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.

RESULTS

Cited2 Is Essential for Sustaining Multilineage Hematopoiesis

Cited2 expression analysis indicated that it is highly expressed in long-term HSCs (LT-HSCs; Linâ–¬Sca-1â–¬c-kit+(LSK)CD34â–¬Flt3â–¬ cells), less abundantly in short-term HSCs (ST-HSCs; LSKCD34â–¬ Flt3â–¬ cells), and profoundly downregulated in lymphoid-primed multipotent progenitors (LMPPs; LSKCD34â–¬Flt3â–¬ cells) (Figure 1A). To investigate a functional requirement for Cited2 in adult hematopoiesis, we generated Cited2∗Mx1-Cre conditional knockout mice (MacDonald et al., 2008), in which treatment with poly(I)-poly(C) (pIpC) induces efficient gene deletion (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.
Critical Role of Cited2 in Adult HSC Maintenance

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) Cited2fl/fl Mx1-Cre and Cited2cre/cre 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 Cited2cre/cre and control mice (mean ± SEM; n = 3).

(D) Total number of BM nucleated cells obtained from two tibias and two femurs of Cited2cre/cre 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 Cited2cre/cre and control mice. Bottom: FACS plot showing CD4 and CD8 staining in thymi from Cited2cre/cre and control mice. Data are shown as mean frequency ± SD (n = 3).

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, Cited2cre/cre BM cells failed to generate colonies in methylcellulose (Figure 3A). To evaluate HSC activity in vivo, we performed limiting dilution cobblestone area-forming cell (CAFC) assays and found that Cited2cre/cre BM cells completely lacked CAFCs (Figure 3B). To assess HSC activity in vivo, we transplanted CD45.2+ BM cells from Cited2cre/cre and Cited2cre/cre control mice (with or without WT CD45.1+ BM competitors) into irradiated congenic CD45.1+ recipients. Without CD45.1+ BM competitors, Cited2cre/cre BM cells did not rescue recipient mice from lethal irradiation (data not shown). Furthermore, CD45.2+ Cited2cre/cre BM cells transplanted with CD45.1+ BM competitor cells did not contribute to multilineage hematopoiesis (Figure 3C). Immunophenotypic analysis of Cited2cre/cre 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 Cited2cre/cre 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 Cited2cre/cre, Mx1-Cre and Cited2cre/cre mice and found no apparent differences (Figures S2A−S2D). These data indicate that plpC-induced Cre activity does not phenocopy Cited2 deletion in HSPCs.
The rapid kinetics of HSC loss upon acute deletion of Cited2 suggest a survival defect. To test this, we deleted Cited2 in cultured LSK cells and demonstrated that the rate of apoptosis was markedly increased in Cited2Δ/Δ cells, as compared to WT cells (Figure S2E). Thus, decreased survival of LSK cells underpins the multilineage BM failure observed in Cited2Δ/Δ mice.

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 Cited2Δ/Δ 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 mice (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 cocultured 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 (Krnac 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 Cited2Δ/Δ MEFs (Figure S4A; Krnac 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 Cited2Δ/Δ Mx1-Cre Trp53+/−, Cited2Δ/Δ 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
Critical Role of Cited2 in Adult HSC Maintenance

**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 knockdown in human CD34+CD38- cells 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 suppressor Inka/Arf and Trp53 regulate multiple HSC fate decisions (Akali 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 Inka/Arf and Trp53 are required for loss of HSCs lacking Cited2. Our results demonstrated that deletion of Inka/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 tibiae 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 tibiae 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 Mx1-Cre or Cited2 Δ/Δ Trp53 Δ/Δ Mx1-Cre 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.
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 (Kracz et al., 2003) and myeloid progenitors (Chen et al., 2007). Bmi-1 maintains HSCs (Lessa 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 (Akaia 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 survival. 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+/− 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 (Laurenti 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 plpC
8- to 12-week-old mice received five to six intraperitoneal injections of plpC (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 FluoReporter 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 × 10^5 cells per well. After 1 to 7 days, cultures were seeded at 2-fold dilutions (2.9 × 10^0–18, 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). 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 × 10^5 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 FACSARiaII (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 × 10^5 cells in all experiments.

Statistical Analysis
Statistical significance was determined via two-tailed Student’s t tests assuming unequal variance.
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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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; e-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 37ºC 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 37ºC 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 Cited2^Δ/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 Cited2^Δ/Δ mice transplanted with wild-type bone marrow. Wild-type bone marrow cells were transplanted into lethally irradiated Cited2^fl/fl Mx1-Cre and Cited2^fl/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<sup>+/+</sup> Mx1-Cre* and *Cited2<sup>fl/fl</sup>* mice. (E) Defective survival of LSK cells lacking *Cited2*.

*Cited2<sup>+/+</sup> Mx1-Cre* and *Cited2<sup>fl/fl</sup>* 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<sup>+/+</sup> Mx1-Cre* and *Cited2<sup>fl/fl</sup>* mice (n=3±SD).

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

(C) CFC assays with BM cells obtained from pIpC-treated *Cited2<sup>+/+</sup> Mx1-Cre* and *Cited2<sup>fl/fl</sup>* mice (n=3±SD).

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

(E) We bred *Cited2* conditional knockout mice with *Rosa26<sup>CreERT<sup>+/</sup></sup>* mice, in which efficient deletion of floxed genes is induced by tamoxifen. We generated *Cited2<sup>0/0</sup> Rosa26<sup>CreERT<sup>+/</sup></sup>* (CKO) and *Cited2<sup>+/+</sup> Rosa26<sup>CreERT<sup>+/</sup></sup>* (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<sup>+</sup> 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<sup>+/+ </sup>Rosa26<sup>CreERT/+ </sup>(WT) and Cited2<sup>fl/fl </sup>Rosa26<sup>CreERT/+ </sup>(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 μM 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<sup>fl/fl </sup>(WT) and Cited2<sup>fl/fl Mx1-Cre </sup>(CKO) mice. The cells were cultured in the presence of IFN-α for 36 hours to induce Mx1-Cre-mediated gene deletion. Q-PCR reactions were performed to determine the relative expression of Cdkn1a (n=3±SD, *p<0.005). Cdkn1a expression was normalised 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> Mxl-Cre Trp53<sup>+/−</sup> mice were set to 1. Mouse genotypes are indicated.