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Histone Acetyltransferase Cofactor TRRAP Is Essential for Maintaining the Hematopoietic Stem/Progenitor Cell Pool

Joanna I. Loizou,† Gabriele Oser,‡ Vivek Shukla,*§ Carla Sawan,* Rabih Murr,*¶ Zhao-Qi Wang,‖ Andreas Trumpp,# and Zdenko Herceg‡*

The pool of hematopoietic stem/progenitor cells, which provide life-long reconstitution of all hematopoietic lineages, is tightly controlled and regulated by self-renewal and apoptosis. Histone modifiers and chromatin states are believed to govern establishment, maintenance, and propagation of distinct patterns of gene expression in stem cells, however the underlying mechanism remains poorly understood. In this study, we identified a role for the histone acetyltransferase cofactor TRRAP in the maintenance of hematopoietic stem/progenitor cells. Conditional deletion of the TRRAP gene in mice resulted in ablation of bone marrow and increased lethality. This was due to the depletion of early hematopoietic progenitors, including hematopoietic stem cells, via a cell-autonomous mechanism. Analysis of purified bone marrow progenitors revealed that these defects are associated with induction of p53-independent apoptosis and deregulation of Myc transcription factors. Together, this study has identified a critical role for TRRAP in the mechanism that maintains hematopoietic stem cells and hematopoietic system, and underscores the importance of TRRAP and histone modifications in tissue homeostasis.

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1 J.I.L., G.O., V.S., C.S., and R.M. performed all the experiments. Z.H. and J.I.L. coordinated the project and wrote the manuscript. Z.Q.W. and A.T. gave conceptual assistance and contributed with planning the experiments. All authors discussed the results and manuscript text.

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3 Abbreviations used in this paper: HSC, hematopoietic stem cell; HAT, histone acetyltransferase; TRRAP, TR(ansformation/tRanscription domain-Associated Protein; p300/CBP-associated factor complex; pIpC, poly(inosinic)-poly(cytidylic) acid; WT, wild type; BM, bone marrow; CKO, conditional knockout; FISH, fluorescence in situ hybridization.

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performing PCR on DNA extracted from tail tissue. For were I3.0 and I3.1 to detect the floxed and wild-type (WT) alleles and G7.0

solved on 2% agarose gels. Hprt or Gapdh mRNA abundance was used as

the c-Myc oncoprotein has been shown to directly interact with Trrap/ HAT complexes and this interaction is required for its transcriptional and transforming activities (17, 20, 21). c-Myc and N-Myc regulate a variety of genes involved in the control of cell proliferation and cell death (22) and their disruption lead to compromised maintenance of HSCs and hematopoietic function (23, 24), although the precise molecular mechanism remains to be elucidated. Furthermore, a recent large-scale RNAi screen in mouse embryonic stem cells revealed that Tip60/p400 HAT is necessary for the maintenance of embryonic stem cell identity (25), although the role of HATs and histone acetylation in establishing and maintaining distinct chromatin signature required for unique stem cell properties remains largely unknown (26).

In the present study, we generated conditional knockout (CKO) mice that allow for inactivation of the Trrap gene in a spatiotemporal manner in vivo after expression of Cre-recombinase, and studied the function Trrp/HAT and histone acetylation in vivo in adult mice and in adult tissue stem cells. This study has revealed an essential function of Trrap in the maintenance of hematopoietic stem/progenitor cells and homeostasis of the hematopoietic system.

Materials and Methods

Generation of Trrap conditional knockout mice

Embryonic stem cells carrying the Trrap “conditional” (floxed); Trrap<sup>wild-type/floxed</sup>, Trrap<sup>-/-</sup>) allele, generated using the Cre-loxP system (27), were injected into blastocysts for the production of chimeric and Trrap “floxed” (Trrap<sup>-/-</sup>) mice. Trrap<sup>-/-</sup> mice were intercrossed with Trrap<sup>+/+</sup> mice (27) to obtain Trrap<sup>+/+</sup> and Trrap<sup>-/-</sup> mice. To inactivate the Trrap “conditional” allele in tissues and organs in an inducible manner, Trrap<sup>+/+</sup> and Trrap<sup>-/-</sup> mice were crossed to transgenic mice expressing Cre recombinase under the control of the Ms-1 promoter, a (polyinosinic-polyribidylic) acid (pIpC)-inducible promoter (28), to produce Trrap<sup>+/Cre<sup>”Cre</sup> and Trrap<sup>+/Cre</sup> mice. To delete Trrap in vivo, mice harboring the Trrap “floxed” allele and Cre transgene acquired an inactivating mutation of the gene upon treatment with three intra-peritoneal injections of 250 μg pIpC (InvivoGen), each 2 days apart, unless otherwise stated. To generate Trrap conditional knockout mice in a p53 null background Tpr53<sup>+/</sup>/pIpC and Tpr53<sup>−/−</sup>/pIpC mice were interbred to generate Tpr53<sup>−/−</sup>/pIpC, each 2 days apart. To generate Trrap conditional knockout mice in a p53 null background Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice were interbred to generate Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice. To delete Trrap in vivo, mice harboring the Trrap “floxed” allele and Cre transgene acquired an inactivating mutation of the gene upon treatment with three intra-peritoneal injections of 250 μg pIpC (InvivoGen), each 2 days apart, unless otherwise stated. To generate Trrap conditional knockout mice in a p53 null background Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice were interbred to generate Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice. To delete Trrap in vivo, mice harboring the Trrap “floxed” allele and Cre transgene acquired an inactivating mutation of the gene upon treatment with three intra-peritoneal injections of 250 μg pIpC (InvivoGen), each 2 days apart, unless otherwise stated. To generate Trrap conditional knockout mice in a p53 null background Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice were interbred to generate Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice. To delete Trrap in vivo, mice harboring the Trrap “floxed” allele and Cre transgene acquired an inactivating mutation of the gene upon treatment with three intra-peritoneal injections of 250 μg pIpC (InvivoGen), each 2 days apart, unless otherwise stated. To generate Trrap conditional knockout mice in a p53 null background Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice were interbred to generate Tpr53<sup>−/−</sup>/pIpC and Tpr53<sup>+/−</sup>/pIpC mice. To delete Trrap in vivo, mice harboring the Trrap “floxed” allele and Cre transgene acquired an inactivating mutation of the gene upon treatment with three intra-peritoneal injections of 250 μg pIpC (InvivoGen), each 2 days apart, unless otherwise stated.

Genotyping of tissue and mice by PCR and Southern blot

The Trrap, Trrp53, and Mxl1-Cre genotypes of mice were determined by performing PCR on DNA extracted from tail tissue. For Trrp53, the primers used for PCR were Neo19, X6.5, and X7.0. For Trrap, the primers used were I3.0 and I3.1 to detect the floxed and wild-type (WT) alleles and G7.0 and I2.1 to detect WT/floxed and δ alleles. For detection of the Mxl1-Cre transgene, the primers B2.1, B2.2, Cre1, and Cre2 were used. Primer sequences are listed in supplementary Table 1. To detect the Trrap floxed, WT, and δ alleles by Southern blotting, DNA was digested overnight with KpnI, resolved on 1% agarose gel, transferred to nitrocellulose, and probed with a 32P-labeled probe as described previously (27).

RT-PCR

Total RNA from bone marrow (BM), spleen, Lin- cells, thymus, and liver was extracted using the RNAeasy mini kit (Qiagen). Between 70 ng and 300 ng, total RNA was used for reverse transcription using 200 U of M-MLV Reverse Transcriptase (Invitrogen). Two microliters of the reverse transcriptase reaction was subsequently subjected to PCR amplification. Primer sequences are shown in supplementary Table I. PCR products were resolved on 2% agarose gels. Hprt or Gapdh mRNA abundance was used as control.

<sup>4</sup>The online version of this article contains supplementary material.

Western blot analysis

Total protein lysates were prepared from lin-BM cells, resolved by electrophoresis on 10% SDS-PAGE gel, and blotted on nitrocellulose membrane (Bio-Rad). The following Abs were used: rabbit anti-c-Myc (1/500; Santa Cruz Biotechnology), rabbit anti-N-myc (1/200; Santa Cruz Biotechnology), and mouse anti-actin (1/1000; Santa Cruz Biotechnology). Proteins were visualized with HRP-conjugated anti-rabbit IgGs (1/5000; Dako-Cytomation) and anti-mouse IgGs (1/2000; DakoCytomation), followed by use of the ECL chemiluminescence system (Amersham).

Blood analysis

For analysis of peripheral blood elements, ~200 μl of blood was collected retro-orbitally into a glass Pasteur pipette containing ~30 μl heparin. Analysis was performed by a commercial company.

Histological analysis of BM and liver

Femur and liver samples were fixed in 4% buffered formaldehyde overnight, and then in 70% ethanol overnight before embedding in paraffin. Femurs were additionally decalcified before performing histological analyses on 3 μm sections stained with H&E.

Assessment of BM, spleen, and thymus cellularity

BM cells were flushed from the long bones of hindlegs and prepared by standard procedures. Cell suspensions of BM, spleen and thymus were filtered through a nylon mesh filter (40 μm), stained with trypan blue, and counted under a light microscope.

CFU assay

BM was cultured in M3434 Methocult (Stem Cell Technologies) containing insulin, transferrin, SCF, IL-3, IL-6, and erythropoietin for 7 to 10 days at 37°C in an atmosphere of 5% CO2 in air. Resulting colonies were scored according to morphology as directed by the manufacturer using an inverted light microscope.

Purification of lin- BM cells

To purify lineage cells, the BM from several mice was pooled and lin- cells for CD3e, CD11b, CD45/B220, Ly-6G and Ly-6C (Gr-1), and Ter119/erythroid cells (Ly-76) were recovered using a biotin-conjugated mouse lineage panel of Abs (BD Biosciences), as recommended by the manufacturer. Dynabeads (Invitrogen) were then used to exclude biotin bound cells as directed by the manufacturer. The enrichment of lin- cells was confirmed by FACS using a c-kit Ab labeled with FITC.

Analysis of apoptosis, cell cycle profile, and proliferation

For cell cycle profiling lin- BM cells were stained with propidium iodide using the CycleTEST PLUS kit according to the recommendations of the manufacturer (BD Biosciences). To detect apoptotic BM cells we stained lin- cells with Annexin-V-Fluos that detects phosphatidyserine on cell membranes as recommended by the manufacturer (Roche). All FACS analyses were performed using a FACScalibur apparatus equipped with CellQuest software (BD Biosciences).

DNA fluorescence in situ hybridization (FISH)

Lin- cells were spun using a cytospin and slides were fixed and hybridized with a 5'-biotinylated DNA probe (Invitrogen) recognizing the mouse major satellite repeats as described previously (5).

Abs and FACS analysis of hematopoietic compartments

Abs against Gr-1, Ter 119, B220, CD41b, CD4, CD8a, CD11a, and CD18 were purified and conjugated in the laboratory following standard protocols. Abs to CD34, CD135, CD117, Scal, CD43, IgM, CD19, CD71, CD93, CD49d, and CD29 were purchased from eBioscience. Protocols used for FACS analysis of hematopoietic cells were described previously (23).

BM transplantation

In the case where mice carrying at least one wild-type allele were used as the recipients, mice were lethally irradiated with 10 Gy of whole-body irradiation and then reconstituted with 3 × 10<sup>5</sup> BM cells by retro-orbital injection. Trrap-CKO BM cells were extracted from the femurs of mice 6 days post deletion. In the case where mice amenable to deletion of Trrap were used as the recipients, ~4-wk-old mice were transplanted with 3 × 10<sup>6</sup> BM cells by retro-orbital injection and after 2 wk administered with three i.p. injections of 250 μg pIpC, each 2 days apart.
Results

Generation of mice with a floxed allele of Trrap and inactivation of Trrap in adult mice

We have previously shown that homozygous mutation of Trrap in mice induced embryonic lethality (27). To circumvent problems of embryonic lethality and to study the function of Trrap and Trrap/HAT-mediated histone acetylation in vivo in adult mice, we have generated Trrap conditional knockout mice that allow inducible deletion of the gene in a spatiotemporal manner. Mouse embryonic stem cells carrying the Trrap conditional (floxed; Trrap\textsuperscript{flox/flox}) allele generated using the Cre-loxP system (27) were injected into blastocysts for the production of chimera and Trrap floxed (Trrap\textsuperscript{flox/flox}) mice. Trrap\textsuperscript{+/-} mice were either intercrossed or crossed with Trrap\textsuperscript{flox/flox} mice (27) to obtain Trrap\textsuperscript{flox/flox} and Trrap\textsuperscript{flox/flox} mice (Fig. 1A). Although Trrap\textsuperscript{flox/flox} embryos did not survive (data not shown), consistent with our previous report (27), Trrap\textsuperscript{flox/flox} and Trrap\textsuperscript{flox/flox} mice were healthy and phenotypically normal, suggesting that the Trrap floxed allele is functional and that one functional allele of Trrap is sufficient to support normal development and viability.

To inactivate the Trrap conditional allele in tissues in an inducible manner, Trrap\textsuperscript{flox/flox} and Trrap\textsuperscript{flox/flox} mice were crossed to transgenic mice expressing Cre recombinase under the control of the Mx promoter (Mx-Cre) (28) to produce Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-} and Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-} mice (Fig. 1A). For different requirements of the experiments, these mice were then either intercrossed or crossed with Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-}, Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-}, or Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-} to produce different genotypes, all of which were healthy and phenotypically normal. To delete Trrap in vivo, mice harboring the Trrap floxed allele and Cre transgene acquired an inactivating mutation of the gene upon treatment with pIpC (28) (Fig. 1A). The MxI promoter was chosen to drive Cre expression as it allows deletion of the gene in IFN-responsive tissues and hence generates a high level of deletion in hematopoietic tissues, including hematopoietic stem cells (28). Cre-mediated deletion of Trrap was monitored by Southern blotting and PCR analysis of genomic DNA. Efficient deletion of the Trrap gene could be induced by treatment of Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+} and Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+} mice with three injections of pIpC at 2-day intervals. We found that 2 days after the third injection of pIpC, the deletion of Trrap was highly efficient in BM and liver (nearly 100%), slightly less efficient in spleen (80%), whereas deletion in thymus was significantly less efficient (25% deletion) (Fig. 1B, and data not shown). The similar efficiency of deletion of Trrap ‘floxed’ allele was observed from homozygous (Trrap\textsuperscript{flox/flox}) and hemizygous (Trrap\textsuperscript{flox/flox}) mice (data not shown). In agreement with these findings, the Trrap mRNA was 90% less abundant in BM and liver of Trrap conditional knockout mice 2 days after the 3rd injection of pIpC (Fig. 1C). These data indicate that pIpC-mediated conditional deletion of the Trrap floxed allele effectively inactivated the Trrap gene in the hematopoietic system, including BM and spleen. Hereafter, Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+} and Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+} mice treated with pIpC are designated as Trrap-CKO mice. The control group (Trrap-CO) was composed of several genotypes (Trrap\textsuperscript{+/-}Cre\textsuperscript{+/-}; Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-}; Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-}Cre\textsuperscript{+/-} and Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-} injected with pIpC; and Trrap\textsuperscript{flox/flox}Cre\textsuperscript{+/-}Cre\textsuperscript{+/-} injected with PBS), all of which carried at least one intact Trrap allele and produced consistent results in all experiments. Within 5 to 6 wk after pIpC treatment, the majority of Trrap-CKO mice, but not control mice including several genotypes, appeared moribund and either died or were killed (Fig. 1, D and E).

Fatal failure of BM after inactivation of Trrap

Analysis of Trrap-CKO mice revealed a dramatic decrease in cellularity of the femur and spleen at days 6 and 12 post deletion (Fig. 2, A and B). Furthermore, analysis of peripheral blood indicated a reduction in platelets, white blood cells, and lymphocytes in Trrap-CKO mice in comparison to control mice (Fig. 2, C–E). Indeed, blood analysis of severely moribund mice revealed a four-fold reduction in RBC, 36-fold reduction in platelets and 21-fold reduction in white blood cells (data not shown). Hence, Trrap-CKO died from severe anemia and cytopenia. A comprehensive
FIGURE 2. Trrap is required to maintain homeostasis of the hematopoietic system in adult mice. Trrap-CKO mice, both at 6 and 12 days, display about a 60% reduction in cells from the BM (A) and about a 30% reduction in cells from the spleen (B) in comparison to control (Co) mice. Analysis of peripheral blood indicates a reduction in white blood cells (C), platelets (D), and lymphocytes (E) in Trrap-CKO mice at 2, 7, and 20 days, in comparison to control (Co) mice. F, Histological analysis by H&E staining reveals that Trrap-CKO mice have reduced nucleated cells in BM but not liver at 2 and 7 days. G, FACS analysis on cells from BM and spleen at 6 and 12 days postadministration of pIpC reveals a dramatic reduction in both the relative numbers of granulocytes (Gr1<sup>+</sup>/CD11b<sup>+</sup>) (FACS scan) and the total number of granulocytes (bar chart) in Trrap-CKO in comparison to Co mice. Additionally, the relative numbers of reticulocytes (Ter119<sup>+</sup>/CD71<sup>+</sup>) and erythrocytes (Ter119<sup>+</sup>/CD71<sup>+</sup>) are down-regulated in the spleen (FACS scan) of Trrap-CKO mice (day 12 postdeletion) as compared with control mice. Intriguingly, analysis of the total number of reticulocytes and erythrocytes (bar chart) in the BM of Trrap-CKO mice (day 12) indicates the erythrocytes to be increased and the reticulocytes to be moderately decreased in comparison to control mice. I, Megakaryocytes were drastically decreased in both the spleen and BM of Trrap-CKO mice both at day 6 and 12 as indicated in the bar chart and FACS scan. J, The relative numbers (FACS scan) and total numbers (bar charts) of mature B cells (CD19<sup>+</sup> IgM<sup>+</sup>) and T cells from the spleen of Trrap-CKO mice were not significantly changed both at 6 and 12 days postdeletion of Trrap as compared with control mice. All FACS scans indicate analyses performed at 6 and 12 days postdeletion. The data shown are representative of two independent experiments.
Histological analysis revealed no apparent abnormalities in any tissues of Trrap-CKO mice except in BM (Fig. 2F; and data not shown) that confirmed the severe decrease in cellularity at 2 and 7 days post deletion (Fig. 2A). As a control, histological analysis of the liver, another target tissue of Mx-Cre, displayed no difference between Trrap-CKO mice and control mice (Fig. 2F). To further investigate the phenotype of these mice, we performed FACS analysis of blood elements on BM and splenocyte cells. We found a vast reduction in the number of granulocytes in the spleen and BM at both 6 and 12 days post deletion in Trrap-CKO mice (Fig. 2G). Similarly, the number of reticulocytes and megakaryocytes was decreased post deletion in the BM and spleen (Fig. 2, H and I), whereas the relative number of mature B/T cells and erythrocytes was unaffected and increased, respectively (Fig. 2, H and J), suggesting that erythroid and lymphoid progenitors might be less dependent on HSCs, at least in the short term. These results indicate that Trrap deletion affected a wide range of mature hematopoietic cells in BM and spleen.

Trrap maintains the hematopoietic system in a cell-autonomous manner

To examine whether the reduced survival of the mice and depletion of hematopoietic cells associated with Trrap deletion was a result of hematopoietic failure, we performed BM reconstitution experiments (Fig. 3A). BM cells from Trrap-CKO mice or control mice were transferred into lethally irradiated Trrap-/-/- or Trrap+/+ recipients and engraftment was allowed to occur. As expected, the mice reconstituted with control BM cells retained 100% survival, however only 30% of those reconstituted with Trrap-CKO BM cells survived (Fig. 3B). Importantly, analysis of the DNA isolated from the blood of those 30% surviving mice revealed only contributing DNA from the Trrap allele but not the host origin (i.e., the Trrap allele) (Fig. 3C), indicating that these mice are “escapers” of deletion. All mice lethally irradiated and mock transplanted (administered with PBS) died within 2 wk postirradiation (Fig. 3B). These results demonstrate that Trrap-deficient hematopoietic stem/precursor cells lost the capacity to repopulate lethally irradiated BM, suggesting that the defect in this compartment may account for the hematopoietic failure and reduced survival of Trrap-CKO mice.

Loss of BM progenitor populations and HSCs after Trrap inactivation

Loss of Trrap results in a reduction of several mature blood elements including granulocytes, megakaryocytes, platelets, and white blood cells, therefore we reasoned that the progenitors of these cells may be affected upon deletion of Trrap. To test this possibility, we have cultured BM cells under conditions that allow for the progenitors to differentiate into varying lineages which can then be identified based on their morphology in vitro. BM from Trrap-containing and Trrap-CKO mice were cultured for 10 days following which colonies with different morphology were scored. This assay revealed that Trrap-CKO BM generated dramatically fewer colonies than did Trrap control BM and most progenitors tested were markedly down-regulated (Fig. 4, A and B). To determine the status of hematopoietic progenitors using a more quantitative assay, phenotypic FACS analysis was performed on cells taken from BM, spleen and thymus. Strikingly, we found that the common myeloid progenitor, common lymphoid progenitor, granulocyte-monocyte progenitor, and megakaryocyte-erythrocyte progenitor were all dramatically reduced in Trrap-CKO mice (Fig. 4, C and D and data not shown). Additionally, Trrap deletion resulted in a loss of the immature PreproB, PreB, and PreB cells (Fig. 4, C and D; and data not shown) as well as lineage negative (lin-) stem/progenitor cells (Fig. 4E). This decrease in progenitors, as determined both by FACS and colony formation assay, could be due to an inability of the progenitors to proliferate or an absence of the progenitors. Considering that in adult BM these progenitors arise from the rare (<0.1%) HSCs (lineage marker c-Kit+ Lin- Sca-1-), we next investigated the number of these cells in the BM by FACS. As early as 2 days postdeletion of Trrap, and even more drastically at 12 days postdeletion, we found the number of both long-term and short-term HSCs to be vastly reduced in comparison to control mice (Fig. 4, F–H). Quantitative analysis revealed that both absolute and relative numbers of either long-term-HSCs, short-term-HSCs, and multipotent progenitor cells were severely reduced in Trrap-deficient BM, whereas relative number of multipotent progenitor at later time-point (day 12) seems to be more strongly affected (Fig. 4H, and data not shown). Furthermore, expression of adhesion molecules CD29, CD49d,
FIGURE 4. Loss of hematopoietic progenitors and hematopoietic stem cells after Trrap deletion. A and B, Trrap-CKO BM was harvested 2 days (A) and 7 days (B) after three intraperitoneal dose of pIpC and cultured in medium that allows for the formation of hematopoietic progenitor colonies. Data are presented as mean number of colonies from triplicate performed assays ± SEM counted 10 days after plating of 10,000 BM cells. C and D, B cell progenitors (common lymphoid progenitor, pre-pro B, proB, and preB) in BM deleted for Trrap were found to be down-regulated in BM as assessed by FACS using CD43, CD93, CD19 and IgM Abs, indicated by FACS (C) scan plot and bar chart (D). E, Lin- cells in BM were down-regulated in Trrap-CKO mice both at 6 and 12 days postdeletion. F–H, HSCs were dramatically reduced in Trrap-CKO BM. F, Gating strategy for quantitative analysis of HSCs in BM. BM cells were stained for lineage markers, gated on the lin− (linnegative) subset, and further stained for c-Kit (CD117) and Sca-1. Numbers on the plot are the frequency of cells in the indicated regions. Hematopoietic stem cells are contained within the c-Kit+ lin− Sca-1+ (KLS cells) population. G and H, HSC (Sca-1+ CD117+) in BM from Trrap proficient (Co) and Trrap-CKO mice was assessed by FACS using specific Abs both at 2 days and 12 days postdeletion. Boxes indicate KLS-HSC populations. H, Quantification of hematopoietic stem/progenitor cells as in G. Data are representative of two independent experiments.
and CD49e on KLS immature cells were unaffected in Trrap-depleted BM at 2 days after Trrap deletion, and only slightly down-regulated 12 days post deletion (data not shown), arguing that changes in the expression of adhesion molecules are unlikely to induce the migration of HSCs out of the niche and subsequently cause their loss in Trrap-CKO mice. These results show that hematopoietic failure in Trrap-deficient mice is most likely due to the loss of HSCs or cell autonomous defects in HSCs, indicating that Trrap plays an essential role in the maintenance of HSCs and the repopulating capacity of hematopoietic stem/progenitor cells.

Apoptosis of BM progenitor populations after Trrap deletion

BM failure and the reduction in hematopoietic cells could be due to a blockage of cell cycle progression or induction of apoptosis in hematopoietic stem/progenitor populations. To address what contributes to this cellular reduction, lin^- cells were purified from total BM and used for further analysis (Fig. 5A). The purity of the lin^- population was verified by performing FACS analysis using an anti-c-kit Ab (Fig. 5B), and the efficiency of Trrap deletion in these cells following plpC injection was analyzed by PCR (Fig. 5C). To examine the proliferation dynamics and apoptotic fraction of lin^- BM cells lacking Trrap, Trrap was deleted in BM and 48 h thereafter the cell cycle profile and cell death of lin^- BM cells were analyzed ex vivo by flow cytometry after staining cells with PI or FITC-conjugated Annexin V, respectively. Although cell cycle profiles were virtually indistinguishable between Trrap-deficient and Trrap-containing BM cells (Fig. 5D), lin^- cells lacking Trrap exhibited a marked increase (over 2-fold) in apoptotic fraction in comparison to Trrap-proficient controls (Fig. 5E; and data not shown). In addition, a fraction of cells with nuclear features of apoptosis (chromatin condensation visualized by FISH staining of major satellite repeats MSR) was significantly increased in Trrap-depleted lin^- cells as compared with Trrap-proficient controls (Fig. 5F, and data not shown). These results indicate that increased apoptosis, and not cell cycle block, in the stem/progenitor compartment is likely to be the cause of BM failure and loss of hematopoietic progenitors in Trrap-deficient mice. We therefore asked whether Trrap controls viability of hematopoietic stem/progenitor cells. For this purpose, we crossed mice harboring floxed alleles of p53 with Trrap-deficient mice and subsequently caused their loss in Trrap-CKO double-mutant mice was analyzed by flow cytometry after Annexin V staining. As shown in Fig. 5F, Trrap-deficient lin^- cells on p53^-/- background exhibited a significantly higher apoptotic fraction compared with lin^- cells from p53^-/- Trrap-Co mice. This increase in apoptotic fraction was comparable to that observed in Trrap-deficient mice on p53^-/-/Trrap-Co background (Fig. 5E), indicating that p53 status does not affect cell death induction associated with loss of Trrap. Together with the findings that p53^-/- Trrap-CKO mice displayed high mortality rates that were similar to Trrap-CKO mice (data not shown), these data indicate that Trrap may prevent apoptosis of hematopoietic stem/progenitor cells through a p53-independent mechanism.

Deregulation of c-Myc, a transcription factor which directly interacts with TRRAP (17), was shown to alter hematopoietic stem cell pools (23, 24). Therefore, to test the expression and function of the Myc transcription factors in hematopoietic stem/progenitor cells lacking Trrap, we measured quantitatively by RT-PCR the expression of c-Myc and N-Myc and the sets of genes known to be the targets of these transcription factors in Trrap-CKO lin^- cells. Loss of Trrap resulted in up-regulation of c-Myc and N-Myc expression in lin^- cells, whereas expression of the anti-apoptotic gene Mcl1 and housekeeping gene hprt remains unchanged (Fig. 5J, and data not shown). Surprisingly, Western blot analysis revealed that c-Myc protein levels were dramatically reduced in Trrap-CKO lin^- cells, whereas N-Myc protein showed a moderate decrease (Fig. 5J), indicating that the protein Trrap is required for the stability of Myc proteins. Furthermore, we found that Trrap deficient lin^- cells exhibited a significant reduction in cyclin D2, Mcm7, and Id2 and concomitant up-regulation of cyclin D1 (Fig. 5, J and K). These results show that loss of Trrap in lin^- cells, despite down-regulation of c-Myc and N-Myc proteins, compromises both transcriptional activation and repression function of Myc transcription factors on their respective targets. Because deregulation of c-Myc and N-Myc was shown to alter hematopoietic stem cell pools (23, 24), these results suggest that deregulation of c-Myc and N-Myc function in the absence of Trrap leads to apoptosis and/or defect in the maintenance of hematopoietic stem/progenitor cells.

Discussion

Recent studies have indicated a role of Tip60-p400 HAT complex in the maintenance of stem cell identity (25), however the underlying mechanism remains poorly understood. In the present study, we conditionally deleted Trrap in bone marrow stem/progenitor cells as a model system to further identify Trrap as a part of the mechanism that promotes the maintenance of stem cell pools. Our study reveals that deletion of Trrap leads to a loss of hematopoietic stem and progenitor cells via a cell autonomous manner without affecting the HSC niche hence Trrap is essential for maintaining the integrity of adult BM. Furthermore, Trrap-deficient hematopoietic stem/progenitor cells exhibited a marked increase in p53 independent apoptosis, arguing that loss of hematopoietic stem/progenitor pool results in BM failure leading to the severe phenotype and frequent death of Trrap-CKO mice. Similar phenotypes have been observed after deletion of several transcriptional regulators and molecular players involved in chromatin modifications including Zfx (30), Foxol3/34 (31), Foxo3a (32), Bmi-1 (33), and Mi2β. These studies suggest that Trrap belongs to a group of molecules involved in transcriptional reprogramming and chromatin reconfiguration that play critical roles in the maintenance of hematopoietic stem/progenitor cell pool. The severe phenotype of TTRAP deficient mice and bone marrow progenitor/stem cells raises the question on the contribution of different TTRAP-dependent processes to the TTRAP-deficient phenotype. TTRAP is a component of several HAT complexes (8) and interacts with transcription factors c-Myc and E2F (17). However, deletion of any single TTRAP-interacting player (such as individual HAT enzyme, c-Myc or E2F) leads to embryonic lethality at only later embryonic stage (c-Myc at E10.5; Gcn5 at
FIGURE 5. Loss of Trrap leads to increased apoptosis and deregulation of the Myc transcription factors in BM stem/progenitor cells. A, Trrap<sup>fl/fl</sup> Cre<sup>+</sup> and Trrap<sup>fl/fl</sup> Cre<sup>−</sup> mice were injected with pIpC and lin<sup>−</sup> cells were purified from BM 2 days later. Cells were then used for Annexin V staining (to detect apoptotic cells), cell cycle profiling, immunofluorescent staining, and RT-PCR analysis. B, The purity of immune negative cells was assessed by FACS using c-kit (CD117) Ab. C, RT-PCR analysis of Trrap depletion efficiency by pIpC-induced Cre recombinase in hematopoietic lin<sup>−</sup> cells. Total RNA was isolated from lin<sup>−</sup> cells purified from control (Co) and Trrap<sup>-CKO</sup> mice and Trrap mRNA levels were analyzed by RT-PCR. HPRT levels were used as internal loading control. D–F, Analysis of cell cycle and apoptosis in Trrap-deficient BM cells. D, BM cells isolated from Trrap<sup>-CKO</sup> and control mice were stained with propidium iodide and analyzed by FACS. E, Flow cytometry analysis of BM cells isolated from Trrap<sup>-CKO</sup> and control after staining with FITC-Annexin V. The numbers indicate the percentage of Annexin V-positive (apoptotic) cells. F, DNA-FISH analysis of the mouse major satellite repeats in lin<sup>−</sup> cells isolated from Trrap-containing (Co) and Trrap-depleted (CKO) BM. Cells were counterstained by DAPI (DNA). G and H, Apoptosis of hematopoietic stem/progenitor cells after Trrap deletion is p53 independent. BM cells from Trrap<sup>-CKO</sup> in Trp53<sup>/−</sup>/Trp53<sup>/−</sup> background were harvested 2 days (G) and 7 days (H) after three intraperitoneal dose of pIpC and cultured in medium that allows for the formation of hematopoietic progenitor colonies. Data are presented as mean number of colonies from triplicate performed assays ± SEM counted 10 days after plating of 10,000 BM cells. I, Analysis of apoptosis in lin<sup>−</sup> BM cells from Trrap deficient mice on p53<sup>/−</sup> background. Lin<sup>−</sup> BM cells from p53<sup>/−</sup>/Trrap<sup>-CKO</sup> and p53<sup>/−</sup>/Trrap<sup>-Co</sup> (after intraperitoneal injection of pIpC) were analyzed by flow cytometry after Annexin V staining. The numbers indicate the percentage of Annexin V-positive cells. J and K, RT-PCR and Western blot analysis comparing expression of Myc transcription factors and their targets in Trrap deficient stem/progenitor cells. J, Total RNA was isolated from lin<sup>−</sup> cells purified from control (Co) and Trrap<sup>-CKO</sup> mice and mRNA levels of indicated genes were analyzed by RT-PCR and Western blotting. All data was normalized against the expression of hprt (RT-PCR) and actin (Western blot). K, Quantification of gene expression after normalization as in J. mRNA levels of indicated genes were analyzed densitometrically and normalized to hprt.
E-10.5) (34–36) or is fully compatible with embryonic development (PCAF; E2F1) (37, 38). Consistent with full or partial redundancy of different HATs, genetic deletion of multiple HATs in mice, for example double knockout of GCN5 and PCAF, shows more severe defects than individual knockouts (36, 38). These findings are consistent with the notion that HATs may have partially overlapping functions, whereas Trrap has nonredundant functions in cellular processes including embryonic development and adult tissue homeostasis.

Induction of apoptosis and the lack of cell cycle changes in hematopoietic stem/progenitor cells after Trrap deletion is surprising because Trrap deletion in mouse embryonic fibroblasts induces striking cell cycle defects in the absence of cell death (Ref. 27 and data not shown). Different outcomes caused by Trrap deletion in hematopoietic stem/progenitor cells and mouse embryonic fibroblasts may reflect different pathways affected and distinct default programs triggered in different cell types lacking Trrap. Although further studies are needed to define the precise underlying mechanisms, it is possible that Trrap is involved in the maintenance of hematopoietic stem/progenitor cells through its interaction with other key regulators such as c-Myc, a transcription factor which directly interacts with Trrap (17). c-Myc and N-Myc play key roles in hematopoietic stem/progenitor cells and mouse embryonic fibroblasts. c-Myc and N-Myc are involved in the regulation of ubiquitination and degradation of specific proteins involved in the regulation of ubiquitination and degradation of Myc proteins. Alternatively, Trrap may be required for the expression and/or activity of gene products involved in the regulation of ubiquitination and degradation of Myc proteins. Although Myc has a pivotal function in apoptosis and their abnormal expression sensitizes cells to apoptosis by a variety of stimuli, it should be noted that induction of apoptosis in lin– cells lacking Trrap may also result from the defect other than Myc de-repression. The transcription of many genes relies on histone acetylation and Trrap is involved in transcription of many genes and molecular pathways (27, 39). Therefore, it is possible that induction of apoptosis in Trrap deficient cells may be a consequence of down-regulation of anti-apoptotic genes or up-regulation of pro-apoptotic genes. Finally, Trrap may mediate expression of specific genes in hematopoietic stem cell niche that are required for survival of stem/progenitor cells. Together, this study has identified a critical role for Trrap in the mechanism that maintains HSCs and hematopoietic system, and underscores the importance of Trrap and histone modifications in tissue homeostasis.

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Disclosures

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