New Insights into the Regulation of Hematopoietic Stem Cell Self-Renewal

By

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Thomas Merton said, "The beginnning of love is the will to let those we love be perfectly themselves, the resolution not to twist them to fit our own image." With this ideal in mind, I dedicate this work to my wife Christine. Without your love, encouragement, and understanding, this journey would not have been possible.

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ABSTRACT

Self-renewal is essential for stem cell maintenance. In the blood system, rare hematopoietic stem cells (HSCs) must maintain their self-renewal potential to sustain long-term homeostasis. HSC self-renewal is tightly regulated, as defective self-renewal can result in stem cell depletion and unrestricted self-renewal is a hallmark of leukemia. In this report, we describe two novel regulators of HSC self-renewal illustrative of the diverse mechanisms that sustain stable long-term blood formation.

Absent, small, or homeotic 1-like (*Ash1l*) is a Trithorax group (TrG) member with a previously uncharacterized physiological function. The TrG is of interest in hematopoiesis, as *Mixed-lineage leukemia 1*, the prototypical TrG member, is required for HSC maintenance and frequently drives human leukemogenesis. Our work demonstrates that HSCs require *Ash1l* for establishing quiescence in the bone marrow, a fundamental process for preservation of HSC self-renewal. In the absence of *Ash1l*, HSCs become depleted in the young adult bone marrow, and HSC function is not detected in transplantation assays. Despite an apparent lack of functional HSCs, *Ash1l*-deficient mice do not progress to hematopoietic failure elucidating a paradoxical preservation of steady-state hematopoiesis despite a lack of transplantable HSC function. Additionally, we found that *Ash1l* cooperates non-redundantly with *Mll1* to maintain hematopoietic homeostasis. This is reminiscent of the functional cooperativity that defines *Drosophila* TrG members and has not been demonstrated in mammals.

Adrenocortical dysplasia (*Acd*), encodes Tpp1, a member of the telomere-protecting shelterin protein complex. Recent work implicates shelterin in a particularly aggressive form of the human bone marrow failure syndrome dyskeratosis congenita, though roles for shelterin proteins in hematopoietic homeostasis have not been fully defined. Our work indicates that *Acd* loss causes acute hematopoietic stem and progenitor cell depletion and severely impaired HSC self-renewal in transplantation assays. Though *Acd* deletion results in p53 target gene activation, *p53* deletion is not sufficient to rescue *Acd*-deficient HSC function. This is contrary to models of *Acd* deficiency in other stem cell systems in which *p53* inactivation largely rescues function. Our data suggest that HSCs are exquisitely sensitive to *Acd* loss and that shelterin proteins have previously undefined context-dependent functions in stem cell biology.

CHAPTER 1. HEMATOPOIETIC STEM CELL DEVELOPMENT, SELF-RENEWAL, AND FUNCTION

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HISTORICAL BACKGROUND TO THE STUDY OF HEMATOPOIETIC STEM CELLS

In the early 1950s, researchers observed that bone marrow transplantation could rescue hematopoietic failure in lethally irradiated animals [1]. This was of particular interest amidst growing concerns about the threat of radiation exposure following the Manhattan Project and the subsequent bombings of Hiroshima and Nagasaki [2]. Initially, hematopoietic rescue was attributed to "humoral factors" that were present in the donor bone marrow and could revitalize the irradiated host's damaged marrow [3]. Prior to establishing an experimental understanding of the nature of this hematopoietic rescue, two reports demonstrated that this finding had translational potential in human medicine. The first described a group of physicists who were accidentally exposed to a high dose of radiation, but recovered faster and better than expected after an infusion of donor bone marrow, probably as a result of transient engraftment [4]. The second report described two cases of leukemia patients who were treated with lethal irradiation and then transplanted with bone marrow from an identical twin sibling [5]. Hematopoietic function was restored by the procedure. While these isolated reports highlighted the promise of

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* Material in this chapter is partially covered in:
Sandy, A. R., Jones, M., and Maillard I., *Notch signaling and development of the hematopoietic system*. In: Notch signaling in embryology and cancer. Jorg Reichrath and Sandra Reichrath (eds.). Springer. 2011.

bone marrow transplantation, there was a limited understanding at this stage about the mechanisms of hematopoietic rescue after bone marrow infusion.

In 1961, seminal experiments by Till and McCulloch demonstrated that the source of hematopoietic rescue in mouse transplantation models was cellular and not humoral [6]. Following irradiation and subsequent bone marrow transplantation, it was noted that discrete colonies could be enumerated in the spleens of murine recipients. These colonies contained multiple lineages of hematopoietic cells, and were directly proportional to the number of nucleated bone marrow cells injected into the animal. Furthermore, the cells responsible for forming these colonies were rare, estimated at ~ 1 in 1,000 bone marrow cells. Further insight into the origin of these hematopoietic colonies was provided by the demonstration that each colony arose from a single cell, suggesting that the bone marrow housed a primitive cell type that could differentiate into multiple blood lineages [7].

The notion that rare, transplantable, multipotent cells could be the source of hematopoietic recovery in transplant recipients laid the groundwork for the study of what became known as hematopoietic stem cells (HSCs). Soon after their initial identification, these rare colony-forming cells were shown to have extensive self-renewal potential [8, 9]. Indeed, when splenic colonies were harvested, processed, and re-transplanted into a secondary recipient, new colonies would form. This suggested that the colony-initiating cells, now termed HSCs, could divide while maintaining their own identity and giving rise to differentiated progeny. This asymmetric mode of cell division is the hallmark of stem cell populations. The serial transplantation approach described in these studies remains similar to gold standard methods for testing HSC self-renewal to this day.

In the 50 years since the discovery of bone marrow HSCs, investigators have made significant progress toward understanding the mechanisms by which HSCs maintain their function. We now know that HSCs utilize many mechanisms to balance self-renewal and differentiation, and that this balance is critical to hematopoietic homeostasis. In this chapter, we will review our current understanding of HSC development and the multifactorial mechanisms through which self-renewal is regulated.

HEMATOPOIETIC STEM CELL EMERGENCE AND DEVELOPMENT

Hematopoiesis is initiated in two distinct waves. The first or "primitive" wave originates in the extra-embryonic yolk sac and is primarily responsible for the development of erythroid cells required for gas exchange during mid-gestation [10]. The second, or definitive, wave results in the formation of the HSC compartment that will be the focus of this discussion. Definitive hematopoietic stem cells are capable of differentiating into all cell lineages found in the hematopoietic system, including lymphocytes (**Figure 1.1**). Early work suggested that these multipotent cells emerge from large embryonic arteries, including the umbilical artery, the vitelline artery, and the omphalomesenteric artery, as well as from a region of the developing embryo known as the aorta-gonad-mesonephros (AGM) [11-15]. Subsequent studies revealed that the placenta might also be a source of definitive HSCs [16, 17]. Among these putative sites of definitive HSC emergence, perhaps the best characterized is the AGM. Elegant microscopy analyses identified that ventral wall endothelial cells of the dorsal aorta, the major AGM blood vessel, undergo a morphologic change that results in the generation of hematopoietic stem and progenitor cells that bud into the aortic lumen (**Figure 1.2**) [18]. The initiation of HSC specification from this specialized hemogenic endothelium was

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shown to require *Notch1* and *Runx1* [19-21]. Since *Notch1* deficiency results in a failure

to up-regulate *Runx1*, and *Runx1* over-expression rescues HSC specification defects in

Notch1-deficient embryos, it seems that Notch signaling is a critical upstream regulator

of definitive HSC emergence [19, 22]. These newly-specified HSCs then migrate through

the vasculature to the fetal liver, the next major site of HSC development and expansion

[23].
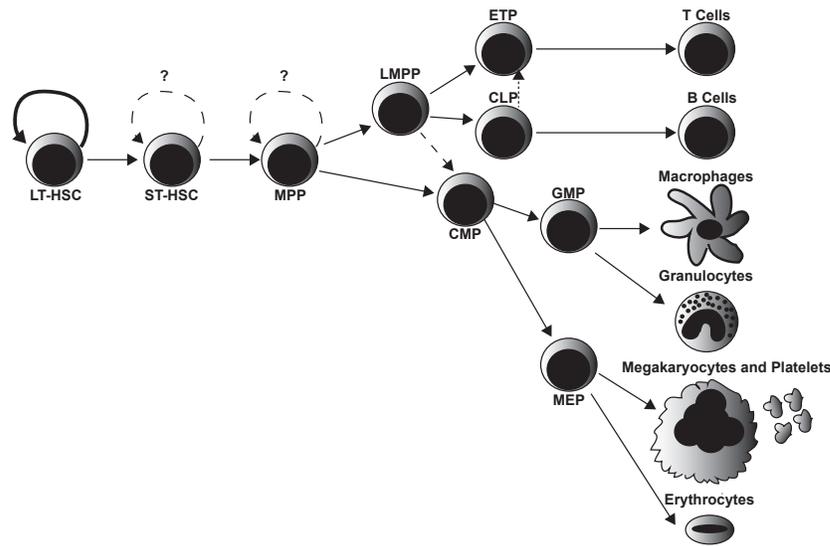
Figure 1.1. The hierarchy of normal hematopoiesis. Long-term hematopoietic stem cells (LT-HSCs) are multipotent and capable of self-renewal. They differentiate into short-term hematopoietic stem cells (ST-HSCs) and then multipotent progenitors (MPPs), which maintain multipotency, but are no longer capable of self-renewal. MPPs differentiate towards either the lymphoid or myeloid/erythroid lineages. Lymphoid-primed multipotent progenitors (LMPPs) differentiate into either early T-lineage progenitors (ETPs) or common lymphoid progenitors (CLPs), before subsequent maturation into T cells or B cells, respectively. Common myeloid progenitors (CMPs) can differentiate into granulocyte-monocyte progenitors (GMPs) or megakaryocyte-erythroid progenitors (MEPs). GMPs further differentiate into macrophages and granulocytes, while MEPs give rise to megakaryocytes and platelets or erythrocytes.

Between embryonic days 12.5 and 16.5 (E12.5-E16.5), murine fetal liver HSCs

expand nearly 40-fold, marking the most prolific expansion of HSCs during

hematopoietic ontogeny [24]. Sustaining such an expansion requires vigorous cell cycle

activity and presumably a propensity towards symmetric, self-renewing divisions. The

extrinsic signals that mediate this expansion remain largely unknown, but fetal HSCs

cell-autonomously require the master transcriptional regulator *Sox17* for proper

development and function [25, 26]. *Sox17* is robustly expressed in HSCs throughout fetal

development and is rapidly extinguished around the time of birth, before becoming

undetectable in adult bone marrow HSCs [25]. This pattern of expression is consistent

with a significant role for Sox17 in fetal, but not adult HSC development. Fetal liver

HSCs fail to expand in the absence of Sox17, and *Sox17* over-expression confers fetal-

like self-renewal properties and cell cycle activity to adult hematopoietic progenitors [25,

26]. This demonstrates that Sox17 is both necessary and partially sufficient to confer fetal

HSC attributes to hematopoietic progenitors. These studies have led to Sox17 being

termed a master regulator of fetal HSC development.

Aorta-Gonad-Mesonephros

E9-10 Dorsal Aorta

% Quiescent: ??

E11-18 Fetal Liver

% Quiescent: 5%

Wk5-Wk12 Bone Marrow

% Quiescent: ~70%
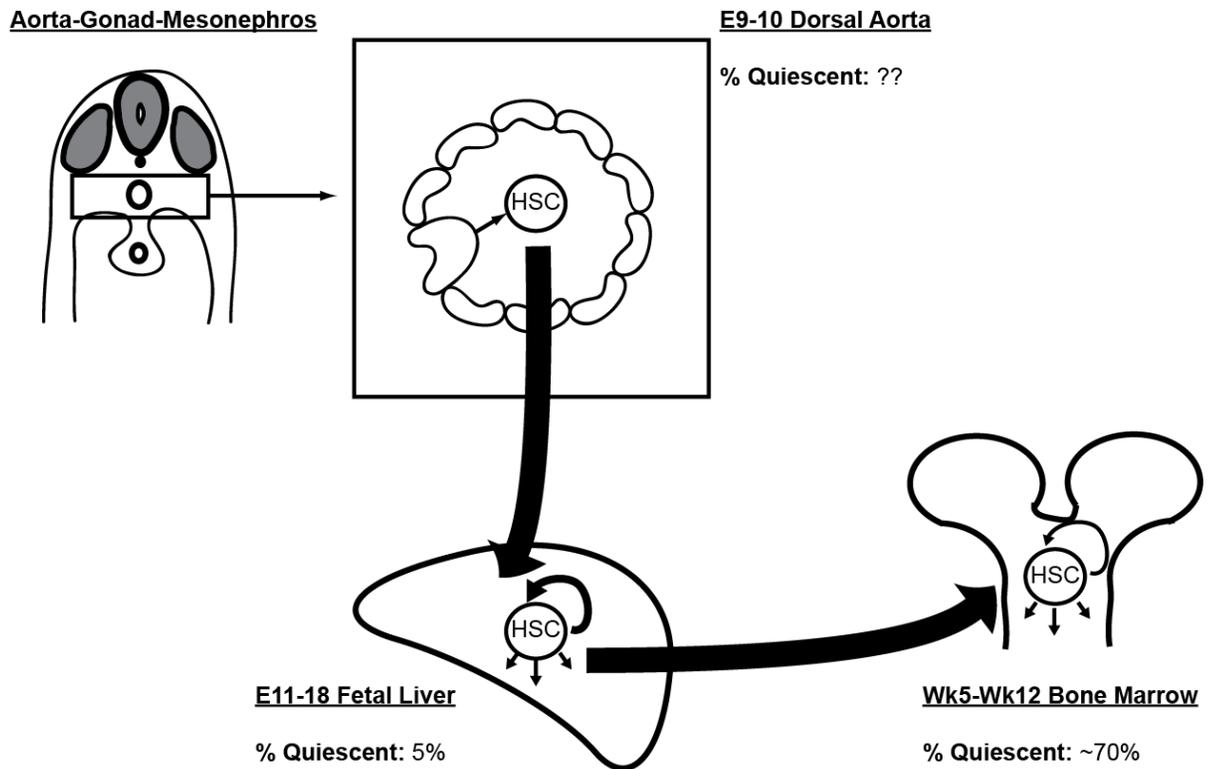
Figure 1.2. Definitive hematopoietic stem cell development. Hematopoietic stem cells emerge from hemogenic endothelium in the dorsal aorta between E9 and E10. These HSCs migrate to the fetal liver and robustly expand through self-renewing divisions between E11 and E18. Soon after birth, HSCs seed the bone marrow, and within 5 weeks, are predominantly found in the G0 phase of the cell cycle. At this point, most HSCs infrequently enter the cell cycle to maintain hematopoietic homeostasis during steady-state conditions.

Fate-tracing studies revealed that fetal liver HSCs migrate to the bone marrow and persist to maintain long-term hematopoiesis throughout life [27, 28]. This HSC migration to the bone marrow marks the onset of a critical developmental transition known as the fetal to adult transition. The predominant feature of this transition is the robust exit of HSCs from the cell cycle within 4 weeks after birth [29]. During fetal life, up to 95% of HSCs are engaged in cell cycle activity to facilitate robust HSC expansion [29]. Conversely, after reaching the bone marrow, between 70% and 90% of HSCs exit the cell cycle and enter into a state of quiescence [29, 30]. Models of hematopoietic stress through chemical myeloablation indicate that BM HSCs can rapidly reenter the cell

cycle, expand through self-renewing divisions, and differentiate in an attempt to reestablish hematopoietic homeostasis [30, 31]. This suggests that HSCs may sense the loss of downstream cell populations through an undefined mechanism. Such a mechanism could be triggered to replenish downstream cells lost to physiological cell turnover, or in stress situations such as major bleeding or infection. Interestingly, following the reestablishment of hematopoietic steady-state conditions, HSCs once again exit the cell cycle and become quiescent [30]. It is believed that this propensity towards quiescence is required for the ability of HSCs to self-renew throughout an organism's lifetime, and is thus essential for HSC longevity.

RESTRICTION OF HSC CELL CYCLE ENTRY: CELL-AUTONOMOUS AND NON-AUTONOMOUS CUES

The finding that HSCs proliferate rapidly in the fetal liver, but then exit the cell cycle within a few weeks of seeding the bone marrow suggests that there may be factors in the bone marrow, so-called niche factors, required for the establishment and maintenance of quiescence. Indeed, several studies have demonstrated that specific bone marrow cytokines or factors may be required for efficient HSC cell cycle exit.

Thrombopoietin (TPO), a cytokine initially described for its role in promoting megakaryocye/platelet formation, is required for BM HSC quiescence and long-term self-renewal [32, 33]. Human patients with mutations in c-mpl, the TPO receptor, initially present with thrombocytopenia, but eventually progress to pancytopenia [34]. This suggests roles for TPO beyond megakaryocyte development. Indeed, HSCs from mice that lack TPO expand normally in the fetal liver, but become depleted in the bone marrow within 1 year of birth [32]. Assessment of HSCs prior to depletion reveals that they fail to

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enter quiescence upon reaching the bone marrow. This finding was linked to an inability to up-regulate expression of the cyclin-dependent kinase inhibitor (CDKI) *p57* [32, 33]. *p57* is critical to the maintenance of HSC quiescence [35, 36]. The transplantation of fetal liver and adult HSCs from *Tpo*-deficient donors revealed strikingly different outcomes [32]. If fetal liver HSCs from *Tpo*-deficient donors were transplanted into *Tpo*-sufficient recipients, they engrafted and maintained long-term hematopoiesis as well as wild-type donor HSCs. If HSCs from the bone marrow of young adult *Tpo*-deficient donors were transplanted, they showed significant functional defects. This suggested that the inability to establish quiescence had severely limited the self-renewal capacity of adult HSCs and that rapidly cycling fetal liver HSCs must have mechanisms to sustain enhanced self-renewal that do not exist in adult BM HSCs. The nature of these mechanisms remains to be determined.

Angiopoietin-1 has similarly been linked to the maintenance of HSC quiescence and preservation of self-renewal activity [37]. Treatment of HSCs with angiopoietin-1 *in vitro* limited cell division, and resulted in preservation of self-renewal activity in hematopoietic transplantation assays after cell culture. Furthermore, Tie-2, the receptor of angiopoietin-1, was found to mark quiescent HSCs *in vivo*, suggesting that angiopoietin-1/Tie-2 signaling could play a role in HSC quiescence *in vivo*. Direct testing of HSC cell cycle status or self-renewal activity in the absence of angiopoietin-1/Tie-2 signaling has not been reported, and is required to further examine the role that this pathway plays in the establishment and/or maintenance of HSC quiescence.

TGF-β signaling, like angiopoietin-1, limits HSC expansion *in vitro* and preserves self-renewal activity in transplantation assays [38]. The presence of TGF-β in culture

restricted HSCs from dividing, and this was found to facilitate stable long-term engraftment in transplant recipients, consistent with enhanced self-renewal. Reduced cell cycle activity was shown to be associated with increased expression of *p57*, again linking a cytokine to cell cycle regulation through this CDKI in the bone marrow [38, 39]. Further support for the role of TGF-β signaling in HSC self-renewal was provided by the finding that the loss of *smad4*, encoding a downstream transducer of TGF-β signaling, resulted in severe impairment of HSC function in transplantation experiments [40]. Together, these data supported a significant role for TGF-β in restricting HSC cell cycle entry and thus in preserving HSC self-renewal potential.

CXCL12, a chemokine expressed on perivascular stromal and endothelial cells in the bone marrow, is essential for HSC homing to the bone marrow, BM retention, and functional maintenance [41, 42]. CXCR4, the receptor for CXCL12 on HSCs, was shown to restrict HSC cell cycle entry and preserve quiescence [43]. *CXCR4*-deficient HSCs lost quiescence and showed increased cell cycle entry, as well as increased sensitivity to treatment with 5-fluoruracil treatment, an antimetabolite that kills dividing cells. Importantly, *CXCR4*-deficient HSCs also demonstrated reduced *p57* gene expression.

The above studies indicate that CXCR4, Angiopoietin-1, TPO, and TGF-β all promote HSC quiescence in addition to expression of *p57*. Given that p57 is known to restrict cell cycle activity, it is attractive to postulate that p57 is a critical, cell-autonomous factor in establishing and/or maintaining HSC quiescence. Direct evidence for this idea was provided in recent studies demonstrating that p57, along with the related CIP/KIP family member p27, cell-autonomously maintains HSC quiescence *in vivo* [35, 36]. p57 played the dominant role in maintaining quiescence, though in the absence of

p57, p27 could partially compensate. In the absence of both p27 and p57, HSCs did not maintain quiescence and were eventually depleted. Transplantation assays revealed that this loss of quiescence significantly impaired self-renewal activity. These data reveal the essential role for CDKI expression in the maintenance of self-renewal, and support a link between niche signaling and the regulation of HSC quiescence.

In addition to niche-mediated regulation of CDKI expression, HSCs actively restrict cell cycle entry in response to mitogenic signals from the phosphatidylinositol-3-OH kinase (PI(3)K) pathway. PI(3)K signaling is critical to mediate the HSC response to pro-proliferative signals. Within this pathway, PTEN serves to attenuate PI(3)K signaling, thus limiting HSC proliferation. When PTEN was deleted, the HSC pool entered the cell cycle and transiently expanded before becoming profoundly depleted [44]. Additionally, *Pten*^{-/-} HSCs had reduced self-renewal potential in bone marrow transplantation assays, suggesting that the increased responsiveness to proliferative stimuli was detrimental to self-renewal. *Pten* deficiency eventually resulted in the development of highly penetrant leukemia in mice. Prior to depletion, *Pten*^{-/-} HSCs up-regulated expression of the tumor suppressors *Cdkn2a* and *p53*, and progression to leukemia was associated with the loss of tumor suppressors through secondary mutations [45]. This suggested a model in which increased proliferative stress on HSCs was detrimental to self-renewal in normal hematopoiesis. At the same time, the accrual of secondary mutations in a cell population that had preexisting self-renewal potential provided a substrate for leukemogenesis.

REGULATION OF HSC SELF-RENEWAL

While extensive self-renewal is a fundamental feature of HSCs and is required for long-term function after transplantation, adult HSC self-renewal capacity is not unlimited. This phenomenon is clearly demonstrated in serial transplantation assays in which HSCs were successively transplanted 4-6 times, showing progressive functional decline [46, 47]. When genes required to promote HSC quiescence are mutated, HSCs often transiently expand but almost inevitably progress to functional decline [44, 48-51]. As indicated by the *Pten*$^{-/-}$ model, this limitation in self-renewal potential may in fact be adaptive to prevent leukemogenesis [25]. In this section, we discuss the diverse mechanisms through which HSCs promote and limit self-renewal activity.

Telomeres and HSC self-renewal

Telomeres, the repetitive genomic sequences that cap linear chromosomes, have long been proposed to be a mitotic clock, limiting the lifespan of various proliferating cell populations [52]. With each round of replication, the inability to completely replicate the 3' ends of DNA strands results in genomic erosion. If left unchecked, this erosion would eventually result in the loss of essential genetic material (the "end replication problem"). The telomere buffers DNA ends from such loss. Certain long-lived cell populations that maintain proliferative potential, including HSCs, express the ribonucleoprotein telomerase which can extend telomeres [53]. This activity is required for the maintenance of HSC self-renewal.

Since mice have long telomeres compared to humans, several generations of breeding are required before the effects of telomere shortening can be observed [54-56]. Initial studies reported that late generation telomerase-deficient mice had significantly

reduced hematopoietic output [56]. This reduced output was linked to decreased HSC function. When early generation telomerase-deficient HSCs were subjected to serial transplantation and thus forced to self-renew extensively, they exhibited severe functional deficits, consistent with an accelerated decline in self-renewal potential [57]. Analysis of telomere length in this system demonstrated that this self-renewal defect occurred concurrently with telomere shortening. These data suggest that in situations of severe hematopoietic stress, telomerase expression and the regulation of telomere length are absolutely required for the maintenance of HSC function. Interestingly, over-expression of telomerase did not by itself increase HSC longevity [58]. This suggests that while telomerase activity is necessary for prolonged HSC function, it is not sufficient to confer enhanced self-renewal capacity.

In addition to telomerase, telomeres require the shelterin protein complex for stability. This six-member protein complex physically protects telomeres from aberrant recognition by cellular DNA damage machinery (reviewed in detail in **Chapter 4**). Recent work indicates that individual components of the shelterin complex may be required for HSC maintenance [59, 60]. Due to differing functions among shelterin proteins in telomere protection, rigorous examination of HSC function in the absence of individual shelterin components must be evaluated to understand how shelterin contributes to HSC homeostasis.

Epigenetic regulation of HSC self-renewal

Many essential epigenetic regulators can be classified into one of two categories: regulators of DNA methylation and regulators of histone modifications. With regard to the former category, several genes have recently been identified that directly impact HSC

self-renewal. DNA methyltransferase 1 (DNMT1) was classified as a maintenance methyltransferase due to its function in reestablishing DNA methylation patterns reflecting those of a complimentary DNA strand after strand replication. *Dnmt1* deletion resulted in defective self-renewal in adult HSCs [61]. Interestingly, *Dnmt1* inactivation in primary mice did not cause major hematopoietic defects in primary mice. However, when wild-type HSCs were transplanted into unirradiated *Dnmt1*$^{-/-}$ mice, they stably engrafted the *Dnmt1* hosts. This suggested that *Dnmt1*-deficient HSCs were not as stably associated with the niche as wild-type HSCs. Additionally, when *Dnmt1*$^{-/-}$ HSCs were transplanted into lethally irradiated recipients, they were functionally deficient, consistent with reduced self-renewal capacity. Since DNA methylation is associated with gene repression, it was predicted that *Dnmt1* loss might lead to de-repression of multiple genetic targets. This was only partially true, as gene expression was both positively and negatively altered after *Dntm1* deletion, indicating a complex function of DNMT1 in gene expression and self-renewal.

DNMT3a and DNMT3b were both classified as *de novo* DNA methyltransferases, referring to the fact that they establish patterns of DNA methylation based on DNA sequence recognition and not on pre-existing complimentary strand methylation patterns. Initial studies demonstrated that HSCs deficient for both DNMT3a/b could not reconstitute lethally irradiated recipients, identifying a critical function for these methyltransferases in HSC self-renewal [62]. Deletion of *Dnmt3a* or *Dnmt3b* alone did not elicit a reported phenotype in these studies. However, recent studies reevaluating the function of DNMT3a in HSC function demonstrated that *Dnmt3a*-deficient HSCs actually had enhanced self-renewal, but impaired differentiation ability [63]. This finding

was reported following bone marrow transplantation experiments that showed HSC expansion out of proportion to their contribution to mature blood lineage reconstitution. In parallel, the authors observed de-repression of several genes associated with HSC identity and a paradoxical loss of expression of genes associated with lineage commitment. The mechanism by which *Dnmt3a* loss both positively and negatively affects gene expression is not clear. It is possible, however, that initial *Dnmt3a* deletion resulted in a de-repression of specific direct genetic targets, while the subsequent loss of lineage specification genes might have been a secondary consequence.

Like DNA methylation, histone modification plays an essential role in the epigenetic regulation of gene expression. Many histone-modifying enzymes are classified into two diverse categories of epigenetic regulators: the Polycomb group (PcG) and the Trithorax group (TrG). The PcG class of epigenetic regulators is typically associated with gene repression. Within this group, the protein product of *Bmi1*, a ubiquitin ligase with histone 2a lysine 119 specificity, plays a critical role in adult HSC self-renewal [64]. *Bmi1*-deficient animals have normal fetal liver HSC development, but progress to hematopoietic failure with HSC depletion by young adulthood. Neither fetal nor adult HSCs are capable of long-term reconstitution in bone marrow transplantation assays, demonstrating a critical role for Bmi1 in HSC self-renewal. This reduced self-renewal potential is partially explained by cell-autonomous de-repression of *Cdkn2a* and *Cdkn2d*, and premature HSC senescence [64]. Additionally, cell non-autonomous effects attributed to *Bmi1* deficiency in the BM niche play a significant role in HSC functional decline independent of *Cdkn2a* and *Cdkn2d* [65]. Thus, Bmi1 has complex cell-autonomous and non-autonomous functions in the maintenance of HSC self-renewal.

Ezh1, a PcG member whose protein product methylates histone 3 lysine 27 residues, has similarly been linked to HSC self-renewal [66]. As with Bmi1 loss, Ezh1 loss prevented stable long-term reconstitution in transplantation assays. In this model, self-renewal defects were rescued by *Cdkn2a* deletion, again suggesting that premature senescence was linked to defective self-renewal. Thus, it seems that PcG members share a common role in suppressing cellular senescence in HSCs, and that this function is required for the long-term maintenance of HSC self-renewal potential.

Whereas PcG members are associated with target gene repression, TrG members are required for the activation or maintenance of gene expression. The role of the TrG in mammalian hematopoiesis is reviewed extensively in **Chapter 2**. Briefly, *Mll1* is a histone 3 lysine 4-specific methyltransferase required for target gene expression. Loss of *Mll1* results in aberrant HSC cell cycle entry and defective self-renewal in bone marrow transplantation assays [67, 68]. Unlike *Ezh1* or *Bmi1* deficiency, which caused self-renewal defects due to premature senescence, self-renewal defects due to *Mll1* deletion correlated with excessive cell cycle entry. This phenomenon is a poorly defined concept known as HSC exhaustion that broadly describes reduced HSC function and self-renewal defects that follow a period of excessive HSC cell cycle activity. The molecular mechanisms that contribute to exhaustion are unclear, though they are believed to relate to increased metabolic stress and DNA damage that occurs due to aberrant cell cycle entry, similar to the prevailing model to explain HSC defects in the absence of PTEN.

Mll5 was defined as a TrG member due to homology to *Mll1*, though SET domain functionality has not been identified. *Mll5* deficiency increased HSC cell cycle entry, though not as robustly as in *Mll1*-deficient HSCs [69-71]. In addition to enhanced cell

cycle entry, *Mll5*-deficient HSCs failed to support long-term hematopoietic reconstitution in transplantation assays, again indicating a functional role for *Mll5* in HSC self-renewal [70, 71]. Our current understanding of the mechanisms by which Mll1 and Mll5 impact self-renewal is reviewed in **Chapter 2**.

Taken together, these data indicate that epigenetic regulation plays an essential, diverse role in the maintenance of HSC self-renewal. Though effects on self-renewal have been identified with respect to Dnmt1, Dnmt3a, Bmi1, Ezh1, Mll1, and Mll5, the molecular mechanisms by which each of these genes contributes to self-renewal remain poorly defined. Furthermore, the potential interactions between different epigenetic regulators in controlling HSC function have not been explored. Such studies will provide insight into the complex means through which epigenetic modifiers affect HSC self-renewal.

ROS, Metabolism, and HSC Self-Renewal

Reactive oxygen species (ROS), a byproduct of oxidative metabolism, critically limit HSC function (reviewed in [72]). It is thought that to control exposure to oxygen toxicity, quiescent HSCs localize to a hypoxic niche [73]. Within this niche, HSCs further suppress ROS accumulation through increased expression of FoxO family members and the DNA damage response protein ATM (encoded by *Ataxia telangectasia mutated*) [74, 75]. Reduced ROS suppression due to the loss of either of these key factors results in compromised HSC self-renewal. Similarly, an inability to mount a Hif-1α-dependent oxidative stress response results in HSC depletion, aberrant cell cycle entry, and reduced self-renewal potential [76]. These data support the concept that HSCs must efficiently limit ROS exposure to maintain self-renewal and normal functionality.

Consistent with a preference for hypoxic conditions, HSCs require efficient

gycolysis for long-term maintenance [77]. HSCs in which *Pyruvate dehydrogenase*

kinase 1 and *2* were deleted to inhibit glycolysis aberrantly entered the cell cycle, became

depleted, and lost self-renewal capacity in transplantation assays. Despite the HSC

preference for low oxygen conditions and glycolysis, recent studies have sought to

evaluate the role of mitochondria and oxidative metabolism in HSC self-renewal.

PTPMT1 is a mitochondrial phosphatase with an unclear physiological function, but that

is required for oxidative metabolism. Supporting the notion that HSCs are more sensitive

to disruption in glycolysis than mitochondrial aerobic respiration, inhibiting

mitochondrial function through *PTPMT1* deletion did not limit HSC self-renewal [78].

Interestingly, *PTMPT1*-deficient HSCs failed to differentiate into mature lineages. This is

hypothesized to be because lineage-committed progenitors require aerobic metabolism,

which was perturbed by PTPMT1 loss. Thus, whereas HSCs are able to survive and self-

renew in the absence of PTPMT1, downstream progenitors dependent on efficient aerobic

metabolism are sensitive to this loss.

While work with PTMPT1 suggested that HSC self-renewal was independent of

oxidative metabolism, studies focused on Lkb1 revealed that mitochondrial function

might still be essential for HSC maintenance. Lkb1 was initially identified as a positive

regulator of AMPK involved in reducing cellular energy expenditure by limiting

macromolecule synthesis. While the significance of Lkb1 regulation of AMPK in HSCs

is unclear, a critical role in HSC mitochondrial function has been elucidated [79-81].

Following *Lkb1* deletion, HSCs lost quiescence and transiently expanded before being

rapidly depleted [79, 81]. *Lkb1*$^{-/-}$ HSCs demonstrated profound self-renewal limitations in

bone marrow transplantation [79-81]. Analysis of the defective *Lkb1*^{-/-} HSCs revealed

reduced mitochondrial mass, mitochondrial membrane potential, and ATP synthesis,

suggesting that perturbed mitochondrial function contributed to the observed HSC

defects. These data suggest that HSCs require a low, basal level of oxidative metabolism.

According to this hypothesis, PTMPT1 loss, while promoting reduced respiration, did not

reduce metabolic output below this level. Lkb1 loss, on the other hand, limited

mitochondrial function so that metabolic output was below even this low threshold,

resulting in HSC defects. Although clarification is needed, these findings illustrate the

need to further explore the metabolic regulation of HSC function.

Inflammation and HSC self-renewal

Recent studies demonstrate that HSCs can enter the cell cycle and robustly

expand in response to bacterial infection [82]. This is hypothesized to facilitate the

expansion of downstream cell populations that are required to combat infection. While

this transient HSC expansion is adaptive, several lines of evidence suggest that chronic

infection has detrimental effects on HSCs.

Interferons are cytokines that play critical roles in the immune response to various

forms of infection. Acute exposure to interferon-α (IFNα), a type I interferon, resulted in

increased HSC proliferation, but did not impair HSC function [83]. When HSCs were

subjected to chronic IFNα exposure, however, self-renewal activity was severely

impaired in transplantation assays, suggestive of HSC exhaustion. Similarly, when HSCs

were chronically exposed to interferon-γ (IFNγ), a type II interferon, during chronic

infection with *Mycobacterium avium,* increased cell cycle entry was observed followed

by impaired self-renewal in transplantation assays [84]. Collectively, these data demonstrate that while an adaptive expansion of HSCs might occur during infection, chronic infection and associated HSC proliferation impair long-term self-renewal.

Additional studies demonstrate that HSCs have evolved intrinsic mechanisms that limit their response to infection. Deletion of the negative regulator of interferon signaling *Irf2* resulted in an IFNα-dependent HSC depletion [85]. Loss of this negative regulator exacerbated HSC exhaustion following chronic IFNα exposure. Similarly, loss of *Igrm1*, another negative regulator of interferon signaling, resulted in defective HSC self-renewal in an interferon-dependent fashion [86]. These mechanisms dampen HSC responsiveness to infections and are required to preserve HSC self-renewal in pathological conditions.

SUMMARY

In this report we describe two novel regulators of HSC maintenance and self-renewal. **Chapter 3** describes *Absent, small, or homeotic 1-like* (*Ash1l*), a member of the TrG, as being an essential regulator of HSC function. We discovered that *Ash1l* plays a critical role in the establishment of HSC quiescence in the bone marrow, at least in part through regulation of CDKI expression. Loss of quiescence in this model results in the absence of detectable long-term self-renewal activity in transplantation assays. We further demonstrate that *Ash1l* functionally cooperates with *Mll1* to maintain hematopoietic homeostasis, thus representing the first identification of *in vivo* cooperativity between TrG members in mammalian developmental biology. Surprisingly, despite a severely compromised HSC compartment, *Ash1l*-deficient mice do not succumb to hematopoietic failure. We observe increased cell cycle activity in downstream

progenitors, suggesting that they may have sufficient self-renewal activity to maintain hematopoiesis in the absence of HSCs.

Chapter 5 describes that Tpp1 (encoded by *Acd*), a member of the shelterin complex of telomere-protecting proteins, is also critical for HSC self-renewal. *Acd* loss results in acute HSC depletion and an inability for HSCs to self-renew in transplantation assays. This functional decline is preceded by p53 activation and cell cycle arrest, though unlike in other stem cell compartments, genetic *p53* ablation does not rescue HSC function. *Acd* deficiency thus provides a novel model of acute telomere deprotection in stem cells and implicates Tpp1 as an essential factor in the preservation of HSC self-renewal potential. These findings contrast previous studies that described a gradual progression to hematopoietic failure following shelterin loss. Our data suggest that acute stem and progenitor dysfunction may underlie hematopoietic failure and thus should be rigorously evaluated in other models of acute telomere deprotection.

CHAPTER 2. THE TRITHORAX GROUP: A CONSERVED CLASS OF EPIGENETIC REGULATORS WITH ESSENTIAL FUNCTIONS IN HEMATOPOIESIS

INTRODUCTION

The function of the trithorax group (TrG) of epigenetic regulators has been of particular interest since the discovery that *Mixed-lineage leukemia-1* (*Mll1*) is a frequent fusion partner in a subset of pediatric and adult leukemias [87-89]. This gene is the mammalian homolog of *Drosophila trithorax*, a TrG member with a well-characterized role in *Drosophila* body patterning. Understanding the conserved mechanisms through which TrG members regulate developmental body patterning has provided important insight into how TrG members regulate normal and malignant hematopoiesis.

THE TRITHORAX GROUP (TRG) IN *DROSOPHILA* BODY PATTERNING

Drosophila anterior-posterior body segmentation requires precise, ordered gene expression at loci encoded within the *antennapedia* and *bithorax* complexes (reviewed in [90]). Together, these clusters encode 8 *homeobox* (*hox*) genes that are expressed along the anterior-posterior body axis in the order in which they are encoded within the cluster. Transcription is initiated from these loci based on a gradient of maternally encoded transcription factors. After this initiation, Polycomb group (PcG) and Trithorax group (TrG) proteins are required for the maintenance of *hox* expression patterns, and thus for proper body segmentation as the fly embryo expands. TrG and PcG members achieve this activity through positive and negative regulation of *hox* targets, respectively.

Inappropriate *hox* gene expression during development is sufficient to transform one body segment into another reflective of the altered *hox* expression ("homeotic transformation").

In flies, members of the TrG were defined by three main characteristics: 1. Mutations in TrG members result in homeotic transformations consistent with *hox* loss-of-function; 2. Combined heterozygous mutations of different group members reveal that they serve as dominant enhancers of one another, increasing the penetrance of homeotic phenotypes; 3. Mutations in TrG members suppressed PcG loss-of-function phenotypes (which reflected *hox* gain-of-function mutations) [91]. *trithorax*, the archetypal TrG member, was originally described in flies with homeotic transformations consistent with loss of *bithorax* cluster expression [92, 93]. This identification came from body patterning screens in which mutations were identified that did not map to a known *hox* locus. Furthermore, mutant phenotypes were consistent with reduced expression of several *hox* loci within the *bithorax* cluster, suggestive of the loss of an upstream regulator. Later work revealed that in addition to causing reduced *bithorax* expression, *trithorax* loss-of-function mutations decreased *antennapedia* complex expression and suppressed the effects of *polycomb* mutations [94]. At the time, it was clear that *polycomb* acted as a negative regulator of the *bithorax* cluster, and together, these data demonstrated that *trithorax* was a positive regulator of *Drosophila hox* genes.

Two additional members of the TrG were identified in studies pursuing a *Drosophila* mutant phenotype characterized by prominent imaginal disc defects [95]. Imaginal discs are the precursors of *Drosophila* appendages and, as for body segmentation, require defined *hox* expression patterns for proper specification. Each set

of appendages (i.e. the antennae, wings, legs, eyes) develops from a pair of imaginal disc precursors. A series of imaginal disc mutants in which the discs were either absent, small, or homeotically transformed into a different disc (i.e. antenna disc to leg disc resulting in leg growth where an antenna should be) were initially described in 1971 [95]. Over a decade later, two genes, termed *absent, small, or homeotic 1* and *2* (*ash1* and *ash2*) were shown to be the causal loci accounting for these mutant phenotypes [96].

The identification of *ash1*, *ash2*, and *trithorax* resulted in the description of the Trithorax group (TrG) [97]. Each member of this group promotes expression of *hox* cluster genes. The combined heterozygosity for loss-of-function mutations in any pair of these genes results in enhancement of homeotic phenotypes. Phenotypes associated with *polycomb* loss-of-function mutations (consistent with *hox* gain-of-function) are suppressed by mutation in any of these TrG members. Together, this report identified the TrG as a group of functionally related proteins that promoted gene expression, and acted in opposition to PcG members to preserve proper body patterning.

A subsequent screen for suppressors of *polycomb* phenotypes revealed that *brahma* also met the criteria for inclusion in the TrG [98]. As was true for other TrG members, *brahma* loss-of-function resulted in phenotypes consistent with loss of *antennapedia* and *bithorax* complex expression. Additionally, *brahma* mutations enhanced phenotypes associated with *trithorax* and *ash1* mutations, demonstrating a functional interaction between *brahma* and *trithorax* and therein fulfilling the criteria for TrG inclusion [98, 99].

While TrG members are functionally related in promoting *hox* gene transcription, the mechanisms through which they do so are diverse. *ash1* and *trithorax* both encode

large proteins that contain a SET domain [100, 101]. This domain, named for its presence in the su(var)3-9, e(z), and trithorax proteins, confers histone methyltransferase activity to ash1 and trithorax (reviewed in [102]). *ash2* does not encode a SET domain, though it may enhance histone methyltransferase activity of other SET domain-containing TrG members [103]. *brahma* is a homolog of a component of the yeast SWI/SNF complex [98]. This complex hydrolyzes ATP to physically move histones, and thus influences transcription through alterations in chromatin structure [104, 105]. These proteins are representative of the diverse mechanisms through which TrG members, of which 13 have been identified in total, promote gene expression from target loci (for additional TrG members, see [106]). While diverse biochemical functions have been identified for TrG members, the role that these functions may play in cooperativity between TrG members has not been identified.

THE TRG IN MAMMALIAN BODY PATTERNING

As in *Drosophila*, mammalian body patterning requires proper *Hox* gene expression. Whereas there are 8 *hox* genes in *Drosophila*, 39 *Hox* loci have been identified in mammals (reviewed in [107] and [108]). Mutations in multiple *Hox* loci cause homeotic transformations of vertebral segments [109-111]. This demonstrates that *Hox* gene expression is required for anterior-posterior segmentation in mammals, and suggests that similar regulatory mechanisms may exist between mammals and *Drosophila*. However, while significant work has been done in describing the roles of *Drosophila* TrG members as upstream regulators of body patterning, relatively little work has been done in the mammalian system. Here, we review our current understanding of how mammalian TrG members regulate the establishment of the body plan.

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Mll1

The mammalian homolog of *trithorax*, *Mixed-lineage leukemia 1* (*Mll1*), was

originally identified for its oncogenic role in pediatric leukemias (discussed below). Mll1

bares significant amino acid sequence homology to Set1 family histone

methyltransferases, a class of lysine modifying enzymes that promote gene expression

through histone 3 lysine 4 methylation [112-114]. Comparison of protein structural

domains between Mll1 and Setd1a, a classical Set1 family member, demonstrates that

both proteins possess a C-terminal SET domain followed by a post-SET domain (Figure

2.1). This C-terminal SET domain is characteristic of SET1-like proteins. Mll1 histone

methyltransferase activity requires interactions with the cofactors WDR5, Ash2l, and

RbBP5 for catalysis and with menin for binding to relevant loci [115-119]. Additional

specificity in Mll1 targeting is provided by its interaction with the PAF transcriptional

elongation complex. This interaction is required for Mll1 targeting to a subset of *Hox*

loci, and suggests an additional role for Mll1 in regulating transcriptional elongation at

individual target genes [120, 121]. In addition to cofactor-mediated targeting, Mll1 itself

contains several domains that promote interactions with DNA and chromatin. The N-

terminus contains 3 closely-spaced AT hooks, which are known to facilitate binding with

the minor groove of DNA [122]. These AT hooks are followed by a series of 3 PHD

fingers and a bromodomain, which bind methylated and acetylated lysine residues,

respectively [123, 124]. Mll1 therefore uses a combination of protein-protein, protein-

DNA, and protein-chromatin interactions for specific targeting to gene loci.
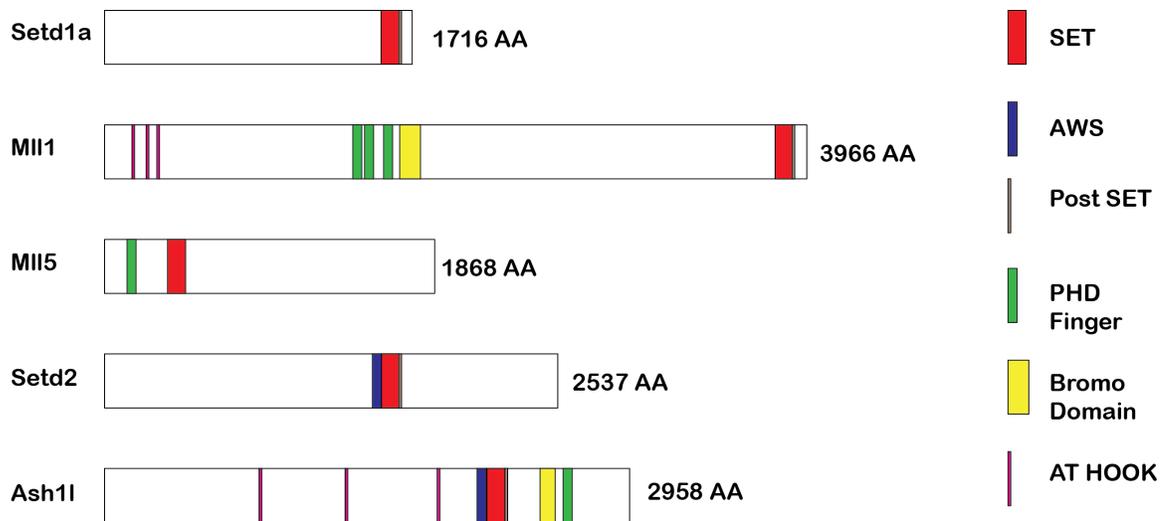
Figure 2.1. Structural organization of selected murine SET domain-containing proteins. Setd1a and Mll1 show conserved C-terminal SET domain localization characteristic of SET1 family histone methyltransferases. Setd2 and Ash1l show internal SET domains characteristic of SET2 family histone methyltransferases. Mll5 does not clearly fit a SET1 or SET2 family classification due to its N-terminal SET domain. All images are drawn to scale and conserved domains were identified using UniProt.

Initial characterization of mice heterozygous for an *Mll1* null allele described prominent homeotic transformations along the vertebral column consistent with reduced *Hox* gene expression [125]. Further analysis revealed that *Hox* boundaries were shifted in heterozygous animals, while animals with a complete loss of *Mll1* expression eventually lost *Hox* expression and died *in utero*. These data established that *Mll1*, like its homolog, is required for proper *Hox* expression and subsequent body segmentation in mammals.

Developmental *Hox* gene defects and consequent homeotic transformations have been linked to the catalytic activity of the Mll1 SET domain [126]. Ablation of the SET domain recapitulated many of the axial skeletal phenotypes and *Hox* expression abnormalities reported in *Mll1* loss-of-function studies. This work established that Mll1 histone methyltransferase activity is required for normal *Hox* gene expression. Of note, SET domain deletion did not cause embryonic lethality, as was reported with the *Mll1* null allele, suggesting that Mll1 may have additional functions in development beyond

SET domain-mediated catalysis [125, 126]. While SET-independent functions remain to be explored, the catalytically inactive *Mll1* revealed that SET domain activity was specific to methylation at histone 3 lysine 4 (H3K4) residues [126]. Subsequent analyses showed that Mll1 is predominantly responsible for H3K4 di- and trimethylation, a histone mark associated with positive regulation of gene expression [115, 127]. Together, these data indicate that Mll1 directly regulates transcriptional output from target genes, including those of the *Hox* cluster.

Further studies demonstrated that *Mll1* is required for the maintenance of *Hox* gene expression in developing mouse embryos [128]. While *Mll1*-deficient fetuses normally expressed *Hox* genes at early developmental time points, *Hox* expression was eventually lost, suggesting that *Mll1* is not required for initiating *Hox* gene expression, but rather for maintaining expression as tissues expand. This reflects a conserved functional paradigm between *Drosophila* and mammals in which Mll1 is required to preserve cellular memory of *Hox* expression patterns during body segmentation.

In addition to positively regulating *Hox* gene expression, Mll1 antagonizes the function of the PcG member Bmi1 [129]. Combined deficiencies in *Mll1* and *Bmi1* suppressed homeotic transformations of the axial skeleton. Studies of gene expression differences in MEFs derived from *Mll1*- and *Bmi1*-deficient embryos identified complementary patterns of *Hox* gene expression where genes that appeared to be de-repressed in the absence of Bmi1 failed to be expressed when Mll1 levels were reduced. This suggests the existence of a highly conserved crosstalk between mammalian TrG and PcG proteins in developmental body patterning and *Hox* gene expression.

Ash1l

Beyond studies of Mll1 in body patterning, little work has been done characterizing the role that other TrG homologs might play in development. Recent evidence suggests that *Ash1-like* (*Ash1l*), the homolog of fly *ash1,* has a significant role in axial skeletal development (Camper et al., personal communication). Mice homozygous for a "gene trap" loss-of-function allele that results in profound hypomorphism for *Ash1l* (described in **Chapter 3**) display prominent rib patterning defects, predominantly in the form of extra thoracic ribs with abnormal sternal attachments. This finding was reminiscent of phenotypes ascribed to *Hox* loss-of-function and suggests that *Ash1l* plays a conserved function in mammalian body patterning, though additional studies will be required to identify the precise molecular targets involved in axial skeletal development.

By analogy to Mll1, it is tempting to speculate that Ash1l functions in positive regulation of *Hox* expression through SET domain catalysis. Several independent groups have associated Ash1l SET domain activity with H3K4, H3K9, H3K36, and H4K20 methylation [130-133]. Recent studies using rigorous biochemistry and crystallography indicated that H3K36 dimethylation is the most likely specificity for the Ash1l SET domain [134, 135]. Supporting this finding, amino acid sequence analysis reveals significant homology to Set2 proteins, a family of histone 3 lysine 36-specific methyltransferases [133, 136]. Histone 3 lysine 36 methylation is a cotranscriptional histone modification that facilitates effective transcription through promoting efficient mRNA splicing and limiting aberrant initiation from cryptic promoters [137-139]. As is characteristic of Set2 family members, the Ash1l SET domain is internal to the protein, as opposed to being at the C-terminus like Mll1 (**Figure 2.1**). Amino acid sequence analysis

reveals an associated-with SET (AWS) domain upstream of the Ash1l SET domain; this feature is shared among Set2 family members. Ash1l also includes 3 AT hooks, a bromodomain, and a PHD finger, thus promoting DNA, acetylated lysine, and methylated lysine binding, respectively. Additional cofactors required for Ash1l targeting to gene loci have not yet been identified. Moreover, direct catalytic function of Ash1l at target loci has not been described *in vivo*. Thus, while it seems that Ash1l directly binds several *Hox* loci in cultured cells, the physiological significance of this binding is unknown [130].

THE TRG AND HEMATOPOIESIS

As implied by the name, *Mll1* (in humans *MLL1*) was originally identified in the context of hematopoiesis, and specifically in leukemia. Work from several groups demonstrated that the *MLL1* locus was involved in chromosomal translocations at the 11q23 locus in pediatric and adult leukemia patients with an increased incidence in infants and patients with secondary leukemias after exposure to topoisomerase II inhibitors [87-89]. Understanding the mechanistic implications of this translocation is significant, as patients with *MLL1* fusions have a particularly poor prognosis. We now know that there are more than 60 gene fusions involving *MLL1* [140]. While these fusions are diverse and often result in the loss of the MLL1 C-terminal SET domain, they uniformly cause increased *Hox* gene expression [141, 142]. These findings suggest a significant role for both *Hox* genes and MLL1 in hematopoiesis.

Initial studies focused on identifying a potential role for Mll1 in hematopoiesis demonstrated critical functions in fetal hematopoietic stem cell development. The assessment of fetal liver hematopoiesis in *Mll1*-deficient mice revealed reduced

cellularity, though mutlilineage differentiation appeared intact [143]. This was initially attributed to proper HSC specification, but subsequent failure in HSC fetal liver expansion. Later studies identified that *Mll1*-deficient HSCs were already significantly reduced in the AGM region, suggesting a role for Mll1 in the emergence of definitive hematopoiesis [144].

Studies using embryoid bodies derived from *Mll1*-deficient embryonic stem cells revealed the role for Mll1 regulation of *Hox* genes in hematopoietic development [145]. Analysis of *Hox* gene expression over the course of hematopoietic specification from embryoid bodies revealed that several genes, including *Hoxa7*, *Hoxa9*, and *Hoxa10* were significantly upregulated during this process. Importantly, these genes have also been identified as targets of MLL1 leukemogenic fusions [141, 142, 146]. *Mll1*-deficient embryoid bodies failed to upregulate expression of these *Hox* genes, and hematopoietic progenitors derived from these embryoid bodies failed to expand [145]. This provided the first evidence that a Mll1-dependent *Hox* program may be required for hematopoietic development, though this evidence is indirect, as a failure to specify normal hematopoietic progenitors for other reasons could result in reduced *Hox* expression.

Mll1-deficient embryos had significant developmental defects outside of hematopoiesis, thus complicating the *in vivo* understanding of Mll1 function. Though the embryoid body studies strongly suggested that Mll1 drives *Hox* expression, additional genetic tools were required to test the cell-autonomous function of *Mll1* in HSC development and function. Such tools were provided when two groups independently developed *Mll1* conditional alleles [67, 68]. Conditional inactivation of *Mll1* revealed that Mll1 was required for the maintenance of HSC self-renewal [67, 68]. Transplantation

assays demonstrated that *Mll1* deficiency was incompatible with long-term reconstitution, the gold standard for testing HSC self-renewal. Furthermore, *Mll1* deficiency resulted in the loss of HSC quiescence prior to depletion, suggesting that *Mll1*-deficient HSCs aberrantly entered the cell cycle [67]. *Mll1*-deficient hematopoietic progenitors lost expression of *Hoxa7*, *Hoxa9*, and *Hoxa10* prior to the observed self-renewal defects. These data expanded our understanding of the function of Mll1 in hematopoiesis and identified that Mll1 function was critical for HSC maintenance and self-renewal.

Intriguingly, the two studies identifying profound HSC defects in the absence of Mll1 reported different phenotypic severities, though both reports described comparable self-renewal defects upon transplantation. Jude and colleagues described a rapid progression to HSC depletion and bone marrow failure shortly after induction of *Mll1* excision [67]. This latter finding was linked to additional roles of Mll1 in myeloid and lymphoid progenitor function. McMahon and colleagues reported that they could stably excise *Mll1* in fetuses and that this did not cause a defect in postnatal hematopoiesis in the absence of transplantation [68]. This study did identify reduced myeloid progenitor function in methylcellulose cultures, indicative of a role for Mll1 downstream of HSCs. Thus, the predominant difference between these two studies was the extent of HSC depletion in primary mice and progression to bone marrow failure. The reason for this difference is unknown, though perhaps alternative means of conditional allele generation or different Cre induction strategies between the groups could account for these findings.

The tumor suppressor menin was demonstrated to be required for both endogenous Mll1 and Mll1 fusion protein targeting to *Hox* loci [118, 147-149]. This function is dependent on the link that menin provides between Mll1 and LEDGF as an

adaptor protein during Mll1 recruitment to target loci [117]. Thus, loss of *Men1* (encoding menin) is predicted to reflect *Mll1* loss-of-function in hematopoietic tissues. Analysis of *Men1* deficiency revealed that, as Mll1, menin is required for HSC self-renewal [150]. Similar to the phenotype reported by McMahon and colleagues, *Men1*deficiency resulted in HSC functional defects in transplantation assays, but not in primary mice. Hematopoietic progenitors had reduced expression of *Hoxa9* and *Hoxa10*, reminiscent of gene expression changes observed in *Mll1* deficiency. These data further support a critical role for Mll1 in HSC self-renewal, and thus in HSC function.

The finding that Mll1-driven leukemogenesis correlates with increased *Hox* expression and that HSC self-renewal defects in the absence of Mll1 were characterized by loss of *Hox* expression suggested that *Hox* genes might play critical roles in self-renewal. Indeed, *Hoxa9* deficiency results in impaired hematopoietic recovery following irradiation, which could reflect underlying HSC self-renewal defects [151]. Defective self-renewal was confirmed in transplantation assays, identifying *Hoxa9* as a key regulator of HSC self-renewal. However, this phenotype is not as severe as that which is observed when Mll1 is lost, consistent with the idea that Mll1 is an upstream regulator of *Hoxa9* and additional targets. Further supporting a role for *Hoxa9* in self-renewal, *Hoxa9* over-expression was capable of imparting increased self-renewal properties on hematopoietic progenitors, though generation of transplantable leukemias required co-expression of the *Hox* co-factor *Meis1* [146, 152, 153].

The TrG member Mll5 is a SET domain-containing protein with limited homology to Mll1 that includes a N-terminal SET domain lacking an identified function (**Figure 2.1**). Mll5 has also been implicated in regulating HSC function [69-71].

Hematopoietic progenitors lacking Mll5 entered the cell cycle at an increased rate, suggestive of a loss of quiescence [69, 71]. Hematopoietic stress induced by sublethal irradiation or 5-fluorouracil injection resulted in the progression to bone marrow failure in *Mll5*-deficient mice [70, 71]. While increased cell cycle activity can contribute to heightened sensitivity to either of these treatments, reduced self-renewal can also limit the expansion of the stem cell pool that is required for recovery. Subsequent testing of self-renewal in transplantation demonstrated severe HSC functional impairment in *Mll5* knockout animals [70, 71]. Further analysis of hematopoietic progenitors revealed that *Mll5* deficiency resulted in reduced expression of *Hoxa7* and *Hoxa9* [71]. Mll5, like Mll1, is therefore required for HSC self-renewal.

Beyond this body of work identifying roles for Mll1 and Mll5 in HSC self-renewal and hematopoietic homeostasis, little is known about the function of additional TrG members in mammalian hematopoiesis. Furthermore, while Mll1 and Mll5 seem to regulate partially overlapping genetic targets, cooperativity between these and any other TrG members in hematopoiesis has not been evaluated. Thus, while focus has been placed on Mll1 because of its role in human malignancy, much work remains in understanding the role that TrG proteins may have in HSC function and hematopoietic homeostasis.

CHAPTER 3. THE TRITHORAX GROUP MEMBER *ASH1L* FUNCTIONALLY COOPERATES WITH *MLL1* TO REGULATE HEMATOPOIETIC STEM CELL QUIESCENCE AND SELF-RENEWAL

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ABSTRACT

Hematopoietic homeostasis requires that rare hematopoietic stem cells (HSCs) balance the processes of differentiation and self-renewal to sustain long-term hematopoietic output. To preserve long-term function, HSCs largely remain outside of the cell cycle in a quiescent state that limits their exposure to replicative stress. In this study, we identify the Trithorax group (TrG) member *Absent, small, or homeotic 1-like* (*Ash1l*) as being required for the induction and maintenance of this quiescent state during the neonatal period. In the absence of *Ash1l*, HSCs actively cycle and eventually become profoundly depleted by young adulthood. This inability to achieve quiescence correlates with reduced expression of *p27* and *p57*, two cyclin-dependent kinase inhibitors required for the maintenance of quiescence. Intriguingly, bone marrow transplantation assays fail to identify HSC function in the fetal or adult *Ash1*-deficient bone marrow. Despite this finding, *Ash1l*-deficient mice do not progress to hematopoietic failure, suggesting that few remaining LT-HSCs or downstream progenitors are sufficient to maintain survival

for more than 6 months in this model. Additionally, we demonstrate that this HSC functional deficit results in niche availability such that *Ash1l*-deficient mice can be engrafted with wild-type HSCs in the absence of irradiation. *Ash1l* functionally cooperates with the TrG member *Mll1* to promote hematopoietic stem cell function and homeostasis, identifying conserved cooperativity between TrG members for the first time in mammals. These data demonstrate that *Ash1l* plays an essential role in the maintenance and function of HSCs.

INTRODUCTION

Sustained hematopoiesis requires that long-term hematopoietic stem cells (LT-HSCs) balance the processes of self-renewal and differentiation. To maintain self-renewal, LT-HSCs must primarily remain outside of the cell cycle, or in a quiescent state. Establishment of quiescence occurs within 4 weeks of homing to the bone marrow, marking a transition from robust cell cycle activity in the fetal liver to quiescence in the adult bone marrow [29]. The failure to establish or maintain quiescence is often linked to LT-HSC depletion, increased sensitivity to myelosuppression, and reduced engraftment in bone marrow transplantation assays [29, 32, 36, 154]. Thus, understanding the mechanisms through which LT-HSCs establish and maintain quiescence is critical to understanding self-renewal and long-term hematopoietic output from these rare cells.

The Trithorax group (TrG) is a diverse class of epigenetic regulators that was originally identified as being required for *Drosophila* body patterning (reviewed in [106]). Collectively, TrG proteins maintain proper expression of the *antennapedia* and *bithorax* complexes to affect proper segmentation during embryogenesis (reviewed in [90]). In *Drosophila,* individual TrG members function non-redundantly to promote gene

expression from these homeobox (*hox*) loci such that combined heterozygous mutations of TrG members function as dominant enhancers of one another [97, 99]. TrG members have a conserved function in developmental body segmentation in mammals. Mice deficient for *Mixed-lineage leukemia 1* (*Mll1*), the mammalian homolog of *trithorax*, have disrupted body segmentation due to a failure of the maintenance of *Hox* gene expression [125]. Additionally, mice deficient for the TrG member *Absent, small, or homeotic 1-like* (*Ash1l*), the mammalian homolog of *Drosophila ash1*, have disrupted body patterning as evidenced by axial skeleton abnormalities attributable to defective *Hox* gene expression (Brinkmeier and Camper, personal communication). While these data suggest that TrG members may share a conserved, non-redundant role in mammalian development, this concept has not yet been evaluated.

Past work demonstrated a critical role for *Mll1* in both normal and malignant hematopoiesis. *Mll1* was originally identified as a frequent partner in genetic fusions driving human leukemias in both infants and adults [87-89]. These leukemias demonstrate a characteristic aberrant upregulation of *Hox* gene expression [141, 142]. Additional work demonstrated that *Mll1* was required for the maintenance of normal hematopoietic stem cells (HSCs) through a critical function in the preservation of HSC self-renewal [67, 68]. *Mll1*-deficient mice had reduced *Hox* gene expression in HSCs, and this was believed to contribute to reduced HSC function. Since *Hoxa9*, a critical target of MLL1 in both leukemia and normal hematopoiesis, only contributes modestly to HSC self-renewal, additional, unidentified genetic targets must be dysregulated in *Mll1*-deficient HSCs to account for the profound functional defects [151]. These factors

remain to be elucidated. Furthermore, the role of additional TrG members in normal and malignant hematopoiesis has not been thoroughly evaluated.

The TrG member *ash1* was originally identified in *Drosophila* genetic screens seeking regulators of imaginal disc development [95]. It was later determined that *ash1* encodes a large protein containing a SET domain with putative histone methyltransferase activity [101]. Mammalian *Ash1l* encodes a similar SET domain-containing protein, and has been demonstrated to associate with actively transcribed gene loci, including at several *Hox* genes [130]. Recently, the Ash1l SET domain has been reported to have histone 3 lysine 36 dimethylase ability using *in vitro* biochemical assays, though the significance of this function *in vivo* has not been evaluated [133-135]. Interestingly, a recent report suggested that *Ash1l* functions in cultured hematopoietic cells and may cooperate with *Mll1* to drive *Hox* gene expression [155]. Neither the function of *Ash1l* nor the idea of cooperativity with *Mll1* has been evaluated rigorously with *in vivo* genetic models.

In this report, we describe an essential role for the TrG member Ash1l in the maintenance of adult, but not fetal HSCs. Our data demonstrate that *Ash1l* is required for HSC self-renewal in the bone marrow and for the establishment of quiescence at the fetal to adult transition. Despite a 5-10-fold reduction in phenotypic BM HSCs and an inability to detect functional HSCs in transplantation assays, *Ash1l*-deficient mice do not progress to hematopoietic failure. This might be attributable to increased proliferation in progenitors downstream of HSCs that maintain hematopoiesis. Additionally, we demonstrate that *Ash1l* cooperates with *Mll1* to maintain hematopoiesis at the level of

HSCs and progenitor cells. This report represents the first genetic demonstration of *in vivo* cooperativity between TrG members in mammals.

RESULTS

Ash1l^{GT/GT} fetal HSCs develop and expand normally

To examine the function of *Ash1l* in hematopoiesis, we utilized a gene trap insertion allele in which a potent splice acceptor cassette was placed in the first intron of the *Ash1l* locus (**Figure 3.1A**). This strategy resulted in a >90% reduction in full-length *Ash1l* transcripts in Lineage⁻Sca-1⁺cKit⁺ (LSK) hematopoietic progenitors both within fetal liver and bone marrow. E14.5 *Ash1l^{GT/GT}* fetal livers had normal cellularity and mature myeloid, erythroid, and B lymphocyte population frequencies compared to wild-type littermates (**Figure 3.1B, C**). To assess LT-HSC content, we utilized the SLAM (CD150⁺CD48⁻LSK) definition of LT-HSCs, as this strategy has been shown to robustly identify both fetal liver and adult bone marrow LT-HSCs [156, 157]. Phenotypic *Ash1l*-deficient LT-HSCs were present at a normal frequency in the fetal liver (**Figure 3.1D**). This demonstrated that *Ash1l^{GT/GT}* fetal LT-HSCs developed and expanded normally, and that they were capable of supporting hematopoiesis.
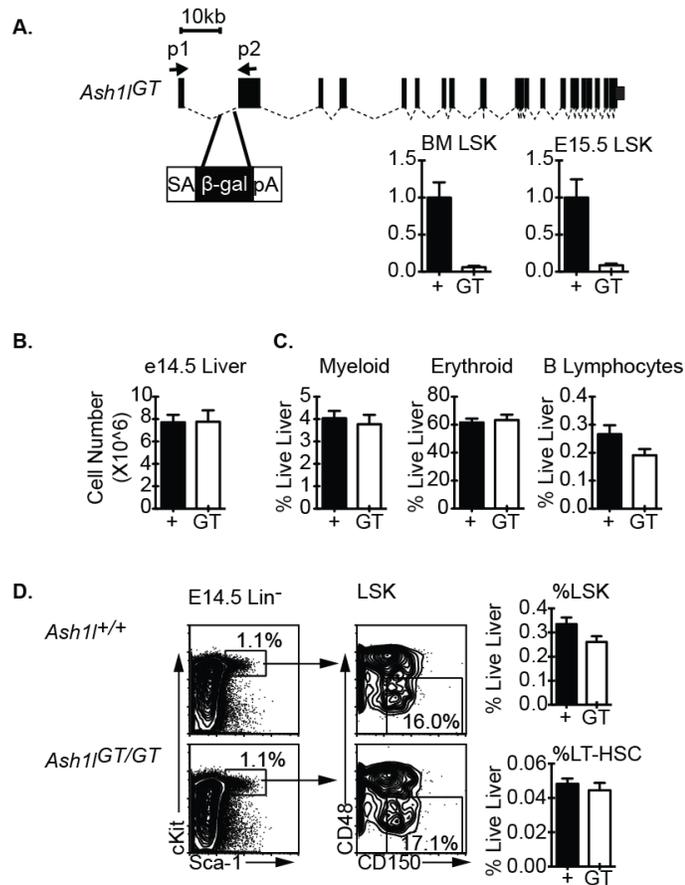
Figure 3.1. E14.5 *Ash1l^{GT/GT}* fetal LT-HSCs expand normally and support hematopoiesis. (**A**) The *Ash1l^{GT}* allele was generated by the insertion of a strong splice-acceptor gene trap cassette into the first intron of *Ash1l*. Homozygosity for this allele resulted in a >90% reduction in wild-type transcripts in fetal and adult LSK progenitors as indicated by qRT-PCR analysis with exon 1-2 spanning primers; (**B**) E14.5 fetal liver cellularity is comparable between WT (+) and *Ash1l^{GT/GT}* (GT) fetuses (N≥4 mice/genotype from 2 independent experiments; mean +/- SEM); (**C**) Flow cytometric determination of myeloid (CD11b$^+$Gr1$^+$), erythroid (Ter119$^+$), and B lymphocyte (CD19$^+$B220$^+$) frequencies in + and GT E14.5 livers showing no differences (N≥4 mice/genotype from 2 independent experiments; mean+/- SEM); (**D**) Flow cytometric analysis of E14.5 fetal livers showing normal frequencies of CD150$^+$CD48$^-$Lineage$^-$Sca-1$^+$cKit$^+$ (SLAM) LT-HSCs in + and GT mice (data are representative of ≥ 4 mice/genotype; mean +/- SEM). Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate.

Ash1l^{GT/GT} LT-HSCs are profoundly depleted in the young adult bone marrow

6-12 week old *Ash1l^{GT/GT}* mice had normal bone marrow cellularity and normal

frequencies of myeloid, erythroid, and B lymphocyte populations in the bone marrow

(**Figure 3.2A, C**). Despite seemingly normal hematopoietic output, flow cytometric

analysis revealed a 5-10-fold reduction in SLAM LT-HSCs compared to wild-type mice

(**Figure 3.2D**). To rule out that an altered surface phenotype was the cause for this reduction we demonstrated a similar 5-10-fold reduction in LT-HSCs using the alternative CD34⁻Flt3⁻ definition (**Figure 3.2E**) [158]. This depletion did not impact myeloid progenitor output in CFU-GM assays, suggesting preserved myeloid progenitor function (**Figure 3.2B**). Surprisingly, despite the profound reduction in phenotypic LT-HSCs, *Ash1l*$^{GT/GT}$ mice did not progress to bone marrow failure by 6 months of age and did not display further reductions in LT-HSC frequency (**Figure 3.2F, G**). These data indicated that *Ash1l*$^{GT/GT}$ LT-HSCs developed normally in fetuses, but became depleted in the young adult bone marrow. Despite this depletion, bone marrow output was preserved for at least 6 months without phenotypic worsening.
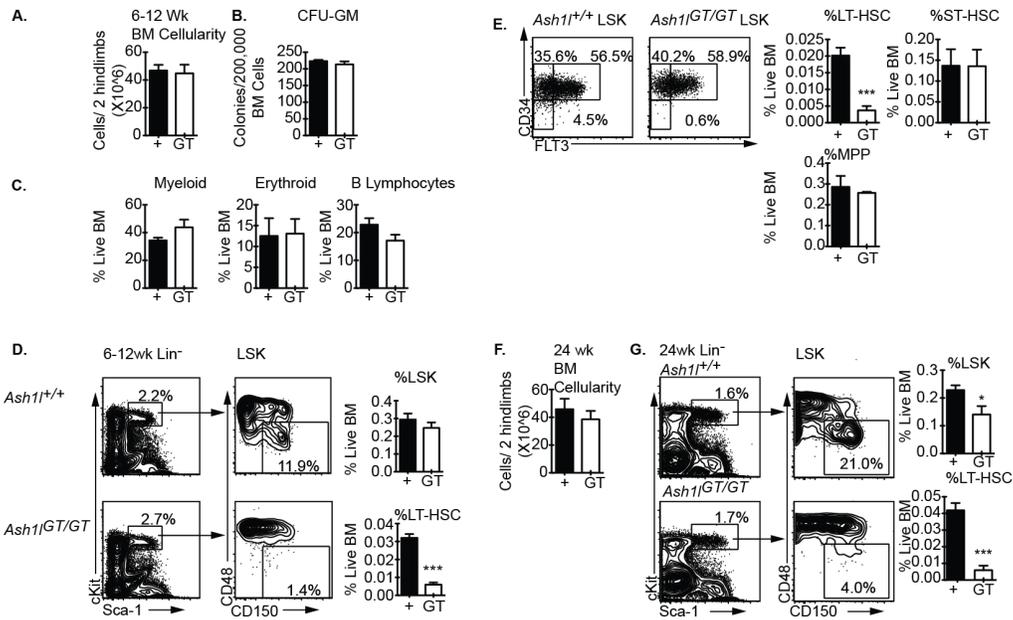
Figure 3.2. *Ash1l^GT/GT* LT-HSCs are profoundly depleted in the BM by 6-12 weeks. (**A**) Young adult (6-12 week) BM cellularity is preserved between + and GT mice (N≥6 mice/genotype from at least 2 independent experiments; means +/- SEM); (**B**) GT BM has normal ability to form myeloid colonies in CFU-GM assays (mean of technical triplicates representative of 2 independent experiments); (**C**) Flow cytometric determination of myeloid (CD11b$^+$Gr1$^+$), erythroid (Ter119$^+$), and B lymphocyte (CD19$^+$B220$^+$) frequencies in + and GT young adult BM showing no differences (N≥2 mice/genotype; means +/- SEM); (**D**) Flow cytometric analysis of young adult mice (6-12 wk) showing GT mice had a >5 fold reduction in SLAM LT-HSCs (data are representative of ≥ 6 mice / genotype; means are shown +/- SEM); (**E**) Flow cytometric analysis demonstrating reduced LT-HSCs (CD34$^-$FLT3$^-$LSK), but not ST-HSCs (CD34$^+$FLT3$^-$ LSK) or MPPs (CD34$^+$FLT3$^+$LSK) in young adult + or GT mice (N≥4 mice/genotype from 2 independent experiments; means +/- SEM); (**F**) Analysis of mice aged at least 24 weeks demonstrates preserved bone marrow cellularity (N=6 mice/genotype from 3 independent experiments; means +/- SEM); (**G**) Flow cytometric analysis reveals a >5 fold reduction in 24 week old GT CD150$^+$CD48$^-$LSK LT-HSCs, as was true in young adult mice (N=6 mice/genotype from 3 independent experiments; means +/- SEM). Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate. *p<0.05, ***p<0.001.

Neither fetal nor adult Ash1l^GT/GT LT-HSCs are capable of sustaining long-term hematopoietic reconstitution

Since *Ash1l^GT/GT* mice were able to survive for at least 6 months with very few phenotypic HSCs, it was possible that a phenotypically abnormal but functional HSC compartment existed in the *Ash1l^GT/GT* bone marrow. To test HSC function independent of phenotypic characteristics, we used whole fetal liver or bone marrow in competitive transplantation assays (**Figure 3.3A**). We chose the fetal liver as our initial source of LT-HSCs since *Ash1l^GT/GT* fetal livers did not have reduced phenotypic LT-HSCs. We found

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that despite normal phenotypic LT-HSCs, $Ash1l^{GT/GT}$ fetal livers were incapable of sustaining myeloid, T cell, or B cell reconstitution in the peripheral blood (**Figure 3.3B**). Since a tri-lineage defect is suggestive of underlying LT-HSC dysfunction, we examined LT-HSC engraftment after 25 weeks. This revealed the absence of $Ash1l^{GT/GT}$ LT-HSCs in the bone marrow of transplant recipients, demonstrating that fetal $Ash1l^{GT/GT}$ LT-HSCs were already dysfunctional and incapable of supporting durable bone marrow hematopoiesis (**Figure 3.3C**)

To determine if the young adult $Ash1l^{GT/GT}$ BM contained a phenotypically abnormal LT-HSC compartment capable of long-term reconstitution, we transplanted donor mice with $Ash1l^{GT/GT}$ or $Ash1l^{+/+}$ BM mixed with an equal amount of competitor BM. We found that as was true with $Ash1l^{GT/GT}$ fetal liver LT-HSCs, $Ash1l^{GT/GT}$ BM LT-HSCs did not support long-term tri-lineage output or durable LT-HSC BM engraftment (**Figure 3.3D, E**). Since there are examples of LT-HSCs performing poorly in competitive BM transplants but stably engrafting the BM in the absence of competition, we examined $Ash1l^{GT/GT}$ LT-HSC function in the absence of competition (**Figure 3.3F**) [48]. We found that 70% of $Ash1l^{GT/GT}$ BM recipients died within 10-150 days after transplantation, consistent with transient radioprotection but LT-HSC dysfunction (**Figure 3.3G**). Analysis of the surviving $Ash1l^{GT/GT}$ BM recipients revealed that these mice survived with host (CD45.1$^+$) reconstitution and not $Ash1l^{GT/GT}$ LT-HSC engraftment (**Figure 3.3H**). Together, these data demonstrated that $Ash1l^{GT/GT}$ fetal liver and BM LT-HSCs were devoid of functional HSCs capable of stably engrafting the BM and sustaining long-term hematopoiesis.
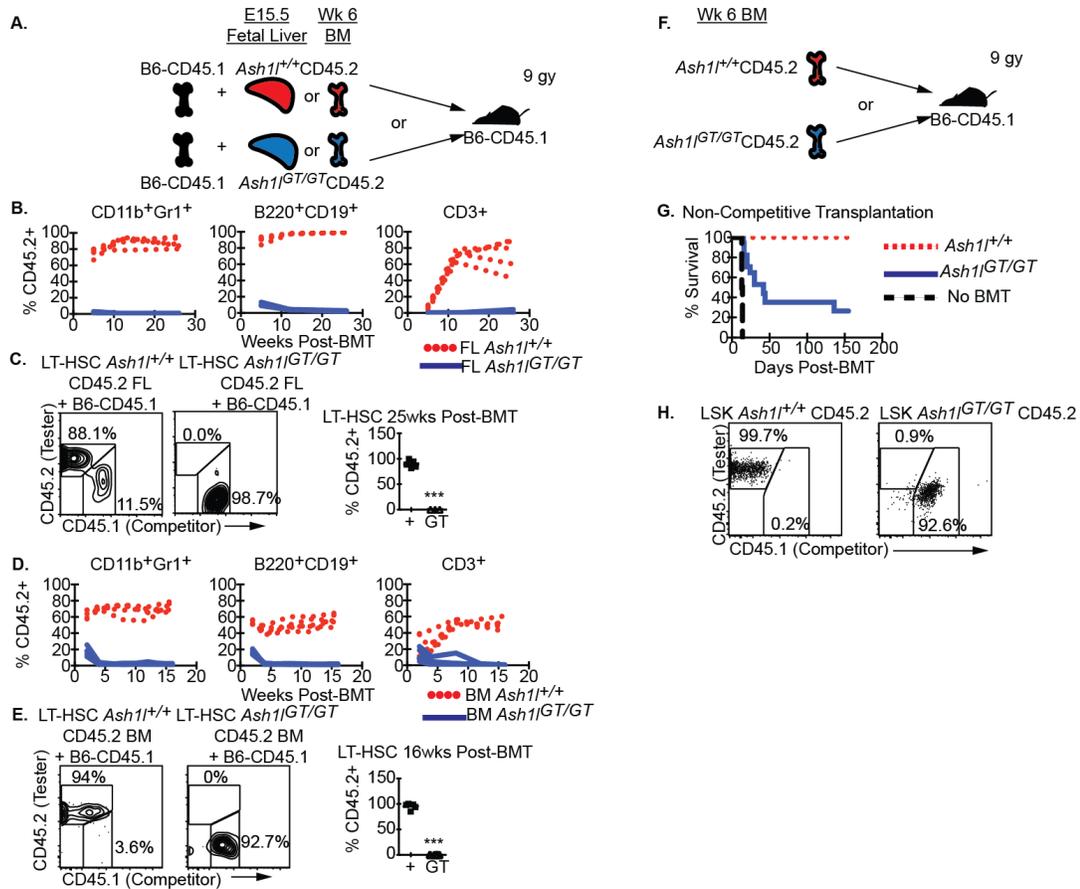
Figure 3.3. Neither *Ash1l*$^{GT/GT}$ fetal liver nor young adult BM has a functional LT-HSC compartment in transplantation assays. (**A**) Experimental strategy: either *Ash1l*$^{+/+}$ or *Ash1l*$^{GT/GT}$ B6-CD45.2 bone marrow was mixed with WT B6-CD45.1 competitor bone marrow (1:1 ratio; 2.5×10^5 for fetal liver or 5×10^5 of each for BM) and injected into lethally irradiated (9Gy) WT B6-CD45.1 recipient mice; (**B**) Flow cytometric analysis of the peripheral blood 4-25 weeks after fetal liver transplantation demonstrates a profound reduction of the *Ash1l*$^{GT/GT}$ bone marrow contribution to myeloid (CD11b$^+$Gr1$^+$), B cell (CD19$^+$B220$^+$), and T cell (CD3$^+$) reconstitution (data are representative of ≥ 3mice/genotype); (**C**) Analysis of CD45.2/CD45.1 chimerism in the CD150$^+$CD48$^-$LSK LT-HSC compartment 25 weeks after transplantation demonstrates an absence of *Ash1l*$^{GT/GT}$ LT-HSCs (data are representative of ≥ 3mice/genotype); (**D**) Flow cytometric analysis of peripheral blood 2-16 weeks after 6 wk-old BM transplantation shows a profound reduction of the *Ash1l*$^{GT/GT}$ BM contribution to myeloid (CD11b$^+$Gr1$^+$), B cell (CD19$^+$B220$^+$), and T cell (CD3$^+$) reconstitution (data are representative of ≥ 4 mice/genotype); (**E**) Analysis of CD45.2/CD45.1 chimerism in the CD150$^+$CD48$^-$LSK LT-HSC compartment 16 weeks after transplantation demonstrates an absence of *Ash1l*$^{GT/GT}$ LT-HSCs (data are representative of ≥ 4 mice/genotype); (**F**) Experimental Strategy: either *Ash1l*$^{+/+}$ or *Ash1l*$^{GT/GT}$ B6-CD45.2 bone marrow cells (10^6 cells of each) were injected into lethally irradiated (9Gy) WT B6-CD45.1 recipient mice. (**G**) Mice were monitored for survival for the indicated period of time; (**H**) Flow cytometric analysis of surviving mice showing that *Ash1l*$^{GT/GT}$ recipients had only CD45.1 (Host)-derived LSK progenitors, while *Ash1l*$^{+/+}$ recipients were reconstituted with CD45.2 (Donor) progenitors (data representative of at least 3 mice/genotype). Representative plots are shown. Numbers indicate the percentage of cells in each gate. ***p<0.001.

Ashl$^{GT/GT}$ *HSCs home to the bone marrow but do not properly establish or maintain a quiescent HSC pool*

Both bone marrow transplantation and the establishment of a BM HSC pool require homing of HSCs to the BM niche: transplantation requires that HSCs home from the site of injection to the bone marrow, while physiologic homing requires that HSCs migrate from the fetal liver to the bone marrow. Since *Ash1l*$^{GT/GT}$ mice lacked a stable HSC pool in both experimental models, it was possible that *Ash1l*$^{GT/GT}$ HSCs exhibited homing defects. To test this possibility, we analyzed the BM LT-HSC compartment in P10 mice by flow cytometry. These experiments revealed that *Ash1l*$^{GT/GT}$ neonatal mice had comparable frequencies of LT-HSCs in their BM, inconsistent with a homing defect (**Figure 3.4A**). We further found that normal *Ash1l*$^{GT/GT}$ LT-HSC frequency was preserved for at least 3 weeks after birth (data not shown).

Assessment of neonatal BM LT-HSCs demonstrated that *Ash1l*$^{GT/GT}$ LT-HSCs had increased expression of cell surface CD34 as compared to wild-type mice (**Figure 3.4B**). CD34 expression has been reported to mark fetal, but not adult mouse LT-HSCs [159, 160]. In addition, CD34 expression is upregulated on the surface of HSCs that are actively engaged in cell cycle activity [30, 159]. To test if *Ash1l*$^{GT/GT}$ BM LT-HSCs maintained a fetal-like transcription program, we crossed our mice with *Sox17-GFP* reporter mice [26]. *Sox17* is a master regulator of the fetal HSC program and is expressed only in fetal HSCs and not in adult BM HSCs. We found that *Ash1l*$^{GT/GT}$ LT-HSCs properly expressed Sox17-GFP in the fetal liver and appropriately extinguished Sox17-GFP by 2 weeks after birth (**Figure 3.4C**). Thus, *Ash1l* deficiency did not result in the maintenance of a fetal-like LT-HSC state.

To test if $Ash1l^{GT/GT}$ HSCs had an abnormal cell cycle status, we used both BrdU and Ki67-based staining strategies. P19 $Ash1l^{GT/GT}$ LT-HSCs showed a trend for increased BrdU incorporation suggesting aberrant cell cycle entry (**Figure 3.4D**). Ki67 staining revealed that $Ash1l^{GT/GT}$ HSCs had a significantly reduced G0 fraction, increased entry into G1, and a trend for more HSCs in the later S-G2-M phases of the cell cycle (**Figure 3.4E**). Since HSCs exit the cell cycle in the BM between P14 and P21, this suggested that *Ash1l*-deficient HSCs failed to establish a normal quiescent stem cell pool [29].

Recent work has demonstrated that HSC BM quiescence is dependent on the expression of *p27*(*Cdkn1b*) and *p57*(*Cdkn1c*) [35, 36]. Gene expression analysis in LSK progenitors showed that P10 $Ash1l^{GT/GT}$ hematopoietic progenitors had markedly reduced expression of these two critical regulators of quiescence, though expression of *p21*(*Cdkn1a*), a CIP/KIP family member not implicated in the establishment of normal HSC quiescence, was not altered (**Figure 3.4F**). Collectively, these data suggested that $Ash1l^{GT/GT}$ HSCs failed to establish normal quiescence in the BM due to reduced *p27* and *p57* expression.
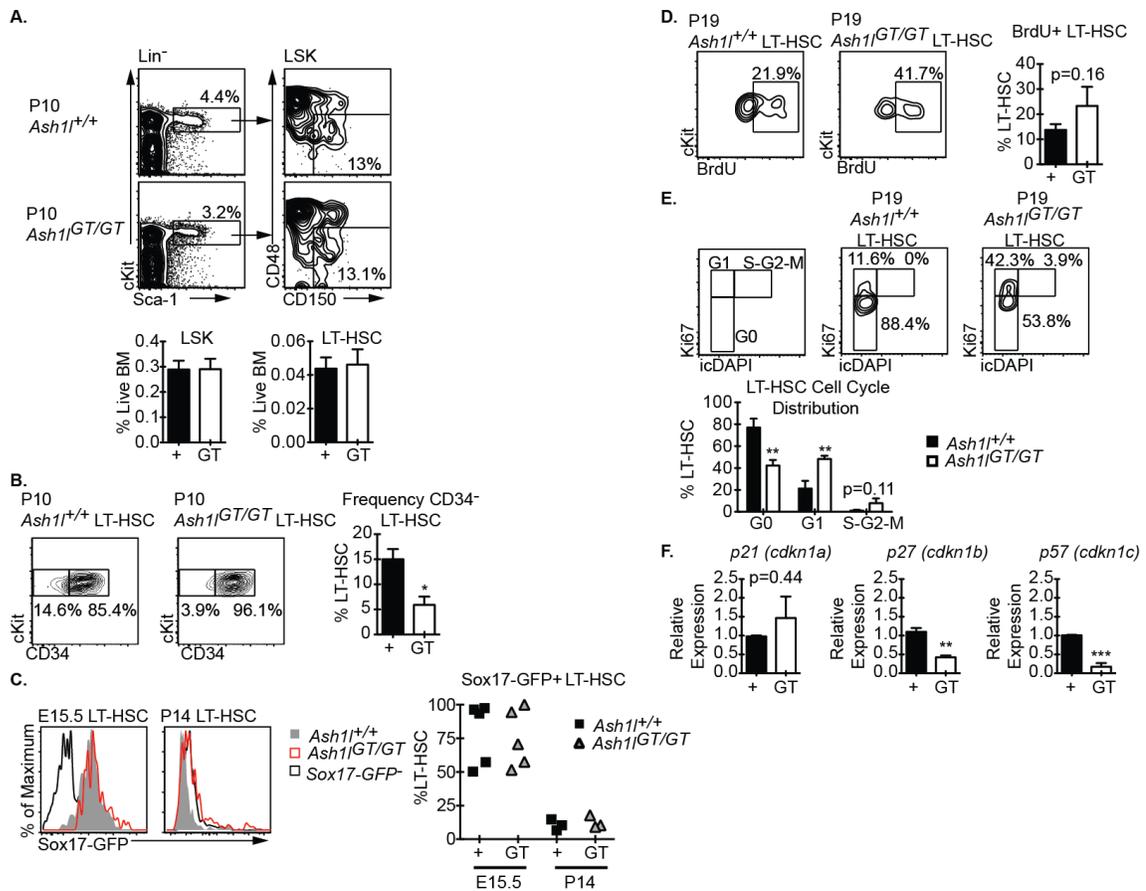
Figure 3.4. *Ash1l^{GT/GT}* LT-HSCs home to the bone marrow but fail to establish quiescence. (**A**) Flow cytometric analysis of P10 BM demonstrated comparable frequencies of SLAM LT-HSCs between *Ash1l^{GT/GT}* and *Ash1l^{+/+}* littermates (data are representative of ≥ 9 mice / genotype; means +/- SEM); (**B**) P10 GT SLAM LT -HSCs had increased cell surface CD34 expression (data are representative of 4 mice/genotype; means +/- SEM); (**C**) Flow cytometric analysis of Sox17-GFP expression in E15.5 fetal liver and P14 BM SLAM LT-HSCs shows that Sox17-GFP is present in fetal liver LT-HSCs and is appropriately extinguished in the P14 BM (N≥3 mice/genotype from 4 independent experiments; data points represent individual mice with bar at mean); (**D**) Representative flow cytometry plots showing P19 *Ash1l^{GT/GT}* SLAM LT-HSCs had increased cell cycle entry as indicated by increased BrdU incorporation (data are representative of ≥ 4 mice / genotype; means are shown + SEM); (**E**) Representative flow cytometry plots showing P19 *Ash1l^{GT/GT}* SLAM LT-HSCs had a reduced G0 (quiescent fraction) and increased distribution into G1 and S-G2-M phases by Ki67 (data are representative of 5 mice/genotype; means are shown +/- SEM); (**F**) Reduced p27 and p57 gene expression in P10 *Ash1l^{GT/GT}* LSK progenitors by qRT-PCR (3 individual sorted samples +/- SEM per genotype; expression relative to *Hprt1*). Representative plots are shown. Numbers indicate the percentage of cells in each gate. *p<0.05, **p<0.01, ***p<0.001.

Ashl^{GT/GT} LT-HSCs and progenitors maintain increased cell cycle activity

The cell cycle analyses described above provide a snapshot of cell cycle activity in LT-HSCs, but cannot provide detailed insight into cell cycle status over time. Since LT-HSCs were described as a slow-cycling cell population compared to differentiated cells in long-term pulse-chase experiments, detailed analysis of cell cycle history can identify putative HSCs with the least cell cycle entry [161]. To examine whether *Ash1l^{GT/GT}* BM harbored cells with such very slow-cycling behavior, we bred *Ash1l^{GT/GT}* mice to mice in which an *H2B-GFP* transgenic allele was inducible by tetracycline through the *M2rtTa* tetracycline activator [161]. This strategy allowed us to perform pulse-chase experiments and examine the long-term cell cycle activity of hematopoietic stem and progenitor cells (**Figure 3.5A**). We found that *Ash1l^{GT/GT}* LT-HSCs had increased cell cycle activity sustained throughout the 6 week chase period as indicated by enhanced GFP dilution (**Figure 3.5B**). This increased cell cycle activity may reflect efforts of the few remaining phenotypic LT-HSCs to maintain hematopoietic homeostasis. Interestingly, the *Ash1l^{GT/GT}* LSK compartment as a whole had an even more robust increase in cell cycle activity than the LT-HSC compartment (**Figure 3.5B**). This suggested that increased proliferation in progenitors downstream of LT-HSCs might be the source of sustained hematopoietic output despite reduced LT-HSCs in *Ash1l^{GT/GT}* mice. This finding could underlie the long-term survival and maintained hematopoietic output in *Ash1l^{GT/GT}* mice.

To further assess *Ash1l^{GT/GT}* LT-HSC cell cycle activity, we challenged *Ash1l^{GT/GT}* and control mice with the antimetabolite 5-fluorouracil (5-FU) (**Figure 3.5C**). 5-FU treatment is toxic to actively dividing cells, but spares quiescent cells including LT-HSCs. Following a single dose of 5-FU, LT-HSCs robustly enter the cell cycle and

proliferate to restore hematopoietic homeostasis [31]. We found that a single treatment with 5-FU resulted in a nearly 2 log reduction in $Ash1l^{GT/GT}$ LT-HSCs compared to controls, thus amplifying the baseline LT-HSC reduction by 10-20-fold (**Figure 3.5D**). This further demonstrated that $Ash1l^{GT/GT}$ BM lacks a quiescent stem cell pool, and that remaining LT-HSCs were persistently engaged in cell cycle activity.
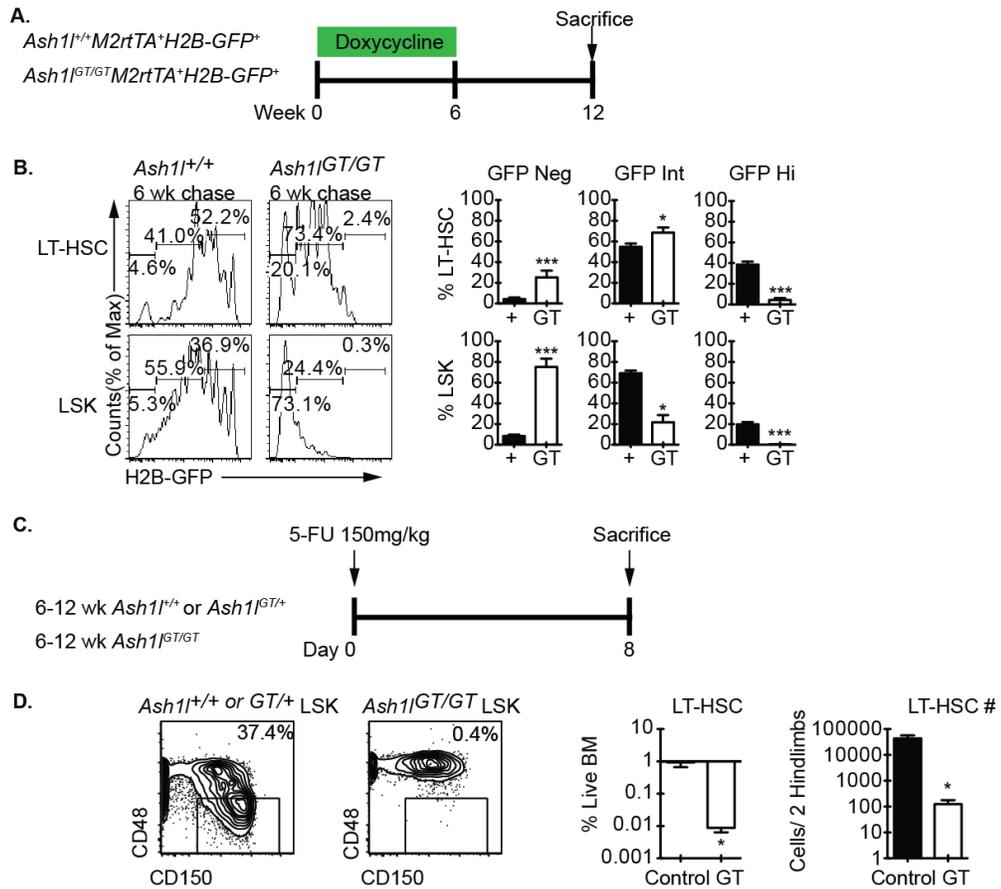
Figure 3.5. $Ash1l^{GT/GT}$ **LT-HSCs and LSK progenitors sustain increased cell cycle activity.** (**A**) Experimental strategy: $Ash1l^{+/+ \text{ or } GT/GT}$ with $M2rtTA$ and $H2B$-GFP were maintained on doxycycline drinking water for 6 weeks to label hematopoietic cells with GFP. GFP dilution was monitored by flow cytometry after a 6 week chase period; (**B**) Flow cytometric analysis after the 6 week chase showing significantly increased GFP dilution in $Ash1l^{GT/GT}$ SLAM LT-HSCs and LSK progenitors compared to controls, demonstrating increased cell cycle activity (data are representative of ≥ 6 mice/genotype; means are shown +/- SEM); (**C**) Experimental Strategy: mice of indicated genotypes were injected with 150mg/kg 5-fluorouracil (5-FU) and sacrificed 8 days later; (**D**) Flow cytometric analysis of SLAM LT-HSCs showing 2-log reduction in frequency and 2.5-log reduction in LT-HSC numbers in $Ash1l^{GT/GT}$ as compared to control mice after 5-FU exposure (N≥4 mice/genotype; graphs show means +/- SEM). Representative plots are shown. Numbers indicate the percentage of cells in each gate. *p<0.05, **p<0.01, ***p<0.001.

The Ash1l^{GT/GT} niche supports donor LT-HSC engraftment in the absence of conditioning

Few reports describe HSC genetic defects that permit donor HSC engraftment in the absence of conditioning [61, 162]. We reasoned that since *Ash1l*$^{GT/GT}$ BM had profoundly reduced LT-HSCs, and remaining phenotypic LT-HSCs were non-functional in transplantation assays, there might be available BM niche space and/or defective HSCs that could be out-competed by wild-type donor BM. Either scenario could permit engraftment of donor BM without conditioning. To examine this possibility, we transplanted wild-type (CD45.2$^+$) or *Ash1l*$^{GT/GT}$ (CD45.2$^+$) mice with wild-type (CD45.1$^+$) bone marrow (**Figure 3.6A**). We found that in 4/5 *Ash1l*$^{GT/GT}$ recipients, stable tri-lineage hematopoietic output from donor BM could be detected in the peripheral blood for at least 12 weeks (**Figure 3.6B**). We further determined that this output resulted from wild-type LT-HSCs stably engrafting the *Ash1l*$^{GT/GT}$ BM (**Figure 3.6C**). As expected, minimal to no donor engraftment was detected in wild-type recipients. These data further support a model in which *Ash1l* is cell-autonomously required for the maintenance of LT-HSCs in the BM niche. The combination of vacant niches and profound LT-HSC dysfunction in *Ash1l*$^{GT/GT}$ BM was suitable for engraftment by donor cells without any conditioning. Importantly, these data demonstrated that the *Ash1l*$^{GT/GT}$ niche was not itself defective, as it was able to support stable engraftment by wild-type donor LT-HSCs.
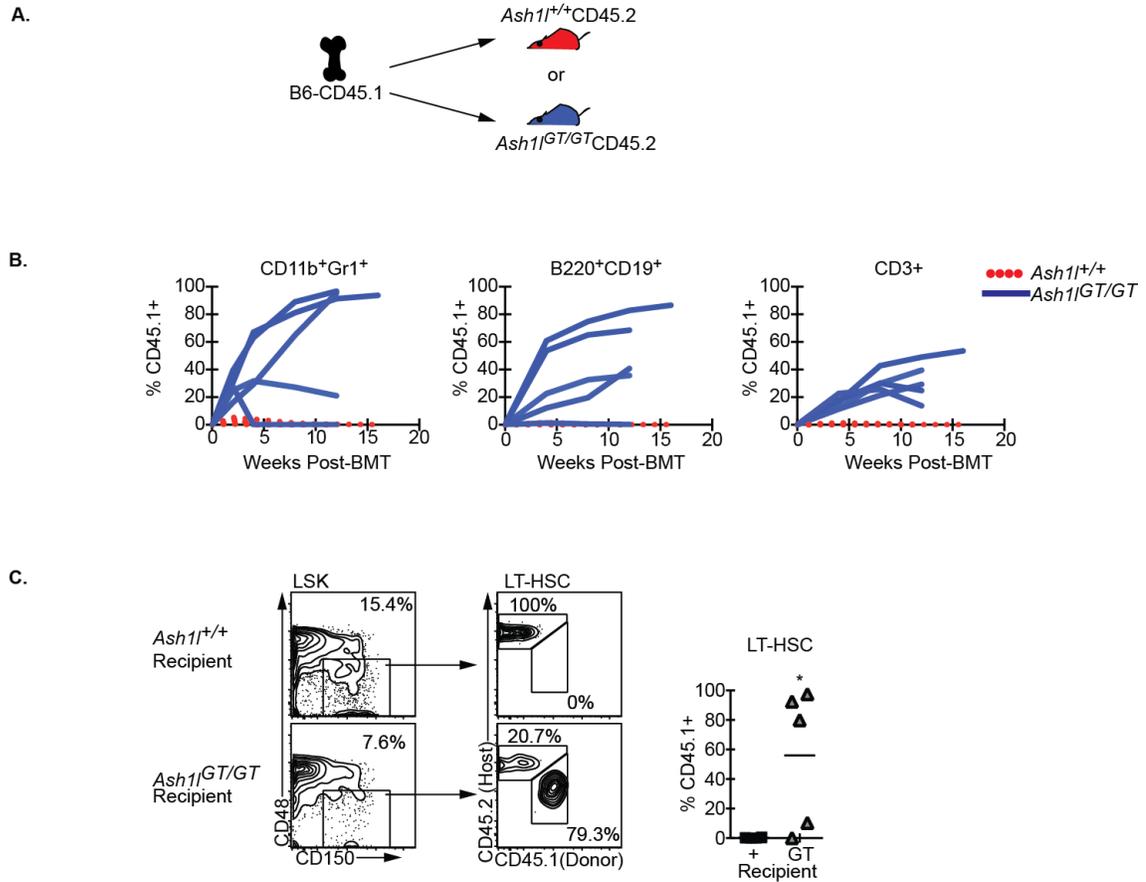
Figure 3.6. *Ash1l* **deficiency allows LT-HSC engraftment in the absence of conditioning. (A)** Experimental strategy: $Ash1l^{GT/GT \text{ or } +/+}$ (CD45.2+) mice were transplanted with 2 doses of 2 X 10^7 BM (CD45.1+) separated by 1 week without irradiation; (**B**) Flow cytometric analysis of peripheral blood of transplant recipients demonstrates significant output from donor BM in myeloid (CD11b+Gr1+), B cell (CD19+B220+), and T cell (CD3+) lineages in 4/5 $Ash1l^{GT/GT}$ recipients, but in 0/6 WT recipients. Reconstitution was sustained for at least 12 weeks (data are representative of ≥ 5 mice/genotype); (**C**) Flow cytometric analysis of LT-HSC compartment demonstrates stable donor (CD45.1+) LT-HSC engraftment in 4/5 $Ash1l^{GT/GT}$ recipients, but in 0/6 WT recipients. Representative plots are shown. Numbers indicate the percentage of cells in each gate. *p<0.05.

Ash1l cooperates with Mll1 to maintain hematopoiesis

Studies in *Drosophila* revealed that members of the TrG can functionally cooperate with one another to maintain *homeobox* gene expression [97]. This phenomenon has not been assessed in mammals *in vivo*. We reasoned that if mammalian TrG members cooperated with one another, they might share a subset of target genes and functional effects. *Mll1*, the mammalian homolog of *Drosophila trithorax*, was shown to

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regulate a number of *Homeobox* genes including *Meis1*, *Hoxa9,* and *Hoxa10*, all of which

are known to be essential for HSC self-renewal. We found that P10 *Ash1l*$^{GT/GT}$ LSK

progenitors had reduced expression of *Hoxa5*, *Hoxa9*, *Hoxa10*, and *Meis1*, with *Hoxa9*

and *Meis1* being the most profoundly affected (**Figure 3.6**). Importantly, *Mll1* levels

were not changed in these progenitors, demonstrating that effects of *Ash1l* deficiency on

Hox gene expression were not secondary to reduced *Mll1* levels. This suggested that

Ash1l and Mll1 shared a subset of genetic targets known to be essential for LT-HSC

function, and thus that they may cooperatively function to maintain hematopoietic

homeostasis.
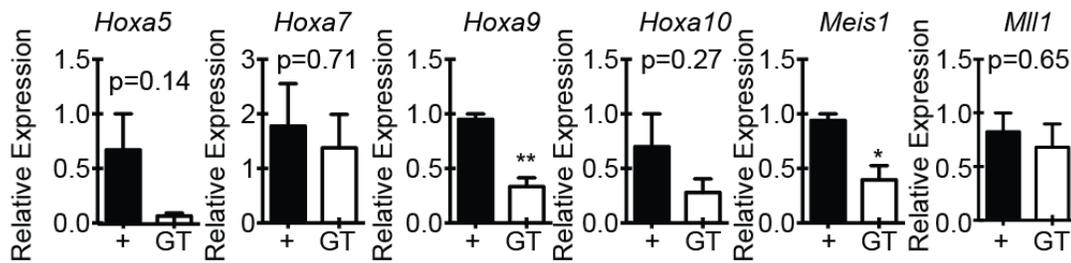
Figure 3.7. *Ash1l*$^{GT/GT}$ **neonatal LSKs have reduced *Hox* gene expression independent of *Mll1*.**
Reduced *Hox* gene expression in P10 *Ash1l*$^{GT/GT}$ LSK progenitors by qRT-PCR (3 individual sorted samples
+/- SEM per genotype; expressed relative to *Hprt1*). *p<0.05, **p<0.01.

To examine if Ash1l and Mll1 cooperate to maintain hematopoietic homeostasis,

we intercrossed *Mll1*$^{fl/fl}$ *Mx1-cre*$^{+}$ mice with *Ash1l*^{GT} [68]. This system allowed us to

delete *Mll1* via poly(I:C) injection (**Figure 3.8A**). Using this model, we found that

combined deficiency for *Mll1* and *Ash1l*$^{GT/GT}$ resulted in an acute reduction in bone

marrow cellularity, suggesting that these mice developed bone marrow failure (**Figure

3.8B**). To determine if loss of hematopoietic progenitor cells underlied bone marrow

failure, we analyzed the LT-HSC and downstream progenitor compartments by flow

cytometry. Flow cytometric analysis of LT-HSCs and hematopoietic progenitors

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demonstrated that *Ash1l*^{GT/GT}*Mll1*^{fl/fl}*Mx1-cre*⁺ mice had profound reductions in LT-HSCs

and LSK progenitors (**Figure 3.8C, D, E**). In addition, $Ash1l^{GT/+}Mll1^{fl/+}Mx1\text{-}cre^{+}$ mice

had fewer LT-HSCs than $Ash1l^{GT/+}$ or $Mll1^{fl/+}Mx1\text{-}cre^{+}$ mice, suggesting that LT-HSCs

had a unique sensitivity to the observed functional interaction between Mll1 and Ash1l.

Flow cytometric analyses further revealed that CD34⁻SLAM LT-HSCs, one of the most

quiescent HSC subsets that can be identified with surface markers, were profoundly

reduced in $Ash1l^{GT/GT}Mll1^{fl/fl}Mx1\text{-}cre^{+}$ mice as well as in $Ash1l^{GT/+}Mll1^{fl/+}Mx1\text{-}cre^{+}$ mice

(**Figure 3.8F**). This suggested that Ash1l and Mll1 cooperate to maintain quiescent

HSCs. Together, these findings identified cooperativity between Mll1 and Ash1l in

maintaining hematopoietic stem and progenitor cells. This is the first *in vivo*

demonstration of a functional interaction between TrG members in mammals.
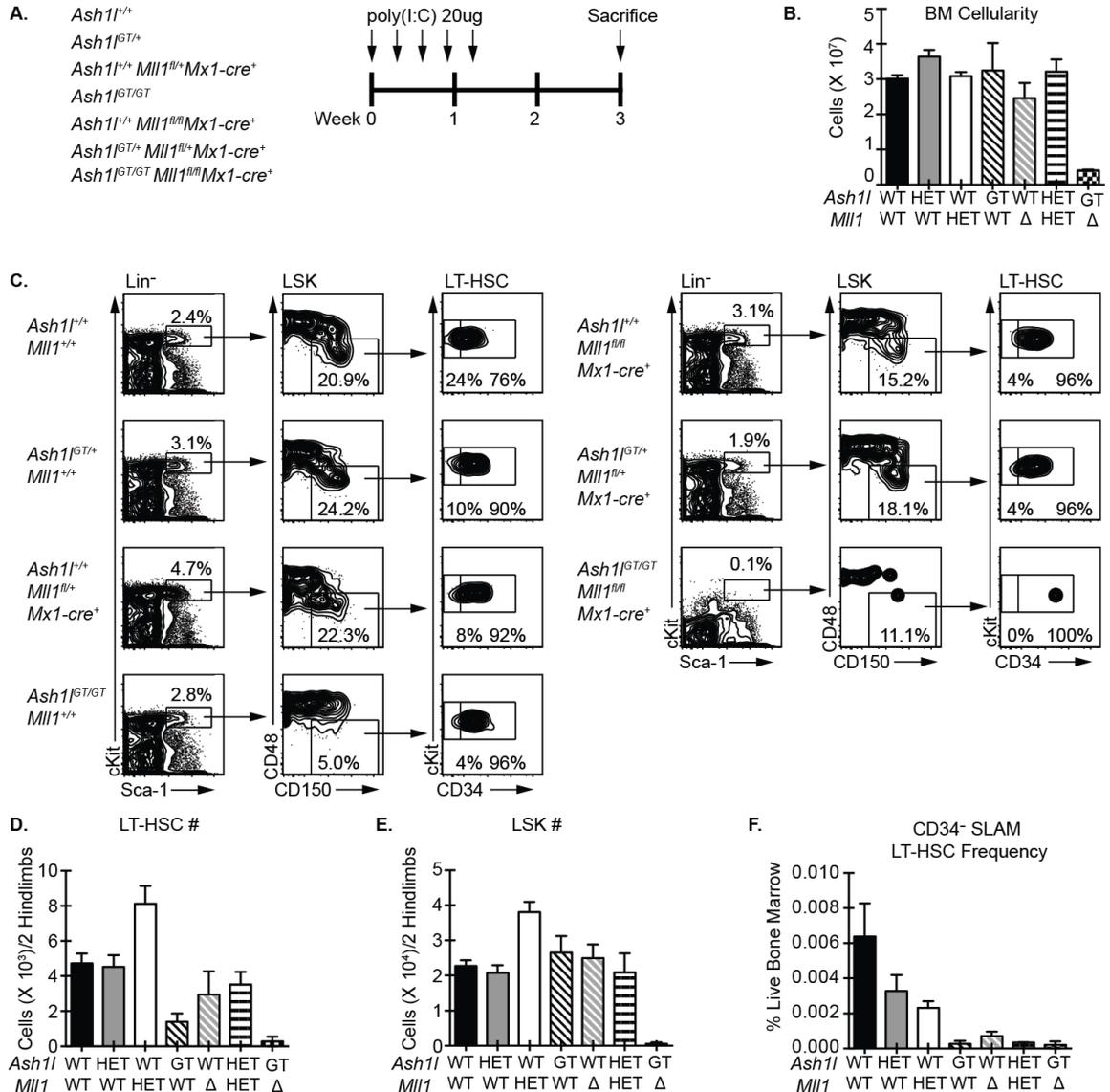
Figure 3.8. Combined *Ash1l* and *Mll1* deficiency results in acute LT-HSC and LSK depletion. (**A**) Experimental strategy: mice of indicated genotypes were injected 5X with 20ug of poly(I:C); (**B**) Reduced bone marrow cellularity in *Ash1l*$^{GT/GT}$*Mll1*$^{fl/fl}$*Mx1-cre*$^+$mice (data are representative of ≥ 2 mice/genotype; mean +/- SEM); (**C**) Flow cytometric analysis showing severe reductions in CD34$^-$ SLAM LT-HSCs, SLAM LT-HSCs, and LSK progenitors in *Ash1l*$^{GT/GT}$*Mll1*$^{fl/fl}$*Mx1-cre*$^+$mice and reduced CD34$^-$ SLAM LT-HSC frequency *Ash1l*$^{GT/+}$*Mll1*$^{fl/+}$*Mx1-cre*$^+$mice; (**D, E**) LT-HSC and LSK absolute cell numbers reflecting profound defect in *Ash1l*$^{GT/GT}$*Mll1*$^{fl/fl}$*Mx1-cre*$^+$mice and reduced LT-HSC numbers in *Ash1l*$^{GT/+}$*Mll1*$^{fl/+}$*Mx1-cre*$^+$mice (data are representative of ≥ 2 mice/genotype; mean +/- SEM); (**F**) Frequencies of CD34$^-$ SLAM LT-HSCs emphasizing reduction in *Ash1l*$^{GT/+}$*Mll1*$^{fl/+}$*Mx1-cre*$^+$ BM. Representative plots are shown. Numbers indicate the percentage of cells in each gate.

Previous studies indicated that the cofactor menin is required for Mll1 targeting to at least a subset of gene loci [117, 118, 147, 148]. Combining the *Ash1l*^{GT} allele with *Men1*$^{fl/fl}$ *Mx1-cre*$^+$ mice thus provided a means to independently verify the described

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functional interaction between Ash1l and Mll1 in hematopoiesis [163]. *Men1* excision

was achieved by poly(I:C) injection, and this allowed us to ablate menin-dependent Mll1

function in the hematopoietic system (**Figure 3.9A**). Analysis of the bone marrow

compartment 3 weeks after the initiation of poly(I:C) revealed a significant reduction in

BM cellularity in *Ash1l$^{GT/GT}$Men1$^{fl/fl}$Mx1-cre$^+$* mice (**Figure 3.9B**). This suggested that

mice progressed to bone marrow failure in the absence of Ash1l and menin/Mll1,

consistent with the development of profound neutropenia and thrombocytopenia (**Figure**

3.9C, D). Additionally, the combination of a single *Ash1l^{GT}* allele with *Men1* inactivation

(*Ash1l$^{GT/+}$Men1$^{fl/fl}$Mx1-cre$^+$*) resulted in reduced platelets as compared to either *Ash1l$^{GT/+}$*

or *Men1$^{fl/fl}$Mx1-cre$^+$* peripheral blood. This suggested that *Men1* loss sensitized the bone

marrow to *Ash1l* haploinsufficiency, further indicating cooperativity between menin/Mll1

and Ash1l.
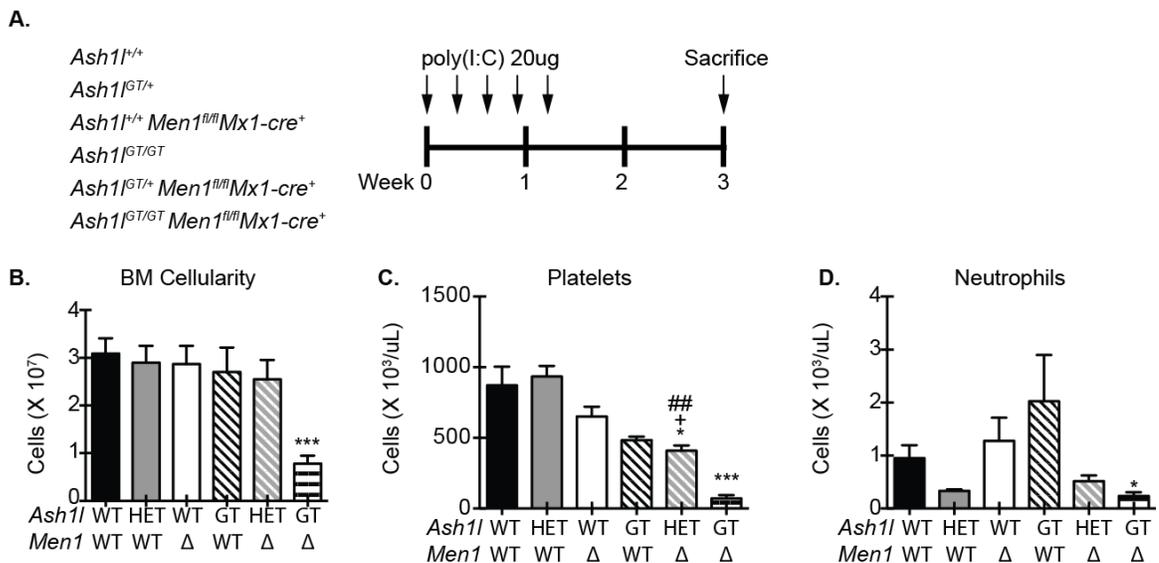
Figure 3.9. Combined deficiency of *Ash1l* and *Men1* results in rapid progression to frank hematopoietic failure. (**A**) Experimental strategy: mice of indicated genotypes were injected 5 X with 20ug of poly(I:C); (**B**) Reduced bone marrow cellularity in *Ash1l$^{GT/GT}$Men1$^{fl/fl}$Mx1-cre$^+$* mice compared to wild-type (data are representative of ≥ 5 mice/genotype; mean +/- SEM); (**C**) Complete blood count analysis of peripheral blood platelets showing thrombocytopenia in *Ash1l$^{GT/GT}$Men1$^{fl/fl}$Mx1-cre$^+$* mice within 3 weeks of poly(I:C) injection (N≥3 mice/genotype from 3 independent experiments; means +/- SEM); (**D**) Complete blood count analysis of peripheral blood neutrophils demonstrating profound neutropenia in

Ashl$^{GT/GT}$*Men1*$^{fl/fl}$*Mx-1-cre*$^{+}$ mice within 3 weeks of poly(I:C) injection (N≥3 mice/genotype from 3 independent experiments; mean +/- SEM). *p<0.05, ***p<0.001 compared to wild-type. For comparisons between *Ashl*$^{GT/+}$*Men1*$^{fl/fl}$*Mx-1-cre*$^{+}$and *Ashl*$^{GT/+}$ or *Men1*$^{fl/fl}$*Mx1-cre*$^{+}$: ## p<0.01 compared to *Ash1l*$^{GT/+}$; + p<0.05 compared to *Men1*$^{fl/fl}$*Mx1-Cre*$^{+}$.

To determine if bone marrow failure occurred concurrently with hematopoietic stem and progenitor cell depletion, as observed above, we analyzed these populations by flow cytometry following *Men1* excision. We found that *Ash1l*$^{GT/GT}$*Men1*$^{fl/fl}$*Mx1-cre*$^{+}$ LT-HSCs were severely decreased, and that this occurred concurrently with a profoundly depleted LSK progenitor compartment (**Figure 3.10A, B**). *Ash1l*$^{GT/+}$*Men1*$^{fl/fl}$*Mx1-cre*$^{+}$ resulted in reduced LT-HSCs compared to either *Ash1l*$^{GT/+}$ or *Men1*$^{fl/fl}$*Mx1-cre*$^{+}$ LT-HSC compartments, demonstrating that menin/Mll1 loss sensitized LT-HSCs to reduced Ash1l levels. Despite the LT-HSC depletion, however, the pool of downstream progenitors was not profoundly depleted unless both alleles of *Men1* and *Ash1l* were mutated. Together, these findings demonstrated that combined *Men1* and *Ash1l* deficiency reproduced the bone marrow failure phenotype observed in the setting of combined *Mll1* and *Ash1l* deficiency. These findings further indicated a robust functional cooperativity between menin/Mll1 and Ash1l in hematopoietic stem and progenitors, and thus a critical role in maintaining hematopoietic homeostasis.
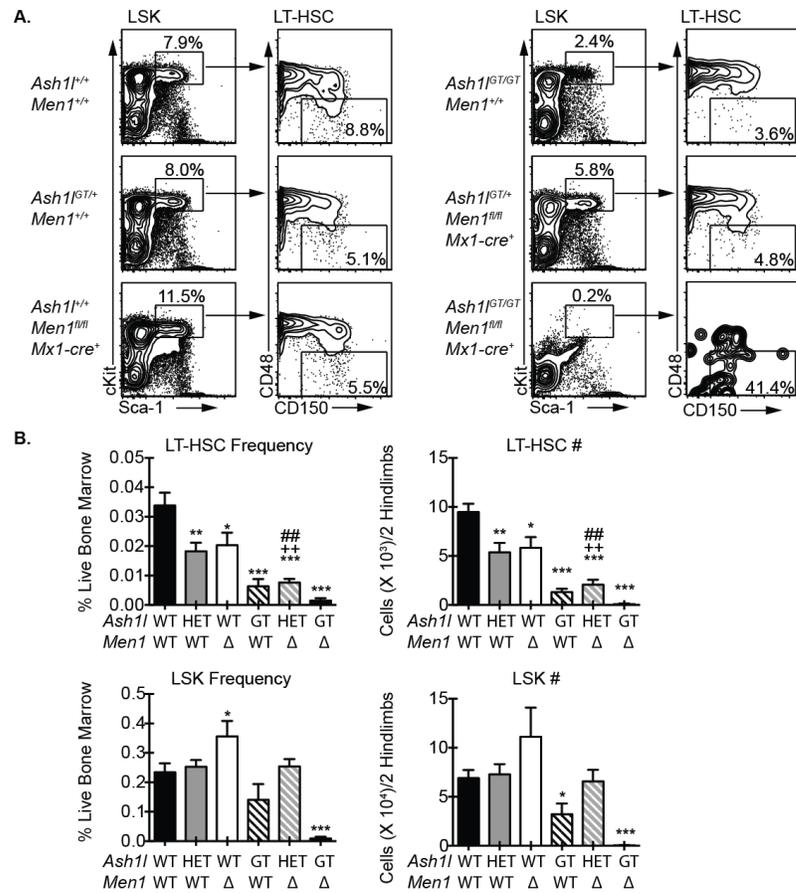
Figure 3.10. Profound LT-HSC and LSK progenitor depletion underlies hematopoietic failure in mice with combined *Ash1l* and *Men1* deficiency. (**A**) Flow cytometric analysis showing severely reduced LT-HSC and LSK progenitor compartments in *Ash1l^{GT/GT}Men1^{fl/fl}Mx1-cre^+* mice and reduced LT-HSC frequency *Ash1l^{GT/+}Men1^{fl/fl}Mx1-cre^+* mice; (**B**) LT-HSC and LSK frequencies and absolute cell numbers reflecting profound defect in *Ash1l^{GT/GT}Men1^{fl/fl}Mx1-cre^+* mice and reduced LT-HSC frequency and absolute numbers in *Ash1l^{GT/+}Men1^{fl/fl}Mx1-cre^+* mice (data are representative of ≥ 5 mice/genotype; mean +/- SEM). Representative plots are shown. Numbers indicate the percentage of cells in each gate. *p<0.05, **p<0.01, ***p<0.001 compared to wild-type. For comparisons between *Ashl^{GT/+}Men1^{fl/fl}Mx-1-cre^+* and *Ashl^{GT/+}* or *Men1^{fl/fl}Mx1-cre^+*: ## p<0.01 compared to *Ash1l^{GT/+}*; ++ p<0.01 compared to *Men1^{fl/fl}Mx1-Cre^+*.

DISCUSSION

Our findings identify that LT-HSC self-renewal is dependent on Ash1l. When

Ash1l levels were profoundly reduced due to homozygosity for our gene trap allele, LT-

HSCs had an impaired ability to establish and maintain quiescence. *Ash1l*-deficient HSCs

developed and expanded normally in the fetal liver and initially seeded the bone marrow.

Once in the bone marrow, these LT-HSCs failed to upregulate the CDKIs *p27* and *p57*,

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leading to markedly reduced numbers of quiescent adult HSCs. Since fetal HSCs are known to actively transit through the cell cycle, it was tempting to speculate that these findings were the result of an inability of HSCs to extinguish the fetal program upon reaching the bone marrow [29, 164]. However, expression of *Sox17*, the master regulator of the fetal HSC transcriptional program, was properly repressed in the bone marrow. This finding indicated that the reduced ability for *Ash1l*$^{GT/GT}$ LT-HSCs to establish quiescence was independent of the transcriptional transition from the fetal state to the adult state. This suggests that Ash1l may be a novel key regulator of the cell cycle changes associated with the fetal to adult HSC transition.

Ash1l$^{GT/GT}$ bone marrow had profoundly reduced LT-HSC numbers, but maintained normal mature hematopoietic cell output. This paradox could have been explained by phenotypically abnormal, but functional HSCs residing in the *Ash1l*$^{GT/GT}$ bone marrow. However, this was disproven as young adult *Ash1l*$^{GT/GT}$ bone marrow could engraft lethally irradiated recipients in neither competitive nor non-competitive transplantation assays. Based on this gold standard approach, we could not detect LT-HSC function in the *Ash1l*$^{GT/GT}$ bone marrow. We thought that this inability to detect LT-HSC function could have been because of the rarity of *Ash1l*$^{GT/GT}$ LT-HSCs; perhaps we transplanted too few bone marrow cells to include adequate LT-HSC numbers. To rule out this hypothesis, we transplanted *Ash1l*$^{GT/GT}$ fetal liver cells. This allowed us to normalize LT-HSC frequency, as both *Ash1l*$^{GT/GT}$ and *Ash1l*$^{+/+}$ had comparable LT-HSC compartments. Strikingly, we could not detect functional LT-HSCs in this transplantation setting either. This demonstrated that *Ash1l*$^{GT/GT}$ LT-HSCs could not stably engraft the bone marrow niche. This could be because *Ash1l*$^{GT/GT}$ LT-HSCs cannot respond to niche

factors required for stable engraftment and quiescence. Among these, thrombopoietin and TGF-β have been linked to promoting quiescence through CDKI regulation, and thrombopoietin has additionally been linked to promoting *Hox* gene expression [32, 33, 38]. It is thus possible that Ash1l may be necessary for signaling downstream of essential niche factors required for bone marrow HSC quiescence and long-term function.

Interestingly, *Ash1l*-deficient mice did not progress to frank hematopoietic failure despite a severely depleted phenotypic LT-HSC compartment and a lack of detectable HSC function in transplantation assays. Increased H2B-GFP dilution exhibited by *Ash1l*$^{GT/GT}$ LT-HSCs may suggest that this severely reduced LT-HSC compartment elevated its proliferative output to maintain hematopoietic homeostasis. Indeed, actively proliferating HSCs have reduced engraftment potential in transplantation assays, and this could explain the observed difference between steady-state hematopoiesis and transplantation models in our system [29, 154]. Alternatively, the lack of detectable HSC function in transplantation assays could suggest that *Ash1l*$^{GT/GT}$ LT-HSCs are in fact non-functional and do not contribute to steady-state hematopoiesis. In this scenario, the robust dilution of H2B-GFP in LSK progenitors could indicate that progenitors downstream of LT-HSCs are responsible for maintained hematopoietic homeostasis. The fact that these progenitors actively proliferated but did not become depleted could suggest that they possess significant self-renewal activity. Such would be the case because if these cells strictly underwent differentiating divisions, they would be predicted to become depleted over time. This self-renewal activity would not be detectable in transplantation assays because quiescence is required for engraftment, as described above. This is an intriguing possibility in light of recent work in thymocytes indicating that in the absence of fresh

progenitors seeding the thymus, downstream progenitors could self-renew to maintain T cell output [165, 166]. This was striking because thymic progenitors were not previously shown to have self-renewal potential, and this was only revealed in the absence of continuous progenitor input. Perhaps the absence of functional LT-HSCs in our model revealed intrinsic self-renewal potential in downstream progenitors, similar to what was observed in the thymus.

Genetic models of HSC dysfunction that support non-ablative transplantation are rare findings in hematopoietic biology [61, 162]. In the $Ash1l^{GT/GT}$ system, successful engraftment in non-ablative transplantation experiments provided critical proof that the $Ash1l$-deficient BM niche was not grossly defective, as it could efficiently support the maintenance of wild-type LT-HSCs. Stable wild-type LT-HSC engraftment into the $Ash1l$-deficient niche suggests that niche spaces were available due to LT-HSC depletion and/or that remaining $Ash1l^{GT/GT}$ LT-HSCs were dysfunctional to an extent that they could be out-competed or displaced by wild-type LT-HSCs. Either scenario is consistent with extreme LT-HSC dysfunction when $Ash1l$ levels are reduced.

The functional interaction between Mll1 and Ash1l in LT-HSC and progenitor maintenance was a novel demonstration of the evolutionarily conserved cooperativity between members of the TrG. This notion has been explored in *Drosophila*, but had not been previously described in mammals. Perhaps the most striking demonstration of this was the finding that a single $Ash1l^{GT}$ allele amplified LT-HSC depletion in *Men1*-deficient and *Mll1* heterozygous mice. This dominant phenotypic enhancement is reminiscent of criteria used to initially identify TrG members in *Drosophila* [97]. Given that both Mll1 and Ash1l are required for *Hox* gene expression, it is possible that both

proteins act non-redundantly to promote transcription. Indeed, both $Ash1l^{GT/GT}$ and $Men1$-deficient hematopoietic progenitors displayed a reduced, but not absent expression of $Hoxa9$, suggesting multifactorial regulation of this locus [150]. Additional studies assessing whether this cooperativity is due to SET domain catalysis or perhaps through promoting the formation of larger, transcription-promoting protein complexes are required. Such studies will provide insight into the complex regulation of hematopoietic development and HSC function.

CHAPTER 4. SHELTERIN PROTEINS: FUNCTIONS IN TELOMERE PROTECTION AND HEMATOPOIESIS

INTRODUCTION

Telomere biology has historically been studied in the context of the end replication problem. This problem arises because the DNA replication machinery requires an initial RNA primer to provide a 3' hydroxyl group to begin DNA strand synthesis. This primer is removed, and as a consequence, the 3' end of the template strand is not efficiently replicated. Consequently, this process could result in the loss of essential genetic material if such material was encoded at the very 3' end of chromosomes. Telomeres provide a molecular buffer to such erosion, and thus protect essential genetic material during replicative stress. In long-lived proliferative stem cell populations, the ribonucleoprotein telomerase maintains telomere length [55-57, 167-170]. In recent years, we have learned that telomeres alone are not sufficient to protect the chromosome ends, as exposed DNA of any form triggers recognition by DNA damage machinery (the "end protection problem"). Six proteins, known as the shelterin complex, bind telomeric ends and prevent this aberrant DNA damage recognition. These proteins, Trf1, Trf2, Rap1, Tin2, Pot1 and Tpp1, not only protect telomeres, but also recruit telomerase and regulate telomere length [171]. In this chapter, we review the functions of individual shelterin proteins and discuss our current understanding of how they contribute to hematopoietic homeostasis.

TELOMERES AND THE DDR

Telomeres consist of a single-stranded, G-rich overhang preceded by a double-stranded region. Shelterin proteins bind to both the double- and single-stranded components (**Figure 4.1**). Without shelterin binding and end protection, telomeres are recognized as chromosomal aberrations and detected by a robust DDR. Exposure of the single-stranded G overhang elicits a robust ataxia telangectasia and Rad3-related (ATR)-mediated DDR [172, 173]. This is because, when uncovered, the single-stranded overhang is indistinguishable from single-stranded DNA that arises from replication fork stalling. Such single-stranded DNA is recognized and bound by replication protein A (RPA) [174]. RPA/ssDNA complexes are bound by ATR-interacting protein (ATRIP), which in turn recruits ATR. Additionally, RPA/ssDNA is bound by the Rad9-Rad1-Hus1 (9-1-1) complex through an interaction with Rad17 [175]. This complex recruits TopBP1, which activates ATR through a poorly defined mechanism [176-178]. Activated ATR phosphorylates Chk1 and can trigger cell cycle arrest through direct regulation of cyclin-dependent kinases or p53 activation (reviewed in [179-181]). This arrest is believed to allow cells the opportunity to repair DNA aberrations.

Exposure of the double-stranded portion of the telomere results in aberrant recognition of the telomere as a double-stranded break. Double-stranded breaks are sensed and bound by the Mre11-Rad50-Nbs1 (MRN complex) [182]. MRN recruitment triggers ataxia telangectasia mutated (ATM) kinase binding and enhances ATM interaction with Chk2 and p53 [182-184]. Activated Chk2 can then enforce cell cycle arrest both directly and through p53 activation (reviewed in [179]). A growing body of work additionally supports that ATM may activate ATR, suggesting that as the DDR

evolves in response to double-stranded breaks, both kinases may be activated to promote more robust cell cycle slowing and repair processes [185].

DDR activation at the telomere primarily proceeds to activation of non-homologous end joining (NHEJ) and homologous recombination (HR), although complex genetic models indicate that additional repair pathways may be activated [186]. A growing body of literature, described in detail below, indicates that exposure of the double-stranded portion of the telomere activates NHEJ repair, while exposure of the single-stranded portion activates HR [172, 173, 187]. As NHEJ attempts to repair DNA damage through re-joining perceived DNA breaks, this results in the fusion of unrelated chromosomes in the context of telomere instability. Since telomeric HR attempts to repair lesions through the use of homologous sister chromatid templates, attempts at HR are reflected by the exchange of material between sister chromatids, or sister chromatid fusions. DNA abnormalities consistent with NHEJ and HR are identifiable in metaphase chromosome preparations depending on the model of telomere deprotection.

THE FUNCTION OF INDIVIDUAL SHELTERIN PROTEINS IN TELOMERE PROTECTION

DDR components, including ATR and ATM, are recruited to telomeres during cellular proliferation to promote efficient telomere replication [188]. These proteins are required to alleviate replication fork stalling triggered by the repetitive telomeric DNA sequence and to promote secondary structure formation required for telomere stability. Studies using murine embryonic fibroblasts (MEFs) showed that individual shelterin proteins have specific functions in suppression of the DNA damage response and telomere regulation (**Figure 4.1**). This complex has three general classes of proteins: those that bind the double-stranded portion of telomeric DNA, those that bind the single-

stranded portion of telomeric DNA, and those that do not bind DNA but instead interact

with other shelterin proteins.
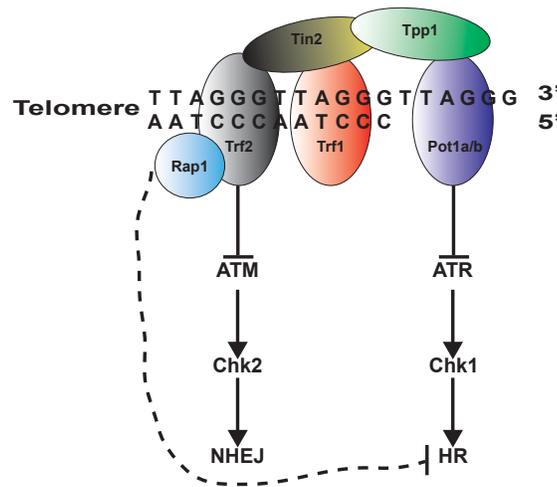
Figure 4.1. The function of individual shelterin proteins in telomere protection. Pot1 (in mice Pot1a) prevents recognition of the single stranded telomeric overhang from an ATR-Chk1 mediated DDR. Recognition by this pathway results in attempts at DNA repair through homologous recombination (HR). Trf2 prevents recognition of the double-stranded portion of the telomere from an ATM-Chk2 mediated DDR. ATM activation results in attempts at telomere repair through nonhomologous end-joining (NHEJ). Pot1 is linked to the shelterin complex through a Tpp1/Tin2 tether. Rap1, a Trf2 interacting protein, plays a poorly defined role in preventing aberrant telomeric HR.

Trf1

Trf1 and Trf2 directly bind doubled-stranded telomeric DNA but play unique

roles in telomere protection [189-192]. Over-expression of Trf1 in a human telomerase

positive cell line resulted in telomere shortening, while a dominant negative Trf1 elicited

a telomere lengthening phenotype [193]. These data suggest that Trf1 is a negative

regulator of telomere length. Additional work led to the proposal that Trf1, along with

Trf2, is not involved in the direct regulation of telomerase expression or activity, but

rather is linked to the formation of higher ordered structures that prevent telomerase

access to telomeres [194]. This telomerase inaccessible, or closed state, was predicted to

be dependent on the number of Trf1/2 proteins bound to the telomeric end. Thus, limiting Trf1/2 abundance or shortening the telomere to a point that restricted the number of bound Trf1/2 proteins should result in telomerase recognition and subsequent telomere lengthening.

Studies in MEFs demonstrated that when *Trf1* was conditionally inactivated, the DNA replication machinery stalled at the telomere, and abundant gaps, known as fragile sites, could be observed in metaphase chromosomes [195, 196]. Such chromosomal aberrations have been linked to chromosomal instability and cancer. This work suggested an interesting role in Trf1-mediate regulation of efficient telomere replication. *Trf1*-deleted MEFs rapidly progressed to cellular senescence in a p53 and retinoblastoma-dependent fashion [195, 196]. Conflicting data exist regarding the upstream components of DNA damage signaling following *Trf1* deletion. One report documented the presence of chromosomal fusions and a robust DDR consisting of both ataxia telangectasia mutated (ATM) kinase and ataxia telangectasia and Rad3-related (ATR) kinase activation [196]. The second report did not document chromosomal fusions and demonstrated an S-phase specific ATR response [195]. The nature of the discrepancy between these reports is unclear, though it may be attributable to different Cre-mediated *Trf1* deletion strategies and slightly different experimental timelines. It is also possible that ATM/ATR activation was not directly due to *Trf1* deletion, but was instead secondary to replication fork collapse and accumulating genetic lesions following *Trf1* deletion.

Mouse models of *Trf1* loss have demonstrated critical roles for Trf1 in developmental and cancer biology. Constitutive deletion of *Trf1* results in embryonic lethality before E6.5 [197]. This lethality did not appear to occur concurrently with

telomere instability, as fragile site formation or chromosomal fusions were not observed. Furthermore, *p53* deletion, which rescued MEF phenotypes, only modestly extended embryo survival and did not rescue embryonic lethality. This suggested a critical role for Trf1 in early development. Deletion of *Trf1* in the bone marrow resulted in a progression to bone marrow failure within 3 weeks (discussed in detail below) [59]. This failure was linked to p53/p21 activation, telomeric DDR activation, telomeric shortening, and eventual cellular senescence. The significance of p53 activation in these processes was not directly tested. Deletion of *Trf1* in the skin resulted in severe epidermal developmental defects and mortality due to the loss of skin barrier function [196]. Interestingly, *p53* deletion in the skin was sufficient to rescue developmental defects. However, this rescue strategy resulted in squamous cell carcinoma development, demonstrating that underlying genetic instability due to Trf1 loss was tumorigenic. These data are intriguing in that they suggest that different developmental processes or cell types may differentially activate DDR signaling in response to shelterin defects. Furthermore, they suggest that limiting the DDR through p53 ablation, though capable of rescuing some developmental processes, can result in sustained chromosomal instability sufficient to initiate tumorigenesis.

Trf2

Trf2 directly protects telomere from an ATM-dependent DDR [172, 173, 198, 199]. Loss of Trf2 triggers telomeric fusions as a result of ATM-mediated activation of non-homologous end-joining (NHEJ) [187, 199, 200]. Interestingly, Trf2 loss was found to activate a DNA damage response in all phases of the cell cycle, though NHEJ activity was restricted to G1 [199, 201]. These data support the idea that Trf2 loss induces

telomeric DDR activation at all times, rather than eliciting a DNA damage response secondary to replication fork stalling during strand replication (unlike Trf1 loss).

Trf2, like Trf1, also seems to play a role in telomere length homeostasis. Overexpression of *Trf2* in telomerase positive human cells resulted in progressive telomere shortening, and telomere elongation was observed when Trf2 levels decreased [194]. This finding was not related to regulation of telomerase expression or activity, and may have been due to decreased telomerase access to telomeres in the presence of increased Trf2, as discussed above.

As seen for Trf1, *Trf2* knockouts are embryonic lethal, suggesting broad roles in early development [198]. Genetic *p53* deficiency did not rescue this embryonic phenotype. Interestingly, overexpression of *Trf2* in the skin resulted in hyperpigmentation and predisposed mice to squamous cell carcinomas [202]. Consistent with the previously described human cell line data, epithelial studies from mice with *Trf2* overexpression revealed prominent telomere shortening. This shortening was not the result of telomerase dysfunction. If telomerase deficiency was coupled with *Trf2* overexpression, tumorigenesis was significantly accelerated [203]. This occurred as the result of enhanced telomere dysfunction and fusion events in the setting of increased *Trf2* and telomerase deficiency. These data suggest that while initial telomere shortening due to increased Trf2 is not due to telomerase dysfunction, the addition of telomerase dysfunction further destabilizes the genome to increase tumorigenesis.

Rap1

Rap1 does not bind telomeric DNA, but instead is localized to the telomere by an interaction with Trf2 [204, 205]. Rap1 was originally identified as suppressing NHEJ at

the telomere [206-208]. Subsequent work failed to identify a function for Rap1 in suppressing NHEJ or in preventing ATM activation [204, 209, 210]. Instead, *Rap1*-deficient MEFs had increased telomeric homologous recombination (HR) events without evidence of DDR activation [209, 210]. These data indicated that Rap1 is not involved in the suppression of a telomeric DNA damage response, although it seems to be involved in regulation of HR at telomeres.

Rap1 deficiency does not result in embryonic death in mice suggesting that vigorous DDR activation in embryonic development is the key determinant of the embryonic lethality observed in *Trf1*- and *Trf2*-deficient mice. [210]. Interestingly, despite seemingly normal development, *Rap1* deletion in the skin results in hyperpigmentation, telomere shortening, and increased DDR activation in adulthood. Together, these findings suggest that Rap1 does indeed play an important role in physiological telomere homeostasis. Furthermore, studies with Rap1 demonstrate the need to consider the physiological function of individual shelterin components in a developmental context rather than solely in cell line-based studies.

Pot1

Pot1 is an evolutionarily conserved protein that directly binds to the single-stranded G overhang of telomeres [211-213]. This binding is dependent on heterodimerization with Tpp1 (discussed below) [172, 214-217]. At telomeres, Pot1 plays two critical roles in homeostasis. The first is to suppress an ATR-mediated DNA damage response that would result in aberrant HR [218]. Work using chicken Pot1 demonstrated that ATR activation causes growth arrest as cells transition into the G2 phase of the cell cycle [219]. The second critical Pot1 function is the regulation of G strand overhang

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length [218]. Following telomere replication, exonucleolytic processing of the 5' C strand is required to generate the 3' G strand overhang. Initial work suggested that Pot1 regulates the extent of 5' resection and that the absence of Pot1 results in excessive 5' resection and extended 3' overhangs. Subsequent studies in mice, discussed below, revealed a different mechanism by which Pot1 prevents this resection [220].

In mice, the *Pot1* gene has undergone a duplication event resulting in two *Pot1* genes: *Pot1a* and *Pot1b* [221]. This duplication established a separation of the two main POT1 functions between murine Pot1a and Pot1b. Biochemical analysis of the murine proteins demonstrated that the N-terminus of Pot1a is the critical element for ATR inhibition, while the C-terminus of Pot1b prevents excessive 5' resection [221, 222]. Pot1b may play a minor role in end protection, as the combined loss of Pot1a and Pot1b elicits a more robust telomeric DDR than Pot1a loss alone [221]. Furthermore, only combined Pot1a/b loss resulted in a significant increase in telomeric instability and homologous recombination [218, 221]. Additionally, Pot1a/b loss caused increased endoreduplication (replication of DNA without cellular division) in MEFs [221]. As a result of this phenomenon, MEFs with >4N DNA content accumulate in culture.

The DDR induced by *Pot1a* loss resulted in a p53-mediated induction of senescence [222]. This increased senescence was demonstrated in the context of growth slowing in *Pot1a$^{-/-}$* MEF cultures and increased p21 and phospho-p53 levels. Furthermore, this senescence could be by *p53* inactivation in *Pot1a$^{-/-}$* MEFs.

Though *Pot1b* loss did not initiate robust DDR activity, it did result in significant telomeric shortening after many cell culture passages [223, 224]. While *Pot1b* loss did not cause chromosomal instability alone, the combination of *Pot1b* loss with telomerase

deficiency resulted in extensive chromosomal fusions [223]. Furthermore, when *Pot1b*^{-/-} MEFs lost a single copy of the gene encoding the telomerase RNA, rendering cells haploinsufficient for telomerase activity, telomere shortening was exacerbated [223, 224]. These MEFs also had a significant increase in chromosomal fusions [223, 224]. *Pot1* loss combined with telomerase haploinsufficiency induced ATR activation, though it is not clear if this was due to acute deprotection or resulted from increased genomic instability due to excessive telomere shortening [224].

Elegant studies recently revealed the mechanism by which Pot1b prevents excessive 5' C-strand resection [220]. Two nucleases, Apollo and Exo1, act to modify telomere length following telomere elongation. Apollo initiates resection, while Exo1 extends 5' resection. Pot1b limits Apollo-mediated resection. Furthermore, Pot1b is involved in the recruitment of the CST complex to the telomeric end. This complex is a DNA polymerase α cofactor that facilitates strand fill-in of the 5' C strand. Briefly, the CST complex aids in the initiation of lagging strand synthesis at the 5' telomeric end after resection (reviewed in [225]). This fill-in thus limits the relative length of the 3' overhang. Pot1b therefore plays two roles in regulating 3' overhang length by limiting 5' resection and facilitating 5' strand fill-in. It remains to be determined if this mechanism is conserved in humans.

In mice, *Pot1a* deletion results in early embryonic lethality, while *Pot1b*-deficient mice are born in mendelian ratios with normal fertility [221, 222, 224]. This suggested a critical role for Pot1a but not Pot1b in early development. Despite initial fertility, *Pot1b*-deficient male mice eventually become infertile with reduced sperm production [223, 224]. Additionally these mice develop skin hyperpigmentation, increased intestinal

apoptosis, and pancytopenia. This phenotype is reminiscent of the human progeroid

syndrome dyskeratosis congenita (reviewed in [226]). Combination of *Pot1b* deficiency

and telomerase haploinsufficiency, as described for MEFs above, resulted in a significant

worsening of all aspects of the *Pot1b*-deficient phenotype and in eventual death of these

mice due to bone marrow failure. The role of shelterin proteins in models of bone marrow

failure will be discussed in detail below. Complete loss of telomerase activity in the

setting of *Pot1b* deficiency resulted in embryonic lethality, perhaps due to excessive

chromosomal shortening during early development.

Tpp1

Pot1 binding requires Tpp1, the protein product of the *Acd* gene [172, 214-217].

Studies in *Acd*^{-/-} MEFs have indeed revealed that many elements of the *Acd*-deficient

phenotype reproduced those observed when *Pot1a/b* were deleted, predominantly

identifying suppression of ATR signaling and HR through Pot1a/b as the principle

function of Tpp1 [215]. Additional studies, however, have implicated Tpp1 in a more

complex role in end protection. Two groups have identified NHEJ-type chromosomal

fusions and not just homologous recombination events in the setting of *Acd* deficiency,

suggesting protective functions for Tpp1 beyond Pot1a/b recruitment [172, 227]. This is

perhaps due to the triggering of an alternative NHEJ pathway that has been described to

be activated at telomeres when Pot1a/b are lost [187]. Using a knockdown approach (as

opposed to the null allele approach used in [215]), it was demonstrated that Tpp1 loss

resulted in an ATM-dependent DNA damage response, compared to an ATR dependent

DNA damage response following *Pot1a/b* knockdown [172]. An additional report

demonstrated that Tpp1 loss in MEFs through a conditional inactivation strategy resulted

in activation of both ATM- and an ATR-mediated DNA damage responses [228]. The reason for the differences in the specific nature of the DDR identified between these studies is unclear, though the use of different models of *Acd* inactivation (genetic deletion vs. knockdown vs. deletion of the Pot1a/b interacting domain), differences in allelic targeting strategies in *Acd* knockout generation, or variations in DDR detection strategies could be responsible for these differences. Regardless, these studies demonstrated that Tpp1 played a critical role in telomeric end protection.

Acd deletion ultimately resulted in p53-mediated growth arrest in MEFs [172, 228]. In these studies, *p53* inactivation significantly improved *Acd*-deficient MEF cell cycle activity. Additional work demonstrated that there might also be p53-independent factors contributing to *Acd*-deficient MEF growth arrest [215]. In this work, MEFs that had been immortalized through SV40 large T antigen, which inactivates both p53 and the tumor suppressor retinoblastoma (Rb), still showed signs of growth arrest. Together, these data demonstrated that p53-depenedent and independent factors contributed to growth arrest and eventual senescence when *Acd* was inactivated.

In addition to being essential for protection from the DDR, Tpp1 plays a multidimensional role in regulating telomerase activity at the telomeric end. Initial work in a human cell lines demonstrated that TPP1 was required for the recruitment of telomerase to the telomeric end [229]. This telomerase recruitment activity was attributed to the oligonucleotide/oligosaccharide binding (OB) fold domain of TPP1. Recent studies identified that a small region in the TPP1 OB fold, termed the TEL patch, was both necessary and sufficient for this telomerase recruitment [230, 231]. This suggests that TPP1 may be required not just for DDR suppression, but also for telomere elongation in

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telomerase-expressing cell types, including embryonic and somatic stem cells and cancer cells.

In addition to telomerase recruitment, TPP1 promotes telomerase processivity [232]. *In vitro* biochemical analysis demonstrated that the presence of TPP1 increases the efficiency with which telomerase extends telomeres. Recent work identified that this activity is tractable to the TEL patch of the OB fold. Thus, the OB fold was linked to both telomerase recruitment and the efficiency of telomerase activity in extending telomeres.

A recent study has identified that TPP1 interacts with the CST complex to limit excessive telomere elongation [233]. This study proposed a model in which the CST complex is in competition with TPP1/POT1 during telomere elongation. At early steps of telomere replication, TPP1 recruits telomerase and promotes telomere extension, as described above. As replication progresses, CST binds the elongating 3' G-strand, preventing further association between telomerase and the G-strand. Additionally, the presence of CST inhibits the ability of TPP1 to enhance telomerase processivity. The authors of this study suggested that CST present at the telomere could promote 5' strand fill-in (discussed above), but did not directly test this phenomenon. This study demonstrated the complex role that TPP1 plays in telomere length homeostasis.

Given the complex role that Tpp1 plays in telomere length homeostasis and DDR repression, it was not surprising that complete *Acd* inactivation results in embryonic lethality [215]. Studies using a spontaneously occurring *Acd* splice variant that results in a hypomorphic *acd* allele demonstrated several essential development functions for *Acd* [234]. Depending on the mouse strain to which the allele was bred, mice homozygous for *acd* displayed adrenocortical dysplasia, caudal truncation, genitourinary abnormalities,

skin hyperpigmentation, and significant strain-dependent embryonic or perinatal lethality. Interestingly, a subsequent study reported that conditional inactivation of *Acd* in the skin resulted in profound hyperpigmentation and eventual mortality due to the loss of skin barrier function [228]. In both skin-specific *Acd* deletion and *acd* homozygous mice, the majority of the phenotypic abnormalities could be rescued by *p53* inactivation [228, 235, 236]. Interestingly, *p53* inactivation failed to rescue strain-specific embryonic mortality in *acd* homozygotes [235]. Together, these data suggested that Tpp1 plays critical roles in mammalian development, and that, as was true in MEF studies, these processes are mediated by p53-dependent and independent mechanisms.

Tin2

Tin2 is an adaptor protein that plays critical roles in stabilizing the shelterin complex. Within the shelterin complex, Tin2 binds both Trf1 and Trf2 and stabilizes Trf1/Trf2 binding to telomeres [237]. Tin2 also binds Tpp1 and is required for Tpp1/Pot1a/b recruitment to the shelterin complex [238]. Loss of Tin2 results in ATR activation, due to the loss of Tpp1/Pot1a, excessive 3' overhang generation, due to loss of Tpp1/Pot1b, and ATM activation, due to Trf2 destabilization. These data highlight the critical function of Tin2 in organizing the shelterin complex.

TIN2 has additional roles outside of telomere homeostasis. TIN2 was detected in the mitochondria in a human cancer cell line, and TIN2 knockdown resulted in the adoption of abnormal mitochondrial morphology [239]. TIN2 loss promoted increased oxidative metabolism and reduced glycolysis in these human cancer cells. These data demonstrate that TIN2 has telomeric and extratelomeric functions, and suggest the possibility that other shelterin components may function away from the telomere.

Tin2 loss causes embryonic lethality in mice [240]. Conditional inactivation of

Tin2 has not been characterized *in vivo*. However, TIN2 mutations were identified in a

cohort of human patients with dyskeratosis congenita [241-244]. These findings are

discussed below, but indicated that TIN2 indeed plays a critical role in telomere

homeostasis *in vivo*.

THE SHELTERIN COMPLEX AND BONE MARROW FAILURE

Perhaps the best demonstration of the significance of the shelterin complex in

bone marrow failure has come from studies of dyskeratosis congenital (DKC). DKC is a

human telomere shortening syndrome that has been linked to mutations in *TERC*

(telomerase RNA), *TERT* (telomerase protein), *DKC1* (encoding dyskerin, an accessory

protein required for *TERC* stability and function), and recently in *TIN2 (*reviewed in

[245]). Patients classically present with the triad of oral leukoplakia, abnormal skin

pigmentation, and nail abnormalities. Up to 85% of these patients develop bone marrow

failure with a variable timeline, thus establishing the need to consider DKC in the context

of hematopoiesis [226]. It is estimated that up to 60% of DKC cases do not have a known

underlying genetic defect [246]. This suggests that our knowledge of the genetic causes

of DKC remains limited, and additional contributing genes must be considered.

Mutations in *TIN2* identify a unique class of DKC patients. Genetic analysis

identified a mutation near the region of TIN2 that interacts with TRF1 [247]. Based on

studies described above, such a mutation could greatly destabilize the shelterin complex

and elicit a robust DDR. These patients had a particularly severe form of DKC

characterized by early symptom onset with broad organ involvement, and an increased

incidence of bone marrow failure [243]. At least two reports documented that these

findings are associated with more severe telomere shortening than other DKC forms [242, 244]. Since TIN2 disruption could result in TPP1/POT1loss and TRF1/TRF2 destabilization, this may induce a robust DDR activation and consequent telomere attrition due to deprotection. DKC arising from TERC or TERT dysfunction, on the other hand, would result in progressive telomere erosion as highly replicative tissues turn over. The differences between these models would predict that *TIN2* mutations would result in a more acute and perhaps widespread form of DKC, while *TERC/TERT* mutations would result in a more progressive and perhaps localized form of DKC. The possibility that other shelterin components could similarly contribute to human DKC has yet to be evaluated.

HSCs from human DKC patients with *TERC* mutations showed that defective self-renewal could significantly contribute to bone marrow failure in DKC [248]. HSC recovery from DKC patients was significantly reduced, but differentiation ability was not impaired in short-term culture. Strikingly, HSC self-renewal assessment in long-term cultures showed severe self-renewal defects and advanced telomere shortening. Together, these data demonstrated that bone marrow failure in DKC patients is not due to defective differentiation, but is instead a direct result of impaired HSC self-renewal. The link between HSC dysfunction and bone marrow failure in other forms of DKC has not been evaluated.

MOUSE MODELS OF DKC

Mouse models of *Trf1, Pot1b, Acd,* and *Rap1* deficiency, along with *Trf2* overexpression all resulted in skin hyperpigmentation [196, 202, 210, 223, 224, 228, 234, 236]. This was reminiscent of classical skin pigmentation abnormalities described in

DKC patients. This similarity prompted the further assessment of the extent to which models of shelterin deficiency recapitulate pathologies associated with human DKC. Of significant interest was whether or not these models reproduce the primary cause of DKC mortality: bone marrow failure.

The first mouse models of DKC developed from observations of aforementioned mice in which *Pot1b* deficiency was combined with *Terc* haploinsufficiency [223, 224]. In addition to skin hyperpigmentation, these mice displayed progressive testicular atrophy, increased apoptosis in intestinal crypts, and reduced lifespan in the context of telomere shortening. Reduced life expectancy was attributed to the development of severe pancytopenia and bone marrow failure. This was significant for being the first demonstration of a progressive bone marrow failure in the setting of shelterin disruption, and thus the first evidence that studies in shelterin could provide models to understand DKC pathophysiology.

It remained to be determined if bone marrow failure in the $Pot1b^{-/-}Terc^{+/-}$ model could be linked to HSC failure. It was possible that progressive telomere shortening resulted in defective differentiation of HSCs to mature cell types, or that shortened telomeres triggered apoptosis in differentiating progenitors as opposed to HSCs. Recent work revealed that significant HSC self-renewal defects were the main cause of bone marrow failure [60]. $Pot1b^{-/-}Terc^{+/-}$ HSCs were reduced in number and failed to compete in bone marrow transplantation assays. Hematopoietic progenitor cells had increased p53 activation with a bias towards an apoptotic response rather than cell cycle arrest. Of particular significance, *p53* inactivation significantly rescued HSC function, demonstrating that p53 played a critical role in reduced $Pot1b^{-/-}Terc^{+/-}$ HSC function.

A recent model of *Trf1* deficiency further demonstrated the significance of shelterin in models of DKC [59]. *Trf1* deletion resulted in a progression to bone marrow failure within 3 weeks of deletion. *Trf1$^{-/-}$* HSCs had impaired self-renewal activity in transplantation assays. Bone marrow failure did not occur in conjunction with increased apoptosis, but significant *p21* up-regulation suggested a role for p53 activation in this model. The role of p53 activation in limiting HSC self-renewal was not tested. Of significance in this study, bone marrow failure was described as occurring in the context of telomere shortening and increased senescence during long-term studies. The authors of this study did not address if remaining hematopoietic progenitors at these late time points were in fact *Trf1$^{-/-}$* or if they represented cells that had escaped Cre-mediated deletion. If relatively few cells escaped and reconstituted hematopoiesis, these HSCs would be exceptionally stressed and could display telomere shortening and increased senescence as a result. This is an important point to resolve experimentally.

Thus, shelterin-based studies revealed two complementary, but significantly different models of DKC and bone marrow failure. *Pot1b$^{-/-}$Terc$^{+/-}$* resulted in a progressive bone marrow failure that demonstrated cooperativity between shelterin and telomerase in regulating HSC self-renewal. Trf1 loss resulted in bone marrow failure that was caused by acute HSC dysfunction. Since this study did not require additional telomerase dysfunction, it suggested that certain shelterin components are uniquely capable of destabilizing telomere homeostasis in a timeline not consistent with telomere erosion. This is not a concept that has been rigorously tested *in vivo*, though the data indicate that it ought to be, as human patients with *TIN2* mutations progress to bone marrow failure in a more acute timeline than is observed with other known DKC

mutations. Therefore, similar to studies in MEFs and cell lines, individual shelterin proteins must be evaluated for their roles in the maintenance of HSC function as it relates to bone marrow failure.

CHAPTER 5. MURINE HEMATOPOIETIC STEM CELLS ARE ACUTELY SENSITIVE TO INACTIVATION OF THE SHELTERIN GENE *ACD*

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ABSTRACT

The shelterin complex plays dual functions in telomere homeostasis by recruiting telomerase and preventing activation of a DNA damage response. Somatic stem cells require telomerase activity, as evidenced by progressive stem cell loss in hereditary dyskeratosis congenita. Recent work demonstrates that dyskeratosis congenita can also arise from mutations in specific shelterin genes, although little is known about shelterin functions in somatic stem cells. Here, we report that hematopoietic stem cells (HSCs) are acutely sensitive to inactivation of the shelterin gene *Acd*, encoding Tpp1. Homozygosity for a hypomorphic *Acd* allele led to profoundly defective fetal HSCs. Upon complete *Acd* inactivation, HSCs expressed p53 target genes, underwent cell cycle arrest and were severely depleted within days, leading to hematopoietic failure. Tpp1 loss induced increased telomeric fusion events. However, unlike in epidermal stem cells, *p53* inactivation did not rescue Tpp1-deficient HSCs, indicating that shelterin dysfunction has unique effects in different stem cell populations. Because consequences of telomere

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shortening are progressive and unsynchronized, acute loss of shelterin function represents an attractive alternative to study telomere crisis in hematopoietic progenitors.

INTRODUCTION

Linear chromosomes are capped with telomeres to protect their ends from the loss of genetic material during strand replication. Disruptions in the stability of this molecular buffer have been linked to organ failure, aging and cancer. Privileged compartments, including the germline and somatic stem cells, express the ribonucleoprotein telomerase to maintain telomere length during replicative stress [53, 167]. When this activity is impaired, stem cell populations become depleted, leading to loss of tissue homeostasis [56]. In addition, telomeres must be protected from the DNA damage response that would perceive telomeres as sites of DNA breaks, a function achieved by the shelterin complex. Together, the six shelterin proteins Trf1, Trf2, Rap1, Tin2, Pot1 and Tpp1 not only protect telomeres, but also recruit and regulate telomerase activity [171]. Understanding the biological functions of these proteins is therefore critical to understanding telomere homeostasis and human diseases related to dysfunctional telomeres.

Studies using murine embryonic fibroblasts showed that individual shelterin proteins have specific functions in suppression of the DNA damage response and telomere regulation. Pot1 binds the single-stranded telomeric overhang and prevents ataxia telangectasia and Rad3-related (ATR) kinase activation [172, 173]. Pot1 binding requires Tpp1, the protein product of the *Acd* gene [172, 214, 215]. In addition to being essential for Pot1 recruitment, Tpp1 recruits telomerase to the telomeric end and is required for telomere extension [229, 232]. Trf1 and Trf2 bind the double-stranded portion of the telomere [189-191]. Trf2 prevents ataxia telangectasia mutated (ATM) kinase from

mistaking telomeric ends for sites of DNA breaks [173, 249]. Tin2 stabilizes Trf1 and Trf2 at the telomere and binds to Tpp1, linking the single-stranded and double-stranded binding portions of shelterin [237, 238]. Rap1 interacts with Trf2 and prevents aberrant non-homologous end joining from occurring at the telomere [206-208]. In mice, studies of the shelterin complex are complicated by the duplication of the *Pot1* gene into *Pot1a* and *Pot1b* [221]. Pot1a prevents ATR activation and Pot1b prevents excessive 5' resection at the telomere and consequent generation of excessive 3' overhangs [221]. In humans, a single POT1 protein accomplishes both of these functions [218]. To obtain complete loss of Pot1 function in mice, either *Pot1a/b* double deficient mice must be used, or *Acd* must be inactivated. In embryonic fibroblasts, both *Acd* inactivation and *Pot1a/b* deletion caused rare telomeric fusion events and proliferative arrest, a phenotype that required p53-driven expression of the cyclin-dependent kinase inhibitor *p21* [172, 214, 215, 221].

Although embryonic fibroblasts have been a useful tool in understanding the molecular functions of the shelterin complex in cell culture systems, the physiological role of shelterin components *in vivo* remains poorly understood, especially in tissues maintained by somatic stem cells. Recent studies demonstrated that when combined with telomerase haploinsufficiency, *Pot1b* deficiency resulted in a gradual decline in tissue homeostasis similar to that observed in late generation telomerase-deficient mice [224]. These mice displayed skin hyperpigmentation and bone marrow failure reminiscent of human patients with the telomere shortening syndrome dyskeratosis congenita (DKC). DKC has been linked to mutations in the telomerase components genes *TERC* and *TERT*, or in *DKC1*, encoding the telomerase accessory protein dyskerin [250]. Recently,

mutations affecting the shelterin gene *TIN2* have been identified in patients with a particularly aggressive form of the disease [241-244]. As HSC loss leading to bone marrow failure is the most frequent cause of lethality in DKC, understanding the importance of telomerase and shelterin genes in hematopoiesis is relevant to human disease. Mice deficient for *Acd* due to homozygosity for a spontaneously arising hypomorphic *acd* allele demonstrate a pleiotrophic phenotype that includes adrenocortical dysplasia, caudal truncation, genitourinary abnormalities, skin hyperpigmentation, and significant strain-dependent embryonic or perinatal lethality [234]. These data indicate tissue-specific functions of Tpp1. Furthermore, the skin hyperpigmentation phenotype observed in *Acd*-deficient mice bares a striking similarity to that observed in DKC patients and in the Pot1b-deficient mouse model. Subsequent studies using complete loss of *Acd* in a skin-specific knockout model indicated that the hyperpigmentation phenotype resulted from functional defects in epidermal stem cells, suggesting a role for shelterin components in stem cell maintenance in this compartment [228]. In both the complete knockout and hypomorphic *Acd* models, defects were linked to telomere dysfunction as well as p53-mediated apoptosis and proliferative arrest [228, 235, 236].

To gain detailed insight into functions of the shelterin complex in hematopoiesis, we studied the impact of *Acd* deficiency on hematopoietic stem cells (HSCs). By targeting *Acd*, we focused on a central component of the shelterin complex that is essential for Pot1a/b function and plays a role in telomerase recruitment. Our approach was designed to capture both acute and long-lasting effects of *Acd* deficiency on hematopoietic stem and progenitor cells. We found that stem cell function was profoundly dependent on

Tpp1 in both fetal and adult hematopoiesis. In mice homozygous for a hypomorphic *Acd* allele, hematopoietic stem and progenitors were generated and maintained during fetal life, but they acquired phenotypic abnormalities, evidence of G2/M arrest and a complete inability to reconstitute irradiated recipients after transplantation, indicating defective function. In the complete absence of *Acd*, HSCs were rapidly depleted and animals progressed to frank hematopoietic failure. This phenotype was cell-autonomous and surprisingly acute, as a complete depletion of the hematopoietic stem and progenitor compartment was observed within 5 days after *Acd* inactivation. Tpp1 loss led to rapid induction of p53 target gene expression. However, *p53* inactivation failed to rescue HSC depletion and function. These findings differed markedly from past observations on hematopoiesis in Pot1b-deficient mice as well as organ development in *Acd* hypomorphic mice and skin stem cell function in the absence of *Acd*, as all these phenotypes were largely rescued by p53 deficiency. Thus, our data identify an essential acute requirement for *Acd* in hematopoietic stem and progenitor cells that differs from its effects in other tissues and stem cell compartments.

RESULTS

Acd deficiency results in cell cycle arrest and impaired function of fetal liver hematopoietic stem cells.

To assess the role of *Acd* in hematopoiesis, we first used mice that were homozygous for a spontaneously occurring hypomorphic allele [234]. This hypomorphic *Acd* variant is caused by a G to A transition within the third intron of the gene (**Figure 5.1A**), resulting in aberrant splicing and either a 7bp insertion after exon 3 or the inclusion of the entire third intron in the mRNA [227, 234]. Both outcomes cause

premature termination of translation and a truncated protein lacking all functional domains. Homozygosity for this allele (henceforth *acd*) decreases expression of wild-type transcripts to ~2% of normal, consistent with a profoundly hypomorphic phenotype [227]. Analysis of the fetal livers from E13.5 *acd* mice revealed an overall mild decrease in fetal liver cellularity compared to control littermates (**Figure 5.1B**). Of note, *acd* fetuses also had a reduction in body weight (data not shown). When cell numbers were normalized in colony forming assays measuring hematopoietic progenitor activity, no defect in the ability of *acd* fetal liver cells to form myeloid colonies was observed (**Figure 5.1C**). Flow cytometric analysis revealed that *acd* fetuses had a normal frequency of Lineage$^-$ fetal liver cells and an increased frequency of Lineage$^-$Sca-1$^+$cKit^{hi} (LSK) cells, containing hematopoietic progenitors (**Figure 5.1D**). Furthermore, *acd* hypomorphism did not cause a significant defect in the overall frequency of CD150$^+$CD48$^-$ LSK cells, the most rigorous phenotypic definition of long-term hematopoietic stem cells (HSCs) [156, 157] (**Figure 5.1D**). Thus, HSC frequencies and myeloid progenitor activity were not compromised in *acd* animals. However, close analysis of the LSK progenitor compartment (containing HSCs) revealed that *acd* LSKs were larger, more granular, and expressed higher levels of the surface protein Sca-1, suggesting an activated phenotype (**Figure 5.1E**). Cell cycle analysis demonstrated an accumulation of *acd* progenitors at the G2/M phases of the cell cycle (**Figure 5.1F**). This phenotype was reminiscent of the G2/M arrest reported in *Acd*-deficient embryonic fibroblasts and epidermal progenitor cells [215, 228].
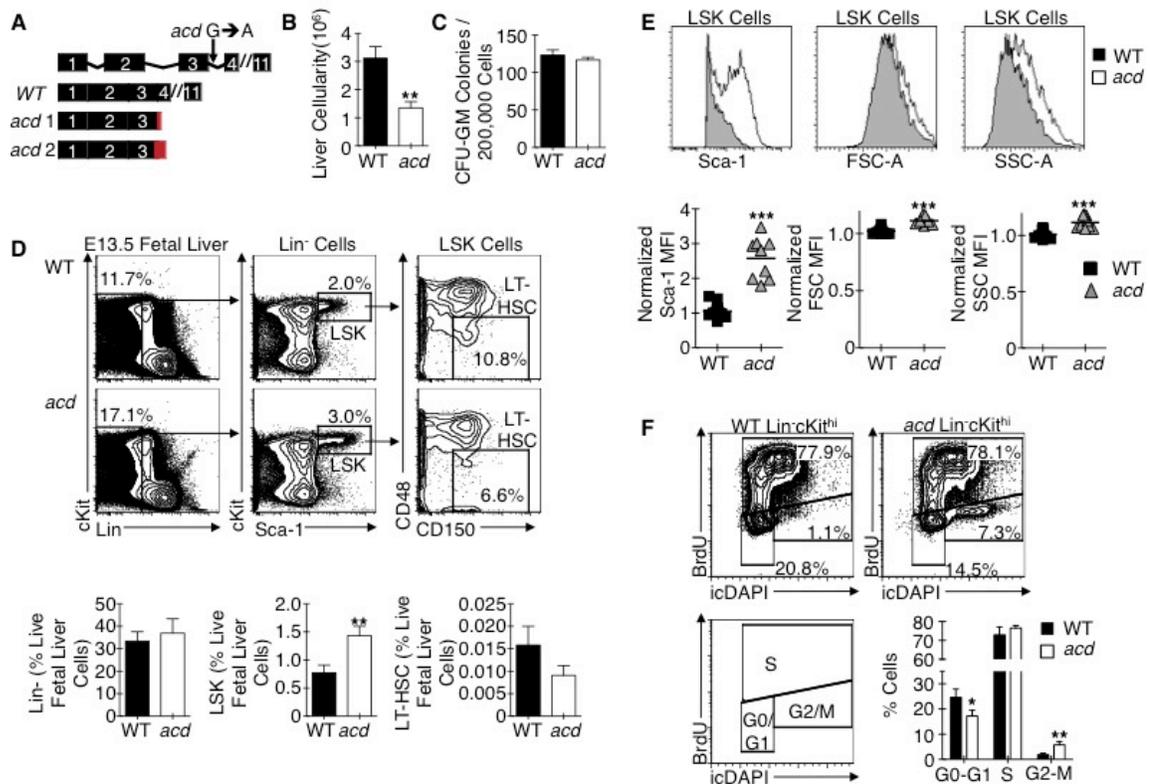
Figure 5.1. Reduced *Acd* expression results in phenotypic abnormalities and G2/M arrest in fetal hematopoietic progenitors. (**A**) Structure of the hypomorphic *acd* allele, a mutant *Acd* allele arising from an intron 3 G→A transition and leading to aberrant splicing. *acd* homozygosity decreased the abundance of normal transcripts to ~2% of wild-type (WT) (33); (**B**) Fetal liver cellularity in E13.5 mice homozygous for the *acd* hypomorphic allele; (**C**) CFU-GM analysis demonstrating preserved granulocyte-macrophage (GM) progenitor activity in E13.5 *acd* fetal liver (representative of 3 experiments with triplicate colony formation assays, mean +/-SEM); (**D**) Preserved overall frequency of phenotypically defined long term hematopoietic stem cells (LT-HSCs), defined as CD150$^+$CD48$^-$ Lin$^-$Sca^{hi}c-Kit^{hi} (LSK) cells (n$\geq$9 mice/group from 5 independent experiments, mean +/- SEM); (**E**) Increased Sca-1 expression in *acd* LSK progenitors. *acd* LSK cells were also larger and more granular by forward (FSC-A) and side scatter (SSC-A) characteristics, respectively (n$\geq$9 mice/group from 5 independent experiments). Gray shading shows data from control littermates, white shading represents *acd* progenitors. MFI: mean fluorescence intensity; (**F**) Cell cycle analysis with BrdU incorporation (12 hour pulse) and intracellular DAPI staining for (icDAPI, DNA content) in E13.5 *acd* Lin$^-$c-Kit^{hi} hematopoietic progenitors, showing accumulation in G2/M phases of the cell cycle (n=3 mice/group from 3 independent experiments, mean +/- SD). Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate. *p<0.05, **p<0.01, ***p<0.001.

Although colony formation assays measure the output of a heterogeneous progenitor pool, they do not evaluate HSC self-renewal. To study the function of *acd* fetal HSCs, we performed competitive transplantation assays in lethally irradiated

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recipients and followed transplanted animals to monitor long-term reconstitution (**Figure 5.2A**). We observed profound defects in trilineage reconstitution from *acd* fetal progenitors as early as 4 weeks post-transplant that persisted for the duration of the experiment (**Figure 5.2B**). When the LSK compartment of primitive hematopoietic progenitors was examined at the termination of the experiment, no contribution of *acd* cells could be detected (**Figure 5.2C**). These findings demonstrate that, despite preserved hematopoietic progenitor frequencies and myeloid progenitor activity, *acd* HSCs had severe functional impairment.
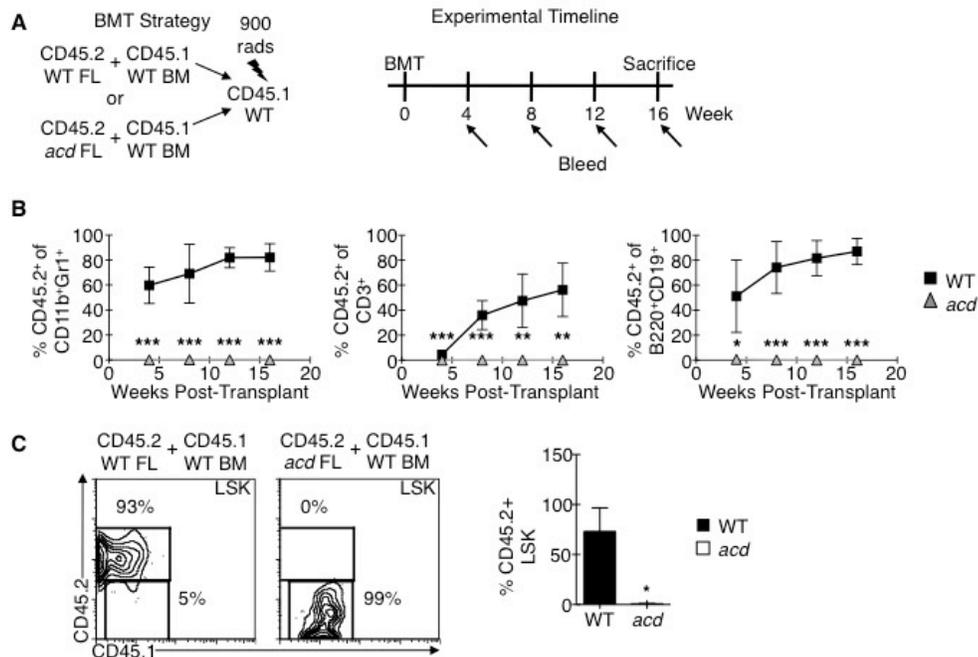
Figure 5.2. *acd* hematopoietic stem cells do not support long-term hematopoietic reconstitution. (**A**) Experimental design: lethally irradiated B6-CD45.1 mice were transplanted with 1:1 mixtures of E13.5 wild-type (WT) fetal liver (CD45.2$^+$) or E13.5 *acd* hypomorphic fetal liver (CD45.2$^+$) and B6-CD45.1 competitor bone marrow cells (5x10^5 cells each); (**B**) Flow cytometric analysis of peripheral blood 4-16 weeks after transplantation showing robust contribution of the wild-type but not *acd* CD45.2$^+$ fetal liver graft to myeloid (CD11b$^+$Gr1$^+$), T cell (CD3$^+$) and B cell compartments (CD19$^+$B220$^+$) (n=3-5/group, mean +/– SD); (**C**) CD45.1/CD45.2 chimerism in Lin$^-$Sca^{hi}c-Kit^{hi} (LSK) bone marrow progenitors 16 weeks after

transplantation. No residual CD45.2$^+$ *acd* progenitors could be detected. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate. Graphs show mean +/ – SEM. *p<0.05, **p<0.01, ***p<0.001.

Acd inactivation leads to acute depletion of adult hematopoietic stem and progenitor cells.

Analysis of the *acd* phenotype revealed profound HSC functional defects, but non-hematopoietic developmental abnormalities in *acd* fetuses could have contributed to these defects through non-cell-autonomous mechanisms. Furthermore, analysis of a hypomorphic phenotype may underestimate the full impact of *Acd* given the presence of residual wild-type transcripts at low levels. To bypass these limitations, we studied the effects of complete *Acd* inactivation in adult hematopoietic tissues using a conditional *Acd* allele that we previously described [215] (**Figure 5.3A**). This strategy facilitated robust, temporally controlled *Acd* excision in hematopoietic cells, and thus allowed us to study the acute effects of Tpp1 loss in hematopoietic tissues. We induced *Acd* inactivation using poly(I:C) injections to activate the interferon-responsive *Mx-Cre* transgene [251] (**Figure 5.3A, B**). Within 48 hours of a single poly(I:C) injection, we achieved at least 80% excision of the floxed *Acd* allele (**Figure 5.3C**). Within 5 days, we found that the hematopoietic progenitor compartment in *Acd*$^{fl/-}$ animals became severely depleted (**Figure 5.3D**), and we could already detect a reduction in total bone marrow cellularity (**Figure 5.3E**). These data indicate that hematopoietic tissues are acutely sensitive to *Acd* inactivation, and that this sensitivity can be traced back to a rapidly failing hematopoietic progenitor compartment.
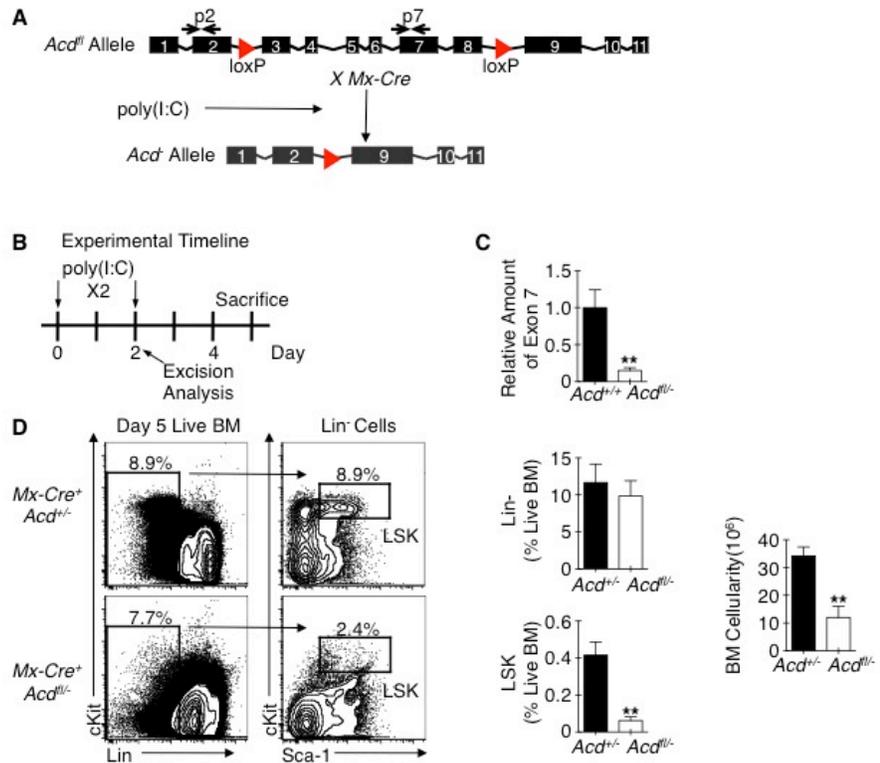
Figure 5.3. Acute depletion of adult hematopoietic progenitors after *Acd* inactivation. (**A**) Structure of the floxed *Acd* allele with *loxP* sites flanking exons 3-8 (*Acd^fl^*). *Mx-Cre* expression was achieved via poly(I:C) injections, leading to *Acd* inactivation into an excised null allele (*Acd^−^*). Arrows indicate p2 and p7 primer pairs used to quantify excision efficiency; (**B**) Experimental timeline to capture the acute effects of *Acd* inactivation; (**C**) Quantitative PCR for *Acd* exon 7 (p7) relative to exon2 (p2) DNA in Lin⁻Sca^hi^c-Kit^hi^ (LSK) progenitors, demonstrating ~80% excision of the floxed region after only a single poly(I:C) injection (qPCR; data are shown as mean ± SEM and are representative of 3 individual mice per group); (**D**) Flow cytometric analysis of hematopoietic progenitors at day 5 after poly(I:C), showing profound depletion of LSK progenitors and decreased bone marrow (BM) cellularity (*Acd^+/−^* n=5; *Acd^fl/−^* n=4). Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate. ****p<0.01.

The type I interferon response resulting from poly(I:C) administration was previously shown to drive quiescent HSCs into the cell cycle, which could enhance the impact of Tpp1 loss in hematopoietic progenitors [83]. To avoid this problem, we bred *CreERT2* x *Acd^fl/−^* mice and inactivated *Acd* with tamoxifen [252] (**Figure 5.4A**). We observed decreased c-Kit expression at day 3 and near disappearance of LSK hematopoietic progenitors at day 5 after starting tamoxifen administration in *CreERT2* x *Acd^fl/−^* mice (**Figure 5.4B, C**). These findings indicate that primitive hematopoietic

progenitors are acutely sensitive to Tpp1 loss even in the absence of an interferon

response.
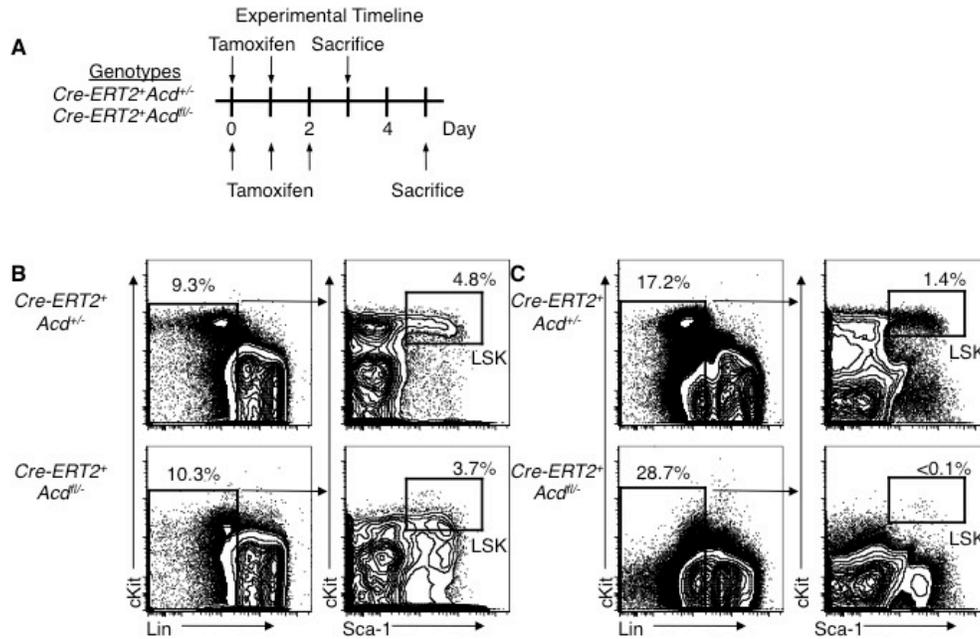
Figure 5.4. *Acd* inactivation with tamoxifen-inducible Cre recombinase results in rapid depletion of hematopoietic stem and progenitor cells. (A) Experimental design: tamoxifen was administered to control *Cre-ERT2⁺ x Acd⁺ᐟ⁻* or *Cre-ERT2⁺ x Acd^{fl/–}* mice (1 mg i.p. daily day 1-3). The *Cre-ERT2* lentitransgene encodes the ubiquitously expressed Tamoxifen-inducible Cre-ERT2 recombinase; (B-C) Flow cytometric analysis of bone marrow at day 3 (B) or day 5 (C) after initiation of tamoxifen. In *Cre-ERT2⁺ x Acd^{fl/–}* mice, the Lineage⁻Sca-1⁺cKit^hi (LSK) compartment (containing hematopoietic stem and progenitors cells) became abnormal within 3 days of tamoxifen administration and was severely depleted within 5 days. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate.

Cell-autonomous mechanisms underlie the requirement for Acd in hematopoiesis.

Although *Mx-Cre* is primarily expressed in the bone marrow, activity can also be

detected in other organs such as liver, kidney and skin. Thus, non-cell autonomous

mechanisms could still contribute to hematopoietic defects in *Mx-Cre⁺ x Acd^{fl/–}* mice. To

exclude this possibility, we generated bone marrow chimeras and studied *Acd/Tpp1*-deficient hematopoiesis in a wild-type environment. Lethally irradiated B6-CD45.1 mice were transplanted with *Mx-Cre*$^+$ x *Acd*$^{fl/-}$ or control *Mx-Cre*$^+$ x *Acd*$^{+/-}$ bone marrow, allowing 6 weeks for full hematopoietic reconstitution before poly(I:C) injection (**Figure 5.5A**). To examine the effects of *Acd* inactivation on overall hematopoiesis, we monitored peripheral blood counts of transplant recipients for 20 weeks following poly(I:C) administration (**Figure 5.5B**). Tpp1 loss led to significant pancytopenia between weeks 1 and 4 after starting poly(I:C). Subsequent analysis showed recovery of peripheral blood counts by ~6 weeks. At week 20, bone marrow cellularity and LT-HSC numbers in *Mx-Cre*$^+$ x *Acd*$^{fl/-}$ recipients had recovered to numbers similar as those in control bone marrow chimeras (**Figure 5.5C**). To identify the source of this reconstitution, bone marrow was plated in methylcellulose cultures and individual colonies were harvested for clonal evaluation of *Acd* inactivation. All colonies analyzed maintained the floxed allele, demonstrating that reconstitution in *Mx-Cre*$^+$ x *Acd*$^{fl/-}$ recipients had occurred exclusively from cells that escaped Cre-mediated gene deletion (**Figure 5.5D**).
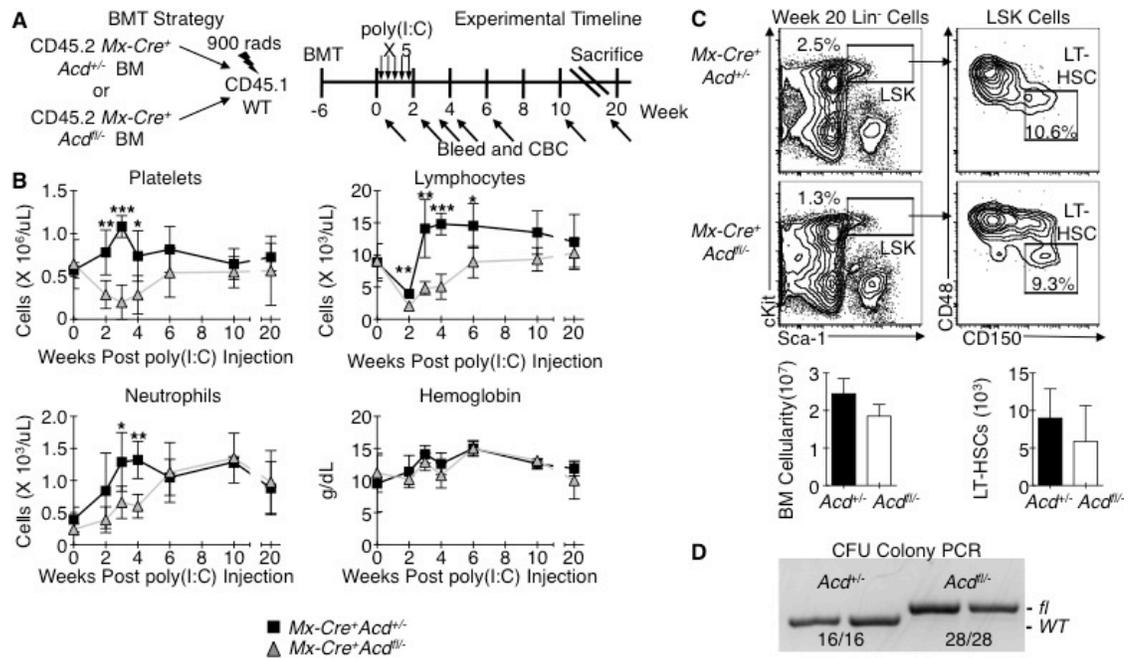
Figure 5.5. Hematopoietic inactivation of *Acd* in a wild-type environment results in pancytopenia followed by strong selection for reconstitution by unexcised progenitors. (**A**) Experimental design: lethally irradiated B6-CD45.1 mice were transplanted with *Mx-Cre*[+]*Acd*^{+/−} or *Mx-Cre*[+]*Acd*^{fl/−} bone marrow (BM) cells (5x10^{5}). Six weeks later, poly(I:C) was administered to induce *Acd/Tpp1* inactivation only in donor-derived hematopoietic cells; (**B**) Complete blood counts at baseline and 1-20 weeks after poly(I:C) injection, showing a transient reduction in platelet, lymphocyte and neutrophil counts (mean +/- SD); (**C**) Flow cytometric analysis of Lin[−]Sca[hi]c-Kit[hi] (LSK) progenitors and long-term hematopoietic stem cells (LT-HSCs) at 20 weeks after poly(I:C) injection, revealing no difference in HSC frequency and BM cellularity of *Mx-Cre*[+]*Acd*^{+/−} and *Mx-Cre*[+]*Acd*^{fl/−} recipients (*Acd*^{+/−} n=5; *Acd*^{fl/−} n=3, mean +/- SEM). Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate; (**D**) Clonal analysis of hematopoietic progenitors by CFU-GM and single colony PCR at week 20. In *Mx-Cre*[+]*Acd*^{fl/−} recipients, all colonies analyzed (28/28) retained the *Acd*[fl] allele and had thus escaped *Mx-Cre*-mediated *Acd* inactivation. Representative PCR results are shown.

In view of the strong selection pressure favoring cells that preserved an undeleted *Acd* allele, we generated competitive bone marrow chimeras in which CD45.2[+] *Mx-Cre*[+] x *Acd*^{fl/−} bone marrow could be studied in the presence of CD45.1[+] wild-type competitors (**Figure 5.6A**). This strategy decreased the selection pressure favoring rare *Mx-Cre*[+] x *Acd*^{fl/−} progenitors escaping *Acd* inactivation, as hematopoiesis was maintained by

CD45.1^{+} competitor cells throughout the experiment. Bone marrow chimeras were given 6 weeks to facilitate recovery of baseline hematopoiesis prior to *Acd* deletion. Within 4 weeks of poly(I:C) administration, no contribution from the *Acd*-deficient CD45.2^{+} graft could be detected among blood myeloid cells, a population characterized by its rapid turnover (**Figure 5.6B**). Peripheral blood B and T cells arising from the *Acd*-deficient graft also became progressively depleted (**Figure 5.6C**). This trilineage defect suggested failure of multipotent stem and progenitor cells. Indeed, no residual *Acd*-deficient HSCs could be detected in the bone marrow at the termination of the experiment (**Figure 5.6D**). Altogether, these data demonstrate that *Acd* is cell-autonomously required by HSCs, with strong selective pressure for rare progenitors that maintain a functional *Acd* gene to support hematopoietic recovery.
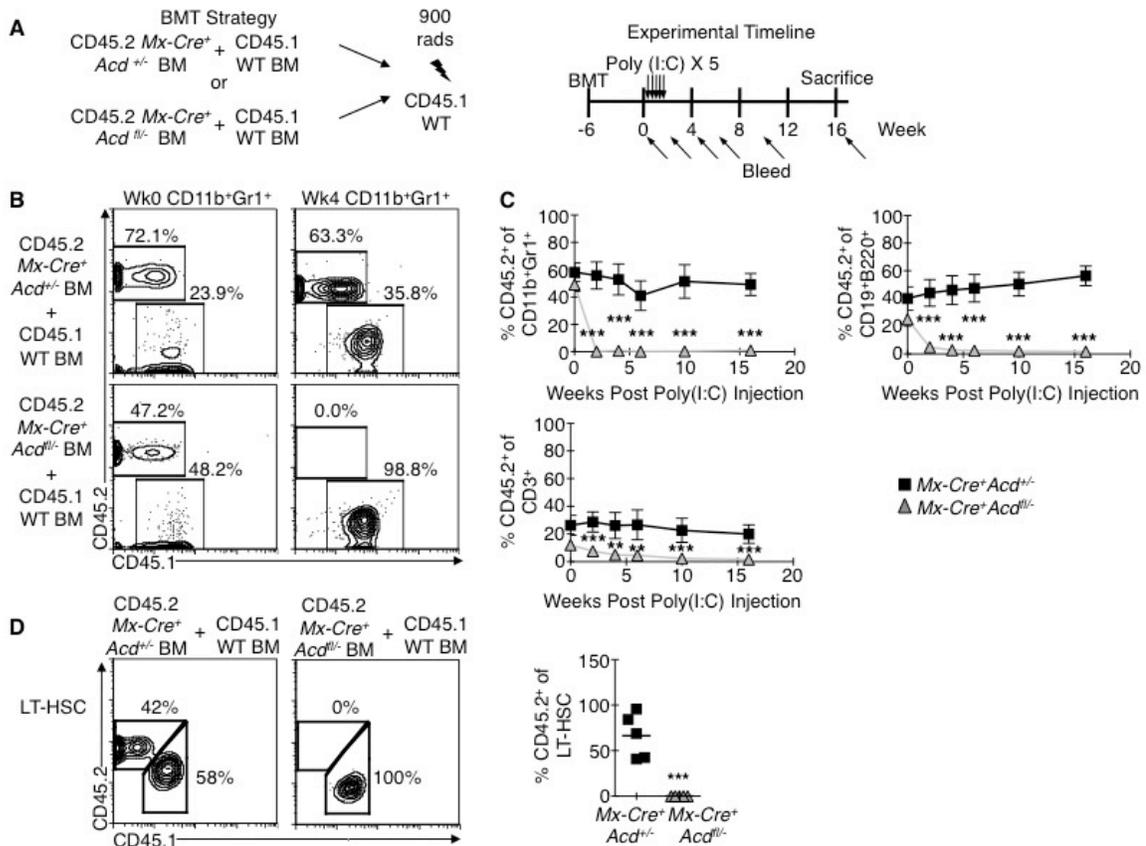
Figure 5.6. *Acd*-deficient HSCs do not support hematopoiesis after competitive bone marrow transplantation. (**A**) Experimental design: lethally irradiated B6-CD45.1 mice were transplanted with 1:1 mixtures of *Mx-Cre*[+]*Acd*^{+/–} or *Mx-Cre*[+]*Acd*^{fl/–} and competitor B6-CD45.1 bone marrow (BM) cells (5×10^5 each). After 6 weeks to allow hematopoietic reconstitution, baseline chimerism was assessed, followed by poly(I:C) administration; (**B**) Flow cytometric analysis of CD45.2/CD45.1 chimerism among CD11b^{+}Gr1^{+} blood myeloid cells at baseline (Wk0) and 4 weeks after poly(I:C) (Wk4). Representative flow cytometry plots are shown (n=5 mice per group, 2 independent experiments); (**C**) Flow cytometric analysis of peripheral blood 0-16 weeks after poly(I:C), showing a rapid drop in contribution of the CD45.2^{+} *Acd*^{fl/–} graft to the myeloid (CD11b^{+}Gr1^{+}), T cell (CD3^{+}), and B cell (CD19^{+}B220^{+}) compartments (mean +/- SEM, 2 independent experiments); (**D**) CD45.2/CD45.1 chimerism in the long-term hematopoietic stem cell (LT-HSC) compartment 16 weeks after poly(I:C) administration, revealing no residual CD45.2[+] *Acd*^{fl/–} HSCs. Representative flow cytometry plots are shown (n=5 mice/group). Numbers indicate the percentage of cells in each gate.

Deletion of Acd in fetal liver hematopoietic stem and progenitor cells results in hematopoietic failure and death.

Fetal and adult HSCs have numerous differences with respect to gene expression

programs, cytokine responsiveness and cell cycle activity. Thus, it was possible that adult

and fetal HSCs had different sensitivities to complete *Acd* inactivation. To address this

question, we bred *Acd*^{fl/fl} mice with *Vav-Cre* transgenic mice (**Figure 5.7A**). Previous

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studies have demonstrated that *Vav-Cre* is expressed specifically in fetal hematopoietic cells starting at ~E10.5, leading to near complete excision of target loci by day E14.5 [21, 253]. No *Vav-Cre*[+] *x Acd*^{fl/fl} mice were born, while other genotypes were present at the expected mendelian frequency (**Fig. 5.7B**). At E14.5, live *Vav-cre*[+] *x Acd*^{fl/fl} fetuses were present, but these mice had pale fetal livers and vasculature, suggesting defective hematopoiesis (**Fig. 5.7C, D**). Flow cytometric analysis revealed that fetuses with hematopoietic-specific *Acd* inactivation had few if any residual hematopoietic LSK progenitors in the liver at E14.5 (**Figure 5.7E**). These data demonstrate that maintenance of both fetal and adult HSCs is acutely dependent on *Acd*.
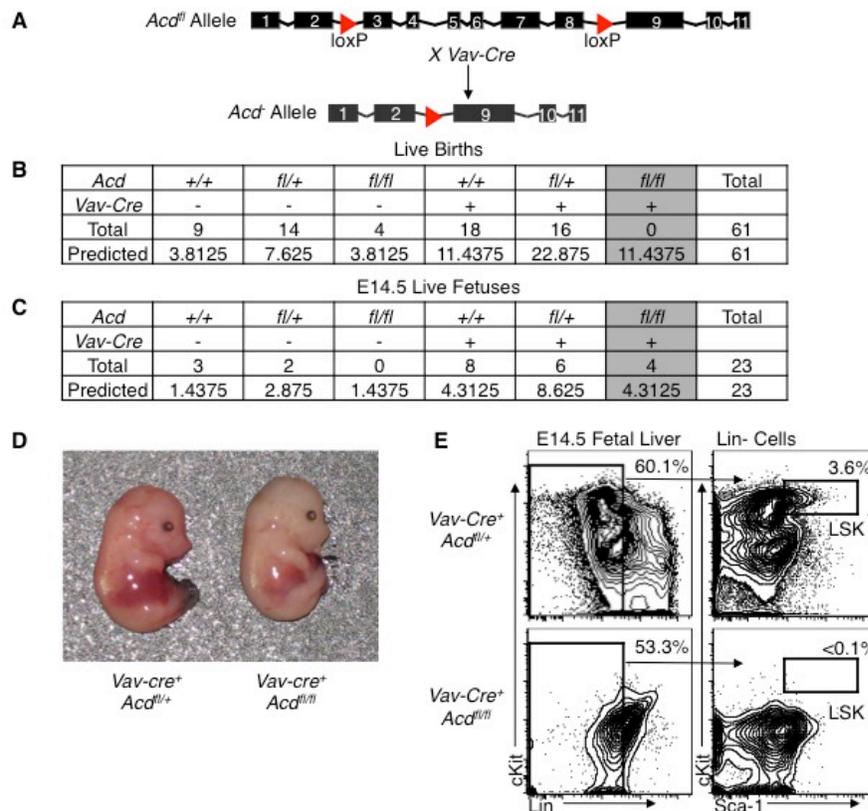
Figure 5.7. Complete *Acd* inactivation in fetal hematopoietic cells is incompatible with survival. (A) Experimental design: *Acd* inactivation restricted to fetal hematopoiesis was achieved using a *Vav-Cre*[+]

transgene. Breedings were established using *Vav-Cre⁺Acd^fl/+* parents; (**B**) Genotyping results demonstrating that no *Vav-cre⁺Acd^fl/fl* were born, while all other genotypes were represented. Genotyping results varied significantly from Mendelian predictions by Chi Squared analysis (p=1.60x10⁻⁵); (**C-D**) At E14.5, live *Vav-cre⁺Acd^fl/fl* fetuses could be identified, however these mice were pale compared to all other genotypes. No difference was detected between observed genotypes and those predicted by Mendelian ratios (p=0.19); (**E**) Flow cytometric analysis of E14.5 fetal livers, demonstrating that *Vav-cre⁺Acd^fl/fl* fetuses had profoundly depleted Lineage⁻Sca-1⁺cKit^hi (LSK) hematopoietic progenitors. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate.

Acd deficiency results in acute p53 activation, but p53-independent cell cycle arrest in hematopoietic progenitors.

Based on work in other cell types such as embryonic fibroblasts and epidermal stem cells, *Acd* inactivation is predicted to activate a DNA damage response due to the loss of Pot1 localization at telomeric ends, with many downstream effects driven by p53 activation. To determine if p53 activation occurred in hematopoietic progenitors following *Acd* inactivation, we assessed expression of the p53 target genes *Puma*, *Noxa* and *p21* within days after induction of Cre recombinase expression, but before hematopoietic progenitors were lost (**Figure 5.8A**). *Acd* deletion resulted in the upregulation of *Noxa* and *p21* (**Fig. 5.8B**) but not *Puma* (data not shown) within 48 hours of poly(I:C) treatment. This effect was p53-dependent, as LSK progenitors from mice deficient for both *Acd* and *p53* did not demonstrate upregulated expression of *p21* and *Noxa*. Next, we assessed the cell cycle status of LSK progenitors shortly after *Acd* inactivation, in the presence or absence of p53 (**Fig. 5.8C, D**). Upon Tpp1 loss, we observed accumulation of hematopoietic progenitors in the G2/M phases of the cell cycle, consistent with the presence of G2/M arrest. Interestingly, this cell cycle arrest was observed even in the absence of p53. These data indicate that while p53 activation occurs acutely following *Acd* deletion, it is not required for G2/M arrest.
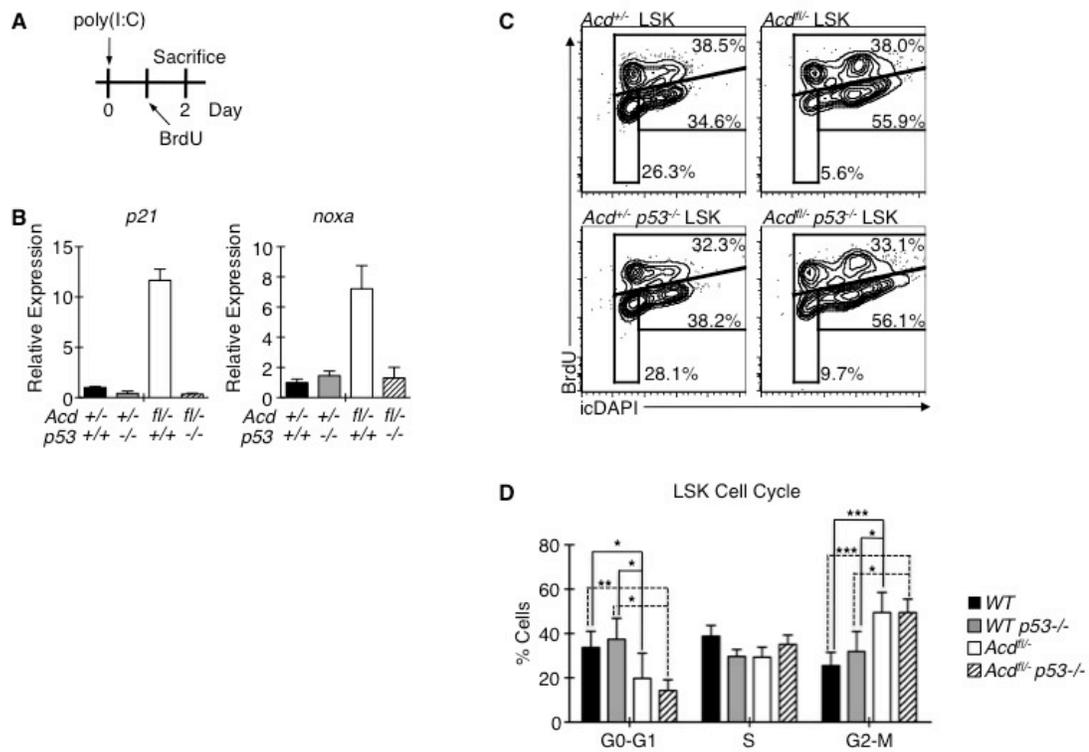
Figure 5.8. *Acd* **inactivation results in acute cell cycle arrest and induction of p53 target genes.**(A) Experimental design: $Mx\text{-}Cre^{+}Acd^{+/-}$, $Mx\text{-}Cre^{+}Acd^{+/-}p53^{-/-}$, $Mx\text{-}Cre^{+}Acd^{fl/-}$ and $Mx\text{-}Cre^{+}Acd^{fl/-}p53^{-/-}$ mice were injected with a single dose of poly(I:C) followed by BrdU, and sacrificed as indicated; **(B)** Relative abundance of *p21* and *Noxa* transcripts in purified $Lin^{-}Sca^{hi}c\text{-}Kit^{hi}$ (LSK) progenitors, demonstrating that *Acd* deletion induced a p53-dependent increase in *p21* and *Noxa* mRNA (qRT-PCR; data are shown as mean ± SEM representative of at least 2 independently sorted samples per group); **(C)** Flow cytometric analysis of progenitors for BrdU incorporation and intracellular DAPI (icDAPI), showing accumulation of $Mx\text{-}Cre^{+}Acd^{fl/-}$ and $Mx\text{-}Cre^{+}Acd^{fl/-}p53^{-/-}$ cells in G2/M phases of the cell cycle. Representative flow cytometry plots are shown from 4 independent experiments; **(D)** Quantification of data shown in **(C)** (n=3 mice/group). $*p<0.05$, $**p<0.01$, $***p<0.001$.

Acd deficiency results in chromosomal fusions at telomeres in the absence of detectable telomere shortening.

Acd-deficient embryonic fibroblasts display an increased rate of rare chromosomal fusion events involving telomeres. To evaluate if this occurred in hematopoietic tissues, we prepared metaphase spreads from control or $Mx\text{-}Cre^{+} \times Acd^{fl/-}$ bone marrow cells within 48 hours after poly(I:C) administration (**Figure 5.9A**). This strategy ensured that dividing hematopoietic progenitors could be examined acutely after

Acd inactivation, while their numbers were still preserved. Efficient Cre-mediated excision was detected at the time of analysis (**Figure 5.9B**). Quantitative PCR showed that the amount of telomere signal was preserved at this stage in *Mx-Cre⁺ x Acd^{fl/−}* bone marrow, consistent with the absence of telomere shortening within days of Tpp1 loss (**Figure 5.9C**). In contrast, FISH analysis revealed an increased frequency of metaphases in which chromosomal fusions were detected with a shared telomeric signal (**Figure 5.9D, E**). These findings are consistent with Tpp1 loss driving the fusion of deprotected telomeric ends in dividing hematopoietic progenitors.

Genotype	Metaphases Analyzed	Chromosome Fusions + Telomere Signal
Mx-Cre⁺ Acd⁺/⁻	49	3 (6.1%)
Mx-Cre⁺ Acd^{fl/−}	30	5 (16.7%)
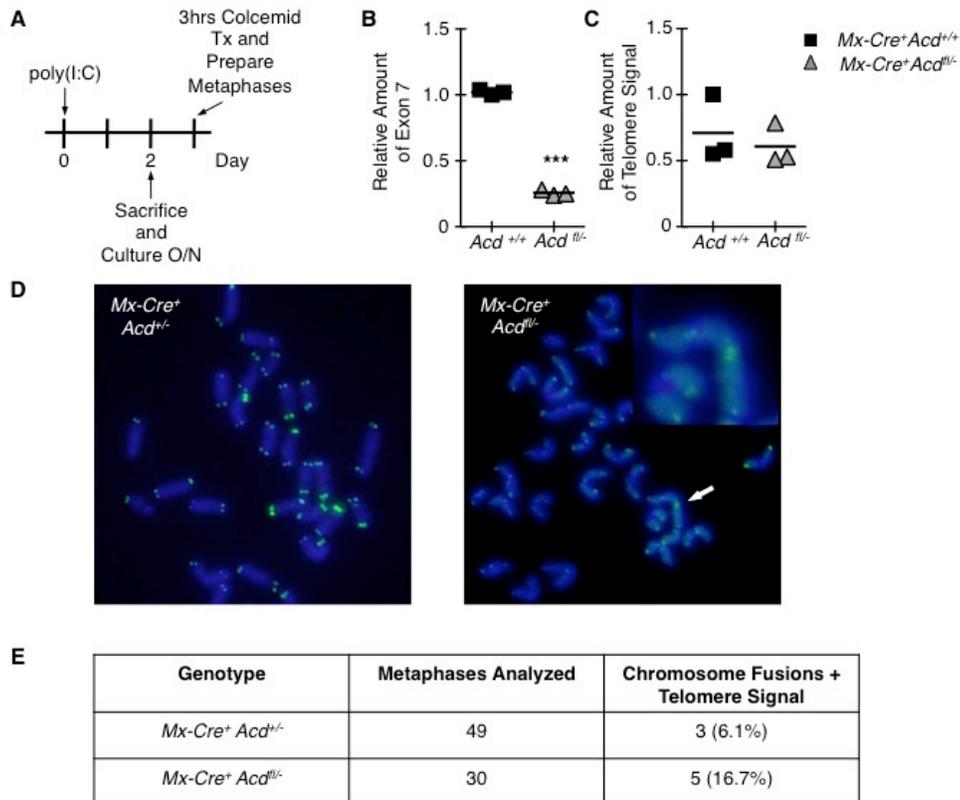
Figure 5.9. *Acd*-deficient hematopoietic progenitors demonstrate acute chromosomal instability in the absence of telomere shortening. (**A**) Experimental design: bone marrow was harvested from *Mx-Cre⁺Acd⁺/⁻* or *Mx-Cre⁺Acd^{fl/−}* mice 48 hours after a single dose of poly(I:C) and cultured overnight with IL-3/IL-6/SCF. Metaphases were prepared after a 3 hour Colcemid treatment; (**B**) Relative abundance of exon 7 DNA signal normalized to exon 2, showing rapid Cre-mediated excision in total bone marrow. ***p<0.001; (**C**) qPCR assessment of telomere length in cultured bone marrow cells, showing preserved abundance of telomeric sequences acutely after *Acd* inactivation; (**D**) Representative pictures of metaphases

stained with DAPI and a FITC-labeled telomeric probe. Insert shows a chromosomal fusion event centered on a telomeric signal; **(E)** Blind scoring of metaphases, showing an increase in chromosome fusions containing a telomere signal among $Mx\text{-}Cre^{+}Acd^{fl/-}$ metaphases.

p53 inactivation does not rescue the function and maintenance of Acd-deficient hematopoietic stem cells.

Previous studies demonstrated that p53 inactivation rescued many features of abnormal development in *acd* hypomorphic mice [235, 236]. Furthermore, p53 deficiency restored epidermal stem cell function following *Acd* inactivation [228]. To investigate if p53 inactivation could rescue HSC function and maintenance in the bone marrow following *Acd* deletion, we generated hematopoietic chimeras with wild-type CD45.1^{+} bone marrow and bone marrow from control CD45.2^{+} $Mx\text{-}Cre^{+}$ x $Acd^{+/-}$ or $Mx\text{-}Cre^{+}$ x $Acd^{fl/-}$ mice, in the presence or absence of *p53* (**Figure 5.10A**). After hematopoietic reconstitution, poly(I:C) was administered to induce *Mx-Cre* expression and *Acd/Tpp1* inactivation specifically in the cohort of CD45.2^{+} hematopoietic cells. Mice transplanted with either $Mx\text{-}Cre^{+}Acd^{fl/-}$ $p53^{+/+}$ or $Acd^{fl/-}$ $Mx\text{-}Cre^{+}p53^{-/-}$ progenitors lost all myeloid cells originating from the CD45.2^{+} graft within 2 weeks of *Acd/Tpp1* inactivation, and at all subsequent time points (**Figure 5.10B**). Additionally, CD45.2^{+} *Acd*-deficient T and B cells were progressively lost, irrespective of the presence of p53. At 16 weeks after poly(I:C) administration, flow cytometric analysis of the long-term HSC compartment demonstrated that *Acd*-deficient HSCs were completely depleted even in the presence of *p53* inactivation (**Figure 5.10C**). In fact, analysis of the bone marrow revealed that numbers of *Acd*-deficient HSCs were already profoundly reduced within 1 week of *Acd* inactivation, regardless of *p53* status (**Fig. 5.11**). Altogether, p53 activation occurred acutely after loss of Tpp1 in hematopoietic progenitors. However, unlike in

epidermal stem cells and other cellular compartments, the maintenance and function of

Acd/Tpp1-deficient HSCs could not be rescued by *p53* inactivation.
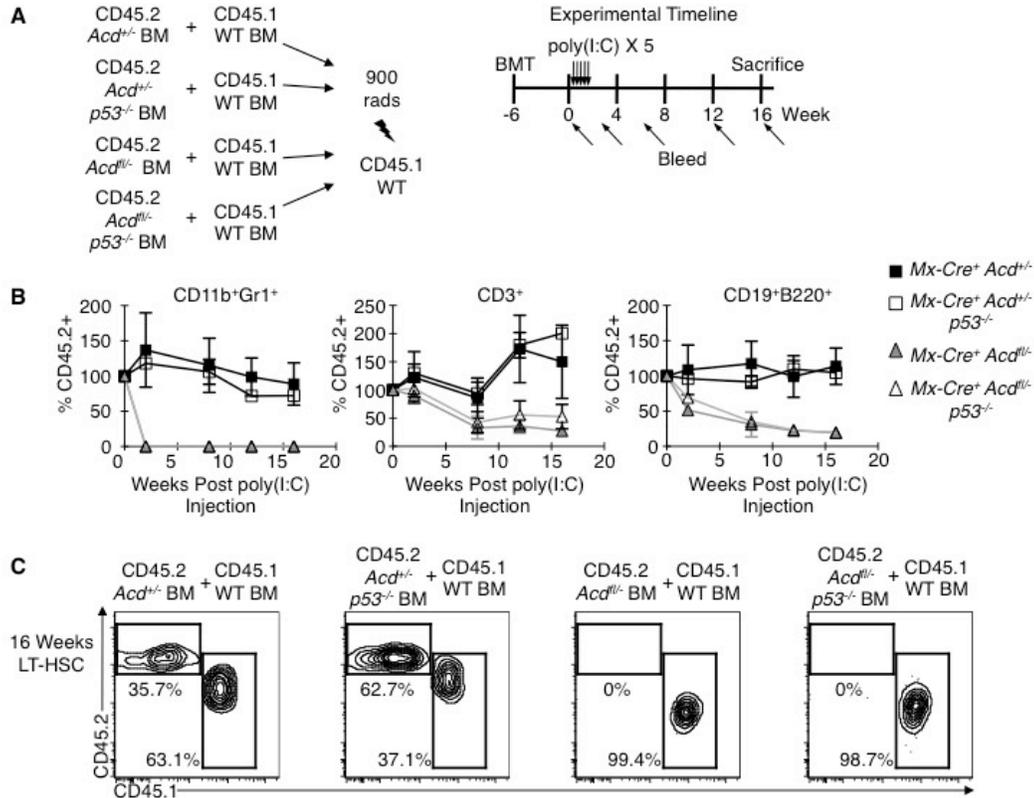
Figure 5.10. *p53* inactivation does not rescue the survival and function of *Acd*-deficient hematopoietic stem cells. (A) Experimental design: lethally irradiated B6-CD45.1 mice were transplanted with bone marrow (BM) from *Mx-Cre$^+$Acd$^{+/-}$p53$^{+/+}$, Mx-Cre$^+$Acd$^{+/-}$p53$^{-/-}$, Mx-Cre$^+$Acd$^{fl/-}$p53$^{+/+}$* or *Mx-Cre$^+$Acd$^{fl/-}$ p53$^{-/-}$* B6-CD45.2$^+$ and wild-type (WT) B6-CD45.1 competitor mice (5x10^5 cells each, 1:1 ratio). After 6 weeks to allow hematopoietic reconstitution, poly(I:C) was administered; **(B)** Flow cytometric analysis of peripheral blood 2-16 weeks after poly(I:C), showing a rapid drop in contribution of the CD45.2$^+$ *Mx-Cre$^+$Acd$^{fl/-}$p53$^{+/+}$* and *Mx-Cre$^+$Acd$^{fl/-}$p53$^{-/-}$* grafts to the myeloid (CD11b$^+$Gr1$^+$), T cell (CD3$^+$) and B cell (CD19$^+$B220$^+$) compartments. Baseline chimerism was normalized to 100% in each mouse. The percentage of *Mx-Cre$^+$Acd$^{fl/-}$p53$^{+/+}$* and *Mx-Cre$^+$Acd$^{fl/-}$p53$^{-/-}$* graft output differed significantly from *Mx-Cre$^+$Acd$^{+/-}$ p53$^{+/+}$* and *Mx-Cre$^+$Acd$^{+/-}$p53$^{-/-}$* output in the myeloid compartment by week 2 (p<0.001), in the T cell compartment by week 12 (p<0.01), and in the B cell compartment by week 8 (p<0.01). Data represent mean +/− SD (n≥4/group); **(C)** CD45.2/CD45.1 chimerism among long-term hematopoietic stem cells (LT-HSC) 16 weeks after poly(I:C) administration, showing no residual CD45.2$^+$ *Mx-Cre$^+$Acd$^{fl/-}$p53$^{+/+}$* or *Mx-Cre$^+$Acd$^{fl/-}$p53$^{-/-}$* HSCs. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate.
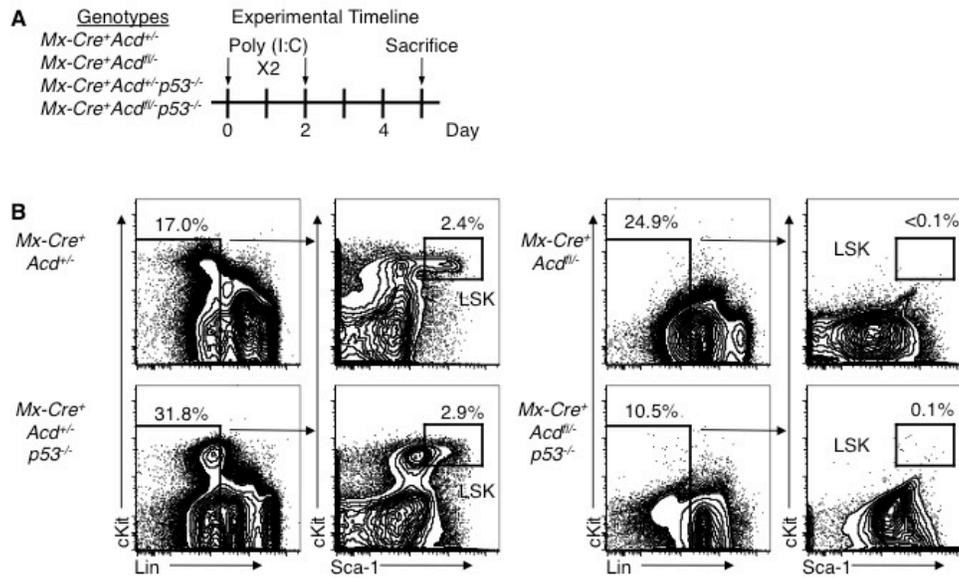
Figure 5.11. *p53* inactivation does not rescue *Acd*-deficient hematopoietic stem and progenitor cells from acute depletion. (**A**) Experimental design: lethally irradiated B6-CD45.1 mice were transplanted with bone marrow from Mx-$Cre^+Acd^{+/-}p53^{+/+}$, Mx-$Cre^+Acd^{+/-}p53^{-/-}$, Mx-$Cre^+Acd^{fl/-}p53^{+/+}$ or Mx-$Cre^+Acd^{fl/-}p53^{-/-}$ B6-CD45.2 and wild-type (WT) B6-CD45.1 competitor mice (10^6 cells each). After 6 weeks to allow hematopoietic reconstitution, poly(I:C) was administered; (**B**) Flow cytometric analysis of bone marrow 5 days after starting poly(I:C) injection, demonstrating an acute depletion of Lineage⁻Sca-1^+cKit^hi (LSK) hematopoietic progenitors in both Mx-$Cre^+Acd^{fl/-}p53^{+/+}$ and Mx-$Cre^+Acd^{fl/-}p53^{-/-}$ recipients (right panels). Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each gate.

DISCUSSION

Our findings demonstrate that hematopoietic stem cells are highly sensitive to shelterin deprotection initiated by *Acd* inactivation. Complete loss of the *Acd* gene product induced a surprisingly acute depletion of the fetal or adult blood-forming stem cell compartments, leading to rapid hematopoietic failure before telomere shortening could be detected. Although Tpp1 and the shelterin complex play dual functions in recruiting telomerase and in shielding telomeric ends from an uncontrolled DNA damage

response, our observations indicate that the overall effects of shelterin dysfunction in hematopoietic progenitors are dominated by acute telomere deprotection. Tpp1-deficient hematopoietic stem cells rapidly upregulated p53 target gene transcription and had evidence of cell cycle arrest and chromosomal instability. However, the maintenance of Tpp1-deficient stem cells was not rescued by *p53* inactivation. These findings contrast to the major effects of Tpp1 loss on epidermal stem cell homeostasis and fetal musculoskeletal development, which are rescued in the absence of p53. Even without complete loss of Tpp1, as modeled by homozygosity for the hypomorphic *acd* allele, hematopoietic stem cell function was severely impaired. This demonstrates that blood-forming stem cells are very sensitive to defects in telomere protection as compared to other tissues.

Telomere deprotection has been shown to trigger a p53/p21-dependent cell cycle arrest [254]. Similarly, p53 activation has been shown to contribute to cellular senescence in response to critical telomere shortening [255-257]. These findings, combined with data demonstrating that *p53* inactivation rescued epidermal stem cell defects owed to *Acd* inactivation led us to investigate the role of *p53* in bone marrow failure in the setting of *Acd* deficiency [228]. We found that while *Acd* inactivation resulted in the increased transcription of p53 targets, indicating p53 activation, p53 deficiency did not rescue any component of the hematopoietic stem and progenitor defects that we tested. This included a failure to rescue acute cell cycle arrest, acute stem and progenitor depletion, and short and long-term functional deficiencies. These data indicate that p53 activation is not the predominant underlying cause of HSC functional defects or bone marrow failure

following *Acd* deletion, and suggests that different stem cell compartment may react with varying DNA damage responses in the setting of a telomere crisis.

Telomere dysfunction has been hypothesized to play a role in multiple contexts including organ failure, aging and cancer. However, dyskeratosis congenita (DKC) is the prototypical human disorder most directly associated with defects in telomere homeostasis. DKC is a hereditary syndrome characterized by progressive telomere shortening. Although bone marrow failure is the most significant organ dysfunction and the leading cause of death in DKC, affected patients can also develop abnormalities in the skin, mucosal tissues, biliary tract and lungs, suggesting that multiple somatic stem cell compartments are affected. Mouse models of DKC have typically described gradual homeostatic dysfunction in rapidly proliferating tissues, and an eventual lethality due to the loss hematopoietic stem cells and bone marrow aplasia [60]. Upon reduced expression of *Terc* or *Tert* genes, progression to telomere crisis and stem cell loss typically occurs only after telomere attrition accumulates through several successive mouse generations [56]. With respect to shelterin components, a mouse syndrome reminiscent of DKC was first reported using combined *Pot1b* deficiency and *Terc* haploinsufficiency [223, 224]. In this model, a relatively mild DNA damage response and gradual telomere shortening have been identified as the drivers of hematopoietic failure. However, the significance of this model for human disease is limited because Pot1b is a mouse-specific shelterin component [218, 221]. Humans have a single POT1 protein that, if deleted, would yield a vigorous DNA damage response resulting not primarily in telomere erosion, but instead in acute telomere deprotection and a robust DNA damage response. Interestingly, human DKC patients with mutations in *TIN2*, the only shelterin

component thus far causally linked to DKC, have a particularly severe form of DKC with exceptionally short telomeres and a very early onset [241, 244, 247]. The acute nature of this form of DKC might be due to the fact that *TIN2* mutations, like our *Acd* inactivation model, result in rapid telomere deprotection and destabilization, rather than the erosion observed in the *Pot1b* mouse model and in DKC patients in whom *TERC* or *TERT* are mutated. In fact, data in mouse embryonic fibroblasts indicate that the critical function of Tin2 is to stabilize the Tpp1-Pot1a/b complex at telomeres [238]. Progression towards stem cell loss may thus be significantly accelerated in the presence of shelterin mutations.

It is estimated that up to 60% of DKC cases do not have a known underlying genetic defect [246]. Thus, it is interesting to speculate whether new shelterin genes mutations might be discovered in human DKC. Based on our results, we would predict that complete loss of shelterin function would not be compatible with life, as evidenced for example by in utero death upon complete loss of *Acd* in fetal hematopoiesis. However, it is possible that relevant mutations may decrease rather than abolish *Acd* expression. In mice, the hypomorphic *acd* splice variant was identified as a spontaneous mutation compatible with survival, but leading to a developmental syndrome with features reminiscent of DKC, including profound HSC dysfunction. These observations suggest that mutations in gene regulatory regions or in introns resulting in impaired gene expression ought to be considered when probing uncharacterized cases of DKC. Furthermore, recent studies have identified a specific protein-protein interface mediating a direct interaction between TERT and TPP1 that is critical for telomerase localization and processivity [230, 258]. It is possible that specific mutations affecting this interaction could affect the ability of TPP1 to recruit telomerase without disrupting its function in

preventing an uncontrolled DNA damage response, leading to a DKC-like phenotype. Finally, shelterin mutations may have to be considered in a broader range of human syndromes including in patients with early onset organ failure (as seen for *TIN2* mutations) and with developmental disorders not previously associated with shelterin dysfunction.

A recent report has linked inactivation of the *Trf1* shelterin gene to an acute form of bone marrow failure in mice [59]. These findings may be relevant to human disease, as *TIN2* mutations in early onset DKC cluster in a region encoding a TRF1-binding site. Interestingly, *Trf1* inactivation led to the rapid loss of hematopoietic progenitors and severe bone marrow failure, initially without detectable telomere shortening. When residual bone marrow cells were studied within 1-3 weeks after induction of Cre recombinase, increased compensatory proliferation was apparent, leading eventually to telomere shortening and *p21* activation. These findings were proposed to underlie the pathogenic effects of shelterin mutations in human DKC through telomere deprotection, leading to rapid HSC loss. Our observations in *Acd*-deficient mice share key features with this report, including the profound HSC depletion independent of telomere shortening. However, near-complete HSC depletion was apparent in our study already within 5 days after *Acd* inactivation, with a detectable DNA damage response and cell cycle arrest evident within 48 hours. To capture the direct cellular and molecular consequences of shelterin deprotection, we focused on very early time points before affected HSCs are depleted, as evidenced for example by our detection of increased telomeric fusion events 2-3 days after Tpp1 loss. Delaying analysis or decreasing the efficiency of target gene inactivation to preserve enough progenitors for study may lead a strong selection for cells

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that escape Cre-mediated excision. In this situation, findings would focus on rare HSCs escaping inactivation and with functional shelterin that undergo major replicative stress, rather than on the direct effects of shelterin deprotection. It is possible that these considerations may account for differences in our observations on *Acd* deficiency and those reported on the effects of *Trf1* inactivation.

While it remains to be determined if *Acd* mutations contribute to human bone marrow failure and DKC, mouse models of telomere deprotection through *Acd* mutation or deletion suggest this possibility. Furthermore, the hyperacute nature of hematopoietic defects following Tpp1 loss provides a practical approach that could shed light on the consequences of telomere crisis in other models of bone marrow failure. Indeed, it is widely assumed that shortened telomeres induce a cellular crisis when their length becomes inadequate to recruit an efficient shelterin buffer, unleashing a DNA damage response that can cause cell cycle arrest, apoptosis or genetic instability. However, it is virtually impossible to study this pathogenic process directly in somatic stem cells of mice and patients, as telomere shortening occurs progressively and asynchronously in different stem cells over years in human patients and over several generations in classical mouse DKC models. Furthermore, relevant stem cell populations are by definition profoundly depleted by the time of diagnosis in patients with bone marrow failure. In contrast, our study provides a well-defined acute window of observation on a cohort of hematopoietic progenitors that simultaneously and acutely undergoes telomere deprotection. Thus, our study presents the first insight into very early hematopoietic defects that may predate bone marrow failure in DKC patients with shelterin mutations

and could be beneficial in developing a more complete understanding of the natural

progression of human bone marrow failure syndromes.

CHAPTER 6. CONCLUSIONS AND PERSPECTIVES

Since Till and McCulloch's initial discovery of hematopoietic stem cells, many researchers have tried to understand how these rare cells maintain hematopoiesis over a lifetime. It is clear that self-renewal plays a fundamental role in this long-term function, and as such significant research has sought to understand this property. In **Chapter 1**, we reviewed our current understanding of the extensive, diverse factors that contribute to HSC self-renewal, and as a result of the original work described in this dissertation, we have added our own novel insights into the regulation of this fundamental stem cell property.

Our work with Ash1l has demonstrated that this understudied TrG member plays an essential function in regulating bone marrow HSC self-renewal (**Chapter 3**). We identified that the paradigm of TrG member cooperation in developmental processes is conserved between flies and mammals. To date, the biochemical nature of this cooperativity has not been studied in any model. Given that both Mll1 and Ash1l are described as having catalytically active SET domains, it is tempting to speculate that cooperative SET domain activity promotes gene expression (**Figure 6.1A**). In this model, Mll1 H3K4 trimethylation activity could cooperate with Ash1l H3K36 dimethylation activity to promote robust target gene expression. Since Ash1l has not been detected in complex with Mll1, it is possible that both proteins are recruited to targets independently. Alternatively, the SET domain of Ash1l may be irrelevant for *in vivo* function. In this

case, Ash1l may be required for the recruitment of additional transcription factor(s) or epigenetic regulators and such factors may cooperate to promote gene expression independent of Ash1l SET domain activity (**Figure 6.1 B**). Since we currently lack good quality antibodies to detect Ash1l, it is possible that we lack sensitivity to detect complexes including Ash1l and Mll1. If this is true, it is possible that Ash1l is required for Mll1 recruitment or vise versa (**Figure 6.1C**). Our data suggest that this is unlikely as combined deficiency for *Mll1* and *Ash1l* resulted in phenotypic worsening beyond the loss of either gene alone. If the products of these genes were required for the recruitment of one another, one would predict that such phenotypic amplification would not occur. These data do not rule out, however, that Ash1l and Mll1 are part of the same complex but do not directly recruit one another (**Figure 6.1D**). Significant biochemical studies will be required to examine the nature of this observed cooperativity and to test these hypothetical models.
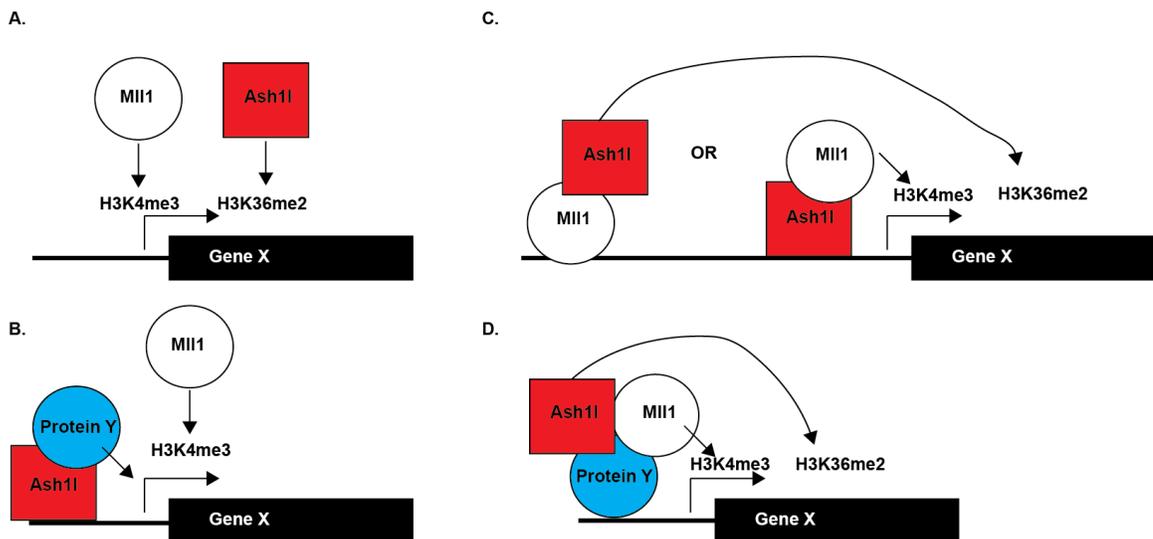
Figure 6.1. Models for Ash1l cooperativity with Mll1. (A) Mll1 and Ash1l are recruited independently to target loci and cooperate through their respective SET domain activities. (B) Ash1l recruits additional transcription factor (s) that cooperates with Mll1 independent of Ash1l SET domain function. (C) Mll1 is required for Ash1l recruitment of vise versa. (D) Ash1l and Mll1 are recruited to the same protein complex which targets SET domain activities to target genes.

Profound hypomorphism for *Ash1l* resulted in a 5-10-fold LT-HSC reduction by young adulthood following an inability to establish a normal quiescent HSC pool. Importantly, our *Ash1l*^{GT} allele is not completely null, although it is profoundly hypomorphic. It is possible that residual *Ash1l* expression from the *Ash1l*^{GT} allele could mask additional phenotypes that would be revealed by the use of a truly null allele. For example, if only a low level of *Ash1l* is required for fetal hematopoiesis, a null allele may reveal additional fetal hematopoietic phenotypes not observed in our model. Assessing hematopoietic development with an *Ash1l* null allele may thus reveal differential sensitivities of fetal and adult hematopoietic stem and progenitor cells to *Ash1l* levels. This concept remains to be tested.

Our data suggest that bone marrow HSC function is profoundly dependent on Ash1l. The 5-10% of normal *Ash1l* transcripts present in *Ash1l*$^{GT/GT}$ HSCs were not sufficient to allow long-term HSC function in transplantation. This observation suggested that *Ash1l*-deficient mice could be destined to hematopoietic failure over time. Surprisingly, however, this did not occur. One could argue that the few remaining HSCs were sufficient to maintain hematopoiesis in primary mice, but could not engraft in the setting of transplantation due to hematopoietic stress or rarity. This latter possibility is unlikely, as even when HSC content was normalized by using fetal liver as the donor source, we could not identify functional *Ash1l*$^{GT/GT}$ HSCs. The former possibility is supported by the finding that residual *Ash1l*$^{GT/GT}$ HSCs diluted GFP more than wild-type in pulse-chase experiments. This could suggest that these rare cells increased their proliferative output to sustain hematopoiesis, though it remains to be determined if this increased proliferation was sufficient to support hematopoietic homeostasis. An

additional and more intriguing possibility is that $Ash1l^{GT/GT}$ hematopoiesis is maintained

predominantly from downstream progenitors that are not true LT-HSCs. Indeed, we

could not detect long-term HSC function in transplantation, and this may reflect the

absence of an LT-HSC compartment in the $Ash1l^{GT/GT}$ model. Our data may therefore

question whether or not steady state hematopoietic homeostasis requires an LT-HSC

population.

The terms long-term and short-term HSC are strictly based on function in bone

marrow transplantation assays. Long-term HSCs were defined as cells capable of

sustaining hematopoietic reconstitution of lethally irradiated mice in bone marrow

transplantation assays for greater than 16 weeks [156, 259, 260]. Short-term HSCs were

identified as providing robust, transient tri-lineage reconstitution but could not sustain

reconstitution for 16 weeks [156, 158, 259, 260]. Due to these findings, LT-HSCs are

viewed as the cells that possess self-renewal potential and maintain long-term

hematopoietic homeostasis. The idea that LT-HSCs are required for steady-state

hematopoiesis has never been addressed in the absence of transplantation, and thus

remains a hypothesis.

A recent model of LT-HSC cell cycle activity indicated that long-term

reconstituting potential was primarily maintained in HSCs that very rarely enter the cell

cycle [161]. Phenotypic LT-HSCs that had divided only a few times had reduced long-

term reconstituting activity compared to undivided cells, and HSCs that had more

extensively divided could not sustain long-term reconstitution. This was hypothesized to

have occurred because dormant HSCs were the true LT-HSC population, and thus had

more robust self-renewal activity. Mathematical models indicate that these dormant LT-

HSCs would divide ~5 times over the lifetime of a mouse. This finding may be inconsistent with dormant LT-HSCs contributing to the day-to-day turnover of hematopoietic tissue, though additional testing and mathematical modeling are required to evaluate this concept.

Transplantation assays suggest that LT-HSC function resides almost exclusively in dormant HSCs, and this observation may parallel their support of steady-state hematopoiesis However, it is also possible that current transplantation methods are not suitable for adequately testing long-term potential or self-renewal in downstream progenitor populations. Standard transplantation protocols do not allow one to distinguish between an inability to engraft and reduced self-renewal; both present as reduced tri-lineage output in the blood and reduced donor HSC detection at transplant termination. Indeed, several groups have reported that cycling HSCs were not as effective at long-term tri-lineage reconstitution as non-cycling HSCs in transplantation assays [29, 154]. If prospective LT-HSCs were cultured and forced into S-G2-M, they engrafted poorly. If the culture conditions were extended such that the HSCs had time to transit through the cycle and re-enter G1, they could again engraft [29]. This suggested that cells did not lose self-renewal potential, but instead could not engraft the bone marrow if they were in late cell cycle stages. It would thus seem that reduced cell cycle entry correlates with better engraftability, and that transplantation assays do not always provide direct measurements of HSC self-renewal or stemness. Since downstream progenitors are actively cycling compared to dormant HSCs, this may impede our ability to test their long-term function in transplantation due to engraftment defects.

Since we have historically defined LT-HSC function in transplantation assays, and we now know that cycling cells fail to achieve robust engraftment in transplantation, it follows that we may have far underestimated the frequency of cells capable of maintaining long-term hematopoiesis in steady-state conditions. We have thus defined LT-HSC potential under stress conditions, either through myeloablation or transplantation, and actually do not know the requirement for LT-HSC function during homeostasis.

By analogy, recent findings in T cell development can be informative when thinking about the requirement for classically defined primitive progenitor pools in developmental biology. A significant body of work based on transplantation studies suggested that thymic output required that the thymus be perpetually seeded with fresh progenitors. Until recently, the prevailing concept in the field was that this progenitor pool was needed because there were no self-renewing progenitors in the thymus, and thus all cells needed to be frequently turned over. Recent work demonstrates that self-renewal potential is actually present in the thymus under certain conditions [165, 166]. In the absence of progenitor import, existing thymic progenitors could self-renew and sustain thymic output. It was determined that in this system, if fresh progenitors were available, they could out-compete older thymic progenitors, promoting thymic turnover. However, if fresh progenitors were unavailable, these older intrathymic progenitors could sustain thymic output.

When considering our *Ash1l*$^{GT/GT}$ model in the context of the data presented in this thesis, we suggest a similar model to the T cell development studies described above. We could not detect functional LT-HSCs in transplantation, yet cells defined as being

ST-HSCs were present at normal frequencies, and hematopoietic output was largely preserved. It is possible that in the absence of fresh progenitors from the LT-HSC compartment, the ST-HSC compartment was capable of self-renewal and thus maintained hematopoietic homeostasis. At least two lines of evidence suggest that this may be true in the setting of *Ash1l* deficiency. Our non-ablative transplantation experiments demonstrated that if a healthy pool of LT-HSCs was made available to *Ash1l*$^{GT/GT}$ mice via transplantation, ST-HSCs were turned-over and these fresh LT-HSCs were then the major contributors to hematopoietic homeostasis. This is similar to what was reported in T cell development, although it has not been described thus far in HSC biology. Additionally, pulse-chase experiments demonstrated that progenitors downstream of LT-HSCs were extensively cycling, presumably to maintain homeostasis. Despite this increased proliferation, these downstream progenitors were not depleted, as would be expected if these cells were strictly undergoing differentiating divisions to maintain mature cell populations. This increased proliferative capacity could thus be reflective of enhanced self-renewal potential. Therefore, loss of LT-HSCs in this model may have enhanced an intrinsic self-renewal capacity in downstream hematopoietic progenitors that cannot be assessed in standard transplantation.

This hypothesis would support a model in which LT-HSCs are capable of differentiating into ST-HSCs (**Figure 7.1**). These fresh ST-HSCs have not experienced the various DNA damage and metabolic stresses that older progenitors that have been engaged in cell cycle activity face. As a result, new ST-HSCs are more fit than older ST-HSCs and outcompete them, resulting in turnover of the compartment. This would essentially suppress any self-renewal potential that ST-HSCs possess as they would not

be long-lived enough to demonstrate it. In the absence of LT-HSCs and this turnover, this block on ST-HSC self-renewal is removed. There are now no fresh progenitors to outcompete the older ones, and thus the self-renewal capacity of ST-HSCs is revealed. This hypothesis challenges the paradigm that LT-HSCs are required for long-term hematopoietic homeostasis under steady-state conditions.
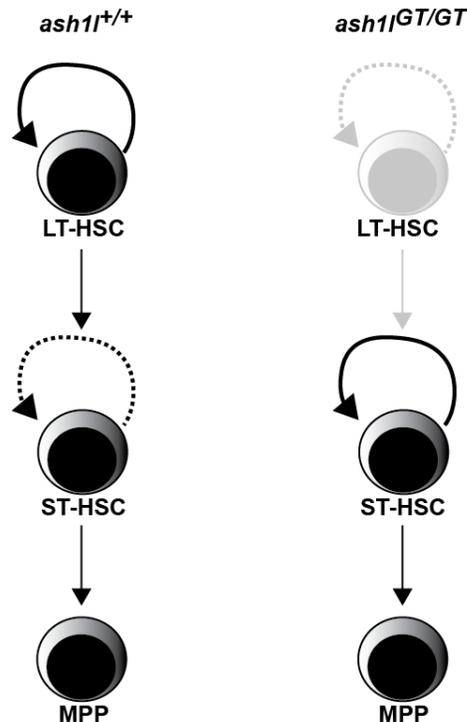
Figure 6.2. Model of enhanced ST-HSC self-renewal in setting of reduced LT-HSC pool. In *Ash1l*-sufficient hematopoiesis, LT-HSCs give rise to ST-HSCs and downstream progenitors. Self-renewal potential resides primarily in the LT-HSC pool. In *ash1l*-deficient hematopoiesis, the depletion of LT-HSCs unmasks self-renewal activity in ST-HSCs, and these ST-HSCs maintain hematopoiesis.

In a field where function in transplantation is the gold standard for identifying self-renewal, examining this hypothesis would require overcoming engraftment defects that are associated with cell cycle activity. One strategy could be retrovirally transducing hematopoietic progenitors with *p27*. *p27* and *p57* have been shown to cooperate in establishing quiescence, and *p57* loss can be compensated by *p27* expression [35, 36]. Since *p57* has been linked to playing diverse roles in development, it is not a viable

candidate for these transduction studies [261]. *p27* has not been linked to such processes, and thus could promote quiescence without disrupting development. We are currently developing reagents to perform such a study.

Quiescence preserves LT-HSC function through limiting exposure to proliferative stress. Such stress manifests itself in ROS accumulation, DNA damage, and leukemogenesis. While quiescence limits this damage, over the course of a lifetime, even largely dormant LT-HSCs sustain insults. Collectively, this damage results in hematopoietic aging (reviewed in [262]). Age-related decline in hematopoietic stem cell function results in reduced lymphoid output and reduced self-renewal activity [263]. One would predict acceleration in this aging process within actively dividing cell populations. The comparatively enhanced cell cycle activity of ST-HSCs relative to LT-HSCs in the setting of *Ash1l* deficiency suggests that our *Ash1l*$^{GT/GT}$ system may offer a new model in which to study aging. In data not shown in this dissertation, we have identified that *Ash1l*$^{GT/GT}$ mice have reduced lymphoid progenitor populations and early thymic involution, both of which are reminiscent of an aged hematopoietic system. Additional work will be required to assess age-related pathologies, including DNA damage, ROS accumulation, and predisposition to leukemia, in the context of *Ash1l* deficiency to determine the extent to which it models physiological aging.

TPP1 and acute HSC depletion

The loss of Tpp1 in LT-HSCs and hematopoietic progenitors resulted in an acute depletion of these cell populations (**Chapter 5**). Transplantation assays revealed a virtual absence of self-renewing LT-HSCs following *Acd* deletion. This acute sensitivity was not predicted by previous hematopoietic models of shelterin deficiency or telomere

dysfunction [57, 60, 224]. In these models, decline in LT-HSC function was gradual and hematopoietic output developed progressively. This was the case because these models required telomere erosion secondary to proliferative stress to elucidate phenotypes. Even loss of the shelterin protein Trf1, while causing a more rapid decline in hematopoietic function than previous models, did not perturb LT-HSCs as acutely as did Tpp1 loss [59]. This leads one to consider that Tpp1 loss causes LT-HSC dysfunction in a manner that differs from previous models.

MEF studies indicated that Tpp1 deficiency should activate an ATR-dependent DNA damage response [215]. However, ATR activation is unlikely to affect such an acute depletion of cells, especially those that are quiescent. ATR is activated by stalled DNA replication forks, and thus requires active DNA replication for activation. This typically means that ATR is activated in S phase. Since adult LT-HSCs do not uniformly enter the cell cycle and are only rarely in later S-G2-M phases of the cell cycle, ATR activation is unlikely to be the sole mode of LT-HSC depletion in the *Acd*-deficient model.

Our initial identification of upregulation of the p53 target genes *p21* and *noxa* following *Acd* deletion suggested that p53 mediated cell cycle arrest and apoptosis could have significantly contributed to LT-HSC functional decline. This was consistent with the p53-dependent functional decline of skin stem cells in the absence of Tpp1, as well as the rescue of many developmental abnormalities attributed to *acd* hypomorphism by *p53* loss [228, 235, 236]. However, despite robust activation of p53 target genes following *Acd* deletion, *p53* deficiency did not rescue LT-HSC function nor did it slow LT-HSC and progenitor depletion. Thus, p53 was not the primary cause of LT-HSC dysfunction

following Tpp1 loss. These findings demonstrate potential tissue-specific functions of shelterin proteins.

One intriguing possibility is that Tpp1 loss results in mitochondrial dysfunction. Telomere shortening has been linked to mitochondrial failure and ROS accumulation, both of which would compromise even quiescent LT-HSCs [264]. ROS accumulation has been shown to trigger cell death independently of p53 through p38/MAPK activation. Indeed the shelterin protein Tin2 has been shown to contribute to mitochondrial function through an unclear mechanism. Preliminary work from our lab has identified activation of the intrinsic (mitochondrial) pathway of apoptosis following *Acd* deletion even in the absence of p53. We are currently developing techniques to assess mitochondrial function to evaluate this possibility.

Understanding the mechanisms through which Tpp1 loss results in hematopoietic failure has direct implications for human disease. The recent identification of *TIN2* mutations in the human bone marrow failure syndrome dyskeratosis congenita necessitates such studies. *TIN2* mutations result in a very aggressive form of the disease with an early onset. It thus does not follow a progressive model similar to other forms of the disease associated with telomere erosion. In the shelterin complex, one of the primary functions of Tin2 is tethering Tpp1/Pot1 to Trf1 and Trf2. Tin2 loss is embryonic lethal in mouse studies, and no conditional allele has been described. Tpp1 loss is therefore the most accurate reflection of what may happen when *TIN2* is mutated in human patients. Understanding how *Acd* deficiency affects stem cell compartments, not just in the hematopoietic system but throughout development and across tissues, may direct therapeutic interventions in treating patients.

CHAPTER 7. MATERIALS AND METHODS

Mice used for general transplantation assays. C57BL/6 (B6, CD45.2$^+$) mice were purchased from Harlan (Indianapolis, IN) and C57BL/6.Ptprca (B6-SJL, CD45.1$^+$) were purchased from the National Cancer Institute (Frederick, MD).

Mice used for *Acd* studies. *acd* hypomorphic mutant mice and mice carrying a floxed conditional *Acd/Tpp1* allele (*Acd^{fl}*) have been described previously [215, 234]. *Acd$^{fl/+}$* mice were crossed with mice carrying a null *Acd* allele (*Acd$^-$*) and *Mx-Cre* [251], *Vav-Cre* transgenic [265]or *Cre-ERT2* lentitransgenic mice [252]. When indicated, mice were crossed onto a *p53$^{-/-}$* background [266]. To fully inactivate *Acd/Tpp1*, Cre-expressing *Acd$^{fl/-}$* mice were used. *Mx-Cre* activation was initially achieved via 5 intraperitoneal injections of 200 μg poly(I:C) (EMD Biosciences, Billerica, MA). To better capture acute changes induced by *Acd/Tpp1* loss in hematopoietic stem and progenitor cells, we used a single 20 μg dose of highly purified poly(I:C) (GE Healthcare Biosciences, Pittsburgh, PA). This allowed detection of hematopoietic progenitors soon after administration with minimal effects on their phenotypic profile from the poly(I:C)-mediated interferon response. *Cre-ERT2* activation was achieved by 2-3 intraperitoneal injections of tamoxifen in corn oil (Sigma, St. Louis, MO; 1 mg/dose). The University of Michigan Committee on Use and Care of Animals approved all experiments.

Mice used for *Ash1l* studies. *Ash1l*^GT^ embryonic stem cells were obtained from the Wellcome Trust Sanger Institute (Hinxton, England). ES cells were injected into blastocysts, and mice were generated by The University of Michigan Transgenic Animal Core. To study the fetal to adult transition, *Ash1l*^GT/+^ mice were bred with *Sox17*^GFP/+^ mice [26]. To perform long-term pulse-chase experiments, *Ash1l*^GT/+^ mice were bred with transgenic animals containing both *Rosa26-rtTA* and *TetOP-H2B-GFP* as previously described [161, 267]. To label hematopoietic cells with GFP, animals were maintained on doxycycline (2mg/ml) for 6 weeks. Following this labeling period, doxycycline drinking water was removed and animals were maintained on normal drinking water for a 6 week chase period to allow GFP dilution in dividing cells.

For experiments examining cooperativity between Ash1l and menin/Mll1, previously described transgenic *Men1*^fl^ mice were bred to generate *Ash1l*^GT/GT^*Men1*^fl/fl^*Mx-Cre*^+^ mice and additional indicated genotypes [163]. *Men1* excision was achieved by intraperitoneal injection of 5 doses of 50ug poly (I:C) administered every other day. The University of Michigan Committee on Use and Care of Animals approved all experiments.

Flow cytometry. Fetal liver and bone marrow specimens were harvested and single cell suspensions were prepared. Red blood cells were lysed with ACK lysis buffer (Cambrex, Walkersville, MD) and counted with a hemacytometer or a Nexcelom AutoT4 Cellometer (Nexcelom, Lawrence, MA). The following antibodies were from BioLegend (San Diego, CA), eBiosciences (San Diego, CA) or BD Biosciences (San Jose, CA): anti-CD3, CD4, CD8, CD11b, CD11c, CD19, CD48, CD150, Gr1/Ly-6G, B220, NK1.1, TCRβ, TCRγδ,

c-Kit and Sca-1. We used the following antibody cocktail to exclude Lineage^{+} cells: anti-CD11b, Gr1, CD11c, B220, CD19, CD3, TCRβ, TCRγδ, CD8, NK1.1 and Ter119. BrdU analysis was performed using a BrdU labeling kit (BD Biosciences). Ki67 staining was achieved using the BD Ki67 Set (BD Biosciences). Annexin-V staining was performed using an Annexin-V labeling kit (BD Biosciences). Analysis was on FACSCanto and sorting on FACSAria II/III (BD Biosciences). Dead cells were excluded with 4′6-diamidino-2-phenylindole (Sigma). Files were analyzed in FlowJo (Tree Star, San Carlos, CA).

Bone marrow and fetal liver cell transplantation. 6-8 week old B6-SJL (CD45.1[+]) recipient mice were lethally irradiated (900 Gy, [37]Cs source). Four hours after irradiation, mice were transplanted with the indicated donor bone marrow or fetal liver cells via tail vein injection. For competitive transplantation, we mixed equal numbers of competitor B6-SJL bone marrow and tester CD45.2[+] bone marrow or fetal liver cells. For non-ablative transplantation, 5-8 week old $Ash1l^{GT/GT}$ or wild-type controls were injected via tail vein 2 X with 2.0 X 10^{7} B6-SJL (CD45.1[+]) bone marrow cells. Injections were separated by 1 week.

Complete blood counts. Blood was obtained through retroorbital bleeding and transferred to EDTA-treated tubes. Complete blood counts were determined by analyzing the samples on the Advia 120 Hematology System (Siemens, Malvern, PA).

Quantitative Real-Time PCR. For gene expression analyses, at least 5000 Lin⁻Sca^hi^c-Kit^hi^ (LSK) hematopoietic progenitors were sort-purified directly into Trizol (Invitrogen, Carlsbad, CA). After RNA extraction, cDNA was generated using the SuperScript III First Strand Synthesis Kit (Invitrogen) or the Nugen Ovation PicoSL WTA System (Nugen Technologies, San Carlos, CA). Relative gene expression was measured using Taqman (Applied Biosystems, Carlsbad, CA) primer and probe sets or SybrGreen (Fisher, Rockford, IL) for the indicated target genes. Reactions were carried out on a Mastercycler realplex (Eppendorf, Westbury, NY). Relative expression was calculated after normalization with *Hprt1* expression using the ΔΔCT method. Primer information is listed in **Table 7.1**.

For quantification of *Acd* excision, 10000 LSK progenitors were sort-purified into Direct Lysis buffer (Viagen Biotech, Los Angeles, CA) with 1 μL Proteinase K. Samples were incubated overnight and Proteinase K was inactivated at 85°C for 1hr. To assess excision, we used a primer pair specific for the floxed *Acd* exon 7 and an exon 2-specific primer pair amplifying a DNA sequence outside the floxed region. Amplification was performed with SybrGreen (Fisher, Rockford, IL) before quantification of exon7/exon2 signal on Mastercycler realplex using the ΔΔCT method. For measurement of telomere length, DNA was extracted with Direct Lysis buffer as described above. Primers specific for telomeric DNA and the reference locus *m36b4* were used with SybrGreen amplification on Mastercycler realplex, as described [268, 269]. The abundance of telomeric DNA normalized to m36b4 signal was calculated using the ΔΔCT method.

Table 7.1. qPCR Primers

Target	Primer Sequence or TaqMan Assay ID	Format
Acd Exon 2	F-TACTCCAGGACTCGGAGACT R-TCTGACACAAGTAACACGGC	SybrGreen
Acd Exon 7	F-CTAACCCTGTCCCAGCTTCT R-TACAAGGACCTTTCAGCACC	SybrGreen
Ash1l	Mm01212715_m1	TaqMan
Hoxa5	Mm00439362_m1	TaqMan
Hoxa7	Mm00657963_m1	TaqMan
Hoxa9	Mm00439364_m1	TaqMan
Hoxa10	Mm00433966_m1	TaqMan
Hprt1	Mm01545399_m1	TaqMan
m36b4	F-ACTGGTCTAGGACCCGAGAAG R-TCAATGGTGCCTCTGGAGATT	SybrGreen
Meis1	Mm00487664_m1	TaqMan
Noxa	Mm00451763_m1	
p21	Mm00432448_m1	TaqMan
p27	Mm00438168_m1	TaqMan
p57	Mm00438170_m1	TaqMan
Puma	Mm00519268_m1	
Telomere	F-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT R-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	SybrGreen

Metaphase preparation and analysis. Bone marrow cells were cultured for 12 hours in

S-clone SF-O3 medium (Iwai, Foster City, CA) with IL-3 (9 ng/ml), IL-6 (5 ng/ml) and

stem cell factor (100 ng/ml) (Peprotech, Rocky Hill, NJ) at 5%CO_2 and 37°C.

KARYOMax Colcemid (Invitrogen) was added at 0.2μg/mL for 3-4 hours at 37°C.

Metaphase spreads were then prepared as described [270]. Samples were stained with a

PNA-TelG-FITC probe (Biosynthesis, Lewisville, TX) and DAPI as previously

described. Metaphases were analyzed and images acquired using a Nikon E800

microscope equipped with an Olympus DP-71 digital camera. Images were scored in a

blinded fashion.

CFU-GM and single-colony PCR. 20000 bone marrow cells were plated per mL of

Methocult GF M3534 (Stem Cell Technologies, Vancouver, BC). Colonies were scored

7-10 days later. Where indicated, individual colonies were removed as a plug with

methylcellulose and washed in PBS before DNA extraction in 100uL lysis buffer

(Viagen) with 1uL proteinase K. Samples were incubated overnight and Proteinase K was

inactivated at 85°C for 1hr. Colony DNA was then genotyped as described (Kibe, 2010).

Statistical analysis. Comparison of two means was performed with 2-tailed unpaired

Student t test. Analysis of more than two means was performed with ANOVA followed

by Bonferroni post-test analysis. Comparisons of observed genotypes to those predicted

by Mendelian ratios were performed with Chi Squared analysis.

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