WASH is required for the differentiation commitment of hematopoietic stem cells in a c-Myc–dependent manner

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Hematopoiesis is fully dependent on hematopoietic stem cells (HSCs) that possess the capacity to self-renew and differentiate into all blood cell lineages. WASH, Wiskott–Aldrich syndrome protein (WASP) and SCAR homologue (WASH) is involved in endosomal sorting as an actin-nucleating protein. Here, we show that conditional WASH deletion in the hematopoietic system causes defective blood production of the host, leading to severe cytopenia and rapid anemia. WASH deficiency causes the accumulation of long-term (LT)-HSCs in bone marrow and perturbs their differentiation potential to mature blood lineages. Importantly, WASH is located in the nucleus of LT-HSCs and associates with the nucleosome remodeling factor (NURF) complex. WASH assists the NURF complex to the promoter of c-Myc gene through its VCA domain-dependent nuclear actin nucleation. WASH deletion suppresses the transcriptional activation of c-Myc gene and impairs the differentiation of LT-HSCs. WASH acts as an upstream regulator to modulate c-Myc transcription for hematopoietic regulation.

Adult hematopoiesis is the process of generating all blood components, which are renewed quickly during the whole life of a host (Dey et al., 2012; Tsi et al., 2013). This renewal depends on rare multipotent BM-resident hematopoietic stem cells (HSCs). HSCs are critical for lifelong blood generation and remain quiescent, self-renew, and differentiate into all types of mature blood cells (Akashi et al., 2003; Sugimura et al., 2012). HSCs comprise long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). Most of the LT-HSCs are in low cell cycle rates but are able to expand extensively under stress (Scheller et al., 2006). ST-HSCs, without self-renewal ability, are doomed to differentiate and give rise to multiple blood lineages. The hematopoietic system is precisely regulated. Many of these genes lead to disorders of the blood system (Park et al., 2003; Hock et al., 2004; Ito et al., 2004; Wilson et al., 2004; Miyake et al., 2006; Tothova et al., 2007; Lieu and Reddy, 2009; Wang et al., 2009; Rossi et al., 2012; Tsai et al., 2013; Will et al., 2013). However, the molecular mechanisms involved in the balance of self-renewal and lineage commitment of HSCs have not been defined yet.

WASH, Wiskott–Aldrich syndrome protein (WASP) and SCAR homologue (WASH), an actin nucleating factor of WASP family, has been reported to participate in endosomal trafficking by generating forces through actin filaments to facilitate fission of vesicles from mother endosomes (Linardopoulou et al., 2007; Derivery et al., 2009; Gomez and Billadeau, 2009; Hao et al., 2013; Park et al., 2013). During this process, WASH, together with several other components of the SHRC complex (the WASH regulatory complex; Jia et al., 2010), works coordinately with a retromer complex to mediate the retrograde transport from early endosomes to Golgi apparatuses. Meanwhile, WASH is essential for division of recycling endosomes (Derivery et al., 2009). WASH deletion in Drosophila causes abnormality of pupae and no mutant flies survive to adulthood (Linardopoulou et al., 2007). WASH deficiency causes early embryonic lethality.

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at embryonic day 7.5 (Xia et al., 2013). We just demonstrated that WASH is localized in autophagosomes that modulates autophagy induction (Xia et al., 2013). WASH-deficient T cells display normal naive TCR signaling and activation, but had defective proliferation (Piotrowski et al., 2013). However, cell autonomous function of WASH in hematopoiesis is still unknown.

Controlling hematopoiesis requires coordinated genetic and epigenetic modulation. In most cases, chromatin presents a barrier to the association of trans-acting factors with DNA. Epigenetic regulations have evolved to modulate the structure of chromatin, and thus access to DNA. Chromatin remodeling is a prerequisite for eukaryotic gene transcription (Krasteva et al., 2012), which depends on ATP-dependent chromatin remodeling complexes. These complexes are classified into four major subfamilies, including SWI/SNF, ISWI, CHD, and INO80, based on a common SWI2/SNF2-related catalytic ATPase subunit (Clapier and Cairns, 2009). A major member of the ISWI subfamily is the nucleosome remodeling factor (NURF), which specifically targets the chromatin through association with sequence-specific transcription factors and modified histones. The nucleosome remodeling factor (NURF) complex is composed of four subunits in Drosophila (NURF301, NURF38, NURF55, and ISWI) and three subunits in mammalian cells (BPTF, Rbhp4/RBAP46/48, and SNF2L; Alkhatif and Landry, 2011). A study showed that NURF complex is required for thymocyte maturation (Landry et al., 2011). It has been reported that the NURF complex also regulates the canonical Wnt pathway probably through modulating the chromatin structures of targeting genes to make transcriptional regulators more accessible (Song et al., 2009). However, it is unclear whether the NURF complex is involved in HSC development. Here, we show that WASH deficiency causes rapid disruption of hematopoiesis and acute anemia of mutant mice. WASH knockout dramatically perturbs the differentiation potential of LT-HSCs to subsequent progenitors. WASH promotes LT-HSC differentiation via a c-Myc-dependent fashion. WASH assists the NURF complex to the promoter of c-Myc through its nuclear actin nucleating activity.

RESULTS

WASH deletion causes disruption of hematopoiesis

We recently reported that full deletion of WASH led to embryonic lethality at embryonic day (E) 7.5 by crossing WASHfloxflox mice with Ella-Cre mice (Xia et al., 2013). We found that WASH inhibits autophagy via suppression of Beclin 1 ubiquitination. To further explore the in vivo role of WASH in adult mice, we generated ER-Cre+; WASHfloxflox mice (Fig. 1 A), which deleted WASH in adult mice with tamoxifen administration. Interestingly, we observed that adult mice with WASH deletion apparently lost their body weight (Fig. 1 B). Additionally, these mice developed severe anemia (Fig. 1 C). Thus, these observations prompted us to examine the role of WASH in hematopoiesis.

We first examined the expression levels of WASH in the blood system. We purified mouse LT-HSCs, ST-HSCs, multipotent progenitor cells (MPPs), common myeloid progenitor cells (CLPs), common lymphoid progenitor cells (CMPs), megakaryocytic-erythroid progenitor cells (MEPs), granulocytic-monocytic progenitor cells (GMPs), and CD8+ T cells and analyzed them by quantitative RT-PCR and immunofluorescence staining. We found that WASH was expressed in all the isolated blood subpopulations (Fig. 1 D). Among them, WASH was expressed with a highest level in the LT-HSC compartment. Interestingly, WASH was mainly localized in the nucleus of LT-HSCs, while it appeared in both the nucleus and cytoplasm of ST-HSCs (Fig. 1 E). In contrast, WASH was mainly located in the cytoplasm of MPPs. These observations were verified by fractionation of the cytoplasm and the nucleus of these three compartments (Fig. 1 F). To further verify the specificity of anti-WASH antibody and the nuclear localization of WASH in HSCs, we performed the aforementioned immunofluorescence and immunoblotting assays by using three anti-WASH antibodies, including the antibody we generated (Xia et al., 2013), and antibodies from previous studies (Derivery et al., 2009; Gomez and Billadeau, 2009). We obtained similar staining patterns by using these three anti-WASH antibodies (unpublished data). In contrast, WASH displayed vesicle staining in MEFs similar to previous works (Derivery et al., 2009; Gomez and Billadeau, 2009; unpublished data).

To define the in vivo role of WASH in hematopoiesis, we crossed WASHfloxflox mice with MxCre mice to generate MxCrend; WASHfloxflox. After being treated with poly[I:C] three times to induce Cre recombinase expression, WASH was completely deleted in BM of these mice (Fig. 2, A and B; hereafter, poly[I:C] treated MxCrend; WASHfloxflox mice are called KO), and MxCrend littermates with poly[I:C] treatment did not alter WASH expression (poly[I:C]-treated MxCrend; WASHfloxflox mice were referred to as Ctrl). Surprisingly, by 8 wk after deletion, WASH KO mice had white feet and ears, as well as white long bones (Fig. 2 C). Moreover, these mice showed severe anemia, peripheral blood cytopenia, thrombocytopenia, and neutropenia (Table 1). During an 8-wk observation time, nearly 60% of WASH KO mice died (Fig. 2 D), suggesting a critical role of WASH in hematopoiesis.

We observed a dramatic decrease of cellularity in BM and in other lymphoid organs from WASH KO mice (Fig. 2, E and F). Additionally, we found that WASH KO mice dramatically lowered red blood cells and other cell types (Fig. 2 G and Table 1). Because the BM is the primary location of adult hematopoiesis, we further assessed the cellular composition of this organ. Dynamic analysis of BM cellularity showed that blood cells in BM declined gradually after WASH deletion (Fig. 2 H), accompanied by declined lineage+ cells (Fig. 2 I). Additionally, the staining for lineage+ cells with permeabilization displayed similar patterns to those of unpermeabilized samples (Fig. 2 J), indicating the decreased numbers of lineage+ cells in WASH KO are not caused by inefficient trafficking of surface markers we analyzed. Moreover, cells positive for differentiated mature cell markers were significantly decreased in WASH KO mice (Fig. 2 K), and these
mature cells did not undergo apparent apoptosis (Fig. 2 L). These data indicate that WASH KO mice had less differentiated mature cells than those of control mice. Collectively, WASH deficiency causes severe cytopenia and WASH is indispensable for adult hematopoiesis.

**WASH deficiency impairs repopulation capacity of HSCs**

Flk2 (also known as Flt3), a receptor tyrosine kinase, is used to stain LSKs for distinguishing between HSCs and MPPs (Christensen and Weissman, 2001). To test HSCs and progenitors after deletion of WASH, we stained LSK cells (Lin⁻/IL-7Rα⁻/Sca-1⁻/c-Kit⁺/CD34⁻/Flk2⁻) for LT-HSCs, Lin⁻/IL-7Rα⁻/Sca-1⁻/c-Kit⁺/CD34⁺/Flk2⁺ for ST-HSCs, Lin⁻/IL-7Rα⁻/Sca-1⁻/c-Kit⁺/CD34⁺/Flk2⁺ for MPPs, Lin⁻/IL-7Rα⁻/Sca-1⁻/c-Kit⁺ for CLPs, Lin⁻/IL-7Rα⁻/Sca-1⁻/c-Kit⁺/CD34⁺/FcγRII/III⁻ for CMPs, Lin⁻/IL-7Rα⁻/Sca-1⁻/c-Kit⁺/CD34⁺/FcγRII/III⁻ for MEPs, and Lin⁻/IL-7Rα⁻/Sca-1⁻/c-Kit⁺/CD34⁺/FcγRII/III⁻ for GMPs. CD8⁺ T cells were sorted from thymuses as a positive control. mRNA levels of WASH were examined by quantitative RT-PCR and standardized to that of CD8⁺ T cells. (E) The indicated cells were stained with anti-WASH antibody (green) and counterstained with propidium iodide (PI; red). Percentage of cells with the indicated localization was calculated and shown on the right. C, cytoplasm; N, nucleus. (F) WASH was detected in cytoplasmic (C) and nuclear (N) cell lysates. Bar, 5 µm. Data are shown as means ± SD. ***, P < 0.001. Data were repeated for three times with similar results.
We then stained BM cells with several surface progenitor markers to explore which subpopulations of the hematopoietic system were affected. WASH deficiency remarkably reduced early committed precursors of all the hematopoietic lineages.
cytometry. No significant difference was observed in WASH KO and control mice (Fig. 4 D). These data suggest that the accumulation of LT-HSCs after WASH deletion is not caused by increased proliferation or survival. Thus, an alternative possibility for LT-HSC accumulation may be a defect in LT-HSC differentiation. We transplanted BM cells from WASH KO mice (CD45.2) to lethally irradiated CD45.1-recipient mice, and the KO was successfully reconstituted in the recipient mice, followed by analysis of HSCs and progenitor cells. We found that LT-HSCs dramatically accumulated (>15-fold increase) in the reconstituted recipient mice compared with control mice (Fig. 4 E). However, ST-HSCs, MPPs, CLPs, and CMPs were remarkably declined. These observations indicate that WASH-deficient BM cells fail to reconstitute early progenitor cells and lose their differentiation potential to mature hematopoietic cells.

To further determine whether WASH directly affects LT-HSC differentiation in vitro, we cultured WASH KO and control LT-HSCs with a differentiation cytokine cocktail containing mSCF, mTPO, mFlt3L, IL-6, IL-7, IL-11, GM-CSF, and EPO (Wilson et al., 2004). After 1 wk, cells were harvested and analyzed for differentiated cells. In control cultures, morphologically differentiated cells expressing myeloid and lymphoid markers were increased (Fig. 4 F), whereas these differentiated cells were almost undetectable in WASH KO LT-HSCs. To test whether WASH KO LT-HSCs can be rescued by ectopic expression of WASH, LT-HSCs were isolated from the BM of WASH KO mice and transfected with a WASH or empty vector. Importantly, we observed that reintroduction of WASH was able to restore the differentiation defects of WASH KO LT-HSCs (Fig. 4 F). Consequently, WASH expression was successfully restored in the WASH deficient LT-HSCs (Fig. 4 G). Moreover, the accumulation of LT-HSCs also existed in the secondary (Fig. 3 F, G). WASH KO mice we analyzed showed complete deletion of WASH (Fig. S1 B). Reduced committed precursors induced by WASH knockout were not caused by inefficient trafficking of the detected surface markers (Fig. 3 H). However, WASH deficiency only significantly increased the percentage of LT-HSCs (Fig. 3, F and G). We checked the long-term repopulation ability of WASH KO LT-HSCs through competitive repopulation experiments. 10^2 CD45.2 donor LT-HSCs together with 3 × 10^5 CD45.1 BM cells were transplanted into lethally irradiated CD45.1 recipient mice (Fig. 3 I, top). The recipient BM was successfully reconstituted 16 wk after transplantation. However, we observed that donor CD45.2 cells were almost lost in the recipient BM (Fig. 3 I, bottom). Additionally, donor CD45.2 cells were also undetectable in other lymphoid organs of recipient mice (Fig. 3 J). WASH KO mice we checked displayed no WASH expression in BM (Fig. S1 C). The apoptosis frequency of LT-HSCs was unchanged in WASH KO versus control mice (Fig. 2 L). In summary, WASH deficiency leads to an expanded pool of LT-HSCs that are, however, functionally defective in their ability to differentiate into all types of mature lineages.

**LT-HSCs abolish differentiation potential in WASH-deficient KO**

To determine whether WASH modulates HSC self-renewal, we analyzed quiescent and actively cycling cells in the LSK and LT-HSC compartments (Fig. 4 A). WASH was completely deleted in BM of WASH KO mice (Fig. S1 D). In both stem cell–containing populations, there was no obvious alteration of the rates of cells in a resting (G0) or a cycling state (S/G2/M) between WASH KO and control mice (Fig. 4, B and C). To look at the turnover rate of LT-HSCs in vivo, the kinetics of BrdU uptake was examined by flow cytometry. No significant difference was observed in WASH KO and control mice (Fig. 4 D). These data suggest that the accumulation of LT-HSCs after WASH deletion is not caused by increased proliferation or survival. Thus, an alternative possibility for LT-HSC accumulation may be a defect in LT-HSC differentiation. We transplanted BM cells from WASH KO mice (CD45.2) to lethally irradiated CD45.1-recipient mice, and the KO was successfully reconstituted in the recipient mice, followed by analysis of HSCs and progenitor cells. We found that LT-HSCs dramatically accumulated (>15-fold increase) in the reconstituted recipient mice compared with control mice (Fig. 4 E). However, ST-HSCs, MPPs, CLPs, and CMPs were remarkably declined. These observations indicate that WASH-deficient BM cells fail to reconstitute early progenitor cells and lose their differentiation potential to mature hematopoietic cells.

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### Table 1. Hematopoietic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MxCre^-; WASH^+/+ (Ctrl)</th>
<th>MxCre^-; WASH^+/-- (KO)</th>
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<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC (×10^3/ml)</td>
<td>12.0 ± 0.5</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>RBC (×10^6/ml)</td>
<td>10.8 ± 0.5</td>
<td>4.6 ± 0.6</td>
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<tr>
<td>Hemoglobin (g/dl)</td>
<td>16.3 ± 0.3</td>
<td>8.5 ± 0.2</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>59.1 ± 1.5</td>
<td>33.0 ± 4.1</td>
</tr>
<tr>
<td>Platelet (×10^3/ml)</td>
<td>1327.0 ± 98.7</td>
<td>125.6 ± 8.7</td>
</tr>
<tr>
<td>Differential WBC (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>69.0 ± 3.4</td>
<td>65.3 ± 3.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>25.9 ± 3.1</td>
<td>28.8 ± 0.4</td>
</tr>
<tr>
<td>Monocytes</td>
<td>4.4 ± 1.7</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1.3 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC/femur (×10^3/ml)</td>
<td>23.9 ± 0.5</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>100.1 ± 3.6</td>
<td>33.6 ± 5.2</td>
</tr>
</tbody>
</table>

Differential WBCs were determined by monitoring the morphology of cells stained with May–Grunewald Giemsa. Data are shown as means ± SD. WBC, white blood cells; RBC, red blood cells.
Figure 3. WASH deficiency impairs repopulation capacity of HSCs. (A) Flow cytometry of HSCs in Ctrl and WASH KO mice. Sca-1+c-Kit+ cells in the Lin- and IL-7Rα- population were gated out (top left) for Flk2 analysis (bottom left). Percentages of the indicated cells in parent populations were calculated and shown on the right. IgG was gated as a negative control. n = 15 for each group. (B) Survival curves of CD45.1 mice transplanted with BM cells from Ctrl (n = 20) and KO mice (n = 21). Lethally irradiated CD45.1 mice were transplanted with BM cells from Ctrl and KO mice (left) and survival rates were calculated (right). Lethally irradiated CD45.1 mice without transfer served as a negative control (n = 13). (C) Equal amounts of CD45.1 (CFSE labeled) and CD45.2 (Violet labeled) lin- BM cells were transplanted into lethally irradiated CD45.1 mice, followed by flow cytometry 18 h after transplantation.
transplantation by using WASH KO LT-HSCs from the first reconstituted mice (Fig. 4 H). WASH is essential for LT-HSC differentiation.

**WASH promotes LT-HSC differentiation in a c-Myc-dependent manner**

We next performed gene expression profiling in LT-HSCs of WASH KO and littermate control mice. We clustered key hematopoiesis-related transcription factors (Moignard et al., 2013). We focused on down-regulated transcription factors caused by WASH deficiency. Among them, c-Myc and its related target genes were apparently down-regulated (Fig. 5 A). c-Myc and the downstream target genes were verified in WASH KO LT-HSCs by quantitative real-time PCR (Fig. 5 B). Interestingly, the phenotype caused by WASH deficiency was quite similar to that of c-Myc-deficient mice (Wilson et al., 2004). Thus, we focused on the role of c-Myc in the regulation of LT-HSC specification.

We next wanted to confirm whether WASH deficiency could directly affect the expression of c-Myc and its related downstream targets. We rescued WASH expression in WASH KO LT-HSCs and transferred them into lethally irradiated CD45.1 recipient mice. Intriguingly, the WASH-rescued LT-HSCs showed the same repopulation ability in the recipient mice as control LT-HSCs (Fig. 5 C), and normally differentiated into mature blood cells (Fig. 5 D). Additionally, the WASH rescued LT-HSCs restored the expression of c-Myc and its related downstream target genes (Fig. 5 E). We proposed that the WASH deficiency-induced accumulation of LT-HSCs may be caused by down-regulation of c-Myc.

We then wondered whether WASH associates with the promoter of c-Myc for its transcriptional regulation. We performed ChIP assays in LT-HSCs with antibody against WASH. We analyzed the immunoprecipitated DNAs using PCR analysis for the promoters of c-Myc, Tbr4, Tncg1, and N-Myc genes. WASH only associated with the c-Myc promoter, not other gene promoters we checked (Fig. 5 F). Additionally, we further mapped the binding region of c-Myc promoter for WASH and identified the binding site was at −750 to −550 bp region upstream from the transcription start site (TSS; Fig. 5 G, H). These data suggest that WASH might regulate the transcription of c-Myc gene as an upstream regulator. If WASH acts upstream of c-Myc in LT-HSCs, down-regulated expression of c-Myc can contribute to the accumulation of LT-HSCs in WASH KO mice. To test this, we rescued c-Myc expression in WASH KO LT-HSCs and transferred them into lethally irradiated recipient mice. c-Myc mutant, failed to bind to DNA but had normal functions (Halazonetis and Kandil, 1991; Fig. 5 I), was rescued as a negative control. Expectedly, c-Myc rescued the WASH KO LT-HSC repopulation ability of recipient mice (Fig. 5 J), and sustained LT-HSCs at a comparable level to control mice. However, c-Myc mutant rescue had no such activity. Moreover, the c-Myc rescued WASH KO LT-HSCs also restored the normal differentiation of mature blood cells (Fig. 5 K). Actually, c-Myc rescued the expression level of c-Myc in WASH KO LT-HSCs comparable to control mice (Fig. 5 L), and restored its related downstream target genes similar to control mice.

**WASH recruits NURF complex to activate c-Myc transcription**

To elucidate the molecular mechanism that WASH regulates differentiation potential of LT-HSCs, we screened a cDNA library using WASH as bait in the yeast two-hybrid system. We identified Rbbp4 as a novel interacting protein of WASH (Fig. 6 A). Rbbp4, also called as RBAP46/48, is a component of several chromatin remodeling complexes (for example NURF, Sin3, and Mi-2/NuRD; Hennig et al., 2005). We validated their interaction between WASH and Rbbp4 by a GST pull-down assay (Fig. 6 B). Importantly, we found that anti-WASH antibody could immunoprecipitate Rbbp4, BPTF, and SNF2L from LT-HSC lysates (Fig. 6 C), but not Mi-2β or Sin3A. These data indicate that WASH associates with the NURF complex, but not the NuRD or the Sin3 complex. Furthermore, WASH co-stained with each component of the NURF complex in the nuclei of LT-HSCs (Fig. 6 D).

The NURF remodeling complex plays a critical role in specific stages of mammalian development, acting in prominent signaling pathways to the nucleus (Ho and Crabtree, 2010). Alkhatib and Landry (2011) reported that the NURF complex regulates chromatin structure and expression of genes important for T cell development. Little is known about the effect of the NURF complex on HSC development. We next wanted to test whether the NURF complex was involved in regulation of c-Myc transcription. We performed
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ChIP assays with antibodies against the NURF components in isolated Ctrl or WASH KO LT-HSCs. Interestingly, the NURF complex associated with the promoter of c-Myc in the control LT-HSCs (Fig. 6 E), but WASH deletion lost their interactions. In contrast, the NURF complex did not bind to the promoter of c-Myc in MPPs (Fig. 6 E). To further test whether the NURF complex regulates c-Myc in vivo, we knocked down each component of the NURF complex in LT-HSCs in vitro, and the silencing efficiency was >80% (Fig. 6 F). We then transplanted these cells together with CD45.1 BM cells into lethally irradiated CD45.1 recipient mice. As expected, LT-HSCs with NURF complex component silencing showed decreased repopulation ability (Fig. 6 G), and reduced expression of c-Myc compared with control cells (Fig. 6 H). In summary, these data indicate that WASH can recruit the NURF complex to promote transcriptional activation of c-Myc gene.

WASH-mediated nuclear actin nucleation is required to assist the NURF complex to c-Myc promoter

Several studies showed that WASH plays an essential role in endosomal sorting in the cytoplasm through facilitating tubule
Figure 5. WASH promotes LT-HSC differentiation in a c-Myc–dependent manner. (A) Sorted Ctrl and WASH KO LT-HSCs were subjected to RNA extraction, followed by gene expression profiling using NimbleGen mouse 12 x 135 K gene expression array. Blue indicates down-regulated genes and red denotes up-regulated genes. (B) Gene expression levels were confirmed in sorted Ctrl or KO LT-HSCs by quantitative RT-PCR. mRNA levels of WASH KO mice were compared with that of Ctrl mice. n = 14 for each group. (C–E) The indicated LT-HSCs infected with lentiviruses encoding GFP or GFP-WASH were transferred to lethally irradiated CD45.1 mice together with 3 x 10^5 CD45.1 BM cells. Percentage of CD45.2 cells and numbers of LT-HSCs were analyzed by flow cytometry 16 wk later. Numbers of the indicated CD45.2+ cells were calculated 16 wk later. (D) B lymphocytes, T lymphocytes, and myeloid cells were obtained from peripheral blood. Erythroblasts were obtained from KO. Markers used: B220 for B lymphocytes, CD3 for T lymphocytes, CD11b for myeloid cells, and TER-119 for erythroblasts. LT-HSCs were sorted and lysed for RNA extraction for detection of mRNA. mRNA levels were normalized to Ctrl cells. WT, n = 15; KO, n = 13. (F) Sorted LT-HSCs were lysed for chromatin immunoprecipitation (ChIP) assays with anti-WASH antibody or control IgG. Promoters of c-Myc, Tlr4, Tcerg1, and N-Myc were also examined. (G and H) DNaseI hypersensitive site (arrows), landmarks of factor occupancy (boxes) and primers used to determine the association fragment of WASH were illustrated. DNA-binding sites were analyzed by PCR. (I) Scheme for DNA-binding–disabled c-Myc mutant. The DNA-binding region of c-Myc was disrupted to abolish its DNA-binding capacity. (J–L) The indicated LT-HSCs infected with lentiviruses encoding control vector, c-Myc, or c-Myc mutant were transferred to lethally irradiated CD45.1 mice together with 3 x 10^5 CD45.1 BM cells. Percentage of CD45.2 cells and numbers of LT-HSCs were checked by flow cytometry 16 wk later. Numbers of the indicated CD45.2+ cells were calculated 16 wk later as described in J. LT-HSCs were lysed for mRNA detection of the indicated genes. mRNA levels were compared with Ctrl cells. WT, n = 14; KO, n = 16. Data are shown as means ± SD. **, P < 0.01; ***, P < 0.001. Data represent three separate experiments.
fission via its VCA domain–dependent Arp2/3 activation (Derivery et al., 2009; Gomez and Billaudeau, 2009; Jia et al., 2010; Gomez et al., 2012; Hao et al., 2013). However, whether WASH nucleates actin in the nucleus is elusive. Our data show that WASH associates with the NURF complex to regulate c-Myc transcription in the nucleus of LT-HSCs, which implies that WASH might play an important role in gene transcription. We performed pyrene actin assays using LT-HSC nuclear or cytoplasmic extracts. Recombinant WASH could induce actin filament formation in both nuclear and cytoplasmic extracts (Fig. 7 A), indicating that nuclear WASH in LT-HSCs is also able to induce actin filaments. To support our hypothesis that the nuclear actin nucleation depends on the VCA domain of WASH, we generated various WASH mutants to perform the pyrene actin assays. Only WASH with the intact VCA domain could induce actin filament formation (Fig. 7 B), whereas other VCA domain mutants had no such activity. Moreover, the components of the Arp2/3 complex, which is essential for the nucleating function of WASH, all existed in both the cytoplasm and the nucleus of LT-HSCs (Fig. 7, C and D). As WASH was able to nucleate nuclear actin in LT-HSCs, we then wanted to explore whether WASH bridges
Moreover, the association of WASH with the NURF complex was dramatically increased during the differentiation of LT-HSCs (Fig. 7 F). More importantly, the association of the NURF complex with the c-Myc promoter was also accelerated undergoing LT-HSC differentiation (Fig. 7 G). Consequently, DNA accessibility of the c-Myc promoter was remarkably decreased in the actin polymerization inhibitor cytochalasin D–treated WT LT-HSCs (Fig. 7 H). Moreover, WASH knockout also exhibited decreased DNA accessibility to the NURF complex. We found that Rbbp4 could associate with actin in control cells, but this association was disrupted in WASH KO LT-HSCs (Fig. 7 E). Additionally, p16, a component of Arp2/3 complex (Welch et al., 1997), exhibited the association with actin in WASH KO and control LT-HSCs as a positive control. In contrast, Atg5, a nonassociated protein of Arp2/3 complex, did not bind to actin as a negative control. These data imply that WASH plays a critical role in linking the actin filaments to the NURF complex. Moreover, the association of WASH with the NURF complex was dramatically increased during the differentiation of LT-HSCs (Fig. 7 F). More importantly, the association of the NURF complex with the c-Myc promoter was also accelerated undergoing LT-HSC differentiation (Fig. 7 G). Consequently, DNA accessibility of the c-Myc promoter was remarkably decreased in the actin polymerization inhibitor cytochalasin D–treated WT LT-HSCs (Fig. 7 H). Moreover, WASH knockout also exhibited decreased DNA accessibility.
of the c-Myc promoter in WASH KO LT-HSCs. These observations suggest that NURF complex enhances transcription of c-Myc in an actin nucleation-dependent manner.

To further validate the critical role of actin nucleation in c-Myc transcriptional activation, we overexpressed WASH or WASH (ΔVCA) mutant in WASH KO LT-HSCs and transferred them into lethally irradiated CD45.1 recipient mice. VCA-truncated WASH (ΔVCA) LT-HSCs failed to rescue the repopulation ability of WASH KO cells (Fig. 7 I), and the expression level of c-Myc (Fig. 7 J), whereas WASH rescued LT-HSCs could restore these repopulation ability. Together, these data indicate that WASH assists the NURF complex to the promoter of c-Myc gene through its nuclear actin nucleating activity.

DISCUSSION
In this study, we show that conditional WASH deficiency in adult hematopoietic system causes defective blood production of the host due to hindered differentiation potential of LT-HSCs leading to severe cytopenia. WASH knockout breaks the balance that controls the self-renewal and differentiation of HSC hematopoiesis. WASH is located in the nucleus of LT-HSCs and associates with the chromatin remodeling complex NURF. WASH assists the NURF complex to the promoter of c-Myc gene by generating actin filaments via its VCA domain. Loss of WASH suppresses the transcriptional activation of c-Myc gene and results in perturbed differentiation of LT-HSCs. WASH is essential for differentiation of LT-HSCs.

Hematopoiesis is fully dependent on a rare population of hematopoietic stem cells that possess the capacity to self-renew and differentiate into all blood cell lineages (Doulatov et al., 2012; Orkin and Zon, 2008). HSCs are finely regulated in their KO niche to keep the numbers of individual components in the blood system at a proper level. Cell cycle regulators are important for HSC self-renewal (Scheller et al., 2006). Constitutive activation of β-catenin causes abnormal HSC cell cycle and disable HSC reconstitution ability (Scheller et al., 2006). Noncanonical Wnt signaling was reported to be critical for the maintenance of HSC self-renewal (Sugimura et al., 2012). Under stresses, noncanonical Wnt signaling is down-regulated, whereas the canonical Wnt signaling is activated, which leads to HSC differentiation. Here, we found that WASH KO LT-HSCs had no obvious alteration of their cell cycles and turnover rates compared with control mice. Moreover, WASH deficiency did not influence expression levels of self-renewal genes. Thus, our data suggest that the accumulation of LT-HSCs after WASH deletion is not due to increased proliferation, survival, or self-renewal. We further confirmed that WASH deficiency impairs differentiation potential of LT-HSCs.

c-Myc, a protooncogene, is a major Myc family member as a transcription factor that is involved in cell growth, division, apoptosis, differentiation, angiogenesis, and regulation of chromatin structure (Pelengaris et al., 2002). Moreover, c-Myc was identified as an important factor in the induced reprogramming of adult fibroblasts into induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Knoepfler, 2008). Accumulating evidence has been shown that c-Myc plays an essential role in the process of hematopoiesis (Wilson et al., 2004; Laurenti et al., 2008; Reavie et al., 2010; Will et al., 2013). Conditional deletion of c-Myc gene in the adult hematopoietic system causes an accumulation of functionally defective HSCs (Wilson et al., 2004). Importantly, c-Myc deficiency results in dramatic accumulation of LT-HSCs, as well as apparent accumulation of ST-HSCs and MPPs. c-Myc-deficient HSCs up-regulate expression of N-cadherin and adhesion receptors, which may restrict the HSCs to the KO niche that perturbs differentiation of HSCs (Wilson et al., 2004). Later, the same group also showed that c-Myc and N-Myc activities are involved in proliferation, differentiation, and survival of HSCs (Laurenti et al., 2008). Furthermore, genes in modulation of c-Myc expression also participate in HSC differentiation. Fbw7, a ubiquitin ligase of c-Myc, was reported to control the stability of c-Myc in the process of hematopoiesis (Reavie et al., 2010). Depletion of Fbw7 causes increased levels of c-Myc in HSCs, leading to decreased LSK numbers due to its enhanced differentiation potential for the high level c-Myc. Furthermore, the expression level of c-Myc is elevated during the differentiation process of HSCs, especially from LT-HSCs to ST-HSCs (Laurenti et al., 2008). Here, we show that the defective hematopoiesis of WASH deficiency is quite similar to that of c-Myc mutants. WASH acts as an upstream regulator to modulate c-Myc gene transcription. However, WASH KO only causes the accumulation of LT-HSCs, but not ST-HSCs or MPPs, which is different from that of c-Myc deficiency.

WASH plays a critical role in endosomal trafficking through its actin nucleation function (Derivery et al., 2009; Gomez and Billadeau, 2009; Jia et al., 2010; Gomez et al., 2012; Hao et al., 2013). They showed that WASH is an endosomal protein that exists in the FAM21-containing multiprotein complex (Derivery et al., 2009; Gomez and Billadeau, 2009). We just demonstrated that WASH is localized in autophagosomes of MEF cells (Xia et al., 2013), which modulates the process of autophagy. In our study, we first demonstrate that WASH is constitutively expressed in the blood cells and mainly localized in the nucleus of LT-HSCs. WASH deletion breaks the balance that controls self-renewal and differentiation of HSCs. WASH KO transfer causes death within 10 d, much faster than that of the c-Myc KO or the c-Myc/N-Myc double KO BM transfer (Laurenti et al., 2008). The HSCs transferred by the c-Myc KO or the c-Myc/N-Myc double KO BM contained LT-HSCs and ST-HSCs populations that may keep the mice alive longer. Another possibility is that WASH may have more roles beyond the regulation of c-Myc transcription. Additionally, the Drosophila homologue of WASH was reported to be associated with the nucleosome remodeling factor (NURF) chromatin remodeling complex, which is localized in the nucleus of Drosophila cells (Hochheimer et al., 2002; Lianardopoulou et al., 2007). These data suggest that WASH may also exert its function in the nucleus of mammalian cells.
Interestingly, we found that nuclear WASH associates with NURF complex to promote the transcription of c-Myc gene through its actin-nucleating domain in LT-HSCs. Depletion of the components of the NURF complex results in disruption of repopulation ability of BM, similar to that of WASH depletion. Additionally, the VCA domain is essential for restoration of repopulation ability of BM. Thus, WASH-mediated actin nuclearization in the nucleus plays a pivotal role in regulation of c-Myc transcription. In contrast, WASH is localized in both of the nucleus and cytoplasm of ST-HSCs, and it is mainly localized in the cytoplasm of MPPs. Therefore, whether WASH exerts different specific functions in different cell types still needs to be further investigated. More recently, WASH was reported to be activated by K63-linked polyubiquitination via MAGEL2-TRIM27 (Hao et al., 2013). Whether the nuclear WASH is intrinsically active or needs further posttranslational modification for its activation also needs to be further elucidated.

Epigenetic regulations are involved in many biological processes, such as gene transcription, DNA replication, DNA repair, and DNA recombination (Berger et al., 2009; Jiang and Pugh, 2009). These processes are composed of posttranslational modulation of histones, DNA methylation, incorporation of histone variants, and nucleosome remodeling activity. The nucleosome remodeling and incorporation of histone variants are largely accomplished by assistance of ATP-dependent chromatin remodeling complexes. The chromatin remodeling complexes have been implicated in maintaining the pluripotency of HSCs (Will et al., 2013). BAF53a, a component of the SWI/SNF-like BAF complex, is indispensable for HSC maintenance and survival (Krasteva et al., 2012). SNF2-like ATPase Mi-2β subunit of the nucleosome remodeling deacetylase (NuRD) complex, is needed for maintenance of HSCs (Yoshida et al., 2008). Loss of Mi-2β causes enhanced cycling of HSCs, leading to decrease in quiescence of HSCs. Here, we show that WASH associates with the NURF complex that assists its binding to the promoter of c-Myc gene, and this association is enhanced once HSCs decide to differentiate into subpopulations. Rbpb4, also called as RBAP46/48, is identified as a subunit of the NURF complex (Song et al., 2009; Alkhatib and Landry, 2011), but it is also associated with the NuRD or the Sin3 complex (Kuzmichev et al., 2002; Lejon et al., 2011). A previous study reported that c-Myc exists in both LT-HSCs and MPPs (Laurenti et al., 2008). Here we showed that c-Myc expression is dependent on WASH and the NURF complex in LT-HSCs. However, the NURF complex could not bind to the c-Myc promoter in MPP cells (Fig 6 E). These data suggest that c-Myc promoter might be regulated differently in LT-HSCs and MPPs. Altogether, WASH preferably associates with the Rbpb4-containing NURF complex in LT-HSCs to control the balance of self-renewal and differentiation of hematopoietic stem cells.

MATERIALS AND METHODS

Antibodies and reagents. A rabbit polyclonal antibody against WASH was generated from the VCA domain of WASH protein as described previously (Xia et al., 2013). Human anti-WASH antibody was a gift from D. Billadeau (Mayo Clinic, Rochester, NY; Gomez and Billadeau, 2009) and mouse anti-WASH antibody was a gift from A. Gautreau (Institut Curie, Centre de Recherche, Paris, France; Derivery et al., 2009). The following commercial antibodies were used: mouse hematopoietic lineage eFlour 450 cocktail (eBioscience; sc-45100), APC-Cy7 conjugated goat anti-rabbit IgG (sc-3869), PE-anti-E1A (sc-33588), anti-histone H3 (sc-8564), and anti-ubiquitin (sc-8017) were from Sigma-Aldrich. Anti-Rbpb4 (9067), anti-BPTF (8038), anti-Atg5 (2630), and anti-Sin3A (8056) were from Cell Signaling Technology. Donkey anti-rabbit IgG secondary antibodies conjugated with Alexa Fluor 488 (A11008), Alexa Fluor 594 (A11012), or Alexa Fluor 405 (A31566) were purchased from Molecular Probes. Donkey anti-mouse IgG secondary antibodies conjugated with Alexa Fluor 488 (A11029) or Alexa Fluor 594 (A11032) were purchased from Molecular Probes. HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Cytochalasin D (C6762) and Bafilomycin A1 (SAB2700857), anti-p21 (anti-ARPC3, SAB2500115), anti-p16 (anti-ARPC5, SAB1100953), and anti-β-actin (A1978) were from Sigma-Aldrich. Anti-EEA1 (sc-14414), anti-p120 (sc-22650), anti-EEA1 (sc-66367), anti-p-actin (sc-37680), and anti-ubiquitin (sc-8017) were from Sigma-Aldrich. Anti-Flt3/CD135 (eBioscience; 56–0193), anti-Gr-1 (eBioscience; 53–5931), anti-CD34 (eBioscience; 22–771), APC-anti-Ly6A/E (Sca-1; eBioscience; 17–5891), PE-anti-CD117 (e-Kit; eBioscience; 12–1171), anti-CD48 (eBioscience; 14–0489), anti-CD150 (eBioscience; 16–1501), anti-CD3 (eBioscience; 50–0032), anti-CD19 (eBioscience; 56–0193), anti-Gr-1 (eBioscience; 53–5931), and anti-CD34 (eBioscience; 56–0431) were purchased from eBioscience. Anti-Flt3/CD135 (FLk2; ab18370), anti-p34 (anti-ARPC2, ab11798) and anti-p20 (anti-ARPC4; ab110770) were obtained from Abcam. Anti-phospho-Ser-Thr/Tyr was purchased from AnaSpec Inc (53775). PerCP-Cy5.5-conjugated goat anti-rat IgG (sc-45100), APC-Cy7 conjugated goat anti-rabbit IgG (sc-3869), PE-E1A (sc-33588), anti-histone H3 (sc-8564), and anti-ubiquitin (sc-8017) were from Santa Cruz Biotechnology. Anti-GST (sc-77891), anti-p40 (anti-ARPC1, SAB2708857), anti-p21 (anti-ARPC3, SAB2860115), anti-p16 (anti-ARPC5, SAB1100953), and anti-β-actin (A1978) were from Sigma-Aldrich. Anti-Rbpb4 (9067), anti-BPTF (8038), anti-SNF2L (12483), anti-Mi-2β (12011), anti-Ang5 (2630), and anti-Sin3A (8056) were from Cell Signaling Technology. Donkey anti-rabbit IgG secondary antibodies conjugated with Alexa Fluor 488 (A11008), Alexa Fluor 594 (A11012), or Alexa Fluor 405 (A31566) were purchased from Molecular Probes. Donkey anti-mouse IgG secondary antibodies conjugated with Alexa Fluor 488 (A11029) or Alexa Fluor 594 (A11032) were purchased from Molecular Probes. HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Cytochalasin D (C6762) and Bafilomycin A1 (B793) was from Sigma-Aldrich.

Cell culture. Mouse multipotent HSC/MPP-like cell line EML cells were cultured in Iscove’s modified Dulbecco’s media, containing 4 mM l-glutamine and 200 ng/ml mouse stem cell factor (SCF), 20% FBS, β-mercaptoethanol, 100 µg/ml streptomycin and 100 µl/ml penicillin. For HSC culture, cells were cultured in StemPro-34 media (Invitrogen), containing 4 mM l-glutamine, 100 µg/ml streptomycin, 100 µ/ml penicillin and the following cytokines (from PeproTech): 10 ng/ml IL-3, 25 ng/ml SCF, 25 ng/ml Flt3L, 10 ng/ml GM-CSF, 25 ng/ml IL-11, 4 U/ml Epo, and 25 ng/ml Tpo. For cytokine withdrawal, HSCs were cultured in above medium deprived of the indicated cytokines. Otherwise, HSCs were plated on a 70% confluent monolayer of OP9-DL1 cells into 6-well plates in α-MEM, containing 20% FBS, 100 µg/ml streptomycin, and 100 µ/ml penicillin and further supplemented with 5 ng/ml rmIL-7 and 5 ng/ml rmFlt3L.

Histology. Mouse spleens were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) for 12 h. Mouse femurs were fixed in PBS buffer. Spleen cells were obtained by mashing the spleen in PBS buffer. Cells were sifted through 50-µm cell strainers after removing. Flow cytometry. Mice were euthanized and KO cells were flushed out from femurs in PBS buffer. Spleen cells were obtained by mashing the spleen in PBS buffer. Cells were sifted through 50-µm cell strainers after removing.
red blood cells by suspending cells in ammonium-based red cell lysis buffer. For flow cytometric analyses, cells were either incubated with fluorophore-conjugated antibodies or incubated with primary antibodies and further incubated with fluorophore-conjugated secondary antibodies, followed by detecting or sorting on an Influx cell sorter (BD). For analysis of HSC cells, 2 × 10^5 events were detected and for 2 × 10^6 events were obtained for differentiated cells. Data were analyzed using the FlowJo 7.6.1 software.

**Immunofluorescence assay.** Hemopoietic cells were placed on 0.01% poly-L-lysine treated coverslips and fixed with 4% PFA for 30 min at room temperature, followed by permeabilization with 0.5% Triton X-100 for 20 min at room temperature. 10% donkey serum was used for blocking and primary antibodies were added for 2 h at RT. After washing with PBS, the coverslips were stained with Alexa Fluor 488-, Alexa Fluor 594-, or Alexa Fluor 408–conjugated secondary antibodies. Images were obtained with laser scanning confocal microscopy (Olympus FV500).

**RNA interference and RT-qPCR.** RNA interference sequences were designed according to pSUPER system instructions (Oligoengme). H1 promoter and targeting sequences were further cloned to the modified lentivirus vector pSIN-EF2. Lentiviruses containing RNA interference sequence were generated by transfecting pSIN-EF2-shRNA and packaging vectors into HEK293T cells, followed by concentration using ultracentrifugation at 50,000 g. EML or sorted LT-HSC cells were infected with lentiviruses for 36 h before examination or transplantation. shRNA sequences were as follows: Rbpb4, 5′-AGAGGAGGATGTCATCG-3′, 2# 5′-GGAGGAGATGACATGGAA-3′, SNFL2, 5′-AGCACAGAGTCCTCAACA-3′, 2# 5′-GCCGCACAGAAAAAGAAGA-3′. Total RNA was extracted from sorted cells using TRIzol reagent and cDNA was reverse-transcribed using SuperScript II (Invitrogen). RT-PCR was performed using the following primers: WASH, forward, 5′-AGTGGGAGCTTTCATCGTACG-3′, reverse, 5′-AGAAGAGGCTCTCCAGGATCG-3′; c-Myc, forward, 5′-GCCGGTGAAGATTCTCGGA-3′, reverse, 5′-ATCGGAGGATGTCATCGACG-3′; Ercc1, forward, 5′-CCTCCATTGTGACGCT-3′, reverse, 5′-GCCGCACAGAAAAAGAAGA-3′. Total RNA was reverse-transcribed using SuperScript II (Invitrogen). RT-PCR was performed using the following primers: mRNA expression was normalized to that of housekeeping gene β-actin.

**Pyrene actin assay.** Cytoplasmic and nuclear extracts were obtained from sorted LT-HSC cells. Extracts were changed to Buffer A (2 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, and 1 mM Na3VO4). Supernatants were collected by centrifugation (15,000 g, 15 min, 4°C), and incubated with the indicated antibodies for 4 h at 4°C following by immunoprecipitation with 30 μl protein A/G agarose. The precipitates were completely washed with RIPA lysis buffer three times and tested by immunoblots. 5% of cell lysates were loaded as input.

**Chromatin immunoprecipitation (ChIP).** ChIP was performed using MAgNAlyt ChIP kit following manufacturers’ instructions. In brief, cells were fixed in 1% formaldehyde for 20 min, and then quenched with 0.125 M lysine, followed by swelling in lysis buffer (50 mM Heps, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% NaDeoxycholate, and Protease Inhibitor Cocktail Set III [Calbiochem]) for 30 min on ice. Chromatin was sheared to a mean length of 400 bp by sonication. After being de-cross-linked by RNaše, protease K, and heat, input genomic DNA was precipitated with ethanol and quantified in a GeneQuant 100 spectrophotometer (GE Healthcare). Chromatin was precleared with protein A/G-agarose (Santa Cruz Biotechnology, Inc.), followed by incubation with indicated antibodies at 4°C overnight and further incubation with protein A/G-agarose for 2 h. Beads were washed with washing buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% NaDeoxycholate, 1 mM EDTA) three times and eluted with elution buffer (50 mM Tris, pH 8.0, 1% SDS, and 1 mM EDTA). Eluates were de-cross-linked by RNaše, protease K, and heat, and DNA was extracted with phenolchloroform, followed by ethanol precipitation. For each ChIP experiment, 2 × 10^6 cells were used. 5% of nuclear extracts served as inputs. Immunoprecipitated DNAs were further analyzed by real-time PCR. Primers for c-Myc promoter mapping were listed below. −2000–1800, forward, 5′-CAAGGCCTGGCCCCATCATCAGGCA-3′, reverse, 5′-CTCTTGCTCGTCTCTCTTTTGGTCTGCA-3′; −1750–1550, forward, 5′-ATCCCTAAAGCTGATTTGCC-3′, reverse, 5′-AAAACCTTCTTTCATCGTAC-3′; −1550–1400, forward, 5′-GCCCTTGAGGGGAGGTTTCGGA-3′, reverse, 5′-CAAGGGGCGTGATGCACTCG-3′; −1350–1150, forward, 5′-TCCAAGCGATGCCG-3′, reverse, 5′-GCCGCAGGTGGTTATAGC-3′; −1150–950, forward, 5′-GGGCTCTCTCGAGGCTCCCAGC-3′, reverse, 5′-TGTTGGAGCCATTTGCAGCC-3′; −950–800, forward, 5′-TTGACCTTGGATTTGACG-3′; −800–750, forward, 5′-GAGGAGGCTGGATTCCACCG-3′; −750–650, forward, 5′-TTCCCTTGGCATGTGCACAG-3′, reverse, 5′-TCTCCCTTCTCGAGC-3′; −450–350, forward, 5′-CTCCCTTCTCGAGC-3′; −350–200, forward, 5′-TCTCCCTTCTCGAGC-3′, reverse, 5′-CTCCCTTCTCGAGC-3′; −200–0, forward, 5′-TCTCCCTTCTCGAGC-3′, reverse, 5′-TCTCCCTTCTCGAGC-3′.

**Generation of conditional WASH knockout mice.** To obtain hematopoietic WASH depleted mice, WASH+/− mice were crossed with MxCre+ mice to get MxCre+; WASH+/− mice. The MxCre+; WASH−/− mice were further crossed with WASH+/− mice to generate MxCre+; WASH−/− mice. MxCre+; WASH−/− mice were injected i.p. with 300 μg poly(I:C) every other day for a total of three times to induce WASH deletion. Ercc1−/−; WASH−/− or Ercc1+/−; WASH−/− mice were generated by crossing Ercc1−/− mice with WASH−/− mice as described above. Adult mice were injected i.p. with tamoxifen (50 mg/kg) for five consecutive days to induce WASH deletion. Mouse experiments were approved by the Institutional Animal Care and Use Committees at the Institute of Biophysics, Chinese Academy of Sciences. Genotyping primers were listed below. For WASH flux identification, primers were as follows: 5′-CCTCTTGTCCCTTTTGTTGCAGTT-3′ and 5′-CCCGGCCATATGGGGCTGCT-3′; WT allele, 100 bp; flux allele, 140 bp. For WASH KO identification, primers were as follows: 5′-CCTCTTGTCCCTTTTGTTGCAGTT-3′ and 5′-GCCCTCCACACCC-3′; flux allele, 2100 bp; KO allele, 330 bp. For Cre identification, primers were as follows: 5′-GCCCTGATATCCGGGCTGATGC-3′ and 5′-CAGGTTTATAGAAGCAATCCC-3′; Cre allele, 550 bp.

**Immunoprecipitation assay.** BM cells were obtained as described above. Cells were labeled with biotin-conjugated antibodies, followed by magnetic labeling with Microbeads (Miltenyi Biotec) against biotin. Lin− cells were collected by depletion of magnetically labeled cells. Lin− cells were further enriched by positive selection using biotin-conjugated antibody against Sca-1. Highly enriched cells were then stained with surface markers and sorted by flow cytometry. Sorted LT-HSC cells (2 × 10^5) or KO cells (2 × 10^5) were lysed with RIPA lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, and 1 mM Na3VO4). Supernatants were collected by centrifugation (15,000 g, 15 min, 4°C), and incubated with the indicated antibodies for 4 h at 4°C following by immunoprecipitation with 30 μl protein A/G-agarose. The precipitates were completely washed with RIPA lysis buffer three times and tested by immunoblots. 5% of cell lysates were loaded as input.
Donor KO cells were separated as described above and injected alone or with recipient KO cells to lethally irradiate (10 Gy) CD45.1 mice. Reconstituted mice were fed with water containing 1 g/liter ampicillin for 2 wk before switching to regular water. For analysis of peripheral blood cells, blood was obtained from mice tail vein and stained as described above.

**Gene expression profiling.** Total RNA was extracted from control and WASH KO LT-HSCs using standard RNA extraction protocol, followed by DNase incubation to remove nuclear DNA. The quality of RNA was monitored by NanoDrop ND-1000 and the integrity of RNA was tested by agarose gel electrophoresis. Total RNA was subjected to labeling and array hybridization using mouse 12 × 155 K gene expression array according to manufacturer’s instructions (NimbleGen). Hybridized array was scanned using the Axon GenePix 4000B microarray scanner (Molecular Devices), followed by grid alignment and expression analysis using the NimbleScan software (version 2.5). Expression data were then normalized to generate Probe level and Gene level files, followed by further analysis using the Agilent GeneSpring GX software (version 11.5.1).

**Statistical analysis.** Student’s t test was used as statistical analysis by using Signamaplot as previously described (Wang et al., 2013).

**Accession nos.** Microarray data is available from Gene Expression Omnibus under accession no. GSE57741.

**Online supplemental material.** Fig. S1 shows validation of WASH deletion in WASH KO cells used in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20140169/DC1.

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The authors declare no competing financial interests.

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