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Epigenetic regulation of T cell exhaustion

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Abstract

Chronic antigen stimulation during viral infections and cancer can lead to T cell exhaustion, which is characterized by reduced effector function and proliferation, and the expression of inhibitory immune checkpoint receptors. Recent studies have demonstrated that T cell exhaustion results in wholescale epigenetic remodeling that confers phenotypic stability to these cells and prevents T cell reinvigoration by checkpoint blockade. Here, we review foundational technologies to profile the epigenome at multiple scales, including mapping the locations of transcription factors and histone modifications, DNA methylation and three-dimensional genome conformation. We discuss how these technologies have elucidated the development and epigenetic regulation of exhausted T cells and functional implications across viral infection, cancer, autoimmunity and engineered T cell therapies. Finally, we cover emerging multi-omic and genome engineering technologies, current and upcoming opportunities to apply these to T cell exhaustion, and therapeutic opportunities for T cell engineering in the clinic.

T cells are a central component of the adaptive immune system and continuously survey the body for the presence of pathogens[1]. Each T cell is equipped with a somatically recombined T cell antigen receptor (TCR). Upon TCR recognition of foreign antigens presented on major histocompatibility complex (MHC) molecules, T cells are activated, clonally expand and kill infected cells using a variety of effector molecules. After clearance of infected cells, effector T cells differentiate into memory cells that persist long term and enable the

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rapid clearance of subsequent reinfections with the same pathogen. In contrast, in settings of chronic infection and cancer, foreign antigens often cannot be easily eliminated, and thus, T cells are chronically stimulated and can adopt a so-called exhausted cell state[2]. Functionally, exhaustion is characterized by low effector cytokine secretion, poor proliferative capacity and persistence, and the expression of inhibitory receptors on the cell surface, all of which can reduce the effectiveness of T cell-mediated immunity[3]. Although T cell exhaustion was initially described in the setting of chronic viral infections, it is now well appreciated that exhausted T cells (T_{EX}) are present in many diseases, including in cancer, and that the gene regulatory programs governing exhaustion are largely conserved across disease settings[4,5]. Importantly, studies have suggested that T cell exhaustion limits the efficacy of immunotherapies, including checkpoint blockade and engineered cell therapies, and therefore, understanding the cellular and molecular regulation of T_{EX} cells has become a central focus of the field[6–9].

In this Review, we provide an in-depth discussion of the molecular regulation of T cell exhaustion with an emphasis on epigenetic mechanisms. We begin by discussing recent technologies that have enabled mapping epigenetic modifications at multiple scales in primary T_{EX} cells, including histone modifications, transcription factors (TFs), chromatin accessibility, DNA methylation and genome conformation. We then describe how these methods have been leveraged to understand the epigenetic hallmarks of T cell exhaustion that have defined T_{EX} cells as a distinct cell state, distinct cellular differentiation trajectories and regulatory pathways underlying the T_{EX} cell fate, and lineage stability of T_{EX} cells after antigen clearance or checkpoint blockade. Finally, we highlight emerging genome engineering and multi-omic technologies that may provide the next wave of insights into T_{EX} cell programming and enable the development of new therapeutic strategies for cancer.

Technologies to interrogate epigenetic regulation

In mammalian cells, epigenetic mechanisms enable cells to differentiate, adapt to changes in the environment and propagate their cellular state after cell division[10]. Each chromosome is organized into megabase-sized topologically associated domains (TADs), which are largely conserved from early stem cells to differentiated cell types. Within this invariant genome scaffold, cell-type-specific and cell-state-specific gene regulatory DNA interactions establish specific gene expression programs, such as T cell exhaustion. This bridge from identical genotype (DNA sequence) to disparate phenotypes (gene expression) is broadly termed ‘epigenetic regulation’ and is coordinated by a complex interplay of *cis*-acting DNA elements (enhancers) and *trans*-acting TFs. The development and application of genome-scale technologies to systematically map features of the epigenome has uncovered principles of T_{EX} cell genomic organization at multiple scales, including histone modifications and TFs, chromatin accessibility, DNA modifications such as DNA methylation and three-dimensional (3D) folding of the genome.

Fundamental DNA elements required for efficient gene transcription are the promoter, transcription start site (TSS) and distant gene regulatory enhancers[11] (Fig. 1a). The promoter is upstream of the gene TSS and provides a platform for the assembly of the general transcription factor (GTF) complex, which is necessary for RNA polymerase II (RNAPII)

recruitment and gene transcription[12]. Enhancers are non-protein-coding DNA elements that may be far away from the gene in the linear genome, but can be bound by cell-type-specific TFs and brought into spatial proximity of the gene TSS by genome conformational changes to activate gene transcription with RNAPII[13]. Dense clusters of enhancers, termed 'super-enhancers,' are defined by high occupancy of the Mediator complex and master TFs[14–16]. Super-enhancers preferentially regulate key lineage-determining TFs and signaling molecules, such as cytokines and cytokine receptors in T cells[16,17].

Central to the spatial organization of the genome is chromatin, the complex of DNA and proteins that structures the genome and packages it into the nucleus[18]. To fit nearly 2 meters of DNA into the ~10-μm nucleus of each single cell, DNA is first packaged into nucleosomes, which consist of ~147 base pairs of DNA wrapped around a histone octamer, and then into larger and larger chromatin fibers that form chromosomes[19]. Chemical modifications of chromatin regulate gene transcription[20]. Post-translational modifications (PTMs) of histone tails can either repress or activate gene transcription (Fig. 1a). For example, lysine acetylation of histone tails, such as acetylated histone H3 Lys27 (H3K27ac) and acetylated histone H3 Lys9 (H3K9ac), is associated with active promoters and enhancers and is deposited by histone acetyltransferases[10]. Lysine acetylation activates transcription via recognition by bromodomain-containing proteins (for example, BRD4), which in turn facilitate transcriptional elongation by interacting with the positive transcription elongation factor complex that phosphorylates the C-terminal domain of RNAPII[21,22]. Conversely, histone deacetylases remove this modification and thereby repress gene expression. Similarly, lysine methylation can represent either repressed (for example, H3K9me3 and H3K27me3) or active (for example, trimethylated histone H3 Lys4 (H3K4me3)) chromatin states and is deposited by histone methyltransferases, such as EZH2, which deposits H3K27me3. Histone methylation can be reversed by histone lysine demethylases (Fig. 1a). Finally, bivalent gene loci are those that are simultaneously marked by activating (for example, H3K4me3) and repressive (for example, H3K27me3) modifications, which signify a 'poised' chromatin state that enables rapid control of gene expression[23]. For example, in naïve $CD8^+$ T cells, gene loci encoding TFs important for differentiation, including the *Tbx21*, *Irf4*, *Gata3* and *Eomes* loci, are maintained in a bivalent state that enables rapid cell fate commitment upon TCR signaling[24].

The location of specific histone modifications and TFs can be mapped genome wide with methods that link antibody-mediated immunoprecipitation or immunocleavage of proteins or PTMs with high-throughput sequencing. One technology developed for this purpose is chromatin immunoprecipitation with sequencing (ChIP–seq), where genomic DNA is crosslinked to associated proteins or PTMs and then fragmented and immunoprecipitated. Sequencing of the DNA pulled down with the target of interest reveals the genome-wide localization of the given protein or histone modification[25–28] (Fig. 1b). Recent adaptations of this method use antibody-tethered micrococcal nuclease (MNase) or Tn5 complexes to cut chromatin at sites proximal to a bound protein, termed chromatin immunocleavage (ChIC)[29]. These cleaved DNA fragments then diffuse out of the nucleus and can be isolated and sequenced. When MNase is used, the method is known as CUT&RUN (or ChIC-seq), and when Tn5 is used, the method is known as CUT&TAG (or ACT-seq)[30–33]. Each of these methods obtain similar information to ChIP–seq without the requirement for cellular

fixation. Further, they often reduce the required input cell number, enabling profiling of primary cell types[34–36] and even single cells, as recently demonstrated with single-cell CUT&TAG and single-cell ChIC-seq[30–33,37,38]. It is important to note that inefficiencies in immunocleavage and signal dropout inherent to sequencing technologies can limit the insights gained from single-cell adaptations of these methods, for example, specific TF-binding sites in each cell, and therefore, aggregation of signals from many single-cell profiles may be required[39].

A fundamental measurement of chromatin structure is accessibility; namely, is a regulatory DNA sequence open (and active) or closed (and inactive)? Inactive genes and regulatory elements are generally organized into densely packed chromatin fibers, called heterochromatin, whereas transcribed genes and active regulatory elements typically remain in loosely packaged euchromatin. Furthermore, some stretches of DNA within euchromatin are depleted of nucleosomes and can directly interact with TFs or other transcriptional machinery to control gene expression. The outcome of this genome structure is that only a small fraction of the genome—approximately 1–2%—is accessible at any given time in a cell, and the identification of these open sites can be used to nominate sites that may regulate gene expression[40].

The location of accessible DNA genome wide can be profiled with technologies such as DNase-seq, MNase-seq and assay for transposase-accessible chromatin with sequencing (ATAC–seq)[41–44]. The common principle underlying each method is the enzymatic cleavage of chromatin, either by DNase-I, MNase or Tn5. These enzymes preferentially cut open chromatin, and therefore, sequencing the resulting DNA fragments identifies genomic locations that are accessible. *Cis*-regulatory sites can be identified by enrichments of reads (for example, representing DNase-I hypersensitive sites in DNase-seq) at specific regions of the genome. The activity of *trans*-factors can be inferred through the identification TF ‘footprints’ within these sites—DNA sequences that are protected from enzymatic cleavage via direct binding of a TF. Thus, the locations of thousands of potential *cis*- and *trans*-elements can be read out genome wide using a single assay. For example, ATAC–seq utilizes a hyperactive prokaryotic transposase (Tn5) to directly transpose sequencing adaptors into regions of accessible chromatin[44]. As with DNase-seq, ATAC–seq profiles can provide insights into several layers of epigenetic regulation from a single assay, including the identification of enhancer and promoter sequences genome wide with base-pair resolution, the precise positioning of nucleosomes, and the inference of TF activity through DNA footprinting of transposase-inaccessible regions[44–46]. However, it is important to note that while TF footprint predictions are often accurate in aggregate (that is, when averaged across the genome), they typically cannot conclusively identify specific TFs that are active at individual DNA positions and require experimental validation to demonstrate a functional role for the TF. Initially, the major advantages of performing ATAC–seq compared to DNase-seq were its lower preparation time (several hours compared to days) and associated cost, and its sensitivity for samples with low cell numbers, or for archival tissues or sections[44,47]. In recent studies, DNase-seq has also been adapted for low-input and archival samples, even down to single-cell resolution[48,49]. Overall, these technological advances in open chromatin analysis have enabled epigenetic profiling of mouse and human T_{EX} cells, including intratumoral T cells in clinical samples, discussed below[6–8,50].

Another important regulator of gene expression involves DNA methylation that occurs upon covalent addition of a methyl group to the pyrimidine ring of cytosine to form 5-methylcytosine (5-mC)[51]. The presence of 5-mC on promoters and enhancers typically indicates repression of gene transcription but has also been reported to facilitate transcription[52]. At repressed genomic regions, 5-mC is bound by methyl-CpG binding proteins, which facilitate chromatin condensation (Fig. 1a). DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and is oxidized by TET enzymes into 5-hydroxymethyl cytosine (5-hmC), which can then be converted back to unmodified cytosine via multiple mechanisms[53]. Among DNMTs, DNMT3 enzymes (DNMT3A and DNMT3B) mediate de novo methylation, while DNMT1 maintains the methylation landscape during cell division[54]. The location of methylation genome wide can be mapped by bisulfite sequencing (bisulfite-seq; Fig. 1b). Bisulfite treatment of DNA chemically converts cytosine to uracil, but cytosines marked by 5-mC or 5-hmC are protected. Therefore, identities of methylated and unmethylated cytosines can be distinguished by sequencing[55–57].

The 3D conformation of chromosomes underlies the spatial interaction of distal regulatory elements with target genes, and recent advances in sequencing-based technologies have improved our understanding of the multi-scale organization of the 3D genome[13,58] (Fig. 1a). These methods typically begin by crosslinking cells, which preserves interactions between neighboring and distant genomic loci. Next, cells are lysed, and DNA is digested with a restriction enzyme followed by proximity ligation to generate small fragments of interacting DNA. The fragments of interest are chimeric; they contain fragments of DNA from two interacting locations in the genome, and these ‘contacts’ are identified as chimeric reads from sequencing (Fig. 1c). Chromosome conformation capture (Hi-C) was the first technology that enabled the unbiased and systematic mapping of all genome-wide DNA contacts[58,59]. Initial Hi-C maps demonstrated that chromosomes can be characterized by spatially separated compartments that can be several megabases in size. Within compartments, TADs represent the next level of genome organization and are self-interacting loci in the range of 0.2–1.0 Mb[60,61]. The main architectural regulators of genome conformation are insulator elements, which bind TF CTCF at TAD boundaries. TADs form via a dynamic process of cohesin-mediated loop extrusion[62], which can proceed either symmetrically (that is, both cohesin rings slide equally along DNA) or asymmetrically, as occurs when one cohesin ring is stuck at a particular location and ‘reels in’ the other ring[63]. Asymmetric extrusion can be caused by tethering of promoters within the loop to super-enhancers, manifesting as a ‘stripe’ on the Hi-C contact map and termed a ‘stripe domain’. Recent adaptations of Hi-C have improved its sensitivity (for example, in situ Hi-C) and have also incorporated a protein-directed immunoprecipitation step (for example, HiChIP)[64–67] (Fig. 1c). These more sensitive technologies have revealed sub-TAD structures, such as specific enhancer–promoter DNA loops, as well as structures that are bound by proteins or chemical modifications of histones. Importantly, newer technologies, including HiChIP, have dramatically reduced the cell number required for profiling protein-directed genome conformation, enabling the application of these technologies to T_{EX} cells, as recently demonstrated in chimeric antigen receptor (CAR) T cells[68]. Altogether, epigenetic modifications, chromatin accessibility and genome conformation provide spatiotemporal control of gene regulation, and the recent development of these

genomic technologies with increased sensitivity, resolution and throughput have enabled new insights into the molecular mechanisms underpinning T cell function.

Distinct epigenetic programs in T_{EX} cells

The central function of T cells is to monitor peptide antigens presented by MHC molecules, discriminate self from foreign antigens and, upon recognition of a foreign antigen, mount an appropriate immune response[1]. The T cell response is directed not only by the TCR–antigen signal (signal 1), but also by co-stimulatory signals (signal 2) and the local cytokine milieu (signal 3)[69]. Collectively, these signals induce the activation and differentiation of naïve T cells into different T cell subsets, each with distinct phenotypes and functions. After activation, naïve $CD8^+$ T cells differentiate into a highly proliferative and cytotoxic effector state[70] (Fig. 2a). If the antigen can be cleared, such as in acute viral infections, most effector cells undergo apoptosis (termed short-lived effector cells or terminal effector (TE) cells), while a small fraction persist and differentiate into memory cells via memory precursor (MP) cells[71]. MP cells and TE cells can be distinguished via reciprocal expression of surface markers KLRG1 and interleukin (IL)-7R, and the fate decision is established by the TF T-bet; high *Tbx21* (which encodes T-bet) expression induces the TE cell fate, while low expression induces the MP cell fate[71,72]. Early epigenetic profiling studies in primary $CD8^+$ T cells demonstrated how DNA methylation, histone modifications and chromatin accessibility regulated the processes of T cell activation, effector response and memory formation during viral infection[24,73,74]. For example, these studies demonstrated that key effector genes, such as *Prf1* and *Gzmb*, are demethylated and gain chromatin accessibility upon antigen recognition, while naïve-associated genes are repressed. After pathogen clearance, MP cells can differentiate into long-lived memory cells by demethylating naïve-associated genes required for survival, such as *Bcl2* and *Il7r*. As a result, memory $CD8^+$ T cells have demethylated and open chromatin at both effector and naïve genes, which enable them to be long-lived, while retaining the ability to rapidly mount an effector response upon pathogen reencounter[73,74].

In contrast to acute infection, in settings of persistent antigen, such as in chronic viral infections or cancer, $CD8^+$ T cells can instead become exhausted. T cell exhaustion is characterized by poor proliferative potential, expression of multiple cell surface inhibitory receptors (for example, PD-1, CTLA-4, TIM3 and LAG3) and a loss of effector function[2]. Although T_{EX} cells were initially characterized in the context of the chronic murine lymphocytic choriomeningitis virus (LCMV) clone-13 infection model, there is a growing appreciation that these cells are conserved across a variety of disease settings, including human infectious diseases, cancer and autoimmune disease[75–79], and are a key determinant of response to immunotherapies, including checkpoint blockade and engineered T cell therapies[5,80–83]. However, whether T cell exhaustion represents a distinct cell state and differentiation program—as opposed to the isolated upregulation of a select few inhibitory receptors, such as PD-1—remained unresolved until recently[84–86]. To address this question, multiple studies profiled LCMV-specific or tumor-specific $CD8^+$ T cells to identify open chromatin sites associated with T cell exhaustion[6–9,87]. In both models, comparative analysis between T_{EX} cells and naïve, effector or memory T cells, or between tumor-infiltrating T_{EX} cells and acutely stimulated or bystander T cells, revealed genome-wide reprogramming

of the T_{EX} cell epigenetic state, comprising thousands of differentially accessible regions, many of which neighbored genes that mediate T_{EX} cell differentiation. For example, T_{EX} cells lacked open chromatin sites present in the *Ifng* locus in T effector and memory cells, which accompanied the diminished expression of *Ifng* in T_{EX} cells. Similarly, several T_{EX} cell-specific open chromatin sites were present in the *Pdcd1* locus, and CRISPR–Cas9 mutagenesis experiments demonstrated that these sites functioned as bona fide enhancers that maintain high levels of PD-1 expression specifically in T_{EX} cells[6]. These findings, taken together with previous studies demonstrating large-scale transcriptional changes in T_{EX} cells[86], support the concept that T_{EX} cells represent a distinct T cell chromatin state, rather than the isolated expression of inhibitory receptors.

Importantly, epigenetic analysis in human chronic infection, such as human immunodeficiency virus (HIV), and in human cancers, has demonstrated a conserved epigenetic profile between murine and human T_{EX} cells[4,6,50,75]. ATAC–seq profiling of T_{EX} cells in the setting of human basal cell carcinoma identified ~4,500 differentially accessible regions in T_{EX} cells[50]. This extent of chromatin remodeling was comparable to that which was observed in other T cell states, such as regulatory $CD4^+$ T cells or effector $CD8^+$ T cells, indicating a large-scale change that is consistent with a distinct cell lineage, as was observed in murine T_{EX} cells. Accordingly, a core T_{EX} cell gene signature derived from transcriptomic and epigenomic profiling of murine T_{EX} cells in chronic LCMV infection was shared in human T_{EX} cells present in chronic infection and cancer[4]. More broadly, a recent study performed a comprehensive reanalysis of over 300 human and mouse ATAC–seq and RNA-sequencing (RNA-seq) datasets from $CD8^+$ T cells in chronic infection and cancer and showed that T cells obtained from both settings exhibited highly similar global chromatin profiles, although precise enhancer sequences in individual gene loci may diverge across organisms[68,75]. Altogether, these results demonstrate that T_{EX} cells exhibit a common differentiation program across species and immune challenges, which suggests that this program may be regulated by common upstream signals and T_{EX} cell-specific TFs.

Subsets of exhausted T cells and key transcription factors

Temporal and T_{EX} cell subset-based analysis has further enabled the dissection of epigenetic and TF programs underlying T_{EX} cell differentiation. A temporal analysis of tumor-infiltrating lymphocyte (TIL) exhaustion over the course of 60 days after the transfer of naïve T cells into the tumor microenvironment (TME) identified two distinct phases of chromatin remodeling[8]. The first phase of remodeling occurred early, within 5 days of T cell transfer, while the second (and final) phase of remodeling occurred approximately 2 weeks after transfer. The number of differentially regulated *cis*-regulatory elements was similar in both phases, but these sites were regulated by different TFs. Comparing chromatin changes in early (day 7) versus late (day 14) TILs revealed that day 7 TILs had increased chromatin accessibility at sites containing AP-1, NFAT and TCF-1 TF motifs, while day 14 TILs had increased accessibility at sites containing E2F and KLF TF motifs. To test the functional importance of these regulatory programs, TILs were isolated at multiple time points for functional studies, which demonstrated that early T_{EX} cells (day 5, PD-1^{hi}CD38^{lo}CD101^{lo} cells) could regain effector function when removed from the TME, while late T_{EX} cells (day 12 or after, PD-1^{hi}CD38^{hi}CD101^{hi}) could not, leading to a model in which PD-1^{lo}

TILs undergo two sequential waves of chromatin remodeling, of which only the early T_{EX} cell epigenetic program may be reversible. Interestingly, transplanting memory T cells into tumor-bearing mice revealed that the TME could induce a similar epigenetic state in naïve and memory T cells.

These results suggested a stepwise acquisition of the epigenetic program of exhaustion, and the early epigenetic changes suggest a distinct cellular T_{EX} cell differentiation trajectory after T cell activation, compared to effector and memory T cells. Indeed, flow cytometry analysis of antigen-specific T cells in chronic LCMV infection identified the presence of a progenitor T_{EX} cell population that developed early during chronic infection, which was defined by its ability to proliferate and self-renew in response to antigen, its preferential localization in lymphoid organs, and the expression of the TF TCF-1 (refs. [5,80,88]). Moreover, after anti-PD-1 immunotherapy, these cells were the primary source of the T cell proliferative burst, suggesting their preserved function, compared to other T_{EX} cell populations[80]. In subsequent studies, two additional subsets of T_{EX} cells have been described: transitory T_{EX} cells, defined by their expression of PD-1 and CX3CR1, and terminal T_{EX} cells, defined by their expression of PD-1, TIM3, LAG3, CD38, CD39 and CD101 (refs. [88–90]) (Fig. 2a). Transitory T_{EX} cells exhibit substantial proliferative and effector function and represent an intermediate cell state between progenitor T_{EX} and terminal T_{EX} cells, which exhibited the most severe functional defects. Importantly, although studies have demonstrated that terminal T_{EX} cells develop from transitory T_{EX} cells[88,91], under certain circumstances—including insufficient $CD4^+$ T cell help[89] or the presence of transforming growth factor-β (TGF-β)[92]— it has also been proposed that cells may progress directly from progenitor T_{EX} cells to terminal T_{EX} cells. In these cases, $CX3CR1^+$ cells have been proposed to represent an alternate endpoint that does not progress to terminal T_{EX} cells[89,92]. Additional lineage tracing studies will be needed to determine whether the $CX3CR1^+$ state is truly bypassed in these situations or the differentiation to terminal T_{EX} cells is simply accelerated.

TCR-based lineage tracing analysis of antigen-specific T cells in chronic LCMV infection supports the concept that a subpopulation of transitory T_{EX} cells may represent an alternate T_{EX} cell differentiation endpoint[93]. This study identified heterogeneity within the $CX3CR1^+$ transitory T_{EX} cell pool and proposes a model in which T_{EX} cells progress through a $CX3CR1^+$ T_{EX} cell intermediate phenotype to either terminal T_{EX} cells or an alternate $CX3CR1^+$ T_{EX} cell state, which is marked by expression of killer cell lectin-like receptors (KLRs; T_{EX}^{KLR})[93]. Therefore, three non-mutually exclusive models of differentiation are emerging: (1) a linear model in which all cells eventually progress to terminal T_{EX} cells (Fig. 2a), (2) a linear model in which certain cells do not progress past transitory T_{EX} cells (for example, certain cells are retained in a $CX3CR1^+$ T_{EX}^{KLR} state) and (3) a divergent model in which cells progress from progenitor T_{EX} cells to either terminal T_{EX} cells or transitory T_{EX} cells (including T_{EX}^{KLR}). The determinants of T_{EX} cell differentiation trajectories may depend on multiple factors. As one example, an important determinant of T cell fate is TCR affinity, which manifests via TCR signal strength[94]. It has been shown that when two antigens of different affinities are present within the same tumor, T cells specific for the weaker antigen are enriched for progenitor T_{EX} cells[95]. In addition, recent complementary studies analyzing polyclonal T cells responding to a single antigen (gp33)[93]

or transgenic T cells responding to three antigens of carefully defined affinities[94], have demonstrated that TCRs with higher affinity may preferentially progress to terminal T_{EX} cells, while lower-affinity interactions induce alternate states including T_{EX}^{KLR} cells. In CAR T cells, repeated antigen exposure has also been shown to induce a natural killer cell-like state, which expresses many of the same genes as T_{EX}^{KLR} cells[96].

To further understand the regulatory programs underlying these functional state transitions, recent studies have performed ATAC–seq analysis of T_{EX} cell subsets in the setting of chronic LCMV infection[91,97,98]. Analysis of TF motif accessibility across subsets identified early-stage and late-stage TF activities, analogous to the observations in exhausted TILs. Progenitor T_{EX} cells showed increased activity of TCF-1 and BACH2, transitory T_{EX} cells showed enrichments in T-bet and RUNX motifs, and terminal T_{EX} cells showed enrichments in NR4A and EOMES motifs, nominating a hierarchy of TF families whose activity may underlie each cell-state transition. Importantly, although transitory T_{EX} cells expressed several transcripts and TFs in common with effector T cells (for example, *Cx3cr1*, *Tbx21*, and so on), these cells were epigenetically distinct, with nearly 5,000 differentially accessible regulatory elements compared to effector cells. Similarly, H3K27ac ChIP–seq analysis demonstrated that progenitor T_{EX} cells exhibited the most distinct active enhancer landscape (2,863 unique enhancers) compared to terminal T_{EX}, MP and TE cells in LCMV infection[99]. A common finding across multiple modalities, including ATAC–seq, ChIP–seq and HiChIP, is the enrichment of AP-1/bZIP family TF motifs in active chromatin of terminal T_{EX} cells[36,68,83], which may represent promising targets for functional follow-up and engineering. Interestingly, comprehensive CUT&RUN analysis of active and repressed chromatin marks in exhausted TILs revealed a decoupling of active histone modifications and active gene expression, as well as an increase in bivalent enhancers, suggesting the presence of altered 3D chromosome conformation in terminal exhaustion[36]. Accordingly, H3K27ac HiChIP in naïve, exhausted (HA-28z) and non-exhausted CAR T (CD19–28z) cells identified T_{EX} cell-specific 3D chromatin conformation, which in many cases exhibited differential chromatin looping despite minimal changes in chromatin accessibility, suggesting a further layer of T_{EX} cell genome regulation that should be investigated in future studies[68]. Altogether, these findings support a distinct lineage trajectory for T_{EX} cells and nominate sequential TF activities that may program the T_{EX} cell epigenetic state.

Despite these advances, the existence and identity of a lineage-determining T_{EX} cell TF remained unknown. By analyzing differentially expressed genes in T_{EX} cells across chronic infection and mouse and human tumors, several studies identified the TF TOX as a key regulator of the T_{EX} cell lineage[99–103]. In chronic infection and in tumors, TOX was rapidly induced by TCR signaling in T_{EX} cells and remained highly expressed, while in acute infections, low levels of TOX were transiently induced but not sustained[101,102]. Importantly, overexpression of TOX in T cells in vitro was sufficient to recapitulate several features of the T_{EX} cell program, including upregulation of the inhibitory receptor genes, *Pdcd1* and *Havcr2*, and *Entpd1*; however, the magnitude of gene expression change was not as large as is observed in T_{EX} cells in vivo. Conversely, genetic deletion of *Tox* led to a decrease in the surface expression of inhibitory receptors and improved proliferation in tumors and during chronic infection but did not impact the development of effector or memory cells during acute infection. Intriguingly, not all aspects of T cell exhaustion were reversed by

the deletion of *Tox*; namely, *Tox*-deficient and wild-type T cells showed varied defects in the production of effector molecules and in their ability to lyse antigen-bearing cells. It remains to be determined whether these discrepancies are due to the different model systems tested or other factors. Moreover, *Tox*-deficient cells persisted less in tumors, compared to wild-type cells, suggesting a decoupling of multiple T_{EX} cell programs (for example, inhibitory receptor expression, effector functions and persistence) downstream of TCR signaling. These data suggest that TOX may work in concert with other key TFs to establish the full T_{EX} cell program.

At the epigenetic level, transcriptional changes observed in *Tox*-deficient cells, or after *Tox* overexpression, were associated with corresponding changes in chromatin accessibility[101–103]. For example, putative regulatory elements in the *Pdcd1*, *Cd38* and *Entpd1* gene loci were less accessible in *Tox*-deficient T cells, compared to wild-type T cells. Globally, approximately 40% of the accessible regions that were significantly decreased in *Tox*-deficient cells were T_{EX} cell specific. In contrast, epigenetic analysis revealed an increase in accessibility in a large fraction of sites near effector genes, including *Klrg1*, *Gzma*, *Gzmb* and *Zeb2*, supporting a role for TOX as a key determinant of the early fate decision between effector and T_{EX} cell lineages. ChIP–seq analysis revealed that the *Tox* locus was bound by NFAT1 and NFAT2, key TFs immediately downstream of TCR signaling, and chronic TCR stimulation and NFAT2 overexpression were sufficient to induce *Tox* expression, while NFAT2-deficient T cells failed to upregulate *Tox*[101]. However, the sustained expression of *Tox* appears to be independent of NFAT2 and may be regulated at least in part by DNA methylation of the *Tox* locus[100].

Taken together with previous epigenetic studies, these results suggest a temporally coordinated TF hierarchy that establishes and maintains the T_{EX} cell state (Fig. 2b). First, immediately downstream of TCR signaling, NFAT proteins tune the balance between productive T cell activation and T cell dysfunction[104]. NFAT:AP-1 heterodimers lead to T cell activation, while ‘partnerless’ NFAT directly binds to and induces expression of inhibitory receptor genes and *Tox*[102,104]. Progenitor T_{EX} cells maintain expression of *Tcf7* (which encodes TCF-1 and is also expressed in naïve T cells), which may initially be driven by BACH2 and enables them to self-renew and proliferate[98]. However, following continued antigen stimulation, T cells proceed to a transitory T_{EX} cell state, which has partial effector activity, driven by the activity of T-bet. Finally, T cells progress to terminal T_{EX} cells, in which sustained expression of TOX induces the upregulation of EOMES and NR4A TFs. These factors in turn regulate the terminal exhaustion program that includes inhibitory receptors, decreased proliferative and effector functions, and increased pro-survival molecules and metabolic adaptations that ensure T cell persistence in the setting of chronic antigen.

Epigenetic stability of T cell exhaustion

The clinical success of immunotherapies targeting inhibitory surface receptors on T cells, including PD-1 and CTLA-4 blockade, has motivated research into the T cell subsets responsible for tumor control and clinical response[80,81]. T cell infiltration and exhaustion have been associated with clinical response, but it has been unclear whether T_{EX} cells are a

cause or a byproduct of tumor regression. Early studies on the effect of PD-1 blockade on $CD8^+$ T cells were performed in the setting of chronic viral infection, where it was shown that treatment could lead to the expansion of highly functional antigen-specific $CD8^+$ T cells, in addition to improved survival and reduced viral load[76,77,105,106]. Adoptive transfer of congenically marked T_{EX} cells induced by chronic infection showed that these cells maintain substantial proliferative capacity, leading to a model in which reinvigoration of preexisting T_{EX} cells directly mediates disease response. In contrast, other functional studies of T_{EX} cells after adoptive transfer demonstrated persistent impairments in effector function and cytotoxicity even after the removal of antigen[107].

Recent studies have revisited this concept with genome-wide transcriptional and epigenetic profiling technologies and from the epigenetic viewpoint of T_{EX} cells. The precise definition of the T_{EX} cell-specific chromatin signature allowed one group to investigate the stability of this chromatin state in the setting of immunotherapy[7]. Strikingly, after PD-1 blockade, only ~10% of the epigenetic landscape was 'reinvigorated' to resemble the effector T cell landscape, suggesting that the durable reacquisition of T cell function may be limited by the stability of the T_{EX} cell-associated chromatin state. In addition, a small subset of chromatin regions (98) diverged even further from the effector state after anti-PD-L1 treatment. The 555 chromatin regions that demonstrated at least partial reversal to the effector state were enriched for NFAT-binding sites, and 'partnerless' NFAT-dependent genes showed reduced expression in anti-PD-L1-treated T_{EX} cells, highlighting that several coordinated TF pathways may underlie T_{EX} cell stability[7,104].

A second series of studies took this question a step further and asked whether the T_{EX} cell chromatin state remains stable even in the absence of antigen[108–110]. In one of these studies, the authors transferred T_{EX} cells from chronic LCMV-infected mice to infection-free mice and analyzed their functional, transcriptional and epigenetic reinvigoration toward T effector cells[108]. Strikingly, even though T_{EX} cells acquired some transcriptional features of the memory T cell program, such as the downregulation of inhibitory receptors and reexpression of *Il7r* and *Tcf7*, these cells were still highly impaired in their ability to proliferate and generate a robust recall response in the context of a new infection. Importantly, ATAC–seq analysis showed that the chromatin state of T_{EX} cells transferred into an infection-free animal still more closely resembled the T_{EX} cell state, rather than the T cell memory state; namely, only 182 regulatory elements changed accessibility after removal of antigen. These results suggest that the T_{EX} cell epigenetic state is highly stable, indicating that chronic antigen exposure leaves persistent 'scars' that are not removed by PD-1 blockade or cessation of antigen exposure. Finally, the authors determined that T_{EX} cells that could proliferate in the recall response were predominantly derived from progenitor T_{EX} cells, supporting the relative reversibility of early T_{EX} cell programs, compared to late T_{EX} cell programs.

Although the precise molecular programs that maintain the T_{EX} cell state are still under investigation, one study has demonstrated that this is mediated at least in part by the de novo DNA methyltransferase, DNMT3A[111]. Whole-genome bisulfite-seq of antigen-specific $CD8^+$ T cells in LCMV clone-13-infected mice identified ~1,200 DNA methylation events that accompanied the T_{EX} cell transition. Analysis of *Dnmt3a*-conditional knockout mice

demonstrated that these methylation events were DNMT3A-dependent and included target genes such as *Eomes*, *Tbx21* and *Tcf7* (ref. [111]). Interestingly, this de novo exhaustion methylation program was not impacted by PD-L1 blockade, but anti-PD-L1 blockade synergized with DNMT3A inhibition to enhance T cell proliferation in chronic infection. Of note, DNMT3A may also represent a promising target for CAR T cell engineering, as DNMT3A-knockout CAR T cells demonstrated enhanced antitumor activity, proliferation and effector function while limiting exhaustion[112].

In summary, these epigenetic studies support several fundamental concepts regarding the regulation of T cell exhaustion. First, that the primary driver of T_{EX} cell lineage commitment is chronic TCR signaling, not environment-specific effects or PD-1 signaling. This concept is supported by the conservation of a common T_{EX} cell program across diverse disease settings with different microenvironment effects[4,5,75], and in CAR T cells[83], the induction of *Tox* expression by chronic TCR signaling or downstream TFs[102,104], the similarity of exhaustion programs in $CD8^+$ and $CD4^+$ T cells that experience chronic TCR signaling[50,113], comparisons of tumor-specific TILs with bystander TILs in the same tumor environment[9,114], and comparisons of antigen-specific T cells responding to variants of chronic LCMV infection that allow the strength and duration of antigen stimulation to be varied in a controlled manner[100,115]. Second, that in many cases, T cell exhaustion may be beneficial for the organism, enabling T cells to continue to persist in the setting of chronic antigen stimulation with reduced function, rather than undergoing activation-induced cell death. Therefore, targeting select, but not all, molecular programs in T_{EX} cells may provide a ‘goldilocks’ approach for improved T cell function in cancer[94].

Targeted perturbation of the epigenome and single-cell technologies

New technologies for targeted genetic and epigenetic perturbations, as well as single-cell and multi-omic profiling methods, are an important frontier for characterizing immune cell function and T cell exhaustion. CRISPR–Cas9-based genome engineering has made genetic and epigenetic perturbations easier, faster and higher fidelity than was previously possible[116,117]. There are two versions of CRISPR–Cas9 that provide the basis for an extensive suite of emerging technologies (Fig. 3a). The first, and original function of Cas9, is to edit the genome at a location programmed by a single guide RNA (sgRNA; Fig. 3a). In most cases, the induced double-strand break is repaired by nonhomologous end joining, which results in a small random insertion or deletion (indel) at the cut site. These indels are typically deleterious and, when targeted to coding regions of the genome, result in disruption of the targeted protein. Alternatively, if an exogenous piece of template DNA is provided with ends homologous to the sequences flanking the cut site, the template may be integrated into the genome via homology-directed repair. A second adaptation of CRISPR–Cas9 is catalytically dead Cas9 (dCas9), which retains the genome targeting capability of wild-type Cas9 but is not able to induce double-stranded breaks[118] (Fig. 3a). Therefore, any protein that can be expressed and function as a fusion construct with dCas9 can be precisely targeted to any genomic location, which is useful for many applications, including transcriptional inhibition (CRISPRi)[118,119] or activation (CRISPRa)[120]. Finally, both Cas9 and dCas9 can be used together with pools of sgRNAs (as opposed to individual sgRNAs) to perform high-throughput screens of coding[120–123] and noncoding regions[124–126]. Although

initial proof-of-concept studies were performed in model systems (for example, cell lines), the development of the Cas9-expressing mice and methods to efficiently deliver Cas9 into primary T cells are enabling broader applications of genome engineering technologies to understand immune regulation[35,126–134]. In the context of T cell exhaustion, recent studies have demonstrated the ability of putative enhancers identified by epigenetic studies to be functionally tested with CRISPR, for example, validating a causal role for several *cis*-regulatory elements in the regulation of PD-1 expression[6,68]. Pooled screening in T_{EX} cells has also enabled functional interrogation of TFs and led to the identification of *Fli1* as a factor that limits T cell function[35]. We envision that future studies will soon enable the large-scale testing of putative regulatory elements and TFs nominated by epigenetic profiling to rapidly uncover functional elements and novel T_{EX} cell biology.

In parallel, single-cell technologies for profiling the transcriptome[135], epigenome[50], immune receptor repertoire[136], surface proteins[137,138] and CRISPR perturbation[139] have matured into streamlined and widely available platforms (Fig. 3b). There have also been substantial advances in combining multiple modalities within the same cell, for example, in CITE-seq (single-cell RNA-seq with surface proteins)[138], SHARE-seq (single-cell RNA-seq with single-cell ATAC–seq)[140], Perturb-seq (single-cell RNA-seq with CRISPR)[141–143], Perturb-ATAC (single-cell ATAC–seq with CRISPR)[144,145] and DOGMA-seq (single-cell RNA-seq, single-cell ATAC–seq and surface proteins)[146]. These multi-omic approaches can be used to discover new cell types[147], uncover precise differentiation trajectories[50,148] and characterize gene or TF regulatory networks[143,144] (Fig. 3b). In the context of T cell biology, these profiling technologies have demonstrated the phenotypic diversity of intratumoral T cells[50,79,82,149–152], TCR repertoire evolution during development and clonal T cell dynamics after checkpoint blockade[82,148,153], and can nominate new molecular regulators and therapeutic targets[99,152].

Opportunities for epigenetic engineering of T cells in the clinic

Improvements in our understanding of T cell exhaustion and the mechanisms of action of currently approved immunotherapies suggest new therapeutic opportunities. In particular, the ability to modify a patient's own T cells ex vivo and reinfuse them offers the opportunity to use engineered T cells as therapeutic agents themselves[154,155] (Fig. 4a). The two most common types of T cell therapies are engineered TCR T cells, where a synthetic TCR is introduced that recognizes, for example, a known cancer antigen (that is, peptide-MHC antigen)[156,157], and CAR T cells, in which a synthetic construct is introduced that combines antibody-mediated antigen recognition with an intracellular signaling domain[154,158]. It is increasingly recognized that exhaustion limits CAR T cell function, but can be ameliorated with various engineering strategies such as using the 4–1BB co-stimulatory domain[159], overexpressing AP-1 factors, such as c-Jun or BATF[83,160], knocking out NR4A family members[161] or TET2 (ref. [162]), and 'resting' the cells by limiting antigen exposure[163]. Historically, nearly all of these engineering efforts have relied on the use of viral delivery —lentiviral or retroviral delivery of a synthetic construct randomly into the genome—but CRISPR–Cas9 has recently enabled targeted genome engineering directly in primary T cell therapies. CRISPR–Cas9-mediated knock-in can be used to insert the antigen-recognition domain directly into the endogenous TCR locus (*TRAC* locus)[131,164], optionally together

with other genes[165]. CRISPR–Cas9 can also be used to inactivate specific genes in clinical-grade cell therapy products, the safety of which was recently demonstrated in a first-in-human trial[166]. In the coming years, we anticipate that the T cell engineering field will identify a diverse set of targets for genome engineering via high-throughput screening, and will expand to encompass more sophisticated engineering approaches such as using dual sgRNAs to knock out specific enhancers, or directly perturbing genome conformation[35,127,133,165,167–169] (Fig. 4b).

Future perspectives

In conclusion, although there has been marked progress in our understanding of the development of T cell exhaustion, its unique features and its importance in diverse disease settings, important questions remain. First, single-cell and longitudinal studies are needed to finely characterize the phenotypic plasticity of these cells at each step in their differentiation trajectory. For example, a recent study in humans with hepatitis C virus infection demonstrated that T_{EX} cells that have undergone years of chronic stimulation have permanent deficits in memory formation after viral clearance[110]. However, T cells in which TCR signaling was stopped earlier—due to viral evolution that caused viral escape from certain TCRs but not complete viral clearance—could completely recover, consistent with findings in CAR T cells that demonstrate that antigen ‘rest’ can improve T cell function[110,163]. Therefore, more precise temporally resolved studies will be needed to understand exactly when T_{EX} cells pass the ‘point of no return’ and their functional deficits become permanent, what transcriptional and epigenetic features define that state, and whether the plasticity can be extended or reversed therapeutically. These studies may benefit from recently developed lineage tracing tools, for example, using TCR sequencing to track specific T cell clones in a polyclonal setting[93,170], or from recently developed tools that are compatible with transgenic TCR models that have a defined specificity[171]. Second, how these molecular programs coalesce with other environmental factors, such as metabolic deficiencies or stresses remains an open question[172]. For example, recent studies have demonstrated that hypoxia may influence the progression to terminal exhaustion[36,173]. Finally, high-throughput CRISPR–Cas9 screening is poised to rapidly expand our understanding of T_{EX} cell biology and therapeutic opportunities by enabling the testing of hundreds to thousands of modifications in parallel. When performed directly in clinically relevant cell therapies such as CAR T cells, genetic ‘hits’ have a direct path to therapeutic relevance, sidestepping the labor-intensive and time-intensive search for conventional therapeutics (for example, chemical or biologic agents), which phenocopy the genetic perturbation. Furthermore, as single-cell sequencing continues to define cell states associated with efficacious cellular therapies, we envision that multimodal readouts such as Perturb-seq and Perturb-ATAC will be used to directly screen for synthetic constructs and gene knockouts that directly tune the phenotype of a particular cellular therapy toward beneficial gene expression programs, and away from dysfunctional or exhausted phenotypes. When paired with individual profiling of additional dysfunction-inducing factors such as TGF-β signaling in the TME, genetic programs tailored to a patient’s own tumor could be installed in the engineered T cell, enabling personalized cellular therapies.

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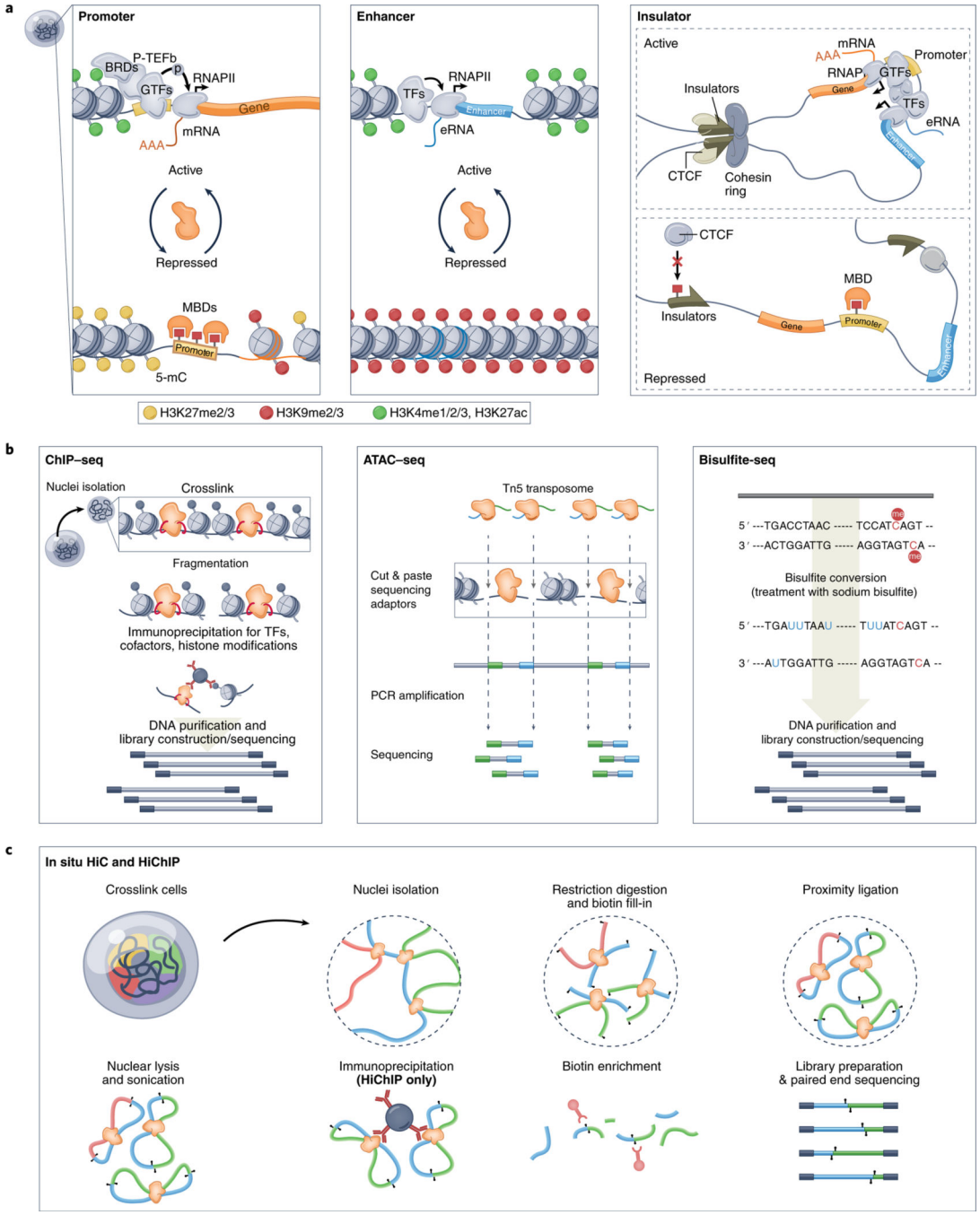

Fig. 1 |. Epigenetic regulation of gene expression.

a, Promoters, enhancers and insulators are shown in their active and repressed states. **b**, Technologies to map protein localization on chromatin (ChIP–seq), accessible chromatin (ATAC–seq) and DnA methylation (bisulfite-seq). **c**, Technologies to map spatial genome architecture. eRnA, enhancer RnA; GTF, general transcription factor; MBD, methyl-CpG binding domain; P-TeFb, positive transcription elongation factor.

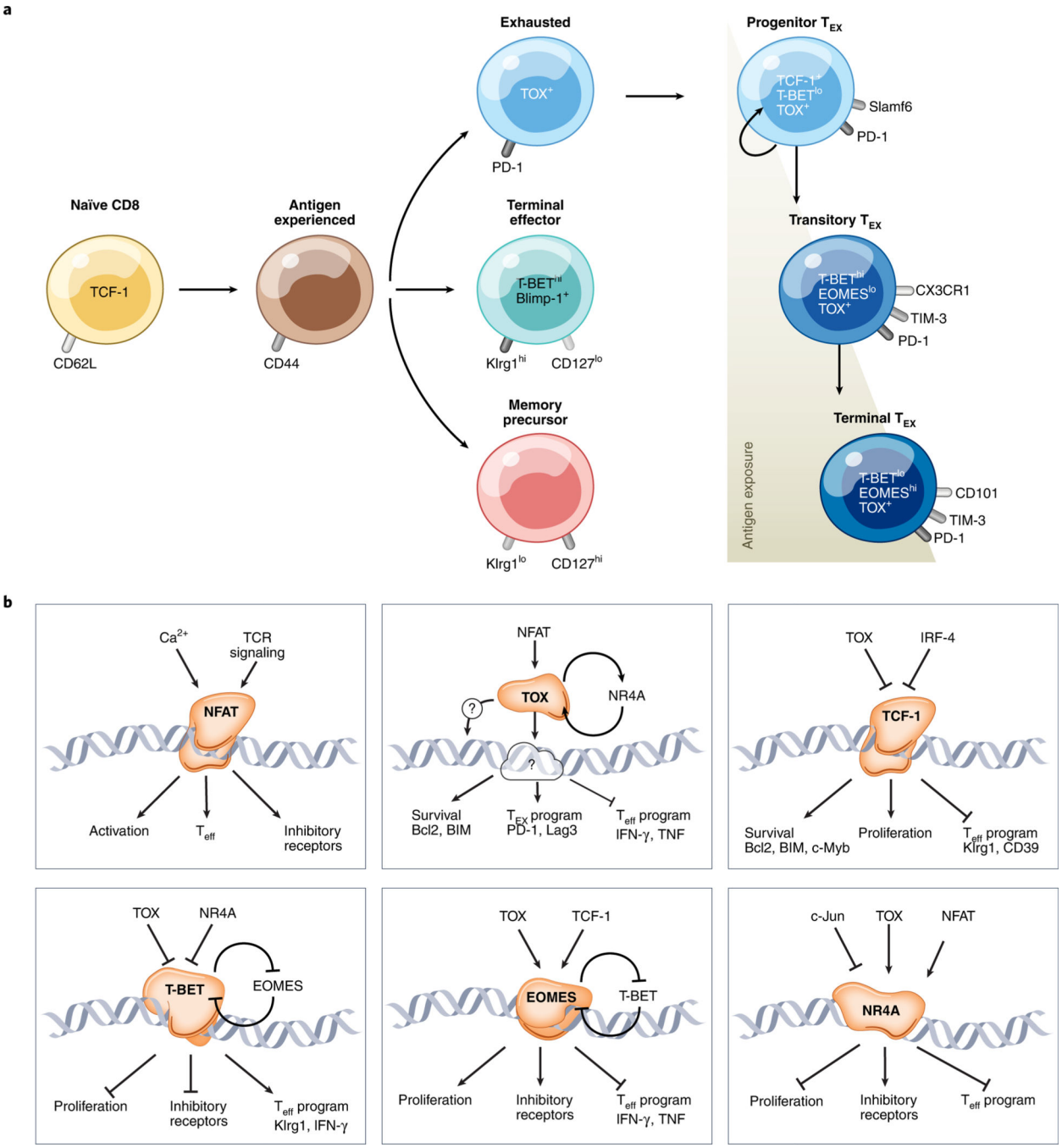

Fig. 2 |. CD8$^+$ T cell development and key mediators of T cell exhaustion.

a, Differentiation trajectory of CD8$^+$ T cells. Important surface markers and TFs are indicated on each cell type. **b**, Key transcriptional regulators of CD8$^+$ T cells and exhausted CD8$^+$ T cells. IFn-γ, interferon gamma; TnF, tumor necrosis factor.

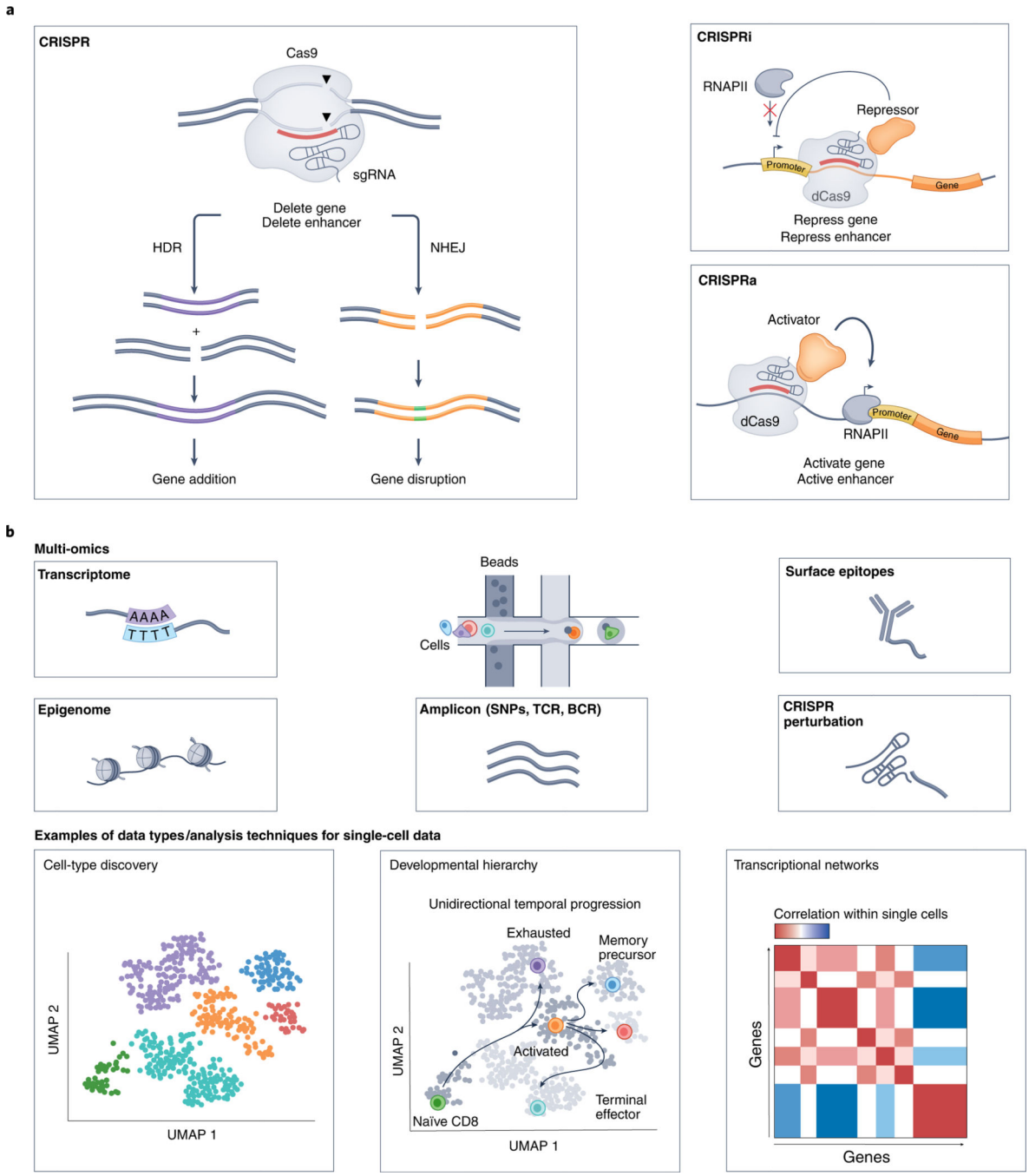

Fig. 3 |. Emerging technologies for studying the epigenome.

a, CRISPR–Cas9-based technologies for perturbing the epigenome. **b**, Multi-omic technologies for cell profiling. HDR, homology-directed repair; nHeJ, nonhomologous end joining.

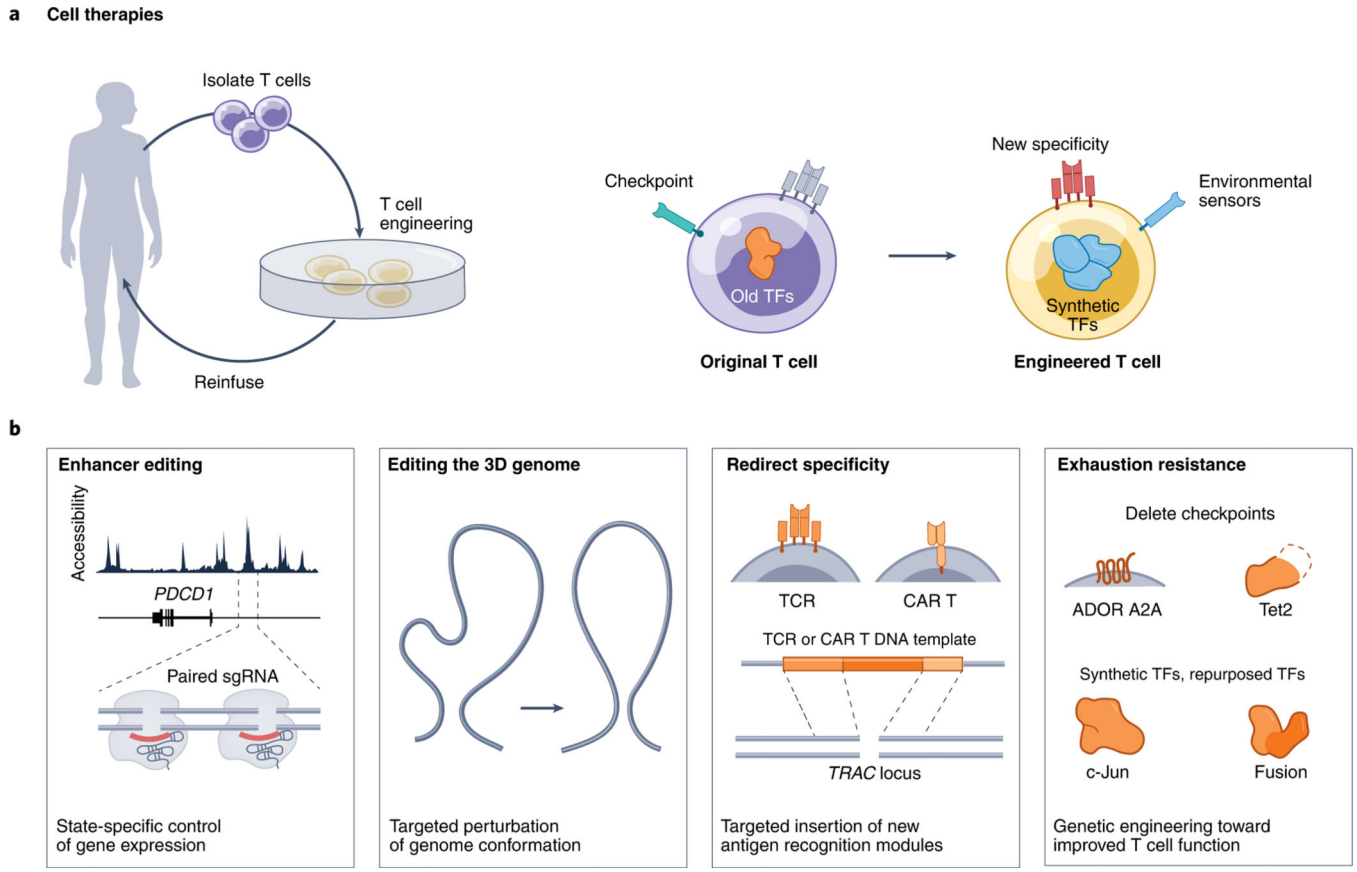

Fig. 4 |. Opportunities for epigenetic engineering in the clinic.

a, Engineered T cell therapies. **b**, Emerging strategies for genetic and epigenetic engineering.