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DNA methylation dynamics in the functional régulation of human T lymphocytes

Vincenzetti Lucia

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DNA methylation dynamics in the functional regulation of human T lymphocytes

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DNA methylation dynamics in the functional regulation of human T lymphocytes

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pour le Doyen
de la Faculté de biologie et de médecine

O. Spertini

Prof. Olivier Spertini

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Index

1. Abstract

1. Abstract

In mammals, the 5' methylation of the cytosine base (5mC) in the genomic DNA is intimately linked with the regulation of gene expression. Despite its stability and heritability across cell division, 5mC erasure is required for the activation of developmental programs associated with extensive functional reprogramming. Once deposited in the genome, 5mC can be removed either through passive dilution during DNA replication, or through active mechanisms characterized by enzymatic oxidation of the methyl mark to 5'-hydroxymethylcytosine (5hmC), which can persist as such or undergo further oxidation and enzymatic removal. However, it is still unclear what is the relative contribution of each mechanism to the epigenetic control in evolving biological systems. Therefore, the overall aim of this thesis is to investigate the contribution of active and passive mechanisms to 5mC and 5hmC removal in a cellular system. To explore this critical issue we used primary human T helper (T_H) lymphocytes, in which two cellular states can be clearly identified: quiescent naive T cells, which are slowly or rarely proliferating, and rapidly proliferating activated T cells upon antigen recognition. First, we found that the 5hmC is dynamically modulated during T cell activation, indeed unstimulated naive T cells maintained higher levels of 5hmC compared to those of all other antigen-experienced T lymphocytes, highlighting that DNA demethylation is a process linked to the activation and not to specific effector T cell subsets. Using compounds that inhibit methylation or enhance demethylation, we found an increased ability of the cells to produce effector cytokines, suggesting that DNA methylation-related processes are required for the efficient differentiation of T cells to effector and memory subsets. By optimizing methods to uncouple T cell activation from proliferation, naive T lymphocytes showed a significant loss of genomic 5hmC upon activation, even in the complete absence of proliferation. These results point towards a role for active processes of DNA demethylation in the first activation of naive T cells. On the other hand replication-dependent dilution was the driving force leading to 5hmC reduction in highly proliferative and already differentiated cells. Finally, by analyzing genome-wide DNA methylation and hydroxymethylation we found that these modifications change dynamically during T cell activation, and 5hmC in particular appeared to be differentially associated with regulatory regions of early and late response immune genes. Our data suggest that the usage of active *vs.* passive mechanisms of DNA demethylation is differentiation and activation stage-dependent. Specifically, active demethylation mechanisms are selectively involved in quiescent naive T lymphocytes prior to cell-cycle entry to maintain regulatory regions poised for rapid responses to physiological stimuli, while passive, replication-dependent dilution of the modified cytosines primarily is at work in memory T lymphocytes.

1. Résumé

Chez les mammifères, la méthylation en 5' de la base cytosine (5mC) de l'ADN génomique est intimement liée à la régulation de l'expression des gènes. Malgré sa stabilité et son héritabilité au cours des divisions cellulaires, la suppression de 5mC est requise pour l'activation de programmes de développement. Une fois déposée sur le génome, 5mC peut être éliminée soit par dilution passive lors de la réplication de l'ADN soit par des procédés actifs caractérisés par l'oxydation enzymatique du groupe méthyl générant la 5'-hydroxyméthylcytosine (5hmC) qui peut persister en tant que tel ou bien subir ultérieurement oxydation et élimination enzymatique. Toutefois, la contribution relative de chacun de ces mécanismes dans le contrôle épigénétique des systèmes biologiques évolutifs demeure incertaine. Dès lors, l'objectif de cette thèse est d'étudier la contribution des mécanismes actifs et passifs de la suppression de 5mC et 5hmC dans un système cellulaire. A cette fin, nous avons utilisé des lymphocytes humains T « helper » (T_H) primaires dans deux états cellulaires distincts: les cellules T naives quiescentes proliférant rarement ou très lentement et les cellules T activées par l'antigène et proliférant vigoureusement. Nous avons établi que la 5hmC est dynamiquement modulée durant l'activation des cellules T, les cellules naives non stimulées maintenant des niveaux de 5hmC plus élevés que ceux de tous les autres lymphocytes T ayant rencontré leur antigène, mettant ainsi en évidence que la déméthylation de l'ADN est un procédé lié à l'activation et non pas à une sous-population de cellules T effectrices spécifiques. Grace à des composés inhibant la méthylation ou augmentant la déméthylation, nous avons pu montrer une capacité accrue des cellules à produire des cytokines effectrices, suggérant ainsi que les procédés relatifs à la méthylation de l'ADN sont requis pour une différentiation efficace des cellules T effectrices et mémoire. Après optimisation des méthodes pour découpler l'activation de la prolifération, les lymphocytes T naifs ont montré une perte significative de la 5hmC génomique lors de l'activation et ce même en totale absence de prolifération, pointant vers un rôle des procédés actifs de déméthylation lors de l'activation des cellules T naives. D'autre part, la dilution dépendant de la réplication de l'ADN est le procédé principal menant à la réduction de la 5hmC pour les cellules hautement prolifératives et déjà différenciées. Enfin, en analysant l'entier du génome, nous avons découvert que ces modifications sont dynamiques durant l'activation des cellules T et que 5hmC en particulier semble être associée à des régions régulatrices de gènes de la réponse immunitaire initiale. Nos résultats suggèrent que l'usage des mécanismes actifs vs passifs de la déméthylation de l'ADN dépend de l'état d'activation et de différentiation. Plus précisément, les mécanismes actifs sont impliqués sélectivement chez les lymphocytes T naïfs avant l'entrée dans le cycle cellulaire afin de maintenir les régions régulatrices prêtes pour une réponse rapide alors que la dilution passive des cytosines modifiées dépendant de la réplication opère principalement chez les lymphocytes T mémoire.

2. Human T lymphocytes

2. Human T lymphocytes

2.1 Introduction

The human immune system responds to invading pathogens through a network of coordinated reactions, constituting the immune response, against antigens, namely molecules recognized as foreign and dangerous. The immune response is based on two major pillars: the first is the innate immune response (involving phagocytic cells, dendritic cells and natural killer cells as cellular components), that is poised to act rapidly and it recognizes conserved molecular patterns; the second is the adaptive immune response that takes longer to develop and it has the specificity for individual unique foreign antigens. An adaptive immune response includes humoral immunity, mediated by antibodies secreted by B lymphocytes, and cellular immunity mediated by T lymphocytes. The focus of my PhD studies is the T cell compartment. T lymphocytes are characterized by the surface expression of antigen-specific receptors encoded by genes that are assembled by somatic rearrangement of germ-line gene elements to form T cell receptors (TCRs), recognizing both the antigenic peptide and the MHC (major histocompatibility complex) molecules involved in peptide displaying. Indeed, while innate immune cells and B lymphocytes recognize native antigens, T cells require processing and presentation of the antigen prior to recognition. Such antigen capture and display to T lymphocytes is accomplished by antigen presenting cells (APCs). Among these, dendritic cells are exceptionally efficient in inducing a T cell response. Dendritic cells through pathways of antigen processing convert protein antigens present in the cytosol (foreign antigens in the cytosol may be the products of viruses or other intracellular microbes that infect such cells, or tumor antigens) into peptides then loaded onto class I MHC molecules and recognized by cytotoxic $CD8^+$ T cells; whereas antigens internalized from the extracellular environment are processed into peptides displayed by class II MHC molecules and recognized by cytokine-producing $CD4^+$ helper T cells (Haskins et al., 1983). The binding of a TCR to its specific peptide-MHC complex triggers the activation and differentiation of antigen-inexperienced naive T cells into effector cells

involved in the elimination of the pathogen. However, while the majority of effector cells are eliminated after accomplishing their functions, some differentiated cells will remain as long-lived memory cells to provide rapid protection upon re-exposure to the same antigen. Memory T cells, according to the profile of cytokine production and tissue localization, are divided into central memory (T_{CM}) and effector memory subsets (T_{EM}) (Sallusto and Lanzavecchia, 2009). In the next chapters I will focus in more detail on T cell differentiation and activation and on how these processes are regulated at the molecular level, specifically in $CD4^+$ T lymphocytes.

2.2 Origin and development

T lymphocytes are the components responsible for the specificity of cell-mediated adaptive immune responses. T cells, like all of the blood cells, initially originate from hematopoietic stem cells. As precursors, they migrate to the thymus where their final development and maturation takes place. Developing T cells in the thymus are called thymocytes.

The process of T lineage commitment includes distinct developmental stages supported by the thymus' environment, which provides all the necessary stimuli needed for proliferation and maturation of thymocytes, such as the expression of MHC molecules, cytokines (IL-7) and chemokines (CCL25, CCL19, CCL21). Each stage of T cell maturation is characterized by a specific localization in the thymus, by the pattern of expression of antigen receptor genes, and by the expression of the CD4 and CD8 coreceptors. The earliest T lineage cells, recently migrated from the bone marrow, are initially located in the thymus outer cortex; they are called double-negative thymocytes (also considered to be at the pro-T cell stage) since they lack the surface expression of both CD4 and CD8 ($CD4^-CD8^-$), and the TCR genes are in their germline state. Subsequently, successful rearrangement first of TCR β chain gene, and then of the TCR α chain gene in the double-positive stage ($CD4^+CD8^+$ thymocytes), leads to the formation of the complete αβ TCR that is expressed on the cell surface together with CD3 and ζ proteins that are accessory chains needed to

transduce signals when TCR binds to antigen-MHC complexes (Ledbetter et al., 1987); TCR chains, CD3 complex and ζ chains constitute the TCR complex.

At this stage, the cells migrate from the thymic cortex to the medulla and undergo processes of positive and negative selection, in order to preserve useful cells in the recognition of foreign peptide antigens, and eliminate potentially harmful ones that could react against the host. Positive selection of $CD4^+CD8^+$ TCR αβ thymocytes retains cells that recognize self-peptides complexed with self-MHC with low avidity; at the same time lineage commitment to CD4 or CD8 expression occurs: thymocytes with MHC class I-restricted TCR become $CD4^-CD8^+$ (single positive CD8), and cells with MHC class II-restricted TCR become $CD4^+CD8^-$ (single positive CD4). On the other hand, negative selection leads to apoptosis of thymocytes whose receptors recognize with high avidity peptide-MHC complexes present in the thymus. This mechanism is at the base of the self-tolerance concept: it eliminates potentially self-reactive T cells preventing autoimmune reactions. Once the T lymphocyte maturation process is complete, cells that acquired the ability to differentiate into either antigen-inexperienced naive $CD4^+$ (generally called 'T helper cells' that activate both humoral and cellular responses) or $CD8^+$ (showing cytotoxic activity against cells infected with intracellular microbes or tumor cells) T cells, migrate through blood circulation to peripheral lymphoid organs and tissues (lymph nodes, spleen, cutaneous and mucosal immune system). Upon first encounter with foreign antigens, these naive cells differentiate into functionally distinct effector subsets tailored to the elimination of those specific antigens (Abbas et al., 2012; Rothenberg et al., 2008).

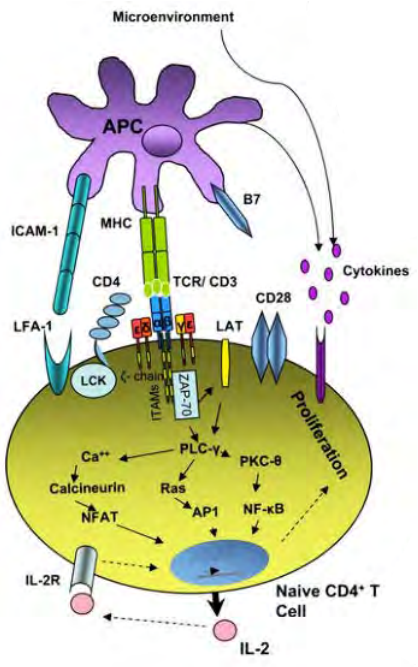
2.3 T lymphocyte activation

Naive T cells are by definition T cells that have not yet encountered a foreign antigen, and as such recirculate throughout the body in a resting state. It is only upon antigen recognition that naïve T cells become activated and acquire functional properties. Activation of naive T lymphocytes occurs in the T cell zone of draining lymph nodes where they normally recirculate. In particular dendritic cells

that encountered pathogens in epithelia or tissues and internalized their protein antigens, migrate there and display these foreign peptides to naive T cells.

When the TCR complex recognizes peptides associated to MHC on an APC, there is the formation of an immunological synapse, a contact region that includes the signaling machinery involved in T cell activation: the TCR complex, the CD4 or CD8 coreceptors and costimulatory receptors (such as CD28). The CD4 or CD8 molecules bind to the MHC and thus recognize part of the same ligand (peptide-MHC complex) that interact with the TCR (Reinherz et al., 1983). The cytoplasmic tails of both CD4 and CD8 bind the Src family kinase Lck, and the ability of these coreceptors to interact with MHC molecules helps these proteins to be drawn adjacent to the TCR that binds the same MHC-peptide complex on the APC. On the other hand, CD3 complex (composed of three polypeptide chains) and ζ chains have a role in signaling and not in antigen recognition. Indeed the capacity of the TCR to transduce signals across the membrane is mediated by the cytoplasmic tails of CD3 and ζ proteins containing ITAMs (immunoreceptor tyrosine-based activating motifs) [Figure 1] (Reth, 1989). The antigen recognition by TCR, resulting in the clustering of coreceptors with the antigen receptor, starts ITAM tyrosines phosphorylation by Lck (June et al., 1990; Samelson et al., 1986), that in turn triggers the activity of downstream tyrosine kinases leading to the activation of multiple signal transduction pathways: DAG (diacylglycerol)-dependent pathway leading to activation of PKC (protein kinase C); PLC (phospholipase C)γ1-calcium dependent pathway leading to the activation of phosphatase calcineurin; and small G protein-MAP (mitogen-activated protein) kinase pathways leading to activation of ERK (extracellular receptor-activated) and JNK (c-Jun N-terminal) kinases. As I will describe in the following sections, in antigen-stimulated T lymphocytes these multiple signaling pathways merge to activate transcription factors (TFs) that induce expression of different genes. Three important TFs are induced by T cell activation and have a central role in most T cell responses. These are NFAT (nuclear factor of activated T cells), AP-1, and NF-κB [Figure 1] (Abbas et al., 2012; Cantrell, 1996).

Antigen-induced signals (so-called signal 1) are however not sufficient for the full activation of naive T lymphocytes, and result in a non-responsive state (anergy) or cell death by apoptosis. Indeed, APCs express on their surface not only antigens but also costimulatory molecules (signal 2), essential for full T cell activation. The best characterized costimulatory molecules expressed on activated APCs are B7-1 (CD80) and B7-2 (CD86). These molecules are recognized by the receptor CD28 expressed on the T cell surface, that is part of the immunological synapse and acts in concert with antigen recognition to trigger the responses of naive T cells (Jenkins et al., 1991). The role of CD28 is on the one hand to amplify signals (Ras/ERK/MAP kinase pathways) from the TCR complex, but also in parallel to induce independent signaling pathways involving PI3 (phosphatidylinositol triphosphate) and Akt kinases. The final contribution of these different signaling cascades is to promote survival of T cells, enhance proliferation, induce cytokines production (such as IL-2) and as a consequence to trigger differentiation of naive T cells (Abbas et al., 2012; Smith-Garvin et al., 2009). Further signals (signal 3) are provided by pathogen-induced inflammatory cytokines that direct and amplify naive T cell differentiation and expansion. For example, a cytokine milieu containing IL-12 or IL-4 can lead to the generation of IFN-γ-producing T_H1 cells or IL-4-producing T_H2 cells [Figure 1] (Curtsinger and Mescher, 2010; Mosmann et al., 1986; Valenzuela et al., 2002).



Adapted from: Simeoni et al, Clin Kidney J 2016

Figure 1. Main signaling pathways coupled to the TCR and co-signaling molecules at the immunological synapse. Upon TCR ligation by MHC/peptide complexes on APCs, an activating signal is triggered within T cells leading to transcriptional activation, IL-2 production and proliferation. Additional receptors (e.g. CD4, CD28, integrins and cytokine receptors) also participate in this process. The orchestration of these signals results in Th differentiation into various effector and memory subsets.

Proliferation and differentiation into effector and memory cells are events following T lymphocytes activation and involve changes in the expression of surface molecules, expression of cytokine receptors and secretion of cytokines. Regarding surface molecules, one of the earliest functional changes occurring is an increase in the expression of CD69, which is an early activation marker expressed within 1-2 hours after activation (Cebrián et al., 1988; Testi et al., 1989). The role of CD69 is to block the exit of recently activated T lymphocytes from lymphoid organs in order to retain them to receive signals necessary to trigger proliferation and differentiation; indeed later, as cells divide, CD69 expression is reduced, allowing activated T cells to exit the lymphoid organs in order to reach peripheral sites of infection. Another essential early functional change occurring upon T cell activation is the induction of the surface expression of CD25 (IL-2Rα), that together with the IL-2Rβ and IL-2Rγ chains constitute the high-affinity receptor for IL-2, a change strictly connected with the rapid production of this cytokine. In fact, IL-2 is the major cytokine produced by naive $CD4^+$ T lymphocytes early after activation representing a survival and proliferation factor (Ahmed et al., 1997). IL-2 is a stimulus for the induction of CD25, creating a positive feedback loop, where IL-2 acts as autocrine growth factor, through which T cell responses amplify themselves [Figure 1]. The consequence of this proliferation is clonal expansion that, starting from a small number of naive antigen-specific lymphocytes, leads to the generation of the large number of cells required to eliminate the pathogen (Abbas et al., 2012; Boyman and Sprent, 2012).

2.4 $CD4^+$ T lymphocyte differentiation: signaling pathways and transcription factors

Following the first recognition of a foreign infectious agent, naive $CD4^+$ helper T lymphocytes receiving both activation and proliferation signals, will generate an array of effector T cell subsets involved in different types of immune response. Helper T cell differentiation is driven by the extracellular cytokine environment produced by activated APCs in response to encounter with different pathogens, along with TCR stimulation. All of these signals contribute to the expression of a distinct set of TFs that activate cytokine gene transcription defining a specific T helper lineage. As I will describe in the next chapter, the establishment of a particular transcriptome depends also on epigenetic changes affecting cytokine gene loci (Bevington et al., 2016), with the term epigenetics describing heritable modifications in gene expression without changing the DNA sequence itself.

The family of the major $CD4^+$ effector T cell subsets has expanded during the past two decades, and currently includes T_H1, T_H2, T_H9, T_H17, T_H22, follicular helper T (T_{FH}) cells, Tr1 and regulatory T (Treg) cells. These different subsets are defined primarily by the cytokines they produce, the specific pathogens they act upon and by the TFs that are necessary for their differentiation. For example, T_H1 cells are primarily important to control intracellular pathogens (such as viruses), T_H2 cells act against helminth parasites and allergens, while T_H17 cells have an important role against extracellular bacteria and fungi. The generation of these effector T cells underlines the specificity of adaptive immunity able to induce responses tailored towards the specific pathogen being recognized. T_H1 differentiation is primed by the cytokines IL-12 and IFN-γ activating the TFs STAT1 and STAT4, which in turn induce the expression of the master TF T-BET. For T_H2 cells the main differentiation stimulus comes from IL-4, activating STAT6 that in turn induces GATA-3 expression. IL-4, in combination with TGF-β, was also found to be required for T_H9 development by inducing the expression of the TFs IRF-4 and PU.1 (Dardalhon et al., 2008). The development of T_H17 cells depends on pro-inflammatory cytokines such as IL-6 and IL-23 that activate the TF STAT3 which functions with RORγt, the master

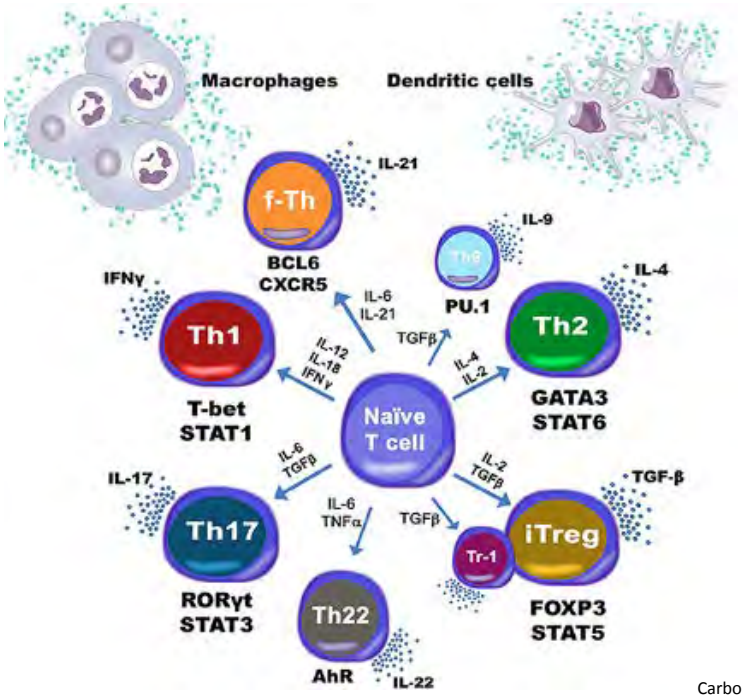
regulator for T_H17 cells. IL-6 together with TNF-α, promotes also the generation of T_H22 cells that produce predominantly IL-22 cytokine (Duhen et al., 2009). In addition to cytokines signals, AhR was found to be the master TF responsible for the development of T_H22 cells (Ramirez et al., 2010) [Figure 2]. Thus, the establishment of each T helper subset is characterized by the combination of specific TFs (Abbas et al., 2012; Kanno et al., 2012).

Helper T cells orchestrate host defense through their ability to produce cytokines that, by acting on target immune and non-immune cells, control effector responses aimed at pathogen elimination. The signature effector cytokine secreted by T_H1 cells is IFN-γ that activates macrophages to kill intracellular microbes. IFN-γ triggers different signaling pathways and TFs (such as STAT1) enhancing microbial killing by stimulating the expression of several enzymes in the phagolysosomes of macrophages. Conversely the functions of T_H2 cells are executed by IL-4, IL-5 and IL-13 involved in the stimulation of reactions mediated by IgE and eosinophils to fight helminthic infections. In particular IL-4 acts on B cells to stimulate the production of helminth-specific IgE antibody promoting the binding of eosinophils; on the other hand IL-5 activates mature eosinophils by inducing JAK2 and STAT3 signaling pathways leading to the release of their granule contents that help to destroy the parasite. T_H2 cells are recognized also for their involvement in allergies and atopic illness. Similarly, IL-9-producing T_H9 cells were shown to act in the development of allergic inflammation (Chang et al., 2010). Most of the inflammatory actions of T_H17 cells are mediated by IL-17 secretion. IL-17 stimulates the production of chemokines and other cytokines by epithelial, endothelial, and other stromal cells inducing recruitment of neutrophils, which in turn represent the main defense against extracellular bacteria and fungi (Wan and Flavell, 2009) [Figure 2].

T cell help to B cells is another fundamental aspect of adaptive immunity. T_{FH} cells localize to B cell follicles and they are the specialized providers of B cell help. In the early 2000s, studies on $CD4^+$ T cells in human tonsils showed that T cells expressing CXCR5 display a superior capacity to induce B cells to produce immunoglobulins *in vitro* as compared to $CD4^+$ T cells lacking CXCR5 expression.

Based on their localization and functions, tonsillar $CD4^+$ T cells were defined as T_{FH} cells. Their signature cytokine is IL-21, and in 2009 the TF Bcl-6 was discovered as an essential factor in defining T_{FH} cells as an independent T_H lineage [Figure 2] (Ueno et al., 2015).

In addition to selective expression of cytokines, another critical aspect of differentiating T helper cells is to maintain immunological unresponsiveness to self-antigens, and to suppress excessive immune responses deleterious to the host (Sakaguchi et al., 2008). Treg cells represent the T cell subset essential in the control of fatal autoimmunity throughout the lifetime of an organism. Foxp3 is the TF expressed by Tregs, representing the master regulator of the regulatory pathway in the development and function of these cells [Figure 2] (Hori et al., 2003). So beyond their ability to become effector cells, $CD4^+$ T lymphocytes can become Foxp3-expressing regulatory T cells that regulate immune homeostasis. Moreover, it was discovered a $CD4^+$ T cell population named type 1 regulatory T cells (Tr1), that differed significantly from $CD4^+Foxp3^+$ Treg cells, largely due to $Foxp3^-$ phenotype. These $CD4^+Foxp3^-$ Treg cells appeared to exert their immunosuppressive functions through the expression of the anti-inflammatory cytokine IL-10 (Groux et al., 1997), however, no uniquely expressed TF has been identified in Tr1 cells so far (Zeng et al., 2015).



Carbo et al, Front Cell Dev Biol 2014

Figure 2. Heterogeneity of CD4$^+$ T cell subsets. In the effector phase of the response, the effector CD4$^+$ T cells respond to antigen by producing cytokines that have several functions. Cytokines inducing Th differentiation, lineage-defining TFs, effector cytokines are indicated.

Differentiated effector cells exit secondary lymphoid organs and enter the circulation migrating toward the site of infection in peripheral tissues. Migration of effector T lymphocytes is allowed by the down-modulation of homing receptors that retain cells in the lymph nodes. One of the major receptor involved in maintaining T cells in the lymph node is the CCR7 chemokine receptor (Förster et al., 1999) that normally, binding to its ligands (CCL19 and CCL21) expressed in the T cell zones of secondary lymphoid organs, retains naive T cells there. Upon activation of T cells, specific chemokines and cytokines produced at the site of infection induce effector T cells to express chemokine receptors and adhesion molecules, and allowing them to preferentially home to inflammatory sites. Once the primary response is completed and the infection is cleared, a drastic contraction of the effector T cell population occurs. This happens because once the antigen is eliminated, the signals that allow activated lymphocytes to survive and proliferate are no longer available.

However, while the majority of effector cells are eliminated after accomplishing their functions, some differentiated cells will persist as long-lived memory T lymphocytes. Compared to their naive counterparts specific for the same antigen, memory T cells have less stringent requirements for subsequent activation via antigenic and costimulatory receptors, an increased proliferative potential, and a more rapid effector response. IL-7 and IL-15 induce the expression of high levels of antiapoptotic proteins and stimulate low-level proliferation, both of which maintain populations of memory T cells during long periods (Surh and Sprent, 2008). Memory cells can traffic not only through draining secondary lymphoid organs, but also through peripheral tissues that are instead poorly accessed by naive T cells. Collectively, these characteristics allow the production of an in situ response to reinfection in a shorter time respect to primary response.

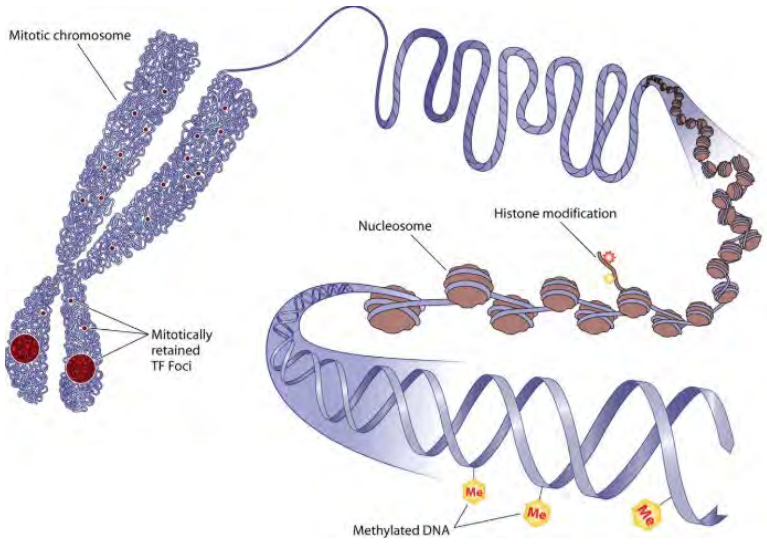
$CD4^+$ memory T cells can be subdivided into subsets according to their different homing characteristics, functions and cytokine profiles (Sallusto et al., 1999, 2004). Central memory T cells (T_{CM}) are characterized by high expression of the CCR7 homing receptor, consequently locating them in secondary lymphoid organs. T_{CM} cells show limited effector functions but they have an increased potential for proliferation upon antigen re-challenge. On the other hand, effector memory T cells (T_{EM}) do not express CCR7 and are thought to recirculate between blood and non-lymphoid tissues, or to remain poised to mobilize to the site of inflammation where they produce effector cytokines, such as IFN-γ, to rapidly eradicate pathogens, although their proliferative potential is more limited. Of note, a new lymphocyte lineage was recently identified and called tissue-resident memory T (T_{RM}) cells, representing T cells that occupy tissues without recirculating (Jiang et al., 2012; Masopust et al., 2010). T_{RM} derive from precursors that enter the peripheral tissues during the effector phase of immune responses, finally remaining positioned within this compartment. T_{RM} provide a first response against infections reencountered at body surfaces, where they accelerate pathogen clearance (Schenkel and Masopust, 2014).

3. Epigenetics

3. Epigenetics

3.1 Definition and epigenetic modifications

Conrad Waddington introduced the term epigenetics for the first time in the early 1940s (Waddington, 2012) defining it as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington, 1968). Over time, with an improving knowledge about genetics, epigenetics has been defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu Ct and Morris, 2001). Thus, currently the term epigenetics refers to all modifications in the genome, ideally heritable from one cell to another, that induce changes in gene expression without affecting the DNA sequence itself (Allis and Jenuwein, 2016). At a molecular level, epigenetic mechanisms include primarily covalent modifications of histone tails and cytosine bases in the DNA, resulting in the modulation of gene expression [Figure 3]. By establishing a distinctive transcriptional profile, epigenetic modifications contribute to the regulation of processes such as differentiation and commitment towards specific cell lineages.

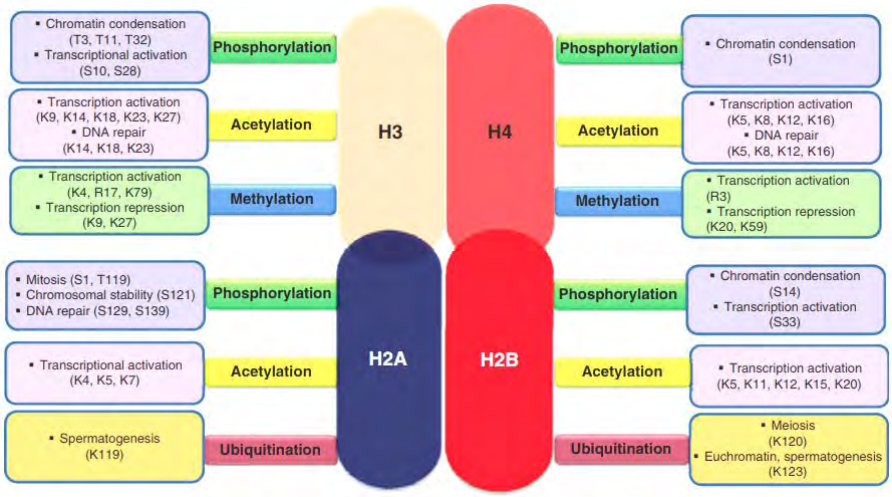


Adapted from: Zaidi et al, Mol Cell Biol 2010

Figure 3. Epigenetic mechanisms involved in the regulation of gene expression. Epigenetic mechanisms include histone modifications and DNA methylation, occurring respectively at the level of histone tails and cytosines. They contribute to the regulation of processes such as differentiation and commitment towards specific cell lineages.

The nucleosome is the fundamental repeating unit of chromatin that is a tightly condensed complex of DNA and proteins forming chromosomes within the nucleus of eukaryotic cells. Each nucleosome unit particle is constituted by an octamer of core histone proteins (H2A, H2B, H3, H4) around which a 147-bp segment of DNA is wrapped. Each histone octamer is composed of two H2.A-H2.B dimers and one H3-H4 tetramer; histone H1 instead is not part of the core nucleosome but it binds to the DNA stabilizing the core structure (Kouzarides, 2007). Nucleosome positioning, namely the localization of a single histone octamer on a specific region of DNA, contains already epigenetic information since with a specific pattern it modulates the accessibility of regulatory proteins and transcriptional machinery to the DNA, hence influencing gene expression. These nucleosome patterns have a dynamic nature, since multi-protein chromatin remodeling complexes can use ATP hydrolysis to slide or disassemble core histones; by doing so they can regulate transcription in a positive or negative manner (Bell et al., 2011; Ho and Crabtree, 2010). Histone proteins are characterized by a flexible amino-terminal tail protruding outward from the nucleosome, and mediating the interaction with other nucleosomes and regulatory factors. In particular, histone tails are subjected to covalent post-translational modifications (PTM) such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ribosylation (Rando and Chang, 2009) occurring at specific amino acidic positions. Such covalent modifications can alter DNA-histone interactions, impacting chromatin structure and recruitment of specific proteins; as a consequence histones PTMs are epigenetic marks with important roles in the regulation of transcription, DNA replication, DNA repair and cell cycle progression (Bhaumik et al., 2007) [Figure 4]. For example, acetylation is one of the most widely studied modifications of histones and it is catalyzed by histone acetyltransferase enzymes (HATs), while histone deacetylases (HDACs) are responsible for the reverse process by removing acetylation from histone tails. Acetylation neutralizes positive amino acids charges, reducing the affinity between histones and DNA and promoting gene activation. Acetylation of lysine 27 on histone 3 (H3K27ac) is indeed a common mark of active enhancers (sequences that can be located thousands of base pairs away from the transcription starting site (TSS) that, when bound by TFs enhance the

transcription of the associated gene). Another example is provided by the methylation of histone tails. This process is mediated by histone methyltransferases and it can influence gene expression both in a positive and negative manner. Indeed, trimethylation of lysine 4 on histone 3 (H3K4me3) is associated with gene activation, while trimethylation of lysine 27 on histone 3 (H3K27me3) is mainly found in silenced genes (Strahl and Allis, 2000). Of note, histones can be modified at multiple sites at the same time, giving rise to a cross-talk among the different marks specifying a particular outcome.



Jayani et al, Methods in Cell Biology 2010

Figure 4. Biological roles of major histone modifications. Here is shown a summary of the best characterized histone post-translational modifications, including histone modified site, modification pattern and proposed biological function.

The only epigenetic modification truly inheritable across cell division remains however the methylation of the fifth carbon of cytosine in the genomic DNA (5'-methylcytosine, 5mC), since this modification can be copied from one generation to the next through the action of the maintenance DNA methyltransferase DNMT1 (Leonhardt et al., 1992a). DNA methylation occurs primarily at the level of CpG (Cytosine-phosphate-Guanine) dinucleotides, and it is deposited by DNA methyltransferase enzymes (DNMT1, DNMT3a, DNMT3b). DNA methylation is conserved among most major eukaryotic groups including plant, fungi and animals (Feng et al., 2010), although the spectrum of DNA methylation levels and patterns is very broad. For example, the nematode worm *Caenorhabditis elegans* lacks detectable 5mC, correlating with the fact that it does not encode DNMT

enzymes, while the fly *Drosophila melanogaster* shows only very low levels of 5mC. The vertebrate genomes have instead generally high levels of DNA methylation, which is globally distributed over most of the genome (Bird, 2002).

As described in more details in the next chapters, DNA methylation plays a role in the control of gene expression. For example, the methylation of promoter regions generally leads to repression of transcription. Inhibition of transcriptional activation may result either from interference of DNA methylation on the recruitment of TFs, or may be caused by repressor proteins that specifically recognize and bind to this modification. Despite its stability, mammalian 5mC can be actively reversed to its unmodified state through enzymatic oxidation of 5mC to 5hmC (5'-hydroxymethylcytosine) and further oxidation products, catalyzed by Ten-eleven translocation (TET) 1-3 enzymes, and then followed by enzymatic removal (Wu and Zhang, 2017). Interestingly, 5hmC is not only an intermediate in the process of DNA demethylation, but it can also act as a stable marker for transcriptional regulation, enriched within gene bodies of highly transcribed genes and at functional regulatory elements such as enhancers (Rasmussen and Helin, 2016).

Aberrant DNA methylation is a common feature of human tumors, which often show extensive genomic hypomethylation and hypermethylation. In particular, DNA hypomethylation could contribute to cancer progression through loss of imprinting, inappropriate cell-type expression, activation of oncogenes, reactivation of transposable elements, and genomic instability. On the other hand, hypermethylation of tumor-suppressor genes blocks their expression triggering uncontrolled cell proliferation (Leoni et al., 2015a). Analogously, decreased expression of TET proteins and lower 5hmC levels are general hallmarks of many cancer types (Leoni et al., 2015b; Lian et al., 2012). However, the precise impact of altered TET activity on the onset, progression, and maintenance of these tumors is largely unknown and remains a topic of active research (Rasmussen and Helin, 2016). Moreover, some biological consequences of altered methylation do not necessarily link to disease. Indeed, *DNMT3A* and *TET2* in particular are frequently mutated in a number of hematological

malignancies, and mice lacking *Dnmt3a* or *Tet2* gene show altered hematopoiesis and can develop malignancies (Quivoron et al., 2011). However, the genes encoding for DNMT3a and TET2 can also be found mutated in healthy, aging individuals (Busque et al., 2012), and 'second hit' mutations in other genes are typically required for progression to a fully malignant phenotype (Ko et al., 2015). Consequently, these evidences point towards a potential role for *DNMT3A* and *TET2* mutations in increasing the risk of developing disease upon acquisition of further driver mutations.

In the next chapters, I will provide a detailed description of the general mechanism of DNA methylation and demethylation, as well as the functional roles that are known for these modifications in mammalian cells.

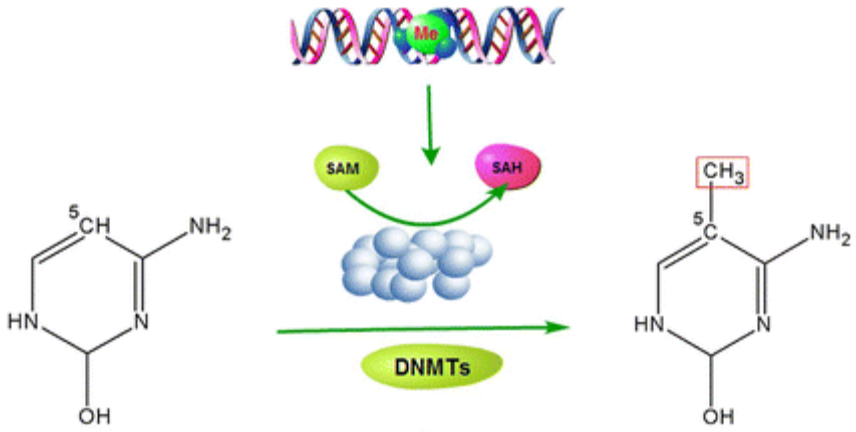
4. DNA methylation

4. DNA methylation

4.1 Writers of DNA methylation

The primary sites of DNA methylation are restricted to the CpG dinucleotide context, although in embryonic stem (ES) and neuronal cells, cytosines outside of CpGs have also been shown to be methylated (Lister et al., 2009). DNA methylation is involved in the regulation of many cellular processes. These include mammalian development, X chromosome inactivation, genomic stability during mitosis, parent-of-origin imprinting, protection against spurious activity of repetitive elements, and regulation of gene expression. The deposition of the methyl mark is mediated by the activity of three DNMT enzymes: DNMT1, DNMT3A and DNMT3B (Smith and Meissner, 2013). DNMT enzymes methylate DNA through a conserved mechanism involving the transfer of the methyl group from S-adenosylmethionine (SAM), acting as methyl donor, to the fifth carbon of a cytosine residue to form 5mC (Santi et al., 1983) [Figure 5]. Although these enzymes are highly conserved across different species and share a similar structure with a N-terminal regulatory domain and a C-terminal catalytic domain, they have unique functions and expression patterns. During DNA replication in the S phase of the cell cycle, DNMT1 acts as the maintenance methyltransferase, primarily responsible for copying the pre-existing DNA methylation pattern from the parental DNA strand on the newly synthesized nascent one. On the other hand, DNMT3A and DNMT3B are *de novo* methyltransferases able to deposit the methyl mark on fully unmethylated DNA. In agreement with this, DNMT1 is constitutively expressed in dividing cells, it prefers hemimethylated DNA as a substrate and it localizes at replication foci, ensuring inheritance of methylation patterns across cell division (Leonhardt et al., 1992b). DNMT1 is recruited to DNA replication forks through interaction with PCNA (proliferating cell nuclear antigen) and UHRF1 (ubiquitin-like with PHD and ring finger domains 1), which directs DNMT1 to hemimethylated sites by binding to the parental methylated strand (Smith and Meissner, 2013). The DNMT3A/B enzymes are required for the genome-wide *de novo* methylation of DNA that occurs after embryo implantation (Li et al., 1992) and in general, the DNMT

family members play an important role during mammalian development and cell differentiation. Indeed, deletion of the *Dnmt1* and *Dnmt3b* genes in mice is embryonically lethal, whereas gene knockout of *Dnmt3a* leads to lethality around four weeks after birth (Okano et al., 1999). In humans, somatic mutations in DNMT enzymes potentially contribute to malignant transformation (Zhang and Xu, 2017). In particular, it was found that genetic aberrations at *DNMT1* locus are rare in human hematopoietic cancers. Similarly, mutations of *DNMT3B* were identified in patients affected by a rare autosomal recessive disease termed ICF (immunodeficiency centromeric instability and facial anomalies) syndrome. Conversely the *DNMT3A* gene was found frequently mutated in myeloid and lymphoid malignancies and associated with poor prognosis (Ko et al., 2015). DNMT3A and DNMT3B can also be distinguished by their expression pattern: DNMT3A expression is ubiquitous and can be readily detected in most adult tissues, whereas DNMT3B is expressed at very low levels in most tissues except testis, thyroid and bone marrow (Xie et al., 1999). A final member of DNMT family is DNMT3L that however lacks the catalytic domain. Although DNMT3L has no catalytic function of its own, it associates with the DNMT3A and DNMT3B and stimulates their methyltransferase activity during the early stages of ES cells development (Jia et al., 2007).



Adapted from: Li et al, J Hematol Oncol 2017

Figure 5. Biochemistry of DNA methylation. Methylation of the carbon 5 (C5) of the aromatic ring of cytosines is catalyzed by DNMTs that transfer the methyl group from S-adenosylmethionine (SAM), acting as methyl donor, resulting in the final production of 5'-methylcytosine and S-adenosylhomocysteine (SAH).

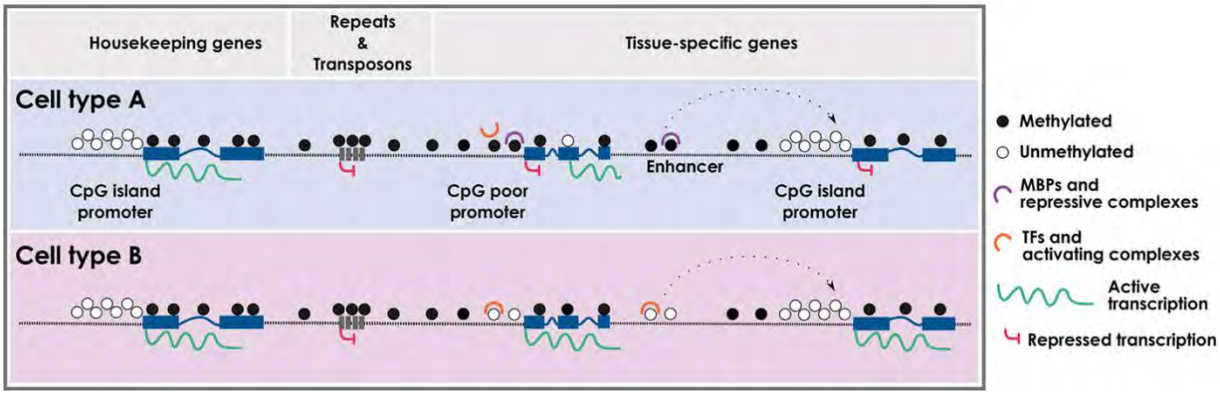
All DNMTs share a similar multi-domain structure composed by a variable N-terminal part including regulatory domains, and a conserved C-terminal region that harbors the catalytic methyltransferase domain (MTase) responsible for DNA chemical modification. The different specific activity of DNMTs enzymes is reflected in their different N-terminal domains (Jurkowska and Jeltsch, 2016). For example, the N-terminal part of DNMT1 contains domains that specify the anchoring to hemimethylated substrates at the replication fork of replicating DNA (Song et al., 2011). On the other hand, the N-terminal part of DNMT3A/B contains principally two functional domains. The ADD (ATRX-DNMT3-DNMT3L) domain is essential for the interaction with H3 histone tails and is blocked by the histone mark for transcriptional activation H3K4me3 (Otani et al., 2009); the PWWP (proline-tryptophane) domain (missing in DNMT3L) was shown to be essential for the targeting of the enzymes to pericentromeric repeats (Chen et al., 2004).

4.2 DNA methylation patterns and function

Genome wide DNA-methylation analysis showed that 60-80% of CpG sites in mammalian genomes are methylated, and about 10% of CpGs occur in regions characterized by an higher density of CpG dinucleotides called CpG islands (Smith and Meissner, 2013). CpG islands are largely resistant to DNA methylation, and are located primarily in the proximity of promoters of actively transcribed genes like housekeeping genes and developmental regulator genes (Deaton and Bird, 2011). Approximately 60% of TSSs correspond to CpG islands. However, a small fraction of CpG island promoters become methylated in a tissue-specific manner during early development or in differentiated tissues, and in these cases methylation is associated with stable transcriptional silencing [Figure 6] (Ambrosi et al., 2017; Smith and Meissner, 2013). DNA methylation plays a key role in a variety of processes that ensure proper gene expression during development, for example in the process of genomic imprinting, in which hypermethylation at one of the two parental alleles leads to monoallelic gene

expression. Imprinted genes include the extensively studied *Igfr2* (insulin-like growth factor 2 receptor) which was identified in 1991 (Barlow et al., 1991), but also several other genes such as *PHLDA2, PEG3, PEG10* and *PLAGL1*, most of which are involved in the control of fetal growth and behaviour after birth, highlighting the importance of DNA methylation-related processes for normal mammalian growth and development (Reik and Walter, 2001). DNA methylation is also essential for the stable silencing occurring during X-chromosome inactivation (XCI) in females; XCI is a mechanism of dosage compensation used in mammals to balance the expression of sex-linked genes between 46,XY males and 46,XX females, resulting in the silencing of the majority of genes on one of the two X chromosomes in each somatic cell in females (Portela and Esteller, 2010). XCI is the result of a combination of several epigenetic mechanisms that act synergistically to maintain the X inactive state. Specifically, XCI involves the action of two non-coding RNAs: *Xist* (X-inactive specific transcript) and its antisense transcript *Tsix. Xist* is expressed exclusively from the inactivated chromosome and works to modulate its epigenetic silencing; it binds to the polycomb repressive complex 2 (PRC2), leading to the deposition of the repressive histone mark H3K27me3 thereby inactivating chromosome expression (Zhao et al., 2008). Expression of *Xist*, which sets the inactivation process, is followed by widespread CpG islands methylation reinforcing the chromosome silencing (Keohane et al., 1996; Wutz and Jaenisch, 2000). On the other hand, *Tsix* expression, that is silenced in the inactivated chromosome, is essential to restrict *Xist* activity on the active copy of the X chromosome, either by antisense-binding of *Xist* or through the recruitment of enzymes catalyzing DNA-methylation of the *Xist* promoter, which results in its silencing (Panning and Jaenisch, 1996). The importance of DNA methylation in contributing to genome stability is further highlighted by its role in the silencing of non-coding sequences, including pericentromeric repeats and repetitive elements, such as long or short interspersed transposable elements (LINEs or SINEs), characterized by a latent transcriptional potential [Figure 6]. The repression of pericentromeric satellite repeats is essential for a correct chromosome alignment, segregation and integrity during mitosis. Transposable elements, since they often contain strong promoters elements, are constitutively hypermethylated to prevent

their activity, which may cause chromosomal instability, translocations and gene disruption (Smith and Meissner, 2013).



Carrio' et al, Front Aging Neurosci. 2015

Figure 6. Schematic representation of the mammalian methylome and role in gene expression. CpG island promoters are usually protected from DNA methylation and are prone to active transcription. CpG-poor regions (intergenic) and repetitive elements are typically methylated, with the exception of enhancers and CpG-poor promoters that can be differentially methylated in a cell type-specific fashion. Intragenic regions can also be differentially methylated leading to specific cell-type transcripts. MBPs: methyl-binding proteins; TFs: transcription factors.

4.3 DNA methylation and its readers: a role in gene expression

While the majority of genomic methylation patterns remain stable in cells after development, modifications of levels of DNA methylation can also occur dynamically at specific regulatory regions, to modulate gene expression during cell differentiation and in response to environmental signals. The functional read-out of DNA methylation is context dependent; in general, CpG island methylation is associated with gene silencing. Indeed DNA methylation was found in most CpG rich promoters and in low-to-intermediate CpG distal regulatory regions such as enhancers of inactive genes (Jones, 2012). In particular, the presence of methylation marks at TSSs is usually associated with lack of gene transcription that could be mediated either through attraction or repulsion of regulatory factors. For example, methylated-CpGs attract methyl-CpG-binding domain (MBD) proteins (MBD1,2,4 and MeCP2) in a sequence-independent manner (Hendrich and Bird, 1998). Binding of MBD proteins could directly prevent TFs from binding because of steric hindrance or through the recruitment of

histone modifying and chromatin remodeling complexes [Figure 6] (Baubec et al., 2013). Conversely other readers recognize methyl-CpGs by zinc-finger domains in a sequence-dependent context; these factors include KAISO, ZBTB4, ZBTB38 and ZFP57 (Filion et al., 2006). For example, by recognizing the methylated TGCCGC hexanucleotide, ZFP57 recruits its cofactor KAP1 and other chromatin and DNA modifiers to methylated, imprinted regulatory regions. This general mechanism resulted to be important in the maintenance of DNA methylation at imprinting control regions during early embryonic development, protecting specific loci against DNA demethylation (Quenneville et al., 2011). On the other hand many factors known to recognize CpG-containing sequences are not able to bind when the cytosine is methylated. For example by analyzing the effect of DNA methylation on TF binding in mouse ES cells, it was shown that NRF1 (nuclear respiratory factor 1) is a methylation-sensitive TF; indeed, the induction of local hypermethylation led to a loss of NRF1 binding, demonstrating the competition between TF binding and CpG methylation (Domcke et al., 2015). Methylation of DNA occurs also at lower CpG density regions called “CpG islands shores” located up to 2 kb upstream of CpG islands (Irizarry et al., 2009). The methylation of CpG islands shores is associated with the transcriptional inactivation of the downstream gene (Portela and Esteller, 2010). Most of the tissue-specific DNA methylation patterns occur at the level of CpG island shores, and 70% of differentially methylated regions during developmental reprogramming were indeed found to be associated with them (Doi et al., 2009). As mentioned before, the read-out of DNA methylation is context-dependent and DNA methylation does not necessarily and invariably correlate with transcriptional repression. For instance, gene body methylation usually positively correlates with gene expression. Specifically, it was shown that DNMT3B, by recognizing H3K36me3 (histone H3 lysine 36 trimethylation), participates in the maintenance of gene body methylation (Baubec et al., 2015), and DNMT3B-dependent intragenic DNA methylation appeared to protect the gene body from spurious RNA polymerase II entry, avoiding cryptic transcription initiation from internal exons (Neri et al., 2017).

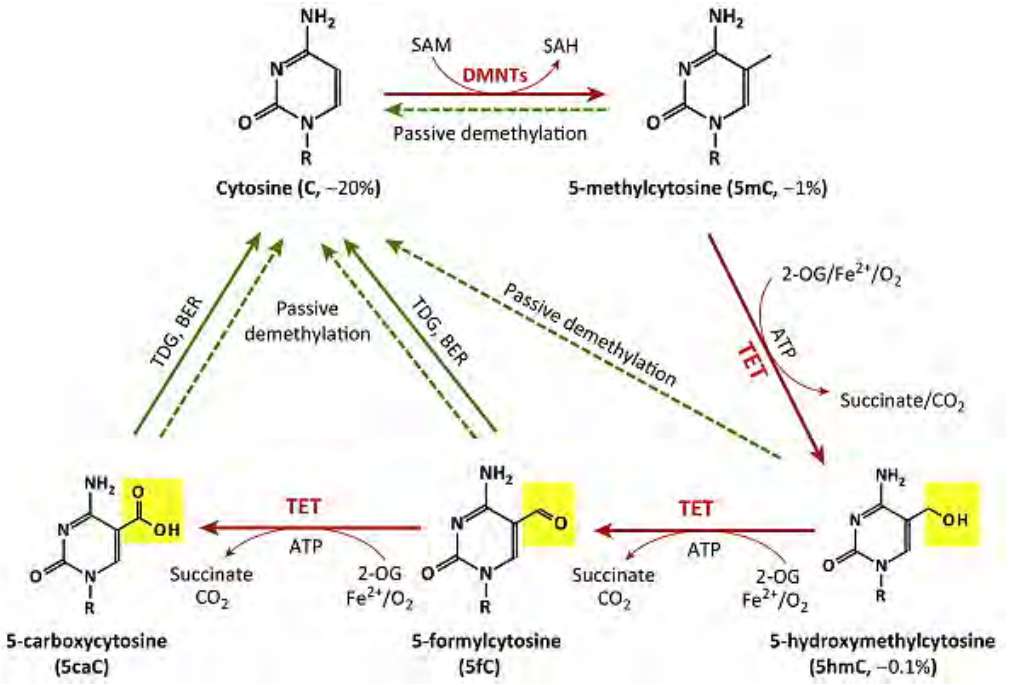
4.4 DNA demethylation

Despite its stability and heritability across cell division, dynamic changes in 5mC deposition are observed during development and differentiation, and are necessary for the establishment of stable cell-specific gene expression programs. Hence, DNA demethylation (removal of the methyl mark) is an integral component of the epigenetic regulatory network (Ambrosi et al., 2017).

4.4.1 Passive *versus* active DNA demethylation

Once deposited in the genome, 5mC can be erased either through active or passive mechanisms [Figure 7]. Passive DNA demethylation is based on the dilution of the methyl mark through DNA replication during cell division. Specifically, DNMT1 is needed to methylate hemimethylated sites that are generated during DNA replication, and the absence of a functional DNA methylation maintenance machinery leads to 5mC dilution during successive rounds of cell divisions (Wu and Zhang, 2017). On the other hand, active DNA demethylation is potentially a replication-independent process involving iterative enzymatic oxidation steps leading to the modification of 5mC and its replacement with an unmodified cytosine. In particular, in mammals active DNA demethylation is mediated by the enzymatic activity of TET (1-3) proteins. The 5mC mark is first oxidized by TET enzymes to 5hmC and then to further oxidation products (5-formylcytosine, 5fC and 5-carboxylcytosine, 5caC) (Ito et al., 2010; Tahiliani et al., 2009). These oxidized products are recognized and excised by the thymine-DNA-glycosylase (TGD) enzyme, creating abasic sites that are replaced with unmodified cytosines through mechanism of base excision repair (BER) (He et al., 2011). The deposition of 5hmC can also have a role in the induction of passive DNA demethylation since DNMT1 does not efficiently recognize 5hmC-modified bases (Valinluck and Sowers, 2007). Consequently TET-mediated oxidation of the methylation mark could trigger passive replication-dependent DNA demethylation (Rasmussen and Helin, 2016; Valinluck and Sowers, 2007).

In the next section I will describe the discovery, catalytic activity and role in gene expression of TET proteins and 5hmC.



Huang et al, Trends Genet. 2014

Figure 7. Overview of DNA methylation and demethylation pathways in mammals. Active demethylation is achieved by iterative oxidation of the methyl group of 5mC by TET proteins and restoration of unmodified cytosines. On the other hand, passive demethylation of modified cytosines, 5mC (TET-independent) or 5hmC (TET-assisted), is based on the dilution of the methyl mark through DNA replication during cell division.

5. DNA hydroxymethylation

5. DNA hydroxymethylation

5.1 Discovery of 5hmC and TET proteins

The first identification of 5hmC in vertebrates occurred in 1972, when it was identified in the DNA of rat, mouse and frog brain and in rat liver and spleen. The occurrence of 5hmC was identified by paper chromatography, UV spectra in acid and alkaline solutions and by its conversion into 5-hydroxymethyluracil (5hmU) (Penn et al., 1972). However, other researchers failed to replicate these initial findings in later studies (Kothari and Shankar, 1976), and 5hmC was thought to be the by-product of oxidative DNA damage with no established functional role (Tardy-Planechaud et al., 1997). It took more than 30 years to confirm the presence of 5hmC as a stable DNA modification and to gain insights into its molecular function as epigenetic mark. A turning point occurred in 2009, when by taking advantage of more advanced analytical techniques, such as 2D thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) coupled to mass spectrometry analysis, 5hmC was rediscovered in Purkinje and granule neuronal nuclei of adult mouse brains (Kriaucionis and Heintz, 2009). At the same time, another research group independently observed 5hmC to be abundant in mouse ES cells (Tahiliani et al., 2009). *Tahiliani et al.* used bioinformatic tools to predict that TET (1-3) proteins would likely oxidize 5mC to 5hmC. Specifically, the prediction that TET enzymes might have DNA-modifying activity was based on the analysis of *Trypanosoma brucei* J-binding protein (JBP) 1 and 2. JBPs are Fe^{2+}- and 2-oxoglutarate (2-OG)-dependent dioxygenases that catalyze the oxidation of the methyl group of thymine resulting in the production of 5hmU. In protists, 5hmU can be further glycosylated producing the so-called “base J” (β-D-D-glucosyl-hydroxymethyluracil). Like 5mC, base J was shown to be associated with gene silencing (Borst and Sabatini, 2008). By performing a computational search for homologs of JBP1 and JBP2, mammalian TET1 enzyme was thereby shown to be involved in the modification of 5mC (Tahiliani et al., 2009). Notably, human TET1 was initially identified in 2003 as a fusion partner with MLL1 (mixed-lineage leukaemia 1) gene; this fusion results from a translocation, found in rare cases of acute myeloid and

lymphocytic leukaemia, between human chromosome 10 and chromosome 11 (Lorsbach et al., 2003). However, despite a potentially important role in malignant transformation, no functional roles were associated to TET1. Following the initial work on the discovery of TET1 as an enzyme able to modify 5mC to 5hmC, the other two TET family proteins, TET2 and TET3, were also shown to possess 5mC oxidizing activity (Ito et al., 2010). In summary, work performed by *Tahiliani et al.* proposed 5mC oxidation products as intermediates in the conversion from 5mC to the unmethylated cytosine, providing the first evidences for a pathway of active DNA demethylation in mammals. So the discovery of 5hmC and TET proteins represents the major breakthrough in the epigenetic field of the last years, providing a new perspective on how DNA modification influences the regulation of gene expression.

5.2 Levels and distribution of 5hmC

Mass spectrometry analyses suggest that, unlike 5mC, the levels of 5hmC are highly variable in different mammalian tissues (Ito et al., 2011). Consistent with the fact that the highest recorded levels of 5hmC were identified in the brain, 5hmC was found to be most abundant in cerebellar Purkinje neurons, where it comprises nearly 40% of the level of 5mC (Kriaucionis and Heintz, 2009). Moreover, in murine ES cells about 5-10% of 5mC is converted to 5hmC (Tahiliani et al., 2009). In contrast, somatic tissues such as kidney, heart, spleen and thymus contain medium to low levels of 5hmC compared to neuronal tissues (Bachman et al., 2014; Globisch et al., 2010). Globally, the content of 5hmC was shown to vary across different mouse tissues regardless of the levels of 5mC observed in those same tissues (Nestor et al., 2012). Such 5mC-independent distribution suggested that 5hmC may be not only an intermediate in the process of DNA demethylation, but it may represent a stable epigenetic mark in its own right. Concordant with this idea a recent study, using stable isotope labelling of cytosine derivatives in the DNA of mammalian cells followed by ultrasensitive tandem liquid-chromatography mass spectrometry, showed that the majority of 5hmC

is a stable modification, as opposed to a transient intermediate (Bachman et al., 2014). In contrast to DNA methylation, which occurs immediately during replication, 5hmC appeared to form slowly during the first 30 hours following DNA synthesis. Moreover isotopic labelling of DNA in mouse tissues confirmed the stability of 5hmC *in vivo* (Bachman et al., 2014).

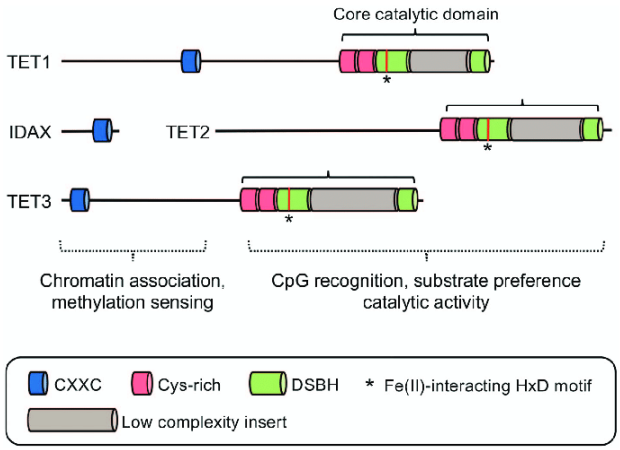
5.3 TET proteins: expression and catalytic activity

Functional evidences suggest that TET proteins are crucial for different biological processes, including zygotic epigenetic reprogramming, pluripotent stem cell differentiation, and hematopoiesis. Accordingly certain mutations in TET proteins were found to be associated with the onset of hematological diseases and with a wide variety of human solid tumors. In particular, TET1 is highly expressed in ES cells and its expression is reduced as soon as the cells are differentiated into embryoid bodies (Koh et al., 2011). On the other hand, TET2 is expressed at lower levels in ES cells but it increases upon cell differentiation, and it shows robust expression in numerous differentiated organs and adult tissues. Finally, TET3 is highly expressed in the zygote (Pastor et al., 2013). Genetic knockout mouse models underlined the physiological importance of TET proteins during development. Even though *Tet1* and *Tet2* were shown to regulate murine ES cell lineage specification, deletions of either *Tet1* (Dawlaty et al., 2011) or *Tet2* (Moran-Crusio et al., 2011) resulted in viable mice with normal embryonic and postnatal development; in contrast, mice with constitutive deletion of *Tet3* died perinatally (Gu et al., 2011). These findings suggested that *Tet3* may have a unique role during early stages of embryonic development that cannot be compensated by the other TET proteins. When generating double-knockout mice deficient for both *Tet1* and *Tet2*, it was noticed that, while some survived and developed normally, the majority died perinatally and showed a wide spectrum of defects such as exencephaly, growth retardation and compromised imprinting (Dawlaty et al., 2013). In contrast, deletion of both *Tet2* and *Tet3* resulted in the rapid onset of an aggressive myeloid leukemia (An et al., 2015). Finally, the successful generation of *Tet1/2/3* triple-knockout (TKO) ES cells underlined how the interplay between the different TET

proteins activity is central for supporting embryonic development. In fact, the combined deficiency of all three TET proteins depleted 5hmC from the genome and impaired ES cell differentiation, leading to the formation of poorly differentiated TKO embryoid bodies and teratomas (Dawlaty et al., 2014). Considering that single-knockout and some double-knockout survive until adulthood, it was hypothesized the possible late onset of phenotypes; indeed, loss of *Tet2* led to normal development, and only around at 1 year of age mice showed dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies (Li et al., 2011), whereas *Tet1* deficiency led to dysregulated B-cell lineage differentiation (Cimmino et al., 2015). In agreement with the mouse data, TET1 and TET2 are involved in cancer development in humans. TET1 was reported to function as an oncogene in MLL-rearranged leukemias (Huang et al., 2013), while TET2 acts as a tumor suppressor in all hematopoietic cell types, since deletions and loss-of-function mutations of the *TET2* gene are associated with the development of lymphoid malignancies, myeloid leukemias, and myeloproliferative neoplasms (Ko et al., 2015).

As for the general structure of TET proteins, these enzymes are 2-OG and Fe^{2+}-dependent dioxygenases. All TET proteins contain a conserved DSBH (double-stranded β-helix) domain, a cysteine-rich domain that wraps around the DSBH core to stabilize the overall structure and TET-DNA interaction, and binding sites for the cofactors Fe^{2+} and 2-OG that together form the catalytic core region in the C-terminus (Iyer et al., 2009). In addition to their catalytic domain, TET1 and TET3 contain an N-terminal CXXC domain (a Zn^{2+}-chelating domain characterized by the signature amino acid sequence $CGXCXXC(X)_NC$ in which X represents any amino acid) through which they are most likely recruited to genomic DNA target sites [Figure 8]. The CXXC domain preferentially binds to unmethylated CpG sequences *in vitro*, and it is frequently found in proteins preferentially binding at CpG-rich promoters and CpG islands (which are mainly unmethylated) (Ko et al., 2013; Xu et al., 2011, 2012; Zhang et al., 2010). Differently from TET1 and TET3, during evolution TET2 underwent a chromosomal inversion event in which the exon encoding for the CXXC domain was detached and became a separate gene encoding IDAX (Ko et al., 2013), resulting to be the interacting partner of

TET2 for the recruitment to unmethylated DNA, predominantly at CpG islands (Iyer et al., 2009). Interestingly, the CXXC domain is able to regulate the levels of DNA methylation by preventing unwanted DNMTs activity in murine ES cells. Specifically, it was shown that TET1 controls levels of DNA methylation in two ways: first by binding to CpG-rich regions through the CXXC domain, hence excluding DNMT1 binding and preventing its methyltransferase activity, and second through the conversion of 5mC to 5hmC (Xu et al., 2011). Apart from site-specific recruitment, the activity of TET enzymes can be modulated at a global level in the presence of specific cofactors and PTMs. For example, vitamin C (ascorbic acid) was shown to stimulate the catalytic activity of TET enzymes in both cultured cells and mouse tissues (Blaschke et al., 2013). The underlying mechanism is likely to involve the interaction of vitamin C with the catalytic domain of TET enzymes, providing a local reducing environment that increases the recycling efficiency of the Fe^{2+} cofactor (Yin et al., 2013). Regarding PTMs, TET enzymes can be monoubiquitinylated at a specific lysine residue (K1299) by the E3 ubiquitin ligase complex CLR4 potentially inducing a conformational change that favors binding of TETs to the DNA (Yu et al., 2013).



An et al, Exp Mol Med. 2017

Figure 8. Domain structure of TET proteins. TET proteins share similar structure subdivided between N-terminal variable region and C-terminal conserved catalytic domain.

5.4 5hmC and regulation of gene expression

Besides serving as demethylation intermediate, 5hmC may carry out additional functions as an epigenetic mark with functional roles in the regulation of gene transcription (Cimmino and Aifantis, 2017). Indeed, as mentioned above, large-scale mapping revealed that the global content of 5hmC in different mouse and human cell types varies in a 5mC-independent manner (Nestor et al., 2012). Along the same line, confirming the concept that 5hmC and its oxidized forms could have an independent function from 5mC, deletion of TET proteins does not always lead to increased levels of 5mC at specific genomic loci (Huang and Rao, 2014).

The recent development of enrichment-based and single base-resolution techniques to analyse 5hmC, provided a more complete picture of its distribution across the mammalian genome, giving insights into the possible functional role of 5hmC in transcriptional regulation. For example, in murine ES cells data showed that 5hmC levels are low at promoters of highly expressed genes, characterized by high CpG density and marked by H3K4me3 (commonly associated with the activation of transcription of nearby genes). In comparison, promoters with low-to-intermediate CpG density have higher levels of 5hmC (Booth et al., 2012; Wu et al., 2011; Yu et al., 2012).

Of note, in ES cells high level of 5hmC is observed at promoters characterized by bivalent histone marks: H3K27me3 (associated to gene downregulation thorough the formation of heterochromatin regions) and H3K4me3. These promoters correspond to genes transcriptionally inactive in ES cells but then activated upon differentiation. The same correlation with bivalent domains is not true for 5mC (Fouse et al., 2008). This evidence suggests the hypothesis of a unique functional contribution of 5hmC: it could be involved in the maintenance of an inactive chromatin configuration, but poised to rapid activation in response to the appropriate signals (Pastor et al., 2011).

Moreover 5hmC showed a deposition in gene bodies from TSSs to transcription termination sites (TTSs), emphasizing its potential correlation with transcriptional elongation (Sun et al., 2015). 5hmC is also enriched at distal regulatory elements such as enhancers, which are often regions of low-to-intermediate CpG density with reduced levels of DNA methylation respect to neighbouring regions

(Stadler et al., 2011). Specifically, single-base resolution mapping highlighted that 5hmC is strongly enriched at flanking regions of TF binding sites, but relatively low at the precise site of binding, further emphasizing that 5hmC may have a regulatory role in transcriptional activation (Yu et al., 2012). However, due to the absence of data on the binding of TF to enhancers in TET-deficient cells, there are still open questions regarding the precise mechanism involving 5hmC and TET enzymes as possible transcriptional regulators. One possibility could be that 5hmC 'opens' the chromatin structure allowing the binding of TFs, or that TFs recruit TET enzymes catalyzing the formation of 5hmC at these regulatory regions. On the other hand, the depletion of 5hmC at the precise TF binding site could be explained by a process of TET-assisted demethylation, or by a physical blockage by TFs of the access of TET enzymes in this region. In agreement with this latter possibility, in differentiating ES cells (Sérandour et al., 2012), 5hmC levels strongly increase at activated enhancers marked also by acetylation of H3K27, a common mark of active enhancers. Consistent with these results, mouse ES cells lacking a functional TDG pathway (thereby with an impaired DNA demethylation machinery) showed an accumulation of 5fC and 5caC at enhancer regions, further corroborating the fact that active demethylation processes take place at this regulatory region (Shen et al., 2013). Along the same line, recent work showed that in ES cells TET1 recruits the SALL4A TF at enhancer regions and, this process allows the subsequent recruitment of TET2, which mediates further oxidation of 5hmC present at SALL4A-bound sites (Xiong et al., 2016). These results suggest that TET1 and TET2 cooperate and are coordinated by SALL4A that in turn recognize 5hmC. Moreover SALL4A was shown to be a genomic region-specific regulator of 5hmC deposition (Xiong et al., 2016). The use of quantitative mass spectrometry-based proteomics techniques further supports the growing evidence of 5hmC as a stable epigenetic mark for the recruitment of chromatin or transcription regulators. Indeed it was shown that specific proteins bind selectively to 5hmC in ES cells, neural progenitor cells and mouse adult brain (Spruijt et al., 2013). These proteins include the neural progenitor cell-specific protein UHRF2, TF such as zinc-fingers and homeoboxes protein 1 (ZHX1), ZHX2 and THAP domain-containing protein 11 (THAP11), and several uncharacterized

proteins (Spruijt et al., 2013). In addition, two methyl-binding proteins, methyl-CpG-binding protein 2 (MeCP2) and methyl-CpG-binding domain protein 4 (MBD4), were shown to recognize and bind 5hmC (Mellén et al., 2012; Otani et al., 2013). In particular, the work of *Mellen et al.* identified a role for MeCP2 in the regulation of chromatin structure, although a mechanism by which MeCP2 binding to 5hmC could regulate chromatin accessibility remains to be determined.

Overall these findings strongly corroborate the potential involvement of TET proteins and 5hmC in the regulation of gene expression, but further investigation is needed to elucidate how proteins that bind 5hmC contribute to gene regulation, and how catalytic activity of TET enzymes is influenced by the interaction with other proteins.

Regarding the activity of TET enzymes, there is increasing evidence showing that these proteins may also work independently of their catalytic activity by repelling or recruiting other factors. For instance, TET1 can be involved in transcriptional regulation through its interaction with the SIN3A histone deacetylase complex (Williams et al., 2011); similarly TET2 can regulate transcription by recruiting histone deacetylase 2 (HDAC2) (Zhang et al., 2015). Along the same line, it was shown that TET proteins in ES cells can modulate transcription in a catalytic-activity-independent manner by recruiting O-linked N-acetylglucosamine transferase (OGT) (Vella et al., 2013).

Further evidence supporting the hypothesis of a catalytic-activity independent function of TET proteins comes from data demonstrating that mutated forms of TET proteins can have biological function although catalytically inactive. For example, in mouse hippocampus, overexpression of a catalytically inactive form of TET1 affects memory in a way similar to the wild-type TET1 (Kaas et al., 2013); during the inflammation resolution of innate myeloid cells, catalytically inactive TET2 recruits HDAC2 and in so doing it represses IL-6 transcription (Zhang et al., 2015); and in HEK 293T cells overexpression of wild-type TET1 and its mutated form showed highly similar transcriptional changes (Jin et al., 2014). Finally, in mast cells, hyperproliferation resulting from TET2 deletion could be rescued by a catalytically dead mutant (Montagner et al., 2016).

Overall these data strongly suggest a role of TET enzymes and 5hmC in the regulation of gene expression, with multiple levels of regulation contributing to the final definition of specific gene expression patterns. Indeed, transcriptional activation or repression can be accomplished depending on the specific genomic region, and also on the activity of interacting factors able to 'read' 5hmC modification, or to influence TET enzymes catalytic activity.

6. Epigenetics in T lymphocytes

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Following antigenic stimulation, antigen-inexperienced naive T cells proliferate and differentiate into an array of effector and memory subsets, with responses specific for the elimination of the pathogen being recognized. The controlled activity of TFs and epigenetic mechanisms define the specific transcriptome of each subset (Bevington et al., 2016). Indeed, several genes controlling immune functions are known to be regulated by epigenetic modifications (Leoni et al., 2015a). In the next section I will describe the impact of DNA methylation and DNA hydroxymethylation in T lymphocytes development, differentiation and acquisition of effector functions.

6.1 Role of DNA methylation in immune-regulation

Mouse models lacking DNMT enzymes showed that DNA methylation can be dynamically modulated during T cell development and lineage-specific gene expression programs. Indeed, the generation of mice lacking *Dnmt1* at sequential stages of T cell development, showed for example that double-negative thymocytes have an impaired activation-induced proliferation; moreover naive T cells in the periphery have increased cytokines expression. This work was the first demonstration that *Dnmt1* and DNA methylation are required for the proper expression of specific genes important to define lineage and dictate function in T cells (Lee et al., 2001). In subsequent work it was also shown that in the absence of DNMT1, murine $CD4^+$ and $CD8^+$ T lymphocytes are not able to silence properly the *Il4* locus, resulting in increased and dysregulated production of T_H2-type cytokine (Makar and Wilson, 2004; Makar et al., 2003). Moreover, T cell activation in the presence of specific cytokines promoting T_H2 polarization, reduced DNMT1 recruitment to the *Il4-Il13* locus, eventually leading to enhanced gene expression (Makar et al., 2003). These results further underline the central role of DNMT1 and DNA methylation for proper cytokine expression and lineage commitment. Dysregulated and promiscuous cytokine expression was observed even in the absence of DNMT3A (but not DNMT3B); indeed murine $CD4^+$ T lymphocytes showed increased and simultaneous expression of IFN-γ and IL-4

after expansion under non-polarizing conditions. In details, DNMT3A was shown to be required for the establishment of a normal pattern of DNA methylation at the *ifng* locus under T_H2 polarizing conditions, but it becomes dispensable for directing the appropriate expression of IFN-γ and IL-4 in already T_H1 and T_H2-polarized cells; however T_H2 cells lacking *Dnmt3a* showed increased expression of IFN-γ under T_H1 polarizing conditions (Gamper et al., 2009).

These results indicate that DNMT3A has a critical role in stabilizing T cell gene expression through the methylation of selective targets. More recently, methylome analysis of murine $CD4^+$ T cells investigated changes in DNA methylation patterns during the process of T cell differentiation, and it emphasized changes in chromatin architecture concomitant with the acquisition of effector/memory phenotype upon naive T cell stimulation. In addition, methylation profiles were different between memory T cell subsets, indicating a link between the status of T cell methylation and T cell differentiation (Hashimoto et al., 2013). A global DNA methylation remodeling accompanying the acquisition of effector functions and repression of the naive cell state was observed also in murine $CD8^+$ T lymphocytes (Scharer et al., 2013).

These observations were confirmed also in human; indeed genome-wide characterization of epigenetic changes in human $CD4^+$ T lymphocytes showed differential methylation between memory and naive cells, connected to differential gene expression following activation. In particular, memory cells were characterized by a progressive global loss of DNA methylation correlating with activation-induced gene expression compared to naive cells (Durek et al., 2016; Komori et al., 2015).

The central role played by DNA methylation in the proper regulation of immune responses was observed also in Treg cells, characterized by the expression of the TF Foxp3. Mutations in the *Foxp3* gene cause the scurfy phenotype in mice and IPEX (immune dysfunction, polyendocrinopathy, X-linked) syndrome in humans (Lal and Bromberg, 2009). The maintenance of Tregs phenotypic stability is strictly connected with a stable Foxp3 expression. There are different mechanisms to preserve Treg cell identity (Feng et al., 2014). In particular, a stable Foxp3 expression was associated with selective demethylation of a regulatory region within the Foxp3 locus called TSDR (Treg-specific

demethylated region); this work demonstrated that epigenetic imprinting of this region is important for the establishment of a stable Treg lineage (Polansky et al., 2008). However, DNA methylation appears to have a complex role in regulating Treg stability and function. Expression of DNMT1 (but not DNMT3A) was shown to be necessary for Treg development and function; in fact, deletion of DNMT1 did not affect methylation patterns within Foxp3, but decreased global DNA methylation altering the Treg expression of many pro-inflammatory genes, and mice with conditional deletion of DNMT1 in Tregs died because of lethal autoimmunity (Wang et al., 2013).

6.2 Role of DNA hydroxymethylation in immune-regulation

Before the discovery of TET proteins, DNA demethylation was thought to occur exclusively through a passive process connected with cell proliferation, where DNMT1 fails to methylate the nascent DNA strand during the S phase of the cell cycle. But growing evidences suggested a possible role for DNA demethylation in immune genes transcriptional regulation, mediated by an active enzymatic mechanism. Indeed, upon activation of murine $CD4^+$ T lymphocytes a rapid and specific demethylation of the *Il2* promoter was observed, in absence of DNA replication and causally affecting *Il2* gene transcription. This work suggested that the T cells possessed an active enzymatic mechanism for demethylation involved in the regulation of immune gene expression (Bruniquel and Schwartz, 2003). Similarly, modifications in the level of DNA methylation were observed at the *Ifng* locus in memory $CD8^+$ T lymphocytes; the *Ifng* gene promoter was partially methylated at resting state, but became rapidly demethylated upon antigenic stimulation in a proliferation-independent manner, inferring again a demethylase activity (Kersh et al., 2006).

The discovery of the TET enzymes (Tahiliani et al., 2009) provided a new perspective on several potential mechanisms by which they might mediate DNA demethylation, suggesting a possible role of DNA hydroxymethylation and TET proteins in the regulation of T cell development and differentiation. Genome-wide mapping of 5hmC distribution in murine thymocytes and peripheral T

cells at several developmental stages, provided a characterization of 5hmC dynamics during thymic development and peripheral T_H1 and T_H2 differentiation. One remarkable finding was the tight connection, observed in differentiating T cells, between gain of gene expression and gain of 5hmC; indeed intragenic 5hmC levels were high in gene bodies of highly expressed genes, and this was correlated with marks of active transcription (H3K4me3, H3K27me3) (Zhou et al., 2011).

In parallel, by investigating 5hmC dynamics at different stages of T cell development, it was found that 5hmC is enriched in active thymus-specific enhancers during the transition from double negative thymocytes to single positive CD4 and CD8, again emphasizing the correlation with gene expression (Tsagaratou et al., 2014). Together, these data provided valuable insights into the potential biological roles of 5hmC and set the bases for the investigation of 5hmC function in immune-regulation. However, germline deletion of mouse *Tet2* led to largely normal B and T lymphocyte development (Tsagaratou et al., 2017), although *Tet2* deletion in early lymphoid progenitors showed a partial reduction of IFN-γ and IL-17 expression in T_H1 and T_H17 cells respectively (Ichiyama et al., 2015), suggesting that TET2 promotes DNA demethylation and activation of cytokine gene expression in T cells. The important discoveries made on the role of TET-5hmC during differentiation of mammalian $CD4^+$ T cells obtained from mouse models, were confirmed also in humans, revealing the importance of DNA demethylation in terms of 5hmC in human $CD4^+$ T cell differentiation. Specifically, *Nestor et al.* generated genome-wide 5hmC and gene expression profiles during *in vitro* differentiation of $CD4^+$ naive T lymphocytes into the T_H1 and T_H2 subsets. They showed a global reduction in genomic 5hmC during both activation and differentiation of $CD4^+$ T cells, associated with a modest gain of 5hmC at specific genomic regions involved in T cell activation. These findings were confirmed in $CD4^+$ memory T cells ex *vivo*, suggesting that early remodeling events persists in long-term differentiated cells (Nestor et al., 2016). These results support the concept of an important role of 5hmC in human gene regulation and lineage commitment. However, 5hmC dynamics upon T cell activation need to be further characterized, in particular in terms of what is the relative contribution of passive and active mechanisms of demethylation in regulating T cell activation, and also which is the specific role of

5hmC in gene transcriptional activation. Further studies will provide a more complete understanding about the role of DNA hydroxymethylation in human immune-regulation.

7. Aim and overview of the study

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Efficient immune responses orchestrated by $CD4^+$ T lymphocytes require both lineage commitment and phenotypic flexibility, allowing the development of responses tailored to invading pathogens. Stability in the expression of subset-specific genes must be reconciled with mechanisms enabling plastic phenotypic changes in response to environmental cues. In mammals, the 5mC modification in the genomic DNA contributes to the dynamic control of gene expression, where the methyl mark erasure is required for the activation of developmental programs. DNA demethylation can occur either through passive dilution during DNA replication, or through active mechanisms mediated by TET enzymes that catalyze oxidation of 5mC to 5hmC, which can be further oxidized. Of note, 5hmC is a stable mark that contributes to the regulation of activation and differentiation of human $CD4^+$ T cells, however 5hmC dynamics upon T cell activation need to be further characterized. In particular, although the basic mechanistic aspects of passive and active demethylation are well described, it is still unclear which is their relative contribution in regulating T cell activation and effector functions. **Therefore, the overall aim of this thesis is to investigate the contribution of active and passive mechanisms of 5mC and 5hmC removal in primary human T lymphocytes, as well as to characterize changes in 5mC and 5hmC both genome-wide and at specific loci relevant for T cell functions.**

The studies I performed to achieve this aim are described in a manuscript addressing the role of passive and active DNA demethylation in the functional regulation of human T lymphocytes **(Manuscript 1)**. This manuscript represents the largest part of my PhD studies and I performed the majority of the experiments described.

I also contributed to a review article describing the role of epigenetics modifications in T lymphocytes **(Manuscript 2)**.

My specific contributions to each manuscript are as follows:

Manuscript 1

The contribution of active and passive mechanisms of 5mC and 5hmC removal in human T lymphocytes is differentiation- and activation-dependent

L. Vincenzetti, C. Leoni, M.Chirichella, I. Kwee, S.Monticelli

This is an author produced version of the manuscript reviewed by Cell Reports.

The expression of immune-related genes is transcriptionally regulated by 5mC and 5hmC in the genomic DNA. In this work, in order to understand the role of such modifications in modulating human T lymphocyte activation and functions, we started by measuring the levels of 5mC and 5hmC in different subsets of T lymphocytes. Resting naive T cells showed higher levels of 5hmC and 5mC compared to their memory counterparts. However, upon activation, all subsets displayed a significant decrease in the overall levels of genomic 5hmC, although the strongest effect was observed in naive T cells, pointing towards a role for 5hmC in the activation of antigen-inexperienced naive T lymphocytes. To further investigate whether genomic 5hmC and 5mC can indeed influence the acquisition of effector functions by T cells, we treated T cells with vitamin C (to enhance DNA demethylation) or decitabine (to block DNA methylation). Both treatments increased the ability of the cells to produce effector cytokines such as IFN-γ, suggesting that DNA methylation-related processes are required for the efficient differentiation of T cells to effector and memory subsets. The reduced levels of genomic 5mC and 5hmC observed upon T cell activation could be due to either enzymatic processes of active DNA demethylation, or to passive dilution due to DNA replication during cell proliferation. To understand the relative contribution of active and passive DNA demethylation during T cell activation, we performed experiments aimed at separating T cell activation from proliferation. We found that the observed reduction in 5hmC do not strictly require TCR engagement, and it is primarily due to passive dilution, although active demethylation processes are also present during the early phase of activation of naive, but not memory, T cells. Active

processes of demethylation appeared to be critical in quiescent naive T lymphocytes for the maintenance of regulatory regions poised for rapid responses to physiological stimuli. Finally, because DNA demethylation correlated with the acquisition of effector functions, we further investigated changes in 5mC and 5hmC both genome-wide and at specific loci relevant for T cell functions. From the data obtained, we hypothesized a model in which in the absence of cell proliferation, 5hmC enables the expression of housekeeping and early response genes, presumably by counteracting DNA methylation at these regions. Conversely, a small subset of effector genes acquired 5hmC upon activation and proliferation, and these changes correlated with gene expression.

This work constituted the major focus of my PhD and therefore I performed the vast majority of the experiments required for this study, apart from: the generation of genome-scale DNA methylation profiles by RRBS (reduced representation bisulfite sequencing), which was outsourced at Diagenode, the MeDIP (methylated DNA immunoprecipitation) and hMeDIP (hydroxymethylated DNA immunoprecipitation) experiments, and the analysis of already published datasets in human naive T cells.

Manuscript 2

Epigenetics of T lymphocytes in health and disease

C. Leoni, **L. Vincenzetti**, S. Emming, S. Monticelli – in Swiss Med Wkly (2015) 145:w14191.

In this manuscript we reviewed the published literature regarding the role of DNA methylation and hydroxymethylation dynamics in the regulation of gene expression, and we mainly focused on their role in coordinating functions of T lymphocytes during normal immune responses, as well as in autoimmune disease. I contributed to the discussion of studies describing DNA methylation dynamics in the modulation of cytokine production upon T cell activation, underlining in particular the possible role of active DNA demethylation in T cells differentiation and effector responses.

8. Discussion

8. Discussion

During their development and until terminal differentiation, T lymphocytes make sequential cell fate choices each one characterized by a specific gene expression program, and once initiated, these programs must be faithfully propagated in an heritable manner from parental cells to their progeny. But, at the same time, stability in the expression of specific genes must be reconciled with mechanisms enabling plastic phenotypic changes in response to environmental cues. Epigenetic mechanisms, including DNA methylation and modifications in chromatin structure, allow T cells to tune the threshold of specific gene expression and thereby help to determine T cell fate and function. Cell cycle progression is considered to be the key mechanism to reprogram and relieve epigenetic repression during differentiation. The work presented in this thesis shows the existence of an active process of 5hmC removal that is operating specifically in naive T cells prior to cell-cycle entry, while passive, replication-dependent dilution of the modified cytosines is at work in memory T lymphocytes. Consequently, we hypothesized that the active removal of cytosine modifications contributes significantly to the first activation of naive T lymphocytes, while on the other hand replication-dependent dilution is the driving force leading to 5hmC reduction in highly proliferative already differentiated cells.

The data presented in this work could provide an answer to the following question: how are naive T lymphocytes, as slowly or rarely proliferating cells, able to remove repressive DNA methylation and become amenable of transcriptional activation and poised for rapid response to stimuli?

Previous studies of 5hmC during differentiation of mouse $CD4^+$ T cells reported a global loss of 5hmC in differentiated T_H cell subsets (Ichiyama et al., 2015; Tsagaratou et al., 2014). These findings were confirmed also in human, showing a global reduction in genomic 5hmC during both activation and differentiation of $CD4^+$ T lymphocytes (Nestor et al., 2016). However, 5hmC dynamics need to be further characterized, in particular in term of what is the relative contribution of passive and active

mechanisms of DNA demethylation in the significant decrease of genomic 5hmC observed upon T cell activation and differentiation, and if there are possible differences between naive and memory T lymphocytes. In this work, first of all we found that unstimulated naive T cells maintained higher levels of 5hmC compared to those of all other antigen-experienced T lymphocytes, suggesting a role in the initial activation of naive but not memory T cells.

Then, we optimized systems to uncouple T cell activation from proliferation by analyzing early time points of T cell activation prior to cell cycle progression, and by stimulating cells in the presence or absence of cell cycle inhibitors. These experimental approaches gave a full characterization about the dynamics of passive and active mechanisms of DNA demethylation upon T cell activation. In particular they were consistent with the idea of a predominant role for proliferation-dependent dilution of 5hmC upon activation of naive T lymphocytes. In these cells, an active mechanism of removal of the 5hmC mark may be at play in a narrow window of time prior to cell cycle entry, contributing significantly to their activation. Our observations are consistent with studies showing that 5hmC-mediated processes may be important in rarely dividing cells like post-mitotic neurons in mammalian brain. In particular, active DNA demethylation has been shown to occur both in cultured neurons (Martinowich et al., 2003) and in various brain regions *in vivo* (Ma et al., 2009), indicating important roles for DNA demethylation dynamics in regulating neuronal plasticity, similarly to our findings in non-proliferating naive T cells.

But more specifically, which is the primary role of 5hmC in gene transcriptional activation in T lymphocytes, especially in naive T cells?

One possibility is that the presence of 5hmC is required to counteract deleterious DNA methylation to regions that are not actively transcribed in resting lymphocytes, but require fast activation upon antigen recognition. In line with this, we found that regulatory regions of several early response genes (genes that can be rapidly transcribed upon activation of resting cells and in the absence of proliferation), were characterized by an enrichment in levels of 5hmC. This is also in agreement with

recent findings showing that the activity of TET proteins is important in ES cells to preserve promoters from *de novo* methylation to ensure robust lineage-specific transcription upon differentiation; this mechanism was thought to facilitate rapid changes of methylation state to activate transcription in a locus-specific manner (Verma et al., 2018). Similarly, TET1 was shown to be not directly involved in initiation of global DNA demethylation during epigenetic reprogramming in gonadal primordial germ cells, but it was found to have a critical role in preventing the spreading of DNA methylation (Hill et al., 2018). Consequently, in T lymphocytes, the main role of 5hmC might be to counteract DNA methylation in particular in resting, non-proliferating naive T cells, which cannot take advantage of passive (cell-cycle dependent) mechanism of dilution to eliminate wrongful methylation.

Another possibility explaining the role of 5hmC in maintaining naive T cells in a state amenable of transcriptional activation comes from the following question: what triggers the opening of chromatin at enhancers to overcome a nucleosomal barrier and start regulatory events? Different hypothesis include the involvement of pioneer TFs that can recruit chromatin modifiers, or they can directly bind to nucleosomal DNA making it competent for the occupancy of other TFs through the repositioning of nucleosomes, or they can protect the enhancer region from DNA methylation (Calo and Wysocka, 2013; Zaret and Carroll, 2011). Moreover the histone modification H3K4me1 was found to be enriched at the level of enhancers (Heintzman et al., 2007), and it was suggested not to be tightly linked to enhancer activity, but to favor it by facilitating nucleosomal mobility and/or binding of pioneer TFs. Interestingly, 5hmC enrichment at enhancers was shown to be characterized by the high concordance with H3K4me1 (Yu et al., 2012), corroborating the idea that 5hmC could participate in the multiple levels of regulation of transcriptional activation. In particular, 5hmC may be part of enhancer activation process in its own right by counteracting transcriptionally restrictive chromatin states or by recruiting yet to be identified effector molecules (Calo and Wysocka, 2013). Considering these observations, in the context of this thesis, 5hmC may have a critical role at specific enhancers in order to create a 'window of time' for enhancer demethylation upon T cell activation, allowing

even quiescent naive T cells to be poised for transcriptional activation. Supporting this possibility, our analysis of available ATAC-seq datasets of human naive T cells (Mumbach et al., 2017) revealed that ATAC-accessible regions were indeed enriched of 5hmC, highlighting the possible interaction between 5hmC, TF binding site accessibility and nucleosome positioning in regulating transcriptional activation. Finally, the fate of 5hmC itself needs to be further explored in the context of T cell terminal differentiation. We found that once the cells overcame their activation threshold and entered the cell cycle, 5hmC modification was then lost by passive dilution. Our hypothesis is that once T lymphocytes differentiate, their phenotype may become stabilized by other mechanisms (such as histone modifications at regulatory regions) and demethylation-dependent mechanisms become in turn relatively less relevant; accordingly, the forced expression of TET enzymes in activated and proliferating T cells revealed only a modest effect on gene expression (Nestor et al., 2016).

Altogether, the work of this PhD thesis supports a model in which the 5hmc mark may be necessary to maintain enhancer accessibility in resting T lymphocytes, hence inhibiting DNA methylation of regions that must retain a poised state for rapid response upon encounter with an antigen.

9. References

9. References

Abbas et al. (2012). Cellular and molecular immunology (Elsevier).

Ahmed, N.N., Grimes, H.L., Bellacosa, A., Chan, T.O., and Tsichlis, P.N. (1997). Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. Proc. Natl. Acad. Sci. U. S. A. *94*, 3627–3632.

Allis, C.D., and Jenuwein, T. (2016). The molecular hallmarks of epigenetic control. Nat. Rev. Genet. *17*, 487–500.

Ambrosi, C., Manzo, M., and Baubec, T. (2017). Dynamics and Context-Dependent Roles of DNA Methylation. J. Mol. Biol. *429*, 1459–1475.

An, J., González-Avalos, E., Chawla, A., Jeong, M., López-Moyado, I.F., Li, W., Goodell, M.A., Chavez, L., Ko, M., and Rao, A. (2015). Acute loss of TET function results in aggressive myeloid cancer in mice. Nat. Commun. *6*, 10071.

Bachman, M., Uribe-Lewis, S., Yang, X., Williams, M., Murrell, A., and Balasubramanian, S. (2014). 5-Hydroxymethylcytosine is a predominantly stable DNA modification. Nat. Chem. *6*, 1049–1055.

Barlow, D.P., Stöger, R., Herrmann, B.G., Saito, K., and Schweifer, N. (1991). The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature *349*, 84–87.

Baubec, T., Ivánek, R., Lienert, F., and Schübeler, D. (2013). Methylation-dependent and -independent genomic targeting principles of the MBD protein family. Cell *153*, 480–492.

Baubec, T., Colombo, D.F., Wirbelauer, C., Schmidt, J., Burger, L., Krebs, A.R., Akalin, A., and Schübeler, D. (2015). Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. Nature *520*, 243–247.

Bell, O., Tiwari, V.K., Thomä, N.H., and Schübeler, D. (2011). Determinants and dynamics of genome accessibility. Nat. Rev. Genet. *12*, 554–564.

Bevington, S.L., Cauchy, P., Piper, J., Bertrand, E., Lalli, N., Jarvis, R.C., Gilding, L.N., Ott, S., Bonifer, C., and Cockerill, P.N. (2016). Inducible chromatin priming is associated with the establishment of immunological memory in T cells. EMBO J. *35*, 515–535.

Bhaumik, S.R., Smith, E., and Shilatifard, A. (2007). Covalent modifications of histones during development and disease pathogenesis. Nat. Struct. Mol. Biol. *14*, 1008–1016.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev. *16*, 6–21.

Blaschke, K., Ebata, K.T., Karimi, M.M., Zepeda-Martínez, J.A., Goyal, P., Mahapatra, S., Tam, A., Laird, D.J., Hirst, M., Rao, A., et al. (2013). Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. Nature *500*, 222–226.

Booth, M.J., Branco, M.R., Ficz, G., Oxley, D., Krueger, F., Reik, W., and Balasubramanian, S. (2012). Quantitative sequencing of 5-methylcytosine and 5-hydroxymethylcytosine at single-base resolution. Science *336*, 934–937.

Borst, P., and Sabatini, R. (2008). Base J: discovery, biosynthesis, and possible functions. Annu. Rev. Microbiol. *62*, 235–251.

Boyman, O., and Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of the immune system. Nat. Rev. Immunol. *12*, 180–190.

Bruniquel, D., and Schwartz, R.H. (2003). Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat. Immunol. *4*, 235–240.

Busque, L., Patel, J.P., Figueroa, M.E., Vasanthakumar, A., Provost, S., Hamilou, Z., Mollica, L., Li, J., Viale, A., Heguy, A., et al. (2012). Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. Nat. Genet. *44*, 1179–1181.

Calo, E., and Wysocka, J. (2013). Modification of enhancer chromatin: what, how, and why? Mol. Cell *49*, 825–837.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Annu. Rev. Immunol. *14*, 259–274.

Cebrián, M., Yagüe, E., Rincón, M., López-Botet, M., de Landázuri, M.O., and Sánchez-Madrid, F. (1988). Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. J. Exp. Med. *168*, 1621–1637.

Chang, H.-C., Sehra, S., Goswami, R., Yao, W., Yu, Q., Stritesky, G.L., Jabeen, R., McKinley, C., Ahyi, A.-N., Han, L., et al. (2010). The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. Nat. Immunol. *11*, 527–534.

Chen, T., Tsujimoto, N., and Li, E. (2004). The PWWP domain of Dnmt3a and Dnmt3b is required for directing DNA methylation to the major satellite repeats at pericentric heterochromatin. Mol. Cell. Biol. *24*, 9048–9058.

Cimmino, L., and Aifantis, I. (2017). Alternative roles for oxidized mCs and TETs. Curr. Opin. Genet. Dev. *42*, 1–7.

Cimmino, L., Dawlaty, M.M., Ndiaye-Lobry, D., Yap, Y.S., Bakogianni, S., Yu, Y., Bhattacharyya, S., Shaknovich, R., Geng, H., Lobry, C., et al. (2015). TET1 is a tumor suppressor of hematopoietic malignancy. Nat. Immunol. *16*, 653–662.

Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R.A., Mitsdoerffer, M., Strom, T.B., Elyaman, W., Ho, I.-C., et al. (2008). IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. Nat. Immunol. *9*, 1347–1355.

Dawlaty, M.M., Ganz, K., Powell, B.E., Hu, Y.-C., Markoulaki, S., Cheng, A.W., Gao, Q., Kim, J., Choi, S.-W., Page, D.C., et al. (2011). Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. Cell Stem Cell *9*, 166–175.

Dawlaty, M.M., Breiling, A., Le, T., Raddatz, G., Barrasa, M.I., Cheng, A.W., Gao, Q., Powell, B.E., Li, Z., Xu, M., et al. (2013). Combined deficiency of Tet1 and Tet2 causes epigenetic abnormalities but is compatible with postnatal development. Dev. Cell *24*, 310–323.

Dawlaty, M.M., Breiling, A., Le, T., Barrasa, M.I., Raddatz, G., Gao, Q., Powell, B.E., Cheng, A.W., Faull, K.F., Lyko, F., et al. (2014). Loss of Tet enzymes compromises proper differentiation of embryonic stem cells. Dev. Cell *29*, 102–111.

Deaton, A.M., and Bird, A. (2011). CpG islands and the regulation of transcription. Genes Dev. *25*, 1010–1022.

Doi, A., Park, I.-H., Wen, B., Murakami, P., Aryee, M.J., Irizarry, R., Herb, B., Ladd-Acosta, C., Rho, J., Loewer, S., et al. (2009). Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat. Genet. *41*, 1350–1353.

Domcke, S., Bardet, A.F., Adrian Ginno, P., Hartl, D., Burger, L., and Schübeler, D. (2015). Competition between DNA methylation and transcription factors determines binding of NRF1. Nature *528*, 575–579.

Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A., and Sallusto, F. (2009). Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat. Immunol. *10*, 857–863.

Durek, P., Nordström, K., Gasparoni, G., Salhab, A., Kressler, C., de Almeida, M., Bassler, K., Ulas, T., Schmidt, F., Xiong, J., et al. (2016). Epigenomic Profiling of Human CD4+ T Cells Supports a Linear Differentiation Model and Highlights Molecular Regulators of Memory Development. Immunity *45*, 1148–1161.

Feng, S., Jacobsen, S.E., and Reik, W. (2010). Epigenetic reprogramming in plant and animal development. Science *330*, 622–627.

Feng, Y., Arvey, A., Chinen, T., van der Veeken, J., Gasteiger, G., and Rudensky, A.Y. (2014). Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. Cell *158*, 749–763.

Filion, G.J.P., Zhenilo, S., Salozhin, S., Yamada, D., Prokhortchouk, E., and Defossez, P.-A. (2006). A family of human zinc finger proteins that bind methylated DNA and repress transcription. Mol. Cell. Biol. *26*, 169–181.

Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23–33.

Fouse, S.D., Shen, Y., Pellegrini, M., Cole, S., Meissner, A., Van Neste, L., Jaenisch, R., and Fan, G. (2008). Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. Cell Stem Cell *2*, 160–169.

Gamper, C.J., Agoston, A.T., Nelson, W.G., and Powell, J.D. (2009). Identification of DNA methyltransferase 3a as a T cell receptor-induced regulator of Th1 and Th2 differentiation. J. Immunol. Baltim. Md 1950 *183*, 2267–2276.

Globisch, D., Münzel, M., Müller, M., Michalakis, S., Wagner, M., Koch, S., Brückl, T., Biel, M., and Carell, T. (2010). Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PloS One *5*, e15367.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., and Roncarolo, M.G. (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature *389*, 737–742.

Gu, T.-P., Guo, F., Yang, H., Wu, H.-P., Xu, G.-F., Liu, W., Xie, Z.-G., Shi, L., He, X., Jin, S., et al. (2011). The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature *477*, 606–610.

Hashimoto, S., Ogoshi, K., Sasaki, A., Abe, J., Qu, W., Nakatani, Y., Ahsan, B., Oshima, K., Shand, F.H.W., Ametani, A., et al. (2013). Coordinated changes in DNA methylation in antigen-specific memory CD4 T cells. J. Immunol. Baltim. Md 1950 *190*, 4076–4091.

Haskins, K., Kubo, R., White, J., Pigeon, M., Kappler, J., and Marrack, P. (1983). The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. J. Exp. Med. *157*, 1149–1169.

He, Y.-F., Li, B.-Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L., et al. (2011). Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science *333*, 1303–1307.

Heintzman, N.D., Stuart, R.K., Hon, G., Fu, Y., Ching, C.W., Hawkins, R.D., Barrera, L.O., Van Calcar, S., Qu, C., Ching, K.A., et al. (2007). Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat. Genet. *39*, 311–318.

Hendrich, B., and Bird, A. (1998). Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol. Cell. Biol. *18*, 6538–6547.

Hill, P.W.S., Leitch, H.G., Requena, C.E., Sun, Z., Amouroux, R., Roman-Trufero, M., Borkowska, M., Terragni, J., Vaisvila, R., Linnett, S., et al. (2018). Epigenetic reprogramming enables the transition from primordial germ cell to gonocyte. Nature *555*, 392–396.

Ho, L., and Crabtree, G.R. (2010). Chromatin remodelling during development. Nature *463*, 474–484.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057–1061.

Huang, Y., and Rao, A. (2014). Connections between TET proteins and aberrant DNA modification in cancer. Trends Genet. TIG *30*, 464–474.

Huang, H., Jiang, X., Li, Z., Li, Y., Song, C.-X., He, C., Sun, M., Chen, P., Gurbuxani, S., Wang, J., et al. (2013). TET1 plays an essential oncogenic role in MLL-rearranged leukemia. Proc. Natl. Acad. Sci. U. S. A. *110*, 11994–11999.

Ichiyama, K., Chen, T., Wang, X., Yan, X., Kim, B.-S., Tanaka, S., Ndiaye-Lobry, D., Deng, Y., Zou, Y., Zheng, P., et al. (2015). The methylcytosine dioxygenase Tet2 promotes DNA demethylation and activation of cytokine gene expression in T cells. Immunity *42*, 613–626.

Irizarry, R.A., Ladd-Acosta, C., Wen, B., Wu, Z., Montano, C., Onyango, P., Cui, H., Gabo, K., Rongione, M., Webster, M., et al. (2009). The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat. Genet. *41*, 178–186.

Ito, S., D'Alessio, A.C., Taranova, O.V., Hong, K., Sowers, L.C., and Zhang, Y. (2010). Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature *466*, 1129–1133.

Ito, S., Shen, L., Dai, Q., Wu, S.C., Collins, L.B., Swenberg, J.A., He, C., and Zhang, Y. (2011). Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science *333*, 1300–1303.

Iyer, L.M., Tahiliani, M., Rao, A., and Aravind, L. (2009). Prediction of novel families of enzymes involved in oxidative and other complex modifications of bases in nucleic acids. Cell Cycle Georget. Tex *8*, 1698–1710.

Jenkins, M.K., Taylor, P.S., Norton, S.D., and Urdahl, K.B. (1991). CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J. Immunol. Baltim. Md 1950 *147*, 2461–2466.

Jia, D., Jurkowska, R.Z., Zhang, X., Jeltsch, A., and Cheng, X. (2007). Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. Nature *449*, 248–251.

Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C., and Kupper, T.S. (2012). Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. Nature *483*, 227–231.

Jin, C., Lu, Y., Jelinek, J., Liang, S., Estecio, M.R.H., Barton, M.C., and Issa, J.-P.J. (2014). TET1 is a maintenance DNA demethylase that prevents methylation spreading in differentiated cells. Nucleic Acids Res. *42*, 6956–6971.

Jones, P.A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat. Rev. Genet. *13*, 484–492.

June, C.H., Fletcher, M.C., Ledbetter, J.A., Schieven, G.L., Siegel, J.N., Phillips, A.F., and Samelson, L.E. (1990). Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. Proc. Natl. Acad. Sci. U. S. A. *87*, 7722–7726.

Jurkowska, R.Z., and Jeltsch, A. (2016). Enzymology of Mammalian DNA Methyltransferases. Adv. Exp. Med. Biol. *945*, 87–122.

Kaas, G.A., Zhong, C., Eason, D.E., Ross, D.L., Vachhani, R.V., Ming, G.-L., King, J.R., Song, H., and Sweatt, J.D. (2013). TET1 controls CNS 5-methylcytosine hydroxylation, active DNA demethylation, gene transcription, and memory formation. Neuron *79*, 1086–1093.

Kanno, Y., Vahedi, G., Hirahara, K., Singleton, K., and O'Shea, J.J. (2012). Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. Annu. Rev. Immunol. *30*, 707–731.

Keohane, A.M., O'neill, L.P., Belyaev, N.D., Lavender, J.S., and Turner, B.M. (1996). X-Inactivation and histone H4 acetylation in embryonic stem cells. Dev. Biol. *180*, 618–630.

Kersh, E.N., Fitzpatrick, D.R., Murali-Krishna, K., Shires, J., Speck, S.H., Boss, J.M., and Ahmed, R. (2006). Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. J. Immunol. Baltim. Md 1950 *176*, 4083–4093.

Ko, M., An, J., Bandukwala, H.S., Chavez, L., Aijö, T., Pastor, W.A., Segal, M.F., Li, H., Koh, K.P., Lähdesmäki, H., et al. (2013). Modulation of TET2 expression and 5-methylcytosine oxidation by the CXXC domain protein IDAX. Nature *497*, 122–126.

Ko, M., An, J., and Rao, A. (2015). DNA methylation and hydroxymethylation in hematologic differentiation and transformation. Curr. Opin. Cell Biol. *37*, 91–101.

Koh, K.P., Yabuuchi, A., Rao, S., Huang, Y., Cunniff, K., Nardone, J., Laiho, A., Tahiliani, M., Sommer, C.A., Mostoslavsky, G., et al. (2011). Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. Cell Stem Cell *8*, 200–213.

Komori, H.K., Hart, T., LaMere, S.A., Chew, P.V., and Salomon, D.R. (2015). Defining CD4 T cell memory by the epigenetic landscape of CpG DNA methylation. J. Immunol. Baltim. Md 1950 *194*, 1565–1579.

Kothari, R.M., and Shankar, V. (1976). 5-Methylcytosine content in the vertebrate deoxyribonucleic acids: species specificity. J. Mol. Evol. *7*, 325–329.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693–705.

Kriaucionis, S., and Heintz, N. (2009). The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science *324*, 929–930.

Lal, G., and Bromberg, J.S. (2009). Epigenetic mechanisms of regulation of Foxp3 expression. Blood *114*, 3727–3735.

Ledbetter, J.A., Gentry, L.E., June, C.H., Rabinovitch, P.S., and Purchio, A.F. (1987). Stimulation of T cells through the CD3/T-cell receptor complex: role of cytoplasmic calcium, protein kinase C translocation, and phosphorylation of pp60c-src in the activation pathway. Mol. Cell. Biol. *7*, 650–656.

Lee, P.P., Fitzpatrick, D.R., Beard, C., Jessup, H.K., Lehar, S., Makar, K.W., Pérez-Melgosa, M., Sweetser, M.T., Schlissel, M.S., Nguyen, S., et al. (2001). A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity *15*, 763–774.

Leonhardt, H., Page, A.W., Weier, H.U., and Bestor, T.H. (1992a). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell *71*, 865–873.

Leonhardt, H., Page, A.W., Weier, H.U., and Bestor, T.H. (1992b). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell *71*, 865–873.

Leoni, C., Vincenzetti, L., Emming, S., and Monticelli, S. (2015a). Epigenetics of T lymphocytes in health and disease. Swiss Med. Wkly. *145*, w14191.

Leoni, C., Montagner, S., Deho', L., D'Antuono, R., De Matteis, G., Marzano, A.V., Merante, S., Orlandi, E.M., Zanotti, R., and Monticelli, S. (2015b). Reduced DNA methylation and hydroxymethylation in patients with systemic mastocytosis. Eur. J. Haematol. *95*, 566–575.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915–926.

Li, Z., Cai, X., Cai, C.-L., Wang, J., Zhang, W., Petersen, B.E., Yang, F.-C., and Xu, M. (2011). Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. Blood *118*, 4509–4518.

Lian, C.G., Xu, Y., Ceol, C., Wu, F., Larson, A., Dresser, K., Xu, W., Tan, L., Hu, Y., Zhan, Q., et al. (2012). Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell *150*, 1135–1146.

Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.-M., et al. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. Nature *462*, 315–322.

Lorsbach, R.B., Moore, J., Mathew, S., Raimondi, S.C., Mukatira, S.T., and Downing, J.R. (2003). TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia *17*, 637–641.

Ma, D.K., Jang, M.-H., Guo, J.U., Kitabatake, Y., Chang, M.-L., Pow-Anpongkul, N., Flavell, R.A., Lu, B., Ming, G.-L., and Song, H. (2009). Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science *323*, 1074–1077.

Makar, K.W., and Wilson, C.B. (2004). DNA methylation is a nonredundant repressor of the Th2 effector program. J. Immunol. Baltim. Md 1950 *173*, 4402–4406.

Makar, K.W., Pérez-Melgosa, M., Shnyreva, M., Weaver, W.M., Fitzpatrick, D.R., and Wilson, C.B. (2003). Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. Nat. Immunol. *4*, 1183–1190.

Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., Fan, G., and Sun, Y.E. (2003). DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science *302*, 890–893.

Masopust, D., Choo, D., Vezys, V., Wherry, E.J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K.A., Barber, D.L., Kawamura, K.S., et al. (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. J. Exp. Med. *207*, 553–564.

Mellén, M., Ayata, P., Dewell, S., Kriaucionis, S., and Heintz, N. (2012). MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system. Cell *151*, 1417–1430.

Montagner, S., Leoni, C., Emming, S., Della Chiara, G., Balestrieri, C., Barozzi, I., Piccolo, V., Togher, S., Ko, M., Rao, A., et al. (2016). TET2 Regulates Mast Cell Differentiation and Proliferation through Catalytic and Non-catalytic Activities. Cell Rep. *15*, 1566–1579.

Moran-Crusio, K., Reavie, L., Shih, A., Abdel-Wahab, O., Ndiaye-Lobry, D., Lobry, C., Figueroa, M.E., Vasanthakumar, A., Patel, J., Zhao, X., et al. (2011). Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell *20*, 11–24.

Mumbach, M.R., Satpathy, A.T., Boyle, E.A., Dai, C., Gowen, B.G., Cho, S.W., Nguyen, M.L., Rubin, A.J., Granja, J.M., Kazane, K.R., et al. (2017). Enhancer connectome in primary human cells identifies target genes of disease-associated DNA elements. Nat. Genet. *49*, 1602–1612.

Neri, F., Rapelli, S., Krepelova, A., Incarnato, D., Parlato, C., Basile, G., Maldotti, M., Anselmi, F., and Oliviero, S. (2017). Intragenic DNA methylation prevents spurious transcription initiation. Nature *543*, 72–77.

Nestor, C.E., Ottaviano, R., Reddington, J., Sproul, D., Reinhardt, D., Dunican, D., Katz, E., Dixon, J.M., Harrison, D.J., and Meehan, R.R. (2012). Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes. Genome Res. *22*, 467–477.

Nestor, C.E., Lentini, A., Hägg Nilsson, C., Gawel, D.R., Gustafsson, M., Mattson, L., Wang, H., Rundquist, O., Meehan, R.R., Klocke, B., et al. (2016). 5-Hydroxymethylcytosine Remodeling Precedes Lineage Specification during Differentiation of Human CD4(+) T Cells. Cell Rep. *16*, 559–570.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247–257.

Otani, J., Nankumo, T., Arita, K., Inamoto, S., Ariyoshi, M., and Shirakawa, M. (2009). Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. EMBO Rep. *10*, 1235–1241.

Otani, J., Arita, K., Kato, T., Kinoshita, M., Kimura, H., Suetake, I., Tajima, S., Ariyoshi, M., and Shirakawa, M. (2013). Structural basis of the versatile DNA recognition ability of the methyl-CpG binding domain of methyl-CpG binding domain protein 4. J. Biol. Chem. *288*, 6351–6362.

Panning, B., and Jaenisch, R. (1996). DNA hypomethylation can activate Xist expression and silence X-linked genes. Genes Dev. *10*, 1991–2002.

Pastor, W.A., Pape, U.J., Huang, Y., Henderson, H.R., Lister, R., Ko, M., McLoughlin, E.M., Brudno, Y., Mahapatra, S., Kapranov, P., et al. (2011). Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature *473*, 394–397.

Pastor, W.A., Aravind, L., and Rao, A. (2013). TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. Nat. Rev. Mol. Cell Biol. *14*, 341–356.

Penn, N.W., Suwalski, R., O'Riley, C., Bojanowski, K., and Yura, R. (1972). The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. Biochem. J. *126*, 781–790.

Polansky, J.K., Kretschmer, K., Freyer, J., Floess, S., Garbe, A., Baron, U., Olek, S., Hamann, A., von Boehmer, H., and Huehn, J. (2008). DNA methylation controls Foxp3 gene expression. Eur. J. Immunol. *38*, 1654–1663.

Portela, A., and Esteller, M. (2010). Epigenetic modifications and human disease. Nat. Biotechnol. *28*, 1057–1068.

Quenneville, S., Verde, G., Corsinotti, A., Kapopoulou, A., Jakobsson, J., Offner, S., Baglivo, I., Pedone, P.V., Grimaldi, G., Riccio, A., et al. (2011). In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. Mol. Cell *44*, 361–372.

Quivoron, C., Couronné, L., Della Valle, V., Lopez, C.K., Plo, I., Wagner-Ballon, O., Do Cruzeiro, M., Delhommeau, F., Arnulf, B., Stern, M.-H., et al. (2011). TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell *20*, 25–38.

Ramirez, J.-M., Brembilla, N.C., Sorg, O., Chicheportiche, R., Matthes, T., Dayer, J.-M., Saurat, J.-H., Roosnek, E., and Chizzolini, C. (2010). Activation of the aryl hydrocarbon receptor reveals distinct requirements for IL-22 and IL-17 production by human T helper cells. Eur. J. Immunol. *40*, 2450–2459.

Rando, O.J., and Chang, H.Y. (2009). Genome-wide views of chromatin structure. Annu. Rev. Biochem. *78*, 245–271.

Rasmussen, K.D., and Helin, K. (2016). Role of TET enzymes in DNA methylation, development, and cancer. Genes Dev. *30*, 733–750.

Reik, W., and Walter, J. (2001). Genomic imprinting: parental influence on the genome. Nat. Rev. Genet. *2*, 21–32.

Reinherz, E.L., Meuer, S.C., and Schlossman, S.F. (1983). The delineation of antigen receptors on human T lymphocytes. Immunol. Today *4*, 5–8.

Reth, M. (1989). Antigen receptor tail clue. Nature *338*, 383–384.

Rothenberg, E.V., Moore, J.E., and Yui, M.A. (2008). Launching the T-cell-lineage developmental programme. Nat. Rev. Immunol. *8*, 9–21.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. Cell *133*, 775–787.

Sallusto, F., and Lanzavecchia, A. (2009). Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. Eur. J. Immunol. *39*, 2076–2082.

Sallusto, F., Lenig, D., Förster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708–712.

Sallusto, F., Geginat, J., and Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol. *22*, 745–763.

Samelson, L.E., Patel, M.D., Weissman, A.M., Harford, J.B., and Klausner, R.D. (1986). Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. Cell *46*, 1083–1090.

Santi, D.V., Garrett, C.E., and Barr, P.J. (1983). On the mechanism of inhibition of DNA-cytosine methyltransferases by cytosine analogs. Cell *33*, 9–10.

Scharer, C.D., Barwick, B.G., Youngblood, B.A., Ahmed, R., and Boss, J.M. (2013). Global DNA methylation remodeling accompanies CD8 T cell effector function. J. Immunol. Baltim. Md 1950 *191*, 3419–3429.

Schenkel, J.M., and Masopust, D. (2014). Tissue-resident memory T cells. Immunity *41*, 886–897.

Sérandour, A.A., Avner, S., Oger, F., Bizot, M., Percevault, F., Lucchetti-Miganeh, C., Palierne, G., Gheeraert, C., Barloy-Hubler, F., Péron, C.L., et al. (2012). Dynamic hydroxymethylation of deoxyribonucleic acid marks differentiation-associated enhancers. Nucleic Acids Res. *40*, 8255–8265.

Shen, L., Wu, H., Diep, D., Yamaguchi, S., D'Alessio, A.C., Fung, H.-L., Zhang, K., and Zhang, Y. (2013). Genome-wide analysis reveals TET- and TDG-dependent 5-methylcytosine oxidation dynamics. Cell *153*, 692–706.

Smith, Z.D., and Meissner, A. (2013). DNA methylation: roles in mammalian development. Nat. Rev. Genet. *14*, 204–220.

Smith-Garvin, J.E., Koretzky, G.A., and Jordan, M.S. (2009). T cell activation. Annu. Rev. Immunol. *27*, 591–619.

Song, J., Rechkoblit, O., Bestor, T.H., and Patel, D.J. (2011). Structure of DNMT1-DNA complex reveals a role for autoinhibition in maintenance DNA methylation. Science *331*, 1036–1040.

Spruijt, C.G., Gnerlich, F., Smits, A.H., Pfaffeneder, T., Jansen, P.W.T.C., Bauer, C., Münzel, M., Wagner, M., Müller, M., Khan, F., et al. (2013). Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized derivatives. Cell *152*, 1146–1159.

Stadler, M.B., Murr, R., Burger, L., Ivanek, R., Lienert, F., Schöler, A., van Nimwegen, E., Wirbelauer, C., Oakeley, E.J., Gaidatzis, D., et al. (2011). DNA-binding factors shape the mouse methylome at distal regulatory regions. Nature *480*, 490–495.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41–45.

Sun, Z., Dai, N., Borgaro, J.G., Quimby, A., Sun, D., Corrêa, I.R., Zheng, Y., Zhu, Z., and Guan, S. (2015). A sensitive approach to map genome-wide 5-hydroxymethylcytosine and 5-formylcytosine at single-base resolution. Mol. Cell *57*, 750–761.

Surh, C.D., and Sprent, J. (2008). Homeostasis of naive and memory T cells. Immunity *29*, 848–862.

Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., et al. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science *324*, 930–935.

Tardy-Planechaud, S., Fujimoto, J., Lin, S.S., and Sowers, L.C. (1997). Solid phase synthesis and restriction endonuclease cleavage of oligodeoxynucleotides containing 5-(hydroxymethyl)-cytosine. Nucleic Acids Res. *25*, 553–559.

Testi, R., Phillips, J.H., and Lanier, L.L. (1989). Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. Requirement for receptor cross-linking, prolonged elevation of intracellular [Ca++] and stimulation of protein kinase C. J. Immunol. Baltim. Md 1950 *142*, 1854–1860.

Tsagaratou, A., Äijö, T., Lio, C.-W.J., Yue, X., Huang, Y., Jacobsen, S.E., Lähdesmäki, H., and Rao, A. (2014). Dissecting the dynamic changes of 5-hydroxymethylcytosine in T-cell development and differentiation. Proc. Natl. Acad. Sci. U. S. A. *111*, E3306-3315.

Tsagaratou, A., Lio, C.-W.J., Yue, X., and Rao, A. (2017). TET Methylcytosine Oxidases in T Cell and B Cell Development and Function. Front. Immunol. *8*, 220.

Ueno, H., Banchereau, J., and Vinuesa, C.G. (2015). Pathophysiology of T follicular helper cells in humans and mice. Nat. Immunol. *16*, 142–152.

Valinluck, V., and Sowers, L.C. (2007). Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res. *67*, 946–950.

Vella, P., Scelfo, A., Jammula, S., Chiacchiera, F., Williams, K., Cuomo, A., Roberto, A., Christensen, J., Bonaldi, T., Helin, K., et al. (2013). Tet proteins connect the O-linked N-acetylglucosamine transferase Ogt to chromatin in embryonic stem cells. Mol. Cell *49*, 645–656.

Verma, N., Pan, H., Doré, L.C., Shukla, A., Li, Q.V., Pelham-Webb, B., Teijeiro, V., González, F., Krivtsov, A., Chang, C.-J., et al. (2018). TET proteins safeguard bivalent promoters from de novo methylation in human embryonic stem cells. Nat. Genet. *50*, 83–95.

Waddington, C.H. (1968). Towards a theoretical biology. Nature *218*, 525–527.

Waddington, C.H. (2012). The epigenotype. 1942. Int. J. Epidemiol. *41*, 10–13.

Wan, Y.Y., and Flavell, R.A. (2009). How diverse--CD4 effector T cells and their functions. J. Mol. Cell Biol. *1*, 20–36.

Wang, L., Liu, Y., Beier, U.H., Han, R., Bhatti, T.R., Akimova, T., and Hancock, W.W. (2013). Foxp3+ T-regulatory cells require DNA methyltransferase 1 expression to prevent development of lethal autoimmunity. Blood *121*, 3631–3639.

Williams, K., Christensen, J., Pedersen, M.T., Johansen, J.V., Cloos, P.A.C., Rappsilber, J., and Helin, K. (2011). TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature *473*, 343–348.

Wu, X., and Zhang, Y. (2017). TET-mediated active DNA demethylation: mechanism, function and beyond. Nat. Rev. Genet. *18*, 517–534.

Wu, H., D'Alessio, A.C., Ito, S., Wang, Z., Cui, K., Zhao, K., Sun, Y.E., and Zhang, Y. (2011). Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes Dev. *25*, 679–684.

Wu Ct, null, and Morris, J.R. (2001). Genes, genetics, and epigenetics: a correspondence. Science *293*, 1103–1105.

Wutz, A., and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol. Cell *5*, 695–705.

Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W.W., Okumura, K., and Li, E. (1999). Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene *236*, 87–95.

Xiong, J., Zhang, Z., Chen, J., Huang, H., Xu, Y., Ding, X., Zheng, Y., Nishinakamura, R., Xu, G.-L., Wang, H., et al. (2016). Cooperative Action between SALL4A and TET Proteins in Stepwise Oxidation of 5-Methylcytosine. Mol. Cell *64*, 913–925.

Xu, Y., Wu, F., Tan, L., Kong, L., Xiong, L., Deng, J., Barbera, A.J., Zheng, L., Zhang, H., Huang, S., et al. (2011). Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Mol. Cell *42*, 451–464.

Xu, Y., Xu, C., Kato, A., Tempel, W., Abreu, J.G., Bian, C., Hu, Y., Hu, D., Zhao, B., Cerovina, T., et al. (2012). Tet3 CXXC domain and dioxygenase activity cooperatively regulate key genes for Xenopus eye and neural development. Cell *151*, 1200–1213.

Yin, R., Mao, S.-Q., Zhao, B., Chong, Z., Yang, Y., Zhao, C., Zhang, D., Huang, H., Gao, J., Li, Z., et al. (2013). Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. J. Am. Chem. Soc. *135*, 10396–10403.

Yu, C., Zhang, Y.-L., Pan, W.-W., Li, X.-M., Wang, Z.-W., Ge, Z.-J., Zhou, J.-J., Cang, Y., Tong, C., Sun, Q.-Y., et al. (2013). CRL4 complex regulates mammalian oocyte survival and reprogramming by activation of TET proteins. Science *342*, 1518–1521.

Yu, M., Hon, G.C., Szulwach, K.E., Song, C.-X., Zhang, L., Kim, A., Li, X., Dai, Q., Shen, Y., Park, B., et al. (2012). Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell *149*, 1368–1380.

Zaret, K.S., and Carroll, J.S. (2011). Pioneer transcription factors: establishing competence for gene expression. Genes Dev. *25*, 2227–2241.

Zeng, H., Zhang, R., Jin, B., and Chen, L. (2015). Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance. Cell. Mol. Immunol. *12*, 566–571.

Zhang, W., and Xu, J. (2017). DNA methyltransferases and their roles in tumorigenesis. Biomark. Res. *5*, 1.

Zhang, H., Zhang, X., Clark, E., Mulcahey, M., Huang, S., and Shi, Y.G. (2010). TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. Cell Res. *20*, 1390–1393.

Zhang, Q., Zhao, K., Shen, Q., Han, Y., Gu, Y., Li, X., Zhao, D., Liu, Y., Wang, C., Zhang, X., et al. (2015). Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL-6. Nature *525*, 389–393.

Zhao, J., Sun, B.K., Erwin, J.A., Song, J.-J., and Lee, J.T. (2008). Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science *322*, 750–756.

Zhou, V.W., Goren, A., and Bernstein, B.E. (2011). Charting histone modifications and the functional organization of mammalian genomes. Nat. Rev. Genet. *12*, 7–18.

Manuscript 1

The contribution of active and passive mechanisms of 5mC and 5hmC removal in human T lymphocytes is differentiation- and activation-dependent

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The contribution of active and passive mechanisms of 5mC and 5hmC removal in human T lymphocytes is differentiation- and activation-dependent

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Running Title: DNA demethylation dynamics in T lymphocytes

Abstract

In mammals, the 5'-methylcytosine (5mC) modification in the genomic DNA contributes to the dynamic control of gene expression. 5mC erasure is required for the activation of developmental programs and occurs either by passive dilution through DNA replication, or by enzymatic oxidation of the methyl mark to 5-hydroxymethylcytosine (5hmC), which can persist as such or undergo further oxidation and enzymatic removal. The relative contribution of each mechanism to epigenetic control in dynamic biological systems still remains a compelling question. To explore this critical issue, we used primary human T lymphocytes, in which two cellular states can be clearly identified, namely quiescent naïve T cells, which are slowly or rarely proliferating, and rapidly proliferating activated T cells. We found that active mechanisms of methylation removal were selectively at work in naïve T cells, while memory T lymphocytes entirely relied on passive, replication-dependent dilution, suggesting that proliferative capacity influences the choice of the preferential demethylation mechanism. Active processes of demethylation appear to be critical in quiescent naïve T lymphocytes for the maintenance of regulatory regions poised for rapid responses to physiological stimuli.

Introduction

The methylation of the cytosine base (5mC) in the genomic DNA is essential for mammalian development and for cell lineage specification, and is intimately linked with the regulation of gene expression (Smith & Meissner, 2013). The bulk genomic methylation patterns are mostly static in differentiated cells and tissues, with large stably methylated regions including the inactive X chromosome, imprinted genes, pericentromeric repeats and other repeated elements, and transposable elements (Bird, 2002). Despite its stability and heritability across cell division, dynamic changes in 5mC deposition are observed during development and differentiation, and are deemed to be necessary for the establishment of stable cell-specific gene expression programs (Wu & Zhang, 2017). Once deposited in the genome, 5mC can be removed either through passive dilution during DNA replication, which occurs if the methyl mark is not copied on the nascent DNA strand, or through active mechanisms mediated by enzymes of the TET (Ten-eleven translocation) family (TET1-3). The 5mC mark is oxidized by TET proteins to 5-hydroxymethylcytosine (5hmC), which can then undergo further oxidation processes (Ito et al, 2010; Tahiliani et al, 2009). 5hmC is however a stable mark that can accumulate to significant levels (up to 0.6% of total nucleotides in post-mitotic Purkinje neurons (Kriaucionis & Heintz, 2009)), contributing to the regulation of gene expression, possibly by recruiting readers of this modification (Wu & Zhang, 2017). Differently from 5mC, 5hmC is enriched within the gene bodies of highly transcribed genes and at functional regulatory elements such as enhancers (Tsagaratou et al, 2014; Tsagaratou et al, 2017; Wu & Zhang, 2017; Yu et al, 2012a), while the vast majority of repetitive elements is enriched with 5mC but not 5hmC (Yu et al, 2012a). The 5hmC modification can therefore act both as an intermediate of active DNA demethylation and as a stable epigenetic mark (Scott-Browne et al, 2017; Wu & Zhang, 2017).

Whereas basic mechanistic aspects of passive and active DNA demethylation are well characterized, it is still unclear what is their relative contribution to the dynamic removal of 5mC and 5hmC in evolving biological systems. Therefore, we set out to address this critical issue in a cellular system, human T helper (T_H) lymphocytes, in which antigen-induced differentiation and activation is associated with extensive functional reprogramming and persistent changes in cellular physiology. Following the first recognition of a foreign infectious agent, antigen-inexperienced naïve T_H cells undergo rapid proliferation, and at the same time differentiate into an array of memory and effector subsets, with responses tailored towards the specific pathogen being recognized (Kanno et al, 2012). At a molecular level, T cell activation and acquisition of effector functions occur through the combinatorial action of transcription factors and epigenetic mechanisms to finally establish transcriptomes distinctive of each subset (Bevington et al, 2016). 5mC and 5hmC have a variable impact on T cell phenotypes, as inferred by mouse models lacking either DNMT or TET enzymes. For example, DNA methylation and DNMT enzymes were shown to influence the expression of the *Il2*, *Il4* and *Ifng* cytokine genes in both $CD4^+$ and $CD8^+$ mouse T cells (Bruniquel & Schwartz, 2003; Gamper et al, 2009; Kersh et al, 2006; Lee et al, 2001; Makar et al, 2003; Makar & Wilson, 2004; Yu et al, 2012b). Genome-wide studies of DNA methylation also highlighted the extent of changes accompanying the acquisition of the effector/ memory phenotype upon stimulation of mouse and human naïve T cells (Durek et al, 2016; Hashimoto et al, 2013; Komori et al, 2015; Scharer et al, 2013), with progressive demethylation correlating with differentiation of memory cells. Finally, DNA methylation changes and histone modifications occurring during cell cycle progression were shown to

control T cell differentiation in the mouse (Bird et al, 1998). As for 5hmC, it is enriched inside the body of highly expressed genes in murine T lymphocytes and at active thymus-specific enhancers, suggesting a possible role during T cell development and differentiation (Tsagaratou et al, 2014). However, germline deletion of mouse *Tet2* led to largely normal B and T lymphocyte development (Ko et al, 2011; Tsagaratou et al, 2017), although *Tet2* deletion in early lymphoid progenitors determined a partial reduction of IFN-γ and IL-17 expression by the T_H1 and T_H17 lymphocyte subsets, respectively (Ichiyama et al, 2015). In humans, T cell activation was associated with an incompletely characterized global reduction in genomic 5hmC, as well as with a modest gain of 5hmC at specific genomic regions associated with T cell activation (Nestor et al, 2016).

Our data now indicate that naïve T cells rely significantly on an active process of 5hmC removal, while active removal of modified cytosines could not be detected in memory lymphocytes, which instead used passive, replication-dependent dilution of the modified cytosines. Our data point to the notion that the preferential usage of active *vs*. passive mechanisms of DNA demethylation is differentiation- and activation stage-dependent, with active demethylation mechanisms being selectively involved in quiescent naïve T cells to maintain regulatory regions poised for rapid responses to stimuli.

Material and Methods

Primary human T cell isolation and culture. Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano (Switzerland), with informed consent from the Swiss Red Cross. Mononuclear cells were separated from peripheral blood using Ficoll-Paque Plus (GE Healthcare). $CD4^+$ or $CD8^+$ T lymphocytes were further isolated by positive selection using magnetic microbeads (Miltenyi Biotec). Naive and memory T cell subsets were then sorted based on the expression of the following surface markers: naïve $CD4^+$ T cells: $CD4^+CD25^-CD45RA^+CCR7^+$; memory $CD4^+$ T cells: $CD4^+CD25^-CD45RA^-CCR7^{+/-}$; T_H1 cells: $CD4^+CD25^-CD45RA^-CCR6^-CXCR3^+$; T_H2 cells: $CD4^+CD25^-CD45RA^-CCR6^-CCR4^+$; T_H17 cells: $CD4^+CD25^-CD45RA^-CCR6^+CCR4^+$, T_{CM} (central memory): $CD4^+CD25^-CD45RA^-CCR7^+$; T_{EM} (effector memory): $CD4^+CD25^-CD45RA^-CCR7^-$; naïve $CD8^+$ T cells: $CD4^-CD45RA^+CCR7^+$; memory $CD8^+$ T cells: $CD4^-CD45RA^{+/-}CCR7^{+/-}$. Cells were sorted using a FACSAria (BD Biosciences). Human T cells were cultured in RPMI-1640 medium supplemented with 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, penicillin, streptomycin, 50mM β-mercaptoethanol and 5% human serum. For *in vitro* stimulation, T lymphocytes were stimulated using plate-bound anti-CD3 (clone TR66; 5 μg/ml) and anti-CD28 (1 μg/ml) antibodies.

T cell proliferation and treatments. To measure proliferation, human T lymphocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE, 5 μM, Thermo Fischer Scientific) for 8 min at 37°C, followed by extensive washing. For experiments of *homeostatic proliferation*, different subsets of $CD4^+$ T lymphocytes were cultured for 6 days in 96-flat bottom microplates in the presence of recombinant IL-2 (300 U/ml), IL-6 (25 ng/ml) and TNF-α (25 ng/ml). $CD8^+$ naïve and memory T cells were cultured in the presence of IL-7 (25 ng/ml) and IL-15 (25 ng/ml). All recombinant cytokines were purchased from Peprotech. For *demethylation experiments*, $CD4^+$ naïve and memory T cells were activated for 5 days in the presence or absence of decitabine (5-aza-2'-deoxycytidine, Sigma-Aldrich) used at 1 μM and 5 μM, or were pre-treated for 48h with vitamin C (50 and 100 μg/ml, Sigma-Aldrich), prior to activation. For experiments using *caspase inhibitors*, $CD4^+$ naïve T lymphocytes were activated for 48 h in the presence of z-LVSR-fmk (inhibitor of MALT1; 40 μM), or z-VAD (inhibitor of caspases; 40 μM), kindly provided by Prof. Margot Thome Miazza (University of Lausanne). For experiments using *cell cycle inhibitors*, $CD4^+$ naïve and total memory T cells were activated for 3 days in the presence of aphidicolin (1 μg/ml, Sigma-Aldrich) or nocodazole (2 μg/ml, Sigma-Aldrich).

Mouse T cell isolation and culture. Spleens were obtained from 8 weeks-old C57BL/6 male mice and homogenized to single cell suspensions. T cells were isolated by negative selection ($CD19^-CD11c^-CD11b^-$). $CD4^+$ or $CD8^+$ naïve T cells were then sorted based on the following markers: naïve $CD4^+$: $CD3^+CD4^+CD8\alpha^-CD62L^{high}CD44^{low}$; naïve $CD8^+$: $CD3^+CD4^-CD8\alpha^+CD62L^{high}CD44^{low}$. Murine T cells were cultured in RPMI-1640 medium supplemented with 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, penicillin, streptomycin, 50mM β-mercaptoethanol and 10% fetal bovine serum (FBS). T lymphocytes were activated with plate-bound anti-CD3 (BioLegend, clone 145_2c11; 2 μg/ml) and anti-CD28 (BioLegend, clone 37.51; 2 μg/ml) antibodies, with addition of 100 U/mL of recombinant IL-2 (Peprotech) after the first 48 hours of stimulation.

Surface and intracellular stainings. To assess the expression of surface markers of early T cell activation, cells were stained with anti-CD25 (Beckman Coulter) and anti-CD69 (BioLegend)

antibodies conjugated to different fluorochromes. To measure cytokine expression, cells were stimulated for 5 h with phorbol 12-myristate 13-acetate (PMA; 200 nM) and ionomycin (1 μg/mL) in the presence of 10 μg/mL brefeldin A (all from Sigma-Aldrich) for the final 2.5 h of culture, followed by fixation and permeabilization. Cells were then stained with anti-cytokine antibodies (all from BioLegend) conjugated to different fluorochromes, and data were acquired using a Fortessa (BD Bioscience) cell analyzer. Flow cytometry data were analyzed with FlowJo.

Cell lines and transfections. Jurkat T cells were cultured in RPMI-1640 medium supplemented with 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, penicillin, streptomycin, kanamycin, 50mM β-mercaptoethanol and 10% FBS. Jurkat cells were transiently transfected with the 100 μl Neon Transfection System kit (Thermo Fischer Scientific), using $3x10^6$ cells and 30 μg of plasmid DNA per condition and the following parameters: 1325 V, 10 ms, 3 pulses.

Plasmids. Expression plasmids encoding for the full-length human TET1, mouse Tet2 and human TET3 were obtained from Addgene (plasmids no. 49792, 41710 and 49446) (Ko et al, 2013; Ko et al, 2010; Tahiliani et al, 2009). The catalytically inactive form of mTet2, containing the amino acid substitutions H1302Y and D1304A, was generated by targeted mutagenesis using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

Quantitative RT-PCR. Total RNA was extracted using TRI reagent (MRC) and was reverse transcribed using the qScript cDNA SuperMix (Quanta Biosciences). Primer sequences for SYBR Green qRT-PCR are listed in the **Suppl. Table 1**. All qPCRs were performed with an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

5hmC and 5mC measurements. For dot blot experiments, genomic DNA was isolated using the QIAamp DNA Micro Kit (Qiagen) and denatured with 1 M NaOH, 25 mM EDTA at 95°C for 10 min. Two-fold dilutions of the denatured DNA were then spotted on a nitrocellulose membrane, from a starting amount of 150-500 ng. Following nucleic acid crosslinking (80°C for 2 h), membranes were incubated with an anti-5hmC antibody (Active Motif) or an anti-5mC antibody (Epigentek), followed by incubation with an HRP-conjugated secondary antibody. Image acquisition was performed with the ImageQuant LAS 4000 (GE Healthcare Life Sciences), and quantification was performed using the Multi Gauge software. The number of pmol of 5hmC (or 5mC) per μg of genomic DNA in the different samples was calculated by comparing the intensity of the signal in the experimental sample with that of a standard with known concentration.

Methylated and hydroxymethylated DNA immunoprecipitation (MeDIP/hMeDIP) and qPCR. Genomic DNA from primary naïve and memory T lymphocytes was isolated using the DNeasy Blood & Tissue kit (Qiagen), after which 2.5 μg of DNA were sonicated (Bioruptor, Diagenode) to produce fragments ranging between 300 and 500 bp. MeDIP was performed using a MeDIP Kit (Zymo Research) following manufacturer's instruction. Briefly, 500 ng of sonicated DNA were denatured for 5 min at 98°C prior to incubation with 5 μg of anti-5mC antibody for 1 h at 37°C. For hMeDIP, sonicated DNA was denatured for 10 min at 95°C, and 1 μg of denatured DNA was incubated overnight at 4°C with 1 μg of anti-5hmC rabbit antibody (Active Motif). For both MeDIP and hMeDIP, 500 ng of each sample of sonicated DNA were set aside as input. DNA-antibody complexes were immunoprecipitated for 3 h at 4°C with 20 μl of Dynabeads Protein G (Invitrogen). Magnetic beads were washed 5 times with 1x immunoprecipitation (IP) buffer (10x IP buffer: 100 mM Na-

Phosphate pH 7.0, 1.4 M NaCl, 0.5 % Triton X-100). Beads were then re-suspended in digestion buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5 % SDS) containing 30 µg of Proteinase K (Ambion) and incubated for 3 h at 50°C. Finally, immunoprecipitated DNA was purified using the E.Z.N.A. Cycle Pure Kit (VWR-International AG). Quantitative PCR was performed with a 7900HT Fast Real Time PCR System (Applied Biosystems) and the percentage of immunoprecipitated hydroxymethylated or methylated DNA was calculated as follows: % input = $2^{(Ct\ input - Ct\ IP)}$ x input dilution factor x 100 (Leoni et al, 2017). *Controls for MeDIP and hMeDIP*. To optimize hMeDIP efficiency and as a quality control for each immunoprecipitation, a control DNA was produced by mixing 1:6 ratio of two otherwise identical PCR products containing either d5hmCTP or dCTP (1ng 5hmC-PCR : 6 ng C-PCR). Prior to IP, 2 ng of control DNA mix were spiked into every sample and after IP, PCR amplification was performed with primers specific for the control DNA. Because an EcoRV restriction site was present only in the 5hmC-PCR, further digestion with EcoRV was used to determine the specific enrichment of the 5hmC-containing product following IP. Similarly, to assess MeDIP efficiency, a control DNA mix containing 1:4 ratio of *in vitro* methylated and non-methylated pUC19 plasmids (MeDIP Kit, Zymo) was added to each sample prior IP. MeDIP efficiency was then evaluated by NcoI digestion exactly as described (Leoni et al, 2017).

Reduced Representation Bisulfite Sequencing (RRBS). Primary naïve and memory T lymphocytes were obtained from two independent donors and were either left resting or were stimulated for 3 days with anti-CD3 and anti-CD28 antibodies. Genomic DNA was purified using the DNeasy Blood & Tissue Kit (Qiagen) and the RRBS experiment itself was outsourced to the Diagenode Premium RRBS technology service. The aligned (to the hg19 human reference genome) output data were processed using methylKit R package (Akalin et al, 2012). Briefly, Bismark outputs coverage2cytosine.txt reports were read by methRead() function. Bases with coverage below 10 reads, as well as the one displaying extremely high read coverage (>99.9th percentile of coverage in each sample), were filtered out. Samples in each group were pooled to obtain one representative methylation profile per sample group and differential methylated CpG dinucleotides were calculated through the calculateDiffMeth() function according to Fisher's exact test. To annotate differential methylated CpGs, only CpGs displaying at least 50% of changes in methylation level and having a q-value (false discovery rate corrected p-value from Fisher's exact test) ≤ 0.05 were retained. Annotation information was retrieved from a BED file containing gene annotation information based on hg19 alignment downloaded from UCSC Genome Browser. The RRBS data have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE113884).

To review GEO accession GSE113884:
Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113884
Enter token gjafwkcwhbglrmb into the box.

Analysis of hMeDIP-seq data. hMeDIP-seq data were obtained from Nestor *et al.* (Nestor et al, 2016). Antonio Lentini (Centre for Personalized Medicine, Linköping, Sweden) kindly provided alignments in *.bam format, which were visualized and analyzed with Integrative Genomics Viewer software (IGV). Early, late and housekeeping genes were defined by analyzing gene expression raw data from Agilent 450k microarray from the same publication (Nestor et al, 2016) using R software and Microsoft Excel. To obtain the list of late genes, the following criteria were used: (i) differential expression (DE) in T_H1 cells between day 0 and day 1 of activation; (ii) DE in T_H1 cells between day

1 and day 5 of activation; (iii) Log_2 DE >1; (iv) increased expression between day 1 and day 5; (v) sum of expression values for raw data in all groups > 34. Housekeeping genes were defined as being (i) not differentially expressed between the various groups, and (ii) with a minimum expression above 6.7 in the naïve T cell group. Gene lists were analyzed using DAVID (Huang da et al, 2009a; Huang da et al, 2009b) and genes related to T cells and immunity gene ontology categories were selected. Finally, the 5hmC levels of the corresponding annotated enhancers for these genes were analyzed. Read counts of 5hmC for these enhancers were obtained with Samtools software for Linux. Analysis was performed on both the "BB" and "BC" set of samples from $CD4^+$ T cells *in vitro* polarized towards the T_H1 or T_H2 subsets. Graphs of read counts were made with GraphPad Prism 7. Enhancers and regulatory regions are indicated on the coverage plots either as GeneHancer Identifiers (GH) from FANTOM database or as CNSs (Conserved Noncoding Sequences). All references are according to the GRCh37/hg19 assembly.

Statistical analysis. Statistical analysis was performed with Prism software (GraphPad). Data are represented as mean ± SEM or SD, and significance was assessed by paired or unpaired Student's t test, two-tailed.

Results

DNA methylation and hydroxymethylation dynamics upon T cell activation. We first determined the global changes of genomic 5mC and 5hmC upon activation of primary human T lymphocytes. Naïve (T_N) and memory (T_{MEM}) cells were separated from the peripheral blood of healthy donors and were either left unstimulated or stimulated for 5 days with anti-CD3 and anti-CD28 antibodies. Levels of genomic 5mC and 5hmC were then determined by dot blot. Global levels of 5mC were about 1.4-fold higher in naïve compared to memory T cells, consistent with a role for DNA demethylation specifically during cell differentiation (**Figure 1a-b**) (Durek et al, 2016). Accordingly, in naïve cells, levels of 5mC were modestly reduced after 5 days of activation, while memory T cells showed negligible changes upon acute stimulation. Compared to 5mC, the dynamic range of changes in genomic 5hmC appeared to be much greater, with a global reduction that affected more than 87% of the initial 5hmC levels in recently activated naïve cells (**Figure 1c**). The overall levels of 5hmC were on average 4.6-fold higher in resting naïve compared to resting memory or activated T cells, effectively separating naïve T cells from all antigen-experienced T lymphocytes (**Figure 1c-d**). Such difference was not justified by the observed differences in 5mC: when calculating the ratio between 5hmC and 5mC, unstimulated naive cells maintained levels of 5hmC that were 4.3-fold higher compared to those of all other T cell subsets (**Figure 1e-f**).

The mammalian genome is heavily and stably methylated, especially at stably heterochromatic regions such as centromeric repeats, transposons and the inactive X chromosome (Bird, 2002), making bulk changes in 5mC difficult to detect, as they are restricted to a much smaller fraction of the genome (active genes and enhancers) than that associated with constitutive methylation. Therefore, on a global scale, changes in 5hmC provide a more sensitive, albeit indirect, readout of the dynamic changes in methylation occurring at gene bodies and regulatory regions (Yu et al, 2012a). Indeed, loss of 5hmC indicates either its passive dilution or the further oxidation followed by base excision and replacement with an unmethylated cytosine. We therefore analyzed changes in levels of genomic 5hmC in various conditions of T cell activation. First, similarly to $CD4^+$ T cells, a robust difference between naïve and memory cells was observed in human $CD8^+$ T cells, which displayed a strong reduction in 5hmC upon activation (**Figure 2a**). The same phenomenon was also observed in mouse $CD4^+$ and $CD8^+$ T cells activated *in vitro* with anti-CD3 and anti-CD28 antibodies for 3 days (**Figure 2b-c**). The high levels of genomic 5hmC characteristic of naïve T cells are likely to ensue during thymocyte development in the transition between the double-positive (DP) and single-positive (SP) stage (**Suppl. Figure 1**), although in peripheral cells they may also derive from ongoing processes of 5mC oxidation.

Within the human memory compartment, effector memory T lymphocytes (T_{EM}) showed the lowest 5hmC content, while central memory T cells (T_{CM}) showed intermediate levels between naive and T_{EM} cells (**Figure 2d**). Such trend was inversely correlated with the acquisition of effector functions, as shown by the ability of T_{EM} cells to express the highest levels of effector cytokines such as IFN-γ and IL-22 (two effector cytokines that are easily detectable in primary T cells not subdivided into more specialized subsets) (**Figure 2e**). Different effector subsets, such as T_H1, T_H2 and T_H17 showed low levels of 5hmC that were comparable to those of T_{EM} cells (less than 1 pmol/ μg of genomic DNA) (**Figure 2f**), indicating that the global 5hmC reduction is a general feature of T cell activation and differentiation.

DNA demethylation favors the acquisition of an effector T cell phenotype. Having established that levels of 5hmC (and to a lesser extent 5mC) are globally reduced in recently activated naïve T cells, we then asked whether the link between DNA demethylation (of which 5hmC represents an intermediate) and acquisition of effector functions might be causal. First, we found that the treatment of naïve and memory human T lymphocytes with decitabine (DAC), an inhibitor of DNA methyltransferases, led to a reduction in global 5mC in proliferating cells that was more prominent in recently activated naïve than in memory T cells (**Figure 3a-b**). The treatment with DAC also led to the acquisition of a more effector-like phenotype by activated naïve cells, which showed a significant increase in the expression of IFN-γ and IL-22 to levels that were fully comparable to those of memory T cells, along with a modest reduction in IL-2 expression (**Figure 3c**). On memory cells, such effect was less apparent, most likely because these cells are already capable of optimal cytokine expression. To further clarify the role of DNA demethylation in regulating the expression of effector cytokines, we enhanced TET-assisted DNA demethylation by over-expressing TET proteins in Jurkat cells. Our initial experiments showed that transfection or transduction of proteins as large as the TET enzymes in primary T cells was sufficient to affect T cell activation and even more proliferation, resulting in confounding consequences on the final biological effects. We therefore transfected the full-length TET proteins in Jurkat T cells, whose proliferation was instead unaffected. The three TET enzymes were variably effective at increasing the levels of genomic 5hmC in these cells, with TET2 and TET1 being the most and the least effective, respectively (**Figure 3d**). Despite its low efficacy in this system, TET1 was expressed by transfected Jurkat cells as expected, it was able to re-activate expression of fully methylated, non-replicable reporter plasmids, and it was functional in immunofluorescence experiments using 5hmC as readout (**Suppl. Figure 2**). However, TET1 has already been shown to be unable to induce global DNA demethylation, but instead to act as a maintenance DNA demethylase that counteracted the spreading of DNA methylation at regulatory regions, potentially explaining its limited efficacy in our system (Jin et al, 2014). Notwithstanding such differences among TET proteins, increased levels of 5hmC invariably correlated with a modest increase in cytokine expression by Jurkat cells (**Figure 3e**). A catalytically inactive version of TET2 (H1302Y and D1304A) (Ko et al, 2010; Montagner et al, 2016), was unable to increase either 5hmC levels or cytokine expression (**Figure 3d-e**), suggesting that the observed effects were indeed dependent on the catalytic activity of TET enzymes.

Vitamin C was reported to enhance TET enzymatic activity, by acting as a co-factor that maintains the essential atom of iron in the catalytic site in a reduced state (Blaschke et al, 2013; Montagner et al, 2016; Yin et al, 2013), and to influence differentiation of regulatory T cells in both human and mouse, enhancing their suppressive capacity (Yue et al, 2016). To further define the role of DNA demethylation-related processes in T cell activation and differentiation, we cultured primary T cells in the presence of vitamin C to assess whether its supplementation in the culture medium was sufficient to counteract the loss of 5hmC observed upon activation. Treatment of naïve and memory T lymphocytes with increasing concentrations of vitamin C led to a corresponding increase in the global 5hmC levels, as assessed by dot blot (**Figure 3f**). Increased 5hmC levels correlated with increased capacity of memory T cells to produce cytokines, as determined by intracellular staining (**Figure 3g**). Contrary to DAC treatment, increased cytokine expression was observed primarily in memory, but

not in naïve T cells, suggesting that vitamin C is sufficient to induce a modest increase in gene expression at genomic loci that are already accessible for transcription, but it is insufficient to significantly alter the differentiation of naïve T cells. Overall, our results indicate that DNA demethylation-related processes are important for the efficient acquisition of effector functions by activated T lymphocytes.

Changes in 5hmC do not strictly require TCR engagement and are predominantly linked to T cell proliferation. We dissected the relative roles of T cell activation and proliferation in the global changes of 5hmC observed upon stimulation of T lymphocytes. To assess whether T cell receptor (TCR) signaling was required for such changes in 5hmC, freshly isolated $CD4^+$ naive, T_{CM} and T_{EM} cells were cultured in the presence of IL-2, IL-6 and TNF-α to induce cytokine-driven homeostatic proliferation (Geginat et al, 2001; Unutmaz et al, 1994) in the absence of TCR engagement. The extent of cell proliferation in each of the subsets (as measured by CFSE dilution) reflected their activation requirements, as naive cells could barely proliferate in the absence of full antigenic stimulation, while T_{EM} cells proliferated efficiently and T_{CM} cells displayed an intermediate phenotype (**Figure 4a**). In each case, the bulk levels of genomic 5hmC inversely correlated with the extent of T cell proliferation (**Figure 4b-c**). A similar result was observed in primary human $CD8^+$ T cells undergoing homeostatic proliferation upon culture with IL-7 and IL-15 (Geginat et al, 2003) (**Figure 4d-e**), suggesting that T cell proliferation, rather than TCR-dependent activation, is the main driver of the observed reduction in genomic 5hmC. Concordant with the fact that TCR signaling is not required *per se* for 5hmC removal, treatment with Z-LVSR, an inhibitor of the MALT-1 paracaspase (Jaworski & Thome, 2016), or with Z-VAD, a general caspase inhibitor, did not influence the levels of 5hmC at early time points of activation (**Figure 4f**). The only difference was observed after 48 h of activation, when untreated (but not treated) cells entered the cell cycle, leading to a reduction in 5hmC (**Figure 4f**, red square).

To further determine whether the reduction in 5hmC that was observed upon T cell proliferation was exclusively linked to passive dilution, we optimized systems to uncouple T cell activation from proliferation. First, we defined the changes in levels of genomic 5hmC at early time points of T cell activation, prior to cell cycle progression. After stimulation with plate-bound anti-CD3 and anti-CD28 antibodies, we monitored T cell activation by measuring the surface induction of the early activation markers CD25 and CD69 (**Figure 5a**). After 30 h of activation, cells were uniformly $CD69^+$ (which is an earlier marker of activation compared to CD25) and 40-60% of them were already also $CD25^+$, indicating effective activation. However, 30 h of stimulation were insufficient for cells to engage in cell division, which occurred only after 48 h (**Figure 5b**). In these experimental conditions, we found that 5hmC levels remained stable in the initial stages of activation (until 24 h after activation), and decreased rapidly and significantly (by about half) as soon as naïve and memory T cells entered cell division (48 h time point); however, we detected a modest but significant decrease in 5hmC in naïve, but not memory T cells prior to cell cycle progression, indicating that an active removal of 5hmC may be at play specifically in naive T lymphocytes (**Figure 5c-d**). To further explore this issue, naïve and memory T lymphocytes were loaded with CFSE and were then stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. After 3 days, cells underwent about three cycles of cell division (**Figure 5e**). To address whether levels of 5hmC diminished with each cycle, we separated cells contained in

each CFSE peak by sorting and we measured the global levels of 5hmC by dot blot. We found that memory T cells showed no detectable sign of active 5hmC removal, as the levels of 5hmC dropped exactly by half within the first cycle and then became somewhat stabilized at very low levels (**Figure 5e**). Naïve T cells showed instead a reduction in 5hmC levels at the first cycle that was higher than expected, pointing once again towards a combination of active and passive mechanisms of removal of this modified base specifically in these cells.

To clarify whether indeed an active process of removal of 5hmC may be operating in naïve T cells, we stimulated naïve and memory T lymphocytes in the presence or absence of the cell cycle inhibitors nocodazole or aphidicolin. Nocodazole interferes with microtubule polymerization, including the mitotic spindle, thereby blocking the cells in the G2/M phase, while aphidicolin is a reversible inhibitor of the DNA polymerase α resulting in a block in the G1/S phase. After 3 days, untreated cells underwent at least three division cycles, while treated cells were for the most part blocked, as determined by CFSE dilution (**Figure 6a**). Despite the block in proliferation, T cells were effectively activated, as shown by the induced expression of CD25 (**Figure 6a**). In memory T cells, inhibition of the cell cycle almost completely counteracted the reduction in genomic 5hmC observed in untreated cells (**Figure 6b-c**), pointing towards a passive, proliferation-dependent mechanism of dilution of 5hmC in activated memory T lymphocytes. Conversely, treated naïve T lymphocytes showed a significant loss of genomic 5hmC upon activation, even in the complete absence of proliferation (**Figure 6b-c**). Overall, our data point towards the existence of active mechanisms of removal that are operating specifically in naïve T cells prior to cell-cycle entry, while memory T lymphocytes rely primarily on mechanisms of proliferation-dependent dilution.

Changes in 5hmC at enhancer regions distinguish early and late response genes. Because DNA demethylation correlated with the acquisition of effector functions, we further investigated changes in 5mC and 5hmC both genome-wide and at specific loci relevant for T cell functions. First, we performed reduced representation bisulfite sequencing (RRBS) to generate genome-scale DNA methylation profiles. This method directly measures the cytosine methylation state at high CpG-density sequences such as promoters and a subset of repetitive sequences (Bock et al, 2010; Meissner et al, 2008). We found that the majority of differentially methylated regions were observed comparing naïve to memory T cells, consistent with previous studies (Durek et al, 2016; Komori et al, 2015), while fewer differences were observed upon short-term activation of memory and naïve T lymphocytes (**Figure 7a** and **Suppl. Figure 3**). The observed differentially methylated regions were primarily located within introns and intergenic regions, although hypermethylation at some promoter regions was acquired by memory T cells, as compared to naïve cells, regardless of short-term stimulation (**Figure 7a** and **Suppl. Figure 3**), possibly contributing to T cell differentiation. Indeed, analysis of the gene ontology (GO) terms (Chen et al, 2013) associated with the differentially methylated promoter regions (defined as -1500bp from the transcription start site (TSS) of the closest gene) identified categories reflecting the underlying T cell biology (**Figure 7b** and **Dataset 1**). For instance, hypo-methylated regions were associated with NF-kB signaling and positive regulation of cytokine and chemokine secretion, while hyper-methylated regions were primarily associated to regulation of transcription, but also to regulation of TCR signaling and of memory T cell differentiation.

Because 5hmC is usually enriched at regulatory regions such as enhancers (Tsagaratou et al, 2014; Yu et al, 2012a), to assess whether changes in 5hmC could more specifically distinguish patterns of gene expression in human T lymphocytes, we analyzed available hMeDIP-seq (hydroxymethylated DNA immunoprecipitation and sequencing) data (Nestor et al, 2016) performed on primary human naïve T cells and *in vitro* differentiated T_H1 and T_H2 subsets. We focused our analysis on two general categories of genes, namely *i)* genes induced in the absence of cell division (including early genes, activated within a few hours after T cell activation, such as *IL2RA*), and *ii)* late genes, such as *IFNG*, that require multiple cell divisions and DNA demethylation-linked processes before being effectively transcribed (Grogan et al, 2001). At all the interrogated regulatory regions of early response genes (*IL2RA*, *CD69*, *ICOS* and *LTA*), we found high levels of 5hmC in resting cells, followed by a general reduction consistent with the global decrease in 5hmC observed in activated, proliferating cells (**Figure 7c** and **Suppl. Figure 4a**). Late genes (*IFNG*, *IL4*, *CD70*, *GZMB* and *CXCL10*) showed instead comparatively low basal levels of 5hmC in their regulatory regions at resting state, followed by an increase during differentiation and proliferation, suggesting a role for 5hmC in facilitating transcription of these genes in differentiated T lymphocytes (**Figure 7c** and **Suppl. Figure 5**). Interestingly, housekeeping genes (genes whose expression did not change in the different conditions analyzed) showed either no change of 5hmC (e.g. *SDHA*) or followed the general reduction in 5hmC observed after activation and proliferation (**Figure 7c** and **Suppl. Figure 4b**).

We then confirmed changes in 5mC and 5hmC at the regulatory regions of specific immunologically relevant genes by methylated and hydroxymethylated DNA immunoprecipation (MeDIP and hMeDIP) followed by qPCR. We selected *IL2* and *IL2RA* as early genes and *IFNG* as a late gene. We observed a variable and generally very modest, if any, reduction in the levels of 5mC at the *IL2* and *IL2RA* enhancers (**Figure 7d**, top). Consistent with previous studies demonstrating a strong correlation between hypomethylation of the proximal *Infg* enhancer and expression of IFN-γ in mouse T_H1 cells (Schoenborn et al, 2007), we found that this regulatory region, corresponding to the mouse CNS-6 (conserved non-coding sequence at -6 kb) was methylated in resting human naïve T cells, and underwent demethylation upon activation (**Figure 7d**, top). The TSS region of *IFNG* appeared to undergo some level of demethylation in activated memory T cells, probably correlating with the high levels of transcription of this gene. As for levels of 5hmC, the *IL2* and *IL2RA* enhancers showed a general 5hmC reduction following activation, consistent with the global loss of genomic 5hmC observed after cell activation and proliferation, while the *IFNG* proximal enhancer region showed a significant 5hmC increase, mirroring the changes in 5mC at this region (**Figure 7d**, bottom). No 5hmC changes were detected at the TSS of the *IFNG* gene. Changes in 5hmC were therefore more dynamic than those in 5mC and appeared to have a distinct behavior in early (5hmC reduction) and late (5hmC increase) response genes.

High levels of 5hmC may be critical especially in non-proliferating cells to maintain regulatory regions in an accessible state, amenable of transcriptional activation even in quiescent cells. To explore this possibility, we analyzed available ATAC-seq (Assay for Transposase-Accessible Chromatin and sequencing) datasets in human naïve T cells (Mumbach et al, 2017). Our analysis revealed that ATAC-accessible regions were indeed enriched of 5hmC (**Figure 7e**). Overall, these data are consistent with a model in which in the absence of cell proliferation, 5hmC enables the expression of housekeeping and early response genes, presumably by counteracting DNA methylation

at these regions. Conversely, a small subset of effector genes acquired 5hmC upon activation and proliferation, and these changes correlated with gene expression.

Discussion

Cell cycle progression is a key mechanism to relieve epigenetic repression during development and differentiation, while terminally differentiated cells acquire a relatively more stable epigenetic landscape. Our data now show that active removal of cytosine modifications contributes significantly to the first activation of naïve T lymphocytes, while replication-dependent dilution is the driving force leading to 5hmC reduction in highly proliferative already differentiated cells.

If 5hmC is diluted predominantly through DNA replication, why is it important for T cells in the first place? One possibility is that the activity of TET proteins is required to counteract the 'spreading' of DNA methylation to regions that are not actively transcribed in resting lymphocytes, but require fast activation upon antigen recognition. Indeed, we found that regulatory regions of several early response genes, namely genes that can be rapidly transcribed upon activation of resting cells and in the absence of proliferation, were characterized by the presence of 5hmC, suggesting that 5hmC-dependent processes may be required to counteract deleterious methylation at these regions. The 5hmC modification at such early response genes was then lost by passive dilution, once the cells overcame their activation threshold and entered the cell cycle. Housekeeping genes, that require expression even in the absence of proliferation, showed either no changes in 5hmC levels or a reduction correlating with the general loss of 5hmC observed in proliferating cells. Finally, activation of late effector genes correlated with an increase of 5hmC at their regulatory regions, likely to be associated with active transcription and the necessity to establish a stably permissive chromatin landscape at these enhancers in differentiated cells. The fact that DNA methylation has the tendency to accumulate to transcriptional inactive genes (Bird, 2002), and at enhancers that are not actively bound by transcription factors (a process that would be extremely deleterious for genes encoding for effector cytokines) was already proposed with the discovery that DNA-binding factors were necessary and sufficient to induce local demethylation, and that loss of transcription factor binding was conversely sufficient for DNA methylation (Calo & Wysocka, 2013; Stadler et al, 2011). This is also in line with recent findings showing that the activity of TET proteins is important to safeguard promoters from *de novo* methylation in embryonic stem cells (ESCs), thereby preserving appropriate lineage-specific transcription upon differentiation (Manzo et al, 2017; Verma et al, 2018). Similarly, TET1 was shown to prevent the spreading of DNA methylation at CpG islands (Jin et al, 2014), and also to be required in germ cells to provide efficient protection from aberrant DNA methylation, thereby stabilizing the acquired epigenetic landscape after reprogramming events (Hill et al, 2018).

In T lymphocytes, the primary role of 5hmC would therefore be to counteract DNA methylation especially in resting, non-proliferating naïve T cells, which cannot exploit passive, cell cycle-dependent mechanisms of dilution as a 'resetting' device of wrongful methylation. Such mechanism may be especially important for human naïve T lymphocytes: differently from the mouse, in which the average lifespan of a naïve T lymphocyte is only 6-10 weeks, individual human naïve T cells can persist for 5-10 years without losing significant functionality (den Braber et al, 2012; Kumar et al, 2018; Thome et al, 2016; van den Broek et al, 2018; Vrisekoop et al, 2008). Similarly to human naïve T cells, 5hmC-mediated processes may be crucial in rarely dividing or post-mitotic cells, such as neurons, in which replication-dependent dilution cannot occur, and that indeed present exceptionally high levels of 5hmC in their genome (Kriaucionis & Heintz, 2009). During mouse brain development,

5hmC was shown to be present at poised (yet dormant) loci, in order to facilitate activation at later developmental stages (Lister et al, 2013).

A second related possibility is that the presence of 5hmC may be required at specific enhancers to somehow create a 'window of opportunity' for enhancer demethylation upon T cell activation, which is no longer required once the cells are appropriately activated and can enter proliferation. Indeed, a model in which 5hmC could be part of enhancer activation processes somewhat independently of 5mC was already proposed (Calo & Wysocka, 2013), in which 5hmC may counteract gene silencing by opposing chromatin states that would impede transcription, for example through the specific recruitment of yet to be identified effector molecules.

Once T lymphocytes become activated and enter a differentiation pathway towards effector cells, they become 'locked' in a less plastic memory/ effector state in which demethylation-dependent mechanisms are likely to become progressively relatively less relevant. Indeed, many mechanisms contribute to the maintenance of the differentiated state, including histone modifications at lineage-specific enhancer genes and specific expression of master transcription factors essential for lineage determination (Kanno et al, 2012; Rothenberg & Zhang, 2012; Wilson et al, 2009). For example, permissive histone modifications can be observed at the murine *Ifng* locus early after T cell activation in both T_H1 and T_H2 polarizing conditions, but a T_H1-specific pattern becomes apparent as early as 48 hours after stimulation (Avni et al, 2002). However, stable commitment to a single lineage was shown to require three-to-four cycles of cell division (Grogan et al, 2001); once this threshold was passed, polarized lymphocytes maintained expression of their specific lineage-appropriate cytokines even upon removal of polarizing stimuli. The fact that DNA-demethylation processes may become less relevant as cell differentiation progresses can be also inferred by the fact that forced expression of TET enzymes in already activated and proliferating T lymphocytes revealed only a modest effect on gene expression (Nestor et al, 2016).

Altogether, our data corroborate a model in which 5hmC may be required to maintain enhancer accessibility in resting T lymphocytes, thereby counteracting DNA methylation of essential regions that must retain a poised state for rapid responses upon encounter with an antigen.

Author Contributions

LV and CL designed and performed experiments and analyzed data; MC and IK analyzed data; SM overviewed the project, analyzed data and wrote the manuscript with input from all authors.

Conflict of interest

The authors declare no competing financial interests.

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References

Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, Mason CE (2012) methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome biology* **13:** R87

Avni O, Lee D, Macian F, Szabo SJ, Glimcher LH, Rao A (2002) T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nature immunology* **3:** 643-651

Bevington SL, Cauchy P, Piper J, Bertrand E, Lalli N, Jarvis RC, Gilding LN, Ott S, Bonifer C, Cockerill PN (2016) Inducible chromatin priming is associated with the establishment of immunological memory in T cells. *The EMBO journal* **35:** 515-535

Bird A (2002) DNA methylation patterns and epigenetic memory. *Genes & development* **16:** 6-21

Bird JJ, Brown DR, Mullen AC, Moskowitz NH, Mahowald MA, Sider JR, Gajewski TF, Wang CR, Reiner SL (1998) Helper T cell differentiation is controlled by the cell cycle. *Immunity* **9:** 229-237

Blaschke K, Ebata KT, Karimi MM, Zepeda-Martinez JA, Goyal P, Mahapatra S, Tam A, Laird DJ, Hirst M, Rao A, Lorincz MC, Ramalho-Santos M (2013) Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. *Nature* **500:** 222-226

Bock C, Tomazou EM, Brinkman AB, Muller F, Simmer F, Gu H, Jager N, Gnirke A, Stunnenberg HG, Meissner A (2010) Quantitative comparison of genome-wide DNA methylation mapping technologies. *Nature biotechnology* **28:** 1106-1114

Bruniquel D, Schwartz RH (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nature immunology* **4:** 235-240

Calo E, Wysocka J (2013) Modification of enhancer chromatin: what, how, and why? *Molecular cell* **49:** 825-837

Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, Clark NR, Ma'ayan A (2013) Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC bioinformatics* **14:** 128

den Braber I, Mugwagwa T, Vrisekoop N, Westera L, Mogling R, de Boer AB, Willems N, Schrijver EH, Spierenburg G, Gaiser K, Mul E, Otto SA, Ruiter AF, Ackermans MT, Miedema F, Borghans JA, de Boer RJ, Tesselaar K (2012) Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. *Immunity* **36:** 288-297

Durek P, Nordstrom K, Gasparoni G, Salhab A, Kressler C, de Almeida M, Bassler K, Ulas T, Schmidt F, Xiong J, Glazar P, Klironomos F, Sinha A, Kinkley S, Yang X, Arrigoni L, Amirabad AD, Ardakani FB, Feuerbach L, Gorka O, Ebert P, Muller F, Li N, Frischbutter S, Schlickeiser S, Cendon

C, Frohler S, Felder B, Gasparoni N, Imbusch CD, Hutter B, Zipprich G, Tauchmann Y, Reinke S, Wassilew G, Hoffmann U, Richter AS, Sieverling L, Consortium D, Chang HD, Syrbe U, Kalus U, Eils J, Brors B, Manke T, Ruland J, Lengauer T, Rajewsky N, Chen W, Dong J, Sawitzki B, Chung HR, Rosenstiel P, Schulz MH, Schultze JL, Radbruch A, Walter J, Hamann A, Polansky JK (2016) Epigenomic Profiling of Human CD4(+) T Cells Supports a Linear Differentiation Model and Highlights Molecular Regulators of Memory Development. *Immunity* **45:** 1148-1161

Gamper CJ, Agoston AT, Nelson WG, Powell JD (2009) Identification of DNA methyltransferase 3a as a T cell receptor-induced regulator of Th1 and Th2 differentiation. *Journal of immunology* **183:** 2267-2276

Geginat J, Lanzavecchia A, Sallusto F (2003) Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* **101:** 4260-4266

Geginat J, Sallusto F, Lanzavecchia A (2001) Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *The Journal of experimental medicine* **194:** 1711-1719

Grogan JL, Mohrs M, Harmon B, Lacy DA, Sedat JW, Locksley RM (2001) Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* **14:** 205-215

Hashimoto S, Ogoshi K, Sasaki A, Abe J, Qu W, Nakatani Y, Ahsan B, Oshima K, Shand FH, Ametani A, Suzuki Y, Kaneko S, Wada T, Hattori M, Sugano S, Morishita S, Matsushima K (2013) Coordinated changes in DNA methylation in antigen-specific memory CD4 T cells. *Journal of immunology* **190:** 4076-4091

Hill PWS, Leitch HG, Requena CE, Sun Z, Amouroux R, Roman-Trufero M, Borkowska M, Terragni J, Vaisvila R, Linnett S, Bagci H, Dharmalingham G, Haberle V, Lenhard B, Zheng Y, Pradhan S, Hajkova P (2018) Epigenetic reprogramming enables the transition from primordial germ cell to gonocyte. *Nature* **555:** 392-396

Huang da W, Sherman BT, Lempicki RA (2009a) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research* **37:** 1-13

Huang da W, Sherman BT, Lempicki RA (2009b) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* **4:** 44-57

Ichiyama K, Chen T, Wang X, Yan X, Kim BS, Tanaka S, Ndiaye-Lobry D, Deng Y, Zou Y, Zheng P, Tian Q, Aifantis I, Wei L, Dong C (2015) The methylcytosine dioxygenase Tet2 promotes DNA demethylation and activation of cytokine gene expression in T cells. *Immunity* **42:** 613-626

Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* **466:** 1129-1133

Jaworski M, Thome M (2016) The paracaspase MALT1: biological function and potential for therapeutic inhibition. *Cellular and molecular life sciences : CMLS* **73:** 459-473

Jin C, Lu Y, Jelinek J, Liang S, Estecio MR, Barton MC, Issa JP (2014) TET1 is a maintenance DNA demethylase that prevents methylation spreading in differentiated cells. *Nucleic acids research* **42:** 6956-6971

Kanno Y, Vahedi G, Hirahara K, Singleton K, O'Shea JJ (2012) Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annual review of immunology* **30:** 707-731

Kersh EN, Fitzpatrick DR, Murali-Krishna K, Shires J, Speck SH, Boss JM, Ahmed R (2006) Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. *Journal of immunology* **176:** 4083-4093

Ko M, An J, Bandukwala HS, Chavez L, Aijo T, Pastor WA, Segal MF, Li H, Koh KP, Lahdesmaki H, Hogan PG, Aravind L, Rao A (2013) Modulation of TET2 expression and 5-methylcytosine oxidation by the CXXC domain protein IDAX. *Nature* **497:** 122-126

Ko M, Bandukwala HS, An J, Lamperti ED, Thompson EC, Hastie R, Tsangaratou A, Rajewsky K, Koralov SB, Rao A (2011) Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. *Proceedings of the National Academy of Sciences of the United States of America* **108:** 14566-14571

Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* **468:** 839-843

Komori HK, Hart T, LaMere SA, Chew PV, Salomon DR (2015) Defining CD4 T cell memory by the epigenetic landscape of CpG DNA methylation. *Journal of immunology* **194:** 1565-1579

Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* **324:** 929-930

Kumar BV, Connors TJ, Farber DL (2018) Human T Cell Development, Localization, and Function throughout Life. *Immunity* **48:** 202-213

Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S, Cherry SR, Tsai JH, Tucker SM, Weaver WM, Kelso A, Jaenisch R, Wilson CB (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* **15:** 763-774

Leoni C, Montagner S, Rinaldi A, Bertoni F, Polletti S, Balestrieri C, Monticelli S (2017) Dnmt3a restrains mast cell inflammatory responses. *Proceedings of the National Academy of Sciences of the United States of America* **114:** E1490-E1499

Lister R, Mukamel EA, Nery JR, Urich M, Puddifoot CA, Johnson ND, Lucero J, Huang Y, Dwork AJ, Schultz MD, Yu M, Tonti-Filippini J, Heyn H, Hu S, Wu JC, Rao A, Esteller M, He C, Haghighi FG, Sejnowski TJ, Behrens MM, Ecker JR (2013) Global epigenomic reconfiguration during mammalian brain development. *Science* **341:** 1237905

Makar KW, Perez-Melgosa M, Shnyreva M, Weaver WM, Fitzpatrick DR, Wilson CB (2003) Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. *Nature immunology* **4:** 1183-1190

Makar KW, Wilson CB (2004) DNA methylation is a nonredundant repressor of the Th2 effector program. *Journal of immunology* **173:** 4402-4406

Manzo M, Wirz J, Ambrosi C, Villasenor R, Roschitzki B, Baubec T (2017) Isoform-specific localization of DNMT3A regulates DNA methylation fidelity at bivalent CpG islands. *The EMBO journal* **36:** 3421-3434

Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454:** 766-770

Montagner S, Leoni C, Emming S, Della Chiara G, Balestrieri C, Barozzi I, Piccolo V, Togher S, Ko M, Rao A, Natoli G, Monticelli S (2016) TET2 Regulates Mast Cell Differentiation and Proliferation through Catalytic and Non-catalytic Activities. *Cell reports* **15:** 1566-1579

Mumbach MR, Satpathy AT, Boyle EA, Dai C, Gowen BG, Cho SW, Nguyen ML, Rubin AJ, Granja JM, Kazane KR, Wei Y, Nguyen T, Greenside PG, Corces MR, Tycko J, Simeonov DR, Suliman N, Li R, Xu J, Flynn RA, Kundaje A, Khavari PA, Marson A, Corn JE, Quertermous T, Greenleaf WJ, Chang HY (2017) Enhancer connectome in primary human cells identifies target genes of disease-associated DNA elements. *Nature genetics* **49:** 1602-1612

Nestor CE, Lentini A, Hagg Nilsson C, Gawel DR, Gustafsson M, Mattson L, Wang H, Rundquist O, Meehan RR, Klocke B, Seifert M, Hauck SM, Laumen H, Zhang H, Benson M (2016) 5-Hydroxymethylcytosine Remodeling Precedes Lineage Specification during Differentiation of Human CD4(+) T Cells. *Cell reports* **16:** 559-570

Rothenberg EV, Zhang JA (2012) T-cell identity and epigenetic memory. *Current topics in microbiology and immunology* **356:** 117-143

Scharer CD, Barwick BG, Youngblood BA, Ahmed R, Boss JM (2013) Global DNA methylation remodeling accompanies CD8 T cell effector function. *Journal of immunology* **191:** 3419-3429

Schoenborn JR, Dorschner MO, Sekimata M, Santer DM, Shnyreva M, Fitzpatrick DR, Stamatoyannopoulos JA, Wilson CB (2007) Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. *Nature immunology* **8:** 732-742

Scott-Browne JP, Cw L, Rao A (2017) TET proteins in natural and induced differentiation. *Current opinion in genetics & development* **46:** 202-208

Smith ZD, Meissner A (2013) DNA methylation: roles in mammalian development. *Nature reviews Genetics* **14:** 204-220

Stadler MB, Murr R, Burger L, Ivanek R, Lienert F, Scholer A, van Nimwegen E, Wirbelauer C, Oakeley EJ, Gaidatzis D, Tiwari VK, Schubeler D (2011) DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature* **480:** 490-495

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* **324:** 930-935

Thome JJ, Grinshpun B, Kumar BV, Kubota M, Ohmura Y, Lerner H, Sempowski GD, Shen Y, Farber DL (2016) Longterm maintenance of human naive T cells through in situ homeostasis in lymphoid tissue sites. *Science immunology* **1**

Tsagaratou A, Aijo T, Lio CW, Yue X, Huang Y, Jacobsen SE, Lahdesmaki H, Rao A (2014) Dissecting the dynamic changes of 5-hydroxymethylcytosine in T-cell development and differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **111:** E3306-3315

Tsagaratou A, Lio CJ, Yue X, Rao A (2017) TET Methylcytosine Oxidases in T Cell and B Cell Development and Function. *Frontiers in immunology* **8:** 220

Unutmaz D, Pileri P, Abrignani S (1994) Antigen-independent activation of naive and memory resting T cells by a cytokine combination. *The Journal of experimental medicine* **180:** 1159-1164

van den Broek T, Borghans JAM, van Wijk F (2018) The full spectrum of human naive T cells. *Nature reviews Immunology*

Verma N, Pan H, Dore LC, Shukla A, Li QV, Pelham-Webb B, Teijeiro V, Gonzalez F, Krivtsov A, Chang CJ, Papapetrou EP, He C, Elemento O, Huangfu D (2018) TET proteins safeguard bivalent promoters from de novo methylation in human embryonic stem cells. *Nature genetics* **50:** 83-95

Vrisekoop N, den Braber I, de Boer AB, Ruiter AF, Ackermans MT, van der Crabben SN, Schrijver EH, Spierenburg G, Sauerwein HP, Hazenberg MD, de Boer RJ, Miedema F, Borghans JA, Tesselaar K (2008) Sparse production but preferential incorporation of recently produced naive T cells in the

human peripheral pool. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 6115-6120

Wilson CB, Rowell E, Sekimata M (2009) Epigenetic control of T-helper-cell differentiation. *Nature reviews Immunology* **9:** 91-105

Wu X, Zhang Y (2017) TET-mediated active DNA demethylation: mechanism, function and beyond. *Nature reviews Genetics* **18:** 517-534

Yin R, Mao SQ, Zhao B, Chong Z, Yang Y, Zhao C, Zhang D, Huang H, Gao J, Li Z, Jiao Y, Li C, Liu S, Wu D, Gu W, Yang YG, Xu GL, Wang H (2013) Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. *Journal of the American Chemical Society* **135:** 10396-10403

Yu M, Hon GC, Szulwach KE, Song CX, Zhang L, Kim A, Li X, Dai Q, Shen Y, Park B, Min JH, Jin P, Ren B, He C (2012a) Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. *Cell* **149:** 1368-1380

Yu Q, Zhou B, Zhang Y, Nguyen ET, Du J, Glosson NL, Kaplan MH (2012b) DNA methyltransferase 3a limits the expression of interleukin-13 in T helper 2 cells and allergic airway inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **109:** 541-546

Yue X, Trifari S, Aijo T, Tsagaratou A, Pastor WA, Zepeda-Martinez JA, Lio CW, Li X, Huang Y, Vijayanand P, Lahdesmaki H, Rao A (2016) Control of Foxp3 stability through modulation of TET activity. *The Journal of experimental medicine* **213:** 377-397

Figures and Figure Legends

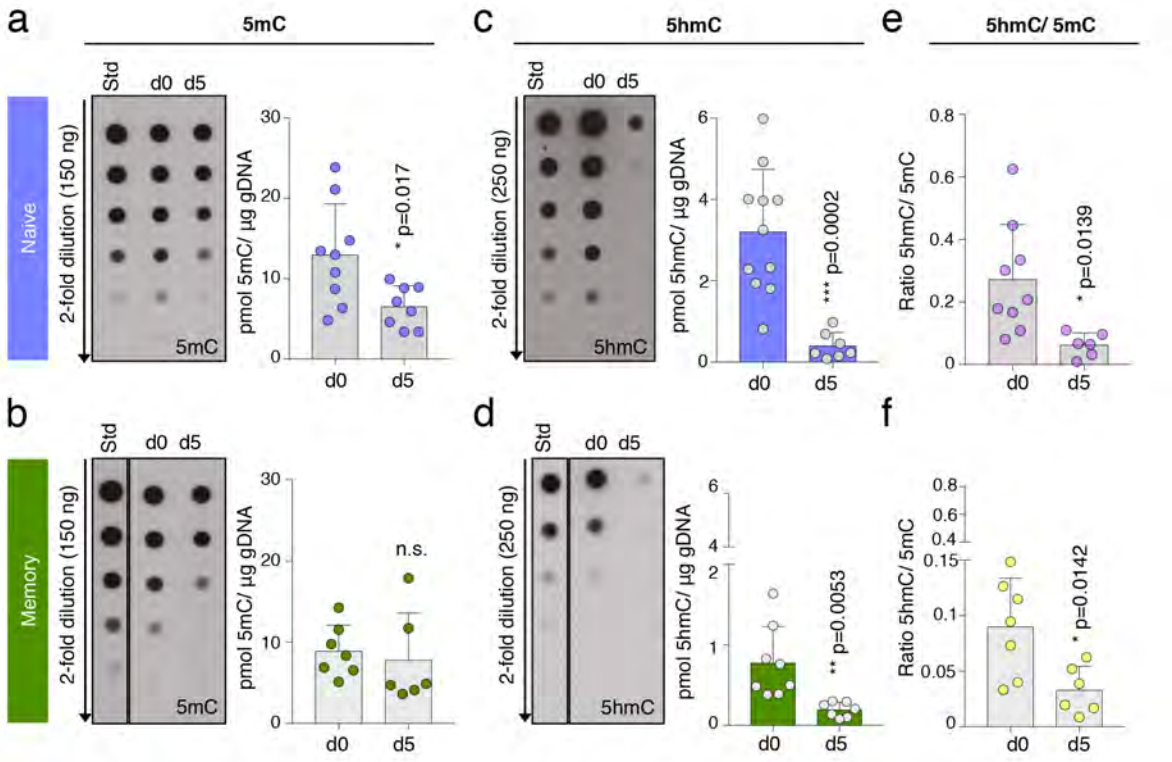


Figure 1. Dynamics of 5mC and 5hmC changes in primary human T lymphocytes. (a) Freshly isolated naïve and **(b)** memory $CD4^+$ T lymphocytes were either left resting or were stimulated for five days with plate-bound anti-CD3 and anti-CD28 antibodies, followed by genomic DNA extraction and measurement of the levels of 5mC and **(c-d)** 5hmC by dot blot. **(e-f)** Ratio between genomic 5hmC and 5mC measured in the previous panels. Each dot represents one individual human donor. Mean ± SD. Unpaired t-test.

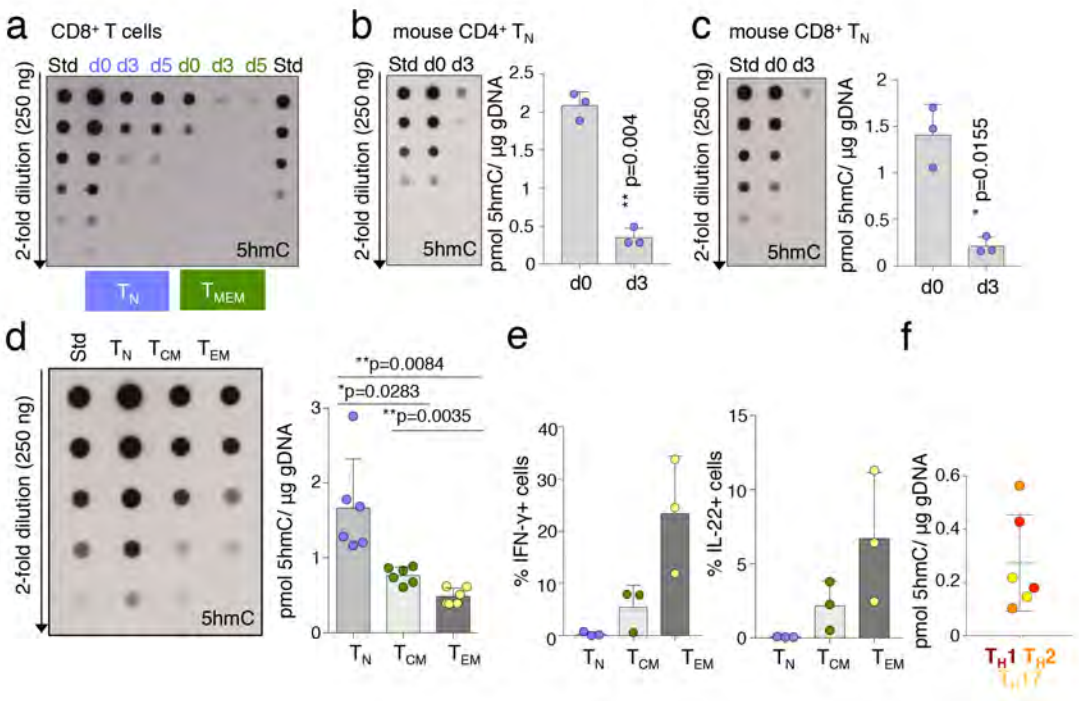


Figure 2. Loss of genomic 5hmC is a general feature of T cell activation. (a) Primary human naïve and memory CD8$^+$ T lymphocytes were either left resting or were stimulated for three and five days with plate-bound anti-CD3 and anti-CD28 antibodies, followed by measurement of the levels of genomic 5hmC. **(b)** Naïve CD4$^+$ and **(c**) CD8$^+$ murine spleen T lymphocytes were either left resting or were stimulated for three days with plate-bound anti-CD3 and anti-CD28 antibodies, followed by genomic DNA extraction and measurement of the levels of 5hmC. Each dot represents one mouse. Mean ± SD. Paired t-test. **(d)** Human naïve, central memory (T_{CM}) and effector memory (T_{EM}) T lymphocytes were isolated from peripheral blood and global levels of 5hmC were measured by dot blot. One representative blot is shown on the left, while the compiled analysis of several donors is shown on the right. Each dot represents one individual donor. Mean ± SD. Paired t-test. **(e)** Cells were isolated as in (d) and the percentage of IFN-γ- and IL-22-producing cells was determined by intracellular staining. Each dot represents one donor. Mean ± SD. **(f)** Individual effector subsets (T_H1, T_H2 and T_H17) were freshly isolated from peripheral blood and global levels of 5hmC were measured by dot blot. Each dot represents one individual donor. Mean ± SD.

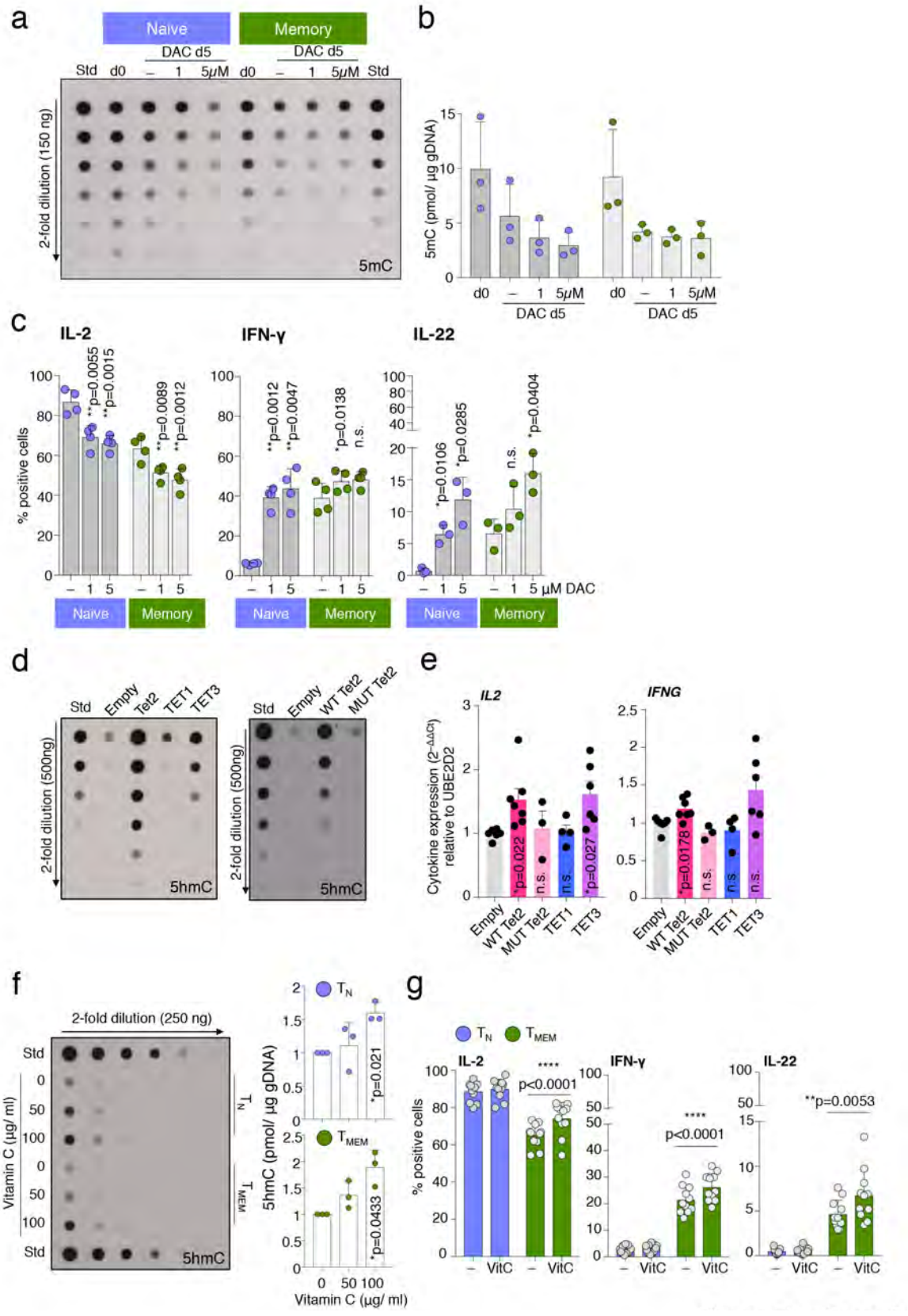


Figure 3. DNA demethylation-related processes augment expression of effector cytokines. (a) Naïve and memory T cells were separated from peripheral blood and either left untreated or were stimulated for five days with plate-bound anti-CD3 and anti-CD28 antibodies, with or without addition of decitabine (1 μM or 5 μM). Genomic DNA was purified and global levels of 5mC were measured by dot blot. **(b)** Same as (a), three independent experiments. Mean ± SD. **(c)** Cells were

treated as in (a), except that the expression of the indicated cytokines was measured by intracellular staining. Each dot represents one independent experiment. Mean ± SD. Paired t-test. **(d)** Jurkat T cells were transiently transfected with plasmids expressing either full-length mouse Tet2 (wild-type or mutated in the catalytic domain), human TET1 or human TET3. 48 h after transfection, genomic DNA was isolated and levels of 5hmC were measured by dot blot. Shown is one representative blot of at least 3 independent experiments. **(e)** Cells were treated as in (d), except that they were also stimulated with PMA and ionomycin for 3 h to induce cytokine transcription, and total RNA was isolated. The expression of the indicated cytokines was measured by qRT-PCR, relative to UBE2D2 (Ubiquitin-conjugating enzyme E2 D2), used as endogenous control, and relative to cells transfected with an empty vector. Each dot represents one independent experiment. Mean ± SEM. Paired t-test. **(f)** Naïve and memory T cells were stimulated for three days in the presence or absence of the indicated concentrations of vitamin C. Levels of genomic 5hmC were measured by dot blot. Compiled results of three independent experiments are shown on the right. Mean ± SD. Paired t-test, compared to untreated cells. **(g)** Cells were treated exactly as in (f), except that the expression of the indicated cytokines was measured by intracellular staining. Shown are the results obtained from cells treated with 100 μg/ ml vitamin C. Each dot represents one individual donor. Mean ± SD. Paired t-test.

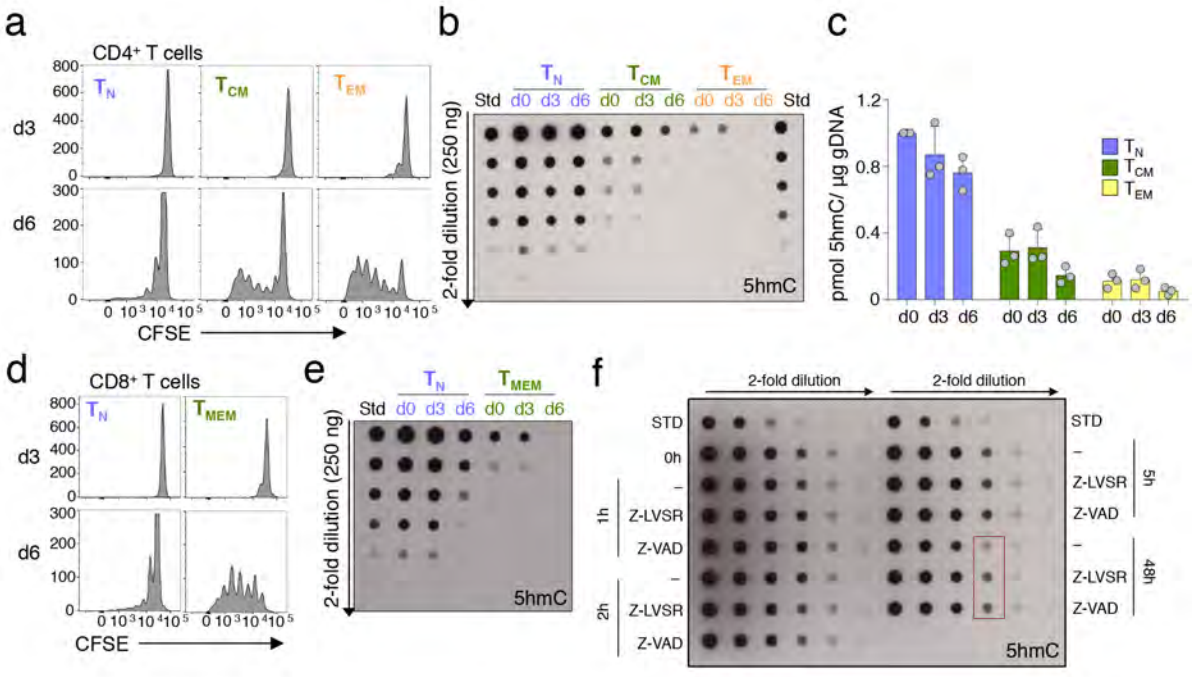


Figure 4. Proliferation, but not TCR engagement, determines the loss of genomic 5hmC. (a) Human naïve, T_{CM} and T_{EM} $CD4^+$ T cells were isolated from peripheral blood, loaded with the cell tracer CFSE to assess proliferation, and cultured in the presence of IL-2, IL-6 and TNF-α. Proliferation was measured after three and six days of culture. **(b)** Genomic DNA was purified from cells as in (a), and levels of 5hmC were measured by dot blot. One representative blot is shown. **(c)** Same as in (b); compiled results of three independent experiments. Mean ± SD. **(d)** Human naïve and memory $CD8^+$ T cells were isolated from peripheral blood and treated as in (a), except that they were cultured in presence of IL-7 and IL-15. **(e)** Dot blot to measure genomic 5hmC of cells treated as in (d). **(f)** Naïve T cells were either left resting or were stimulated with anti-CD3 and anti-CD28 antibodies, in the presence or absence of 40 µM z-LVSR or z-VAD inhibitors. Genomic DNA was collected at the indicated times after stimulation and levels of 5hmC were measured by dot blot. Representative of two independent experiments.

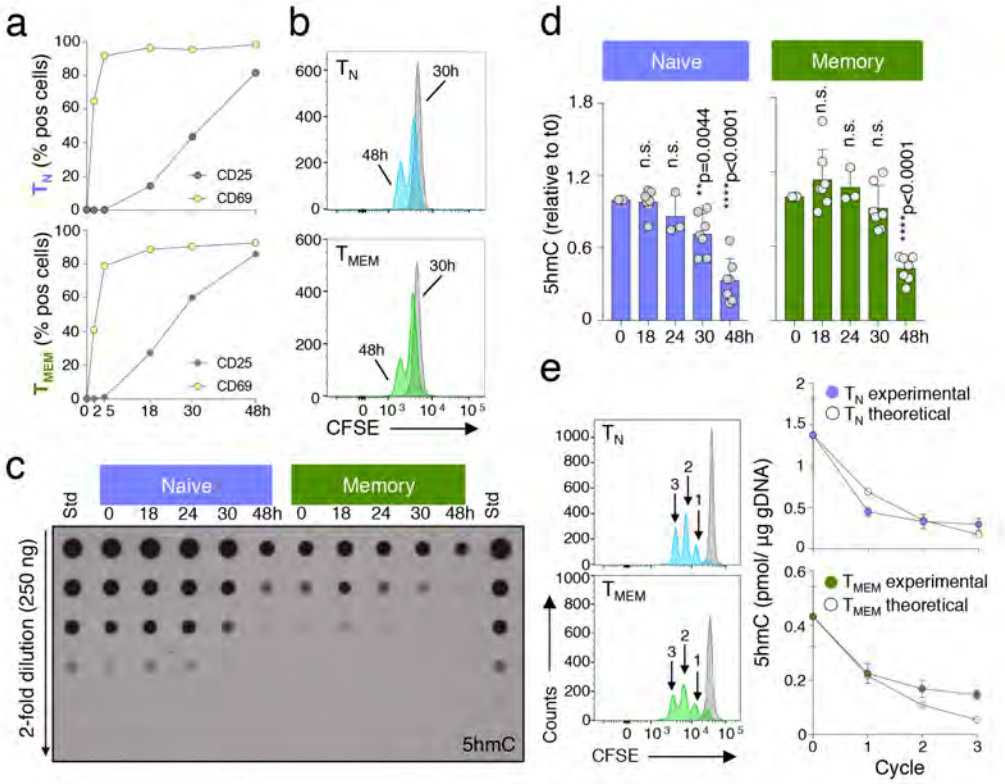


Figure 5. Active DNA demethylation in naïve, but not memory T lymphocytes. (a) Naïve and memory T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for the indicated times. The surface expression of early activation markers (CD25, CD69) was determined at each time point by FACS staining. **(b)** Same as (a), except that cells were loaded with CFSE prior to stimulation to measure the extent of cell proliferation at each time point. **(c)** Same as (a), except that genomic DNA was extracted at the indicated time points, and levels of 5hmC were measured by dot blot. **(d)** Compiled results of individual experiments performed as in (c). Each dot represents one donor. Mean ± SD. Paired t-test, compared to unstimulated cells. **(e)** Cells were loaded with CFSE, followed by stimulation with plate-bound anti-CD3 and anti-CD28 antibodies. After 3 days, each indicated peak was sorted, and levels of 5hmC were measured by dot blot in comparison to unstimulated cells. Shown are the results of n=4 independent experiments. Mean ± SD.

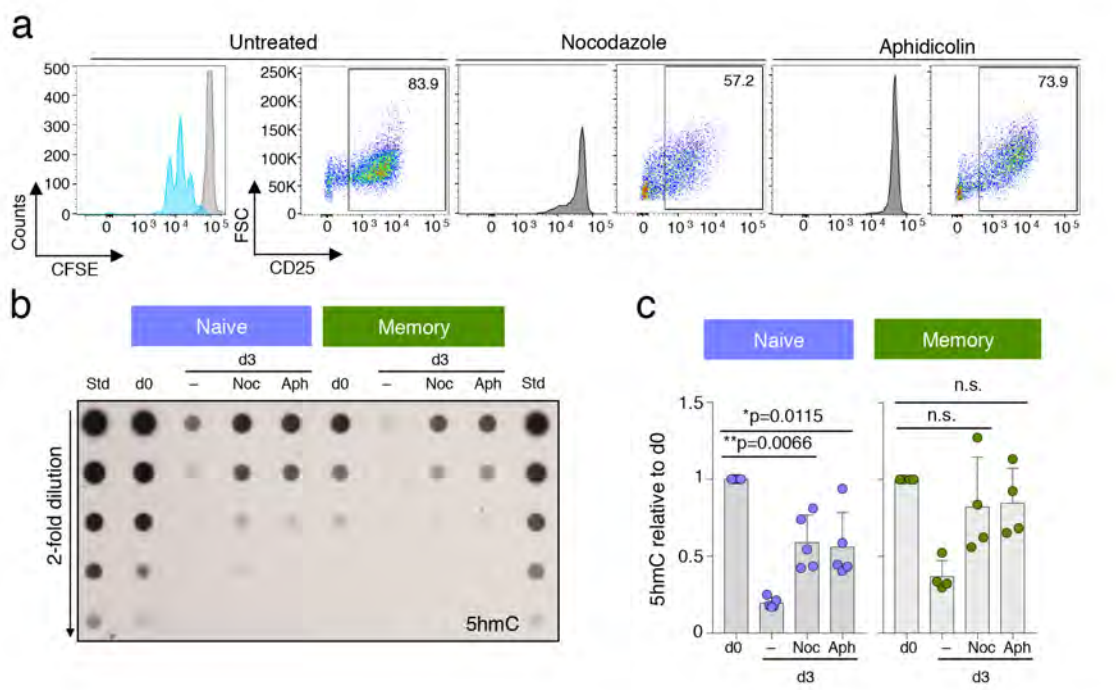


Figure 6. Inhibition of cell division reduces the loss of 5hmC. (a) Naïve and memory T lymphocytes were loaded with CFSE and activated for three days with anti-CD3 and anti-CD28 antibodies in the presence or absence of the cell cycle inhibitors aphidicolin and nocodazole. The extent of cell proliferation was determined by CSFE dilution, while cell activation was evaluated by measuring cell size (FSC) and surface expression of CD25. Shown is one representative result for naïve T cells. **(b)** Cells were treated as in (a), and levels of 5hmC in the genomic DNA were measured by dot blot. **(c)** Compiled results of individual experiments performed as in (b). Each dot represents one donor. Mean ± SD. Paired t-test, compared to unstimulated cells.

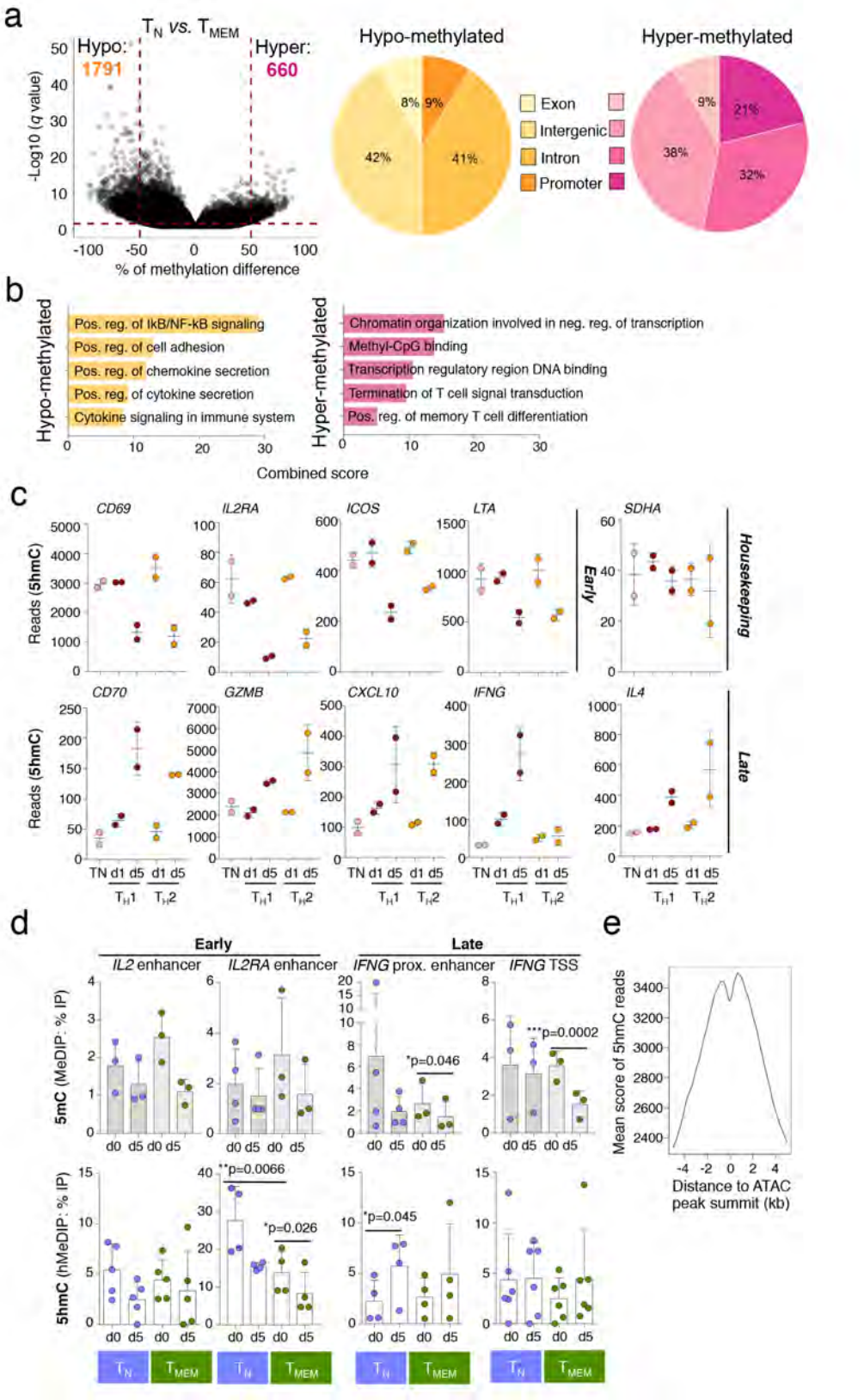
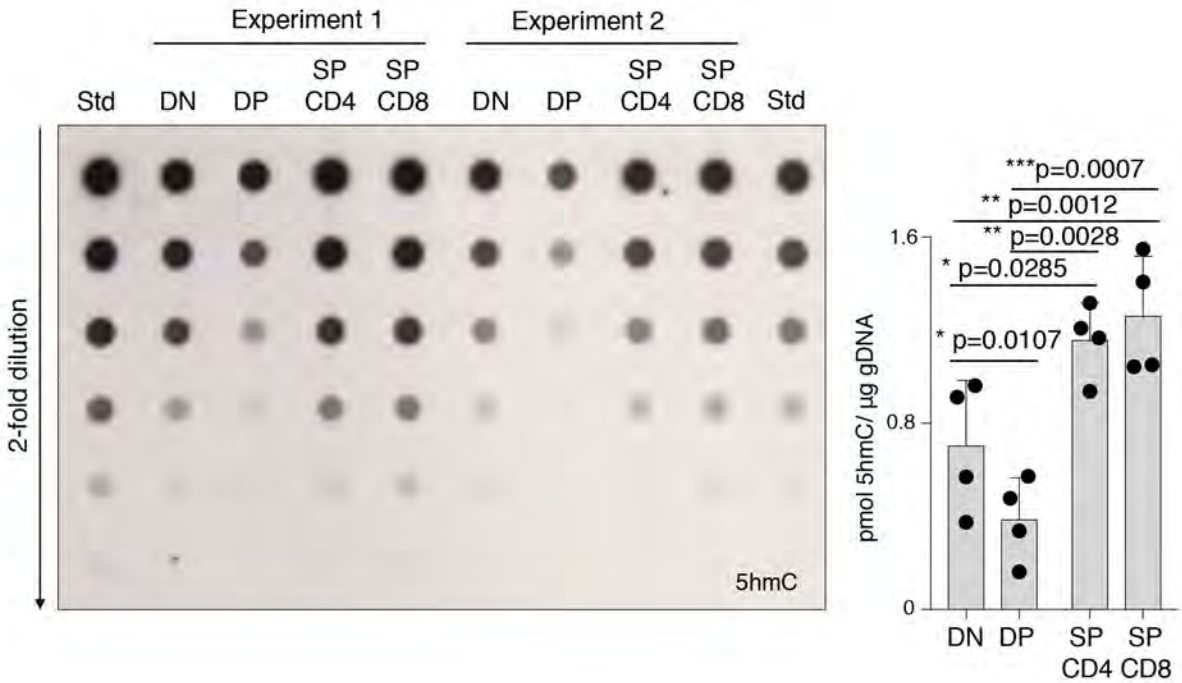


Figure 7. Dynamic changes of 5mC and 5hmC at regulatory regions of immune-related genes. **(a)** Left: volcano plot showing the differentially methylated regions observed by RRBS between freshly isolated naïve and memory T lymphocytes. Right: the differentially methylated regions shown in (a) were analyzed for their genomic location. **(b)** Selected GO terms significantly associated with hypo- or hyper-methylated promoter regions are shown. Differentially methylated regions located in

the region upstream of the TSS (0 to -1500bp), with q-value ≤0.2 and with a methylation difference of at least 33% were selected for the analysis, which was performed using EnrichR (Chen et al, 2013). **(c)** Levels of 5hmC in differentiated T cells at the indicated loci as assessed by hMeDIP-seq (Nestor et al, 2016). **(d)** MeDIP (top) or hMeDIP (bottom) experiments were performed on naïve and memory T cells either resting or stimulated for 5 days with plate-bound anti-CD3 and anti-CD28 antibodies. The enrichment for the indicated regulatory regions was measured by qPCR. Each dot represents one donor. Mean ± SD. Paired t-test. **(e)** Correlation of 5hmC signal with ATAC-accessible regions in a window of 10 kb in human naïve T lymphocytes. A score matrix of 5hmC score around ATAC peak positions was built starting from available data (Mumbach et al, 2017; Nestor et al, 2016).

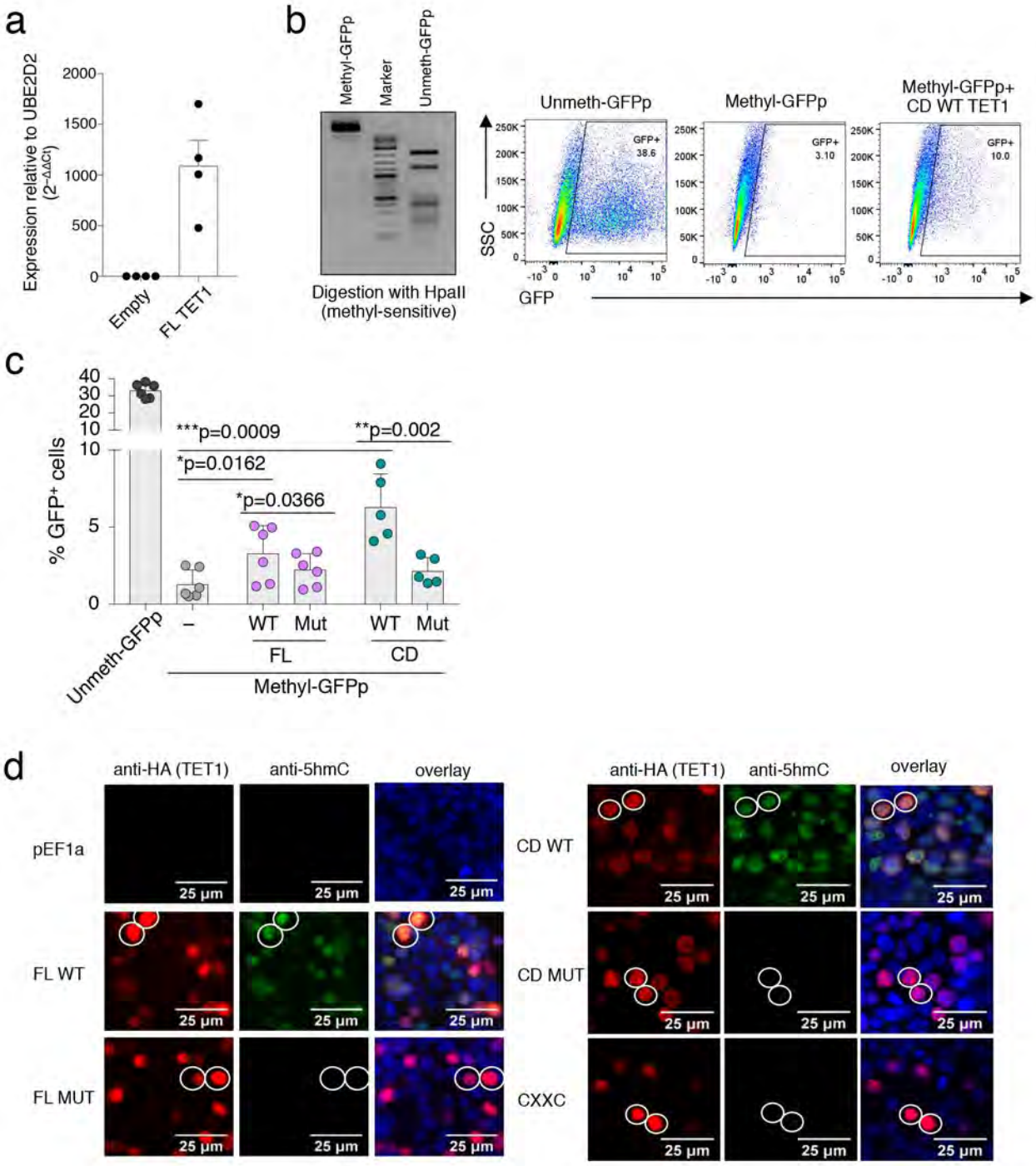
Supplemental Information

Table of contents:



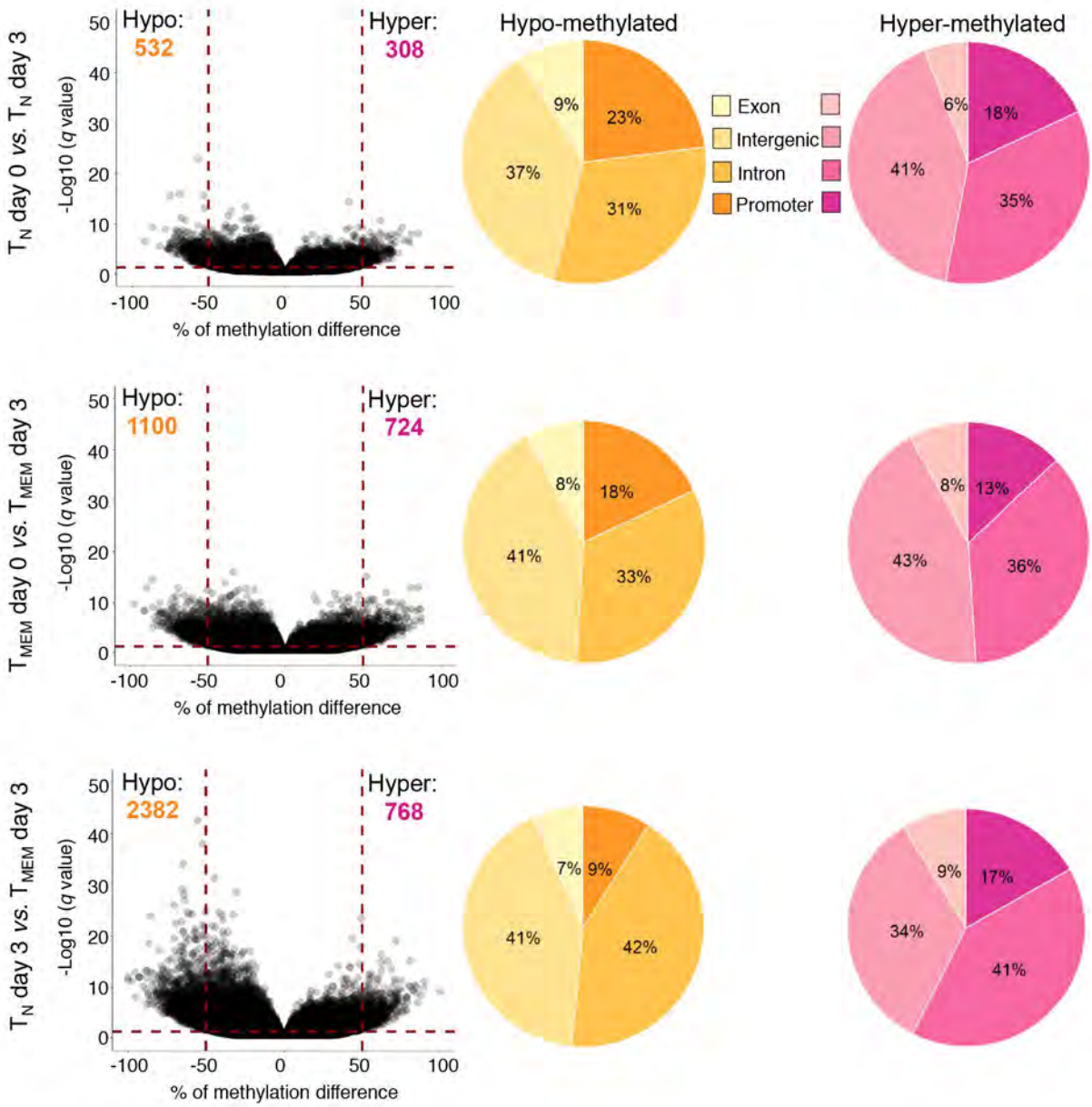
Vincenzetti – Suppl Fig 1

Suppl. Figure 1. Dynamic changes of 5hmC during thymocyte development. Thymi were obtained from 8 weeks-old C57BL/6 male mice and homogenized to single cell suspensions. Thymocytes were stained with anti-CD4 and anti-CD8 antibodies and separated by sorting in the following subsets: double-negative (DN) cells: $CD4^{-}CD8\alpha^{-}$; double-positive (DP) cells: $CD4^{+}CD8\alpha^{+}$; single-positive (SP) $CD4^{+}$: $CD4^{+}CD8\alpha^{-}$; SP $CD8^{+}$: $CD4^{-}CD8\alpha^{+}$. Genomic DNA was extracted and levels of 5hmC measured by dot blot. Two representative experiments are shown on the left, while the compiled results obtained from four independent mice are shown on the right. Mean ± SD. Paired t-test.



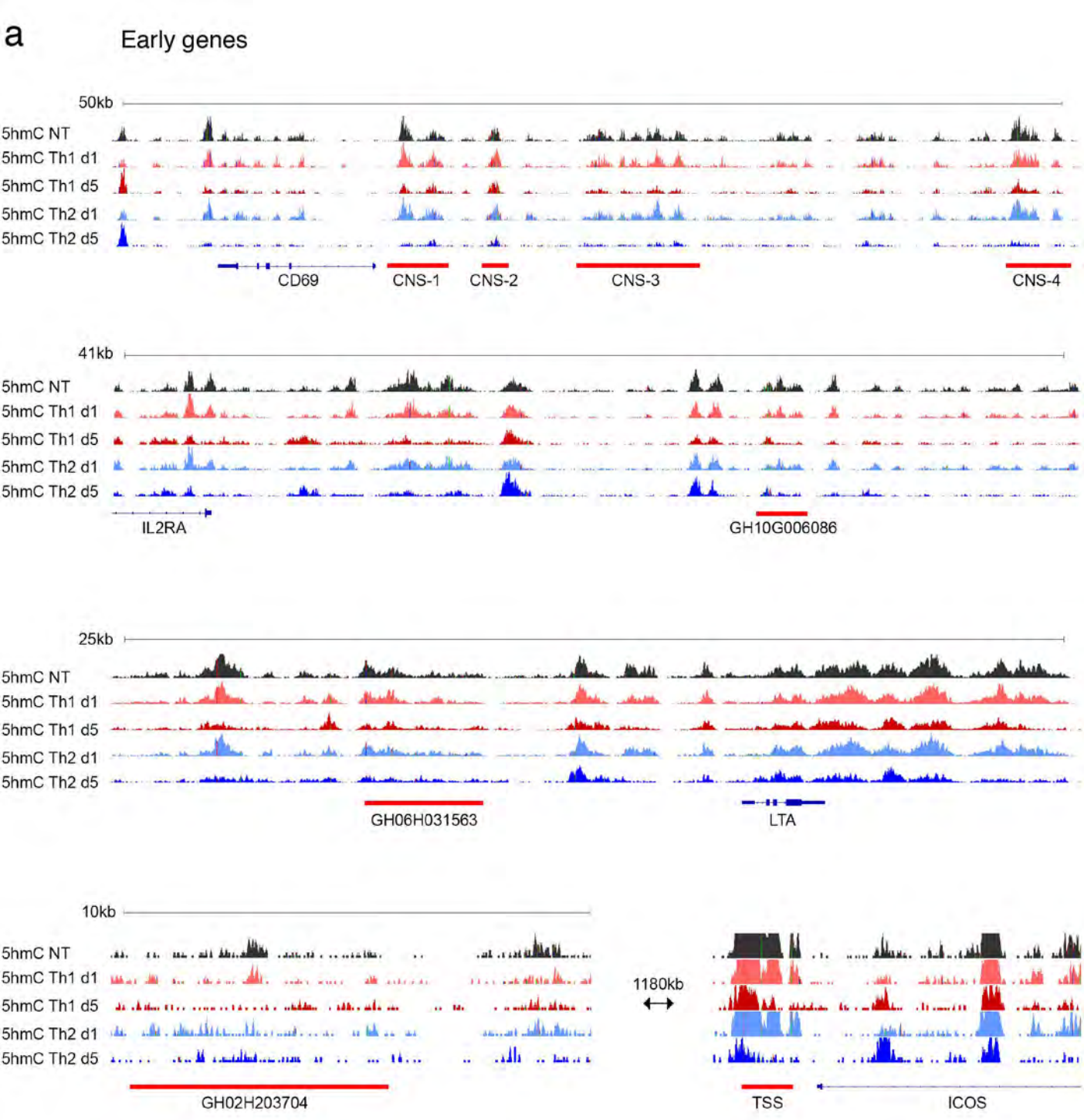
Vincenzetti – Suppl Fig 2

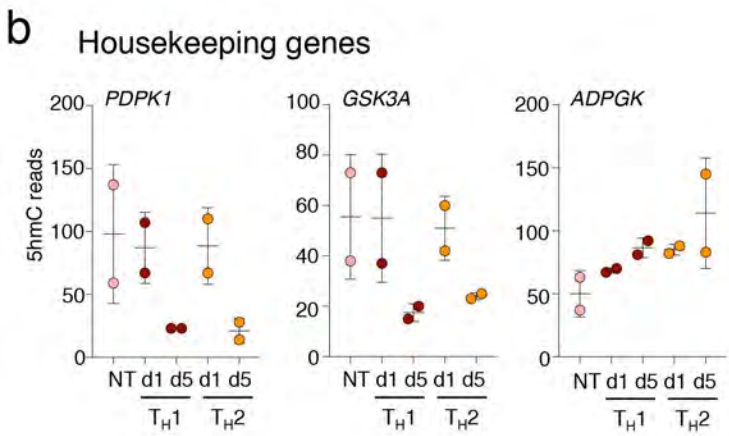
Suppl. Figure 2. Expression and functionality of transfected TET1 plasmids. (a) Jurkat cells were transiently transfected with a vector expressing full-length human TET1 or the corresponding empty vector as control. Total RNA was extracted 48 h after transfection and levels of *TET1* were measured by qRT-PCR. **(b)** To determine TET1 functionality, we performed a demethylation reporter assay as described (Guo et al., 2011). Briefly, 10 μg of a GFP-expressing plasmid were incubated with 20 units of M.SssI (CpG methyltransferase, New England BioLabs) and 1600 μM of S-adenosylmethionine (SAM) for 3 h at 37°C, followed by re-addition of the same amount of M.SssI and SAM and incubation for another 3 h. The reaction was stopped by heating at 65°C for 20 min, followed by DNA purification. Successful methylation was confirmed by the inability of HpaII (a methyl-sensitive restriction enzyme) to digest this plasmid in comparison with the same unmethylated plasmid (left panel). The fully methylated GFP-expressing plasmid was used to transfect HEK293 cells together with a plasmid expressing the wild-type catalytic domain (CD) of TET1 at a 1:6 ratio. GFP expression was measured by FACS 96 h after transfection. One representative experiment is shown on the right. **(c)** Exactly as in (b), except that the fully methylated GFP-expressing plasmid was co-transfected with different expression constructs for TET1 (full-length (FL) wild-type or mutated in the catalytic domain; CD wild-type or mutated). All plasmids were generated using common cloning procedures starting from FL WT TET1 (Addgene plasmid no. 49792). CD TET1 corresponds to amino acids 1247-2136 of TET1; the mutated form of TET1 contains the catalytic inactivating substitutions H1672Y and D1674A, and was generated by targeted mutagenesis using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). **(d)** TET1 expression and functionality from the same plasmids was also assessed by immunofluorescence. HEK293 cells were transfected in a 6 wells-plate with 2.5 μg of plasmid using polyethylenimine (PEI) and standard protocols. Plasmids were exactly as in (c), with addition of a plasmid expressing the truncated CXXC domain of TET1 (lacking the catalytic domain and corresponding to amino acids 1-1412 of TET1). For immunofluorescence, transfected cells were fixed with 4% paraformaldehyde for 15 min at RT and then permeabilized for 15 min with 0.2% Triton X-100 in PBS. The DNA was denatured by treating the slides with 2N HCl for 30 min followed by pH neutralization with 100 mM Tris-HCl buffer (pH 8.0) for 10 min. After 1 h of blocking with 1% BSA, cells were stained with rabbit anti-5hmC antibody (Active Motif) and mouse anti-HA (Santa Cruz Biotechnology), followed by incubation with secondary antibodies conjugated with different fluorochromes (Thermo Fisher Scientific). For microscope image acquisition, a Nikon Eclipse E800 upright microscope with a 80x magnification objective was used. Images were acquired using VisiView software.



Vincenzetti – Suppl Fig 3

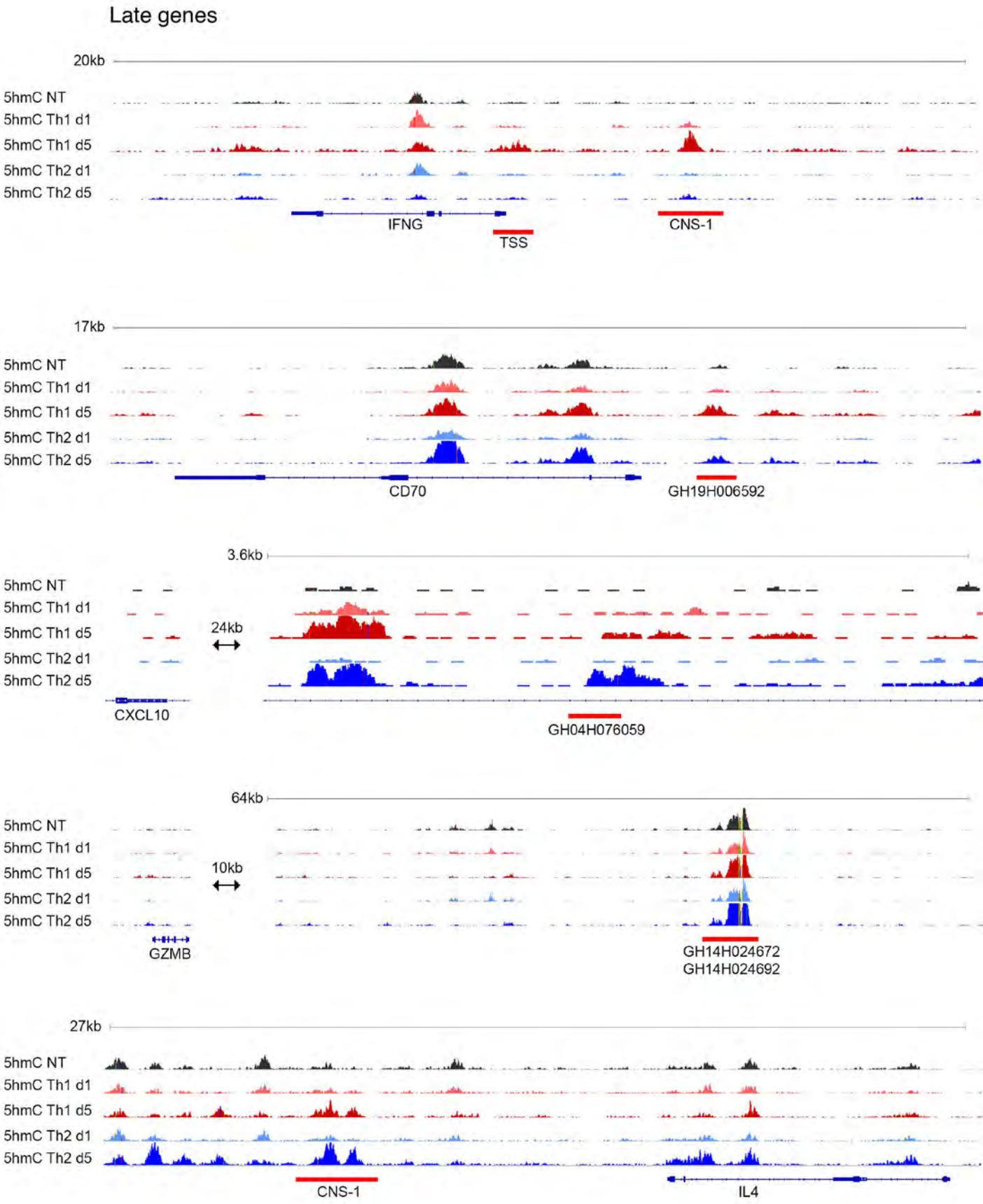
Suppl. Figure 3. RRBS analysis of primary human T lymphocytes. Volcano plots showing the differentially methylated regions observed by RRBS between: i) naïve T cells unstimulated and stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 antibodies; ii) memory T cells unstimulated and stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 antibodies; iii) naïve and memory T lymphocytes stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 antibodies. In each case the differentially methylated regions (either hyper- or hypo-methylated) were analyzed for their genomic location (promoter, exon, intron, intergenic).





Vincenzetti – Suppl Fig 4

Suppl. Figure 4. Illustrative snapshots of hMeDIP data (early genes) and analyses of supplementary housekeeping genes. (a) Illustrative snapshots of hMeDIP data for early genes presented in Figure 4. Red bars represent enhancers and regulatory regions. **(b)** Analyses of regulatory regions of housekeeping genes, supplementary to the one shown in Figure 4.



Vincenzetti – Suppl Fig 5

Suppl. Figure 5. Illustrative snapshots of hMeDIP data (late genes). Illustrative snapshots of hMeDIP data for early genes presented in Figure 4. Red bars represent enhancers and regulatory regions.

Supplementary Table 1. SYBR Green qPCR primer sequences.

SYBR Green primers		
Gene name	**Forward**	**Reverse**
IL2	5'-CCCAGGGACTTAATCAGCAA-3'	5'-GGTTGCTGTCTCATCAGCAT-3'
IFNG	5'-CGAGATGACTTCGAAAAGCTG-3'	5'-CAGTTCAGCCATCACTTGGA-3'
TET1	5'-GAACCATTGGATTCACTCAGC-3'	5'-GCACACTGGTTAGGTGGAGAG-3'
IL2 enhancer	5'-GCTAACTACCACGTGTGTCATACC-3'	5'-TGCAACACAGTGAGCAATCTC-3'
IFNG enhancer	5'-ACTGCAGAATAAGTCACCATCAAGA-3	5'-TGTGTCATGTATAACCATAGCCAGT-3'
IFNG TSS	5'-TGGGTGTTGTAGTTAGAGTTTCCTT-3'	5'-TCAATGTGCTTTGTGAATGAAGAGT-3'
IL2RA enhancer	5'-GCAGTTTTCCTCCTGGAAGTT-3'	5'-TGCAGTCTGAGACCACTCCTT-3'
UBE2D2 (endogenous control)	5'-GATCACAGTGGTCTCCAGCA-3'	5'-CGAGCAATCTCAGGCACTAA-3'

Supplementary References

Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell* **145:** 423-434

Manuscript 2

Epigenetics of T lymphocytes in health and disease

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Epigenetics of T lymphocytes in health and disease

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Summary

The risk of developing autoimmune diseases depends on both genetic and environmental factors, with epigenetic mechanisms of regulation potentially translating environmental cues into stable modifications in gene expression. Such stable memory of a functional state has been deciphered into a number of molecular mechanisms that collectively define the epigenetic status of a cell. In recent years, it has become increasingly clear that epigenetic modifications are highly dynamic and are able to adapt to the changing environment, with important impact on the onset and development of a number of diseases. Here, we describe some of the epigenetic mechanisms of regulation of cellular functional states in T lymphocytes, with a particular focus on DNA methylation. We will also discuss current knowledge on the role of epigenetics in autoimmunity and consider open questions in the field.

Key words: *autoimmunity; epigenetics; T lymphocytes; DNA methylation; microRNAs*

DNA methylation and hydroxymethylation dynamics in the regulation of gene expression

"Epigenetic" classically described inheritable phenotypes that are not the result of alterations in the DNA sequence [1, 2]. Here, however, we will utilise a broader interpretation of such a definition to include all mechanisms that can provide stability to a given phenotype, including those that can be important to impart short-term memory of an environmental signal [3, 4]. Mechanisms providing such cellular memory of gene expression include histone modifications, as well as regulation mediated by microRNAs (miRNAs) [3], although we will mainly focus on DNA methylation dynamics and their role in regulating functions of T lymphocytes during normal immune responses, as well as in autoimmunity.

DNA methylation

DNA methylation occurs most commonly at the 5' position of the cytosine ring in the context of CpG dinucleotides, and is mediated by DNA methyltransferase enzymes (DNMT1, DNMT3a, DNMT3b), which show variable affinities for unmethylated and hemimethylated DNA. The importance of proper DNA methylation throughout mammalian development is highlighted by the consequences of germline deletions of DNMTs: *Dnmt1* and *Dnmt3b* deletions in mice are embryonically lethal, and *Dnmt3a*-null mice die perinatally [5, 6].

The relevance of these enzymes can also be inferred from the fact that they are often found mutated in a variety of

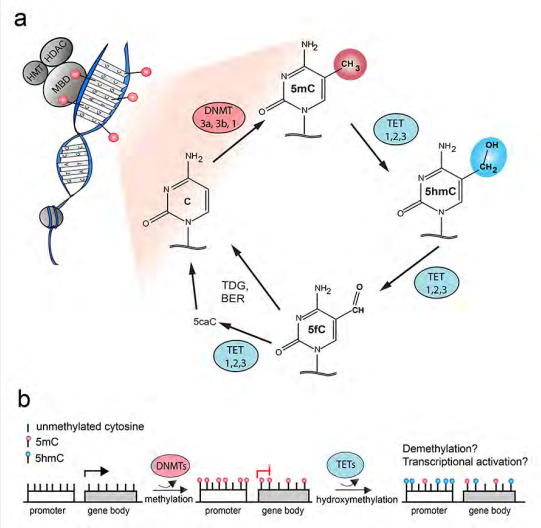

Figure 1

DNA methylation and hydroxymethylation. Cytosine modifications in DNA have functional consequences on gene expression. **(a)** Cytosine can be methylated by DNMT enzymes to give rise to 5mC, which can be oxidised by TET enzymes to 5hmC and further products (5fC and 5caC). In an active demethylation pathway, these products of TET enzymatic activity are likely to be excised and repaired by the thymine-DNA glycosylase enzyme and base excision repair mechanisms to regenerate the unmodified cytosine. Methylated DNA can be recognised by MBD proteins that can recruit histone modifying complexes (HMTs and HDACs). **(b)** DNA methylation occurs at regulatory regions (such as promoters), but also in gene bodies. DNMT enzymes are responsible for 5mC deposition, often resulting in the transcriptional repression of the methylated promoter. TET proteins activity can lead to demethylation with a potential for transcriptional activation. 5caC = 5-carboxylcytosine; 5fC = 5-formylcytosine; 5hmC = 5-hydroxymethylcytosine; 5mC = 5-methylcytosine; BER = base excision repair; DNMT = DNA methyltransferase; HDAC = histone deacetylase; HMT = histone methyltransferase; MBD = methyl-CpG-binding domain protein; TDG = thymine-DNA glycosylase; TET = ten-eleven translocation

diseases. For example, *DNMT3a* is frequently mutated in a number of haematological malignancies, and upon transplantation of haematopoietic stem cells lacking DNMT3a, mice develop a spectrum of malignancies similar to those seen in patients with mutations in this enzyme, supporting the crucial role of DNA methylation in disease [7, 8]. Mutations in the gene encoding for DNMT3b are instead found in patients with ICF (immunodeficiency, centromere instability and facial anomalies), a recessive autosomal syndrome, and were shown to lead to reduced DNA methyltransferase activity [9].

Methylated DNA provides a platform for the binding of several methyl-CpG-binding domain (MBD) proteins, which can coordinate downstream processes by recruiting multiprotein complexes containing histone deacetylases (HDACs) and histone methyltransferases (HMTs), leading to chromatin modifications and alterations in gene expression [5, 10]. However, histone tails can also influence recruitment of DNMTs, which was in fact shown to be independent of pre-existing DNA methylation [11]. Indeed, methylation at lysine 4 of histone H3 (H3K4) was shown to inhibit DNMT3a recruitment [12, 13], and H3K36me3 modulated DNMT3b binding and consequent DNA methylation specifically to transcribed gene bodies [11]. Importantly, accessibility of selected transcription factors is also influenced by DNA methylation, since the methylation status of a binding site can define binding affinity and even specificity for a given transcription factor [14–17].

Despite such prominent progress in the field and decades of intensive studies, the precise role of DNA methylation in regulating gene expression is still not completely understood, and many concepts about DNA methylation have had to be reconsidered in recent years. DNA methylation was classically associated with gene silencing, and this is indeed usually the case at transposable elements and gene promoters, but methylation downstream of the transcriptional start site (TSS) usually does not block elongation [18]. Moreover, DNA methylation was not necessarily considered of general importance, since model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* show very low or essentially no cytosine methylation [19]. However, while cytosine methylation represents the dominant DNA modification in humans, very recent work showed how organisms with virtually no cytosine methylation show significant levels of other DNA modifications, such as N6-methyladenine (6mA), further highlighting a possible widespread regulatory role of DNA methylation (reviewed in [20]). Finally, DNA methylation was thought to be substantially irreversible, other than through passive mechanisms of dilution, but the recent discovery of pathways leading to DNA demethylation have led to a revision of this idea.

An effort to describe the methylome of up to 17 eukaryotic genomes was able to show that DNA methylation is highly conserved in eukaryotes, and that is was likely present in a common ancestor [19, 21, 22]. Interestingly, methylation of the gene body was a highly conserved feature across genomes, with exons being usually more methylated than introns and with reduced methylation at both the promoter region and the 3' end of genes. While gene expression was mostly inversely correlated with promoter methylation, gene body methylation showed a parabolic correlation with expression: modestly expressed genes are more likely to be methylated, while both high and low expressers are the least likely to be methylated [19, 21, 22]. This generally conserved pattern indicates a role of DNA methylation in regulating multiple steps of transcription, including initiation, elongation, termination and even splicing [19, 23]. Indeed, genome-wide studies showed that removal of one DNMT enzyme leads to both hypo- and hypermethylation at specific loci, underlying the complexity of the regulation mediated by DNA-modifying enzymes and further highlighting the fact that DNA methylation does not always equate to gene silencing [24, 25].

Overall, these studies clearly challenged the general dogma that DNA methylation invariably corresponds to transcriptional silencing, and put forward the idea that the final outcome on transcriptional regulation relates to the context and spatial distribution of DNA methylation [10]. Therefore, DNA methylation appears to be intimately associated with gene transcription.

Reversal of DNA methylation by hydroxylation

Once thought to be a stable chromatin modification, DNA methylation is now understood to have a much more dynamic nature, as its distribution is influenced by DNA demethylation processes, which can occur either passively, by dilution upon DNA replication at each cell division, or through an active process initiated by ten-eleven translocation (TET1-3) enzymes [26] (fig. 1). These enzymes are responsible for the catalytic conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and to further oxidation products, including 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [27, 28]. Similarly to DNMT enzymes, TET proteins are frequently found mutated in a number of diseases. Specifically, mutations in the *TET2* gene are common in many haematological disorders, and mice lacking TET2 show expansion of the pool of haematopoietic stem cells, with a bias towards myelopoiesis, which in some cases can turn to myeloproliferative disorder [29–33].

Although the biological significance of such oxidation derivatives of 5mC remains to be fully elucidated, several lines of evidence have determined their importance in transcriptional regulation. Indeed, they are likely to be intermediates in processes of DNA demethylation and they modify the binding patterns of several chromatin regulators and transcription factors, thereby influencing transcription. A recent work undertook the challenge of investigating the identity of a large number of 5mC and 5hmC-interacting proteins in the mouse, revealing that each cytosine modification is able to recruit a distinct set of proteins with little overlap, and also that a large number of proteins preferentially interact with unmethylated cytosines [15]. These findings further indicate that the role of DNA methylation is integral to regulation (both positive and negative) of transcription. In general, because many of the methods used to study DNA methylation in the past could not distinguish between 5mC and 5hmC [34], it will be interesting to see how many of the functions attributed in the past to 5mC are actually dependent (or not) on the presence of 5hmC in the genome.

Epigenetic regulation of T cell functions

T lymphocytes are central to the orchestration of immune responses to invading pathogens, but their activity has to be carefully balanced and controlled to avoid tissue damage and pathology. Following antigenic stimulation, naïve T cells proliferate and differentiate into a number of effector and memory subsets, each characterised by the ability to produce specific signature cytokines. For example, CD4+ T helper (Th) cells differentiation to the Th1 subset is characterised by the production of high levels of interferon-gamma (IFNγ). Th2 cells instead mainly produce interleukin (IL)-4 and IL-13, while Th17 cells are characterised by the expression of IL-17. Th1 and Th17 cells in particular, and their associated cytokines, have been implicated in the pathogenesis of autoimmune diseases such as multiple sclerosis (MS) [35–37].

Several genes controlling immune functions are known to be regulated by DNA methylation, although our understanding of the impact of epigenetic modifications in the regulation of T cell functions is currently still limited. The fact that DNA methylation can be dynamically modulated during T cell responses to an antigen is suggested by a number of observations. For instance, the *Il2*, *Il4* and *Ifng* cytokine genes are known to be regulated by DNA methylation in T cells [38–40]. In the absence of DNMT1 or DNMT3a (but not DNMT3b), murine CD4+ T lymphocytes were unable to silence properly the *Ifng* and *Il4* loci under appropriate culture conditions, resulting in increased and promiscuous cytokine expression [41–43]; even T cells lacking MBD2 (methyl-CpG-binding domain protein 2), a reader of methylated DNA, showed a similar phenotype [44]. T cell activation in Th2-polarising conditions was shown to reduce substantially recruitment of DNMT1 to the *Il4-Il13* locus in proliferating cells, eventually leading to reduced DNA methylation of this locus and enhanced gene expression [39]. Dysregulated Th2-type cytokine expression in the absence of DNMT1 was observed even in CD8+ T cells, indicating defective gene silencing [45]. Moreover, DNMT3a expression was shown in the mouse to be increased by T cell receptor (TCR) engagement. Accordingly, *Dnmt3a*-null murine T cells showed hypomethylated cytokine loci and dysregulated cytokine expression [41]. More recently, genome-wide methylation studies investigated changes in DNA methylation in effector and memory murine T cells compared with their naïve counterparts, highlighting the extent of chromatin remodelling events accompanying the acquisition of the effector/memory phenotype upon stimulation of a naïve T cell [46, 47].

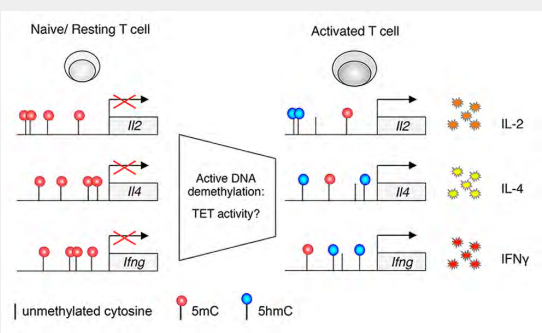

Figure 2

DNA methylation dynamics regulate cytokine production in T lymphocytes. DNA methylation modulates cytokine production upon T cell activation, and active demethylation processes (independent of cell replication) have been shown to act at some cytokine loci. Hypothetically, these processes may involve TET proteins as an underlying mechanism of demethylation.
5hmC = 5-hydroxymethylcytosine; 5mC = 5.methylcytosine; IFNγ = interferon-gamma; IL = interleukin; TET = ten-eleven translocation

Prior to the discovery of TET proteins, models for DNA demethylation placed emphasis on a passive process due to a failure of DNMT1 to methylate the newly synthesised DNA strand during the S phase of the cell cycle. However, the rapid and DNA replication-independent demethylation of the *Il2* locus that was observed upon activation of CD4+ T cells and that causally affected *Il2* gene transcription was inconsistent with such a model [40]. Along the same line, dynamic methylation of the *Ifng* locus was described for memory CD8+ T lymphocytes: while this locus was partially methylated at resting state, it underwent demethylation within 5 hours of antigenic stimulation [38]. This process was independent of DNA replication and cell division, and the involvement of a yet unidentified demethylase activity was proposed [38] (fig. 2). Interestingly, characterisation of the genome-wide distribution of 5hmC in mouse CD4+ T cells could not lead to a definitive answer about the role of 5hmC in T cell differentiation, although it showed a clear effect of TET2 in modulating effector responses in some T cell subsets, with reduced IL-10, IFNγ and IL-17A production in the absence of TET2 [48].

Among the various T cell subsets required to regulate immune responses properly, regulatory T cells (Tregs) are essential to suppress fatal autoimmunity throughout the lifetime of an organism. These cells are characterised by the expression of the transcription factor Foxp3, which plays a key role in their differentiation and function. Indeed, mutations or deficiency of the *Foxp3* gene lead to the development of autoimmune disease, causing the *scurfy* phenotype in mice and IPEX (immune dysfunction, polyendocrinopathy, X-linked) syndrome in humans [49]. Stable Foxp3 expression is essential to maintain Treg cell identity and functional integrity, and mechanisms are in place to preserve Treg cell lineage stability [50]. In particular, regulatory regions in the Foxp3 locus have to be maintained free of methylation in order to confer phenotypic stability to Tregs and, accordingly, inhibition of DNA methylation using 5-azacytidine or 5-aza-deoxycytidine led to stable Foxp3 expression [49–51]. By modulating the stability of the Treg phenotype, DNA methylation could therefore also have an impact on the onset of autoimmune diseases, and DNMT inhibitors were proposed as potentially useful tools to enhance Foxp3 expression in an attempt to limit inflammation [49]. Expression of DNMT1, but not DNMT3a, was, however, shown to be essential for the appropriate differentiation and function of Tregs, and mice with conditional deletion of DNMT1 in Tregs died of lethal autoimmunity [52]. DNA methylation therefore appears to have a complex role in regulating Treg stability and function that may hinder the development of epigenetic-based therapies.

DNA methylation in autoimmune and autoinflammatory diseases

Although the aetiology of MS and other autoimmune diseases is still largely unknown, it involves T cell-mediated processes [35]. Genome-wide association studies (GWAS) on MS patients have identified several genetic loci associated with a greater risk of developing the disease. Of these, the most commonly identified risk locus in MS and other autoimmune diseases is the major histocompatibility complex (MHC), pointing towards a fundamental role for antigen presentation, but many of the other loci associated with MS risk are related to immune functions, including cytokines and cytokine receptors, costimulatory receptors and signal transduction molecules [53].

Epigenetic mechanisms of regulation of gene expression may partly explain why only a proportion of genetically susceptible individuals may end up manifesting the disease. The complexity of such regulation and its impact on disease is exemplified by investigations on monozygotic twins: despite sharing identical genetic material, the risk of developing MS in a twin of an MS patient is only 25%, although it remains higher than in dizygotic twins or non-twin siblings [54]. Similarly, besides genetic predisposition, epigenetic factors appear to be important for the onset of systemic lupus erythematosus (SLE), since monozygotic twins are often discordant also for this disease [55]. To assess the relative contribution of heritable vs nonheritable influences on a variety of immunological parameters, a recent work investigated responses of 210 healthy twin pairs [56]. Interestingly, immune variation in this system was dominated by nonheritable determinants, some of which also became more divergent with age, suggesting a cumulative effect of environmental exposure [56].

Despite the clear evidence of an environmental component in disease development, it has not been easy to pinpoint the exact impact of epigenetics in autoimmune diseases; for example, a study investigating three monozygotic twin pairs discordant for MS found no reproducible differences in DNA methylation, single nucleotide polymorphisms or gene expression in CD4+ T cells [57]. A more recent study evaluated genetic and epigenetic determinants of 21 autoimmune diseases, observing that most causal variants map to enhancers rather than protein-coding genes and frequently coincide with regions bound by transcription factors and depleted of nucleosomes [58]. On the other hand, DNA methylation in cells of monozygotic twins discordant for the disease was also analysed in the case of type 1 diabetes (T1D), and the differentially methylated regions included several genes involved in immune responses [59]. Interestingly, some of the DNA methylation sites associated with T1D could be detected years before clinically overt T1D, suggesting a role in increasing the risk of developing this disease [59]. Changes in DNA methylation have been demonstrated also in SLE patients, with T lymphocytes from these patients showing overall genomic hypomethylation compared with controls [55]. Most importantly, exposing T cells to demethylating drugs resulted in the induction of a lupus-like disease in mice [60]. Although the mechanisms by which hypomethylated T cells induce SLE are not fully understood, recent evidence points towards a possible dominant role for IL-10 and IL-17 production in T cells, dependent on altered methylation status at these loci [61, 62]. Altered global DNA methylation and dysregulation at specific genetic loci have also been reported for rheumatoid arthritis (reviewed in [63, 64]), although a comprehensive view of the role of DNA methylation in autoimmunity and whether in each case it represents a cause or consequence is still lacking.

DNA methylation in disease: cause, consequence or correlation?

Aberrant DNA methylation is a pervasive feature of human tumours, which often show extensive genomic hypomethylation together with hypermethylation of tumour-suppressor genes (reviewed in [65]). As cancer progresses, the level of global methylation decreases, and DNA hypomethylation may contribute to cancer progression through chromosomal instability, reactivation of transposable elements and loss of imprinting [65]. *Vice versa*, hypermethylation of tumour-suppressor genes can have a major impact in suppressing the expression of these genes and therefore unleash uncontrolled cell proliferation. Similarly, global loss of 5hmC is a hallmark of a number of different tumours [66-68], although in spite of the diagnostic and prognostic implications, the clear biological significance of such altered methylation patterns remains to be fully understood. Importantly, alterations in the distribution of histone modifications have been described for a number of autoimmune diseases [63], and given the interplay between post-translational modification of histones and DNMT enzymes [69], at least part of the altered DNA methylation patterns observed in disease may potentially be linked to histone modifications.

While a causal link between mutations in DNA-modifying enzymes, altered methylation patterns and disease has been worked out in some cases [7, 8, 66], it has to be highlighted that some biological consequences of altered methylation do not necessarily link directly to disease. Indeed, changes in DNA methylation and hydroxymethylation are imperfectly maintained during replication and are subject to considerable epigenetic drift, that is, gradual increase or decrease at specific loci [70]. Such stochastic drift not only creates epigenetic mosaicism within populations, but it is also part of the normal process of aging, with aging cells and tissues showing considerable DNA hyper- or hypomethylation over time [70, 71]. Moreover, while some mouse models lacking DNMT3a or TET2 develop haematopoietic malignancies [7, 8, 30, 31, 33], this is not the case for mice lacking other DNMT or TET family members, suggesting that altered methylation patterns do not necessarily lead straight to disease. They can however predispose to its onset, and the lack of DNMT3a in particular appeared to confer flexibility to the methylome of haematopoietic stem cells, allowing cellular transformation after the acquisition of additional mutations [7, 8].

Interestingly, although the underlying mechanism remains unclear, chronic inflammation can be an important inducer of epigenetic drift, and potentially predisposing to the onset of disease. For example, *Helicobacter pylori* is a well-known inducer of chronic inflammation and gastric cancer, and altered patterns of DNA methylation are associated

with *H. pylori* infection. In a gerbil model of gastric cancer, *H. pylori* infection was sufficient to induce DNA methylation in gastric epithelial cells, which could be partially reversed by *H. pylori* eradication or blocked by immunosuppressive drugs [72], suggesting that inflammation induced by the infection was a critical determinant of hypermethylation. Other determinants of methylation variations remain poorly understood [70].

MicroRNAs, immune regulation and autoimmunity

MicroRNAs have recently emerged as important regulators of both transcriptional and epigenetic programmes, with pivotal roles in regulating early T cell development, lineage specification and function [73]. As a result of their stability, miRNAs are most suitable to maintain memory of altered cellular states [3] and it is becoming increasingly clear that they are involved in regulating various aspects of autoimmunity (reviewed in [74, 75]), as well as in the regulation of DNA-modifying enzymes. For instance, miR-21 and miR-148a were shown to be highly expressed in patients with SLE, and to promote hypomethylation by repressing DNMT1 expression [76]. This in turn led to increased expression of autoimmune-associated genes like CD70 and LFA-1, surface markers that were shown to play a role in lupus [55].

A growing number of studies also identified miRNAs as potential novel therapeutic targets in animal models of MS as well as in primary cells derived from MS patients (table 1). For example, miR-155 and miR-29ab expression was found to be elevated in mouse CD4+ T cells during experimental autoimmune encephalomyelitis (EAE), and miR-155– or miR-29ab–deficient mice had a delayed course and reduced severity of disease [77, 78]. Another relevant example is provided by miR-326, whose expression was highly correlated with disease severity in patients with MS and in mice with EAE. Accordingly, modulation of miR-326 expression altered disease severity [79]. *Vice versa*, miR-20 was shown to be downregulated during

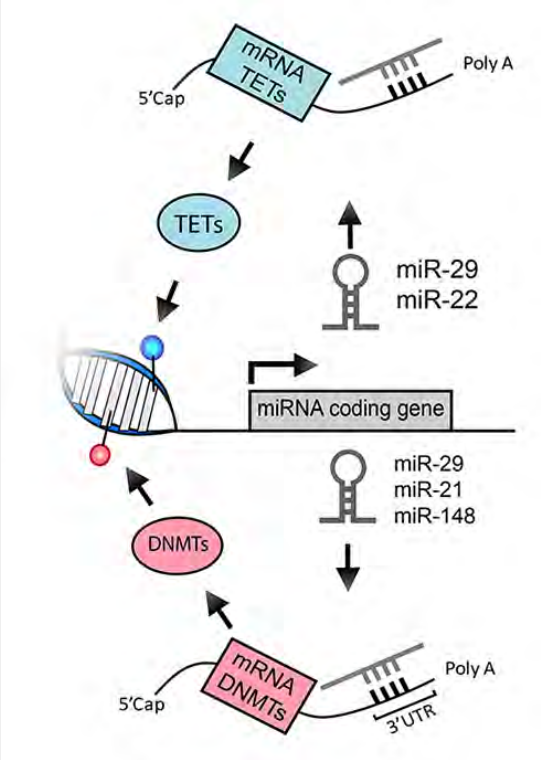

Figure 3

Interplay between miRNAs and the DNA methylation machineries. MiRNAs are both target and effector of epigenetic modifications. Expression of miRNAs can be modulated by levels of DNA methylation in the gene locus of any given miRNA. In turn, mature miRNAs bind the 3'UTR of target mRNAs leading to a reduction in protein output. DNMT and TET mRNAs can be targeted by miRNAs such as miR-29, miR-22 and others, thereby modulating the levels of DNA methylation and hydroxymethylation. DNMT = DNA methyltransferase; miRNA = micro RNA; mRNA = messenger RNA; TET = ten-eleven translocation protein; UTR = untranslated region

Table 1: Common miRNAs dysregulated in autoimmune diseases. Examples of some common miRNAs that have been reported to influence autoimmunity in general, MS or its experimental model (EAE) in humans (Hsa), mouse (Mmu) or rat (Rno).

miRNA	Expression change	Target/ Function	Model system	Outcome	PMID
– (Dicer/Drosha deletions)	All miRNAs down	Elimination of components of the miRNA machinery in Tregs	Mmu	Fatal autoimmunity	18725525 18725526 18725527
Let-7e	Up	Dysregulated cytokine production	Mmu	Silencing ameliorated EAE	23079871
miR-10	Up	Bcl-6, T cell plasticity	Mmu	Delayed onset of EAE	22544395
miR-17~92	Up	Treg functions	Mmu	Treg-specific loss of miR-17~92 results in exacerbated EAE	23858035 20148420
miR-20b	Down	Disease severity, Th17 responses	Mmu	Overexpression leads to reduced severity of EAE	24842756
miR-23b	Down	Suppresses inflammatory cytokine-mediated signaling	Hsa/ Mmu	Overexpression leads to reduced severity of EAE	22660635
miR-29	Up in MS and EAE	T-bet, IFNg, Th1 differentiation	Hsa/ Mmu	Knock-out mice have reduced EAE	22772450
miR-132/212	Up	Th17 differentiation	Mmu	Exacerbated EAE	23818645
miR-155	Up	Positive regulator of inflammation	Mmu	Knock-out mice have reduced EAE	20888269 21788439
miR-181	Up	Regulates threshold of TCR activation	Hsa/ Rno	Upregulated in CD4+ T cells of strains susceptible to EAE and in cerebrospinal fluid of MS patients	17382377 25775432 23514736 23077021
miR-301	Up	Th17 differentiation	Mmu	Exacerbated EAE	22517757
miR-326	Up	Disease severity, Th17 responses	Hsa/ Mmu	Expression correlates with disease	19838199

EAE, and its overexpression led to reduced disease severity in mice [80].

In MS patients, distinct miRNA expression profiles were found in CD4+ and CD8+ T cells as well as B cells as compared with healthy donors, and miR-17-5p, another miRNA known to be involved in autoimmunity, was up-regulated in CD4+ T cells from MS patients [81]. Further highlighting the importance of miRNAs in regulating various aspects of human disease, a role has even been proposed in the regulation of blood-brain barrier endothelial tightness in MS patients [82]. Moreover, miRNAs have been investigated as promising biomarkers for disease, being able to discriminate MS from other neurological diseases, as well as different disease courses of MS [83].

Despite encouraging results underscoring the role of miRNAs in autoimmune diseases, the biological function of most of these miRNAs and their relevance for disease in the context of MS is mostly unknown. MiR-181a is a miRNA known to regulate the threshold of TCR activation in developing thymocytes and to contribute to positive selection in the thymus [84–86]. Interestingly, miR-181a was found to be elevated upon EAE induction in lymph nodes of EAE-susceptible Dark Agouti rats as compared with the EAE-resistant rat strain Piebald Virol Glaxo [87]. Another miRNA of the same family, miR-181c, was shown to be increased in the cerebrospinal fluid of patients with MS compared with other neurological diseases, and to differentiate relapsing-remitting from secondary progressive MS courses [83]. MiR-181c–containing extracellular vesicles were also shown to trigger the breakdown of the blood-brain barrier in a model of brain metastatic cancer [88]. Finally, miR-181b was shown to be elevated in CD4+ T cells from SLE patients, as compared with healthy controls, in a manner that was potentially linked to DNA hypomethylation in these patients [89]. To understand the biological relevance of this miRNA in regulating the activation and function of T cells, and therefore a possible role in disease onset or progression, our lab investigated the role of miR-181a in the activation of primary human T cells upon recognition of specific antigens [90]. We found that miR-181a expression regulated the threshold of activation of primary human T helper cells, especially of the Th17 type, known to be involved in MS pathogenesis [35, 37]. Moreover, we showed that, given two memory T cells bearing the same identical TCR (therefore with the same antigen recognition capacity), the cell expressing the highest levels of miR-181a became able to respond with full-blown activation and overt proliferation to the lowest concentrations of cognate antigen, regardless of the phenotype, strongly suggesting the possibility that miRNAs may also be important regulators of the threshold of T cell responses toward autoantigens in autoinflammatory diseases [90]. More studies in this direction will enable us to understand the specific roles played by these regulatory molecules in the modulation of autoimmune responses.

Interplay between DNA-modifying enzymes and miRNAs

To fully grasp the level of complexity of the mechanisms involved in fine-tuning the regulation of gene expression, one should also consider the interplay between DNA methylation and miRNA expression. Indeed, miRNAs can play a role in the modulation of DNA methylation both as targets (that is, DNA methylation can alter miRNA expression and consequently expression of downstream genes) and as effectors (that is, miRNAs reduce the expression of target DNA-modifying enzymes) (fig. 3).

For instance, the miR-29 family was shown to target DNMT1, DNMT3a and DNMT3b, leading to global DNA hypomethylation and re-expression of tumour suppressor genes in acute myeloid leukaemia cells [91]. Interestingly, miR-29 family members were also shown to be able to target TET enzymes, suggesting that this family of miRNAs acts primarily by protecting cells against perturbations in the existing DNA methylation landscape [92]. Similarly, miR-22 was shown to target directly TET proteins and to contribute to leukaemia development and metastatic potential of cancer cells [93, 94]. By utilising an unbiased high-throughput 3' untranslated region activity screen of 460 miRNA constructs, a recent work showed that TET2 is under extensive regulation by more than 30 miRNAs (including miR-29b, miR-29c, miR-101, miR-125b, and miR-7) able to reduce TET2 expression and levels of genomic 5hmC and to disrupt normal haematopoiesis [95].

Looking at the molecular interplay between DNA methylation and miRNAs in the opposite direction, the list of miRNA genes that can be silenced by DNA methylation, especially in cancer, is constantly growing [96], and given the impact of miRNAs in regulating virtually every single aspect of T cell biology [73], it would not be surprising to identify miRNAs whose methylation-dependent silencing leads to dysregulated immune responses.

An interesting aspect of miRNA-mediated regulation is also related to the fact that selected miRNAs can be exchanged through exosomes during immune synapse formation between T cells and antigen-presenting cells (APCs) [97]. The flow of exchange was shown to be unidirectional, from the T cell to the APC, and to be able to influence gene expression in the recipient cell. Such mechanism of exchange was also shown to occur between Tregs and various immune cell types, and to suppress proliferation and cytokine secretion by pathogenic Th1 cells [98]. Importantly, miRNAs were transferred to Th1 cells both *in vitro* and *in vivo*, and such transfer significantly contributed to suppression and prevention of systemic inflammation in mice [98]. Whether exosome-mediated miRNA exchange is also able to modulate the epigenetic status of the receiving cell remains to be evaluated, and is an exciting question that will undoubtedly be addressed in work to come (box 1). Intriguingly, RNA molecules can also be methylated, and methylation of primary miRNAs (pri-miRNAs) was recently shown to act as a post-transcriptional mark promoting further miRNA processing [99]. Whether such "epitranscriptomic" modifications [100] will become relevant also in the context of disease will be an exciting topic for the future.

Box 1: Future challenges and outstanding questions
- What is the pattern of DNA methylation and hydroxymethylation in T cells *in vivo*?
- How does epigenetic variation impact disease? Is there a role for 5hmC in autoimmune diseases?
- What are the mechanisms that dictate the activity of DNA-modifying enzymes at specific genomic loci?
- Are there any DNA methylation-independent functions for DNMT and TET proteins?
- Are small molecules interfering with 5hmC dynamics going to be effective and useful in the clinic?
- Are miRNAs able to significantly alter the epigenome *in vivo* and in disease?

Diet and metabolism

T cell metabolism

The role of metabolism in regulating T cell differentiation and function is becoming increasingly clear (reviewed in [101, 102]). Specifically, naïve, antigen-inexperienced T cells have a quiescent metabolism and generate energy primarily through oxidative phosphorylation (OXPHOS). Upon activation, effector T cells undergo a profound metabolic reprogramming and induce aerobic glycolysis, which is energetically less efficient, but supplies metabolic intermediates for biosynthesis of molecules required to sustain intense proliferation. Finally, memory T cells preferentially engage OXPHOS fuelled by the catabolism of fatty acids [101]. Metabolic pathways can also bias T helper cell differentiation towards specific subsets, thereby potentially influencing the outcome of an inflammatory response or autoimmune pathology [103]. Given the importance of metabolic pathways in regulating T cell responses, it is not surprising that pharmacological inhibition of some crucial metabolic regulators such as AMPK (AMP-activated protein kinase) have shown some effects in mouse models of EAE [102]. While the rodent models of EAE are extremely useful to dissect the mechanisms leading to or exacerbating disease, confirmation in the human system will be required to translate such potentially important findings to the clinic.

Dietary habits and epigenetic modifications

There is currently a great deal of interest regarding the possible link between diet, epigenetic modifications and disease. Vitamins and trace elements are important cofactors of a wide variety of enzymes as well as donors of chemical groups for epigenetic modifications. Folate is an essential B vitamin whose derivatives are main effectors in methylation reactions. Specifically, derivatives of folate are crucial to maintain appropriate methionine levels in the cells, which in turn is utilised to generate S-adenosylmethionine (SAM), the universal donor of methyl group [104]. Dietary folate intake is one of the few established links between diet and epigenetic modifications in both mouse (such as in the *Agouti* mouse model) and humans, as shown in rural Gambian women whose diet changes drastically according to the rainy and dry seasons [105, 106]. These studies showed that maternal folate intake is able to impact the establishment on 5mC patterns in the offspring.

Vitamin C is an essential nutrient for humans and primates, which have lost the capacity to synthesise it from glucose. Vitamin C acts as an antioxidant, able to maintain the redox state of iron-containing enzymes, and consequently to maintain the activity of dioxygenases such as TET enzymes [107]. As a result, vitamin C treatment of cultured cells led to a striking increase in the levels of genomic 5hmC, strongly influencing gene expression and differentiation potential [108]. However, whether altered levels of these cofactors can impact directly immune responses remains unclear. *Vice versa*, among the environmental factors known to impact the risk of MS are levels of vitamin D, whose serum levels are inversely correlated with the risk of MS. Vitamin D also appears to influence the course of MS, as lower levels are associated with increased severity of the disease [109]. Despite the fact that dietary habits and environmental factors can clearly have an influence of epigenetic modifications as well as on disease susceptibility, a precise link between these factors is still somewhat loose and the magnitude of these effects remains to be fully determined.

Epigenetic "resetting" of autoimmunity?

Although two major demethylating agents (decitabine and its analogue azacitidine) are now commonly used in the clinic for treatment of myelodysplastic syndrome and some leukaemias, the mechanisms underlying their clinical efficacy remain to be fully elucidated [110]. While genetic alterations are permanent, epigenetic modifications are potentially reversible, opening the possibility to utilise epigenetic drugs to at least relieve a certain phenotype. One of the major obstacles in this direction is, however, linked to the fact that there is still a paucity of information about the impact on disease of global changes in methylation versus gene-specific alterations. For example, despite the global hypomethylation observed in SLE patients, gene-specific hypermethylation cannot be ruled out. Moreover, the impact of other DNA modifications, such as 5hmC and its further products of oxidation, on gene transcription and disease remains for the most part to be uncovered. In general, a better understanding of the mechanisms that drive aberrant methylation and their pathological importance will be crucial to be able to exploit them for therapeutic purposes.

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References

1 Bird A. Perceptions of epigenetics. Nature. 2007;447(7143):396–8.

2 Berger SL, et al. An operational definition of epigenetics. Genes Dev. 2009;23(7):781–3.

3 Monticelli S, Natoli G. Short-term memory of danger signals and environmental stimuli in immune cells. Nat Immunol. 2013;14(8):777–84.

4 Natoli G. Maintaining cell identity through global control of genomic organization. Immunity. 2010;33(1):12–24.

5 Cullen SM, et al. Hematopoietic stem cell development: an epigenetic journey. Curr Top Dev Biol. 2014;107:39–75.

6 Okano M, et al. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99(3):247–57.

7 Mayle A, et al. Dnmt3a loss predisposes murine hematopoietic stem cells to malignant transformation. Blood. 2015;125(4):629–38.

8 Celik H, et al. Enforced differentiation of Dnmt3a-null bone marrow leads to failure with c-Kit mutations driving leukemic transformation. Blood. 2015;125(4):619–28.

9 Xu GL, et al. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature. 1999;402(6758):187–91.

10 Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. Cell. 2012;150(1):12–27.

11 Baubec T, et al. Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. Nature. 2015;520(7546):243–7.

12 Guo X, et al. Structural insight into autoinhibition and histone H3-induced activation of DNMT3A. Nature. 2015;517(7536):640–4.

13 Ooi SK, et al. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. Nature. 2007;448(7154):714–7.

14 Rishi V, et al. CpG methylation of half-CRE sequences creates C/EBPalpha binding sites that activate some tissue-specific genes. Proc Natl Acad Sci U S A. 2010;107(47):20311–6.

15 Spruijt CG, et al. Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized derivatives. Cell. 2013;152(5):1146–59.

16 Spruijt CG, Vermeulen M. DNA methylation: old dog, new tricks? Nat Struct Mol Biol. 2014;21(11):949–54.

17 Baubec T, Schubeler D. Genomic patterns and context specific interpretation of DNA methylation. Curr Opin Genet Dev. 2014;25:85–92.

18 Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet. 2012;13(7):484–92.

19 Jeltsch A. Molecular biology. Phylogeny of methylomes. Science. 2010;328(5980):837–8.

20 Heyn H, Esteller M. An Adenine Code for DNA: A Second Life for N6-Methyladenine. Cell. 2015;161(4):710–3.

21 Zemach A, et al. Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science. 2010;328(5980):916–9.

22 Feng S, et al. Conservation and divergence of methylation patterning in plants and animals. Proc Natl Acad Sci U S A. 2010;107(19):8689–94.

23 Yearim A, et al. HP1 is involved in regulating the global impact of DNA methylation on alternative splicing. Cell Rep. 2015;10(7):1122–34.

24 Challen GA, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 2012;44(1):23–31.

25 Challen GA, et al. Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. Cell Stem Cell. 2014;15(3):350–64.

26 Pastor WA, Aravind L, Rao A. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. Nat Rev Mol Cell Biol. 2013;14(6):341–56.

27 Tahiliani M, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324(5929):930–5.

28 Ito S, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science. 2011;333(6047):1300–3.

29 Ko M, et al. Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. Proc Natl Acad Sci U S A. 2011;108(35):14566–71.

30 Moran-Crusio K, et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell. 2011;20(1):11–24.

31 Li Z, et al. Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. Blood. 2011;118(17):4509–18.

32 Cimmino L, et al. TET family proteins and their role in stem cell differentiation and transformation. Cell Stem Cell. 2011;9(3):193–204.

33 Quivoron C, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell. 2011;20(1):25–38.

34 Huang Y, et al. The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One. 2010;5(1):e8888.

35 Simmons SB, et al. Modeling the heterogeneity of multiple sclerosis in animals. Trends Immunol. 2013;34(8):410–22.

36 Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol. 2005;23:683–747.

37 Peters A, Lee Y, Kuchroo VK. The many faces of Th17 cells. Curr Opin Immunol. 2011;23(6):702–6.

38 Kersh EN, et al. Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. J Immunol. 2006;176(7):4083–93.

39 Makar KW, et al. Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. Nat Immunol. 2003;4(12):1183–90.

40 Bruniquel D, Schwartz RH. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat Immunol. 2003;4(3):235–40.

41 Gamper CJ, et al. Identification of DNA methyltransferase 3a as a T cell receptor-induced regulator of Th1 and Th2 differentiation. J Immunol. 2009;183(4):2267–76.

42 Lee PP, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity. 2001;15(5):763–74.

43 Yu Q, et al. DNA methyltransferase 3a limits the expression of interleukin-13 in T helper 2 cells and allergic airway inflammation. Proc Natl Acad Sci U S A. 2012;109(2):541–6.

44 Hutchins AS, et al. Gene silencing quantitatively controls the function of a developmental trans-activator. Mol Cell. 2002;10(1):81–91.

45 Makar KW, Wilson CB. DNA methylation is a nonredundant repressor of the Th2 effector program. J Immunol. 2004;173(7):4402–6.

46 Scharer CD, et al. Global DNA methylation remodeling accompanies CD8 T cell effector function. J Immunol. 2013;191(6):3419–29.

47 Hashimoto S, et al. Coordinated changes in DNA methylation in antigen-specific memory CD4 T cells. J Immunol. 2013;190(8):4076–91.

48 Ichiyama K, et al. The Methylcytosine Dioxygenase Tet2 Promotes DNA Demethylation and Activation of Cytokine Gene Expression in T Cells. Immunity. 2015.

49 Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood. 2009;114(18):3727–35.

50 Feng Y, et al. Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. Cell. 2014 ;158(4):749–63.

51 Polansky JK, et al. DNA methylation controls Foxp3 gene expression. Eur J Immunol. 2008;38(6):1654–63.

52 Wang L, et al. Foxp3+ T-regulatory cells require DNA methyltransferase 1 expression to prevent development of lethal autoimmunity. Blood. 2013;121(18):3631–9.

53 International Multiple Sclerosis Genetics, C., et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476(7359):214–9.

54 Willer CJ, et al. Twin concordance and sibling recurrence rates in multiple sclerosis. Proc Natl Acad Sci U S A. 2003;100(22):12877–82.

55 Ballestar E, Esteller M, Richardson BC. The epigenetic face of systemic lupus erythematosus. J Immunol. 2006;176(12):7143–7.

56 Brodin P, et al. Variation in the human immune system is largely driven by non-heritable influences. Cell. 2015;160(1-2):37–47.

57 Baranzini SE, et al. Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. Nature. 2010;464(7293):1351–6.

58 Farh KK, et al. Genetic and epigenetic fine mapping of causal autoimmune disease variants. Nature. 2015;518(7539):337–43.

59 Rakyan VK, et al. Identification of type 1 diabetes-associated DNA methylation variable positions that precede disease diagnosis. PLoS Genet. 2011;7(9): p. e1002300.

60 Quddus J, et al. Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice. J Clin Invest. 1993;92(1):38–53.

61 Hedrich CM, et al. Stat3 promotes IL-10 expression in lupus T cells through trans-activation and chromatin remodeling. Proc Natl Acad Sci U S A. 2014;111(37):13457–62.

62 Rauen T, et al. cAMP-responsive element modulator (CREM)alpha protein induces interleukin 17A expression and mediates epigenetic alterations at the interleukin-17A gene locus in patients with systemic lupus erythematosus. J Biol Chem. 2011;286(50):43437–46.

63 Picascia A, et al. Epigenetic control of autoimmune diseases: from bench to bedside. Clin Immunol. 2015;157(1):1–15.

64 Glant TT, Mikecz K, Rauch TA. Epigenetics in the pathogenesis of rheumatoid arthritis. BMC Med. 2014;12:35.

65 Esteller M. Epigenetics in cancer. N Engl J Med. 2008;358(11):1148–59.

66 Lian CG, et al. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell. 2012;150(6):1135–46.

67 Leoni C, et al. Reduced DNA methylation and hydroxymethylation in patients with systemic mastocytosis. Eur J Haematol. 2015.

68 Ko M, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature. 2010;468(7325):839–43.

69 Smith ZD, Meissner A. DNA methylation: roles in mammalian development. Nat Rev Genet. 2013;14(3):204–20.

70 Issa JP. Aging and epigenetic drift: a vicious cycle. J Clin Invest. 2014;124(1):24–9.

71 Maegawa S, et al. Widespread and tissue specific age-related DNA methylation changes in mice. Genome Res. 2010;20(3):332–40.

72 Niwa T, et al. Inflammatory processes triggered by Helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res. 2010;70(4):1430–40.

73 Monticelli S. MicroRNAs in T helper cell differentiation and plasticity. Semin Immunol. 2013;25(4):291–8.

74 Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. J Autoimmun. 2009;32(3-4):189–94.

75 Wei B, Pei G. microRNAs: critical regulators in Th17 cells and players in diseases. Cell Mol Immunol. 2010;7(3):175–81.

76 Pan W, et al. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. J Immunol. 2010;184(12):6773–81.

77 Murugaiyan G, et al. Silencing microRNA-155 ameliorates experimental autoimmune encephalomyelitis. J Immunol. 2011;187(5):2213–21.

78 Smith KM, et al. miR-29ab1 deficiency identifies a negative feedback loop controlling Th1 bias that is dysregulated in multiple sclerosis. J Immunol. 2012;189(4):1567–76.

79 Du C, et al. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. Nat Immunol. 2009;10(12):1252–9.

80 Zhu E, et al. miR-20b suppresses Th17 differentiation and the pathogenesis of experimental autoimmune encephalomyelitis by targeting RORgammat and STAT3. J Immunol. 2014;192(12):5599–609.

81 Lindberg RL, et al. Altered expression of miR-17-5p in CD4+ lymphocytes of relapsing-remitting multiple sclerosis patients. Eur J Immunol. 2010;40(3):888–98.

82 Reijerkerk A, et al. MicroRNAs regulate human brain endothelial cell-barrier function in inflammation: implications for multiple sclerosis. J Neurosci. 2013;33(16):6857–63.

83 Haghikia A, et al, Regulated microRNAs in the CSF of patients with multiple sclerosis: a case-control study. Neurology. 2012;79(22):2166–70.

84 Ebert PJ, et al. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. Nat Immunol. 2009;10(11):1162–9.

85 Li QJ, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell. 2007;129(1):147–61.

86 Neilson JR, et al. Dynamic regulation of miRNA expression in ordered stages of cellular development. Genes Dev. 2007;21(5):578–89.

87 Bergman P, et al. Next-generation sequencing identifies microRNAs that associate with pathogenic autoimmune neuroinflammation in rats. J Immunol. 2013;190(8):4066–75.

88 Tominaga N, et al. Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood-brain barrier. Nat Commun. 2015;6:6716.

89 Zhao M, et al. DNA methylation and mRNA and microRNA expression of SLE CD4+ T cells correlate with disease phenotype. J Autoimmun. 2014;54:127–36.

90 Mele F, et al. ERK phosphorylation and miR-181a expression modulate activation of human memory T17 cells. Nat Commun. 2015;6:6431.

91 Garzon R, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood. 2009;113(25):6411–8.

92 Morita S, et al. miR-29 represses the activities of DNA methyltransferases and DNA demethylases. Int J Mol Sci. 2013;14(7):14647–58.

93 Song SJ, et al. MicroRNA-antagonism regulates breast cancer stemness and metastasis via TET-family-dependent chromatin remodeling. Cell. 2013;154(2):311–24.

94 Song SJ, et al. The oncogenic microRNA miR-22 targets the TET2 tumor suppressor to promote hematopoietic stem cell self-renewal and transformation. Cell Stem Cell. 2013;13(1):87–101.

95 Cheng J, et al. An extensive network of TET2-targeting MicroRNAs regulates malignant hematopoiesis. Cell Rep. 2013;5(2):471–81.

96 Lopez-Serra P, Esteller M. DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. Oncogene. 2012;31(13):1609–22.

97 Mittelbrunn M, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun. 2011;2:282.

98 Okoye IS, et al. MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. Immunity. 2014;41(1):89–103.

99 Alarcon CR, et al. N6-methyladenosine marks primary microRNAs for processing. Nature. 2015;519(7544):482–5.

100 Lee M, Kim B, Kim VN. Emerging roles of RNA modification: m(6)A and U-tail. Cell. 2014;158(5):980–7.

101 O'Sullivan D, Pearce EL. Targeting T cell metabolism for therapy. Trends Immunol. 2015;36(2):71–80.

102 Lochner M, Berod L, Sparwasser T. Fatty acid metabolism in the regulation of T cell function. Trends Immunol. 2015;36(2):81–91.

103 Berod L, et al. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. Nat Med. 2014;20(11):1327–33.

104 Moen EL, et al. New themes in the biological functions of 5-methylcytosine and 5-hydroxymethylcytosine. Immunol Rev. 2015;263(1):36–49.

105 Dominguez-Salas P, et al. Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. Nat Commun. 2014;5:3746.

106 Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. Nat Rev Genet. 2007;8(4):253–62.

107 Monfort A, Wutz A. Breathing-in epigenetic change with vitamin C. EMBO Rep. 2013;14(4):337–46.

108 Blaschke K, et al. Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. Nature. 2013;500(7461):222–6.

109 Koch MW, Metz LM, Kovalchuk O. Epigenetics and miRNAs in the diagnosis and treatment of multiple sclerosis. Trends Mol Med. 2013;19(1):23–30.

110 Christman JK. 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene. 2002;21(35):5483–95.

Figures (large format)

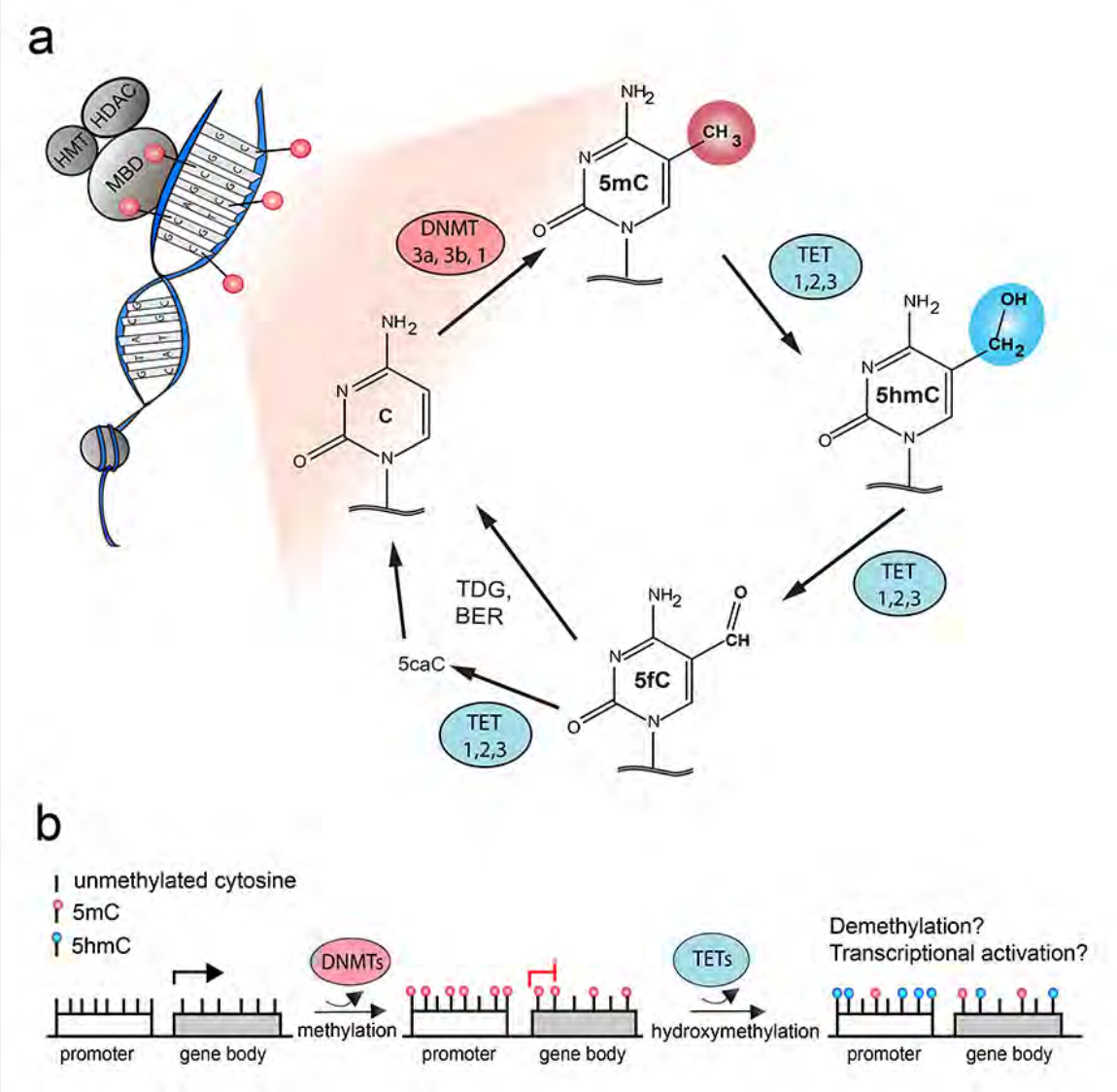

Figure 1

DNA methylation and hydroxymethylation. Cytosine modifications in DNA have functional consequences on gene expression. **(a)** Cytosine can be methylated by DNMT enzymes to give rise to 5mC, which can be oxidised by TET enzymes to 5hmC and further products (5fC and 5caC). In an active demethylation pathway, these products of TET enzymatic activity are likely to be excised and repaired by the thymine-DNA glycosylase enzyme and base excision repair mechanisms to regenerate the unmodified cytosine. Methylated DNA can be recognised by MBD proteins that can recruit histone modifying complexes (HMTs and HDACs). **(b)** DNA methylation occurs at regulatory regions (such as promoters), but also in gene bodies. DNMT enzymes are responsible for 5mC deposition, often resulting in the transcriptional repression of the methylated promoter. TET proteins activity can lead to demethylation with a potential for transcriptional activation.
5caC = 5-carboxylcytosine; 5fC = 5-formylcytosine; 5hmC = 5-hydroxymethylcytosine; 5mC = 5-methylcytosine; BER = base excision repair; DNMT = DNA methyltransferase; HDAC = histone deacetylase; HMT = histone methyltransferase; MBD = methyl-CpG-binding domain protein; TDG = thymine-DNA glycosylase; TET = ten-eleven translocation

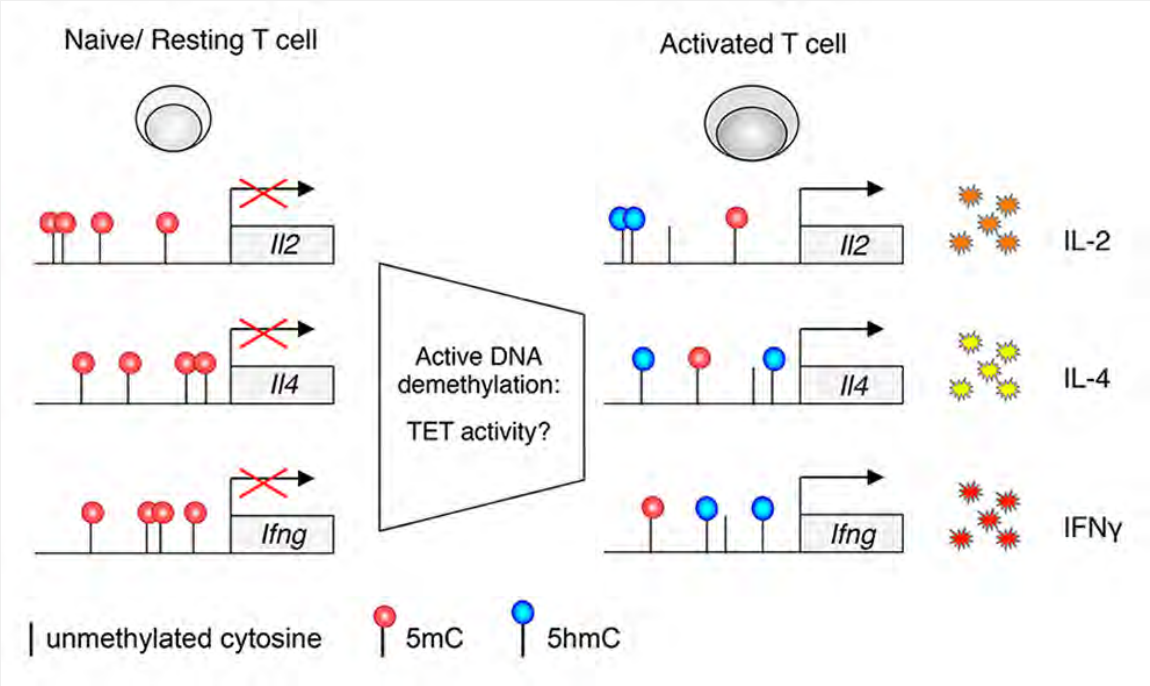

Figure 2

DNA methylation dynamics regulate cytokine production in T lymphocytes. DNA methylation modulates cytokine production upon T cell activation, and active demethylation processes (independent of cell replication) have been shown to act at some cytokine loci. Hypothetically, these processes may involve TET proteins as an underlying mechanism of demethylation.
5hmC = 5-hydroxymethylcytosine; 5mC = 5.methylcytosine; IFNγ = interferon-gamma; IL = interleukin; TET = ten-eleven translocation

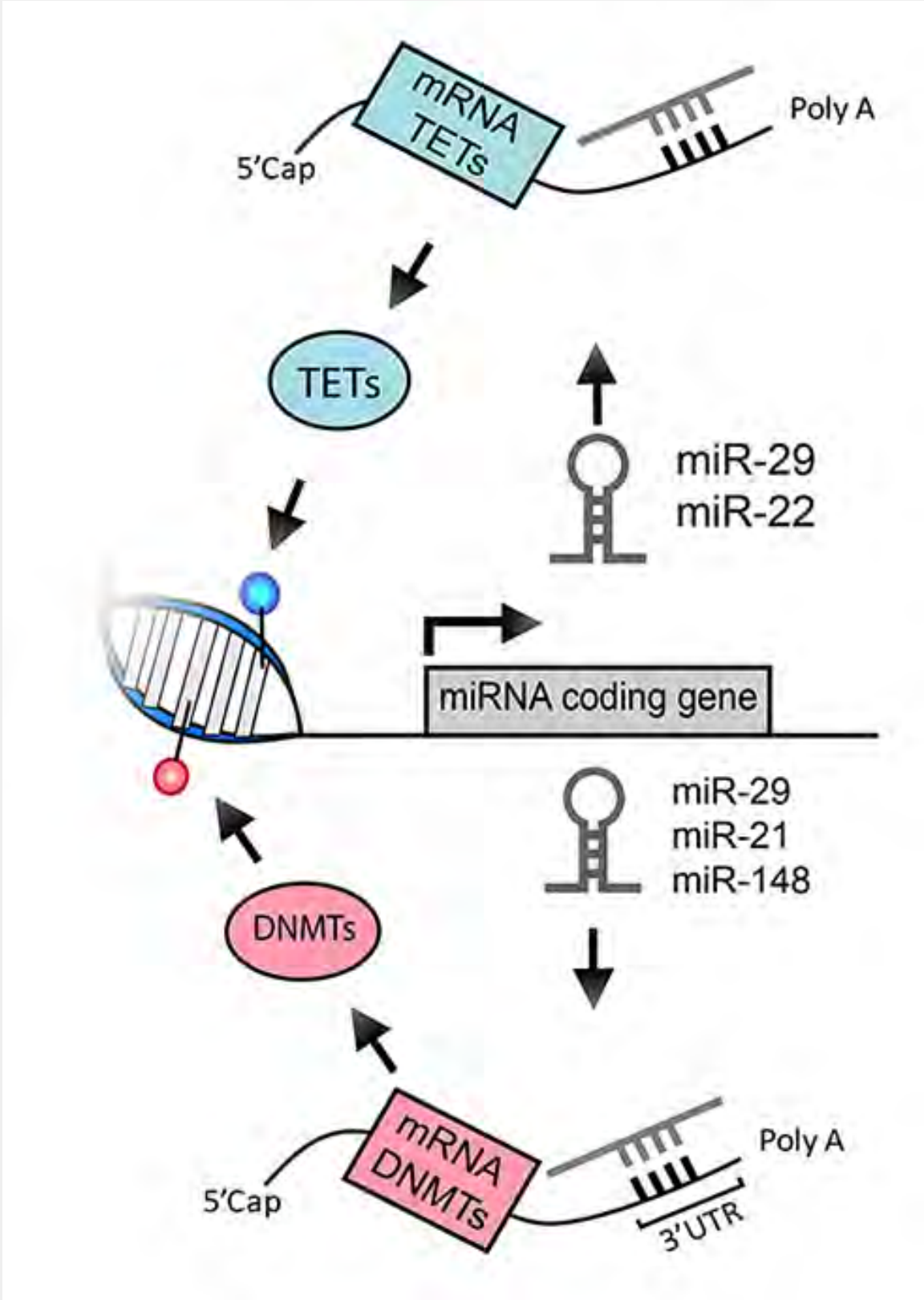

Figure 3

Interplay between miRNAs and the DNA methylation machineries. MiRNAs are both target and effector of epigenetic modifications. Expression of miRNAs can be modulated by levels of DNA methylation in the gene locus of any given miRNA. In turn, mature miRNAs bind the 3'UTR of target mRNAs leading to a reduction in protein output. DNMT and TET mRNAs can be targeted by miRNAs such as miR-29, miR-22 and others, thereby modulating the levels of DNA methylation and hydroxymethylation.
DNMT = DNA methyltransferase; miRNA = micro RNA; mRNA = messanger RNA; TET = ten-eleven translocation protein; UTR = untranslated region