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Transcriptional and epigenetic networks that drive helper T cell identities

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Abstract

The discovery of the specification of $CD4^+$ helper T cells to discrete effector "lineages" represented a watershed event in conceptualizing mechanisms of host defense and immunoregulation. However, our appreciation for the actual complexity of helper T cell subsets continues unabated. Just as the Sami language of Scandinavia has 1000 different words for reindeer, the range of fates available for a $CD4^+$ T cell is numerous and may be underestimated. Added to the crowded scene for helper T cell subsets is the continuously growing family of innate lymphoid cells (ILCs), endowed with common effector responses and the previously defined "master regulators" for $CD4^+$ helper T cell subsets are also shared by ILC subsets. Within the context of this extraordinary complexity are concomitant advances in the understanding of transcriptomes and epigenomes. So what do terms like "lineage commitment" and helper T cell "specification" mean in the early 21st century? How do we put all of this together in a coherent conceptual framework? It would be arrogant to assume that we have a sophisticated enough understanding to seriously answer these questions. Instead, we will review the current status of the flexibility of helper T cell responses in relation to their genetic regulatory networks and epigenetic landscapes. Recent data have provided major surprises as to what master regulators can or cannot do, how they interact with other transcription factors and impact global genome-wide changes and how all these factors come together to influence helper cell function.

Introduction: functional specification of $CD4^+$ helper T cells

The existence of T cells was first recognized in the 1960's (1, 2) and their division into helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells was appreciated in 1970's (1–5). It was not

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until the late 1980's that the dualism between type 1 and 2 responses of $CD4^+$ helper T cell subsets was first proposed (6, 7). Type 1 helper T (Th1) cells produce the signature cytokine interferon gamma (IFN-γ), and play a pivotal role in mounting immunity against intracellular pathogens (8, 9). Type 2 helper T (Th2) cells produce interleukin-4 (IL-4), IL-5 and IL-13, and are important against helminth infections and for helping B-cells to produce IgE antibodies (10).

Just as T and B cells, or $CD4^+$ and $CD8^+$ T cells were viewed as distinct lineages, the notion that these subsets of cytokine-secreting $CD4^+$ T cells were distinct lineages was driven by the recognition that with repeated rounds of stimulation the distinctive cytokine production was stabilized concomitant with extinction of alternate cytokine programs. This view was strengthened in the late 1990's and early 2000's by the findings that each subset expressed a master regulator transcription factor (TF) that was necessary and sufficient for fate determination. (11–15). First came the identification of GATA-3 in Th2 cells followed by T-bet in Th1 cells, RORγt in Th17 cells and Foxp3 in Treg cells. Thus, a helper T cell lineage paradigm evolved to be viewed as having at least two key attributes – expression of a signature cytokine and a master regulator TF. Depending upon your perspective though, it was either edifying or perplexing that the expression of the master regulators was controlled by the signature cytokines: the process is clearly self-reinforcing (16). In addition, it was appreciated that the gene expression programs for Th1 and Th2 cells extended beyond just cytokines, since differentiating Th1 and Th2 cells down-regulated TFs and receptors for cytokines that promoted alternative fates (IL-4R in Th1 cells and IL-12R in Th2 cells) (17, 18).

As recognized by the noted American philosopher, Yogi Berra, "you can observe a lot just by watching". And so it was with $CD4^+$ T cell subsets – immunologists began to observe a number of new options available for $CD4^+$ T cells. This recognition, which continues at a dizzying pace, began with the designation of T helper 17 (Th17) cells (15, 19–21). As implied by the name, these cells produce IL-17A and IL-17F, but also IL-21 and IL-22. They may also express the immunoregulatory cytokine IL-9, which can also be expressed by Th2 and Th9 cells; however, its functional significance for Th17 cells is uncertain (22–26). Th17 cells can also express the immunoregulatory cytokine IL-10 perhaps as a self-imposed negative feedback loop that can be seen in Th1 cells as well (27, 28). Identification of a subset of T cells that produce IL-17 was notable for a number of reasons. As one of the evolutionarily oldest cytokines, IL-17 is important for host defense against extracellular bacteria and fungi; this is vividly illustrated in the disease Job syndrome (29–31). IL-17 is also important for activation of complement and increase of IgA production from B cells (32, 33). Moreover, Th17 cells provided an important "missing link" in pathogenesis of autoimmunity (34–36). Surprisingly, in a mouse model of arthritis, IL-17A is crucial for autoantibody formation (37). Interestingly, within the Th17 lineage, there is heterogeneity manifested as different degrees of pathogenicity (38, 39). With the recognition of Th17 cells, it was edifying that that they too expressed a master regulator, retinoid orphan receptor γt (RORγt, encoded by *Rorc*); although another related factor RORα can also contribute, with a minor role, to differentiation of IL-17-producing cells (40). Whereas IL-22 is produced by Th17 cells, another T cell subset, termed Th22 cells, selectively produces this

cytokine (41–43). Th22 cells differ from “conventional” Th17 cells, since they express low levels of RORγt, high levels of T-bet and mediate protection against *Citrobacter rodentium* (44). In addition, a new subset closely related to Th2 cells, termed Th9 cells, has been identified, which participates in regulation of allergic inflammation, tumor immunity and, recently, immunopathology (45, 46). As indicated by the name, these cells produce IL-9, expression of which is dependent upon TGF-β and IL-4. They express a different “master regulator”, PU.1 along with IRF4 and GATA-3 (47–49).

The preceding lineages of helper T cells were all defined by their production of an eponymous cytokine; however, one effector subset is not defined in this manner. Such cells are called follicular helper T cells (Tfh cells); unlike other subsets, Tfh cells are defined by their location. They are found in B cell follicles and germinal centers and provide help for an efficient antibody production. When dysregulated, Tfh cells can contribute to autoantibody formation as exemplified in the *sanroque* mutant mouse (50). Their signature cytokine is IL-21, but this cytokine is produced by other cells and thus Tfh cells cannot be uniquely defined by their production. Likewise, they express a master regulator transcription factor, Bcl6, but the expression of this factor is by no means absolutely limited to Tfh cells (51–58).

Any student of immunology will appreciate that in addition to the array of immune cells with effector functions, there are also many types of “suppressor” cells. Although suppressors cells have a checkered history (59), it is now understandable in retrospect given the multitude of cells and mechanisms that mediate immunosuppressive functions. This is certainly true of $CD4^+$ T cells, with multiple subsets of $CD4^+$ T cells being endowed with repressive functionality (60). The phenomenology of regulatory function was simplified by the recognition of Forkhead Box P3 (FoxP3) as the master transcription factor that is necessary for the development of these critical regulatory T cells (Treg cells) (61). These cells can arise in the thymus (thymic Treg or tTreg cells), periphery (pTreg cells) or can be induced in vitro (62). Treg cells defied the emerging master regulator/signature cytokine view of specification – they are functionally critical, but the molecular basis of their regulatory activity remains incompletely understood. Furthermore, there is evidence that a network of transcription factors is required for the Treg cell gene expression program (63, 64). Moreover, FoxP3-expressing Treg cells are not the only regulatory T cells; multiple types of Foxp3-negative regulatory T cells have been identified and termed Th3, Tr1 or Tr35 cells (65–68), although the identity of these cells remains somewhat imprecise. These cells produce critical anti-inflammatory cytokines like TGFβ, IL-10 or IL-35 but these are by no means signature cytokines. On the contrary, many cells, including effector T cells, broadly produce these cytokines (69). Even among $CD4^+CD25^+Foxp3^+$ Treg cells, there is heterogeneity. For example, there are fat- and muscle-resident Treg cells etc. (70, 71).

Added to this complexity is the recognition that molecules, like perforin, which are expressed by effector cells have regulatory functions, serving to mediate and limit effector function (72, 73). Thus, what defines the identity of regulatory cells and precisely how they exert their immunosuppressive effect encompasses a variety of factors acting in diverse cells that employ different mechanisms to exert regulatory function.

CD4+ T cells have issues with boundaries

Despite views of different T cell subsets as stable, self-reinforcing, terminally differentiated lineages, there was also evidence early on of a more fluid view of immunoregulation (74–76). Much has been written on this topic and there are many examples of flexibility, so only a few striking cases will be pointed out. Even though “Th17 cells” were quickly anointed as a separate lineage, it is well-known that they can make IFN-γ, a Th1 cytokine (77–82). Indeed, the current view is that Th17 cells represent a heterogeneous collection of cells, some of which are pathogenic and express T-bet, GM-CSF and other factors and others which express IL-10 and are not pathogenic (38, 39). Th2 cells exhibit plasticity too, and can be reprogrammed into GATA-3$^+$T-bet$^+$ cells, that produce both IL-4 and IFN-γ following viral infection (83). By their nature, iTreg cells are prone to Foxp3 instability and can produce effector cytokines (84). The extent to which tTreg cells are plastic is still the subject of some debate, although as will be discussed, epigenetic mechanisms have been identified that help explain their stability (85–87).

Tfh cells are among the hardest cells to characterize as a simple, distinct “lineage”. They do not have a unique pattern of signature cytokine secretion and have the ability to produce cytokines of other lineages. Tfh-like cells generated *in vitro* can be re-programmed to make IFN-γ (88) and Tfh-like features are present early in Th1 differentiation (55). This flexibility is not limited to *in vitro* differentiation. During helminthic infections, IL-4-producing cells in the lymph nodes are located in germinal centers, blurring the boundary of Tfh and Th2 cells (57, 89, 90). Conversely, during a Th1-type bacterial infection, Tfh cells express IFN-γ (57). While this complicates a simple view of helper T cell differentiation, it also makes some sense – after all, a major role of CD4$^+$ helper T cell cells is to provide help in particular for B cells to mount humoral responses. They need not help B cells in just one way, using a limited palette of cytokines.

Though the emerging consensus is that many differentiated CD4$^+$ T cells retain at least some degree of plasticity, it has been assumed that the boundary between CD4$^+$ and CD8$^+$ T cells constitute a more formidable boundary and these two subsets are true lineages. However, even these “terminally differentiated” cells show more flexibility than previously assumed. CD4$^+$ T cell commitment *per se* appears not to be fixed and helper cells can acquire cytolytic functions; more on this shortly (91, 92). Suffice it to say, that it is increasingly difficult to argue that differentiated CD4$^+$ T cells necessarily produce a selective, fixed transcriptomic program.

CD4+ T cells – you are not alone!

An additional development in the field that needs to be considered in discussions of helper T cell lineage commitment is that they are no longer the only lymphoid cell subset that exhibits selective cytokine production. Along with CD4$^+$ T cells, multiple innate lymphoid cell (ILC) subsets have been recently identified and divided in three main groups corresponding to Th1-, Th2- and Th17-associated cytokine production (93, 94) (Figure 1). Long recognized as professional IFN-γ producers are conventional natural killer (NK) cells, which represent the first Type 1 ILC (ILC1) described. Initially identified for their

spontaneous cytotoxic activity (95–98) (99). NK cells represent a major innate source of IFN-γ produced rapidly before the onset of an adaptive immune response. *In vivo* studies have demonstrated that NK cell-produced IFN-γ is important against infections by intracellular bacteria, parasites and viruses (100–102). In addition to conventional NK cells, other tissues contain IFN-γ producing lymphoid cells endowed with lower or no killing activity (103–105); these cells are also termed ILC1 (94). Thus, in addition to IFN-γ-producing Th1 cells, the IFN-γ producing lymphocytes include: α/β $CD8^+$ T cells, NKT cells and γ/δ T cells (106, 107).

The innate source of Th2 cytokines has been of interest for a number of years. Basophils and mast cells can produce IL-4 (108–111), as well as NKT cells (112). More recently, ILC that produce IL-13 and IL-5 have been identified by 3 independent groups and termed nuocytes, natural helper cells and innate type 2 helper cells (ILC2 cells), although the cells identified do not necessarily correspond precisely to the same subsets (113–116). ILC2 functions can be elicited by IL-25 and IL-33 and can amplify type 2 responses (117). Similarly, whereas mast cells are known to produce IL-9 (118–120), ILC2 are now recognized as the major producers of IL-9 in the lung (121).

Production of IL-17 and IL-22 in ILC was characterized in 2009 and it is now recognized as ILC3 represent an important source of these two cytokines in the earlier phases of infection (122–124). Lymphoid Tissue inducer (LTi) cells and cells expressing NKp46 (currently named NCR^+ ILC3) belong to these groups and altogether participate in the development of lymphoid tissues, regulation of epithelium barrier function, host defense against *Citrobacter rodentium*, and shape T cell responses (125–131). Beyond ILC, and other lymphocytes, such as γδ-T cells and NKT cells, expression of “type 3” cytokines has been described also in neutrophils (132–140)

Production of IL-10 is not limited to T cells, but includes many other cells such as myeloid, B and NK cells all produce this key cytokine (69, 141–144). Bone marrow-derived stromal cells also produce IL-10 and have suppressor functions. Parenthetically, it is worth adding that IL-2, the prototypic T cell growth factor is produced by non-T cells including dendritic cells (145), and by a specific ILC1 subset (146). So the bottom line is that selective cytokine production is hardly the sole domain of $CD4^+$ T cells – it appears that no cytokine is produced exclusively by T cells and furthermore $CD4^+$ T cell “lineages” are not the only immune cells that have the capacity to selectively produce restricted cytokine programs. This appreciation has profound implications for the concept of cell identity and specification, and the role of transcription factors as we consider exactly what it is required for helper T cell differentiation.

Transcription factors acting across immune cell fates

It is famously stated, accurately or otherwise, that Eskimos have more than 100 words for snow and ice. Similarly, Sami speakers of Lapland are said to have hundreds of words to describe reindeer as well as snow. This may be a reasonable metaphor for immunologists in the early 21^{st} century. We have become very good at paying attention to the enormous range of subtle and not so subtle differences among populations of immune cells. The challenge,

of course, is to move beyond simple descriptions, and provide solid molecular and mechanistic explanations that explain and predict the actions of lymphoid cells in terms of the patterns of gene expression and regulatory networks.

For this reason, it is useful in thinking about the specification of $CD4^+$ T cells to keep firmly in mind that innate and adaptive lymphoid cells share common bone marrow progenitors and share many functionalities. This is certainly true with respect to selective production of cytokines. The fact that so many immune cells have the capacity to discriminately express virtually all of the cytokines produced by helper T cells implies that this capacity and the attendant machinery is in place prior to the specification of ILCs and T cells (including γ/δ, NKT, $CD4^+$ and $CD8^+$ cells). In other words, the capacity to effect specialized gene expression as it relates to cytokines genes must arise earlier in ontogeny than diversification of lymphocytes from other products of hematopoietic stem cells (HSC). The functionalities of T cells and ILCs are likely to be superimposed upon pre-existing programs.

While defining the precise relationships between the different cells is still a work in progress we do know the identities of a number of transcription factors (TFs) that are fundamentally important for HSC development and fitness. Those factors set the stage for generation of differentiated immune cells. Factors include: Ikaros, E2A, Pu.1, Bcl11a, as well as Hox, Runx, and Gata family members, and all are important contributors to early events in hematopoiesis (147–151) and lymphocyte specification as well.

Deciphering lineage specification

The extraordinary variety of immune cells is coordinated by the regulatory network of TFs, which shapes cell features and identity. In this network some TFs can define and/or preserve boundaries among lineages. However, the same TFs can be “recycled” during differentiation by switching “on” and “off” their expression, serving distinct functions at different times. They can be also shared among the different lineages, making the boundaries of lineage-defining TFs blur and difficult to distinguish (Figure 2).

A major determinant of T cell development is Notch1; T cell differentiation is completely blocked in the absence of this factor, with resultant expansion of B cells in the thymus (152, 153). A constitutively active form of Notch promotes expansion of T cells in the bone marrow at the expenses of B cells (154). Notch signaling though is not just important for T cells, but also ILC, DC, and splenic marginal zone B cells are also affected by the absence of Notch. T cell factor 1 (TCF-1, encoded by *Tcf7*) is induced by Notch and it is also required for generation of T cells, and specific ILC subsets (155–158).

If Notch in some extent is the switch for B/T cell fate, E2A, a basic helix–loop–helix (bHLH) TF controls T/ILC bifurcation. Multiple steps of T cell development in the thymus require the activity of this TF or the related protein HEB (159–163). Bcl11b is yet another factor that is important for double negative thymocytes, repressing genes associated to stem cells and preventing the expression of NK cell lineage genes (164). Fate choice between a helper versus cytotoxic T cells is controlled by the mutually antagonistic actions of Th-inducing POZ-Kruppel factor (ThPOK), encoded by the zinc finger and BTB domain containing 7b (*Zbtb7b*) gene, and the related protein LRF (165) and Runx (166–169); (170).

By inhibiting the transcriptional activity of E proteins, the inhibitor of DNA binding (Id)-2 (a bHLH protein) promotes generation of all ILC (114, 171–173). Deletion of E2A in $Id2^{-/-}$ mice is sufficient to restore generation of NK cells (174), while overexpression of Id3 promotes NK cell development at the expense of T/B lymphocytes in an *in vitro* system (175). Unlike ILCs, *Id2* deletion is not sufficient to abrogate development of thymic invariant NKT (iNKT) cells, due to the redundant role of *Id3* in promoting iNKT lineage specification (176).

The basic leucine zipper TF encoded by *Nfil3* (also called E4bp4) was initially proposed as the first TF specifically required for NK cell development (177, 178). Surprisingly, during viral infection, activating receptors and pro-inflammatory cytokines can drive generation of fully competent NK cells in absence of *Nfil3* (179). However, requirement of Nfil3 is not restricted to NK cells and broadly contributes to the differentiation of other ILC1 subsets, and $CD8\alpha^+$ DC, IgE class switching in B cells and regulation of cytokine production in $CD4^+$ T cells (180–186). Moreover, an expanded role has been attributed to the signature TF for iNKT development, PLZF (Promyelocytic Leukemia Zinc Finger protein, encoded by *Zbtb16*, a member of the POK family). It is expressed by a precursor that generates all helper ILCs, with the exception of NK and LTi cells (187). Finally, thymocyte selection-associated high mobility group box (Tox) is a factor that is important for both $CD4^+$ T cells and LTi cells (188, 189). Tox−/− mice show decreased LNs and Peyer's patches, and absence of NK cells; whether other ILC are affected has not been investigated.

STATs and lymphoid development and differentiation

Cytokine signaling is a critical determinant of the lymphoid differentiation programs. Signal transducer and activator of transcription (STAT) family includes 7 members, (STAT1-4, STAT5A, STAT5B and STAT6) able to transmit signals from most cytokines and to regulate unique spectra of gene sets. The advent of ChIP-seq technology, which has rapidly advanced over the last few years has quickly expanded the knowledge of the molecular functions of STATs on T cell. Here, we will review some of the main concepts concerning the role of STATs in lymphocyte differentiation.

Among the different STATs, STAT3 and STAT5 cover a wide spectrum of functions, even beyond the hematopoietic system. There are two Stat5 genes, *Stat5a* and *Stat5b*, which play a nonredundant role in mammary gland development and growth hormone signaling, respectively (190–193). Deletion of both alleles typically results in growth retardation and perinatal lethality due to anemia (194). STAT5 is a critical factor for the hematopoietic system and the entire lymphoid compartment, controlling HSC fitness, lymphoid cell development/homeostasis and, later on, Th polarization (195–197). Its relevance relates to the importance of c-kit (stem cell factor) and IL-7 signaling for HSC and lymphoid development, and IL-15 signaling for generation of conventional NK cells and homeostasis of memory T cells (198, 199).

STAT5 Chip-seq data revealed that regulation of homeostasis during Th polarization occurs through direct binding of STAT5 to genes important for proliferation and anti-apoptotic activity (including cyclin genes and *Bcl2*). STAT5 controls Treg homeostasis and

generation, directly by regulating the *Il2ra* and *Foxp3* genes generating a positive loop in which stable expression of Foxp3 is influenced by expression of IL-2 receptor (200). STAT5 is essential for both Th1 and Th2 cell differentiation by transmitting IL-2 signals (201). On the other hand, IL-2 through STAT5 suppresses formation of Tfh and Th17 cells (202–206). STAT5 can directly inhibit *Il17a* and promotes FoxP3 expression by competing with STAT3 (207). In summary, STAT5 is a critical TF for lymphocytes at all stages of their differentiation. Elucidation of the stage-specific versus unique functions of STAT5 is still being resolved.

Many of the paradigms concerning the role of STATs have been developed by the plethora of evidence concerning helper T cell polarization (208). In the initial rigid monolithic view of Th polarization, each STAT (except STAT2) was argued to be associated with a given T cell fate. While some STATs are more easily linked to particular T cell subset (e.g. STAT4 and STAT6 with Th1 and Th2, respectively), it is now recognized that each subset can be influenced by multiple STATs. A good example is provided by Tfh cell development, which is promoted by the complementary actions of STAT1, STAT3, and STAT4 (55, 209–214).

Among the ways STATs promote specific helper features is through direct interaction and activation of “master regulator” TF genes. Like STAT5 and Foxp3, STATs directly regulate *Tbx21*, *Gata3* and *Rorc*. STATs regulate hundreds of other genes, including many other “lineage-specific” loci including cytokines, cytokine receptors, chemokines and chemokine receptors (215–217) microRNAs (218) and lincRNAs (219, 220) (see section below).

Although the role of STATs has been relatively poorly characterized in ILC, especially in terms of defining targets by Chip-seq, it is likely that they will regulate many of the key loci that contribute to ILC function, especially those that are shared with T cells. It will be of great interest to dissect shared and unique actions. NK cells express high basal levels of STAT4 and their effector functions are highly affected in STAT4-deficient mice (221). At the same time STAT3 deficiency in ILC3 impairs their ability to produce IL-22 and IL-17 (222). Whether STAT6 can participate in regulation of effector functions in ILC2 has not been characterized yet. However, the two main cytokines involved in ILC2 activation, IL-25 and IL-33, do not use STAT6 for their signaling.

Function of helper cell master regulators beyond Th differentiation

The classical helper T cell “master regulators”, T-bet, Gata-3 and Rorγt, have functions beyond this restricted role. Even though T-bet (encoded by *Tbx21* gene), initially described as a Th1 specific TF (11) and an important factor for acquisition of type 1 features in Th cells, it is also expressed in $CD8^+$ T cells, NKT cells, conventional NK cells/ILC1, specific ILC3 subsets, myeloid cells and B cells (223). Th1 responses and development of tissue specific ILC1, along with effector functions of $CD8^+$ T cells, conventional NK cells and NKT cells are all T-bet dependent (224–229). Global profiling of T-bet binding and its impact on transcription and epigenetics has now been accomplished (230). T-bet binds to the *Ifng* locus and promotes its expression, as well as the loci for *Il12rb2* and *Cxcr3*. The integration of T-bet binding and transcriptional profiling in T-bet deficient cells suggests

that only 6% of genes bound by T-bet are transcriptionally regulated by this factor, but overall the number of genes positively or negatively regulated by T-bet are comparable.

Interestingly, T-bet also seems to be important for IL-22 production in Th22 cells (44) and it is relevant for generation of NCR^+ ILC3 (224, 231). ILC3 expressing T-bet can acquire the ability to produce IFN-γ and can convert to "pure" type 1 ILC, but the requirement for development implies a function beyond regulation of IFN-γ (107).

$CD8^+$ T cells and conventional NK cells illustrate the importance of another, non-redundant T-box TFs, Eomesodermin (Eomes). In $CD8^+$ T cells, the fine-tuned regulation of T-bet and Eomes expression can direct fate to the memory vs. effector cells (232). High expression of Eomes is a hallmark of conventional NK cells among the other ILC1, expressing T-bet only and differing for cytokine production (146, 229, 233).

Gata-3 plays a broad role in lymphoid development. During T cells development in the thymus, expression of *Gata3* is finely regulated. Notch, Tcf1 and TCR signaling are important for its induction while E2A proteins restrain Gata-3 expression (234). Beyond its role in T cell lineage commitment, Gata-3 is important to drive generation of $CD4^+$ T cells at the expense of $CD8^+$ T cells, both by inducing ThPOK expression (168) and by repressing Runx3 (235). GATA-3's role in Th2 cells is well appreciated, being induced by IL-2 and IL-4 in a STAT5 and STAT6-dependent manner respectively. GATA-3 is also important for ILC2 cell differentiation and is also required for maintenance and maturation of a lineage-specific ILC2 precursor in the bone marrow (236–238). Global gene expression analysis reveals similar function of GATA-3 in ILC2 and Th2 cells regulating the same pattern of cytokines and receptors (239).

An unbiased analysis of GATA-3 in Th2 cells suggests that 60% of the genes that require GATA-3 for transcription also exhibit GATA-3 binding, arguing for a direct mode of action in a relatively large proportion of genes (240).

Rorγt, encoded by *Rorc*, is essential for generation of Th17 cells, but like T-bet and Gata-3, it too has broad functions in ILCs and other cells (131). Rorγt is important for survival of DP thymocytes and expression of Bcl-xL (241, 242). It is also important for the lymphoid organogenesis and generation of ILC3 (104, 241–243), NKT (244) and γδ-T cells (15). It is also expressed in non-lymphoid cells, including neutrophils, another source of IL-17 (133). The genome-wide characterization of Rorγt binding argues that this protein has a relatively focused mode of action serving as modulator rather than a master transcription factor in the conventional sense. In fact, Rorγt binding is associated with modest changes in gene expression in Th17 cells relative to Th0 cells (217). The atypical nuclear factor I kappa B family member, IkBζ, encoded by *Nfkbiz*) acts in concert with Rorγt to promote Th17 differentiation (245). The role of *Nfkbiz* in ILCs has not been explored, but it would not be surprising if it is relevant for these cells.

The importance of Foxp3, other Forkhead Box proteins and their actions have been intensively reviewed and will not be discussed here; interested readers are referred to many other outstanding reviews of this important topic (246–248).

Repressors abound

Also of interest in terms of helper T cell function are three key repressors Blimp-1, Bach2, and Bcl6 (249, 250). Identified first in B cells, these TFs are in fact expressed in many cell types. Perhaps more interestingly, they create a transcriptional network that can regulate one another (251). In B, T and NK cells, Blimp-1 is associated with terminally differentiated cells (252–258). In B cells, it is the master regulator of plasma cell formation, suggesting that Blimp-1 controls gene programs that drive a highly differentiated state (259, 260). Both Bcl6 and Bach2 can repress Blimp-1, suggesting early and inappropriate activation of Blimp-1 is detrimental to the cellular differentiation process (52, 261, 262). In the absence of Bach2, plasma cells form too early, and both germinal center responses and class switch recombination are impaired (263, 264).

Recently, a critical role for Bach2 was described in T cells, where Bach2 acts to restrain effector T cell differentiation by suppressing Blimp-1 and other targets (265, 266). This is especially critical in Treg cells, where increased Bach2 levels control effector T cell genes and prevent the development of a lethal autoimmunity (265, 266).

In contrast to Blimp-1, Bcl6 is considered to be the master regulator of germinal center reactions (267) (268). In addition to controlling the DNA damage response and cell cycle checkpoints in GC B cells, a major role of Bcl6 is to suppress Blimp-1 and plasma cell development until somatic hypermutation and class switch recombination are completed (249, 269, 270). In T cells, Bcl6 is proposed as the master transcription factor required for Tfh cell formation (52–54). Blimp-1 can also repress Bcl6, and overexpression of Blimp-1 results in severely impaired Tfh responses (52). Mutations in all three of these TFs are associated with lymphomagenesis, further emphasizing the critical role these factors play in controlling cellular differentiation (249).

Although these factors are members of different families, they work in a similar fashion. All have N-terminal protein-protein interaction domains, with C-terminal DNA binding domains. Blimp-1 recruits co-repressors such as G9a and HDAC1/2 and induces repressive marks like H3K9 methylation (271–273). Bach2 and Bcl6 both have BTB protein-protein binding domains that is known to mediate protein-protein interaction, and function as homodimers, or interact with each other (274). In addition, they bind other TFs and recruit co-repressor complexes (275, 276). Bach2 was identified in B cells in a pull-down with MafK, and has a bZIP DNA binding domain that can bind DNA elements that are well known to also bind AP-1 family members (276, 277). While mainly described as a repressor, examples of Bach2 acting as an activator have been described (278). Bcl6 has a zinc finger DNA binding domain, and recruits the co-repressor complexes SMRT, NCOR and BCOR (279, 280). New models suggest Bcl6 can repress transcription by two distinct but simultaneous mechanisms (281). Bcl6 can repress promoter regions by depletion of activating marks, and addition of repressive marks via a ternary complex with BCOR and SMRT/NCOR. A second mechanism acts on a different set of genes to switch enhancers from an active to a poised configuration by recruiting the deacetylating SMRT-HDAC3 complexes and opposing the action of the histone acetyltransferase (HAT) p300 (281). Although the basics of how these factors repress have been established, the target genes they

each act on in specific cell types and conditions are still unclear. Far more work is needed to fully understand the role these factors play by modulating the epigenetics of chromatin to control gene expression and cellular differentiation. In addition, it is important to note that HATs and HDACs may have additional substrates in addition to histones.

More players in the TF network

T cell receptor (TCR) requirement marks a fundamental demarcation line between T cells and ILC. TCR signals are essential for initiation of $CD4^+$ T cell differentiation and signal strength biases T cell programming toward divergent differentiating directions. In this setting, nuclear factor of activated T-cells (NFAT), adaptor-related protein complex 1 (AP-1, encoded by *Fos* and *Jun*) and nuclear factor κB (NF-κB) among other TFs are important regulators of gene expression (282). While ILC do not express antigen receptors, a variety of receptors including Ly49, NKG2, and integrin family members can provide signals that activate that the aforementioned TFs, which presumably activate many of the same target genes (283, 284).

Other TFs including basic leucine zipper transcription factor (BATF), which can form AP-1 complexes, and a ternary complex with interferon regulatory factor 4 (IRF4), are also essential for Th differentiation (285–290). In Th17 cells, BATF and IRF4 are globally co-localized in the genome and both required for remodeling chromatin landscape for deposition of other TFs (217, 291). Along with STAT3, BATF and IRF4, influence genome-wide histone acetyltransferase p300 occupancy in Th17, whereas RORγt has minimal effects (217). Increasing evidence suggests BATF and IRF4 are “pioneer factors” for permission of lineage specification. However, how these “pioneer TFs” from TCR signaling interact with “polarizing TFs” regulated by cytokines to tune the gene expression remains unclear.

Maf was originally identified as a Th2-associated TF, but is induced by IL-6, IL-27 and STAT3 and so is expressed in Th17, Tfh and Tr1 cells (292, 293). Maf has been reported to be a positive regulator of IL-10 (294). It is also induced by TGFβ and directly inhibits *Il22* (295).

Due to the exposure to the mucosal barrier, generation of Th17 cells and ILC3 subsets share many other common features, such as dependency on bacteria, environmental factors and dietary components. Aryl hydrocarbon receptor (AHR) can affect expression of IL-17 and IL-22 in T cells and it is also required for the generation of ILC3 cells (296–300). Dietary stress, such as vitamin A deprivation, highly impacts ILC3 generation (301), while Th17 generation is favored (302). Finally, dietary salt can enhance IL-23-mediated Th17 differentiation by regulating serum glucocorticoid kinase 1 (SGK1). One action of SGK1 is to deactivate the transcription factor Foxo1 (303, 304). SGK1 can also promote Th2 and repress Th1 cell differentiation (305).

At the risk of overwhelming readers, it should be clear from the above that numerous TFs work in concert to drive gene expression. While it may seem like an impossibility to sort out their discrete, cell- and stage-specific functions, Chip-seq technology does provide a high throughput means to experimentally identify potential direct targets of TFs. Using genome-editing technology it should be feasible to introduce specific binding-site mutations and

prove causality of some of these DNA-binding events. We are in our infancy of such studies and the data and work ahead will be overwhelming; nonetheless, it should be possible to identify precise functions amidst this apparent cacophony. But wait, it's not just about TFs acting on protein-coding genes……

Gene expression and epigenetic controls

While key transcription factors working in a combinatorial fashion are essential elements for cell specification, their "substrate", DNA, is anything but a passive participant with respect to control of gene expression. DNA is packaged into nucleosomes and chromatin, and variety of DNA and chromatin modifications contribute to the accessibility of DNA. The regulatory mechanisms that promote or restrict DNA accessibility include: DNA methylation, histone modifications, nucleosome positioning or remodeling, chromatin insulators and long-distance chromatin interactions. All of these factors weave a complicated network now referred to as the epigenome that contributes each unique cell identity and fate determination. By analogy, the RNA within a cell is neither linear nor naked; therefore, RNA-binding proteins and the epitranscriptome will need to be considered.

A major challenge in the field though is to understand how epigenetic modifications allow or prevent TF access to key sites in the genome. Alternatively, TFs can also modify the epigenetic landscapes (so-called "pioneer" factors) (306, 307) (Figure 3). In addition, it is now well appreciated that the control of lineage-specific programming extends far beyond the small portion of the genome that encodes conventional genes that give rise to proteins. Only a tiny portion of the genome encodes such genes (< 2%); a considerably greater portion of the genome is transcribed and these diverse RNAs generate many products large and small, including microRNAs (miRNAs), enhancer RNAs (eRNAs) and long non-coding RNAs (lncRNAs). Emerging data indicate that these products themselves are important in controlling gene expression. In addition, chromatin accessibility and architecture also function as "switches" that regulate distal gene activities by facilitating or excluding TF binding to *cis*-regulatory elements including promoters and enhancers. Promoters are DNA sequences located upstream of transcription start sites (TSS) and are essential for transcription by recruiting the transcriptional apparatus. Enhancers regulate gene expression also by recruiting TFs and are "distal" in terms of linear distance from genes; because of looping of DNA and higher order chromatin conformations, enhancers can bring TFs to promoters. An important goal is to integrate the action of TF networks with modifications of epigenetic landscapes, signaling pathways and cellular metabolism. This is an active area of research that has already yielded a number of surprises. Traditionally, defining how gene transcription was influenced by the epigenetic landscape was a significant challenge; however, thanks to the development of deep-sequencing technology and bioinformatic methods, nowadays it is reasonably straightforward to measure genome-wide gene expression for both coding and non-coding RNAs, transcription factor binding, and epigenetic dynamics. We will briefly summarize the current views of epigenetic regulation and their roles in programming cellular differentiation using examples pertinent to lymphocyte biology and provide instances in which we have begun to understand how TFs modify the epigenome.

DNA methylation

DNA methylation modifies cell development and differentiation by attracting specific proteins or making DNA less accessible to TF binding (308). DNA methylation at the fifth carbon of cytosine (5mC) occurs mainly at CpG dinucleotides that are abundant across the genome (~70% of promoters contain high frequency of CpG sites, termed CpG islands). DNA methylation can repress gene activity through recruiting repressor complexes that contain methyl CpG-binding domain. It can also simply prevent interaction with some DNA-binding proteins that can either activate or repress transcription. Methylated CpG islands also influence nucleosome positioning (309, 310). Methylation of cytosine is catalyzed de novo by DNA methyltransferases (DNMT) 3A and DNMT3B, and then maintained by DNMT1 during mitosis. Absence of DNMT1 in naive $CD4^+$ T cells results in abnormal cytokine expression (311).

Methylated DNA has been argued to be among the most stable epigenetic marks; however, there are multiple examples in lymphocytes of rapid or active demethylation. For instance, the *Il2* locus is quickly demethylated upon T cell activation (312) and the *Ifng*, *Il4* and *Il17* loci are demethylated during differentiation of Th1, Th2 and Th17 cells respectively (313–317). These loci remain methylated in cells that are differentiated to opposing fates (e.g. the *Ifng* locus is methylated in Th2 cells). In addition, demethylation of *Foxp3* and other Treg signature genes is important for stabilization of Treg fate (318–320). At present, there are no comprehensive, genome-wide comparisons of DNA methylation among the different helper T cell subsets and the consequences on transcription is not well known.

The role of DNA methylation has also been studied in ILCs, but no comprehensive maps have been provided. DNA methylation is important for the regulation of *Ly49* genes in NK cells, a collection of loci clustered on chromosome 6 (321, 322). These genes are variably expressed by different mouse strains (323) and are subject to allelic exclusion (324). However, precisely how other characteristic features of ILCs are or are not controlled by methylation has not been determined.

The biochemical basis of DNA demethylation has been elusive and while it has been proposed that the loss DNA methyl-groups could occur simply by dilution during cell division, this does not explain the rapid demethylation of the *Il2* locus that occurs independent of cell proliferation (312, 325). Recently, new insights into the processing of methylated DNA have emerged. Instead of a simple erasure of the methyl group, 5mC is sequentially converted into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) through oxidation by ten-eleven translocation (TET) proteins-catalyzed oxidations (326). In mouse embryonic stem cells (ESCs), Tet1 and Tet2 are highly expressed, whereas in differentiated cells Tet2 and Tet3 are the major TET enzymes. The 5fC and 5caC are further removed by thymine DNA glycosylase (TDG) and the base excision repair (BER) pathway.

The dynamic 5mC oxidation forms can regulate gene expression by further modulating protein binding landscapes. For instance, 5mC but not 5hmC can be recognized by repressive complexes recruiter, methyl CpG-binding domain protein 1 (MBD1) and MBD2,

whereas both 5mC and 5hmC are recognized by MeCP2. MBD2 has been linked to regulate demethylation of immune-related genes (327–329). MBD2 mediates demethylation and TET2 binding of a CpG-rich region upstream FoxP3, which is critical for FoxP3 expression in thymic Treg (tTreg) cells (328, 329). Hence, dynamic 5mC oxidation forms and the proteins each of them recruits can be important for lineage specification.

Despite our very incomplete understanding of the genome-wide state of DNA methylation in lymphocytes in health and diseases, a number of new techniques are now or becoming available that will help fill the gaps in our knowledge. Currently, genome-wide DNA modification of each 5mC oxidation form can be identified at single base resolution by combining bisulphite-based chemical reactions with deep-sequencing, including bisulphite-sequencing (BS-seq), oxidative bisulphite-sequencing (oxBS-seq), Tet-assisted bisulfite sequencing (Tab-seq) and chemical modification-assisted bisulfite sequencing (fCAB-seq) (reviewed by W.A. Pastor et al (326)). Interestingly, the demethylation intermediates are enriched at regulatory elements (330–332). For instance, 5hmC is enriched at promoters with “bivalent” histone modifications as well as active enhancers (333–337). Comprehensive measurement of genome-wide methylation remains technically challenging and is costly. As a result, a comprehensive DNA methylation map of relevant cytokine-producing subsets is lacking; however, it will surely be the case that many immune response-related genes are tightly regulated by DNA methylation/demethylation.

It should be emphasized that alterations in DNA methylation are not just relevant to our basic understanding of helper T cell differentiation but also may be relevant to the pathogenesis of immunologic diseases (338), especially systemic lupus erythematosus (SLE) (339–342). Of particular note is that drugs that affect DNA methylation can cause lupus in humans and also in mouse models (343, 344).

Nucleosome positioning and histone modifications

Nucleosomes are the basic units of chromatin that contains a histone octamer wrapped by 147 base pairs of DNA. The dynamic nucleosome positioning and histone modifications play key roles in determining chromatin accessibility to transcription factor binding. At *cis*-regulatory elements, such as promoters and enhancers, nucleosomes are usually depleted or replaced by more dynamic histone variants like H2A.Z and H3.3. Therefore, *cis*-regulatory elements are usually more accessible and sensitive to DNA nucleases and can be predicted by DNaseI or Micrococcal nuclease hypersensitivity.

The mechanisms for nucleosome positioning are complicated and not fully understood. In addition to DNA sequence preference, both ATP-dependent chromatin remodelers and transcription machinery are also involved in the localization of nucleosome positioning (reviewed by Struhl and Segal (345)). SWI/SNF complex, one of the ATP-dependent chromatin remodelers, can loosen nucleosomes using the energy from ATP hydrolysis and cause nucleosome depletion or sliding. SWI/SNF complexes are essential for remodeling chromatin at multiple stages of T cell development in thymus while receiving external signals from pre-TCR and TCR (346) and for the bifurcation of CD4/CD8 SP T cells by silencing CD4 expression (347). This mechanism is also relevant to helper T cell

differentiation. In Th1 differentiation, Brahma-related gene 1 (BRG1), one of SWI/SNF complex subunits, targets *Ifng* locus for nucleosome remodeling in a STAT4-dependent manner (348). In cooperation with STAT4, BRG1 also regulates *Il12rb2* gene expression in Th1 cells (349). BRG1 is also required for full activation of Treg ability to suppress autoimmunity (350).

Post-translational modifications of histone proteins create epigenetic codes that mark distinct chromatin status and function (351–353). Histone H3 lysine 4 tri-methylation (H3K4me3) and H3K36me3 mark active transcription; H3K4me3 modification is highly enriched at TSS regions, while H3K36me3 modification preferentially spreads across the transcribing gene body. Conversely, histone methylation of H3K9, H4K20 and H3K27 are linked to gene repression. High H3K4 mono-methylation (H3K4me1) and low H3K4me3 modifications are recognized as general features of enhancers, in which “active” enhancers can be distinguished from “poised” enhancers by H3K27 acetylation and acetyltransferase, p300 and/or CBP binding. The roles of other histone acetyltransferases such as PCAF and GCN5 are not yet known in T cells. Using these marks, studies have revealed global enhancer landscape that will be discussed later. In addition, histones can be modified by many other post-translational modifications including phosphorylation, ubiquitination, sumoylation – we are just beginning to decipher all of the elements of the “histone code”. It is important to note that although particular histone marks has been associated with either promoting or repressing transcription, and even splicing, genetic evidence in mammals is for the most part lacking.

H3K27 trimethylation is catalyzed by histone methyltransferase Ezh1 or Ezh2, a subunit of polycomb repressive complex 2 (PRC2). Ezh2 has been linked to various types of cancers including prostate cancer, breast cancer, and leukemia (354–356). In $CD4^+$ T cells, Ezh2 is important for modulating *Tbx21* and *Gata3* expression in Th1 and Th2 cells, respectively (357, 358). It suppresses Eomes expression (358) and stabilizes T-bet levels through both transcriptional and post-translational regulation in Th1 cells (359). In Th9 cells, TGF-β activated Smad proteins displace Ezh2 from the *Il9* locus promoting expression of the encoded cytokine (360). In Treg cells, Ezh2 is induced and recruited to FoxP3-bound regions of the genome following inflammatory stimuli. This results in increased H3K27 trimethylation, and repression of nearby genes (361). In Th17 cells, a DNA-binding protein called Jarid2 is required for recruitment of PRC2 to its chromatin targets, which include *Il22*, *Il10*, *Il9* and *Atf3* (362). PRC2 is generally thought to lead to PRC1 recruitment; however, the role of PRC1 in T cells is not known. In addition to nuclear functions, however, Ezh2 also controls TCR-dependent actin polymerization (363).

H3K9me3 is another important repressive mark that recruits heterochromatin protein (HP) for gene silencing. During T cell differentiation, Th2 cell commitment requires H3K9me3 involved repression of Th1 loci (364). In addition, H3K9me3 also controls $CD8^+$ T cell memory progression by Blimp-1-dependent recruitment of G9a histone methyltransferase to the *Il2ra* and *Cd27* loci (271).

The genome-wide enumeration of permissive and repressive histone marks in helper T cells has been obtained and helps explain several features of distinctive gene expression in helper

T (365). As expected, characteristic genes associated with lineage commitment have the predicted accessible marks in their respective lineage and repressive marks in opposing lineages. However, genes that encode key regulatory transcription factors including *Tbx21*, *Gata3*, *Rorc*, *Prdm1*, etc. have more complex features. The combination of H3K4me3 and H3K27me3 modifications, so called “bivalent” domains, is indicative of genes that are poised for expression (365). In principle, this could provide an explanation for the plasticity of transcription factor expression. Other transcription factors like Bcl6 reveal a different pattern; the epigenetic marks surrounding this locus show that the *Bcl6* gene accessible in all subsets (55). This helps explain the fact that multiple T cell subsets can acquire features of Tfh cells. Thus, the epigenetic landscape of genes encoding master regulators may allow flexibility in expression and thereby permit the blurring lineages, allow fine tuning or provide sub-specialization.

As discussed, a critical issue is defining the factors responsible for creation and modification of epigenetic landscapes. STAT proteins are one important class of transcription factors that regulate lineage-specific expression profiles by shaping histone modification patterns. In Th1 cells, STAT4 is essential for promoting genome-wide H3K4me3 modification for activated genes, whereas in Th2 cells, a major aspect of STAT6’s action is to influence the removal of repressive H3K27me3 modification on poised loci (216). In addition, analysis of epigenetic marks and transcription activities of STAT4 target genes reveals that STAT4 can regulate histone modifications or transcription independently. That is, for STAT4-bound genes, only a very small proportion (4%) is STAT4-dependent in terms of both histone modifications and transcription. In contrast, 11% shows STAT4-dependence with respect to transcription only and another 20% shows STAT4-dependence for epigenetic modifications only. These observations suggest STAT4 impacts cell phenotype in various ways. This point is particularly important because STATs were first identified as gene activators; however, identification of STAT4-dependent repressive markers with genome-wide analysis suggests a role for STAT4 as a transcriptional repressor as well as its more widely recognized role as a transcriptional activator.

Epigenetic modifications communicate with transcriptional machinery through certain “histone code readers.” For example, bromodomain and extraterminal (BET) proteins can recognize acetylated histones. BET proteins, including BRD2, BRD3, BRD4 and BRDT, provide a bridge on chromatin to connect histone modifiers, chromatin remodelers and Mediator complex to for gene regulation (366). BRD4, for instance, can recruit the positive transcription elongation factor b (P-TEFb) complex to promote phosphorylation of paused RNA polymerase II for mRNA elongation. BET proteins have been proposed to be target for cancer therapy because they regulates oncogenesis-related growth factors such as c-myc in cancer cells (367). Of note, BET proteins also play a role in the regulation of pro-inflammatory cytokines and chemokines as well as T cell differentiation. Targeting BET proteins with small molecule inhibitors suppresses the production of IL-1β, IL-6, IL-12α, CXCL9 and CCL12 from bacterial endotoxin stimulated macrophages (368). In addition, BRD2 and BRD4 control Th17 differentiation through direct binding to *Il17* locus (369). Treatment of BET inhibitors suppresses both Th1- and Th17-induced autoimmune pathology in mice (369, 370).

Enhancer landscapes

Enhancers are DNA elements essential for gene regulation by controlling promoter activity from a distance as far as a megabase away. It is believed that enhancers are brought into proximity of promoters by looping of DNA and in this way contribute to the precise spatial and temporal regulation of gene expression profiles during development and differentiation. Therefore, identifying functional enhancers and understanding the mechanisms for their dynamic activities is likely to be key in deciphering basis of cellular specification and the acquisition of specialized functions. The other side of the coin is to characterize transcriptional repressor or silencer elements in DNA.

For many years, the identification of enhancer elements was an arduous task. One strategy to identify candidate enhancers was through computational approaches, seeking conserved non-coding sequences. An alternative approach was DNase hypersensitivity assays based on the property of enhancers as being nucleosome-depleted to allow for transcription factor binding. This though, was only done on small portions of the genome and was validated by cloning candidate sequences into reporter constructs that may not reflect the endogenous chromatin context. In a limited number of circumstances, their *in vivo* function was established genetically by deleting the sequences in engineered mouse models.

In lymphocytes, the cytokine loci are regulated by complicated enhancer structure that fine-tunes gene expression under various stimulations or defines lineage specificity (371). For instance, enhancer activity from CNS2 on *Il4* locus is critical for IL-4 expression specifically in Tfh cells but not in Th2 cells (372). Similarly, CNS1 on *FoxP3* locus is the enhancer required for differentiation of pTreg cells but not for tTreg cells (318).

The identification of chromatin signatures at enhancers using high throughput sequencing has profoundly affected the field of chromatin biology. As described previously, enhancers are highly associated with high H3K4me1 and low H3K4me3 modifications (373) and the activity of these enhancers are reflected by H3K27Ac modification and deposition of the acetyltransferase p300 (373–376). These enhancer characteristics have been used to identify numerous putative enhancers and to track the dynamics of enhancer activity during cell development and reprogramming (377, 378). For instance, comparing H3K4me1 and p300 binding patterns in macrophages with or without lipopolysaccharide (LPS) treatment suggests that LPS-induced enhancers marked by p300 are labeled with H3K4mel prior to LPS stimulation (378). Genome-wide analysis of H3K27 acetylation has been used to track dynamic enhancer activity in heart, brain and liver tissues during mouse development (377). Recently, H3K27 acetylation has been used to identify a cluster of lineage-specific enhancers (379), which will be discussed later.

With the ability to enumerate one class of distal enhancers, questions arise as to what factors are responsible for the appearance of these sites and what factors employ these sites to exert their effect. At present, the answers to these questions for lymphocytes are limited. Nonetheless, some surprises have already emerged. Master regulatory factors, or lineage-determining TFs (LDTFs), have been argued to be important for determining the lineage-specific enhancer landscape (380). These LDTFs recognize essential *cis*-regulatory elements

and mark them through histone modification and/or nucleosome positioning that alters the accessibility for other factors. For instance, PU.1, a key LDTF essential for development of hematopoietic cells, can coordinate with other regulatory factors to “prime” enhancer candidates for complete composition of active enhanceosome (381). In addition, it has been characterized that PU.1 can maintain enhancer structure through maintaining H3K4me1 modification (378). As appealing as this model is, the situation for $CD4^+$ T cells is more complicated.

During $CD4^+$ T cell differentiation, the expectation might be that LDTFs like T-bet, GATA-3, Rorγt, and FoxP3 might be the major drivers of the selective enhancer landscapes. In fact though, the lack of these factors had minimal impact on the global profiles of enhancer landscape in Th1, Th17 and Treg cells, respectively (217, 300, 382, 383). This calls into question whether these factors are indeed master regulators since they are subservient to STATs and Foxo1, for example. Based on current data, it appears that the LDTFs for T helper cell subsets exert their effect on a preset chromatin landscape. Indeed some master regulators, like T-bet, have limited action on distal enhancers and preferentially exert their affect more proximally directly on genes. Similarly, Foxp3 binds to regions that are already accessible in Naïve $CD4^+$ T cells, the stage prior to Treg differentiation and FoxP3 expression. However, FoxP3 leads the road for Ezh2 to mark FoxP3-bound regions with H3K27me3 once Ezh2 is up-regulated upon inflammatory stimuli. Therefore, FoxP3 is not the “pioneer” factor to permit chromatin accessibility, rather, is one of the “directing” factors for selective gene expression and cell fate. Given the limited ability of LDTFs to shape the enhancer landscape for T helpers (382, 383), a useful strategy was to identify computationally factors that generated the accessibility of LDTFs. A recently developed assay of transposase accessible chromatin, ATAC-seq, which allows evaluation of chromatin accessibility as well as transcription factor footprints on small amount of cells, provides a new avenue to assess the identity and hierarchy of gene regulators (384).

If master regulators are not the major factors that drive creation of the distinctive “switches” in T cells, then who are the drivers and what are the master regulators doing? Interestingly STATs were found to have a much more profound effect on lineage-specific chromatin landscape than T cell master regulators. More specifically, STAT1/STAT4 and STAT6 binding motifs are enriched in Th1- and Th2- specific active enhancers, respectively, in both mouse and human (382, 385). Within the more than 9000 murine Th1-specific active enhancers, only 17% are T-bet-dependent, while 60% are STAT1- and/or STAT4-dependent (382). Importantly, exogenous expression of T-bet or GATA-3 fails to fully rescue the defective chromatin landscapes caused by STAT deficiency. Similarly, during Th17 differentiation, the presence of STAT3 as well as BATF and IRF4 is more critical for the establishment of lineage-specific enhancer landscapes than the presence of RORγt (217).

With advanced bioinformatic assistance, a new family of enhancers called “super” or “stretch” enhancers (SEs) have been recently identified (379, 386, 387). SEs represent sequences across several kilobases that contain multiple discontinuous enhancer domains bound by key TFs, Mediator complex and intense deposition of p300 or H3K27 acetylation. Mutation of the Mediator complex, inhibiting Brd4 or any key TFs results in reduced expression of SE-related genes. Comparison of SEs patterns in various cell types revealed

that SEs play a significant role in defining cell identity (379, 386, 387). For instance, in embryonic stem cells (ESCs), SEs are enriched at genes essential for ESCs. Therefore, it is intriguing to utilize SE patterns to distinguish diverse hematopoietic lineages, especially for $CD4^+$ T and ILC subsets. With the ability to identify enhancers genome-wide, an obvious next question is what they regulate – hold that thought for now. We will return to this issue.

Non-Coding RNAs

Although only 2% of genome encodes messages for proteins, recent whole transcriptome RNA sequencing data suggest that over 80% of genome is actively transcribed {Ecker: 2012ji}. While there is considerable debate surrounding this topic, it clearly begs the question why there are so many RNAs generated that do not produce proteins. This question has been partially answered by discovery of new RNA roles within various important biological processes (388). Arrays of small RNAs (<30nt), including microRNA (miRNA) and piwi-associated RNA (piRNA), function as gene repressors by binding to complementary RNA sequences and recruiting silencing complexes that either act at the post-transcriptional or translational level, respectively (389). Recently, a new focus of the RNA field is deciphering the function of enhancer RNAs (eRNAs) and long non-coding RNAs (lncRNAs) that are largely unknown.

lncRNA

LncRNAs are transcripts longer than 200 nucleotides that lack a functional open reading frame. Most lncRNAs are believed to be produced in the similar way as mRNAs in the sense that both of them are transcribed by RNA polymerase II, modified by 5′ capping and 3′ polyadenylation and undergo splicing and sometimes exported to the cytoplasm. Recently, the maturation of high throughput RNA-seq methods enhanced the progress of lncRNA identification and brings us to a new level of viewing fundamental biology in the cell. More than 10,000 lncRNAs have been identified in mammals, but only a few have been functionally characterized (390, 391).

Despite this paucity of knowledge, the criticality of lncRNAs has been established. Perhaps the most striking example is the role of Xist, a lncRNA essential for X chromosome inactivation (392). In addition, lncRNAs have roles in imprinting, chromatin remodeling and constructing chromatin architecture. Recently, several lncRNAs were identified to function as scaffold for recruiting histone modifiers. For instance, HOTTIP, a ~4 kb lincRNA transcribed upstream of *HoxA* gene clusters, can regulate its target genes through direct interactions by chromatin loop formation and through introduction of histone methyltransferase MLL complex by direct interactions with WDR5, a subunit of MLL complex. These actions drive H3K4 trimethylation and facilitate transcription of HOTTIP target genes (393). LncRNAs can also antagonize protein or miRNA function through physical interactions. LncRNA GAS5, for instance, binds to the DNA-binding domain of the glucocorticoid receptor (GR) to inhibit GR-induced gene activation {Kino:2010dt}. ecCEBPA RNA can physically target DNA methyltransferase DNMT1 to prevent local DNA methylation (394). Recently, a new class of circularized lncRNA molecules that are abundant can “sponge up” miRNAs in the cells to neutralize their activity (395, 396).

Disruption of novel lncRNAs by knockdown in vitro or knockout *in vivo* results in cell abnormality or death, arguing that lncRNAs are functionally essential rather than just byproducts from transcription machinery (390, 397).

Emerging data are beginning to show just how important lncRNAs are essential for immune cells. During lymphocyte development, expression of lncRNAs on antigen receptor loci (also called germline transcription or sterile transcription) is essential for recombinase accessibility to target recombination signal sequences in order to reassemble V(D)J gene segments (398). In germinal center B cells, sterile transcription of switch regions are predictive of immunoglobulin isotype class switch recombination (399). NeST (also known as TMEVPG1 or LincR-Ifng-3′AS), a 45kb lincRNA located adjacent downstream *Ifng* locus, controls susceptibility to Theiler's virus and *Salmonella* infection in mice through epigenetic regulation of the interferon-γ (IFN-γ) locus (400). NeST is expressed specifically in Th1 and $CD8^+$ T, but not NK cells, and the expression is dependent on Th1 factors STAT4 and T-bet (401). Like HOTTIP, NeST regulates gene expression through the recruitment of WDR5 and its associated H3K4 methylation (400). In Th2 cells, an antisense lncRNA, lincR-Ccr2-5′AS, is important for regulating gene expression across this chemokine locus, which contains the *Ccr1*, *Ccr2*, *Ccr3* and *Ccr5* genes. These chemokines are required for Th2 migration to lung and are down-regulated after knocking down LincR-Ccr2-5′AS (220). Another lncRNA that is involved in immune responses is lincRNA-Cox2, which positively and negatively regulates distinct clusters of immune genes. lncRNA-Cox2 can repress genes through its interaction with heterogeneous nuclear ribonucleoprotein A/B and A2/B1 (402).

The array of lncRNAs produced by subsets of T cells has recently been cataloged by deep sequencing of both poly-A^+ and total transcriptomes within differentiating T cells at various stages and 1524 lncRNAs were identified in total (220). Among these lncRNAs, 464 were expressed by double-negative thymocytes, 515 in double- and single- positive thymocytes, and 646 in naïve and/or differentiated $CD4^+$ helper T cell subsets. The expression of these lncRNAs was highly dynamic during thymocyte development and helper T cell differentiation as compared to mRNA expression, and therefore provides a new way of thinking about functional cell identity. Of note, a number of these newly identified lncRNAs are STAT-dependent in their expression.

eRNAs

Another exciting discovery in RNA field is the identification of transcripts originated from enhancers, termed eRNAs. eRNAs are non-coding RNAs transcribed bidirectionally from enhancers and are generally 5′-capped, non-spliced and non-polyadenylated (403–406). eRNAs are essential for transcriptional regulations as well as loop formation for enhancer-promoter interactions. The expression of eRNAs can be induced by external stimuli and their expression correlates well with neighbor gene expression (407–410). The evolving view is that eRNAs are active participants in established accessibility of protein-coding genes. Using cap analysis of gene expression (CAGE), the FANTOM project has mapped genome-wide transcription start site (TSS) across hundreds of cell types (411). Interestingly, enhancers that identified by the combination of H3K27ac, H3K4me1 and p300 correlate

well with the production of bidirectional eRNAs, while TSSs for protein coding genes are more biased towards one direction (Andersson et al, 2014). Therefore, the expression of eRNAs can be another indicator for the prediction of active enhancers.

miRNAs

Numerous miRNAs are recognized as critical regulators to fine-tune gene expression. They are encoded in the genome and transcribed by RNA polymerase II to generate primary miRNA (pri-miRNA) transcripts, which are then processed sequentially by two members of RNase III type endonucleases, Drosha and Dicer. The mature ~21mer miRNAs are bound by Argonaute proteins to form miRNA-induced silencing complexes (miRISCs) to target complementary mRNAs in a sequence-specific fashion. miRNAs modulate target mRNA levels through various mechanisms including blocking translation, mRNA deadenylation followed by 5′ decapping, and enhancing mRNA degradation. (389). Importantly, each of these “tiny pieces” can target more than one gene; vice versa, each gene can be regulated by more than one miRNA, therefore, creating a complicated regulatory network. Thus, the regulatory logic of miRNAs is analogous to transcription factors except that as far as we know miRNAs repress gene expression in general.

miRNAs have been shown to dramatically influence the homeostasis of immune systems. T-cell specific deletion of Drosha or Dicer causes abnormal T cell differentiation and autoimmunity (412–414). Interestingly, in the absence of Dicer, Th2 differentiation cultures contain T cells that aberrantly express IFN-γ, suggesting that one or more miRNAs restrict Th2 cell plasticity (412). Individual miRNAs also have been shown to influence effector cell differentiation and stability. miR-155, for instance, is involved in the development of Th17 and Treg cells under the regulations of key regulators like STAT3 (218) and FoxP3 (415). miR-155 regulates IL-2 production for Treg cell maintenance by suppressing cytokine signaling 1 (*Socs1*), a negative regulator of IL-2 signaling (416). miR-155 also controls TGF-β signaling molecules SMAD2 (417) and SMAD5 (418), Ets1, a negative regulator of Th17 differentiation, (419), c-Maf and Jarid2 (362, 420). Of note, miR-155 deficient mice are protected from EAE and CIA (416, 421–423) but develop enteric and lung inflammation (420). miR-146a and miR-29 are essential for suppression of Th1 differentiation; miR-29 does this by directly targeting IFNγ, T-bet and Eomes (424, 425); miR-146a inhibits Th1 responses through regulating Treg cell activity. More specifically, miR-146a keeps STAT1 expression in check, which would otherwise unleash IFNγ expression. Deficiency of miR-146a in T cells leads to over-expression of IFNγ and Th1-mediated pathology (426). miR-146a also targets IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), two molecules involved in NFκB activation. De-repression of IRAK1 and TRAF6 leads to NFκB-mediated TCR hyper-responsiveness, followed by up-regulation of IFN-γ in effector T cells (427).

Other miRNAs, mir-10a, miR-181, miR-210, and miR-17~92 cluster are also involved in various immune regulations. miR-10a can restrain conversion of iTreg into Tfh by targeting Bcl-6 and is also involved in suppression of Th17 differentiation (428). miR-181 modulates T cell responses mainly by targeting several phosphatases critical for TCR signaling (429–431). miR-210 regulates Th17 differentiation in hypoxia by targeting HIF-1α, a key TF for

Th17 polarization (432). Finally, miR-17~92 cluster regulates IL-10 production in Treg cells and Tfh differentiation (433, 434).

Higher order chromatin conformation

Beyond the previously mentioned epigenetic mechanisms, another aspect of chromosome biology is also critical for gene expression and cell identity, namely the three-dimensional chromatin conformation. It has been appreciated that enhancers regulate gene activity through physical interactions with promoters. These interactions require chromatin folding that excludes intervening genes and specifies enhancer targets. As the enhancers can function in a location-independent manner, analyzing enhancer-promoter interactions has become critical for identifying putative targets of an enhancer without getting into laborious genetic modifications. More importantly, the three billion base pair, two-meter long genome is complexly packaged in nuclei that are only a couple micrometers in diameter (reviewed by Gibcus and Dekker (435)). How this compact architecture permits the tightly-regulated gene expression is intriguing in terms of understanding what switches regulate which circuits. Mapping these connections is key to deciphering the logic of lymphocyte function.

Currently, chromosome conformation capture (3C) and its derivative methods are prevalently used for determining chromatin spacial organization. In the past decade, the development of 3C-based methods, including 4C, 5C, Hi-C and ChIA-PET, has broadened our access to chromatin architecture from local loops to global interactions (436). (4C: chromosome conformation capture-on-chip or circular chromosome conformation capture, using inverse PCR to genome-widely identify regions interacting with interest bait [one-to-all]; 5C: chromosome conformation capture carbon copy, using multiplex primers during ligation-mediated amplification [many-to-many]; HiC, amplifying ligation junction by introducing biotin and pulling down [all-to-all]; ChIA-PET: *chromatin* interaction analysis with paired-end tag sequencing, combining chromatin immunoprecipitation and Hi-C). The basis of 3C involves formaldehyde-crosslinking and ligation of DNA fragments that are nearby in three-dimensional space in the nucleus. The advantages of 3C technology include that it can detect DNA folding at molecular level (high resolution as compared to imaging three-dimensional fluorescence in situ hybridization [3D-FISH]) and it can be incorporated with modern sequencing techniques to study genome-wide chromosome topology (436). Furthermore, 3D-FISH is low throughput and can only look at a few genes at a time.

Global mapping of DNA proximity reveals a hierarchic chromatin organization that aggregates active and inactive genes in euchromatin and heterochromatin compartments, respectively (437). Within these compartments are megabase-scale globules termed topologically associated domains (TADs) that have stable boundaries that are invariant within different cell types and are conserved between species (437–439). Within each TAD are numerous submegabase-scale long-distance interactions that are dynamic and cell-type specific (440). TADs that contain repressive genes are often associated with nuclear peripheral lamina regions as well as H3K9 and H3K27 methylation (439, 441, 442). Hence, identification of cell-type specific interactome is informative for understanding the regulation of gene expression and cell specification.

With regard to the mechanisms, both transcription factors and global chromatin organizers are essential for the formation of cell-type specific chromatin architecture. It has been shown that the long-distance structure domains consist of colocalizing of CTCF and cohesin, whereas dynamic enhancer-promoter interactions are regulated by Mediator and cohesin (440). Master transcription factors and Polycomb proteins are also reportedly involved in the formation of cell-type specific chromatin architecture. In mouse pluripotent stem cells, lineage-specific master transcription factors, Nanog, Sox2, and Oct4, orchestrate chromatin conformations with the help of Polycomb proteins. Depletion of one master regulator or Polycomb subunit disrupts local DNA contacts, but not the large-scale chromosome topology (443, 444).

Several studies have demonstrated cell type-specific and stimulus-inducible chromatin architectures on cytokine loci (445–449). For instance, the Th2 cytokine (*Il4*, *Il5* and *Il13*) locus forms a cell-type specific interacting center that recruits the promoters of these genes in $CD4^+$ T and NK cells but not in B cells or fibroblasts (447). Interestingly, upon Th2 activation, this locus further develops from basal status with limited contacts into a more complicated “cage-like” chromatin architecture in a special AT-rich sequence binding protein 1 (SATB1)-dependent manner (448). Similarly, the *Ifng* locus possesses lineage-specific DNA contacts across 100kb specifically in Th1 cells that facilitate IFN-γ expression (445, 446). The Th1-specific interacting hub on *Ifng* locus is framed by two CTCF/cohesin binding sites anchor to another CTCF/cohesin site within the first intron of *Ifng* gene. Knockdown of CFCF or cohesin results in reduction of long-distance interactions and IFNγ production (445, 446). T cell lineage-specific transcription factors, T-bet and GATA-3, respectively, are also essential for the looping on Th1- and Th2 cytokine loci (445, 447). Based on the chromatin signature, *Ifng* gene is surrounded by multiple enhancers (a good example of a super-enhancer) and most of which are within the loop created by CTCF/cohesin, suggesting this factor can help define the boundaries of super-enhancer architecture.

The *Ifng* and *Il4/Il3/Il5* loci contrast with genes rapidly activated by TNF in which the enhancer-promoter interactions are present prior to stimulation, suggesting that the chromatin conformation sets the stage for rapid responses of extrinsic stimuli (449). Furthermore, the genome-wide mapping of promoter-enhancer interactomes reveals that global gene expression is fine-tuned by tissue-specific enhancers, even for those genes that are not cell-type specific. For instance, within near five thousand promoter interactions shared by B cells and ES cells, up to 90% use at least one cell type-specific enhancer (450). These enhancers, however, are associated with lineage-determining factors.

Evidence also reveals that expression of co-regulated genes can be coordinated through inter-chromosomal interactions (451). During mouse T cell differentiation, the dynamic inter-chromosomal interactions between cytokine loci provide a new mechanism for genomic regulation. For example, *Ifng* locus on chromosome 10 interacts with Th2 cytokine on chromosome 11 in naïve $CD4^+$ T cells, in which both genes are inactive. This interaction further dissociates once the cell differentiated into Th1 or Th2 cells, suggesting a co-regulation or “poised” nuclear organization for lineage-specific genes (452). Similarly, the Th2 locus is also shown to interact with *Il17* locus to restrain Th17 differentiation (453).

Conclusions

More than three decades ago, the term “master regulator” was introduced to describe “ a gene that occupies the very top of a regulatory hierarchy” (454). This concept was introduced roughly at the same time when “lineages” of $CD4^+$ helper T cells were first recognized. “Master regulator” tacitly implies that these factors dominantly specify cell lineage. The classical example is the myogenic transcription factor MyoD, which is essential for muscle cell differentiation but can turn on myogenic genes when introduced into heterologous cells. Initially, it seemed appropriate to view helper T cell lineages and cognate master regulators in the same way. However, much has changed over the last 30 years. There are many more fates for $CD4^+$ T cells and likewise the array of cytokines produced by ILCs has also expanded. These discoveries highlight the limitations of a one lineage-one master regulator model for explaining the diversity of functions of lymphoid cells. More accurate is the appreciation that the establishment of each immune cell type requires multiple key TFs that coordinately regulate aspects of their specialized functions (Figure 4). In this way, more than one “master regulator” can be expressed in more than one cell type. Moreover, multiple cells can exhibit the same functionality (e.g. production of IFN-γ or IL-17) and not surprisingly these cells express many of the same factors. However, master regulators like T-bet appear to be functionally critical in different ways in different cells. GATA-3 and Rorγt are important at multiple steps in lymphocyte differentiation – their function is not limited to cytokine production alone. Therefore, the notion of master regulators, at least based on the traditional definition, needs to be revised with respect to diverse immune cell populations that have distinct functions and gene expression. Superimposed upon selective cytokine production are other functionalities of immune cells and their ability to localize in diverse tissues. Consequently, lymphoid populations express more than one “master regulator” and diverse types of cells can express the same “master regulator”; this limits the notion that a single transcription factor defines a specific cell population. A more accurate view is to think about the superimposition of functionalities that can coexist. Thus, the combinatorial action of TFs is probably a more appropriate way of thinking about how these factors specify gene expression programs.

In addition to thinking about how TFs act on genes, one also needs to consider how chromatin states affect the action of TFs. Accumulating evidence indicates that cell identity is established by converging signals provided by epigenetic traits accumulated from the action of pioneer TFs, not master regulators, and the consequence of past environmental stimuli that alter the epigenetic landscape to imprint “memory” and in this way alter transcription factor deposition. For instance, the process of hematopoietic stem cells to differentiate into effector immune cells requires multiple steps of chromatin remodeling and epigenetic reprogramming. However, the connections between these events are only partially understood. An important challenge will be to track the dynamic appearance of epigenetic marks along cell differentiation and activation, to understand the interpretation of each epigenetic mark, to identify the hierarchy and/or the combination of transcription factors for cell identity.

Finally, genes represent only a tiny portion of the genome; most of the genome represents different kinds of switches, many of which are themselves transcribed into RNA, but not

into proteins. Understanding what factors are responsible for the creation of these switches and what controls their state is clearly an important challenge. Clarifying the role of key TFs in creating the switches or how the switches influence TFs access to the genome are important questions to resolve. The advent of deep sequencing technologies now allows comprehensive, genome-wide views of chromatin states in lymphocytes, along with assessment of transcription factor binding and measurements of the transcriptome that go far beyond the small portion of the genome that encodes conventional protein coding genes. With improved ability to edit the genome with efficient technologies like TALENs or Crispr/Cas9, along with rich resources like ENCODE, (http://www.encode-roadmap.org) enumeration and functional dissection of the switches is now within reach. Defining TF networks and how they affect or employ enhancer landscapes will undoubtedly provide a more sophisticated understanding of diverse lymphoid populations in health and disease.

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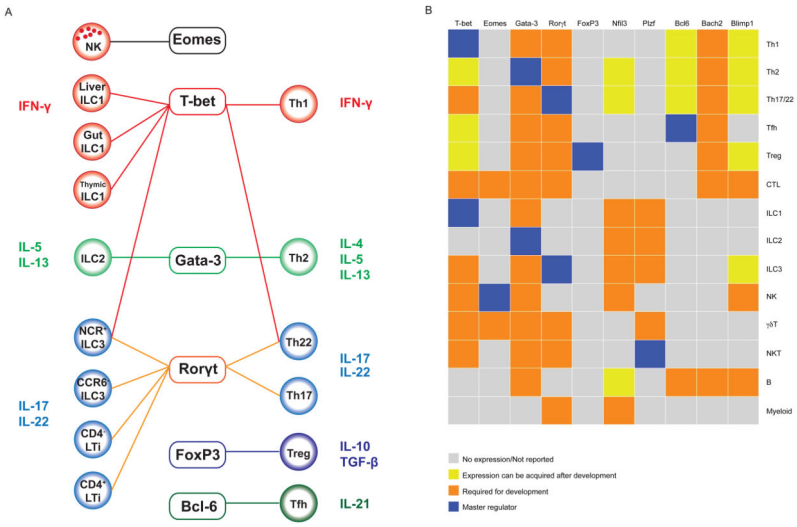

Figure 1.

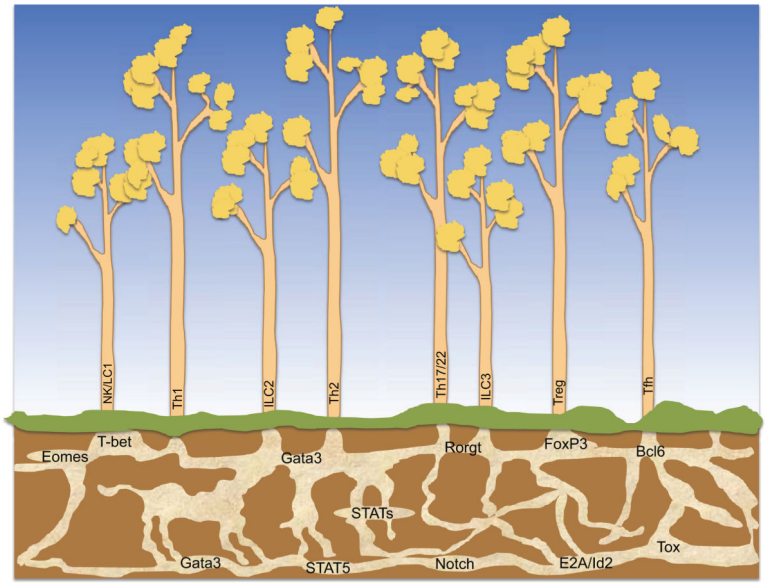

Figure 2.

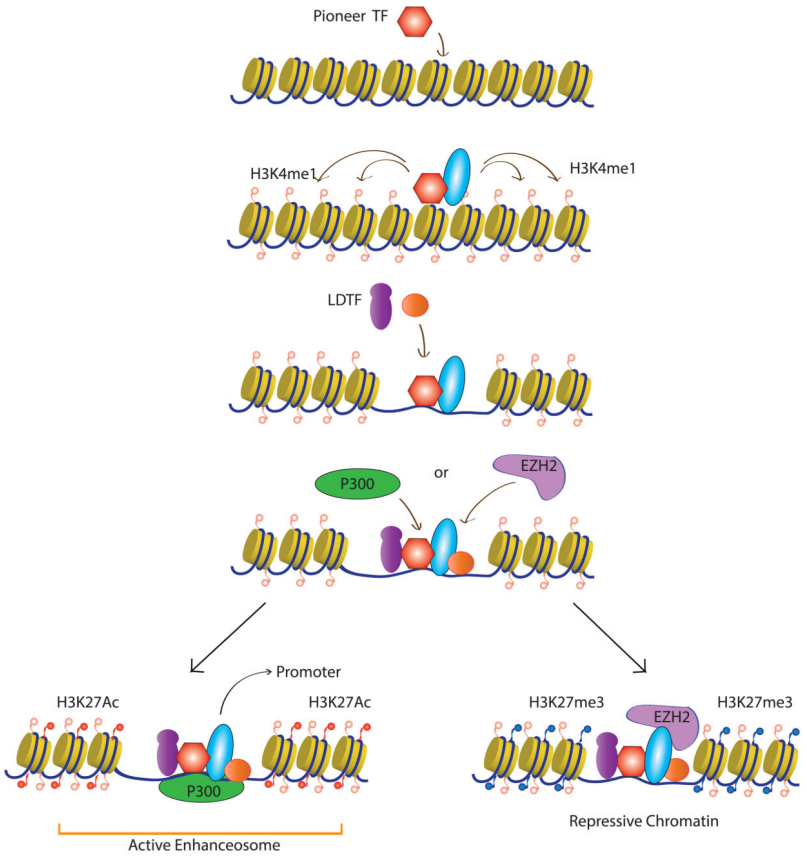

Figure 3.