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Eliciting the T cell fate with Notch

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Abstract

Multipotent progenitors arrive at the thymus via the blood. Constraining the non-T cell fates of these progenitors while promoting the T cell fate is a major task of the thymus. Notch appears to be the initial trigger for a developmental program that eventually results in T cell lineage commitment. Several downstream targets of Notch are known, but the specific roles of each are poorly understood. A greater understanding of how Notch and other thymic signals direct progenitors to a T cell fate could be useful for translational work. For example, such work could eventually allow for the generation of fully competent T cells in vitro that could supplement the waning T cell numbers and function in the elderly and boost T cell-mediated immunity in patients with immunodeficiency and after stem cell transplantation.

Keywords

Hematopoietic stem cell; Selective thymic seeding; Early T progenitors; T-cell lineage commitment; Notch signal

1. Many extrathymic progenitors with T lineage potential

All blood cell types originate from multipotent, self-renewing hematopoietic stem cells (HSCs), via non-renewing multipotent progenitors (MPPs), cell types that reside predominantly in the bone marrow [1,2]. As self-renewing HSCs are not present in the thymus, T cell development must be sustained by the continuous importation of hematopoietic precursors that travel via blood from the bone marrow [3].

The precise identity of the progenitors that physiologically settle the thymus from blood remains unknown, because the number of such thymus-settling progenitors is thought to be very small [4–6]. Multiple progenitors in BM have been shown to possess T lineage potential when placed in the thymus, including HSCs and MPPs [7]. A fraction of cells with an MPP (LSKFlt3⁺) phenotype also express RAG genes; these cells have been termed early lymphoid progenitors (ELPs) [8]. ELPs are efficient lymphoid progenitors that retain a degree of myeloid lineage potential [8]. They overlap extensively with populations of lympho-myeloid progenitors termed lymphoid-primed multipotential progenitors (LMPPs) (LSKFlt3^{hi}) [1,9].

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Further downstream of LMPPs, common lymphoid progenitors (CLPs) ($\text{Lin}^{-}\text{Sca1}^{\text{lo}}\text{Kit}^{\text{lo}}\text{Flt3}^{\text{hi}}\text{IL7R}\alpha^{+}$) also possess T cell potential [10–12]. Very recently, it has been demonstrated that CLPs in BM are heterogeneous; efficient T cell potential resides in a subset expressing low levels of RAG-1, whereas RAG-1^{hi} cells are instead B lineage-specified [13–15].

2. The ability to settle the thymus is selective

For BM progenitors to contribute to T cell development, they must egress the BM, circulate in the bloodstream, and finally settle in the thymus. It has become clear in recent years that only selective BM progenitors have the capacity to settle the thymus from blood [16]. In particular, HSC and MPP appear to be normally excluded from thymic settling [17]. This indicates that only a subset of the many BM progenitors with T-lineage potential can physiologically contribute to T cell development. Much work from several groups has converged on the previously defined LMPP/ELP and CLP populations as containing physiologically relevant T cell progenitors [15,18–22]. Identification of homing molecules important for thymic recruitment of BM-derived progenitors has helped to better define the most relevant thymic settling progenitors. It is hypothesized that thymic settling progenitors enter the thymus by utilizing similar mechanisms as mature lymphocytes entering lymph nodes [23]. In this model, selectin molecules first slow the progenitor and cause it to roll along the endothelial surface. Chemokine receptor signaling then activates integrin dimers into high-affinity conformations. Finally, these integrins mediate the firm arrest and subsequent extravasation of the cell across the endothelium. Indeed, the selectin/ligand pair P-selectin/PSGL-1 have been demonstrated to play a role in thymic settling [24]. The chemokine receptors CCR7 and CCR9 each independently support the migration of progenitors into the thymus [21,22,25,26]. Furthermore, the absence of both CCR7 and CCR9 on progenitors leads to their near-absolute restriction from thymic settling. Finally, limited evidence suggests that the integrin subunits $\alpha 4$ and $\beta 2$ integrin may also be involved in this process [21,23,25–28]. Together these data indicate that cells bearing one or more of these molecules are likely to represent some, if not all, of the cells that settle the thymus. Cells expressing P-selectin, CCR7, or CCR9 can indeed be found in the BM and constitute fractions of both the lymphoid-primed multipotent progenitor (LMPP) and common lymphoid progenitor (CLP) pools [2,8,9,17,19].

Although the precise identity of thymus settling progenitors remains to be better resolved, it seems clear that many progenitors entering the thymus are not yet committed to the T-lineage, but instead retain multi-lineage potential [29–31]. As we discuss, intrathymic Notch signals play a key role in initiating a developmental program that ultimately results in T cell lineage commitment. It has also been demonstrated that T cell commitment can occur extrathymically following irradiation [32,33] and in normal mice [32,34]. In the circumstance of irradiation, the generation of extrathymic T-lineage committed progenitors also relies on Notch signals [35]. In this review, we describe the cells that undergo commitment to the T cell lineage, and the molecular mechanisms governing this process within the thymus.

3. Intrathymic progenitors maintain non-T lineage potentials

Multipotent progenitors are suggested to enter the thymus via the blood vessels near the corticomedullary junction [36]. Following extravasation out of the circulation, these cells come into contact with thymic stromal cells, an interaction that will ultimately induce commitment to the T cell lineage and suppress alternative lineage potentials [36,37]. The earliest hematopoietic progenitors within the thymus have the ability to proliferate extensively and to generate all thymocyte populations, including both $\alpha\beta$ and $\gamma\delta$ T cells [38]. These early T-cell progenitors (ETPs) are minimally defined as lineage-marker ($\text{Lin}^{\text{lo}}\text{CD25}^{-}\text{Kit}^{+}$) and constitute approximately 0.01% of the young adult mouse thymus, though the frequency of these cells

declines with age [7,38,39]. ETPs are multipotent and can generate T, B, natural killer (NK) cells, myeloid and dendritic cells (DC) [40–43].

Though the ETP population is small, it is heterogeneous, suggesting multiple incoming progenitor types and/or that developmental transitions exist within this population. Subsets of ETPs can be identified based on expression of Flt3, CD24, and CCR9 [18,41,42]. Flt3⁺ ETPs are developmentally less mature than their Flt3^{lo} counterparts [41]. These Flt3⁺ ETPs overlap with CCR9 expressing ETPs, and this CCR9⁺Flt3⁺ population contains rare cells with B cell potential [18,41]. CCR9⁺Flt3⁺ ETPs proceed down the T-lineage pathway by differentiating into CCR9⁺Flt3^{lo} ETPs, and subsequently into CD4/CD8 double-negative 2 (DN2) cells. DN2 cells, which are defined phenotypically as Lin⁺CD25⁺Kit⁺, retain NK and DC lineage potentials. Depending on cell surface expression of Kit, DN2 cells are subdivided into DN2a (Kit^{hi}) and DN2b (Kit^{mid}) populations; recent evidence shows that DC potential is lost in DN2b cells [44]. DN2b cells further downregulate expression of CD44 and Kit, and differentiate into DN3 cells, which are now fully committed to the T cell lineage. DN3 cells subsequently differentiate into CD4⁺CD8⁺ double-positive cells, which undergo positive and negative selection and then migrate into the periphery to generate the mature immunocompetent T cell pool [45]. Hence, T cell commitment is a prolonged process, and loss of all non-T lineage options only occurs after prolonged intrathymic residence, by the DN3 stage.

4. Notch acts after thymic settling to promote the T cell fate

Notch signaling is an essential component of T lineage commitment, and is necessary and sufficient to impose the T cell fate on multipotential hematopoietic progenitors. Expression of constitutively active Notch1 in HSCs results in extrathymic T cell development and suppression of BM B cell development [46] indicating that Notch signals are sufficient for T-lineage development. Conditional knockout mice have been developed in which Notch1 is inactivated in BM precursors using the Cre-loxP targeting strategy. The loss of Notch1 in hematopoietic progenitors results in a thymus of markedly reduced overall cellularity containing mainly B cells [47]. When Notch signaling was blocked by constitutively expressing a dominant-negative form of the critical Notch signaling intermediary Mastermind-like 1 (DNMAML), ETPs were no longer detectable, though upstream progenitors in BM and blood appeared intact [41,45,48]. These results indicate a requirement for Notch in T cell development only after thymic settling, though the precise basis for this requirement remains unknown.

Canonical Notch signaling requires ligand-receptor interaction. Interactions between Notch and its ligands are regulated by stage-specific expression of the fringe glycosyltransferases [49–51]. In mammals, 5 Notch ligands have been identified which belong to two different families, Jagged (Jag1 and Jag2) and Delta-like (DLL1, DLL3, and DLL4) [52]. Expression of nearly all of these ligands has been detected in thymic epithelial cells [53–57]; however, mice deficient in Jagged1 and Jagged2 do not have gross perturbations in T cell development [58,59]. Additionally, when ectopically expressed on bone marrow stromal cell lines, DLL1 and DLL4, but not Jag1 or Jag2, can support T cell development in vitro up to the CD4/CD8 double positive (DP) stage [60]. Thus, T cell development appears to depend largely on Notch1 signals delivered by members of the Delta-like ligands. Surprisingly, conditional inactivation of Dll1 in thymocytes and/or thymic epithelial cells (TECs) does not inhibit T cell development [61]. This result could be accounted for by overlapping or redundant functions of DLL1 with DLL4, though DLL4 binds with higher affinity to Notch1 than does DLL1 [62]. Recently, however, several groups have independently explored the function of DLL4 within the thymus using conditional loss of function approaches. Dll4^{lox/lox} control mice were crossed to mice expressing Cre recombinase under the control of the Foxn1 gene, which is highly expressed in thymic epithelial cells, to generate Dll4-floxed:Foxn1-Cre mice [56,57]. Inactivation of Dll4

within the thymic epithelium led to a complete block in T cell development and an accumulation of thymic B cells, thus recapitulating the phenotype observed when Notch1 itself is deleted. These data provide evidence that progenitors require intrathymic DLL4 Notch ligands to undergo T cell lineage commitment, and further strengthen the idea that Notch signals are required very early after progenitor entry into the thymus.

In addition to its essential role shortly after entry into the thymus, Notch signaling is critical at multiple steps in thymic T cell development. In Notch1^{+/-}:Notch1^{+/-} mixed bone marrow chimeras, the degree of Notch1^{+/-} cell chimerism declined with each progressive developmental step in the thymus. These results indicate that Notch1 signaling is necessary at several early stages of intrathymic T cell development [63]. As mentioned, ETPs and also some DN2 cells can adopt non-T-lineage fates, but this only occurs readily if Notch signals are withdrawn [30,31,64,65]. Final T cell commitment does not occur until the DN3 stage, coincident with the down regulation of the PU.1 transcription factor. The reason non-T cell lineage potentials are retained for such a prolonged period after thymic settling is puzzling. One suggestion is that early T cell development requires transcription factors and other genes that are also required for development of alternative lineages; this economy may come with a consequence of developmental instability [66]. Another possibility discussed in more detail in subsequent sections is that T cell progenitors may normally contribute to non-T cell lineages within the thymus [30,31].

5. Notch signals constrain ETPs from adopting alternative fates

Evidence that Notch is required to constrain alternative fates in T cell development derives from the observation that cells of alternative lineages expand in the thymus when Notch signaling is inhibited. Previous work established that very few ETPs are evident in the thymus when Notch signaling is prevented [41,63]. Instead, B cell populations expand when Notch1 is conditionally ablated in bone marrow progenitor populations [47,67–69]. Cells in which Notch1 is deleted at the ETP stage also adopted the DC fate, suggesting that Notch1 is also required to restrain early progenitors from adopting DC fates [68]. It is important to understand the limitations of these studies as such observations establish only that some of the incoming progenitors can adopt B cell or DC fates in the absence of intrathymic Notch signals. These studies cannot establish whether progenitors with B potential are the only or even the dominant, progenitor population for T cells that settles the thymus. Although some thymic B cells likely derive from Notch-ablated T cell progenitors in these experiments, it is apparent that the majority of B cells expanding in the thymus after Notch ablation do not derive from T lineage progenitors [68]. Instead, ablating Notch1 and so blocking T cell development may allow the thymus to be invaded by other progenitor populations that give rise to B cells. The thymus is able to support B cell development, as it contains lymphopoietic cytokines such as IL-7; however, it may be less permissive for myeloid development or development of other lineages [70]. Hence, it may be important that Notch signals suppress B cell potential very soon after thymic entry of multipotent progenitors, as the thymus would otherwise support their development and expansion down the B cell lineage. The majority of ETPs have no detectable B lineage potential, which is consistent with evidence that Notch1 signaling is a very efficient and rapid mechanism for suppressing B cell development [55]. An alternative explanation for the paucity of ETPs with B cell potential is that some ETPs may derive from progenitors that lose B cell potential prethymically [42]; this possibility needs to be further explored.

Although B cell lineage potential is lost early in T cell development, by the ETP stage, complete loss of other non-T lineage potentials and full T cell commitment is not achieved until the DN3 stage. DN2 cells possess DC and NK potential [44,55]. In addition, the majority of ETPs and some DN2 cells also retain myeloid lineage potential [30,31]. Single ETPs when cultured in appropriate conditions can generate both T- and myeloid lineage progeny. While the myeloid

potential of ETPs at the population level has been long recognized [64,71,72], recent reports demonstrate the presence of T/myeloid bi-potent progenitors in the thymus. Notably, these myeloid lineage potentials of thymocyte progenitors were most readily revealed when ETP or DN2 progenitors were removed from Notch signals. Ectopic expression of PU.1 in DN3 cells was previously shown to induce the formation of Mac1+ dendritic cells whereas expression of C/EBP α induces the generation of functional macrophages. This induced differentiation was also inhibited when DN cells were transduced with essential T cell transcriptional regulators GATA-3 or with constitutively active intracellular Notch1 (ICN) [73,74]. These results provide further evidence that Notch1 signaling in the thymus may constrain alternative lineage potentials. Other past work has established that Notch1 signaling was able to restrict myeloid development by inducing GATA-2 expression from multipotential progenitors including embryonic stem cells, fetal liver and adult bone marrow [74–76]; the recent observations that most early thymocyte progenitors possess a degree of myeloid potential suggest that this ability of Notch signals to suppress myeloid outcomes may be functionally important for T cell development. The mechanisms downstream of Notch signaling that prevent myeloid development in the thymus are at present poorly understood.

Whereas mechanisms clearly exist by which Notch signals inhibit myeloid and other non-T cell lineage development from early progenitor populations, it is also evident that some intrathymic progenitors normally escape these inhibitory effects of Notch, and adopt non-T cell fates within the thymus. A lineage-tracing approach was used to show that a population of thymic granulocytes derives from RAG-expressing and hence lymphoid-specified progenitors [31]. How this occurs is unknown. It is possible that some cells express higher levels of molecules such as Numb, Nrarp (Notch-related ankyrin repeat protein) or Deltex that are negative regulators of Notch signaling [37]. Alternatively, some progenitors might occupy progenitor niches in which Notch ligands are not present. Whether it is important for normal intrathymic T cell development that some myeloid lineages derive from early progenitor populations such as ETPs is also unclear at this time.

6. Molecular mechanisms of Notch1-mediated T lineage commitment

Following Notch receptor-ligand interaction at the cell surface, sites sensitive to proteolytic cleavage in the receptor are exposed, resulting in cleavage of the receptor. This cleavage event allows for the translocation of intracellular Notch (ICN) into the nucleus [37]. Inside the nucleus, ICN interacts with CSL/RBP-J κ , and recruits additional transcriptional co-activators to regulate the expression of target genes [37,77]. MINT (Msx2-interacting nuclear target protein) and NKAP (NF- κ B activating protein) are recently discovered molecules that can act as transcriptional co-repressors of Notch [78,79]. Some Notch target genes in T cell progenitors have been identified, contributing to our limited understanding of the molecular basis for Notch-mediated T cell commitment. Included in the identified targets are: bHLH transcriptional repressor Hairy and enhancer of split1 (HES-1), Deltex1, Nrarp, Ptcra, the IL-2 receptor component CD25, and GATA-3 [37]. Among known Notch target genes, Deltex, and Nrarp play inhibitory roles in the Notch signaling pathway although the mechanism by which they inhibit signaling is not clearly understood [80–82]. Knockouts of each gene possess no abnormal phenotype in T cell development [83]. We discuss some of the other individual players below. At this time the extent to which any of these targets of Notch signaling are involved in T cell commitment versus T lineage progression is still not clear.

One known Notch target gene is GATA-3, which is an attractive candidate to play a role downstream of Notch signals in lineage commitment. GATA-3 deficient progenitors fail to generate normal numbers of ETPs, recapitulating the Notch deficient phenotype. Interestingly, B cells do not expand in the thymus when progenitors lack GATA-3 however, other lineages have not been examined. GATA-3 can replace Notch and prevent the induction of myeloid

development in DN3 cells retrovirally transduced with CEBP/α. Recent work using a new hypomorphic strain is consistent with a potential role for GATA-3 in lineage commitment in vivo [84]. Adoptive transfer experiments using hematopoietic cells reconstituted from GATA-3 hypomorphic strain show that GATA-3 is required for ETP generation in a cell autonomous manner. Furthermore, GATA-3 controls differentiation rather than cell survival or proliferation during these early stages of T cell development [84–87].

Another critical transcription factor downstream of Notch that is upregulated shortly after thymic entry is the canonical Notch target gene HES-1. A known transcriptional repressor [88,89], HES-1 was initially an attractive candidate for suppression of genes crucial for alternative fates. Hes1 overexpression has been shown to perturb B cell and myeloid cell development [90]. The majority of HES-1 deficient embryos lack a thymus, and the thymus is much smaller in those that do have one [91]. HES-1 deficient progenitors fail to make significant numbers of thymic T lineage progenitors when transplanted into RAG-deficient mice and those T lineage cells that do develop are blocked at the DN stage [91]; however, cells of alternative lineages including B cells did not expand, suggesting HES-1 is not necessary to prevent adoption of alternative cell fates. This may be because of compensation by other HES genes, as mice engineered to lack both HES-1 and HES-5 in early thymocytes do show a small degree of B cell expansion in their thymus [92]. When HES-1 was retrovirally overexpressed in progenitors placed into fetal thymic organ culture (FTOC), increased numbers of T lineage progeny were recovered compared with control [93]. These studies support the idea that HES-1 plays a major role in early thymocyte proliferation rather than lineage commitment. Taken together, these studies suggest that additional mechanisms apart from HES-1 must exist downstream of Notch1 signaling that inhibit B cell potential of entering progenitors.

Bcl11b is another transcriptional repressor that is highly up regulated during the early ETP to DN2 transition, both in vivo and in vitro [66,94–96]. Such up-regulation occurs in response to Notch signaling, and also requires Runx1 activity, but the function of this molecule with regard to early T cell development remains unclear [95]. Germline deletion of Bcl11b alters early stages of T cell development, causing a block at the DN3 stage in mice and enhanced susceptibility to apoptosis reflected by reduced total thymocyte numbers [97]. Overexpression of mouse Bcl11b under the control of a heat shock promoter contributes to a dramatic increase in CCR9 chemokine receptor expression in the fish embryo [98]. Moreover, the Gfi-1 zinc finger protein repressor also appears to be important for T lineage development, since loss of Gfi-1 inhibits the generation of DN2 cells, and Gfi-1 overexpression can inhibit the development of DP cells from DN precursors [99–101]. Gfi-1 in hematopoietic stem and progenitor cells, including MPPs, promotes B cell fates by repressing PU.1; however, whether it plays a similar role in the thymus remains to be determined [102].

TCF-1 plays a critical, nonredundant role in adult thymocytes in progression from ETP to DN2 [87,103]. Expression of TCF-1 is restricted to the T cell lineage in adult mice, and TCF-1 has been found to play an important role in thymocyte development. TCF-1 belongs to TCF/LEF gene family, characterized by the presence of a high mobility group (HMG) box that recognizes a consensus DNA sequence and is able to induce DNA binding [104]. TCF-1 by itself lacks transcriptional activity and its functions are thus dictated by two groups of transcription factors with which it associates. In the presence of Wnt signaling, TCF-1 may interact with beta-catenin, though the significance of this interaction is unclear [105,106]. In the absence of Wnt signaling, TCF-1 interacts with Groucho (Grg1-5 in mouse), which confers TCF-1 with the ability to repress transcription. The absence of TCF-1 causes a severe defect in thymic cellularity. TCF-1 may be required to regulate trophic factors, such as IL-7Rα and BCL-XL, and T lineage specific genes, like CD3ε and CD4, at later stages of T lineage development. During earlier stages, TCF-1 may be important in repressing PU.1 in DN3 cells; however, B lineage cells and DC do not expand in the thymus in the absence of TCF-1. It is unknown

whether TCF-1 and GATA-3 are direct Notch targets, or whether indirectly regulated by Notch signals.

In concert with Notch, multiple transcription factors, including Ikaros [107], c-Myb [108], and Runx family transcription factors in complex with CBF β , [109] appear to be required for early progenitors to undergo T cell lineage commitment [96]. One Ikaros family member, Helios, is expressed selectively in hematopoietic stem cells and in cells of the T cell lineage, with increasing expression during T cell specification and decreasing expression during B cell development [110]. Ikaros may also repress Notch target gene expression in developing T cells. [111,112]. Basic helix-loop-helix (bHLH) transcription factors are also crucial for thymocyte development. Members of the bHLH family include ubiquitously expressed factors E2A and HeLa E-box binding protein (HEB). HEB-deficient mice exhibit reduced thymic cellularity and display a block in T cell differentiation at the DN to DP stage [113,114]. Another form of HEB (HEBAIt), which is expressed in early T cell precursors, is specifically required for the efficient generation of early T cell precursors [79,113,115]. The precise roles played by these molecules remains to be better described.

As previously mentioned, the majority of ETPs, DN2 and DN3 cells fail to generate B lineage cells even when removed from the thymus and cultured in vitro in the absence of Notch1 signals, suggesting suppression of the B cell fate is a very early event in the thymus. Notch signaling may transiently downregulate E2A activity to promote loss of the B cell fate [88, 116]. It appears possible that Notch enacts permanent epigenetic modifications leading to the silencing of B cell-specific genes such as EBF1 and Pax5 [117]; at this time, the underlying mechanisms are only beginning to be explored.

7. Conclusions

Significant progress has been made recently regarding the identity and properties of cells migrating to the thymus, and the signals utilized for this migration. However, much remains to be understood regarding the mechanisms downstream of Notch signals that mediate T cell commitment. As we have discussed, no single known Notch target gene, alone or in combination, is known to replace the effects of Notch signaling. It seems likely that collaboration between many of these genes is necessary to effect T cell lineage commitment and T lineage progression. Recent work extends our knowledge of co-repressors that may play critical roles in modifying the outcome of Notch signals in T cell development; however, additional mechanisms may remain to be discovered. The ultimate goal is to understand how Notch orchestrates T cell development by integrating multiple transcriptional programs. This challenging goal appears tractable in the coming years.

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Fig. 1. Cellular substrates and molecular signals in T cell development

Within the bone marrow, hematopoietic stem cells (HSCs) differentiate into multipotential progenitors (MPPs). A subset of MPPs that are Flt3^{hi} termed lymphoid-primed multipotential progenitors (LMPPs). HSCs, MPPs and LMPPs have the Lin⁻Sca-1^{hi}c-Kit^{hi} (LSK) phenotype. Subsequent progenitors include common lymphoid progenitors (CLPs). Both LMPPs and CLP express CCR7 and high level of Notch1. All of the bone marrow progenitors express PSGL-1 and circulate in the blood. Circulating T cell progenitors (CTPs) have also been identified in blood and are candidate T cell progenitors. CTP may feed directly to downstream stages of thymocytes development without going through an ETP to DN2 stage. The ability of circulating progenitors to settle within the thymus is regulated and requires the molecules chemokine receptor CCR9, CCR7 and P-selectin glycoprotein ligand-1 (PSGL-1). Thymus settling progenitors generate Flt3⁺ Early thymic progenitors (ETPs) and Flt3^{lo} ETPs. Double-Negative 2 (DN2), Double-Negative 3 (DN3) cells arise from ETPs. T cell commitment through these stages (ETP to DN3) depends on a set of regulators include both T-cell specific and shared transcription factors and signaling molecules. "Lin" refers to a lineage cocktail containing a mixture of antibodies to surface antigens expressed by differentiated cell types and contains antibodies to CD8α, CD8β, TCRβ, TCRγ, CD3ε, NK1.1, Mac1 (CD11b), Gr1, B220, CD19, CD11c, and Ter119.