Cited2 Is an Essential Regulator of Adult Hematopoietic Stem Cells

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SUMMARY

The regulatory pathways necessary for the maintenance of adult hematopoietic stem cells (HSCs) remain poorly defined. By using loss-of-function approaches, we report a selective and cell-autonomous requirement for the p300/CBP-binding transcriptional coactivator Cited2 in adult HSC maintenance. Conditional deletion of Cited2 in the adult mouse results in loss of HSCs causing multilineage bone marrow failure and increased lethality. In contrast, conditional ablation of Cited2 after lineage specification in lymphoid and myeloid lineages has no impact on the maintenance of these lineages. Additional deletion of Ink4a/Arf (encoding p16Ink4a and p19Arf) or Trp53 (encoding p53, a downstream target of p19Arf) in a Cited2-deficient background restores HSC functionality and rescues mice from bone marrow failure. Furthermore, we show that the critical role of Cited2 in primitive hematopoietic cells is conserved in humans. Taken together, our studies provide genetic evidence that Cited2 selectively maintains adult HSC functions, at least in part, via Ink4a/Arf and Trp53.

INTRODUCTION

Adult hematopoiesis depends on rare multipotent bone marrow (BM)-resident hematopoietic stem cells (HSCs) (Orkin and Zon, 2008). HSCs may remain quiescent, self-renew, undergo apoptosis, or differentiate into multiple blood lineages. Tight regulation of these fates is essential to maintain the adult HSC pool, and studies in mice have revealed some of the key regulators of HSC maintenance. To identify novel regulators of adult HSC maintenance, we and others employed comparative global gene expression approaches. These studies identified the p300/CBP-binding transcriptional coactivator Cited2 as a candidate regulator of adult HSCs (Gomes et al., 2002; Mansson et al., 2007; Zhong et al., 2005), but functional validation remains to be performed.

CITED2 mutations are found in patients with congenital heart disease (Sperling et al., 2005), lending clinical significance in trying to understand CITED2 function. Cited2 physically interacts with the histone acetyltransferase p300/CBP (Bhattacharya et al., 1999), coactivates DNA-binding transcription factors (Bamforth et al., 2001; Chou et al., 2006; Glenn and Maurer, 1999; Tien et al., 2004), and represses HIF-1-mediated transcription (Bhattacharya et al., 1999). Cited2 has oncogenic properties (Sun et al., 1998) and controls proliferation of mouse embryonic fibroblasts (MEFs) via polycomb group genes Bmi-1 and Mel18 and the tumor suppressor Ink4a/Arf (Kranc et al., 2003). Cited2 deletion in mice is embryonic lethal, causing multiple developmental defects (Bamforth et al., 2001; Yin et al., 2002), including impaired fetal liver hematopoiesis (Chen et al., 2007). Severe fetal liver malformations (Qu et al., 2007) precluded defining a cell-autonomous role for Cited2 in HSC function and hematopoiesis, although these findings suggest a potential role for Cited2 in fetal HSC regulation. In this study, we use a conditional knockout strategy to establish a requirement for Cited2 in adult HSCs. Further, we demonstrate a role for CITED2 in human hematopoiesis by RNA interference in CD34+ cord blood (CB) cells.

RESULTS

Cited2 Is Essential for Sustaining Multilineage Hematopoiesis

Cited2 expression analysis indicated that it is highly expressed in long-term HSCs (LT-HSCs; Lin- Sca-1+c-kit+(LSK)CD34−Flt3− cells), less abundantly in short-term HSCs (ST-HSCs; LSKCD34+ Flt3− cells), and profoundly downregulated in lymphoid-primed multipotent progenitors (LMPPs; LSKCD34+Flt3− cells) (Figure 1A). To investigate a functional requirement for Cited2 in adult hematopoiesis, we generated Cited2fl/fl Mx1-Cre conditional knockout mice (MacDonald et al., 2008), in which treatment with poly(I)-poly(C) (pIpC) induces efficient gene deletion and provides a robust strategy to establish a requirement for Cited2 in adult HSCs. Further, we demonstrate a role for CITED2 in human hematopoiesis by RNA interference in CD34+ cord blood (CB) cells.
Cre-mediated recombination, a lacZ expression cassette comes under the control of the endogenous Cited2 promoter (MacDonald et al., 2008), and efficient gene deletion was demonstrated by abundant lacZ expression in Cited2\(^{−/−}\) BM cells (Figure S1A available online). Furthermore, Cited2 mRNA was undetectable in Cited2\(^{−/−}\) BM cells (Figure 1C). Within 6 to 15 days after initiation of plpC treatment, most Cited2\(^{−/−}\) mice became moribund and were sacrificed, in contrast to control mice, which survived normally (Figure S1B). BM analysis revealed severely reduced cellularity in Cited2\(^{−/−}\) mice (Figure 1D) and strikingly reduced frequencies of mature myeloid (Mac-1\(^{+}\)Gr-1\(^{+}\)) and B-lymphoid (CD19\(^{+}\)B220\(^{+}\)) cells in Cited2\(^{−/−}\) BM, as compared to control mice (Figure 1E). Conditional loss of Cited2 also reduced T cell frequencies (Figure 1E). These data support an essential role for Cited2 in sustaining adult multilineage hematopoiesis.

Mx1-Cre mediates gene deletion in both hematopoietic and nonhematopoietic tissues (Kuhn et al., 1995), so we assessed the contribution of Cited2 deletion in nonhematopoietic tissues to morbidity. We transplanted wild-type (WT) BM cells into Cited2\(^{+/+}\) Mx1-Cre and Cited2\(^{−/−}\) mice, and 12 weeks after transplantation, recipients received plpC. We observed no lethality in either cohort of mice (Figure S1C), indicating that BM failure in Cited2\(^{−/−}\) mice is the primary cause of mortality.

**Cited2 Is Dispensable for the Maintenance of Committed Blood Lineages**

The multilineage defects observed in Cited2\(^{−/−}\) mice could reflect a requirement for Cited2 in the maintenance of committed hematopoietic lineages. To test this hypothesis, we used Cd19-Cre, LysM-Cre, and Cd4-Cre strains to delete Cited2 in B cell, myeloid, and T cell lineages, respectively. Cd19-Cre efficiently excised Cited2 in CD19\(^{+}\)B220\(^{+}\) cells but did not affect their frequency in the thymus (Figure 2C). Therefore, Cited2 is expendable for the maintenance of these committed lineages.

**Cited2 Is Required for the Maintenance of Adult HSCs**

Next, we addressed the impact of Cited2 deletion on HSC and progenitor cell activity. In colony-forming cell (CFC) assays, Cited2\(^{−/−}\) BM cells failed to generate colonies in methylcellulose (Figure 3A). To evaluate HSC activity in vitro, we performed limiting dilution cobblestone area-forming cell (CAFC) assays and found that Cited2\(^{−/−}\) BM completely lacked CAFCs (Figure 3B). To assess HSC activity in vivo, we transplanted CD45.2\(^{+}\) BM cells from Cited2\(^{−/−}\) and control mice (with or without WT CD45.1\(^{+}\) BM competitors) into irradiated congenic CD45.1\(^{+}\) recipients. Without CD45.1\(^{+}\) BM competitors, Cited2\(^{−/−}\) BM cells did not rescue recipient mice from lethal irradiation (data not shown). Furthermore, CD45.2\(^{+}\) Cited2\(^{−/−}\) BM cells transplanted with CD45.1\(^{+}\) BM competitor cells did not contribute to multilineage hematopoiesis (Figure 3C). Immunophenotypic analysis of Cited2\(^{−/−}\) BM revealed a near complete loss of cells in the LSK compartment (Figure 3D) that contains LT-HSCs, ST-HSCs, and LMPPs. The frequency of Lin\(^{−}\)Sca-1\(^{−}\)c-Kit\(^{+}\) (LK) myeloid progenitor cells was also profoundly decreased in Cited2\(^{−/−}\) mice. To exclude the effects of plpC-induced Cre-mediated toxicity on hematopoietic stem and progenitor cells (HSPCs), we compared the immunophenotypic and functional properties of HSPCs from Cited2\(^{−/−}\) Mx1-Cre and Cited2\(^{−/−}\) mice and found no apparent differences (Figures S2A–S2D). These data indicate that plpC-induced Cre activity does not phenocopy Cited2 deletion in HSPCs.

**Figure 1. Conditional Deletion of Cited2 Results in Multilineage Bone Marrow Failure**

(A) Relative expression of Cited2 mRNA in LT-HSC, ST-HSC, and LMPP populations sorted from WT C57BL/6J mice. Data are mean ± SEM (n = 3).

(B) Cited2\(^{−/−}\) Mx1-Cre and Cited2\(^{−/−}\) mice received six injections of plpC on alternate days and analyzed 5 days after the last injection.

(C) Relative expression of Cited2 mRNA in total BM cells from Cited2\(^{−/−}\) and control mice (mean ± SEM; n = 3).

(D) Total number of BM nucleated cells obtained from two tibias and two femurs of Cited2\(^{−/−}\) and control mice. The results are presented as mean number of cells ± SD (n = 5). *p < 0.0001.

(E) Top and middle: Frequencies of B-lymphoid and myeloid cells, respectively, in BM from Cited2\(^{−/−}\) and control mice. Bottom: FACs plot showing CD4 and CD8 staining in thymi from Cited2\(^{−/−}\) and control mice. Data are shown as mean frequency ± SD (n = 3).
was markedly increased in cultured LSK cells and demonstrated that the rate of apoptosis suggests a survival defect. To test this, we deleted system, we mixed CD45.2+ BM cells from untreated caused by pins the multilineage BM failure observed in cells (Figure S2E). Thus, decreased survival of LSK cells under-

**Cited2 Functions in a Cell-Autonomous Manner in HSCs**

To independently examine whether loss of Cited2Δ/Δ HSCs is caused by Cited2 deletion specifically in the hematopoietic system, we mixed CD45.2+ BM cells from untreated Cited2fl/fl Mx1-Cre or Cited2fl/fl mice with CD45.1+ WT BM competitor cells and transplanted them into irradiated recipients. Eight weeks after transplantation, the mice received pIpC and five days after the last dose the percentage of the donor-derived CD45.2+ cells was analyzed in the BM. The percentage of CD45.2+ Cited2Δ/Δ cells in LSK and LK compartments was significantly reduced compared to CD45.2+ Cited2fl/fl cells (Figure 3E). These data indicate a cell-autonomous requirement for Cited2 in HSC maintenance.

**CITED2 Is a Regulator of Primitive Hematopoietic Cell Function in Human Cord Blood**

The high evolutionary conservation of Cited2 in mammals (Bhat-tacharya et al., 1999) suggests a conserved role for Cited2 in HSC function. We generated a lentivirus expressing short-hairpin RNA (shRNA) targeting human CITED2 (Figures S3A–S3D) and performed assays to enumerate LTC-ICs, the most primitive human progenitors assessable in vitro. CB CD34+ cells transduced with shRNA and control lentiviruses were co-cultured on stromal cells. CITED2 knockdown in CD34+ cells led to a severe reduction in cellularity over time, compared to CD34+ cells transduced with a control lentivirus (Figure S3E). Furthermore, CITED2 knockdown in CD34+ cells strikingly impaired primitive hematopoietic cell activity, as judged by LTC-IC assays (Figure 3F). Thus, our data indicate that CITED2 is a conserved regulator of primitive hematopoietic cell function in mammals. Furthermore, with this Mx1-Cre-independent model system, we corroborate the data obtained in our conditional mouse model.

**Intact Ink4a/Arf and Trp53 Are Required for the Loss of Cited2Δ/Δ HSCs**

We previously showed that Cited2 null MEFs senesce prematurely and have increased levels of p16ink4a and p19arf (Kranck et al., 2003), whereas ectopic expression of Cited2 represses p16ink4a and p19arf, enhancing MEF proliferation. Deletion of Ink4a/Arf or Trp53 (encoding p53, a downstream target of p19arf), rescued defective proliferation in Cited2fl/fl MEFs (Figure S4A; Kranck et al., 2003). Ink4a/Arf and Trp53 are essential in maintaining HSC function (Akala et al., 2008), so we hypothesized their involvement in the loss of Cited2Δ/Δ HSCs. Consistent with this, Cited2 deletion in LSK cells resulted in an increased expression of p19arf and p53 proteins and a p53 target gene Cdkn1a (Figures S4B–S4D). Next, we generated Cited2fl/fl Mx1-Cre Trp53+/−, Cited2fl/fl Mx1-Cre Trp53−/−, Cited2fl/fl Mx1-Cre Ink4a/Arf+/−, and control mice and treated them with pIpC. Q-PCR confirmed that Cited2 was not expressed in Cited2Δ/Δ BM cells, regardless of Ink4a/Arf and Trp53 status (Figure S4E). Deletion of one Ink4a/Arf allele or one or two alleles of
Trp53 restored total BM cellularity in Cited2Δ/Δ mice to the levels observed in Cited2fl/fl control mice (Figures 4A and 1D). Ablation of one allele of Trp53 also rescued B cell and myeloid development in Cited2Δ/Δ BM (Figure 4B). Furthermore, deletion of one allele of Ink4a/Arf or one or two alleles of Trp53 restored BM Cited2Δ/Δ LSK cells (Figure 4C). BM cells from Cited2Δ/Δ Trp53+/Δ, Cited2Δ/Δ Trp53+/−, and Cited2Δ/Δ Ink4a/Arf Δ/Δ, but not Cited2Δ/Δ mice efficiently generated multilineage colonies in methylcellulose (Figure 4D). After confirming that Cited2Δ/Δ Trp53+/Δ, Cited2Δ/Δ Trp53+/−, and Cited2Δ/Δ Ink4a/Arf Δ/Δ cells from primary colonies lacked Cited2 expression, we demonstrated efficient generation of secondary colonies (data not shown).

To examine whether HSCs lacking both Cited2 and Trp53 have long-term repopulating capacity, we transplanted Cited2Δ/Δ Trp53+/Δ, Cited2Δ/Δ Trp53+/−, and Cited2Δ/Δ Trp53−/− total BM cells (mixed with WT support BM cells) into irradiated recipients and analyzed peripheral blood (PB) 16 weeks after transplantation. Cited2Δ/Δ Trp53+/Δ BM cells failed to repopulate recipients (Figure 4E), whereas BM cells lacking both Cited2 and Trp53 repopulated recipients to a similar extent as those lacking Trp53 with intact Cited2. To corroborate this, we transplanted BM cells from untreated Cited2Δ/Δ Mx1-Cre Trp53+/Δ and Cited2Δ/Δ Mx1-Cre Trp53−/− mice into irradiated recipients (Figure 4F). After reconstitution, the recipients were treated with plpC and analyzed 8 weeks after administration of the last dose. We measured the percentage of donor cell chimerism in PB nucleated cells or myeloid cells of recipients by using lacZ as a marker of Cited2-deficient cells. Whereas Cited2Δ/Δ Trp53+/Δ cells failed to sustain hematopoiesis, those lacking Cited2 and one allele of Trp53 showed significant donor-derived contribution (Figure 4G). Together, these data provide genetic evidence that the loss of HSCs in Cited2Δ/Δ mice is, at least in part, mediated by Ink4a/Arf and Trp53.

**DISCUSSION**

In this report, we investigate the requirement for Cited2 in adult HSCs maintenance and committed hematopoietic lineages. By using an inducible conditional knockout approach in adult mice, we demonstrate that Cited2 deletion results in an acute loss of HSCs, at least in part via apoptosis, subsequently causing multilineage BM failure. Specific deletion of Cited2 within the hematopoietic system demonstrates a cell-autonomous requirement for Cited2 in maintaining adult HSC integrity, whereas deleting Cited2 in committed lymphoid and myeloid lineages has no impact. Furthermore, CITED2 knocked down in human CD34+CB reveals a conserved requirement for Cited2 in HSC maintenance. Together, our data provide evidence that Cited2 functions in a cell-autonomous manner to maintain HSCs.

Genetic evidence indicates that the tumor suppressors Ink4a/Arf and Trp53 regulate multiple HSC fate decisions (Akala et al., 2008; Liu et al., 2009; Oguro et al., 2006). One function of p19Arf is to stabilize p53 (Pomerantz et al., 1998), and the activation of the p19Arf-p53 pathway results in loss of HSCs (Park et al., 2003). We showed that loss of Cited2 increased p19Arf and p53 expression in the LSK compartment. Based on this observation, we used a genetic rescue approach to test whether Ink4a/Arf and Trp53 are required for loss of HSCs lacking Cited2. Our results demonstrated that deletion of Ink4a/Arf or Trp53 restored functionality of HSCs lacking Cited2, implying that Cited2 maintains HSCs,
Figure 4. Genetic Deletion of Trp53 or Ink4a/Arf Restores HSC Functions and Rescues Bone Marrow Failure in Cited2 Δ/Δ Mice

Mice of indicated genotypes were treated with pIpC.
(A) Total BM cellularity from two tibias and two femurs. The results are presented as mean number of cells ± SD (n = 3 per genotype). *p < 0.002 versus remaining genotypes.
(B) Graphs show total number of BM CD19+B220+ cells (B cells) and Mac-1+Gr-1+ cells (myeloid cells) in two tibias and two femurs per mouse. Mean values ± SD (n = 4). *p < 0.005 versus remaining genotypes.
(C) Frequencies of the BM LSK cells from mice of indicated genotypes. FACS plots are representative of three independent experiments.
(D) CFC assay. Nucleated BM cells were plated in methylcellulose medium. Cultures were assessed on day 10 for granulocyte (CFC-G), macrophage (CFC-M), granulocyte-macrophage (CFC-GM), erythroid (E), and mixed (Mix) colony formation. The data are representative of three independent experiments and are shown as the mean ± SD (n = 2 mice per genotype).
(E) Contribution of donor cells from Cited2 Δ/Δ Trp53 Δ/Δ, Cited2 Δ/Δ Trp53 Δ−/−, and Cited2 Δ/Δ Trp53 Δ−/Δ mice to the myeloid compartment of PB 16 weeks after transplantation. BM cells from mice of the indicated genotypes were mixed with support WT BM cells and transplanted into irradiated recipients. The graph shows the mean (±SD) percentage of CD45.2+ cells in myeloid compartment of recipient mice (n = 3 per group). *p < 0.0003 versus remaining genotypes.
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at least in part, via Ink4a/Arf and Trp53. These data support the postulate that deletion of Cited2 in HSCs results in activation of the p19ARF-p53 pathway and thereby leads to their loss.

It is of interest to relate Cited2 to other critical regulators of HSC maintenance. Cited2 is required for Bmi-1 expression in MEFs (Krac et al., 2003) and myeloid progenitors (Chen et al., 2007). Bmi-1 maintains HSCs (Lessard and Sauvageau, 2003; Park et al., 2003) and directly represses Ink4a/Arf (Bracken et al., 2007), whereas deletion of Ink4a/Arf (Oguro et al., 2006) or Trp53 (Akala et al., 2008) restores Bmi-1+/− HSC function. Genetic evidence indicates distinct roles for Bmi-1 and Cited2 in HSC fate decisions. Whereas Bmi-1 mediates HSC self-renewal, our results are compatible with a requirement for Cited2 in HSC maintenance. In agreement with this, acute Cited2 deletion in HSCs does not affect the expression of Bmi-1 (data not shown), suggesting that downregulation of Bmi-1 expression is not responsible for the loss of Cited2+/Δ HSCs. However, this does not exclude the possibility that Cited2 controls Bmi-1 in other contexts in HSCs. Conditional deletion of Cited2 generates a stem cell phenotype reminiscent of conditional inactivation of Tel/Etv6 and Mcl-1 (Hock et al., 2004; Opferman et al., 2005). Like Tel/Etv6 (Hock et al., 2004), Cited2 appears to be selectively required for HSC maintenance, but dispensable for mature lineages. Mcl-1, however, also plays critical roles in mature T and B cell survival (Opferman et al., 2003), revealing a broader spectrum of hematopoietic function than Cited2. Conditional deletion of Apc and combined deficiency of c-Myc and N-Myc (but not ablation of N-Myc alone) results in loss of HSCs (Laurent et al., 2008; Qian et al., 2008). Although the expression of Apc and c-Myc is unaltered in Cited2-deficient HSCs, the expression of N-myc is decreased (data not shown). Although this observation alone does not explain the loss of Cited2-deficient HSCs, N-Myc may mediate some functions of Cited2 in HSCs. Finally, Cited2 binds p300 and its paralog CBP (Bhattacharya et al., 1999). Although CBP is essential for adult HSC maintenance, p300 appears dispensable for HSC maintenance but required for multilineage hematopoietic differentiation (Kung et al., 2000; Rebel et al., 2002). It will be of interest to clarify the roles of CBP-Cited2 and p300-Cited2 interactions in adult HSC maintenance and hematopoiesis, and the relationship between Cited2 and other critical stem cell regulators remains an open question meriting future investigation.

In conclusion, we provide genetic evidence that Cited2 is an essential and cell-autonomous regulator of adult mammalian HSC maintenance. Our data, together with the sufficiency of Cited2 to maintain undifferentiated embryonic stem cells (Pritsker et al., 2006), suggest that it is a critical master regulator of stem cell fate. Understanding Cited2 functions at the molecular level will offer insights into the similarities and differences in the transcriptional circuitry of embryonic and somatic stem cells.

**EXPERIMENTAL PROCEDURES**

**Mice**

We backcrossed Cited2+/+ and Cited2+/− mice (Bamforth et al., 2001; MacDonald et al., 2008) to C57BL/6J for ten generations to generate cosogenic mice. Mx1-Cre, Cd19-Cre, and LysM-Cre mice were purchased from the Jackson Laboratory. Cd4-Cre mice were purchased from Taconic. Ink4a/Arf+/− and Trp53−/− mice were obtained from B. Hassan and M. van Lohuizen, respectively. All experiments on animals were performed under UK Home Office authorization.

**Administration of pIpC**

8- to 12-week-old mice received five to six intraperitoneal injections of pIpC (GE Healthcare; 0.2–0.3 mg per dose) every alternate day. Deletion efficiency was determined by Q-PCR or lacZ expression analysis (via a FluorReporter lacZ Flow Cytometry Kit, Invitrogen).

**Murine CAFC Assay**

Stromal layers were prepared from the BM of C57BL/6J mice, irradiated at 15 Gy, and subcultured in 96-well flat-bottom plates at a density of 2 × 10⁴ cells per well. After 1 to 7 days, cultures were seeded at 2-fold dilutions (2.9 × 10³–18,125 per well) of nucleated BM cells from each genotype. CAFCs were scored at week 5.

**CFC Assays**

H4434 and M3434 media (StemCell Technologies) were used to enumerate human and mouse colony-forming cells, respectively. Two replicates were used per group in each experiment. Colonies were tallied at day 10–14.

**Q-PCR**

RNA extraction and Q-PCR reactions were performed as previously described (Mansson et al., 2007). For specific TaqMan Assays-on-Demand probes used, see Supplemental Experimental Procedures. Reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR System in normal mode for 50 cycles. All experiments were performed in triplicate. Differences in input cDNA were normalized with a combination of Hprt, Gapdh, Actb, Ubc, and B2m expression with qBase 1.3.5 software (http://medgen.ugent.be/qbase/).

**Lentiviral Transductions**

CITED2 shRNA was subcloned from the pLKO.1 puro vector (Open Biosystems) into the pLKO.1 GFP vector (gift from J. Larsson). Lentiviral Transductions (Hock et al., 2004) were scored at week 5. Cited2 shRNA was subcloned from the pLKO.1 puro vector (Open Biosystems) into the pLKO.1 GFP vector (gift from J. Larsson). Lentivirus production and transduction of human CD34+ CB cells are described in Supplemental Experimental Procedures.

**Human Long-Term Cultures on Stroma and LTC-IC Assays**

CB CD34+ cells (StemCell Technologies) were isolated by MiniMACS (Miltenyi Biotec) selection. After transduction, 3 × 10⁴ cells were cultured on M5 stromal cells in Long-Term Culture medium (see Supplemental Experimental Procedures). Cultures were demidepopulated weekly for analysis. LTC-IC numbers were enumerated by overlaying M5 stromal cocultures at week 5 with H4434 medium, followed by counting colonies 2 weeks later.

**FACS**

All samples were analyzed on a CyAn ADP flow cytometer (Dako). Sorts were performed on FACSAriaIIu (BD) or MoFlow (Dako) cell sorters. Antibodies are described in Supplemental Experimental Procedures.

**Competitive Repopulation Assay**

CD45.2+ test donor BM cells were mixed with CD45.1+ competitor BM cells in a 1:1 ratio and injected intravenously into lethally irradiated (9 Gy) B6.SJL CD45.1+ recipients. The competitor cell number was 5 × 10⁶ cells in all experiments.

**Statistical Analysis**

Statistical significance was determined via two-tailed Student’s t tests assuming unequal variance.

(F) Schematic of experimental design.
(G) Contribution of donor cells of the indicated genotypes to PB. Percentage of lacZ+ donor cells was analyzed by flow cytometry in total PB mononuclear compartment and myeloid (Mac-1+Gr-1+) compartment of recipients (n = 5 per group). *p < 0.002; **p < 0.0008.
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SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00574-8.

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REFERENCES


**Supplemental Data**

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**Supplemental Experimental Procedures:**

**Quantitative RT-PCR probes:**

TaqMan Assays-on-Demand probes used were: Cited2: Mm00516121_m1; Hprt: Mm00446968_m1; B2m: Mm00437762_m1; Gapdh: Mm99999915_g1; Actb: Mm00607939_s1; Ubc: Mm01201237_m1; Bmi-1: Mm00776122_gH; Mel18: Mm00464756_m1; Apc: Mm00545877_m1; c-Myc: Mm00487803_m1; N-Myc: Mm00476449_m1; Cdkn1a: Mm00432448_m1; CITED2: Hs00366696_m1; ACTB: Hs99999903_m1; B2M: Hs00187842_m1; GAPDH: Hs99999905_m1; HPRT1: Hs99999909_m1.

**Lentiviral transductions**

The CITED2 RNA hairpin was NdeI-SpeI subcloned from the pLKO.1 puro vector (Open Biosystems, Oligo ID: TRCN0000015654) into the pLKO.1 GFP vector (gift from J. Larsson, Lund University). The sequence of scrambled oligonucleotide used to generate shRNA was TTCTCCGAACGTGTCACGTT. 2.5x10^6 293T Human Embryonic Kidney cells were transfected with 3 µg pCMV Δ8.91, 0.7 µg VSV-G and 3 µg of either pLKO.1 scrambled shRNA (gift from J. Larsson, Lund University), pLKO.1 CITED2 shRNA, IRES-GFP (control) or CITED2-IRES-GFP vectors. After 24 hours medium was changed to HPGM (Cambrex, Verviers, Belgium) and after 12 hours supernatant containing lentiviral particles was harvested and stored at -80°C. Cord blood CD34^+ cells were
isolated with MiniMACS columns and subsequently cultured in HPGM supplemented with human c-KIT ligand, FLT3 ligand (both from Amgen, USA) and TPO (100 ng/ml each) for 16 hours at 37°C and 5% CO₂. Cord blood CD34⁺ cells were transduced in 2 consecutive rounds of 8 to 12 hours with lentiviral supernatant supplemented with c-Kit ligand/FLT3 ligand/TPO (100 ng/ml each) and Polybrene (4 µg/ml). Transduction efficiency was measured by FACS analysis and knock-down was assessed by Q-PCR.

**FACS**

Antibodies used for cell surface staining were CD19 (1D3), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b/Mac-1 (M1/70), CD4 (H129.9), CD8a (53-6.7), SCA-1 (E13-161.7), c-KIT (2B8), CD45.2 (104), CD34 (RAM34), FLT3 (AZF10.1) (all from BD Pharmingen). Polyclonal goat-anti-rat Tricolor was purchased from Caltag.

**Human cord blood cell culture conditions**

Long Term Culture medium (αMEM supplemented with 12.5% FCS, 12.5% Horse serum (Gibco), 1% penicillin and streptomycin, 200 mM Glutamine, 57.2 µM β-mercaptoethanol (Sigma) and 1 µM hydrocortisone (StemCell Technologies)) was used to co-culture cord blood cells with MS5 stromal cells. Cultures were kept at 37°C and 5% CO₂.
Figure S1. Deletion of Cited2 in adult mice using Mx1-Cre results in premature lethality which can be rescued by wild-type bone marrow.

(A) LacZ expression in bone marrow cells from Cited2Δ/Δ and control mice. As Cre-mediated deletion of exon 2 brings the lacZ cassette under control of the endogenous Cited2 promoter, the analysis of lacZ expression allows the confirmation of deletion of conditional alleles of Cited2.

(B) Kaplan-Meier survival curve for cohorts of 10 mice of each genotype injected with pIpC. Mice were treated with pIpC according to schematic in Figure 1B.

(C) Survival curve of Cited2Δ/Δ mice transplanted with wild-type bone marrow. Wild-type bone marrow cells were transplanted into lethally irradiated Cited2fl/fl Mx1-Cre and Cited2fl/fl control mice (n=9 for each genotype). 12 weeks after transplantation recipient mice were treated with 300 μg pIpC as indicated in Figure 1B. The graph shows the percentage of surviving mice.
Figure S2. (A-D) Properties of stem and progenitor cells from Cited2^{+/+} Mx1-Cre and Cited2^{fl/fl} mice. (E) Defective survival of LSK cells lacking Cited2.

Cited2^{+/+} Mx1-Cre and Cited2^{fl/fl} mice were treated with pIpC according to the schematic in Figure 1B and BM was analysed 5 days after the last pIpC administration.

(A) BM cellularity in pIpC-treated Cited2^{+/+} Mx1-Cre and Cited2^{fl/fl} mice (n=3±SD).

(B) Immunophenotypic analysis of the BM LSK compartment in pIpC-treated Cited2^{+/+} Mx1-Cre and Cited2^{fl/fl} mice (n=3±SD).

(C) CFC assays with BM cells obtained from pIpC-treated Cited2^{+/+} Mx1-Cre and Cited2^{fl/fl} mice (n=3±SD).

(D) Competitive repopulation assay. BM cells from pIpC-treated Cited2^{+/+} Mx1-Cre and Cited2^{fl/fl} mice were mixed in a 1:1 ratio with competitor CD45.1+ BM cells and transplanted into lethally irradiated CD45.1+ recipients. Reconstitution was analysed 16 weeks after transplantation. Data are shown as percentage of CD45.2+ cells in peripheral blood (n=6 mice per group).

(E) We bred Cited2 conditional knockout mice with Rosa26^{CreERT/+} mice, in which efficient deletion of floxed genes is induced by tamoxifen. We generated Cited2^{fl/fl} Rosa26^{CreERT/+} (CKO) and Cited2^{+/+} Rosa26^{CreERT/+} (WT) mice and sorted bone-marrow LSK populations from these mice. The cells were cultured in the presence of 1 μM tamoxifen and apoptosis was detected by Annexin-V staining. Graph shows percentage of Annexin-V+ cells of indicated genotypes.
Figure S3. Human cord blood CD34+ cells with CITED2 knockdown fail to sustain hematopoiesis in vitro.

(A) 293T HEK cells were infected with control scrambled and shRNA lentiviruses. The efficiency of knockdown was determined by western blotting. Top panel: western blot of total cell lysates was probed with an anti-CITED2 monoclonal antibody (JA22, Abcam, Cambridge, UK). Bottom panel: western blot was re-probed with an anti-β-actin (C4, MP Biomedicals, UK) monoclonal antibody to confirm equal loading.

(B) Relative expression of CITED2 mRNA in CD34+ cord blood cells infected with lentiviruses expressing CITED2 shRNA and a control lentivirus (scrambled shRNA). The data are presented as the mean ± SEM of triplicate assays in which CITED2 expression was normalised to the expression of GAPDH.

(C) CFC assay. Human CD34+ CB cells were infected with shRNA and control lentiviruses and cultured in methylcellulose for 2 weeks. The graph shows representative data (mean colony number ± SD) of 3 independent experiments performed in duplicate.

(D) CD34+ cord blood cells were transduced with CITED2-IRES-GFP (expressing a human full-length CITED2 driven by human EF1α promoter) and control IRES-GFP lentiviruses. Transduced GFP+ cells were then superinfected with shRNA and scrambled control lentiviruses and cultured in methylcellulose for two weeks. The graph shows the mean numbers of colonies (± SD) and is a representative of two independent experiments performed in duplicate.

(E) MS5 co-cultures of human cord blood CD34+ cells transduced with shRNA and control lentiviruses. 30,000 cells were plated in MS5-precoated T25 culture flasks in 5 ml Long-Term Culture medium. Half of the cultures were harvested weekly and fresh medium was added to the culture. Cumulative cell numbers for a representative growth curve are shown (n=4).
Figure S4. Cited2 functions genetically upstream of Ink4a/Arf and Trp53.

(A) MEFs obtained from embryos at 13.5 dpc were serially passaged according to the 3T3 protocol. Cell proliferation is shown as a plot of cumulative population doubling (CPD) versus passage number. Genotypes are indicated.

(B and C) BM LSK populations were sorted from Cited2\(^{+/+}\) Rosa26\(^{CreERT/+}\) (WT) and Cited2\(^{fl/fl}\) Rosa26\(^{CreERT/+}\) (CKO) mice. The cells were cultured in StemSpan medium in the presence of SCF, FLT3 ligand and TPO (100 ng/ml each) and 0.5 \(\mu\)M tamoxifen for 2-3 days. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X100 and the expression of p19 Arf and p53 was detected using Ab80 (Abcam) and CM5 (Novocastra) antibodies, respectively. Data are representative of 2 experiments.

(B) Percentage of p19 Arf-expressing cells cultured in the presence of tamoxifen for 2 days.

(C) Percentage of p53-positive cells cultured with tamoxifen for 3 days.

(D) Expression of Cdkn1a in LSK cells lacking Cited2. LSK cells were sorted from Cited2\(^{fl/fl}\) (WT) and Cited2\(^{fl/fl}\) Mx1-Cre (CKO) mice. The cells were cultured in the presence of IFN-\(\alpha\) for 36 hours to induce Mx1-Cre-mediated gene deletion. Q-PCR reactions were performed to determine the relative expression of Cdkn1a, normalized to the expression of Hprt and Gapdh.

(E) Relative expression of Cited2 mRNA in total bone marrow samples obtained from mice used for experiments in Figure 4A, 4C and 4D. The data are presented as the mean.
± SEM of triplicate assays in which *Cited2* expression was normalised to the expression of *Hprt*. Expression levels in *Cited2*<sup>+/−</sup> *Mx1-Cre Trp53<sup>−/−</sup>* mice were set to 1. Mouse genotypes are indicated.