Pontin is essential for murine hematopoietic stem cell survival

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ABSTRACT

Pontin is a highly conserved DNA helicase/ATPase which is a component of several macromolecular complexes with functions that include DNA repair, telomere maintenance and tumor suppression. While Pontin is known to be essential in yeast, fruit flies and frogs, its physiological role in mammalian organisms remains to be determined. We here find that Pontin is highly expressed in embryonic stem cells and hematopoietic tissues. Through germline inactivation of *Ruvbl1*, the gene encoding Pontin, we found it to be essential for early embryogenesis, as *Ruvbl1* null embryos could not be recovered beyond the blastocyst stage where proliferation of the pluripotent inner cell mass was impaired. Conditional ablation of *Ruvbl1* in hematopoietic tissues led to bone marrow failure. Competitive repopulation experiments showed that this included the loss of hematopoietic stem cells through apopotosis. Pontin is, therefore, essential for the function of both embryonic pluripotent cells and adult hematopoietic stem cells.

Key words: Pontin, regulation, hematopoietic stem cell, embryogenesis, adult.

Citation: Bereshchenko O, Mancini E, Luciani L, Gambardella A, Riccardi C, and Nerlov C. Pontin is essential for murine hematopoietic stem cell survival. Haematologica 2012;97(9):1291-1294. doi:10.3324/haematol.2011.060251

Introduction

Pontin and Reptin (also known as Tip49/Ruvbl1 and Tip48/Ruvbl2, respectively) are homologous ATPases with DNA helicase activity that have been identified as participants in macromolecular complexes carrying out key functions in trancription and gene regulation. These include DNA helicases (Ino80 complex),[1] histone modification (Tip60 complex)[2] and nucleosome remodeling (SWI/SNF complex). In addition, Pontin/ Reptin participate in complexes important for SnoRNA biogenesis (R2TP complex) and telomere maintenance (Telomerase core complex).[3] Finally, Pontin interacts directly with the TATA-binding protein,[4] and acts as co-factor for specific transcription factors and oncoproteins, such as c-Myc[5] and β-catenin,[6] both of which play essential roles in regulating hematopoietic and other stem cells. These multiple interactions of Pontin/Reptin suggest an essential physiological role, and studies in *S. Cerevisiae* have demonstrated that yeast orthologs of both Reptin and Pontin are required for cell viability without overall shutdown of transcription.[7,8] Essential deveopmental roles in Drosophila and Xenopus have also been observed.[9,10] However, the only information currently available about their role in mammalian cells is that knockdown of Pontin in human diploid fibroblasts causes proliferation arrest,[11] and that knockdown of *Ruvbl1* (and other Tip60 complex components) in embryonic stem (ES) cells induces loss of pluripotency.[12] We here use genetic ablation of the *Ruvbl1* gene, encoding Pontin, in the mouse germline to demonstrate that this gene is essential for embryogenesis at an early stage. No *Ruvbl1*$^{-/-}$ embryos were retrieved post-implantation, and outgrowth of pluripotent cells from *Ruvbl1*$^{-/-}$ blastocysts was not achieved. To address the role of Pontin in hematopoietic stem cells (HSCs), we conditionally ablated Ruvbl1 from the hematopoietic system using the *Mx1-Cre* transgene. This led to complete hematopoietic failure, including apoptotic loss of hematopoietic stem cells. Pontin is, therefore, essential for both early embryogenesis and adult hematopoiesis.

Design and Methods

The *Ruvbl1* gene was targeted by homologous recombination in E14.1 ES cells.[13] Breeding to deleterFlp[14] and deleterCre[15] mice generated the conditional and null alleles, respectively. Genotyping of mice and embryos was as described in the *Online Supplementary Design and Methods*. Conditional gene inactivation was achieved by activating the *Mx1-Cre* transgene[16] through polyIC injection.[17] Bone marrow cells were counted from femur, tibia and ilium. Peripheral blood counts and flow cytometric analysis, as well as flow cytometric analysis of bone marrow were performed as previously described[17,18] (antibodies and dilutions used are described in the *Online Supplementary Design and*

The online version of this article has a Supplementary Appendix.

Acknowledgements: the authors would like to thank Dr. Bruno Amati for supplying Tip48 and Tip49 antibodies.
Funding: the work was supported through the FP6 EuroStemCell and EuroCSC consortia, and by an MRC Program Grant (grant n. 93339) and MRC Strategic Award to CN. OB was the recipient of an HFSP postdoctoral fellowship, and is a recipient of a José Carreras Young Investigator Fellowship.
Manuscript received on December 11, 2011. Revised version arrived on February 1, 2012. Manuscript accepted on February 15, 2012.
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Methods). Western blotting was performed on cell/tissue extracts (*Online Supplementary Appendix*) using Tip48, Tip49[19], PCNA (Sigma) and β-tubulin (Sigma) antibodies as described.[20]

Results and Discussion

To address the physiological role of Pontin we first generated a *Ruvbl1* null allele by targeting the mouse germline (*Online Supplementary Figure S1*). Intercrossing of *Ruvbl1*$^{+/-}$ mice did not generate any viable offspring, and no post-implantation *Ruvbl1*$^{-/-}$ embryos could be retrieved (Figure 1A). A few *Ruvbl1*$^{-/-}$ blastocysts were identified. However, upon culturing, which results in outgrowth of the pluripotent inner cell mass, no proliferating cultures were observed to be *Ruvbl1*$^{-/-}$. We conclude from this that Pontin is required for embryogenesis at a very early stage, possibly involving the proliferation of pluripotent inner mass cells.

To address the role of Pontin after development, we analyzed the Pontin and Reptin expression patterns in the adult mouse and selected cell lines. Overall, the expression of Pontin, but not of Reptin, correlated with that of proliferating cell nuclear antigen (PCNA), consistent with Pontin playing a specific role in cell proliferation. In particular, we found Pontin to be highly expressed in hematopoietic tissues (bone marrow, spleen, thymus, lymph nodes), as well as pluripotent cells/tissues (ES cells, testis), with low levels in liver, brain and lung (Figure 1B). To address Pontin function in the hematopoietic system, a conditional *Ruvbl1* allele (*Online Supplementary Figure S1*) was combined with the *Mx1-Cre* transgene, which deletes with high efficiency in hematopoietic tissues after induction with polyIC[17]. At two weeks after polyIC induction of *Ruvbl1*$^{fl/fl}$;*Mx-Cre*$^{tg/+}$ mice (Pontin^{cKO} mice) and *Ruvbl1*$^{fl/fl}$;*Mx-Cre*$^{+/+}$ controls (Pontin^{Con} mice), we observed specific lethality of Pontin^{cKO} mice (Figure 1C), associated with a significant decrease in Pontin^{cKO} peripheral blood cells (Figure 1D). Within eight

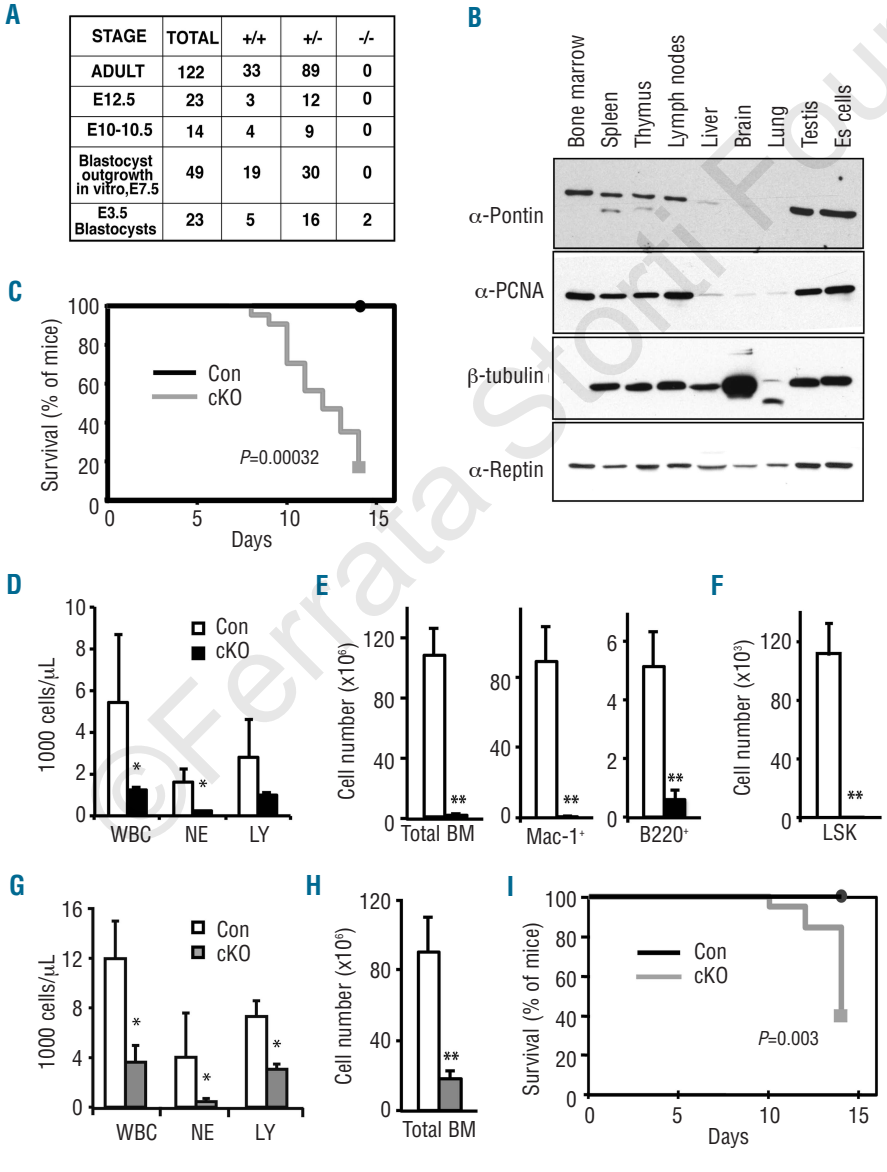

Figure 1. Pontin is essential for mouse development and definitive hematopoiesis. (A) *Ruvbl1*$^{+/-}$ mice were intercrossed and litters and embryos of the indicated developmental stages were genotyped. Number of live animals/embryos with the 3 *Ruvbl1* genotypes are shown for each developmental stage. Embryos were collected from at least 3 females at each stage. (B) Western blot of Pontin, Reptin and PCNA expression in various mouse tissues and E14.1 ES cells. The same filter was probed using antibodies specific for the indicated proteins, and an antibody against β-tubulin as a loading control. (C) Kaplan Meier plot of survival of Pontin^{cKO} (n=14) and Pontin^{Con} (n=12) mice following injection of polyIC to induce Pontin deletion. The statistical significance of the difference in survival, determined by the log rank method, is indicated in the plot. Death was associated with pancytopenia. (D) Peripheral blood counts of live Pontin^{cKO} (n=3) and Pontin^{Con} mice (n=3) at Day 8 after first polyIC injection. Mean ± SEM. *P<0.05. (E) Total bone marrow (BM) cell count, and number of Mac-1^{+} and B220^{+} bone marrow cells determined by flow cytometry in Pontin^{cKO} (n=6) and Pontin^{Con} mice (n=8) mice on Day 8 after first polyIC injection, from 3 independent experiments. Mean ± SEM. **P<0.01. (F) Number of LSK cells in the bone marrow of Pontin^{cKO} (n=3) and Pontin^{Con} mice (n=3) mice on Day 8 after first polyIC injection. Mean ± SEM. **P<0.01. (G) Peripheral blood counts of live recipeint mice transplanted with Pontin^{cKO} (n=5) and Pontin^{Con} bone marrow (n=4) at Day 8 after first polyIC injection. PolyIC injections were initiated eight weeks after transplantation. Mean ± SEM. *P<0.05. (H) Total number of bone marrow cells in Pontin^{cKO} (n=5) and Pontin^{Con} (n=4) non-competitively transplanted mice at Day 11 after first polyIC injection. Mean ± SEM. **P<0.01. (I) Kaplan-Meier plot of survival analysis of Pontin^{cKO} (n=5) and Pontin^{Con} (n=4) non-competitively transplanted mice following polyIC injection. The statistical significance, determined by log rank test, of the difference in surivival is indicated in the plot. Death was associated with pancytopenia.

days post-injection, surviving Pontin^{cKO} mice displayed a massive loss of bone marrow cellularity (Figure 1E). This involved the coordinated loss of myeloid and lymphoid cells (Figure 1E) and was preceeded by the complete loss of Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) HSCs (Figure 1F). To determine whether the observed effects could be attributed to an intrinsic hematopoietic requirement for Pontin, we transplanted CD45.2 Pontin^{cKO} and Pontin^{Con} bone marrow non-competitively into irradiated CD45.1/2 recipients. Subsequent polyIC induction resulted in loss of hematopoietic cells (Figure 1G and H) and lethality (Figure 1I) similar to that observed in non-transplanted Pontin^{cKO} mice.

The severe phenotype made it difficult to assess the direct effects of Pontin loss on quiescent hematopoietic stem cells, since a strong depletion of mature cells and progenitors would activate HSCs, potentially rendering them more susceptible to the loss of Pontin. To circumvent this issue, we performed competitive transplantation of CD45.2 Pontin^{cKO} and Pontin^{Con} bone marrow along with CD45.1 competitor into irradiated CD45.1/2 recipients, allowing wild-type CD45.1 hematopoietic cells to maintain hematopoiesis after deletion. To minimize indirect effects of *Ruvbl1* deletion, we used a 3-fold excess of CD45.1 competitor bone marrow. We achieved similar levels of Pontin^{cKO} and Pontin^{Con} CD45.2 chimerism prior to polyIC induction (Figure 2A). At four days after induction, the frequency of Pontin^{cKO} HSCs (defined as either LSK, LSKCD150$^+$ or Lin$^-$Sca-1$^+$CD48$^-$CD150$^+$) was significantly decreased compared to Pontin^{Con}-transplanted mice (Figure 2B-E), and a very high proportion of the remaining Pontin^{cKO} HSCs were apoptotic, as measured by staining for AnnexinV and intracellular DNA (Figure 2F). From this we conclude that Pontin is required for HSC viability and that in its absence HSCs undergo apoptosis.

These results demonstrate an essential role for Pontin in early mammalian development, consistent with its presence in multiple complexes carrying out essential cellular functions. In addition, the definitive hematopoietic system in general, and HSCs in particular, were critically dependent on Pontin for its maintenance, with HSC apoptosis and depletion being an immediate consequence of *Ruvbl1* inactivation. This is in contrast to the proliferation arrest observed after *in vitro Ruvbl1* knockdown.[11] Conditional hematopoietic inactivation of both *Myc* and *Mycn* results in

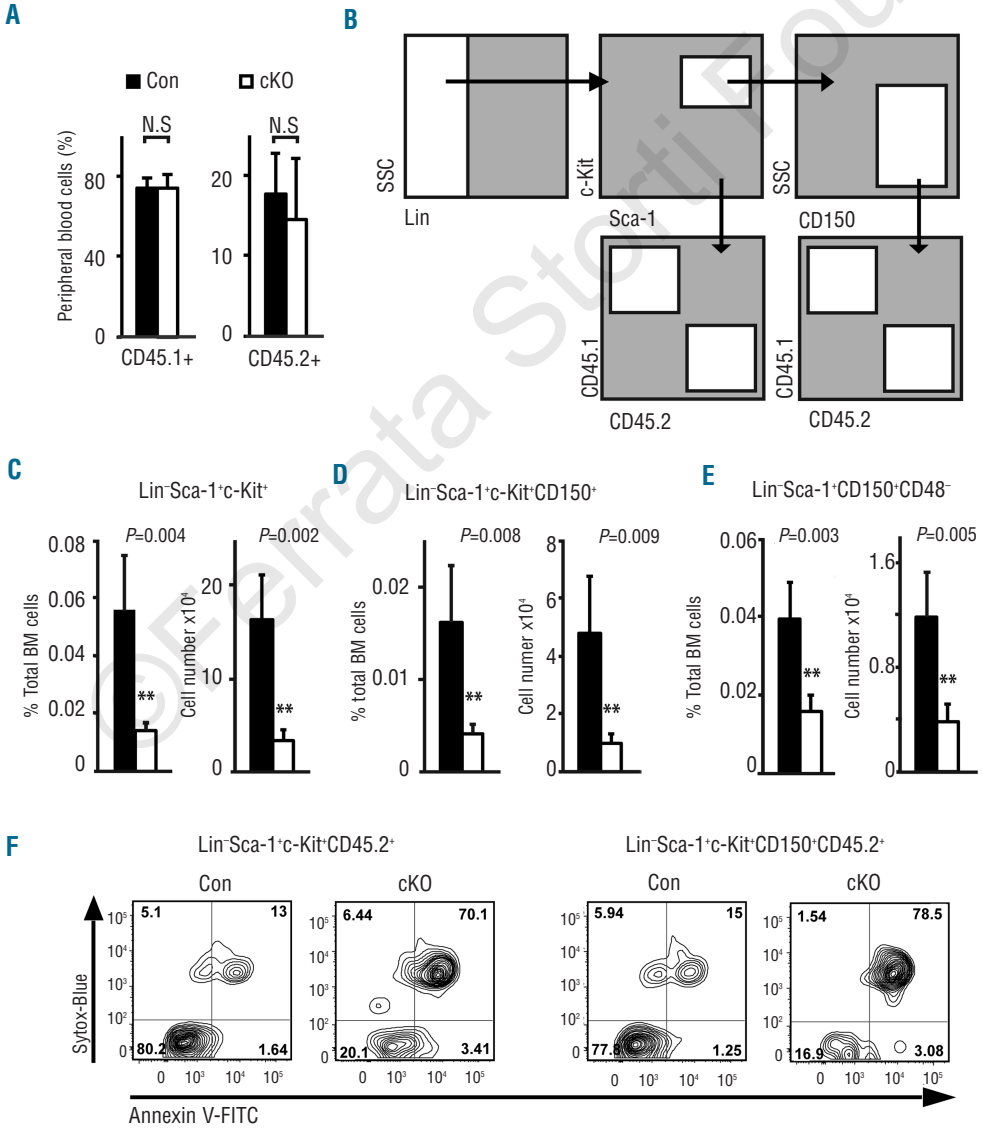

Figure 2. Pontin is required for HSC survival. (A) Reconstitution levels of competitor (CD45.1) and experimental donor cells (CD45.2) in irradiated recipients reconstituted with 1,500,000 helper and 500,000 donor cells (either Pontin^{cKO} or Pontin^{Con}). Pontin^{cKO}: n=4; Pontin^{Con}: n=5. N.S.: no statistically significant difference. (B) Scheme of the gating for the flow cytometric analysis of HSC compartment in competitively transplanted mice. (C) Frequency (left panel) and cell number (right panel) of CD45.2$^+$ LSK cells in BM of competitively transplanted mice at Day 4 after a single polyIC injection. Black bars: Pontin^{Con} (n=4); white bars: Pontin^{cKo} (n=4). Mean ± SEM. **P<0.01. (D) Analysis of the number of LSKCD150$^+$ BM cells in the cohort from panel (C). Mean ± SEM. **P<0.01. (E) Analysis of the number of Lin$^-$Sca-1$^+$CD150$^+$CD48$^-$ BM cells in the cohort from panel (C). Mean ± SEM. **P<0.01. (F) Analysis of apoptosis of Pontin^{cKO} and Pontin^{Con} HSCs at Day 4 after polyIC injection by surface staining using AnnexinV and measurement of cell permeability through staining of intracellular DNA (Sytox-Blue dye). Contour plots show AnnexinV and Sytox-Blue staining of LSK and LSKCD150$^+$ cells, gated on the experimental CD45.2 population. Four mice were analyzed for each condition and representative plots are shown.

a very similar phenotype to loss of Pontin.[21] Since Pontin was shown to directly interact with c-Myc and control its oncogenic activity,[5] loss of Myc function may contribute significantly to the hematopoietic *Ruvbl1* null phenotype. Embryonic lethality in the absence of Pontin occurred at a stage of development consistent with a defect in the pluripotent inner cell mass. This phenotype is consistent with *Ruvbl1* being essential for ES cell maintenance, most likely due to Pontin being required for the function of the Tip60 complex.[12] However, due to the pleiotropic functions of Pontin, other molecular interactions could prove critical for the developmental processes observed to be affected by its absence. Pontin and Reptin functionally interact with proteins playing prominent roles in cancer, such as β-catenin, c-Myc and telomerase. Furthermore, Pontin and/or Reptin overexpression was found in a number of solid tumors, as well as in several hematologic malignancies.[22] This suggests that Pontin/Reptin proteins could be involved in tumorigenesis and may represent a potential target for cancer therapy. Based on our own and other published data, inhibition of Pontin expression or potentially its enzymatic activity represents a plausible way to impede tumor growth and induce apoptosis in proliferating cancer cells. Use of these mice in hemizygocity may provide an insight into the dosage-dependent requirent of Pontin in various types of cancer. Overall, our results establish Pontin as a critical regulator of both embryonic and adult hematopoietic stem cells.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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