Ott1(*Rbm15*) has pleiotropic roles in hematopoietic development

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Edited by Janet D. Rowley, University of Chicago Medical Center, Chicago, IL, and approved February 9, 2007 (received for review October 12, 2006)

***OTT1*(*RBM15*) was originally described as a 5′ translocation partner of the *MAL*(*MKL1*) gene in t(1,22)(p13;q13) infant acute megakaryocytic leukemia. *OTT1* has no established physiological function, but it shares homology with the *spen*/*Mint*/*SHARP* family of proteins defined by three amino-terminal RNA recognition motifs and a carboxyl-terminal SPOC (Spen paralog and ortholog carboxyl-terminal) domain believed to act as a transcriptional repressor. To define the role of *OTT1* in hematopoiesis and help elucidate the mechanism of t(1,22) acute megakaryocytic leukemia pathogenesis, a conditional allele of *Ott1* was generated in mice. Deletion of *Ott1* in adult mice caused a loss of peripheral B cells due to a block in pro/pre-B differentiation. There is myeloid and megakaryocytic expansion in spleen and bone marrow, an increase in the $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ compartment that includes hematopoietic stem cells, and a shift in progenitor fate toward granulocyte differentiation. These data show a requirement for *Ott1* in B lymphopoiesis, and inhibitory roles in the myeloid, megakaryocytic, and progenitor compartments. The ability of *Ott1* to affect hematopoietic cell fate and expansion in multiple lineages is a novel attribute for a *spen* family member and delineates *Ott1* from other known effectors of hematopoietic development. It is plausible that dysregulation of *Ott1*-dependent hematopoietic developmental pathways, in particular those affecting the megakaryocyte lineage, may contribute to *OTT1-MAL*-mediated leukemogenesis.**

lymphocyte | megakaryocytic leukemia | hematopoietic stem cell

Infant acute megakaryocytic leukemias in non-Down's syndrome children harbor a unique chromosomal translocation, t(1,22)(p13;q13) (1, 2). This translocation fuses two novel genes believed to be transcription factors, *OTT1* (*RBM15*) and *MAL* (*MKL1*, *BSAC*, or *MRTF-A*), and results in expression of a chimeric protein (3, 4). The breakpoint occurs in the first intron of *OTT1* and in either the third or fourth intron of *MAL*. The chimeric protein OTT1-MAL (RBM15-MKL1) contains almost the entire coding sequence of both genes. Neither gene had previously been described. Although *MAL* has recently been implicated in serum response, thus far no physiologic roles have been elucidated in hematopoiesis for either gene (5).

Ott1 is ubiquitously expressed and belongs to a larger gene family related to *Drosophila spen* (*split ends*) (3, 4). The *spen* family is defined by three amino-terminal RNA recognition motifs and a carboxyl-terminal SPOC (Spen paralog and ortholog carboxyl-terminal) transactivation/repression domain (6). *spen* is involved in a wide range of developmental processes including neural and eye development and was identified as a positive regulator of *egfr* and *ras* pathways (7, 8). *spen* modulates *hox* gene expression in head and thorax development and down-regulates the Notch effector *suppressor of hairless* (9, 10). *spen* is thought to regulate developmental cell fate and spatial positioning processes through these pathways (8–11).

The human and murine homologs of *spen*, *SHARP* (SMRT/HDAC1-associated repressor protein), and *Mint* (Msx-2-interacting nuclear target protein) are thought to provide similar functions in humans and mice, respectively (12, 13). *SHARP* is a component of a corepressor complex including the proteins SMRT (Silencing mediator for retinoic and thyroid hormone receptors) and NCoR (Nuclear receptor Corepressor), which are required for RBP-Jκ-mediated repression (14–16). *Mint* acts as a transcriptional repressor in concert with the homeodomain protein Msx-2 (13). Mint also interacts with RBP-Jκ and is a suppressor of Notch signaling through a competitive binding mechanism (17). Structural analysis of the SHARP SPOC domain suggests that the regions responsible for binding SMRT/NCoR are highly conserved between all *spen* family members, including *OTT1* (18, 19). SHARP also associates with SRA (steroid receptor RNA coactivator) and modulates steroid response (12). SHARP, OTT1, and OTT3 associate with EB2 (Epstein–Barr virus early protein), an RNA export factor (20). Although *in vitro* studies of Mint demonstrate repressor activity on the *Osteocalcin* promoter, transcriptional up-regulation is observed when tested with a basal promoter, raising the possibility of dual repressor/transactivator function (13).

Mint deficiency in mice is lethal around embryonic day 13.5 (E13.5) (17). Defects are noted in the heart, pancreas, and skin and the size of the fetal liver, but the frequency of hematopoietic CFU from fetal liver cells is normal. When fetal liver cells are transplanted into lymphocyte-deficient recipients, there is an increase in splenic marginal zone B lymphocytes at the expense of follicular B lymphocytes that is thought to reflect loss of *Notch2* signaling by *Mint*. Taken together, the products of the *spen* family have similar and overlapping functions.

We have generated a murine allele enabling conditional deletion of *Ott1 in vivo* to gain an understanding of the physiologic role of *OTT1* in hematopoiesis and its contribution to *OTT1-MAL*-associated acute megakaryocytic leukemia.

Author contributions: G.D.R., T.M., O.A.B., and D.G.G. designed research; G.D.R., T.M., H.S., I.R.W., and D.E.C. performed research; K.A. and O.A.B. contributed new reagents/analytic tools; G.D.R., T.M., H.S., I.R.W., K.A., O.A.B., and D.G.G. analyzed data; and G.D.R. and D.G.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte–monocyte progenitor; HSC, hematopoietic stem cell; LSK, $Lin^{-}IL\text{-}7R^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$; MEP, megakaryocyte–erythroid progenitor; plpC, polyinosinic-polycytidylic acid; E*n*, embryonic day *n*.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0609041104/DC1.

Table 1. Genotype of embryos from *Ott1*$^{null/wt}$ × *Ott1*$^{null/wt}$ mice

Days after conception*	No. of litters	No. of embryos	No. of wt/wt mice (%)	No. of null/wt mice (%)	No. of null/null mice (%)
8.5	4	28	9 (32)	13 (46)	6 (22)
9.5	8	73	19 (26)	38 (52)	16 (22)
10.5	10	75	22 (29)	31 (41)	21† (28)
11.5	1	7	3 (43)	2 (28.5)	2† (28.5)
12.5	1	8	1 (12.5)	4 (50)	3† (37.5)
13.5	2	13	5 (38)	8 (62)	0 (0)
Term	10	65	18 (28)	47 (72)	0 (0)

*The day of the vaginal plug is counted as E0.5.
†All embryos observed undergoing degeneration.

Results

Generation of a Conditional *Ott1* Allele in Mice. *Ott1* comprises two exons and is expressed as two major transcripts differing by a facultative intron with all but 23 bp included in the first exon. Therefore, by flanking the first exon with *loxP* sites, conditional deletion of *Ott1* can be achieved through expression of *Cre* recombinase [supporting information (SI) Fig. 5*A*]. The *Ott1* allele was targeted in ES cells (SI Fig. 5 *B* and *C*), and chimeric mice were produced capable of germ-line transmission of the conditional allele, *Ott1*^{flox}. Mice with the *Ott1*^{flox} allele were crossed with transgenic *EIIa-Cre*, which excises broadly in the early embryo, generating mice with complete excision of the floxed allele, *Ott1*^{null} (SI Fig. 5 *D* and *E*).

Deletion of *Ott1* Is Embryonic Lethal. Mice heterozygous for the *Ott1*^{null} allele were bred together to generate homozygous null animals (Table 1). No viable *Ott1*$^{null/null}$ offspring were obtained. In timed matings, there was expected Mendelian frequency of viable *Ott1*$^{null/null}$ embryos through E9.5. However, *Ott1*$^{null/null}$ embryos after E9.5 showed degeneration and resorption, indicative of an essential embryonic requirement for *Ott1* during E9.5–E10.5.

Deletion of *Ott1* in Adult Mice Causes Peripheral B Cell Lymphopenia. *Ott1*^{flox} animals were crossed with *Mx1-Cre* transgenic mice that enable efficient conditional induction of Cre expression with polyinosinic-polycytidylic acid (pIpC) in the hematopoietic compartment of adult mice (21). *Ott1*$^{flox/null}$ *Mx1-Cre* mice were bred in which a single excision event was required to generate homozygosity of the null allele.

Six-week-old mice were treated with pIpC and monitored with weekly nonlethal retroorbital eyebleeds. Total WBC gradually decreased over a 6-week period in *Ott1*$^{flox/null}$ *Mx1-Cre* mice, compared with pIpC-treated littermates with at least one wild-type *Ott1* allele (Fig. 1*A*). No abnormalities were observed between wild-type and hemizygous animals, or with *Mx1-Cre* alone (data not shown). The decrease in WBC resulted from a striking decline in lymphocytes (Fig. 1*A*). There were no significant differences in neutrophil count, hematocrit, or platelet counts (data not shown).

Flow-cytometric analysis of peripheral blood 6 weeks after pIpC induction showed a dramatic decrease in CD19$^+$/B220$^+$ peripheral B cells in *Ott1*$^{flox/null}$ *Mx1-Cre* mice (Fig. 1 *B* and *C*). Although the absolute numbers of B220$^+$ cells were decreased 20-fold, the relative percentages of IgM$^+$IgD$^+$ and IgM$^-$IgD$^+$ were preserved (Fig. 1*B*). CD4$^+$ and CD8$^+$ T cell populations were not significantly changed, nor were cells with monocytic and granulocytic markers (Fig. 1*C* and SI Fig. 6).

After 8 weeks, the lymphocyte counts in *Ott1*$^{flox/null}$ *Mx1-Cre* mice began to rise and eventually returned to baseline levels (data not shown). Although the *Ott1*^{flox} allele was undetectable by PCR in DNA isolated from the peripheral blood 1 week after pIpC, the allele reappeared after 8 weeks (Fig. 1*D*). The correlation between the rise in lymphocytes and the recurrence of the *Ott1*^{flox} allele indicates a strong negative selective pressure in the B lymphocyte population with deletion of *Ott1*.

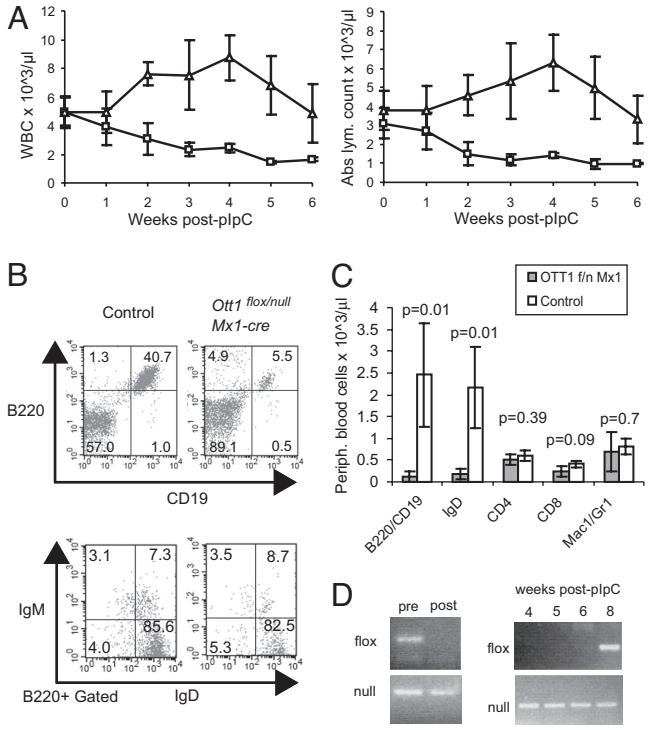

Fig. 1. Loss of peripheral B lymphocytes after deletion of *Ott1* in adult mice. *Ott1*$^{null/flox}$ *Mx1-Cre* mice and control littermates with a wild-type allele were induced with pIpC and bled on a weekly basis. (*A*) Total leukocyte count (WBC). (*B*) Absolute lymphocyte count. Open squares, *Ott1*$^{null/flox}$ *Mx1-Cre* ($n = 4$); open triangles, littermates ($n = 5$). (*B*) Representative flow-cytometry plots of peripheral blood at 6 weeks after pIpC induction with percentage of cells noted in each quadrant. (*C*) Absolute numbers of peripheral blood subsets at 6 weeks after pIpC. Gray bars, *Ott1*$^{null/flox}$ *Mx1-Cre* ($n = 3$); white bars, littermates ($n = 5$). (*D*) Agarose gel electrophoresis of PCR from peripheral blood DNA samples to detect *Ott1* flox or null alleles. (*Left*) PCR from before and 1 week after pIpC induction blood DNA. (*Right*) PCR from weeks 4–8 after pIpC.

***Ott1* Is Required for the Development of Pro/Pre-B Cells.** Flow cytometry of the bone marrow B cell compartment showed that induced *Ott1*$^{flox/null}$ *Mx1-Cre* mice had equivalent numbers of B220$^+$/CD43$^+$ pro- to early pre-B cells (Fig. 2 *A* and *B*). Later pre-B development is characterized by loss of CD43 and acquisition of CD19 and CD25 (22). *Ott1*$^{flox/null}$ *Mx1-Cre* mice show a >50% decrease in B220$^+$CD43$^-$, B220$^+$CD19$^+$, and B220$^+$CD25$^+$ populations (Fig. 2 *A* and *B*). Bone marrow from induced *Ott1*$^{flox/null}$ *Mx1-Cre* mice showed ≈10-fold fewer pre-B cells *in vitro* when plated in IL-7-containing methylcellulose medium (Fig. 2*C*). Single-colony PCR of the few pre-B colonies that did develop from *Ott1*$^{flox/null}$ *Mx1-Cre* mice yielded exclusively unexcised *Ott1*$^{flox/null}$ genotypes, suggesting a strong selective advantage for cells with intact *Ott1* (data not shown). In contrast, *Ott1*$^{flox/wt}$ *Mx1-Cre* pre-B colonies showed complete excision within the pre-B compartment, with 100% conversion of *Ott1*^{flox} into *Ott1*^{null} (data not shown). Excision of all bone marrow cells was >95% as assessed by Southern blot. Splenic excision, however, was incomplete, consistent with prior *Mx1-Cre* studies showing reduced efficiency within the spleen (SI Fig. 7*A*) (21). Taken together these data indicate that *Ott1* is essential for progression beyond the pro/pre-B stage.

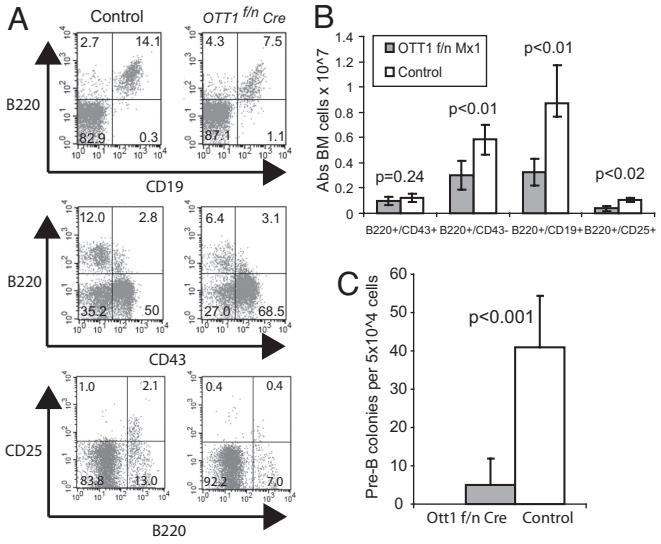

Fig. 2. *Ott1* deletion results in a block of pre-B lymphocyte differentiation. (*A*) Representative flow-cytometry plots of *Ott1*$^{null/flox}$ *Mx1-Cre* and littermate control bone marrow 6 weeks after pIpC. (*B*) Absolute numbers of bone marrow cell subsets. B220/CD19, *Ott1*$^{null/flox}$ *Mx1-Cre*, $n = 5$; control, $n = 7$. B220/CD43, *Ott1*$^{null/flox}$ *Mx1-Cre*, $n = 7$; control, $n = 6$. CD25/B220, $n = 3$. (*C*) Pre-B colonies from 5×10^4 bone marrow cells of mice 6 weeks after pIpC plated in duplicate in IL-7-containing methylcellulose. Gray bar, *Ott1*$^{null/flox}$ *Mx1-Cre* ($n = 4$); white bar, littermate controls ($n = 9$).

Deletion of *Ott1* Results in Megakaryocytic Expansion. Spleen histopathology from *Ott1*$^{flox/null}$ *Mx1-Cre* mice 6 weeks after pIpC treatment showed expansion of the splenic red pulp (Fig. 3*A*). Of particular interest, a ≈5-fold increase in megakaryocytes was observed in the red pulp (Fig. 3*C*). The megakaryocytes also showed abnormal clustering (Fig. 3*A Inset*), a feature associated with myelodysplasia (23). Likewise, an ≈2-fold increase in megakaryocytes was observed in *Ott1*$^{flox/null}$ *Mx1-Cre* bone marrow (Fig. 3 *B* and *C*).

Bone marrow from *Ott1*$^{flox/null}$ *Mx1-Cre* mice 6 weeks after pIpC treatment was plated in a collagen-based medium with IL3, IL6, IL11, and TPO to assess numbers of CFU-megakaryocyte (CFU-Meg). Unexpectedly, equivalent numbers of CFU-Megs were found in comparison to pIpC-treated littermate controls despite increased numbers of megakaryocytes observed *in vivo* (Fig. 3*D*). These data indicate that loss of *Ott1* results in megakaryocyte expansion but suggest that there may be cell-nonautonomous contributions to this phenotype *in vivo*.

Myeloid Expansion After *Ott1* Deletion. Myeloid expansion was observed in the bone marrow of *Ott1*$^{flox/null}$ *Mx1-Cre* mice after pIpC treatment (Fig. 3*B*). Flow cytometry of the spleen or bone marrow demonstrated a 100% increase and a 26% increase, respectively, in Mac-1$^+$/Gr-1$^+$ cells, consistent with histopathology (Fig. 4 *A* and *B* and SI Fig. 7*B*). No tissues other than the spleen showed extramedullary hematopoiesis (data not shown). Although the hematocrit did not differ between *Ott1*$^{flox/null}$ *Mx1-Cre* and control animals (data not shown), there was an increase in erythroid precursors, marked by Ter119, in spleens derived from *Ott1*$^{flox/null}$ *Mx1-Cre* animals and a comparable decrease in Ter119$^+$ cells in *Ott1*$^{flox/null}$ *Mx1-Cre* bone marrow (Fig. 4*A*).

In vitro CFU assays of bone marrow were used to determine whether an increase in precursors could explain the myeloid expansion. Bone marrow from *Ott1*$^{flox/null}$ *Mx1-Cre* mice 6 weeks after treatment with pIpC or littermate controls was plated in methylcellulose to assess myeloid and erythroid CFU potential (Fig. 4*B*). Absolute numbers of nucleated bone marrow cells did not significantly differ between the two groups (data not shown). A 60% increase in total colonies was observed by using the *Ott1*$^{flox/null}$*Mx1-Cre* bone marrow (Fig. 4*B*). The difference in colony number was attributable to increases in monocytic, granulocytic, and combined granulocyte-macrophage (GM) colonies but not erythroid or granulocyte, erythroid, monocyte, and megakaryocyte colonies.

Single-colony PCR of individual myeloid colonies showed 100% conversion of *Ott1*$^{flox/null}$ genotype colonies to a homozygous *Ott1* $^{null/null}$ genotype (SI Fig. 7*C*). These data indicate that *Ott1* is not required for terminal myeloid differentiation in adult hematopoiesis and may inhibit myeloid development and/or proliferation.

Deletion of *Ott1* Results in Increased Numbers of Lin$^-$IL-7R$^-$Sca-1$^+$c-Kit$^+$ (LSK) Cells and a Proclivity for Granulocytic Maturation. Because *Ott1* deletion affected multiple hematopoietic lineages, we studied the hematopoietic stem cell (HSC) and progenitor populations, including LSK, common lymphoid progenitor (CLP), common myeloid progenitor (CMP), megakaryocyte–erythroid progenitor (MEP), and granulocyte–monocyte progenitor (GMP) (24–26). Bone marrow from *Ott1*$^{flox/wt}$ *Mx1-Cre* mice 6 weeks after pIpC treatment, and corresponding pIpC-treated littermates, was lineage-depleted (Lin$^-$) and labeled with anti-Sca-1, c-Kit, IL-7R, and CD34 to separate the LSK/progenitor populations (Fig. 4*C*) (26).

The LSK compartment, which encompasses the HSC population, showed a 48% ($P < 0.01$) increase in *Ott1*$^{flox/null}$ *Mx1-Cre* bone marrow (Fig. 4 *C* and *D*). There were no significant changes in the CLP, CMP, or MEP populations; however, numbers of GMPs rose by 50% ($P = 0.01$). These correlated with the colony-forming potential of a statistically significant increase in CFU-G/M/GM colonies from whole bone marrow that derive from GMPs (Fig. 4*B*).

To assess clonogenicity after *Ott1* deletion, LSK cells from *Ott1*$^{flox/null}$ *Mx1-Cre* mouse bone marrow 6 weeks after pIpC treatment were sorted onto methylcellulose containing IL-3, IL-6, SCF, Epo, Tpo, and GM-CSF. Equivalent colony numbers were obtained between the control group and the *Ott1*$^{flox/null}$ *Mx1-Cre* group (data not shown). Single-colony PCR showed >80% excision efficiency in granulocytic, monocytic, erythroid, and megakaryocytic progeny (data not shown). These data imply that *Ott1* does not impede the ability of LSK cells to differentiate and is not required for myeloid differentiation.

Interestingly, there was an increase in granulocytic differentiation from *Ott1*$^{flox/null}$ *Mx1-Cre* LSK cells compared with controls (25.7 ± 3.4% vs. 6.0 ± 2.0%; $P < 0.01$) (Fig. 4*E*). The increase in granulocytic maturation occurred at the expense of combined granulocyte/monocyte colonies (51.5 ± 4.1% compared with 63.8 ± 6.9%; $P < 0.01$). These data suggest that *Ott1* inhibits granulocytic maturation at the GMP stage or earlier. Megakaryocyte hyperplasia *in vivo* does not appear to be due to increased numbers of MEPs or a "steal" phenomenon from the erythroid lineage, because clonogenicity of the LSK cell toward erythroid and megakaryocytic cells is identical regardless of the presence of *Ott1* (Fig. 4 *B* and *E*).

Discussion

Ott1 is essential for embryonic development beyond E9.5. Loss of a single allele resulted in expected numbers of viable embryos and normal phenotype indicating no gene–dosage effect in early development. In contrast, deletion of *Mint* in mice results in embryonic lethality occurring later around E13.5, signifying a nonredundant developmental function between *Mint* and *Ott1* (17). *Ott3* is more closely related to *Ott1* than *Mint*, but these data show that Ott3 does not have functional redundancy with *Ott1* in early development.

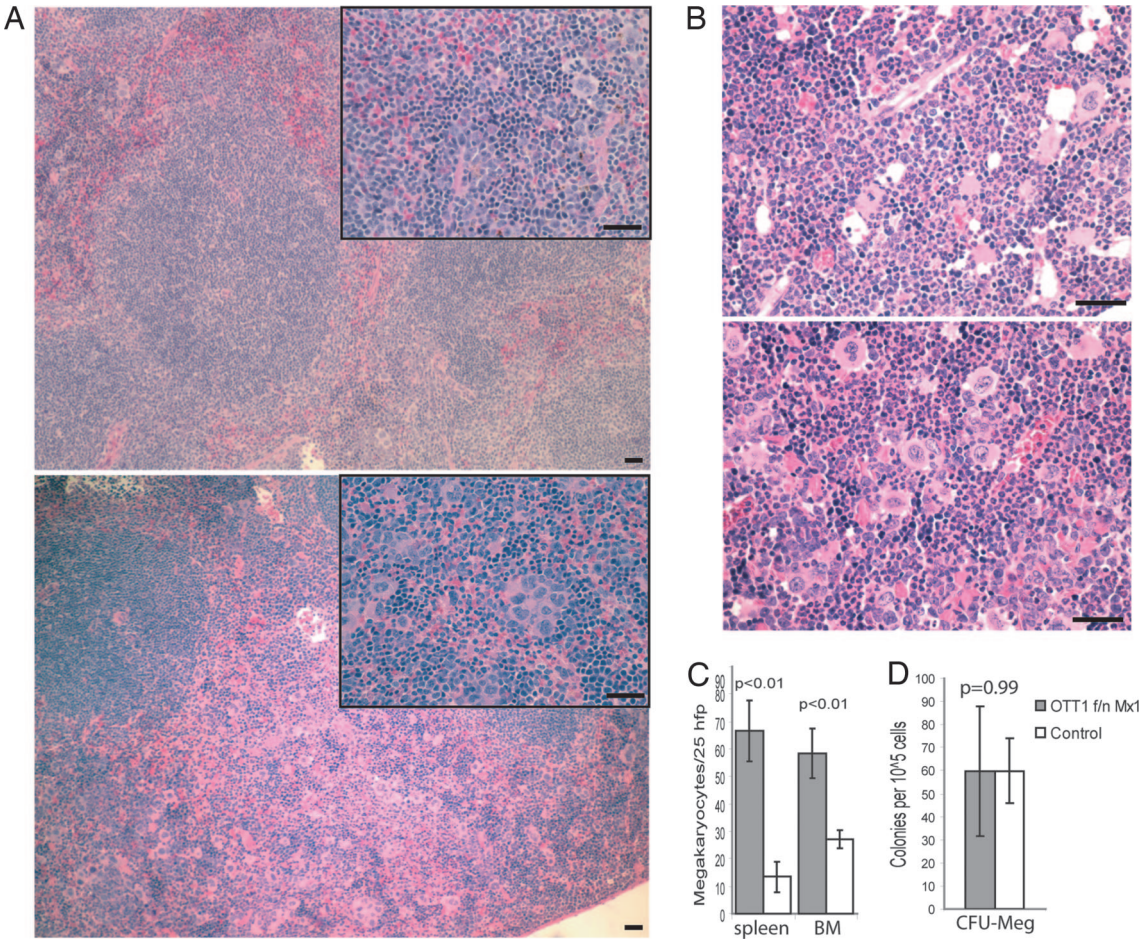

Fig. 3. Megakaryocytic expansion in spleen and bone marrow after *Ott1* deletion. Samples from *Ott1*$^{null/flox}$ *Mx1-Cre* and control littermate mice 6 weeks after pIpC. Shown are photomicrographs of splenic (*A*) and femoral bone marrow (*B*) H&E-stained sections of *Ott1*$^{null/flox}$ *Mx1-Cre* and control littermate mice. (Scale bars: 100 μm.) (*C*) Graph of megakaryocyte density in spleen and bone marrow histology sections. Megakaryocytes were scored by morphology in 25 random hpf ($\times$600) of splenic red pulp (*Ott1*$^{null/flox}$ *Mx1-Cre*, $n = 3$; control, $n = 5$) or bone marrow ($n = 4$ for both). (*D*) Number of CFU-Meg obtained from 1×10^5 bone marrow cells plated in duplicate in MegaCult-C assay (*Ott1*$^{null/flox}$ *Mx1-Cre*, $n = 3$; control, $n = 5$).

We examined the role of *Ott1* in hematopoiesis using a conditional allele to bypass embryonic lethality. *Ott1*-deleted bone marrow has normal numbers of pro-B cells, but the later pre-B population identified by CD19, CD25, and loss of CD43 significantly declines, suggestive of a requirement for survival or proliferation. The defect is cell-autonomous in that *Ott1*-deleted bone marrow cannot form pre-B colonies *in vitro*. Although there was efficient excision in this compartment, rapid reconstitution of B lymphopoiesis with cells that contained an intact floxed allele underscores a marked selective advantage for B cells with intact *Ott1*.

The pro- to pre-B transition is regulated by several processes including cell fate commitment and maintenance using the transcription factors *E2A*, *EBF*, *Pax5*, and *Lef1* (22, 27); *Rag* function and nonhomologous end joining pathways mediating successful V(D)J recombination (28, 29); and proliferation via signaling from the pre-BCR complex (30). Interference with any of the above pathways can result in a block similar to that observed after deletion of *Ott1*.

Ott1 could also be an antagonist of inhibitors of B cell development, such as Id or the Notch pathway (31, 32). Transgenic expression of Id1 and Id2 early lymphoid cells results in an increase of the bone marrow B220$^+$CD43$^+$ population but loss of maturing B220$^+$CD43$^-$ cells similar to loss of *Ott1* (33). Notch1 is less likely as a target of Ott1 because overexpression of Notch1 in the lymphoid compartment results in an abundance of bone marrow T cells and a complete block before an identifiable B cell precursor (34).

In contrast to B cells, myeloid cells had no selection for unexcised cells and instead expanded within the spleen and bone marrow. Furthermore, there was an increase in the number of CFU-G/M/GM, and *in vitro* clonogenicity of *Ott1*-deleted HSCs demonstrated a preference for granulocytic maturation with a reciprocal loss of monocytic differentiation. Negative regulation of *Id* proteins by *Ott1* could explain both the increase in granulocytic cell fate and pre-B cell block upon *Ott1* loss (35). C/EBPα has also been shown to play a critical role in granulocytic vs. monocytic lineage decision (36, 37). In addition, Id1 is up-regulated by C/EBPα, and deletion of C/EBPα causes an increase in LSK/HSC repopulation that could explain *Ott1* effects on pre-B and LSK cells, respectively (38). There was an increase in LSK cells in *Ott1*-deficient animals. Sorted LSK cells were capable of forming megakaryocytic, erythroid, and myeloid colonies in the absence of *Ott1*; therefore, *Ott1* is dispensable for terminal differentiation.

Ott1 loss thus affects the LSK compartment, granulocytic, monocytic, megakaryocytic, and B lymphoid lineages. No known single pathway explains these pleiotropic effects in adult hematopoiesis. The extensive role of *Ott1* in hematopoietic development is unique among *spen* family members, and study of

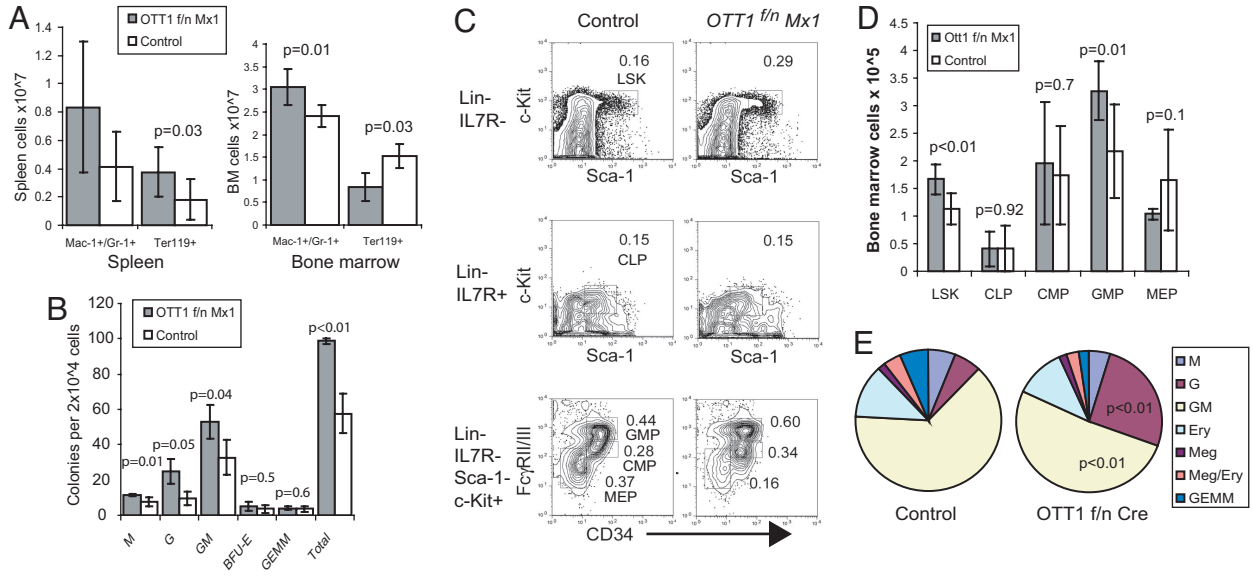

Fig. 4. Myeloid expansion and analysis of progenitors and LSK clonogenicity after *Ott1* deletion. Shown are bone marrow or spleen samples from *Ott1*$^{null/flox}$ *Mx1-Cre* and control littermate mice 6 weeks after pIpC. (*A*) Absolute numbers of Mac-1$^+$/Gr-1$^+$ and Ter119$^+$ cells. Gray bars, *Ott1*$^{null/flox}$ *Mx1-Cre* ($n = 5$); white bars, controls ($n = 7$). (*B*) Myeloid colony formation from duplicate platings of 2×10^4 bone marrow cells in methylcellulose culture. Gray bars, *Ott1*$^{null/flox}$ *Mx1-Cre* ($n = 3$); white bars, control ($n = 7$). (*C*) Representative flow-cytometry plots denoting the LSK, CLP, CMP, GMP, and MEP populations. Numbers are percentages of total bone marrow cells. (*D*) Absolute numbers of bone marrow progenitor cells. Gray bars, *Ott1*$^{null/flox}$ *Mx1-Cre* ($n = 5$); white bars, control ($n = 4$). (*E*) Clonogenicity of sorted LSK cells. One hundred double-sorted LSK cells were plated onto methylcellulose medium plus growth factors in triplicate. Colony type was assessed by morphology after 7–10 days in culture ($n = 3$).

interactions involving the RNA recognition motif and SPOC domains should yield new mechanistic insights.

In regard to the OTT1-MAL fusion in acute megakaryocytic leukemia, deletion of *Ott1* results in an increased number of megakaryocytes in the bone marrow and spleen, suggesting that *Ott1* is a negative regulator of megakaryocyte development. One hypothetical mechanism would be that the OTT1-MAL fusion contributes to the acute megakaryocytic leukemia phenotype by impairing OTT1 function possibly through a dominant-negative effect rather than solely through dysregulation of MAL pathways. The increase in megakaryocyte proliferation was not observed *in vitro*, suggesting a TPO-independent or cell-nonautonomous mechanism.

In summary, *OTT1* plays an important role in hematopoietic development, with pleiotropic effects in the stem cell, lymphoid, and myeloid compartments. Further analysis of the interplay between *Ott1* and other alleles involved in hematopoietic development are needed to improve our understanding of lineage determination and inform our understanding of the pathogenesis of leukemia initiated by the *OTT1-MAL* fusion gene.

Materials and Methods

Generation of Targeted Mice and Cre Deletion. The targeting vector (SI Fig. 5) was electroporated into R-1 ES cells and injected into C57BL/6 blastocysts to produce chimeric animals that were bred for germ-line transmission. The floxed *Ott1* allele was excised in the germ line by breeding with *EIIa-Cre* transgenic mice (The Jackson Laboratory, Bar Harbor, ME). Mice heterozygous for the null allele were bred with *Mx1-Cre* transgenic mice (The Jackson Laboratory) to generate *Ott1*$^{null/wt}$ *Mx1-Cre* animals that were bred with *Ott1*$^{flox/wt}$ or *Ott1*$^{flox/flox}$ mice to produce *Ott1*$^{flox/null}$ *Mx1-Cre* animals. Conditional excision in 4- to 6-week-old mice was achieved by i.p. injections of 250 μg of pIpC (Sigma–Aldrich, St. Louis, MO) every other day for three doses. Animals were maintained in microisolator cages, and experiments were performed with the approval of the Institutional Animal Care and Use Committee.

Southern and PCR Genotyping. Genomic DNA from tail snips or ES cells (Puregene DNA isolation kit; Gentra Systems, Minneapolis, MN) was used to confirm a homologous recombination into the 5′ and 3′ ends of the *Ott1* gene by PCR and Southern hybridization, respectively (SI Fig. 5). DNA from peripheral blood was obtained by using a Blood MiniAmp Kit (Qiagen, Valencia, CA). Single-colony DNA was generated by picking colonies into Eppendorf tubes, washing with PBS, resuspending into 50 μl of dH_2O, boiling 5 min, and then using 5 μl for PCR. Floxed or endogenous alleles were detected by using primers 5′-TGCTGTGACCAAGAGAGTTTGGC-3′ and 5′-CTTCTAAGACTGAGTAGAGAATG-3′, which span the 5′ *loxp* site, giving 250-bp and 216-bp products, respectively. The fully excised null allele was detected with the same forward primer and the reverse primer 5′-TTGTAACAAAGACAAGAGGGGAA-3′ based 3′ to the final *loxp* site and amplifying a 320-bp product.

Flow-Cytometric Analysis. Single-cell suspensions of bone marrow and spleen were prepared as described (39) and frozen in 90% FCS and 10% dimethyl sulfoxide. Thawed cells were washed and blocked with 2.4G2 hybridoma supernatant (American Type Culture Collection, Manassas, VA). Flow reagents with the exception of allophycocyanin-CD4 (Caltag, Burlingame, CA) and phycoerythrin-streptavidin (Immunotech, Marseille, France) were purchased from BD Pharmingen (San Diego, CA). Analysis was performed on a four-color FACSCalibur cytometer (Becton Dickinson, Mountain View, CA) with CELLQUEST software, and a minimum of 10,000 events per experiment were acquired.

Blood and Tissue Analysis. Blood was obtained through nonlethal eyebleeds under anesthesia with isoflurane per institutional guidelines. Complete blood counts were obtained with a Hemavet cell counter (Drew Scientific, Oxford, CT). Bone marrow was obtained from both femurs and tibias of each mouse. Organs from killed

animals were preserved in PBS with 10% neutral, buffered formalin and then processed to H&E slides.

***In Vitro* CFU Assays.** Megakaryocyte CFUs were assayed by using MegaCult-C (StemCell Technologies, Vancouver, BC, Canada). Whole bone marrow was plated according to the manufacturer's instructions with 10 ng/ml rmIL-3, 20 ng/ml rmIL-6, 50 ng/ml rhIL-11, and 50 ng/ml rmTPO (StemCell Technologies). After 6 days, slides were dehydrated, fixed, and stained with acetylthiocholiniodide (Sigma–Aldrich).

Myeloid CFUs were quantitated by using Methocult M3434 (StemCell Technologies) methylcellulose medium containing 3 units per ml Epo, 10 ng/ml rmIL-3, 10 ng/ml rmIL-6, and 50 ng/ml rmSCF. Duplicate plates containing 2×10^4 bone marrow cells were incubated at 37°C with 5% CO_2 for 10 days. Colonies were scored by morphology and verified by Wright–Giemsa stains of single-colony cytospins. Pre-B colony assays were performed by plating bone marrow cells in Methocult M3630 (StemCell Technologies) that contains IL-7 and scoring after 6 days of incubation.

Progenitor Analysis. Bone marrow cells were lineage-depleted and stained for progenitor analysis as described (25, 40) and then analyzed on a modified double-laser FACS (Moflo-MLS; Cytomation, Fort Collins, CO). Cell populations were identified as follows: CMP, $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+Fc\gamma RII/III^{lo}$; GMP, $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+Fc\gamma RII/III^{hi}$; MEP, $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^-Fc\gamma RII/III^{lo}$; HSC, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$; and CLP, $Lin^-Sca\text{-}1^{lo}c\text{-}Kit^{lo}IL\text{-}7R^+$.

LSK clonogenicity was determined by double-sorting 100 LSK directly onto plates containing Methocult M3234 (StemCell Technologies) supplemented with 3 units per ml Epo, 10 ng/ml rmIL-3, 10 ng/ml rmIL-6, 50 ng/ml rmSCF, 10 ng/ml rhIL-11, 10 ng/ml rmGM-CSF, and 10 ng/ml rmTPO (StemCell Technologies). Triplicate plates were incubated as above for 7–10 days and scored by morphology.

Statistics. *P* values were calculated by using a two-tailed Student's *t* test, and error bars represent one SD.

We thank John Manis for critical review of the manuscript and Liz McDowell and Sandy Moore for technical assistance. This work was supported in part by National Institutes of Health Grants K08 CA111399 (to G.D.R.) and R01 CA66996 (to D.G.G.) and the Leukemia and Lymphoma Society (D.G.G.). T.M. is the recipient of a Special Fellow Award from the Leukemia and Lymphoma Society. O.A.B. is supported by Institut National de la Santé et de la Recherche Médicale and Ligue Nationale Contre le Cancer–Comité de Paris. D.G.G. is an Investigator for the Howard Hughes Medical Institute.

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