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Transcriptional repressors, corepressors and chromatin modifying enzymes in T cell development

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Abstract

Gene expression is regulated by the combined action of transcriptional activators and transcriptional repressors. Transcriptional repressors function by recruiting corepressor complexes containing histone-modifying enzymes to specific sites within DNA. Chromatin modifying complexes are subsequently recruited, either directly by transcriptional repressors, or indirectly via corepressor complexes and/or histone modifications, to remodel chromatin into either a transcription-friendly 'open' form or an inhibitory 'closed' form. Transcriptional repressors, corepressors and chromatin modifying complexes play critical roles throughout T cell development. Here, we highlight those genes that function to repress transcription and that have been shown to be required for T cell development.

1. Introduction

Gene expression requires the binding of transcription factors to promoters and enhancers, resulting in the recruitment of RNA polymerase and the initiation of transcription. While this process is a well established mechanism of gene regulation, it has become apparent that epigenetic mechanisms, which alter the structure of chromatin by methylation of DNA and covalent modification of histones, also exert a critical effect on gene expression (for a detailed review, please see [1]). In eukaryotic cells, 147 nucleotides of genomic DNA wraps around an octamer of histones (H2A, H2B, H3 and H4) to form the nucleosome. Nucleosomes are assembled into higher order structures to form chromatin. The N-terminal tails of histones are largely unstructured and subject to a wide array of reversible covalent modifications which modulate placement and packaging of nucleosomes, thereby regulating accessibility of DNA to transcriptional activators and RNA polymerase (for a detailed review, please see [2]). Eight distinct types of histone modifications have thus far been identified: acetylation, methylation, ubiquitylation, and sumoylation of lysine residues; methylation and deimination of arginine residues; serine and threonine phosphorylation; glutamic acid ADP ribosylation; and proline isomerization. Modifications have been detected on 60 different amino acids amongst the core histone proteins. Together, this set of histone modifications is commonly referred to as the 'histone code', which has only begun to be understood. While the function of many modifications are not yet known, a few have been shown to correlate with active or inactive transcription. For example, methylation of lysine 4 of histone H3 (H3K4 methylation) correlates with active transcription, while

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methylation of lysine 27 in the same protein (H3K27 methylation) correlates with decreased transcription [3]. The regulation of chromatin structure is mediated by the coordinated function of transcriptional repressors, corepressors and chromatin modifying complexes (for a comprehensive review, please see [4]). Transcriptional repressors are sequence-specific DNA binding proteins generally thought to function by recruiting corepressor complexes, which contain multiple proteins including histone modifying enzymes. Subsequently, chromatin modifying enzymes, which alter nucleosome packaging, are recruited either by direct associations with corepressor complexes or by recognizing the histone modification catalyzed by these complexes. Epigenetic regulation of this sort plays a critical role in T cell development.

The generation of T cells in the thymus proceeds through a well-defined series of intermediates, which will be summarized briefly here (see [5] for an excellent review of this subject). The earliest T cell precursor in the thymus is the early thymic progenitor (ETP, [6]), which arises from the entrance of c-Kit expressing multipotent hematopoietic cells into the thymus at the cortico-medullary junction. Acquisition of CD25 at DN2 (CD4, CD8 'double negative' – 2 stage) is associated with loss of B cell potential. The downregulation of c-Kit and the rearrangement of the TCR β gene at DN3 is associated with commitment to the $\alpha\beta$ T cell fate and the inability to generate non-T cell lineages. Progression through DN3 is dependent on a quality control checkpoint for TCR β rearrangement (termed β -selection) generated by signals through the pre-TCR (comprised of TCR β and an invariant pre-TCR α chain). Mutations of genes required for TCR/pre-TCR signaling, including Lck, ZAP-70, LAT and SLP-76, result in a severe block at the DN3 stage. Once an appropriate signal is received, cells upregulate CD27, increase their metabolism correlating with an increase in cell size, and initiate several rounds of proliferation. CD25 expression is lost at the DN4 stage, as cells transition to CD4 and CD8 expressing double positive ('DP') T cells. At the DP stage, the TCR α chain is rearranged and the TCR is expressed on the cell surface. Positive and negative selection of the TCR to promote the generation of T cells with low affinity for MHC, and to eradicate cells that recognize self peptide/MHC, results in the generation of thymocytes that express either CD4 or CD8, designated single positive (SP) T cells, which can then exit the thymus and migrate into peripheral lymphoid organs.

This review will examine the role of transcriptional repressors, corepressor complexes, and chromatin remodeling complexes during T cell development, focusing specifically on those genes which have been shown, using genetically altered mice, to be required for T cell development. Figure 1 presents an overview of T cell development, illustrating how transcriptional repressors, corepressor complexes and chromatin modifying enzymes have essential roles at every stage. As there are already many excellent reviews on the subject (such as [7]), the role of the transcriptional repressor Foxp3 in the development of regulatory T cells will not be covered.

2. Transcriptional repressors in T cell development

Transcriptional repressors, including members of the Runx/CBF, Ikaros, Gfi, Bcl11a/Bcl11b, and BTB-ZF/POZ-ZF families, as well as NKAP, have critical functions throughout T cell development. In some cases, such as with Ikaros and the related genes Helios and Aiolos, there is functional redundancy. Expression of a dominant negative isoform of Ikaros which interferes with the function of all Ikaros family members, has a more severe phenotype than deficiency in Ikaros during T cell development [8,9]. In other cases, such as with ThPOK and MAZR of the BTB-ZF family, related proteins have non-overlapping, and actually, antagonistic functions [10,11].

2.1 Runx/CBF β

Core Binding Factors (CBFs) are comprised of two subunits: Runx (also known as CBF α) which binds directly to DNA, and a non-DNA binding subunit, CBF β , that increases the affinity of Runx factors for DNA (reviewed in [12]). The Runx family is composed of three members in mammalian cells: Runx1 (CBF α 2), Runx2 (CBF α 1), and Runx3 (CBF α 3), which are all expressed during T cell development [13,14]. Runx proteins inhibit transcription through several mechanisms. The c-termini of Runx proteins contain a conserved VWRPY motif that mediates association with the transcriptional repressor TLE, a Groucho homolog [15]. Runx proteins also associate with several histone deacetylase enzymes (HDACs) [17] and with the corepressor mSin3a, through a site distinct from the VWRPY motif [16]. The relative importance of the interaction with TLE/Groucho or with HDACs for transcriptional repression is variable depending on the target gene and/or cellular context. For example, while interaction with HDACs is critical for Runx2-mediated inhibition of p21 Cip1/Waf1 expression [18], interaction with TLE/Groucho is important for T cell development [19].

Runx1-deficient and CBF β -deficient animals die in utero due a complete failure of hematopoiesis [20-22]. Runx2 [23] and Runx3 deficient [24] mice die perinatally due to non-hematopoietic defects. Consequently, the role of the Runx genes in T cell development has been analyzed using conditional knockout approaches or bone marrow reconstitutions. Runx1 is critical to iNKT cell development [25]. Runx2 is not required for T cell development, but both Runx1 and Runx3 regulate CD4 expression, albeit at different stages of T cell development [14]. During normal T cell development, repression of CD4 in DN and CD8 SP cells is dependent on the CD4 silencer, and disruption of CD4 silencer function leads to inappropriate CD4 expression [26]. In the absence of Runx3, there was incomplete repression of the CD4 gene in CD8 SP cells both in the thymus and in the periphery, but not in the DN compartment [14]. In contrast, in Lck-cre Runx1 conditional knockout mice, CD4 was aberrantly expressed in the DN population, but there was no defect in repression of CD4 in CD8 SP thymocytes or peripheral T cells [14]. Therefore, Runx1 and Runx3 have non-redundant roles in suppressing CD4 expression in DN and CD8 SP thymocytes, respectively. In addition to regulating CD4 expression, Runx/CBF β also repress transcription of ThPOK in the CD8 lineage [27]. ThPOK is required for CD4 helper T cell development, as overexpression of ThPOK drove thymocytes expressing MHC-Class I restricted TCRs into the CD4 lineage [11,28]. Consistent with negative regulation of ThPOK, mutation of Runx1 and Runx3 forced MHC Class I restricted T cells to develop into CD4 SP cells [27].

In addition to regulating CD4 expression, Runx1 also has critical roles at earlier stages in T cell development. While Runx1 is required for the initiation of fetal definitive hematopoiesis [29], inducible Mx1-cre Runx1 conditional knockout mice were viable [30,31]. Thymic cellularity was decreased 10-fold in Mx1-cre Runx1 conditional knockout mice and there was a block in T cell development at the DN2 to DN3 transition [30,31]. In Lck-cre Runx1 conditional knockout mice, thymic cellularity was also decreased by approximately 6-fold [32]. There was no alteration in the absolute number of DN2 and DN3 cells in these mice, but DP and SP cells were reduced owing to decreased proliferation after β -selection in DN4 [32]. Development of $\gamma\delta$ T cells was normal. In CD4-cre Runx1 conditional knockout mice, thymic cellularity was maintained, however there were fewer CD4 and CD8 SP thymocytes. The loss of Runx1 resulted in decreased IL-7R α expression, which led to defects in T cell survival and homeostasis rather than a block in SP T cell development [32]. The effects on CD4 and CD8 SP cells were exacerbated upon simultaneous deletion of Runx1 and Runx3 using CD4-cre [32]. Similarly, severe defects on T cell development were observed in mice with a hypomorphic allele of CBF β [33]. There were severe defects in the ETP to DN2 transition, as well as the DN2 to DN3 transition, resulting in a near complete block in T cell

development prior to the DP stage [33]. The ETP to DN2 block was attributed to a defect in T cell specification as there were significant alterations in the expression of GATA3 and TCF7 [34]. The ETP to DN2 block in T cell specification in CBF β mutant mice occurred at an earlier point than the block caused by deletion of Runx1 alone [30,31]. Since all three Runx family members are expressed throughout T cell development [14], it is likely that either Runx2 or Runx3 can compensate for deletion of Runx1 in early T cell development. Conditional deletion of CBF β using Lck-cre and CD4-cre resulted in defects in thymic cellularity, depression of CD4, and the production of mature SP thymocytes, similar to the results of Runx1 and Runx3 deletions [35].

2.2 Ikaros family

Ikaros is the founding member of a family of zinc-finger containing DNA-binding proteins that, along with its relatives Helios and Aiolos, is expressed in many cells in the immune system (reviewed in [36]). All of these proteins have several alternatively spliced forms, with differences in function and DNA binding, and can heterodimerize. Through the association with histones and chromatin modifying enzymes, Ikaros functions as a transcriptional repressor. In a yeast-two-hybrid screen, Ikaros was shown to associate with the corepressor mSin3A [37]. mSin3A and mSin3B also allow Ikaros to associate indirectly with HDACs. Repression by Ikaros requires association with HDACs, as the HDAC inhibitor Trichostatin A as shown to block Ikaros-mediated repression of a reporter [37]. Ikaros, through association with the ATPase Mi-2 β , associates primarily with the NuRD chromatin remodeling complexes and directs these complexes to DNA upon T cell activation [38]. Ikaros has also been shown to associate with the SWI/SNF complex [39].

Ikaros deficiency, but not Helios or Aiolos deficiency [40,41], resulted in a defect in T cell development, suggesting it is the only member of this family required for this process [9]. However, mice that express a non-DNA binding, dominant negative mutant of Ikaros (Ikaros DN) displayed more severe defects in hematopoiesis and T cell development than Ikaros null mice [8]. The most severe effects were observed in very young mice, as the loss of Ikaros function lead to the development of clonal aggressive T cell leukemias at 2-3 months of age, complicating analysis of older mice [42]. In Ikaros-deficient mice, all fetal T cell development was blocked [9], which may have been due to defects earlier in hematopoiesis in the generation of lymphoid-primed multipotent progenitors (LMPP, [43]). Thymocytes first appeared between day 3 and 6 after birth, and were present in 100- to 300-fold fewer numbers than in wild type littermates. Between 3 to 4 weeks, thymic cellularity was decreased (between 3- to 9-fold as compared to wild type), and CD4/CD8 profiles were skewed [44]. An enhancement in the proportion of CD4 SP cells was observed, although the absolute number was decreased compared to wild type [44]. Enhanced positive selection towards the CD4 lineage was also observed upon crossing to DO11.10 TCR transgenics [44]. Ikaros also plays a role in lineage choice, as CD4 SP cells developed in Rag1-deficient, female H-Y transgenic mice, which are normally positively selected into the CD8 lineage, when they were also Ikaros-deficient [44]. Further, negative selection is also dependent on Ikaros, as clonal deletion was inefficient in Ikaros-deficient, male H-Y transgenic mice [44]. It was also found that dendritic epidermal $\gamma\delta$ T cells and intestinal intraepithelial lymphocytes were absent in Ikaros-deficient mice [9], though vaginal epithelial $\gamma\delta$ T cells were present, showing that Ikaros contributes to the development of certain $\gamma\delta$ T cell subsets.

2.3 Gfi family

Gfi1, and the related proteins Gfi1b, Snail and Slug, are transcriptional repressors that contain six carboxy-terminal zinc fingers that mediate direct binding to DNA and an N-terminal SNAG domain that is required for transcriptional repression (reviewed in [45]).

Chromatin modification and transcriptional repression by Gfi1 is mediated by its association with HDAC1 and the histone lysine methyltransferase G9a [46]. Gfi1 can positively regulate transcription as well, though by an indirect mechanism. Gfi1 associates with a complex of Stat3 and its inhibitor PIAS3 [47], promoting the release of Stat3 and enhancing Stat3-mediated transcriptional activation.

Gfi1-deficient mice have a severe block in T cell development, resulting in a 90% decrease in thymic cellularity [48]. Development of both $\alpha\beta$ and $\gamma\delta$ T cell lineages was decreased in the absence of Gfi1. Viability of c-Kit⁺ ETP and DN2 thymocytes was reduced, as demonstrated by a 25% increase in Annexin V positive cells in Gfi1 knockout mice relative to wild type [48]. While expression of a bcl2-transgene in Gfi1-deficient mice restored the viability of c-Kit⁺ ETP and DN2 thymocytes, there was a minimal rescue of thymic cellularity, revealing an additional requirement for Gfi1 in T cell development. Examination of lin⁻ thymocytes revealed a block in T cell development at an intermediate stage between DN1 and DN2, characterized by high expression of CD44 and intermediate CD25 staining [48]. This was not a result of the cells adopting an alternative fate, as they did not express NK1.1, $\gamma\delta$ TCR, Mac-1, Gr-1, CD19 or CD11c. Later in T cell development, there was a bias towards development of CD8 SP cells [48]. Transcription profiling of Gfi1-deficient thymocytes demonstrated upregulation of the E-protein inhibitors Id1 and Id2, indicating that the block in T cell development may be caused by inhibition of E-protein function [48]. However, T cell development was not rescued in either Gfi1-deficient mice in which Id2 was knocked down or in Gfi1^{-/-}Id2 heterozygotes, albeit B cell and myeloid development was partially rescued [49]. Mice doubly deficient in Gfi1 and Id2 were not viable [49]. Whether Id1 deficiency, alone or in combination with Id2 deficiency, would diminish the the block in T cell development observed in the absence of Gfi1 remains to be determined. Gfi1b is not required for T cell development [50].

2.4 BTB-ZF/POZ-ZF

Members of the BTB-ZF family of transcriptional repressors contain c-terminal zinc fingers required for DNA binding, and a conserved BTB domain (reviewed in [51,52]). The BTB domain was initially identified in the Drosophila proteins Broad complex, Tramtrack and Bric-a-brac, and is also known as a POZ domain (found in poxvirus and zinc finger proteins). The BTB domain has several functions, including mediating protein-protein interactions and conferring nuclear localization [53–55]. Through the BTB domain, BTB-ZF proteins can form homodimers or heterodimers with other BTB-ZF proteins, although there is specificity in heterodimer formation [53]. The BTB domain also mediates interactions with components of corepressor complexes, including SMRT, NCoR, BCoR, and mSin3a, which function in part by recruiting HDACs [56–58]. The HDAC inhibitor Trichostatin A can block PLZF- or Bcl6-mediated repression [57]. However, not all BTB-ZF transcriptional repressors associate with corepressors through the BTB domain; additional non-conserved regions of BTB-ZF proteins can also contribute to transcriptional repression [59]. Disruption of the BTB domain/corepressor interaction by point mutation, peptide inhibitors or small molecule inhibitors abrogates function [60–62].

Several BTB-ZF family members are co-expressed in T cells, including PLZF, MAZR, ThPOK (also known as cKROX or Zfp67), PLZF (also known as FAZF, ROG or TZFP), Bcl6, and Bcl6b (also known as BAZF). T cell development was normal in mice deficient for PLZF [63,64], Bcl6 [65] and Bcl6b [66], but deficiency in either PLZF, MAZR or ThPOK resulted in defects in the development of specific T cell lineages. The role of ThPOK in CD4 T cell development was discovered upon analysis of the spontaneous mouse mutant line HD ('helper-deficient'), which has a point mutation resulting in a single amino change in the second zinc finger of ThPOK [11]. These mice have few CD4 SP thymocytes, and development of CD4 helper T cells was not rescued by ectopic expression of a CD4

transgene, indicating that development of the lineage, rather than just CD4 expression, was abrogated [11]. Consistent with its role as a master regulator of the CD4 lineage, overexpression of ThPOK drove T cells with MHC Class I restricted TCRs into the CD4 lineage [11,28], and loss of ThPOK drove T cells with MHC Class II restricted TCRs into the CD8 lineage [67]. ThPOK was found to repress Runx3 expression [68], suggesting that there is a reciprocal antagonistic relationship between Runx3 and ThPOK which drives differentiation into the CD8 and CD4 lineages, respectively. MAZR is required for differentiation of CD8 cytotoxic T cells [10]. In MAZR-deficient mice, thymocytes bearing MHC Class I restricted TCRs are redirected into the CD4 helper T cell lineage [10]. As MAZR was found to associate with the Th-POK silencer and negatively regulates its expression, MAZR likely promotes CD8 differentiation by inhibition of ThPOK [10]. PLZF knockout mice displayed a block in the development of functional iNKT (invariant NKT) cells and innate $\gamma\delta$ T cells, but not in development of conventional CD4 or CD8 T cells [69–72].

2.5 Bcl11a/Bcl11b

The related transcriptional repressors CTIP1/Bcl11a and CTIP2/Bcl11b were cloned by association with the transcriptional repressor COUP-TF on a yeast-two-hybrid screen [73]. Bcl11a and Bcl11b bind DNA directly, through two conserved C₂H₂ zinc fingers [74]. Mice deficient in either Bcl11a or Bcl11b were found to die within one day of birth, although the cause of lethality was not characterized [75,76]. Both T cell and B cell development was altered in Bcl11a-deficient mice [75]. While T cell development was largely normal in Bcl11a-deficient embryos at e18.5, T cell development was blocked when Bcl11a-deficient bone marrow was used to reconstitute irradiated recipients [75]. In such reconstituted mice, thymic cellularity was greatly decreased, and very few DP cells were found, indicating that Bcl11a is required at the DN to DP transition. DN subsets were not characterized to further define the developmental block. Additional studies are needed to understand the function of Bcl11a in T cell development, and to determine whether the differences observed at e18.5 and in radiation chimeras reflect differential requirements for Bcl11a in fetal vs adult mice.

A severe defect in T cell development was also observed in mice lacking Bcl11b, while development of B cells and $\gamma\delta$ T cells was unaffected [76]. At birth, thymic cellularity was reduced to 10% of wild type littermates, with a severe block prior to the development of DP cells and an accumulation of DN2, DN3, and CD8 immature single positive (ISP) cells [76]. The block at the DN2 stage was due to a failure to commit to the T cell lineage and suppress alternative fates [77–79]. Once committed to the T cell lineage, Bcl11b-deficient DN3 thymocytes did not express the TCR β chain [76,80]. Analysis of rearrangement at the TCR β locus showed that while D β to J β rearrangements occurred, there were few rearrangements between V β to DJ β [76,80]. As Bcl11b is a transcriptional repressor that associates with the NuRD chromatin remodeling complex [81,82], the failure of TCR β rearrangement might be explained by altered chromatin accessibility at the locus. However, no alteration in H3 acetylation was observed [80]. As additional histone modifications were not examined, it remains possible that Bcl11b regulates chromatin accessibility through modifications other than H3 acetylation. Introduction of a rearranged DO.11.10 TCR β or TCR $\alpha\beta$ transgene did not restore T cell development, indicating that Bcl11b regulates pathways in addition to V β to DJ β rearrangement, such as signaling downstream of the pre-TCR [80]. This would be consistent with results from CD4-cre Bcl11b conditional knockout mice, which have a block in the development of SP thymocytes due to altered positive selection [83]. Erk phosphorylation, calcium mobilization and ZAP-70/SLP-76 phosphorylation were defective in Bcl11b-deficient thymocytes upon TCR crosslinking [83]. How Bcl11b regulates TCR signaling is not known.

2.6 NKAP

NKAP, a protein with no previously characterized functional domains, was identified on a genetic complementation screen for novel regulators of T cell activation [84]. NKAP associates with HDAC3, and represses transcription in reporter assays. Truncation analyses using Gal4-DBD (DNA binding domain) fusion proteins demonstrated that the transcriptional repressor function of NKAP maps to the HDAC3-binding region in the c-terminus [84]. NKAP also associates with CIR, which is a component of the Notch corepressor complex. This complex is recruited to Notch-regulated promoters by the transcription factor RBP-J κ (also known as CBF-1 or CSL) and represses their transcription in the absence of Notch activation (reviewed in [85]). Conditional deletion of NKAP using Lck-cre demonstrated that NKAP is required for $\alpha\beta$ T cell development, although $\gamma\delta$ T cell development was unaffected by NKAP deficiency [84]. Lck-cre NKAP conditional knockout mice exhibited a 25-fold decrease in the number of DP T cells, which was shown to result from a block in T cell development at the DN3 to DP transition. Consistent with the biochemical characterization of NKAP as a negative regulator of Notch signaling, expression of three genes regulated by Notch (Hes1, Deltex1 and CD25) was significantly increased in DP T cells from Lck-cre NKAP conditional knockout mice. The block between DN3 and DP was not due to a failure of pre-TCR signaling/ β -selection, as TCR β expression by intracellular staining was normal, as was the transition from pre-selection DN3a to post-selection DN3b [84]. A decrease in cell cycle progression was observed, indicating that decreased proliferation after β -selection may have contributed to the loss of DP T cells. NKAP has been shown to be part of a DNA binding complex by chromatin immunoprecipitation [84,86]. However, NKAP does not contain any previously characterized DNA binding domains and has not been shown to bind DNA directly. Thus, further work is required to determine whether NKAP should be classified as a repressor, or a corepressor that binds DNA indirectly.

3. Corepressor complexes in T cell development

Transcriptional repressors recruit corepressor complexes, leading to histone modifications and ultimately chromatin remodeling. In certain circumstances, association with a corepressor complex can turn a transcriptional activator into a repressor (such as AML-ETO recruitment by E proteins [87]). In this section, three common components of corepressor complexes, NCoR/SMRT, Sin3 and HDACs, will be described. In some circumstances, all of these components can function together at a single promoter to regulate transcription [88–90].

3.1 NCoR/SMRT

NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) are related corepressors, initially identified by their regulation of nuclear hormone receptors (reviewed in [4]). They contain three repressor domains that function, at least in part, to recruit HDACs, and a conserved bipartite nuclear receptor interaction domain. Their functional activity, however, is not limited to the nuclear hormone receptor family. NCoR and SMRT are constituents of larger corepressor complexes for MAD [91], RBP-J κ /CSL [92], and homeodomain factors [93]. Deletion of NCoR resulted in embryonic lethality, by E15.5 [91]. Analysis of T cell development using fetal thymic organ culture demonstrated an 8-fold decrease in thymic cellularity relative to wild type when NCoR was deleted. There was a severe block in T cell development at the DN3 stage, which was not due to a failure of TCR β rearrangement or lack of CD3 expression [91], showing that NCoR is required for the progression from DN to DP. The block observed in NCoR-deficient mice was likely caused by defective signaling of the pre-TCR complex, as injection of anti-CD3 into these mice resulted in the generation of DP T cells. SMRT

deficient mice die during embryogenesis [94], and it unlikely that a direct role for SMRT in T cell development will be found as it is not expressed in the thymus [91].

3.2 mSin3A

Sin3 is another common component of corepressor complexes (reviewed in [95]). Mice contain two closely related Sin3 genes, mSin3A and mSin3B [96]. Sin3-containing complexes associate with HDACs [88-90], histone methyltransferases, and O-linked N-acetylglucosamine transferases [97,98], all of which can modify histones to promote transcriptional repression. Mice deficient in mSin3A die embryonically, between implantation and E10.5 [99]. Conditional deletion of mSin3A using Lck-cre resulted in a 3-fold decrease in overall thymic cellularity, a block in $\alpha\beta$ T cell development at the DN3 stage, and reductions in DP and SP thymocyte populations. Development of $\gamma\delta$ T cells was normal [99]. Deletion of mSin3A in mouse embryonic fibroblasts (MEFs) resulted in decreased proliferation. Consequently, it was hypothesized that the reduced cellularity in Lck-cre mSin3A conditional knockout mice resulted from decreases in the proliferative burst induced by pre-TCR signaling between the DN3 and DP stages, though this was not examined explicitly. Additionally, the paucity of thymic and peripheral CD8 SP cells in mSin3A conditional knockout mice suggests that mSin3A may be specifically required in the CD8 but not the CD4 lineage. The decrease in CD8 SP cells was not abrogated upon crossing to the MHC-Class I specific TCR transgenic OT-I. mSin3A associates with Ikaros [37], which is required for CD8 α expression [100], and disruption of this interaction is a likely mechanism for the defect in CD8 T cell development caused by mSin3A deletion. mSin3A-deficient thymocytes cultured *ex vivo* exhibited, relative to wild type, increased apoptosis as measured by Annexin-V staining and a greater percentage of cells in sub-G1 by analysis of DNA content. Hence, decreased survival may also contribute to the developmental defect in mSin3-deficient T cells. The block in T cell development caused by mSin3A deletion may have been incomplete due, at least in part, to co-expression of the related gene mSin3B in thymocytes, and mSin3B protein levels were in fact increased upon mSin3A deletion. mSin3B-deficient mice die perinatally and exhibit defects in erythrocyte and granulocyte differentiation, though effects on T cell development remain to be examined [101].

3.3 HDACs

Acetylation of the ϵ -amino group of lysine residues in histones, catalyzed by histone acetyltransferase enzymes, correlates with transcriptional activation. Conversely, removal of such acetyl groups by HDAC enzymes is a critical step in chromatin remodelling by corepressor complexes, leading to transcriptional repression of target genes (reviewed in [102]). Pharmacological treatment with HDAC inhibitors such as Trichostatin A can block the function of transcriptional repressors (such as the BTB-ZF family members described earlier). At least eighteen HDACs exist in mammals, with unique and overlapping functions. Though HDACs are an integral part of corepressor complexes required for T cell development, analysis of their role in T cell development has been complicated both by early embryonic lethality and by coexpression of functionally redundant HDAC family members. HDAC7 has been shown to regulate T cell survival by modulating expression of Nur77 [103]. However, HDAC7 deficiency causes embryonic lethality at E11 and T cell specific ablation has not yet been reported [104]. While HDAC1-deficient mice display embryonic lethality at E10.5 [105], normal T cell development was observed in CD4-cre HDAC1 conditional knockout mice. However, HDAC2 protein levels were upregulated, which may have had a compensatory effect [106]. In fact, there is functional redundancy between HDAC1 and HDAC2 during B cell development [107]. While conditional deletion of either HDAC1 or HDAC2 had minimal effect, conditional deletion of both genes resulted in a nearly complete block in B cell development prior to generation of B220⁺CD19⁺ pro-B

cells (Hardy Fraction B) [107]. Further, HDAC2 protein expression was increased in mb1-cre HDAC1 conditional knockout B cells [107]. A similar redundancy may also occur in T cell development, which may be demonstrated upon the generation of compound HDAC conditional knockout mice crossed to a T-lineage cre transgenic.

4. Chromatin remodeling complexes and DNA modifying enzymes in T cell development

Accessibility of loci to transcriptional activators is dependent on chromatin conformation. Alterations in chromatin structure are modulated by chromatin remodeling complexes, including SWI/SNF and NuRD. The Polycomb complex plays an important role in the maintenance of epigenetic marks. Inhibition of transcription is mediated by direct methylation at CpG dinucleotides by DNA methyltransferases.

4.1 SWI/SNF Complex

The activation of gene transcription requires nucleosome remodeling to permit access of cis-regulatory elements to transcriptional regulators. The SWI/SNF complex (also known as the BAF complex) alters nucleosome association within chromatin, either by translocating nucleosomes across DNA [108] or by mediating dissociation of DNA from histones [109]. This complex is evolutionarily conserved (reviewed in [110]) and contains 8 to 15 subunits. However, while yeast contain a single, invariant complex, there is substantial variation in the SWI/SNF complex in mammals as many of the subunits are encoded by multiple genes. As nucleosome remodeling requires energy, all SWI/SNF complexes contain either one of the two related ATPases Brm (also known as SNF α or Smarca2) or Brg (also known as SNF2b or Smarca4). These proteins have been found to have, at least in part, non-redundant functions in T cell development. Brg deficient embryos die at the implantation stage [111]. When Brg was conditionally deleted in T cells using Lck-cre, a dramatic block in T cell development at the DN3 to DP transition was observed, resulting in a 20-fold drop in thymic cellularity [112]. The CD4/CD8 profile was altered in these mice, including the near complete loss of DP cells and the appearance of an immature CD3⁻ CD4 SP population; this result is consistent with previous evidence that the SWI/SNF complex mediates transcriptional repression of the CD4 gene and activation of the CD8 gene. SWI/SNF complexes directly associate with the CD4 transcriptional silencer, as both Brg and BAF57, another SWI/SNF component, bound to the CD4 silencer in chromatin immunoprecipitation assays [113]. TCR β recombination and expression were unaltered in Lck-cre Brg conditional knockout mice, showing that the block at the DN3 to DP transition was not due to a failure in pre-TCR expression. Introduction of a Bcl-x_L transgene partially compensated for loss of Brg, leading to an increase in cellularity. However, the severe block in differentiation remained, demonstrating that the phenotype is not simply a result of increased apoptosis [112]. Anti-CD3 treatment failed to generate DP T cells, implying that the defect is likely downstream of pre-TCR signaling [112]. Unlike Brg-deficiency, loss of Brm did not alter T cell development [113]. Further, Brm expression was not increased in Brg-deficient mice, clearly showing that Brm cannot substitute for Brg during T cell development. Recent data has shown that Brm and Brg regulate accessibility of D β J β during TCR β rearrangement [114]. Though TCR β rearrangement was unaffected in Brg-deficient T cells, mice with combined deletion of Brg and Brm in the T cell lineage have not been generated. Thus, Brg and Brm may still be shown to have additional roles in T cell development that have previously been masked by functional redundancy.

Dysregulation of CD4 and CD8 is also observed upon overexpression of two separate dominant negative mutants of BAF57, a DNA binding component of SWI/SNF complexes. Using a transgene under the control of the Lck proximal promoter, it was found that

mutations in BAF57 that disrupt DNA binding impaired the silencing of CD4 and the expression of CD8 [113]. However, unlike the result of Brg-deficiency, only minimal effects were observed in total thymic cellularity. Hence, distinct Brg-containing SWI-SNF complexes, lacking BAF57, must be critical at the DN3 to DP transition in T cell development.

4.2 NuRD complex

In addition to the SWI/SNF chromatin remodeling complex, mammalian cells also contain the NuRD (*nucleosome remodeling histone deacetylase*) complex, which is highly expressed in thymocytes (reviewed in [115]). Similar to SWI/SNF, the NuRD complex contains multiple subunits, and the energy for remodeling is generated through ATPases which are distantly related to Brm and Brg. These ATPases are designated Mi-2 α (also known as CHD3) and Mi-2 β (also known as CHD4), and Mi-2 β is the predominant isoform expressed during T cell development [116]. Conditional deletion of Mi-2 β in thymocytes using Lck-cre led to a three-fold drop in absolute thymic cellularity, with decreased numbers of DP and CD4 SP cells, and increased numbers of CD8 SP cells [116]. Mi-2 α was upregulated upon deletion of Mi-2 β , which may account for the milder defect in T cell development compared to that produced upon deletion of the NuRD-associated proteins Ikaros and Aiolos. Analysis of surface markers indicated that the increase in CD8 SP cells was caused by a failure of DP T cells to upregulate CD4, rather than a true increase in mature CD8 SP cells. Mi-2 β associates with the histone acetylase p300, and with HEB, a member of the E-protein family of transcriptional activators. When bound to the CD4 enhancer, this complex promotes CD4 expression, demonstrating a positive effect on gene expression for chromatin remodeling by NuRD complexes. In addition to the misexpression of CD4 in Mi-2 β conditional knockout mice, a block at the DN3 to “DP” transition was observed, which was likely the cause of the three-fold drop in thymic cellularity. Expression of preT α and TCR β mRNA, as well as intracellular expression of TCR β in DN3 and DN4 populations, was unaltered upon Mi-2 β deletion, indicating that alterations in accessibility/rearrangement of TCR β did not cause the phenotype. Upon receipt of a pre-TCR signal, DN3 cells become blasts and initiate rapid proliferation. However, no alterations in either cell size or cell cycle progression were noted in the Lck-cre Mi-2 β conditional knockouts, indicating that the block was not due to a failure of pre-TCR signaling. An accumulation of DN4 cells was observed, indicating that while passage through β -selection at DN3 was normal, the differentiation into “DP” cells was blocked. The molecular mechanism responsible for this remains to be determined.

4.3 Polycomb family

Polycomb genes (PcG) were originally identified as regulators of segmentation during *Drosophila* embryogenesis, but were later found to have a larger role in maintaining epigenetic modifications which regulate transcription (reviewed in [117,118]). There are two distinct regulatory complexes formed by PcG which are conserved evolutionarily from flies to mammals, polycomb repressive complexes 1 and 2 (PRC1 and PRC2).

4.3.1 PRC1—PRC1 and PRC1-like complexes are comprised of members of the Ring (Ring 1/2), PH (PH1/2), Bmi1/Mel-18 and Cbx families (such as M33/Cbx2). One function of PRC1 complexes is to mediate ubiquitylation of H2A. As discussed below, deficiencies in Bmi1, Mel-18 and M33 alter T cell development. However, deficiencies in other components of PRC1 and PRC1-like complexes do not cause similar consequences. For example, T cell development was unaffected in mice deficient in Rae28, a member of the PH family [119]. Deficiency in Ring1B did not alter thymocyte cellularity [120], and Ring1A-deficient mice were phenotypically normal [121]. The lack of effects may stem from functional redundancy, or it may be that only specific PRC1 complex(es) function during T cell development.

Bmi1 and Mel-18: Bmi1 and Mel-18 are RING finger domain-containing proteins that are 93% identical [122], yet have separate and distinct roles in T cell development. Mice deficient in Bmi1 have a cell-intrinsic defect in T cell development at the DN3 stage, resulting in a severe block in the generation of DP T cells [123,124]. No alterations were observed in TCR β rearrangement, intracellular TCR β expression, or expression of preT α or CD3, indicating that the block was not caused by defects in the generation or expression of the pre-TCR [124]. Loss of Bmi1 leads to cell cycle arrest due, in part, to upregulation of the cyclin dependent kinase inhibitors p16Ink4a and p19Arf, which are both encoded by the Ink4a locus [125,124]. The block in thymocyte development was substantially rescued in mice doubly deficient in the Ink4a locus and Bmi1 [125]. In subsequent analysis, it was found that the block in T cell development was caused specifically by upregulation of p19Arf and not p16Ink4a, as p19Arf deficiency alone rescued most of the defects caused by Bmi1 deficiency [124]. It was also demonstrated that upregulation of p19Arf leads to increased apoptosis of immature thymocytes [124]. The effect on p19Arf expression was likely direct, as H3K27 (Histone 3 lysine 27) trimethylation at the Ink4a locus was reduced in the absence of Bmi1. Therefore, Bmi1 is required during T cell development, at least in part, to repress expression of p19Arf. A severe defect in T cell development was also observed in mice deficient in Mel-18 [126,127]. Thymic cellularity was reduced to 2–10% of wild type, although the phenotype was modulated by strain background. In the initial description of these mice on a mixed 129xB6 background, a complete block in T cell development at the DN stage was reported [126]. Upon backcrossing to C57Bl/6, the decrease in cellularity was maintained, but thymic profiles appeared normal [128,127]. Examination of DN1–4 populations did not reveal a specific stage in which T cell development was blocked, suggesting that arrest may initiate prior to or within DN1 [129]. The absolute number of ETPs (CD25[−]CD44⁺c-kit⁺) was decreased by 97% compared to wild type [127]. In fetal thymic organ culture, Mel-18-deficient progenitors did not expand to similar levels as wild type, indicating an alteration in proliferation or survival. Cell cycle analysis of DN1 and DN2 cells did not reveal any differences between Mel-18-deficient and wild type mice. However, a 2-fold increase in apoptotic cells was detected by TUNEL staining, indicating that Mel-18-deficient thymocytes are more susceptible to cell death [127]. Examination of genes important for regulating early T cell development did not indicate alterations in levels of GATA-3, TCF1, LEF1, E2A, Notch, RBP-Jk or Deltex in Mel-18- deficient DN1 and DN2 populations, although the level of Hes1 was significantly decreased [127]. The similarities in thymic phenotypes between Mel-18- and Hes1-deficient mice suggests that reduced Hes1 expression may be responsible for the block in T cell development in Mel-18 thymocytes [130,131]. There is some functional redundancy between Bmi1 and Mel18, as doubly deficient mice died earlier in gestation than singly deficient mice [132]. Simultaneous deletion of both genes specifically in T cells has not yet been reported.

M33: Mice deficient in M33 (also known as Cbx2, a member of the Cbx family) have a severe defect in T cell development [133,134]. At 3- to 4- weeks of age, M33-deficient mice exhibited a pronounced, cell-intrinsic decrease in thymic cellularity, of approximately 100-fold compared to wild type [133]. There was a block at the DN to DP transition, but no accumulation of cells at the DN3 stage [133]. Decreases were observed in all populations, indicating a defect at the earliest stages of T cell development. The block in T cell development was likely not due to increased apoptosis, as it was maintained upon interbreeding to a bcl2 transgenic [134]. It was hypothesized that the defect may have been due to altered proliferation in the DN population. In fact, M33-deficient MEFs displayed decreased proliferation and upregulation of the cyclin dependent kinase inhibitor p16 Ink4a. As with Bmi1 deficiency, there was increased expression of p16Ink4a and p19Arf in thymocytes from M33-deficient mice as shown by semi-quantitative PCR. This increase may have suppressed proliferation, leading to reduced cellularity. It remains to be determined

if, as in Bmi1/p19Arf double knockout mice [124], deficiency in either p16Ink4a and/or p19Arf can rescue the T cell development defect caused by M33 deficiency.

4.3.2 PRC2—PRC2 complexes are comprised of RBAP46/48, SUZ12, Ezh1/2 and members of the EED family (EED 1-4). The catalytic subunit of PRC2, which mediates histone H3 lysine 27 methylation (mono-, di- and tri-), a key signature of silenced chromatin, is either Ezh1 or Ezh2, although both SU(Z)12 and EED are needed for histone methyltransferase activity. The mechanism for recruitment of PRC2 complexes to DNA is subject to debate. However, the vast majority of targeted promoters contain GC-rich sequences or CpG islands [118]. There are functional differences in PRC2 complexes containing either Ezh1 or Ezh2; Ezh1-containing PRC2 complexes can compact polynucleosomes, whereas Ezh2-containing PRC2 complexes cannot [135]. In contrast, Ezh2-containing PRC2 complexes have stronger histone methyltransferase activity than Ezh1-containing complexes [135]. Ezh2 is critical for T cell development, as deletion of Ezh2 in hematopoietic stem cells led to a 10 to 100 fold decrease in thymic cellularity [136]. Specifically, a severe block at the DN3 stage was observed. This block was not due to impaired TCR β rearrangement, as it was not rescued upon crossing to either MHC Class I restricted H-Y TCR transgenic mice or MHC Class II restricted DO11.10 TCR transgenic mice [136]. Instead, it was likely caused by defects in pre-TCR signaling, as anti- CD3 treatment failed to generate DP T cells. There was not, however, a general deficiency in proliferation because Ezh2-deficient thymocytes proliferated in response to either IL-7 or PMA stimulation [136]. Ezh2, and the additional PRC2 complex components EED and SU(Z)12, were found to associate with the GTP/GDP exchange factor Vav1, which is also required for T cell development at the DN3 to DP transition. Vav1 deficiency results in only an incomplete DN3 block, which is a less severe effect than that of Ezh2 deficiency [137]. However, thymocytes contain three related Vav family members (Vav1-3) and deletion of all three results in a severe DN3 block [138]. While it is not known if Ezh2 can associate with Vav2 or Vav3, a disrupted interaction with two or more Vav family members could conceivably explain the effect of Ezh2 deficiency. Deletion of Ezh2 using CD4-cre did not alter subsequent T cell development or the generation of mature T cells [136]. The effect of Ezh1-deficiency on T cell development is not yet known. Mice deficient in EED and SUZ12 exhibited embryonic lethality prior to the onset of hematopoiesis [139,140], so T cell development was not examined. Mice with a hypomorphic allele of EED are viable, and exhibited a minor defect in T cell development at the DN3 stage [141].

4.4 DNA methylation at CpG dinucleotides

DNMT1—DNA methylation on cytosines at CpG dinucleotides represses gene expression, and is mediated by three DNA methyltransferases (DNMTs) in mammals. DNMT1 controls methylation after DNA replication, while DNMT3a and DNMT3b regulate de novo DNA methylation. DNMT1, DNMT3a and DNMT3b are all co-expressed in T cells, but have different patterns of expression during thymocyte development [142]. DNMT1 is expressed throughout development (at the DN, DP and SP stages), while DNMT3a is turned on only when thymocytes reach the DP stage [142]. DNMT3b expression is regulated more dynamically, being expressed at the DN and SP stages, but being turned off at the intervening DP stage [142]. The functions of these three related proteins are non-redundant, as deficiency in each alone causes defects in thymocyte development [143–145]. DNMT1-deficient mice die at gastrulation [146]. In Lck-cre DNMT1 conditional knockout mice, there was an approximately 10-fold reduction in thymic cellularity and a severe defect in $\alpha\beta$ T cell development, though $\gamma\delta$ T cell development was unaffected [144]. The thymus was primarily comprised of DN cells, indicating a severe block in the DN to DP transition. A three-fold decrease in proliferation was observed by BrdU incorporation. In addition, AnnexinV staining of thymocytes from Lck-cre DNMT1 conditional knockout mice was

increased relative to wild type, indicating a defect in survival in addition to proliferation. In support of this, thymic cellularity in Lck-cre DNMT1 conditional knockout mice was substantially restored by overexpression of a Bcl-x_L transgene [144]. Deletion of DNMT1 later in thymic development using CD4-cre did not alter T cell development or cellularity [144]. DNMT1 associates with the histone methyltransferase G9a, which may allow concomitant methylation of DNA and histones to regulate gene expression [147]. However, T cell development was unaffected in Lck-cre G9a conditional knockout mice, suggesting that the function of DNMT1 during T cell development is not dependent on its association with G9a [148].

DNMT3a/DNMT3b—Point mutations in DNMT3b are the cause of ICF syndrome, a rare autosomal recessive disease characterized by severe immunodeficiency including reduced numbers of T cells [149–151]. DNMT3b deficiency results in embryonic lethality in mice [152]. Knock-in mice with DNMT3b mutations equivalent to those found in ICF patients survive gestation but exhibit perinatal lethality, indicating that the DNMT3b mutations are hypomorphic alleles [143]. At birth, thymic cellularity was found to be reduced approximately 10-fold in mice with ICF mutations in DNMT3b, with an increased proportion of DN cells. Almost half the thymocytes present at birth were AnnexinV+, and strong TUNEL staining in thymic sections was observed, suggesting that the reduced cellularity was due to apoptosis [143]. Therefore, DNMT3b is required to maintain thymocyte viability. Mice deficient in DNMT3a are runted, and die within 4 weeks of birth [152]. A severe defect in T cell development was observed in 2.5 to 3.5 week old DNMT3a-deficient mice [145], including a 10-fold decrease in thymic cellularity and an enhanced proportion of DN cells. This defect could not be attributed to reduced proliferation, as DNMT3a-deficient thymocytes proliferated similar to wild type thymocytes upon anti-CD3 stimulation. Thymocyte viability was not examined.

5. Future Directions and Conclusions

Although this review takes a reductionist approach to analyzing the role of transcriptional repressors, corepressors and chromatin modifying complexes during T cell development, the end result is dependent on the coordinated action of all of these proteins. How the interplay of such proteins regulates specific loci largely remains to be elucidated. For example, although it has been 10 years since the initial report that absence of the corepressor NCoR causes a block in T cell development [91], the genes whose dysregulation leads to the block at the DN3 to DP transition have yet to be identified. Further, while many mice with deficiencies in specific repressors, corepressors or chromatin modifying enzymes phenocopy one another, it is not clear whether or not the blocks are due to altered expression of the same target genes. What is clear is that chromatin modifications, and the proteins that regulate them, are critical mediators of developmental decisions and that characterization of alterations in gene expression in their absence will provide greater insight into their roles in T cell development.

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Figure 1. Transcriptional repressors, corepressors and chromatin modifiers in T cell development

Shown is a schematic of T cell development, listing negative regulators of transcription required at different stages of T cell development as demonstrated by knockout or conditional knockout mice. The names of transcriptional repressors are shown in red, corepressors are shown in blue, and chromatin modifiers are shown in green. Please note that as analysis of DN4 and ISP cells was not consistently performed in the studies discussed, a simplified scheme is presented in which DN3 cells transition directly into DP cells. Please refer to the text for specific details. In addition, as *bcl11a* was shown to be required prior the development of DP T cells, but DN subsetting was not performed to identify which stage(s) were altered, it has therefore has been placed at each stage of DN development with a question mark.