### **ORIGINAL ARTICLE**

### Developmentally induced Mll1 loss reveals defects in postnatal haematopoiesis

T Gan<sup>1,3</sup>, CD Jude<sup>1,3</sup>, K Zaffuto<sup>1</sup> and P Ernst<sup>1,2</sup>

<sup>1</sup>Department of Genetics, Norris Cotton Cancer Center, Dartmouth Medical School, Hanover, NH, USA and <sup>2</sup>Department of Microbiology and Immunology, Norris Cotton Cancer Center, Dartmouth Medical School, Hanover, NH, USA

The mixed lineage leukemia (MLL) gene is disrupted by chromosomal translocations in acute leukemia, producing a fusion oncogene with altered properties relative to the wild-type gene. Murine loss-of-function studies have shown an essential role for MII in developing the haematopoietic system, yet studies using different conditional knockout models have yielded conflicting results regarding the requirement for MII during adult steady-state haematopoiesis. In this study, we used a loxP-flanked MII allele ( $MII^{F}$ ) and a developmentally regulated, haematopoietic-specific VavCre transgene to reassess the consequences of MII loss in the haematopoietic lineage, without the need for inducers of Cre recombinase. We show that VavCre;MII mutants exhibit phenotypically normal fetal haematopoiesis, but rarely survive past 3 weeks of age. Surviving animals are anemic, thrombocytopenic and exhibit a significant reduction in bone marrow haematopoietic stem/progenitor populations, consistent with our previous findings using the inducible Mx1Cre transgene. Furthermore, the analysis of VavCre mutants revealed additional defects in B-lymphopoiesis that could not be assessed using Mx1Cremediated MII deletion. Collectively, these data support the conclusion that MII has an essential role in sustaining postnatal haematopoiesis.

*Leukemia* (2010) **24**, 1732–1741; doi:10.1038/leu.2010.171; published online 19 August 2010

Keywords: haematopoiesis; MLL; Vav; interferon; stem cells

#### Introduction

The treatment of childhood leukemia has improved dramatically in the last five decades. However, patients with certain cytogenetic abnormalities do not effectively respond to the conventional chemotherapy, particularly those defined by translocations in the *mixed lineage leukemia* (*MLL*) locus at 11q23. It remains unclear why this group has a particularly poor outcome independent from patient age.<sup>1</sup> Significant advances have been made in understanding the molecular consequences of 11q23 translocations using mouse models, but the need for new strategies to target this cytogenetically defined group of leukemias justifies further investigation into molecular pathways regulated by *MLL*.<sup>2</sup>

The protein encoded by the *MLL* gene (*Mll1* in mouse) is a large nuclear chromatin-modifying protein that encodes histone methyltransferase activity.<sup>3,4</sup> In addition to its methyltransferase activity, MLL possesses sequence-nonspecific DNA recognition

motifs<sup>5–7</sup> and chromatin recognition motifs.<sup>8,9</sup> Much of the chromatin-targeting activity is retained in the N-terminus and is thus shared between MLL and oncogenic MLL fusion proteins. Therefore, it is not surprising that many genes identified as overexpressed in cells harboring *MLL* translocations are also natural *MLL* target genes.<sup>10</sup> The extent to which MLL fusion proteins simply hyperactivate a natural MLL-dependent haematopoietic program or alternatively, exhibit neomorphic activity (acquiring and deregulating new target genes), remains unclear, but this distinction is critical for the development of targeted therapeutics.

To understand the normal role of wild-type *Mll* gene in the haematopoietic system, our group and others have performed gene disruption experiments to generate loss-of-function alleles using a variety of strategies.<sup>11–15</sup> Owing to the large and complex nature of the *Mll1* locus, none of these gene disruptions produce null alleles, and all (with the exception of the SET (*Su*(*var*)*3-9*, *enhancer of zeste*, *trithorax*) domain deletion<sup>14</sup>) result in embryonic lethality when homozygous. The age of embryonic lethality varies amongst alleles, suggesting that residual function of the different alleles may cause the variable results reported. Furthermore, *Mll* expression in the embryo is dynamic and widespread and the cause of lethality has not been clearly elucidated. For these reasons, we and others,<sup>15</sup> have developed conditional knockout alleles to more precisely assess the role of *Mll* in adult haematopoietic cell types and in other tissues.

To produce a conditional loss-of-function allele, our group generated an MII allele in which exons 3 and 4 are loxP-flanked  $(MII^{F})$ .<sup>16</sup> We demonstrated that the excision of exons 3 and 4 (producing the  $Mll^{\Delta N}$  allele) results in an in-frame deletion and the resulting MLL<sup> $\Delta N$ </sup> protein can not be imported into the nucleus because of the loss of nuclear localization motifs. The cytoplasmic localization was demonstrated in cells expressing an epitope-tagged version of the  $MLL^{\Delta N}$  protein as well as in MII-deleted primary cells.<sup>16</sup> In addition, the AT hooks,<sup>5</sup> methylation status-specific DNA recognition motifs,<sup>6,7</sup> and subnuclear targeting motifs<sup>17</sup> are lacking in the MLL<sup> $\Delta N$ </sup> protein. These motifs are essential for transformation in the context of MLL-ENL (eleven; nineteen leukemia) fusion oncoproteins. In an independent study, McMahon et al.<sup>15</sup> generated an Mll<sup>F</sup> allele, in which exons 8 and 9 are excised, also resulting in an in-frame deletion. The protein produced from this allele is likely unstable; MLL is undetectable by immunoblot in mutant fetal liver extracts. Interestingly, both gene disruption strategies result in several very similar phenotypes such as embryonic lethality of germline homozygotes beginning at ~E12.5, inefficient fetal liver haematopoiesis and adult bone marrow cells that fail to engraft secondary recipients. However, one significant phenotypic difference distinguished these two conditional knockout models. In the McMahon et al.<sup>15</sup> study, the pan-haematopoietic, VavCre-mediated deletion resulted in efficient Mll excision



Correspondence: Dr P Ernst, Department of Genetics, Norris Cotton Cancer Center, Dartmouth Medical School, 725 Remsen, HB7400, Hanover, NH, 03755, USA.

E-mail: patricia.ernst@dartmouth.edu

<sup>&</sup>lt;sup>3</sup>These authors contributed equally to this work.

Received 14 January 2010; revised 13 June 2010; accepted 6 July 2010; published online 19 August 2010

in all haematopoietic cells, with no effect on steady-state haematopoietic populations. Despite normal steady-state haematopoiesis, bone marrow cells from these animals exhibited a severe defect in engrafting secondary recipients. Thus, based on this mouse model, the authors concluded that *Mll* is only required for the process of regenerating the haematopoietic system but not for steady-state haematopoiesis. In contrast, our studies using the inducible *Mx1Cre* transgene<sup>18</sup> demonstrated an absolute requirement for *Mll* in steady-state as well as regenerative haematopoiesis. We observed a significant reduction in haematopoietic stem cell function as early as 4 days after deletion of *Mll* followed by a rapid decline in most bone marrow cells and lethality 2–3 weeks after deletion.<sup>16</sup>

Two major differences between these mouse models may account for the different conclusions. First, the proteins encoded by the two different alleles may retain some quantitatively or gualitatively different residual functions. Second, the method of excision (Mx1Cre versus VavCre) may impact on the effects of Mll loss. The Mx1Cre transgene is widely used to evade embryonic lethality and introduce Cre-mediated gene manipulations into the haematopoietic system.<sup>19</sup> The induction of Cre expression is initiated in adult animals by the injection of polyinosinic-polycytidylic acid (pI:pC), a double stranded NA analog. Toll-like receptor 3 mediated recognition of double stranded RNA initiates the expression of type I interferons (IFNs), which result in the activation of the Mx1Cre transgene.<sup>18</sup> Systemic IFNs affect many developing and mature haematopoietic cell types, often limiting the use of the Mx1Cre model to periods after recovery from transient pl:pC-initiated nonspecific effects. Type I IFNs are potent inhibitors of normal haematopoietic cell progenitor growth including lymphoid, 20,21 erythroid and myeloid cell types.<sup>22,23</sup> In addition, a proproliferative role of IFNa on haematopoietic stem cells has been demonstrated recently.<sup>24,25</sup> Therefore, any assessment of haematopoietic phenotypes using the Mx1Cre model should take into account the potential for combined effects of the engineered mutation and IFN signaling.

As targeting MLL fusion oncoproteins may include strategies that inadvertently target endogenous MLL, it is important to determine whether the inhibition of endogenous MLL activity could be tolerated. Therefore, the objective of this study was to reassess the role of *Mll* in adult and fetal haematopoiesis using a method not requiring induction of IFNs. To this end, we generated and analyzed animals in which haematopoietic-specific *Mll* excision is achieved using a *VavCre* transgene. In this study, we show that the developmentally regulated excision of our *Mll*<sup>F</sup> allele largely phenocopies the acute excision using the *Mx1Cre* transgene, supporting the conclusion that *Mll* is required to sustain adult steady-state haematopoiesis.

### Materials and methods

### Animals

Mice were maintained in compliance with the Dartmouth Animal Resources Center and Institutional Animal Care and Use Committee policies.  $MII^F$  alleles and gene targeting have been described previously.<sup>13</sup>  $MII^{\Delta N}$  animals were generated by crossing *Ella-Cre* transgenics (Jackson Labs, Bar Harbor, ME, USA) to  $MII^F$  animals and selecting progeny that transmitted the deleted ( $MII^{\Delta N}$ ) version of the allele. *VavCre* mice were obtained from Hanna Mikkola (University of California, Los Angeles, CA, USA) with permission from Thomas Graf (Center for Genomic Regulation, Barcelona, Spain). *Rosa26-YFP (RosaYFP)* reporter mice were obtained from Jackson Labs (stock no. 006148). Embryo age was defined as day 0.5 at 0800 hours the day of plug observation. To screen out germline and mosaic expression of the *VavCre* transgene, embryos harboring the *RosaYFP* allele were imaged after dissection or non-haematopoietic tissues were assessed for *Mll* excision using a quantitative genomic PCR assay<sup>16</sup> using tail, limb bud, somite or head genomic DNA. A similar approach was used to screen adult *VavCre* animals, using ear, tail or liver genomic DNA.

### Flow cytometry and differential blood counts

Bone marrow cells were prepared and lineage staining was performed as previously described<sup>16,</sup> except that the F' ab<sub>2</sub> antirat conjugate was a PE-Cy5.5 reagent (Invitrogen, Carlsbad, CA, USA). Splenocytes and thymocytes were prepared by trituration through a 40-micron nylon filter. For splenocytes and bone marrow, RBC lysis buffer (eBioscience, San Diego, CA, USA) was used to remove red blood cells. For adult bone marrow, lineage cocktail consisted of unlabeled rat anti-Gr-1, Mac-1, interleukin-7Ra, Ter119, CD3, CD4, CD8, B220 and CD19, all from Invitrogen except that interleukin-7Ra antibody was obtained from eBioscience. For the fetal liver analyses, lineage cocktail lacked the Mac-1 antibody. In some experiments, goat anti-rat APC (BD Biosciences, San Jose, CA, USA) was used in conjunction with Sca-1 biotin, c-Kit PE, CD48 FITC and streptavidin-PECy5.5 all from BD Biosciences. T cell analyses utilized anti-CD3, CD4, CD8, CD25, CD44, CD45.1 and CD45.2 from BD Biosciences or eBioscience. Flow cytometry was performed with a FACSCalibur or FACSAria (BD Biosciences) and data was analyzed using FlowJo Software (TreeStar Inc., Ashland, OR, USA). Peripheral blood collected from the periorbital sinus was analyzed using a Hemavet 950 blood analyzer (Drew Scientific, Inc, Dallas, TX, USA).

### In vitro *colony assays and transplantation experiments* Colony assays to enumerate myeloerythroid colony-forming

units were performed by plating unfractionated fetal liver or bone marrow cells in 1 ml M3434 (Stemcell Technologies, Vancouver, BC, Canada) in duplicate. Total colonies were enumerated 6–7 days after plating. Competitive transplantation assays were performed using C57Bl/6 female mice irradiated with a split dose of 950 Rads. Each recipient received  $2 \times 10^5$ protective C57Bl/6 bone marrow cells and either  $5 \times 10^4$  or  $5 \times 10^5$  fetal liver cells. Engraftment was assessed by the presence of Ly5.1<sup>+</sup> (fetal donor) cells in the peripheral blood of recipients by flow cytometry.

### Statistical analysis and software

Unless otherwise indicated, unpaired Student's *t*-tests were used to calculate *P*-values. All error bars represent 95% confidence intervals unless otherwise indicated in the figure legend. Graphs and statistical tests were performed using Prism (Graphpad, La Jolla, CA, USA) or Excel (Microsoft Corporation, Redmond, WA, USA).

### Histology and imaging

For bone marrow sections, the sternum was removed, cleaned and transferred to Bouin's fixative overnight at 4 °C. Fixed samples were processed by the Dana-Farber/Harvard Cancer Center Rodent Histopathology Core Facility. Samples were imaged on a Nikon Optiphot-2 microscope using  $\times 10$  and  $\times 40$  objectives for a total magnification of  $\times 100$  and  $\times 400$ .

Images were manipulated using Adobe Photoshop and Illustrator software (Adobe Systems, Inc., San Jose, CA, USA). Embryo images were acquired on a Leica MZFIII stereomicroscope using a GFP-470 filter (Chroma Technology, Bellows Falls, VT, USA), Color Mosaic 11.2 camera and Spot Insight 4.0 software (Diagnostic Instruments, Sterling Heights, MI, USA). Total magnification was 6.3X–8X.

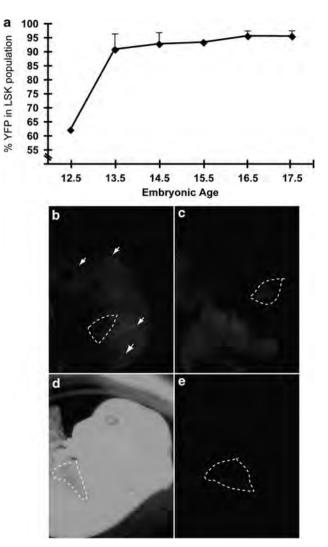
### Results

# VavCre-mediated gene deletion becomes fully penetrant by embryonic day (E) 13.5

Several groups have generated Cre recombinase-expressing transgenic animals using regulatory elements of the vav1 gene.<sup>26-29</sup> To determine the first point at which VavCremediated excision becomes fully penetrant in an haematopoietic stem cell-enriched population, we analyzed fetal livers from wild-type VavCre embryos harboring the RosaYFP reporter gene. Excision of RosaYFP increased rapidly from E12.5 in lineage-negative, Sca-1+c-Kit+ (LSK) cells, approaching 100% at E13.5 (Figure 1a). Depending on the regulatory elements used, *vav* transgenes have been observed to exhibit variegated or ectopic expression.<sup>26,28,29</sup> We observed that up to 20% of the progeny of *VavCre;MII<sup>F/+</sup>* X *MII<sup>F/F</sup>* crosses had undergone either germline excision (most tissues had undergone Mll excision) or mosaic excision (patchy excision outside the haematopoietic system). The remaining animals exhibited faithful expression of the VavCre transgene exclusively in the fetal liver at E13.5. The aberrant excision was most apparent when embryos harbored the RosaYFP allele to visualize all tissues in which Cre expression had occurred (Figures 1b-e). To reduce the mosaic and germline expression of the VavCre transgene, an alternative breeding strategy was also used ( $VavCre;Mll^{\Delta N/+} \times Mll^{F/F}$ ). In this strategy, the animal that harbored the Vav transgene also had one 'pre-deleted' Mll allele (Mll<sup>4N</sup>), thus avoiding Mll excision through ectopic VavCre activity in germ cells, as had been previously reported.<sup>26,28</sup> This strategy increased the number of animals exhibiting haematopoietic-specific MII excision and also increased the penetrance of the phenotypes observed.

## Normal fetal haematopoietic expansion in VavCre;Mll mutant embryos

Using germline *Mll* alleles, several groups have reported inefficient fetal liver haematopoiesis.<sup>15,30</sup> To determine whether the loss of Mll exclusively in developing haematopoietic cells results in a similar effect on fetal liver cellularity, we analyzed fetal haematopoiesis in VavCre; MII<sup>F/F</sup> embryos from E13.5 to E17.5, with and without the RosaYFP allele. Although full penetrance of Cre excision in the fetal liver is achieved by E13.5, no reduction in cellularity was observed, even by E15.5 in either VavCre; Mll<sup>F/F</sup> or VavCre; Mll<sup>4N/F</sup> embryos (Figures 2a, d and g). Furthermore, the haematopoietic stem cell-enriched LSK/CD48-negative population<sup>31</sup> was also not reduced (Figures 2b, c, e and f). In contrast to the significant decrease in B cells in adult mutant mice (see Figures 5-6), a comparable number of B220<sup>+</sup>CD19<sup>+</sup> cells were observed in VavCre;Mll mutant relative to wild-type fetal livers (Figure 2h). This was also true within the YFP-gated population, further demonstrating that cells that had expressed Cre recombinase were equally able to generate B-lineage cells in vivo (Figure 2i). Thus, haematopoietic-specific loss of MII during development does not hinder

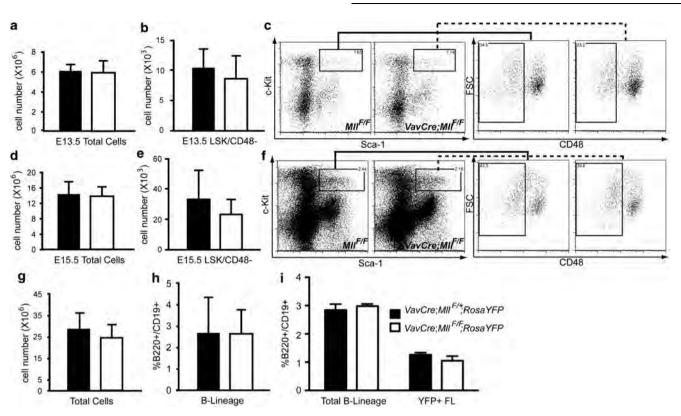


**Figure 1** History of *VavCre* transgene expression in fetal liver LSK cells. (a) Embryos resulting from *VavCre* X *RosaYFP* crosses were collected at the embryonic days indicated on the x-axis and the YFP<sup>+</sup> percentage within the LSK population of the fetal liver was determined by flow cytometry. Each point represents the average of individual embryos (n = 1-4, error bars represent standard deviation). Lower panels, three distinct patterns for *VavCre* expression were observed. (b) mosaic expression, characterized by the lack of liver-specific YFP signal in conjunction with patches of YFP signal throughout the embryo, (c) true, haematopoietic-specific expression of *VavCre*, exemplified by a bright YFP signal in the fetal liver only, obscuring the normally red liver, (d) germline excision was clearly visible as a completely bright YFP<sup>+</sup> embryo, and (e) an embryo that did not inherit the *VavCre* transgene is shown as a negative control. The liver is outlined by dashed white lines. Embryos between E13.5 and E16.5 are used as examples.

haematopoietic expansion or the generation of B-lineage cells in the fetal liver.

### Function of MII-deficient fetal haematopoietic cells

The surprisingly normal fetal haematopoietic development in *VavCre;Mll* mutants prompted us to test these cells in several quantitative functional assays, including myeloerythroid colony assays and competitive transplantation assays. Similar to our observations using adult bone marrow, <sup>16</sup> we found that *Mll*-deficient fetal liver cells yielded 2.6-fold reduced



**Figure 2** Normal cell expansion in *Mll*-deficient fetal livers. Fetal liver cells were analyzed at E13.5 (**a**–**c**), 14.5 (**i**) E15.5 (**d**–**f**, **g**–**h**, **j**). One of three breeding strategies was used to produce *Mll*-deficient embryos:  $VavCre;Mll^{P/F}$  (panels **a**–**f**),  $VavCre;Mll^{F/F} \times Mlf^{F/F}$  (panels **g**–**h**) or  $VavCre;Mll^{F/F} \times Mlf^{F/F}$  (panels **i**). (**a**) Average total number of fetal liver cells per E13.5 embryo. Black bars represent control littermates (Cre-negative and  $VavCre;Mll^{F/F}$  animals) and white bars represent  $VavCre;Mll^{P/F}$  embryos in panels **a**–**f** or  $VavCre;Mll^{F/F}$  embryos in panels **g**–**i**. (**b**) Average number of LSK/CD48-negative cells in E13.5 fetal livers.(**c**) Representative example of LSK/CD48-RACS data used in panel **b**. Cells shown in the first plot are gated as lineage negative and the solid or dashed lines indicate the gate for the second plots (forward scatter versus CD48). (**d**) Average number of LSK/CD48 FACS data used in panel **e**. Gating strategy is the same as in panel **c**. (**g**) Average total number of E15.5 fetal liver cells. (**h**) Average percentage of B220<sup>+</sup>/CD19<sup>+</sup> cells within the E15.5 fetal liver shown in panel **g**. (**i**) Average percentage of B220<sup>+</sup>/CD19<sup>+</sup> cells within the E15.5 fetal liver shown in panel **g**. (**i**) Average percentages of B220<sup>+</sup>/CD19<sup>+</sup> cells within the E15.5 fetal liver shown in panel **g**. (**i**) Average percentages of B220<sup>+</sup>/CD19<sup>+</sup> cells within the E15.5 fetal liver shown in panel **g**. (**i**) Average percentages of B220<sup>+</sup>/CD19<sup>+</sup> cells within the E15.5 fetal liver shown in panels **a**–**i**, n=3-7 embryos and P>0.1.

colony-forming units relative to littermate controls (Figure 3a). We also performed competitive engraftment assays using *VavCre;MII<sup>F/F</sup>* fetal liver cells. In these experiments, we could not detect *VavCre;MII<sup>F/F</sup>* embryo-derived cells in the peripheral blood of recipients, even as early as 4 weeks after transplant (Figures 3b–c). These data illustrate that despite the normal phenotype and developmental expansion of fetal haematopoietic cells, *MII*-deficient stem and progenitor cells are functionally impaired, particularly in their ability to engraft into an adult environment.

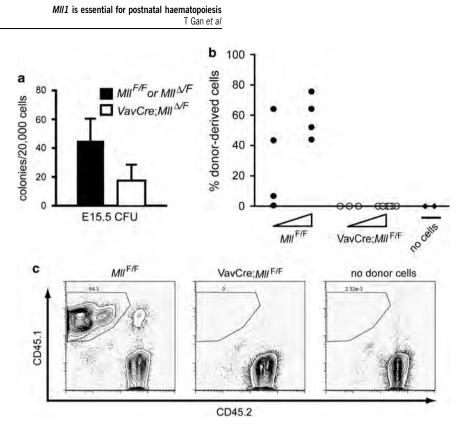
## Postnatal multilineage haematopoietic defects in VavCre;Mll mutants

In contrast to the embryonic lethality observed in germline MII mutants<sup>11,13,15</sup> VavCre;MII mutants were born (Table 1), but postnatal differences between these mutants and their littermates became immediately apparent. Our first attempts to produce adult mutant animals used the VavCre; $MII^{F/+}$  X  $MII^{F/F}$  breeding strategy. The genotypes of 3-week old animals revealed less than half the expected Mendelian ratio of VavCre; $MII^{F/F}$  animals (Table 1). The surviving mutant animals were smaller than their littermates (Supplementary Figure S1) and exhibited incomplete gene excision in bone marrow and thymocyte populations (Supplementary Figure S2–S3), suggesting that cells escaping MII deletion contributed to animal

survival. Close observation of pups born from this breeding strategy demonstrated that most mutants were moribund or died between 2–3 weeks of age (unpublished data).

Histological analysis of bone marrow sections illustrated the overall reduced cellularity and also the paucity of megakaryocytes in VavCre; MII<sup>F/F</sup> animals (Figures 4a-d, arrows), similar to what we had observed after inducing MII deletion in adult bone marrow.<sup>16</sup> Enumerating LSK cells in the bone marrow revealed a sixfold reduction in VavCre; MIIF/F animals (Figure 4e). Examination of the more penetrant  $VavCre;MII^{\Delta N/F}$ animals revealed a >100-fold reduction in all lin<sup>-</sup>/c-Kit<sup>+</sup> cells (Supplementary Figure 4). As a result, all stem and progenitor populations defined by c-Kit expression were severely reduced, and these animals did not survive beyond 3 weeks of age. In contrast, some VavCre; MII<sup>F/F</sup> animals survived beyond this age. Despite the incomplete excision of *Mll* in these surviving VavCre; MIIF/F animals, they afforded an opportunity to assess the effects of Mll loss on multiple lineages in the haematopoietic system without the potentially confounding effects of pl:pC, as had limited our previous studies using Mx1Cre;Mll inducible mutants.

Analysis of the peripheral blood of surviving 3- to 5-week old  $VavCre;Mll^{F/F}$  mice showed significant deficiencies in white blood cells, red blood cells and platelets, as well as an increased mean red blood cell corpuscular volume (Figure 5). These defects were also observed in pl:pC-induced  $Mx1Cre;Mll^{F/F}$ 



**Figure 3** Defects in *VavCre;Mll* mutant fetal haematopoietic cells. (**a**) Haematopoietic colonies obtained by plating  $2 \times 10^4$  total E15.5 fetal liver cells from control or *VavCre;Mll*<sup>AN/F</sup> embryos as indicated in the legend. Cells were plated in methylcellulose supplemented with cytokines to support myeloerythroid cell types (M3434) and colonies were counted 7 days after plating. Averages represent duplicate plates per embryo and three embryos per genotype. The *P*-value is 0.005 for this comparison. Mosaic and germline embryos were excluded from all analyses as indicated in the Materials and methods. (**b**) Results of competitive transplantation experiments using *VavCre;Mll<sup>F/F</sup>* E16.5 embryo fetal liver cells. Donor fetal liver cells (CD45.1<sup>+</sup>) were transplanted into lethally irradiated C57Bl/6 (CD45.2<sup>+</sup>) recipients at two doses ( $5 \times 10^4$  or  $5 \times 10^5$ ) indicated by the triangle below each set of data points. Fetal liver cells were transplanted together with  $2 \times 10^5$  protective C57Bl/6 bone marrow cells, and each donor was transplanted into four to eight recipients. The data shown represents percentage of donor-derived (CD45.1<sup>+</sup>) cells in the peripheral blood of individual recipient animals at 4 weeks after engraftment as determined by flow cytometry. (**c**) Representative example of flow cytometry results used in panel **b**. Similar results were obtained in two independent experiments representing four individual donor embryos and 40 recipients. No cells represents animals transplanted with protective C57Bl/6 bone marrow only.

 Table 1
 Survival of VavCre; mixed lineage leukemia (MII) mutant animals

Genotype	Observed	Expected	Percentage (%)
VavCre;Mll <sup>F/F</sup>	33	64	52
VavCre;Mll <sup>ΔN/F</sup>	8	16	50

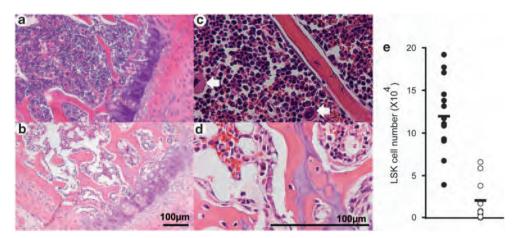
In the first row, the progeny of crosses consisting of *VavCre;Mll<sup>F/+</sup>* animals mated to *Mll<sup>F/F</sup>* animals (with or without the *RosaYFP* allele) were genotyped at 21 days of age (observed) and compared with the number based on Mendelian inheritance (expected). The second line represents the same analyses for the breeding strategy using one germline excised *Mll* allele (*Mll<sup>AN/</sup>*) in the female, *VavCre;Mll<sup>AN/+</sup>*, crossed to *Mll<sup>F/F</sup>* males. The percentage of homozygous mutant animals genotyped at 21 days is indicated.

mice approximately 2 weeks after inducing *MII* deletion (unpublished data and reference <sup>16</sup>). Interestingly, analysis of the peripheral blood of *VavCre;MII*<sup>F/F</sup> animals demonstrated a significant decrease in the percentage of circulating B cells, with a slightly elevated percentage of circulating T cells (Figures 5e–f).

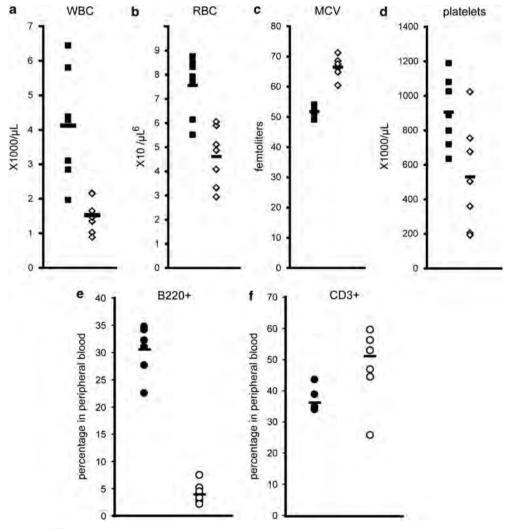
To determine the consequences of *VavCre*-mediated *Mll* deletion on developing lymphocytes and lymphocyte progenitors, populations in the bone marrow and thymus were analyzed

in the same surviving animals as described above. Total B220<sup>+</sup> in the bone marrow were reduced ninefold (Figure 6a). Common lymphoid progenitors (lin<sup>-</sup>/interleukin-7Ra<sup>+</sup>/c-Kit<sup>+</sup>/ Sca-1<sup>+</sup>) were also reduced over fivefold in *VavCre;Mll<sup>F/F</sup>* animals (Figure 6b). More detailed analysis of developing B cells revealed consistent reductions throughout B cell development (Figures 6c–e, Supplementary Figure 5). However, these data likely underestimate the severity of haematopoietic phenotypes because of the poor and variable penetrance of the *VavCre* transgene particularly in the B cell lineage of *VavCre;Mll* mutants (Supplementary Figure 2).

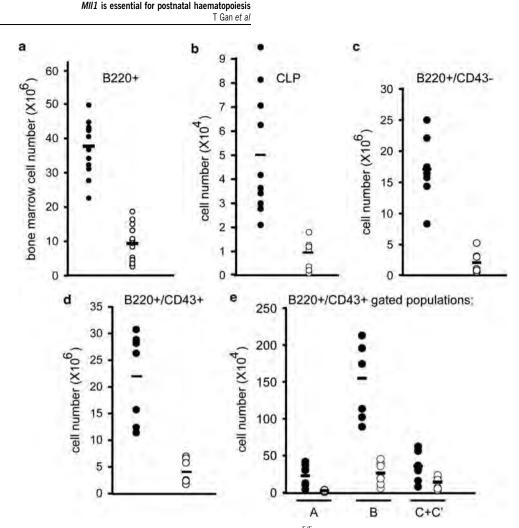
Within the thymus, the reduction in thymocyte number (4.6-fold) was largely attributable to the CD4<sup>+</sup>/CD8<sup>+</sup> doublepositive population (Figure 7a). In contrast to B-lineage cells, thymocytes consistently demonstrated high levels of *Mll* excision (unpublished data) and *RosaYFP* excision (Supplementary Figure 3). Within the double-negative (DN) population, a specific reduction in CD25<sup>+</sup> cells was observed (Figure 7b). As thymus-seeding cells are within the bone marrow LSK population<sup>32</sup> and this population is significantly reduced in *VavCre;Mll*<sup>F/F</sup> animals, (Figure 4e) it is likely that input of progenitors from the bone marrow limits T cell development. In support of this hypothesis, excision of *Mll* at the DN3–DN4 transition with an *LckCre* transgene<sup>16</sup> or with a *Rag1Cre* knock-in (Gan *et al.* in preparation) had no effect on thymocyte numbers.



**Figure 4** Severe reduction in haematopoietic stem and progenitor cells in *Mll*-deficient bone marrow. Representative hematoxylin and eosin stained sternum sections of 3-week old control (**a**, **c**; *Mll*<sup>*F/F*</sup>) and mutant (**b**, **d**; *VavCre;Mll*<sup>*F/F*</sup>) animals. Sections are shown at  $\times 100$  (**a**, **b**) and  $\times 400$  total magnification (**c**, **d**). White arrows indicate megakaryocytes in panel **c**. (**e**) Lineage-negative, c-Kit<sup>+</sup>, Sca-1<sup>+</sup> (LSK) cells in absolute number per two hindlimbs is shown. Filled circles represent littermate controls (*Mll*<sup>*F/F*</sup>) and mutants (*VavCre;Mll*<sup>*F/F*</sup>) are represented by open circles.



**Figure 5** Adult *VavCre;Mll<sup>F/F</sup>* mice exhibit multilineage defects in steady-state haematopoiesis. Peripheral blood cell counts were determined for 3- to 5-week old control  $Ml^{F/F}$  animals (black symbols) and littermate *VavCre;Mll<sup>F/F</sup>* animals (white symbols). Mosaic animals were excluded from this analysis as described in the Materials and methods. White blood cells (WBC), red blood cells (RBC), mean corpuscular volume (MCV) and platelets are represented. (**e–f**) Flow cytometry analysis was performed to quantify (**e**) B (B220<sup>+</sup>) and (**f**) T cells (CD3<sup>+</sup>) in the peripheral blood. At least seven animals of each genotype were analyzed. Averages are represented by horizontal bars.



**Figure 6** Developing lymphoid populations are reduced in  $VavCre;Mll^{F/F}$  animals. (a) Quantification of developing B cells in the bone marrow; total number of B220<sup>+</sup> cells in both hind limbs is shown.(b) Common lymphoid progenitor (CLP; lineage negative, interleukin-7R $\alpha^+$ , c-Kit<sup>+</sup>, Sca-1<sup>+</sup>) absolute number per two hindlimbs is shown. (c–e) B cell populations indicated below each graph are shown as absolute cell number per two hindlimbs. Hardy fractions A-C' are defined by BP1 and CD24 staining as indicated in Supplementary Figure 5. All data was obtained using control  $Mll^{F/F}$  animals (filled symbols) and littermate  $VavCre;Mll^{F/F}$  animals (open symbols) with 6–15 animals representing each genotype. The average values are represented by horizontal bars.

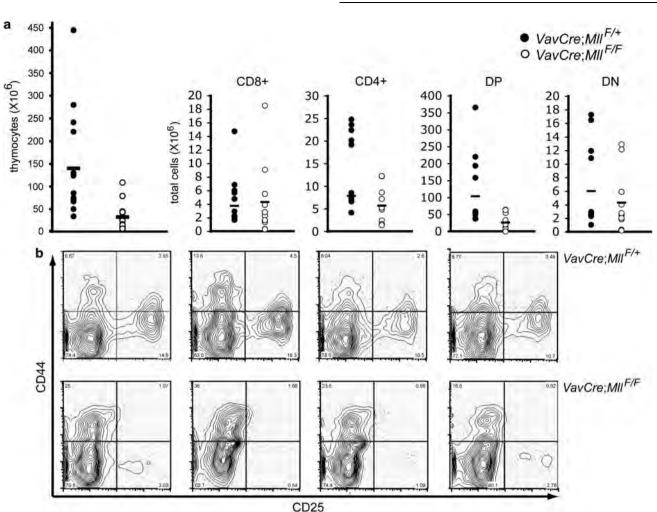
In summary, the developmental excision of *Mll* in haematopoietic cells results in substantial multi-lineage haematopoietic defects that only become apparent after birth, or by transplanting cells into an adult animal. Using a breeding strategy that results in the more penetrant phenotypes (*VavCre;Mll<sup>Δ/F</sup>*), most animals succumb to bone marrow failure between birth and 3 weeks of age. However, examination of adult animals harboring a less penetrant allele combination (*VavCre;Mll<sup>F/F</sup>*) revealed additional defects in B cell development. Collectively, these data support the conclusion that *Mll1* is essential for the establishment and maintenance of adult haematopoiesis.

### Discussion

The primary goal of this study was to reassess the severity of *Mll* deficiency in the absence of the potential confounding effects of systemic IFNs. Using the developmentally regulated *VavCre* transgene, we demonstrate that animals lacking *Mll* specifically in haematopoietic cells exhibit a range of haematopoietic phenotypes very similar to those we observed upon acute excision of *Mll* using the *Mx1Cre* transgene. These include hypocellular bone marrow, a dramatic reduction in the number

of primitive (c-Kit<sup>+</sup>, lineage negative) haematopoietic cells, anemia, thrombocytopenia and reduced numbers of developing lymphocytes. The consistency between these two conditional knockout approaches indicates that the haematopoietic failure observed in  $Mx1Cre;MII^{F/F}$  mutants was likely because of MII loss and not the combined effect of MII loss and IFN signaling. Therefore, we conclude that MII has an essential, non-redundant role during the establishment of adult steady-state haematopoiesis.

In this study, we found that the *in vivo* expansion of *VavCre;Mll<sup>F/F</sup>* fetal haematopoietic cells proceeds normally. However, *Mll*-deficient fetal liver haematopoietic cells exhibit reduced colony-forming potential and competitive engraftment capability, revealing an inherent functional deficit in these cells. The fact that we find no haematopoietic-intrinsic defect in fetal liver cellularity or phenotype is consistent with the results of McMahon *et al.*<sup>15</sup>, who also reported defects in fetal haematopoiesis only in the context of their germline knockout of *Mll* and not their *VavCre* conditional knockout. Therefore, the reduction in fetal liver cellularity observed using traditional germline knockouts may be because of the cell-extrinsic contributions of *Mll* loss in non-haematopoietic tissues such as endothelial or other stromal support cells. Alternatively, the



**Figure 7** Developing T cell populations in the thymus of  $VavCre;Mlf^{F/F}$  animals. (a) Populations of thymocytes were resolved by flow cytometry using CD4 and CD8 antibodies and the absolute number of each subset is shown for control (filled circles) or *Mll* mutants (open circles). Single-positive CD4 (CD4<sup>+</sup>) or CD8 (CD8<sup>+</sup>) are indicated, as are CD4/CD8 double positive (DP) and CD4/CD8 double-negative (DN) cells. (b) Resolution of DN1–4 populations. CD4- or CD8-expressing thymocytes were excluded from the analysis, and CD44 versus CD25 expression is shown on the remaining DN cells. Data from four representative animals is shown for the genotypes indicated at the right of each row.

severity of germline mutants may be due to an early developmental role for *Mll* such as in the hemangioblast, which would be affected in germline but not *VavCre Mll* mutants. This *VavCre* transgene is not expressed in endothelial cells or hemangioblasts, making it a valuable tool to exclude contributions from these tissues.<sup>29,33</sup> More detailed analyses using endothelialspecific and temporally regulated transgenes will be necessary to account for the differences between germline and *VavCre* mutants.

The differences between our results and those of McMahon *et al.* with respect to the role of *Mll* in steady-state adult haematopoiesis are most easily explained by differences in the severity of the knockout allele, not the method of deletion. Both mutant alleles are associated with loss-of-function *Mll* phenotypes, yet the *Mll*<sup>AN</sup> allele exhibits more severe defects in haematopoiesis. The molecular mechanism behind the severity of the *Mll*<sup>AN</sup> allele likely lies in the fact that it is not only retained outside of the nucleus but also lacks important functional motifs for targeting chromatin. Although the allele used in the McMahon *et al.* study is thought to produce no protein, given the technical challenges of detecting MLL by immunoblot in primary cell types, it is possible that this

approach produces a low level of partially active protein that can access the nucleus.

An important observation from our VavCre;Mll<sup>F/F</sup> animals that was obscured using the Mx1Cre inducible transgenic model is the selective reduction in developing and peripheral B cells. A nonspecific reduction in B cells persists up to 15 days after pl:pC injection using our experimental conditions (unpublished data), thus it is likely that the effect of Mll loss was obscured by the global B cell reduction in pI:pC injected animals. The selective reduction in B-lymphopoiesis observed in VavCre mutants recalls the clinical observation that MLL-rearranged acute lymphocytic leukemia (ALL) is predominantly precursor-B cell ALL and is rarely T cell ALL.<sup>34,35</sup> In fact, among patients with MLL translocations, the T-ALL group has a much more favorable outcome than the B-ALL group.<sup>36</sup> Therefore, the selective and persistent reduction in B lymphocytes observed in VavCre; Mll<sup>F/F</sup> mutants suggests that MLL controls unique and important pathways in developing B cells that may be deregulated by MLL fusion oncoproteins. Future studies in which Cre recombinase is expressed specifically in early lymphocyte populations, thus sparing all other Mll-dependent cell types in the bone marrow, will be essential for dissecting a potential B-lymphocyte specific function of MLL.

Our data have important implications for the development of strategies to target MLL associated leukemia. First, the results presented here further support our conclusion<sup>16</sup> that targeting oncogenic MLL fusion proteins through direct inhibition of the N-terminus, which is shared with endogenous wild-type MLL may have the undesirable effect of suppressing ongoing normal haematopoiesis. This is particularly problematic, because structure-function relationships in the N-terminus have been extensively interrogated, and molecules that directly target the N-terminus of MLL could be used against a wide variety of MLL fusions differing in C-terminal fusion partner. An ideal targeted therapeutic would act exclusively on the aberrant activity of the oncogenic fusion protein, yet this degree of selectivity is difficult to achieve. Thus the viability of an MLL N-terminal targeting molecule may depend on identifying a therapeutic index distinguishing the activities of MLL fusion proteins from those of endogenous MLL. Mll loss-of-function models will help define the pathways required for normal haematopoiesis relative to those deregulated by MLL fusion proteins, perhaps guiding alternative strategies for selectively targeting the actions of leukemogenic MLL fusion proteins.

### **Conflict of interest**

The authors declare no conflict of interest.

### Acknowledgements

We thank our laboratory members, David Traver and Christopher Dant for insightful comments and suggestions, Dr Roderick Bronson, Dr Benjamin Lee and Dr Glen Raffel for histolology advice, and Dr Hanna Mikkola, Dr Matthias Stadtfeld and Dr Thomas Graf for *VavCre* mice and advice for breeding and analyzing this strain. We are grateful to Nancy Speck for advice and use of her microscopes. This work was supported by the NIH grant HL090036 to PE and NCRR COBRE grant P20RR016437 to William Green.

#### References

- 1 Behm FG, Raimondi SC, Frestedt JL, Liu Q, Crist WM, Downing JR *et al.* Rearrangement of the MLL gene confers a poor prognosis in childhood acute lymphoblastic leukemia, regardless of presenting age. *Blood* 1996; **87**: 2870–2877.
- 2 Liedtke M, Cleary ML. Therapeutic targeting of MLL. *Blood* 2009; **113**: 6061–6068.
- 3 Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD *et al.* MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell* 2002; **10**: 1107–1117.
- 4 Nakamura T, Mori T, Tada S, Krajewski W, Rozovskaia T, Wassell R et al. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol Cell* 2002; **10**: 1119–1128.
- 5 Zeleznik-Le NJ, Harden AM, Rowley JD. 11q23 translocations split the 'AT-hook' cruciform DNA-binding region and the transcriptional repression domain from the activation domain of the mixedlineage leukemia (MLL) gene. *Proc Natl Acad Sci USA* 1994; **91**: 10610–10614.
- 6 Slany RK, Lavau C, Cleary ML. The oncogenic capacity of HRX-ENL requires the transcriptional transactivation activity of ENL and the DNA binding motifs of HRX. *Mol Cell Biol* 1998; 18: 122–129.
- 7 Ayton PM, Čhen EH, Cleary ML. Binding to nonmethylated CpG DNA is essential for target recognition, transactivation, and myeloid transformation by an MLL oncoprotein. *Mol Cell Biol* 2004; **24**: 10470–10478.

- 8 Yokoyama A, Cleary ML. Menin critically links MLL proteins with LEDGF on cancer-associated target genes. *Cancer Cell* 2008; **14**: 36–46.
- 9 Bach C, Mueller D, Buhl S, Garcia-Cuellar MP, Slany RK. Alterations of the CxxC domain preclude oncogenic activation of mixed-lineage leukemia 2. *Oncogene* 2009; **28**: 815–823.
- 10 Daser A, Rabbitts TH. Extending the repertoire of the mixedlineage leukemia gene MLL in leukemogenesis. *Genes Dev* 2004; 18: 965–974.
- 11 Yu BD, Hess JL, Horning SE, Brown GA, Korsmeyer SJ. Altered Hox expression and segmental identity in Mll-mutant mice. *Nature* 1995; **378**: 505–508.
- 12 Ayton P, Sneddon SF, Palmer DB, Rosewell IR, Owen MJ, Young B *et al.* Truncation of the MII gene in exon 5 by gene targeting leads to early preimplantation lethality of homozygous embryos. *Genesis* 2001; **30**: 201–212.
- 13 Ernst P, Fisher JK, Avery W, Wade S, Foy D, Korsmeyer SJ. Definitive hematopoiesis requires the mixed-lineage leukemia gene. *Dev Cell* 2004; **6**: 437–443.
- 14 Terranova R, Agherbi H, Boned A, Meresse S, Djabali M. Histone and DNA methylation defects at Hox genes in mice expressing a SET domain-truncated form of Mll. *Proc Natl Acad Sci USA* 2006; 103: 6629–6634.
- 15 McMahon KA, Hiew SY, Hadjur S, Veiga-Fernandes H, Menzel U, Price AJ *et al.* Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. *Cell Stem Cell* 2007; 1: 338–345.
- 16 Jude CD, Climer L, Xu D, Artinger E, Fisher JK, Ernst P. Unique and independent roles for MLL in adult hematopoietic stem cells and progenitors. *Cell Stem Cell* 2007; 1: 324–337.
- 17 Yano T, Nakamura T, Blechman J, Sorio C, Dang CV, Geiger B et al. Nuclear punctate distribution of ALL-1 is conferred by distinct elements at the N terminus of the protein. Proc Natl Acad Sci USA 1997; 94: 7286–7291.
- 18 Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science* 1995; 269: 1427–1429.
- 19 Jude CD, Gaudet JJ, Speck NA, Ernst P. Leukemia and hematopoietic stem cells: balancing proliferation and quiescence. *Cell Cycle* 2008; **7**: 586–591.
- 20 Baron ML, Gauchat D, La Motte-Mohs R, Kettaf N, Abdallah A, Michiels T et al. TLR ligand-induced type I IFNs affect thymopoiesis. J Immunol 2008; 180: 7134–7146.
- 21 Demoulins T, Abdallah A, Kettaf N, Baron ML, Gerarduzzi C, Gauchat D et al. Reversible blockade of thymic output: an inherent part of TLR ligand-mediated immune response. J Immunol 2008; 181: 6757–6769.
- 22 Despres D, Goldschmitt J, Aulitzky WE, Huber C, Peschel C. Differential effect of type I interferons on hematopoietic progenitor cells: failure of interferons to inhibit IL-3-stimulated normal and CML myeloid progenitors. *Exp Hematol* 1995; 23: 1431–1438.
- 23 Mizutani T, Tsuji K, Ebihara Y, Taki S, Ohba Y, Taniguchi T *et al.* Homeostatic erythropoiesis by the transcription factor IRF2 through attenuation of type I interferon signaling. *Exp Hematol* 2008; **36**: 255–264.
- 24 Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA *et al.* IFNalpha activates dormant haematopoietic stem cells *in vivo. Nature* 2009; **458**: 904–908.
- 25 Sato T, Onai N, Yoshihara H, Arai F, Suda T, Ohteki T. Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. *Nat Med* 2009; **15**: 696–700.
- 26 Ogilvy S, Metcalf D, Gibson L, Bath ML, Harris AW, Adams JM. Promoter elements of vav drive transgene expression *in vivo* throughout the hematopoietic compartment. *Blood* 1999; **94**: 1855–1863.
- 27 de Boer J, Williams A, Skavdis G, Harker N, Coles M, Tolaini M et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur J Immunol 2003; **33**: 314–325.
- 28 Georgiades P, Ogilvy S, Duval H, Licence DR, Charnock-Jones DS, Smith SK et al. VavCre transgenic mice: a tool for mutagenesis in hematopoietic and endothelial lineages. *Genesis* 2002; 34: 251–256.
- 29 Stadtfeld M, Graf T. Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. *Development* 2005; **132**: 203–213.
- 30 Yagi H, Deguchi K, Aono A, Tani Y, Kishimoto T, Komori T. Growth disturbance in fetal liver hematopoiesis of Mll-mutant mice. *Blood* 1998; **92**: 108–117.

- 31 Kim I, He S, Yilmaz OH, Kiel MJ, Morrison SJ. Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood* 2006; **108**: 737–744.
- 32 Bhandoola A, Sambandam A. From stem cell to T cell: one route or many? *Nat Rev Immunol* 2006; **6**: 117–126.
- 33 Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* 2009; **457**: 887–891.
- 34 Abshire TC, Buchanan GR, Jackson JF, Shuster JJ, Brock B, Head D et al. Morphologic, immunologic and cytogenetic studies in children with acute lymphoblastic leukemia at diagnosis and

relapse: a Pediatric Oncology Group study. Leukemia 1992; 6: 357-362.

- 35 Huret JL, Brizard A, Slater R, Charrin C, Bertheas MF, Guilhot F et al. Cytogenetic heterogeneity in t(11;19) acute leukemia: clinical, hematological and cytogenetic analyses of 48 patients– updated published cases and 16 new observations. *Leukemia* 1993; 7: 152–160.
- 36 Pui CH, Chessells JM, Camitta B, Baruchel A, Biondi A, Boyett JM *et al.* Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. *Leukemia* 2003; **17**: 700–706.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

### **Supplementary Figure Legends**

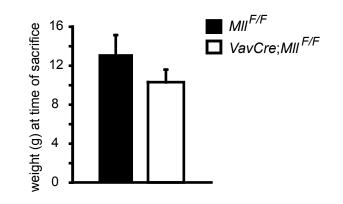
**Figure S1**. *VavCre;Mll*<sup>F/F</sup> survivors are smaller than their *Mll*<sup>F/F</sup> littermates. Animal weight was determined at the time of sacrifice, which was between 3 and 5 weeks of age. The number of animals analyzed and p value is shown below the figure. Animals exhibiting evidence of mosaic or germline *Mll* excision were removed from the analysis (see Methods).

**Figure S2**. Evidence for a selective advantage of non-excised cells in bone marrow populations of *VavCre;Mll*<sup>F/F</sup>;*RosaYFP* adult mice. Bone marrow cells from 3-5 week old animals of the genotype indicated at the top of each column were analyzed to determine the percent YFP<sup>+</sup> cells within each population shown on the right of each pair of histograms. LSK, lineage-negative, Sca-1<sup>+</sup>, c-Kit<sup>+</sup> cells; other populations are defined by the antibodies indicated on the right. This example is representative of 3-5 animals of each genotype analyzed.

**Figure S3**. High levels of gene excision in *VavCre;Mll*<sup>F/F</sup>;*RosaYFP* adult thymocytes. Thymocytes from 3-5 week old animals of the genotype indicated at the top of each column were analyzed to determine the percent YFP<sup>+</sup> cells within each population shown on the right of each pair of histograms. DN, CD4- and CD8-negative cells; other populations indicated by the single or combination of antibodies shown on the right of the histograms. This example is representative of 3-5 animals of each genotype analyzed. **Figure S4**. Over 100-fold reduction in  $lin^{-}/c-Kit^{+}$  cells in the bone marrow in *VavCre;Mll*<sup> $\Delta N/F$ </sup> mice. **a)** Absolute number of  $lin^{-}/c-Kit^{+}$  cells in the bone marrow of three-week old *VavCre;Mll*<sup> $\Delta N/F$ </sup> (black bar) and control, *VavCre;Mll*<sup>F/+</sup> mice (white bar). Three to four mice were analyzed per group, standard deviation is represented by the error bars, p=0.026. **b)** Representative flow cytometry plots of bone marrow  $lin^{-}/c-Kit^{+}$  populations in the groups shown in **a**.

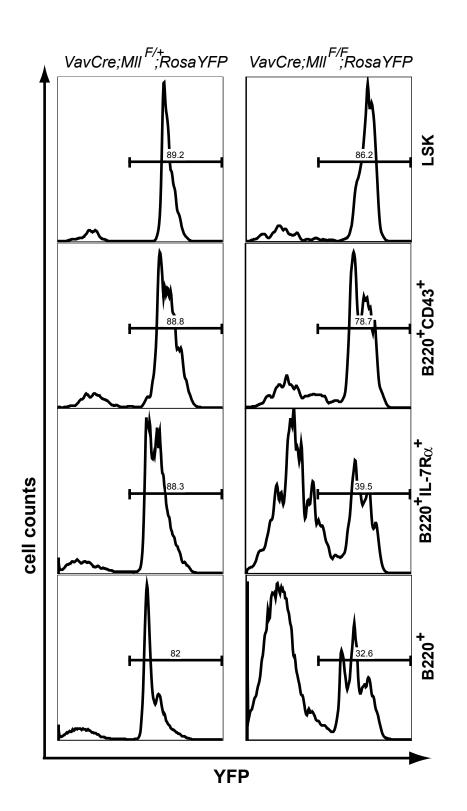
**Figure S5.** Example of gating strategy used to analyze B cell populations quantified in Figure 6. Bone marrow B cells were first resolved into  $CD43^+/-B220^+$  populations then the  $CD43^+B220^+$  population was further analyzed using anti-BP1 and anti-CD24 antibodies to quantify Hardy fractions A-C' as indicated on the flow cytometry plot.

Supplementary Figure 1 Gan et al.

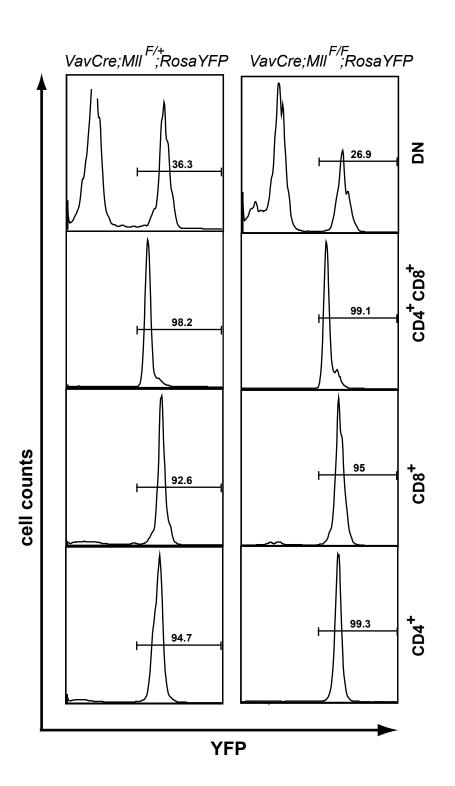


n=13-14, p=0.019

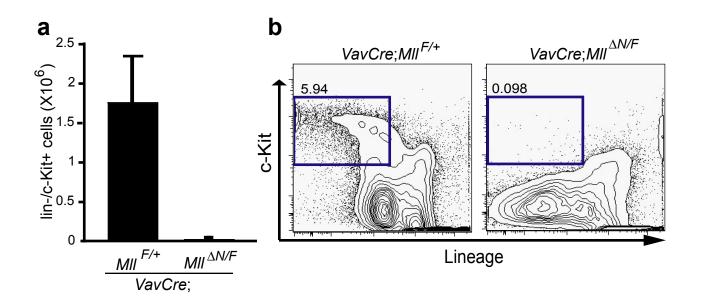
Supplementary Figure 2 Gan et al.



Supplementary Figure 3 Gan et al.



Supplementary Figure 4 Gan et al.



Supplementary Figure 5 Gan et al.

