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 (Runx1Q307X) models genetic lesions observed in patients with leukemia and myeloproliferative disorders. The Runx1Q307X 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, Runx1Q307X 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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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, Runx1Q307X, 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

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**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.
(NMIF) preparations of HeLa cells transduced with wild-type Runx1 or Runx1Q307X were examined by in situ immunofluorescence microscopy. Expression of the truncated Runx1Q307X (denoted ΔC in figures) was confirmed by western blot (Fig. 2A). In whole cell preparations, Runx1Q307X retained the characteristic punctate nuclear foci observed with wild-type Runx1 (Fig. 2B). However, the Runx1Q307X 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 Runx1Q307X is able to activate target promoters. The GM-CSF promoter was activated in the presence of wild-type Runx1, but not Runx1Q307X, in both HeLa and K562 cells (Fig. 2C). Runx1Q307X forms stable protein-DNA complexes in electrophoretic mobility shift assay (EMSA) (Fig. 2D). Thus, while Runx1Q307X 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 Runx1Q307X 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 Runx1Q307X died at E12.5 from central nervous system hemorrhage, similar to the Runx1 RHD mutant knockout mice (1). The presence of the Runx1Q307X 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 Runx1Q307X 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 Runx1Q307X heterozygous intercrosses.

<table>
<thead>
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<th>Age</th>
<th>Embryos</th>
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<td>97</td>
<td>12</td>
<td>27</td>
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</tr>
<tr>
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<td>19*</td>
<td>5</td>
<td>9</td>
<td>10</td>
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Genotyping was performed by Southern blot as described in Figure 2 or by PCR using the following primers, forward primer ACT CTG GCA GTG TAG GAA GCC and reverse primer AGG CGC CGT AGT ATA GAT GGT A. *At 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 sinuses of Runx1Q307X 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 Runx1Q307X 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 Runx1Q307X 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 Runx1Q307X homozygous mice, we performed RT−PCR analysis to assess the effects of Runx1Q307X 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 Runx1Q307X 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 Runx1Q307X homozygous mice, confirming the hematopoietic specificity of the mutation. Signaling proteins that are indirect targets were also affected in Runx1Q307X mice (Fig. 5E). Interestingly, VEGF was upregulated in the Runx1Q307X 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 Runx1Q307X 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 Runx1Q307X 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 Runx1Q307X mouse contains a precise mutation that
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<sup>Q307X</sup> is predicted to trigger a cascade of deregulation.

In this study, we show that Runx1<sup>Q307X</sup> 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<sup>Q307X</sup> 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<sup>Q307X</sup> 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<sup>Q307X</sup> embryos die at E12.5 from lack of definitive hematopoiesis and central nervous system hemorrhages. Hence, Runx1<sup>Q307X</sup> 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<sup>Q307X</sup> 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<sup>Q307X</sup> mouse establish that the subnuclear targeting and transactivation functions of Runx1 are essential for hematopoietic development and are consistent
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 Fugene 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 Runx 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 Runx1Q307X, and 5 ng pHLR-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). Luciferase activity was normalized to Renilla luciferase, 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.

Electrophoretic mobility shift assay (EMSA)

Runx consensus oligonucleotide 5’-CGA GTA TTG TGG TTA ATA CG-3’ was 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 Runx1Q307X expression vector

Wild-type Runx1 in the pcDNA3.1/HISa expression vector (46) was mutated to Runx1Q307X by site-directed mutagenesis using a QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) with the following primers: forward CGG CGA CCC ACG CTA GTT CCC TAC TCT G, reverse AGG AAG CTT TTC GAT CTC CTT CCT CCA GTG TCT G, Raf1 GCG GCC GCG ATC ACG GAG AGT GCC TCT GAC AC; RAR1 GAG TAG GGA ACT AGC GTG GG; RAF2 CCC ACG CTA GGT 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 Clal site to obtain the entire 4.0 kb fragment. The 3.97 and 4.0 kb fragments were cloned in tandem into the pGEM-SZI(+) 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 Runx1Q307X allele

The targeting vector was linearized with Ascl 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 Runx1Q307X allele was established by Southern blot analysis using restriction sites and probes external to the targeting vector. Hybridization was carried out using the PerfectHybTM 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 Runx1Q307X null mice

The PC3 ES cell clone with a targeted Runx1Q307X 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 germline 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 CTT GCA GTC TAG GAA GCC, reverse AGG CGC CGT AGT ATA GAT GGT A). Runx1Q307X heterozygous mice were crossed to generate Runx1Q307X 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 mM 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 semidyferms 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 [FU.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 with PBST, blots were incubated for 1 h with goat 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 (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 CCG 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 CGT CTG; Gfi1 forward AGG AGG CAC CGA GAG ACT CA, reverse GGG AGG AGG CAG AGA AGA CAT C; GM-CSF forward TAT CAG AGG AGG GTC GTC TG, 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; Cebp 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 C; p21 forward CTG CTT 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 CAC T; p57 forward GTTC 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 ATCG AGC AGG GTC TG, reverse ACC AGC CCC TAC TCC AGC AA; p19arf forward TCT TTG GTG AAC TTC GTG CGA TCC, reverse ACG TGA AGC TTG CCC ATC ATC ATC.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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