Cohesin is a multimeric protein complex that is very well conserved throughout evolution and across species and is critically important in mediating proper sister chromatid cohesion (SCC) and separation from S phase to M phase during mitosis (Hirano, 2005; Nasmyth and Haering, 2009). The complex consists of four proteins Rad21, Smc1a, Smc3, and Stag2 (also known as SA-2) that form a ring structure that can physically wrap around the chromatin (Gruber et al., 2003). During the different phases of cell division, additional regulator proteins (e.g., NIPBL, HDAC8, and WAPL) are required for its proper function (Haarhuis et al., 2014). Cohesin’s ring structure is also essential for its additional functions, namely DNA repair and three-dimensional chromatin looping. The latter has been studied extensively in embryonic stem (ES) cells where cohesin controls core pluripotency genes by assisting the looping of enhancers to specific promoters (Kagey et al., 2010). Genome-wide studies have shown that cohesin predominantly co-occurs with CTCF on the chromatin of mammalian cells (Parelho et al., 2008; Wendt et al., 2008). Sites that are bound by both CTCF and cohesin were proposed to serve as anchoring points for long-range genomic interactions (Dowen et al., 2014), suggesting that cohesin together with CTCF dictates higher-order chromatin structure (Holwerda and de Laat, 2012). For instance, in ES cells it
was shown that CTCF and cohesin help to establish borders of topologically associated domains (TADs), and these structures have been shown to play a major role in delimiting regulatory interactions (Dixon et al., 2012; Phillips-Cremins et al., 2013; Dowen et al., 2014). Not surprisingly, suppression of cohesin leads to unfolding and relaxation of topological domains (Sofueva et al., 2013; Mizuguchi et al., 2014). This implies that cohesin is an important regulator of transcription through genome-wide chromatin organization. Another way that cohesin regulates transcription is by acting as a docking site for transcription factors in cells that exit mitosis. Cohesin is one of the last protein complexes to leave the condensing chromatin in mitosis, serving as a cellular memory for transcription factors in cells that exit mitosis. Cohesin silencing leads to increased replating capacity in vitro (Xu et al., 2010; Seitan et al., 2011). We observed that induction of the shRNA in vivo resulted in an ~80% loss of mRNA/protein expression that strikingly did not lead to adverse effects on proliferation. Moreover, cohesin knockdown leads to a shift in the hematopoietic stem compartment and increases the replating capacity of these cells. Over time, cohesin knockdown mice developed clinical features of myeloproliferative disorders/neoplasms. We did not observe genomic instability in cohesin knockdown cells, suggesting that these phenotypes are caused by misregulation of cohesin’s role on gene expression and genome organization. These findings provide the first in vivo evidence that cohesin governs adult stem cell homeostasis and myeloid development, potentially functioning as tumor suppressor in the hematopoietic system.

RESULTS
AML-derived cohesin mutations can impair core complex formation
To determine the effect of mutations found in AML patients on cohesin complex formation, we initially modeled selected mutations in vitro. We overexpressed wild-type and AML mutant cohesin proteins in 293T cells and used immunoprecipitation to pull down the endogenous cohesin complex. As expected, all wild-type cohesin proteins strongly interact with the other members of the core complex. Strikingly, several AML-derived mutations completely abrogated complex formation. This is most clear in the case of Rad21 (Fig. 1 A) and Stag2 (Fig. 1 B). The underlying reasons why these proteins no longer interact with the other members of the complex could be diverse; in the case of Rad21, the short fragments appear to be unstable (Zhang et al., 2013). For Stag2, the C terminus of the protein has been reported to be important for protein function as it is the location of many phosphorylation sites and potentially the nuclear localization signal (Hauf et al., 2005; Solomon et al., 2011). Truncation of Stag2 will hence cause loss of the phosphorylation and potential loss of nuclear localization. For Smc3, we noticed that one mutation (R245*) causes complete loss of complex formation (Fig. 1 C) and a milder phenotype for some of the other mutations.

Cohesin silencing leads to increased replating capacity in vitro
We have shown that many recurrent cohesin mutations found in AML patients are likely to result in loss of function of the protein involved (Fig. 1). This is supported by large-scale sequencing efforts in AML samples (Kon et al., 2013), suggesting such a function for cohesin mutations. For this reason, we chose an RNAi-mediated knockdown strategy to suppress endogenous cohesin proteins. We created between five and nine retroviral shRNA vectors for each of the cohesin core components of cohesin has proven to be incompatible with cellular proliferation and survival, as a result of their essential role in SCC (Xu et al., 2010). For example, homozygous knockout mouse models for cohesin exhibit early embryonic lethality, therefore limiting comprehensive investigations of cohesin in vivo (Xu et al., 2010; Seitan et al., 2011). In this study, we present the first successful in vivo loss of function murine models for distinct cohesin members. We have created RNAi mouse models that inducibly knock down Rad21, Smc1a, and Stag2 systemically.
intervening lanes were spliced out. After immunoprecipitation with Flag-beads, Western blot analysis was performed for endogenous Rad21, Smc1a, and Stag2 protein. Dashed line indicates possibly the case for Q801* as well (Solomon et al., 2011). (C) Flag-tagged wild-type and AML patient mutants of Stag2 were transfected in 293T cells. After immunoprecipitation with Flag-beads, Western blot analysis was performed for endogenous Rad21, Smc1a, and Stag2 protein. Dashed line indicates intervening lanes were spliced out.

complex genes: Rad21, Smc1a, Smc3, and Stag2 (Table S1). We chose a vector that expresses GFP in combination with the shRNA, enabling us to track cells expressing the shRNA. After cloning, these vectors were tested for their knockdown efficiency (not depicted). We selected two shRNA vectors for each Rad21, Smc1a, Smc3, and Stag2 gene that suppressed the endogenous mRNA efficiently but not completely (75–90%), as we expected that a complete knockdown would be detrimental to the cells. First, we investigated what the effect of cohesin knockdown is on hematopoietic stem and progenitor cells. Therefore, we transduced mouse bone marrow c-Kit+ hematopoietic progenitor cells with retroviral shRNA vectors for Rad21, Smc1a, Smc3, and Stag2. The infected cells were plated in methylcellulose for CFU assays (Fig. 2 A). Although control infected cells (shRNA targeting Renilla) yielded colonies for only two rounds of plating, all cohesin knockdown cells continued forming colonies after at least five rounds of plating in methylcellulose. This phenotype was reproducible and was validated using multiple independent shRNAs for the cohesin subunits (not depicted). Furthermore, the selected cells proliferated in liquid culture for prolonged periods of time (not depicted). We confirmed by quantitative real-time PCR (qRT-PCR) that shRNA-infected cells indeed demonstrated a strong reduction of endogenous cohesin transcripts (Fig. 2 B). Cohesin knockdown demonstrated decreased expression of the differentiation markers Cd11b and Gr1 (Fig. 2 C). This finding was coupled with increased c-Kit and Sca1 expression as early as the first plating (Fig. 2 D).

Microscopically, we observed progressing cytomorphologic changes in the CFU assays. After several rounds of plating in methylcellulose, the cohesin knockdown cell population demonstrated a left shift in myelopoiesis, including a mild increase in mast cells, basophils, and eosinophils. The precursor cells exhibited slightly abnormal granulation, including lucent primary granules in conjunction with variable amounts of azurophilic/orangeophilic granules, whereas the more mature elements demonstrated a decrease in secondary granules. Mild nuclear cytoplasmic asynchrony as well as occasional myeloid/monocytoid elements with cytoplasmic vacuolization were also noted, compatible with G-CSF effect from the culture medium (Fig. 2 E).

To gain a better understanding of the underlying changes in gene expression changes, we performed RNA sequencing of cells obtained from the first and fifth plating in methylcellulose (Fig. 2 F and Table S2). In agreement with protein expression data and the cellular phenotypes reported, we observed higher expression of c-Kit and Sca1 mRNA in cohesin knockdown cells compared with Renilla control cells. In addition, we observed that knockdown of all four cohesin members of the cohesin complex (Rad21, Smc1a, Smc3, and Stag2) resulted in comparable changes in gene expression, as is to be expected of members of such a nonredundant complex with presumably similar function (Fig. 2 F).

Although a prior study confirmed that cohesin gene mutant AML samples are largely cytogenetically normal (Cancer Genome Atlas Research Network, 2013), we wanted to rule out that cohesin knockdown causes severe effects in proper sister chromatid separation, leading to genomic instability. We thus analyzed metaphase spreads from cells from the first plating in methylcellulose and observed some metaphase spreads that showed typical loss of SCC (Fig. 2 G). Next, we quantified the number of metaphases that demonstrate this loss of SCC phenotype, which appeared to be a minority of cells in the culture (<15%; Fig. 2 H). This was not surprising as the majority of the cells were actively cycling and we did not observe massive cell death (not depicted). Moreover, we noticed that the loss of SCC phenotype seems to be counterselected over time (not depicted). These observations could explain the absence of any significant karyotype changes in cells that were obtained from the first methylcellulose plating (in the course of the experiment, these cells were proliferating...
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Figure 2. Cohesin knockdown cells acquire replating capacity in vitro. (A) Mouse c-Kit+ cells were infected with retroviral shRNA vectors targeting Rad21, Smc1a, Smc3, and Stag2. Infected cells were seeded in methylcellulose and replated for five passages. (B) qRT-PCR to assess knockdown of Rad21, Smc1a, Smc3, and Stag2 shRNA–infected cells after the first plating in methylcellulose. (C and D) Quantification of flow cytometry analysis of Smc1a knockdown cells after the first (P1) and fourth plating (P4). Cells were stained for myeloid differentiation markers (Cd11b and Gr1; C) and stem cells markers c-Kit and Sca1 (D). (E) Morphology of Renilla and cohesin knockdown cells (as indicated) after the first and third plating in methylcellulose. (F) RNA-seq analysis of cohesin knockdown cells after the first (CFU P1) and fifth (CFU P5) plating in methylcellulose. Heat map shows genes that are significantly differently expressed (P < 0.05). (G) Metaphase spreads of control and Smc1a knockdown cells after the first plating in methylcellulose. (H) Quantification of fraction of metaphases that show loss of SCC in control and cohesin knockdown cells after the first plating in methylcellulose. (I) Quantification of number of chromosomes as counted in metaphase spreads of control and cohesin knockdown cells after the first plating in methylcellulose. Error bars indicate SD.

for ∼14 d; Fig. 2 I). Overall, these studies suggest that not only hematopoietic stem and progenitor cells could survive when the expression of distinct cohesin members was significantly (but not totally) reduced, yet these same cells appeared to acquire the ability to self-renew in vitro. Such de novo replating capacity is frequently reported when putative AML tumor suppressors have been altered or silenced (Lee et al., 2007; Moran-Crusio et al., 2011; Quivoron et al., 2011).
Generation of in vivo loss of function cohesin animal models

Encouraged by the results from our in vitro experiments using retroviral-mediated cohesin knockdown, we decided to model cohesin loss of function in vivo. First, we analyzed cohesin expression in a subset of cell types of the mouse bone marrow. As expected, for a protein complex that is essential for mitosis, little variation was detected in hematopoietic stem cells (HSCs), immature progenitors, and myeloid progenitors (Fig. 3 A). Strikingly, large differences were observed in more mature cell populations within the bone marrow. Cohesin expression was markedly lower in myeloid cells (Cd11b+/Gr1+) and moderately significantly increased in basophilic erythroblasts (CD71+/Ter119+) when compared with B cells (B220§; Fig. 3 B).

To achieve effective and reversible cohesin knockdown in vivo, we used a recently developed shRNA mouse approach (Premsrirut et al., 2011; Takiguchi et al., 2013; Bolden et al., 2014). In these animals, a single copy of an shRNA embedded in the 3’ untranslated region (UTR) of a GFP transgene is knocked in at the collagen (Col1a1) locus downstream of a tetracycline (Tet)-responsive element (TRE; Fig. 3 C). This shRNA animal model can subsequently be crossed to variants of Tet transcriptional activators (tTAs) or reverse tTAs (rtTAs). The resulting mouse model allows for inducible, reversible, and traceable expression of shRNAs in vivo. We generated shRNA mice for three members of the cohesin complex, Rad21, Smc1a, and Stag2. These cohesin shRNA mice were crossed to a ROSA26(M2rtTA/+ ) mouse to achieve ubiquitous and inducible systemic cohesin knockdown. To test this system, we provided doxycycline-enriched nutrition and checked for GFP expression in the bone marrow after 10 d. In all three cohesin shRNA mouse models GFP expression was robust (Fig. 3 D). No obvious differences were observed between control (Renilla) and cohesin shRNA in bone marrow, peripheral blood, spleen, and thymus (Fig. 3, D and E). As we used a systemic rtTA model, we also confirmed GFP (shRNA) expression (by immunohistochemical evaluation) in a variety of nonhematopoietic organs, including liver, skin, and intestine (not depicted). Next, we tested whether these high levels of GFP expression corresponded to efficient knockdown
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Figure 4. Efficient in vivo silencing of cohesin does not lead to acute phenotypes. (A) GFP+ cells were sorted from total bone marrow from Renilla(shRNA/+), Rad21(shRNA/+), Smc1a(shRNA/+), and Stag2(shRNA/+), mice (n = 2). Protein was extracted and blotted for the indicated antibodies. (B) Erythroid progenitors (GFP+; CD71+/Ter119+) were FACS sorted from bone marrow of mice (n = 3) that were exposed to doxycycline for 10 d, qRT-PCR was performed (using Actin B as reference), and cohesin knockdown was compared with Renilla control animals. (C) Animals (n = 9) were exposed to doxycycline for 10 d and blood was drawn. FACS analysis is shown for B (B220+), T (CD3+), and myeloid cells (Cd11b+/Gr1+). (D) c-Kit+ cells from Renilla(shRNA/+) and Smc1a(shRNA/+) mice were cultured for 6 d, Smc1a protein was immunoprecipitated and blotted for indicated antibodies (IP, immunoprecipitation; FT, flow through). (E) Bone marrow of Renilla(shRNA/+), Rad21(shRNA/+), Smc1a(shRNA/+), and Stag2(shRNA/+), mice (n = 3) was plated in methylcellulose containing doxycycline and replated for four passages. Error bars indicate SD.

in various cellular compartments. We tested knockdown of the mRNA in the thymus (T cells and progenitors) by Western blot analysis (Fig. 3 F).

In addition, we tested knockdown of the mRNA and protein in the bone marrow using Western blot and qRT-PCR (Fig. 4, A and B). Strikingly, in the Smc1a(shRNA/+) mice we not only observed a strong reduction of the intended mRNA (Smc1a), but also a significant decrease of the associated protein members of the cohesin complex, reminiscent of studies in different cellular systems (Vass et al., 2003; Laugsch et al., 2013). Despite this significant reduction of cohesin protein levels, FACS analysis of control and cohesin knockdown mice did not detect significant differences in peripheral blood cellular/mononuclear elements (Fig. 4 C). Likewise, no differences in complete blood counts of cohesin mice were observed at this stage (not depicted).

Furthermore, we did not detect gross chromosomal instability when we compared karyotype analysis of c-Kit+ progenitor cells of Renilla(shRNA/+), Rad21(shRNA/+), Smc1a(shRNA/+), and Stag2(shRNA/+) animals (not depicted). This can be explained by the fact that despite efficient knockdown of Rad21, Smc1a, or Stag2, the remaining proteins will continue to form a functional cohesin complex. We then evaluated this hypothesis with Smc1a and confirmed that the remaining Smc1a protein formed a complex with Smc3, allowing proper sister chromatid separation (Fig. 4 D). Finally, we sought to verify that the in vivo reduction of Rad21, Smc1a, and Stag2 is comparable with retroviral knockdown in our previous experiments. To test this, we plated bone marrow from Rad21(shRNA/+), Smc1a(shRNA/+), and Stag2(shRNA/+) mice in methylcellulose. Bone marrow from all three cohesin shRNA mice replated for four passages (compared with two for Renilla bone marrow; Fig. 4 E). These results indicate that significantly reduced expression of cohesin can be tolerated in vivo, providing us with the ability to further probe for cohesin functions in stem cell differentiation and transformation.

Cohesin silencing leads to alterations in myeloid/erythroid differentiation

To monitor progressive changes in the hematopoietic system of cohesin knockdown mice, we generated a cohort of Tet-on rtTA shRNA animals that were continuously exposed to doxycycline starting at 6 wk of age. At several time points we performed whole body necropsy on these animals followed by a thorough analysis of the hematopoietic system. First, we confirmed that GFP (and therefore shRNA) expression is stable over time in the mouse models we generated. We analyzed frequency of GFP-expressing cells in the hematopoietic organs of mice that were exposed to doxycycline for 30 d. In all organs, GFP expression remained stable; interestingly, we
observed an increase in frequency of GFP-expressing cells in the spleen of Smc1aΔRNA/+ animals (Fig. 5 A). This prompted us to further investigate the lineages present in the spleen, bone marrow, and blood of Rad21ΔRNA/+ , Smc1aΔRNA/+ , Stag2ΔRNA/+ , and RenillaΔRNA/+ control mice. Indeed, lineage skewing was apparent by FACS analysis (Fig. 5 C) and peripheral blood mononuclear cell differential counts (Fig. 5 C) in Rad21ΔRNA/+ , Smc1aΔRNA/+ , and Stag2ΔRNA/+ animals compared with RenillaΔRNA/+ mice. Morphologically, several animals displayed splenomegaly, which was most apparent in Smc1aΔRNA/+ mice (Fig. 5 D). FACS analysis of the spleens of Rad21ΔRNA/+ , Smc1aΔRNA/+ , and Stag2ΔRNA/+ animals showed a decrease in the proportion of B cell population (B220+), coupled to an increase in myelomonocytic (Cd11b+/Gr1+) and erythroid cells (CD71+/Ter119+) (Fig. 5 E).

A marked erythroid expansion with increased amounts of early erythroid elements (CD71+/Ter119+) was observed in the spleen of Rad21ΔRNA/+ and Smc1aΔRNA/+ animals (Fig. 5, F and G). These results suggested the presence of erythroid colonies in spleen samples of these animals. In addition, we found a significant increase of Lineage−/c-Kit+ cells in spleens of Rad21ΔRNA/+ and Smc1aΔRNA/+ mice (Fig. 5 H). The combined findings, namely the increase of both erythroid and c-Kit+ cells, indicate myeloid metaplasia, also known as extramedullary hematopoiesis, in the splenic parenchyma, a phenomenon observed in several preleukemic mouse models (Moran-Crusio et al., 2011; Celik et al., 2015).

Next, we investigated the bone marrow, where we observed a decrease in the Ter119+ cell population in the GFP+ bone marrow of Rad21ΔRNA/+ and Smc1aΔRNA/+ animals (Fig. 6 A). The increase in myeloid element in the spleen and bone marrow prompted us to investigate the progenitor compartment in the bone marrow of affected animals. Here, we noticed an expansion in the frequency of myeloid/erythroid lineage colonies in spleens of Rad21ΔRNA/+ and Smc1aΔRNA/+ mice (Fig. 6 B). The increase in myeloid element in the spleen and bone marrow prompted us to investigate the progenitor compartment in the bone marrow of affected animals. Here, we noticed an expansion in the frequency of myeloid/erythroid lineage colonies in spleens of Rad21ΔRNA/+ and Smc1aΔRNA/+ mice (Fig. 6 B). This was accompanied by a decrease in megakaryocyte-erythocyte progenitors (Lineage−/c-Kit+ cell populations) (Fig. 6 C). An additional phenotype we observed was an increase in nuclear size in sorted Lineage−/c-Kit+ progenitor cells (Fig. 6 D). The exact cause and/or consequence of this phenomenon is unknown, but it has been previously associated with cohesin loss of function in other cell types (Hoque and Ishikawa, 2002; Sofueva et al., 2013). The apparent increase in nuclear size was also seen in other elements of the hematopoietic system of cohesin knockdown mice (not depicted). Of note, the increase in nuclear volume was not associated with increase of genomic material or instability, as confirmed by conventional cytogenetic analysis on metaphase spreads.

**Figure 5.** Cohesin knockdown leads to changes in the myeloid/erythroid lineage in blood and spleen. (A) Frequency of GFP+ cells as measured by flow cytometry (*, P < 0.05). (B) Flow cytometry analysis of peripheral blood from RenillaΔRNA/+ , Rad21ΔRNA/+ , Smc1aΔRNA/+ , and Stag2ΔRNA/+ mice (n = 3) for the indicated antibodies. (C) Absolute numbers of lymphocytes and neutrophils/monocytes are plotted for the indicated genotypes (*, P < 0.05). (D) Representative spleens from Smc1aΔRNA/+ mice ex vivo stained with antibody to CD34. (E) Flow cytometry analysis of spleen from RenillaΔRNA/+ , Rad21ΔRNA/+ , Smc1aΔRNA/+ , and Stag2ΔRNA/+ mice (n = 3; *, P < 0.05) for the indicated antibodies. (F) Representative FACS analysis of the spleen of GFP+ basophilic erythroblasts (CD71+/Ter119+) and orthochromatophilic erythroblasts (Ter119+) for the indicated genotypes. (G) Quantification of GFP+ basophilic erythroblasts (CD71+/Ter119+) and orthochromatophilic erythroblasts (Ter119+) in spleens (n = 3) in animals of the indicated genotype that were exposed to doxycycline for 30 d (*, P < 0.05). (H) Frequencies of GFP+/Lineage−/c-Kit+ cells as measured in the spleens of the indicated genotypes (n = 3; *, P < 0.05). Error bars indicate SD.
Cohesin knockdown induces differentiation skewing of HSCs

The changes found in the differentiated and progenitors cells prompted us to address the impact of cohesin knockdown on HSCs. We decided to focus on two out of the three mouse models we created, Smc1a(shRNA/+) and Stag2(shRNA/+)]. We chose Smc1a and Stag2 to include one core ring component, Smc1a, and one associated protein, Stag2. Indeed, even after a short period of cohesin gene knockdown (10 d), we observed distinct changes in the HSC compartment of Smc1a(shRNA/+) and Stag2(shRNA/+) mice (Fig. 7 A). First, we noted that cohesin knockdown did not significantly impact the Lineage− Sca1+c-Kit+ (LSK) cells, a population which is composed of HSCs and multipotent progenitors (MPPs; not depicted). Next, we analyzed the effect of cohesin knockdown in long-term HSCs (LT-HSCs) and MPPs. A striking reduction of shRNA-expressing phenotypic long-term (CD150+) and short-term (CD150−/CD48−) HSC subsets was observed, evaluated by expression, frequency (Fig. 7 B), and absolute counts (Fig. 7 C). In the Smc1a(shRNA/+) mouse model, this loss of HSCs was compensated by a gain in MPPs and specifically the CD150+/CD48+ MPP subset (Fig. 7 C). A similar phenotype has been described previously in an Flt3-ITD animal model (Chu et al., 2012).

Next we wanted to identify the transcriptional changes, which underlie the phenotypic changes that we observed in the Lineage− Sca1+c-Kit+ compartment (LSK subset, comprised of HSCs and MPP cells) of Smc1a and Stag2 knockdown mice. We performed gene expression analysis (using RNA sequencing) of purified LSK cells (GFP+/Lineage−/c-Kit+/Sca1−) from Renilla (shRNA/+) and Smc1a(shRNA/+) and Stag2(shRNA/+) mice. We again confirmed that the shRNA-expressing cells (as measured by expression of GFP) indeed verifying a normal diploid karyotype (not depicted). Collectively, these results demonstrate that knockdown of cohesin subunits can alter hematopoiesis with an apparent bias toward the myeloid hyperplasia, suggesting a role for cohesin in myeloid differentiation.

Figure 6. Cohesin knockdown induces myeloid progenitor skewing and increases the size of the nucleus.
(A) Quantification of flow cytometry of bone marrow of animals (n = 3) of the indicated genotype (*, P < 0.05). (B) FACS analysis of bone marrow of cohesin knockdown mice (doxycycline 30 d, n = 3) for myeloid progenitors (GFP+/Lin−/c-Kit+/Sca1−). (C) Quantification of myeloid progenitors in the bone marrow of mice (n = 3) with the indicated genotype (*, P < 0.05). (D) GFP+ c-Kit+ cells were sorted from mouse bone marrow of control and cohesin knockdown (10 d of doxycycline). Cells were fixed on glass slides and stained with DAPI. (E) Quantification of nuclear size of GFP+ c-Kit+ cells for the indicated genotypes. Surface area of nuclei was measured using DAPI intensity with ImageJ (National Institutes of Health; *, P < 0.05). Error bars indicate SD.
compared with the Renilla control. In the Stag2(shRNA/+) LSK cells, we found up- and down-regulation of 390 and 480 genes, respectively, compared with Renilla control. By comparing the gene expression profiles of Smc1a(shRNA+) with Stag2(shRNA/+), few differentially expressed genes were found (53 down and 125 up). Indeed, Smc1a and Stag2 appear to regulate an have a very potent knockdown of the intended target genes, Smc1a and Stag2 (not depicted). Next, we focused on the significantly differentially expressed genes (FPKM > 5, fold change [FC] > 1.5, and P < 0.05) in the various genotypes (Table S3). In the Smc1a(shRNA/+) LSK cells, we found up-regulation and down-regulation of 257 and 338 genes, respectively, compared with the Renilla control. In the Stag2(shRNA/+) LSK cells, we found up- and down-regulation of 390 and 480 genes, respectively, compared with Renilla control. By comparing the gene expression profiles of Smc1a(shRNA/+) with Stag2(shRNA/+), few differentially expressed genes were found (53 down and 125 up). Indeed, Smc1a and Stag2 appear to regulate an
identical expression program in LSK cells (Fig. 7, D and E). Interestingly, genes found up-regulated in cohesin knockdown LSK cells are associated with a myeloid differentiation program, including the Fgr3 and Cebpa genes, encoding key regulators of myelopoiesis (Fig. 7 D). At the same time, we observed down-regulation of genes involved in lymphoid development (including Blink, Lax1, and Cd86). These data suggested that cohesin gene knockdown LSK cells are primed to differentiate toward the myeloid lineage, in agreement with our in vivo phenotype of the studied animals. This was further confirmed by an unbiased analysis of the gene expression changes by gene set enrichment analysis (GSEA). Such analysis showed that LSK cells from Smc1a(shRNA/+), and Stag2(shRNA/+), mice showed an aberrant and premature commitment to the myeloid lineage based on their gene expression signature (Fig. 7 F). In agreement with the loss of phenotypic HSCs in these animals, LSK cells have lost expression of genes that normally comprise a signature of LT-HSCs. These data show that cohesin knockdown in LSK cells leads to transcriptional changes that are associated with a differentiation bias during early stages of hematopoiesis.

Cohesin silencing leads to aberrant chromatin accessibility

Cohesin is known to be involved in several processes that regulate transcription and gene expression. Cohesin binds to CTCF and is found together with CTCF on chromatin (Parelho et al., 2008; Wendt et al., 2008). More recently, it was shown that cohesin clusters are found at sites with high chromatin accessibility measured by DNase I hypersensitivity (Yan et al., 2013) assays. Therefore we decided to apply ATAC sequencing, a genome-wide mapping of chromatin accessibility (Buenrostro et al., 2015). ATAC-seq analysis showed a large number of changes between Renilla(shRNA/+), and Stag2(shRNA/+), mice to map changes in chromatin accessibility (Buenrostro et al., 2015). ATAC-seq analysis showed a large number of changes in chromatin accessibility (Buenrostro et al., 2015). ATAC-seq analysis showed a large number of changes between Renilla(shRNA/+), and Stag2(shRNA/+), samples (Fig. 8, A and B; and Table S4). Changes in chromatin accessibility were pronounced in genes that were up-regulated in cohesin knockdown LSK cells, like Fcγ-receptor (Fgr3 and Fgr4) and Myeloperoxidase (Mpo; Fig. 8, C and D). These genes are highly expressed in myeloid cells, suggesting that the cohesin complex plays a role in the regulation of myeloid differentiation through chromatin accessibility. In addition, we found that genes that were expressed in lower levels in cohesin knockdown cells (like Cd74) lose DNA accessibility (Fig. 8, C and D). Next, we compared RNA expression and chromatin accessibility in an unbiased way. We found that increased chromatin accessibility around promoter regions indeed correlates with significant increased expression, and the reverse is also true (Fig. 8 E). Furthermore, we performed GSEA with the ATAC-seq peaks enriched in either Renilla(shRNA/+), or Stag2(shRNA/+), LSK cells. Here we observed that peaks enriched in Stag2(shRNA/+), LSK cells correlate with the GMP signature that we found before to be enriched in the RNA sequencing data (Fig. 8 F). Furthermore, we used an unbiased approach to test whether the changes in chromatin accessibility reflected a change in the cellular state (McLean et al., 2010). Regions that were more accessible in the Stag2 knockdown cells correlated with GO terms for myeloid differentiation and erythroid development, in line with our findings in vivo (Fig. 8 G and Table S5). This is specific for cohesin knockdown cells, as we don’t find the same enrichment in the regions that are more accessible in Renilla control LSK cells (Table S5). GSEA and GO term analysis confirmed the notion that cohesin knockdown LSK cells are committed to differentiate along the myeloid lineage. Finally, we analyzed the genomic sequence in the more accessible regions of the cohesin cells for enrichment of transcription factor motifs (Imrichová et al., 2015). We found that the most enriched transcription factor motif is the GATA factor motif (Fig. 8 H). GATA1 is a master regulator of primitive and definitive hematopoiesis, and erythropoiesis and is often mutated in cancer (Crispino, 2005). In our ATAC-seq data, we also found increased accessibility in the GATA1 locus in Stag2 knockdown LSK cells (Fig. 8 C). Collectively, these data suggest that cohesin knockdown leads to changes in chromatin accessibility in genes that are expressed in the myeloid lineage, possibly through involvement of GATA transcription factors.

Silencing of cohesin complex genes leads to an MPN/myeloid disorder

The ageing cohort of cohesin gene knockdown was monitored by FACS analysis of peripheral blood at regular intervals. During these routine examinations, we found that several animals showed a marked expansion of myeloid elements (granulocytic > monocytic) in the peripheral blood, consistent with myeloid hyperplasia (Cd11b+/Gr1+; Fig. 9, A and B [I–III]). Necropsy was performed on a subset of the animals to allow for a more in-depth analysis. A first gross morphological indication for an underlying hematopoietic disorder was the presence of varying degrees of splenomegaly. Fig. 9 depicts an example of the phenotype (organomegaly) of an Smc1a(shRNA/+), animal in comparison to an age-matched Renilla(shRNA/+), control (Fig. 9 C). Histological evaluation confirmed that the splenic architecture was compromised by marked to moderate red pulp expansion, exhibiting myeloid metaplasia (extramedullary hematopoiesis; Fig. 9 B, IV). The hematopoietic elements were composed of numerous myelomonocytic cells, including frequent immature forms, alternating with variable amounts of erythroid precursors, forming occasional erythroid islands and frequent megakaryocytes (Fig. 9 B, V and VI). Gross evaluation of bone specimens of the affected Smc1a(shRNA/+), animals demonstrated “pale appearing” bones and histological evaluation showed a hypercellular bone marrow with erythroid and megakaryocytic hypoplasia and marked myeloid hyperplasia (Fig. 9, B [VII] and D). The myeloid elements were predominantly medium to large in size, with open chromatin, crisp and irregular nuclear membrane, and variable amounts of amphophilic cytoplasm, focally imparting myelomonocytoid features (Fig. 9 B, VIII and IX). Peripheral blood differential count analysis confirmed a significant increase in neutrophil numbers (Fig. 9 E).
Figure 8. Cohesin silencing leads to changes in chromatin accessibility in LSK cells. (A) Venn diagrams of ATAC-seq peaks (peak score > 25) found in LSK cells isolated from Renilla^{shRNA/}\textsuperscript{-}\textsuperscript{-} and Stag2^{shRNA/}\textsuperscript{-}\textsuperscript{-} mice. (B) Heat map of peaks found significantly enriched (FC(abs) > 2, P < 0.05) Renilla^{shRNA/}\textsuperscript{-}\textsuperscript{-} and Stag2^{shRNA/}\textsuperscript{-}\textsuperscript{-} ATAC-seq centered on the peak maximum. (C) Changes in chromatin accessibility in key myeloid differentiation genes (Mpo, Fcgr3/4, and GATA1). Tracks showing increase in ATAC-seq signal on MPO and FCGR3/4 genes in LSK cells from Renilla^{shRNA/}\textsuperscript{-}\textsuperscript{-} and Stag2^{shRNA/}\textsuperscript{-}\textsuperscript{-} mice. Loss of ATAC-seq signal in the stem cell gene CD74 in LSK cells from Renilla^{shRNA/}\textsuperscript{-}\textsuperscript{-} and Stag2^{shRNA/}\textsuperscript{-}\textsuperscript{-} mice. (D) Changes in gene expression in LSK cells from cohesin knockdown mice. Indicated are FPKM values for MPO, FCGR3, and CD74 as obtained from RNA sequencing in LSK cells for the indicated genotypes (*, P < 0.05). (E) RNA expression correlates with chromatin accessibility. Changes in RNA expression (RNA sequencing) of genes that have either increased (Up) or decreased (Down) chromatin accessibility (ATAC-sequencing) at their promoter are plotted (*, P < 0.05). (F) GSEA of Stag2 knockdown enriched ATAC-seq peaks. (G) GO term enrichment of biological processes with peaks (FC(abs) > 2, P < 0.05) that are unique in the Stag2^{shRNA/}\textsuperscript{-}\textsuperscript{-} ATAC-seq. (H) Result from transcription factor motif enrichment in peaks (FC(abs) > 2, P < 0.05) unique in the Stag2^{shRNA/}\textsuperscript{-}\textsuperscript{-} ATAC-seq. Error bars indicate SD.
Figure 9. MPNs in aged Smc1a(shRNA+/+) mice. (A) FACS analysis of peripheral blood of age-matched Renilla(shRNA+/+) control and Smc1a(shRNA+/+) mouse (antibodies are indicated). (B) Histological analysis of peripheral blood, spleen, and bone marrow from Smc1a(shRNA+/+) mouse. Peripheral blood was stained with Wright-Giemsa. Spleen and bone marrow were stained with H&E. (C) Representative spleen of aged Smc1a(shRNA+/+) mouse. (D) Representative femur and tibia of aged Smc1a(shRNA+/+) mouse. (E) Blood count of diseased Smc1a(shRNA+/+) mice. WBC, white blood cells; NE, neutrophils; LY, lymphocytes; and MO, monocytes (*, P < 0.05). (F and G) FACS analysis of bone marrow and spleen of representative aged Smc1a(shRNA+/+) mice. (left) GFP expression. (middle) Antibody staining for myeloid (CD11b+/Gr1+), B (B220+), T (CD3+), and erythroid cells (CD71+/Ter119+ and Ter119+) is indicated. (right) FACS staining for myeloid progenitors (GFP+/Lin−/c-Kit−/Sca1−) is plotted. Error bars indicate SD.
in the affected animals, further supporting that Smc1a silencing promotes a clinical picture consistent with an MPN. Flow cytometry analysis performed on peripheral blood spleen and marrow specimens revealed similar but more pronounced phenotypes in these animals compared with the younger animal cohort. All three compartments demonstrated a marked increase in myeloid cells (CD11b+/Gr1+), which was accompanied by a decrease in lymphoid elements, particularly B220+/B220- cells (Fig. 9, A, F, and G). Composition of medullary and extramedullary hematopoiesis differed from case to case, with a subset of spleen specimens showing trilineage maturing hematopoiesis with erythroid predominance and intact erythroid islands, immunophenotypically confirmed by increased CD71+/Ter119+ cells, whereas bone marrow hematopoietic elements demonstrated erythroid hypoplasia confirmed by a decrease in CD71+/Ter119+ cells (Fig. 9, F and G). Notably, a striking increase of GMPs (GFP+/Lineage-1/c-Kit+/Sca-1+/Cd34+/FcRII/III+) was found in these bone marrow specimens (Fig. 9, F, right), in agreement with previous experiments (Fig. 6 C). In spleen specimens of Smc1a(shRNA+/-) mice, the immature myelomonocytic proliferation was immunophenotypically confirmed by an increase in GFP+/Lineage-1/c-Kit+/Sca-1-1 cells (Fig. 9 G, right).

Encouraged by these results, we decided to generate mice with a more potent disruption of the cohesin complex. Our rationale for this is the fact that SMC1A and STAG2 mutations found in patients potentially generate strong loss of function alleles because these genes are located on the X chromosome. In addition, simultaneous down-regulation of the mRNA level of multiple cohesin members has been observed in AML samples (Thota et al., 2014). To obtain compound shRNA mice that target two cohesin subunits simultaneously, we intercrossed Smc1a(shRNA+/-) and Stag2(shRNA+/-) mice. Compound Smc1a(shRNA+)/Stag2(shRNA-) did not show any deleterious phenotypes, although an effective knockdown was confirmed (Fig. 10 A). Short-term exposure to doxycycline (22 d) caused splenomegaly (not depicted) and lineage skewing (Fig. 10 B). Over time, Smc1a(shRNA+)/Stag2(shRNA-) animals showed again significant lineage skewing in the peripheral blood, namely myeloid hyperplasia (CD11b+/Gr1+) and lymphopenia (decrease in B220+ cells; Fig. 10 C). FACS analysis of these animals revealed a very similar yet more pronounced phenotype compared with the Smc1a(shRNA+/-) mouse (not depicted). Spleens of aged compound Smc1a(shRNA+)/Stag2(shRNA-) animals were enlarged compared with control animals and showed disrupted morphology (Fig. 10, D and E). Diseased animals demonstrated signs of anemia, as exemplified by peripheral blood counts (Fig. 10 F).

In summary, these experiments effectively demonstrate that silencing of the cohesin complex alters stem cell homeostasis, predominantly affecting the myeloid component, leading to disturbances in myeloid proliferation and differentiation closely resembling the clinical picture of a myeloid disorder/myeloid neoplasm. In addition, our study describes the first in vivo models that demonstrate a tumor-suppressive role for the cohesin complex.

**DISCUSSION**

AML is a genetically diverse disease. Fusion genes and mutations in many lineage-specific transcription factors and epigenetic regulators have been uncovered. Recently, mutations in the cohesin (Rad21, Smc1a, Smc3, and Stag2) complex have been identified in de novo and secondary AML samples, as well as many solid tumors (Leiserson et al., 2015). Many of these mutations are predicted (and shown here) to result in loss of function of the affected protein. This is very surprising, as all cohesin genes are essential in every organism tested from yeast to mice. Indeed, Rad21-/- animals are not viable (Xu et al., 2010); however, conditional knockout of Rad21 in postmitotic thymocytes does not affect survival (Seitan et al., 2011). These findings support the notion that cohesin proteins are present at high levels both in actively cycling and quiescent tissues (Wendt et al., 2008). In tumors, mutations in Smc3 and Rad21 are usually heterozygous, leaving one copy of the gene unaffected. Stag2 and Smc1a reside on chromosome X and are therefore not compensated by a wild-type protein when mutated in male patients. Similar in females, all mutations found in Stag2 were reported to be on the active (transcribed) X chromosome (Solomon et al., 2011). Surprisingly, even in an essential gene like Smc1a, a portion (~10%) of the alterations were found to be nonsense and/or frame-shift mutations, predicting a complete loss of protein function (http://www.cbioportal.org). How cells deficient for a cohesin subunit manage to correctly separate their chromosomes remains to be understood.

To model the loss of cohesin as seen in human malignancies, we generated three independent loss of function cohesin animals. As standard mouse knockout techniques create complete null alleles, we opted to generate three mouse models with inducible shRNA targeting cohesin complex members in vivo (Premririt et al., 2011). In these animals, we managed to efficiently suppress cohesin transcripts and reduce cohesin protein expression and function similar to that seen in human malignancies. Surprisingly, even in mice where we induced systemic cohesin knockdown, we did not observe detrimental effects, although we never reached a complete loss of expression in this model. We were surprised by the initial lack of survival phenotypes in cells with a very efficient reduction of the cohesin complex members. One explanation is the fact that during metaphase only a very small fraction of cohesin is required for SCC (Waizenegger et al., 2000). Apparently, the small amount of cohesin protein that remains in our shRNA mouse is most likely sufficient for proper SCC and successful mitosis. This is consistent with the fact that we did not observe overwhelming defects in SCC in cohesin knockdown cells. Another process cohesin is known to play a role in is DNA damage repair (Watrin and Peters, 2006). At this moment, no consensus exists on whether mutations in cohesin in human malignancies lead to genomic instability (Solomon et al., 2011; Balbás-Martínez et al., 2013; Li et al., 2015), although using our cohesin knockdown cells we did not observe changes of the overall karyotype. Similarly, human AML samples carrying cohesin mutations do
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Adding a new layer of complexity to an already complicated disease. The chromatin accessibility data and correlation with gene expression changes presented here support this hypothesis.

AML is a disease that often originates in stem cells’ hematopoietic system (Shlush et al., 2014). During the progression of the disease, clonal evolution is a common occurrence (Ding et al., 2012). Recently, several studies have identified mutations in the originating stem cell in AML (Genovese et al., 2014). Cohesin genes are among the genes that are mutated in the stem cells that are found in the bone marrow of AML patients (Ding et al., 2012; Jan et al., 2012). Here, we provide evidence that loss of cohesin indeed leads to increased self-renewal of stem cells in vitro. Cohesin knockdown mice display a skewing in their stem cell compartment in the bone marrow and show signs of extramedullary hematopoiesis in the spleen. All these phenotypes have been reported before in mouse models for AML (Lee et al., 2007; Moran-Crusio et al., 2011).

This is the first example of cohesin loss of function in a mammalian system. Strikingly, efficient (but not complete) silencing of cohesin is not detrimental to cellular homeostasis but leads to myeloproliferative disease, suggesting that there not have an overt genomic instability phenotype (Cancer Genome Atlas Research Network, 2013).

As no differences are observed in steady state SCC and DNA damage response, we hypothesize that loss of cohesin complex member expression in human disease mainly affects higher-order chromatin structure and gene expression. The genome of eukaryotes is organized into structural domains that help establish regulatory interactions (Lieberman-Aiden et al., 2009; Dixon et al., 2012). The cohesin complex is one of the main factors involved in the stability of the three-dimensional organization of the chromatin. When cohesin is removed from cells, a general unfolding and disorganization of the chromatin is observed with associated changes in gene expression (Seitan et al., 2013; Sofueva et al., 2013; Mizuguchi et al., 2014). Changes in higher-order chromatin structure have been observed in disease, including cancer (Rickman et al., 2012). It is therefore not unconceivable that loss of cohesin in cancer leads to disruption of chromatin organization. Cohesin serves as a docking station for transcription factors, and its absence could therefore cause transcription factors to aberrantly activate genes (Yan et al., 2013). We postulate that cohesin mutations in AML mainly impact genome organization, adding a new layer of complexity to an already complicated disease. The chromatin accessibility data and correlation with gene expression changes presented here support this hypothesis.

AML is a disease that often originates in stem cells’ hematopoietic system (Shlush et al., 2014). During the progression of the disease, clonal evolution is a common occurrence (Ding et al., 2012). Recently, several studies have identified mutations in the originating stem cell in AML (Genovese et al., 2014). Cohesin genes are among the genes that are mutated in the stem cells that are found in the bone marrow of AML patients (Ding et al., 2012; Jan et al., 2012). Here, we provide evidence that loss of cohesin indeed leads to increased self-renewal of stem cells in vitro. Cohesin knockdown mice display a skewing in their stem cell compartment in the bone marrow and show signs of extramedullary hematopoiesis in the spleen. All these phenotypes have been reported before in mouse models for AML (Lee et al., 2007; Moran-Crusio et al., 2011).

This is the first example of cohesin loss of function in a mammalian system. Strikingly, efficient (but not complete) silencing of cohesin is not detrimental to cellular homeostasis but leads to myeloproliferative disease, suggesting that there
are defined thresholds of cohesin expression and activity. This notion is consistent with the findings illustrated in the accompanying paper in this issue by Viny et al., where complete depletion of another cohesin member, Smc3, leads to lethal bone marrow aplasia, whereas Smc3 haploinsufficiency leads to enhanced self-renewal and AML in the background of Flt3-ITD. Our study is the first evidence that mutation of cohesin is a driving event in the initiation and progression of cancer and not merely a bystander. Furthermore, this suggests that cohesin loss could be therapeutically exploited. Cells carrying mutations in the cohesin complex are potentially more vulnerable to certain stressors or insults. The identification of cohesin synthetic lethal interactions could aid the development of new drugs, specifically targeting cohesin mutant cells. For this to be successfully introduced into the clinic, it is a prerequisite that AML patients are preselected based on their cohesin mutation status. Such a new class of drugs is very welcome in a disease such as AML, where very few targeted therapies are available at this moment.

MATERIALS AND METHODS

Western blot. Protein was extracted from equal cell numbers in a high urea buffer (48% urea, 15 mM Tris-HCl, pH 7.5, 8.7% glycerol, 1% SDS, 0.004% bromophenol blue, and 143 mM urea buffer (48% urea, 15 mM Tris-HCl, pH 7.5, 8.7% glycerol, 1% SDS, 0.004% bromophenol blue, and 143 mM urea buffer). Protein levels were measured using the Bradford assay. Equal amounts of protein were loaded on NuPAGE Novex 4–12% Bis-Tris protein gel (NP0321; Life Technologies) and run with NuPAGE MOPS buffer (NP0001; Life Technologies). Proteins were transferred to Immobilon P (IPVH00010; EMD Millipore). Membrane was blocked in 5% nonfat milk in TBST and incubated with antibodies for Rad21 (H-210; Santa Cruz Biotechnology, Inc.), Smc1a (A300-055A; Bethyl Laboratories, Inc.), Smc3 (ab9263; Abcam), Stag2 (I8015; Sigma-Aldrich) and labeled with CD117-MicroBeads (130-091-224; Miltenyi Biotec) for 15 min at 4°C. Cell suspension was selected on AutoMacs (Miltenyi Biotec) and labeled with CD117-MicroBeads (130-091-224; Miltenyi Biotec) for 15 min at 4°C. Cell suspension was selected on AutoMacs (Miltenyi Biotec), and c-Kit+ cells were seeded at 500,000/ml in Opti-MEM (31985-070; Life Technologies) supplemented with 10% FCS, 55 µM β-mercaptoethanol (21985-028; Gibco), 50 ng/ml SCF (250-05; PeproTech), 50 ng/ml Flt3-Ligand (250-31H; PeproTech), 10 ng/ml IL-3 (213-13; PeproTech), and 10 ng/ml IL-6 (216-16; PeproTech). Virus was produced 24 h after cell seeding in growth media with 8 µg/ml polybrene. Viral transduction was monitored 48 h after virus addition by FACS analysis.

qRT-PCR. RNA was isolated using RNeasy mini plus kit (Qiagen). cDNA was synthesized with a high-capacity RNA-to-cDNA kit (4387406; Life Technologies). The qRT-PCR reaction was performed in a volume of 10 µl with SYBR green (4887352001; Roche) and run on a Light-Cycler 480 machine (Roche) in 384-well plates. The following qRT-PCR primers (all for mouse) were used in this study: ACTB-F, 5'-GGCTGTTATTTCCCTTCATGC-3'; ACTB-R, 5'-CCAGTTGGTGAACAATGCCATGT-3'; Rad21-F, 5'-GCCCATGTTATTTAGTGCGCA-3'; Rad21-R, 5'-ACTCCCCAGGAAGGTGC-3'; Smc1a-1-F, 5'-TATCTATGC-TGAGAGGCCCC-3'; Smc1a-R, 5'-TGGTTGTTTCTCTGGCTCCG-3'; Smc3-F, 5'-TCCGGAATGTACCGAGACCA-3'; Smc3-R, 5'-GTTGTTCTTTCCAGACGCA-3'; Stag2-F, 5'-CAGCTGAATGCATCCTC CCC-3'; and Stag2-R, 5'-GCAAACAGCTGATGTTCCTTG-3'.

RNA sequencing and analysis. RNA was isolated using RNeasy mini plus reagents (Qiagen), and RNA sequencing was performed using the NEXTag Rapid Directional RNA-Seq kit (5138-07; Boao Scientific). Libraries were barcoded with NEXTag adapters and quantified using qPCR and Qubit (Life Technologies). Libraries were sequenced at a HiSeq 2000 (illumina). Next, sequencing data analysis was performed as follows. Reads were aligned to mm9 reference genome using TopHat, and Cuffdiff was performed to confirm GFP expression and for markers of differentiation. The following antibodies were used at 1/10,000 dilution Cd11b (clone M1/70), Gr1 (clone RB6-8C5), c-Kit (clone 2B8), and Sca-1 (clone D7). For serial replating experiments 2,000 GFP+ cells were seeded as above.

Metaphase spreads. Cells were grown in the presence of 0.1 µg/ml nocodazole for 1–4 h. Cells were harvested and resuspended in ice-cold 0.5% KCl. Cells were fixed in ice-cold methanol/glacial acetic acid (3:1) solution and dropped in cold glass slides from circa 50 cm. Slides were air-dried and stained with DAPI. Metaphases were acquired using ECL (P134077 and P134095; Thermo Fisher Scientific).

Retrovirus production and infection of c-Kit+ cells. Virus was produced in 293T cells. Cells were transfected with 20 µg of plasmids using the calcium phosphate protocol. Supernatant containing the virus was harvested 36, 48, and 60 h after transfection. Virus was cleared using a 20-µm filter and concentrated (Amicon Ultra-15, 100 kD MWCO) to ~1/10 volume. Virus was snap frozen and stored at −80°C. Mouse bone marrow cells were isolated from femur, tibia, and pelvic bones. Cells were blocked with rat IgG (8015; Sigma-Aldrich) and labeled with CD117-MicroBeads (130-091-224; Miltenyi Biotec) for 15 min at 4°C. Cell suspension was selected on AutoMACS (Miltenyi Biotec), and c-Kit+ cells were seeded at 500,000/ml in Opti-MEM (31985-070; Life Technologies) supplemented with 10% FCS, 55 µM β-mercaptoethanol (21985-028; Gibco), 50 ng/ml SCF (250-03; PeproTech), 50 ng/ml Flt3-Ligand (250-31H; PeproTech), 10 ng/ml IL-3 (213-13; PeproTech), and 10 ng/ml IL-6 (216-16; PeproTech). Virus was added 24 h after cell seeding in growth media with 8 µg/ml polybrene. Viral transduction was monitored 48 h after virus addition by FACS analysis.

CFU assay. To address self-renewal capacity, 2,000 retrovirally infected GFP+ or 20,000 total bone marrow cells were seeded in 0.5 ml (per well in 24-well plate) methylcellulose (M3434; STEMCELL Technologies). After 7-d incubation time, GFP+ colonies were counted. FACS analysis was performed to confirm GFP expression and for markers of differentiation. The following antibodies were used at 1/10,000 dilution Cd11b (clone M1/70), Gr1 (clone RB6-8C5), c-Kit (clone 2B8), and Sca-1 (clone D7). For serial replating experiments 2,000 GFP+ cells were seeded as above.

shRNA mouse generation and handling. To generate shRNA mice for cohesin, we tested 10 shRNAs for Rad21 and Qdub (Life Technologies). Libraries were sequenced at a HiSeq 2000 (illumina). Next, sequencing data analysis was performed as follows. Reads were aligned to mm9 reference genome using TopHat, and Cuffdiff was used to find differentially expressed genes (Trapnell et al., 2012). Heat maps were generated in multiple experiment viewer (MEV; http://www.tm4.org). GSEA was performed by running RNA sequencing data from LSK cells isolated from Renilla(shRNA/+), Smc1a(shRNA/+), and Stag2(shRNA/+) against the most differentially expressed genes from the Immunological Genome Project (Table S6; Shay and Kang, 2013).

shRNA sequences as used in shRNA mice in this study are as follows: Rad21-920, 5'-GATGCATGTTAGATTGTTTGCAATAGTGAAGCCACAGATGT-3'; Smc1a-3921, 5'-GATCAGAG-TCGAGATGACAATAGTGAAGCCACAGATGT-3'; and Stag2-744, 5'-CCAGTTGGTGAACAATGCCATGT-3'.
through a 70-μm strainer with a plunger. Cells were blocked with rat IgG (B015; Sigma-Aldrich) for 20 min on ice. Staining for cell surface antigens was performed by adding fluorochrome- or biotin-conjugated antibodies on ice between 20 min and overnight on ice in the dark. Following antibodies were used in this study: anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), and anti–Sca-1 (D7) all from BD; anti-CD71 (R17217), anti–Ter-119 (116228), anti–c-Kit (CD117, 2B8), anti-CD150 (TC15-12F12.2), and anti–CD48 (HM481), all from BioLegend; and anti–CD3 (145-2C11) from Thermo Fisher Scientific.

**Histology.** For histological analyses, mouse tissues were fixed in 10% buffered formalin. Fixed tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) according to standard laboratory protocols.

**Statistical tests and error bars.** All p-values were calculated using unpaired two-tailed Student’s t test. Statistically significant differences are indicated with asterisks in figures with the accompanying p-value in the legend. Error bars in figures indicate SD for the number of replicates as indicated in the figure legend.

**ATAC-seq.** ATAC sequencing was performed as described earlier (Buennrostro et al., 2015). The resulting library was sequenced in HiSeq2000. Reads were aligned with bowtie on mm9. Peaks were called with PeakDeCk (McCarthy and O’Callaghan, 2014). HTSeq was used to measure count density at peak sites (Anders et al., 2015). Areas with differential accessibility were identified with DESeq (Anders and Huber, 2010). GO term biological enrichment was obtained by entering the enriched areas from either Renilla or Stag2 knockdown ATAC-seq in GREAT (McLean et al., 2010). Transcription factor motifs were discovered by entering enriched regions in i-cisTarget (Imrichović et al., 2015).

**Accession numbers for high-throughput sequencing experiments.** Unprocessed and processed data for all high-throughput sequencing experiments in this study can be found in the GEO super series GSE72370: specifically, ATAC sequencing (GSE72367), RNA sequencing in CFU replating cells (GSE72368), and RNA sequencing in LSK cells (GSE72369).

**Online supplemental material.** Table S1, included as a separate Excel file, lists targeting sequences of shRNAs used in retroviral plasmids. Table S2, included as a separate Excel file, lists differentially expressed genes in control (Renilla) and cohesin (Rad21, Smc1a, Smc3, and Stag2) cells from the first (P1) and fifth (P5) plating from the CFU assay; only genes with expression levels of FPKM > 5 are listed. Table S3, included as a separate Excel file, lists differentially expressed genes in control (Renilla) and cohesin (Smc1a and Stag2) knockdown LSK cells sorted from mice treated with doxycycline for 10 d. Table S4, included as a separate Excel file, lists differentially expressed genes in control (Renilla) and cohesin (Rad21, Smc1a, Smc3, and Stag2) cells from the first (P1) and fifth (P5) plating from the CFU assay; only genes with expression levels of FPKM > 5 are listed. Table S5, included as a separate Excel file, shows peaks from ATAC sequencing on sorted LSK cells from Renilla and Stag2 shRNA mice treated with doxycycline for 10 d. Table S6, included as a separate Excel file, shows GO factors as found by GREAT analysis of regions that are specifically enriched in Stag2(shRNA+) LSK cells and GO factors as found by GREAT analysis of regions that are specifically enriched in Renilla(shRNA+) LSK cells (top 50 are shown for each). Table S7, included as a separate Excel file, shows genes set used to analyze RNA-seq from LSK cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20151323/DC1.

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**Author contributions.** J. Mullenders and I. Aifantis conceived the experiments and analyzed the data and wrote the manuscript. J. Mullenders, K. Wang, and C. Kayembe managed the shRNA mouse colony. J. Mullenders performed in vitro CFU experiments. J. Mullenders and P.P. Rocha performed karyotype analysis. P. Loumouad, M. Keller, J. Pae, B. Aranda-Orgilles, P.K. Premirsirut, and L. Cimmino provided technical assistance. B. Aranda-Orgilles, R. Raviram, Y. Gong, and A. Tsirigos provided bioinformatics support. D. Hoehn analyzed the histology of diseased animals. J.A. Skok and R. Bonneau are supervising R. Raviram.

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