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The epigenetic landscape of fate decisions in T cells

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Specialized T cell subsets mediate adaptive immunity in response to cytokine signaling and specific transcription factor activity. The epigenetic landscape of T cells has an important role in facilitating and establishing T cell fate decisions. Here, we review the interplay between transcription factors, histone modifications, DNA methylation and three-dimensional chromatin organization to define key elements of the epigenetic landscape in T cells. We introduce key technologies in the areas of sequencing, microscopy and proteomics that have enabled the multi-scale profiling of the epigenetic landscape. We highlight the dramatic changes of the epigenetic landscape as multipotent progenitor cells commit to the T cell lineage during development and discuss the epigenetic changes that favor the emergence of $CD4^+$ and $CD8^+$ T cells. Finally, we discuss the inheritance of epigenetic marks and its potential effects on immune responses as well as therapeutic strategies with potential for epigenetic regulation.

T cells are central to adaptive immunity, patrolling the body for pathogens and eliminating threats including tumors. They can also contribute to autoimmunity by erroneously targeting self-antigens. T cell differentiation into distinct effector subtypes is a complex process, shaped by interactions with innate immune cells and driven by both T cell receptor (TCR) recognition of antigens and a diverse cytokine milieu. Cytokines are proteins that can bind to cells expressing their receptors, triggering a cascade of intracellular signaling pathways. These signaling events propagate to the nucleus and induce the expression of transcription factors that instruct the gene expression programs required for immune responses. Although the molecular mechanisms by which transcription factors govern T cell responses have been difficult to decipher, the field of epigenetic regulation has recently yielded crucial insights. Epigenetics can be interpreted in various ways in the scientific community, with numerous articles exploring and debating its definitions[1–4]. Here, we use the term 'epigenetics' to refer to changes in gene expression that affect cell identity or function without altering the underlying DNA sequence. In this Review, we delve into how studying the epigenome gives us a mechanistic understanding of T cell development and differentiation. We introduce the major epigenetic regulators in T cells, explain the techniques used to study the epigenome and share the latest findings on epigenetic regulation during T cell development, the specialization of $CD4^+$ helper T cells and $CD8^+$ T cell responses.

Key modulators of epigenetic changes

Epigenetic regulation primarily occurs within the nuclear compartment and targets chromatin—the complex of DNA and proteins that constitutes chromosomes. The fundamental unit of chromatin is the nucleosome, consisting of DNA wrapped approximately twice around a histone octamer composed of two copies of core histones H2A, H2B, H3 and H4. (Fig. 1a). Histones are decorated by a variety of posttranslational modifications, also referred to as 'epigenetic marks'. Acetylation and methylation of the histone H3 are the two most prominent and well-studied histone posttranslational modifications in T cells (Fig. 1b). Histone acetylation destabilizes the binding of DNA to histones by shielding the positive charge of lysine residues on histone tails. This modification increases chromatin accessibility at *cis*-regulatory

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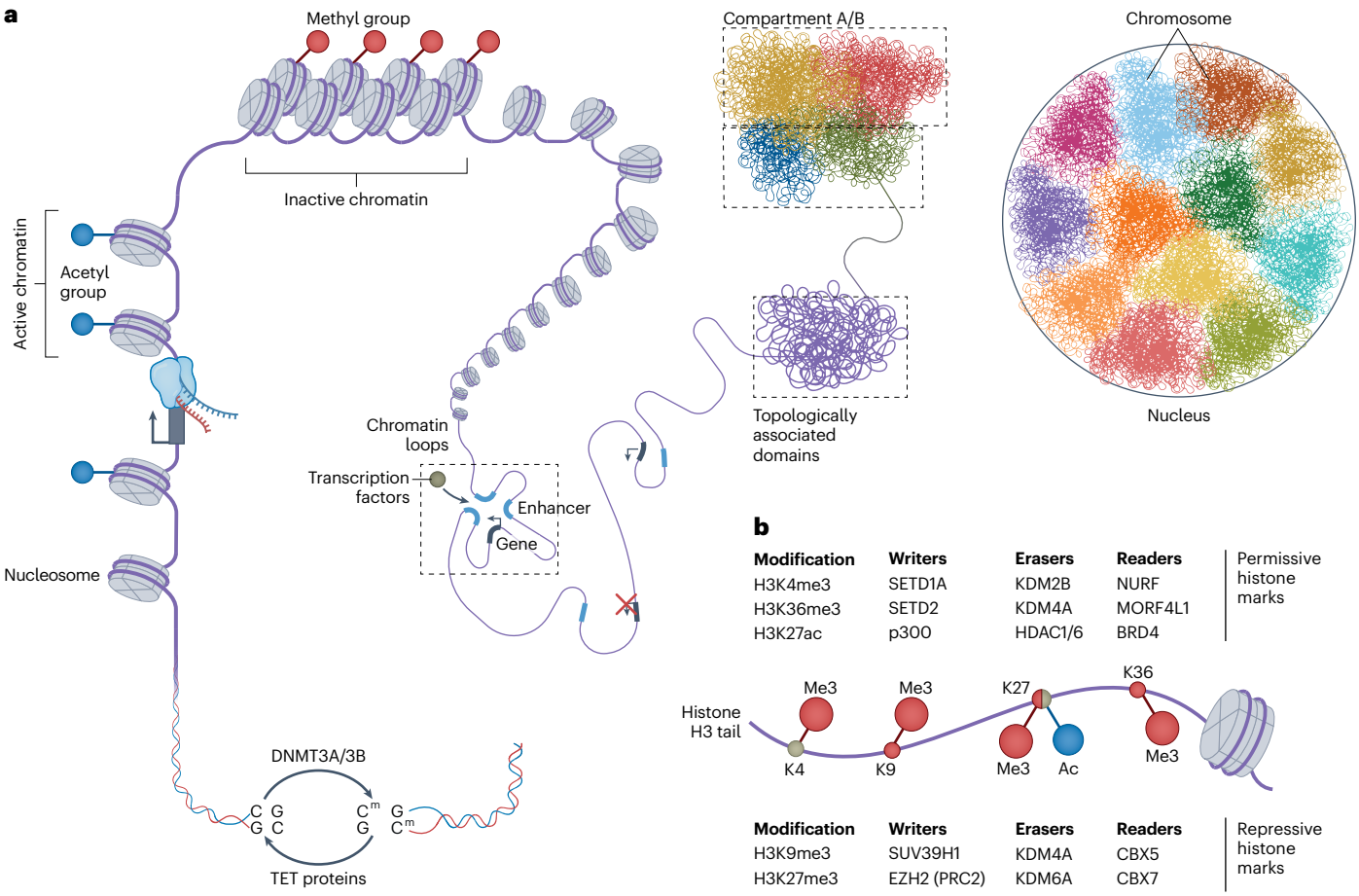

Fig. 1 | Chromatin organization in the nucleus. a, Epigenetic modification can occur at the level of the DNA, such as methylation of cytosine residues in DNA CpG islands by DNMTs such as DNMT3A and DNMT3B to form 5mC required for gene repression; while TET enzymes act to reverse the activity of DNMTs and activate gene expression, or at the level of the histone components of the nucleosome, which are acetylated and methylated, that further instruct gene expression. Chromatin loops into topologically associated domains, which regulate local gene expression by facilitating enhancer interactions with gene promoters, and represent functional units within compartments, which can be either regions of active gene expression (compartment A) or regions of repressed gene expression (compartment B). This hierarchy defines chromatin organization within the nucleus. **b**, Histone modifications occur at four main lysine residues on the histone H3 tail—K4, K9, K27 and K36. Permissive histone marks such as H3K4me3, H3K36me3 and H3K27ac increase chromatin accessibility and promote gene expression through the activity of histone readers NURF, MORF4L1 and BRD4, respectively. SETD enzymes and p300 facilitate the acetylation of K4, K27 and K36, while KDM enzymes remove the acetylation marks. Repressive histone marks such as H3K9me3 and H3K27me3 modify the tail of histone H3 to reduce chromatin accessibility and gene expression. The trimethylation of K9 and K27 is mediated by SUV39H1 and EZH2, respectively, while the KDM family of enzymes aids in the demethylation of both K9 and K27.

elements, enabling binding of transcription factors and polymerase enzymes that control gene expression[5]. Histone methylation increases the basicity and hydrophobicity of arginine and lysine residues, and as such favors interactions between DNA and histones. Depending on the residue targeted and the degree of methylation, methylated histones can activate or repress gene expression. Trimethylated histone H3 Lys4 (H3K4me3), trimethylated histone H3 Lys36 (H3K36me3), acetylated histone H3 Lys27 (H3K27ac) and acetylated histone H3 Lys9 (H3K9ac) associate with gene activation, while trimethylated histone H3 Lys9 (H3K9me3) and trimethylated histone H3 Lys27 (H3K27me3) are hallmarks of gene repression[6]. A bivalent state can also be established by simultaneous deposition of H3K4me3 and H3K27me3, which primes developmentally important genes for activation or repression[7]. DNA methylation at the transcriptional start site is an epigenetic mechanism that represses gene transcription by inhibiting the binding of transcription factors and regulatory proteins[8]. Genome-wide DNA methylation analysis of human effector memory $CD8^+$ T cells revealed decreased methylation at gene promoters associated with increased expression during $CD8^+$ T cell differentiation, and increased methylation at promoters of silenced genes[9]. Genetic screening has also identified DNA methylation as a key factor in silencing the *Cd4* locus during the development of helper T cell subsets[10]. Besides the chemical modifications of DNA and histone proteins, the three-dimensional (3D) architecture of the nucleus facilitates physical interactions between enhancers, which are short DNA sequences that serve as binding sites for transcription factors and can regulate gene expression over long genomic distances, and their target promoters[11]. Together, packaging of DNA into chromatin is tightly regulated to control gene expression.

Two classes of proteins are central to the dynamic modification of chromatin: chromatin remodelers, which mediate alterations in nucleosome positioning and regulate chemical modifications of histones and DNA, and transcription factors, which confer sequence specificity via DNA binding, thereby influencing gene expression programs. Histone posttranslational modifications are catalyzed by 'writer' and 'eraser' chromatin remodeler enzymes, and are recognized by 'reader' proteins that bind the acetyl or methyl groups (Fig. 1b). For example, permissive histone modifications such as H3K4me1, H3K4me3 and H3K36me3, which correspond to actively transcribed regions, are deposited by methyltransferases such as SETD1A and SETD2 (ref. 12), to establish and maintain an open chromatin state, facilitating transcriptional

activation. Histone modifications such as H3K4me3 can also regulate the methylation of DNA at cytosine residues in CpG nucleotides to affect the activity of DNA-binding factors and gene expression[13]. DNA methyltransferases (DNMTs) such as DNMT3a and DNMT3b methylate cytosine to 5-methylcytosine (5mC) when H3K4 is unmethylated to repress expression[13], while TET enzymes counteract DNMTs to demethylate DNA and increase expression[13].

Transcription factors can interact with chromatin remodelers to modulate chromatin structure, histone modification, DNA methylation and nucleosome positioning, thereby controlling gene expression programs. Transcription factors can also modulate 3D genome organization. Local changes in histone acetylation mediated through the interactions of transcription factors and histone acetyl-transferases can recruit cohesin, a protein that enables genome folding in a process called loop extrusion[14]. Loop extrusion also requires the recruitment of the structural protein CTCF, which halts cohesin movement, leading to stable loop formation, a process linked to large-scale changes in 3D genome organization[15–17]. Together, the cooperation of transcription factors with chromatin remodeling enzymes and structural proteins such as CTCF defines the epigenetic architecture that dictates cellular identity and responses to environmental cues.

Technologies to measure the epigenome

Sequencing, microscopy and proteomics technologies have been crucial for dissecting the roles of histone modifications, transcription factor binding and chromatin accessibility within the epigenetic landscape. The assay for transposase-accessible chromatin with sequencing (ATAC-seq), which uses the Tn5 transposase to fragment and tag open chromatin regions, has revolutionized high-resolution profiling of chromatin accessibility[18]. Tn5 has also been adapted to other protocols, such as single-cell chromatin accessibility profiling or mapping histone modifications and transcription factor binding[19]. Using ATAC-seq to profile chromatin accessibility across T cells, B cells and NK cells has identified transcription factors that control T cell fate through altering the chromatin accessibility landscape. Recognition sites for TCF-1 are enriched within T cell-specific open chromatin regions[20] and TCF-1 can bind closed chromatin and facilitate chromatin accessibility during T cell development[20]. In various developmental programs, proteins like TCF-1, which have an intrinsic ability to bind closed chromatin, have been referred to as 'pioneer factors'[21]. Similarly, analysis of chromatin accessibility measurements using ATAC-seq across different $CD8^+$ T cell subsets showed the enrichment of recognition sites for NR4A1, NR4A2 and Nr4A3 proteins within genomic regions that were selectively accessible in exhausted T cells[22,23]. Corroborating this observation, the ectopic expression of NR4A1, NR4A2 or NR4A3 can modify the chromatin accessibility landscape of $CD8^+$ T cells, inducing changes characteristic of T cell exhaustion[24,25]. Other important protocols for T cell biology are chromatin immunoprecipitation (ChIP) and cleavage under targets and release using nuclease (CUT&RUN)[26,27], which measure chromatin regions with specific histone modifications, such as H3K27me3, or genomic DNA bound by transcription factors.

Recent cutting-edge technologies with great potential to advance T cell biology are long-read sequencing and spatial genomics. One such long-read sequencing technology from Oxford Nanopore Technologies uses a platform that passes individual nucleic acid molecules through nanometer-sized protein pores embedded in a polymer membrane[28]. As the molecules pass through the nanopore, changes in current act as a direct readout of base identity and DNA or RNA modifications, without the need for pre-treatments such as fragmentation or amplification, which are hallmarks of short-read next-generation sequencing techniques[28]. Methods such as long-read TET-assisted pyridine borane sequencing can reveal the methylation status of genomic V(D)J regions to profile the TCR repertoire without the DNA degradation and fragmentation. Such modifications pose a challenge in conventional DNA methylation analyses[29] that rely on bisulfite treatment to distinguish between methylated and unmethylated cytosines. Long-read sequencing of the transcriptome in 29 human immune subsets including T cells, B cells and innate immune cells purified from the peripheral blood of a healthy donor has enabled mapping of transposable elements (mobile DNA sequences capable of replicating themselves within genomes independently of the host cell DNA[30]. This study found a strong association between splicing events and the insertions of transposable elements[30], suggesting a role of transposable elements in conferring isoform diversity to immunological genes. Spatial genomics platforms, such as spatial CUT&Tag RNA sequencing and RNase H-dependent PCR-enabled T cell receptor sequencing (rhTCR-seq), allow the visualization of T cell interactions with neighboring cells, while defining gene expression at a single-cell resolution[31,32] (Table 1). Other methods of interest include PacBio HiFi sequencing[33], another long-range sequencing platform, and additional spatial genomics techniques such as 10x Visium[34], spatial ATAC-RNA-seq[35], Slide-TCR-seq[32] and Stereo-seq[36,37] (Table 1).

Unlike sequencing-based techniques, which require cell lysis, microscopy-based assays retain the native subcellular structures and enable their visualization at multiple scales from single cells to tissues[38,39] (Table 1). Until recently, observations of chromatin fibers were restricted to electron cryo-microscopy, as measurements fell below the diffraction limit of visible light[40,41]. Super-resolution microscopy can now precisely image nanoscale chromatin domains and observe the kinetics of chromatin interactions. Single-molecule localization microscopy techniques compatible with standard wide-field microscopes, such as stochastic optical reconstruction microscopy (STORM), can visualize folding patterns in DNA and investigate the roles of histone proteins in chromatin compaction[42–45]. STORM has been used in an array of immune contexts, including visualization of the interaction between the HIV envelope glycoprotein, Env, and T cell plasma membranes, which revealed how Env clustering determines fusion with the membranes[46]. Three techniques that hold great potential in uncovering epigenetic mechanisms in T cells are expansion microscopy, sequential fluorescence in situ hybridization (FISH) and single-molecule tracking. Expansion microscopy enables super-resolution visualization of subcellular structures, such as the nucleus, through isotropic physical expansion of the cell[47]. Expansion microscopy-based SCEPTRE has visualized the colocalization of H3K4me3, RNA polymerase (Pol) II and the *GAPDH* locus in cell lines derived from retinal epithelial cells[48], while chromatin expansion microscopy has been used to visualize the binding of NANOG, a transcription factor that maintains the pluripotency of embryonic stem cells, to silent chromatin and the recruitment of RNA Pol II[49]. Oligonucleotide-based FISH assays, such as OligoSTORM, and sequential FISH methods, such as ORCA (Table 1), can sequentially hybridize a variety of targets with a few spectrally distinct fluorophores to visualize chromatin and RNA in cells[50–53]. Because the FISH assays are compatible with immunofluorescence, it is possible to simultaneously visualize 3D chromatin structure in different epigenetic states, such as how compact chromatin endowed with H3K27me3 forms higher chromatin packing density[54]. To determine the dynamics of chromatin interactions in live cells, single-molecule tracking can measure the kinetics of nuclear proteins using stable fluorescent probes[55]. This ability to visualize the dynamics of nuclear proteins in real time has been used in live mouse embryonic stem cells to determine the transcription repression activity of PRC1 (ref. 56), a repressive complex that mediates gene silencing through the deposition of repressive H3K27me3.

Another major technology with potential for epigenomic profiling is proteomics (Table 1). Proteomics-based methods offer functional insights on the role of molecules involved in chromatin organization. Pull-down assays that involve bait proteins to purify targets of interest, followed by mass spectrometry or immunoblotting, have been adapted to study these molecules in complex. Co-immunoprecipitation[57], ChIP[58] and RNA immunoprecipitation[59] exploit antibodies to pull down a target protein and molecules in complex with the target and allow the

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Table 1 | Sequencing, microscopy and proteomics technologies for epigenomic profiling

Common next-generation sequencing platforms	Technology	Application	Detection method	Read length	Accuracy	Ref.
	Illumina sequencing	Short-read sequencing	Bridge amplification and sequencing by synthesis	50–300 bp	>99.9%	33
	Oxford Nanopore Technologies nanopore sequencing	Long-read sequencing	Single-molecule nanopore detection	20 bp to >4 Mb	87–98%	33
	PacBio HiFi sequencing	Long-read sequencing	Single-molecule real-time sequencing using zero-mode waveguide	500 bp to 20 kb	>99%	33
Spatial next-generation sequencing	Technique	Target	Resolution (μm) /tissue thickness (μm)	Capture efficiency	Sequencing area	Ref.
	10x Visium	Poly(A) capture and antibody-derived proteins	55/10	15,377 UMIs/55 μm^2	6.5 mm×6.5 mm	34
	Spatial ATAC-RNA-seq	Poly(A) RNA capture (deterministic barcoding) and transposed chromatin	50/10	~12,000 UMIs, ~4,000 genes/50 μm^2	16 mm×16 mm	31,35
	Slide-TCR-seq	Poly(A) RNA capture and TCR gene sequences	10/10	59 UMIs/10 μm^2	3-mm diameter	32
	Stereo-seq	Poly(A) RNA capture	0.22/10	1,450 UMIs/10 μm^2	132 mm×132 mm	36,37
Chromatin organization microscopy	Method	Spatial resolution (lateral/axial; nm)	Temporal resolution	Imaging depth (micrometres)	Used in immune cells or tissues	Ref.
	STED	90–110/90–110	Seconds to minutes	<20	Yes	136
	STORM	20–50/40–100	Seconds to minutes	1–10	Yes	42
	ExM	25–70/70–200	Seconds to minutes	200	Yes	47
	SCEPTRE	~75	Seconds to minutes	Unreported	No	48
	ChromExM	~3–15	Seconds to minutes	Unreported	No	49
	OligoSTORM	<20	Seconds to minutes	1–10	No	50
	ORCA	Diffraction limited (genomic–2 kb)	Minutes to days	1–10	No	51
	MERFISH	Diffraction limited	Seconds to minutes	1–10	Yes	52
	seq-FISH+	Diffraction limited	Seconds to minutes	≤5	No	53
Proteomics	Technique	Target	Detection	Method	Readout	Ref.
	Co-immunoprecipitation	Protein	Proteins that interact with the primary target	Antibody-based purification of target protein and interacting proteins	SDS–PAGE Immunoblotting Mass spectrometry	57
	ChIP	DNA-binding proteins	DNA regions that target protein binds	Antibody-based purification of DNA-binding proteins crosslinked to DNA targets	PCR or sequencing	58
	RNA immunoprecipitation	RNA-binding proteins	RNA that interacts with target protein	Antibody-based purification of RNA-binding proteins and bound targets	RT–PCR or RNA-seq	59
	Top-down mass spectrometry	Intact whole histone proteins	Combinatorial histone modifications	Mass spectrometry analysis of intact histone proteins	Mass spectrometry	61
	Middle-down mass spectrometry	Intact histone tails	Combinatorial histone modifications	Mass spectrometry analysis of partially digested histone tails	Mass spectrometry	61

UMI, unique molecular identifier.

study of interactions between a target protein with other proteins, DNA or RNA. RNA pull-down assays have been used to study RNA–protein and RNA–DNA interactions through targeting RNA. These pull-down assays use biotinylated antisense probes and leverage hybridization to bind and purify RNA along with other molecules in complex. While yet to be applied widely in T cells, another major utility of proteomics in epigenetic research is the interrogation of histone modifications through mass spectrometry[60]. Top-down and middle-down mass spectrometry, which analyze intact and large peptides, respectively, offer the advantage of identifying combinatorial histone modifications[60,61]. Mass spectrometry methods can also be used to study transcription factor structure and dynamics. In $CD4^+$ T cells, mass spectrometry has shown that the transcription factors that govern naive T cell quiescence have high turnover, enabling their quick degradation when naive T cells get activated[62]. Together, cutting-edge techniques in genomics, microscopy and proteomics can uncover new epigenomic regulation in T cells.

Epigenetics of T cell development

T cell development is a tightly regulated process driven by sequential changes in transcriptional programs and chromatin architecture. As multipotent hematopoietic progenitors migrate from the bone marrow to the thymus, Notch signaling and other extracellular cues initiate a cascade of epigenetic and transcriptional events that guide lineage commitment. The early stages comprising early thymic progenitors (ETPs), CD4 and CD8 double-negative stages DN2a, DN2b, DN3 and DN4, are marked by dynamic chromatin remodeling, which enables the activation of T cell-specific gene programs while progressively restricting alternative lineage potential (Fig. 2a). The transition from the double-negative to the CD4 and CD8 double-positive stage represents a critical juncture, where global shifts in chromatin accessibility and histone modifications reinforce commitment to the T cell fate[63,64]. CD4 or CD8 single-positive thymocytes emerge with lineage-defining transcriptional profiles, ensuring the establishment of mature $CD4^+$ and $CD8^+$ T cells. These developmental transitions are actively orchestrated by transcription factors in coordination with chromatin remodelers and higher-order genome organization. Below, we highlight key insights into the epigenetic mechanisms that shape T cell development.

A combinatorial action of transcription factors establishes T cell fate in the thymus[65]. Bone marrow progenitors entering the thymus feature a gene expression program controlled by transcription factors inherited from hematopoietic progenitors, such as BCL11A, PU.1 (also known as SPI1) and LYL1 (ref. 66), which remain expressed in ETPs and support early growth and expansion of T cells before gradually being repressed during T cell lineage commitment. Bone marrow progenitors that enter the thymus are also exposed to Notch ligands expressed by specialized thymic epithelial cells, leading to the activation of Notch signaling and the generation of ETPs[67]. Notch signaling in the thymus triggers a developmental cascade that locks progenitors on to the T cell lineage, while also restricting alternative lineages. E protein transcription factors such as E2A and HEB, which are already active in pre-thymic bone marrow progenitors, increase the expression of *Notch1* and *Notch3* in ETPs, with their deletion leading to impaired Notch signaling and early arrest of T cell development[68]. Control of E proteins is regulated by the Id family of transcription factors, which bind to E proteins to form E protein–Id dimers that cannot bind DNA (Fig. 2b). E proteins, Id2 and Id3 form a regulatory axis that tunes the induction of Notch feedback loops throughout thymic development. For instance, Id2 expression is low whereas E2A expression is high, leading to strong Notch signaling through induction of *Notch1* at the ETP stage, while Id3 expression is upregulated to repress *Notch1* and abrogate Notch signaling in DN3s, which undergo β-selection[68,69].

Studies modulating transcription factors via genetic deletion or overexpression have largely shaped our understanding of T cell development. Advances in epigenetic technologies are now elucidating the detailed molecular mechanisms of how transcription factors shape the chromatin, hence the identity, of T cells in the thymus. Access to T cell gene programs in progenitors entering the thymus is limited, as chromatin regions that are open in mature $CD4^+$ and $CD8^+$ are initially inaccessible, needing to be established throughout development[20,70]. Measuring chromatin accessibility by ATAC-seq at all stages of T cell development revealed three large waves of chromatin remodeling at the ETP, DN2b and single-positive stages[20]. Searching for transcription factor footprints within these genomic regions led to the discovery that TCF-1 creates the accessible chromatin landscape during T cell development. TCF-1 (encoded by *Tcf7*) is the earliest T cell-specific transcription factor to be induced by Notch signaling in ETPs and is persistently expressed throughout T cell maturation. TCF-1 binds to its target regions in silent chromatin using its disordered effector domain[71] and creates regions of accessibility[20,70]. At a subset of TCF-1-bound de novo open chromatin sites, such as *Ccr7* and *Rorc*, loss of repressive H3K9me3 and H3K27me3, along with gain of permissive H3K27ac, suggests that TCF-1 can reshape the chromatin landscape by modifying histone marks[20]. The mechanism behind these functions remains unclear. However, the ability of TCF-1 to bind H3K9me3 and H3K27me3 heterochromatin stands out as an exceptional feature[72]. Importantly, the expression of TCF-1 leads to the induction of other transcription factors essential for T cell development, such as GATA3 and BCL11B. Many other transcription factors, such as RUNX1 (ref. 73) and ETS1 (refs. 74,75) cooperate with TCF-1, BCL11B and GATA3 at different stages of T cell development. Some of these transcription factors, such as BCL11B, which are necessary for T cell lineage commitment, interact with major chromatin complexes including SWI/SNF, NuRD and PRC1 (refs. 63,76). The biology of BCL11B underscores the synergy between transcription factors and chromatin remodelers in guiding genome reorganization.

An integral event in shaping the identity of T cells comes during the DN2-to-DN3 transition, a stage at which T cells become locked into the T cell lineage[64]. Epigenetic analysis of the DN2-to-DN3 transition showed that this commitment is characterized by abrupt global changes in chromatin accessibility, flipping of chromatin compartments and 3D genome reorganization, suggesting an epigenetic shift that locks T cells into their fate[64]. These shifts tend to repress alternative lineage or multipotency programs, with loci harboring genes such as *Hmga2*, *Meis1*, *Mef2c*, *Lmo2* and *Bcl11a* having a decrease of long-range chromatin interactions, accompanied by decreased gene expression[64]. Conversely, a subset of genes important for T cell development, such as *Bcl11b*, *Ets1* and *Tcf7*, show an increase in chromatin interactions accompanied by increased gene expression[64]. The mechanisms that underlie this shift have yet to be fully determined, but repression of PU.1 and increased activity of BCL11B are key. PU.1 is active in alternative lineage pathways and facilitates multipotency in pre-commitment T cells. Similar to TCF-1, PU.1 is a pioneer factor and its binding sites show local recruitment of histone methyltransferases and it is linked to H3K4me2 modifications[63]. Silencing of PU.1 during the DN2-to-DN3 transition is concordant with closing of many of its binding sites, along with the downregulation of other transcription factors specific for progenitors, such as *Cd34* and *Bcl11a*[77], suggesting the role of PU.1 in maintaining accessibility for pre-commitment expression programs. PU.1 repression also allows redistribution of RUNX1 and SATB1 at PU.1-dependent genes, such as *Il31ra*, *Cd44* and *Cd48*, which contributes to chromatin reconfiguration, along with a shift in expression programs[78]. Conversely, *Bcl11b* becomes highly expressed during the DN2-to-DN3 transition and drives epigenetic changes by recruiting remodelers, such as SWI/SNF, to genomic sites. BCL11B also seems to facilitate 3D chromatin organization, with analysis of sites with commitment gain of loops and interactions showing enrichment of BCL11B binding[64] (Fig. 2c). Silencing of *Bcl11b* in naive $CD4^+$ T cells shows reduction of chromatin interactions near these sites[64]. TCF-1 has also been linked to deposition of H3K27ac modification in DN3 T cells, leading to facilitation of long-range interactions in previously insulated neighborhoods[79]. These studies suggest mechanisms by which transcription factors control 3D genome organization in T cell development through modification of the chromatin.

The assembly of TCR genes through V(D)J recombination defines T cell identity during development. Recombination is mediated by expression of RAG1 and RAG2, which act on target recombination signal sequences to cleave double-stranded DNA at TCR gene loci. *RAG* gene expression is tightly regulated through epigenetic mechanisms. A T cell lineage-specific enhancer, named Rag-T cell enhancer, was described as necessary for induction of *RAG* gene expression during development[80]. Rag-T cell enhancer acts as a binding site for the E protein E2A, which activates enhancers to induce *RAG* gene expression[80]. E2A activity also orchestrates 3D chromatin rearrangement at the *RAG* loci, by compacting and bringing enhancer elements closer to the *RAG* gene bodies to further drive expression[80]. In addition to regulation of expression, the functions of RAG proteins are also subject to epigenetic controls.

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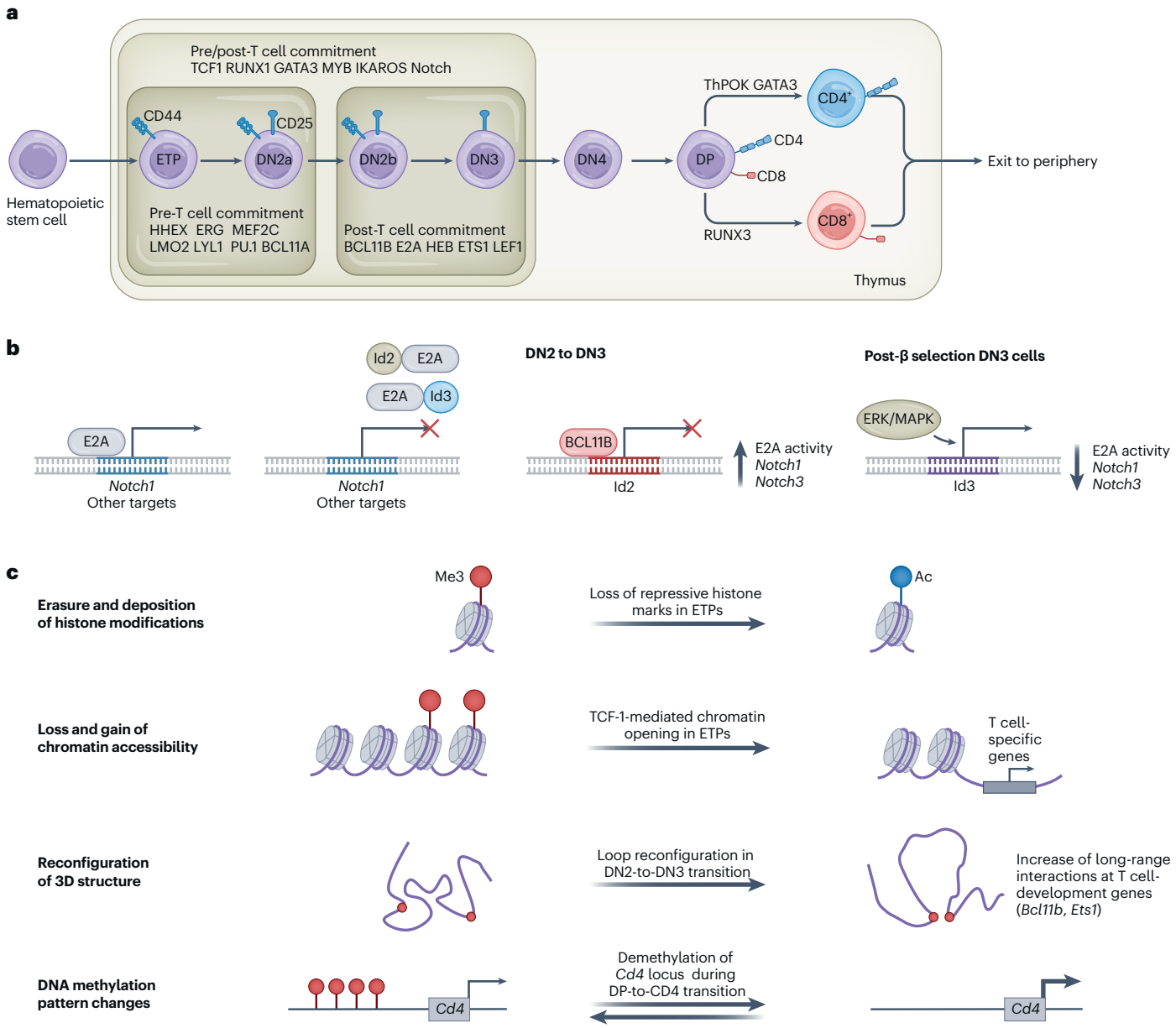

Fig. 2 | Epigenetic mechanisms in thymic T cell development. a, Transcription factors expressed at the pre-T cell commitment stage, which encompasses the CD8 and CD4 double-negative DN1 and DN2a stages, such as HHEX, EEG, MEF2C, LMO2, LYL1, PU.1 and BCL11A, are inherited from bone marrow progenitors. Transcription factors expressed at the post-T cell commitment stage (DN2a and DN3), are either upregulated during T cell commitment (BCL11B, ETS1 and LEF1) or active throughout, but become more upregulated during commitment checkpoint (E2A and HEB). Transcription factors such as TCF-1, RUNX1, GATA3, MYB family factors, IKAROS family factors and Notch signaling are active from the double-negative DN1 to double-positive (DP) stages, to facilitate transitions. **b**, The interplay between the E proteins E2A and the Id proteins Id2 and Id3, which dimerize with E2A to inhibit its DNA-binding activity, determine the expression of E2A target genes such as *Notch1*. At the double-negative DN2 to DN3a stages, BCL11B-mediated repression of Id2 increases E2A activity and expression of *Notch1* and *Notch3*, whereas in post-β-selection DN3b cells, ERK/MAPK-mediated induction of Id3 has the opposite effect. **c**, Epigenetic events control T cell differentiation. Histone modifications can be repressive (Me3) or permissive (Ac) to gene expression. Gain of chromatin accessibility mediated by transcription factors such as TCF-1 in ETPs enables increased expression of genes at opened loci. Reconfiguration of chromatin 3D structure to increase long range interactions at T cell-specific genes during T cell commitment leads to upregulation of important transcription factors such as BCL11B and ETS1. DNA methylation pattern changes such as demethylation at the *Cd4* locus during the transition from double positive (DP) to CD4 single positive (SP) lead to increased *Cd4* expression.

The selection of recombination signal sequences for cleavage by RAG1 or RAG2 is modulated by the accessibility of TCR loci.

Beyond histone modifications, DNA methylation and the activity of DNMT enzymes also regulate T cell development. This occurs particularly during the selection of CD4 single-positive or CD8 single-positive thymocytes from double-positive thymocytes, which occurs through the selective expression of transcription factors such as ThPOK (for $CD4^+$ T cells)[81,82] or RUNX3 (for $CD8^+$ T cells)[83]. CD4 and CD8 single-positive cells have 5-hydroxymethylcytosine DNA modifications in the gene bodies of actively transcribed genes, including specific enrichment at loci of lineage-determining factors, such as *Zbtb7b* (encoding ThPOK), *Gata3* and *Runx3* (ref. 84). Disruption of DNA methyltransferase 1 (DNMT1), using either short hairpin RNA or genetic models, leads to loss of RUNX1-mediated or RUNX3-mediated

silencing of CD4 during CD8 commitment, marked by loss of DNA methylation at the *Cd4* locus[85]. Analysis of DN3 and double-positive stages shows that repressive 5mC hypermethylation of the *Cd4* locus is already present in DN3 T cells[10], indicating stage-specific mechanisms in which the methylation of the *Cd4* locus is sustained during the transition from the double-positive to CD8 single-positive stage, and is selectively demethylated during the transition from the double-positive to CD4 single-positive stage.

A growing body of work indicates that the chromatin rearrangement that occurs early in T cell development primes the function of peripheral T cells. Comparison of chromatin landscapes during T cell development and activated peripheral T cells showed that a large component of the active enhancer landscape in effector T cells is poised early during T cell development through a highly coordinated interaction between chromatin remodelers from the SWI/SNF family in addition to RUNX1, PU.1 and BCL11B[86]. This observation fits with an emerging view that transcription factors can alter chromatin configuration before mediating transcriptional control. Given that transcription factors such as TCF-1 (ref. 79) and BCL11B[64] facilitate loop formation during development, it will be interesting to assess what degree of the essential 3D genome landscape specific for peripheral T cells is encoded in early thymocytes.

Epigenetics of CD4$^+$ helper T cells

After maturation, CD4$^+$ T cells leave the thymus and migrate to the periphery, entering the naive T cell compartment. Once naive CD4$^+$ T cells encounter their cognate antigens, they undergo clonal expansion and, depending on a series of key environmental signals from the TCR, co-stimulatory proteins and cytokines, they can differentiate into distinct helper T lineages, including the T helper 1 (T_H1), T_H2 and T_H17 subsets of helper T cells, follicular helper T cells and induced regulatory T cells (Fig. 3a). Genome-wide profiling of H3K4me1 and acetylated chromatin identified more than 20,000 genomic regions that gain accessibility at enhancer sites after CD4$^+$ T cell activation[87]. A relatively large number of enhancers become acetylated and some, such as the *Ifng* and *Il4-Il13* enhancers, gain accessibility after CD4$^+$ T cell activation[87]. STAT transcription factors change the enhancer landscape of CD4$^+$ helper T cells[87] in part by recruiting the acetyltransferase p300 (ref. 88). For example, STAT5 and STAT6 binding deposits the permissive histone marks H3K9ac and H3K4me3 at the promoters of the *Il4*, *Il5* and *Il13* genes, required for their transcription[89]. Long-range chromatin interactions, spanning approximately 50 kb to 1 Mb, also increase during CD4$^+$ helper T cell differentiation, while short-range 1-kb to 10-kb interactions decrease[90]. The chromatin interaction profile of the *Il12rb2* gene showed that loops newly established at late-stage (72 h) CD4$^+$ T cell polarization were specific to CD4$^+$ helper T cell differentiation and facilitated long-distance interactions between putative enhancers and promoters[90]. Comparison of naive-versus-activated CD4$^+$ T cells also indicates loss of active enhancers meticulously established during T cell development[91]. Examination of transcription factor-binding sites enriched within regions that lose accessibility upon CD4$^+$ T cell activation and the reduction of TCF-1 expression in activated CD4$^+$ T cells suggest TCF-1 is required to maintain accessible chromatin at these regions. Although the analysis of naturally occurring polymorphisms in transcription factor-binding motifs in F_1 hybrid C57BL/6-Cast mice indicated TCF-1 accessible sites were lost during CD4$^+$ T cell activation[91], the direct role of TCF-1 in maintaining the accessibility of genes that lose expression during CD4$^+$ T cell activation remains to be elucidated. In addition, a subset of genomic regions selectively accessible in naive CD4$^+$ T cells that lose accessibility after T cell activation regain accessibility in CD4$^+$ memory T cells[91]. The chromatin landscape of memory CD4$^+$ T cells shares similarities with that of effector CD4$^+$ T cells, including chromatin accessibility at enhancers of many cytokine-encoding genes, despite the lack of active transcription in memory CD4$^+$ T cells[92,93]. These observations suggest the importance of poised chromatin for memory responses. Although it has been suggested that for some cytokines, pre-transcribed cytokine-encoding mRNA is stored in memory T cells and a translational block leads to low cytokine expression in memory T cells[94], the latest bulk and single-cell RNA-seq profiling in T cells suggests limited transcription for genes encoding cytokines in memory T cells[23,95].

Epigenetics of CD8$^+$ T cells

CD8$^+$ T cell responses are critical for controlling a wide range of infections and mediating cancer immunity. Upon antigen challenge and in the presence of a specific cytokine milieu, naive CD8$^+$ T cells differentiate into effector CD8$^+$ T (T_{eff}) cells, which control infection through the production of cytotoxic molecules. Once the primary challenge has been cleared, the CD8$^+$ T_{eff} cell population contracts, while a subset of antigen-specific CD8$^+$ memory T (T_M) cells persists in lymph nodes and other peripheral tissues such as the skin, gut and lungs as effector memory, tissue-resident memory, stem cell memory and central memory CD8$^+$ T cells (Fig. 3b).

Loss of open chromatin regions associated with naive CD8$^+$ T cells after activation, major gain of de novo open chromatin regions in effector CD8$^+$ T cells and similarity between CD8$^+$ T_M cells with both naive and effector CD8$^+$ T cells in regards to open chromatin regions are consistently reported in mice[22,23,96,97]. Besides the gain and loss in chromatin accessibility, other epigenetic mechanisms, such as DNA methylation, lock in the CD8$^+$ T_{eff} cell and CD8$^+$ T_M cell fates. Loss of memory potential in CD8$^+$ T_{eff} cells has been linked with selective epigenetic silencing of pro-memory genes such as *Tcf7* and *Bach2* during CD8$^+$ T_{eff} cell differentiation[98]. Endogenous expression of Id2 in activated CD8$^+$ T cells by TCR signaling inhibits the E2A-mediated expression of memory-associated genes such as *Ccr7* and *Sell* in memory precursor effector cells, leading to loss of memory CD8$^+$ T_M cell differentiation[99]. E2A in turn regulates the chromatin accessibility of enhancers associated with genes related to memory establishment and maintenance, including Id3 (ref. 100). E47, one of the alternatively spliced variants of E2A, maintains accessibility of naive CD8$^+$ T cell enhancers, which allows a fraction of effector CD8$^+$ T cells to develop into memory CD8$^+$ T cells after infection. Expression of Id3 induces the differentiation of long-lived CD62L$^+$CD127$^+$CD8$^+$ T_M cells, while expression of Id2 drives the induction of short-lived CD8$^+$ T_{eff} cells[101–103]. Another transcription factor critical for the establishment of CD8$^+$ T_M cell fate is FOXO1 (ref. 104). The overexpression of FOXO1, but not TCF-1, can increase the antitumor activity of human chimeric antigen receptor T cells[105], indicating that FOXO1 can effectively reprogram CD8$^+$ T_M cells, possibly through inducing increased chromatin accessibility at memory-associated loci such as *Il7r*[105].

Tracking CD8$^+$ T cells at the single-cell level using time-resolved transcriptomics and quantitative live-cell imaging after pathogen clearance has indicated that effector CD8$^+$ T cells may regain a KLRG1$^-$CD127$^+$CD62L$^+$ memory phenotype, even if this potential was initially lost[106]. This flexibility was attributed to a stochastic *cis*-epigenetic switch that silences TCF-1 (ref. 106). Analysis of DNA methylation changes at the *Pdcd1* locus encoding PD-1 in virus-specific CD8$^+$ T cells during acute infection indicated that cells eventually giving rise to CD8$^+$ T_M cells acquired new DNA methylation patterns at the *Pdcd1* locus in naive CD8$^+$ T cells while undergoing demethylation at the same locus in CD8$^+$ T_{eff} cells[107]. One interpretation of these results has been that CD8$^+$ T_M cells arise from CD8$^+$ T_{eff} cells that have undergone unique methylation changes that prime them for long-term survival and functionality. How CD8$^+$ T_M cells arise remains a controversial topic, primarily because different models inspired different interpretations.

In contrast to the default trajectory for CD8$^+$ T cells, extended stimulations of TCR signaling from chronic antigens or tumors can force CD8$^+$ T cells to follow an alternative path, called T cell exhaustion. Epigenomic mappings have been critical in establishing

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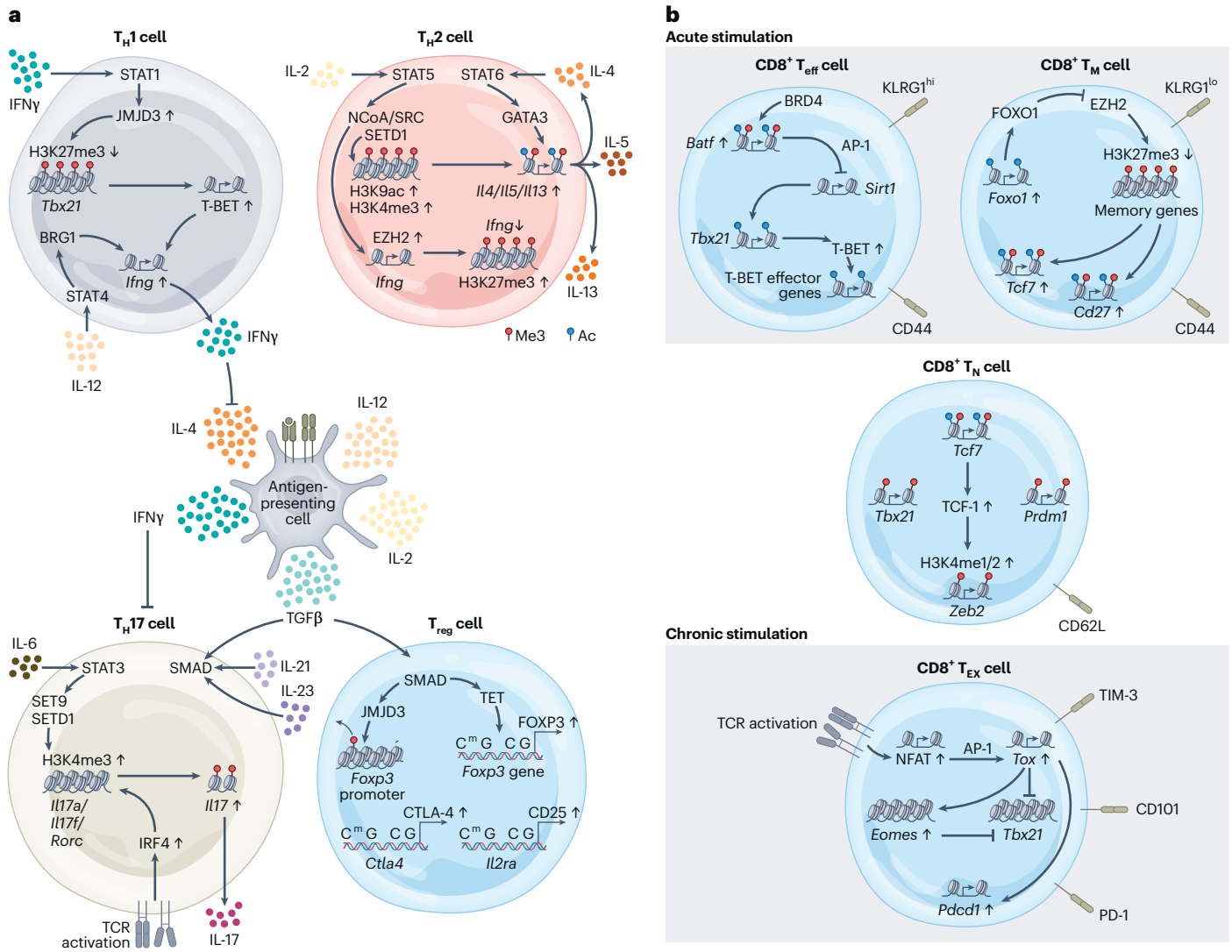

Fig. 3 | Epigenetics of CD4⁺ and CD8⁺ T cell differentiation. a, In the CD4⁺ T cell lineage, naive CD4⁺ T cells differentiate into specialized effector lineages depending on cytokines and antigen exposure. T-BET-expressing type 1 helper T (T_H1) cells first undergo demethylation of the *Tbx21* gene, which encodes T-BET, through the activity of STAT1-induced JMJD3. In conjunction with the activity of STAT4 and BRG1, T-BET binds to the promoter of *Ifng* and upregulates IFNγ. GATA3-expressing T_H2 cells are induced by the cytokine IL-4 and the upregulation of STAT6. T_H1 gene programs are suppressed in T_H2 cells by the activity of EZH2, which increases H3K27me3 repressive marks in the *Ifng* gene, repressing its expression. RORγt-expressing T_H17 cells are triggered by IL-6 expression, which induces STAT3-dependent upregulation of SET9 and SETD1, which deposits permissive H3K4me3 at the *Il17* locus to increase expression and secretion of IL-17. FOXP3-expressing T_{reg} cells are activated by TGFβ, which increases the expression of SMAD, which initiates JMJD3 to demethylate H3K4 in the *Foxp3* promoter and upregulate its expression. SMAD also increases the activity of DNA demethylase TET at the *Ctla4* and *Il2ra* loci. **b**, In the CD8⁺ T cell lineage, naive CD8⁺ T (T_N) cells are defined by TCF-1 activity, which deposits permissive H3K4me1 and H3K4me2 at the *Tbx21*, *Zeb2* and *Prdm1* loci. Upon acute antigen stimulation, CD8⁺ T_N cells differentiate into KLRG1^{hi}CD44⁺CD8⁺ T_{eff} cells driven by BRD4, which reads histone H3 lysine acetylation at the *Batf* locus. BATF upregulation leads to AP-1 activation, which further increases the activity of T-BET and T-BET effector genes to establish a terminal effector state. Following acute stimulation, KLRG^{lo}CD44⁺CD8⁺ T_M cells emerge through the increased activity of FOXO1. FOXO1 inhibits the EZH2-mediated deposition of repressive H3K27me3 marks at memory genes, which increases the expression of genes such as *Tcf7* (TCF-1) and *Cd27*. Under conditions of chronic antigen stimulation, terminally exhausted TIM-3⁺CD101⁺PD-1⁺CD8⁺ T_{EX} cells differentiate due to the TCR activation-mediated upregulation of NFAT, which in turn drives the expression of TOX. TOX expression in conjunction with increased levels of EOMES suppresses *Tbx21*, which encodes T-BET, a hallmark of CD8⁺ T_{eff} cells.

that exhausted T (T_{EX}) cells represent a developmentally distinct program[23,96,108,109]. Considering the importance of exhausted T cells in cancer immunity, multiple reviews are dedicated to the epigenetic landscape of this T cell subset[110]. In terminally exhausted T-BET^{lo}EOMES^{hi}TOX⁺CD8⁺ T cells, a substantial fraction of genes whose active chromatin landscape does not correlate with active transcription was linked to hypoxia in the tumor microenvironment[111], indicating CD8⁺ T_{EX} cells can attain altered functions driven by the environmental factors. Many transcription factors that regulate various subpopulations of CD8⁺ T_{EX} cells, such as progenitor, transitory and terminal T_{EX} cells, have been identified, including the HMG-protein TOX, NFAT and NR4A family proteins[24,109,112–115].

T cell enhancers

Enhancers are crucial *cis*-regulatory elements controlling gene expression through long-range interactions with their target genes in a cell-type-specific and developmental stage-specific manner. Sequence variation within enhancers has been implicated in various T cell-mediated diseases, including allergy, asthma and autoimmune disorders, highlighting their importance in maintaining proper T cell

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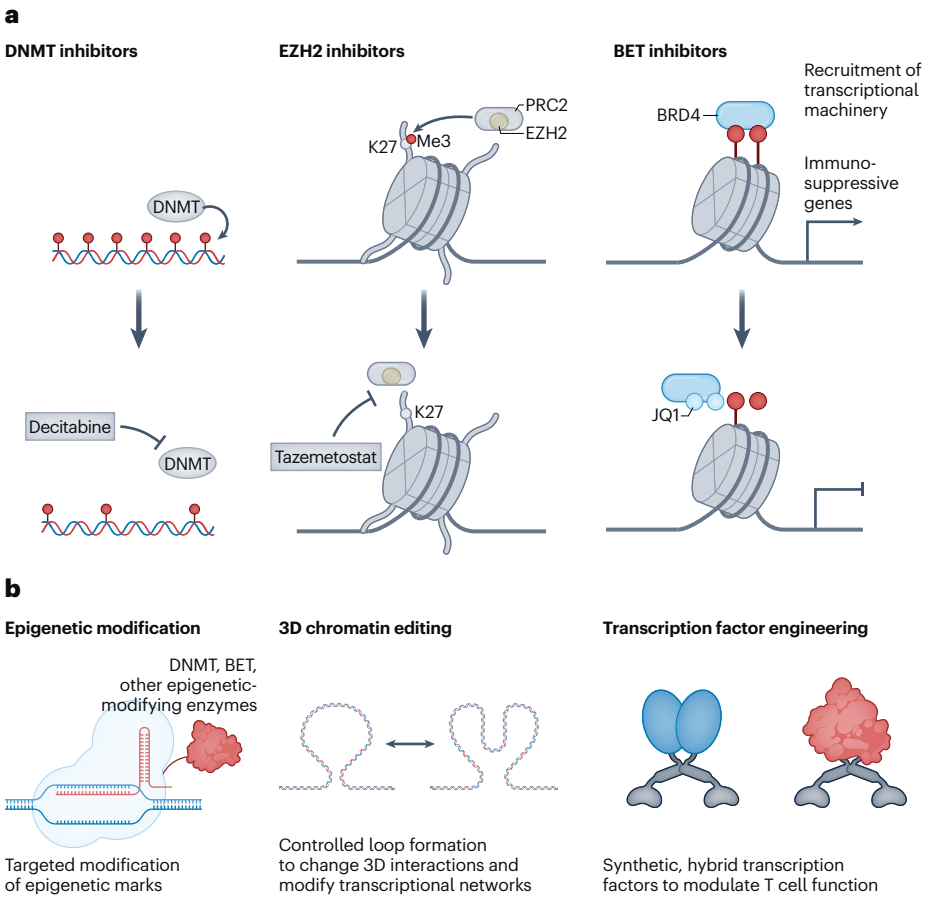

Fig. 4 | Opportunities for epigenetic T cell therapies. a, Current epigenetic drugs aiming to modulate the immune response include inhibitors of DNMTs (decitabine), which can reverse disease-associated DNA methylation patterns, inhibitors of the histone methyltransferase EZH2 (tazemetostat), which can block the deposition of H3K7me3 marks and JQ1, and an inhibitor of bromodomain and extra-terminal domain (BET) family proteins such as BRD4, which can block upregulation of targets related to T cell exhaustion. **b**, Future epigenetic drug development directions include catalytically inactive Cas9 (dCas9), which can be fused to proteins that make epigenetic modifications, such DNMTs, to make targeted epigenetic changes at loci, DNA editing strategies, such as genome editing via CRISPR, to shape 3D chromatin organization, and hybrid proteins engineered by fusing different DNA-binding domains and effector domains, which can lead to the creation of synthetic transcription factors with novel functions.

function. Before advances in epigenomic techniques, genomic locations of enhancers were annotated based on sequence conservation and their distance to the promoter of genes encoding critical cytokines or transcription factors such as *Ifng*, *Il4*, *Il5*, *Foxp3*, *Bcl11b*, *Il17f* and *Il17a* (reviewed in ref. 116). For example, the composition, size and maintenance of T_{reg} cells are controlled by conserved noncoding DNA sequence (CNS) elements at the *Foxp3* locus[117]. Despite advances in epigenomic mapping that have facilitated large-scale profiling of putative enhancers across diverse T cell subsets, demonstrating the necessity of these enhancers for T cell responses has proven difficult. Genetic engineering of enhancers in mice is a standard strategy to evaluate if a regulatory enhancer contributes to T cell development or T cell responses in vivo. Considering that only one element can be deleted at a time, candidate enhancers are typically prioritized and selected for deletion based on various epigenomic and genetic criteria. These include high sequence conservation, strong enhancer activity marked by high levels of H3K27ac modification at super-enhancers[118,119], high level of long-range interactions in 3D forming hyperconnected hubs[120–122] or the enrichment of single-nucleotide polymorphisms associated with complex immune diseases[118]. For example, the evaluation of H3K4me1 at the *Ifng* locus in naive $CD4^+$ T cells identified the CNS-28 silencer, a noncoding element that restricts the transcription of *Ifng* by reducing the enhancer–promoter interactions within the *Ifng* locus mediated by GATA3 binding to CNS-28 (ref. 123). Deletion of CNS-28 results in increased IFNγ production in polarized T_H0 and T_H1 cells from CNS-28-deficient mice compared to wild-type mice, which leads to hyperactivation of immune responses and chronic inflammation. Mapping of enhancer interactions using H3K27ac HiChIP in thymocytes identified multi-enhancer connectivity in the *Ets1-Fli1* locus, which harbours *Ets1*, a transcription factor required for $CD4^+$ helper T cell differentiation[120]. In addition to multi-enhancer connectivity at the *Ets1-Fli1* locus, a 25-kb long noncoding RNA annotated as *Gm27162* served as a major anchor for enhancer connectivity to the *Ets1* promoter (hence named '*Ets1-SE*') and was enriched for single-nucleotide polymorphisms associated with type 2 immune diseases, such as allergy. Ablation of the *Ets1-SE* resulted in loss of multi-enhancer interactions between the *Ets1* promoter and other enhancers with a reduction in the expression of *Ets1* in naive $CD4^+$ T cells, which also impaired the differentiation of T_H1 cells in vitro and in vivo[124]. *Ets1-SE*$^{-/-}$ mice had an overt response to a house dust mite challenge relative to wild-type mice due to compromised T_H1 cell differentiation failing to dampen allergic inflammation[124]. This work suggests the importance of long-range enhancer–promoter interactions in the control of gene dosage required for $CD4^+$ helper T cell differentiation[121]. We believe that the unbiased analysis of 3D genome and comparisons of genome organization between human and mice[125] have the potential to reveal mechanisms through which sequence variation within enhancers of T cells change transcriptional control, causing autoimmunity or allergic inflammation.

Epigenetic memory and immune responses

One of the most compelling yet underexplored dimensions of T cell biology is the role of epigenetic memory in shaping immunological responses. Epigenetic memory refers to the stable maintenance and inheritance of epigenetic marks that underlie gene expression patterns through cell divisions[126]. In T cells, epigenetic memory can be triggered by environmental signals, such as prior cytokine exposure, leaving stable epigenetic marks independently of antigen recognition through TCR signaling, similarly to the mechanisms that induce trained immunity in innate cells[127]. These epigenetic marks have the potential to affect immune responses. During naive $CD4^+$ and $CD8^+$ T cell activation, the transcription factors NFAT and AP-1 are induced downstream of TCR signaling to generate thousands of new accessible sites in chromatin stably occupied by ETS1 and RUNX1 (ref. 92). These open chromatin sites are maintained after T cell activation and after T cell replication, and act as inducible enhancers for rapid reactivation of different classes of T cell response genes such as cytokines (*Il1*, *Il2*) and chemokines (*Cxcl3*, *Ccl2)* in stimulated $CD4^+ T_M$ cells[92]. Genes encoding cytokines are strongly involved in this epigenetic priming, with a subset of open chromatin sites initially induced by the IL-2-triggered recruitment of AP-1 and STAT5 and later maintained by other cytokines, such as IL-7 (ref. 93). A subset of naive $CD4^+$ T cells that experienced increased exposure to IL-4 driven by helminth infection in vivo had dampened immune responses to challenge with specific antigens in mice[128]. While the dampening of the antigen-induced response was transient, with a recovery to uninfected baseline levels in 3 weeks after infection, and the epigenetic changes were not profiled[128], it is possible that cytokine exposure elicited a dynamic change at IL-4-responsive genes. These observations suggest that T cells might have an inherent epigenetic plasticity, which can be modulated by environmental cues, such as cytokines, tuning their overall immune response. Determining to what degree epigenetic memory can be modified in T cells holds profound implications for optimizing immune memory or reversing autoimmunity.

Future perspectives

Insights into the epigenetic regulation of T cells have created opportunities for novel intervention strategies. Epigenetic-associated therapeutics are tested to improve T cell function, often in the context of combating T cell exhaustion. These drugs are typically inhibitors that target enzymes involved in DNA or histone modifications or other transcription regulators. In $CD8^+$ T cells, DNMT and EZH2 inhibitors have been used to improve antitumor immunity by blocking the deposition of epigenetic marks at memory and proliferation-associated genes such as *Tcf7*, *Bcl6*, *Lef1* and *Il7r*[129–131]. A bromodomain and extra-terminal domain inhibitor, JQ1, which represses histone acetylation reader proteins, can combat $CD8^+$ T cell exhaustion by suppressing the expression of exhaustion-related genes such as those encoding PD-1 and TIM-3 (ref. 132; Fig. 4a). While promising, given the global effects of these inhibitors, understanding the specific context of their use is important to weigh in their functional outcome. Beyond global inhibition, a tempting future therapeutic direction lies in the targeted use of the epigenetic machinery. DNA methylation can be edited in a targeted manner using methods that fuse the catalytically inactive dCas9 to TET1 or other enzymes that act as epigenetic regulators[133,134]. An example is the creation of stable repressive DNA hypermethylation at the *CDKN2B* (p15) promoter that can be inherited throughout immune development from $CD34^+$ progenitor cells to $CD33^+$ myeloid cells and $CD19^+$ lymphoid cells[133,134]. Another example is the engineering of transcription factors that can finely control specific transcriptional networks and customize T cell responses. An example includes synthetic *Notch* receptors that express the DNA-binding domain from the transcription factor GAL4 (ref. 135) at the Notch intracellular domain, which enables new cell–cell contact signaling pathways (Fig. 4b). As we continue to unravel the complexities of the T cell epigenome, we anticipate major advances in our understanding of immune regulation, leading to not only more effective immunotherapies but also potential breakthroughs in the treatment of autoimmune disorders.

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