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T-cell identity and epigenetic memory

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Summary

T cell development endows cells with a flexible range of effector differentiation options, superimposed on a stable core of lineage-specific gene expression that is maintained while access to alternative hematopoietic lineages is permanently renounced. This combination of features could be explained by environmentally responsive transcription factor mobilization overlaying an epigenetically stabilized base gene expression state. For example, "poising" of promoters could offer preferential access to T-cell genes, while repressive histone modifications and DNA methylation of non-T regulatory genes could be responsible for keeping non-T developmental options closed. Here we critically review the evidence for the actual deployment of epigenetic marking to support the stable aspects of T-cell identity. Much of epigenetic marking is dynamically maintained or subject to rapid modification by local action of transcription factors. Repressive histone marks are used in gene-specific ways that do not fit a simple, developmental lineage-exclusion hierarchy. We argue that epigenetic analysis may achieve its greatest impact for illuminating regulatory biology when it is used to locate cis-regulatory elements by catching them in the act of mediating regulatory change.

1.1 The problem of T-cell identity

Cohorts of T-lineage cells develop from hematopoietic precursors throughout fetal and much of postnatal life in mammals. Basic T-lineage properties, including the gene rearrangements leading to expression of a clonally individual T-cell receptor for antigen (TCR), are conferred by differentiation in the thymus (T lineage commitment). However, T cell development also continues after these properties are established. Not only are mature T cells long-lived cells with extensive proliferative potential, but also they continue to specialize after leaving the thymus. In response to antigen, they select and mobilize any of a variety of gene expression programs for effector responses, and then reinforce these programs for preferential access during stimulation events in the future (effector subset commitment). The distinct effector programs that mature T-cells can deploy in response to antigen challenge (O'Shea and Paul 2010, Zhou et al. 2009, Zhu et al. 2010, Spits and Di Santo 2011) are of great medical significance, since they determine not only what the T-cells are likely to do, but also what intercellular interaction molecules they will express to influence the functions of other immune cells. An early split, occurring within the thymus, separates the mostly $CD4^+$ TCR$\alpha\beta^+$ "helper" cells from the mostly $CD8^+$ TCR$\alpha\beta^+$ "cytotoxic" cells. Then among the $CD4^+$ cells, antigen-triggered functional specialization generates distinct cytokine-producing T-cell subsets which have been designated Th1, Th2, Th17, and Treg cells. In each of these major effector programs, the $CD4^+$ T cell has its differentiation guided by a combination of subset-specific cytokine receptor signaling, subset-specific STAT factor mobilization, and subset-specific "master" transcription factor action [rev. by (O'Shea and Paul 2010, Zhu et al. 2010, Murphy and Stockinger 2010, Wilson et al. 2009, Zhou et al. 2009)]. Thus, a set of divergent gene networks can be

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mobilized for antigen-dependent differentiation, each influenced by intrinsic, autocrine, and paracrine environmental effects. There is considerable interest in how stably or reversibly cells commit to any of these subsets, and some of the emerging answers are reviewed briefly below.

There are actually multiple subtypes of T cells besides these major $CD4^+$ TCRαβ$^+$ "Th" subsets. Some are additional variants of $CD4^+$ effectors, such as Th9, Th22, and Tfh cells, which may diverge from the other effector types through mechanisms that are under intense discussion (Murphy and Stockinger 2010, Spits and Di Santo 2011). In addition, several more divergent T-cell lineages branch off from all major TCRαβ lineages in the thymus. These include TCRγδ subsets that not only use different genes to encode their receptors but also have distinctive homing, cytokine expression, and response threshold properties; and in addition, NKT and possibly also CD8αα "innate type" T cells that follow different triggering rules than conventional TCRαβ $CD4^+$ T cells (Meyer et al. 2010, Park et al. 2010, Kreslavsky et al. 2009, Spits and Di Santo 2011, Das et al. 2010, Gangadharan et al. 2006). The initial determinant spurring choice of one of these developmental pathways is often the particular TCR the cell expresses, and its interaction with particular ligands expressed in the thymic environment. Nevertheless, the result in each case is to assemble within the cell a particular gene regulatory network that will be the framework for all the cells' responses to future stimulation. These diverse variants of T cells are all testimony to the versatility and dynamism of "the T cell program". However, the developmental problem posed by T-cell differentiation is actually broader and even more challenging than the distinctions among these subsets.

Despite the flexibility, the initial programming that enables a precursor to become a T-lineage cell at all creates a major core identity that is not flexible, and is apparently irreversibly set. The activation-dependent specialization differences are superimposed on this background of stability. Not only do rearranged TCR genes stay rearranged (as they must), but the cells also faithfully preserve a stable program of lineage-specific gene expression that has nothing to do with gene rearrangement, maintaining transcription of genes that encode the invariant TCR complex signaling components CD3γ, δ, ε and TCRζ, the lineage-specific kinases Lck, ZAP70, and Itk, and the crucial signaling adaptor molecules Lat, SLP76 (Lcp2), and GADS (Grap2). These genes are all largely or completely T-cell specific in their RNA expression (http://www.immgene.org)(Heng et al. 2008). Thus, they must be maintained by a specific aspect of T-lineage regulatory state that is held constant, even while the cells dynamically alter their transcriptional program choices in response to their environments. The contrast between stable T-lineage identity functions and versatile, multi-option, actively modulated effector functions is much more obvious in the case of T cells than in the case of B cells. Moreover, wherever they migrate and whatever signals they encounter, mature T cells do not regain access to genes associated with some of the alternative developmental programs that were available to their early precursors, such as the myeloid or B-cell programs[1]. Thus, some fundamental aspects of the T-cell developmental program are not only established earlier, but also established much more robustly, than effector function specialization. How does this work?

In this review, we start with two general models, combinatoriality and hit-&-run regulation, for understanding the T-cell state. The take-home lessons from well-established paradigms of effector gene expression in later T-cell development are reviewed. We then evaluate more critically how transcription factor action and epigenetic modification intersect, using more recent data from genome-wide analyses. Finally, the principles that emerge are applied to

[1]An interesting partial exception is the progenitor cell, B cell, and myeloid cell associated transcription factor PU.1, which is discussed below.

consider the current evidence for the regulatory origins of the T-cell gene expression program, and the crucial questions that need to be tackled in the future.

1.2 Combinatoriality

Stability of some gene expression patterns while others are changing can be explained simply by the use of different transcription factor combinations to control stable and dynamically regulated genes. Gene expression as a rule depends on combinations of factors both in development and in physiological responses to activation signals, and the precise combinations of inputs needed are dictated by the structure of gene-specific cis-regulatory elements. Thus, whether or not a particular factor is rate-limiting for activation of a given target gene generally depends on what other factors are available to collaborate, and a different requirement may apply at other target genes of the same factor. An extreme case is that of the T-cell cytokine gene, *Il2*, which depends on a complex ensemble of differentially-activated transcription factors, all of which can make rate-limiting contributions to its expression (Bunting et al. 2006, Rothenberg and Ward 1996, Jain et al. 1995). Like other transiently activated cytokine genes, it requires inputs from the acutely mobilized signal-response transcription factors AP-1, NFAT, and an NF-κB family member such as c-Rel. When *Il2* is induced, transcription terminates almost immediately when cyclosporin A or FK506 is used to interrupt availability of one of these factors, and in these cases the entire transcription factor ensemble rapidly dissociates from the promoter-proximal enhancer of the gene (Garrity et al. 1994, Chen and Rothenberg 1994, Rao et al. 2001). Thus, if it were the case that all T cells stably expressed certain core transcription factors, then it is theoretically possible that any genes that need to be expressed stably could simply require combinations of these constant factors. Then, in this scenario, genes involved in dynamic subset specialization would be distinguished by their requirement for different combinations of transcription factors which might need to include at least one factor that was not part of the core, but rather dependent on context or environmental signals. Indeed, this is a way that STAT factors are used to regulate T effector subset differentiation genes, as noted below. Such a combinatorial gene control model is consistent with known features of gene regulation (Davidson 2006) and does not necessarily require any hierarchy in mechanisms leading to different degrees of expression stability among different genes.

1.3 Epigenetics and hit-&-run gene regulation

There is an alternative way that some gene expression “decisions” could be made more permanent than others, however, and over recent years this alternative has attracted great interest. The constraint that some transcription factors need to be expressed with perfect stability or excluded continuously can be removed if the genome itself can be selectively masked by modifications of chromatin that are passively maintained from generation to generation. If intrathymic differentiation could position specific epigenetic modifications so as to block or favor particular genes for expression in the future, then these components of the T-cell program would be selectively buffered against regulatory change while others could vary freely during immune responses.

Epigenetic mechanisms were first described to explain the hit-and-run action of the factors that establish Hox complex gene expression patterns, which are then sustained for long-term organization of body plans in embryonic development of many kinds of animals (Grimaud et al. 2006, Schwartz and Pirrotta 2007). Epigenetic modifications of chromatin domains are also found associated with the long-term silencing of repeated gene arrays (Garrick et al. 1998), and implicated in position effect variegation of transgenes inserted near heterochromatin (Williams et al. 2008). Several examples in T-cell gene regulation have set additional precedents for the ability of epigenetic regulatory mechanisms to mediate durable

changes in gene accessibility, raising or lowering these genes' thresholds for future activation. Silencing of CD4 expression in the $CD8^+$ lineage depends on Runx factor repressive activity within the thymus, but after the cells are mature, repression of CD4 is less sensitive to Runx/silencer interactions (Taniuchi et al. 2002, Telfer et al. 2004). Conversely, while activation of CD4 in mature $CD4^+$ lineage cells depends on a proximal enhancer, once maturation has occurred CD4 expression continues even if this enhancer is deleted (Chong et al. 2010). Effector response genes in memory $CD8^+$ cells become easier to activate than in naïve T cells as well, due to epigenetic modifications of the chromatin at these loci that preserve a partially open state even when the genes are not currently induced (Araki et al. 2009, Araki et al. 2008). The idea of a hit and run mechanism for cell type specification would be particularly appealing to explain T-cell differentiation, because the key events in the establishment of a T-cell identity occur during interaction with a transient Notch pathway signal in the thymus, which is then discontinued and becomes dispensable after commitment (Petrie and Zuniga-Pflucker 2007, Rothenberg et al. 2008).

Well-studied examples of epigenetic changes occur during commitment of a mature, antigen-activated $CD4^+$ T cell to one or another effector subtype (Cuddapah et al. 2010, Murphy and Stockinger 2010, Amsen et al. 2009, Wei et al. 2009, Wilson et al. 2009, Nakayama and Yamashita 2009, Ansel et al. 2006, Reiner et al. 2003), as summarized below. During $CD4^+$ T cell effector polarization, genes of the favored pathways become easier to activate and those of the disfavored pathway become harder to activate than in the naïve cells. In these cases, clear shifts are seen in the patterns of CpG DNA methylation and in chromatin compaction restricting DNase sensitivity around subset-specific cytokine genes. In addition, covalent modification of histone proteins in nucleosomes appear to mediate many of the effects of transcription factors on chromatin, enabling prior transcription factor activity to influence future nucleosome conformation, DNA methylation, and ultimately gene expression.

Most histone marks are thought to favor or reinforce the regulatory effects (activation, repression, etc.) in the course of which they were first deposited (Kouzarides 2007). Thus they are thought to stabilize the induced gene expression pattern in a kind of positive feedback, helping to make differentiation irreversible. "Activating" marks like histone H3 K(9,14) acetylation or H3 K4 trimethylation, "accessibility" marks like H3 K4 mono- or di-methylation, and "repressive" marks like H3K27 trimethylation or H3K9 trimethylation can then affect the tightness of nucleosome packing and the ease of access of transcription factors or RNA polymerase II to their target sequences in the DNA. "Repressive" histone marks also recruit DNA methyltransferases which strengthen repression by methylating local CpG residues. Not only is this associated with recruitment of chromatin condensation proteins, but also it can directly block future recognition of target sites by activating transcription factors (Polansky et al. 2010, Maier et al. 2003). At the same time, combinations of "activating" and "repressive" histone marks can occur at the same loci ("bivalent" marks), and these are strongly associated with instability of expression, perhaps increasing the sensitivity of the gene to future activating or repressive signals (Cui et al. 2009, Bernstein et al. 2006).

1.4 Interplay between cellular history and current activation state: examples from cytokine gene control

Cytokine gene regulation in mature T cells has yielded strong case studies for how epigenetic modifications can mediate the interplay between prior and current transcription factor activity. This extensive literature has been authoritatively reviewed (Wilson et al. 2009, Cuddapah et al. 2010, Murphy and Stockinger 2010, Balasubramani et al. 2010, Zhu et al. 2010, Amsen et al. 2009, Ansel et al. 2006) and is only briefly summarized here. The

main take-home lessons have come from the Th2 cytokine gene cluster, including *Il4, Il13,* and *Il5*, and from the extensive array of elements that regulate the signature Th1 cytokine gene, *Ifng*. Both the Th2 and the Th1 cytokine loci are activated by TCR signaling, but in mutually exclusive patterns in polarized effector cells. In contrast, naïve T cells respond to TCR activation by activating other loci preferentially, such as the *Il2* locus; they can only weakly and slowly induce expression of either the Th1 or Th2 signature cytokine loci. Yet the transcription factors activated by TCR signaling that directly induce all these genes, including the Ca^{2+}/calcineurin-dependent factor NFAT, are apparently the same in all cases. Thus, the difference in inducibility among Th1 cytokine, Th2 cytokine, and *Il2* loci appears to reside in the prior accessibility marking that these loci undergo during differentiation from naïve to antigen-activated polarized cells.

The IL-4 mobilized transcription factor Stat6 plays a major role in establishing the permissiveness of the Th2 cytokine gene complex for activation (Lee and Rao 2004, Wei et al. 2010), which is then sustained by binding by the product of another Stat6 target gene, the Stat6-upregulated transcription factor GATA-3 (Onodera et al. 2010, Ouyang et al. 2000, Yamashita et al. 2004). As cells differentiate into Th2 effectors, new, subset-specific DNase hypersensitive sites appear that are opened and maintained by Stat6 and GATA-3 binding. Importantly, these maintain a distinct condition of “active” chromatin that persists much later, between bouts of TCR-signaling, in the Th2 memory state. Thus, the active chromatin configuration around the locus is not simply an adjunct to active transcription nor dependent on ongoing NFAT recruitment to the promoter. The permissive state is associated not only with DNase hypersensitive sites but also with depletion of CpG methylation across the Th2 cytokine gene promoters, as well as the presence of “accessible” histone marks. This permissiveness presumably underlies the preferential recruitment of factors like NFAT to the *Il4* promoter in these cells, the next time TCR signaling is reactivated. The Th1 cytokine genes are not equivalently clustered, but the regulation of the “signature” Th1 cytokine gene *Ifng* has now been described in detail (Balasubramani et al. 2010, Schoenborn et al. 2007, Chang and Aune 2007). At the *Ifng* locus, an array of elements, extending up to about 60 kb upstream and about 50 kb downstream of the transcription unit itself, turns out to be required for correct, fully efficient expression. Again, DNase hypersensitivity of these elements is greatly enhanced by Th1 differentiation and reduced by Th2 differentiation. Several of these key elements are directly engaged by the Th1 transcription factors T-bet (Tbx21) and Stat4 (Schoenborn et al. 2007, Wei et al. 2010, Balasubramani et al. 2010). These cases illustrate chromatin modifications that go along with lineage-specific memory and stable differentiation, and which can be separated from activation of pol II-dependent transcription *per se*.

1.5 Reversibility and plasticity of histone marks

Nevertheless, histone marks are clearly subject to modification in response to changes in engagement of sequence-specific transcription factors [rev. by (Natoli 2010)]. It is notable that across the genome the great majority of the “positive” marks, H3K4me2 and H3K4me3, are localized in discrete islands, with clear peaks implying a site-specific deposition mechanism (see examples in Fig. 1). Indeed, changes in histone marks can be a symptom of current transcription factor binding. For example, activation of the combination of factors that turns on *Il2* transcription can rapidly increase DNA accessibility to DNase and restriction endonuclease digestion (Ward et al. 1998, Rao et al. 2001) and demethylate CpG residues in the promoter-proximal region (Bruniquel and Schwartz 2003, Murayama et al. 2006). This coincides with *de novo* acetylation of histone H3 over several kilobases upstream of the promoter (Adachi and Rothenberg 2005, Chen et al. 2005).

Alteration of local histone modifications is a general correlate of transcription factors at work. Binding of the crucial myeloid and B cell factors PU.1 and EBF1 can rapidly induce mono- and/or di-methylation of H3K4, respectively, when they bind their target sites (Ghisletti et al. 2010, Heinz et al. 2010, Treiber et al. 2010). Nucleosome remodeling and local conversion of H3K4me1 to H3K4me2 or me3 marking, causing the H3K4me1 marked region to flatten and "spread", is also seen as a rapid, direct response to the local binding of E2A, EBF1, or PU.1 (Heinz et al. 2010, Lin et al. 2010).

Repressive marks as well as activation marks can be dynamic. The specific loss of "open" H3K4me3 marks can be triggered quickly by the withdrawal of Notch signaling, which causes the Notch-sensitive transcription factor RBP-Jκ (or CSL=CBF1, Suppressor of Hairless, Lag-1) to switch from binding a coactivator to binding a corepressor complex containing the H3K4me3-specific demethylase KDM5A (Jarid1a, RBP-2) (Liefke et al. 2010). Conversely, conversion of naïve T cells to Th2 cells involves elimination of long-standing H3K27me3 marks from an upstream regulatory region of *Gata3* (Fang et al. 2007, Amsen et al. 2007) (Fig. 1B). The transcription factor Tbx21 (T-bet) can directly strip H3K27me3 repressive marks from the promoters of some of its target genes, by recruitment of demethylases like Jmjd3 and Utx (Miller and Weinmann 2010). Finally, even the supposedly prohibitive modification of CpG DNA methylation has elegantly been shown to be removable by the combined action of transcription factors E2A, EBF1, and Runx1 in early B cell development (Maier et al. 2004). Clearly, in order to promote these epigenetic modifications, the factors must have access to the DNA and the previous marks cannot be viewed as deterministic.

These considerations challenge the picture that histone modification patterns give past transcription factor action a lasting dominance over the scope of future transcription factor action. Such marks may not, for example, distinguish prohibited from temporarily quiet genes in a given cell type. Instead, they may offer access to a different, more focused, more developmentally interesting kind of insight. Histone marking patterns in a steady state condition reflect the site-specific integrations of prior and current regulatory inputs. Thus, the location of *changes* in histone marking, across the time of a developmental transition, will be highly selective, and will reveal specific cis-elements where key transcription factors must be acting within that time frame to promote that change.

1.6 Combinatorial transcription factor action and epigenetic modification

Genome-wide methods allow new light to be shed on the rules governing the reciprocal interaction of transcription factors with the epigenetic modification apparatus. One question is to evaluate globally how much transcription factor binding may really be limited by prior epigenetic modification, if that can be separated from the concurrent action of other transcription factors. Another is what determines the epigenetic consequences of a transcription factor's binding in a given cellular and DNA sequence context.

The foundational determinant of transcription factor binding occupancy is of course target site sequence recognition. Powerful high-throughput technologies for assessing binding by purified transcription factors in vitro have now provided a greatly improved measure of the quantitative preferences that particular transcription factors show for diverse site variants, when they are binding on their own (Berger and Bulyk 2009). However, the actual distribution of sites that transcription factors bind in vivo is not always congruent with the set of sites that they would be predicted to bind even based on these improved bioinformatic grounds. For most factors, only a subset of canonical sites are bound in vivo at any one time, and also there is significant recruitment to sites where no predicted target site exists (Badis et al. 2009, Gordân et al. 2009). These findings suggest that the sites where factors actually

work in a given cell type can be affected positively by interaction with other factors, not just limited by site masking or through chromatin constraints.

Although transcription factors generally bind to many fewer sites than could be predicted on sequence grounds alone, the sites they bind are not simply predictable by degree of match to a measured position weight matrix. More importantly, they are cell type specific. A factor such as PU.1 is expressed at different levels in B lineage cells, myeloid lineage cells, and early T cells, but it binds to distinct patterns of sites in all three (Heinz et al. 2010)(J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished results). Although myeloid lineage cells express the highest amounts of PU.1, they fail to engage PU. 1 at a number of genomic sites where it is bound in the much lower-expressing B or early T-lineage cells (Heinz et al. 2010) (J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished results). This implies that classical mass-action is not the sole discriminant for site selection. In a similar way, E2A binding in pre-pro B cells occupies some sites that it will not occupy later in definitive pro-B cells (Lin et al. 2010), despite increasing overall expression of E2A at the later stage. Is the site discrimination in these cases caused by masking through repressive chromatin? To date, the only genome-wide assessment of chromatin “closing” can be provided by the mapping of repressive histone marks. In our own studies of early T-cell precursors, PU.1 site selectivity does not appear to result from masking by H3K27me3 marks in general (J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished results).

Instead, some contextual information that may be provided by other transcription factors determines where a factor will bind. One example is the developmental role of EBF1 in recruitment of E2A to a large number of new sites in early B cell precursors, sites where E2A can bind in close proximity to EBF1 and where new H3K4 methylation marks are formed (Lin et al. 2010). Another likely example is the selective binding and activity profile of EBF1 when ectopically expressed in a hematopoietic, as opposed to a nonhematopoietic, cellular context (Treiber et al. 2010). Finally, the B and myeloid lineage factor PU.1 is recruited to different spectra of binding sites in myeloid and B cell contexts, in large part because of its need to interact with C/EBP family factors in the former and with E2A and/or Oct family factors in the latter (Heinz et al. 2010). C/EBP factors and PU.1 can each recruit the other to bind to joint cis-elements. The implication is that a large fraction of binding is itself combinatorially defined.

Whether binding by interacting factors must be strictly coordinate or whether it can be mediated through a “pioneer” mechanism seems to vary according to the factors involved and the cis-regulatory sequences that are in play. When constitutively expressed transcription factors bind DNA, they may create a favored recruitment site for later-mobilized transcription factors, mediated through local histone modifications. For example, constitutively expressed PU.1 in myeloid cells can guide activation-dependent factors selectively to myeloid-specific response genes, very likely because it can induce permissive local H3K4 monomethylation and nucleosome remodeling at sites where it has bound (Natoli et al. 2011, Ghisletti et al. 2010, Heinz et al. 2010). Although the PU.1 binding is insufficient to turn on these activation-dependent target genes itself, it can focus the activity of the activation-dependent factors on a subset of their potential genome-wide targets for efficient and cell-type specific responses. In these cases, combinatorial transcription factor action is still important, but epigenetic changes allow different participants to be brought to the site asynchronously.

Although factors such as PU.1, E2A, and EBF1 often induce “accessibility” histone marks at their binding sites, for each of these factors there is also a minority of sites bound in vivo where these marks are induced barely if at all, even with strong transcription factor

occupancy (Lin et al. 2010)(J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished results). Why can these factors induce H3K4 modification in some but not all cases? The combinatorial binding analyses of Lin et al. (Lin et al. 2010) suggest that E2A binding alone has a different functional role than E2A binding together with cofactors such as FoxO1 and EBF1. Thus, factor-factor interactions beyond those needed to stabilize occupancy may explain how recruitment of Set7/9 and MLL-type H3K4 methyl transferases, which are thought to generate mono- and dimethyl H3K4 respectively (Allis et al. 2007), may fail to occur at some sites. A crucial open question is whether these H3K4-undermethylated sites are simply nonfunctional sites, or whether they reflect deployment of the transcription factor for a subset of their functional roles that may not involve "accessibility", such as chromosome looping or repression. There is a clear precedent from the activation and repression of different classes of target genes in erythroid cells by GATA-1, in collaboration with different partners and with the induction of different epigenetic marks (Yu et al. 2009, Fujiwara et al. 2009, Tripic et al. 2009, Cheng et al. 2009).

1.7 A problem of repression

The stability of repression is central to some of the most important questions about the inheritance of cellular identity. Cellular identity clearly involves prohibiting certain genes from being expressed, even while other genes can be on or off at different times, and it would be valuable to understand the biochemical distinction between permanent repression and conditional repression, if the mechanisms are actually different. For activating marks, the functional connection is easy to make. Acetylated histone H3 and H3K4 trimethylation not only are highly correlated with the sites of currently active promoters across the genome (Wang et al. 2008, Orford et al. 2008, Heintzman et al. 2007, Barski et al. 2007, Johnson et al. 2007, Roh et al. 2006); these are marks that are actively emplaced by the coactivator complexes recruited by positive regulatory factors, including p300/CBP, GCN5, and PCAF (KAT2A, B and KAT3A,B), and MLL factors (KMT2 family), respectively, and they are then functionally implicated in the recruitment of RNA pol II cofactors (rev. by (Allis et al. 2007, Kouzarides 2007)). In contrast, H3K27me3 only indicates one type of Polycomb Repression Complex (PRC) 2-dependent repression. Other repressive marks have been described, but none are comprehensive (Barski et al. 2007). Those repression marks that are present may also be less sharply localized than activating marks. It is more frequent for an entire transcription unit or extended stretch of intergenic DNA to be associated with H3K27me3 marks than with H3 acetylation or H3K4 di- or trimethylation "activating/accessibility" marks.

When a transcription start site is modified by H3K27me3 and/or H3K9me3 in the absence of overt activating marks, then the gene is normally silent. However, this is not the only form of silencing that is found, in part because a gene can be silent due to a lack of local activators, interaction with a remote silencing element, or sequestration in a "silencing" compartment of the nucleus whether or not its promoter-proximal region has been modified with these marks. Filion et al. (Filion et al. 2010) have shown that the genome of *Drosphila melanogaster* can be divided among five types of histone marked chromatin, where most of the genes that are transcriptionally silent are in domains that lack all known "repressive" as well as "activating" marks. This unmarked fraction is considerably larger in mammalian cells (Barski et al. 2007)(J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished results), where it includes a major fraction if not a majority of the silent loci. These "empty" genes, untranscribed but lacking both activating and known repressive marks, provide little insight into the mechanisms that have turned them off originally or keep them silent afterwards.

It is not clear whether all silent genes should require active repression or direct promoter blockade in order to stay silent. Many genes could remain silent passively, simply because the transcription factors that they require are themselves repressed. Interestingly, in our own studies, silent transcription factor genes are much more likely to bear H3K27 or H3K9 trimethylation markings than the average for silent genes in the genome overall (J. Zhang, unpublished data). This may be a common feature of development from Drosophila to human, as an overwhelming majority of the PRC-repression target genes conserved between *D. melanogaster* and human appear to encode transcription factors (Schuettengruber et al. 2007). It is more likely for non-transcription factor target genes to remain silent without defined repressive marks.

It is likely that some transcriptional repressor proteins used in T-cell development work as a rule by specific mechanisms that bypass a need for PRC2-type complexes. In our own analyses of developing T-cell precursors (J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished results), repressive H3K27me3 marks are not present on the CD4 intronic silencer (Taniuchi et al. 2002) in DN cells, at stages when this silencer is acting, nor on the intergenic silencer between Rag1 and Rag2 (Yannoutsos et al. 2004) at stages when these genes are also repressed. The CD4 silencer seems not to be maintained by DNA methylation either (Zou et al. 2001). Since both CD4 and Rag1/Rag2 silencers are Runx dependent, it is possible that Runx factors as a rule use a repressive mechanism other than H3K27me3 or DNA methylation. Recent evidence suggests that Runx-mediated repression can involve interchromosomal interactions, which occur in particular nuclear membrane-associated compartments of the nucleus (Collins et al. 2011), possibly mediated by the known nuclear matrix binding activity of Runx factor C-terminal domains (Zeng et al. 1998). In any case, these exclusions mean that the absence of H3K27me3 (or H3K9me3) marks need not mean the absence of a repressive mechanism.

These findings make it difficult to use a single “snapshot” of a long-repressed gene to locate the regulatory element(s) through which the initial repression occurred. The power of epigenetic analysis will be greatly enhanced when diagnostic indicators for additional classes of repression can be discovered. However, valuable information can emerge nonetheless when time-resolved changes in repressively marked domains are detected.

1.8 Epigenetic marking events in T-lineage gene activation from stem-cell precursors

For T-cell development, the challenge is to explain the combination of persistence of central identity together with plasticity of functional characteristics. What kind of information about the programming of T-cell identity can we expect to obtain through mapping of epigenetic changes? If data are only available from mature T cells, the “activation” marks at annotated promoters are likely to agree with current transcriptional activity, and are not particularly helpful to explain the nature of the process that established this active gene expression. But we can ask whether and where distinctive epigenetic marks may have been deposited through the mechanisms that explain the long-term exclusion of non-T lineage properties. Also, looking beyond the transcription start sites themselves, we can use epigenetically marked regions to locate candidate sites for cis-regulatory elements of both T and non-T lineage genes. And most powerfully, if we can examine immature T cells, as well, to track the pattern of epigenetic marking across the genome through developmental time, we may be able to explain the separate mechanisms that operate during the distinctive staging of the T-cell specification and commitment process.

The origin of the “persistent” T-cell properties may itself have an interesting epigenetic component because of the way the T-cell program is activated in developing thymocytes. It

requires many days of Notch pathway signaling, under the influence of Notch ligands in the thymus, in order to establish T-cell gene expression and lineage commitment (rev. by (Rothenberg et al. 2008, Petrie and Zuniga-Pflucker 2007). However, afterwards the Notch signal becomes dispensable for $CD4^+$ and $CD8^+$ cell production (Wolfer et al. 2001, Tanigaki et al. 2004). Thus, even the onset of T cell identity raises the question of why such a sustained exposure to Notch pathway signals in the thymus is needed, persisting over days, in order to activate the T-cell program and eventually to block off other potential programs forever. The highly staged progression toward lineage commitment preceding TCR gene rearrangement would be consistent with a stepwise mechanism in which initial regulatory factor induction could lead to epigenetic changes that make cis-regulatory elements of a few target genes more accessible to additional regulatory factors, thus allowing the targets to be turned on or off once these additional factors are available (cf. refs. (Heinz et al. 2010, Hagman and Lukin 2005)). If some target genes in these cases encode transcription factors themselves, then this process can be iterated several times, reaching more and more target genes as distinct sets of regulatory genes come into play. But is this true? At present, the published literature offers a few tantalizing clues.

Not all T cell genes begin from an inaccessible state in stem and progenitor cells. In multipotent hematopoietic stem cells, low-level gene expression appears to proceed simultaneously at a range of loci that are ordinarily associated with mutually exclusive cell fates, a phenomenon known as multilineage priming (Ji et al. 2010, Ng et al. 2009, Weishaupt et al. 2010, Månsson et al. 2007, Pronk et al. 2007, Miyamoto et al. 2002, Hu et al. 1997). Some of the earliest hints for poised chromatin as a mechanism for multilineage priming in stem cells included DNase hypersensitivity of T-lineage specific elements in multipotent cells (Wotton et al. 1989). A recent study has now compared multiple histone marks and pol II occupancy patterns across the genome in hematopoietic stem cells, multipotent progenitors, and more restricted progenitors on the one hand and mature peripheral T cells on the other hand (Weishaupt et al. 2010). A number of genes with T-cell specific expression patterns and strong activation marks in mature T cells were found to be poised for activation with H3K4me3 marks in stem and progenitor cells long before transcription began, while others had their promoters bivalently marked (with H3K4me3 or H3Ac, and H3K9me3 or H3K27me3). Still other T-cell specific genes were apparently devoid of any marks in the stem and progenitor compartment (Weishaupt et al. 2010). The prevalence of poised genes and genes not obviously silenced raises the question of what trigger these genes are waiting for. However, this inference does not reveal the timing and ordering of epigenetic modification relative to gene activation.

T-cell gene expression is induced in several waves during the differentiation of pro-T cells both in vivo and in vitro (Yui et al. 2010, David-Fung et al. 2009, Tydell et al. 2007, Kawazu et al. 2007, Dik et al. 2005, Taghon et al. 2005, Tabrizifard et al. 2004). There is a general trend to full mobilization of a “T-cell identity” gene expression program by the DN3 stage (Rothenberg et al. 2008), but no single “on switch” activates T-cell genes at a stroke. Thus there could be a role for epigenetic constraints that need to be removed stepwise at certain key loci. Our own studies are tracking the shifting patterns of epigenetic modification across the genome as they correlate with transcriptional changes at successive stages in this process (J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished results). To date, however, in the published literature, epigenetic changes during murine T-cell specification itself have been traced most closely at the level of changes in DNA CpG methylation (Ji et al. 2010). The major increase in T-cell gene expression in the DN2 and DN3 stages is marked by dramatic demethylation of CpG sites around genes involved in T-cell identity, including *Lck*, *Tcf7* (encoding TCF-1), and *Bcl11b* (Ji et al. 2010)(http://charm.jhmi.edu/hsc/]. The demethylation of *Bcl11b* in particular appears tightly coupled with the activation of this highly T-lineage specific gene (Yui et al.

2010). Thus, the transcription factors activated during and immediately after the DN1 stage can specifically recruit demethylating enzymes to these DNA sequences, or else selectively block maintenance methylation of these sites during proliferation, opening up access for recognition by the next tier of DNA-binding proteins. This rich trove of evidence is likely to yield considerable insight into the gene regulation mechanisms in early T cells. However, there is still much to learn about the component processes that interlock to activate the T-cell program.

1.9 Epigenetic repression of non-T cell genes during T-lineage commitment

T lineage commitment depends in principle on both positive and negative regulation. T-cell essential positive regulatory factors need to be activated and their expression stabilized to maintain T-cell identity gene regulation. In parallel, regulatory genes used in alternative pathways need to be repressed or kept permanently silent. Although effects on alternative-lineage cytokine receptor gene expression and homing receptor expression are also likely to be important for shaping the process of lineage restriction in vivo, as discussed elsewhere (Rothenberg 2011), a particularly central cell-intrinsic aspect is the silencing of transcription factors that could provide access to non-T cell developmental programs (Table 1). These alternative-fate regulatory genes in fact are likely to be silenced through several distinct waves of repression, because access to different non-T fates is lost at distinct stages in T-cell specification (Rothenberg 2011). One alternative, the NK cell program, remains closely linked to the T-cell program in regulatory terms and arguably continues to underlie mature cytolytic T cell function. Only one transcription factor yet described appears to distinguish NK cells from T cells, the zinc finger factor Zfp105 (Chambers et al. 2007, Li et al. 2010b) (http://www.immgen.org). But other fate alternatives have clearly distinct regulatory features that separate them from virtually all mature T cells. Thus, T-lineage commitment can be predicted to involve establishment of durable, robust repressive mechanisms at a specific set of important regulatory loci.

Loss of access to the B cell program should entail loss of inducibility of the crucial B-cell specific factors EBF1 and Pax5 (Mandel and Grosschedl 2010, Lukin et al. 2008, Cobaleda et al. 2007). Loss of access to the dendritic cell program is likely to involve loss of PU.1 (Carotta et al. 2010, Lefebvre et al. 2005), just as loss of access to other myeloid programs should entail loss of PU.1 and C/EBP family factors (Laiosa et al. 2006, Wölfler et al. 2010). Another program that must be repressed is the pluripotent stem/progenitor cell state itself, since pre-commitment T-cell precursors initially express a group of regulatory factors that are strongly implicated in stem and progenitor cell proliferative expansion or self-renewal. These factors include products of the known proto-oncogenes *Tal1* (SCL), *Lyl1*, *Hhex*, *Lmo2*, *Bcl11a*, *Erg*, and *HoxA* cluster genes as well as the Hox cofactor Meis1, PU.1 (*Sfpi1*) and Gfi1b (Yui et al. 2010, Rothenberg et al. 2010), all of which are implicated in stem and progenitor cell development and maintenance. Thus, silencing of these genes may play a role in the irreversibility of T-lineage choice. Expression of the stem/progenitor cell regulatory state is evidently fairly stable, as it persists into the DN2 stage when pro-T cells have visibly responded to Notch signaling and have already begun upregulating T cell identity genes. The stem/progenitor regulatory genes must then be actively repressed through a T-lineage specific mechanism, because in Bcl11b-deficient pro-T cells they continue to be expressed abnormally (Li et al. 2010a). Given this integral role in early T-cell precursors, it is particularly interesting to consider how the stem/progenitor regulatory genes are eventually taken out of action.

Published datasets for mature T cells indicate that some of the alternative-lineage genes are indeed repressed through the imposition of epigenetic silencing marks (Fig. 2, Table 1). *Pax5* and *Ebf1* are heavily marked with H3K27me3 across their 5′ ends and known

regulatory elements in multiple effector subsets of mouse and human $CD4^+$ T cells (Weishaupt et al. 2010, Wei et al. 2009, Barski et al. 2007)(Table 1; e.g., Fig. 2C). The single NK-specific regulatory gene that is not expressed in any known subsets of T cells, namely Zfp105, is covered with repressive marks in mature T cells as well. Similarly, stem/progenitor-cell genes *Gata2, Tal1*, *Lmo2*, and *Bcl11a* have all acquired strong repression marks in mouse and human mature $CD4^+$ T cells (e.g. Fig. 2A, D). However, a role for alternative modes of repression is evident in the cases of PU.1 (*Sfpi1* gene)(Fig. 2B). PU.1 is silenced during T-lineage commitment in the DN2-DN3 transition, but is devoid of known repressive marks in mature $CD4^+$ T cells (Weishaupt et al. 2010, Wei et al. 2009, Barski et al. 2007). This is potentially associated with the ability of PU.1 to be "reawakened" during differentiation into the Th9 type of effector T cells, one response path for mature $CD4^+$ cells after antigen stimulation (Chang et al. 2010). However, Th9 cells constitute a rare subset, and for the overwhelming majority of T cells PU.1 remains permanently silent. Several other genes similarly appear to be silenced without strong repressive marks, and for these it is currently difficult to use epigenetic information to elucidate the timing and sites of action of T-lineage specific repressive mechanisms.

For the genes that are silenced with H3K27me3 deposition, two interesting questions arise. First, is the deposition of this mark an integral part of transcriptional silencing, or may it simply be allowed to occur once the actual transcriptional repression event has taken place? Can appearance of repressive marks follow afterwards, in the aftermath of developmental repression? For example, there are genes that have their promoters flanked by islands of strong H3K27me3 modification even when they are expressed, similar to the case of *Gata3* (Fig. 1B). In these cases, conceivably the "open" state maintained at the promoter might depend on a continuous "antirepression" mechanism that limits the spread of this negative regulatory feature. If these genes stop being actively induced, it is possible that negative marks could be propagated across cis-elements of the gene simply as a result of prolonged absence of the activating complexes that previously kept them at bay.

Second, is the repression denoted by H3K27me3 marking truly permanent? In fact our own results (J. Zhang, A. Mortazavi, B. Williams, B. J. Wold, and E. V. Rothenberg, unpublished) provide a number of cases where these marks can be removed rather quickly when developmentally important genes are activated. Furthermore, there are some surprising examples from the data of Wei et al. (Wei et al. 2009) where H3K27me3 marking appears to be partially reversible (Table 1). In these cases, genes that are fully repressed in thymocytes (J. Zhang et al., op. cit., unpublished data) and naïve T cells alike may have their repression marks seemingly attenuated during antigen-dependent effector specialization in Th1 or Th2 cells, even if the genes are not re-expressed. It is interesting that T-cell activation appears to result in the transfer of the histone H3K27 methyltransferase Ezh2 to the cytoplasm (Su et al. 2005, Hobert et al. 1996). If this indeed depletes the nuclear pool, then T cell activation could temporarily create a less restrictive chromatin environment for repressed genes with each round of DNA replication. Thus, something else besides the presence of the repressive marks defines their permanence in cases like Pax5, where no known reactivation mechanism appears to exist.

1.10 Concluding remarks: open prospects

To explore how different regulatory mechanisms, durable and reversible ones, may be superimposed upon each other in T cell development, epigenetic analysis offers a glorious harvest of detailed, gene-specific observations. However, it still presents some difficulties for explaining developmental programs. The information conveyed by specific histone and DNA methylation marks at a given moment in time is purely correlative, and so far, much of the use of this information has been to ratify what is already observed at the RNA

expression level. For prediction or understanding of future gene activity and its constraints, there is much to be learned still. The H3K(9,14)Ac mark is tightly correlated with active promoters through its functional connection with RNA pol II recruitment, while the H3K4me3 modification marks both active and potentially active promoters (Heintzman et al. 2007). However, these give no clear way of distinguishing an inactive promoter that will be activated soon from one that was active but will never be active again. For repression, the H3K9me3 and H3K27me3 marks are highly correlated with silencing, but there are many silenced genes and possibly multiple silencing mechanisms that they miss. More poignantly, some genes that are covered with repressive marks and invested with CpG methylation in one stage may be strongly activated at a later stage, and we lack ways to distinguish prospectively the cases in which this is or is not possible.

Much interest has focused on the epigenetic status of known promoter regions that can be associated with known gene transcripts. Bioinformatically, due to rich genome annotation, this is the low hanging fruit. However, the greatest payoff from epigenetic mapping is likely to come from the ability to visualize regulatory regions that are not part of known promoters or included in known transcription units. Some of these may actually be promoters of novel genes, including noncoding RNA genes, while others are distal cis-elements for known genes. In either case, the pattern of cis-elements that are in play is cell type specific and stage-specific, profoundly influenced by the way stage-specific transcription factors can affect histone modifications at their binding sites, as already described for PU.1, EBF1, and T-bet. Epigenetic marking patterns around developmentally regulated genes often suggest the presence of cis-elements that can be up to 100 kb away from the promoter or more, but visible because of histone modifications paralleling those at the promoter. Such elements may be crucial in order eventually to account for the regulation of the gene in various biological contexts.

With respect to T cells, a particularly interesting question is whether there is any link between different mechanisms of gene silencing and hierarchies of “fate exclusion” in development. The T-cell developmental pathway originates from hematopoietic committed cells in which lineage-irrelevant genes are permanently silent. Cells then appear to undergo an early exclusion of the erythroid and megakaryocytic developmental fates, followed by restrictions on myeloid fate, exclusion of the B cell fate, and then elimination of dendritic-cell and remaining myeloid potential together with loss of “stem/progenitor” properties (Rothenberg 2011). The NK-cell alternative may be eliminated last. One might imagine two extreme scenarios to account for these events. In the first, the depth of repression marking could be cumulative, so that non-hematopoietic and erythroid genes would be most heavily marked and NK cell genes the least heavily marked. Alternatively, at the opposite extreme, the heaviest repression marks might need to be applied in order to halt ongoing transcription, but eventually become dispensable as not only the gene itself but also the positive regulatory factors that once promoted its expression remain untranscribed. The incompleteness of our tools to detect different modes of repression limits the conclusions that can be drawn, but there are already data in hand to call into question both extreme models. The stem-cell genes *Lmo2* and *Gata2* carry repression marks in mature T cells, while the erythroid transcription factor gene *Gata1* remains in apparently open chromatin; yet H3K27me3 marks bury the B-cell specific *Pax5* and *Ebf1* genes, while the later-silenced PU.1 gene (*Sfpi1*) remains “open” (Table 1)(Barski et al. 2007, Wei et al. 2009, Weishaupt et al. 2010).

These results imply that distinct silencing marks may be less indicators of the “silent state” as a whole, or of genealogical hierarchy, than traces of the highly specific mechanism that acted to repress a given gene during a specific gene network transaction. In this way, they may be bringing us closer than we expected to the actual regulatory factors that are the causal forces in developmental lineage commitment.

The scope of evidence provided by genomic mapping of epigenetic marks in a given cell type is enormous, but the ability to use this evidence to answer specific developmental questions is enhanced and greatly focused when maps are compared from cells at successive stages of development. This follows from the goal of explaining changes in gene expression, which are necessarily derivatives of transcriptional activity with respect to time. The marked histone peaks that are relevant to the *change in expression* of a particular gene are most likely to be those that undergo a *change in modification* in parallel. Thus, rather than simply creating an impossibly large amount of data to analyze, sequential epigenetic analysis across a developmental process helps to narrow the field of focus to those particular transcription units and epigenetically marked regions that change status between stages. Cis-regulatory analysis of mammalian genes has not been as prominent in the past decade as it was earlier, partly because many strategies based on "Ockham's razor" logic have proven disappointing. However, by selecting the full set of candidate cis-elements identified as islands of dynamically specific epigenetic marking, and using modern gene transfer methods such as BAC transgenesis to assess their function, important regulatory questions should now come within range of solution.

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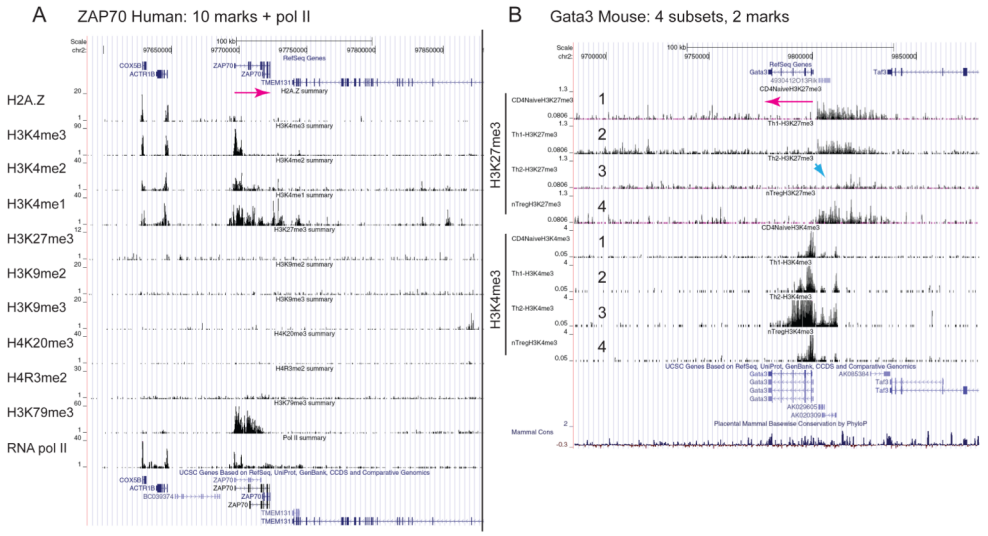

Figure 1.

Examples of histone marking patterns in neighborhoods of strongly-expressed T-cell genes. (A) *ZAP70*, in human CD4$^+$ T cells (data from (Barski et al. 2007)). (B) *Gata3*, in four subsets of murine CD4$^+$ T cells: 1, naïve T cells; 2, Th1 T cells; 3, Th2 T cells, and 4, "natural" regulatory T cells (nTregs)(data from (Wei et al. 2009)). Plots show enrichments of chromatin immune precipitation with antibodies against the indicated modified histones and RNA polymerase II, displayed as histograms against the human (hg18, panel A) and murine (mm9, panel B) genomes on the UCSC genome browser (http://genome.ucsc.edu). WIG files for (A) were downloaded from http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.html. WIG files for (B) were constructed from raw data deposited in a public repository by Wei et al., and converted from mm8 to mm9 coordinates before plotting. H2A.Z, H3K4me1, H3K4me2, and H3K4me3 are all markers for accessibility and/or activation. H3K27me3, H3K9me2, H3K9me3, and H4K20me3 are all implicated in repression. H3K79me3 is associated with actively elongating polymerase. The repression mark H3K27me3 and the activation mark H3K4me3 are particularly useful, as shown in (B). Magenta arrows indicate the genes of interest and their directions of transcription. In (B), blue arrow shows site of upstream regulatory element for *Gata3* that is selectively demethylated in Th2 cells.

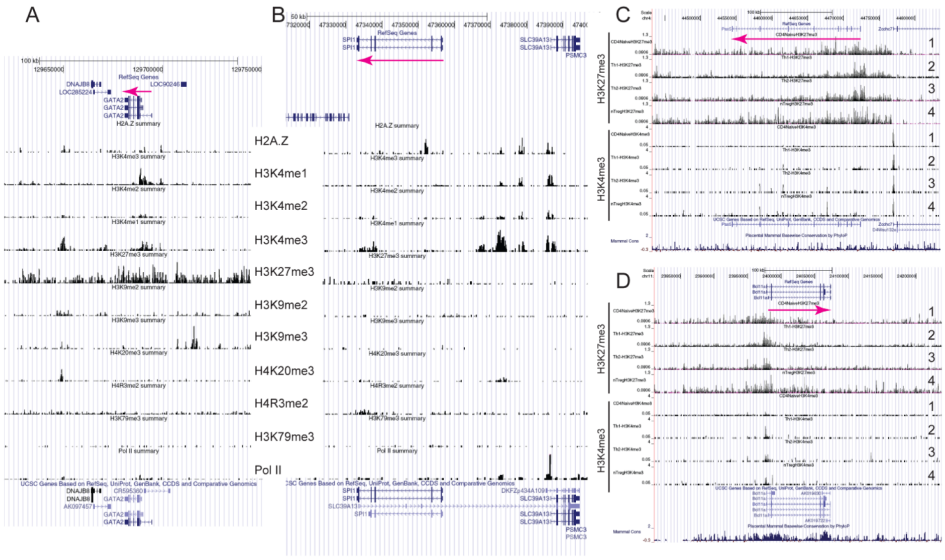

Figure 2.

Distinct modes of repression of lineage-inappropriate genes in T cells. (A) *GATA2* in human CD4$^+$ T cells: an example of "broad" H3K27me3-mediated repression of a gene that was expressed in the earliest precursor stages. (B) *SPI1* in human CD4$^+$ T cells: an example of repression without any repressive marks. Note the lack of any of the marks, H3K27me3, H3K9me2, H3K9me3, and H4K20me3, which are thought to mediate repression. (C) *Pax5* in four subsets of murine CD4$^+$ T cells: an example of "broad" H3K27me3-mediated repression of a gene which may never have been expressed in T-cell precursors. (D) *Bcl11a* in four subsets of murine CD4$^+$ T cells: an example of promoter-associated H3K27me3-mediated repression of a gene that is expressed in T cells until DN3 stage. In (C, D): 1, naïve T cells; 2, Th1 T cells; 3, Th2 T cells, and 4, nTregs. Magenta arrows indicate the genes of interest and their directions of transcription. Data for (A, B) were from Barski et al. 2007, as in Fig. 1A. Data for (C, D) were from Wei et al. 2009, as in Fig. 1B.

TABLE 1

SILENCING OF "INAPPROPRIATE" GENES IN MATURE T-LINEAGE CELLS

Alternative program	Stage fate excluded	Gene	Repression mark in mature T cells	Comments
Erythroid	Prethymic	*Gata1*	None	"Empty"
B cell	Thymic entry	*Ebf1*	H3K27me3, promoter > body	Silent in multipotent progenitors
B cell	Thymic entry	*Pax5*	H3K27me3, broad	Silent in multipotent progenitors
Myeloid	DN2	*Cebpa (C/EBPα)*	H3K27me3, bivalent	
Myeloid/ progenitor	DN2-DN3	*Sfpi1 (SPI1, PU.1)*	None	"Empty"
Stem/ progenitor	DN2-DN3	*Tal1 (SCL)*	H3K27me3, broad	
Stem/ progenitor	DN2-DN3	*Lmo2*	H3K27me3, broad	No expression after DN1 stage
Stem/ progenitor	DN2-DN3	*Gata2*	H3K27me3	No expression after DN1 stage
Stem/ progenitor	DN2-DN3	*Hhex*	H3K27me3, broad	Bivalent in Th1, Th2
Stem/ progenitor	DN2-DN3	*Gfi1b*	Light H3K27me3	
Stem/ progenitor	DN2-DN3	*Bcl11a*	H3K27me3, mostly promoter-focused	Some expression until end of DN3 stage
NK	DN2-DN3	*Nfil3*	H3K27me3, promoter	Derepressed in Th1, Th2
NK	DN2-DN3	*Zfp105*	H3K27me3, broad	Gene silent in all known T cells
NK, CD8	DN2-DN3	*Eomes*	Bivalent	Silent in DN; not used in CD4 T
Immature T	β-selection (DN to CD4+CD8+)	*Hes1*	H3K27me3	Notch target, no expression after DN3
Immature T	Positive selection	*Rag1-Rag2*	Very light H3K27me3	
Immature T	Positive selection	*Dntt*	Very light H3K27me3; bivalent	No repression on promoter
CD8 T	Positive selection	*Cd8b1 (CD8B)*	No marks	Not used in CD4 T
CD8 T	Positive selection	*Cd8a*	H3K27me3	Not used in CD4 T

Timing of program exclusions taken from references reviewed in (Rothenberg 2011, Rothenberg et al. 2010), supplemented with gene expression data from J. Zhang et al. (op. cit., unpublished data) and www.immgen.org. Data for modifications associated with genes in CD4+ mature T cells were mined from (Weishaupt et al. 2010, Wei et al. 2009) for murine genomes and from (Barski et al. 2007) for human genomes. Raw reads for murine data were converted to WIG files, processed to convert mm8 coordinates to mm9 equivalents, and uploaded onto the UCSC Genome Browser (www.genome.ucsc.edu) for visualization relative to gene annotations. Some data on status of genes in multipotent prethymic progenitors was obtained from (Weishaupt et al. 2010). See Figures 1, 2 for examples.