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Potential Pitfalls of the *Mx1-Cre* System: Implications for Experimental Modeling of Normal and Malignant Hematopoiesis

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SUMMARY

Conditional knockout mice are commonly used to study the function of specific genes in hematopoiesis. Different promoters that drive *Cre* expression have been utilized, with the interferon-inducible *Mx1-Cre* still being the most commonly used "deleter strain" in experimental hematology. However, different pitfalls associated with this system could lead to misinterpretation in functional studies. We present here two of these issues related to the use of *Mx1-Cre*: first, a high spontaneous recombination rate when applying commonly used techniques in experimental hematology, and second, undesired short-term consequences of the use of polyinosinic:polycytidylic acid, including changes in cellular phenotypes that, however, resolve within days. Our studies emphasize therefore that proper controls are crucial when modeling gene deletion using the *Mx1-Cre* transgene.

INTRODUCTION

Conditional knockout (cKO) mice based on *Cre*-mediated excision (Jiang and Gridley, 1997) are frequently used when studying normal and malignant hematopoiesis. Most cKO models are generated by flanking a gene segment of interest with *loxP* sites, which allows for its removal in a cell- or tissue-specific manner by *Cre* recombinase. The *Mx1-Cre* model (Kuhn et al., 1995) represents the most commonly used "deleter strain" in experimental hematology. In this strain, the Mx dynamin-like GTPase 1 (*Mx1*) promoter is activated in an interferon-dependent manner following injection of polyinosinic:polycytidylic acid (pIpC), which results in downstream expression of *Cre* recombinase. Despite the wide use of the *Mx1-Cre* strain, caution has to be entertained due to caveats that have not been well described. These include spontaneous recombination and undesired side effects caused by the pIpC itself.

In experimental hematology, bone marrow (BM) transplantation represents a key approach in the investigation of the role of specific genes. Most often, this is performed with cells obtained from cKO mice. We show here that the transplantation assay and expression of an activated receptor tyrosine kinase (FLT-3^{ITD}) can trigger spontaneous *Mx1-Cre*-driven deletion of floxed genes, which substantially exceeded the 2%–3% originally reported (Kemp et al., 2004; Kuhn et al., 1995; Mupo et al., 2013). In addition, we observed transient changes in phenotype and frequency of hematopoietic stem and progenitor cells (HSPCs) after pIpC injection, which could lead to incorrect identification of subsets of cells in short-term studies. Finally, we propose a potential alternative strategy for gene deletion using an ex vivo Tat-Cre-recombination approach. Awareness of these shortcomings has high relevance for hematopoietic research because it can aid in experimental design and avoid potential errors in the interpretation of results.

RESULTS

Transplantation Induces Uncontrolled Recombination in Hematopoietic Cells from *Mx1-Cre* Mice

To study the influence of HIF-1α in the initiation of leukemia, we retrovirally introduced different oncogenes into *Mx1-Cre*; *Hif-1α*$^{flox/flox}$ cKIT^{+} cells and transplanted them into wild-type (WT) recipients (Velasco-Hernandez et al., 2014). Surprisingly, we observed an unexpectedly high deletion frequency of the floxed gene prior to pIpC injection, ranging between 30% and 50% (Figure 1A).

To identify experimental procedures that contribute to the spontaneous *Mx1-Cre* activation, we crossed Rosa26-Lox-Stop-Lox-YFP (YFP^{LSL}) reporter mice (Srinivas et al., 2001) with the *Mx1-Cre* mice. HSPCs from these mice were next subjected to a routine protocol for transduction/transplantation experiments, using retroviral vectors encoding fluorescence proteins (Figures 1B–1G). Isolated unmanipulated cells from donor mice displayed a spontaneous recombination close to 20% (Figures 1C, 1D, 1E, 1H, and S1). Analyses after transplantation indicated that

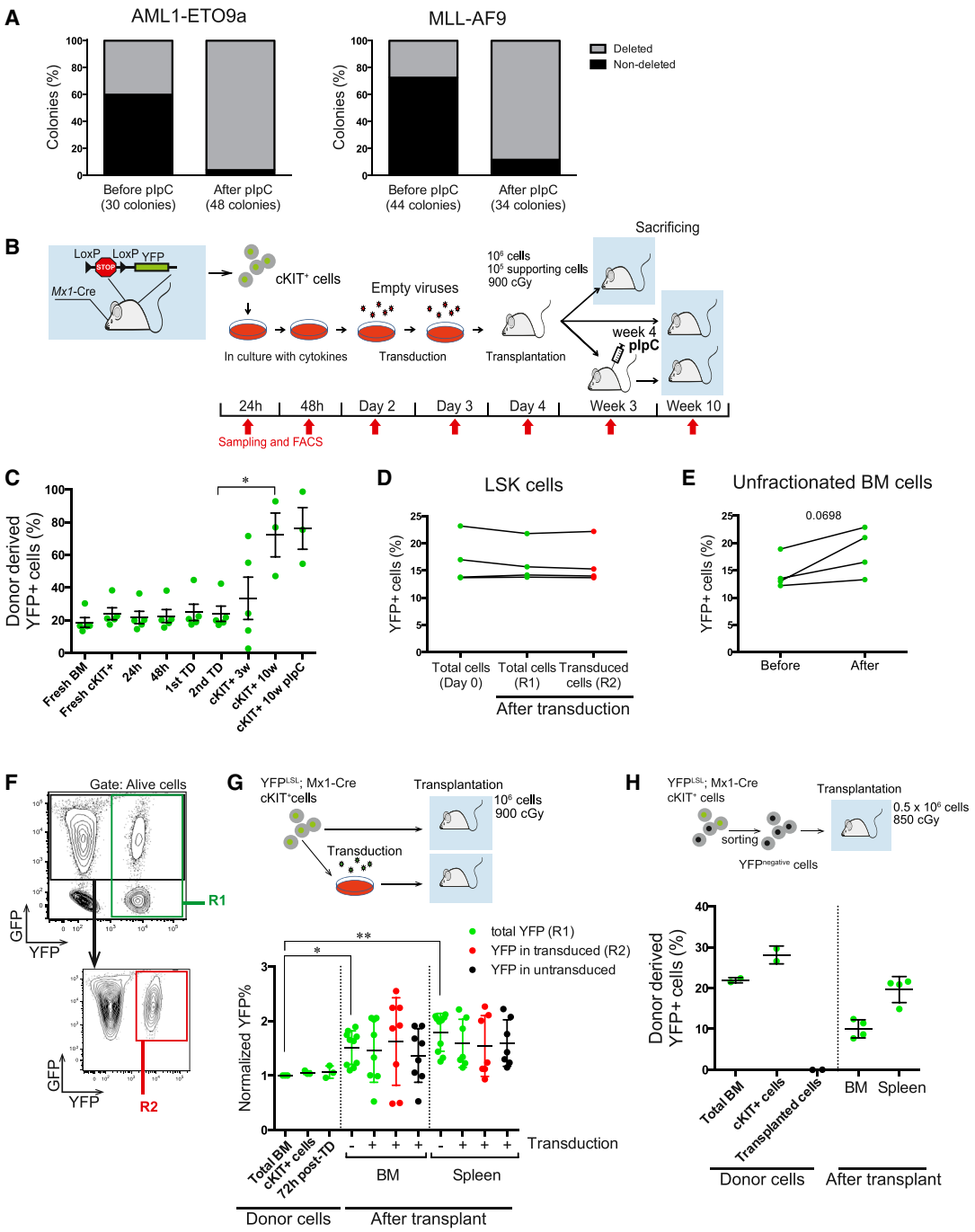

Figure 1. Spontaneous Deletion of Floxed Genes in Hematopoietic Cells from *Mx1-Cre* Mice after Transplantation

(A) Frequency of HIF-1α-deleted colonies from peripheral blood cells derived from animals transplanted with transduced *Mx1-Cre; Hif-1α*$^{flox/flox}$ HSPCs before (week 3 post-transplantation) and after pIpC injection (pIpC injection at week 4 and analysis at week 7). Plots show two independent experiments, using either AML1-ETO9a or MLL-AF9 expressing donor cells.

(B) Experimental setup for analysis of spontaneous deletion in a commonly used transduction/transplantation protocol. Donor cells were collected from five separate animals.

(C) Variation in frequency of recombined cells (YFP+) along the different steps of the protocol described in (B). Percentage of recombined cells is calculated within the donor-derived population after the transplantation step. *p < 0.05.

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the percentage of YFP+ (recombined) cells in the donor-derived compartment had increased significantly at week 10 (Figures 1C and S1). To address if this was due to the transduction or transplantation procedures, we performed the same protocol with or without transduction (Figures 1G and S1). Analysis of BM and spleen cells 15 days after transplantation indicated that the levels of recombination were similar in both groups. However, the frequency of recombined cells increased by 50% as a consequence of the transplantation, despite the lack of an evident positive selection pressure in favor of the recombined cells. Investigations into the effect of retroviral transduction in a more primitive and homogeneous population ($Lin^{-}SCA1^{+}cKIT^{+}$, LSK cells) also failed to reveal increments in recombination 48 hr after transduction (Figures 1D and S2). Furthermore, no significant increase in recombination was observed when culturing unfractionated BM cells, including more mature populations that could include interferon-producing cells (Figure 1E). Finally, to conclusively exclude a potential positive selection of already recombined cells that could increase in vivo after transplantation, we sorted cKIT+ YFP− cells from YFP^{LSL}; *Mx1-Cre* mice and transplanted them into WT recipients without pIpC injection (Figure 1H). This revealed 10%–20% of YFP+ donor cells 15 days after transplantation in donor BM and spleen cells of transplanted mice.

These results demonstrate that the major cause of the unexpectedly high levels of spontaneous gene deletion is caused by the transplantation procedure per se.

Constitutively Active *Flt-3* Induces Spontaneous Recombination in *Mx1-Cre* Mice

We recently crossed *Hif-1α* cKO mice with $Flt\text{-}3^{ITD}$ knockin mice. We observed that a large fraction of hematopoietic cells in these mice showed a spontaneous deletion prior to pIpC injection ((Velasco-Hernandez et al., 2015) and Figure 2A), consistent with previous publications (Mead et al., 2013; Mupo et al., 2013). To test if this was present also early in development, we analyzed deletion in fetal liver (FL) cells. FL hematopoietic cells showed either no deletion or only a minor portion of deleted cells in a few colonies (Figures 2A and S3), indicating that spontaneous *Mx1-Cre*-mediated excision is a cumulative effect during the lifespan of these animals. Since it has been reported that floxed alleles have different sensitivities to *Cre*-induced recombination (Liu et al., 2013), we investigated spontaneous recombination in a different ($Phd2^{flox/flox}$; *Mx1-Cre*; $Flt\text{-}3^{ITD/+}$) model. Using this, colonies derived from BM cells were analyzed and consistently presented a high rate of recombination in the absence of pIpC injection (Figures 2B and S3). Because FLT-3 signaling is critical for dendritic cell (DC) differentiation and proliferation (Gilliet et al., 2002; Karsunky et al., 2003; Maraskovsky et al., 1996; O'Keeffe et al., 2002), and plasmacytoid DCs (pDCs) are prominent producers of interferon (Colonna et al., 2004), we analyzed BM and spleen in $Flt\text{-}3^{ITD}$ mice for pDCs and classical DCs (cDCs) (Figures 2C, 2D, and S3). In both organs, we observed increments in pDCs and cDCs when comparing $Flt\text{-}3^{ITD/+}$ with WT mice. These data imply that spontaneous recombination can be induced by $FLT\text{-}3^{ITD}$ expression, probably in an interferon-dependent manner, illustrating that this phenomenon is not unique to the transplantation system.

The Immunophenotype of HSPCs Is Transiently Perturbed Following pIpC Administration

Interferon signaling has previously been shown to enhance proliferation of HSPCs in vivo (Baldridge et al., 2010; Essers et al., 2009). To evaluate this, we took advantage of an in vivo approach in which a histone 2B-mCherry fusion protein can be conditionally expressed in a doxycycline-inducible manner (Sawen et al., 2016).

Col1a1-tetO-H2B-mCherry mice were administered doxycycline to label the pool of HSPCs, followed by assessments of mCherry intensities in phenotypically defined subsets at different time points (days 1, 3, 8, and 24) after pIpC or PBS (control) injections. While proliferation of hematopoietic stem cells (HSCs, $Lin^{-}SCA1^{+}cKIT^{+}CD150^{+}CD48^{-}$) was not appreciably altered at any time point after pIpC injections (Figure 3A), lower mCherry intensities

(D) Recombined cell frequency in YFP^{LSL}; *Mx1-Cre* LSK cells at harvesting/sorting time (day 0) or 48 hr after transduction with a GFP virus (MIGR1): YFP+ cells from total cells (R1 population) and YFP+ cells within transduced cells (R2 population) (n = 4). Transduction levels were 10%–20%.

(E) Recombined cell frequency in unfractionated YFP^{LSL}; *Mx1-Cre* BM cells before and after 4 days culture (n = 4).

(F) Gating strategy used to analyze R1 and R2 population within transduced cells.

(G) Analysis of the effect of the transplantation alone on the spontaneous recombination. YFP^{LSL}; *Mx1-Cre* cKIT+ cells were isolated and directly transplanted or transduced with GFP virus before transplantation. Recombination levels were analyzed 15 days after transplantation (donor mice, n = 3; transplanted mice, n = 10). Recombination levels are normalized to initial YFP+ cells values from each independent donor. Transduction levels were between 10% and 50%. Pooled data from two independent experiments. $*p < 0.05$, $**p < 0.01$.

(H) Recombined cell frequency of donor-derived BM and spleen cells 15 days after transplantation with sorted YFP^{LSL}; *Mx1-Cre* cKIT+ YFP− (n = 4). Plots show mean ± SEM. TD, transduction. See also Figures S1 and S2.

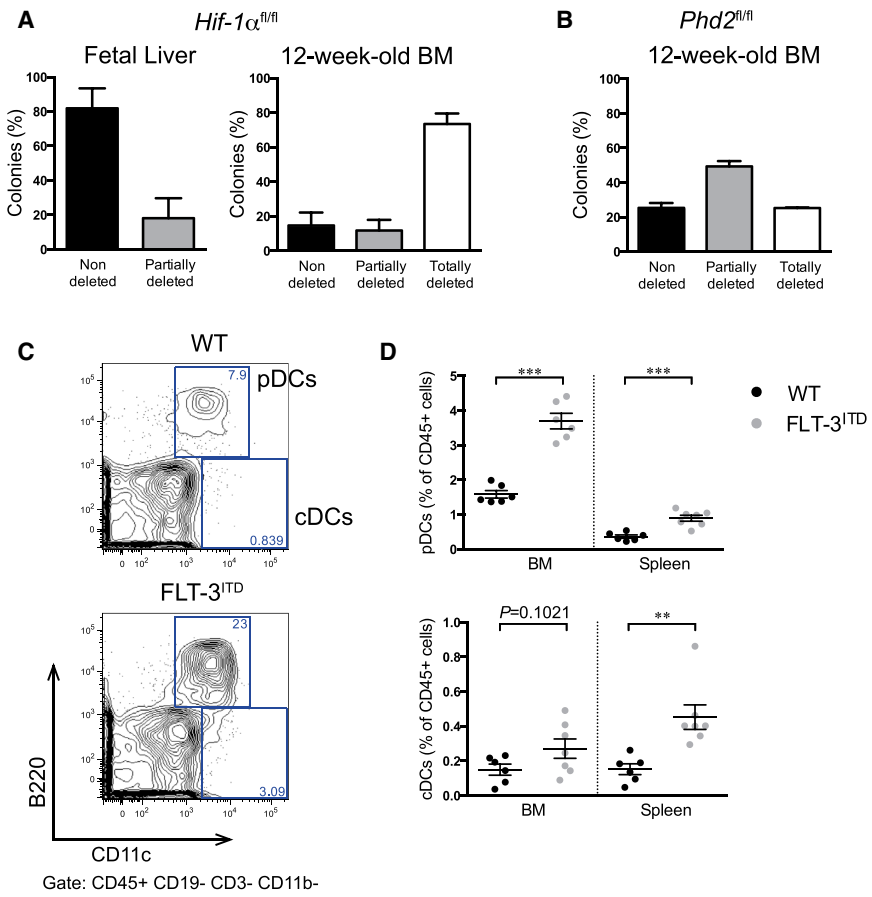

Figure 2. Spontaneous Deletion of Different Floxed Genes in Hematopoietic Cells from *Mx1-Cre* Mice Expressing *Flt-3*^{ITD}

(A) Evaluation of *Hif-1α* deletion in colonies derived from fetal liver (E14.5) (n = 4) or BM (12-week-old mice) (n = 3) cells from *Flt-3*$^{ITD/+}$; *Hif-1α*$^{flox/flox}$; *Mx1-Cre* embryos/mice.

(B) Evaluation of *Phd2* deletion in colonies from BM (12-week-old mice) cells (n = 2) from *Flt-3*$^{ITD/+}$; *Phd2*$^{flox/flox}$; *Mx1-Cre* mice.

(C and D) Flow cytometry analysis of dendritic cells (pDCs [CD45$^+$CD19$^-$CD3$^-$CD11b$^-$B220$^+$ CD11c$^{low/int}$] and cDCs [CD45$^+$CD19$^-$CD3$^-$CD11b$^-$B220$^-$CD11c$^+$]) (C) in BM and spleen (D) from FLT-3$^{ITD/+}$ and WT animals (n = 6). Plots show mean ± SEM. **p < 0.01, ***p < 0.001. See also Figure S3.

suggested enhanced proliferation of granulocyte/macrophage lymphoid progenitors (GMLPs, Lin$^-$SCA1$^+$cKIT$^+$CD150$^-$CD48$^+$) and more committed pre-granulocyte/macrophage progenitors (preGMs, Lin$^-$cKIT$^+$SCA1$^-$CD150$^-$CD105$^-$) in the short term (days 1 and 3) after pIpC administration (Figure 3A). However, this effect was not observed at later time points. While this potentially could reflect an elimination of more rapidly proliferating cells at later times, the frequencies of different HSPC subsets (Figure S4) were dramatically altered early after pIpC injections, but largely normalized after 3 days (Figures 3B and 3C). We noted transient elevations in the levels of SCA1 1 day after pIpC injections, leading to higher expression and frequency of SCA1$^+$ cells (Figure 3D). This declined to levels observed in untreated mice relatively rapidly. Therefore, pIpC doses typically used for *Cre* induction do not cause any pronounced effects on HSPC proliferation, but pIpC-mediated induction of SCA1 can complicate more refined phenotypic analyses of these cells by "pushing" SCA1$^{-/low}$ progenitor cells into the pool of more primitive LSK cells. These data highlight an additional and unwanted transient effect of pIpC administration on hematopoiesis that can lead to erroneous conclusions on cell identities in short-term studies (before day 8).

An Alternative In Vitro Cre-Recombination Strategy Using Ex Vivo Tat-Cre Treatment

Finally, to explore an alternative approach to the *Mx1-Cre*-mediated recombination, we subjected HSPCs from Rosa26-LoxP-Stop-LoxP-RFP(TdTomato) (TdTomato^{LSL}) mice to a brief in vitro exposure to recombinant Tat-Cre. This revealed an efficient induction of RFP expression, with no spontaneous deletion in the absence of Tat-Cre (Figures 4A and 4B). We next applied this deletion strategy to investigate the impact of an activating KRAS^{G12D} mutation on MLL-ENL-driven leukemia in vivo. This KRAS^{G12D} model has previously been shown to associate with a relatively high level of spontaneous activation when crossed to *Mx1-Cre* mice (Chan et al., 2004; Sabnis et al., 2009). After crossing doxycycline-inducible MLL-ENL mice (Ugale et al., 2014) to the KRAS$^{LSL-G12D}$ strain, we sorted HSCs and subjected these to Tat-Cre or sham recombination, followed by transplantation. As expected (Ugale et al., 2014), recipients of HSCs expressing only the MLL-ENL oncogene (no Tat-Cre) failed to

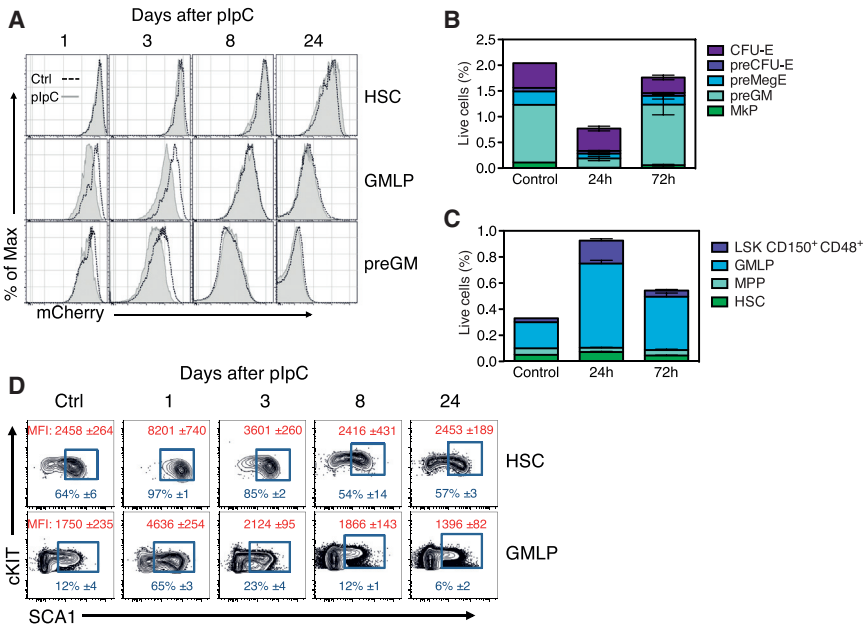

Figure 3. Alterations in Phenotype of BM Stem and Progenitor Subsets after pIpC Injections

(A) *Col1a1*-tetO-H2B-mCherry mice fed with doxycycline-containing food were injected with pIpC and analyzed 1 (n = 4), 3 (n = 4), 8 (n = 3), and 24 days (n = 3) after the last injection. Representative histograms show H2B-mCherry label retention in HSPCs at the indicated time points after pIpC injection.

(B and C) Frequencies of (B) phenotypic myeloid progenitors and (C) LSK HSPC subsets 24 or 72 hr following one pIpC injection.

(D) SCA1 expression in phenotypically defined HSCs and GMLPs. In red, mean fluorescent intensity (MFI) values of SCA1 and, in blue, average percentage of $SCA1^+$ cells (blue gating). Plots represent mean ± SD. Ctrl, control; HSC, hematopoietic stem cell; GMLP, granulocyte/macrophage lymphoid progenitors; preGM, pre-granulocyte/macrophage progenitors; MkP, megakaryocyte progenitor; preMegE, pre-megakaryocyte–erythrocyte progenitors; preCFU-E, pre-colony-forming units-erythrocytes; CFU-E, colony-forming units-erythrocytes; MPP, multipotent progenitor; LSK, $Lin^-SCA1^+cKIT^+$. See also Figure S4.

develop leukemia, while all recipients with combined activation of $KRAS^{G12D}$ and MLL-ENL (+ Tat-Cre) developed myeloid leukemia within 10 weeks (Figure 4C). To test this strategy in a non-leukemic model in which recombined cells have no selective advantage, we isolated LSK cells from the YFP^{LSL} model (lacking *Mx1-Cre*) and applied the same protocol (Figures 4D and 4E). We observed recombination rates of 2%–8%, also present in BM and spleen cells 15 days after transplantation. We did not observe any undesired recombination in sham-recombined cells. These results demonstrate the utility of an alternative recombination strategy that can circumvent some of the technical caveats associated with the *Mx1-Cre* system, although we have not rigorously shown that recombination occurs in stem cells with this system.

DISCUSSION

In experimental hematopoiesis, the requirement of specific genes for normal HSC function, initiation, and maintenance of leukemia, using either transplantation alone or the retroviral transduction/transplantation model, has been tested extensively using specific cKO mice (Kocabas et al., 2012; Takeishi et al., 2013; Wang et al., 2010; Zhang et al., 2004). However, the requirement of a specific gene for leukemia initiation will be difficult to evaluate if the gene to be tested is deleted in an uncontrolled manner.

It is a general assumption that deletion of floxed genes using the *Mx1-Cre* system occurs by pIpC injections either in steady-state hematopoiesis or after engraftment into recipients. Yet, despite its widespread use, little information is available on baseline levels of recombination and the influence of transplantation itself on spontaneous gene deletion. When we investigated the role of HIF-1α in leukemia initiation by transducing HSPCs from *Hif-1α* cKO mice with leukemic oncogenes, we observed a 40% gene deletion frequency after transplantation, but prior to pIpC injections. This led us to further investigate this phenomenon because of its broader importance for hematopoiesis and leukemia research, in which these methods are commonly used.

In the literature, a spontaneous recombination rate of 2%–3% when using the *Mx1-Cre* deleter strain has been described (Kemp et al., 2004; Kuhn et al., 1995; Mupo et al., 2013). Even a low recombination rate can lead to a high gene deletion frequency if the cells with the deleted gene have a growth advantage; for example, when investigating leukemia initiation. Based on our findings, *Mx1-Cre* mice not injected with pIpC should be included in all experiments using this model and deletion assessed before initiation of experiments. This is not unique to the transplantation scenario, as we observed that activation of *Mx1-Cre* can also be triggered by an oncogene (constitutive active *Flt-3*). Activation of this pathway, which leads to excessive generation and activation of interferon-producing DCs, is likely induced also by other activated receptor

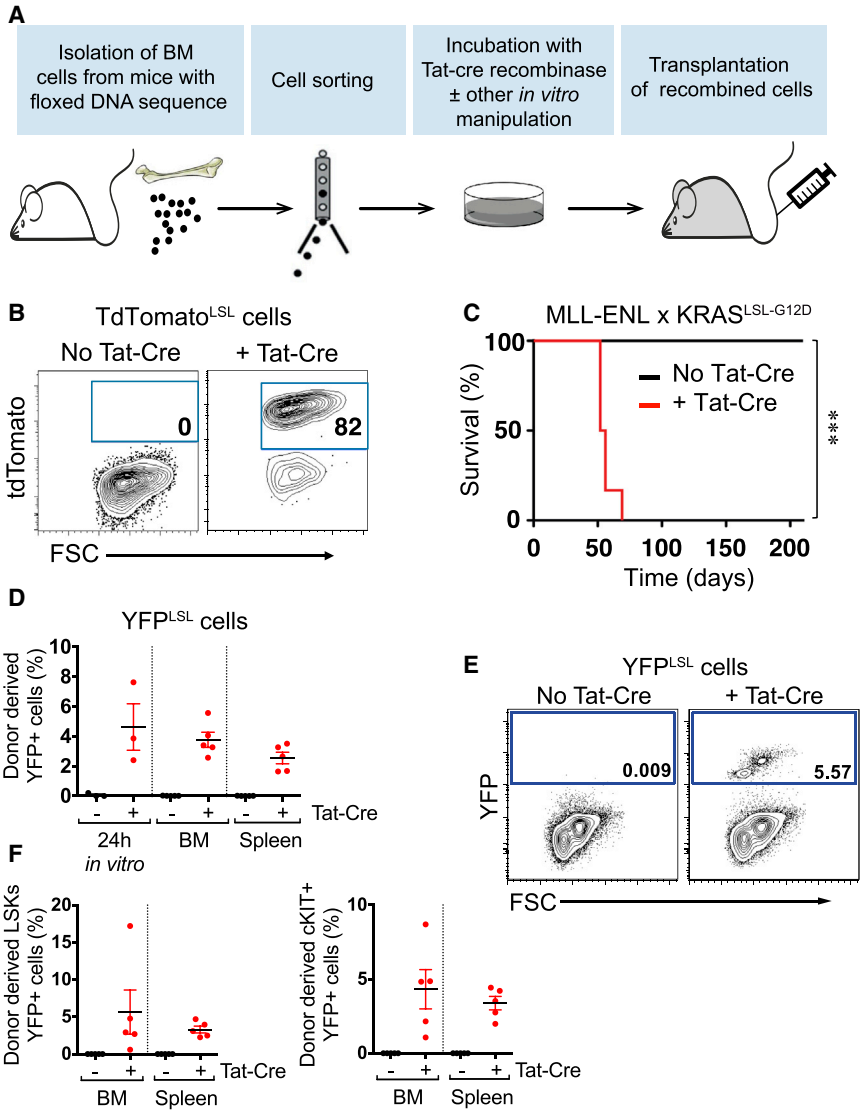

Figure 4. Alternative Strategy for Cre-Mediated Excision Using Ex Vivo Treatment with Tat-Cre Recombinase

(A) Schematic outline of the in vitro Cre-recombination alternative strategy using Tat-Cre recombinase.

(B) TdTomato^{LSL} HSCs were treated with Tat-Cre in vitro. Representative fluorescence-activated cell sorting (FACS) plots of TdTomato expression in cells after 3 days in culture are shown.

(C) *Col1a1*-tetO-MLL/ENL x KRAS$^{LSL-G12D}$ HSCs were induced in vitro with Tat-Cre to promote the activation of KRAS^{G12D}. Kaplan-Meier curves depict the survival of mice transplanted with Tat-Cre-induced (n = 6) or uninduced (n = 7) HSCs, under continuous MLL-ENL activation with doxycycline-containing food. ***p < 0.001.

(D) YFP expression in BM and spleen of transplanted mice with YFP^{LSL} LSK cells, Tat-Cre or sham treated, 15 days after transplantation (donor mice, n = 3; transplanted mice, n = 5).

(E) Representative FACS plots of YFP expression in donor BM cells.

(F) YFP expression in LSK and cKIT+ cells in the transplanted mice shown in (D).

Plots show means ± SEM.

tyrosine kinases, and might therefore be particularly problematic in combination with the *Mx1-Cre* system.

The *Mx1-Cre* system can be potentially associated with additional problems. For instance, pIpC administration has been suggested to trigger HSC cycling, similar to interferon itself (Essers et al., 2009; Gidali et al., 1981; Sugiyama et al., 2006). While we found less evidence for a dramatic effect of pIpC in inducing HSPC proliferation, we noted that pIpC induced rapid and transient changes (that resolved by day 8) in cellular phenotypes, which could affect the interpretation of experimental results.

Several alternatives to the *Mx1-Cre* model can be found in the literature (Joseph et al., 2013; Kemp et al., 2004; Lewandoski, 2001; Rossi et al., 2012). In addition, we provide evidence for the use of Tat-Cre recombinase (Peitz et al., 2002) as an alternative method (De Santa et al., 2007; Gordon et al., 2012; Li et al., 2014). Because Tat-Cre-mediated excision represents an exogenous expression system, cells are not susceptible to undesired recombination. However, this method also has limitations since it relies on an ex vivo step for the treatment with Tat-Cre, and is therefore not compatible with steady-state studies or for the study of genes necessary for homing. In addition, we have observed a significant variation in the recombination rate depending on the floxed gene, as described previously for Cre strains in general (Liu et al., 2013). Although the data we report herein displayed a rather low recombination rate, deletion efficiencies can be substantially increased in a cell-type-dependent and procedure-specific manner (data not shown). However, it is perhaps unlikely that the procedure will be able to reach 100% efficiencies. The titratable recombination rates can, however, also be beneficial, for

instance in leukemogenesis studies, in which only a small percentage of recombined cells is desired.

In summary, we present evidence from multiple experimental conditions and procedures that can contribute to the induction of high spontaneous recombination in *Mx1-Cre*-based cKO mice. Being up to 20-fold higher than the ones reported in the literature for steady-state conditions, our studies highlight the importance of proper controls when applying this system to study gene function in the hematopoietic system.

EXPERIMENTAL PROCEDURES

Mice

The following mouse strains were used: *Flt-3*^{ITD} (Lee et al., 2007) (kindly provided by G. Gilliland), *Mx1-Cre* (Kuhn et al., 1995), *Hif-1α*$^{flox/flox}$ (Ryan et al., 2000), *Phd2*$^{flox/flox}$ (kindly provided by P. Carmeliet), YFP^{LSL} (Srinivas et al., 2001) (JAX 006148), *Col1a1*-tetO-H2B-mCherry (Egli et al., 2007) (JAX 014602), TdTomato^{LSL} (Madisen et al., 2010) (JAX 007905), *Col1a1*-tetO-MLL/ENL (Ugale et al., 2014), and KRAS$^{LSL-G12D}$ (Jackson et al., 2001) (JAX 008179). To activate *Mx1-Cre* in vivo, 400 μg of pIpC (Sigma-Aldrich) was either injected three times every second day or once (analyzed samples at days 1 and 3). Mice were maintained at the animal facilities of the Biomedical Center at Lund University. All experiments were performed with consent from a local ethics committee.

Statistical Analysis

Data were analyzed using Microsoft Excel (Microsoft) and Prism 6 (GraphPad Software). Data are expressed as means ± SEM or SD. "n" always stands for individual mice. Differences between groups were assessed by two-tailed Student's t test. Survival rates were plotted using the Kaplan-Meier method (significance values were calculated by Mantel-Cox log rank test). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.06.002.

AUTHOR CONTRIBUTIONS

T.V. and P.S. performed the experiments, analyzed the data, and generated the figures. T.V., P.S., D.B., and J.C. designed the experiments and wrote the paper.

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Supplemental Information

Potential Pitfalls of the *Mx1-Cre* System: Implications for Experimental Modeling of Normal and Malignant Hematopoiesis

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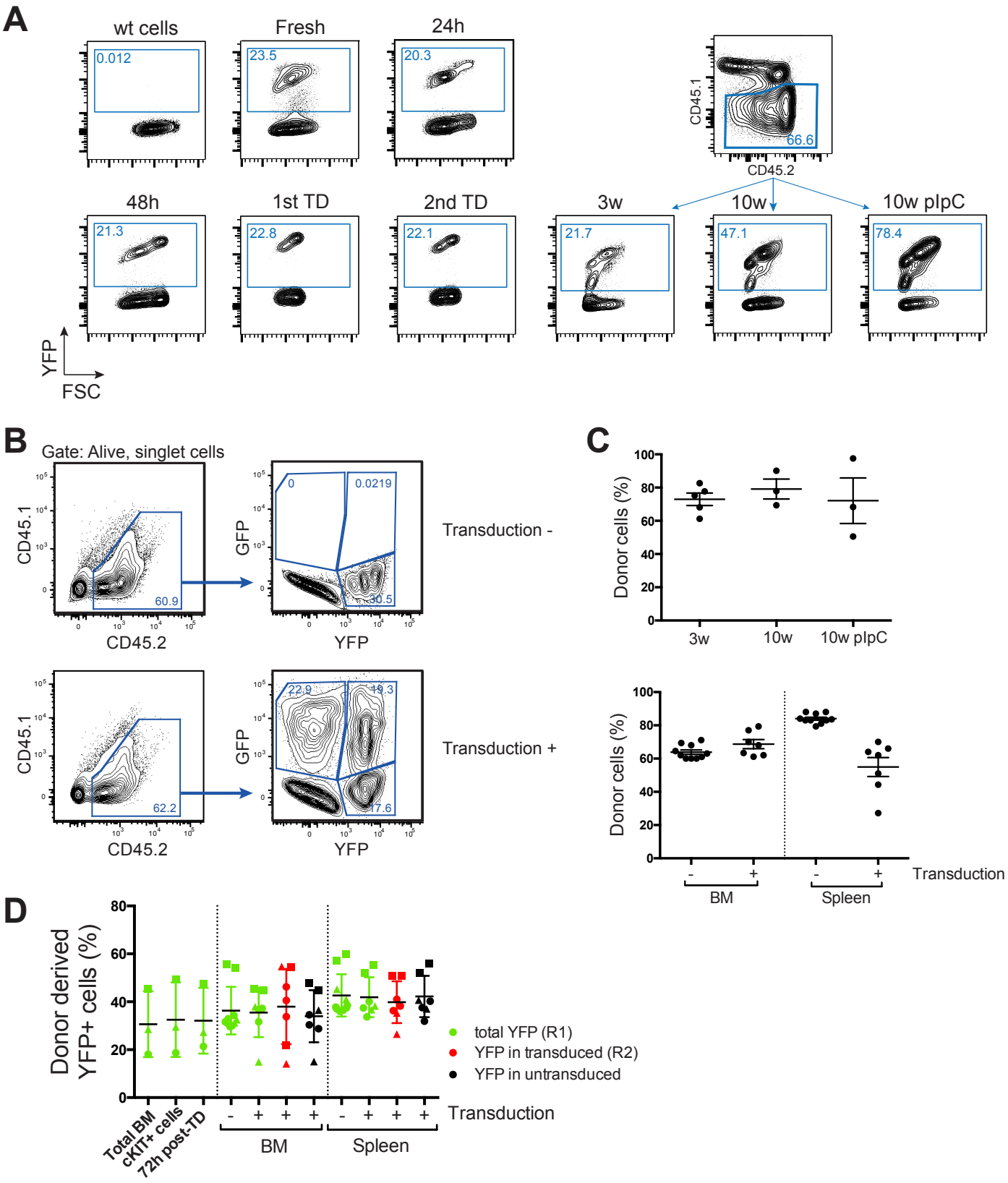
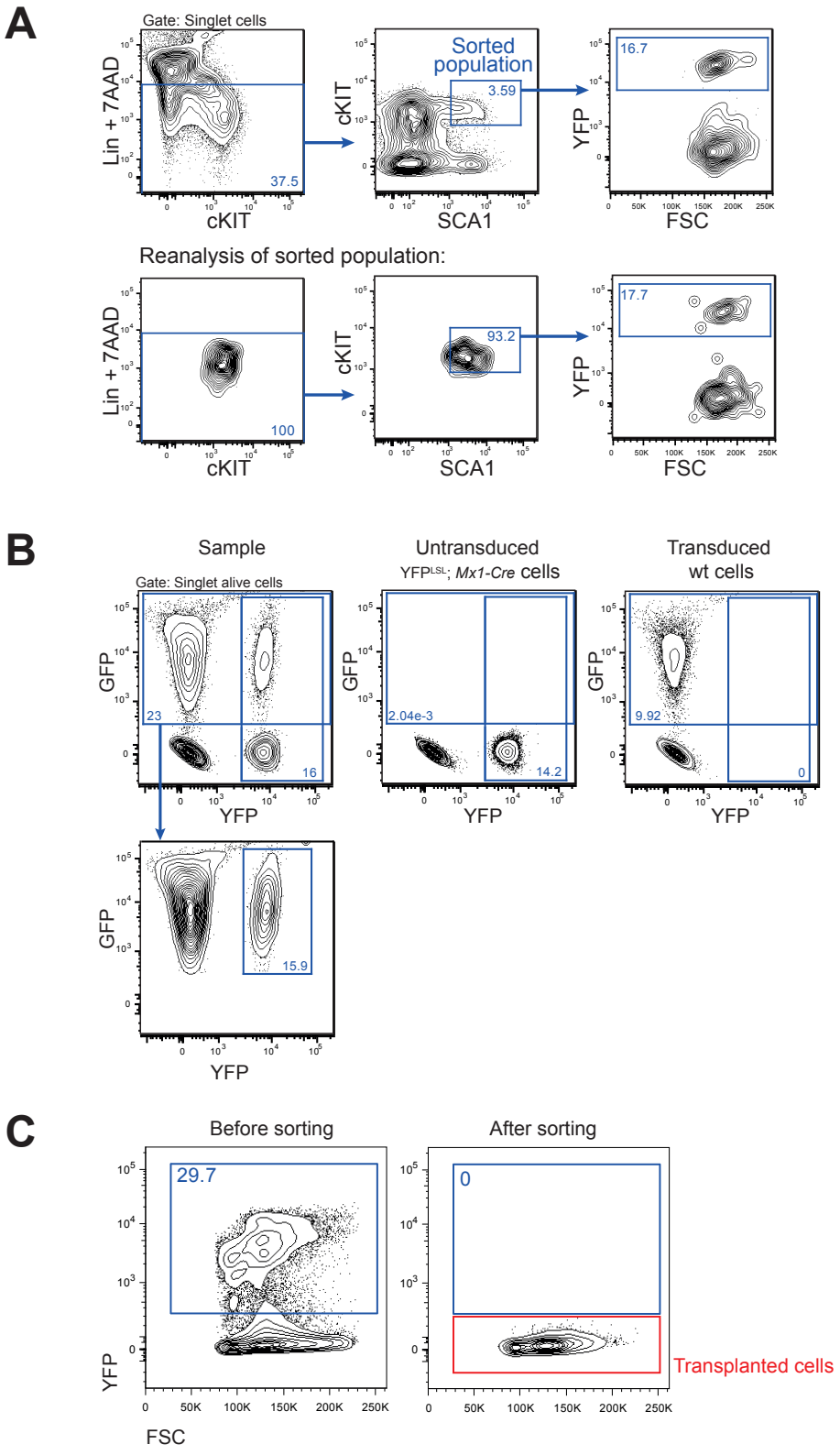
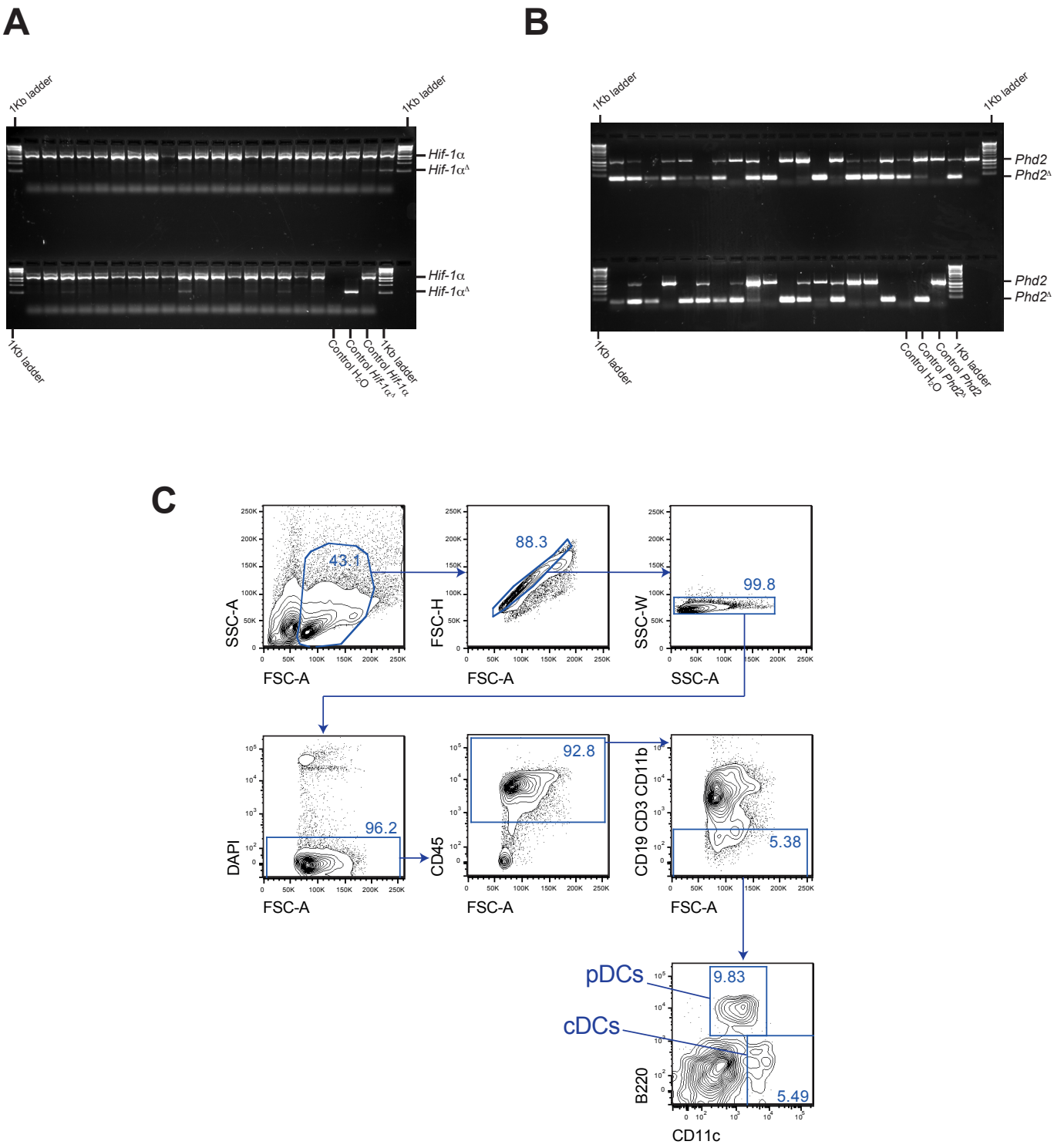


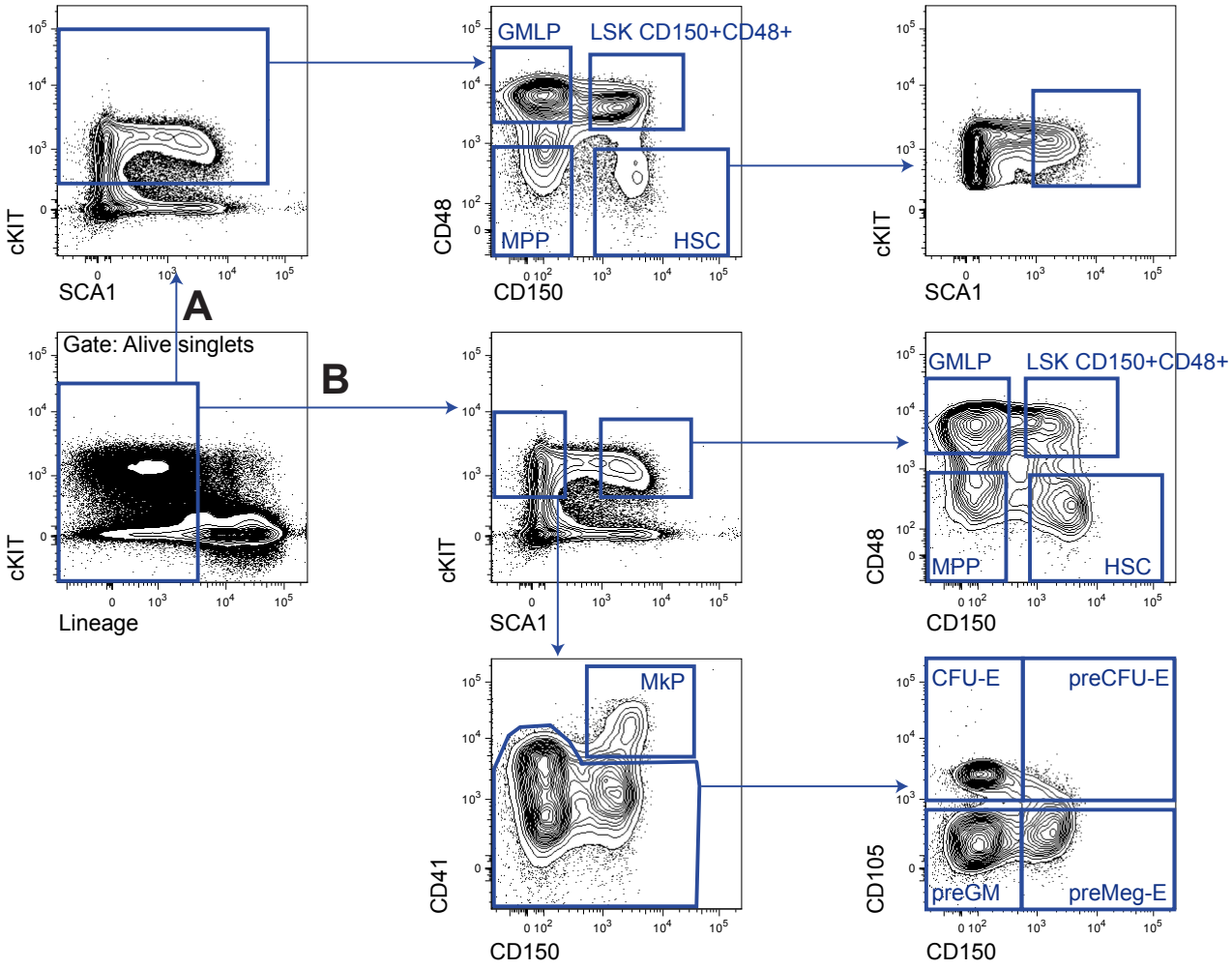
Supplemental Figure S1, related to Figure 1. (A) Flow cytometry gating used to identify YFP+ cells in Figure 1C. (B) Flow cytometry gating used to identify the different populations in Figure 1G. (C) Donor contribution in the analyzed samples in Figures 1C and 1G, respectively. (D) Recombined (YFP+) percentage of cells corresponding to normalized data of Figure 1H. Different shapes (squares, circles and triangles) indicate each used donor and the recipient mice transplanted with it. Plots show mean ± SEM. TD: transduction.



Supplemental Figure S2, related to Figure 1. (A) Flow cytometry gatings used to identify the LSK population during the sorting and reanalysis of the sorted cells in Figure 1D. (B) Flow cytometry gatings used to identify the recombined cells (YFP+) and transduced cells (GFP+) after the transduction and culture of the LSK cells. (C) YFP negative cells sorted and used for transplantation in Figure 1H. Cells derived from one donor mouse are shown.



Supplemental Figure S3, related to Figure 2. (A) Colony analysis derived from cKO-*Hif-1α* fetal liver cells. Fetal liver cells (E14.5) from *Hif-1α*$^{flox/flox}$; *Mx1-Cre*; *Flt-3*^{ITD} embryos were plated in methylcellulose medium. After 7 days, colonies were picked and genomic DNA from individual colonies analyzed by PCR for *Hif-1α* deletion (n=40). PCR results from colonies derived from one of the analyzed embryos are shown. (B) Colony analysis derived from cKO-*Phd2* BM cells. BM cells from 12-week-old *Phd2*$^{flox/flox}$; *Mx1-Cre*; *Flt-3*^{ITD} mice were plated in methylcellulose medium. After 7 days, colonies were picked and genomic DNA from individual colonies analyzed by PCR for *Phd2* deletion (n=39-40). PCR results from colonies derived from one of the analyzed mice are shown. (C) Gating strategy used for the identification of dendritic cells. For the identification of plasmocytoid dendritic cells (pDCs) and classical dendritic cells (cDCs) we used the above gating strategy: cell-size events, singlets (FSC-A vs FSC-H), singlets (SSC-A vs SSC-W), alive cells (DAPI^{-}), CD45^{+} cells, lineage negative cells (CD19^{-}, CD3^{-} and CD11b^{-}) and CD11c and B220 expression.



Supplemental Figure S4, related to Figure 3. Gating strategy used for the identification of the different subsets of HSPCs. FACS plots derived form a control mouse (day 1, no pIpC treatment) are shown. The strategy A was used in Figure 3D for the analysis of SCA1 expression in HSCs and GMLPs. The strategy B was used in Figure 3A-C for the identification of the different subsets of HSPCs. HSC, hematopoietic stem cell; GMLP, granulocyte/macrophage lymphoid progenitors; preGM, pre-granulocyte/macrophage progenitors; MkP, megakaryocyte progenitor; preMegE, pre-megakaryocyte–erythrocyte progenitors; preCFU-E, pre-colony forming units-erythrocytes; CFU-E, colony forming units-erythrocytes; MPP, multipotent progenitor; LSK, Lin-SCA1+cKIT+.

Supplemental Experimental Procedures

Cell culture

Colony assay and clonal analysis: Peripheral blood (PB), BM or fetal liver samples were harvested at indicated time points, erythrocytes lysed with ammonium chloride (Stem Cell Technologies) (PB samples), plated into methylcellulose (Methocult GM3434, Stem Cell Technologies) and incubated for 7 days at 37°C. Single colonies were picked and genomic DNA extracted. PCR was performed to identify the deletion of *Hif-1α* or *Phd2*.

In vitro culture conditions: cKIT+ or LSK cells were stimulated for transduction in StemSpan SFEM medium (StemCell Technologies) supplemented with penicillin/streptomycin (Invitrogen), mIL3 (20 ng/mL), hIL6 (50 ng/mL), hTPO (50 ng/mL) and mSCF (50 ng/mL).

Tat-Cre recombination: For *in vitro* Cre recombination, cells were washed and incubated for 4 hours at 37°C in 900 ml StemSpan (StemCell Technologies) supplemented with mSCF (100 ng/ml) and hTPO (20 ng/ml) (Peprotech), 50 μM β-mercaptoethanol and penicillin/streptomycin (Invitrogen) with 50 μg/ml recombinant Tat-Cre (Excellgen). In the case of *Col1a1*-tetO-MLL/ENL x $KRAS^{LSL-G12D}$ cells, 1 mg/ml doxycycline (Sigma) was also added. In the case of $TdTomato^{LSL}$ cells, TdTomato expression was evaluated after 3 days in culture.

HSC transplantation

AML1-ETO9a and MLL-AF9: Transduction and transplantation of these models were performed as previously described (Velasco-Hernandez et al., 2014).

YFP^{LSL} cells: For analysis of spontaneous recombination after transplantation, BM c-kit+ cells were isolated using a magnetic separation system (MACS) (Miltenyi Biotech). Transduction of cells was performed as described previously (Velasco-Hernandez et al, 2014) with RFP viruses (generated from a producer cell line, 293GPG-pCAG-RFP-WPRE) or GFP viruses (MIGR1 empty retroviral vector). After transduction, $1x10^6$ cells were transplanted together with $1x10^5$ support cells (no supporting cells in figure 1G) by tail-vein injection into lethally irradiated wt mice (850-900 cGy). For analysis of Tat-Cre recombination, LSK cells were isolated by sorting and transplanted (10,000 LSKs/recipient) into CD45.1 irradiated recipients (850 cGy) without supporting cells.

MLL-ENL x $KRAS^{LSL-G12D}$ cells: sorted HSCs (lineage-negative, $SCA1^+$, $cKIT^+$, $CD150^+$ and $CD48^-$) from *Col1a1*-tetO-MLL/ENL x $KRAS^{LSL-G12D}$ mice (CD45.1-CD45.2) were transplanted (450 cells/recipient) together with $3x10^5$ unfractionated BM support/competitor cells (CD45.1) into lethally irradiated (950 cGy) C57BL/6 mice. Recipient mice were administered doxycycline containing food pellets 5 days prior to transplantation (2 g/kg, Ssniff Spezialdiäten GmbH) and throughout the experiment.

Flow cytometry

For YFP^{LSL} cell analysis, transduction rate and Cre-mediated recombination (YFP^+ cells) were monitored by flow cytometry of BM cells. For dendritic cell analysis the following antibodies were used: CD45.2-FITC (104), CD3e-PE (142-2C11), Gr-1-PE (RB6-8C5), CD11b-PE (M1/70), B220-APC (RA3-6B2) (BioLegend) and CD19-PE (1D3), CD11c-PECy7 (HL3) (BD Biosciences). DAPI (Sigma) was used to exclude dead cells. For HSPCs phenotype analysis and sorting, HSPCs were isolated as described (Ugale et al., 2014). Shortly, the following antibodies were used: CD4 (GK1.5), CD8a (53-6.7), Gr1 (RB6-8C5), CD11b (M1/70), B220 (RA3-6B2), Ter119 (Ter-119), cKIT (2B8), SCA1 (D7), CD150 (TC15-12F12.2), CD48 (HM 48-1), CD41 (MWReg30), CD105 (MJ7/18), CD45.1 (A20) (BioLegend), and CD45.2 (104) (eBioscience). Samples were analyzed using a FACSCantoII, a LSRII, a LSRFortessa (BD Biosciences) or a FACS Aria III cell sorter (BD Biosciences) and analyzed with FlowJo software (TreeStar).