GATA-3 is required for early T lineage progenitor development

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Most T lymphocytes appear to arise from very rare early T lineage progenitors (ETPs) in the thymus, but the transcriptional programs that specify ETP generation are not completely known. The transcription factor GATA-3 is required for the development of T lymphocytes at multiple late differentiation steps as well as for the development of thymic natural killer cells. However, a role for GATA-3 before the double-negative (DN) 3 stage of T cell development has to date been obscured both by the developmental heterogeneity of DN1 thymocytes and the paucity of ETPs. We provide multiple lines of in vivo evidence through the analysis of T cell development in Gata3 hypomorphic mutant embryos, in irradiated mice reconstituted with Gata3 mutant hematopoietic cells, and in mice conditionally ablated for the Gata3 gene to show that GATA-3 is required for ETP generation. We further show that Gata3 loss does not affect hematopoietic stem cells or multipotent hematopoietic progenitors. Finally, we demonstrate that Gata3 mutant lymphoid progenitors exhibit neither increased apoptosis nor diminished cell-cycle progression. Thus, GATA-3 is required for the cell-autonomous development of the earliest characterized thymic T cell progenitors.

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Abbreviations used: CLP, common lymphoid progenitor; DN, double negative; DP, double positive; ES, embryonic stem; ETP, early T lineage progenitor; Fr.A, Hardy fraction A; HSC, hematopoietic stem cell; LMP, lymphoid-restricted MPP; LSK, Lin+ Sca1+c-Kithi; MPP, multipotential hematopoietic progenitor; PI, propidium iodide; PIR, paired immunoglobulin receptor; SP, single positive; TSP, thymus-seeding globulin receptor.

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GATA-3 is a zinc finger transcription factor that was first recognized as a possible key determinant of T cell development when it was originally cloned (Yamamoto et al., 1990; Ko et al., 1991). Gata3 null mutant mice fail to survive beyond embryonic day 11 (e11) because of cardiac dysfunction that develops as a secondary consequence of noradrenaline deficiency (Pandolfi et al., 1995; Lim et al., 2000; Moriguchi et al., 2006). GATA-3 has also been shown to control, either directly or indirectly, the development of T cells (Ting et al., 1996), thymic NK cells (Vosshenrich et al., 2006), the Wolffian duct, and kidney epithelium (Lim et al., 2000; Hasegawa et al., 2007), as well as the luminal epithelium of the mammary gland (Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007). GATA-3 is indispensable for T cell development during β selection at the CD44+CD25+ DN3 stage and for the generation of CD4 SP thymocytes (Pai et al., 2003). In addition, GATA-3 has been termed a master regulator of differentiated Th2 CD4+ T cell function (Zheng and Flavell, 1997; Zhu et al., 2004).

Several studies have shown that GATA-3 is critical for early T lymphopoiesis, but the exact stage at which GATA-3 activity is required during T cell specification and commitment has not been determined. GATA-3 has been detected at low levels in prethymic Lin−Sca1+c-Kithi (LSK) bone marrow cells, in thymic ETPs, and in DN2 cells (Akashi et al., 2000; Sambandam et al., 2005; Lai and Kondo, 2007). Furthermore, suppression of GATA-3 activity in fetal liver progenitors resulted in a significant loss of Thy-1+ T cells after expansion in fetal thymus organ culture (Hattori et al., 1996; Hozumi et al., 2008). Conversely, when GATA-3 was retrovirally transduced into fetal liver progenitors or immature thymocytes, it arrested cell expansion and Thy-1+ cell development (Chen and Zhang, 2001; Taghon et al., 2001; Anderson et al., 2002; Taghon et al., 2007). Thus, although these studies underscored the general impression that GATA-3 function is important during early stages of fetal T lymphopoiesis, they do not provide insight into the stages or processes that are affected.

In chimeric mice generated using Gata3 mutant embryonic stem (ES) cells, lacZ-expressing heterozygous germ-line mutant (Gata3+) cells were able to contribute to the CD4−CD8−CD44+CD25− (DN1) population (~2% of total DN1 cells were β-galactosidase−), whereas the contribution from lacZ-expressing (Gata3−) null mutant ES cells was indistinguishable from background (<0.5% of DN1; Hendriks et al., 1999). However, more recent studies have revealed that DN1 cells (as defined by the same surface antigens) are quite heterogeneous and contain a mixture of mature and immature NK, dendritic, B, and myeloid cells as well as T lineage progenitors (Allman et al., 2003; Porritt et al., 2004; Balcurnaite et al., 2005; Anderson, 2006). Another recent study also revealed that GATA-3 is expressed in and is required for the generation of thymic NK cells (Vosshenrich et al., 2006). Thus, the paucity of ETPs and DN2-stage cells (comprising ~0.01 and 0.02% of total thymocytes in the adult mouse, respectively), as well as the cellular heterogeneity of the DN1 compartment (comprising ~0.5% of total thymocytes in the adult mouse) and the contribution of genuine T progenitors to that compartment have all conspired to prevent a clear assessment of the roles that GATA-3 might play in the earliest phases of T cell development.

We reported previously that the early embryonic lethal phenotype conferred by Gata3 germline null mutation could be complemented to well past e11, their usual time of embryonic demise, by feeding pregnant heterozygous intercrossed dams with catecholamine intermediates (Lim et al., 2000) or by specifically restoring GATA-3 expression in sympathoadrenal lineage cells using the human dopamine β-hydroxylase gene promoter to direct GATA-3 transgene expression (Moriguchi et al., 2006). However, the fact that these rescued Gata3 null mutant embryos are athymic precluded the possibility of determining the contribution, if any, of GATA-3 to early T lymphopoiesis in vivo, because the thymic epithelium and mesenchyme provide the microenvironment that is absolutely required for T cell differentiation.

To examine GATA-3 contributions to various aspects of organogenesis in adult tissues, we recently generated a new conditional Gata3 allele (Gata3fl) in which a chimeric mouse GATA-3.eGFP fusion cDNA that was flanked by loxp sites was inserted into exon 2 of the Gata3 locus (unpublished data). Unexpectedly, this germline Gata3fl allele (before excision) resulted in hypomorphic activity as a consequence of lower accumulated abundance of the GATA-3.eGFP fusion protein; homozygous mutants bearing this allele (Gata3flfl) exhibited perinatal lethality as a probable consequence of craniofacial anomalies that led to severely deficient respiration (unpublished data). Interestingly, even the reduced expression of the GATA-3 fusion protein in the pharyngeal arches (from which the thymus originates) was sufficient to generate a diminutive embryonic thymus, thus allowing us to examine the contribution of GATA-3 to T cell development while avoiding additional complications that might be attributed to the absence of a thymus (as in the Gata3 null mutants; unpublished data; Lim et al., 2000; Moriguchi et al., 2006).

Our preliminary analysis of GFP fluorescence detected in the Gata3fl/fl adult mice indicated that the vast majority of GATA-3–expressing cells in the DN1 fraction of the thymus express NK1.1, a lineage marker for NK cells that is not expressed on the earliest T cell progenitors but is abundantly expressed on c-Kit− cells of the CD4−CD8−CD44+ (DN1 plus DN2) population (Laurent et al., 2004; Balcurnaite et al., 2005). Although the most immature multipotential cells in the thymus have been characterized as c-Kit−NK1.1−, the c-Kit+ NK1.1+ cell population has both NK and T lineage potential at the fetal stage (Carlyle et al., 1997; Michie et al., 2000). On the other hand, almost none of the c-Kithi DN thymocytes express NK1.1 at the adult stage (Balcurnaite et al., 2005). This observation prompted us to reexamine the contribution of GATA-3 to very early stages of fetal and adult T cell development both in vivo and in vitro. In this...
paper, we report that the absolute number of ETPs and DN2 cells was reduced by 80–92% in the Gata3 hypomorphic mutant embryos, was reduced by ~90% in recipient mice reconstituted with Gata3 null mutant hematopoietic progenitors, and was reduced by ~97% in mice in which the Gata3 gene was conditionally ablated at the adult stage, whereas the development of Gata3 mutant hematopoietic cells into prethymic progenitors was unaffected. Furthermore, neither increased apoptosis nor impaired cell-cycle progression was observed in Gata3 null Lin−CD27+ c-Ki67−CD25− lymphoid progenitors that had been induced to differentiate into T cells in vitro for 4 d in OP9-DL1 co-cultures. We conclude that GATA-3 is required for the generation of very early ETPs in the thymus via differentiation pathways that are independent of apoptosis or cell-cycle alteration.

RESULTS
GATA-3 is required for fetal T cell development at or before the DN2 stage
Although diminished GATA-3 activity in fetal liver cells was reported to result in a loss of Thy-1+ T cells after expansion in fetal thymus organ culture (Hattori et al., 1996; Hozumi et al., 2008), the specific affected stages remained unclear. To examine the developmental potential of Gata3 null mutant (lacZ knockin, Gata3z/z; van Doorninck et al., 1999) T cell progenitors in vitro, we isolated and co-cultured fetal liver cells from e12.5/e13.5 catecholamine-rescued Gata3z/z embryos on OP9-DL1 cells (Schmitt and Zúñiga-Pflücker, 2002) to promote T cell development. This process was monitored by flow cytometric examination of the cell-surface expression profiles of CD4, CD8, CD44, and CD25. As shown in Fig. 1 and Table I, Gata3z/z fetal liver cells generated significantly fewer DN2, DN3, and DP cells (0.4, 0.7, and <0.1%, respectively, of wild-type control cells). These results demonstrate that GATA-3 is critical for in vitro T cell development at or before the DN2 stage.

Development of T lymphoid cells in Gata3 hypomorphic mutant fetuses
Gata3 null mutant mice fail to generate even a rudimentary thymus (Lim et al., 2000; Moriguchi et al., 2006), whereas Gata3 conditionally mutant mice that were crossed to mice bearing an lck-directed Cre transgene, and therefore lacked Gata3 at the DN3 stage and thereafter, were reported to have normal numbers of DN-stage cells per thymus (DN1 through DN4 total) but reduced numbers of DP, CD4 SP, and CD8 SP cells (Pai et al., 2003). We recently generated a Gata3 hypomorphic mutant (Gata3g/g) mouse that develops a diminutive thymus (unpublished data). The abundance of the GATA-3-eGFP fusion protein generated from the Gata3g allele was ~8% of the wild-type protein in thymocytes (Fig. S1), whereas the two proteins displayed similar transcriptional activation potential in transient cotransfection reporter gene assays (not depicted). To gain further insight into the contribution of GATA-3 to fetal T cell development, we analyzed the total thymocyte count in e15.5 and e18.5 Gata3g/g embryos. We found that they were reduced to approximately one third of that observed in wild-type embryos (Fig. 2A). We next...
characterized these thymocytes using anti-CD4 and anti-CD8 antibodies, and found that although the mutants displayed a slight temporal delay in DP cell generation, Gata3<sup>+/+</sup> mice were able to develop to the DP stage. However, the absolute numbers of thymocytes at all stages of T cell development were reduced in comparison to e18.5 wild-type or heterozygous mutant embryos (Fig. 2, B–D). Additionally, the absolute number of γδ TCR<sup>+</sup> cells was reduced in Gata3<sup>+/+</sup> embryos (Fig. 2 C). We further examined the distribution of cells during the DN stages of lymphoid development using antibodies directed against c-Kit and CD25 in addition to various lineage surface antigens. As shown in Fig. 2 (E and F) and Table I, Gata3<sup>+/+</sup> mice generated lower number of ETPs and DN2 cells at e18.5 (12.5 and 8.6%, respectively) and at e15.5 (15.7 and 19.6%, respectively) compared with wild-type embryos. The GATA-3-eGFP fusion protein was expressed in fetal ETPs and DN2 cells (Fig. S2 A). Forced expression of GATA-3 was previously shown to induce c-Kit expression (Anderson et al., 2002; Taghon et al., 2007). To ask whether the observed reduction in the number of ETPs and DN2 cells might be caused by reduced c-Kit expression, we analyzed CD44 and CD25 within the Lin<sup>lo</sup> population in Gata3<sup>+/+</sup> mice, because gating on Lin<sup>lo</sup> thymocytes had been shown to enrich for bona fide ETPs that are found in the complex hematopoietic compartment described by the CD44<sup>+</sup>CD25<sup>−</sup>DN1 population (Sambandam et al., 2005). As shown in Fig. S3 (A–C), the absolute number of Lin<sup>lo</sup>CD44<sup>+</sup>CD25<sup>−</sup> cells per thymus was also reduced (to 21.9% [e15.5] or 14% [e18.5] of wild-type controls). This result reinforced the conclusion that GATA-3 is critical for fetal ETP generation. The lack of c-Kit<sup>hi</sup>/IL-7R<sup>+</sup> cell accumulation in this fraction demonstrates that the reduction in the number of ETPs was not a consequence of reduced cell-surface expression of c-Kit. The reduction in the number of DN3 and DN4 cells per thymus was far less pronounced in e15.5 and e18.5 embryos (Fig. 2 F). The ratios of CD4 SP, CD8 SP, DP, and DN thymocytes beyond the DN3 stage are almost normal, implying that the effect of reduction in GATA-3 protein abundance is confined to the early T cell stages (Fig. 2, B and E).

Because B lymphoid cells develop normally in OP9 co-culture of Gata3<sup>−/−</sup> null fetal liver cells (Hozumi et al., 2008), it was anticipated that other hematopoietic lineages would develop normally in Gata3<sup>+/−</sup> embryos. As expected, erythroid (TER119<sup>+</sup>), myeloid (Gr1<sup>+</sup>Mac1<sup>+</sup>), and B lineage (B220<sup>+</sup>CD19<sup>+</sup>) cells were normal in ratio in e18.5 Gata3<sup>+/−</sup> livers and spleens (Fig. S4).

These observations show that either GATA-3 is not required for the development of progenitors in non–T cell lineages or that the reduced transcriptional activity conferred by the Gata3 hypomorphic allele is sufficient to promote normal development of hematopoietic stem cells (HSCs) and non–T cell lineages during fetal hematopoiesis. Furthermore, the remaining GATA-3 functional activity borne in the GATA-3-eGFP fusion protein is insufficient for ETP and DN2 T lineage development, whereas later stages of T cell development are less affected by the hypomorphic GATA-3 activity. Importantly, these findings reveal a pivotal developmental role in vivo for GATA-3 at a stage of T cell development earlier than DN3, which could not be addressed using the strategy pursued by Pai et al. (2003).

### Table I. Summary: mutant cell number in comparison to controls

<table>
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<tr>
<th>Gata3 genotype</th>
<th>CD27&lt;sup&gt;c-Kit&lt;sup&gt;hi&lt;/sup&gt;/CD25&lt;sup&gt;−&lt;/sup&gt;</th>
<th>ETP</th>
<th>DN2</th>
<th>DN3</th>
<th>DN4</th>
<th>DP</th>
<th>CD4 SP</th>
<th>CD8 SP</th>
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<td>2.6</td>
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<td>OP9-DL1 co-culture, day4</td>
<td>g/g</td>
<td>12.5</td>
<td>8.6</td>
<td>23</td>
<td>18.7</td>
<td>34.8</td>
<td>26.2</td>
<td>43.9</td>
<td>Fig. 2</td>
</tr>
<tr>
<td>Fetal thymus, e15.5</td>
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<td>19.6</td>
<td>53.9</td>
<td>42.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fig. 2</td>
</tr>
<tr>
<td>Fetal thymus, e18.5</td>
<td>g/g</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>18.7</td>
<td>34.8</td>
<td>26.2</td>
<td>43.9</td>
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<tr>
<td>Conditional KO</td>
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<td>-</td>
<td>2.6</td>
<td>&lt;0.1</td>
<td>21.7</td>
<td>40.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adoptive transfer</td>
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<td>0.4</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adoptive transfer</td>
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<td>38</td>
<td>338.6</td>
<td>200.7</td>
<td>27.9</td>
<td>29.1</td>
<td>20</td>
<td>34</td>
</tr>
</tbody>
</table>

Controls were compared with wild type except in the case of the conditional KO (the control for which was Gata3<sup>flox−/−; Mx1Cre</sup>−/−), not applicable or not determined.

### Summary: mutant cell number in comparison to controls

The first wave of T progenitor cell migration out of the embryonic fetal liver into the thymic primordium is observed at around e12 to e13 in mice (Owen and Ritter, 1969; Jotereau et al., 1987). Although MPPs have been characterized as the most efficient T cell progenitors at the adult stage (Schwarz and Bhandoola, 2004; Schwarz et al., 2007), the precise characteristics and phenotype of the most efficient prethymic T progenitor cells during fetal development are unknown. T lineage–restricted progenitors in the fetal liver have been reported (Rodewald et al., 1994; Kawamoto et al., 1997) and have been described within the Lin<sup>−/−</sup>c-Kit<sup>hi</sup>IL-7Rα<sup>+</sup>pared immunoglobulin receptor (PIR)<sup>+</sup> fraction (Masuda et al., 2005). Of particular note, the developmental relationship between fetal prethymic T-restricted progenitors and fetal thymic ETPs is presently obscure.

To analyze the status of this prethymic T-restricted progenitor population, we isolated fetal liver cells from e12.5 Gata3<sup>+/−</sup> embryos and analyzed the number of Lin<sup>−/−</sup>c-Kit<sup>hi</sup>IL-7Rα<sup>+</sup>PIR<sup>+</sup> progenitor cells. The total number of e12.5 fetal
Figure 2. Fewer immature T cells in Gata3 hypomorphic mutant embryos. (A) The absolute numbers of total thymocytes in Gata3+/+, Gata3+/−, and Gata3−/− fetal thymi (7–20 mice of each genotype) were quantified. Data represent the summary of at least four independent experiments for each stage (means ± SEM). *, P < 0.01. (B) Thymocytes from e18.5 Gata3+/+ and Gata3−/− embryos were analyzed for CD4 and CD8 surface expression. Data are representative of six to eight embryos of each genotype from five independent experiments. Numbers in boxed areas indicate the mean percentage of cells in those quadrants. (C) The absolute numbers of DN, DP, CD4 SP, CD8 SP, and γδTCR+ cells per thymus in e18.5 Gata3+/+, Gata3+/−, and Gata3−/− embryos are shown. Data represent the summary of five independent experiments, and the mean of six to eight embryo in each genotype with SEM. *, P < 0.05. (D) The absolute number of DP cells per thymus in Gata3+/+ embryos compared with controls (both Gata3+/− and Gata3−/−) embryos. An average of two to six mice of each genotype were shown. (E) Surface expression of c-Kit and CD25 on Lin− thymocytes isolated from e15.5 and e18.5 Gata3+/+ and Gata3−/− embryos. Data are representative of four to eight embryos of each genotype from at least two independent experiments for each stage. Numbers in the boxed areas indicate the mean percentage of cells in those sectors. (F) The absolute numbers of ETPs, DN2, DN3, and DN4 cells per thymus in e15.5 and e18.5 Gata3+/+, Gata3+/−, and Gata3−/− embryos. Data represent the summary of at least two independent experiments for each stage and an average of four to eight embryos in each genotype with SEM. *, P < 0.05.
liver cells as well as the absolute number of Lin<sup>−</sup>c-Kit<sup>+</sup>IL-7Rα<sup>+</sup>PIR<sup>+</sup> cells per liver was virtually identical in the Gata<sup>3<z>⁄</z></sup> and wild-type embryos (Fig. 3). These data indicate that either GATA-3 is not required for the generation of the fetal liver Lin<sup>−</sup>c-Kit<sup>+</sup>IL-7Rα<sup>+</sup>PIR<sup>+</sup> cells, or the remaining GATA-3 fusion protein activity in the Gata<sup>3<z>⁄</z></sup> hematopoietic progenitor cells is adequate to generate this pool but is insufficient to generate a wild-type cohort of ETPs (Fig. 2 F). This result reinforces the contention that the development of multipotent HSCs and progenitor cells in Gata<sup>3<z>⁄</z></sup> embryos is normal.

**A hematopoietic cell–autonomous role of GATA-3 for fetal ETP development**

To confirm that the reduction of ETPs and their progeny in the Gata<sup>3<z>⁄</z></sup> mice was attributable predominantly to a cell-autonomous deficiency in hematopoietic cells, the developmental potential of Gata<sup>3<z>⁄</z></sup> prethymic progenitors was examined in OP9-DL1 co-culture. As shown in Fig. 4 and Table I, progenitor cells derived from e12.5 Gata<sup>3<z>⁄</z></sup> fetal livers developed a reduced number of DN2 and DN3 cells (4.4 and 16.7% of wild-type control), in agreement with the in vivo observations (Fig. 2 F). Because complete developmental arrest of DN2 cells from ETPs was not observed in vivo (Fig. 2 F), this result suggested that GATA-3 fulfills a cell-autonomous role in the development of fetal ETPs as well as in more mature DN T cells.

**Expression of GATA-3 in DN1 fractions**

Although it is known that the adult and fetal stages of T cell development share similarities, they nonetheless differ in numerous and well-defined features (for reviews see Kincade et al., 2002; David-Fung et al., 2006). We previously used a Gata<sub>3</sub> lacZ knockin allele that has proven to be an extremely useful tool to monitor cells that express GATA-3 (van Doorninck et al., 1999; Lim et al., 2000). Hendriks et al. (1999) found that fewer cells express β-galactosidase in the classical DN1 (CD<sub>4<sup>+</sup></sub>CD<sub>8<sup>−</sup></sub>CD<sub>44<sup>−</sup></sub>CD<sub>25<sup>−</sup></sub>) compartment of Gata<sup>3<z>⁄</z></sup> (null) ES chimeras in comparison to heterozygous Gata<sup>3<z>⁄</z></sup> ES chimeric mice, which originally suggested an early deficiency in adult T cell development in the absence of GATA-3. However, more recent studies have revealed that DN1 cells are actually quite heterogeneous in nature, and contain a mixture of mature and immature NK, DC, B, and myeloid lineage cells, as well as rare T cell progenitors (Allman et al., 2003; Porritt et al., 2004; Balciunaite et al., 2005; Anderson, 2006). A recent study also revealed that GATA-3 is expressed in and is required for the generation of thymic NK cells (Vosshenrich et al., 2006).

To examine the detailed expression of GATA-3 in DN1 thymocytes, we took advantage of the fusion protein fluorescence generated by the Gata<sup>3<z>⁄</z></sup> allele. As shown in Fig. 5 A, GFP fluorescence in the DN, DP, CD4 SP, and CD8 SP thymocytes isolated from 6-wk-old Gata<sup>3<z>⁄</z></sup> mice was generally reflective of the reported profile for wild-type GATA-3 expression (Hendriks et al., 1999). As anticipated, a majority of the NK1.1<sup>+</sup> and γδTCR<sup>+</sup> cells expressed abundant GATA-3.eGFP fusion protein. Detailed analysis of the complex DN1

![Figure 3. Development of T-restricted progenitors in the e12.5 fetal liver.](image)

![Figure 4. A hematopoietic cell–autonomous role for GATA-3 in the production of fetal DN thymocytes.](image)
population revealed that (on average) 83.1 ± 2.9% of the eGFP+ cells were also NK1.1+, thus identifying those cells as mature or immature natural killer cells, NKT cells, or T/NK progenitors (Fig. 5 B). Furthermore, ~50% of DN1 cells express NK1.1 (Fig. 5 B). Almost all c-Ki67+ DN thymocytes, including the earliest T progenitors, do not express NK1.1 at the adult stage (Balciunaite et al., 2005). These results collectively indicated that the contribution of GATA-3 to early T cell progenitor stages would be impossible to assess in the classically defined DN1 (CD4−CD8−CD44−CD25−) cell population, and therefore, we sought to identify more finely delineated populations of T cell progenitors to precisely address questions regarding the unique contributions of GATA-3 to early thymopoiesis.

**GATA-3 plays an essential role in ETP development in the adult thymus**

Because GATA-3 is expressed in adult ETPs (Fig. S2 B; Sambandam et al., 2005; Lai and Kondo, 2007) and loss of GATA-3 activity affected the development of ETPs during fetal thymopoiesis (Fig. 2 F), we next asked whether GATA-3 was involved in ETP development during adulthood. To this end, we performed adoptive transfer experiments using hematopoietic cells reconstituted from Gata3z/z or wild-type donors (Fig. 6 A). Because GATA-3 null ES cells can contribute to myeloid and B lymphoid lineage cells in blastocyst chimeras (Ting et al., 1996), reconstitution efficiency was periodically monitored in peripheral blood myeloid (Gr1+Mac1+) and B lymphoid (B220+) cells, and was found to be the same for fetal liver donors of both genotypes (Fig. 6 B).

Thymocytes and bone marrow cells were isolated from recipient mice over the course of 10 wk after transplantation and analyzed by flow cytometry. As shown in Fig. 6 (C and D) and Table I, the contributions of Gata3z/z cells to ETPs and later stages was lower than that from Gata3+/+ cells. Similarly, the contribution of Gata3g/g cells to ETPs was lower than population revealed that (on average) 83.1 ± 2.9% of the eGFP+ cells were also NK1.1+, thus identifying those cells as mature or immature natural killer cells, NKT cells, or T/NK progenitors (Fig. 5 B). Furthermore, ~50% of DN1 cells express NK1.1 (Fig. 5 B). Almost all c-Ki67+ DN thymocytes, including the earliest T progenitors, do not express NK1.1 at the adult stage (Balciunaite et al., 2005). These results collectively indicated that the contribution of GATA-3 to early T cell progenitor stages would be impossible to assess in the classically defined DN1 (CD4−CD8−CD44−CD25−) cell population, and therefore, we sought to identify more finely delineated populations of T cell progenitors to precisely address questions regarding the unique contributions of GATA-3 to early thymopoiesis.

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![Figure 5. Gata3 expression in adult thymocytes.](https://journals.ashp.org/publications/jem/delivering/2009/v206n12/a2993/image.html)
from Gata3+/+ cells (Fig. S5 and Table I). Additionally, the contribution of Gata3+/+ cells to γδTCR+ cells was significantly lower than from Gata3+/+ cells (Fig. 6 E).

Lymphoid-primed MPPs (LMPPs; defined as Flt3hiLSK cells) in the bone marrow are likely to contain some of the cells that acquire a migratory signal that allows thymic homing.
Two early B cell progenitor populations, the common lymphoid progenitor (CLP) and subsequent Hardy fraction A (Fr.A), retain residual potential for the generation of T cells (for review see Hardy et al., 2007). Because GATA-3 is expressed in these fractions (Akashi et al., 2000; Rumfelt et al., 2006), the developmental potential of Gata3+/- cells in these fractions was examined in adoptive transfer experiments. As shown in Fig. 6 F, the contributions of Gata3+/- cells to CLP and Fr.A were not statistically different from those of Gata3++ cells in the adoptive transfer recipients.

To independently confirm the requirement for GATA-3 in the development of adult ETPs, we intercrossed Gata3 conditional knockout (Gata3^floxed) mice (Kurek et al., 2007) with mice bearing the Mx1^Cre transgene (Kühn et al., 1995), followed by induction of Cre recombinase by pl-pC. Thymocytes from compound mutant Gata3^floxed;Tg^Mx1Cre (f/f) experimental and Gata3^floxed;Tg^Mx1Cre (f/+ ) control mice were isolated 3 wk after Cre induction and analyzed by flow cytometry. As shown in Fig. 7 and Table I, the number of ETPs and DN2 cells per thymus in Cre-treated f/+ mice was reduced to 2.6 and 0.99% of control (f/+ ) thymocytes, respectively. The absolute number of Lin^-CD44+CD25- cells (enriched for ETPs) per thymus was also reduced to 12.3% of f/+ controls (Fig. S3 D). These results unequivocally demonstrate that GATA-3 is required for the development of adult ETPs.

A function for GATA-3 in ETP development

To address the possible function of GATA-3 and its requirement for proper ETP development, we used an OP9-DL1 co-culture system. These cultures have advantages for a cell-autonomous analysis of ETP development rather than cell survival or proliferation pathways during these earliest stages of T cell development. First, fewer cells expressed lacZ in the CD4+CD8^-CD44+CD25^-DN1 compartment of Gata3^-/- (null mutant) ES chimeras than in Gata3^+/+ (heterozygous mutant) knockin mice (Hendriks et al., 1999). However, because the DN1 population is heterogeneous and only a very small fraction therein has T lineage potential, in retrospect this experiment was interesting although the final analysis was inconclusive. Second,
This observation suggested that a detailed reevaluation of the contributions of GATA-3 to early T cell development was warranted.

The present data show that GATA-3 activity is required for the development of embryonic and adult thymic ETPs. This conclusion was drawn after the execution of four independent genetic assays: analysis of Gata3 hypomorphic mutant embryos, analysis of hematopoietic reconstitution assays using two types of Gata3 mutant donor cells, and analysis of mice in which Gata3 was conditionally ablated at the adult stage. Both adult and fetal ETPs express GATA-3. Mice in which Gata3 was conditionally deleted during the adult stage generated <3% of ETPs and DN2 cells compared with control.

**Figure 8. A function for GATA-3 in ETP generation.**
(A) Lineage-depleted fetal liver cells isolated from e14.5 Gata3<sup>z/z</sup> and control Gata3<sup>z/+</sup> embryos were induced to undergo T cell differentiation in OP9-DL1 co-culture for 4 d, and then analyzed for surface expression of c-Kit and CD25 in the Lin<sup>−</sup>CD27<sup>+</sup> population. Data are representative of four embryos of each genotype from one independent experiment. Numbers in each quadrant indicate the mean percentage of cells in those sectors. (B) The absolute number of Lin<sup>−</sup>CD27<sup>+</sup>, Lin<sup>−</sup>CD27<sup>+cKit<sup>+</sup>CD25<sup>+</sup>}, and Lin<sup>−</sup>CD27<sup>+cKit<sup>−</sup>CD25<sup>−</sup> cells 4 d after OP9-DL1 co-culture from Gata3<sup>z/z</sup> or Gata3<sup>z/+</sup> e14.5 Lin<sup>−</sup>fetal liver cells. Data represent one independent experiment and an average of four embryos of each genotype with SEM. A similar reduction of Gata3<sup>z/z</sup> Lin<sup>−</sup>CD27<sup>+c-Kit<sup>−</sup>CD25<sup>−</sup> cell numbers was observed when e12.5 Lin<sup>−</sup>fetal liver cells were cultured on OP9-DL1 cells for 4 d under the same culture conditions. *, P < 0.005. (C) Profile of annexin V and PI staining in total cells and Lin<sup>−</sup>CD27<sup>+c-Kit<sup>−</sup>CD25<sup>−</sup> cells 4 d after OP9-DL1 co-culture beginning with e14.5 Lin<sup>−</sup> fetal liver cells. Data are representative of four embryos of each genotype. Numbers in the boxed areas indicate the mean percentage of cells in those sectors. (D) Ratio of annexin V<sup>+</sup>PI<sup>−</sup> and annexin V<sup>−</sup>PI<sup>−</sup> cells in Lin<sup>−</sup>CD27<sup>+c-Kit<sup>−</sup>CD25<sup>−</sup> cells 4 d after OP9-DL1 co-culture in Gata3<sup>z/z</sup> or Gata3<sup>z/+</sup> e14.5 Lin<sup>−</sup> fetal liver cells. Data are representative of four embryos of each genotype. Numbers in the boxed areas indicate the mean percentage of cells in those sectors. (E) Cell-cycle analysis in Lin<sup>−</sup>CD27<sup>−c-Kit<sup>−</sup>CD25<sup>−</sup> cells 4 d after OP9-DL1 co-culture from e12.5 Lin<sup>−</sup> fetal liver cells. Data are representative of three to six embryos of each genotype from two independent experiments. Numbers in the boxed areas indicate the mean percentage of cells in those sectors. (F) Ratio of cells in the G0/G1, S, and G2+M phases in the Lin<sup>−</sup>CD27<sup>−c-Kit<sup>−</sup>CD25<sup>−</sup> population 4 d after OP9-DL1 co-culture from Gata3<sup>z/z</sup> [gray bars] or Gata3<sup>z/+</sup> [black bars] e12.5 Lin<sup>−</sup> fetal liver cells. Data represent the summary of two independent experiments and an average of three to six embryos of each genotype with SEM. *, P < 0.006.
mice. The number of ETPs and DN2 cells generated in Gata3 hypomorphic mutant embryos was <20% of their wild-type counterpart in absolute number per thymus. Hematopoietic reconstitution transplant studies demonstrated the prominent cell-autonomous contribution of GATA-3 to ETP generation. Hematopoietic cells from Gata3 null mutants contributed less efficiently to ETPs than did wild-type cells, whereas the contributions were directly comparable within the bone marrow HSC, MPP, and LMPP populations. Adoptive transfer experiments conducted with Gata3+/+ hypomorphic cells demonstrated the requirement for high-level expression of GATA-3 for ETP development.

These data collectively indicate that GATA-3 activity is required for the development of a functional ETP compartment from LMPPs, therefore raising the question: where and in which early progenitor is GATA-3 activity required for ETP generation? LMPPs in the bone marrow are likely to contain some of the cells that acquire a migratory signal that allows thymic homing (Schwarz and Bhandoola, 2004; Adolfsson et al., 2005; Lai and Kondo, 2007; Schwarz et al., 2007). One immediate possibility is that GATA-3 is required for the homing step from the fetal liver or bone marrow to the thymus. We feel that this is unlikely to be a primary cause of the reduction in the number of ETPs and later stage T cells in Gata3 hypomorphic mutant embryos, because fetal liver cells isolated from the hypomorphs generated fewer DN2 and DN3 stage cells than did wild-type cells in vitro, and a developmental arrest of Gata3 hypomorphic ETPs progressing to the DN2 stage was not observed in vivo; however, these observations do not definitively rule out the possibility that GATA-3–deficient progenitors suffer from a homing deficiency. To date, we have not yet tested the cell developmental potential of bone marrow progenitor cells that completely lack GATA-3 either in vitro or by intrathymic injection. Recently, it was shown that the VCAM-1– pool of adult LMPPs express GATA-3 mRNA, but GATA-3 in this subfraction is very low when compared with its abundance in ETPs (Lai and Kondo, 2007), so the relevance of this observation is unclear. Introduction of GATA-3 before Notch signaling activation fails to promote T cell development (Hozumi et al., 2008). In the absence of GATA-3, bone marrow immature NK cells develop normally, whereas thymic NK cells are not generated (Samson et al., 2003; Vosshenrich et al., 2006). These data suggest a function for GATA-3 in a lymphocyte progenitor population after thymic entry and exposure to Notch ligands. A second possibility is that GATA-3 activity is required at the very beginning of ETP generation, a developmental stage at which very rare thymus-seeding progenitors (TSPs) are thought to proliferate and develop to form the initial ETP population. A current candidate for the TSPs resides in the pool of ETPs expressing both Flt3 and CCR9 (Benz et al., 2008). Because the generation of ETPs was almost completely blocked in mice that harbored a conditionally inactivated Gata3 gene at the adult stage, we speculate that GATA-3 activity is required at this step. Because Flt3-expressing early ETPs retain B, myeloid, dendritic, and NK cell potential (Porritt et al., 2004; Balciunaite et al., 2005; Sambandam et al., 2005), it is possible that TSP preferentially develops to these non–T lineages in Gata3 mutants. To date, we have not yet tested the development of each lineage in the thymus or in co-culture. In relation to the increase of non–T lineage development, it should be repeated that though Lin-CD27-c-Kit+CD25- cells predominated 4 d after co-culture, the absolute number of this population was comparable with control. A third obvious possibility is that GATA-3 activity is required for ETP maintenance and/or viability, but the data presented in this paper do not support this possibility. Neither increased apoptosis nor slowed cell-cycle progression was observed in Gata3+/+ progenitor cells. However, we could not exclude the possibilities that there are defects in survival or proliferation early in the co-culture that have been missed. In summary, these results deductively suggest that GATA-3 controls the differentiation of TSPs into ETPs, although further studies are clearly required to confirm or refute this hypothesis.

Although the adoptive transfer experiments and OP9-DL1 co-culture experiments presented in this study clearly demonstrate a cell-autonomous function for GATA-3 in ETP generation, these data do not exclude a noncell-autonomous function, if such exists. Indeed, GATA-3 is expressed in the third pharyngeal arch of e10.5 mouse embryos, from which the thymus is known to arise (Minot, 1884), and GATA-3 activity in the first through the fourth arches is required for the development of several different organs derived from the pharyngeal arch mesenchyme during early embryogenesis (unpublished data; Lim et al., 2000).

Gata3+/+ hypomorphic cells showed aberrant T cell development at both fetal and adult stages, although the phenotypes differ in each stage. In Gata3+/+ hypomorphic embryos, ETP/DN2 cells were significantly reduced, whereas DN3 stage cells were only modestly reduced in absolute number. In irradiated adult recipients rescued with Gata3+/+ hypomorphic embryos, ETPs were reduced, whereas DN2/DN3 cells actually increased in absolute number. We thus surmise that the threshold level requirement for GATA-3 activity is different at the fetal and adult stages. Similar phenotypes to Gata3+/+ hypomorphic mutant embryos are observed in Flt3 ligand–deficient (Sambandam et al., 2005) and E2A null mutant (Dias et al., 2008) mice. There are several possibilities to account for the increased number of Gata3+/+ DN2/DN3 stage cells compared with ETPs. First, Gata3 hypomorphic cells may proliferate excessively. In support of this possibility, Gata3+/− Lin-CD27-c-Kit+CD25- cells developing from Lin− fetal liver cells 4 d after OP9-DL1 co-culture cycle more actively than Gata3+/+ cells. Furthermore, our preliminary investigations showed that e18.5 Gata3+/+ ETP, DN2, and DN3 thymocytes are more actively cycling than Gata3+/+ cells. A second possibility is that, through either hematopoietic cell–autonomous or noncell–autonomous mechanisms, expression of the hypomorphic Gata3 allele somehow leads to an increased number of DN3 cells via an ETP-independent pathway. In fact, production of DN3-like and later stage cells in the presence of few classical ETPs is observed shortly after
transplantation (Maillard et al., 2006). A third possibility is the existence of a developmental arrest and/or delay phenotype that causes increased accumulation of Gata3<sup>+/−</sup> DN3 cells in comparison to Gata3<sup>+/+</sup> DN3 cells.

Finally, we note that the factors that play important roles in ETP development in collaboration with GATA-3 have not yet been identified. Perhaps because of significance within this context, Notch signaling has been documented to induce thymocyte development (Hozumi et al., 2008). When Lin<sup>−</sup> c-Kit<sup>+</sup> fetal liver cells are cultured on OP9-DL1 cells, Notch target genes are induced on day 1, whereas in contrast, GATA-3 was induced only at day 3 in synchrony with CD25, pT<sub>α</sub>, and CD3 (Taghon et al., 2005). Although examples exist, at least showing that Notch signaling controls GATA-3 expression at other stages, for example in Th2 cells (Amsen et al., 2007; Fang et al., 2007), in contrast, expression of GATA-3 in bone marrow LSK progenitors actually precedes the activation of Notch signaling (Sambandam et al., 2005; Lai and Kondo, 2007), and Notch signaling is dispensable for HSC function in adult mice (Maillard et al., 2008). Thus, the relationship between GATA-3 and Notch signaling during very early T lymphoid development remains unclear. Although the Flt3 cytokine receptor (Sambandam et al., 2005; Schwarz et al., 2007; Sitnicka et al., 2007), IL-7 signal<sup>−</sup> (Sitnicka et al., 2007), CCR9 activity (Schwarz and Bhandoola, 2004; Svensson et al., 2008), E2A (Dias et al., 2008), Me2<sup>−</sup> (Steinhilbing-Sun et al., 2009), and RUNX activity (Talebian et al., 2007) are all known to be important for the generation of ETPs and/or their progenitors, much more extensive characterization will be required to validate the relationships between and epistatic hierarchy among these factors during the earliest stages of T cell development.

MATERIALS AND METHODS

Mice. The Gata3<sup>−/−</sup> allele (van Doorninck et al., 1999) and Mx<sup>Icre</sup> transgenic mice (provided by M. Yamamoto, Tohoku University Medical School, Sendai, Japan; Kuhn et al., 1995) have been previously described. Gata3<sup>+/−</sup> mice were backcrossed for more than five generations onto a C57BL/6 genetic background. Gata3<sup>+/−</sup> embryos were rescued by norepinephrine administration as previously described (Kauffman et al., 2003). The Gata3<sup>+/+</sup> allele was provided by F. Grosveld (Erasmus Medical Center, Rotterdam, Netherlands; Kurek et al., 2007). C57BL/6-Ly<sup>5K</sup> (CD45.2) mice and C57BL/6-Ly<sup>5B6</sup> (CD45.1) mice were purchased from the Jackson Laboratory. All animal experiments were approved by the University Committee on Use and Care of Animals of the University of Michigan, and were performed according to their guidelines (approval no. 8611).

Flow cytometry. Single-cell suspensions of thymocytes, bone marrow, fetal liver, or fetal spleen cells were first incubated with FcBlock (BD) and then incubated with various combinations of antibodies (BD or eBioscience). The following antibodies were used: CD4 (RM4-5), CD8 (53-6.7), c-Kit (2B8), CD44 (IM7), CD25 (PC61), CD45.2 (104), CD45.1 (A20), CD3 (145-2C11), TCR<sub>β</sub> (H57-597), γδTCR (GL3), NK1.1 (PK136), CD11c (N418), B220 (RA3-6B2), CD19 (1D3), Mac-1 (M1/70), Gr-1 (RB6-8C5), TER119 (TER119), IL-7Ra (A7R34), PIR-A/B (6C1), Scal (D7), Flt3 (A2F10), CD93 (AA4.1), Ly-6c (AL-21), CD49b (DX5), and CD27 (LG.7F9). Immature T cells were analyzed as previously described (Talebian et al., 2007). The following cocktail of antibodies was used to exclude mature hematopoietic lineage cells (Lin<sup>−</sup>) in the thymus and the cells cultured in the OP9-DL co-culture system: CD8, CD3, TCR<sub>β</sub>, γδTCR, NK1.1, CD11c, Mac-1, Gr-1, B220, CD19, and Ter119. In some experiments, CD8<sup>−</sup> cells were removed before flow cytometric analysis. T-restricted progenitors in the fetal liver were analyzed as previously described (Masuda et al., 2005). A cocktail containing antibodies recognizing TER119, Gr-1, B220, CD19, NK1.1, and Thy1.2 was used to exclude Lin<sup>−</sup> cells in the fetal liver. Progenitors in the bone marrow were analyzed as previously described (Sambandam et al., 2005; Kunfelt et al., 2006). A cocktail containing antibodies recognizing TER119, Gr-1, B220, CD19, CD3, and Mac-1 was used to exclude Lin<sup>−</sup> cells in the bone marrow for the analysis of HSCs, MPPs, and LMPPs. A cocktail containing antibodies recognizing TER119, Mac-1, CD19, CD3, NK1.1, CD49b, Ly-6c, and Gr1 was used to exclude Lin<sup>−</sup> cells in the bone marrow for the analysis of CLP and F<sub>A</sub>. Stained cells were analyzed or sorted using flow cytometers (FACScanto II or FACSaria; BD). Doublets were excluded with forward-scatter area versus width pulses. Dead cells were excluded by analyzing DAPI or propidium iodide (PI). The resulting cell distribution files were analyzed using FACSdeva (BD), Wessel (WEHI Biotechnology Centre), or FlowJo (Tree Star, Inc.) software.

OP9-DL1 co-culture. To induce T cell development, 1–2 × 10<sup>5</sup> total fetal liver or Lin<sup>−</sup>depleted fetal liver cells recovered from one embryo were cultured on a feeder layer of OP9-DL1 cells (Schantz and Zfitiga-Pflücker, 2002) in the presence of 5 ng/ml IL-7 and 5 ng/ml Flt3<sup>-ligand</sup> (R&D Systems). Partial media change was performed every 3–4 d. After 4–8 d of co-culture, cells were harvested and seeded onto new plastic culture dishes for 20 min to allow OP9-DL1 cells to adhere, and the nonadherent hematopoietic cells were processed and stained for flow cytometry.

Annexin V staining. Annexin V was purchased from BD. 4 d after induction of OP9-DL1 co-culture, the cells were harvested and stained with cell-surface antibodies followed by annexin V staining, and then analyzed by flow cytometry.

For fetal liver single-cell preparations, Gata3<sup>+/−</sup> embryos were recovered from norepinephrine-administered pregnant dams (Kauffman et al., 2003). After PCR genotyping, fetal livers were recovered, minced, and passed several times through a 5-ml syringe and 26-gauge needle; the single-cell suspension was then subjected to magnetic lineage cell-surface marker depletion before initiation of the OP9-DL1 co-cultures. All of the preceding steps were performed on the same day, because when the fetal livers in DMEM containing 10% FBS were left on ice overnight for completion of the procedure on the following day, we observed an increased ratio of annexin V<sup>−</sup> cells in wild-type progenitor cells and an even greater increase in Gata3<sup>+/−</sup> progenitor cells after the usual 4 d of co-culture.

Cell-cycle analysis. A BrdU flow kit was purchased from BD, and staining was performed according to the manufacturer’s instructions with minor modifications. 4 d after induction of OP9-DL1 co-culture, the cells were cultured with BrdU for 1 h. A PE-conjugated anti-BrdU antibody (BioLegend) and DAPI were used.

Induction of Cre activity in Mx<sup>Icre</sup> transgenic mice. 6–8-wk-old mice were intraperitoneally injected with 300 µg pl-pC (Sigma-Aldrich) at days 1, 3, and 5. Thymi were analyzed 3 wk after the first injection.

Adoptive transfer. Gata3<sup>−/−</sup> mice were backcrossed onto a C57BL/6-Ly<sup>5B6</sup> (CD45.2) genetic background. Gata3<sup>+/−</sup> or Gata3<sup>+/+</sup> donor cells were isolated from e14.5 fetal livers. Ly<sup>5B6</sup> × Ly<sup>5K</sup> F1 (CD45.1/CD45.2 heterozygous) mice were irradiated with two doses of 550 rads each, 3–4 h apart, in a cesium-137 γ irradiator, and then retroorbitally injected with 5 × 10<sup>5</sup> donor CD45.2 fetal liver cells and CD45.1 bone marrow cells. To monitor reconstitution, a small amount of peripheral blood was periodically recovered from the tail veins of recipient mice, subjected to ammonium chloride
red cell lysis, and stained for flow cytometry. Anti-B220 antibody (RA3-6B2) was used to monitor reconstitution in peripheral B lineage cells. It should be noted that the B220 epitope is expressed on early to mature B lineage cells as well as on some activated T, dendritic, and NK cells (Watanabe and Akaie, 1994; Huntington et al., 2007).

**Statistical analysis.** Statistical significance was assessed by the Student’s t test compared with wild type unless otherwise noted in the figures.

**Online supplemental material.** Fig. S1 shows the protein expression level from the Gata3 hypomorphic alleles. Fig. S2 shows expression of GATA-3 in embryonic and adult ETPs and DN2 thymocyte. Fig. S3 shows fewer immature T cells in Gata3 mutant thymus. Fig. S4 shows the development of myeloid and B lymphoid lineage cells in Gata3 hypomorphic embryos. Fig. S5 shows the hematopoietic reconstitution assay with Gata3<sup>−/−</sup> cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090934/DC1.

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Figure S1. *Protein expression level from the Gata3 hypomorphic allele.* (A) A representative Western blot is shown. Protein samples prepared from tissues (thymi of Gata3^+/+, Gata3^+/−, and Gata3^-/- individual neonates) and cells (293T human embryonic kidney cells that were mock transfected or transfected with eukaryotic expression plasmid directing GATA-3.eGFP or GATA-3 cDNAs) were first subjected to SDS-PAGE and then Western blotting analysis using a rabbit anti–GATA-3 antibody (not depicted). Anti–GATA-3 immunoreactivity was detected using a fluorescently labeled goat anti–rabbit secondary antibody. Fluorescent signals were detected and quantified using an infrared imaging system (Odyssey; LI-COR Biosciences). The migration positions of wild-type GATA-3 (48 kD) and GATA-3.eGFP (75 kD) proteins are indicated. The antibody also reacted with nonspecific proteins. (B) Thymic protein samples from individual Gata3^-/- neonates (n = 4) were subjected to SDS-PAGE and Odyssey Western blotting analyses. The fluorescent signals of GATA-3.eGFP fusion and wild-type GATA-3 protein bands of each animal were quantified. Native GATA-3 protein levels were arbitrarily set at 100%. Similar results were obtained using a second set of Gata3^-/- neonates (means ± SEM).
Figure S2. **Expression of GATA-3 in embryonic and adult ETPs and DN2 thymocytes.** (A) Expression profiles of eGFP fluorescence in Lin<sup>hi</sup>-Kit<sup>hi</sup>CD25<sup>-</sup> (ETP) and Lin<sup>hi</sup>-Kit<sup>hi</sup>CD25<sup>-</sup> (DN2) populations in Gata3<sup>g/g</sup> embryos at e15.5 (top) and e18.5 (bottom) compared with wild type. Data are representative of four to eight embryos of each genotype from at least two independent experiments for each stage. (B) Expression profiles of eGFP fluorescence in fetal liver donor-derived ETP and DN2 populations in Gata3<sup>g/g</sup> hematopoietic chimeras compared with Gata3<sup>+/+</sup> hematopoietic chimeras 10 wk after transplantation. Data are representative of seven or eight embryos of each genotype from two independent experiments.
Figure S3. Fewer immature T cells in Gata3 mutant thymi. (A and B) The surface expression of CD44 and CD25 on Lin$^+$ thymocytes from Gata3$^{g/g}$, Gata3$^{g/+}$, and Gata3$^{+/+}$ embryos at either e15.5 (A) or e18.5 (B). Lin$^+$CD44$^+$/CD25$^-$ subpopulations, enriched for bona fide ETPs, were analyzed for surface expression of c-Kit and CD24. Data are representative of four to eight embryos of each genotype from at least two independent experiments for each stage. Numbers in boxed areas indicate the mean percentage of cells in those sectors. (C) The absolute number of Lin$^+$CD44$^+$/CD25$^-$ cells per thymus in Gata3$^{g/g}$, Gata3$^{g/+}$, and Gata3$^{+/+}$ embryos at e15.5 (top) and e18.5 (bottom) are shown. Data represent the summary of at least two independent experiments for each stage and an average of four to eight embryos of each genotype are shown with SEM. *, P < 0.05. (D) The absolute number of Lin$^+$CD44$^+$/CD25$^-$ cells per adult thymus in Gata3$^{f/f}$:Tg Mx1.cre compound mutant mice compared with Gata3$^{f/+}$:Tg Mx1.cre mice 3 wk after pl-pC injection is depicted. Data represent the summary of three independent experiments and an average of four mice of each genotype with SEM. *, P < 0.05.
Development of myeloid and B lymphoid lineage cells in Gata3 hypomorphic mutant embryos. (A and B) Liver (A) and spleen (B) hematopoietic cells isolated from e18.5 Gata3<sup>g/g</sup> or Gata3<sup>g/+</sup> embryos were analyzed for surface expression of multiple lineage markers. Cells stained with anti-TER119 antibody (shaded histograms) are shown with cells stained with the same amount of isotype-matched IgG control conjugated with the same fluorochrome (open histograms). Representative flow data examining four to eight mice of each genotype from at least three independent experiments and gatings are shown. (C) The mean ratios of B lymphoid (B220<sup>+</sup>CD19<sup>+</sup>), myeloid (Mac1<sup>+</sup>Gr1<sup>+</sup>), and erythroid (TER119<sup>+</sup>) lineage cells in e18.5 Gata3<sup>g/g</sup>, Gata3<sup>g/+</sup>, or Gata3<sup>g/-</sup> fetal livers or spleens are shown. Data represent the summary of at least three independent experiments for each stage and an average of four to eight mice of each genotype with SEM.
Figure S5. A hematopoietic cell–autonomous role for GATA-3 in the generation of adult ETPs. (A) Experimental strategy for the hematopoietic reconstitution assay with Gata3<sup>+/+</sup> cells is schematically illustrated. (B) Peripheral blood cells were analyzed by flow cytometry at 4, 7, and 10 wk after transplantation for expression of lineage-specific markers and CD45. The mean ratios of fetal liver donor-derived cells (CD45.2<sup>+</sup>CD45.1<sup>-</sup>) in the T lymphoid (CD3<sup>+</sup>), B lymphoid (B220<sup>+</sup>), and myeloid (Mac1<sup>+</sup>Gr1<sup>+</sup>) compartments are shown. Data represent the summary of two independent experiments and mean ratios of seven to eight recipient mice in each group with SEM. *, P < 0.002. (C) Thymocytes were isolated and analyzed for the expression of CD45.2 and CD45.1 in the ETP, DN2, DN3, and DN4 populations 10 wk after transplantation. Data are representative of seven to eight recipients for each genotype from two independent experiments. Numbers in boxed areas indicate the mean percentage of cells in those sectors. (D and E) Fetal liver donor-derived CD45.2<sup>+</sup>CD45.1<sup>-</sup> cell ratios (D) and absolute numbers (E) in each thymocyte or bone marrow cell population were determined by flow cytometry. Data represent the summary of two independent experiments and mean ratios of seven to eight recipient mice in each group with SEM. *, P < 0.05. The contribution of Gata3<sup>+/+</sup> cells to ETPs was lower than that from Gata3<sup>+</sup>/Gata3<sup>-/-</sup> cells; in contrast, the reconstitution ratios were not statistically different in bone marrow LSK and peripheral myeloid and B cells. The reconstitution ratios were comparable at the DN2 and DN3 stages. One possibility for this differential effect on ETPs versus DN2/DN3 cells is that hypomorphic ETP cells proliferate excessively, thereby overcoming the ETP deficiency by the time they differentiate into DN2/DN3 thymocytes. Another possibility is that, through either hematopoietic cell–autonomous or noncell–autonomous mechanisms, expression of the hypomorphic Gata3 allele somehow leads to an increased number of DN2/DN3 cells via an ETP–independent pathway. A third possibility is the existence of a developmental arrest and/or delay phenotype that causes increased accumulation of Gata3<sup>+/+</sup> DN2/DN3 cells in comparison to Gata3<sup>+/+</sup> DN2/DN3 cells. In accord with a previously reported requirement for GATA-3 activity for β selection (DN3 to DN4 development; Pai et al., 2003), the contribution of Gata3<sup>+/+</sup> cells was low in DN4 and subsequent stages compared with DN3.