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Chromatin structure and gene regulation in T cell development and function

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Abstract

During their development from haematopoietic progenitors, lymphocytes go through a series of progressive cell fate decisions. The underlying changes in gene expression programs are controlled in part by the assembly of transcription factor complexes on regulatory regions of their target genes. The propagation of gene expression programs through mitosis in a heritable manner – and hence cellular identity – is achieved by epigenetic processes that affect the accessibility of transcription factors to regulatory regions [1–4]. In mammals, epigenetic information is encoded by differences in the methylation of DNA on cytosines, composition of histone proteins, post-translational histone modifications, and higher-order chromatin structure. Here we discuss recent advances in the epigenetic control of T cell development and function.

Chromatin, histone modifications, and DNA methylation

In the nucleus DNA is contained in chromatin. The basic subunit of chromatin is the nucleosome, comprised of ~160 bp of DNA wrapped around an octamer consisting of two copies each of histones H2A, H2B, H3 and H4 [3]. Nucleosomes are dynamic structures that can be ‘remodeled’ to facilitate or impede transcription factor binding and transit of RNA polymerases [5]. Transcriptional accessibility is also influenced by post-translational histone modifications. For example, at the 5' ends of active genes H3 is typically acetylated on lysines 9 and 14 (H3K9/K14Ac) [6]. Lysine residues can also be methylated. For example, H3 trimethylated on lysine 4 (H3K4me3) co-localizes with H3K9/14Ac, while H3K4me2 is a mark of transcriptional competence present both within genes and at more distant regulatory regions [2,6]. Histone arginine methylation is also a characteristic of active genes. Conversely, H3K9me3 or H3K27me3 is associated with “closed” chromatin and silenced genes. Histones can also be modified by phosphorylation, ubiquitination, and ADP-ribosylation, but the effects of these modifications are less well characterized.

How histone modifications influence transcriptional competence is incompletely understood. Acetyl groups reduce the positive charge of histones, thereby reducing strength of binding to negatively-charged DNA, and also create binding sites for bromodomain-containing proteins, including histone acetyl transferases (HATs) [2]. Methylation of histones does not affect their charge, but can create binding sites for proteins that influence chromatin accessibility, e.g., heterochromatin protein 1 (HP1) binds to H3K9me3, the Polycomb protein/H3K27 methyltransferase EZH2 binds to H3K27me3, and WDR5-containing/H3K4 methyltransferase complexes bind to H3K4me2 [2]. These proteins in turn help to sustain the marks to which they bind, providing site-specific cellular memory of transcriptional competence. The multiple modifications possible for each histone molecule could potentially act in a combinatorial

manner - a concept known as the “histone code”. Whether specific combinations of histone modifications actually encode discrete transcriptional states is controversial [7] but resolution may come from comprehensive genomic analyses of epigenetic modifications [6].

Histone acetylation is dynamic - HATs and histone deacetylases (HDACs) rapidly add or remove acetyl groups. Histone methylation was thought to be more stable until the recent discovery of enzymes that demethylate lysine (LSD1) and arginine (PADI4) [2]. LSD1 can demethylate mono- or dimethyl-H3K4 or -H3K9. H3K4me3 or H3K9me3 may be removed by H3 replacement [8]. A variant of H3 - H3.3 - can be incorporated into nucleosomes in a replication-independent manner and typically shows transcriptionally favorable histone modifications [9].

Methylation of cytosines in CpG dinucleotides is mediated by DNA methyltransferases (Dnmts) [4]. DNA methylation can repress gene expression through direct mechanisms, by blocking the binding of certain transcription factors, and by recruitment of methyl-CpG-binding proteins like MeCP2, MBD1 and MBD2. Methyl-CpG-binding proteins can exclude transcription factors and recruit HDACs and H3K9 methyltransferases, linking DNA methylation to of repressive histone modifications. The only established mechanism by which to demethylate cytosines is through its passive loss during DNA replication. While numerous reports suggest active demethylation, a molecular mechanism for enzymatic cytosine demethylation has not been defined in mammalian cells.

DNA methylation, histone modifications and higher order chromatin structure operate in concert to yield stable programs of gene expression. Higher order genome organisation and RNA interference-directed DNA and histone methylation may help to target these processes to specific sites.

Higher order genome organisation

The one-dimensional sequence of our genome - about 2 metres of DNA - is packed into a nucleus only a few microns across and brought to life by transcription factors, chromatin proteins, machines that remodel chromatin, transcribe DNA into RNA, process RNA transcripts or replicate DNA in preparation for cell division. This may sound chaotic, but a closer look reveals a considerable level of organisation. Important activities like transcription and DNA replication are centralised [10] and loci assume positions within the nucleus according to their expression status.

Nuclear positioning reflects gene activity and silencing

The localisation of chromosomes in the interphase nucleus reflects gene density, so that gene-rich chromosomes occupy more central positions [11]. Many active genes are located centrally, but others associate with nuclear pore proteins at the nuclear envelope [12]. Silent – but not active – genes often localise to heterochromatic areas around centromeres [13,14]. In DP thymocytes, repositioning to centromeric heterochromatin precedes the stable silencing of the Tdt locus [14,15] and the spreading of repressive histone modifications from the Tdt promoter to the surrounding chromatin [15]. Similarly, the CD4/CD8 lineage choice is reflected in locus repositioning prior to overt differentiation into CD4 or CD8 SP cells [16,17].

Intrachromosomal interactions: control of gene expression by contacts between distant regulatory elements

Genes in higher eukaryotes are typically controlled by multiple regulatory elements, often separated by many basepairs from each other and the coding region [18]. Techniques like chromosome conformation capture, 3C [19], reveal that regulatory sites interact in 3-dimensional nuclear space [20–25]. Analysis of the DNA sequences [25] and the DNA binding

proteins involved [26] points to a scenario where transcription factor binding - directly or indirectly - bridges distant regulatory elements to regulate gene expression. For example, GATA-1 and its cofactor FOG-1 are required for the interaction between the β -globin locus control region (LCR) and the β -major globin promoter [26].

From intra- to interchromosomal interactions: contacts between loci on different chromosomes

In addition to interactions *in cis*, sequences located on different chromosomes can come together *in trans*. Examples include the pairing of homologous chromosomes and the coalescence of centromeres in *Drosophila* and the formation of nucleoli by several hundred ribosomal (rDNA) genes on different chromosomes in mammalian cells [27]. Inactive genes on different chromosomes can colocalise in association with polycomb proteins [28] or in the vicinity of centromeric heterochromatin [13] and active genes from different chromosomes colocalise at shared sites of transcription [29]. Such sites had been predicted to exist as so-called 'transcription factories' [10]. A recent study found co-localisation even without transcription: in naive helper T cells, the IFN γ locus on mouse chromosome 10 apparently colocalises with the Th2 cytokine gene cluster (comprising IL-4, IL-15 and IL-13) on chromosome 11 [30]. Significantly, this interaction occurs in naive T cells, which have the potential to express low levels of both IFN γ and Th2 cytokines upon activation. In contrast, Th1 and Th2 cells express either IFN γ or Th2 cytokines (but not both) and interactions between IFN γ and the Th2 loci are lost.

Fluorescence *in situ* hybridisation (FISH) of interphase nuclei shows that individual chromosomes form distinct territories – as opposed to a random 'spaghetti' arrangement. This compartmentalisation of the nucleus appears to be at odds with the numerous cases where chromosomal loci appear to behave as individuals, for example the interchromosomal interactions discussed above [30] and the co-localisation of transcribed [29] or silenced loci [13,14,28]. One explanation for this paradox is that genes represent a relatively small proportion of our chromosomes and the probes used to define territories are therefore mainly directed at intergenic regions. The more genes are found on a stretch DNA (and the more 'open' its chromatin structure) the more often it is found outside its chromosome territory [11]. This results in a significant degree of overlap between territories (Branco and Pombo, personal communication).

Lymphoid tumours are characterised by recurrent chromosomal translocations, which typically bring genes that promote proliferation and/or survival under the control of active regulatory regions. Such translocations confer a selective advantage to cells in which they occur, and - even if they occur rarely - will often be seen in malignancies. In addition, genes involved in recurring translocations have a tendency to co-localise even in normal cells. In B lymphocytes, immunoglobulin genes are often close to Myc and Bcl-2 loci, perhaps foreshadowing Ig/Myc and Ig/Bcl-2 translocations B cell leukaemias [31,32]. Analysis of the entire set of chromosomes correlates the probability of overlap between chromosome territories with the frequency of lymphocyte-specific translocations (Branco and Pombo, personal communication). These data link genome organisation to neoplastic transformation and suggest that translocations arise when double-strand breaks occur in areas of overlap between chromosome territories and are repaired by non-homologous end joining.

Transcriptional and posttranscriptional silencing by small RNAs

Double stranded RNA molecules are emerging as mediators of chromatin structure and gene expression at the transcriptional and post-transcriptional level by RNA interference (RNAi). Central to this pathway are the RNase III enzymes Drosha and Dicer, which process double stranded RNAs, and Argonaute, the executioner of RNAi [33]. RNAi can block mRNA

translation and initiate mRNA degradation, as widely exploited by researchers for siRNA-mediated post-transcriptional gene silencing [34,35].

siRNA, transcriptional silencing and the structure of heterochromatin

In plants, the fission yeast *S. Pombe* and several other organisms, RNAi mediates the establishment of a repressive chromatin structure, the transcriptional silencing of constitutive heterochromatin (such as pericentromeric repeats) and transposable elements in [36]. The discovery that transcriptional silencing by RNAi requires RNA polymerase II [37,38] provides a mechanism for localising RNAi to the nucleus, and for selectively directing it to DNA sequences targeted by RNAi. The concept that siRNA-mediated transcriptional silencing requires transcription may seem paradoxical at first. One attractive explanation is that RNAi has evolved in response to transcriptionally active transposable elements and other invaders, whose silencing would have been advantageous to the host [39].

In organisms from yeast to mammals, defects in RNAi result in the accumulation of centromeric transcripts. Nuclear run-on assays showed that these transcripts also occur in wild type cells, but are rapidly processed into siRNAs [40]. Centromeric transcripts accumulate in Dicer deficient vertebrate cell lines [41,42] but it is unclear whether this is due to increased transcription or to defective processing in the absence of Dicer. In several model organisms, the repressive chromatin structure of pericentromeric repeats clearly depends on the RNAi pathway: without it the structural integrity of centromeres is lost and chromosomes no longer segregate properly in mitosis [36]. However, the role of RNAi for the maintenance of a repressive chromatin structure at centromeres in vertebrate cells is less clear-cut [40,41–45]. This may be due to the presence in vertebrates of redundant silencing mechanisms, including CpG methylation.

Is transcriptional silencing limited to repetitive DNA sequences or does it regulate protein-coding genes involved in cellular differentiation and function? Earlier reports in *S. pombe* have recently been called into question [46,47]. While transfection of mammalian cells with siRNAs complementary to promoter regions can induce DNA methylation and transcriptional silencing [48,49], most known endogenous double stranded RNAs target not promoters but 3' untranslated regions (3'UTR) and affect gene expression at the post-transcriptional level.

miRNAs regulate translation and RNA stability

In contrast to siRNAs, micro RNAs (miRNAs) do not arise from centromeric repeats, RNA viruses or transposable elements but from specialised miRNA genes [33,50]. Since each miRNA can target 3' UTRs of many different transcripts - affecting translation and sometimes RNA stability [51,52] - a sizeable proportion of protein coding transcripts is regulated by miRNAs [50,52]. miRNAs expression is cell type- and developmental stage-specific and distinguishes normal from cancer cells, including lymphoid malignancies [53,54]. Chromosomal translocations (see above) in leukaemias frequently involve miRNA genes, and miRNAs of the miR-17-92 cluster can cooperate with oncogenes to promote murine leukaemia [54] as well as restrict oncogene-driven proliferation by limiting E2F1 expression [55].

Epigenetic Regulation of T cell development

The influence of RNAi and other epigenetic mechanisms on T cell development is gene-, process-, and developmental stage-dependent. Enforced expression of one particular miRNA, miR-181, in haematopoietic progenitors biases lymphoid differentiation towards the B cell lineage at the expense of T cells [63]. And although the RNAi pathway is required for mouse development [64], its role in T cells is being assessed using conditional alleles and Cre transgenes driven by lineage- and developmental stage-specific promoters. Deletion of Dicer

early in T cell development results in the depletion of miRNAs by the DP stage and a 10-fold reduction in $\alpha\beta$ - but not $\gamma\delta$ - thymocytes [44]. Surprisingly, the CD4/CD8 lineage choice appears largely unaffected: CD4- and CD8-lineage specific genes are expressed correctly and Tdt is appropriately silenced [44]. Dicer deletion later in development results in reduced numbers of peripheral T cells affecting the CD8 more than the CD4 lineage [45].

Similar to the effects of Dicer deletion, conditional deletion of Dnmt1 early in T cell development leads to progressive DNA demethylation and a profound reduction in numbers of TCR $\alpha\beta$ but not TCR $\gamma\delta$ lineage cells [56]. A similar phenotype is also seen in thymocytes lacking lymphocyte specific helicase [57], which remodels chromatin and facilitates DNA methylation, and in thymocytes lacking mSin3a [58], which recruits HDACs. Together, these studies indicate that RNAi, DNA methylation, histone acetylation and chromatin remodeling complexes [5,59] are important for proper homeostasis of TCR $\alpha\beta$ but not TCR $\gamma\delta$ lineage progenitors. Perhaps surprisingly, V(D)J recombination does not appear to be affected in thymocytes with any of these defects.

Chromatin remodeling complexes, HDACs, and HP1 also contribute to permanent silencing of CD4 as DP thymocytes differentiate into CD8 SP cells [5,59]. Conversely, Mi-2 β , which normally recruits HDACs, facilitates CD4 expression by recruiting the p300 HAT to the proximal CD4 enhancer [59]. Similarly, Runx proteins and BAF-containing chromatin remodeling complexes bind to enhancers E8_I, E8_{II} and E8_V, recruit HATs to these regions and activate *CD8* expression [60,61]. While DNA methylation does not appear to play an important role in CD4 regulation, it does regulate CD8 expression in T cell progenitors: Aberrant CD8 α and CD8 β expression occurs in a large fraction of Dnmt1-deficient TCR $\gamma\delta$ lineage cells [56]; variegated expression of CD8 in DP thymocytes from E8_I plus E8_{II} knockout mice is associated with increased CpG methylation within E8_V and the *CD8b* promoter, and conditional deletion of Dnmt1 partially reverses variegated CD8 expression [62]. Nonetheless, mechanisms other than DNA methylation are sufficient to silence *CD8* after commitment to the CD4 lineage, since CD4⁺CD8⁺ T cells are not found in the periphery of conditional Dnmt1 knockout mice [56].

Epigenetic Regulation of T cell cytokine production

IL-2 is produced abundantly by naïve T cells, so one would predict a fully accessible *Il2* locus in these cells. Contrary to this prediction, the *Il2* locus is contained in inactive chromatin in resting naïve T cells, but undergoes dynamic and reversible chromatin remodeling and histone acetylation in response to activation [65]. Moreover, Bruniquel et al showed that CpG cytosines in the proximal *Il2* promoter are completely methylated in naïve CD4 T cells, but some of these cytosines are rapidly demethylated after T cell stimulation but before entry into S phase, suggesting active demethylation [66]. This finding is controversial, since Thomas et al found a much lower degree of cytosine methylation in naïve T cells (<33%) and only a modest 2-fold reduction following activation *in vitro* [65]. In any case, promoter demethylation appears to be required for optimal *Il2* promoter activity *in vitro* [19] and activated Dnmt1-deficient T cells express ~5-fold more IL-2 than controls [67].

Dnmt1-deficient naïve T cells also express ~5-10-fold more IFN- γ following activation *in vitro*, although expression remains responsive to other cues – increasing in Th1 conditions and diminishing in Th2 conditions [67]. Consistent with these findings, naïve T cells lacking the methyl-CpG binding protein MBD2 produce increased amounts of IFN- γ and exhibit a Th1 bias *in vivo* [68]. As a consequence, MBD2-deficient mice on the susceptible BALB/c background show increased resistance to infection with *Leishmania major*, and on a B6/129 background have impaired Th2-dependent immunity against *Trichuris muris*. These findings

suggest that the dominant effect of DNA methylation and MBD2 on T cell cytokine production *in vivo* is repression of IFN- γ .

Since CpGs in the murine *Ifng* promoter are constitutively demethylated in naïve T cells [69], it is likely that DNA methylation and MBD2 repress IFN- γ production either indirectly or through actions at other regulatory regions of the *Ifng* gene. Evolutionarily conserved nucleotide sequence elements (CNSs) extend >50kb upstream and downstream of *Ifng* [70–72]. Two of these CNSs, one located ~5kb upstream and the other ~18kb downstream from the ATG start codon, contain Th1 and CD8 effector T cell-specific DNase HS sites, demethylated DNA, acetylated H3 and H4 and methylated H3K4, and exhibit *in vitro* enhancer activity [72,73]. The importance of these CNSs in *Ifng* regulation is also supported by the finding that the upstream CNS is approximated by chromatin looping to the *Ifng* promoter in naïve, Th1 and Th2 cells, whereas the downstream CNS is approximated to the promoter in Th1, but not naïve and Th2 CD4 T cells [30].

Similarly, there are multiple DNase HS sites within the Th2 cytokine locus, many – but not all – of which are within CNSs. A number of these HS sites and the *Il4*, *Il13* and *Il5* promoters are physically approximated in T and NK cells through intrachromosomal interactions forming a ‘hub’ that may facilitate their coordinate expression [24]. Two groups recently identified additional Th2-specific DNase HS sites in the 3' region of the ubiquitously expressed *Rad50* gene, which is located between the *Il5* and *Il13* genes [74,75]. One of these sites (RHS7) is a Th2-specific enhancer/locus control region [25]. Deletion of RHS7 impairs intrachromosomal interactions within the Th2 locus in T cells, interchromosomal interactions between the Th2 locus and the *Ifng* locus, and the expression not only of Th2 cytokines, but also of IFN- γ [30]. Another HS site, HS_{IV}, located downstream of the *Il4* gene, silences inappropriate expression of IL-4 and IL-13 in naïve and Th1 T cells [76], and in these cells exhibits an unusual pattern of permissive (acetylated H3 and methylated H3K4) plus repressive (methylated H3K27) histone modifications; the permissive marks are retained but methylated H3K27 is selectively lost under Th2 culture conditions [77].

Like HS_{IV}, DNA methylation and MBD2 act as non-redundant repressors of inappropriate Th2 cytokine expression. Dnmt1 is recruited to *Il4/Il13* gene regulatory regions during S phase to maintain CpG methylation under non-polarizing or Th1 conditions, but is excluded from these regions in Th2 conditions [78]. Exclusion of Dnmt1 results in passive, slow DNA demethylation, which may help stabilize rather than initiate high-level Th2 cytokine expression. However, one specific CpG in RHS7 undergoes complete demethylation within 48 hr of activation in Th2 conditions in a manner suggesting active rather than passive DNA demethylation, and may thereby facilitate early Th2 cytokine production [74]. Consistent with this possibility, Dnmt1-deficient CD4 and CD8 T cells express ~10-fold and ~1000-fold more IL-4, IL-13, and IL-5, respectively, than control cells in the first 3 days after activation in non-polarizing conditions [20]. Dnmt1-deficient and MBD2-deficient T cells also express increased amounts of IL-4, even in Th1 conditions [67,79]. Together with findings regarding IL-2 and IFN- γ , these results support a model in which DNA methylation, methyl-CpG binding proteins, and histone modifications collaborate to silence inappropriate Th1 and Th2 cytokine expression and to maintain Th1, Th2 and CD8 effector T cell lineage commitment.

Th1 and Th2 cells also have distinct patterns of miRNA expression [45,80]. Repression of IFN- γ appears to be defective in *Dicer*-deficient T cells under Th2 culture conditions [45], but it is not clear if this is due to transcriptional silencing of the *Ifng* locus, post-transcriptional silencing of *Ifng* message, or indirect effects.

Conclusions

Although the past few years have provided a number of insights regarding chromatin and gene regulation in T cells, these insights have in turn generated new questions that require clarification: While a marked increase in IFN- γ expression is seen in Dicer-, MBD2- and Dnmt1-deficient T cells, does this mean that RNAi targets DNA methylation to the *Ifng* locus? To address this question and to understand the role of RNAi in T cell development and function, we need to mechanistically link the phenotypes observed in mice with T cell lineage-specific disruption of the RNAi pathway to the loss of specific double-stranded RNAs and to other epigenetic processes. It also remains to be shown whether miRNAs contribute to the control of cytokine RNA stability by co-stimulatory signals and RNA storage in memory T cells. We also need to learn more about how epigenetic modifications are targeted to or excluded from the regulatory regions of genes governing T cell development and function, whether T cell activation results in active DNA demethylation in cytokine gene regulatory regions, and if so, by what enzymatic mechanism. Work in the near future should illuminate the extent to which ongoing expression of transcription factors versus epigenetic processes determine lineage fidelity as T cell develop, differentiate and die.

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Figure 1.

Schematic representation of chromatin modifications and intrachromosomal and interchromosomal interactions in the Th2 locus on murine chromosome 11 and in the *Ifng* locus on murine chromosome 10. In naïve CD4 T cells, these cytokine loci are in a poised configuration. Repressive histone modifications are limited (minimal, localized H3K27^{me} at the Th2 locus; there are no data regarding H3K27^{me} at the *Ifng* locus) or absent (H3K9^{me}). DNA methylation is present at distal regulatory elements in these loci but is absent from the promoter region of the *Ifng* gene and is present in distal but not proximal regions of the *Il4* promoter. The two chromosomes interact, so that the *Il5* and *Ifng* promoters are approximated to each other and to the RHS6 DNase HS site of the Th2 locus control region (located in the 30' end of the *Rad50* gene). In Th1 and Th2 cells, the interchromosomal interactions between the *Ifng* locus and Th2 locus are lost. In Th1 cells, the *Ifng* locus acquires transcriptionally favorable histone modifications (H3/H4ac and H3K4me), the *IfngCNS1* and *IfngCNS2* distal regulatory elements undergo progressive DNA demethylation and acquire DNase hypersensitivity sites, and *IfngCNS2* is brought into proximity to the *Ifng* promoter. In the Th2 locus, H3K27me and DNA methylation increase and spread upstream and downstream from the *Il4* gene. Conversely, in Th2 cells changes inverse to those seen in Th1 cells occur, although there are no published data regarding H3K27me at the *Ifng* locus. The *Rad50* gene is constitutively active in each of these cell types.