Notch/Delta signaling constrains reengineering of pro-T cells by PU.1

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PU.1 is essential for early stages of mouse T cell development but antagonizes it if expressed constitutively. Two separable mechanisms are involved: attenuation and diversion. Dysregulated PU.1 expression inhibits pro-T cell survival, proliferation, and passage through β-selection by blocking essential T cell transcription factors, signaling molecules, and *Rag* gene expression, which expression of a rearranged T cell antigen receptor transgene cannot rescue. However, Bcl2 transgenic cells are protected from this attenuation and may even undergo β-selection, as shown by PU.1 transduction of defined subsets of Bcl2 transgenic fetal thymocytes with differentiation in OP9-DL1 and OP9 control cultures. The outcome of PU.1 expression in these cells depends on Notch/Delta signaling. PU.1 can efficiently divert thymocytes toward a myeloid-like state with multigene regulatory changes, but Notch/Delta signaling vetoes diversion. Gene expression analysis distinguishes sets of critical T lineage regulatory genes with different combinatorial responses to PU.1 and Notch/Delta signals, suggesting particular importance for inhibition of E proteins, Myb, and/or Gfi1 (growth factor independence 1) in diversion. However, Notch signaling only protects against diversion of cells that have undergone T lineage specification after Thy-1 and CD25 up-regulation. The results imply that in T cell precursors, Notch/Delta signaling normally acts to modulate and channel PU.1 transcriptional activities during the stages from T lineage specification until commitment.

gene regulation | hematopoiesis | lineage commitment | T cell development | transcription factors

T lymphocyte lineage determination is a remarkably protracted process. After T cell precursors segregate from other hematopoietic precursors by migrating to the thymus, after multiple rounds of cell division within the thymus, and even after initiating a T lineage gene expression program, the cells still preserve access to seemingly very different fates, such as myeloid cell differentiation (reviewed in refs. 1–3). The positive regulatory events that initiate the T cell differentiation program *in vivo* are temporally and mechanistically distinct from the regulatory mechanisms that make it irreversible. A possible basis for the delay in T lineage commitment is that the early stages of T cell development depend on factors such as the Ets-family transcription factor PU.1 (4–7), which is also an activator of myelomonocytic differentiation (8, 9). Normal, essential roles of PU.1 in early T cells may come at a cost of developmental instability.

Gene expression and functional perturbation data suggest that PU.1 down-regulation may normally be rate limiting for T lineage commitment *in vivo*. The earliest stage intrathymic precursors, double-negative (DN) 1 (Thy-1^{low} c-*kit*$^{2+}$ CD44^{+} CD25^{-}), express high levels of PU.1 (2, 10, 11) and have multilineage developmental potential. Potential to give rise to natural killer cells, dendritic cells, and macrophages persists through the DN2 (Thy-1^{+} c-*kit*$^{+}$ CD44^{+} CD25^{+}) stage, along with lower but continuing PU.1 expression. Commitment, the loss of these last alternative developmental potentials, occurs at the DN3 (Thy-1^{+} c-*kit*$^{-}$ CD44^{-} CD25^{+}) stage as the cells stop dividing and undergo T cell antigen receptor (TCR) gene rearrangement. Only cells successful in TCR-β rearrangement then continue development and pass through β-selection. They down-regulate CD25 (DN4 stage) and express cell-surface CD4, CD8, and TCR, finally becoming double-positive (DP) cells (summarized in Fig. 1*C*). An alternative pathway from DN3 leads to development of TCR-γδ T cells (12). Commitment at the DN3 stage coincides with an ≈100-times drop in the expression of PU.1 RNA (2, 12).

Lymphoid precursors can be diverted into other hematopoietic lineages by ectopic expression of transcription factors or ectopic cytokine receptor stimulation (for example, refs. 13–15; reviewed in ref. 3). Similarly, forced high-level expression of PU.1 can redirect T cell precursors to a myeloid-like or dendritic cell fate (16, 17). But PU.1 is not ectopic to T lineage differentiation: instead, until commitment, it is essential. If PU.1 shutoff explains loss of myeloid potential at the DN3 stage, the question remains how precursors that normally express high levels of PU.1 ever arrive at that stage. It follows that under normal circumstances, the T lineage differentiation program leading up to commitment must also include regulatory constraints that channel PU.1 effects to exclude alternative developmental outcomes.

This report shows that the pro-T cell development program indeed incorporates such constraints on PU.1 activity. Notably, these constraints are not cell autonomous, as is Pax-5 in B cell specification (18); rather, they depend on environmental signaling. We show that once T lineage differentiation initiates, Notch/Delta signaling selectively restricts the actions of PU.1.

Results

Pro-T Cells Forced to Express PU.1 Cannot Be Rescued by a TCR Transgene. Forced expression of PU.1 from a bicistronic retroviral vector in developing T cell precursors leads to reduced cell numbers, severe attenuation of cells progressing through β-selection to the DP stage, and, in some conditions, diversion of T cell precursors to a myeloid fate (11, 16, 17, 19). Examples are shown in Fig. 1 *A* and *B*, where bicistronic retroviral vector (LZRS) was used to transduce PU.1 into T cell precursors from embryonic day (E)-14.5 fetal thymus. Using the coexpressed GFP marker to track vector expression and CD45 to distinguish lymphoid precursors, subsequent development of the transduced cells could be tested by coculture with OP9-DL1 stromal cells, which promote T cell

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Abbreviations: TCR, T cell antigen receptor; DP, double positive; DN, double negative; E*n*, embryonic day *n*.

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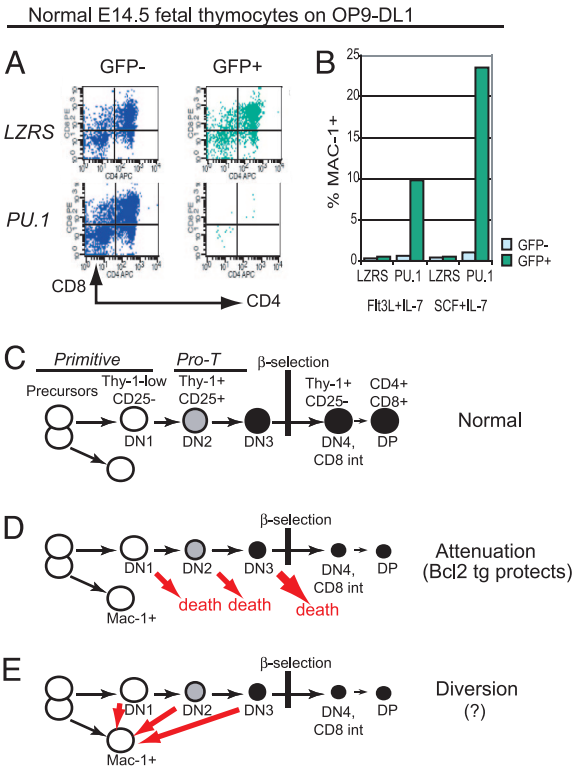

Fig. 1. Effects of PU.1 on normal fetal thymus pro-T cell development in OP9-DL1 culture. (*A*) Transduction with PU.1 (PU.1 GFP$^+$) inhibits generation of CD4$^+$ CD8$^+$ DP cells as compared with empty vector control transductants (LZRS GFP$^+$) or nontransduced cells (GFP$^-$). (*B*) Greatly increased percentage of Mac-1$^+$ cells in PU.1-transduced GFP$^+$ cells in OP9-DL1 culture (*y* axis) as compared with empty vector (LZRS) GFP$^+$ transductants or nontransduced GFP$^-$ cells. Results for two different transduction conditions are shown. (*C*) Scheme of normal early T cell development up to the DP stage. Cells beyond the β-selection checkpoint and γδ cells (not shown) are pre-T cells. (*D* and *E*) Two models for possible modes of interference with T cell development by PU.1.

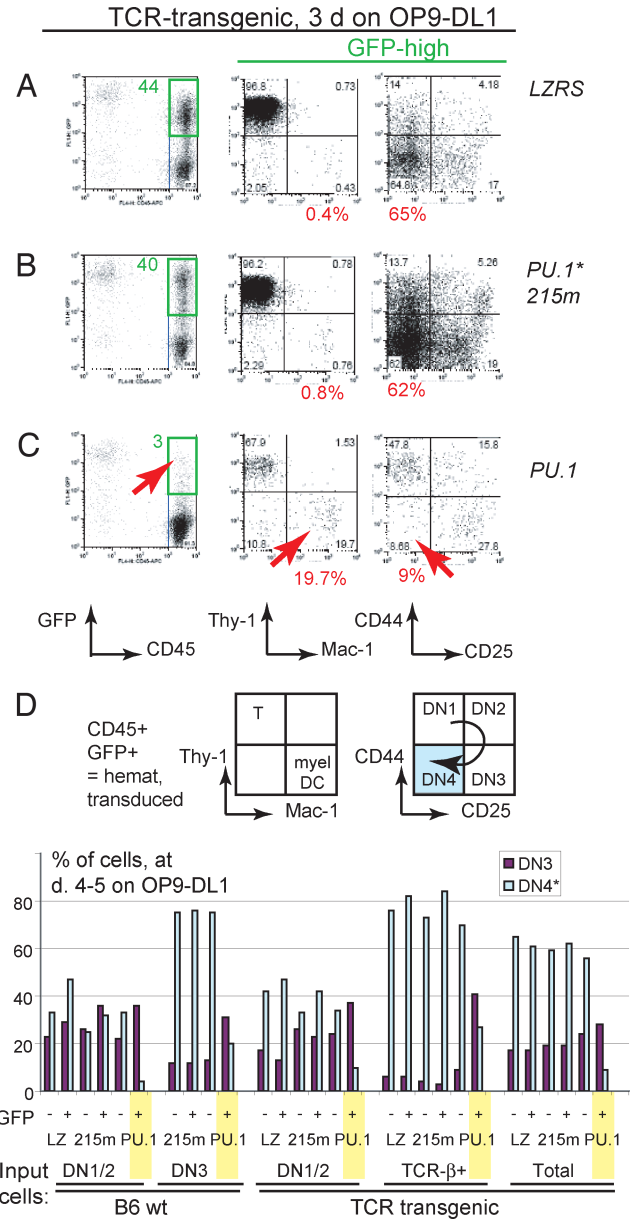

Fig. 2. Expression of a rearranged TCR transgene does not protect pro-T cells from PU.1 effects in OP9-DL1 culture. (*A–C*) Transduction of PU.1 into total E14.5 TCR transgenic thymocytes reduces cellularity (green numbers, *Left*), enhances the production of Thy-1$^-$ Mac-1$^+$ cells (red arrow and red numbers, *Center*), and blocks appearance of DN4-stage and later cells (red arrow and red numbers, *Right*) after 3 days in OP9-DL1 culture. Effects of PU.1 (*C*) compared with vector (*A*) and non-DNA-binding PU.1 mutant (*B*) controls are shown. (*D*) Schematics showing stages corresponding with staining phenotypes in *A–C*. The CD44/CD25 phenotype corresponding to passage through β-selection and γδ-selection, DN4, is highlighted in blue. (*E*) PU.1 retards progression from pre-β-selection (DN3) to postselection stages (DN4* indicates DN4 to DP stages) in 4-day OP9-DL1 culture, whether from TCR transgenic thymocytes or from equivalent WT cells (details are shown in Fig. 6). Results for PU.1-transduced GFP$^+$ cells are highlighted in yellow.

development by presenting the Delta family Notch ligand DL1 (20). In this system, as in our previous reports using a fetal thymus organ culture system (11, 19), PU.1 blocks the development of CD4$^+$ CD8$^+$ DP cells and greatly enhances the appearance of cells with the myeloid marker Mac-1 (Fig. 1 *A* and *B*). Fig. 5, which is published as supporting information on the PNAS web site, shows that PU.1 blocks at the DN3 stage and does not divert the cells to B or natural killer fates.

The relationship between the myeloid-like cell generation and T cell developmental arrest effects of PU.1 could be either selective (Fig. 1*D*) or directive (Fig. 1*E*). Selective emergence of Mac-1$^+$ cells could result if PU.1 generally inhibited T lineage cell viability or proliferation before or during β-selection (Fig. 1*D*, "attenuation"). Alternatively, it could result from diversion of immature T lineage cells to a nonlymphoid fate (Fig. 1*E*), emptying the T cell precursor pool as a byproduct. In the first case, PU.1 levels might not have a direct causal relationship to lymphocyte developmental plasticity, whereas the second case would support a role of PU.1 as a major endogenous regulator of lineage plasticity in pro-T cells. We therefore tested the effect of forcing PU.1 expression in precursors with two kinds of transgenes that are expected to preserve pro-T cell viability and differentiation in different ways: (*i*) a TCR transgene that bypasses the requirement for TCR gene rearrangement for β-selection and (*ii*) a Bcl2 transgene.

Fig. 2 and also Fig. 6, which is published as supporting information on the PNAS web site, show that E14.5 fetal thymocytes from mice with the DO11.10 TCR transgene are not protected from the effects of PU.1 overexpression as measured after 3–5 days of differentiation on OP9-DL1 cells. TCR transgenic fetal thymocytes transduced with an empty vector (LZRS, Fig. 2*A*) or a vector encoding a non-DNA-binding mutant of PU.1 (215m, Fig. 2*B*) (11, 16) were overwhelmingly Thy-1$^+$ and Mac-1$^-$, and >60% successfully passed through β-selection to DN4 or later stage (compare key to stages in Fig. 2*D*; "TCR transgenic total" in Fig. 2*E*). In contrast, TCR transgenic cells transduced with a vector encoding WT PU.1

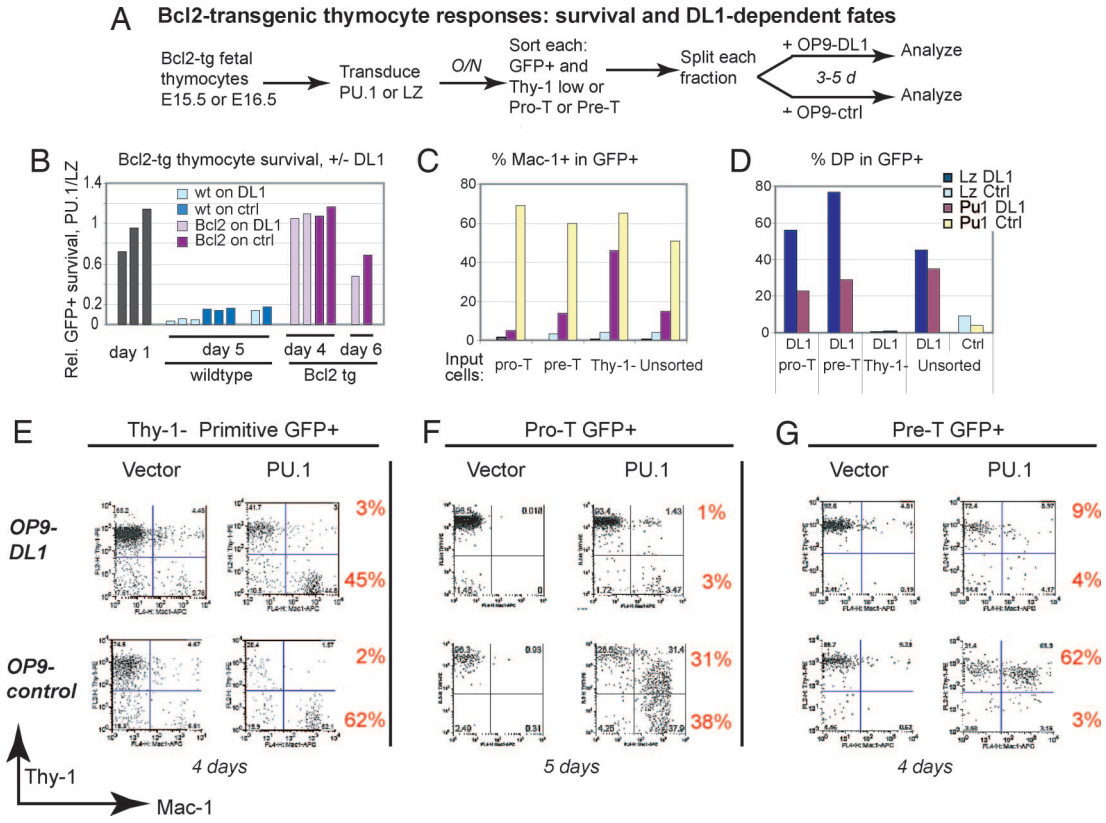

Fig. 3. PU.1 effects on Bcl2 transgenic fetal thymocytes: enhanced survival and Notch/Delta-dependent regulation of developmental choice. (*A*) Experimental scheme for PU.1 effects on subsets of Bcl2 transgenic thymocytes. O/N, overnight; LZ, empty vector. (*B*) Effect of the Bcl2 transgene on recovery of PU.1-transduced cells: ratios of GFP$^+$ cell recovery at indicated time points in PU.1-transduced vs. control-transduced cells. Representative data were pooled from four experiments with E14.5–16.5 fetal thymocytes from B6D2 F_2 (WT) and Bcl2 transgenic (Bcl2) embryos. Day 1 indicates WT transductants before OP9 culture (compare Fig. 7). (*C* and *D*) Effects of PU.1 on total Mac-1$^+$ cell generation (*C*) and DP cell generation (*D*) by Bcl2 transgenic thymocytes in OP9-DL1 culture (dark blue and purple bars) or OP9 control culture (aqua and yellow bars). Additional results and conditions are shown in Fig. 8. (*E–G*) Representative data showing Mac-1$^+$ cell differentiation induced by PU.1 from the indicated thymocyte subsets in the presence (*Upper*) or absence (*Lower*) of DL1. (*E*) E15.5 Thy-1^{low}. (*F*) E16.5 Thy-1$^+$ CD25$^+$. (*G*) E16.5 Thy-1$^+$ CD25$^-$. Red numbers show the percentages of Mac-1$^+$ Thy-1$^+$ and Mac-1$^+$ Thy-1$^-$ cells.

(Fig. 2*C*) showed a >10-times reduction in transduced cell recovery within 3 days (GFP$^+$ CD45$^+$ cells, green numbers). Note that this PU.1 vector gives equivalent or higher initial transduction frequencies than the LZRS control (Fig. 7, which is published as supporting information on the PNAS web site). Losses of GFP$^+$ cells occurred throughout the DN stages with particularly sharp depletion from the DN4 and later stages (red arrow in Fig. 2*C Right*). Despite the TCR transgene, which remained prominently expressed on the surface of most of the surviving PU.1-transduced cells (Fig. 6*A*), a substantial population of Thy-1$^-$ Mac-1$^+$ cells was also generated (red arrow, red numbers in Fig. 2*B Center*). These effects closely resemble those of PU.1 in nontransgenic cells (Fig. 5). The inability of cell-surface TCR expression to protect against PU.1-mediated inhibition was confirmed by purifying cohorts of WT or TCR transgenic thymocytes from stages before or after TCR expression and comparing their kinetics of differentiation after PU.1 transduction (Fig. 6 *B–D* and Fig. 2*E*). TCR transgenic DN1/2 cells were as severely inhibited as WT DN1/2 cells. Even more advanced TCR transgenic cells that were already expressing cell-surface TCR-β before transduction were blocked in development to DP cells (Fig. 6*D*). Thus, high-level PU.1 inhibits T lineage development through some regulatory mechanism other than TCR gene rearrangement.

A Bcl2 Transgene Protects T Cell Development During Constitutive PU.1 Expression in the Context of Notch/Delta Signaling. The effect of a constitutively expressed Bcl2 transgene showed that PU.1 inhibits pro-T cells through an attenuation mechanism (Fig. 1*D*). In striking contrast to the results with normal or TCR transgenic thymocytes (Figs. 1 and 2) (11, 19), Bcl2 transgenic fetal thymocytes were protected from PU.1 effects on viability (Fig. 3*B*). Bcl2 expression promoted high recovery of PU.1-transduced cells from all subsets of E15.5–16.5 thymocytes, spanning the developmental range from primitive (Thy-1^{low}, mostly DN1) to pro-T (Thy-1$^+$ CD25$^+$, DN2/3) to pre-T (post-β-selection or $\gamma\delta$-selection, Thy-1$^+$ CD25$^-$). Protected by the Bcl2 transgene, PU.1-transduced thymocytes could even generate some DP cells in OP9-DL1 culture as well (Fig. 3*D*; see also Fig. 8 *C* and *D*, which is published as supporting information on the PNAS web site). The Bcl2 transgene does not remove normal signaling requirements for development, however; DP cell generation still depended on the presence of the Notch ligand DL1, with or without PU.1 (Fig. 3*D*, "unsorted"). These results imply that for normal fetal thymocytes in OP9-DL1 culture, overexpression of PU.1 attenuates viability and/or proliferation of the T lineage cells through a mechanism that can be counteracted in large part by guaranteeing expression of Bcl2.

The ability of Bcl2 to keep virtually all PU.1-transduced thymocytes alive made it possible to address rigorously the question of how and whether PU.1 instructs lineage choice of early thymocytes (Fig. 1*E*). If PU.1 only promoted outgrowth of Mac-1$^+$ cells due to preferential death of T lineage cells, then Bcl2 transgenic thymocytes, which do not die, should yield a greatly reduced percentage of Mac-1$^+$ cells when forced to express PU.1; however, this is not what occurs. Within the first day of transduction with PU.1, a shift to increased Mac-1 expression was already visible in the pro-T (Thy-1$^+$ CD25$^+$) fraction of Bcl2 transgenic thymocytes (Fig. 7*A*, red arrows), even though pro-T cell survival was not affected. The

developmental shifts caused by PU.1 in the absence of cell death were at least as pronounced as in WT cells (Fig. 7*B*). As described below, PU.1 induced Mac-1$^+$ cells to develop from Bcl2 transgenic thymocytes but only under specific conditions. This system provided robust and stable PU.1 expression in the transductants as shown below, enabling the other conditions needed for lineage diversion to be dissected.

Notch/Delta Signaling Constrains PU.1-Induced Mac-1 Expression on Thy-1$^+$ Fetal Thymocytes. Fig. 3*C* reveals that contact of PU.1-transduced thymocytes with DL1 has a dramatic, specific, and reproducible effect on their ability to generate Mac-1$^+$ cells (yellow vs. purple bars). PU.1 overexpression in normal thymocytes inhibits survival on OP9 control as well as on OP9-DL1 stroma (Fig. 5), but the Bcl2 transgene enables PU.1-transduced cells to survive almost as well as controls in both conditions (Fig. 8*A*, yellow vs. aqua bars). Fig. 3 *E–G Lower* shows that PU.1 could induce prolific Mac-1 cell-surface expression in majorities of primitive Thy-1^{low} cells, pro-T cells, and even pre-T cells, provided that the cells were maintained in OP9 control culture without DL1 (Fig. 3*C*, yellow bars). These Mac-1$^+$ cells were large and expressed CD11c, F4/80, and, in some cases, macrophage colony-stimulating factor receptor, but not Gr-1 (Fig. 9*A*, which is published as supporting information on the PNAS web site). The Mac-1$^+$ cells emerging from pro- and pre-T cells were all initially Thy-1$^+$ (Fig. 9 and data not shown). Then, many of those emerging from pro-T cells (Fig. 3*F*), although not from pre-T cells (Fig. 3*G*), down-regulated Thy-1 expression between days 3 and 5 on OP9. Importantly, PU.1-transduced Mac-1$^+$ cells activated a broad spectrum of myeloid genes while they were still Thy-1$^+$ and continued to express appreciable CD3ε and CD3γ RNA (Fig. 9 *B* and *C*). In contrast, the same transduced Thy-1$^+$ cells did not generate a Mac-1$^+$ population at all if cultured on OP9-DL1 instead (Fig. 3 *F* and *G Upper* and *C*, purple bars). Absolute numbers as well as percentages of Mac-1$^+$ cells were dramatically reduced. Thus, the presence of the Notch/Delta signal blocked activation of the myeloid program and down-regulation of Thy-1 in these cells.

This effect of Notch/Delta signaling was developmentally specific, for in primitive cells that were still Thy-1$^-$ when transduced (Fig. 3*E*), the effect was much weaker or absent. These primitive cells were not committed myeloid precursors, because they developed quickly into T lineage progeny on OP9-DL1 stroma (≈90%) (Fig. 3*E Upper Left*). But when transduced with PU.1, these cells generated profuse myeloid-like Thy-1$^-$ Mac-1$^+$ cells with similar efficiency in either OP9-DL1 or OP9 control culture (Fig. 3*E Right* and *C*). The response of this primitive subset of fetal thymocytes presumably accounts for the Mac-1$^+$ cells generated when bulk populations of PU.1-transduced thymocytes are cultured on OP9-DL1 (Figs. 1*B* and 2*C*).

The results in Fig. 3 were robust in diverse culture conditions with thymocyte subsets from E15.5 or E16.5 (Fig. 8*B*). Thus, once initiated, the T lineage specification process marked by expression of Thy-1 brings the myeloid diversion activity of PU.1 under control of a veto from Notch/Delta signaling.

Notch/Delta Signaling as a Specific Modifier of PU.1 Actions on Target Genes. To define the molecular mechanism through which PU.1 and Notch compete for lineage specification of pro-T cells, we analyzed the earliest gene expression changes induced in fetal thymocytes in response to PU.1 transduction with and without the rescuing effects of Notch/Delta signaling. We sought three kinds of system components in an attempt to determine the components that are most likely to be central to a lineage control switch: (*i*) gene expression effects that might explain T lineage attenuation by PU.1, (*ii*) the earliest myeloid-associated responses, and (*iii*) identification of the responses most highly sensitive to Notch/Delta signaling. We therefore monitored known pro-T cell differentiation genes involved in pre-TCR assembly and signaling, Notch target genes, and known essential T lineage regulatory genes (reviewed in refs. 21–24) as well as myeloid marker and regulatory genes.

Fig. 4 shows effects of PU.1 on gene expression in Bcl2 transgenic cells that were transduced overnight and then returned to culture on either OP9-DL1 or OP9 control stroma for an additional day before preparatively sorting the GFP$^+$ Thy-1$^+$ transductants (Fig. 4*B*). The major effects seen at 40 h (Fig. 4*A*) actually begin within the first 20 h after transduction, and all depend on the integrity of the PU.1 DNA-binding domain (Fig. 10 *A* and *B*, which is published as supporting information on the PNAS web site). Because baseline gene expression changes substantially between the DN1 and DN4 stages (2, 12, 25, 26), pro-T regulatory genes, including Notch 1, Notch 3, and others, were also analyzed separately in control and PU.1-transduced Thy-1^{low}, pro-T, and pre-T cells (Fig. 10*C*). Except as noted, gene-specific effects of PU.1 were similar across subsets.

The basis of attenuation and β-selection blockade was obvious. PU.1 overexpression (Fig. 4*A*, far left) inhibited expression of known essential T lineage regulators, including TCF-1 (T cell factor 1), Ets1, Ets2, Gfi1 (growth factor independence 1), Myb, and E protein genes E2A and HEB, as well as expression of Rag-1 and the crucial signaling molecules Zap70, Lck, and LAT (linker for activation of T cells) (Fig. 4*A*). Weaker but still significant effects were seen on FOG-1 (friend of GATA 1), CD3γ and ε, and Ikaros (in the Thy-1$^+$ subsets only; Fig. 10*C*). Response specificity is shown by the minimal changes in other pro-T cell genes (e.g., Runx3, Runx1, and GATA-3).

The negative effects on pro-T cell genes preceded significant effects on most myeloid genes, but Mac-1, macrophage colony-stimulating factor receptor, and, at a low level, endogenous PU.1 itself were induced. Also induced were myeloid-compatible regulatory genes Id2, Egr2, and Bcl11a (Fig. 4*A* and Fig. 11, which is published as supporting information on the PNAS web site). However, at this time point, PU.1 caused little if any up-regulation of the myeloid-driving transcription factors of the C/EBP family (15, 27); they remained at very low levels (Fig. 4*A*) and were mostly affected by the presence or absence of Notch/DL1 signaling.

Notch 1, Notch 3, and Notch signal delivery were all unaffected by PU.1 in these Thy-1$^+$ cells (Figs. 4*A* and 10*B*). Notch targets HES-1 (hairy enhancer of split 1), Deltex1, and pTα were all remarkably sensitive to the presence or absence of DL1 (Fig. 4*A*, baseline vs. yellow bars and red vs. black bars), but Deltex1 and pTα were virtually indifferent to the addition of PU.1 (Fig. 4*A*, baseline vs. red bars and black bars vs. yellow bars; Fig. 4*C*, group 3). HES-1 was sensitive to PU.1 as well as dependent on DL1 (Fig. 4*A*), indicating more complex regulation. Thus, in Thy-1$^+$ cells, PU.1 and Notch signaling can provide independent regulatory inputs. However, this important feature did not hold in Thy-1^{low} cells, where PU.1 also down-regulated Notch 1 and Notch 3 (Fig. 10*C*).

The gene expression effects of PU.1 in general were blunted by Notch/DL1 signaling (Fig. 4*A*, red vs. purple bars). However, different genes fell into distinct patterns of response to PU.1 ± DL1, as summarized in Fig. 4*C* (*P* values are given in Table 1, which is published as supporting information on the PNAS web site). The positive regulatory effects of PU.1 on macrophage colony-stimulating factor receptor, endogenous PU.1, Bcl11a, and Egr2 were least affected, and even Mac-1 was strongly induced in the presence of DL1 (Figs. 4*C*, group 1, and 11). DL1 protected most pro-T cell genes from maximal inhibition, but many were still significantly down-regulated by PU.1 in the presence of DL1 (Fig. 4*C*, group 2). These genes included Rag-1, several signaling genes, and TCF-1 as described above, and also the Ets family factor Ets1, Bcl11b, and the GATA-3 interaction partner FOG-1 (2, 24) (Fig. 11).

The genes showing the most dramatic influences of Notch/Delta signaling on the response to PU.1 were almost entirely pro-T cell regulatory genes (Fig. 4*C*, groups 4–6). One set was repressed by PU.1 and also dependent on DL1, with additive (logarithmic scale) down-regulation when PU.1 and loss of DL1 were combined (Fig.

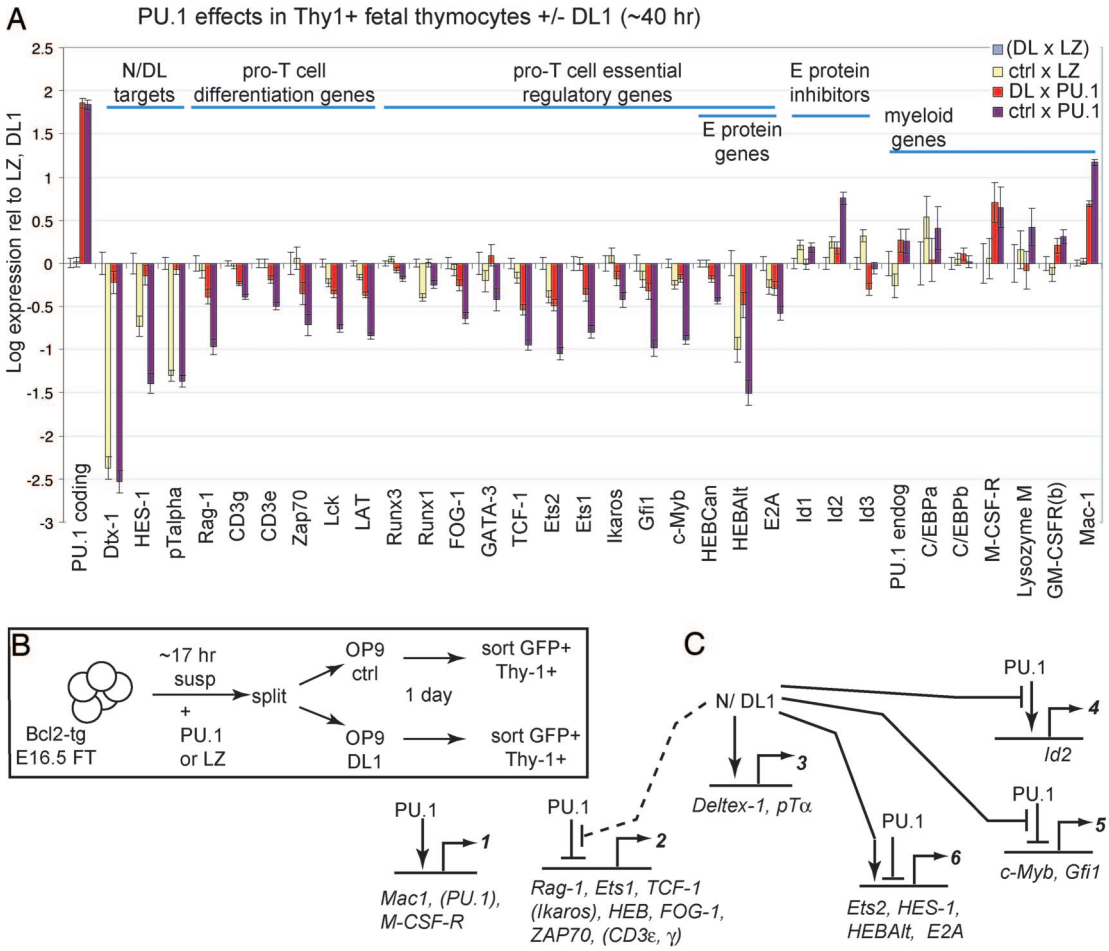

Fig. 4. Effects of PU.1 on gene expression in pro-T cells: selective modulation by Notch/Delta signaling. (*A*) Effects of PU.1 on gene expression in Thy-1$^+$ cells after 40 h of transduction in the presence or absence of Notch/Delta signaling. Means ± SEM of $\log_{10}$-transformed data from three independent experiments are shown normalized to levels in empty vector controls in OP9-DL1 culture. For each gene, (DL × LZ) = log1 = 0. Red bars, PU.1-transduced, OP9-DL1 culture; yellow bars, empty vector-transduced, OP9 control culture; purple bars, PU.1-transduced, OP9 control culture. In the gene list, ''PU.1 endog'' is endogenous PU.1 (3′ untranslated). ''PU.1 coding'' includes exogenous. (*B*) Generation of samples for *A*. (*C*) Schematic of inferred topologies of regulatory inputs for six groups of pro-T cell genes. Genes that are weakly affected are shown in parentheses. In group 1, only Mac-1 is sensitive to DL1 (other genes are shown in Fig. 11). Genes not shown may fall between categories. See text for details.

4*C*, group 6). These genes include HES-1, Ets2, the alternative HEB isoform HEBAlt, and, to a weaker overall extent, E2A. More extreme relationships between Notch and PU.1 effects were shown by c-Myb, Gfi1, and Id2 (Fig. 4*C*, groups 4 and 5). These genes were minimally affected by PU.1 in the presence of DL1 or by removal of DL1 alone but could be substantially repressed (c-Myb and Gfi1) or activated (Id2) by PU.1 if and only if DL1 was absent. DL1 also blocked the weaker repression of Ikaros and canonical-form HEB by PU.1. Thus, although Notch signaling did not appear to regulate Id2 or c-Myb directly, it exerted profound control over the ability of PU.1 to activate Id2 (Fig. 4*C*, group 4) or repress c-Myb and Gfi1 (Fig. 4*C*, group 5) and perhaps others. These genes are thus identified as particularly interesting candidates for controllers of the diversion response.

Discussion

Developmental plasticity is sustained throughout early T cell differentiation in the thymus, as shown when differentiating pro-T cells are removed from the thymic microenvironment. Here, we show that environmental Notch/Delta signaling can be a major lineage fidelity factor for pro-T cells from T lineage specification up to TCR-dependent selection by selectively preventing PU.1 from exerting a subset of its transcriptional activities even when, in other respects, PU.1 activity remains intact. This Notch activity is seen long after Notch signaling blocks B cell development and starts the T lineage specification process. Notch/Delta interaction is remarkably powerful at preventing diversion, even when PU.1 is forcibly expressed in pro-T cells at levels that significantly hamper proliferation and developmental progression within the T lineage. Our results show that PU.1 effects on a variety of regulatory and signaling genes may contribute to the developmental arrest in response to forced PU.1 expression in the presence or absence of Notch signaling. However, Notch-dependent protection of overall E protein activity, c-Myb, and possibly Gfi1 and HES-1 from PU.1 repression is tightly correlated with lineage fidelity, suggesting key roles for these factors as brakes on a lineage switch.

At high levels, PU.1 should interfere with TCR gene rearrangement through its inhibition of Rag gene expression, and its normal expression pattern, reciprocal to Rag-1, may constrain the timing of rearrangement *in vivo*. The spectrum of gene expression effects of forced high-level expression of PU.1 is consistent with partial retrograde differentiation; many of the genes affected would normally peak in expression during the DN3 stage (2). Multiple regulatory and signaling genes are down-regulated by PU.1, most even when DL1 is present, and these effects undoubtedly contribute to population attenuation. However, in the presence of DL1, all

these effects are leaky, as shown by the ability to rescue with Bcl2. Also, cells can survive all these effects and return to normal differentiation if they express PU.1 only transiently (Fig. 8*D*) (11). The reversibility, stage dependence, and environmental sensitivity of PU.1 effects probably represent the reasons that PU.1 can be successfully harnessed for a role in normal pro-T cell development.

Notch/Delta signaling protects pro-T cells through target gene-specific modulation of PU.1 activities. There is a blunting of PU.1 effects generally, and repressive effects especially. However, genes such as c-Myb, Gfi1, and Id2 stand out for their switch-like, Notch-dependent logical processing of inputs from PU.1. Such regulatory genes, and possibly the pro-T genes Ets2, HES-1, Ikaros, and HEBAlt as well, are the best of the candidates tested for participants in a lineage switch. All-or-none control of lineage diversion by PU.1 is also found in a clonal pro-T cell line model system, where strong effects on c-Myb, HEBAlt, and Id2 also are seen (16).

Net activities of basic helix–loop–helix (bHLH) E proteins (E2A and HEB) and their antagonist Id2 seem to be particularly tightly correlated with lineage fidelity when considered as a group rather than individually. The effective level of bHLH activity in developing lymphocytes is normally titrated through shifting Id:E protein balances (28). It is striking that all of the positively acting E proteins tested here are significantly repressed by PU.1 when DL1 is absent and that only then does PU.1 activate their collective antagonist Id2. The effect overall should be multiplicative: a large cumulative decrease in bHLH activity caused by PU.1, specifically when Notch/DL1 signaling is absent. This E protein inhibition could contribute directly to the myeloid or dendritic cell potential of the cells (29, 30). Our evidence that Notch signaling positively regulates bHLH activity in pro-T cells could also explain Notch effects on T vs. natural killer cell development (31, 32).

In summary, PU.1 serves as a probe to discern underlying developmental transitions in pro-T cell regulatory biology that control their lineage plasticity. Once cells pass through initial T lineage specification, Notch expression becomes insensitive to PU.1 levels, and the normal role of PU.1 in uncommitted pro-T cells becomes constrained by Notch/Delta signaling effects, which selectively antagonize reprogramming. Furthermore, the results imply a fundamental asymmetry between T and B cell development: The lineage fidelity role played by the intrinsic, cell-autonomous transcription factor Pax-5 in B cell development (18, 33) appears to be ''outsourced'' to an environmental signaling mechanism throughout the early stages of T cell development. Thus, the thymus is not only important for inducing the T cell differentiation program and for nurturing developing T cells through growth and viability functions (23), but thymic Notch ligands also protect T cell development from the regulatory hazard of the stem/progenitor cell factors it depends on in its early stages, such as PU.1.

Materials and Methods

For detailed methods, see *Supporting Methods*, which is published as supporting information on the PNAS web site.

Mice. C57BL/6 (B6) and Eμ-Bcl2–25 (Bcl2-tg) mice were from our colony (16). Normal B6D2 F_2 embryos were obtained from the California Institute of Technology Genetically Engineered Mouse Service (11). For Bcl2-tg or TCR-tg fetal thymocytes, homozygous Bcl2-tg or TCR transgenic DO-11.10 mice from The Jackson Laboratory (Bar Harbor, ME) were mated with B6 mice.

Transduction of Cells. Non-tissue culture-treated plates were coated with RetroNectin (24 μg/ml) and then with retroviral stocks described in refs. 11 and 16 (the same stocks were used for all experiments). Cells were then incubated on the coated plates for 4–20 h in OP9 culture medium with 0.1 mM 2-mercaptoethanol and also IL-7 and Flt3L or stem cell factor at 5 ng/ml each. For variant conditions, see Figs. 7 and 8.

OP9-DL1 Cultures. Lymphocyte differentiation cultures on OP9-DL1 and OP9 control stroma are described in detail in refs. 12 and 34. Differentiation cultures always contained Flt3L and IL-7.

Cell Staining and Flow Cytometry. Cell staining methods and antibodies were as reported in refs. 12, 26, and 34, except that CD45 staining was used together with GFP detection to gate all analyses of transduced thymocytes from OP9 cultures. For details, see *Supporting Methods*.

Gene Expression Analysis. Quantitative real-time RT-PCR analysis was carried out as described in refs. 2, 12, 16, and 26 with modifications and using primers described in *Supporting Methods*.

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