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The Role Of Lnk Adaptor Protein In Hematopoietic Stem Cell Genome Stability And Self-Renewal

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Abstract
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This thesis examines the role of an adaptor protein known as LNK (SH2B3), which negatively regulates a central cytokine signaling axis in HSCs, in regulating HSC self-renewal and genome stability. HSCs in Lnk-/- mice are expanded, and endowed with enhanced proliferative and self-renewal capabilities. Given this superiority, this thesis first investigates the impact of LNK deficiency in context of a bone marrow failure syndrome, Fanconi Anemia (FA), where HSCs accumulate fatal levels of genomic insults and cannot function. Superimposed on the deletion of a central gene in FA, FANCD2, Lnk deficiency rescues HSC function through restoring genome stability at sites of stress encountered during DNA duplication.

Second, using a model that is capable of tracking HSC divisions in vivo, this thesis investigates the in vivo self-renewal dynamics of Lnk deficient HSCs. On a population level, HSCs exist along a continuum of states between fully functional HSCs and progenitors, and LNK deficiency tips the balance towards HSCs. This is a cell-intrinsic process, and may be regulated by gene expression-dependent and –independent functions of LNK.

Taken together, the data presented in this thesis describes a novel role for cytokine signaling in HSC genome stability, and deepens our understanding of how LNK influences self-renewal and genome stability. Hopefully, these findings can contribute to the foundation of work that may result in the development of novel therapeutic approaches to treat bone marrow failure and genome instability in HSCs.

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THE ROLE OF LNK ADAPTOR PROTEIN IN HEMATOPOIETIC STEM CELL GENOME STABILITY AND SELF-RENEWAL

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DEDICATION

This thesis is dedicated to my first educators and role models: my great aunts Floriana and Joanna and my brother Sebastian. I look to your courage and dedication and I find my own.

Come my friends
'Tis not too late to seek a newer world.
Rush off, and sitting well in order smite
The sounding furrows; for my purpose holds
To sail beyond the sunset, and the baths
Of all the western stars, until I die.
It may be that the gulfs will wash us down:
It may be that we shall touch the Happy Isles,
And see the great Achilles, whom we knew,
Tho' much is taken, much abides; and tho'
We are not now that strength which in old days
Moved earth and heaven; that which we are, we are;
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.

Ulysses

Alfred Tennyson

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ABSTRACT

THE ROLE OF LNK ADAPTOR PROTEIN IN HEMATOPOIETIC STEM CELL GENOME STABILITY AND SELF-RENEWAL

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Hematopoietic stem cells (HSCs) are rare cells that reside in bone marrow. HSCs function to give rise to all blood cells through proliferation and differentiation, but also to HSCs in a tightly regulated process known as self-renewal. It is for these abilities that stem cells stand out from progenitors and other short-lived cells and which allows them to last a lifetime. Self-renewal remains mechanistically enigmatic but central to the biology and long lifespan of HSCs. Because HSCs are so long-lived they face numerous genomic insults and therefore mechanisms of genome stability are also central to HSC function throughout life.

This thesis examines the role of an adaptor protein known as LNK (SH2B3), which negatively regulates a central cytokine signaling axis in HSCs, in regulating HSC self-renewal and genome stability. HSCs in *Lnk-/-* mice are expanded, and endowed with enhanced proliferative and self-renewal capabilities. Given this superiority, this thesis first investigates the impact of LNK deficiency in context of a bone marrow failure syndrome, Fanconi Anemia (FA), where HSCs accumulate fatal levels of genomic insults and cannot function. Superimposed on the deletion of a central gene in FA, FANCD2, *Lnk* deficiency rescues HSC function through restoring genome stability at sites of stress encountered during DNA duplication.

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CHAPTER 1

Introduction

The hematopoietic system is a highly proliferative organ, producing and replacing billions of hematopoietic cells every day. This is sustained through production of hematopoietic cells from a population of hematopoietic stem cells (HSCs). These are rare cells with the two defining properties: 1) being able to repopulate an entire hematopoietic system (multi-lineage repopulation capacity), and 2) being able to produce additional HSCs (self-renewal) (Fig 1.1). HSCs are long-lived and therefore how HSCs handle genotoxic insults is especially important to their longevity and health. This thesis focuses on understanding the role of cytokine signaling in genome protection through a model of augmented cytokine signaling.

1.1 Hematopoietic stem cells

1.1.1 Functional Identification of HSCs

In order to study HSCs, it is essential to identify them. This has not been a trivial undertaking. HSCs reside primarily in the bone marrow, in specialized niches near slow-flowing blood vessels with low oxygenation [1]. HSCs were first described functionally, through the successful reconstitution of a recipient by transplant of donor bone marrow (BMT) [2 3 4]. This revelation led to a field of study dedicated to unraveling the inner workings of HSCs and their roles in normal and malignant hematopoiesis.

The gold standard for functional identification of HSCs remains the BMT where an HSC is defined as a single cell capable of reconstituting a conditioned recipient indefinitely [5]. To test for the total number of functional HSCs, total bone marrow is harvested from donors and injected into lethally irradiated recipients. HSC function is measured in the peripheral blood monthly for four months. Generally, HSC activity is scored if there is reconstitution above 1% across all lineages for the entire four month period [6 7 8 9]. An expected ratio of lymphoid to myeloid production is produced based on whether the HSC is lineage-biased or balanced [10]. Several doses are tested and by this method, it is possible to quantitate the number of functional HSCs in bone marrow. Similarly, reconstitution by BMT is used to test whether HSCs are present in a specific population of interest.

Transplantation is also used to directly test the second key aspect of HSC identity: self-renewal. At the end of the first BMT, phenotypic HSC – assessed by surface marker profiles that have been correlated with functional HSCs – are enumerated in the BM. This provides a first-approximation estimate of the extent to which HSCs self-renewed over the course of the transplant. However, self-renewal is rigorously assessed by secondary transplant of BM from the recipients of the primary BMT [11 12 13 14].

Self-renewal ensures a continued HSC pool throughout the lifespan of the organism. HSC self-renewal has been described at the moment of cell division as an inheritance of HSC fate in one or both daughter cells through asymmetric or symmetric division, respectively [15 16]. However, HSCs are found to undergo division rarely, existing in a state

of quiescence [17] [18] [19]. During hematopoietic depletion or immune challenge, HSCs respond to the stress by proliferating and differentiating to produce hematopoietic effector cells and then return to quiescence [20,21]. However, the proliferative capacity of HSCs is also limited and prolonged stress results in bone marrow failure [22-24]. Together self-renewal and reconstitution capacity constitute the definition of functional HSCs.

1.1.2 Phenotypic Identification of HSCs

To study HSCs, it is necessary to prospectively identify and isolate them from the progenitors and stromal cells that also reside in the bone marrow. One way to do so has been based on determining the surface marker expression profile of cells with reconstitution capacity by flow cytometry. Mature lineage-specific cells are excluded by a combination of empirically-determined surface markers associated with each of the lineages and includes Ter-119 (red blood cells), Gr-1, Mac-1 (myeloid), CD3, CD4, CD8a (lymphoid), and CD19 and B221 B cells), which are collectively termed Lin markers. The remaining cells are enriched for HSC and progenitors [25,26] . This population is further enriched for HSCs by selecting for cells expressing c-kit and Sca-1 [27,28] [29] [25]. Further separation between short-term repopulating cells and long-term repopulating HSCs (LT-HSCs) has been achieved by addition of other surface markers: CD48-CD150+ (SLAM LSK) [11,30] or CD34-Flk2- [5,31], ESAM [32,33], side-population [34] or most recently Sca1^{hi}EPCR^{hi} LSK [35]. These surface marker schemes are capable of enriching HSCs to 1 in every 3 cells, or 1 in 2 cells, respectively.

Though these surface markers do enrich HSCs, there are several important caveats about phenotypic HSC markers. The first is that any given surface marker of an HSC may or may not contribute to HSC function. For example, c-kit or Sca-1 ablation negatively impacts HSC function [28,29]. Second, because not all surface markers are coupled to HSC function, there is not a perfect correlation between surface markers and functional HSCs. For example, some cells marked by CD150+ within CD48-LSK, are not functional HSCs, while some CD150- are [36]. In this way, there are several distinct and partially overlapping surface marker schemes that enrich HSCs, and none can encompass all HSCs. Finally, the expression level of surface markers also correlates with function. High cell surface expression of either Sca1 or CD150 is associated with functional HSCs [35,37], while high expression of c-kit is associated with short-term HSCs [38,39]. Together these caveats contribute to the difficulty of phenotypic isolation of HSCs.

1.2 Cytokine signaling in HSCs

1.2.1 Cytokines involved in HSC self-renewal

HSCs are regulated through multiple cell- autonomous and –nonautonomous mechanisms. One crucial cell-autonomous mechanism is through cytokines. Cytokines are short hydrophilic peptides that are recognized by cognate receptors on the cell surface and trigger a series of signaling events inside the cell. In general, cytokines trigger HSC and progenitor survival, proliferation, differentiation, and lineage commitment. Two cytokines have been shown to be essential to the maintenance of HSCs: stem cell factor (SCF) and Thrombopoietin (TPO) [27-29,40-42].

This thesis focuses on the contribution of TPO signaling to HSC maintenance. TPO binds and signals through its cognate receptor (TPOR). TPOR lacks intrinsic kinase activity, and is associated with the tyrosine kinase JAK2. TPO/TPOR/JAK2 signaling is essential to HSC survival, proliferation, and self-renewal [43] [44]. Mice deficient for TPO or TPOR have a contracted HSC compartment, and these HSCs are severely functionally impaired in transplant assays [41,45-47] [48]. Furthermore, JAK2 deficiency is embryonic lethal [49] [50]. Therefore TPO/TPOR/JAK2 is an important pathway in the study of HSC self-renewal.

1.3 Role of Lnk deficiency in HSCs

LNK is the third member in the SH2 domain containing family of adaptor proteins. This family of proteins function in a variety of cell contexts to curb cytokine signaling specifically in glucose metabolism and immune cell activation {Devalliere:2011fl} [51,52]. LNK is the main member of this family to be expressed in HSCs. In HSCs, one of the main targets of LNK is JAK2. LNK directly binds to and inhibits the activity of JAK2, and therefore negatively regulates TPO/TPOR/JAK2 signaling [53]. Consequently LNK deficiency increases TPO mediated cytokine signaling in cells (Fig 1.2).

1.3.1 *Lnk* deficient HSCs

In *Lnk* deficient animals, *Lnk* deficiency results in a progressive HSC expansion, such that the HSC compartment is 10-fold expanded in young adult animals. Furthermore,

Lnk deficient HSCs provide enhanced reconstitution in recipients [54-56]. Importantly, *Lnk-/-* HSCs show enhanced self-renewal *in vivo* and *in vitro* [8,56]. However, in contrast to the deleterious consequences observed in mice with activating mutations in components of TPO/TPOR/JAK2 signaling such as JAK2 V617F [57 58 59] or constitutive AKT activity [60], *Lnk* deficiency does not lead to premature depletion of HSCs [61] nor to uncontrolled proliferation and neoplasm. In fact, the expanded HSC compartment in *Lnk*-/- mice is mostly quiescent [56] (Fig 1.3). Taken together, *Lnk* deficient HSCs show many enhancements in HSC function, rendering Lnk as a potentially valuable therapeutic target in the expansion and transplantation of HSCs.

Though the immediate signaling consequences of *Lnk* deficiency on TPO signaling have been described, much of the downstream consequences of the augmentation in TPO signaling on HSC biology remain elusive.

1.4 Fanconi Anemia

1.4.1 Fanconi Anemia clinical manifestations

Fanconi Anemia (FA) is a hereditary set of disorders that affect genome stability in cells, first described over 85 years ago [62]. Though there is a spectrum of physical deformations and extent to which FA may affect an individual, a feature that is nearly ubiquitous among FA patients is bone marrow failure (BMF) [63]. FA is the most commonly inherited BMF syndrome, and is universally associated with a shortened life span [64]. FA is caused by a bi-allelic mutation in one of 21 Fanconi genes that cooperate to maintain

genome integrity and repair and include tumor suppressors Brca1 and Brca2 [64,65]. To date there is no cure for FA.

However, the BMF in FA is now treatable. Until recently, there was no treatment for BMF in FA patients because of the risk of inducing cancerous mutations in other tissues by chemotherapeutics that would clear space for donor bone marrow. Recent advances in recipient conditioning through gentler agents has enabled bone marrow transplant (BMT), which allows for lifespan extension in FA patients [66-68]. Nevertheless, FA patients are at a higher risk for developing leukemia and epithelial cancers [69-71].

1.4.2 FA proteins; FANCD2

FA family proteins function in a variety of roles. The best-known role of FA proteins is in context of DNA crosslink repair, in which a damaging agent covalently crosslinks two DNA strands [72,73]. This is known as an interstrand crosslink (ICL). Upon replication, this crosslink halts DNA replication and is then recognized by the core complex, which ubiquitinates and activates FANCD2 (D2) in the FANCD2-FANCI (I-D2) recognition complex [74]. The activated I-D2 complex then recruits downstream effectors that process the crosslink to a double stranded break (Fig 1.4A). This in turn activates strand repair machinery to repair the DSB through homologous recombination and ultimately, the resumption of replication [75]. In the absence of a FA protein, this network falls apart and repair is diverted to error-prone processes such as non-homologous end joining, while genome instability is concurrently dramatically elevated [76 77].

If crosslinks are not resolved they are lethal to cells. Unsurprisingly, FA cells have a high level of genome instability and a hallmark aberrant chromosomal profile in metaphase spreads [64] [78], a higher rate of spontaneous apoptosis in culture, and are extremely sensitive to crosslinking agents, such as Mitomycin (MMC) [78] [72]. Collectively, these defects result in apoptosis, shrinking of the HSC pool and ultimately BMF.

1.4.3 FANCD2 role in maintaining replication fork stability

In addition to the ICL repair complex, FANCD2 is a central component of a second FA complex, which is less extensively characterized. In this role, FANCD2 is recruited to stalled replication forks – due to any cause [79] – and is indispensible in maintaining the integrity of the stalled replication fork, preventing new origin firing during stalling, and ultimately, in restarting stalled forks [80] [79]. Among binding other effectors, FANCD2 binds RAD51, a DNA binding protein essential for replication fork stability [81-84] (Fig 1.4B). Though it is unclear how FANCD2 and RAD51 interact, RAD51 overexpression is sufficient to overcome the fork replication associated defects in FancD2 deficient cells [80], underscoring both the essential role of RAD51 and the importance of further dissection into the regulation of this interaction.

In FANCD2 deficient cells, upon stalling, replication forks are extensively resected by the nuclease MRE11 [85], which also plays a role in normal fork restarting [86] [87]. This progresses to a double stranded break, which then must be repaired by an error prone

mechanism. Though not the most commonly mutated gene in FA, mutations in FANCD2 are associated with the most severe FA and the earliest onset BMF in patients. Moreover, *Fancd2* deficient cells have more genome instability in mice than FANCC or FANCG deletion [88]. Additionally, there is never complete absence of FANCD2 protein in patient cells [75], underscoring the importance of FANCD2 to cell survival. Additionally, in context of BRCA1 or BRCA2 deficiency, FANCD2 deletion dramatically increases genome instability [89,90]. Undoubtedly, FANCD2 is central to genome stability and HSC survival.

1.4.4 Mouse models of FA

FA has been modeled in mice and there exists a few germline deleted lines of FA genes: *Fanca* [91], *Fancc* [92 93 94], *Fancg* [95] and *FancD2* [96-98]. These mouse models recapitulate the cellular hallmarks associated with FA: sensitivity to MMC, high spontaneous rate of apoptosis, and genomic instability. Additionally, in *FancD2-/-* mice the HSC and progenitor compartment – LSK – is two-fold contracted relative to normal mice, and there is evidence of accelerated neoplasia [99,100].

However, the hematological manifestations of FA are not observed in mice. FA mice do not develop spontaneous BMF, and rarely spontaneous leukemia. It is speculated that given the short lifespan of mice, their clean housing conditions and limited exposure to hematopoietic stressors such as infection, there is simply no opportunity to develop BMF spontaneously [63].

Which functions of FA proteins contribute to maintenance of BM is still an active area of investigation (Fig 1.5). For FANCD2, studies have revealed an important relationship: both roles of FANCD2 are known to contribute towards preventing BMF. Several studies have shown that the role of FANCD2 in crosslink repair is essential: *FancD2-/-* mice in combination with deletion of Aldehyde dehydrogenase 2 or Alcohol dehydrogenase 5 (Aldh2, Adh5, respectively) do spontaneously develop BMF or leukemia [101] [102] [103] [104]. Equally importantly, prolonged stress hematopoiesis also induces BMF in *FancD2-/-* mice [105] [106], especially chronic proliferation induced by the pro-inflammatory double stranded DNA mimic polyiosinic:polycytidylic acid (pI:pC) [105]. Here, in contrast to crosslink- induced damage, BMF occurs as a result of proliferation-induced RNA damage in LT-HSCs, which are repeatedly forced into cell cycle by the injections and accrue damage over time.

In this study, we have chosen to use *FancD2-/-* mice as a model for FA diseases because of its centrality to FA roles with demonstrated roles in maintaining HSC integrity.

1.5 Scope and findings of this thesis

The main purpose of this thesis is to explore how LNK deficiency confers enhanced stem cell properties to HSCs. This functionality is explored in two ways: first, by its role

in ameliorating hematopoietic defects in FANCD2 deficient mice, and second in its role in regulating self-renewal in HSCs.

1.5.1 To define mechanism by which LNK deficiency rescues HSPC function in FA

There have been a few reports suggesting that control over inflammatory signaling via TGFb attenuation [107] can ameliorate FA in mice. Notwithstanding, there are no curative interventions for FA. However, given the sufficiency of RAD51 overexpression in correcting *FancD2-/-* cells [80], it is clear that FANCD2 deficiency can be overcome molecularly.

This thesis presents compelling evidence that Lnk deficiency ameliorates HSC function in *FancD2-/-* mice. Lnk deficiency does not impact crosslink repair in *FancD2-/-* cells, but does completely rescue instability in stalled replication forks. This process is JAK2 and TPOR –dependent, demonstrating for the first time a direct role for cytokine signaling in genome stability.

1.5.2 To deepen the understanding by which Lnk deficiency modulates HSC self-renewal

Second, this thesis presents a model in which to study the enhanced HSC self-renewal conferred by LNK deficiency *in vivo* using the H2B-GFP mouse model. Phenotypic HSCs show stratification in HSC function based on division *history in vivo*, and therefore this

model provides a functional way to prospectively examine HSC function. Our *in vivo* and *in vitro* data show that a gradual cell-intrinsic change in HSCs takes place with each *in vivo* division. Our genome wide expression profiling further corroborates these findings and offers insight into potential pathways that may be affected by LNK deficiency.

1.6 REFERENCES

1. Acar, M. *et al.* Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature* **526,** 126–130 (2015).

2. GENGOZIAN, N., URSO, I. S., CONGDON, C. C., CONGER, A. D. & MAKINODAN, T. Thymus specificity in lethally irradiated mice treated with rat bone marrow. *Proc. Soc. Exp. Biol. Med.* **96,** 714–720 (1957).

3. NOWELL, P. C., COLE, L. J., HABERMEYER, J. G. & ROAN, P. L. Growth and continued function of rat marrow cells in x-radiated mice. *Cancer Research* **16,** 258–261 (1956).

4. FORD, C. E., HAMERTON, J. L., BARNES, D. W. & LOUTIT, J. F. Cytological identification of radiation-chimaeras. *Nature* **177,** 452–454 (1956).

5. Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273,** 242–245 (1996).

6. Uchida, N., Dykstra, B., Lyons, K. J., Leung, F. Y. K. & Eaves, C. J. Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Experimental Hematology* **31,** 1338–1347 (2003).

7. Miller, C. L. & Eaves, C. J. Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc Natl Acad Sci USA* **94,** 13648–13653 (1997).

8. Ema, H. *et al.* Quantification of Self-Renewal Capacity in Single Hematopoietic Stem Cells from Normal and Lnk-Deficient Mice. *Developmental Cell* **8,** 907–914 (2005).

9. Dykstra, B. *et al.* High-resolution video monitoring of hematopoietic stem cells cultured in single-cell arrays identifies new features of self-renewal. *Proc Natl Acad Sci USA* **103,** 8185–8190 (2006).

10. Dykstra, B. *et al.* Long-Term Propagation of Distinct Hematopoietic Differentiation Programs In Vivo. *Cell Stem Cell* **1,** 218–229 (2007).

11. Kiel, M. J. *et al.* SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. *Cell* **121,** 1109–1121 (2005).

12. Harrison, D. E., Astle, C. M. & Delaittre, J. A. Loss of proliferative capacity in immunohemopoietic stem cells caused by serial transplantation rather than aging. *J Exp Med* **147,** 1526–1531 (1978).

13. Harrison, D. E., Stone, M. & Astle, C. M. Effects of transplantation on the

primitive immunohematopoietic stem cell. *J Exp Med* **172,** 431–437 (1990).

14. Jones, R. J., Celano, P., Sharkis, S. J. & Sensenbrenner, L. L. Two phases of engraftment established by serial bone marrow transplantation in mice. *Blood* **73,** 397–401 (1989).

15. Faubert, A., Lessard, J. & Sauvageau, G. Are genetic determinants of asymmetric stem cell division active in hematopoietic stem cells? *Oncogene* **23,** 7247–7255 (2004).

16. Morrison, S. J. & Kimble, J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441,** 1068–1074 (2006).

17. Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118,** 149–161 (2004).

18. Cheshier, S. H., Morrison, S. J., Liao, X. & weissman, I. L. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci USA* **96,** 3120–3125 (1999).

19. Kiel, M. J. *et al.* Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. *Nature* **449,** 238–242 (2007).

20. Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C. & Goodell, M. A. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* **465,** 793–797 (2010).

21. Challen, G. A., Boles, N. C., Chambers, S. M. & Goodell, M. A. Distinct Hematopoietic Stem Cell Subtypes Are Differentially Regulated by TGF-β1. *Stem Cell* **6,** 265–278 (2010).

22. Hao, S., Chen, C. & Cheng, T. Cell cycle regulation of hematopoietic stem or progenitor cells. *Int J Hematol* **103,** 487–497 (2016).

23. Trumpp, A., Essers, M. & Wilson, A. Awakening dormant haematopoietic stem cells. *Nature Reviews Immunology* **10,** 201–209 (2010).

24. Pietras, E. M. *et al.* Re-entry into quiescence protects hematopoietic stem cells from the killing effect of chronic exposure to type I interferons. *Journal of Experimental Medicine* **211,** 245–262 (2014).

25. Spangrude, G. J., Heimfeld, S. & weissman, I. L. Purification and characterization of mouse hematopoietic stem cells. *Science* **241,** 58–62 (1988).

26. Morrison, S. J. & weissman, I. L. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1,** 661–673 (1994).

27. Miller, C. L. *et al.* Studies of W mutant mice provide evidence for alternate mechanisms capable of activating hematopoietic stem cells. *Experimental Hematology* **24,** 185–194 (1996).

28. Ikuta, K. & weissman, I. L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci USA* **89,** 1502–1506 (1992).

29. Li, C. L. & Johnson, G. R. Stem cell factor enhances the survival but not the self-renewal of murine hematopoietic long-term repopulating cells. *Blood* **84,** 408–414 (1994).

30. Forsberg, E. C. *et al.* Differential expression of novel potential regulators in hematopoietic stem cells. *PLoS Genet* **1,** e28 (2005).

31. Adolfsson, J. *et al.* Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity* **15,** 659–669 (2001).

32. Yokota, T. *et al.* The endothelial antigen ESAM marks primitive hematopoietic progenitors throughout life in mice. *Blood* **113,** 2914–2923 (2009).

33. Ooi, A. G. L. *et al.* The adhesion molecule esam1 is a novel hematopoietic stem cell marker. *Stem Cells* **27,** 653–661 (2009).

34. Goodell, M. A., Brose, K., Paradis, G., Conner, A. S. & Mulligan, R. C. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* **183,** 1797–1806 (1996).

35. Wilson, N. K. *et al.* Combined Single-Cell Functional and Gene Expression Analysis Resolves Heterogeneity within Stem Cell Populations. *Stem Cell* **16,** 712–724 (2015).

36. Weksberg, D. C., Chambers, S. M., Boles, N. C. & Goodell, M. A. CD150- side population cells represent a functionally distinct population of long-term hematopoietic stem cells. *Blood* **111,** 2444–2451 (2008).

37. Morita, Y., Ema, H. & Nakauchi, H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *Journal of Experimental Medicine* **207,** 1173–1182 (2010).

38. Matsuoka, Y. *et al.* Low level of c-kit expression marks deeply quiescent murine hematopoietic stem cells. *Stem Cells* **29,** 1783–1791 (2011).

39. Shin, J. Y., Hu, W., Naramura, M. & Park, C. Y. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J Exp Med* **211,** 217–231 (2014).

40. Kimura, S., Roberts, A. W., Metcalf, D. & Alexander, W. S. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci USA* **95,** 1195–1200 (1998).

41. Alexander, W. S., Roberts, A. W., Nicola, N. A., Li, R. & Metcalf, D. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood*

87, 2162–2170 (1996).

42. Solar, G. P. *et al.* Role of c-mpl in early hematopoiesis. *Blood* **92,** 4–10 (1998).

43. de Graaf, C. A. & Metcalf, D. Thrombopoietin and hematopoietic stem cells. *Cell Cycle* **10,** 1582–1589 (2014).

44. Seita, J. & Weissman, I. L. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* **2,** 640–653 (2010).

45. Gurney, A. L., Carver-Moore, K., de Sauvage, F. J. & Moore, M. W. Thrombocytopenia in c-mpl-deficient mice. *Science* **265,** 1445–1447 (1994).

46. Murone, M., Carpenter, D. A. & de Sauvage, F. J. Hematopoietic deficiencies in c-mpl and TPO knockout mice. *Stem Cells* **16,** 1–6 (1998).

47. Qian, H. *et al.* Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells. *Cell Stem Cell* **1,** 671–684 (2007).

48. Kaushansky, K. Thrombopoietin: the primary regulator of platelet production. *Blood* **86,** 419–431 (1995).

49. Parganas, E. *et al.* Jak2 is essential for signaling through a variety of cytokine receptors. *Cell* **93,** 385–395 (1998).

50. Neubauer, H. *et al.* Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* **93,** 397–409 (1998).

51. Maures, T. J., Kurzer, J. H. & Carter-Su, C. SH2B1 (SH2-B) and JAK2: a multifunctional adaptor protein and kinase made for each other. *Trends in Endocrinology & Metabolism* **18,** 38–45 (2007).

52. Ahmed, Z. & Pillay, T. S. Functional effects of APS and SH2-B on insulin receptor signalling. *Biochem. Soc. Trans.* **29,** 529–534 (2001).

53. Tong, W. Lnk Inhibits Tpo-mpl Signaling and Tpo-mediated Megakaryocytopoiesis. *Journal of Experimental Medicine* **200,** 569–580 (2004).

54. Seita, J. *et al.* Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. *Proc Natl Acad Sci USA* **104,** 2349–2354 (2007).

55. Buza-Vidas, N. *et al.* Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. *Genes & Development* **20,** 2018–2023 (2006).

56. Bersenev, A., Wu, C., Balcerek, J. & Tong, W. Lnk controls mouse hematopoietic stem cell self-renewal and quiescence through direct interactions with JAK2. *J Clin Invest* **118,** 2832–2844 (2008).

57. Tefferi, A. & Gilliland, D. G. Oncogenes in myeloproliferative disorders. *Cell Cycle* **6,** 550–566 (2007).

58. Wernig, G. *et al.* Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood* **107,** 4274–4281 (2006).

59. Lacout, C. *et al.* JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. *Blood* **108,** 1652–1660 (2006).

60. Kharas, M. G. *et al.* Constitutively active AKT depletes hematopoietic stem cells and induces leukemia in mice. *Blood* **115,** 1406–1415 (2010).

61. Bersenev, A. *et al.* Lnk deficiency partially mitigates hematopoietic stem cell aging. *Aging Cell* no–no (2012). doi:10.1111/j.1474-9726.2012.00862.x

62. Lobitz, S. & Velleuer, E. *Guido Fanconi (1892-1979): a jack of all trades. Nature reviews. Cancer* **6,** 893–898 (Nature Publishing Group, 2006).

63. Garaycoechea, J. I. & Patel, K. J. Why does the bone marrow fail in Fanconi anemia? *Blood* **123,** 26–34 (2014).

64. Brosh, R. M., Bellani, M., Liu, Y. & Seidman, M. M. Fanconi Anemia: A DNA repair disorder characterized by accelerated decline of the hematopoietic stem cell compartment and other features of aging. *Ageing Res. Rev.* **33,** 67–75 (2017).

65. Mamrak, N. E., Shimamura, A. & Howlett, N. G. Recent discoveries in the molecular pathogenesis of the inherited bone marrow failure syndrome Fanconi anemia. *Blood Rev.* (2016). doi:10.1016/j.blre.2016.10.002

66. Ebens, C. L., MacMillan, M. L. & Wagner, J. E. Hematopoietic cell transplantation in Fanconi anemia: current evidence, challenges and recommendations. *Expert Rev Hematol* **10,** 81–97 (2017).

67. Bonfim, C. *et al.* Haploidentical Bone Marrow Transplantation with Post-Transplant Cyclophosphamide for Children and Adolescents with Fanconi Anemia. *Biol. Blood Marrow Transplant.* **23,** 310–317 (2017).

68. Alhuraiji, A. *et al.* Hematopoietic Stem Cell Transplant in Adolescent and Young Adults With Fanconi Anemia Is Feasible With Acceptable Toxicity, With Those Surviving 100 Days Posttransplant Having Excellent Outcomes. *Exp Clin Transplant* **14,** 660–664 (2016).

69. Alter, B. P., Greene, M. H., Velazquez, I. & Rosenberg, P. S. Cancer in Fanconi anemia. *Blood* **101,** 2072–2072 (2003).

70. Alter, B. P. Cancer in Fanconi anemia, 1927-2001. *Cancer* **97,** 425–440 (2003).

71. Alter, B. P. Fanconi anemia and the development of leukemia. *Best Practice & Research Clinical Haematology* **27,** 214–221 (2014).

72. Moldovan, G.-L. & D'Andrea, A. D. How the Fanconi Anemia Pathway Guards the Genome. *Annu. Rev. Genet.* **43,** 223–249 (2009).

73. Kennedy, R. D. & D'Andrea, A. D. The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes & Development* **19,** 2925–2940 (2005).

74. Rajendra, E. *et al.* The genetic and biochemical basis of FANCD2 monoubiquitination. *Molecular Cell* **54,** 858–869 (2014).

75. Patel, K. J. & Joenje, H. Fanconi anemia and DNA replication repair. *DNA Repair* **6,** 885–890 (2007).

76. Adamo, A. *et al.* Preventing nonhomologous end joining suppresses DNA repair defects of Fanconi anemia. *Molecular Cell* **39,** 25–35 (2010).

77. Kee, Y. & D'Andrea, A. D. Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes & Development* **24,** 1680–1694 (2010).

78. D'Andrea, A. D. & Grompe, M. The Fanconi anaemia/BRCA pathway. *Nat. Rev. Cancer* **3,** 23–34 (2003).

79. Lossaint, G. *et al.* FANCD2 Binds MCM Proteins and Controls Replisome Function upon Activation of S Phase Checkpoint Signaling. *Molecular Cell* **51,** 678–690 (2013).

80. Jasin, K. S. H. W. M., Wu, H. & Jasin, M. A Distinct Replication Fork Protection Pathway Connects Fanconi Anemia Tumor Suppressors to RAD51-BRCA1/2. *Cancer Cell* **22,** 106–116 (2012).

81. Chen, Y.-H. *et al.* ATR-Mediated Phosphorylation of FANCI Regulates Dormant Origin Firing in Response to Replication Stress. *Molecular Cell* **58,** 323–338 (2015).

82. Hashimoto, Y., Chaudhuri, A. R., Lopes, M. & Costanzo, V. Rad51 protects nascent DNA from Mre11-dependent degradation and promotes continuous DNA synthesis. *Nature Structural & Molecular Biology* **17,** 1305–1311 (2010).

83. Chen, X., Bosques, L., Sung, P. & Kupfer, G. M. A novel role for non-ubiquitinated FANCD2 in response to hydroxyurea-induced DNA damage. *Oncogene* **35,** 22–34 (2015).

84. Taniguchi, T. S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. *Blood* **100,** 2414–2420 (2002).

85. Ying, S., Hamdy, F. C. & Helleday, T. Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1. *Cancer Research* **72,** 2814–2821 (2012).

86. Trenz, K., Smith, E., Smith, S. & Costanzo, V. ATM and ATR promote Mre11 dependent restart of collapsed replication forks and prevent accumulation of DNA breaks. *EMBO J* **25,** 1764–1774 (2006).

87. Bryant, H. E. *et al.* PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. *EMBO J* **28,** 2601–2615

(2009).

88. Reliene, R., Yamamoto, M. L., Rao, P. N. & Schiestl, R. H. Genomic Instability in Mice Is Greater in Fanconi Anemia Caused by Deficiency of Fancd2 than Fancg. *Cancer Research* **70,** 9703–9710 (2010).

89. Michl, J., Zimmer, J., Buffa, F. M., McDermott, U. & Tarsounas, M. FANCD2 limits replication stress and genome instability in cells lacking BRCA2. *Nature Structural & Molecular Biology* **23,** 755–757 (2016).

90. Kais, Z. *et al.* FANCD2 Maintains Fork Stability in BRCA1/2- Deficient Tumors and Promotes Alternative End- Joining DNA Repair. *CellReports* **15,** 2488–2499 (2016).

91. Cheng, N. C. *et al.* Mice with a targeted disruption of the Fanconi anemia homolog Fanca. *Human Molecular Genetics* **9,** 1805–1811 (2000).

92. Carreau, M. *et al.* Bone marrow failure in the Fanconi anemia group C mouse model after DNA damage. *Blood* **91,** 2737–2744 (1998).

93. Chen, M. *et al.* Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. *Nat Genet* **12,** 448–451 (1996).

94. Whitney, M. A. *et al.* Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. *Blood* **88,** 49–58 (1996).

95. Yang, Y. *et al.* Targeted disruption of the murine Fanconi anemia gene, Fancg/Xrcc9. *Blood* **98,** 3435–3440 (2001).

96. Parmar, K. *et al.* Hematopoietic Stem Cell Defects in Mice with Deficiency of Fancd2 or Usp1. *Stem Cells* **28,** 1186–1195 (2010).

97. Zhang, Q. S. *et al.* Fancd2-/- mice have hematopoietic defects that can be partially corrected by resveratrol. *Blood* **116,** 5140–5148 (2010).

98. Vinciguerra, P., Godinho, S. A., Parmar, K., Pellman, D. & D'Andrea, A. D. Cytokinesis failure occurs in Fanconi anemia pathway–deficient murine and human bone marrow hematopoietic cells. *J Clin Invest* **120,** 3834–3842 (2010).

99. Houghtaling, S. Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes & Development* **17,** 2021–2035 (2003).

100. Houghtaling, S. *et al.* Heterozygosity for p53 (Trp53+/-) accelerates epithelial tumor formation in fanconi anemia complementation group D2 (Fancd2) knockout mice. *Cancer Research* **65,** 85–91 (2005).

101. Garaycoechea, J. I. *et al.* Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. *Nature* 1–7 (2012). doi:10.1038/nature11368

102. Langevin, F., Crossan, G. P., Rosado, I. V., Arends, M. J. & Patel, K. J. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. *Nature* **475,** 53–58 (2011).

103. Rosado, I. V., Langevin, F., Crossan, G. P., Takata, M. & Patel, K. J. Formaldehyde catabolism is essential in cells deficient for the Fanconi anemia DNA-repair pathway. *Nature Structural & Molecular Biology* **18,** 1432–1434 (2011).

104. Pontel, L. B. *et al.* Endogenous Formaldehyde Is a Hematopoietic Stem Cell Genotoxin and Metabolic Carcinogen. *Molecular Cell* **60,** 177–188 (2015).

105. Walter, D. *et al.* Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. *Nature* **520,** 549–552 (2015).

106. Kaschutnig, P. *et al.* The Fanconi anemia pathway is required for efficient repair of stress-induced DNA damage in haematopoietic stem cells. *Cell Cycle* **14,** 2734–2742 (2015).

107. Zhang, H. *et al.* TGF-β Inhibition Rescues Hematopoietic Stem Cell Defects and Bone Marrow Failure in Fanconi Anemia. *Stem Cell* **18,** 1–34 (2016).

1.7 Figures

Figure 1.1

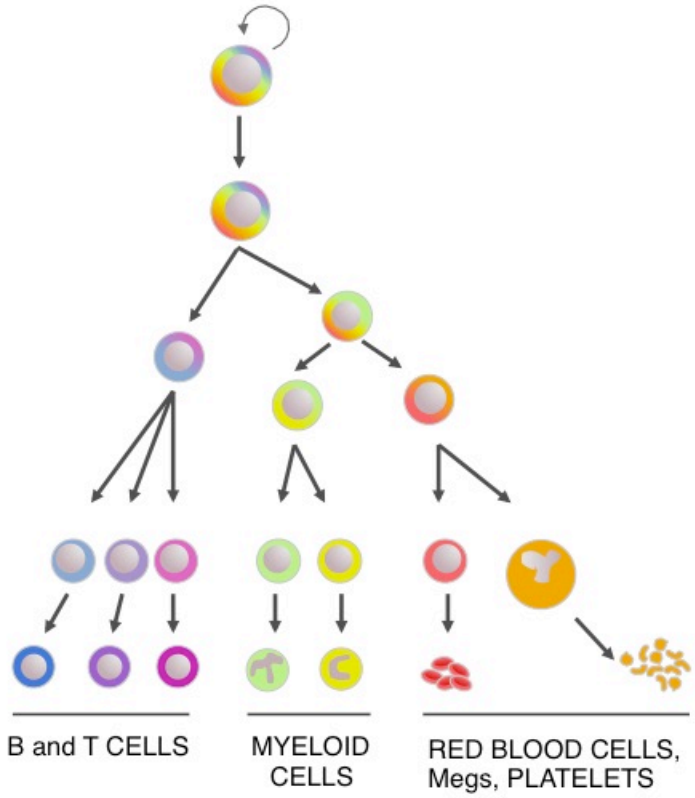
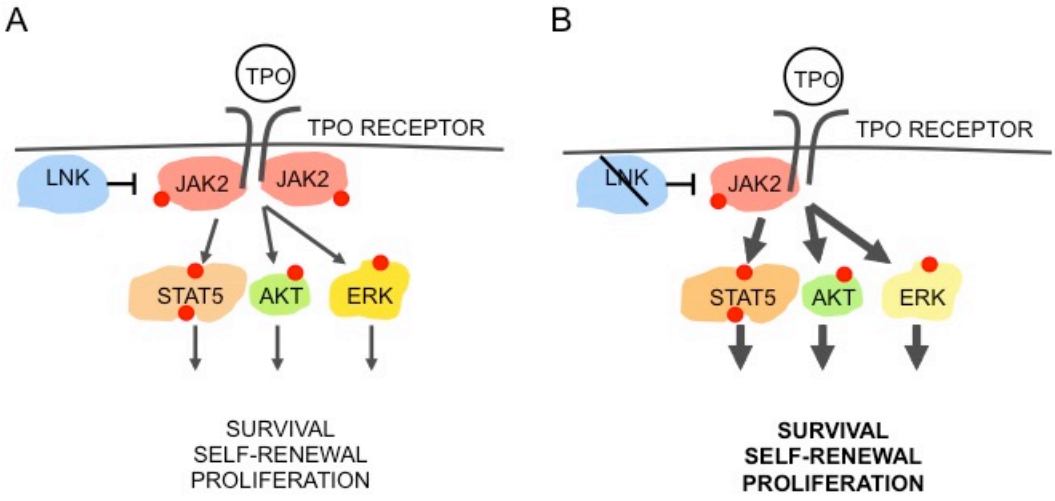


Figure 1.2


A
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SELF-RENEWAL
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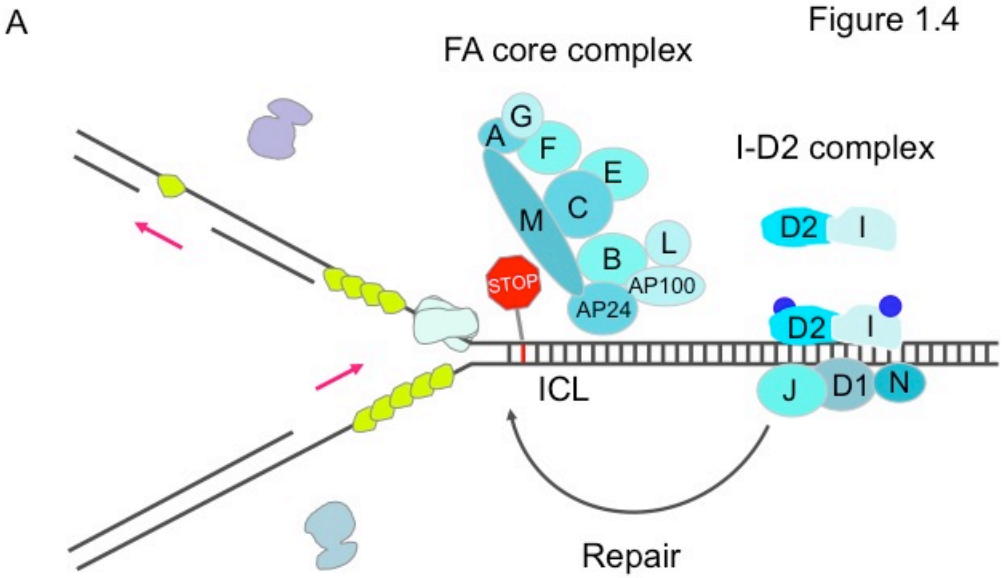
B
TPO
TPO RECEPTOR
LNK
JAK2
STAT5
AKT
ERK
SURVIVAL
SELF-RENEWAL
PROLIFERATION

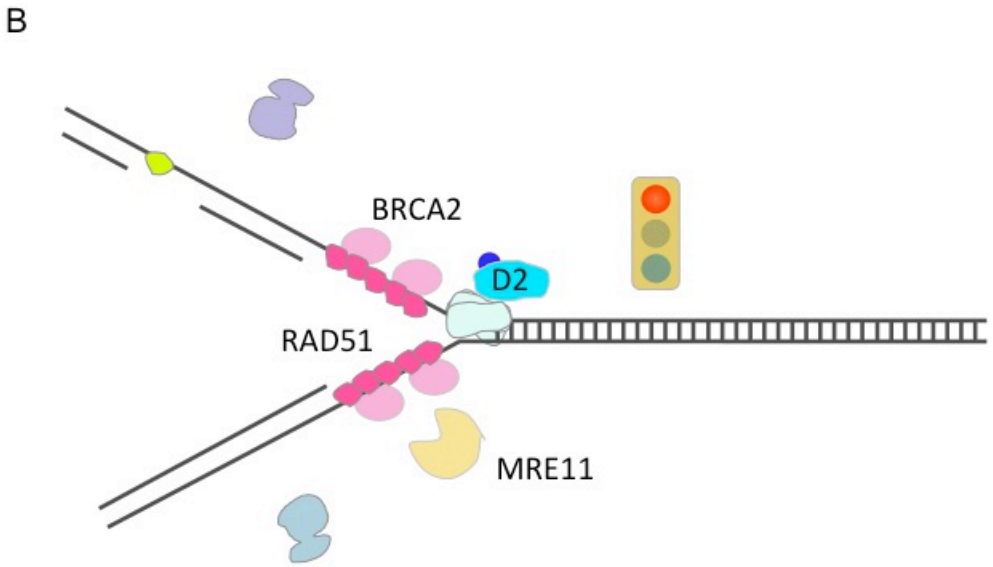
Figure 1.3

Lnk-/-

Blood composition	3x expanded white blood cells and platelets
HSC Frequency	**10x expanded**
Self-renewal	**Increased**
HSC Lifespan	**Unchanged**
% cells Quiescent	**Increased**

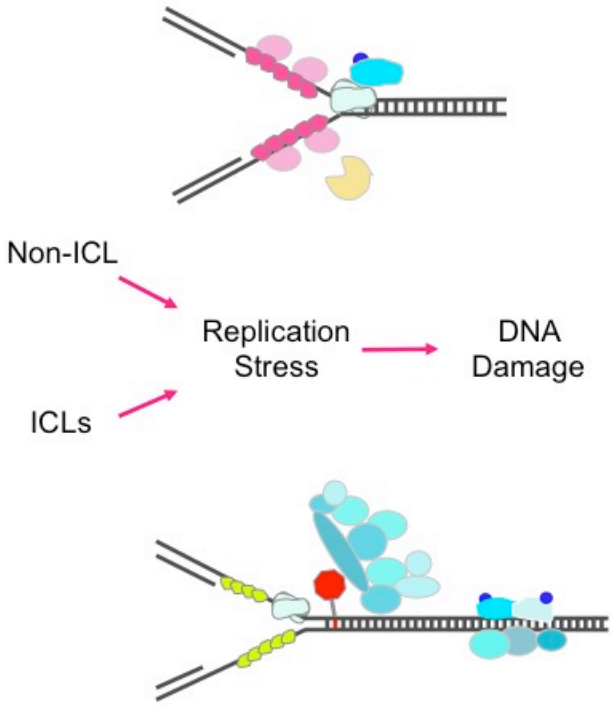
Figure 1.4





1. Prevent resection of DNA by MRE11 nuclease
2. Prevent new origin firing
3. Restart replication

Figure 1.5



1.8 Figure Legends

Figure 1.1: HSCs give rise to all hematopoietic cells and self-renew. HSCs sit atop the hematopoietic hierarchy, producing all hematopoietic cells. Arrows indicate cell identity transitions, which are not exhaustively drawn. Curved arrow indicates self-renewal.

Figure 1.2: TPO signaling is enhanced in absence of LNK. (A) shows a model of TPO signaling in HSCs, and the inhibitory effect of LNK. Signal transduction is depicted by arrows. Phosphorylations are represented as red circles. (B) shows augmented signaling in *Lnk* deficient cells, as indicated by thickened arrows. Thickness is not directly proportional to signal augmentation.

Figure 1.3: Several HSC properties are enhanced in *Lnk* deficient mice.

Figure 1.4: FA proteins play two distinct roles in protecting genome stability. (A) shows the FA proteins involved in ICL repair. FA proteins are noted by the single-letter code of their complementation groups. (B) shows the FA proteins involved in stalled replication fork repair. The hovering traffic light indicates stalled replication fork conditions.

Figure 1.5: Relative importance of the two roles of FA complex in HSC survival.

CHAPTER 2

Lnk/Sh2b3 Deficiency restores Hematopoietic Stem Cell function and genome integrity in Fanconi Anemia mutant mice

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2.1 Abstract

Fanconi Anemia (FA) is an inherited bone marrow failure syndrome that arises due to mutations in a network of FA genes. FA proteins cooperate in a genome stability pathway that is essential for repair of DNA interstrand crosslinks (ICL) and tolerance of replication stress. Mutations in FA genes severely compromise hematopoietic stem cell (HSC) capacity, culminating in bone marrow failure and cancer predisposition. Importantly, interventions to mitigate HSC defects in FA do not exist, aside from allogeneic stem cell transplantation. Remarkably, we show here that loss of the negative regulator of JAK2 kinase, *Lnk* (*Sh2b3*), restores HSC function in *Fancd2* knockout mice without further accelerating neoplastic transformation. *Lnk* deficiency did not directly impact ICL repair but instead ameliorated replication stress by stabilizing replication forks in a manner dependent on cytokine-mediated JAK2 signaling. *Lnk* deficiency restored cell proliferation and survival of *Fancd2*-deficient HSCs to wildtype levels, while reducing replication stress and genomic instability associated with FA. These findings reveal coordination between extracellular/cytoplamic signals and processes that converge on replication associated genome maintenance. They also illuminate the diversity of mechanisms underlying the origin of bone marrow failure in FA patients and have implications for therapeutic strategies to treat FA associated bone marrow failure.

2.2 Introduction

Hematopoietic stem cells (HSCs) are characterized by their ability to self-renew and differentiate into multilineage blood cells[1,2]. They are the source of all circulating blood cells throughout life, and disruption of HSC homeostasis is associated with a variety of human disorders[1,2]. Faithful maintenance of genome integrity in hematopoietic stem and progenitor cell (HSPC) populations is crucial to hematopoiesis and suppression of blood-derived cancers. In humans, DNA repair deficiency, prominently illustrated by Fanconi Anemia (FA) syndromes, results in multiple congenital anomalies, progressive bone marrow failure (BMF) and cancer susceptibility[3,4]. Mutations within nineteen genes have been identified as causative for FA. These genes cooperate in a genome stability network that is essential for repair of DNA interstrand crosslinks (ICLs) and relief of replication stress[5]. Cells derived from FA patients are hypersensitive to ICL-inducing agents such as Mitomycin C (MMC) and cisplatin, and exhibit DNA damage checkpoint and mitosis defects[5]. Unrepaired DNA damage in FA HSPCs increases genome instability and leukemia/cancer. The loss of HSPCs in FA is a consequence of multiple mechanisms, including impaired HSPC function, genotoxicity from the endogenous ICL agent, aldehydes[6,7], physiological proliferative stress[8], elevated p53 levels[9], hypersensitivity to inflammatory cytokines[10,11], oxidative stress[12], and a hyperactive TGFbeta pathway[13]. However, how the FA pathway controls HSPC function remains enigmatic. Importantly, other than allogeneic transplantation, therapeutic interventions that mitigate the HSPC defects in FA do not exist.

The FA pathway involves monoubiquitination of FANCD2-FANCI proteins by the FA core complex in addition to a parallel or downstream function of homologous recombination (HR) proteins, including the breast cancer suppressor BRCA2 and BRCA1[14]. In addition to their established roles in DNA repair of ICL-damage, the FA/BRCA protein network is also highly activated by replication stalling from depletion of nucleotide pools, such as from hydroxyurea (HU). Work by Dr. Maria Jasin has uncovered a DNA repair-independent requirement for FA proteins, including *FANCD2* and *BRCA1*,2 in protecting stalled replication forks from degradation[15,16]. These additional functions of the FA/BRCA proteins also play a critical role in preventing genomic instability and suppressing tumorigenesis [17]. However, it remains to be determined if their roles in protecting stalled replication fork contribute to HSC attrition or BMF in FA.

HSPC homeostasis is under the control of cytokine signaling pathways. One such important signaling axis is initiated by thrombopoietin (TPO) and its receptor, MPL that activates the JAK2 tyrosine kinase signaling pathway[18]. *Tpo*$^{-/-}$ and *Mpl*$^{-/-}$ mice exhibit marked reduction in HSC activity in supporting HSC self-renewal[19,20]. Patients with congenital amegakaryocytic thrombocytopenia (CAMT), many of whom have *MPL* loss-of-function mutations, progress into BMF in childhood [21], indicating the crucial role of the TPO/MPL pathway in HSC homeostasis in humans.

A critical negative regulator of the TPO/MPL pathway in HSCs is the adaptor protein LNK (or SH2B3)[22-24]. Lnk deficiency leads to a >10-fold *increase* in HSC numbers owing to superior HSC self-renewal[23,25]. Furthermore, Lnk deficiency strongly mitigates HSC

aging and delays HSC exhaustion in serial transplants[26]. We reported that LNK directly interacts with phosphorylated JAK2 in a TPO-dependent manner, and *Lnk* deficiency potentiates JAK2 activation and signaling in HSPCs[22].

In this work, we set out to test if *Lnk* deficiency ameliorates HSC defects associated with FA by generating mice double nullizygous to Fancd2 and Lnk. Remarkably, we found that loss of the adaptor protein Lnk restores HSC function in *Fancd2*$^{-/-}$ mice without accelerating neoplastic transformation. Our results indicated that LNK does not play an overt role in ICL repair. Instead, *Lnk* deficiency notably reduces spontaneous DNA damage and genome instability. Strikingly, we demonstrated that Lnk deficiency mitigates replication stress by stabilizing stalled replication forks, and that this effect is dependent upon cytokine-mediated JAK2 signaling. Hence, our studies shed light on the underlying origin of BMF in FA patients and have implications for new therapeutic strategies.

2.3 Methods and Materials

Mice

Fancd2$^{-/-}$ mice were generous provided by Dr. Alan D'Andrea (Dana Farber Cancer Institute) [27] and *Lnk*$^{-/-}$ mice by Dr. Tony Pawson [28] (Samuel Lunenfeld Research Institute, Canada), respectively. All mice were on C57/B6J background (CD45.2). Transplant competitor cells were from SJL (CD45.1) mice and transplant recipients were progeny of SJL x C57/B6J (F1). Both sexes mice of age 2-6 months old were used in the studies. The protocol (#2016-7-781) for this work is approved by Institutional Animal Care and Use committee (IACUC) of Children's Hospital of Philadelphia (CHOP).

Genotyping

Mice were genotyped by PCR of genomic DNA from tail snips of weanling mice. Tails were digested at 55°C overnight in buffer containing 100mM Tris pH 8.0, 5mM EDTA pH 8.0, 0.2% SDS and 200mM NaCl. DNA was precipitated in propanol and washed in 70% EtOH and then resuspended in water. Mice were genotyped for Lnk (wild-type: 5'-gtccgactctctggctatgtggta-3', neo insertion: 5'- cgcatcgccttctatcgcct-3', common: 5'-gaagaggagtccatgtcatagtcc-3') or FancD2 (FancD2 2F: 5'-catgcatataggaacccgaagg-3', FancD2 2R: 5'caggacctttggagaagcag-3', V76F: 5'-cttgcaaaatggcgttacttaagc-3').

Antibodies

Antibodies used for HSC FACS and sorting were: Lineage (biotin-conjugated anti-Gr-1 (RB6-8C5), -Mac1 (M1/70), -B220 (RA3-6B2), -CD19 (eBio1D3), -Ter110 (TER-119), -

CD5 (53-7.3), -CD4 (GK1.5), -CD8 (53-6.7)), -c-kit (2B8), -Sca1 (E13-161.7 or D7) -CD48 (HM48-1), -CD150 (TC15-12F12.2), -CD34 (RAM34) and –Flk2 (A2F10.1). Peripheral blood was analyzed using anti-CD45.1 (A20), -CD45.2 (104), -CD19 (eBio1D3), -CD3e (145-2C11), -Gr-1 (RB6-8C5), and –Mac1 (M1/70). Cell cycle was assessed by Ki67 (SolA15). FACS antibodies were purchased from eBioscience, BD Biosciences or BioLegend. BrdU analog CldU was detected by anti-BrdU (Abcam BU1/75) and AF488 goat anti rat (Life Tech), and IdU by anti-BrdU (BD B44) and AF568 goat anti mouse (Life Tech). Full antibody information is listed in Supplementary Table 1.

Flow Cytometry and Cell Sorting

For peripheral blood analysis, 50μL of peripheral blood was collected by retro-orbital bleed in heparinized microcapillary tubes (Drummond 1-000-7500-HC/5). Red blood cells were lysed with ACK lysis buffer (0.8% ammonium Chloride, 10 μM EDTA, pH 7.5 with sodium bicarbonate). Cells were stained for donor reconstitution (CD45.1, CD45.2) in myeloid (Gr-1, Mac1) or lymphoid (CD19, CD3) lineages. All peripheral blood data was acquired using BD Canto flow cytometer and FACS DiVa software. At least 20k live events were recorded for each sample. APC-Cy7-conjugated anti-CD45.1, Lineage (biotin-Ter-119, -Mac-1, -Gr-1, -CD4, -CD8α, -CD5, -CD19 and -B220), and the HSPC panel: -c-kit-APC, -Sca1-PE, -CD150-PE-Cy7, -CD48-FITC, followed by staining with streptavidin-PE-TexasRed. Data for bone marrow analysis was collected on the BD Fortessa flow cytometer and FACS DiVa software. For HSPC subset analysis, CD45.1 was omitted and anti- CD34, and –Flk2 antibodies were added to the FACS panel. All flow cytometry data was analyzed using FlowJo v8.7 for MAC.

For sorting, HSPCs were pre-enriched using a lineage depletion kit (Miltenyi, 130-090-858) and magnetic separation column (Miltenyi, 130-042-401) following manufacturer's instructions. Briefly, bone marrow from individual mice was resuspended in 150µL of 2mM EDTA 0.5% BSA PBS pH 7.2 (depletion buffer) and 40µL of Lineage antibody cocktail. Cell were incubated at 4°C for 15 minutes, then washed in 10 mL of the depletion buffer. Cells were resuspended in 250µL depletion buffer and 70µL Biotin conjugated microbeads, and incubated and washed again. Cells were resuspended in 1 mL depletion buffer and passed through a 30µm nylon mesh into the separation column. Columns were washed with 3x3mL depletion buffer. LT-HSCs (SLAM LSK) were stained with anti- c-kit, -Sca-1, -CD48, and -CD150 antibodies, as described above. 150 LT-HSCs were sorted using a drop envelope of 1.25 using a BD Aria sorter into individual wells of a 96 well plate containing 100µL of StemSpan SFEM (STEMCELL Technologies, 09600) with 2% fetal bovine serum (FBS) (SAFC Biosciences, 12103C-500mL). Competitor cells were added directly to each well in 50µL of PBS and then transplanted as described above. HSPCs (LSK) for in vitro assays were pre-enriched and sorted into the same media in 1.7mL tubes using the MoFlo Astrios EQ on the "purify" setting.

Bone Marrow Transplant (BMT)

Total bone marrow or sorted LT-HSCs (CD150+CD48-c-kit+Sca1+Lin-) from donor mice were mixed with $3x10^5$ freshly isolated competitor (B6.SJL) cells and injected retro-orbitally into lethally-irradiated (10Gy, split dose, Orthovoltage Precision X-Ray) F1

recipient mice. At 4, 8, 12 and 16 weeks post transplantation, peripheral blood of recipients was analyzed for donor reconstitution in myeloid, T- and B- lineages by flow cytometry. At 16 weeks post transplant, recipients were sacrificed and donor reconstitution in various HPSC compartments was analyzed using flow cytometry. BM cells from primary transplants were harvested and 2 million cells were injected into each secondary recipient. Tertiary transplants were similarly performed.

pI:pC

Mice were injected with 5 mg/kg pI:pC (InvivoGen) i.p. twice weekly for 4 weeks. On day 28, BM cells were isolated and HSCs were quantified by FACS. One or two million total bone marrow from pI:pC treated mice was transplanted into each lethally-irradiated recipients.

Colony Forming Assay

Total BM or sorted LSK were plated onto M3434 semi-solid methylcellulose media (STEMCELL Technologies). Plates were seeded in triplicate and colonies were counted 7 days after plating. For transient drug treatment, freshly sorted LSK were plated in Mitomycin C (MMC) (Sigma) as indicated for 4 hours, then washed and plated in M3434 media and counted as described.

Liquid culture of primary HSPCs and splenic B cells

Sorted LSK were cultured in 96 well plates containing 100µL StemSpan supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies, 15140-122), 1% L-glutamine (Life Technologies, 25030-081), 100µM β-mercaptoethanol (Sigma, M7522), 10 ng/mL murine TPO (Peprotech Inc, 315-14), and 50 ng/mL murine SCF (Peprotech Inc, 250-03). Cells were seeded at a density of 100,000/mL in triplicate and maintained at less than 1 million/mL throughout culture by subculture into fresh media. Viable cells were counted using a Hemacytometer (Hausser Scientific, 1475) and viability dye Trypan Blue (STEMCELL technologies, 07050). Cell number was recorded at day 5, 7, and 14 of culture. For culture in MMC, cell culture media was supplemented with fresh MMC when cells were subcultured.

Splenic B cells were cultured in 6 well plates containing 2.5mL RPMI (Gibco, 31800-022) supplemented with 10% calf serum (HyClone, SH30072.03), 1% penicillin/streptomycin, 1% L-glutamine, 1% nonessential amino acids (Life Technologies, 11140-050), 1% sodium pyruvate (Life Technologies, 11360-070), 50 µM β-mercaptoethanol, 5 ng/mL murine IL-4 (Life Technologies, PMC0045), RP105 (BD Pharmingen, 552128), and 25 µg/mL LPS (Sigma, L4391).

Cell Cycle and Apoptosis assays

Cell cycle analysis on proliferating cells in vitro was performed using a BrdU kit (BD Biosciences, 559619). Cells were exposed to BrdU for 30 minutes and BrdU incorporation was assessed according to manufacturer's instructions. For analysis of

apoptosis, cells were incubated with anti-Annexin V antibody (Biolegend cat 556420) in Annexin V binding buffer (BD Biosciences, 556454) for 15 minutes. Cells were then stained for viability by incubation with 7-AAD (BD Biosciences, 559925). Both BrdU and AV staining data was acquired using a BD Canto flow cytometer and FACS DiVa software and analyzed using FlowJo v8.7 for MAC.

Cell cycle analysis on HSPCs from BM was performed with freshly isolated HSPCs. Lin- depleted BM cells were stained for HSPC markers, then fixed and permeabilized followed by staining with anti-Ki-67-APC antibodies. Cell cycle analysis is performed along with DAPI staining for DNA content and assessed on a BD Fortessa flow cytometer.

Metaphase spreads

Splenic B cells or LSK were cultured for 3 or 7 days respectively, and then treated with 0.5 μM Nocodazole (Calbiochem, 487928) for three hours to arrest in metaphase. Cells were incubated in 75mM potassium chloride (Sigma P9541) at 37°C for 20 minutes to swell cell volume, and subsequently fixed with 3:1 methanol: acetic Acid for 10 minutes on ice. Metaphase spreads were then prepared by dropping cells onto methanol-washed positively charged slides (Globe Scientific Inc 3591W). Slides were stained with Giemsa stain and sealed. Images of 100-150 metaphase spreads from 4-6 individual animals were captured using 100x objective, and chromosomal breaks and radial chromosomes were visually counted using FIJI software.

DNA fiber assay

DNA fiber assay was performed similarly to published protocols [17,29]. Splenic B cells or sorted LSK were cultured for 3 days then replicating DNA was labeled by a 30 minute pulse with 50μM IdU (Sigma I7125-5G) followed by a 30 minute pulse with 250μM CldU (Sigma C6891-100MG). Cells were washed then treated with HU (Sigma H8627) (4mM for spleen B cells and 2mM for BM HSPCs) for three hours. Cells were counted, and resuspended to a concentration of $2.5x10^5$/ mL in PBS, then mixed 1:1 with unlabeled cells. 2.5μL of the cell suspension was mixed with 7.5 μL of lysis buffer (200 mM TrisHCL pH 7.4, 50 mM EDTA, 0.5% SDS) directly on positively charged slides and incubated for eight minutes at room temperature. The slides were then tipped to 30° and dried. Slides were fixed in 3:1 methanol:acetic acid overnight, then rehydrated in PBS. DNA was denatured using 2.5M HCl for one hour at room temperature, followed by washes in PBS to renormalize pH. Slides were blocked in 2% BSA, 0.1% Tween 20 PBS for one hour, then incubated with primary and secondary antibody for 2.5 hours and one hour at room temperature, respectively. DNA fibers were captured using 60x objective on a Nikon Eclipse 80*i* fluorescent microscope and quantified using FIJI software.

Statistical analysis

For all cell culture and BMT experiments, two-tailed Student's t-tests were performed. Graphs are presented as mean ± SEM. For metaphase analysis and DNA fiber labeling, statistical comparisons were made using one-tailed ANOVA and Tukey test in PRISM software. The P value of less than 0.05 will be considered statistically significant.

2.4 Results

Lnk deficiency fully restores phenotypic HSCs in Fancd2$^{-/-}$ mice

Mouse models of FA recapitulate cellular DNA repair defects and impaired HSPC function. Mice deficient for FancD2, a central component of the FA signaling pathway, are reported to have an ~50% reduction in HSPCs as indicated by Lineage^{-}Kit^{+}Sca1^{+} (LSK) cells[27,30]. However, the long-term (LT-) HSCs are only about 5% of the LSK fraction. To further pinpoint the defects in various HSPC compartments, we utilized a panel of cell surface markers to differentiate HSCs from multipotent progenitors (MPPs). Our studies reveal that *Fancd2$^{-/-}$* mice on a pure C57/B6J background exhibit a 50% reduction in MPPs while the phenotypic HSCs are trending lower than wildtype (WT) **(Fig. 1)**. Notably, loss of *Lnk* fully restored phenotypic HSCs in *Fancd2$^{-/-}$* mice as defined by SLAM marker (CD150+CD48-LSK)[31], or CD34-Flk2-LSK [32] markers, or the most stringent and current markers for LT-HSCs (CD150+CD48-CD34-Flk2-LSK)[33,34] **(Fig. 1A-C, and Suppl. Fig. 1A)**. In fact, *Fancd2$^{-/-}$;Lnk$^{-/-}$* double mutant mice had more phenotypic HSCs that WT animals **(Fig. 1A-C and Suppl. Fig. 1A)**.

Lnk deficiency rescues the reconstitution defects in Fancd2$^{-/-}$ BM cells

To assess if *Lnk* deficiency rescues the functional defects in *Fancd2$^{-/-}$* BM cells, we transplanted a graded number of unfractionated BM cells into lethally-irradiated recipients. *Fancd2$^{-/-}$* BM cells showed markedly compromised reconstitution in bone marrow transplantation (BMT) assays **(Fig. 1D)** consistent with previous reports,

although the phenotypes we observed on the pure B6 background is more severe than previous reports on mixed background [27,30]. Strikingly, the reconstituting ability of *Fancd2*$^{-/-}$*Lnk*$^{-/-}$ BM cells was restored to WT levels **(Fig. 1D)**. To test the self-renewal ability of HSCs, we performed serial BMTs. *Lnk* deficiency largely restored HSC self-renewal in *Fancd2*$^{-/-}$ BM cells, albeit slightly inferior to WT cells **(Fig. 1E-G and suppl. Fig, 1B)**. *Fancd2*$^{-/-}$ BM cells had near *zero* reconstitution in the transplants, underscoring the profound functional defects of FA cells as well as the significance of the rescue by *Lnk* deficiency. Of note, none of the serially transplanted mice developed leukemia. Nor did we observe any malignancy in cohorts of *Fancd2*$^{-/-}$*;Lnk*$^{-/-}$ mice by 12 months of age (data not shown).

Lnk deficiency restores HSC functions in Fancd2*$^{-/-}$ *mice

Since *Fancd2*$^{-/-}$*;Lnk*$^{-/-}$ double mutant mice have an increased phenotypic HSC number as assessed by cell surface markers, total BM transplants might reflect the number rather than the functions of HSCs. Thus, we purified HSCs through Fluorescence- activated cytometric sorting (FACS) and injected them into irradiated host animals. Our data showed that *Fancd2*$^{-/-}$ HSCs had a cell-intrinsic defect in reconstituting the hematopoietic system, while *Lnk* deficiency rescued the HSC functional defects associated with FA **(Fig. 1H)**.

Lnk deficiency does not restore HSC quiescence in Fancd2*$^{-/-}$ *mice

We reported previously that *Lnk*$^{-/-}$ HSCs are more quiescent[22], which protects HSCs from regenerative stress. In contrast, FA-deficient HSPCs show decreased quiescence and compromised self-renewal [27,30]. It is plausible that the rescued HSC compartment in double mullizygous mice simply reflects an accumulating quiescent HSC population. To address this possibility, we quantified *in vivo* cell cycle kinetics in HSPC subsets. We found that Lnk deficiency does *not* restore HSC quiescence in *Fancd2*$^{-/-}$ mice **(Fig. 1I-J)**. Thus, the rescue of *Fancd2*$^{-/-}$ HSC homeostasis and activity is not due to protection of HSC quiescence. Rather, *Fancd2*$^{-/-}$*;Lnk*$^{-/-}$ HSCs do proliferate.

Lnk deficiency does not rescue MMC hypersensitivity in Fancd2*$^{-/-}$ *BM progenitors

The hallmark of FA in humans is the hypersensitivity to ICL-inducing reagents. This molecular feature is recapitulated in all FA mouse models. To explore the mechanisms by which *Lnk* deficiency ameliorates HSPC defects associated with FA, we first examined the MMC sensitivity of BM progenitor cells. Using both liquid culture growth assay and clonogenic survival assays, we found that both *Fancd2*$^{-/-}$*Lnk*$^{-/-}$ and *Fancd2*$^{-/-}$ BM progenitors are strongly sensitive to MMC in comparison to WT progenitors (**Fig. 2**). *Lnk*$^{-/-}$ single KO BM cells show similar MMC sensitivity to that of WT (data not shown). Thus, *Lnk* does not appear to play an overt role in ICL repair.

Lnk deficiency rescues ex vivo growth and restores genome stability of Fancd2*$^{-/-}$ *progenitors

BM progenitors from BMF syndromes show growth retardation even in the absence of DNA damage-inducing reagents [35]. Thus, we subjected BM progenitor cells from *Fancd2*$^{-/-}$ mice to liquid cultures in the presence of cytokines. Indeed, *Fancd2*$^{-/-}$ HSPCs showed progressive loss of growth ability in comparison to that of WT or *Lnk*$^{-/-}$ HSPCs **(Fig. 3A)**. Importantly, Lnk deficiency rescued the growth disadvantage of *Fancd2*$^{-/-}$ BM cells as well as colony-forming-unit (CFU) progenitors **(Fig. 3B)**. *FA* cells are observed to have a higher rate of apoptosis [36]. Indeed, we observed the percentage of apoptotic cells was doubled in *Fancd2*$^{-/-}$ culture relative to WT **(Fig. 3C)**, but the cell cycle progress remain little perturbed **(Suppl. Fig. 2)**. Importantly, the percentage of apoptotic cells in DKO was on par with WT **(Fig 3C)**, indicating that *Lnk* deficiency ameliorates the survival defect of *Fancd2*$^{-/-}$ cells.

The increased apoptosis in *Fancd2*$^{-/-}$ cells is attributable to a higher level of genome instability, and suppression of genomic instability is a major role of the FA pathway. Thus, we examined chromosomal abnormality in *ex vivo* cultured progenitors. We arrested cultured BM HSPCs in mitosis with nocodazole and subsequently prepared metaphase spreads as described [37]. At least 30 metaphases from each sample were scored for the presence of sister chromatid breaks, gaps and radial chromosome aberrations, which are hallmarks of ICL repair deficiency in FA. Chromosomal abnormalities were elevated in *Fancd2*$^{-/-}$ cells as expected, which was partially rescued by *Lnk* loss (**Fig. 3D-E**). Primary splenic B cells have been widely used to study molecular and cellular defects in FA mouse models [17]. To assess if Lnk deficiency restores genome stability in cell types other than BM HSPCs, we examined chromosomal aberrations in B cells. We found that in these cells, genome instability in

Fancd2$^{-/-}$ is also significantly elevated relative to WT, while *Lnk* deficiency restored it to WT levels **(Fig. 3E and suppl. Fig. 3)**, reinforcing our conclusion and extending the effect of Lnk deficiency to cell populations beyond HSPCs. Together these results showed that *Lnk* deficiency rescues growth and apoptotic defects and reduces genome instability in the *Fancd2* deficient cells. To our knowledge, this is the first genetic model of reduced genome instability in FA cells.

Lnk deficiency restores HSPC function upon physiological stress-induced proliferation

FA cells are known to be impaired by the damage resulting from physiological stress, such as forced proliferation induced by transplantation and by administration of polyinosinic:polycytidylic acid (pI:pC) [8]. To determine whether *Lnk* deficiency rescues *Fancd2*$^{-/-}$ HSPC function in context of endogenous replication stress, we induced HSPC proliferation *in vivo* by repeated treatment with pI:pC. We then examined phenotypic HSC by FACS and HSC functions by BMT **(Fig. 4A)**. *Fancd2*$^{-/-}$ mice exhibited a reduction in HSCs upon pIpC stress compared to WT, while *Lnk*$^{-/-}$ mice had more HSCs than WT **(Fig. 4B-C)**. Importantly, *Lnk* deficiency restored HSC numbers in *Fancd2*$^{-/-}$ mice **(Fig. 4B-C)**. Functionally, *Fancd2*$^{-/-}$ BM cells repopulated to a very limited extent **(Fig. 4D and suppl. Fig, 4)**. Remarkably, BMs from double nullizygous mice repopulated on par with WT **(Fig. 4D and suppl. Fig, 4)**, indicating robust LT-HSC activity. Taken together, these data show that *Lnk* deficiency preserves HSC function in *Fancd2*$^{-/-}$ mice upon physiological replication stress.

Lnk deficiency stabilizes replication fork upon replication stress in Fancd2$^{-/-}$ progenitors

Cells deficient in FA/BRCA are sensitive to replication poisons, such as hydroxyurea (HU) or poly(ADP-ribose) polymerase (PARP) inhibitors. To investigate a potential role of LNK in replication stress, we first treated freshly isolated HSPCs with PARP inhibitor AZD881. PARP is required for Mre11 localization to stalled forks, and its loss protects against genome instability in Brca mutant backgrounds [17,38,39]. *Fancd2*$^{-/-}$ HSPCs showed a marked reduction in CFU progenitors upon PARPi, and *Lnk* deficiency rescued progenitors' clonogenic ability in *Fancd2*$^{-/-}$ mice **(Fig. 5A)**. In contrast, *Lnk* deficiency failed to rescue clonogenic ability of *Fancd2*$^{-/-}$ HSPCs upon MMC treatment **(Fig. 5A)**. Thus, our data suggest that *Lnk* deficiency alleviates replication stress but not ICL-induced genotoxic stress associated with FA.

To examine the mechanisms by which Lnk loss ameliorates replication stress associated with FA, we treated BM progenitors with HU and examined the stabilization of stalled replication forks by single-molecule DNA fiber analysis [29]. Replication tracts of log-phase cultured HSPCs were pulse labeled with IdU and CldU sequentially before replication fork stalling by HU **(Fig. 5B)**. Single DNA fibers were spread onto microscope slides before immunofluorescence staining with antibodies against IdU and CIdU to measure relative fork length (CidU/IdU ratio). The relative shortening of the IdU tract after HU treatment serves as a measure of replication fork degradation. WT HSPCs as well as splenic B cells showed a mean IdU/CldU tract ratio close to 1 **(Fig. 5B-D)**. However, *Fancd2*-deficient cells exhibited a 30–45% reduction in the relative IdU tract length **(Fig.**

5B-C). Importantly, we discovered that loss of *Lnk* protected stalled replication fork from degradation upon replication stress in *Fancd2*$^{-/-}$ progenitors as well as B cells (**Fig. 5B-C**). Furthermore, we demonstrated that Lnk deficiency conferred resistance to HU in clonogenic survival of HSPCs **(Fig. 5G)**.

Cytokine-JAK2 signaling is important in stabilizing stalled replication forks

Since LNK negatively regulates the cytokine receptor-associated JAK2 kinase, we next asked whether its role in fork protection was cytokine signaling-dependent. Since BM HSPCs dependent upon cytokines for their survival in culture, we were unable to withdraw cytokines from BM culture. By removing IL-4 from the splenic B cell culture, it is possible to evaluate the role of cytokine-JAK signaling in isolation while still providing mitogenic stimuli to proliferating B cells through Toll-like receptor signaling. In these conditions, *Lnk* deficiency failed to rescue *Fancd2*- null B cells from replication degradation upon HU stress **(Fig. 5E)**, suggesting that cytokine/JAK signaling plays a role in replication fork stabilization.

We next attempted to decipher the signaling pathways that contribute to replication fork stability in *Lnk* null cells. LNK is a cytoplasmic adaptor protein that is not known to associate with chromatin. We previously reported that *Lnk* deficiency potentiates JAK2 activation in HSPCs, which in part accounts for its role in HSC self-renewal[22-24,28]. To define the role of JAK2 in replication fork stability, we subjected WT and *Lnk*$^{-/-}$ HSPCs to JAK inhibitors (Ruxolitinib) and examined its effects on replication fork stability. We found that WT HSPCs were sensitive to JAKi in stabilizing stalled replication fork, while

Lnk$^{-/-}$ HSPCs were more resistant to JAKi **(Fig. 5F)**. Furthermore, MRE11 inhibitor Mirin was able to rescue replication fork degradation in WT HPSCs upon JAK inhibition. Our data suggest that JAK signaling contributes to replication fork stabilization in HSPCs through MRE11-mediated nuclease activity.

2.5 Discussion

This report demonstrated that loss of *Lnk* restores FA HSPC functions by alleviating replication stress. To our knowledge, these findings represent a rare *in vivo* example of genetic suppression of FA-associated HSPC defects. There are very few examples of animal models in which there are elevated HSC numbers and function[40]. Understanding how Lnk deficiency imparts these remarkable HSC properties will likely advance our understanding of stem cell biology and offer insights into strategies for the treatment of FA and other BMF diseases in general.

We show that Lnk deficiency increases phenotypic and functional HSCs in *Fancd2*$^{-/-}$ mice. It has been showed recently forced proliferation in vivo by repeated pIpC treatment leads to HSC exhaustion and BMF in FA mice. We found that Lnk deficiency mitigates HSC exhaustion upon physiological stress. Our *ex vivo* growth results are also consistent with the *in vivo* BMT data, as reconstitution of myelo-ablated animals is a form of "forced" proliferation. Furthermore, we showed that *Lnk* deficiency does not protect HSC quiescence in FA, rather it allows *Fancd2*$^{-/-}$ HSPCs to proliferate and self-renew. Previous work demonstrated that p53 activation contributes to the HSPC decline and BMF in FA[9]. However, p53 levels remain unchanged and p53 signaling remains intact in *Lnk*$^{-/-}$ cells (data not shown), suggesting that the rescue of FA by Lnk deficiency cannot be attributed to p53 inhibition. While the survival of *Fancd2*$^{-/-}$ cells can be improved by inactivation of apoptotic signaling with deletion of p53 [9] or inflammatory signaling with inactivation of TNFa signaling[41], the genomic instability in *Fancd2*$^{-/-}$ cells has not been demonstrably suppressed by these pathways. We showed here that *Lnk*

deficiency mitigates replication stress-incurred chromosomal aberrations in FA mice and prevents HSPCs attrition without incurring leukemic transformation. Understanding mechanisms by which *Lnk* deficiency suppresses FA phenotypes will facilitate development of strategies that have the dual benefit of ameliorating BMF and simultaneously reducing genome instability.

Our studies raise the question on the cause of stem cell attrition and BMF in FA patients. It has been suggested that endogenous aldehydes, in the forms of acetaldehydes from cellular lipid peroxidation and formaldehydes from histone, DNA, and RNA demethylation, leads to ICL DNA damage [6,42]. FA patients and mice are thus more sensitive to deficiencies in the aldehyde detoxification enzymes, such as Aldh2 and Adh5 [6,42]. We found that deficiency in Lnk protects stalled replication forks from degradation and rescues the survival and stabilizes genome integrity of *Fancd2*$^{-/-}$ HSPCs without restoring ICL-induced DNA repair. Therefore, our results imply that replication stress may be a more important mechanism for HSC attrition and BMF in FA than previously appreciated. *Lnk* deficiency increases stem cell fitness and alleviates HU-incurred replication stress. This translates to a superior HSC self-renewal and serial transplantability upon forced proliferation in vivo or in vitro. We speculate this DNA repair-independent mechanism is a more probable cause of HSC exhaustion in FA, which is illustrated by the rescue of *Lnk* deficiency in mitigating replication stress.

We show that *Lnk*$^{-/-}$ HSPCs are hypersensitive to MMC-induce DNA damage, indicating that LNK does not play an overt role in ICL repair. A more recently described role for FA

proteins is in replication fork stability [15]. The FA/BRCA pathway is recruited to stalled replication forks and is essential in preventing fork destabilization and resection by Mre11 nuclease [15,16]. Without fork stabilization by RAD51, Mre11 degrades stalled replication forks, results in double stranded breaks, which can lead to chromosomal rearrangements and genome instability. It has been shown recently that protection of nascent DNA from degradation provides a mechanism that can promote synthetic viability in *Brca*-deficient cells and causes drug resistance in *Brca*-deficient cancers without restoring HR at DSBs [17]. These results suggest that defects in replication fork stabilization upon replication stress rather than DNA repair deficiency in FA/BRCA mutated tumors contribute to genome instability and transformation to cancer. We found that *Lnk* deficiency rescues fork stability and genome instability in B cells in addition to BM HSPCs, suggesting a potential broad role for LNK in replication and perhaps that outside the hematopoietic system.

LNK is a cytoplasmic adaptor protein, which is not known to associate with chromatin and all known associated proteins are cytoplasmic. Moreover, a recent large-scale proteome effort identified hundreds of proteins enriched in nascent chromatin, however, LNK was not among them[43]. Given LNK's role in limiting cytokine signaling, we hypothesize that LNK regulates the activity of kinase cascades that transmit their signals to the nucleus to affect the expression or activity of DNA replication and repair proteins. Indeed, our results highlight the importance of cytokine-mediated JAK2 signaling in replication stress. This is the first example, to our knowledge, that cytokine/JAK signaling plays a role in promoting replication fork stability. Future efforts are warranted to

elucidate the mechanisms by which LNK-regulated JAK signaling in replication stress alleviation.

Taken together, *Lnk* deficiency mitigated replication stress-incurred DNA damage in FA mice and prevented HSPCs attrition without incurring leukemic transformation. Understanding mechanisms by which *Lnk* deficiency suppresses FA phenotypes will facilitate development of strategies that have the dual benefit of ameliorating BMF and simultaneously reducing genome instability. Our research sheds light on the underlying the origin of BMF in FA patients and has implications for new therapeutic strategies.

AUTHOR CONTRIBUTIONS

JB, JJ, QJ, WT and RAG designed experiments and interpreted results. JB, WT and RAG wrote and edited the manuscript. JB, JJ, QJ, WZ performed experiments.

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2.6 References

1 Jordan, C. T., McKearn, J. P. & Lemischka, I. R. Cellular and developmental properties of fetal hematopoietic stem cells. *Cell* **61**, 953-963 (1990).

2 Morrison, S. J., Uchida, N. & Weissman, I. L. The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol* **11**, 35-71 (1995).

3 Parmar, K., D'Andrea, A. & Niedernhofer, L. J. Mouse models of Fanconi anemia. *Mutat Res* **668**, 133-140, doi:10.1016/j.mrfmmm.2009.03.015 (2009).

4 Kee, Y. & D'Andrea, A. D. Molecular pathogenesis and clinical management of Fanconi anemia. *J Clin Invest* **122**, 3799-3806, doi:10.1172/JCI58321 (2012).

5 Kennedy, R. D. & D'Andrea, A. D. The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes Dev* **19**, 2925-2940, doi:10.1101/gad.1370505 (2005).

6 Garaycoechea, J. I. *et al.* Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. *Nature* **489**, 571-575, doi:10.1038/nature11368 (2012).

7 Langevin, F., Crossan, G. P., Rosado, I. V., Arends, M. J. & Patel, K. J. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. *Nature* **475**, 53-58, doi:10.1038/nature10192 (2011).

8 Walter, D. *et al.* Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. *Nature* **520**, 549-552, doi:10.1038/nature14131 (2015).

9 Ceccaldi, R. *et al.* Bone marrow failure in fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. *Cell Stem Cell* **11**, 36-49, doi:S1934-5909(12)00247-0 [pii]
10.1016/j.stem.2012.05.013 (2012).

10 Haneline, L. S. *et al.* Multiple inhibitory cytokines induce deregulated progenitor growth and apoptosis in hematopoietic cells from Fac-/- mice. *Blood* **91**, 4092-4098 (1998).

11 Li, J. *et al.* TNF-alpha induces leukemic clonal evolution ex vivo in Fanconi anemia group C murine stem cells. *J Clin Invest* **117**, 3283-3295, doi:10.1172/JCI31772 (2007).

12 Du, W., Adam, Z., Rani, R., Zhang, X. & Pang, Q. Oxidative stress in Fanconi anemia hematopoiesis and disease progression. *Antioxid Redox Signal* **10**, 1909-1921, doi:10.1089/ars.2008.2129 (2008).

13 Zhang, H. *et al.* TGF-b Inhibition Rescues Hematopoietic Stem Cell Defects and Bone Marrow Failure in Fanconi Anemia. *Cell Stem Cell*, doi:10.1016/j.stem.2016.03.002 (2016).

14 Moldovan, G. L. & D'Andrea, A. D. How the fanconi anemia pathway guards the genome. *Annu Rev Genet* **43**, 223-249, doi:10.1146/annurev-genet-102108-134222 (2009).

15 Schlacher, K., Wu, H. & Jasin, M. A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. *Cancer Cell* **22**, 106-116, doi:10.1016/j.ccr.2012.05.015 (2012).

16 Schlacher, K. *et al.* Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. *Cell* **145**, 529-542, doi:10.1016/j.cell.2011.03.041 (2011).

17 Ray Chaudhuri, A. *et al.* Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* **535**, 382-387, doi:10.1038/nature18325 (2016).
18 Kaushansky, K. Thrombopoietin: a tool for understanding thrombopoiesis. *J Thromb Haemost* **1**, 1587-1592 (2003).
19 Kimura, S., Roberts, A. W., Metcalf, D. & Alexander, W. S. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci U S A* **95**, 1195-1200 (1998).
20 Solar, G. P. *et al.* Role of c-mpl in early hematopoiesis. *Blood* **92**, 4-10 (1998).
21 Ballmaier, M. *et al.* c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. *Blood* **97**, 139-146 (2001).
22 Bersenev, A., Wu, C., Balcerek, J. & Tong, W. Lnk controls mouse hematopoietic stem cell self-renewal and quiescence through direct interactions with JAK2. *J Clin Invest* **118**, 2832-2844, doi:10.1172/JCI35808 (2008).
23 Seita, J. *et al.* Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. *Proc Natl Acad Sci U S A* **104**, 2349-2354 (2007).
24 Buza-Vidas, N. *et al.* Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. *Genes Dev* **20**, 2018-2023 (2006).
25 Ema, H. *et al.* Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. *Dev Cell* **8**, 907-914 (2005).
26 Bersenev, A. *et al.* Lnk deficiency partially mitigates hematopoietic stem cell aging. *Aging Cell* **11**, 949-959, doi:10.1111/j.1474-9726.2012.00862.x (2012).
27 Parmar, K. *et al.* Hematopoietic stem cell defects in mice with deficiency of Fancd2 or Usp1. *Stem Cells* **28**, 1186-1195, doi:10.1002/stem.437 (2010).
28 Velazquez, L. *et al.* Cytokine signaling and hematopoietic homeostasis are disrupted in Lnk-deficient mice. *J Exp Med* **195**, 1599-1611 (2002).
29 Jiang, Q. *et al.* MERIT40 cooperates with BRCA2 to resolve DNA interstrand cross-links. *Genes Dev* **29**, 1955-1968, doi:10.1101/gad.264192.115 (2015).
30 Zhang, Q. S. *et al.* Fancd2-/- mice have hematopoietic defects that can be partially corrected by resveratrol. *Blood* **116**, 5140-5148, doi:10.1182/blood-2010-04-278226 (2010).
31 Kiel, M. J., Yilmaz, O. H., Iwashita, T., Terhorst, C. & Morrison, S. J. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121 (2005).
32 Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242-245 (1996).
33 Wilson, A. *et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* **135**, 1118-1129, doi:10.1016/j.cell.2008.10.048 (2008).
34 Foudi, A. *et al.* Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nature biotechnology* **27**, 84-90, doi:10.1038/nbt.1517 (2009).
35 Rio, P. *et al.* In vivo proliferation advantage of genetically corrected hematopoietic stem cells in a mouse model of Fanconi anemia FA-D1. *Blood* **112**, 4853-4861, doi:10.1182/blood-2008-05-156356 (2008).
36 Ridet, A. *et al.* Deregulated apoptosis is a hallmark of the Fanconi anemia syndrome. *Cancer Res* **57**, 1722-1730 (1997).

37 Tang, J. *et al.* Acetylation limits 53BP1 association with damaged chromatin to promote homologous recombination. *Nat Struct Mol Biol* **20**, 317-325, doi:10.1038/nsmb.2499 (2013).
38 Ding, X. *et al.* Synthetic viability by BRCA2 and PARP1/ARTD1 deficiencies. *Nat Commun* **7**, 12425, doi:10.1038/ncomms12425 (2016).
39 Bryant, H. E. *et al.* PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. *EMBO J* **28**, 2601-2615, doi:10.1038/emboj.2009.206 (2009).
40 Rossi, L. *et al.* Less Is More: Unveiling the Functional Core of Hematopoietic Stem Cells through Knockout Mice. *Cell Stem Cell* **11**, 302-317, doi:S1934-5909(12)00484-5 [pii]
10.1016/j.stem.2012.08.006 (2012).
41 Zhang, H. *et al.* TGF-beta Inhibition Rescues Hematopoietic Stem Cell Defects and Bone Marrow Failure in Fanconi Anemia. *Cell Stem Cell* **18**, 668-681, doi:10.1016/j.stem.2016.03.002 (2016).
42 Pontel, L. B. *et al.* Endogenous Formaldehyde Is a Hematopoietic Stem Cell Genotoxin and Metabolic Carcinogen. *Mol Cell*, doi:10.1016/j.molcel.2015.08.020 (2015).
43 Alabert, C. *et al.* Nascent chromatin capture proteomics determines chromatin dynamics during DNA replication and identifies unknown fork components. *Nat Cell Biol* **16**, 281-293, doi:10.1038/ncb2918 (2014).

2.7 Figures

Figure 2.1

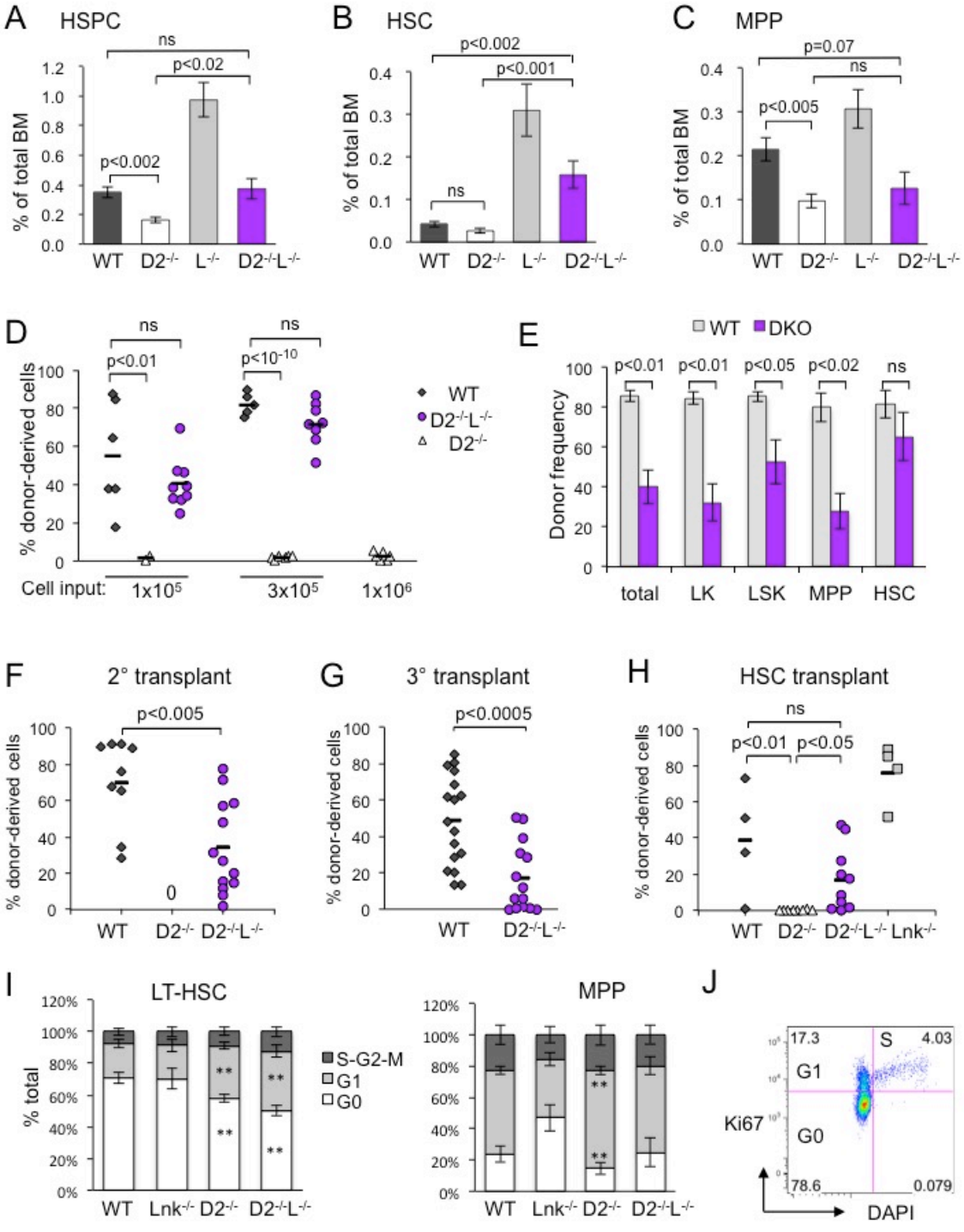


Figure 2.2

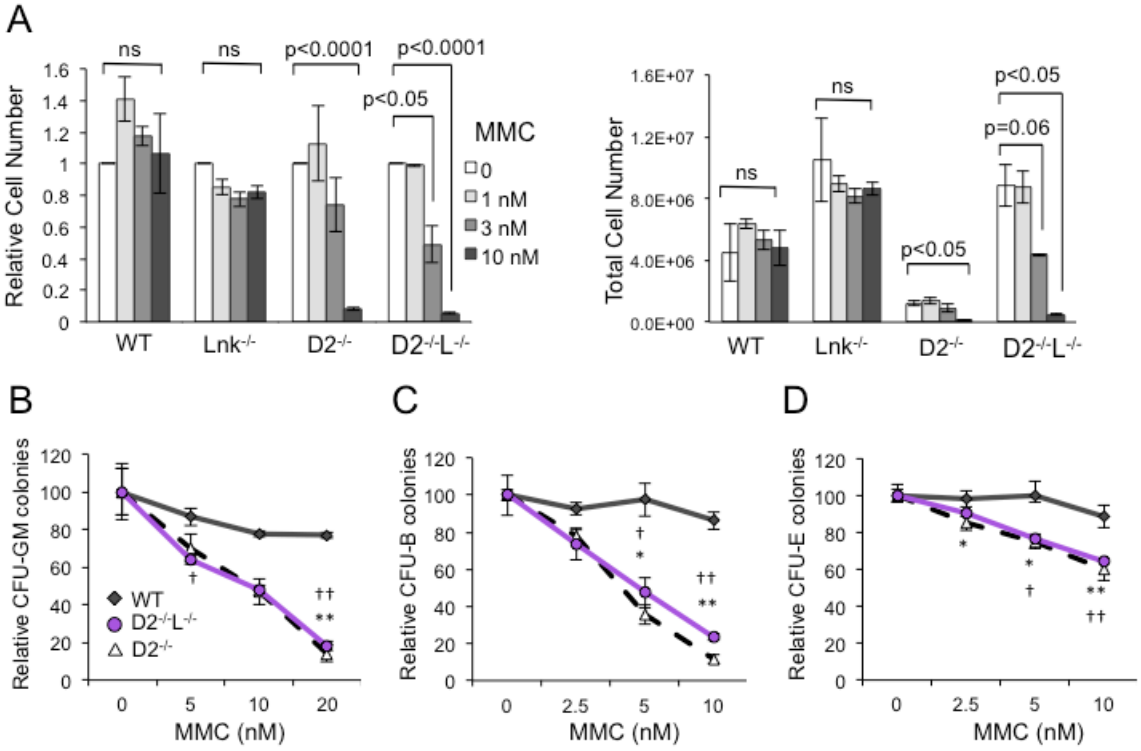

A
Relative Cell Number
ns
ns
p<0.0001
p<0.0001
p<0.05
MMC
0
1 nM
3 nM
10 nM
WT
Lnk-/-
D2-/-
D2-/-L-/-
Total Cell Number
ns
p<0.05
p=0.06
p<0.05
B
Relative CFU-GM colonies
WT
D2-/-L-/-
D2-/-
MMC (nM)
C
Relative CFU-B colonies
MMC (nM)
D
Relative CFU-E colonies
MMC (nM)

Figure 2.3

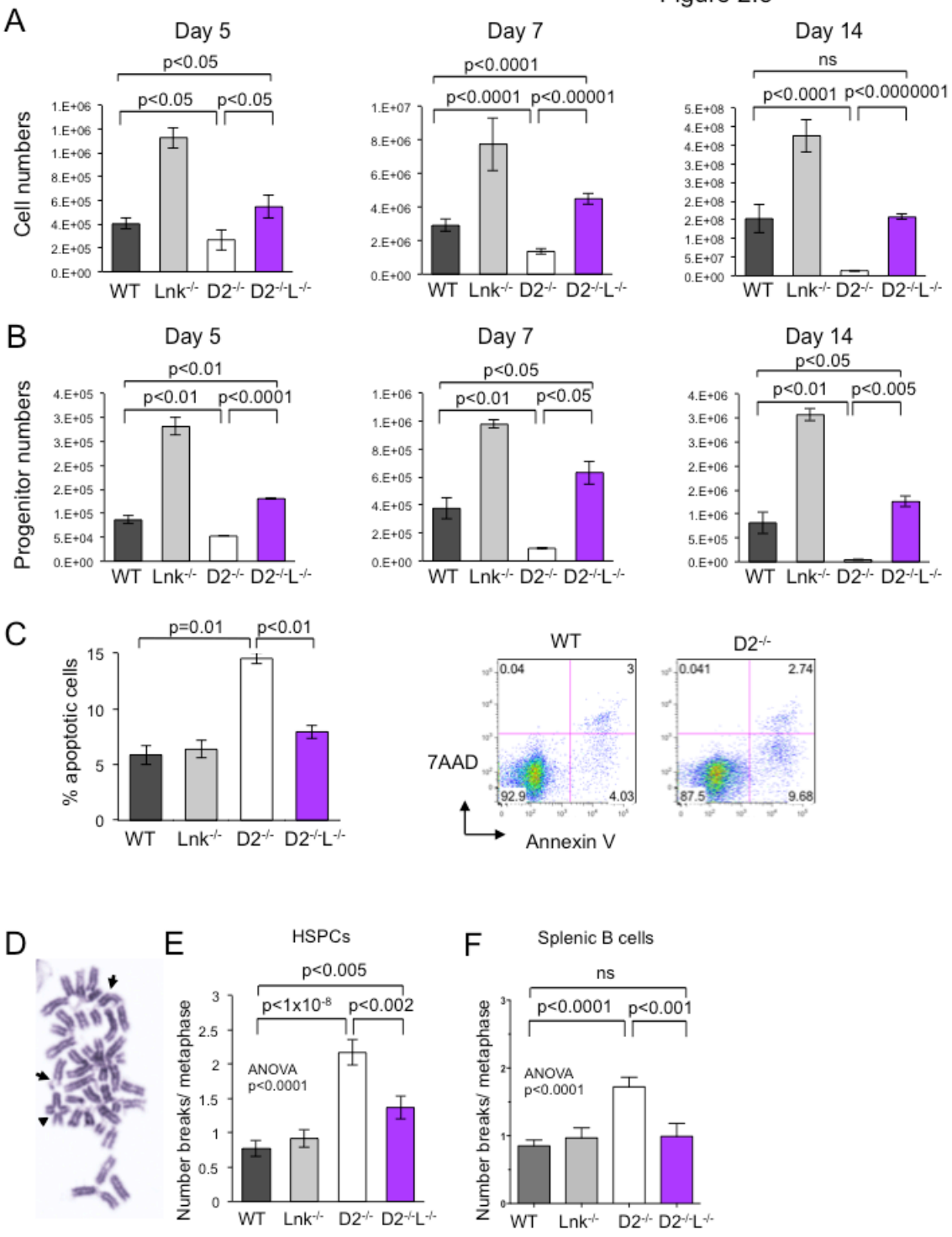

A
Day 5
Day 7
Day 14
Cell numbers
p<0.05
p<0.05 p<0.05
p<0.0001
p<0.0001 p<0.00001
ns
p<0.0001 p<0.0000001
WT Lnk-/- D2-/- D2-/-L-/-
B
Day 5
Day 7
Day 14
Progenitor numbers
p<0.01
p<0.01 p<0.0001
p<0.05
p<0.01 p<0.05
p<0.05
p<0.01 p<0.005
C
% apoptotic cells
p=0.01 p<0.01
WT
D2-/-
7AAD
Annexin V
0.04
3
92.9
4.03
0.041
2.74
87.5
9.68
D
E
HSPCs
Number breaks/ metaphase
p<0.005
p<1x10-8 p<0.002
ANOVA p<0.0001
F
Splenic B cells
ns
p<0.0001 p<0.001
ANOVA p<0.0001

Figure 2.4

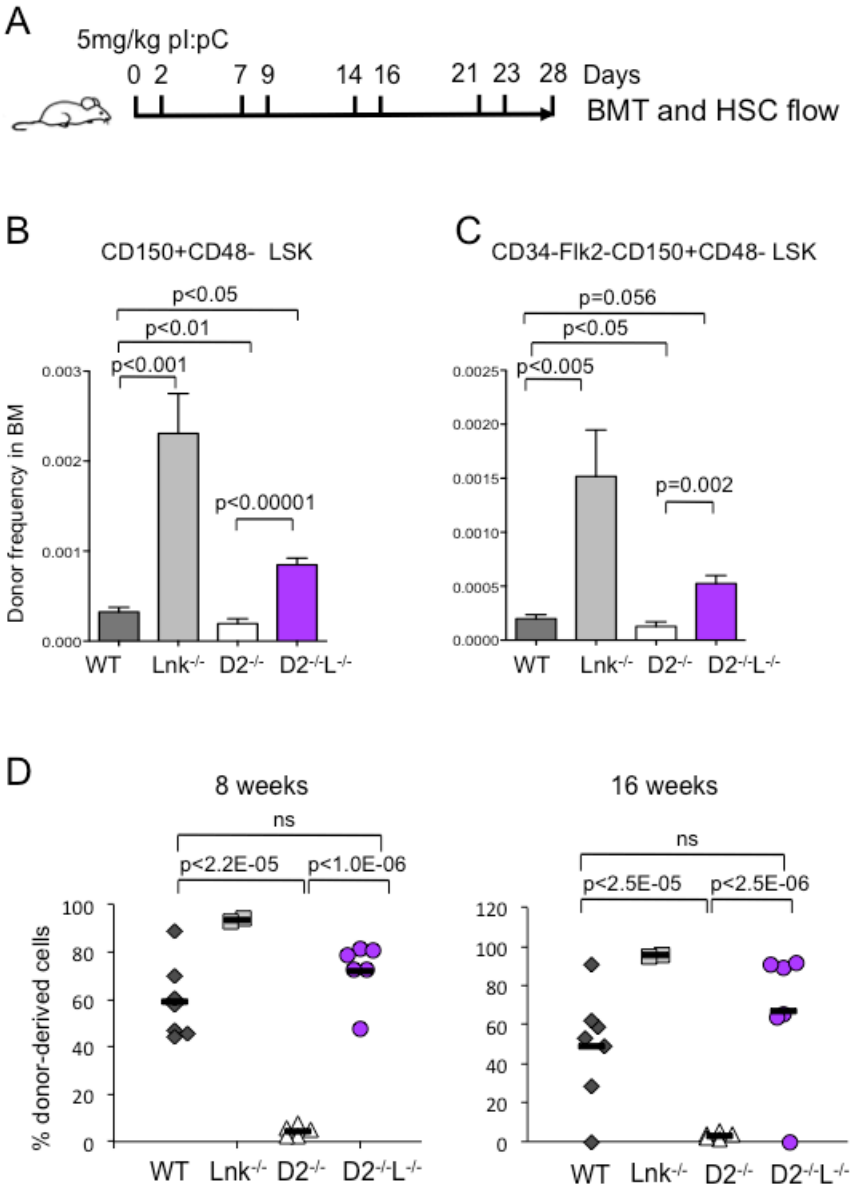

A
5mg/kg pI:pC
0 2 7 9 14 16 21 23 28 Days
BMT and HSC flow
B
CD150+CD48- LSK
p<0.05
p<0.01
p<0.001
p<0.00001
Donor frequency in BM
0.003
0.002
0.001
0.000
WT Lnk-/- D2-/- D2-/-L-/-
C
CD34-Flk2-CD150+CD48- LSK
p=0.056
p<0.05
p<0.005
p=0.002
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
WT Lnk-/- D2-/- D2-/-L-/-
D
8 weeks
ns
p<2.2E-05
p<1.0E-06
% donor-derived cells
100
80
60
40
20
0
WT Lnk-/- D2-/- D2-/-L-/-
16 weeks
ns
p<2.5E-05
p<2.5E-06
120
100
80
60
40
20
0
WT Lnk-/- D2-/- D2-/-L-/-

Figure 2.5

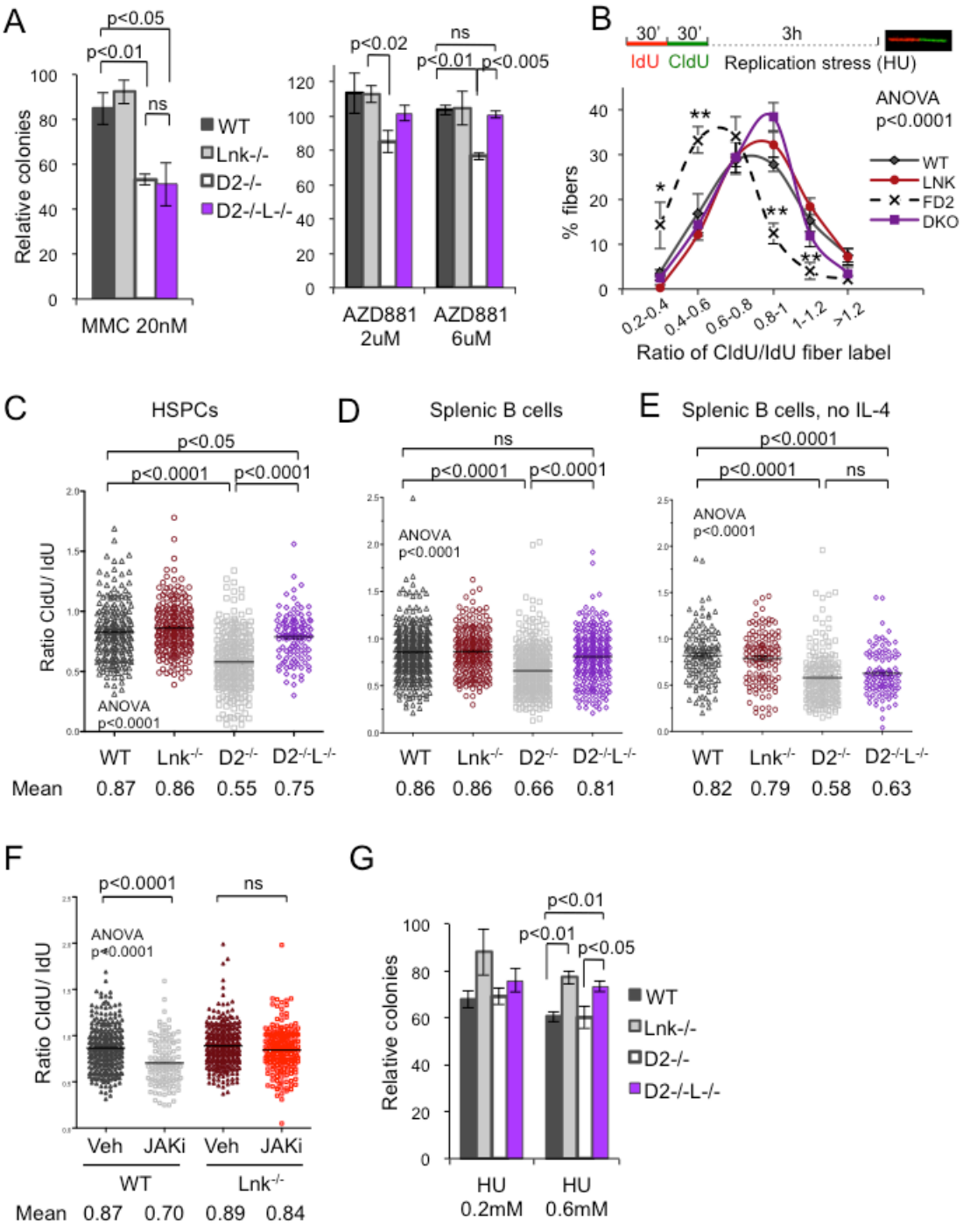
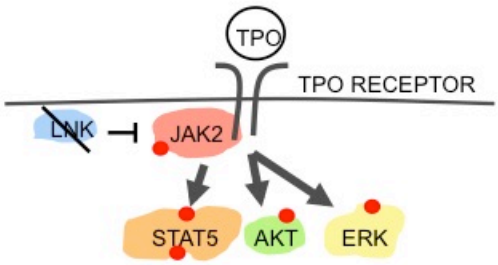
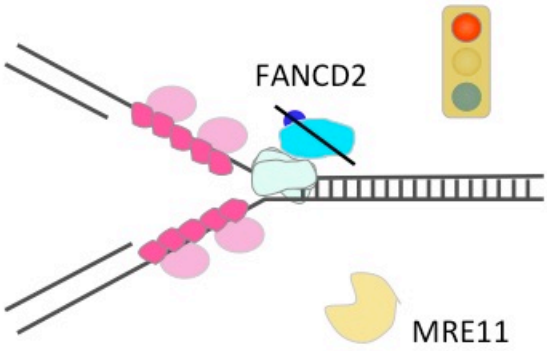

A
p<0.05
p<0.01
ns
Relative colonies
WT
Lnk-/-
D2-/-
D2-/-L-/-
MMC 20nM
p<0.02
ns
p<0.01
p<0.005
AZD881 2uM
AZD881 6uM
B
30'
30'
3h
IdU
CldU
Replication stress (HU)
ANOVA p<0.0001
% fibers
WT
LNK
FD2
DKO
0.2-0.4
0.4-0.6
0.6-0.8
0.8-1
1-1.2
>1.2
Ratio of CldU/IdU fiber label
C
HSPCs
p<0.05
p<0.0001
p<0.0001
Ratio CldU/ IdU
ANOVA p<0.0001
WT
Lnk-/-
D2-/-
D2-/-L-/-
Mean
0.87
0.86
0.55
0.75
D
Splenic B cells
ns
p<0.0001
p<0.0001
ANOVA p<0.0001
WT
Lnk-/-
D2-/-
D2-/-L-/-
0.86
0.86
0.66
0.81
E
Splenic B cells, no IL-4
p<0.0001
p<0.0001
ns
ANOVA p<0.0001
WT
Lnk-/-
D2-/-
D2-/-L-/-
0.82
0.79
0.58
0.63
F
p<0.0001
ns
ANOVA p<0.0001
Ratio CldU/ IdU
Veh
JAKi
Veh
JAKi
WT
Lnk-/-
Mean
0.87
0.70
0.89
0.84
G
p<0.01
p<0.01
p<0.05
Relative colonies
WT
Lnk-/-
D2-/-
D2-/-L-/-
HU 0.2mM
HU 0.6mM

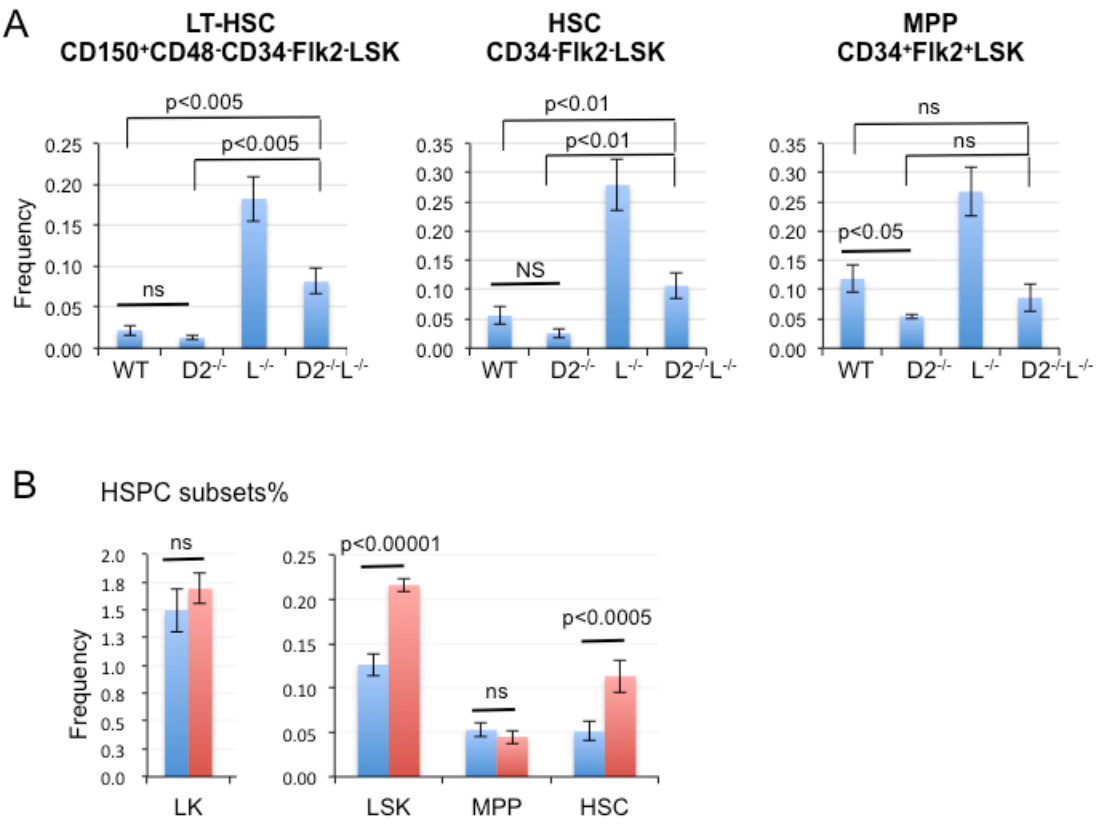
Figure 2.6



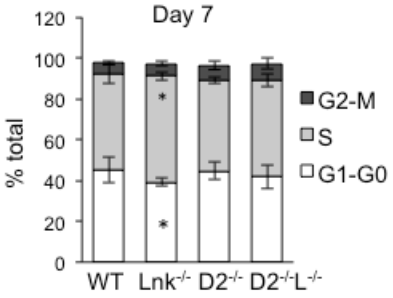
1. Rescues replication fork stability
2. Mitigates genomic instability
3. Rescues HSC function



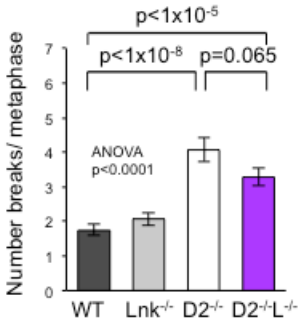
Supplemental Figure 2.1



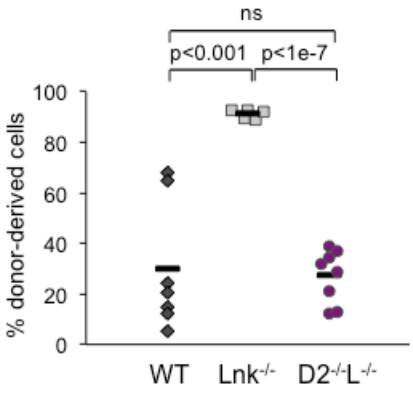
Supplemental Figure 2.2



Supplemental Figure 2.3



Supplemental Figure 2.4



Supplementary Table 1

		supplier	clone	conjugation	Cat #
Lineage	Gr1	eBio	RB6-8C5	biotin	13-5931-86
	Mac1	eBio	M1/70	biotin	13-0112-86
	B220	eBio	RA3-6B2	biotin	13-0452-86
	CD19	eBio	eBio1D3	biotin	13-0193-86
	Ter119	eBio	TER-119	biotin	13-5921-85
	CD5	eBio	53-7.3	biotin	13-0051-85
	CD4	eBio	GK1.5	biotin	13-0041-86
	CD8	eBio	53-6.7	biotin	13-0081-86
	Streptavidin	Life Technologies		PE-TexasRed	SA1017
HSC FACS and sorting	c-kit	eBio	2B8	APC	17-1171-83
	c-kit	eBio	2B8	APC-Cy7	47-1171-82
	Sca1	eBio	D7	Per-CP-Cy5.5	45-5981-82
	Sca1	BD Bioscienes	E13-161.7	PE	553336
	CD48	BioLegend	HM48-1	FITC	103404
	CD150	BioLegend	TC15-12F12.2	PE-Cy7	115914
	CD34	eBio	RAM34	eFluor 660	50-0341-82
	Flk2	BD	A2F10.1	PE	553842
Peripheral Blood FACS	CD45.1	eBio	A20	PE-Cy7	25-0453-82
	CD45.2	BD	104	FITC	553772
	CD19	eBio	eBio1D3	APC	17-0193-82
	CD3	eBio	145-2C11	PE	12-0031-85
	Gr1	eBio	RB6-8C5	PE	12-5931-85
	Mac1	eBio	M1/70	APC	17-0112-83
Ki67	Ki-67	eBio	SolA15	eFluor 660	50-5698-80

		supplier	clone	host	Cat #	dilution
DNA fibers	BrdU (CldU)	abcam	BU1/75	rat	ab6326	1:100
	BrdU (IdU)	BD	B44	mouse	347580	1:100
	AF488 anti rat	Life Tech		goat	A-11006	1:200
	AF568 anti mouse	Life Tech		goat	A-11031	1:200

2.8 Figure legends

Figure 2.1: Lnk deficiency restores phenotypic and functional HSCs in *FancD2*$^{-/-}$ mice.

(**A-C**) Quantification of various HSPC compartments by flow cytometry, Lin-kit+Sca1+ (LSK) HSPC population (**A**), LT-HSCs (CD48+CD150-CD34-LSK) (**B**) and MPP (CD48+CD150-LSK) (**C**) in WT, FancD2$^{-/-}$ (D2$^{-/-}$), Lnk$^{-/-}$ (L$^{-/-}$) and FancD2$^{-/-}$;Lnk$^{-/-}$ (D2$^{-/-}$L$^{-/-}$) mice. **(D-G)** show serial transplantation of total bone marrow cells from WT, D2$^{-/-}$ and D2$^{-/-}$L$^{-/-}$ mice. A graded number of BM cells were mixed with competitors and transplanted into lethally-irradiated host animals. (**D**) Donor chimerisms in the peripheral blood of the recipient mice 16 weeks after transplant are shown. (**E**) At the end of primary transplant. Donor frequency in the host BM and various HSPC populations were quantified by flow cytometry. The 3x10^5 group was transplanted into secondary and tertiary recipients. Donor chimerism in peripheral blood after secondary transplant (**F**) and tertiary transplant (**G**) are shown. (**H**) 100 purified SLAM LSK HSCs were mixed with 3x10^5 competitors and transplanted into lethally-irradiated recipients. Donor chimerisms in peripheral blood 16 weeks after transplant are shown. Each symbol represents an individual mouse; horizontal lines indicate mean frequencies; error bars indicate SE. p values determined by two-tailed student's t-test are shown. ns: not significant. **(I-J)** shows quantifications of cell cycle analysis of LT-HSCs (**D**) and MPPs (**E**) in mice of different genotypes. **: $p<0.01$ compare to WT, two-tailed student's t-test.

Figure 2.2: Lnk deficiency does not rescue ICL hypersensitivity associated with FancD2 deficiency. (**A**) HSPCs were cultured in different concentrations of MMC for 7

days. Total (right) and relative cell numbers to untreated cells (left) are shown. Statistical analysis using two-tailed student's t-test of comparisons among untreated and MMC-treated cells within each genotype are indicated. (**B-D**) Fresh BM cells were plated in semi-solid methylcellulose media containing various concentrations of MMC and assessed for progenitors of myeloid (**B**), B cell (**C**), and erythroid lineages (**D**). Relative colony forming units relative to untreated are shown. * $p<0.05$, ** $p<0.01$, two-tailed student's t-test, denotes comparison between WT and D2; † $p<0.05$, †† $p<0.01$, denotes WT vs DKO.

Figure 2.3: Lnk deficiency rescues the growth and survival defect of FancD2$^{-/-}$ HSPCs and mitigates genome instability. (**A-B**) HSPCs were cultured in TPO and SCF containing media for 14 days. (**A**) Cumulative cell growth at days 5, 7 and 14 is shown. (**B**) At each corresponding day of culture, cells were plated for quantification of colony-forming progenitors. Cumulative progenitor cell numbers were calculated and plotted. P values indicate two-tailed student's t-test. (**C**) On day 7, percentage of apoptotic cells was assessed by Annexin V staining and shown. For panels A-C one representative experiment of three independent replicates is shown. (**D-F**) HSPCs cultured 7 days (**E**) or splenic B cells cultured 3 days (**F**) in cytokines were assessed for chromosomal aberrations. (**D**) shows examples of chromosomal breaks (arrows) and radial chromosomes (arrowhead) scored on metaphase spreads. Quantifications of mean aberrations for 100-150 metaphase spreads from 4-6 animals are shown, and error bars indicate SE. Comparisons among all 4 genotypes were calculated by one-way ANOVA are shown for each graph. p-values using Tukey's t-test for each indicated comparison pair are shown.

Figure 2.4: Lnk deficiency rescues HSC defects associated with FA upon forced *in vivo* proliferation. Proliferation of HSPCs *in vivo* was induced by pI:pC injection. (**A**) depicts a schematic overview of pI:pC injection. Mice were injected i.p. with 5mg/kg pI:pC twice per week over four weeks, followed by analysis for HSPC frequency and function in bone marrow at day 28. LT-HSC frequency determined by SLAM LSK (**B**) or CD34-Flk2-SLAM LSK (**C**) marker schemes is shown. Bars indicate mean of frequencies from four to six mice, and error bars indicate SE. Statistics were calculated by two-tailed student's t test. (**D**) Two million total BM cells from treated mice were mixed with $3x10^5$ competitors and transplanted into lethally-irradiated host animals. Donor chimerism in peripheral blood of recipients 16 weeks after transplant is shown. Each symbols represents an individual recipient animal, horizontal bars represent the mean of each group. p values from two-tailed students' t-test are shown.

Figure 2.5: Lnk deficiency stabilizes stalled replication forks in FancD2$^{-/-}$ HSPCs through cytokine-JAK2 signaling. (**A**) Freshly-isolated HSPCs were treated for 4 hours with MMC or AZD881 (PARP inhibitor), and subsequently plated in semi-solid methylcellulose media. Colony forming progenitor numbers relative to the vehicle-treated group (mean± SE) were enumerated and graphed. ** $p<0.01$, two-tailed student's t-test. (**B**) The top panel shows the experimental overview of the fork protection assay. **(B-C)** Bone marrow HSPCs were subjected to fork protection assay. The frequencies of different replication tract ratios are plotted **(B)**. **(C)** The distribution of CldU/IdU fiber ratios is shown with the horizontal lines indicating median fiber ratios, and the medians

shown at the bottom of the graph. (**D-E**) B cells were cultured in RP-105 and LPS with **(d)** or without IL-4 **(E),** and the median of fiber ratios are shown. (**F**) HSPCs were cultured in the presence of 1μM JAK2 inhibitor ruxolitinib (JAKi) or vehicle alone (Veh) and the distributions of fiber ratios are shown. In panels (**B-F**) each individual symbol represents one CldU/IdU labeled fiber and the horizontal line indicates the mean of each group. The mean of each distribution is written under each plot. Statistical significance of each set of conditions was calculated using one-way ANOVA and comparisons between individual groups were calculated using Tukey's t-test. (**G**) Freshly-isolated HSPCs were treated for 4 hours with HU and colony forming potential was assessed. Representative of 4 independent experiments are shown. Statistics were calculated using two-tailed student's t-test.

Figure 2.6: Model. TPO signaling impacts FANCD2 function at stalled replication forks.

Supplemental Figures:

Figure S1: *Lnk* deficiency restores phenotypic HSCs but not MPPs in *Fancd2*$^{-/-}$ mice. (A) Quantification of various HSPC subsets by flow cytometry using the CD34 and Flk2 surface marker scheme: LT-HSCs (CD150+CD48-CD34-Flk2-LSK), HSCs (CD34-Flk2-LSK) and MPP (CD34+Flk2+LSK). (B) Quantification of various HSPC subsets in BM of transplanted mice by flow cytometry. P values are calculated using two-tailed student's t-test.

Figure S2: *Lnk* deficiency does significantly alter cell cycle status in *Fancd2*$^{-/-}$ HSPCs in a short-term ex vivo cell culture. Quantification of cell cycle status in HSPCs using Ki67 and DAPI after seven days in culture. Bars indicate mean and error bars indicate SE. *: $p<0.05$ compared to WT, student's t-test.

Figure S3: *Lnk* deficiency partially mitigates genome instability after long-term culture in *Fancd2*$^{-/-}$ HSPCs. Quantification of chromosomal aberrations by metaphase spread in HSPCs after 10 days of culture. Results from 100-150 metaphase spreads of 4-6 animals are shown. Bars indicate mean and error bars indicate SE. p-values using student's t-test for each indicated comparison pair are shown. Comparisons among all 4 genotypes calculated by one-way ANOVA is also shown.

Figure S4: Lnk deficiency restores *Fancd2*$^{-/-}$ HSPCs function after forced in vivo cell proliferation. Peripheral blood reconstitution by donor derived cells 16 weeks after transplant of one million total BM from pI:pC –treated mice is shown. Each symbols represents an individual recipient animal, horizontal bars represent the mean of each group. p values from two-tailed students' t-test are shown.

CHAPTER 3

Investigation of *in vivo* dynamics of self-renewal in murine *Lnk-/-* Hematopoietic Stem Cells

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3.1 Abstract

Hematopoietic stem cells (HSCs) are rare cells in the bone marrow that continually generate and replenish all hematopoietic lineages in the blood throughout life and are distinguished by their multilineage differentiation and self-renewal capacities. Adult bone marrow HSCs possess a unique cell cycle status, which is termed quiescence, and infrequently exit quiescence to proliferate and self-renew. Self-renewal is defined as the inheritance of HSC properties by one or both daughter cells and is regulated by HSC division kinetics.

Deficiency of the adaptor protein LNK, a negative regulator of the Thrombopoietin signaling pathway in HSCs, results in a 10-fold expanded HSC pool. *Lnk-/-* HSCs have superior reconstitution potential, enhanced self-renewal and do not exhaust prematurely. This augmentation in HSC function is due, in part, to an increased propensity for HSC self-renewal in *Lnk-/-* HSCs. This property has been observed at a single cell level *in vitro*, but it is unclear how LNK deficiency influences self-renewal propensity.

Using a label retention model that tracks HSC division *in vivo*, we demonstrate a divisional heterogeneity in a highly enriched stem cell population. We demonstrate that HSC function is inversely correlated with each *in vivo* division and that *Lnk* deficiency both enhances the stem cell properties of undivided HSCs, and increases the frequency of bona-fide HSCs in subsets of cells that have divided several times *in vivo*. Genome-wide expression profiling reveals a transcriptional status correlated with active RNA synthesis and signal transduction in undivided cells, and an increase in DNA synthesis

and mitotic machinery as cells accrue *in vivo* divisions. We identify progressive changes in expression of several genes related to self-renewal, which may be targets of enhanced Tpo signaling in *Lnk-/-* HSCs. Additionally, our study uncovers that LNK deficiency retards changes in cell surface expression of several HSC-associated proteins, suggesting a transcription-independent role for LNK deficiency in regulating self-renewal.

Taken together our findings provide an unprecedented view of the relationship between *in vivo* HSC division and self-renewal and illuminate several potential mechanisms involved in self-renewal that might be impacted by *Lnk* deficiency.

3.2 Introduction

Hematopoietic cells are generated and maintained throughout life by a relatively rare population of stem cells. There is an inverse relationship between the frequency of division and the long-term repopulating capacity of HSCs (LT-HSCs). The exact mechanisms behind this relationship between the frequency and fate determinants of division remain poorly understood; however, they are immensely important as they regulate the size of the HSC pool, and indirectly, the ability of HSCs to maintain a constant, homeostatic hematopoietic supply.

Lnk-/- HSCs have enhanced self-renewal capacity. *In vitro*, *Lnk-/-* HSCs show a propensity towards symmetric self-renewing divisions [1] but it has not been possible to study this propensity on a single cell level *in vivo*. Additionally, it is not well understood how Lnk deficiency impacts the propensity for self-renewal in HSCs.

In this manuscript, we investigate HSC self-renewal *in vivo* using the M2R-tTA; H2B-GFP mouse model, in which H2B is fused with GFP and its expression is induced in response to doxycycline administration (Fig 3.S1A). Virtually all cells are labeled during a six-week pulse period, and upon doxycycline withdrawal, GFP signal is diluted two-fold with each division as each cell inherits half of the labeled H2B. Long-term chase experiments have identified HSCs as those cells that retain the GFP label [2,3]. By using a relatively short chase period, we hoped to catch a population of stem cells with sufficient heterogeneity, such that there would be a mix of true LT-HSCs and some ST- or non-HSCs. We anticipated that undivided cells – the d0 population – would contain almost

exclusively HSCs. As cells undergo division we expected to see a dilution in transplantation capacity such that by d5+ there would be few, if any, HSCs.

Naturally, the choice of cell population would be very important – too many or too few HSCs and we would not catch this heterogeneity. We therefore chose to study HSC self-renewal in SLAM LSK. First, the SLAM LSK HSC population has been extensively characterized [4,5]. Second, SLAM LSK cells represent a significant portion of all label-retaining cells. As previously shown, label-retaining cells are enriched by markers associated with HSCs. The stem and progenitor compartment defined by LSK markers considerably enriches for label retaining cells relative to Lin-c-kit+ alone. SLAM LSK further enriches for label retaining cells and represents approximately half of all label-retaining cells (Fig 3.S1B). Finally, since 1 in every 3 cells in SLAM LSK is a LT-HSC [4], there is a high likelihood this population contains a mix of cells suitable to our study. We hypothesized that division status might further stratify LT-HSCs from other cells within SLAM LSK. To this end, we were encouraged to find that SLAM LSK cells are comprised of a heterogeneous cell population with respect to division.

Here we show that HSCs in SLAM LSK are further dissected functionally from early progenitors and non-HSCs by *in vivo* division status, and demonstrate the propensity of *Lnk-/-* HSCs to self-renew *in vivo* using this system. We highlight trends with division that correlate with self-renewal propensity and identify transcriptional changes genome-wide expression in *Lnk-/-* SLAM LSK across divisions, which may shed light on how Lnk deficiency promotes self-renewal.

3.3 Results

HSCs exist in a continuum of repopulating capacity over *in vivo* division and this continuum is skewed by LNK deficiency

Given the inverse relationship between division and self-renewal capacity, we were curious to know whether division status could explain the functional heterogeneity observed within the SLAM LSK compartment. To address this question, we first visualized the division history of SLAM LSK using FACS. After four weeks of chase, SLAM LSK cells exist as heterogeneous population distributed by their division history (Fig 3.S1B). We were able to discern seven distinct division states (division 0 – 6+). We hypothesized that the number of functional LT-HSCs would be decreasing with in vivo division. To test this, we sorted individual division subsets of SLAM LSK (d0, d2, d4, or d5+) and assayed their repopulating capacity from WT and *Lnk-/-* donors by competitive transplant. Indeed, in both genotypes, we observed a decreasing trend in donor reconstitution in peripheral blood with division, indicating that later divisions contained fewer functional LT-HSCs than earlier ones (Fig 3.1A).

We then examined repopulation between WT and *Lnk-/-* cells. *Lnk-/-* cells repopulated recipients significantly better than their WT counterparts within each division subset (Fig 3.1A). Strikingly, recipients of d5+ WT cells did not reach 1% peripheral donor chimerism (the cutoff for functional HSCs) [6 7 1 8], while 4/5 recipients of d5+ *Lnk-/-* cells did (Fig 3.1A), indicating that at least 1/100 d5+ SLAM LSK is still a functional HSC in *Lnk-/-* mice. This was more evident in secondary transplant, where 3/5 recipients of d5+ *Lnk-/-* cells repopulated robustly. Additionally, secondary recipients of d4 *Lnk-/-* cells had donor

chimerism on par with those of d0 cells, while recipients of WT counterparts were significantly less reconstituted, on average (Fig 3.1B). Individually, only 2/5 recipients showed robust reconstitution in the WT group, in contrast with 7/7 recipients of *Lnk-/-* cells. Together this data indicates the presence of bona fide HSCs capable of self-renewal in d4 and d5+ subsets of *Lnk-/-* SLAM LSK.

Secondary transplant and phenotypic HSCs frequency in primary transplant recipient indicate HSC self-renewal. To look directly at this, we quantified the donor SLAM LSK in bone marrow of primary recipients. The donor SLAM LSK frequency of *Lnk-/-* cells showed a marked expansion over WT counterparts. Additionally, Lnk d0 and d2 SLAM LSK expanded significantly over WT d0 cells and expansion was on par between WT d0 in *Lnk-/-* d4 subsets (Fig 3.1C). The expansion was most evident in d2 and d4 recipients, in which *Lnk-/-* cells expanded 8.11x and 13.4x over their WT counterparts, respectively, exceeding the fold expansion by d0 cells.

Another important metric of HSC function is lineage choice. The predominant LT-HSC subtype is the β-HSC, which produces a B-cell heavy lineage distribution [9]. Single lineage repopulation indicates a committed progenitor, while myeloid skewing indicates HSC subtypes that are either partially committed or nearing exhaustion [9]. We therefore next assayed the lineage distribution of primary and secondary transplant recipients. In primary recipients, we found no significant differences in lineage distribution through d4 in both genotypes (Fig 3.1D). In division 5+ of *Lnk-/-* cells, there was a T-cell skew, indicating some lineage-committed cells (Fig 3.1E). Similarly, in secondary transplant,

though there was significantly more myeloid skewing than in primary transplant, there wasn't a significant lineage bias until d5+ in recipients of *Lnk-/-* cells. Therefore, Lnk deficiency slants HSCs along the continuum towards self-renewal and reconstitution.

In vivo division imparts cell intrinsic changes to HSCs

A distinguishing feature of LT-HSCs is their quiescent status, which is evident at the transcriptional and cellular level. *In vitro*, LT-HSCs are demonstrated to enter division later than their ST-HSC and MPP counterparts, resulting in a net proliferative delay, which may help prevent unnecessary proliferation and depletion of LT-HSCs. This delay has been attributed to LT-HSCs exiting quiescence later, having to activate transcriptional and translational machinery for responsiveness to growth stimuli. Indeed, the second *in vitro* division occurred after the same interval regardless of cell identity [10 8].

We asked how *in vivo* division history influences exit from quiescence in SLAM LSK with various *in vivo* division histories. To investigate this, we sorted single cells into wells of a 96 well plate and manually inspected the wells every two hours over a period of 72 hours and scored the number of cells per well. We wanted to maximize our chances of observing HSCs, so we performed this assay in Lnk deficient SLAM LSK, where there are more HSCs than in WT SLAM LSK. To further increase our chances, we chose a 6 week chase. We reasoned that in shorter chase times early division subsets (d0-2) are more likely to also include progenitors that have recently started dividing, whereas longer chase times would enrich for HSCs in these subsets. This is an important consideration since transplant only tests whether long-term repopulating activity is

present in the donor cells, and is not influenced by contaminating cells. In contrast, a single cell division assay reads every cell in the population and therefore its composition is more important. Indeed, after 6 weeks a greater proportion of SLAM LSK are in division subsets 6+ (Fig 3.2A). However, by transplant, cells in d0, d2, and d4 reconstitute recipients on par with cells after 4 week chase (Fig 3.2B), indicating that HSC distribution among subsets is similar between 4 weeks and 6 weeks of chase.

Strikingly, we found a strong negative correlation between every *in vivo* division and time to first division *in vitro* (Fig 3.2C). Cells in d0 divided significantly later (38 hours) than any other subsets. In fact, there was a step-wise shortening of time to division *in vitro* with division *in vivo*: cells in d1 and d2 subsets divided on par with one another, and significantly later than those in d4 (Fig 3.2D). The net effect of this spread in time to division was that later division subsets produced more cells by the end of the observation period than earlier division subsets. This was largely owing to the difference in time to first division, as the rate of second division was more similar (Fig 3.2E), and cell death was negligible (data not shown). Taken together these data demonstrate that *in vivo* division primes HSCs towards proliferation, even after a single *in vivo* division.

Lnk deficiency delays the exit from quiescence of undivided cells

To study how Lnk deficiency impacts cell exit from quiescence, we performed a 4 week chase followed by time to first division assay on WT and *Lnk-/-* d0, d2 and d4 cells. Here a clear negative correlation between division and time to *in vitro* division is also evident (Fig 3.3A), albeit less dramatic among d2 and d4 subsets. In both genotypes, d2 and d4

cells divide earlier than d0 (Fig 3.3B). Interestingly, however, Lnk deficient d0 cells divide significantly later than their WT counterparts (38.4 vs 35.8 hours), suggesting a deeper quiescent status (Fig 3.3C). Strikingly, *Lnk-/-* and WT cells in d2 and d4 subsets divide on par with one another (Fig 3.3B). By the end of the observation period, there was more net change in growth between *Lnk-/-* and WT cells in d2 or d4 subsets (Fig 3.3D). Taken together these findings demonstrate that Lnk deficiency widens the difference in behavior between divided and undivided HSCs.

Genome wide gene expression correlates with continuum of HSC functional states *in vivo*

HSCs exist on a transcriptional continuum *in vivo* [11] and division-related processes are the major source of expression variation among HSCs [12]. However, there has never been a direct study of the relationship between *in vivo* division history and transcriptional profile. We therefore set out to examine how transcription changes with *in vivo* division. To do this we profiled RNA expression in SLAM LSK across d0, d2, d4, and d5+ division subsets by RNAseq. We recovered high percentage (Table 3.S1) of high quality reads, with tight clustering between replicates, with a greater Pearson correlation distance between samples than between biological replicates (Fig 3.4A). We next performed differential expression analysis using DESeq2. 1852 genes were significantly changed among all comparisons, with the greatest number changed between d0 and d5 (Fig 3.4B). Interestingly and consistently with their similar behavior *in vitro,* there were no significant expression changes between d2 and d4 (Fig 3.4B). Unsupervised clustering of gene expression by k-means clustering revealed four clusters: one set of genes with

high expression in d0 only (Cluster 1), one set gradually decreasing in expression from d0 to d5+ (Cluster 2), another gradually increasing (Cluster 3), and one with high expression in d5+ only (Cluster 4) (Fig 3.4C).

To better understand the genes within each cluster functionally, we performed Gene Ontology (GO) analysis on each of the four clusters. We chose to display only the top 15 enriched categories for each cluster. Cluster 1 is enriched for processes related to negative regulation of differentiation (GO:0051254, GO:0051241, GO:0051093) (Fig 3.4D), consistent with previous reports of transcriptional profiles in quiescent cells [13] [14]. Interestingly, several categories related to transcription, and RNA biosynthesis and processing are also enriched in Cluster 1(GO:0045944, GO:0051254, GO:1902680, GO:1903508, GO: 0045893, GO:0010628). Given the well-documented observation that quiescent cells have low RNA content [15] [16], it is possible that there is high turnover in RNA in d0 cells. This is in agreement with findings that demonstrate quiescent HSCs produce transcripts rich in intronic sequences and only express processing machinery as they activate and proliferate [17]. Finally, several categories related to protein signaling are also enriched (GO:0006468, GO:0018193, GO:0035556, GO:0016310), indicating that these cells are actively engaged in signaling, potentially with the niche [18].

In Cluster 2, there is enrichment in RNA processing, and cytokine and tyrosine kinase signaling categories (Fig 3.4D), as in Cluster 1. Notably, there is robust enrichment in TGFβ regulation (GO:0017015, GO:1903844), which is consistent with previous reports showing that TGFβ signaling promotes HSC quiescence *in vivo* [19]. Additionally, there are

several categories associated with cell motility (GO:0010810, GO:0051272, GO:000147, GO:0030335, GO:0040017). Though contradictory at first, it is consistent with our unpublished observation that after the first *in vitro* division d0 and d2 daughter cells have high motility, and move apart from one another while daughters of d4 cells or after *in vitro* divisions do not. Similar observations were previously made in a study where HSCs were imaged every three minutes through time lapse microscopy and shown to have fewer cellular processes than more differentiated cells [8].

In Clusters 3 and 4 the majority of enrichments fall into categories associated with DNA replication and cell division (Fig 3.4D). Interestingly, Cluster 3 is enriched primarily for RNA biogenesis, DNA synthesis and DNA repair, and this is evident transcriptionally as early as the d2 population. However, the strongest enrichment for actual cell division is in Cluster 4, which is only highly enriched in d5+. This is consistent with the decreased repopulating capacity that we observe in divisions 4 and 5, and underscores the pro-proliferative, largely non-HSC composition of these populations.

Gene Set Enrichment (GSEA) is a widely used independent gene expression analysis tool that ranks gene expression in sets against a null distribution, and can thus detect changes in gene expression based on whole pathways. Using GSEA, we found evidence of similar trends and patterns as in the cluster analysis. Relative to d0, every division subset showed enrichment for biosynthetic and division processes (Myc_targets, E2F_targets, G2M_Checkpoint), which again underscores that *in vivo* division poises cells for proliferation [20]. Consistently, this trend was gradual across division subsets:

comparing between d2 and d5+, d5+ cells were enriched for additional gene sets related to active division, indicating a more robust proliferative transcriptional profile (Fig 3.4E).

Taken together, our transcriptional findings reflect and parallel our *in vivo* and *in vitro* findings that there is a gradual, cell-intrinsic transition in HSCs throughout division.

Next we manually inspected the differentially expressed gene lists. Sca1 (Ly6a) expression decreases with division, which correlates well with our surface expression data. We then examined the cell surface expression of other surface markers with significantly changed expression between d0 and d5. Two especially interesting candidates emerged. One is *Alcam* (CD166), a surface glycoprotein important for cell-cell contacts that plays a role in HSC repopulation capacity [21]. We found a progressive inverse change in surface expression of ALCAM with division (Fig 3.S2A, B). Another two significantly changed genes are CD41 (*Itgab*) and CD61 (*Itgb3*) (data not shown). CD41/CD61 forms a functional integrin complex. We find that CD41 shows a strong positive correlation with division (Fig 3.S2C, D), while CD61 has high cell surface expression throughout division (data not shown).

Lnk deficiency retards the change in surface expression of HSC markers that occurs throughout division

Recently there have also been reports showing that expression level of surface markers correlates with HSC function. Within the c-kit-gated population c-kit surface expression is

distributed across an approximate 10-fold range. Within that, high potency HSCs are found in the lower portion of that range, while cells in the higher range are only capable of intermediate term reconstitution of recipients [22-24]. Given the strong negative correlation in HSC function that we have shown with each division, we asked whether the surface expression range of c-kit would be correlated with division status. Indeed, we find a strong positive correlation between c-kit expression and division in WT SLAM LSK (Fig 3.5A, left). In *Lnk-/-* SLAM LSK, this correlation is also present (Fig 3.5A, right), but notably d3 and d4 have a significantly lower relative c-kit expression, indicating more potent HSCs, than their WT counterparts (Fig 3.5B). These findings offer further evidence of the role of LNK deficiency in promoting the HSC identity, and also may point towards a mechanism.

Based on this observation, we asked whether other surface markers correlated with division and whether the correlation was changed by Lnk deficiency. We first looked at well-known HSC markers Sca1 (Fig 3.5C, D), CD150 (Fig 3.5E, F), and CD48 (Fig 3.5G, H). Sca1 surface expressions showed a very strong negative correlation with division in WT SLAM LSK, consistent with a recent report correlating high Sca1 surface expression with HSC function [13]. Interestingly, this progression is significantly tempered in *Lnk-/-* SLAM LSK (Fig 3.5D). CD150 and CD48 expression changes have not conclusively been linked to functional outcomes for HSCs, nevertheless there is a quantifiable change in surface expression with division (Fig 3.5F, H). In these populations, Lnk deficiency also tempers expression changes (Fig 3.5F, 5H). Taken together, these data show changes in cell surface receptors surface expression that correlates with division

status and HSC function. This suggests that Lnk could be involved in regulating stem cell identity through cell surface receptor stability in a transcription-independent way.

In vivo division history marks HSCs independently of surface marker profiles

Prospective HSC purification remains an elusive goal. One other potential application of this work is to identify a surface marker scheme with high specificity and sensitivity; that is, a marker scheme that identifies only the entire population of functional HSCs. SLAM LSK markers enrich HSCs to 1 out of 3 and only partially overlap with other marker schemes such as CD34-Flk2-LSK. Additionally, CD150- side-population also repopulate hosts [25], further underscoring the inadequacy of surface markers to accurately reflect the entire HSC pool.

By FACS analysis, there is a clear population of d0 cells in CD150- (Fig 3.6A), and CD34+ (Fig 3.6B) subsets of CD48-LSK. Some CD34-Flk2- LSK are also CD150-, so we expected there to be repopulating activity in the CD150- CD48- LSK compartment. Since we have shown that *in vivo* division history strongly negatively correlates with HSC function, we wondered whether those HSCs would be found in the d0 subset. We therefore assessed the repopulation capacity of CD150+ or CD150- d0 cells from WT or Lnk-/- mice by transplant. Strikingly we found that both compartments were capable of long-term reconstitution (Fig 3.6C). Similarly, some SLAM LSK are CD34+. To test whether those cells were found in d0 subset, we performed a similar experiment on CD34+Flk2- LSK gated cells. Similarly, we found that CD34+ in d0 cells also reconstituted hosts (Fig 3.6D). We then also investigated the expression of a known

marker of intermediate-term HSCs, CD49b (Fig 3.6E). Consistently, CD49b+ cells in d0 were able to repopulate recipients from both WT and Lnk mice (Fig 3.6F). For comparison, we also transplanted CD49b+ or CD49b- cells from d4 and neither was capable of repopulation (Fig 3.6F). Taken together these data strongly demonstrate that division status is a better determinant of HSC function than any single surface marker scheme.

3.4 Discussion

Here we show that *in vivo*, HSCs exist in a continuum of repopulating and self-renewing capacity and that this continuum is organized by their *in vivo* division history. We devise and characterize a system by which to study the enhanced self-renewal capacity associated with Lnk deficiency *in vivo* and we molecularly probe the transcriptional and surface expression changes between division subsets in Lnk deficient HSCs.

In this manuscript we provide several lines of evidence to demonstrate that *in vivo* division imparts cell-intrinsic changes to HSCs. In both WT and *Lnk* deficient mice there is a dilution of repopulating and self-renewal capacity as well. Using this model we also demonstrate that *Lnk* deficiency retards dilution of HSC function with division, retaining a frequency of at least 1/50 functional HSCs in d4. These are bona-fide stem cells, with self-renewal capacity as evidenced by robust reconstitution of secondary recipients.

The decreasing reconstitution capacity of SLAM LSK with *in vivo* division correlates well with our *in vitro* findings that d0 cells exit quiescence later than cells in other subsets. Furthermore, we demonstrate that each *in vivo* division progressively changes cells, with each division shifting cells towards an earlier response to mitogenic stimuli. Even one *in vivo* division is sufficient to significantly reduce the lag in responsiveness to growth signals. It is intriguing to hypothesize that there may be an underlying functional advantage to activated but non-dividing HSCs. A parallel is seen in muscle stem cells, were activation of stem cells in one location induces a transient state of responsiveness to stimuli in muscle stem cells in a second, distant location [26], raising the intriguing

possibility that infrequent HSC division may be a mechanism to maintain sensitivity in HSCs. It would be interesting to further probe functional changes with each *in vivo* division.

Finally, we see the same trend in genome wide transcriptional profiling of division subsets. The findings from our *in vitro* study are reflected in the profiling data, which shows that cells in later *in vivo* division subsets have a transcriptional program tuned for division. The data is validated by surface expression changes and agrees with published findings. Notably, d0 subsets overlap transcriptional profiles with a core set of genes enriched in functional HSCs, which was described from individual transcription profiles of cells that had been sequenced and correlated with functional output. This study also identified high expression of Sca1 and EPCR on LT-HSCs [13], and we also find correlation between these markers and division by gene and cell surface expression. Another noteworthy correlation is with a study that identified CDK6 as a driver of HSC proliferation and differentiation [10], blockade of which could protect HSCs from exhaustion due to proliferative stress [27]. We find that CDK6 is significantly upregulated in d5+ cells. Our work adds an extra dimension to these studies, providing some insight into the order of transcriptional changes as cells lost self-renewal capacity.

Future studies will aim to dissect the molecular underpinnings of how Lnk deficiency influences HSC self-renewal propensity *in vivo*. We have identified several potential future directions from the transcriptional data. First, changes in ALCAM or CD41 may be drivers of self-renewal propensity. Though we have not yet compared ALCAM and CD41

surface expression between *Lnk-/-* and WT mice, it is intriguing to speculate that one of these functional surface proteins may be altered by LNK deficiency and lead to a preference for self-renewal. Notably, in contrast to its embryonic role [28], CD41 surface expression enriches for progenitors in adult BM [29]. The binding partner of CD41, CD61, forms another integrin complex, CD51 (Itgav), which is highly and uniformly expressed among division subsets at a gene expression level (data not shown). CD61/CD51 surface expression is associated with long term repopulation capacity in HSCs while CD61/CD41 surface expression is associated with short term progenitors [30]. Crucially, CD61/CD51 is activated by TPO signaling through which LT-HSC activity is preserved during *in vitro* culture [30]. Integrins participate in orienting the cell division axis, and therefore influence cell fate decisions {Streuli:2009jo}. In addition, LNK deficiency has been shown to directly regulate integrin activity in endothelial cells [31], and specifically the CD41/CD61 integrin complex in platelets [32]. Therefore, it would be interesting to determine whether LNK regulation of HSC integrins is an important mechanism in its role in regulating self-renewal.

A second potential driver is through the Retinoic Acid (RA) signaling pathway, activation of which has been reported to promote LT-HSC repopulating capacity [33] and be required for the maintenance of dormancy in HSCs [20]. In d0 we see significant enrichment of retinoic acid response elements Rxra and Rxrb, but also Cyp26b1, the enzyme responsible for degradation of all-trans retinoic acid (ATRA). Conversely, in d5+ we see upregulation of ATRA synthesis, by enrichment of *Aldh1a1* expression (the rate-limiting step enzyme), though ALDH1A1 has been shown to be nonessential in HSCs [34]. Stat5, the transcriptional effector of Tpo/TpoR/Jak2 signaling in HSCs, is known to bind the

same sites as some retinoic acid elements and synergize with RA mediated transcription [35]. Ongoing work is aimed at probing the effect of *Lnk* deficiency on this signaling intersection.

Additionally, our data show evidence of a potential role for *Lnk* deficiency to impact HSCs at a protein level. Our data convincingly shows the changes in surface expression with division of several HSC-associated proteins is decelerated by *Lnk* deficiency. It has previously been demonstrated that the level of c-kit surface expression is regulated by CBL, a ubiquitin ligase that targets proteins for degradation. Higher CBL activity was associated with lower c-kit surface expression and therefore higher HSC potency [23]. Intriguingly, by mass spectrometry, we have found that LNK binds CBL (unpublished data), and therefore LNK may alter CBL activity and protein expression at the surface and intracellularly, thereby increasing the likelihood of self-renewing division.

There are two key challenges going forward in this study. The first is that, though *in vivo* division history is traced by H2B-GFP dilution, neither the timing of division nor the relationship between division subsets can be directly inferred. It is certainly possible that some cells in d4 have initiated and undergone rapid division very close to the end of the 4 week chase period, while other d4 cells have divided once every 4 weeks. Though our *in vitro* data would suggest that d4 cells behave relatively uniformly to division stimulus, it is clear by transplant that the d4 subset is a heterogeneous population. Given that self-renewal often occurs as an asymmetric division during homeostasis [36 37], we expect that HSC function might be lost before surface marker profiles begin to change. Another

possibility is that the surface marker expression profile among cells in different division subsets of SLAM LSK is not constant, meaning that, for example, some cells in d4 did not originate from a d0 SLAM LSK but later acquired the SLAM LSK surface marker profile. While these questions could be explored with the help of another biomarker, such a second pulse-chase label that could be applied at variable intervals during the H2B-GFP pulse-chase, this would ultimately lead to the same population-based data.

The second challenge is that there are significant limitations to studying HSCs by surface markers. Foremost is that many surface markers are not functional. Second, surface markers only enrich HSCs. By the most up to date surface marker scheme, only 1 in 2 cells are bona-fide HSCs. Additionally, recent evidence shows that in steady state *in vivo* progenitors contribute extensively to maintaining hematopoiesis, and not the cells designated "LT-HSCs" [38]. Finally, HSCs exist as a functionally heterogeneous population at the single cell level, as a mix of cells with propensities towards a particular lineage [9]. All this calls into question the study of HSCs as a population and based on a phenotypic description and highlights the necessity to study HSCs at a single cell level.

This approach will allow for several advantages over population-level profiling. First, rigorous quantification of HSCs within each division subset in WT and *Lnk-/-* mice by limiting dilution assay will determine the nature of the dilution in repopulation capacity that we observe – whether HSC quantity or quality change, or both. Together with single-cell transcriptional data, it will be possible to independently cluster and map how closely cells within each division subset are related to one another. Second this

approach will be able to determine whether *Lnk-/-* HSCs preserve a specific transcriptional program regardless of division history. Finally, this approach will be able to answer whether the impact of Lnk deficiency is at a transcriptional or post-transcriptional level. At present, it is thought that transcription may be only one component of self-renewal determination. Since self-renewal is defined at the moment of division, which often involves the asymmetric inheritance of HSC function into only one daughter cell [36] [37], it is not unreasonable for an HSC-niche interaction or asymmetric protein distribution to potentially be the actual driver of this asymmetrical inheritance of function. Indeed, multiple HSC-niche interactions such as with E-SELECTIN [39] or CXCL4 and niche megakaryocytes [40] [41] [42], drivers of asymmetric protein distributions such as LIS1 [43], and even level of protein synthesis [44] have been reported to impact HSC function. Therefore, future studies should also include single cell level protein profiling such as by the recently developed mass cytometry [45], particularly in paired cells after division.

Taken together, we show LNK deficiency shifts the *in vivo* HSC continuum towards the stem cell side, and establish several leads that may elucidate the mechanisms behind enhanced self-renewal in *Lnk -/-* HSCs.

3.5 Materials and Methods

Mice

Rosa M2R- tTA; collagen H2B-GFP (H2B-GFP) mice were kindly provided by Dr. Hanno Hock (Massachusetts General Hospital and Harvard University, Boston, MA) [2]. *Lnk-/-* mice by Dr. Tony Pawson (Samuel Lunenfeld Research Institute, Canada). H2B-GFP mice were bred with *Lnk-/-* mice to create M2R; H2B-GFP; *Lnk-/-* animals. All mice were on C57/B6J background (CD45.2) and both male and female mice were used in experiments. Transplant competitor cells were from SJL (CD45.1) mice and transplant recipients were progeny of SJL x C57/B6J (F1). To induce H2B-GFP expression, 6-8 week old mice were given water supplemented with 2mg/mL Doxycycline (Sigma D9891) and 5% sucrose three times a week for a period of six weeks ("label" period). At the end of the label period, mice were returned to normal drinking water ("chase" period). After four or six weeks, mice were sacrificed and bone marrow was harvested from tibias, femurs and ilia. Bone marrow was flushed using 0.5% bovine serum albumin (BSA) (Fisher, BP1600-100) in PBS. Cells were passed through a 30μm nylon filter to make a single cell suspension.

Genotyping

Mice were genotyped by PCR of genomic DNA from tail snips of weanling mice. Tails were digested at 55°C overnight in buffer containing 100mM Tris pH 8.0, 5mM EDTA pH 8.0, 0.2% SDS and 200mM NaCl. DNA was precipitated in propanol and washed in 70% EtOH and then resuspended in water. Mice were genotyped for *Lnk* (wild-type: 5'-gtccgactctctggctatgtggta-3', neo insertion: 5'- cgcatcgccttctatcgcct-3', common: 5'-

gaagaggagtccatgtcatagtcc-3'), M2R-tTA (5'-aaagtcgctctgagttgttat-3', 5'-gcgaagagtttgtcctcaacc-3', 5'-ggagcgggagaaatggatatg-3'), and H2B-GFP (5'-gcacagcattgcggacatgc-3', 5'-ccctccatgtgtaccaagg-3', 5'-gcagaagcgcggccgtctgg-3').

Sorting

Sorts for transplant and RNAseq were performed on one of two different instruments. First, HSPCs were pre-enriched using a lineage depletion kit (Miltenyi, 130-090-858) and magnetic separation column (Miltenyi, 130-042-401) following manufacturer's instructions. Briefly, bone marrow from individual mice was resuspended in 150μL of 2mM EDTA 0.5% BSA PBS pH 7.2 (depletion buffer) and 40μL of Lineage antibody cocktail. Cells were incubated at 4°C for 15 minutes, then washed in 10 mL of the depletion buffer. Cells were resuspended in 250μL depletion buffer and 70μL Biotin conjugated microbeads, and incubated and washed again. Cells were resuspended in 1 mL depletion buffer and passed through a 30μm nylon mesh into the separation column. Columns were washed with 3x3mL depletion buffer. LT-HSCs (SLAM LSK) were stained with anti- c-kit, -Sca-1, -CD48, and -CD150 antibodies. GFP divisions were resolved by individual peaks, and verified by Geo Mean (two-fold dilution after each division; $GeoMean_{dX} = GeoMean_{d0}/2^{X}$). For the paired WT and *Lnk -/-*transplant, cells were double sorted on a BD Aria sorter with a 70μm nozzle. On the first sort, LSK were enriched using the "yield" setting, then highly purified LT-HSCs in division 0, 2, 4, or 5+ were sorted using a "single cell" drop envelope into 1.7mL tubes containing StemSpan SFEM (STEMCELL Technologies, 09600) with 2% fetal bovine serum (FBS) (SAFC Biosciences, 12103C-500mL). For RNAseq analysis, division subsets were sorted only

once on a BD Aria sorter using the “single cell” drop envelope directly into 1.7mL tubes containing Trizol LS (Thermo Fisher 10296028). For all other transplants and for *in vitro* division sorts, LT-HSCs were double sorted using the MoFlo Astrios EQ with on the “low pressure” setting. First LSK were enriched using the “enrich” setting, then LT-HSCs in different division subsets were sorted into individual wells of a 96 well plate containing 100μL of 2% FBS SFEM. Competitor cells were added directly to each well in 50μL of PBS and then transplanted as described below.

Bone Marrow Transplant

Sorted LT-HSCs separated by division status from donor mice were mixed with 350,000 freshly isolated competitor (B6.SJL) cells or 400,000 Sca1-depleted competitor cells and injected retro-orbitally into lethally irradiated (10Gy, split dose, Orthovoltage Precision X-Ray) F1 recipient mice in 100-150μL of PBS per recipient. At 4, 8, 12 and 16 weeks post transplantation, peripheral blood of recipients was analyzed for donor reconstitution in myeloid, T-, and B- lineages by flow cytometry. At 16 weeks post transplant, recipients were sacrificed and donor reconstitution in various HPSC compartments was analyzed using flow cytometry. BM cells from primary transplants were harvested and 2 million cells were injected into each secondary recipient.

Flow Cytometry

For peripheral blood analysis, 50μL of peripheral blood was collected by retro-orbital bleed in heparinized microcapillary tubes (Drummond 1-000-7500-HC/5). Red blood

cells were lysed with ACK lysis buffer (0.8% ammonium Chloride, 10 μM EDTA, pH 7.5 with sodium bicarbonate). Cells were stained for donor reconstitution (CD45.1, CD45.2) in myeloid (Gr-1, Mac1) or lymphoid (CD19, CD3) lineages. All peripheral blood data was acquired using BD Canto flow cytometer and FACS DiVa software. At least 20k live events were recorded for each sample. For donor reconstitution in bone marrow of transplant recipients, cells were stained with fluorophore-conjugated anti-CD45.1, Lineage (biotin-Ter-119, -Mac-1, -Gr-1, -CD4, -CD8α, -CD5, -CD19 and -B220), -c-kit, -Sca1, -CD150, -CD48, followed by staining with streptavidin-PE-TexasRed. For evaluation of additional surface markers, LT-HSCs were pre-enriched and stained with SLAM LSK or CD34 and Flk2 LSK panels as described, and additionally with anti-CD41, or –ALCAM antibodies. Data for bone marrow analysis was collected on the BD LSR Fortessa flow cytometer and FACS DiVa software. All flow cytometry data was analyzed using FlowJo v8.7 for MAC. All antibodies used for flow cytometry are detailed in supplemental table 2.

In vitro division assay

For time to first division *in vitro*, one single cell was sorted into each well of a round bottom 96 well plate (Costar 3799) containing 100μL of 10% FBS SFEM supplemented with 1% penicillin/streptomycin (Life Technologies, 15140-122), 1% L-glutamine (Life Technologies, 25030-081), 100μM β-mercaptoethanol (Sigma, M7522), 10 ng/mL murine Tpo (Peprotech Inc, 315-14), and 50 ng/mL murine SCF (Peprotech Inc, 250-03). Media was filtered through a 0.22μm membrane (Millipore SLGV013SL) prior to aliquotting into plates. Cells were incubated at 37°C with 5% CO2. Starting from 14-16

hours after sorting, each well was inspected individually using an inverted light microscope every two hours until the end of the 72h observation period. Wells in which zero or more than one cells was initially observed were excluded from analysis. The time to first division was counted as the first hour during which two separate cells were visible in a well. The time to second division was counted as the first hour during which four separate cells were visible in a well.

RNA seq

Two replicates of RNA libraries were prepared from 20,000-40,000 single sorted cells from five mice each. 500µL of Qiazol (Qiagen 79306) was added to each sample. Samples were spun for 15 minutes at 4°C 12,000xg and the liquid phase was transferred to a new tube and mixed with 1.5x volume 200 proof ethanol. RNA was isolated using the miRNeasy (Qiagen 217004) kit according to manufacturer's instructions, with the following modifications: columns were replaced by ones from the MinElute cleanup kit (Qiagen 28204), and flow through after column loading was collected and loaded a second time to increase yield. DNA was digested using DNase I (Qiagen 79254). Beijing Genomics Institute (BGI) at CHOP performed sequencing library construction and sequencing. Briefly, BGI prepared amplified cDNA using SPIA amplification (NuGEN) and created a paired-end sequencing library using the Ovation Ultralow Library System (NuGEN). Samples were sequenced on an Illumina HiSeq 2500 Sequencing System (Illumina) using a PE100 flow cell to a depth of 80-100 million reads per sample.

Bioinformatics

RNAseq samples were analyzed for differential expression as follows. Reads were aligned using STAR 2.4.2a with default parameters [46]. STAR genome indices were generated using the UCSC mm9 genomic sequence, and genes annotated using the UCSC mm9 RefSeq GTF files (RefFlat version). Reads were tabulated using HTSeq-0.6.1p1 [47]. Using R.3.2.4 and DESeq2 v3.2 [48], differentially expressed genes were called with adjusted p-values less that 0.1. Graphic outputs used regularized logarithm adjusted data. Gene Set Enrichment Analysis (GSEA) [49] [50] was performed using MSigDB v6.0 and enrichments were significant called at FDR≤0.2 and/ or p≤0.05.

Statistical analysis

All statistical comparisons across samples were calculated by two tailed-student's t-test.

3.6 Figures

Fig 3.1

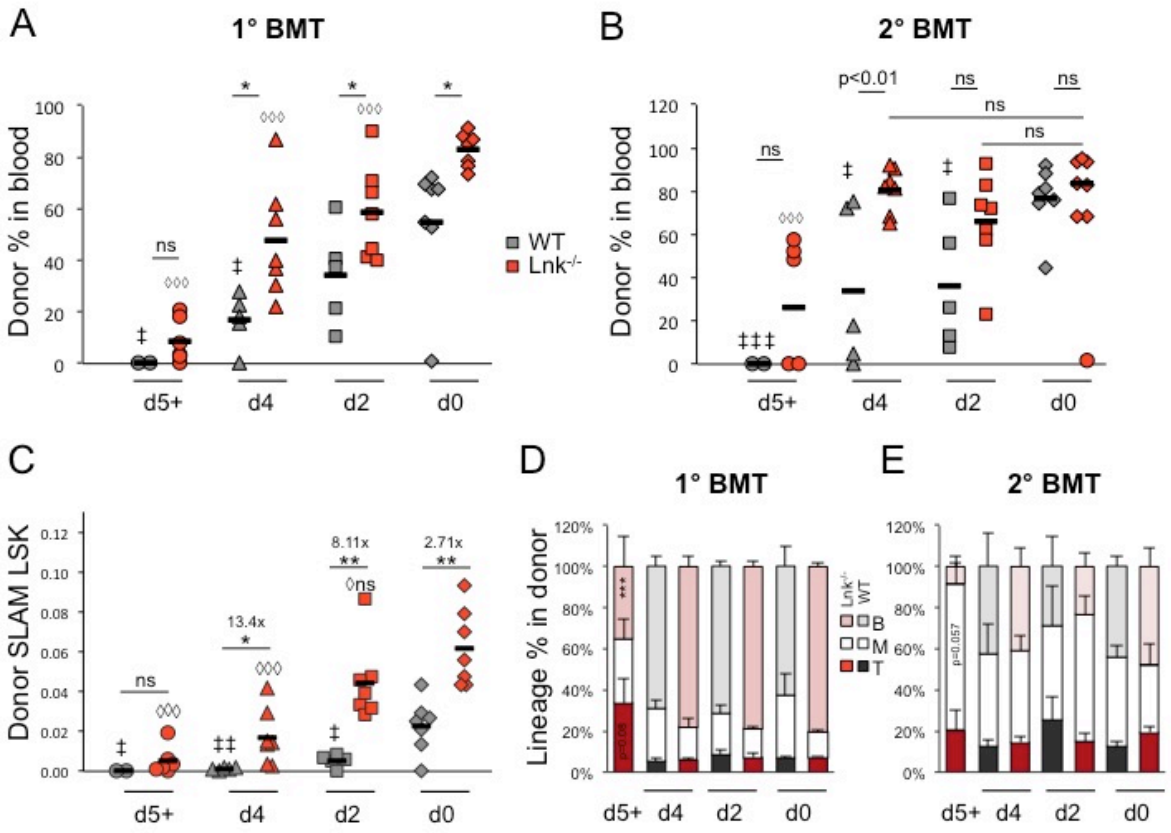


Fig 3.2

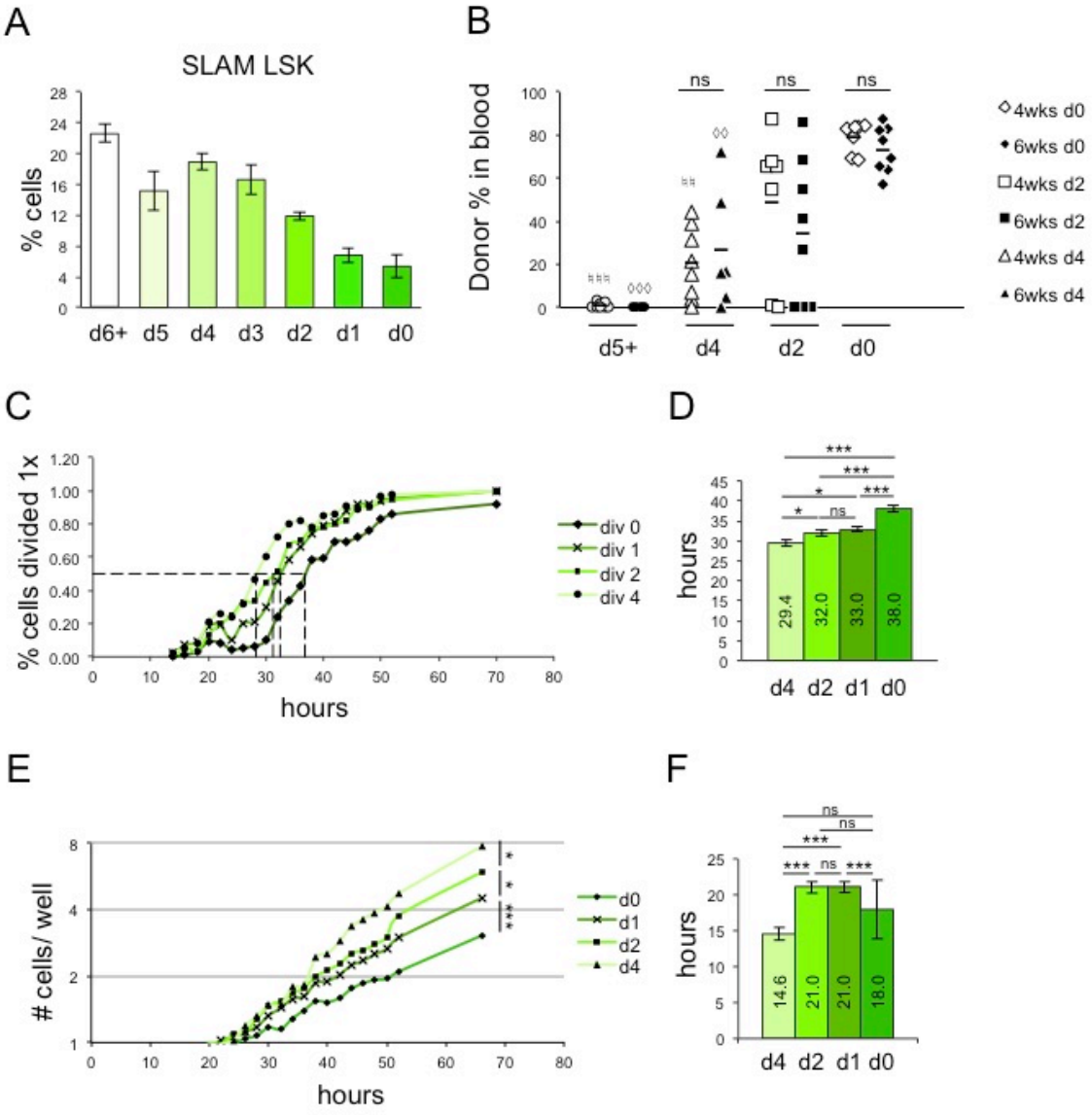

A
SLAM LSK
% cells
d6+ d5 d4 d3 d2 d1 d0
B
Donor % in blood
ns
d5+ d4 d2 d0
4wks d0
6wks d0
4wks d2
6wks d2
4wks d4
6wks d4
C
% cells divided 1x
hours
div 0
div 1
div 2
div 4
D
hours
29.4
32.0
33.0
38.0
d4 d2 d1 d0
E
cells/ well
hours
d0
d1
d2
d4
F
hours
14.6
21.0
21.0
18.0
d4 d2 d1 d0

Fig 3.3

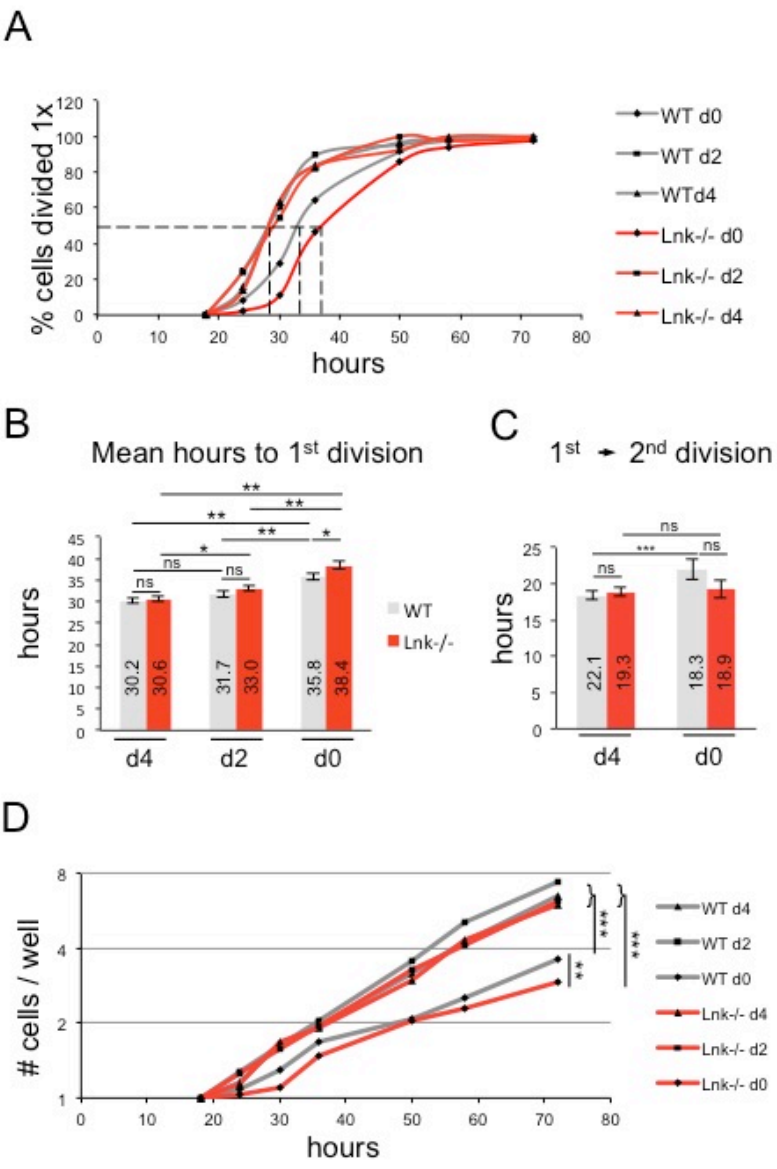
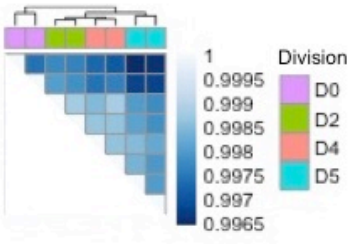

A
% cells divided 1x
hours
WT d0
WT d2
WTd4
Lnk-/- d0
Lnk-/- d2
Lnk-/- d4
B
Mean hours to 1st division
hours
WT
Lnk-/-
d4
d2
d0
C
1st → 2nd division
hours
d4
d0
D
cells / well
hours
WT d4
WT d2
WT d0
Lnk-/- d4
Lnk-/- d2
Lnk-/- d0

Fig 3.4

A

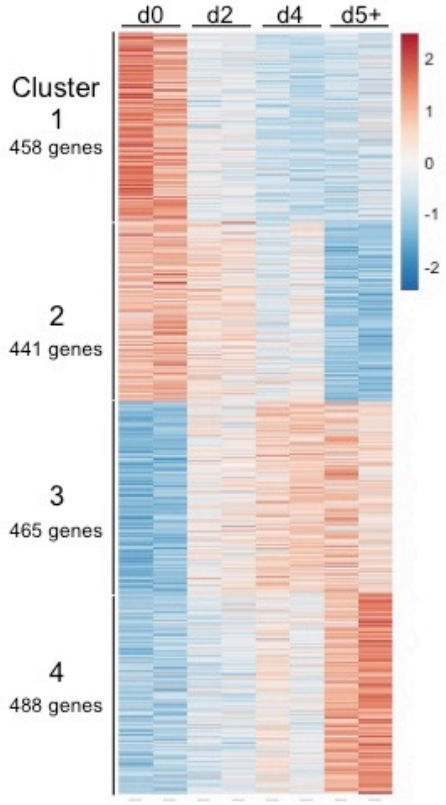


B

Significantly changed genes

v	d0	d2	d4	d5+
d0		182	790	1532
d2			0	247
d4				8

C



D

	Gene Ontology Term and ID	Fold Enrichment	p-value
Cluster 1	peptidyl-amino acid modification (GO:0018193)	2.53	2.87E-02
	protein phosphorylation (GO:0006468)	2.51	7.58E-03
	positive regulation of transcription from RNA polymerase II promoter (GO:0045944)	2.48	4.18E-05
	negative regulation of cell differentiation (GO:0045596)	2.48	4.10E-02
	intracellular signal transduction (GO:0035556)	2.45	1.53E-05
	positive regulation of RNA metabolic process (GO:0051254)	2.39	9.53E-07
	negative regulation of developmental process (GO:0051093)	2.33	1.11E-02
	positive regulation of nucleic acid-templated transcription (GO:1903508)	2.31	2.24E-05
	positive regulation of transcription, DNA-templated (GO:0045893)	2.31	2.24E-05
	positive regulation of RNA biosynthetic process (GO:1902680)	2.3	2.63E-05
	circulatory system development (GO:0072359)	2.29	3.93E-02
	phosphorylation (GO:0016310)	2.25	1.39E-02
	locomotion (GO:0040011)	2.22	1.43E-02
	negative regulation of multicellular organismal process (GO:0051241)	2.17	1.11E-02
	positive regulation of gene expression (GO:0010628)	2.14	2.30E-05
Cluster 2	regulation of transforming growth factor beta receptor signaling pathway (GO:0017015)	6.64	3.59E-03
	regulation of cellular response to transforming growth factor beta stimulus (GO:1903844)	6.5	4.50E-03
	regulation of cell-substrate adhesion (GO:0010810)	4.18	4.21E-02
	transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)	3.52	1.51E-03
	enzyme linked receptor protein signaling pathway (GO:0007167)	3.14	1.07E-04
	positive regulation of cellular component movement (GO:0051272)	3.12	9.53E-04
	cell morphogenesis involved in neuron differentiation (GO:0048667)	3.12	2.05E-02
	cellular response to cytokine stimulus (GO:0071345)	3.11	4.62E-03
	blood vessel morphogenesis (GO:0048514)	3.11	1.28E-02
	positive regulation of cell motility (GO:2000147)	3.1	1.81E-03
	positive regulation of cell migration (GO:0030335)	3.09	3.16E-03
	negative regulation of transcription from RNA polymerase II promoter (GO:0000122)	3	9.69E-07
	cardiovascular system development (GO:0072358)	2.94	1.26E-03
	vasculature development (GO:0001944)	2.92	2.27E-03
	positive regulation of locomotion (GO:0040017)	2.9	6.56E-03
Cluster 3	nucleosome organization (GO:0034728)	5.64	1.96E-02
	DNA replication (GO:0006260)	5.15	2.42E-04
	DNA recombination (GO:0006310)	4.71	8.93E-04
	RNA localization (GO:0006403)	4.31	2.89E-02
	ribosome biogenesis (GO:0042254)	3.57	2.36E-02
	DNA metabolic process (GO:0006259)	3.34	3.39E-08
	RNA splicing (GO:0008380)	3.33	1.20E-02
	establishment of protein localization to organelle (GO:0072594)	3.31	3.92E-02
	DNA repair (GO:0006281)	3.3	5.55E-04
	mRNA processing (GO:0006397)	3.29	1.00E-03
	ribonucleoprotein complex biogenesis (GO:0022613)	3.22	4.21E-03
	mRNA metabolic process (GO:0016071)	3.21	4.16E-05
	mitotic cell cycle process (GO:1903047)	3.03	1.27E-02
	protein localization to organelle (GO:0033365)	3.03	2.73E-04
	regulation of apoptotic signaling pathway (GO:2001233)	2.98	1.67E-02
Cluster 4	mitotic chromosome condensation (GO:0007076)	20.58	4.73E-02
	regulation of mitotic cell cycle spindle assembly checkpoint (GO:0090266)	20.58	4.73E-02
	regulation of mitotic spindle checkpoint (GO:1903504)	20.58	4.73E-02
	centromere complex assembly (GO:0034508)	14.82	3.53E-02
	chromosome condensation (GO:0030261)	14.11	1.27E-03
	mitotic sister chromatid segregation (GO:0000070)	12.48	4.37E-14
	spindle localization (GO:0051653)	11.29	6.70E-03
	mitotic nuclear division (GO:0140014)	11.06	2.31E-16
	mitotic spindle assembly (GO:0090307)	10.97	8.25E-03
	cytoskeleton-dependent cytokinesis (GO:0061640)	10.97	3.67E-04
	mitotic metaphase plate congression (GO:0007080)	10.13	1.48E-02
	sister chromatid segregation (GO:0000819)	10.13	8.21E-13
	mitotic spindle organization (GO:0007052)	10.03	1.02E-05
	metaphase plate congression (GO:0051310)	9.88	9.63E-04
	regulation of mitotic sister chromatid separation (GO:0010965)	9.26	7.01E-03

E

d0 v d2

Up in d0

GS DETAILS	NES	NOM p-val	FDR q-val
HALLMARK_KRAS_SIGNALING_UP	1.68	0	0.026
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.57	0.003	0.066
HALLMARK_NOTCH_SIGNALING	1.56	0.018	0.049
HALLMARK_WNT_BETA_CATENIN_SIGNALING	1.53	0.028	0.044
HALLMARK_ANGIOGENESIS	1.44	0.027	0.081
HALLMARK_INTERFERON_ALPHA_RESPONSE	1.44	0.025	0.069
HALLMARK_APICAL_JUNCTION	1.42	0	0.069
HALLMARK_INTERFERON_GAMMA_RESPONSE	1.39	0.004	0.085
HALLMARK_P53_PATHWAY	1.25	0.046	0.245

Up in d2

GS DETAILS	NOM p-val	FDR q-val
HALLMARK_E2F_TARGETS	0	0
HALLMARK_G2M_CHECKPOINT	0	0.001
HALLMARK_PANCREAS_BETA_CELLS	0.026	0.052
HALLMARK_KRAS_SIGNALING_DN	0.012	0.133
HALLMARK_HEDGEHOG_SIGNALING	0.089	0.109
HALLMARK_MYC_TARGETS_V1	0.025	0.127

d0 v d4

Up in d0

GS DETAILS	NES	NOM p-val	FDR q-val
HALLMARK_ANGIOGENESIS	1.83	0	0.005
HALLMARK_INTERFERON_ALPHA_RESPONSE	1.62	0.002	0.039
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.6	0	0.029
HALLMARK_WNT_BETA_CATENIN_SIGNALING	1.56	0.029	0.029
HALLMARK_NOTCH_SIGNALING	1.44	0.041	0.072
HALLMARK_MYOGENESIS	1.42	0.003	0.069
HALLMARK_UV_RESPONSE_UP	1.42	0.006	0.061
HALLMARK_KRAS_SIGNALING_UP	1.39	0.003	0.072
HALLMARK_P53_PATHWAY	1.37	0.003	0.074
HALLMARK_APICAL_JUNCTION	1.36	0.017	0.076
HALLMARK_TGF_BETA_SIGNALING	1.35	0.066	0.078
HALLMARK_INFLAMMATORY_RESPONSE	1.31	0.014	0.097
HALLMARK_HYPOXIA	1.28	0.017	0.122
HALLMARK_IL2_STAT5_SIGNALING	1.25	0.045	0.145
HALLMARK_INTERFERON_GAMMA_RESPONSE	1.21	0.084	0.19

Up in d4

GS DETAILS	NOM p-val	FDR q-val
HALLMARK_E2F_TARGETS	0	0
HALLMARK_G2M_CHECKPOINT	0	0
HALLMARK_MYC_TARGETS_V1	0	0

d0 v d5

Up in d0

GS DETAILS	NES	NOM p-val	FDR q-val
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.64	0	0.027
HALLMARK_INTERFERON_ALPHA_RESPONSE	1.5	0.007	0.077
HALLMARK_TGF_BETA_SIGNALING	1.5	0.016	0.053
HALLMARK_APICAL_JUNCTION	1.39	0.005	0.115
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	1.37	0.013	0.11
HALLMARK_KRAS_SIGNALING_UP	1.36	0.015	0.098
HALLMARK_INTERFERON_GAMMA_RESPONSE	1.36	0.018	0.087
HALLMARK_MYOGENESIS	1.29	0.022	0.133
HALLMARK_NOTCH_SIGNALING	1.29	0.115	0.123
HALLMARK_ANGIOGENESIS	1.22	0.174	0.193
HALLMARK_UV_RESPONSE_UP	1.22	0.079	0.176
HALLMARK_IL2_STAT5_SIGNALING	1.2	0.059	0.193

Up in d5

GS DETAILS	NOM p-val	FDR q-val
HALLMARK_E2F_TARGETS	0	0
HALLMARK_G2M_CHECKPOINT	0	0
HALLMARK_MYC_TARGETS_V1	0	0
HALLMARK_MYC_TARGETS_V2	0	0
HALLMARK_MTORC1_SIGNALING	0	0.001
HALLMARK_OXIDATIVE_PHOSPHORYLATION	0	0.004
HALLMARK_FATTY_ACID_METABOLISM	0	0.022
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0.016	0.043
HALLMARK_DNA_REPAIR	0.036	0.142
HALLMARK_HEME_METABOLISM	0.059	0.225
HALLMARK_GLYCOLYSIS	0.067	0.258
HALLMARK_COAGULATION	0.095	0.24

F

d2 v d4

Up in d2

GS DETAILS	NES	NOM p-val	FDR q-val
HALLMARK_MYOGENESIS	1.54	0	0.177

Up in d4

GS DETAILS	NOM p-val	FDR q-val
HALLMARK_E2F_TARGETS	0	0
HALLMARK_G2M_CHECKPOINT	0	0
HALLMARK_MYC_TARGETS_V1	0	0
HALLMARK_OXIDATIVE_PHOSPHORYLATION	0	0.006

d2 v d5

Up in d2

Up in d5

GS DETAILS	NOM p-val	FDR q-val
HALLMARK_E2F_TARGETS	0	0
HALLMARK_MYC_TARGETS_V1	0	0
HALLMARK_G2M_CHECKPOINT	0	0
HALLMARK_OXIDATIVE_PHOSPHORYLATION	0	0
HALLMARK_MYC_TARGETS_V2	0	0
HALLMARK_MTORC1_SIGNALING	0	0.001
HALLMARK_DNA_REPAIR	0	0.01
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0	0.022
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY	0.043	0.048
HALLMARK_PEROXISOME	0.03	0.079
HALLMARK_PI3K_AKT_MTOR_SIGNALING	0.04	0.121
HALLMARK_ANGIOGENESIS	0.113	0.169
HALLMARK_ADIPOGENESIS	0.057	0.187

d4 v d5

Up in d4

Up in d5

GS DETAILS	NOM p-val	FDR q-val
HALLMARK_MYC_TARGETS_V1	0	0
HALLMARK_E2F_TARGETS	0	0.003
HALLMARK_G2M_CHECKPOINT	0	0.003
HALLMARK_MYC_TARGETS_V2	0	0.004
HALLMARK_ANGIOGENESIS	0.008	0.014
HALLMARK_MTORC1_SIGNALING	0.002	0.031
HALLMARK_OXIDATIVE_PHOSPHORYLATION	0.005	0.037
HALLMARK_ADIPOGENESIS	0.005	0.094
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY	0.072	0.126
HALLMARK_COAGULATION	0.052	0.166
HALLMARK_UV_RESPONSE_UP	0.042	0.169
HALLMARK_FATTY_ACID_METABOLISM	0.054	0.23
HALLMARK_APOPTOSIS	0.075	0.25

Fig 3.5

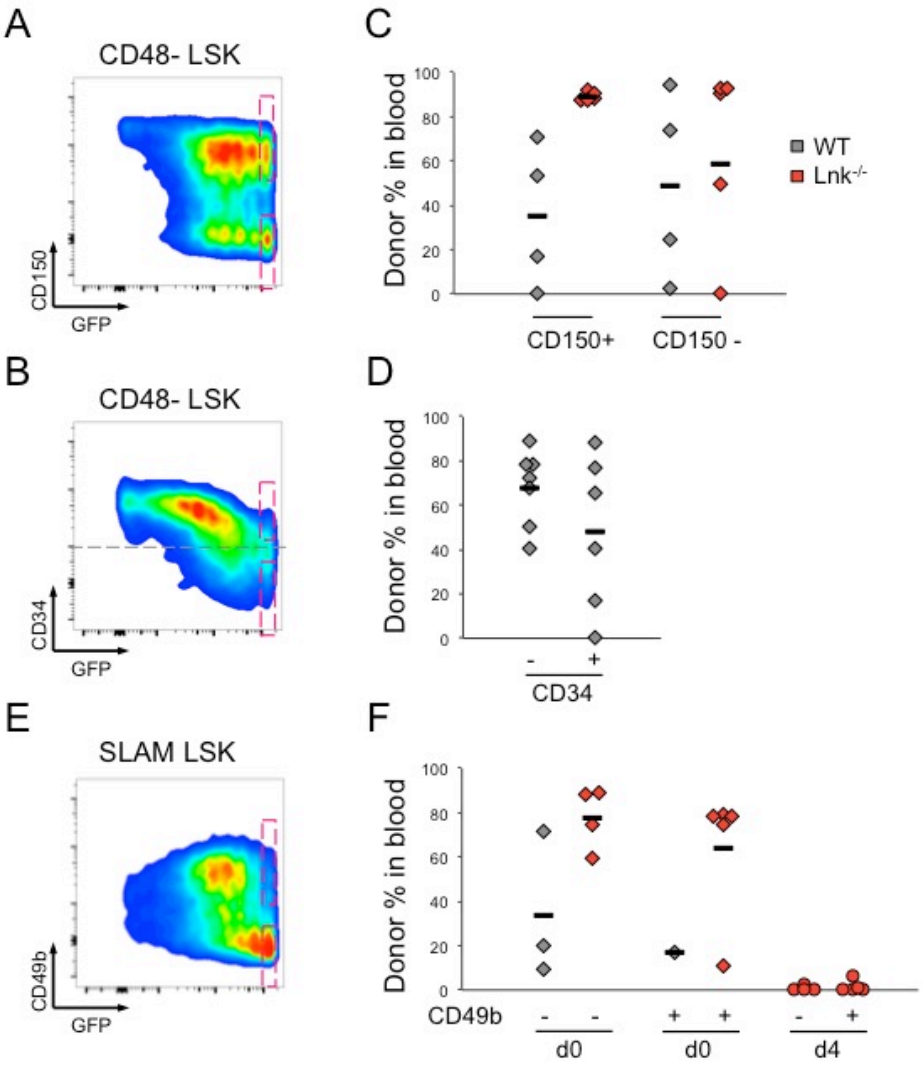

A
CD48- LSK
CD150
GFP
B
CD48- LSK
CD34
GFP
C
Donor % in blood
100
80
60
40
20
0
WT
Lnk-/-
CD150+
CD150 -
D
Donor % in blood
CD34
-
+
E
SLAM LSK
CD49b
GFP
F
Donor % in blood
CD49b
d0
d0
d4

Fig 3.6

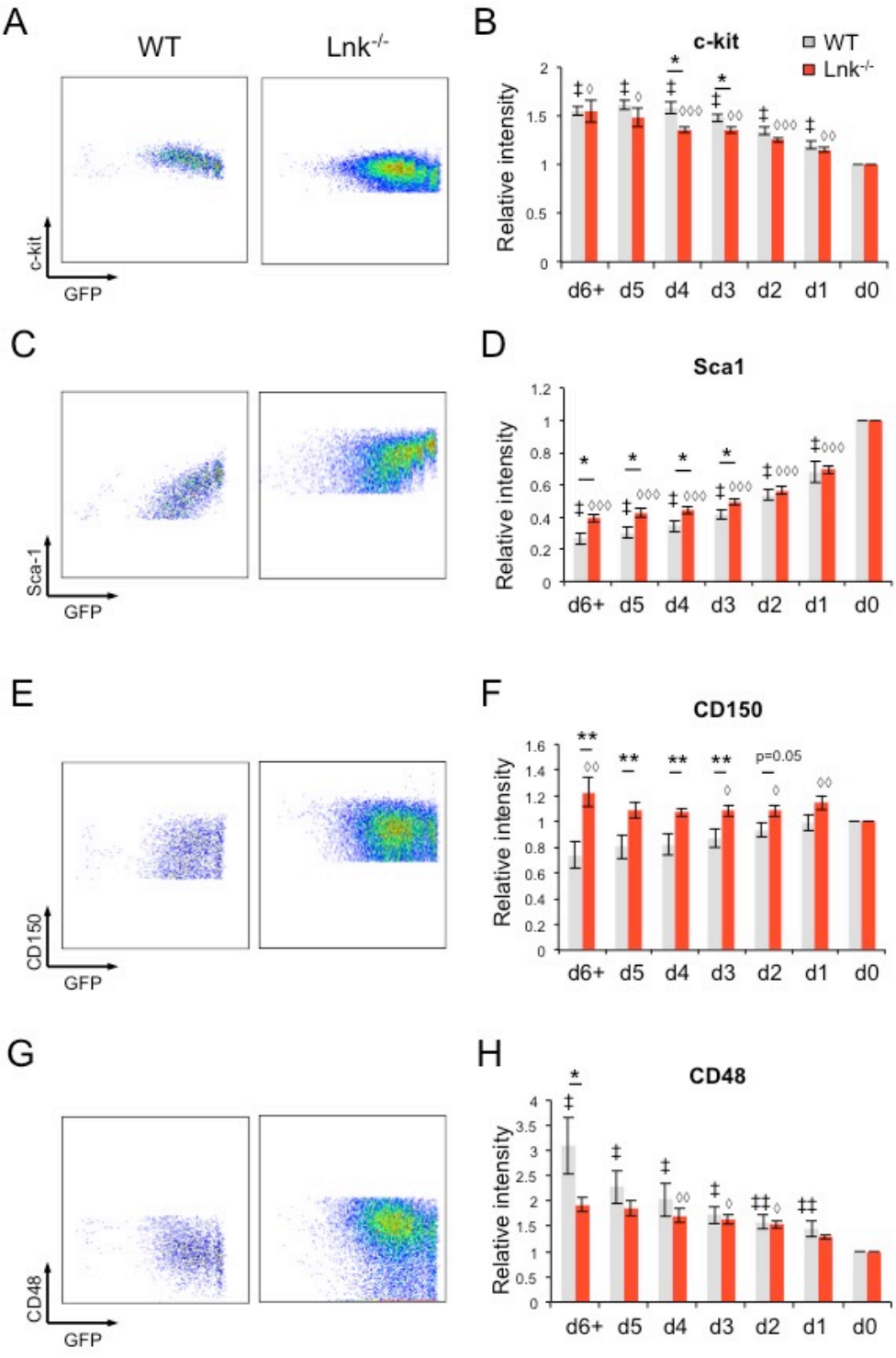

A
WT
Lnk-/-
c-kit
GFP
B
c-kit
WT
Lnk-/-
Relative intensity
d6+ d5 d4 d3 d2 d1 d0
C
Sca-1
GFP
D
Sca1
Relative intensity
d6+ d5 d4 d3 d2 d1 d0
E
CD150
GFP
F
CD150
p=0.05
Relative intensity
d6+ d5 d4 d3 d2 d1 d0
G
CD48
GFP
H
CD48
Relative intensity
d6+ d5 d4 d3 d2 d1 d0

Fig 3.S1

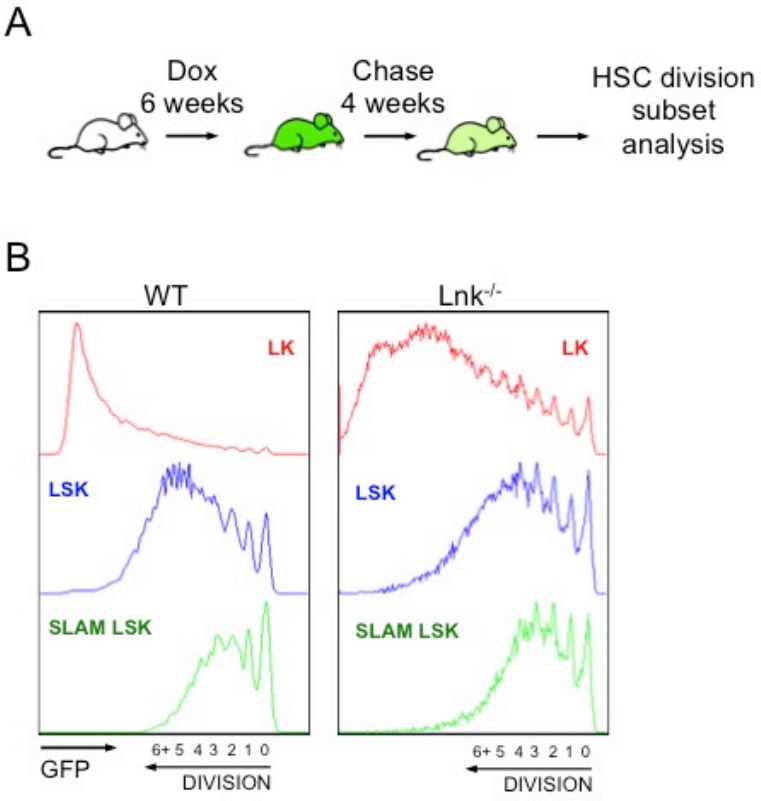
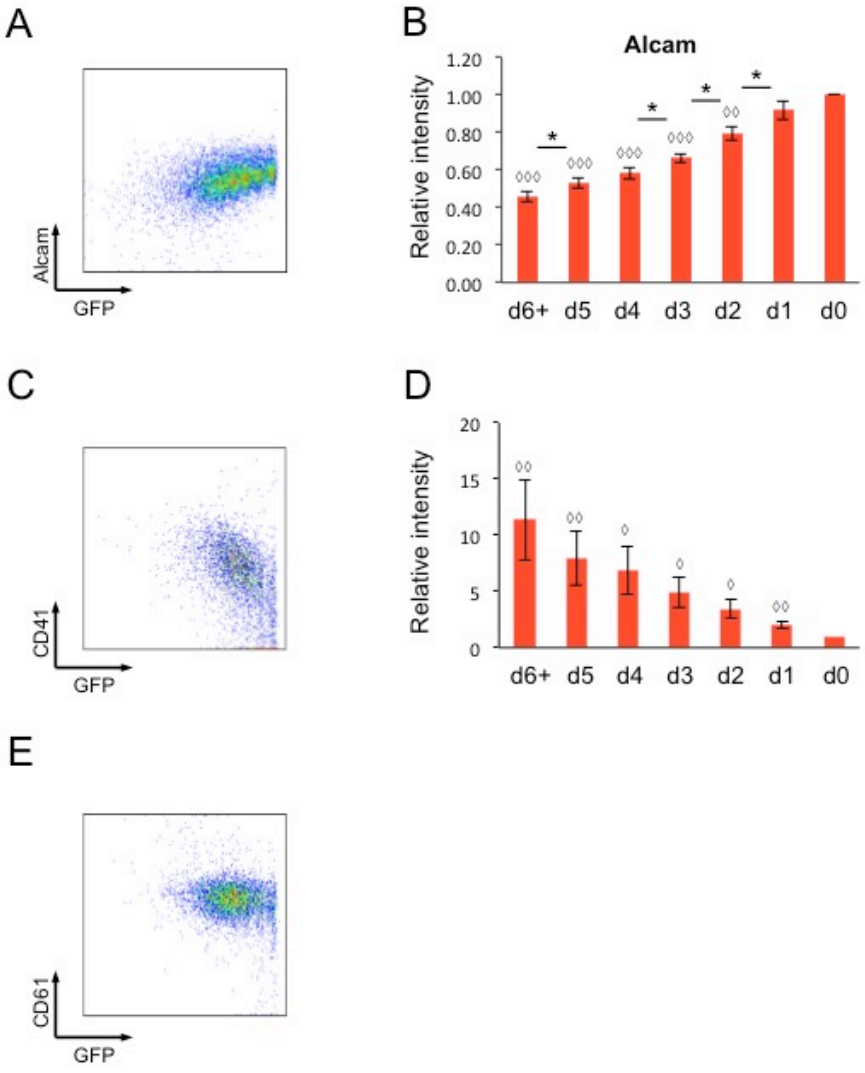

A
Dox
6 weeks
Chase
4 weeks
HSC division
subset
analysis
B
WT
Lnk-/-
LK
LSK
SLAM LSK
GFP
6+ 5 4 3 2 1 0
DIVISION

Fig 3.S2


A
Alcam
GFP
B
Alcam
Relative intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
d6+ d5 d4 d3 d2 d1 d0
C
CD41
GFP
D
Relative intensity
20
15
10
5
0
d6+ d5 d4 d3 d2 d1 d0
E
CD61
GFP

Supplemental Table 1

Column1	d0.1	d0.2	d2.1	d2.2	d4.1	d4.2	d5.1	d5.2
Input	60092817	54388515	54998450	56058827	62443383	53068567	55737730	50455160
Uniquely mapped	52186975	48288308	49961374	56058827	55879617	48535930	50113220	44291345
Multimapped ok	2252102	1678200	1364102	1477042	1915741	1287215	1679717	1739231
sum	54439077	49966508	51325476	57535869	57795358	49823145	51792937	46030576
% input	90.59%	91.87%	93.32%	102.63%	92.56%	93.88%	92.92%	91.23%

Supplemental Table 2

	Marker	Supplier	Clone	Conjugation	Cat#
HSC FACS and sorting	c-kit	eBio	2B8	APC	17-1171-83
	c-kit	eBio	2B8	APC-Cy7	47-1171-82
	Sca1	eBio	D7	Per-CP-Cy5.5	45-5981-82
	CD48	BioLegend	HM48-1	APC	
	CD150	BioLegend	TC15-12F12.2	PE-Cy7	115914
	CD34	eBio	RAM34	eFluor 660	50-0341-82
	Flk2	BD	A2F10.1	PE	553842
Peripheral Blood FACS	CD45.1	eBio	A20	PE-Cy7	25-0453-82
	CD45.2	BD	104	FITC	553772
	CD19	eBio	eBio1D3	APC	17-0193-82
	CD3	eBio	145-2C11	PE	12-0031-85
	Gr1	eBio	RB6-8C5	PE	12-5931-85
	Mac1	eBio	M1/70	APC	17-0112-83

3.7 Figure Legends

Figure 3.1: The HSC pool is a continuum of HSC subtypes with distinct cell cycle kinetics and self-renewal/ differentiation properties. (**A**) Peripheral blood analysis 16 weeks after transplant of 200 WT SLAM LSK (gray) or 100 *Lnk-/-* SLAM LSK (red) into lethally irradiated recipients together with 400k competitor cells is shown. For all transplant data, each symbol represents one animal, black bars represent the mean of each group. Horizontal lines indicate direct comparison. ‡ indicates comparison to WT d0 and ◊◊◊ indicates comparison to *Lnk-/-* d0. ‡ or ◊ signify $p<0.05$, ‡‡ or ◊◊ signify $p<0.01$, and ‡‡‡ or ◊◊◊ signify $p<0.001$. (**B**) Analysis of peripheral blood 16 weeks after secondary transplant of 1 million total BM from primary recipients is shown. (**C**) The donor derived SLAM LSK frequency in bone marrow of primary recipients after 16 weeks is shown. Donor frequency was calculated as follows: (donor%)*(SLAM LSK%). (**D**) Lineage composition of donor derived cells is shown in Myeloid (M), B cells (B) and T cells (T) in the peripheral blood of primary recipients 16 weeks after transplant. Mice with donor reconstitution below 1% in all lineages were excluded from this analysis. All comparisons are not significant. (**E**) Lineage composition of donor-derived cells is shown in M, B, and T cells in the peripheral blood of secondary recipients 16 weeks after transplant.

Figure 3.2: Cell intrinsic properties conferred by in vivo division are maintained *in vitro*. (**A**) The division status of SLAM LSK in *Lnk-/-* mice after six weeks of chase was assessed by flow cytometry. The distribution of cells by division is shown, n=3. (**A, D, E**) Bars indicate means, and vertical lines indicate SE. (**B**) 50 SLAM LSK in division subsets d0, d2, and d4 were transplanted into lethally irradiated recipients together with 350,000 total bone marrow competitor cells. Shown is donor reconstitution of peripheral blood 16 weeks after transplant. (**C**) Individual cells were sorted into wells of round bottom 96 well plates and visualized every two hours for division. Time of division was noted as the first hour during which two individual cells were visible in a well. Median time to first division is charted by dotted lines. Each symbol represents the average number of cells per well. d0, d1, d2, and d4 are indicated by a diamond, cross, square and circle, respectively. The number wells counted is n=93, n=86, n=90, n=88 for d0, d1, d2, and d4 respectively. (**D**) The mean time to first division is quantified, and the numerical value is written in each bar. (**E**) The number of hours between the first and second *in vitro* division is quantified. (**F**) The average number of cells per well throughout the 72h observation period is shown.

Figure 3.3: Lnk HSCs have slower division kinetics *in vitro*. (**A**) Individual cells in division 0, 2 or 4 from WT and *Lnk-/-* mice were sorted into round bottom 96 well plates and visualized for division every two hours. Cells were tracked for 72h. Time to first division in hours is shown, plotted as the percent of wells in which the single cell has divided at least once. Time of division was noted as the first hour during which two individual cells were visible in a well. Median time to first division is charted by dotted lines. Each symbol represents the average number of cells per well. Only seven time points are displayed for clarity. n=3 for *Lnk-/-* and n=1 for WT, results from one paired experiment are shown. d0, d2 and d4 cells are indicated by diamonds, triangles and circles, respectively. The number wells counted for WT cells is n=82, n=86, n=89 for d0, d2, and d4 respectively. The number wells counted for *Lnk-/-* cells is n=84, n=83, n=81 for d0, d2,

and d4 respectively. (**B**) The time in hours to first division for d0, d2, and d4 from WT and Lnk-/- mice cells is shown. (**B, C**) Bars represent the mean and vertical lines represent SE throughout all graphs. The numerical value of the mean is displayed inside each bar. The result of statistical comparison of means by two-tailed student's t-test is indicated above the bars, ns indicates not significant p value, * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$. (**C**) displays the mean number of hours between the 1st and 2nd divisions *in vitro* for d0 and d4 cells from WT and *Lnk-/-* mice. (**D**) Cell number over the 72h observation period is plotted for d0, d2, and d4 cells from WT and *Lnk-/-* mice.

Figure 3.4: **Genome wide gene expression correlates with HSC division continuum**. (**A**) shows unsupervised clustering using Spearman correlation among biological replicates and samples from d0 (purple), d2 (green), d4 (orange), and d5+ (teal) SLAM LSK subsets. (**B**) Differential gene expression was calculated using DESEq2 and the number of significantly changed genes from each comparison is shown in the table. (**C**) K-means clustering analysis shows four clusters of differentially expressed genes. Each gene is scaled relative to expression across subsets by Z-score. Clusters are indicated by vertical lines. Color indicates level of gene expression; low expression is colored in blue, while high expression is colored in red. Division subsets are indicated at the bottom of the heatmap, biological replicates are indicated by horizontal lines. (**D**) shows top 15 Gene Ontology terms for each Cluster. GO terms are ranked by fold enrichment. (**E, F**) GSEA enrichment for each pairwise comparison between division subsets is shown for Hallmark Genesets (h.all.v6.0).

Figure 3.5: HSC in vivo division history best represents HSCs. (**A**) CD150 intensity is plotted against GFP intensity in CD48-LSK cells from *Lnk-/-* mice. Clear CD150 positive and negative fractions are visible in division zero. n= 16, one representative image is shown. (**B**) CD34 intensity is plotted in CD48- LSK. n=5, one representative image is shown. The gray dotted line represents the cutoff between positive and negative set by a fluorescence minus one control. Dotted fuchsia lines indicate sort gates. (**C, D, F**) CD48- LSK were gated on division 0 and additionally selected by CD150, CD34 or CD49b as indicated in each panel. 20 double-sorted cells were transplanted into lethally irradiated recipients together with 400,000 Sca-1 depleted competitor cells. Donor reconstitution in peripheral blood is shown 16 weeks after transplant. (**C**) shows donor reconstitution for CD150 positive or negative CD48- LSK from WT or *Lnk-/-* donors. (**D**) shows donor reconstitution for CD34 positive or negative CD48- LSK from WT donors. (**E**) CD49b is plotted against GFP in SLAM LSK cells. n=6, one representative image is shown. (**F**) shows donor reconstitution for CD49b positive or negative SLAM LSK from WT or *Lnk-/-* donors. In addition, CD49b positive or negative SLAM LSK cells in division 4 were transplanted from *Lnk-/-* donors.

Figure 3.6: Lnk deficiency sustains stem cell markers throughout division. (A, C, E, G) Surface marker intensity was plotted against GFP in SLAM LSK cells from bone marrow of WT and *Lnk-/-* mice. (**B**, **D, F, H**) The geometric mean was calculated for each division subset and normalized relative to the intensity at division 0. Bars represent means and vertical lines represent SE. Statistical comparisons between WT and *Lnk-/-* cells with $p \leq 0.05$ are indicated above the bars. * indicates $p<0.05$, and ** indicates $p<0.01$. Comparisons with d0 within each genotype is

indicated by a double cross (‡) or diamond (◊) for WT or *Lnk-/-,* respectively. ◊ or ‡ indicate $p<0.05$, ◊◊ or ‡‡ indicate $p<0.01$, and ◊◊◊ or ‡‡‡ indicate $p<0.001$. Four recognized stem and progenitor -associated cell surface markers were analyzed. (**A)** The intensity of c-kit is plotted and (**B**) quantified, n= 9 for *Lnk-/-* and n=5 for WT. (**C**) The intensity of Sca-1 is plotted and (**D**) quantified, n= 7 for *Lnk-/-* and n= 5 for WT. (**E**) The intensity of CD150 is plotted and (**F**) quantified, n=16 for *Lnk-/-,* n=5 for WT. (**G**) The intensity of CD48 is plotted and (**H)** quantified, n=8 for *Lnk-/-* and n=5 for WT.

Figure 3.S1: H2B-GFP captures the heterogeneous state of division of SLAM LSK cells. (**A**) H2B-GFP expression is induced by doxycycline administered in drinking water during "pulse" period for 6 weeks. Signal is diluted during "chase" period for four weeks. Signal retention is then examined in bone marrow. (**B**) Histogram plots demonstrating that label retention by GFP intensity is enriched in stem cell populations. Pictured are LK, LSK and SLAM LSK populations of WT and *Lnk-/-* mice. (**C**) Label retention is quantified in SLAM LSK between WT and *Lnk-/-* mice (n=6, and n=17, respectively). *= $p<0.05$, **=$p<0.01$.

Figure 3.S2: **Division impacts several cell surface markers**. (**A**) shows a representative plot of ALCAM surface expression across division subsets in SLAM LSK of *Lnk-/-* mice. (**B**) Mean of ALCAM intensity is quantified across division subsets relative to division 0. n=4 mice. (**C**) shows a representative plot of CD41 surface expression across division subsets in SLAM LSK of *Lnk-/-* mice. n=4. (**D**) Mean CD41 intensity is quantified across division subsets relative to division 0. (**E**) shows a representative plot of CD61 surface expression across division subsets in SLAM LSK of *Lnk-/-* mice. n=4, one plot is shown. Bars indicate mean. Statistical comparisons between surface marker expression relative to d0 are indicated above bars. ◊ indicates $p<0.05$, ◊◊ indicates $p<0.01$, and ◊◊◊ indicates $p<0.001$. Individual pair-wise comparisons are indicated by a horizontal bar, and $p<0.05$ is indicated by *.

Supplemental Table 1: **High quality sequencing reads are obtained from input.** Table shows total reads, uniquely and multiple mapped reads, and mapped reads as percentage of total reads for each replicate and sample submitted for RNA sequencing.

Supplementary Table 2: Antibody clones and suppliers

AUTHOR CONTRIBUTIONS

JB, and WT designed experiments and interpreted results. JB and WT wrote and edited the manuscript. JB, NH, RD, and AB performed experiments.

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3.8 References

1. Ema, H. *et al.* Quantification of Self-Renewal Capacity in Single Hematopoietic Stem Cells from Normal and Lnk-Deficient Mice. *Developmental Cell* **8,** 907–914 (2005).

2. Foudi, A. *et al.* Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol* **27,** 84–90 (2008).

3. Wilson, A. *et al.* Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell* **135,** 1118–1129 (2008).

4. Kiel, M. J. *et al.* SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. *Cell* **121,** 1109–1121 (2005).

5. Oguro, H., Ding, L. & Morrison, S. J. SLAM Family Markers Resolve Functionally Distinct Subpopulations of Hematopoietic Stem Cellsand Multipotent Progenitors. *Stem Cell* **13,** 102–116 (2013).

6. Uchida, N., Dykstra, B., Lyons, K. J., Leung, F. Y. K. & Eaves, C. J. Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Experimental Hematology* **31,** 1338–1347 (2003).

7. Miller, C. L. & Eaves, C. J. Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc Natl Acad Sci USA* **94,** 13648–13653 (1997).

8. Dykstra, B. *et al.* High-resolution video monitoring of hematopoietic stem cells cultured in single-cell arrays identifies new features of self-renewal. *Proc Natl Acad Sci USA* **103,** 8185–8190 (2006).

9. Dykstra, B. *et al.* Long-Term Propagation of Distinct Hematopoietic Differentiation Programs In Vivo. *Cell Stem Cell* **1,** 218–229 (2007).

10. Laurenti, E. *et al.* CDK6 Levels Regulate Quiescence Exit in Human Hematopoietic Stem Cells. *Stem Cell* **16,** 302–313 (2015).

11. Macaulay, I. C. *et al.* Single-Cell RNA-Sequencing Reveals a Continuous Spectrum of Differentiation in Hematopoietic Cells. *CellReports* **14,** 966–977 (2016).

12. Tsang, J. C. H. *et al.* Single-cell transcriptomic reconstruction reveals cell cycle and multi-lineage differentiation defects in Bcl11a-deficient hematopoietic stem cells. *Genome Biology* **16,** 1–16 (2015).

13. Wilson, N. K. *et al.* Combined Single-Cell Functional and Gene Expression Analysis Resolves Heterogeneity within Stem Cell Populations. *Stem Cell* **16,** 712–724 (2015).

14. Passegue, E. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *Journal of Experimental*

Medicine **202,** 1599–1611 (2005).

15. Hüttmann, A., Liu, S. L., Boyd, A. W. & Li, C. L. Functional heterogeneity within rhodamine123(lo) Hoechst33342(lo/sp) primitive hemopoietic stem cells revealed by pyronin Y. *Experimental Hematology* **29,** 1109–1116 (2001).

16. Cheung, T. H. & Rando, T. A. Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol* **14,** 329–340 (2013).

17. Bowman, T. V. *et al.* Differential mRNA processing in hematopoietic stem cells. *Stem Cells* **24,** 662–670 (2006).

18. Forsberg, E. C. *et al.* Differential expression of novel potential regulators in hematopoietic stem cells. *PLoS Genet* **1,** e28 (2005).

19. Leiva, M., Quintana, J. A., Ligos, J. M. & Hidalgo, A. Haematopoietic ESL-1 enables stem cell proliferation in the bone marrow by limiting TGFβ availability. *Nature Communications* **7,** 10222 (2016).

20. Cabezas-Wallscheid, N. *et al.* Vitamin A-Retinoic Acid Signaling Regulates Hematopoietic Stem Cell Dormancy. *Cell* 1–37 (2017). doi:10.1016/j.cell.2017.04.018

21. Jeannet, R., Cai, Q., Liu, H., Vu, H. & Kuo, Y.-H. Alcam Regulates Long-Term Hematopoietic Stem Cell Engraftment and Self-Renewal. *Stem Cells* **31,** 560–571 (2013).

22. Matsuoka, Y. *et al.* Low level of c-kit expression marks deeply quiescent murine hematopoietic stem cells. *Stem Cells* **29,** 1783–1791 (2011).

23. Shin, J. Y., Hu, W., Naramura, M. & Park, C. Y. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J Exp Med* **211,** 217–231 (2014).

24. Grinenko, T. *et al.* Clonal expansion capacity defines two consecutive developmental stages of long-term hematopoietic stem cells. *Journal of Experimental Medicine* **11,** R106 (2014).

25. Weksberg, D. C., Chambers, S. M., Boles, N. C. & Goodell, M. A. CD150- side population cells represent a functionally distinct population of long-term hematopoietic stem cells. *Blood* **111,** 2444–2451 (2008).

26. Rodgers, J. T. *et al.* mTORC1 controls the adaptive transition of quiescent stem cells from G0 to GAlert. *Nature* **510,** 393–396 (2014).

27. He, S. *et al.* Transient CDK4/6 inhibition protects hematopoietic stem cells from chemotherapy-induced exhaustion. *Science Translational Medicine* **9,** eaal3986 (2017).

28. Ferkowicz, M. J. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* **130,** 4393–4403 (2003).

29. Emambokus, N. R. & Frampton, J. The Glycoprotein IIb Molecule Is Expressed on Early

Murine Hematopoietic Progenitors and Regulates Their Numbers in Sites of Hematopoiesis. *Immunity* **19,** 33–45 (2003).

30. Umemoto, T. *et al.* Integrin-αvβ3 regulates thrombopoietin-mediated maintenance of hematopoietic stem cells. *Blood* **119,** 83–94 (2012).

31. Devalliere, J. *et al.* LNK (SH2B3) is a key regulator of integrin signaling in endothelial cells and targets -parvin to control cell adhesion and migration. *The FASEB Journal* (2012). doi:10.1096/fj.11-193383

32. Takizawa, H. *et al.* Lnk regulates integrin alphaIIbbeta3 outside-in signaling in mouse platelets, leading to stabilization of thrombus development in vivo. *J Clin Invest* **120,** 179–190 (2010).

33. Purton, L. E., Bernstein, I. D. & Collins, S. J. All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. *Blood* **95,** 470–477 (2000).

34. Levi, B. P., Yilmaz, O. H., Duester, G. & Morrison, S. J. Aldehyde dehydrogenase 1a1 is dispensable for stem cell function in the mouse hematopoietic and nervous systems. *Blood* **113,** 1670–1680 (2009).

35. Si, J. & Collins, S. J. IL-3-induced enhancement of retinoic acid receptor activity is mediated through Stat5, which physically associates with retinoic acid receptors in an IL-3-dependent manner. *Blood* **100,** 4401–4409 (2002).

36. Takano, H., Ema, H., Sudo, K. & Nakauchi, H. Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. *J Exp Med* **199,** 295–302 (2004).

37. Morrison, S. J. & Kimble, J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441,** 1068–1074 (2006).

38. Busch, K. *et al.* Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature* **518,** 542–546 (2015).

39. Winkler, I. G. *et al.* Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nature Medicine* **18,** 1651–1657 (2012).

40. Norozi, F., Shahrabi, S., Hajizamani, S. & Saki, N. Regulatory role of Megakaryocytes on Hematopoietic Stem Cells Quiescence by CXCL4/PF4 in Bone Marrow Niche. *Leukemia Research* **48,** 107–112 (2016).

41. Bruns, I. *et al.* Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nature Medicine* **20,** 1315–1320 (2014).

42. Zhao, M. *et al.* Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. *Nature Medicine* **20,** 1321–1326 (2014).

43. Zimdahl, B. *et al.* Lis1 regulates asymmetric division in hematopoietic stem cells and in

leukemia. *Nat Genet* **46,** 245–252 (2014).

44. Signer, R. A. J., Magee, J. A., Salic, A. & Morrison, S. J. Haematopoietic stem cells require ahighly regulated protein synthesis rate. *Nature* 1–16 (2014). doi:10.1038/nature13035

45. Spitzer, M. H. & Nolan, G. P. Mass Cytometry: Single Cells, Many Features. *Cell* **165,** 780–791 (2016).

46. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29,** 15–21 (2013).

47. Anders, S., Pyl, P. T. & Huber, W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31,** 166–169 (2015).

48. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15,** 31–21 (2014).

49. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* **102,** 15545–15550 (2005).

50. Mootha, V. K. *et al.* PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* **34,** 267–273 (2003).

CHAPTER 4 Discussion

Together this body of work investigates the impact of *Lnk* deficiency in HSCs. In chapter 2, we show that in context of FA bone marrow failure, *Lnk* deficiency rescues the functional HSPC defects associated with *Fancd2* deficiency and crucially, mitigates genome instability. The second is an investigation of the mechanism underlying increased HSC self-renewal in *Lnk-/-* HSCs. The findings presented here shed light on the mechanism of Lnk function and impact on HSC biology and offer a potential opportunity to manipulate HSCs through transient incapacitation of LNK.

4.1 The impact of Lnk deficiency on FA

4.1.1 Summary of research findings and conclusions

There is much effort devoted to elucidating the roles of known FA proteins and identifying new members. As more information becomes available, it becomes increasingly clear that FA family members participate in a diverse set of roles. FANCC has been shown to mediate an extra-nuclear role in detoxifying reactive oxygen species [1]. Equally importantly, FANCC and FANCD2 have also been reported to promote autophagy through which they exert regulation over protein quality control and this role independently contributes to cell viability [2]. FANCD2 is also important in resolving microbridges during anaphase and preventing microsattelite nuclei [3 4 5]. The impact of these emerging roles for FA proteins is less clear, but the roles are as varied as they are numerous.

Though BMF in FA is currently treatable by BMT, a more permanent approach towards FA phenotype correction, of course, is through gene editing to directly correct the causative mutations. Recent advances in genome editing techniques, most notably via the bacterial-derived CRISPR-Cas9 system, make genome editing a possibility in FA cells. However before such an approach can be a viable therapeutic modality, several challenges must be overcome. First, the ability to effect gene correction by CRISPR-Cas9 necessarily proceeds through a homologous recombination, the very mechanism that is defective in many subsets of FA. This creates an obvious roadblock in the efficient and safe integration of gene correcting sequences into the genome. Second, cells that can be removed from the body, modified, and then returned- such as HSCs- may be effectively targeted by a gene editing mechanism. Unfortunately, most cells would be not be targetable through this approach, leaving them still vulnerable to the hallmark FA-associated susceptibility to cancer. Epithelial head and neck, cancers, for example, have been documented in 5% of FA patients in the FA database [6] and the probability of solid tumor development is 76% in patients without leukemia or aplastic anemia [6]. Together these drawbacks of direct gene correction therapy demonstrate the importance of independent genome instability –reducing interventions.

Two main studies demonstrate improvement in FA manifestations by non-curative means. The first study demonstrates that apoptosis in FA cells is p53 dependent [7]. p53 is basally activated in FA cells from patients as well as in BM cells from Fancg deficient mice. p53 mediates a cellular response that involves G1/G0 arrest in FA cells and

ultimately activates cell death through apoptosis. Predictably, knockdown of p53 improves cell survival, which is also correlated with a rescue in LSK frequency in FancD2 deficient mice. What is especially noteworthy is that human cells with knockdown of p53 and FancD2 show improved reconstitution of recipients over reconstitution by FancD2 knockdown cells. It is important to note, however, that the reconstitution decreases over time, indicating cell exhaustion. Though p53 deletion as a treatment for FA is of course out of the question due to the genomic instability and cancer predisposition conferred by p53 inactivation, this study demonstrates for the first time that overcoming the consequences of accumulating DNA damage alone has significant impact on FA HSC function and lays the groundwork for the feasibility of rescuing HSPCs function in the context of FA without bone marrow transplantation or direct gene correction.

The second study addresses another signaling arm that is over-activated in FA cells: TGFβ. Here, the findings also demonstrate that overcoming the cellular consequences of FA deficiency is sufficient to improve HSC function [8]. Small molecule inhibition or genetic deletion of key TGFβ components results in suppression of bone marrow failure after forced *in vivo* HSC proliferation and a rescue in colony forming capacity. Importantly, the amount of DNA damage after forced *in vivo* proliferation is decreased after TGFβ blockade, as evidenced by γH2AX accrual. Additionally, suppression of TGFβ shifts DNA repair towards HR utilization by restoring transcription of HR component genes, thereby increasing the odds of error-free DNA repair in FANCD2 deficient cells and improving genome stability.

Our findings parallel the conclusions that non-curative interventions can dramatically improve FA HSC function. In the context of FANCD2 deficiency, we demonstrate robust restoration of hematopoietic stem cell function in a rigorous serial transplantation assay by deletion of Lnk. Both HSC numbers and transplantability are restored to WT levels, simultaneously highlighting the importance of replication-associated stress in BMF and the impact of non-curative treatments on ameliorating the manifestations of FA. Crucially, in context of chronic proliferation induced by pI:pC we also demonstrate LNK deficiency rescues FA HSC reconstitution potential. Importantly, LNK deficiency reduces genome instability, and this is through augmenting stalled replication fork stability – a direct rescue of a FANCD2 function. In combination with another approach capable of either directly restoring crosslink repair or detoxifying damaging intermediates, Lnk deficiency highlights potential targets of therapeutic interventions in FA.

4.1.2 Future Perspectives

The role of LNK in human FA cells remains undertermined. This question deserves immediate attention. In humans, inactivating mutations in LNK protein are associated with JAK2-dependent neoplasms [9] or JAK2-independent erythrocytosis [10,11], indicating a similar function in suppression of cell proliferation in humans as in mice. Additionally, human HSPCs modified to suppress LNK expression show increased erythroid cell production [12], demonstrating that LNK does play a role in regulating human HSPCs. The role of LNK deficiency in the expansion and self-renewal of human HSCs is ongoing in

the lab, as well as a parallel study on the impact of Lnk deficiency on HSC function and genome stability of human FA HSCs.

Given the unexpected impact of LNK deficiency on replication fork stability in *FancD2-/-* cells, and by extension of cytokine signaling, it is easy to imagine that cytokine signaling might impact other functions of FA proteins. Our data conclusively rules out an impact on crosslink repair, but the impact of LNK deficiency on redox status and autophagy remain unexplored. In genome-wide profiling of LT-HSCs, we have previously shown an alteration in metabolic gene pathways in *Lnk-/-* HSCs, including enrichment in antioxidant genes [13]. To that end, the role of Lnk deficiency in context of *Fancg*-/- or *Fancc*-/- will yield a definitive answer. Independent activity on redox homeostasis could be another way by which LNK deficiency prevents HSPC attrition, and was not explored in this work. If this is the case, however, then suppression of LNK would be a useful way to decrease DNA damage in FA cells.

Cytokine signaling may play a role in genome-protection more broadly than only between LNK and FA. This role would not be unprecedented. TPO treatment increases radioprotection in IR-treated BM cells while LNK inhibits it [14 15]. Given the role of cytokine signaling in protecting stalled replication forks it would be interesting to speculate whether Lnk deficiency could mitigate genome instability in context of deficiency of other fork-associated components, particularly BRCA2. BRCA2 mutated cells show high levels of genome instability, decrease in which decreases the risk for development of cancer [16 17 18].

In this regard, it would be especially important to investigate whether LNK deficiency can impact genome instability in extra-hematopoietic tissues. In BM, Lnk is expressed in several progenitor populations [19] but Lnk is also expressed in other progenitor populations, which include the brain and adipocyte progenitors [20 21]. In brain, LNK deficiency is associated with improved proliferation of brain neural precursor cells after injury [20].

Together such findings would add to the case for developing a way to suppress LNK in vivo as a therapeutic intervention.

4.2 Cell autonomous changes throughout *in vivo* division and role of LNK in regulating self-renewal

4.2.1 Summary of research findings and conclusions

Self-renewal of HSCs remains an open topic in stem cell biology both for its enigmatic mechanism and for its potential therapeutic applications. Though several key players have been discovered, a thorough understanding of how self-renewal in HSCs occurs is still missing. Deletion of key self-renewal components often leads to extreme consequences for HSCs. As mentioned in the introduction, deletion of TPOR, for example, leads to rapid depletion of HSCs, and impaired reconstitution potential. At the other extreme, some deletions preserve self-renewal at the cost of differentiation, such as is the case for DNMT3 [22,23]. Others deletions or gains of function such as JAK2V617F enhance self-renewal and initiate malignant transformation. In this context, LNK

deficiency offers a unique perspective into self-renewal because it simultaneously increases self-renewal capacity in HSCs without impinging on their capacity to differentiate and therefore provides an insight into studying self-renewal in a way that results in enhanced HSC function without impairment.

HSCs are different from progenitors transcriptionally. Several studies have reported enrichment in mitogenic and proliferative signaling pathways in progenitors, setting HSCs apart by their quiescence [24] [25] [26]. More recently, single cell profiling has more finely dissected these findings. One study sequenced single cells from populations enriched for stem cells [27]. Their surface expression profiles during sorting were indexed and later compared against the transplantability of cells with identical surface profiles. This enabled an extrapolation of correlation between gene expression profile and functional output at a single cell level. Independent clustering revealed a common gene set associated with HSC function. Interestingly, these cells showed higher than average Sca1, and CD150 expression and lower than average CD48 expression, which correlates well with our d0 expression profile.

Another profiling study, in which single HSCs were profiled based on label retention and surface markers against non-label retaining cells, also makes similar conclusions. This study demonstrates that dormant HSCs and activated HSCs exist in a continuum of states, aligned along a biosynthetic axis [28]. Dormant HSCs are identified as label retaining and SLAM LSK, while active HSCs are non-label retaining in SLAM LSK, which is essentially like our profiling of d0 vs d5+ SLAM LSK. Indeed our findings are in

extensive agreement with this study. Interestingly, the study finds that all-trans retinoic acid maintains dormancy in HSCs. Changing trends in retinoic acid metabolism are also reflected in our gene expression profiling and represents an intriguing target for manipulation of self-renewal by LNK deficiency. Our study builds on this information by even more finely dissecting transcriptional profiles by each in vivo division and begins to investigate the relationship between cells after successive divisions.

4.2.2 Future Perspectives

Transcriptional profiling alone falls short of describing self-renewal completely. We have previously transcriptionally profiled the bulk SLAM LSK population and the resulting information was disappointingly scant: transcriptionally, *Lnk-/-* SLAM LSK are very similar to their WT counterparts. As is reflected in our data, fold change in expression of differentially expressed genes from cells across division subsets, where function vastly varies, actually vary only by a small amount. Interestingly, transcriptional profiles are also relatively stable through aging [29,30]. This is true for LNK deficient HSCs as well [13]. Whether the transcriptional changes we see are drivers or consequences of a cell fate decision is not distinguishable. The question arises, then, to what extent regulation of HSC self-renewal is post-transcriptional.

There is mounting evidence that at least in part, self-renewal determination might be made at the post-transcriptional level. Self-renewing divisions are associated with asymmetrical inheritance of fate by asymmetrical inheritance of proteins in daughter

cells [31] [32] [33] [34]. We have previously shown that LNK binds a deubiquitinating complex, BRISC [35]. JAK2 is marked by K63-linked ubiquitin after TPO stimulation, which serves as a mark for signaling activation. BRISC disassembles K63-linked ubiquitin chains, which attenuates JAK2 signal transduction. The cellular distribution of JAK2 at division has not been explored, but it is certainly possible that loss of Lnk could affect the distribution of Jak2 during cell division.

Another, still emerging, self-renewal driver might be metabolic. The substrate for palmitoylation and the membrane anchors that establish polarity are metabolic intermediates. The availability of these intermediates can determine function. Cholesterol, for example, accumulates into local densities known as lipid rafts. These areas of membrane are stiffer than regular phospholipids in the membrane. Lipid rafts serve as organizing centers for signal transduction, and lipid rafts promote TPOR and c-kit clustering, sensitizing cells to signal transduction [36] [37]. Furthermore, decreasing cholesterol level in HSCs suppresses their ability to proliferate and mobilize [38,39]. Additionally, lipid composition has been shown to participate in orienting the cell division axis, which provides fate cues [40]. Moreover, In our bulk SLAM LSK gene profiling, metabolic processes related to glycolysis, cholesterol synthesis and redox status dominated the enrichment categories in Lnk deficient HSCs [13].

Finally, microenvironment may contribute to self-renewal choices. Several studies show the influence of niche interactions in cell fate decision, with daughter cells poised for differentiation dividing away from the niche [32]. In this sense, cell surface proteins such as

integrins may help to determine whether a cell will be capable of receiving a self-renewal signal to begin with. If Lnk deficiency stabilizes cell surface expression of HSC markers, perhaps LNK deficiency improves the ability of HSCs to receive those self-renewal signals.

Relatedly, another open question in *Lnk-/-* HSC biology is the localization of the expanded HSC compartment. It remains unknown whether *Lnk-/-* HSCs crowd the same number of niches or whether they simply create new ones. It would be interesting to determine whether *Lnk -/-* HSCs have enhanced self-renewal due to co-opting niches.

4.3 Lnk deficiency as a mode of therapeutic intervention in HSCs

Taken together, increased genome stability and enhanced self-renewal and repop

ulation advantages make Lnk deficiency an attractive target for improving efficiency of gene therapy or transplant. Currently, two main challenges limit gene therapy: low engraftment after transplant and low editing efficiency [41] [42]. Given our findings, LNK deficiency may help address both issues.

A second important application for *LNK -/-* HSCs is to improve engraftment and reconstitution during transplant. Myeloablative conditioning is a hard on recipients, especially in context of genome instability as in FA, where clearing the bone marrow space needs to be balanced against producing excess damage in other tissues. With the advantage LNK deficiency provides, transplants in which LNK is temporarily inhibited

might be able to produce a more robust reconstitution in recipients, and might reduce the need for severe conditioning. Of course, how LNK deficiency impacts human HSCs remains to be seen.

Additionally, LNK is not considered a classically “druggable” target in itself since it lacks kinase activity. Therefore this highlights the need for further unraveling the mechanism of HSC fitness and expansion in LNK deficiency in order to discover such a target, or to look towards alternative strategies that might temporarily target LNK protein expression, such as inducible shRNAs or modified antisense oligos.

4.5 References

1. Saadatzadeh, M. R., Bijangi-Vishehsaraei, K., Hong, P., Bergmann, H. & Haneline, L. S. Oxidant Hypersensitivity of Fanconi Anemia Type C-deficient Cells Is Dependent on a Redox-regulated Apoptotic Pathway. *J Biol Chem* **279,** 16805–16812 (2004).

2. Sumpter, R., Jr *et al.* Fanconi Anemia Proteins Function in Mitophagy and Immunity. *Cell* **165,** 867–881 (2016).

3. Nalepa, G. *et al.* Fanconi anemia signaling network regulates the spindle assembly checkpoint. *J Clin Invest* **123,** 3839–3847 (2013).

4. Vinciguerra, P., Godinho, S. A., Parmar, K., Pellman, D. & D'Andrea, A. D. Cytokinesis failure occurs in Fanconi anemia pathway–deficient murine and human bone marrow hematopoietic cells. *J Clin Invest* **120,** 3834–3842 (2010).

5. Abdul-Sater, Z. *et al.* FANCA safeguards interphase and mitosis during hematopoiesis in vivo. *Experimental Hematology* **43,** 1031–1046.e12 (2015).

6. Alter, B. P. Cancer in Fanconi anemia, 1927-2001. *Cancer* **97,** 425–440 (2003).

7. Ceccaldi, R. *et al.* Bone Marrow Failure in Fanconi Anemia Is Triggered by an Exacerbated p53/p21 DNA Damage Response that Impairs Hematopoietic Stem and Progenitor Cells. *Stem Cell* 1–14 (2012). doi:10.1016/j.stem.2012.05.013

8. Zhang, H. *et al.* TGF-β Inhibition Rescues Hematopoietic Stem Cell Defects and Bone Marrow Failure in Fanconi Anemia. *Stem Cell* **18,** 1–34 (2016).

9. Campregher, P. V., Santos, F. P. de S., Perini, G. F. & Hamerschlak, N. Molecular biology of Philadelphia-negative myeloproliferative neoplasms. *Rev Bras Hematol Hemoter* **34,** 150–155 (2012).

10. Spolverini, A. *et al.* Infrequent occurrence of mutations in the PH domain of LNK in patients with JAK2 mutation-negative 'idiopathic' erythrocytosis. *Haematologica* **98,** e101–2 (2013).

11. Lasho, T. L., Pardanani, A. & Tefferi, A. LNK mutations in JAK2 mutation-negative erythrocytosis. *N Engl J Med* **363,** 1189–1190 (2010).

12. Giani, F. C. *et al.* Targeted Application of Human Genetic Variation Can Improve Red Blood Cell Production from Stem Cells. *Stem Cell* **18,** 73–78 (2016).

13. Bersenev, A. *et al.* Lnk deficiency partially mitigates hematopoietic stem cell aging. *Aging Cell* no–no (2012). doi:10.1111/j.1474-9726.2012.00862.x

14. de Laval, B. *et al.* Thrombopoietin-Increased DNA-PK-Dependent DNA Repair Limits Hematopoietic Stem and Progenitor Cell Mutagenesis in Response to DNA Damage. *Stem Cell* 1–12 (2012). doi:10.1016/j.stem.2012.10.012

15. Louria-Hayon, I. *et al.* Lnk adaptor suppresses radiation resistance and radiation-

induced B-cell malignancies by inhibiting IL-11 signaling. *Proc Natl Acad Sci USA* **110,** 20599–20604 (2013).

16. Kim, T. M., Son, M. Y., Dodds, S., Hu, L. & Hasty, P. Deletion of BRCA2 exon 27 causes defects in response to both stalled and collapsed replication forks. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **766-767,** 66–72 (2014).

17. D'Andrea, A. D. & Grompe, M. The Fanconi anaemia/BRCA pathway. *Nat. Rev. Cancer* **3,** 23–34 (2003).

18. Fridlich, R., Annamalai, D., Roy, R., Bernheim, G. & Powell, S. N. BRCA1 and BRCA2 protect against oxidative DNA damage converted into double-strand breaks during DNA replication. *DNA Repair* **30,** 11–20 (2015).

19. Bagger, F. O. *et al.* BloodSpot: a database of gene expression profiles and transcriptional programs for healthy and malignant haematopoiesis. *Nucleic Acids Research* **44,** D917–24 (2016).

20. Ahlenius, H. *et al.* Adaptor Protein LNK Is a Negative Regulator of Brain Neural Stem Cell Proliferation after Stroke. *Journal of Neuroscience* **32,** 5151–5164 (2012).

21. Lee, J. H. *et al.* Lnk is an important modulator of insulin-like growth factor-1/Akt/peroxisome proliferator-activated receptor-gamma axis during adipogenesis of mesenchymal stem cells. *Korean J. Physiol. Pharmacol.* **20,** 459–466 (2016).

22. Tadokoro, Y., Ema, H., Okano, M., Li, E. & Nakauchi, H. De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *J Exp Med* **204,** 715–722 (2007).

23. Challen, G. A. *et al.* Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* **44,** 23–31 (2011).

24. Grün, D. *et al.* De Novo Prediction of Stem Cell Identity using Single-Cell Transcriptome Data. *Stem Cell* **19,** 266–277 (2016).

25. Macaulay, I. C. *et al.* Single-Cell RNA-Sequencing Reveals a Continuous Spectrum of Differentiation in Hematopoietic Cells. *CellReports* **14,** 966–977 (2016).

26. Tsang, J. C. H. *et al.* Single-cell transcriptomic reconstruction reveals cell cycle and multi-lineage differentiation defects in Bcl11a-deficient hematopoietic stem cells. *Genome Biology* **16,** 1–16 (2015).

27. Wilson, N. K. *et al.* Combined Single-Cell Functional and Gene Expression Analysis Resolves Heterogeneity within Stem Cell Populations. *Stem Cell* **16,** 712–724 (2015).

28. Cabezas-Wallscheid, N. *et al.* Vitamin A-Retinoic Acid Signaling Regulates Hematopoietic Stem Cell Dormancy. *Cell* 1–37 (2017).

doi:10.1016/j.cell.2017.04.018

29. Chambers, S. M. *et al.* Aging Hematopoietic Stem Cells Decline in Function and Exhibit Epigenetic Dysregulation. *Plos Biol* **5,** e201 (2007).

30. Beerman, I. *et al.* Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proc Natl Acad Sci USA* **107,** 5465–5470 (2010).

31. Faubert, A., Lessard, J. & Sauvageau, G. Are genetic determinants of asymmetric stem cell division active in hematopoietic stem cells? *Oncogene* **23,** 7247–7255 (2004).

32. Morrison, S. J. & Kimble, J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441,** 1068–1074 (2006).

33. Takano, H., Ema, H., Sudo, K. & Nakauchi, H. Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. *J Exp Med* **199,** 295–302 (2004).

34. Zimdahl, B. *et al.* Lis1 regulates asymmetric division in hematopoietic stem cells and in leukemia. *Nat Genet* **46,** 245–252 (2014).

35. Rozenova, K. *et al.* MERIT40 deficiency expands hematopoietic stem cell pools by regulating thrombopoietin receptor signaling. *Blood* **125,** 1730–1738 (2015).

36. Yamazaki, S. *et al.* Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells. *EMBO J* **25,** 3515–3523 (2006).

37. Mahammad, S., Dinic, J., Adler, J. & Parmryd, I. Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation. *BBA - Molecular and Cell Biology of Lipids* **1801,** 625–634 (2010).

38. Yvan-Charvet, L. *et al.* ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation. *Science* **328,** 1689–1693 (2010).

39. Yvan-Charvet, M. W. S. G.-A. A. M. A. S. S. C. R. L. A. T. L. *et al.* Regulation of Hematopoietic Stem and Progenitor Cell Mobilization by Cholesterol Efflux Pathways. *Stem Cell* **11,** 195–206 (2012).

40. Nelson, W. J. Adaptation of core mechanisms to generate cell polarity. *Nature* **422,** 766–774 (2003).

41. Dever, D. P. *et al.* CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. *Nature* **539,** 384–389 (2016).

42. Genovese, P. *et al.* Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* **510,** 235–240 (2014).