounting medium (Invitrogen Molecular Probes). Fluorescence and transmitted light images were captured using a Zeiss Axioplan 2 microscope equipped with a digital charged-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ, USA; Cat. No. C4742-95) interfaced with the MetaMorph Imaging

System (Universal Imaging Corporation Ltd, Marlow, Buckinghamshire, UK).

Luciferase assays

K562 erythroleukemia or HeLa cells were transfected with Eugene 6 (Roche) following manufacturer's instructions. For both cell types, each well (~200 000 cells) of a 6-well plate was transfected with 500 ng of GM-CSF promoter luciferase reporter (pGL3) (18); 200 ng of pcDNA3.1/HISa (Invitrogen) empty vector, Runx1 wild-type or Runx1^{Q307X}; and 5 ng pHRL-null promoter-less *Renilla* luciferase (44). Luciferase activity was measured, 24 h after transfection, using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA; Cat. No. E1960) in a Glomax Luminometer (Promega) with Glomax 1.6 software. Values plotted are the ratios of firefly to *Renilla* luciferase, normalized to empty vector. Error bars represent the standard deviation among samples, $n = 4$ (K562) or 3 (HeLa).

Electrophoretic mobility shift assay (EMSA)

Runx consensus oligonucleotide 5'-CGA GTA TTG TGG TTA ATA CG-3' was end-labeled as described previously (45). Nuclear extracts from HeLa cells (7.5 µg) transduced with empty vector, wild-type Runx1 or Runx1^{Q307X} were used in the binding reaction. Reaction mixtures were prepared using 50 fmol of probe, 50 mM KCl, 1 mM magnesium chloride, 1 mM DTT, 2 mM sodium fluoride, 2 mM sodium vanadate, 10% glycerol, 2 µg of poly(dI-dC)•poly(dI-dC) and DNA-binding reactions were carried out at 25°C for 20 min. Aliquots were separated in a 4% non-denaturing polyacrylamide gel for 1.5 h at 200 V. The gel was dried and subjected to autoradiography.

Construction of the Runx1^{Q307X} expression vector

Wild-type Runx1 in the pcDNA3.1/HISa expression vector (46) was mutated to Runx1^{Q307X} by site-directed mutagenesis using a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the following primers: forward CGG CGA CCC ACG CTA GTT CCC TAC TCT G, reverse CAG AGT AGG GAA CTA GCG TGG GTC GCC G.

Construction of the Runx1^{Q307X} targeting vector

We targeted the mouse Runx1 locus by homologous recombination using a 3.97 kb *SacII*–*NotI* PCR fragment of intron 7 (left arm) and a 4.0 kb *NotI*–*SalI* PCR fragment of intron 7–exon 8 (right arm). Both fragments were generated from mouse AB2.2 genomic DNA by PCR using specific primer pairs (primers 5'–3': LAF1 CCG CGG GGC ATC TCT CTC CTT CCT CCA GTG TCT; LAR1 GAG GGG ATC GAA AAG CTT CCT; LAF2 AGG AAG CTT TTC GAT CCC CTC; LAR2 GCG GCC GCG ATC ACG GAG AGT GCC TCT GAC AC; RAF1 GCG GCC GCG TGG GCA GGA GCA CTC GCT GT; RAR1 GAG TAG GGA ACT AGC GTG GG; RAF2 CCC ACG CTA GTT CCC TAC TC; RAR2 GAC CAC CCA GAT GCA AAC AGG; RAF3 CGC ACC TTA TCG ATT GCA A; RAR3 GTC GAC CCG

ACC AAC AGC CAA ACC CAC CAA). The left arm was created by using two primer pairs that produce 1.3 and 2.67 kb fragments (LAF1 to LAR1 and LAF2 to LAR2, respectively) which were ligated using an internal *HindIII* site to obtain the entire 3.97 kb fragment. The right arm was created using three different primer pairs that produce two overlapping fragments of 1.0 kb containing the stop codon mutation (RAF1 to RAR1 and RAF2 to RAR2) and 3.0 kb (RAF3 to RAR3) which were ligated using an internal *ClaI* site to obtain the entire 4.0 kb fragment. The 3.97 and 4.0 kb fragments were cloned in tandem into the pGEM-5Zf(+) vector (Promega). We then inserted a 2.0 kb *NotI*–*NotI* cassette containing a floxed neomycin gene (LoxP site–PGK promoter–Neo cDNA–LoxP site) and a 2.2 kb *SalI*–*SalI* cassette with the thymidine kinase gene (PGK promoter–TK cDNA) (Fig. 2E). Vectors containing the Neo and TK cassettes were provided by the Transgenic Animal Modeling Core Facility of the University of Massachusetts Medical School. The final targeting vector and intermediate constructs were subjected to DNA sequencing.

Screening of mouse embryonic stem cells with a Runx1^{Q307X} allele

The targeting vector was linearized with *AscI* and electroporated into PC3 (129S5/SvEvBrd) ES cells (Transgenic Animal Modeling Core Facility, University of Massachusetts Medical School) (i.e. 10⁷ ES cells were transfected with 20 µg linearized construct at 230 V and 500 µF). Positive selection was started 24 h after electroporation by addition of 180 µg/ml of G418 (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA). Antibiotic resistant clones were transferred into and cultured in 96-well plates. Homologous recombination of the Runx1^{Q307X} allele was established by Southern blot analysis using restriction sites and probes external to the targeting vector. Hybridization was carried out using the PerfectHyb™ Plus Hybridization kit (Sigma-Aldrich, St Louis, MO, USA). Southern blot analysis identified a single clone with a correctly targeted mutation of the Runx1 locus (Supplementary Material, Fig. S1).

Generation of the Runx1^{Q307X} null mice

The PC3 ES cell clone with a targeted Runx1^{Q307X} allele was micro-injected into C57BL/6 blastocysts. Chimeric mice with a significant ES cell contribution (as determined by agouti coat color) were mated with wild-type C57BL/6 and germ line transmission of the mutant allele was determined by Southern blot genotyping of tail DNA from offspring and confirmed by PCR (Primers 5'–3': forward ACT CTG GCA GTC TAG GAA GCC, reverse AGG CGC CGT AGT ATA GAT GGT A). Runx1^{Q307X} heterozygous mice were crossed to generate Runx1^{Q307X} homozygous mice and offspring were subjected to genotyping by PCR and Southern blot analysis.

Histology

Embryos (E12.5) were embedded in paraffin after a sample of tail was taken for genotyping. Six micron sections were stained with hematoxylin and eosin by standard procedures.

Images were captured using a Axioskop 40 (Carl Zeiss, Inc., Maple Grove, MN, USA) equipped with a AxioCam HRC and AxioVision Rel. 4.7 software (Zeiss).

Western blotting

Embryos (E12.5) were homogenized with a Dounce homogenizer in 2.0 ml direct lysis buffer [2% SDS, 2 M urea, 10% glycerol, 10 mM Tris-HCl (pH 6.8), 0.002% bromophenol blue, 10 mM DTT, 1× Complete protease inhibitor (Roche), 25 μM MG132 and 1 mM PMSF]. HeLa cells were lysed with 500 μl direct lysis buffer per confluent well of a 6-well plate. Cell lysates were boiled for 5 min and equal amounts of protein (embryos) or sample volumes (HeLa cells) were electrophoresed in an 8% SDS-polyacrylamide gel, at 100 V for 1.5 h. Separated proteins were transferred to an Immobilon Membrane (Millipore, Billerica, MA, USA) by semidry transfer for 30–45 min at 10 V. Membranes were blocked for at least 1 h in 5% non-fat dry milk in PBST (PBS with 0.1% Tween 20) and then probed for 1 h with primary antibody diluted 1:1000 [PU.1, YAP, Cdk2, LaminB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or AML1(RHD) (Oncogene Science)]. After four 5 min washes with PBST, blots were incubated for 1 h with goat anti-rabbit (or donkey anti-goat) IgG-HRP secondary antibody diluted 1:4000, washed four times for 5 min in PBST and detected with ECL (Perkin-Elmer, Waltham, MA, USA).

Colony forming unit assays

Fetal livers of E12.5 pups were isolated, placed in Iscove's Modified Dulbecco's Medium and passed three times through a 26 gauge needle to homogenize the mixture. Cells (20 000) from each liver were plated in duplicate in 35 mm dishes containing MethocultTM methyl cellulose medium (StemCell Technologies Vancouver, BC, Canada; Cat. No. M3434), incubated at 37°C and colonies were counted by visual inspection on day 14.

RT-qPCR

RNA was prepared from E12.5 embryos using TRIzol following the manufacturer's protocol (Invitrogen). RNA was treated with DNaseI and 1 μg was subjected to reverse transcription with oligo dT primers. Quantitative PCR was performed on the resulting cDNA using the following primers, 5'–3': Runx1 forward CCA GCA AGC TGA GGA GCG GCG, reverse TGA CGG TGA CCA GAG TG; PU.1 forward TAT CAA ACC TTG TCC CCA GC, reverse GCG AAT CTT TTT CTT GCT GC; MPO forward ATG CAG TGG GGA CAG TTT CTG, reverse GTC GTT GTA GGA TCG GTA CTG; Gfi1 forward AGG AGG CAC CGA GAG ACT CA, reverse GGG AGG CAG GGA AGA CAT C; GM-CSF forward TAT TCG AGC AGG GTC TAC GG, reverse GAG TCA GCG TTT TCA GAG GG; Csf1R forward GCG ATG TGT GAG CAA TGG CAG T, reverse AGA CCG TTT TGC GTA AGA CCT G; VEGF forward ACT GGA CCC TGG CTT TAC TG, reverse GGC AGT AGC TTC GCT GGT AG; YAP forward CGA TCA GAC AAC AAC ATG GC, reverse ATC CTG AGT CAT GGC TTG CT; Cebpδ

forward TCG ACT TCA GCG CCT ACA TTG, reverse CGC TTT GTG GTT GCT GTT GA; BMI1 forward TCC AGG TTC ACA AAA CCA GAC, reverse GTA GTG GGC CAT TTC TTC TCC; p21 forward CTT CTC CCA TTT CTT AGT AGC AG, reverse CCA CGG TAT TCA ACA CTG AG; p27 forward TCT AAA GCC CAC TTA TAA CCC AG, reverse CCT GTG CCA TCT CTA TAT TCC T; p57 forward GTC TGA GAT GAG TTA GTT TAG AGG, reverse TGC TAC ATG AAC GAA AGG TC; BCL-2 forward TAC CGT CGT GAC TTC GCA GAG, reverse GGC AGG CTG AGC AGG GTC TT; MCL-1 forward TAA GGA CGA AAC GGG ACT GG, reverse ACC AGC CCC TAC TCC AGC AA; p19arf forward TTC TTG GTG AAG TTC GTG CGA TCC, reverse ACG TGA ACG TTG CCC ATC ATC ATC.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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