A gene regulatory network armature for T lymphocyte specification

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Choice of a T lymphoid fate by hematopoietic progenitor cells depends on sustained Notch–Delta signaling combined with tightly regulated activities of multiple transcription factors. To dissect the regulatory network connections that mediate this process, we have used high-resolution analysis of regulatory gene expression trajectories from the beginning to the end of specification, tests of the short-term Notch dependence of these gene expression changes, and analyses of the effects of overexpression of two essential transcription factors, namely PU.1 and GATA-3. Quantitative expression measurements of >50 transcription factor and marker genes have been used to derive the principal components of regulatory change through which T cell precursors progress from primitive multipotency to T lineage commitment. Our analyses reveal separate contributions of Notch signaling, GATA-3 activity, and down-regulation of PU.1. Using BioTapestry (www.BioTapestry.org), the results have been assembled into a draft gene regulatory network for the specification of T cell precursors and the choice of T as opposed to myeloid/dendritic or mast-cell fates. This network also accommodates effects of E proteins and mutual repression circuits of Gfi1 against Egr-2 and of TCF-1 against PU.1 as proposed elsewhere, but requires additional functions that remain unidentified. Distinctive features of this network structure include the intense dose dependence of GATA-3 effects, the gene-specific modulation of PU.1 activity based on Notch activity, the lack of direct opposition between PU.1 and GATA-3, and the need for a distinct, late-acting repressive function or functions to extinguish stem and progenitor-derived regulatory gene expression.

GATA-3 | Notch | PU.1 | T cell development | transcriptional regulation

Exclusion of alternative fates is integral to cell-type specification and one of the key features explained by the gene regulatory networks for development in well studied embryological systems. Cell type-specific gene activation is tightly coupled with blockade of alternative gene programs, through three basic elements of gene network architecture: positive autoregulation of major cell type-specific transcription factors, feed-forward relationships between these factors and their collaborators, and mutual antagonisms between the drivers of alternative cell fates. The collective impact of these mechanisms is usually to create within tight spatial and temporal boundaries a swift cascade of regulatory changes that become effectively irreversible (1). Yet this is not the only way that cell type specification can occur. In stem cell-based systems like those in adult mammals, multipotency is actively maintained over many cell cycles. Even as differentiation of these precursors begins, there can be considerable delay before the cell fate decision is determined. For example, many of the cell fate decisions of mouse hematopoietic stem cell progeny may be controlled by dynamic balances of regulatory factors such as PU.1, C/EBPα, and GATA-2 throughout the intermediate stages of the process. Even in collaboration, these factors appear to drive up to four different cell fates depending on the ratios and fluxes of their activities (2, 3). This behavior is a clue that a distinctive gene network architecture may lie at the core of stem cell-based cell-type specification.

An extreme case of this mode of specification is mammalian T lymphocyte development. In T cell specification, cells preserve a variety of developmental options and a capacity for extensive proliferation throughout and even after commitment to a T cell fate. T cell development begins with the migration of multipotent hematopoietic precursors into the thymus, where these cells adopt T lineage characteristics and gradually give up the ability to give rise to other kinds of blood cells. Lineage exclusion is not only slow but discontinuous for T cell precursors in the thymus: there is a delay of multiple cell cycles between the time cells lose certain non-T options (red blood cell, B cell) and the time they finally become committed to a T cell fate (reviewed in ref. 4). Unlike B cell specification, a feed-forward cascade with critical roles for two lineage-specific transcription factors (5), T cell specification appears to use few, if any, dedicated factors (reviewed in refs. 4 and 6). T cell identity emerges through the combined activities of at least eight, mostly lineage nonspecific, transcription factors under the influence of Notch pathway signals from the thymic microenvironment. The challenge has been to understand the mechanisms operating in this multicomponent system.

Here, we seek to make explicit the regulatory structures and some aspects of combinatorial control that underlie T lineage specification in mice. This synthesis combines evidence from: (*i*) purifying staged T cell precursors from the stem cell through the commitment stage; (*ii*) defining multiple transcription factor gene expression changes that distinguish these stages *in vivo*; (*iii*) characterizing the impacts of Notch signaling on gene expression at individual stages, using *in vitro* culture systems to control delivery of Notch signals; and (*iv*) a perturbation analysis based on manipulation of two key transcription factors that are thought to drive opposing network subcircuits in the T cell development process. We compare the likely inputs of three regulators on the developmental trajectory of the cells and present a combinatorial map of regulatory connections as a testable framework for reconstructing the full process.

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Table 1. Gene expression changes marking transitions in T-cell development

Gene category	Initiation, stem to DN1 increase	DN2 entry, DN1 to DN2 increase	Specification, DN1–DN3 continuous		Commitment, DN3 stage		β-Selection	
			Increase	**Decrease**	Increase	**Decrease**	Increase	**Decrease**
Transcription factors	GATA-3 TCF-1 HES1	Bcl11b HEB-alt	GATA-3 TCF-1 (Tcf7) HEB (Tcf12) Ikaros Gfi1 Runx1 Ets1	**PU.1** **SCL** **GATA-2** **Id2** **Runx2** **Runx3** **Gfi1B** **C/EBPα** **(& others)**	Ets2 Pou6f1 SpiB HES1 LEF-1 Id3	**PU.1** **SCL**	Aiolos SATB1 Tbet	**SpiB** **HES1** **Myb** **Runx1** **HEB-alt** **Erg** **Id2** **(and others)**
Targets/other	Deltex1	CD3γ CD3ε	IL-7Rα CD25 Rag-1 CD3ε Lck LAT ZAP70	**Mac-1**	Deltex1 pTα (Ptcra) Rag-1 Notch3 Lck LAT ZAP70	**c-Kit**	TCR-Cα ZAP70	**Deltex1** **IL-7Rα** **pTα (Ptcra)** **Rag-1** **CD25** **Bcl2** **Notch1**

Results and Discussion

Early T Cell Developmental Progression Through Regulatory Gene Expression Space. Mouse T cell precursors go through canonical stages between entry into the thymus and full commitment to the T cell lineage, distinguishable by changes in surface markers and quantitatively distinct patterns of gene expression. The same stages are used in fetal T cell development, adult T cell development, and when T cell precursors are induced to differentiate in stromal culture *in vitro*, although the kinetics of the progression differ among fetal, adult, and *in vitro* development (7–9) (D.D.S.-A., unpublished data). Cells proliferate in each of the first two or three stages for several days before moving on to the next one. Thus, unlike embryonic systems where autonomous progression from state to state is "hard-wired" in the regulatory circuitry, T cell specification is inherently discontinuous: progression between states may depend on repeated microenvironmental stimulation (10).

The characteristic gene expression patterns of these stages are benchmarks for comparison of normal and perturbed versions of T cell development (Table 1). To represent these complex changes in a simpler form, we have combined the expression profiles of >140 genes (7–9, 36) to compute principal components of regulatory change across the T lineage specification process [see supporting information (SI) Text and Tables S1–S4]. For gene expression levels, we used quantitative real-time PCR data for cells from the earliest, still multipotent intrathymic stage (DN1 cells) through commitment (to DN3a), and through the first T cell receptor signaling response, "β-selection" (to DN4). Partial least-squares analysis of the gene expression data was used to calculate the principal components of variance that best separate the c-Kit$^+$"DN1" or "early T cell precursor" (ETP) stage; the c-Kit$^+$ CD25$^+$ CD44$^+$"DN2" stage; the CD25$^+$ CD44$^-$ CD27-low "DN3a" stage; the CD25$^+$ CD44$^-$ CD27$^+$"DN3b" stage; and the CD25$^-$ CD44$^-$"DN4" stage (11) (E.-S.D.-F. and M.A.Y., unpublished work). Tables S2–S4 list the coordinates for each gene and each stage along axes representing the first four principal components.

Each developmental stage can be viewed as a unit vector in one of five orthogonal dimensions. The path connecting the ends of the five DN-stage vectors in sequential order describes the developmental trajectory of thymocytes. Projected onto 2D principal component plots, a DN stage vector appears long if the selected principal component axes capture most of the difference between that DN stage and others. Genes with relatively constant expression are placed near the origin (center of the graph). Genes with the highest change in expression are furthest from the center. The more similar the patterns of expression of two genes, the smaller the angle between them from the center.

Fig. 1*A* and Fig. S1 show that the first principal component (*x* axis) accounts for most of the gene expression differences between the DN1 and the DN3a/3b stages. The second principal component (*y* axis) accounts mostly for differences between DN3a cells and β-selected cells. Subtler changes are captured in the third and fourth principal components. The first part of the developmental path toward commitment is nearly parallel to the first principal component axis, as the cells abandon their use of prethymically expressed regulatory genes such as *Sfpi1* (encoding PU.1), *Tal1* (encoding SCL), *Gata2*, and *Gfi1b* (at the DN1/2 end of the axis). Then, successful T cell receptor expression triggers a shift along the second principal component axis. The intermediate DN2 stage involves a distinct excursion along the third axis (Fig. S1). This trajectory is what should be explained by transcriptional linkages in a gene regulatory network for T cell specification. Three vital nodes of the network are examined here.

PU.1 and GATA-3 as Regulatory Inputs. The transcription factors PU.1 and GATA-3 are both required for early T cell development. Loss of either transcription factor from a prethymic stage virtually eliminates T cell development, but overexpression of either one in early intrathymic stages is also inhibitory, blocking the generation of cells capable of undergoing β-selection. When overexpressed, both can push DN thymocytes toward alternative hematopoietic developmental fates: PU.1 toward the dendritic cell or monocytic lineages, GATA-3 toward the mast-cell lineage. High doses of either PU.1 or GATA-3 inhibit particular sets of T cell genes while activating distinct sets of non-T genes (12–14).

These factors have been expected to oppose one another in T cell development, based on a key precedent in other blood cell fate decisions. The related GATA factor GATA-1 and PU.1 apparently act as mutually inhibitory competitive antagonists in a bistable switch to control the choice between erythroid or megakaryocytic fates, on the one hand, and all myeloid or lymphoid cell fates, on the other hand (reviewed in ref. 15–17). GATA-1 and PU.1 proteins titrate each other's activities antagonistically, while enhancing their own respective expression by positive transcriptional autoregulation. To adapt such a model to the genetic requirement for both PU.1 and GATA-3 in T cell development, PU.1 might support gene expression associated

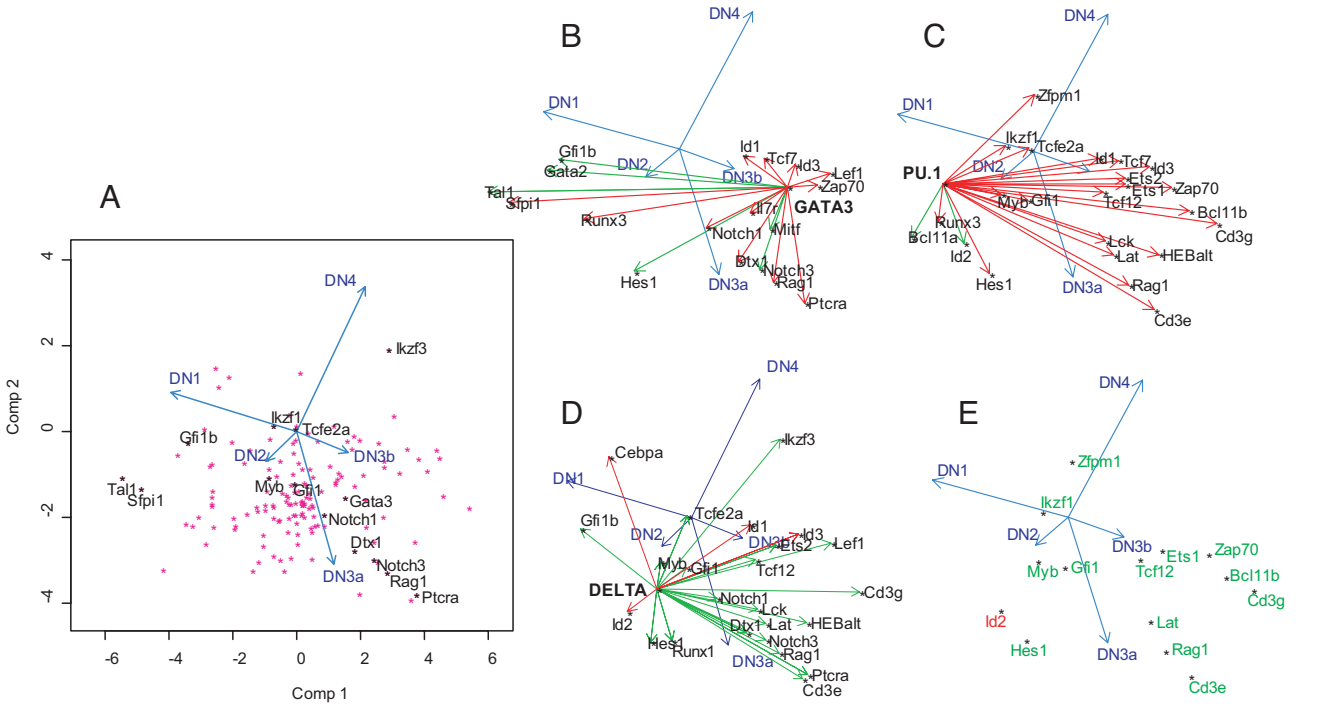

Fig. 1. Gene expression changes during normal T lineage specification and regulatory perturbation: depiction in principal component space. (*A*) Coordinates of key genes (black or magenta stars) and gene expression signatures of DN1, DN2, DN3a, DN3b, and DN4 stages, projected on axes representing the first two principal components of gene expression change (full data in Fig. S1 and Tables S2–S4). (*B*) Targets of GATA-3 overexpression in fetal thymocytes, positioned relative to first two principal component axes shown in *A* (14). The coordinates of the normal, adult DN1–DN4 phenotypes are shown for orientation by blue vectors. Genes positively affected by GATA-3 overexpression are targets of green arrows, with negatively affected genes indicated by red arrows. (*C*) Targets of PU.1 overexpression in fetal thymocytes (13), depicted as in *B*. (*D*) Effects of short-term exposure to Notch–Delta signaling in fetal thymocytes. These effects, compiled from refs. 13 and 14, are calculated independently of effects of PU.1 or GATA-3 in the experiments. (*E*) Supraadditive modulation of PU.1 effects by Notch–Delta signaling ($P < 0.05$; Tables S5 and S6). Genes that are protected from repression supraadditively by Notch–Delta signaling are shown in green. A gene that is prevented from up-regulation by Notch–Delta signaling is shown in red.

with "immaturity," whereas GATA-3 could promote gene expression associated with T lineage commitment. The expression patterns and functions of PU.1 and GATA-3 are indeed divergent (6, 8, 9, 18): from a high initial level of expression, PU.1 is sharply and permanently down-regulated during T lineage commitment (DN1–DN3a) *in vivo*, whereas GATA-3 rises gradually and functions repeatedly throughout T cell development (Table 1). These expression patterns fall near opposite ends of the first principal component axis in Fig. 1*A*; analyzing detailed impacts of exogenous PU.1 or GATA-3 should detect whether opposition between these two factors actually controls the position of cells along this axis.

As T cell development is blocked when either PU.1 or GATA-3 is overexpressed, and because either factor at high level can sequester the other, double overexpression experiments may not prove that antagonism is relevant *in vivo*. However, if PU.1 and GATA-3 did titrate each other in normal thymocytes, each should normally be limiting the other's activity during the DN1 and DN2 stages when both are present. Then, experimentally increased expression of either factor should oppose the distinctive pattern of gene expression effects attributable to the other factor (Fig. 1 *B* and *C*). The magnitudes of the PU.1 and GATA-3 effects and the statistical significances determined by ANOVA are shown in Fig. S2 and Tables S5 and S6 (negative T statistic values indicate repression). Fig. 1 *B–E* show interactions that exceed a *P* value threshold of 0.05 (after correction for multiple hypothesis testing).

The overexpression effects of PU.1 generally oppose the trends of DN1 to DN3a differentiation (Fig. 1*C*), as PU.1 represses a wide range of genes used in the DN3 stages. Although these may not all be direct targets, they support the idea that high-level endogenous PU.1 in the DN1 stage could help to delay gene expression changes associated with T lineage progression, perhaps to allow continued self-renewal. Conversely, high-level GATA-3 does increase expression of a T lineage supporting gene, *Hes1*, and also represses PU.1 (*Sfpi1*) RNA expression in thymocytes (12, 14, 19)(Fig. 1*B*). This effect is confirmed by GATA-3 loss-of-function phenotype (D.D.S.-A., unpublished work) and by effects of GATA-3 coexpression with a PU.1 *cis*-regulatory sequence reporter in myeloid cells (M.A.Z., unpublished work). Although additional regulatory inputs are likely needed to explain the steepness of PU.1 repression in thymocytes (20, 21) (M.A.Z., unpublished work), GATA-3 may be a substantial contributor to the repression mechanism.

However, high-level GATA-3 does not show other effects expected for a T lineage promotion factor. Even setting aside its activation of "non-T" mast cell genes (14) and focusing on T lineage genes only, many effects of high-level GATA-3 also oppose DN3a-specific gene expression (Fig. 1*B*). Consider T cell genes *Myb*, *Gfi1*, *Cd3e*, *Lck*, *LAT*, *Rag1*, *Tcf7* (encoding TCF-1), *Ets1*, and *Ets2*, which are induced from DN1 to DN3a (Table 1). All of these genes are down-regulated by exogenous high-level PU.1, but not enhanced by higher GATA-3: they are either unaffected or frankly down-regulated by high-level GATA-3 as well. Furthermore, *Lef1*, another DN3-stage up-regulated gene, is actually inhibited by GATA-3 but unaffected by PU.1. Two additional features of the PU.1: GATA-3 subnetwork contrast with those of PU.1/GATA-1 interaction. First, PU.1 shows little or no ability to inhibit GATA-3 expression in thymocytes (13). Also, GATA-3 cannot enhance its own expression in these cells (14).

These data imply that the relationship between PU.1 and GATA-3 in T cell development differs from the bistable switch

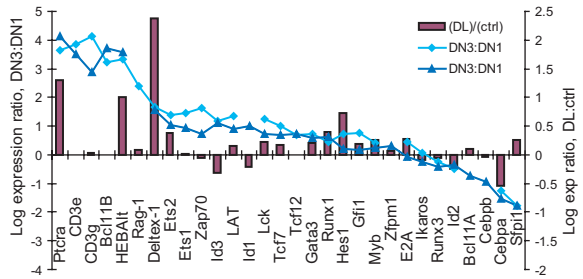

Fig. 2. Direct Notch regulation of early T cell genes compared with developmental regulation during the DN1 to DN3 transitions. Line graphs show the $\log_{10}$ of the ratio of expression of indicated genes in adult DN3a cells relative to DN1 cells in two independent studies (7, 8) (E.-S.D.-F. and M.A.Y., unpublished results); breaks indicate genes not included. Bars show the effect of 24-h Notch–Delta signaling on gene expression in Thy-1$^+$ embryonic day 15.5 fetal thymocytes after a 16-h preculture without Delta. Secondary *y* axis gives $\log_{10}$ of the expression ratio with and without restored Notch–Delta signaling. For additional results see Fig. S3. Data are from empty vector controls in ref. 13.

model imputed to PU.1 and GATA-1. If GATA-3 promotes progression to DN3a, it probably does so not by titrating PU.1 directly to relieve PU.1 repression of T cell genes, but rather by helping to repress transcription of PU.1 itself. GATA-3 clearly provides other unique regulatory functions for early T cell development, but these are likely governed by essential GATA-3 dose–response requirements at specific target genes rather than by opposition of GATA-3 to PU.1.

Notch–Delta Signaling as a Direct Regulator of T Lineage Specification Genes. T cell specification depends completely on Notch pathway triggering by interaction with Delta-class ligands in the environment. Notch–Delta signaling is needed from the earliest DN1 stage through commitment and into β-selection. Some of its most direct effects are seen by incubating defined populations of immature thymocytes with stromal cells that either do (OP9-DL1) or do not (OP9-control) express Delta (22), then reisolating the thymocytes and measuring gene expression. Results from experiments that measured these effects and PU.1 or GATA-3 effects are shown in Fig. S3. Fig. 1*D* shows that many of the regulatory effects, direct and indirect, of Notch–Delta signaling in thymocytes are to activate genes associated with the DN3–DN4 stages. The aggregate effects of Notch signaling on gene expression broadly appear "opposite" to those of PU.1 overexpression in Fig. 1*C*. Thus Notch–Delta signaling appears to be more closely associated with DN3-specific regulatory events than does GATA-3 (Fig. 1*B*).

Is the DN3a stage phenotype then simply a reflection of increasing activation of direct Notch target genes or must additional factors play a rate-limiting role? Fig. 2 shows a direct comparison of the developmental regulation and Notch–Delta dependence of multiple thymocyte genes; additional data are shown in Fig. S3. In Fig. 2, ratios of gene expression in DN3a to DN1 are shown in order of highest to lowest DN3 up-regulation (line graphs: results in two independent experiments) (7, 8), with the effects of short-term Notch signaling on the same genes superimposed (bars) (13, 14). The patterns do not match. Known direct Notch target genes *Deltex1*, *Hes1*, and *Ptcra* (T cell receptor surrogate α chain, pre-Tα) that peak in expression at the DN3a stage are strongly affected by Notch–Delta interaction. However, this is not true for other T lineage genes, such as the regulatory genes *Gata3* and *Bcl11b*, and the genes encoding T cell receptor complex proteins *Cd3g* and *Cd3e* (8, 9). These genes, too, are only induced in hematopoietic precursors through a Notch-dependent regulatory cascade (9, 23), but once activated in Thy-1$^+$ thymocytes (DN2 stage or beyond), these landmark genes and others with maximal expression at the DN3a stage become much less dependent on Notch–Delta interaction (Fig. 2). Thus, regulatory inputs besides Notch (and GATA-3) control the gene expression trajectory through the DN2 and DN3 stages, and these must be included in a T cell gene regulatory network.

Modification of Regulatory Factor Effects by Interactions with Notch. Notch signaling makes another kind of contribution to the regulatory state of the developing cells: to modulate the effects of other regulators in a gene-specific and factor-specific way. In prethymic hematopoietic precursors, Notch signaling synergizes with the effects of the basic helix—loop—helix factor E2A to enhance activation of T lineage-associated genes such as *Ptcra* (24). In fetal thymocytes, Notch signaling modulates the effects of PU.1 selectively to relieve its repression of T lineage genes (13). Note that cells can receive both Notch and PU.1 regulatory inputs independently without direct antagonism. Notch–Delta signaling does not repress *Sfpi1* (PU.1) itself, and even at high levels PU.1 in thymocytes does not inhibit expression of Notch1 or Notch3, nor interfere with Notch–Delta-dependent induction of *Ptcra* or *Deltex1*. However, when effects of PU.1 are assessed in the presence or absence of Notch–Delta signaling, a statistically strong interaction is seen, such that Notch/Delta signals block PU.1 effects on many genes.

Of 23 early T cell genes affected by PU.1 in our analysis, as many as 11 of them (*Myb*, *Hes1*, *Ikzf1*, *Gfi1*, *Cd3e*, *Rag1*, *Lat*, *Bcl11b*, *Zap70*, *Ets1*, and *Tcf12*) were protected supraadditively from PU.1 by Notch–Delta signals ($P < 0.05$; Fig. 1*E* and Table S5). In addition, Id2 was blocked from induction by PU.1 by Notch–Delta signals at $P = 0.06$. The Notch-protected genes were particularly associated with the DN3 states, and the interaction was specific, because it did not apply to other genes regulated by PU.1 and Notch, such as genes associated with myeloid lineage redirection (13). In contrast, effects of high-level GATA-3 were less influenced by Notch signaling. Of 22 early T lineage genes affected by GATA-3, Notch–Delta signaling only modified three in a supraadditive way, reducing the positive effects of GATA-3 on *Hes1* and *Mitf* and protecting *Tcf7* from repression (Table S6).

Thus, PU.1 effects on the progression of thymocytes from DN1 to DN3a can only be evaluated in relation to the status of Notch signals, whereas GATA-3 effects essentially depend on level.

Assembly of a Framework for the T Cell Specification Gene Regulatory Network. We have used BioTapestry software (www.BioTapestry.org) (25) to make explicit the network of regulatory relationships that appear to operate as committed early T cell precursors emerge from hematopoietic stem cells and other pluripotent progenitors. Such a network integrates the available data on regulatory inputs into each of the important genes in a process. It provides a validation map for assessing to what extent available information can account for the pattern of expression of individual genes and for the coordination of expression of groups of genes through the course of the process. Although yet incomplete, this network provides a useful armature for the regulatory relationships involved in T cell specification.

To construct this model, we have combined gene expression data for normal thymocyte subsets and perturbation data for PU.1, GATA-3, and Notch regulatory effects. Because PU.1 and GATA-3 can promote lineage redirection to myeloid/dendritic and mast-cell fates, respectively, regulatory pathways involved in these fate alternatives (26, 27) are also included. Also incorporated is evidence from the literature on some additional Notch inputs (28), possible linkages both upstream [TCF-1 (*Tcf7*), Runx (20, 21)] and downstream [Gfi1, Egr2 (2)] of PU.1, and additional data on changes in gene expression that follow commitment, during TCR-dependent selection in the DN3b and DN4 stages (8, 11, 29). Of special interest are also connections

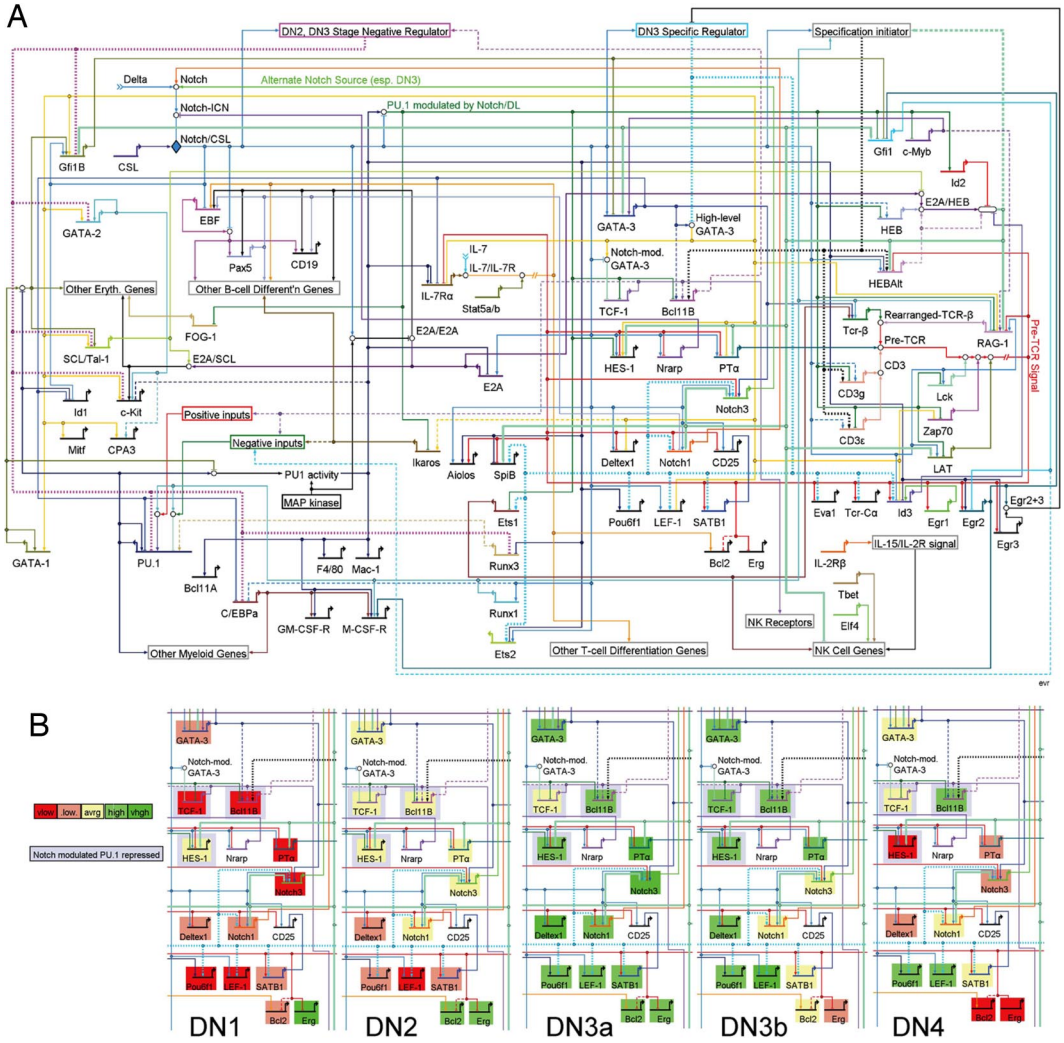

Fig. 3. Gene regulatory network model for T cell specification. (*A*) View from all nuclei: comprehensive map of relationships included in the network, integrating over all stages. For sources of each link, see Table S7. For expanded size figure, see Fig. S4*A*. For predicted differential activity of different network links at different stages, see Fig. S4 *G–K*. (*B*) Close-ups of one region of the network with background highlighting indicating differential gene expression levels at five different developmental states. For full network versions, see Fig. S4 *B–F*.

involving the basic helix—loop—helix E proteins. These contribute to the DN3 stage checkpoint, TCR gene rearrangement, and inhibition of alternative NK and myeloid fates (24, 30–35). A static view of this BioTapestry network is shown in Fig. 3. Full-sized screen shots and full annotation of the individual links are provided in Fig. S4 and Table S7. In the interactive BioTapestry viewer posted on line (www.its.caltech.edu/~tcellgrn), we also provide dynamic views of changing network states through the DN1 to DN4 transitions, all of the raw data from our group's publications on which the network links are based, and continuous updating of the annotations of data for individual links.

The biology of this system has required three modifications of the usual gene regulatory network depiction (25). First, the effects of Notch signaling on the activities of other regulators have had to be taken into account. Besides the independent *cis*-regulatory inputs of Notch and its transcription factor CSL, our results require "processing" of the effects of transcription factors such as PU.1 by some agents of Notch–Delta signaling. Second, GATA-3 is a particularly stark case showing that the same factors make different network links when expressed at different concentrations. For example, GATA-3 at high level shuts off *Il7ra*, which is normally coexpressed with *Gata3*, while it induces other genes (*Tal1*, *Gata2*, *Gfi1b*) that would normally be turned off at stages when *Gata3* expression peaks.

Third, the data reviewed above identifies three obvious gaps in using Notch, GATA-3, PU.1, or other known factors, to account for T cell development. These have been filled by "placeholders." A specification inducer, not Notch/CSL itself, is needed to account for the first up-regulation of *Bcl11b*, *HEBalt*, and the *Cd3* genes at the DN2 stage. A DN3-specific gene activator seems necessary, beyond known effects of Notch signaling (Fig. 2) and E protein activity (24, 30, 32), to account for the full pattern of DN3a-stage gene activation. Finally, a repressive T lineage commitment function is needed to account for the timing of repression of *Sfpi1*, *Tal1*, *Gfi1b*, and other non-T lineage-promoting regulatory genes during the DN2 to DN3 transition. By placing these functions in a network context, regulatory pathways involving known factors may be discovered that are equivalent to those involving these placeholders, and thus help to establish the molecular identities of these agents.

Concluding Remarks

T cell specification is not governed by a few dedicated transcription factors operating in a simple regulatory pathway. It can only

be understood in gene network terms. It depends on multiple transcription factors, almost every one of which is also used, in other combinations, for other hematopoietic programs. The close linkage of T cell development to other hematopoietic fates is hammered home by the ease of diverting T cell precursors to other lineages, when the same transcription factors that are normally part of the T cell program are overexpressed. This sharing is typical for hematopoietic lineage decisions, in which the same transcription factors expressed in different ratios or in different temporal orders yield different cell types.

However, this kind of system also makes gene network construction difficult. The regulatory meaning of every transcription factor completely depends on level and context. To explain how factors like GATA-3 can be used for T cell development at all, it has been crucial to build into the network diagram dose-sensing nodes, which channel transcription factor input to different downstream genes at high factor levels than at low factor levels. Such nodes are not commonly needed in the embryonic specification gene networks developed to date, where transcription factors can act in a quasi-Boolean way because of strongly forward-driving network architecture. For T cell development, a most important part of the context is provided by Notch pathway signaling throughout T cell specification. Notch not only provides its own transcriptional input but also modifies the effects of both high-level GATA-3 and PU.1. Thus, to explain the use of PU.1, it has been crucial to include a node for filtering this transcription factor's regulatory effects through transformations, still poorly understood, that depend on Notch–Delta signaling.

The network presented here is a framework, not a complete solution. Only a limited number of perturbations have been tested so far in the temporally defined and stage-specific way that is needed to discern proximal downstream regulatory targets. The ultimate goal of explaining biology on the basis of internal network structure must await identifying the targets of other regulators. Also, as we have emphasized, there are a number of stage-specific functions required for T cell development that remain to be identified, as they cannot be accounted for by effects of Notch signaling or GATA-3, and do not appear to be explained by the expression patterns of other known factors. Two positive T lineage-promoting functions are needed at different stages, and at least one lineage exclusion function is needed for commitment. Whether these are mediated by single factors or network subcircuits remains to be defined. The network armature developed here nevertheless shows just where additional regulators must be invoked. The completion of these links will ultimately reveal how the architecture of the T cell network explains the distinctive mode of T lineage specification.

Materials and Methods

See full materials and methods in *SI Text*. Regulatory and marker gene expression was measured by quantitative real-time PCR as described (7, 9, 29). Four representative sample series of DN subsets were used for Fig. 1. Expression levels were normalized to β-actin expression in the same samples, $\log_{10}$ transformed, and then submitted to partial least squares analysis and ANOVA (corrected for multiple hypothesis testing) as described in *SI Text*.

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