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Epigenetic Control of Cytokine Gene Expression: Regulation of the *TNF/LT* Locus and T Helper Cell Differentiation

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Abstract

Epigenetics encompasses transient and heritable modifications to DNA and nucleosomes in the native chromatin context. For example, enzymatic addition of chemical moieties to the N-terminal “tails” of histones, particularly acetylation and methylation of lysine residues in the histone tails of H3 and H4, plays a key role in regulation of gene transcription. The modified histones, which are physically associated with gene regulatory regions that typically occur within conserved noncoding sequences, play a functional role in active, poised, or repressed gene transcription. The “histone code” defined by these modifications, along with the chromatin-binding acetylases, deacetylases, methylases, demethylases, and other enzymes that direct modifications resulting in specific patterns of histone modification, shows considerable evolutionary conservation from yeast to humans. Direct modifications at the DNA level, such as cytosine methylation at CpG motifs that represses promoter activity, are another highly conserved epigenetic mechanism of gene regulation. Furthermore, epigenetic modifications at the nucleosome or DNA level can also be coupled with higher-order intra- or interchromosomal interactions that influence the location of regulatory elements and that can place them in an environment of specific nucleoprotein complexes associated with transcription. In the mammalian immune system, epigenetic gene regulation is a crucial mechanism for a range of physiological processes, including the innate host immune response to pathogens and T cell differentiation driven by specific patterns of cytokine gene expression. Here, we will review current findings regarding epigenetic regulation of cytokine genes important in innate and/or adaptive immune responses, with a special focus upon the tumor necrosis factor/lymphotoxin locus and cytokine-driven CD4⁺ T cell differentiation into the Th1, Th2, and Th17 lineages.

1. THE COMPONENTS OF EPIGENETIC TRANSCRIPTIONAL REGULATION

Each human cell, with the exception of enucleated red blood cells, contains roughly 2 m of genomic DNA, which is compacted into a space approximately 10 μm in diameter within the cell's nucleus. Lengths of genomic DNA are wound tightly around nucleosomes comprised of an octamer of histone proteins (consisting of two molecules each of histone H2A, histone H2B, histone H3, and histone H4; Luger, Dechassa, & Tremethick, 2012; Fig. 2.1).

Nuclease digestion and sedimentation gradient assays, respectively, showed that ~145 bp of genomic DNA wraps around each nucleosome, resulting in a nucleoprotein complex of ~206 kD. Cloning the component proteins of the nucleosome revealed that they were members of the highly basic histone family, which is strongly conserved in eukaryotes (Kornberg & Lorch, 1999). Finally, X-ray crystallographic analysis revealed that the nucleosome consists of a disc of histones that is encircled by a left-handed superhelical turn of DNA along its perimeter, such that the relatively unstructured N-terminal ends of the histones are exposed to the outer surface (Luger et al., 1997; Fig. 2.1). This finding that was consistent with biochemical studies, which indicated that the N-terminal "tails" were targets of a range of posttranscriptional modifications (Kornberg & Lorch, 1999).

Nucleosome packaging of DNA presents a physical barrier to the initiation of transcription. When DNA is tightly associated with histones, forming a "closed" nucleosomal configuration, the RNA polymerase complex is prevented from binding to the start site of transcription proximal to the coding region of a gene, and transcription factors are precluded from interacting with their cognate binding sites in gene regulatory regions. However, in response to enzymatic modification of specific histone residues, a nucleosome can adopt an "open" configuration, rendering the DNA accessible to polymerases and transcription factors (Luger et al., 2012). This open nucleosomal conformation is primarily due to electrostatic repulsion between newly acetylated (and thus negatively charged) histone tails and the negatively charged phosphate backbone of DNA (Luger et al., 2012). Histone acetylation is directly coupled to activation of transcription, and a number of general transcription factors (e.g., TFIID) and global coactivator proteins (e.g., CBP and p300) function as histone acetyltransferases (HATs). Conversely, deacetylation of histones, which is mediated by a class of enzymes termed histone deacetylases (HDACs), is coupled to repression of transcription (Medzhitov & Horng, 2009; Wilson, Rowell, & Sekimata, 2009).

An experimental technique that has been instrumental for assaying histone modifications such as acetylation at endogenous genes is chromatin immunoprecipitation, or ChIP (Orlando, Strutt, & Paro, 1997). This technique was initially used for mapping the position, within a gene locus, of histones (Braunstein, Rose, Holmes, Allis, & Broach, 1993; Dedon, Soultis, Allis, & Gorovsky, 1991; Hebbes, Thorne, & Crane-Robinson, 1988; Solomon, Larsen, & Varshavsky, 1988; Solomon & Varshavsky, 1985) and other chromosomal proteins (Dedon et al., 1991; Hecht, Strahl-Bolsinger, & Grunstein, 1996; Orlando & Paro, 1993). Later, ChIP was adapted to detect the association of transcription factors with regulatory sequences at endogenous gene loci or in plasmid DNA (Botquin et al., 1998; Falvo, Parekh, Lin, Fraenkel, & Maniatis, 2000; Koipally, Renold, Kim, & Georgopoulos, 1999; Parekh & Maniatis, 1999; Tomotsune, Shoji, Wakamatsu, Kondoh, & Takahashi, 1993). Proteins recruited to regulatory sequences through interactions with DNA-bound

transcription factors, such as the coactivator protein CBP, were also detected in ChIP assays (Agalioti et al., 2000; Chen, Lin, Xie, Wilpitz, & Evans, 1999). As antibodies became available for the detection of histones bearing specific post-translational modifications, ChIP was employed to examine how unique histone modifications corresponded to differences in endogenous gene regulation, including responses to various stimuli (Braunstein et al., 1993; Hebbes et al., 1988; Kuo et al., 1996; Parekh & Maniatis, 1999; Solomon et al., 1988). For example, ChIP was used to show that histones H3 and H4 were hyperacetylated in the vicinity of the interferon- β (*IFNB1*) promoter in HeLa cells following exposure to Sendai virus (Parekh & Maniatis, 1999). ChIP assays have since been adapted to whole-genome analysis, where a “ChIP-on-chip” technique is utilized in which immunoprecipitated DNA is hybridized to a panel of microarray chip-mounted oligonucleotides (Ren et al., 2000). ChIP-on-chip has been applied to the investigation of global histone modifications in yeast (Kurdistani, Tavazoie, & Grunstein, 2004; Vogelauer, Wu, Suka, & Grunstein, 2000) and mammalian cells (Bernstein et al., 2005), revealing broad correlations between specific histone modifications and gene transcriptional activity, depending on the position of the nucleosome relative to the gene (Ong & Corces, 2012; Rowell, Merckenschlager, & Wilson, 2008).

The ChIP assay, in combination with the DNase I hypersensitivity assay (DHA) and with bioinformatic analyses of comparative sequencing between species, has revealed that conserved noncoding sequences (CNSs) in regulatory regions of cytokine gene loci are associated with inducible and constitutive hypersensitive sites (HSs), or regions of DNA accessibility, and with specific histone modifications. In some cases, these regions are subject to further regulation at the level of DNA modification, specifically by methylation at CpG dinucleotides, which represses transcription, and/or by their organization into higher-order chromatin structures through intra- or intrachromosomal interactions, which can place genes into regions of active or inactive transcription within the nucleus (Amsen, Spilianakis, & Flavell, 2009; Falvo, Tsytzykova, & Goldfeld, 2010; Lee, Kim, Spilianakis, Fields, & Flavell, 2006; Medzhitov & Horng, 2009; Ong & Corces, 2012; Rowell et al., 2008; Williams, Spilianakis, & Flavell, 2010; Wilson et al., 2009). Histone modification, DNA methylation, and higher-order chromatin interactions thus present key mechanisms of epigenetic control, and these will be discussed in the context of specific cytokine loci that play critical roles in the immune response.

1.1. Histone modifications

1.1.1 Covalent modifications—In addition to acetylation, a range of other post-translational histone modifications have been described, including methylation, phosphorylation, and ubiquitylation (Bannister & Kouzarides, 2011; Kouzarides, 2007; Rando, 2012; Tan et al., 2011). The specific combination of these distinct “histone marks” was postulated to mediate distinct patterns of transcriptional regulation, and thereby biological processes; this is known as the “histone code” hypothesis (Jenuwein & Allis, 2001; Strahl & Allis, 2000). While the histone code could theoretically extend to all possible combinations of post-translational modifications in the histone tails, the accumulated data suggests that only a limited number of such combinations occur in nature, and that these are mainly associated with activation or repression of transcription (Rando, 2012). This

limitation arises, in part, from the interplay between histone modifications within the same nucleosome, since specific modifications can favor or oppose the occurrence of another modification (Bannister & Kouzarides, 2011; Kouzarides, 2007). Another level of complexity arises, however, from the observation that the positioning of a nucleosome within the context of a gene locus (e.g., enhancer, promoter, coding region, boundary element, or adjacent region) and the transcriptional state of the gene (actively transcribed, recently transcribed or “primed,” “poised” for transcription, or silenced) correspond to characteristic sets of histone marks (Ong & Corces, 2012; Rowell et al., 2008). Thus, the histone code is dynamic and influenced by the activation state of a cell and by the ambient concentration of factors in the nucleus. Cytokine genes, with their tightly controlled expression patterns prior to and in response to cellular stimuli, present a particularly pertinent example of how temporal changes in histone modification state correspond to gene expression.

As outlined above, histone acetylation is normally associated with activation of transcription due to its effect of loosening the DNA-histone interaction within the nucleosome, and it is thus an “activating” or “permissive” histone mark (Table 2.1). Acetylation occurs at the ϵ -amino groups of specific lysines (i.e., at the terminus of the side chain) in the N-terminal histone tails of histones H3 and H4. For example, certain histone H3 tail acetylation sites are associated with active gene expression, including lysines 9, 14, and 27 (H3K9ac, H3K14ac, and H3K27ac, respectively) (Bannister & Kouzarides, 2011). Lysine 56 of histone H3 (H3K56), which lies in the globular domain of the histone near its interface with the DNA major groove, is also a target of acetylation in yeast (Xu, Zhang, & Grunstein, 2005) and humans (Tjeertes, Miller, & Jackson, 2009). Acetylated histone lysines also provide a docking site for specific protein domains: bromodomains (BRDs), found in a number of HATs and chromatin-remodeling complex proteins, such as Swi2/Snf2 of the SWI/SNF complex; and plant homeodomain (PHD) domains, found in D4 zinc and double PHD fingers family 3b (DPF3b) of the Brg1/Brm-associated factor (BAF) chromatin-remodeling complex (Bannister & Kouzarides, 2011).

The effects of lysine methylation of histones upon gene transcription are more complicated, and depend upon both the lysine residue involved and the number of methyl moieties—one, two, or three—that are coupled to the ϵ -amino group, which is also predominantly restricted to the N-terminal tails of histones H3 and H4 (Table 2.1). Unlike acetylation, methylation does not change the net charge of the modified histone residue. Rather, methylated histone lysines provide an interaction surface for chromatin-modifying proteins and other regulatory proteins, specifically those containing PHD domains or chromo-like domains (chromodomain, Tudor, MBT and PWWP domains), the latter found in the Tudor “royal” protein family (Bannister & Kouzarides, 2011; Kouzarides, 2007).

Broadly speaking, methylation of lysine 4 of histone H3 (H3K4) correlates with transcriptional activation, while methylation of lysine 9 or lysine 27 of histone H3 (H3K9 and H3K27, respectively) correlates with transcriptional repression (Bannister & Kouzarides, 2011; Kouzarides, 2007; Medzhitov & Horng, 2009; Ong & Corces, 2012; Rowell et al., 2008). Monomethylated H3K4 (H3K4me1) is primarily associated with enhancers that are poised or actively involved in transcriptional activation (Creyghton et al.,

2010; Ghisletti et al., 2010; Heintzman et al., 2007; Rada-Iglesias et al., 2011; Zentner, Tesar, & Scacheri, 2011). Trimethylated H3K4 (H3K4me3) is enriched at transcription start sites (TSSs) and is linked to active gene transcription (Ng, Robert, Young, & Struhl, 2003; Santos-Rosa et al., 2002). Furthermore, dimethylated H3K4 (H3K4me2) has been found to recruit HATs to regions downstream of gene promoters, leading to histone acetylation at the linked promoters and, in turn, efficient RNA polymerase II (RNA Pol II) elongation (Kim & Buratowski, 2009). By contrast to the role that some methyl marks play in gene activation, trimethylated H3K27 (H3K27me3) and dimethylated and trimethylated H3K9 (H3K9me2 and H3K9me3, respectively) are typically associated with inactive or repressed gene transcription (Kondo, Shen, & Issa, 2003; Kondo, Shen, Yan, Huang, & Issa, 2004; Okamoto, Otte, Allis, Reinberg, & Heard, 2004; Peters et al., 2003; Plath et al., 2003; Rougeulle et al., 2004). We note, however, that there is some evidence that methylation of H3K9 can also occur within or adjacent to actively transcribed regions (Vakoc, Mandat, Olenchok, & Blobel, 2005). Other N-terminal lysine residues that are methylation targets include lysine 36 of histone H3 (H3K36), which is involved in maintaining histone integrity in coding regions of actively transcribed genes and suppressing spurious cryptic transcripts (Carrozza et al., 2005; Joshi & Struhl, 2005; Keogh et al., 2005; Kizer et al., 2005; Li et al., 2002), and lysine 20 of histone H4 (H4K20), which has been associated with active repression of proinflammatory gene expression (Stender et al., 2012). Lysine 79 of histone H3 (H3K79), which is linked to active transcription, lies within the histone globular domain. Methylation of H3K79 only occurs when lysine 120 of histone H2B (H2BK120)—corresponding to lysine 123 of histone H2B (H2BK123) in yeast—is ubiquitinated (Bannister & Kouzarides, 2011; Kim et al., 2009; Lee et al., 2007).

Arginine residues in histones H3 and H4 can also be targets of methylation, and can be monomethylated at the ω -guanidino group, dimethylated symmetrically (via monomethylation of both terminal guanidino nitrogens), or dimethylated asymmetrically (via dimethylation of one of the terminal guanidino nitrogens) (Bannister & Kouzarides, 2011). Histone arginine methylation can influence transcription by promoting or inhibiting interactions between HATs and histone methyltransferases and their targets at nearby residues. For example methylation of arginine 2 of histone H3 (H3R2) inhibits methylation at H3K4, while methylation of arginine 3 of histone H4 (H4R3) can promote acetylation at lysines 8 and 12 of histone H4 (H4K8 and H4K12, respectively) (Arrowsmith, Bountra, Fish, Lee, & Schapira, 2012; Bannister & Kouzarides, 2011). Furthermore, symmetric dimethylation of H3R2 has been associated with active transcription (Migliori et al., 2012), while asymmetric dimethylation of this residue has been linked to transcriptional repression (Guccione et al., 2007; Kirmizis et al., 2007). Methylation of H3R17 has also been linked to gene activation (Selvi et al., 2010), and methylation of H4R3 is associated with both transcriptional activation when present in the asymmetric state (Balint, Gabor, & Nagy, 2005; Balint, Szanto, et al., 2005; Li et al., 2010) and transcriptional repression when present in the symmetric state (Dhar et al., 2012; Zhao et al., 2009).

Histone phosphorylation, another modification linked to gene activation, has been detected at serine, threonine, and tyrosine residues, predominantly within N-terminal tails. Serine, threonine, or tyrosine phosphorylation introduces a negatively charged moiety and thus, like histone acetylation, alters the net charge of the modified residue and disrupts the

nucleosome structure. Serine phosphorylation, in particular, provides a recognition motif for regulatory factors of the 14-3-3 protein family (Bannister & Kouzarides, 2011; Kouzarides, 2007). Notably, the phosphorylated serine 10 of histone H3 (H3S10p) mark has been characterized in the greatest detail, and its presence is linked to activation of immediate early response genes (Cheung et al., 2000; Li et al., 2002; Saccani, Pantano, & Natoli, 2002; Thomson, Clayton, & Mahadevan, 2001), including the *IL10* gene in human monocytes (Hofmann et al., 2012), as well as chromosome condensation during mitosis (Wei et al., 2009; Table 2.1).

Two lysine residues in the nucleosome, lysine 119 of histone H2A (H2AK119) and lysine 120 of histone H2B (H2BK120), which corresponds to lysine 123 of histone H2B (H2BK123) in yeast, are targets of ubiquitylation. The addition of a molecule of ubiquitin, a 76-amino acid polypeptide, is a much larger covalent modification than those previously mentioned, and it can inhibit or promote the recruitment of other factors, as well as disrupt local and higher-order chromatin compaction (Bannister & Kouzarides, 2011; Kouzarides, 2007; Luger et al., 2012). Monoubiquitylation of H2AK119 (H2AK119ub1) has been linked to repression of expression of several genes (Medzhitov & Horng, 2009; Wang et al., 2004; Zhou et al., 2008), and it appears to rely on prior methylation of H3K27 in the same nucleosome (Cao, Tsukada, & Zhang, 2005). Conversely, monoubiquitylation of H2BK120 (H2BK120ub1) in mammals and H2BK123 (H2BK123ub1) in yeast has been linked to transcriptional activation and, as was mentioned above, is a prerequisite for H3K79 methylation, as well as for H3K4me3 methylation. The disruptive effect of H2BK ubiquitylation upon chromatin compaction may be a broadly conserved mechanism underlying its activating function (Bannister & Kouzarides, 2011; Fierz et al., 2011; Kim et al., 2009; Kouzarides, 2007; Lee et al., 2007).

Sumoylation (from SUMO, small ubiquitin-like modifier) of histones has also been reported. As is the case with ubiquitylation, sumoylation adds a large (~100 amino acids, with some variation in isoforms) covalent modification to lysine residues in histones, and it can potentially have similar effects with respect to steric hindrance or protein recruitment. Although limited data are available, histone sumoylation has been tied to transcriptional repression (Nathan et al., 2006; Shiio & Eisenman, 2003), perhaps due to prevention of acetylation and/or ubiquitylation at lysine residues already occupied by SUMO. While sumoylation of all four component histones occurs in yeast, in mammals this modification has only been detected on histone H4 (Kalocsay, Hiller, & Jentsch, 2009; Nathan et al., 2006; Shiio & Eisenman, 2003). Finally, a range of other histone modifications, including deamination (conversion of arginine to citrulline), addition of β -N-acetylglucosamine to serine and threonine residues, ADP ribosylation of glutamate and arginine residues, biotinylation of lysine residues, and clipping of the N-terminal tail itself, have not been specifically linked to transcriptional regulation (Bannister & Kouzarides, 2011), and the most recently identified histone modification, lysine crotonylation (the crotonyl group is $\text{CH}_3\text{—CH=CH—CO—}$), marks active transcription of sex chromosome-linked genes in postmeiotic male germ cells (Tan et al., 2011).

1.1.2 Histone modifying enzymes and associated factors—Proteins that regulate histone modifications and link these modifications to changes in chromatin structure can be

thought of as belonging to three classes: “writers” that add chemical moieties to histone residues, such as the HATs and methyltransferases; “erasers” that remove these modifications, such as the HDACs and demethylases; and “readers” that recognize specific modifications (Arrowsmith et al., 2012; Ruthenburg, Allis, & Wysocka, 2007).

1.1.2.1 Histone acetyltransferases: Type-A HATs participate in transcriptional regulation, typically through acetylation of the N-terminal tails of histones H3 and H4, while type-B HATs, which are homologous to yeast HAT1, direct transient acetylation of newly translated histones prior to their deposition in nucleosomes (Kleff, Andrulis, Anderson, & Sternglanz, 1995; Kuo et al., 1996; Parthun, Widom, & Gottschling, 1996; Sobel, Cook, Perry, Annunziato, & Allis, 1995; Verreault, Kaufman, Kobayashi, & Stillman, 1996, 1998). Type-A HATs are divided into three categories: the GNAT (Gcn5-related N-acetyltransferase) superfamily, which includes Gcn5 and PCAF; the MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) family; and the CBP/p300 protein family. HAT activity is exhibited by other factors, including the nuclear receptor coactivators, which modulate the transcriptional response to hormone signals (Chen et al., 1997; Spencer et al., 1997). Subunits of TFIIC, which directs RNA polymerase III transcription initiation (Hsieh, Kundu, Wang, Kovelman, & Roeder, 1999; Kundu, Wang, & Roeder, 1999), and the largest TBP-associated factor (TAF) that comprises the TFIID complex, TAF_{II}250 (Mizzen et al., 1996), also exhibit HAT activity.

With respect to cytokine gene transcription, the Gcn5/PCAF complex and CBP/p300 are the most relevant HATs. CBP/p300 acetylates H3K14, H3K18, H3K27, H4K5, and H4K8 (as well as H2AK5, H2BK12, and H2BK15), while Gcn5/PCAF acetylates H3K9, H3K14, and H3K18 (Jin et al., 2011; Schiltz et al., 1999; Tie et al., 2009). Gcn5 and p300 have also been implicated in the acetylation of H3K56 (Bannister & Kouzarides, 2011; Das, Lucia, Hansen, & Tyler, 2009; Tjeertes et al., 2009; Table 2.2). Acetylation of histone lysine residues also leads to recruitment of proteins that possess the BRD, which is a conserved four-helix bundle-containing interaction module that specifically interacts with ϵ -N-acetylated lysine residues (Dhalluin et al., 1999; Hassan et al., 2007). The BRD family includes, in addition to Gcn5, PCAF, CBP, and p300 themselves, the bromo and extra terminal (BET) proteins, as well as a number of other transcriptional regulators. *In vitro* binding studies with acetylated histone peptides indicate that in addition to the above, PCAF interacts with H3K9ac, H3K14ac, H3K36ac, H4K8ac, H4K16ac, and H4K20ac, while GCN5 also interacts with H2AK5ac, H3K9ac, H3K14ac, H3K9ac/K14ac, H4K8ac/K14ac, H4K16ac, and H4K5ac/K8ac/K12ac/K16ac (Dhalluin et al., 1999; Filippakopoulos & Knapp, 2012; Hassan et al., 2007; Hudson, Martinez-Yamout, Dyson, & Wright, 2000; Zeng, Zhang, Gerona-Navarro, Moshkina, & Zhou, 2008; Table 2.2). Gcn5/PCAF-mediated histone acetylation has been specifically linked to recruitment of transcription elongation factors to target genes (Medzhitov & Horng, 2009; Wilson et al., 2009).

The BRDs of CBP/p300 interact with acetylated H2BK85, H3K9/K14, H3K14, H3K36, H3K56, H3S10/K14/K18, H4K12, H4K20, and H4K44 (Filippakopoulos & Knapp, 2012; Kouskouti & Talianidis, 2005; Zeng et al., 2008). CBP/p300-mediated histone acetylation, in turn, creates a docking site for histone readers, such as the aforementioned components of the SWI/ SNF and BAF complexes, which promote an open chromatin conformation and

stimulate transcription. The BET protein BRD4 also binds with high affinity to diacetylated and tetraacetylated H4 peptide and diacetylated H3 peptide (Dey, Chitsaz, Abbasi, Misteli, & Ozato, 2003).

1.1.2.2 Histone deacetylases: In 1997, a series of studies in yeast and human cells (Alland et al., 1997; Hassig, Fleischer, Billin, Schreiber, & Ayer, 1997; Heinzel et al., 1997; Kadosh & Struhl, 1997; Laherty et al., 1997; Nagy et al., 1997; Zhang, Iratni, Erdjument-Bromage, Tempst, & Reinberg, 1997) showed that transcription factors that were bound to gene promoters can recruit protein complexes consisting of Sin3 proteins and histone deacetylases 1 and 2 (HDAC1 and HDAC2), or their yeast homolog reduced potassium dependency 3 (Rpd3), leading to transcriptional repression (Pazin & Kadonaga, 1997; Rosenfeld, Lunyak, & Glass, 2006). The general action of HDACs is to counteract HAT-mediated histone acetylation at H3 and H4, serving as the “eraser” counterpart to the HAT “writers,” and to date, a total of eighteen HDACs have been identified in mammals.

HDACs are divided into five classes, which have all been implicated in regulation of cytokine gene transcription (Medzhitov & Horng, 2009; Rajendran, Garva, Krstic-Demonacos, & Demonacos, 2011; Villagra, Sotomayor, & Seto, 2010). Class I is composed of HDACs 1, 2, 3, and 8, which have homology to Rpd3; Class IIa and Class IIb consist of HDACs 4, 5, 7, and 9 and HDACs 6 and 10, respectively, which have homology to yeast histone deacetylase 1 (Hda1); Class III contains sirtuins 1 through 7 (SIRT1-7), homologues of yeast silent information regulator 2 (SIR2), which use NAD⁺ as a cofactor; and Class IV, which has only one member, HDAC11 (Jüngel et al., 2011; Rajendran et al., 2011; Schneider, Krämer, Schmid, & Saur, 2011; Villagra et al., 2010). Some HDACs target specific lysine residues. For example, SIRT6 interacts with the transactivating nuclear factor κ B (NF- κ B) subunit p65 (RelA) and specifically deacetylates H3K9 at a subset of NF- κ B-dependent genes, resulting in attenuated NF- κ B signaling (Kawahara et al., 2009); furthermore, SIRT1 counteracts the p300-mediated acetylation of p65 (Bourguignon, Xia, & Wong, 2009; Finkel, Deng, & Mostoslavsky, 2009; Medzhitov & Horng, 2009; Salminen, Kauppinen, Suuronen, & Kaarniranta, 2008; Yeung et al., 2004). In addition, SIRT2 specifically targets H4K16 for deacetylation (Kouzarides, 2007; Vaquero et al., 2006).

HDAC specificity can also be influenced by the proteins with which they partner to form complexes. For example, HDAC1 and HDAC2 can form complexes with the transcriptional repressors nuclear receptor corepressor (NCoR) and REST corepressor (CoREST). Large-scale binding and transcription profiling has shown that these repressors, in turn, regulate primary response genes, which have GC-rich promoters, but not secondary response genes (Medzhitov & Horng, 2009; Wilson et al., 2009; Table 2.2).

1.1.2.3 Histone methyltransferases and demethylases: Histone lysine methyltransferases and demethylases have a stricter specificity than most of the HAT and deacetylases. Histone lysine methyltransferases include MLL1-5, SET1A, SET1B, and ASH1, which target H3K4; G9a, SUV39H1, SUV39H2, ESET/SETDB1, EuHMTase/GLP, CLL8, and RIZ1, which modify H3K9; SET2, NSD1, and SYMD2, which methylate H3K36; DOT1, which targets H3K79; SET 7/8, SUV420H1, and SUV420H2, which methylate H4K20; and EZH2, which modifies H3K27 (Arrowsmith et al., 2012; Medzhitov & Horng, 2009; Wilson et al., 2009).

Histone lysine demethylases are divided into two classes: the lysine demethylase 1 (KDM1) family, which was first described in 2004, and the jumonji C containing protein (JmjC) family, which was discovered in 2006 (Shi et al., 2004; Tsukada et al., 2006). In the KDM1 family, H3K4 is targeted by KDM1A, KDM1B, KDM2B, and KDM5A-D; H3K9 is demethylated by KDM1A and KDM4A-D; and KDM2A, KDM2B and KDM4A-D target H3K36. In the JmjC family, H3K9 is demethylated by JHDM1D and PHF8; and JHDM1A, UTX, UTY, and JMJD3 target H3K27 (Table 2.2). Another member of the JmjC family, JMJD6, has been reported to be a histone lysine arginine demethylase that demethylates H3R2 and H4R3 (Chang, Chen, Zhao, & Bruick, 2007), although other reports indicate that JMJD6 primarily functions as a lysyl hydroxylase, both of nuclear proteins involved in RNA splicing (Webby et al., 2009) and of histones (Unoki et al., 2013).

1.1.2.4 Histone serine kinases: As noted above, phosphorylation of H3S10 is an activating histone mark, functioning through electrostatic disruption of nucleosome structure and recruitment of regulatory proteins of the 14-3-3 family. H3S10 phosphorylation has been shown to depend on the p38 mitogen-activated protein kinase (MAPK) pathway, although it remains to be determined whether H3S10 is a direct substrate for p38 or for a p38-regulated kinase, such as mitogen- and stress-activated kinase 1 (MSK1) or MSK2 (Cano, Hazzalin, Kardalidou, Buckle, & Mahadevan, 1995; Lau & Cheung, 2011; Soloaga et al., 2003; Strelkov & Davie, 2002; Thomson et al., 1999; Table 2.2). H3S10 phosphorylation can also be induced by RSK2 (Kouzarides, 2007; Sassone-Corsi et al., 1999) and by a component of the NF- κ B pathway, I κ B kinase- α (IKK- α), when that kinase is recruited to gene promoters (Anest et al., 2003; Duncan, Anest, Cogswell, & Baldwin, 2006; Yamamoto, Verma, Prajapati, Kwak, & Gaynor, 2003). The link between NF- κ B activation and H3S10 phosphorylation is strengthened by the observation that, following LPS stimulation, H3S10p was detected at the gene promoters of interleukin 6 (IL-6), IL-12p40 and CC-chemokine ligand 2 (CCL2, also known as MCP-1), but not TNF and CCL3 (Saccani et al., 2002). With respect to *TNF* gene regulation, a recent report did describe enrichment of H3S10p downstream of the *TNF* promoter early after LPS activation of murine macrophages (Thorne, Ouboussad, & Lefevre, 2012); however, unlike the genes encoding IL-6 and IL-12p40, *TNF* transcriptional initiation is independent of NF- κ B (Falvo et al., 2010), suggesting that a kinase other than IKK phosphorylates H3S10 in this case.

H3S10p is in turn recognized by 14-3-3 ζ , which is a member of the 14-3-3 family of regulatory proteins (Macdonald et al., 2005). Notably, it has been reported that 14-3-3 ζ interaction with H3S10p is enhanced *in vivo* by simultaneous acetylation of H3K9 and/or H3K14 and that this interaction is required for induction of HDAC1 gene expression (Winter et al., 2008). H3S10p has also been implicated in the recruitment of the transcription elongation factor pTEF-b (Ivaldi, Karam, & Corces, 2007; Zippo et al., 2009). Finally, phosphorylation of S10 in histone H3 molecules that possess an adjacent H3K9me_{2/3} mark displaces heterochromatin protein 1 (HP1) from the genome during mitosis, illustrating a mechanism by which phosphorylation of H3S10 counteracts an epigenetic mark of repression (Fischle et al., 2005).

1.1.2.5 Histone ubiquitylation: H2AK119 ubiquitylation is controlled by the polycomb complex-associated transcriptional repressors Bmi and Ring1A (Cao et al., 2005; Wang et al., 2004) or the U3 ligase 2A-HUB (Zhou et al., 2008). By contrast, H2BK120 ubiquitylation is regulated by the E3 ubiquitin ligase RNF20/ RNF40 in conjunction with WAC and UbcH6 (Zhang & Yu, 2011; Zhu et al., 2005). As noted above, ubiquitylation of H2B at lysine 120 (lysine 123 in yeast) is an activating mark for transcription, as it is required for (tri) methylation of H3K4 and H3K79. Furthermore, it has been implicated in stimulating the function of a histone chaperone and elongation factor, facilitates chromatin transcription (FACT; Pavri et al., 2006). By contrast, H2AK119 ubiquitylation is not conserved in yeast and has been shown to be associated with transcriptional repression of several chemokine genes in mammals, including CCL5, CXC-chemokine ligand 10 (CXCL10) and CXCL2, but not CXCL1, in the mouse RAW 264.7 macrophage cell line (Zhou et al., 2008). Ubiquitylation of H2AK119 by 2A-HUB (also known as DZIP3) blocks FACT recruitment to the gene promoters, suppressing RNA Pol II transcriptional elongation; LPS treatment leads to inhibition of 2A-HUB, and thus to reduced H2A ubiquitylation and concomitant recruitment of FACT (Zhou et al., 2008).

Post-translational histone modifications are thus part of a complex network of factors that write, erase, and read the histone code, and these factors and their interaction partners provide even greater levels of regulation, which result in specific programs of gene transcription. As discussed below, several chromatin-modifying proteins and their associated factors play key roles in the regulation of key cytokine loci and transcription of genes that are key in the innate and adaptive immune response.

1.2. DNA methylation

In addition to methylation at lysine and arginine residues in histones, another epigenetic modification influencing cytokine gene expression is DNA methylation itself. In mammals, DNA methylation occurs on CpG dinucleotides at the 5-carbon position of cytosine, and is directed primarily by three DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosyl-L-methionine (AdoMet) to cytosine (Bestor & Ingram, 1983; Bestor, Laudano, Mattaliano, & Ingram, 1988; Okano, Xie, & Li, 1998; Turek-Plewa & Jagodzinski, 2005; Vaissière, Sawan, & Herceg, 2008). *De novo* DNA methylation, which consists of incorporation of methyl groups at CpG dinucleotides within regions of unmethylated DNA and is widespread during early embryonic development, is controlled by DNMT3A and DNMT3B. By contrast, maintenance of methylation in somatic cells, particularly during cell division following each round of DNA replication, is directed by DNMT1 (Delcuve, Rastegar, & Davie, 2009; Miranda & Jones, 2007; Turek-Plewa & Jagodzinski, 2005; Vaissière et al., 2008). It has been appreciated for nearly forty years that conversion of cytosine to 5-methylcytosine (m⁵C) in DNA is associated with control of gene expression (Holliday & Pugh, 1975; Riggs, 1975). CpG methylation has primarily been linked to transcriptional repression, and consistent with this observation, gene promoter regions in particular tend to be devoid of m⁵C (Bird, 2002; Bird, Taggart, Frommer, Miller, & Macleod, 1985; Gardiner-Garden & Frommer, 1987; Lee, Sahoo, & Im, 2009; Meissner et al., 2008; Vaissière et al., 2008). While 60–90% of CpG sites are methylated across the genome, in CG-rich sequences known as CpG islands, which are present upstream of about

40% of human genes, CpG sites are typically unmethylated (Bird, 2000; Meissner et al., 2008; Miranda & Jones, 2007; Lee, Sahoo, et al., 2009; Turek-Plewa & Jagodziński, 2005).

One major mechanism for transcriptional repression by DNA methylation is occlusion of transcription factor binding sites by CpG methylation. Notably, DNA methylation and demethylation at specific loci, and its linked impact upon the ability of transcriptional activators to bind to regulatory elements, is a key feature of T cell lineage commitment (Barnes, 2011; Lee, Sahoo, et al., 2009; Li, 2002). A second major mechanism of transcriptional repression by DNA methylation involves the recruitment of HDACs to gene promoters by methyl-CpG-binding proteins (MeCPs), including MeCP2 and MBD2 (Feng et al., 2001; Jones et al., 1998; Nan et al., 1998; Ng et al., 1999). MeCPs can also recruit additional factors, including HP1 (which recruits several repressive factors including histone methyltransferases) and the histone H3K9 methyltransferases SUV39H1 and SETDB1, which can amplify suppression of gene activation (Feng & Zhang, 2001; Fujita et al., 2003; Ichimura et al., 2005; Jones et al., 1998; Nan et al., 1998; Ng et al., 1999; Vaissière et al., 2008; Zhang et al., 1999). There is also evidence that the acetylation state of adjacent histones can influence DNA methylation. For example, HDAC inhibitors can enhance DNA methylation, and DNA demethylating agents like 5-azacytidine and 5-aza-2'-deoxycytidine can, reciprocally, induce histone acetylation (Selker, 1998; Takebayashi et al., 2001; Zhu, Lakshmanan, Beal, & Otterson, 2001). Furthermore, histone H3 hypoacetylation and H3K9 methylation have been observed to precede DNA methylation during gene silencing (Strunnikova et al., 2005; Mutskov & Felsenfeld, 2004); for example, the tumor suppressor gene *RASSF1A* is progressively inactivated in proliferating human mammary epithelial cells, and this process initially coincides with decreases in histone H3ac and increases in H3K9me3 at the *RASSF1* promoter, and is only linked to DNA methylation of the promoter at later stages (Strunnikova et al., 2005).

1.3. Higher-order chromatin interactions

An important aspect of epigenetic regulation at the chromatin level that has been recently appreciated is the role of higher-order, long-range interactions in modulating gene expression. For many years, it was thought that gene promoters and enhancers operate in *cis* with TSSs, with regulatory sequences influencing neighboring upstream or downstream genes. This is a straightforward model in the case of promoters, which lie adjacent to the TSS. However, in the case of enhancers that are separated from the TSS by a few thousand base pairs, a prevailing model for their function, advanced by Ptashne based on findings with the bacteriophage lambda model system, was that the intervening DNA would be looped out, bringing enhancer DNA-bound transcriptional activators into close proximity with the transcription machinery at the target gene's TSS (Ptashne, 1986). A number of experiments with simple enhancer-promoter systems supported this looping model. For example, looping at a distance induced by interaction between the lambda repressor and the bacterial RNA polymerase complex was detected by examining perturbations in the DNA structure using DNase footprinting (Hochschild & Ptashne, 1986), and these loops were observed directly by electron microscopy (Griffith, Hochschild, & Ptashne, 1986). ChIP assays in yeast cells also provided evidence for activation-induced DNA looping, as immunoprecipitation of an enhancer-associated factor in fixed chromatin also pulled down

both enhancer and promoter/TSS DNA fragments under conditions of transcriptional competence (de Bruin, Zaman, Liberatore, & Ptashne, 2001).

The ability of an intervening sequence of DNA to loop is constrained by both its length and by the biophysical properties of chromosomal DNA. A loop of naked DNA, for example, requires at least ~0.5 kb to form, while a loop of uninterrupted chromatin fiber requires at least ~10 kb. However, looping of chromosomal DNA can be facilitated by acetylation of histones and by the presence of nucleosome-free regions; thus, chromatin-modifying factors can promote the formation of chromatin loops that are relatively smaller by inducing and/or taking advantage of open chromatin configurations. Such a region of open chromatin configuration can thus be thought of as a “hinge” that permits the formation of tight chromatin loops (Göndör & Ohlsson, 2009; Li, Barkess, & Qian, 2006; Rippe, 2001).

Another mechanism whereby proteins can facilitate transcription via structural changes in DNA involves remodeling upon the binding of “architectural” proteins. For example, proteins of the high mobility group (HMG) box family can facilitate transcription by inducing sharp bends in the DNA between regulatory elements (Alvarez, Rhodes, & Bidwell, 2003; Carey, 1998; Paull, Haykinson, & Johnson, 1993; Pil, Chow, & Lippard, 1993). The HMG box protein lymphoid enhancer-binding factor-1 (LEF-1), in particular, induces a dramatic bend of ~117° over 15 base pairs in its cognate DNA motif, and this promotes enhancer complex formation at the T cell receptor α (TCR α) gene (Giese, Kingsley, Kirshner, & Grosschedl, 1995; Giese, Pagel, & Grosschedl, 1997; Love et al., 1995). Thus, protein-mediated alterations in DNA structure in the context of chromatin can bring distant enhancer complexes into contact with the general transcription machinery. These findings underscore the need to consider the role of distant enhancers when analyzing mechanisms of gene transcription.

As discussed below in the sections reviewing epigenetic control of gene regulation at specific cytokine loci, DNA-looping interactions in the context of chromatin typically involve transcription factors and architectural proteins binding at CNSs that undergo epigenetic modifications at the histone or DNA level, or both. Identifying such regulatory CNSs is not always straightforward, however, as primary DNA sequence does not necessarily reflect the physical proximity or distance of gene regulatory regions and their target genes *in vivo*. Simply scanning directly upstream or downstream of a TSS for putative regulatory regions disregards the potential role of much more distal regions (Dekker, 2008). Furthermore, multiple upstream and downstream distal enhancers can make long-distance interactions with a specific gene, even, as will be discussed below, if these enhancers lie on different chromosomes.

A straightforward approach for examining long-range looping events at endogenous gene loci, chromosome conformation capture (3C), was introduced by Dekker, Rippe, Dekker, and Kleckner (2002). The basic steps of the 3C assay involve fixation of chromosomal regions that lie in close proximity via formaldehyde-induced protein-DNA crosslinking, digestion with a specific restriction endonuclease, and ligation under dilute conditions to favor intramolecular ligation of crosslinked fragments over random intermolecular ligation. After purification the ligated DNA fragments serve as templates for PCR with primers that

recognize widely separated DNA sequences of interest in order to quantify long-range interactions, both intrachromosomal and interchromosomal. Furthermore, addition of an immunoprecipitation step allows for selection of DNA fragments that interact with a specific protein (Dekker, 2003, 2006). To examine long-range interactions on a more global level, and without *a priori* knowledge of the location of potential interacting sequences, a range of other 3C-based methods have been developed. These extensions of the original 3C protocol allow a more unbiased, quantitative approach to determining interactions between a specific genomic site and sites throughout the genome. One innovation is the ligation of oligonucleotide linkers to immunoprecipitated fragments, followed by sequencing, to identify direct or indirect DNA contact sites of a given protein (Osborne, Ewels, & Young, 2011; Sanyal, Baù, Martí-Renom, & Dekker, 2011; van Steensel & Dekker, 2010). Much as ChIP-on-chip extended the range of the ChIP assay to a genomic scale, interactions between a given locus and the rest of the genome can be determined by 4C (either “circular chromosome conformation capture” or “chromosome conformation capture-on-chip”), which involves ligating a known segment of DNA to an array of purified 3C products, amplifying the resulting population of circular DNA molecules with inverse PCR, and analyzing the PCR products by high-throughput sequencing (Simonis et al., 2006; Würtele & Chartrand, 2006; Zhao et al., 2006).

These approaches have revealed higher-order chromatin interactions at a range of gene loci in mammalian cells that correlate with epigenetic regulation secondary to cellular stimulation and differentiation, including cytokine loci. Within the murine β -globin locus control region (LCR), for example, enhancer regions that lie within the ~200 kb murine β -globin LCR were shown to interact with active, but not inactive, genes within the locus that are located 40–60 kb away. These long-range intrachromosomal looping interactions occur in erythroid cells, which express globin genes, but not in brain tissue, in which β -globin is not expressed (Patrinos et al., 2004; Tolhuis, Palstra, Splinter, Grosveld, & de Laat, 2002). The interactions are both activation-dependent and dynamic, as they change over the course of erythroid differentiation (Palstra et al., 2003). In addition, this spatial re-organization during differentiation was shown to be driven by the transcription factors erythroid Krüppel-like factor (EKLF), CCCTC-binding factor (CTCF), GATA-binding factor 1 (GATA-1), and friend of GATA-1 (FOG-1; Palstra et al., 2003; Splinter et al., 2006; Vakoc, Letting, et al., 2005). These fluctuating, multi-loop structures have been termed “active chromatin hubs,” in which active nucleoprotein complexes are juxtaposed with TSSs, increasing the local concentration of factors to direct transcription (de Laat & Grosveld, 2003; Williams, Spilianakis, & Flavell, 2010).

Long-range interactions also come into play when interactions between transcriptional initiation and termination sites circularize a gene, creating a conformation optimal for re-initiation of transcription. This was first observed in yeast (Ansari & Hampsey, 2005; O’Sullivan et al., 2004) and in studies of mammalian mitochondrial rDNA (Martin, Cho, Cesare, Griffith, & Attardi, 2005), and, as will be described in the following section, was first observed for mRNA transcription in a higher eukaryote at the *TNF/LT* locus¹

¹Gene names are capitalized when referring to loci in general and human loci specifically, and are in lowercase when referring to murine loci.

(Tsytyskova, Falvo, et al., 2007). 3C and 4C assays have also lent support to an earlier concept, derived from immunofluorescence-based assays, which postulates that genes physically cluster into subnuclear regions of active transcription known as “transcription factories” (Cook, 2010; Iborra, Pombo, Jackson, & Cook, 1996; Jackson, Hassan, Errington, & Cook, 1993; Osborne et al., 2004; Simonis et al., 2006). Based on 3C and 4C analysis of the maternal allele of the *insulin-like growth factor 2 (Igf2)/H19* locus, it also appears that genes can be sequestered away from interaction with active enhancers into “inactive chromatin loops,” a process that requires CTCF (Kurukuti et al., 2006; Ling et al., 2006; Murrell, Heeson, & Reik, 2004; Zhao et al., 2006). High-throughput 3C-based assays have provided global maps of areas where active enhancers colocalize with their target genes (Baù et al., 2011; Sanyal, Lajoie, Jain, & Dekker, 2012), leading to the idea of “neighborhoods” of active and inactive transcription, which can be further grouped into compartments of active and inactive transcription in the nucleus (Sanyal et al., 2011).

These findings have led to a model of the genome as a fractal globule, allowing for dense packing without formation of knots (the classic “nucleosomal beads on a DNA string” further folded into “yarns”). In this model, the genome is partitioned into chromatin interaction domains, termed “topological domains” or “topologically associating domains (TADs)” which can be megabases in length (Dixon et al., 2012; Lieberman-Aiden et al., 2009; Mirny, 2011; Nora et al., 2012; Sanyal et al., 2011, 2012). In the discussion below of how higher-order chromatin organization participates in the regulation of cytokine gene expression, it is helpful to consider how these models inform understanding of the underlying mechanisms that control the rapid and/or persistent three-dimensional association and dissociation of enhancer regions with their target genes.

2. CYTOKINE GENE REGULATION

In the following sections, we will discuss in detail key examples of epigenetic regulation of cytokine gene expression in cells of the innate and adaptive immune systems: (i) the *TNF/lymphotoxin (TNF/LT)* locus, which encodes factors that are key components of the immediate early innate immune response; and (ii) the *interferon- γ (IFNG)* locus, Th2 cytokine locus (which includes *IL4*, *IL5*, and *IL13*, which encode interleukin-4, -5, and -13), and the *interleukin-17A/interleukin-17F (IL17A/IL17F)* locus, which reflect CD4⁺ T cell differentiation into the Th1, Th2, and Th17 lineages, respectively. Finally, epigenetic modifications that control expression at other loci involved in innate and adaptive immunity will be briefly summarized.

2.1. Innate immunity: The *TNF/LT* locus

In humans, the coding regions for *TNF*, *LTA*, and *LTB* (encoding the tumor necrosis factor, lymphotoxin- α , and lymphotoxin- β genes, respectively) lie within a ~13 kb region of the *TNF/LT* locus, which itself occupies ~40 kb within the *MHCIII* locus on the p arm of chromosome 6 (Browning et al., 1993; Nedospasov et al., 1986; reviewed in Falvo et al., 2010; Shebzukhov & Kuprash, 2011; Fig. 2.2A). The transcriptional orientation of *LTB* is opposite to that of *TNF* and *LTA*, an arrangement that is strikingly conserved in vertebrates, from placentals to the frog (*Xenopus tropicalis*; diverging ~360 million years ago; Cross et al., 2005; Deakin et al., 2006; Kono et al., 2006). However, in teleost fish (*Takifugu*

rubripes) and zebrafish (*Danio rerio*), the *TNF* homologue is in tandem with the *TNF/LT*-related gene *TNFN* (Savan, Kono, Igawa, & Sakai, 2005).

TNF was initially described as a product of macrophages (Beutler & Cerami, 1986; Rubin et al., 1985). However, later studies established that *TNF* transcription and *TNF* expression was also found in T cells, B cells, and fibroblasts (Cuturi et al., 1987; Goldfeld, Doyle, & Maniatis, 1990; Goldfeld & Maniatis, 1989; Goldfeld, Strominger, & Doyle, 1991; Goldfeld et al., 1992; Niitsu et al., 1988; Steffen, Ottmann, & Moore, 1988; Sung, Bjorndahl, Wang, Kao, & Fu, 1988; Sung, Jung, et al., 1988; Turner, Londei, & Feldmann, 1987).

Furthermore, it was demonstrated that *TNF* is immediate early gene, and that it is transcribed within minutes following activation of T and B cells or stimulation of monocytes and macrophages (Goldfeld et al., 1991, 1992; Goldfeld, McCaffrey, Strominger, & Rao, 1993). In T cells, *TNF* is one of the first genes to be expressed after cellular activation and is one of the few genes that can be induced by signaling through the T cell receptor in the absence of protein synthesis and a CD28 costimulatory signal (Goldfeld et al., 1993). Indeed, calcium influx alone can induce *TNF* transcription in T cells (Goldfeld et al., 1993). This activation was found to be cyclosporin A-sensitive, and, through an early application of a chemical genetics approach, was also found to be dependent upon the phosphatase activity of calcineurin (Goldfeld et al., 1993, 1994). This led to the discovery of the role of the calcineurin-dependent transcription factor family, nuclear factor of activated T cells (NFAT), in the activation of *TNF* gene transcription in T cells and B cells (Boussiotis, Nadler, Strominger, & Goldfeld, 1994; Goldfeld et al., 1993; McCaffrey, Goldfeld, & Rao, 1994; Tsai, Jain, Pesavento, Rao, & Goldfeld, 1996; Tsai, Yie, Thanos, & Goldfeld, 1996).

The proximal region of the *TNF* promoter (~200 bp upstream of the TSS) mediates initiation of *TNF* transcription in response to a wide range of stimuli in multiple cell types, including T cell and B cell activation (Goldfeld et al., 1994; Tsai, Jain, et al., 1996; Tsai, Yie, et al., 1996; Tsytyskova & Goldfeld, 2000, 2002), calcium ionophore (Goldfeld et al., 1993; Goldfeld et al., 1994), LPS (Goldfeld et al., 1990; Tsai et al., 2000), virus infection (Falvo, Ugliarolo, et al., 2000; Goldfeld et al., 1990), *TNF* (Brinkman, Telliez, Schievella, Lin, & Goldfeld, 1999), *Mycobacterium tuberculosis* (MTb; Barthel et al., 2003), and osmotic stress (Esensten et al., 2005). The proximal *TNF* promoter is very highly conserved in mammals (Cross et al., 2005; Goldfeld, Leung, Sawyer, & Hartl, 2000; Kuprash et al., 1999; Leung et al., 2000; Shakhov, Collart, Vassalli, Nedospasov, & Jongeneel, 1990) and almost completely conserved in higher primates (Baena et al., 2007; Leung et al., 2000). Depending on cell type and stimulus, discrete sets of transcription factors and coactivators assemble at the proximal *TNF* promoter to form higher-order nucleoprotein complexes called enhanceosomes, which drive transcription of the gene (Barthel et al., 2003; Falvo, Brinkman, et al., 2000; Falvo et al., 2008; Falvo, Ugliarolo, et al., 2000; Tsai et al., 2000; Tsytyskova & Goldfeld, 2002). This cell type- and stimulus-specific activation of *TNF* gene transcription is also a key feature of epigenetic regulation of the gene (Tsytyskova, Rajsbaum, et al., 2007; Biglione et al., 2011).

A number of constitutive and inducible DNase I hypersensitive sites (HSs), which occur within evolutionarily conserved sequences, have been detected across the *TNF/LT* locus in a cell type-specific fashion (Barthel & Goldfeld, 2003; Biglione et al., 2011; Ranjbar,

Rajsbaum, & Goldfeld, 2006; Taylor et al., 2008; Tsytsykova, Rajsbaum, et al., 2007; Fig. 2.2A). For example, strong HSs are present at the *TNF* and *LTA* promoters in multiple cell types. In addition, a number of these sites are enhanced in response to cellular activation, bind to distinct activators, and are cell type-specific (Barthel & Goldfeld, 2003; Biglione et al., 2011; Ranjbar et al., 2006; Tsytsykova, Rajsbaum, et al., 2007).

The activation of *TNF* transcription is associated with multiple HATs, including the CBP/p300 coactivators (Barthel et al., 2003; Falvo, Brinkman, et al., 2000; Tsai et al., 2000). Notably, CBP is specifically required for *TNF* gene transcription in response to T cell activation (Falvo, Brinkman, et al., 2000). The first sequence-specific DNA-binding transcription factor to be identified as a HAT, activating transcription factor 2 (ATF-2; Kawasaki et al., 2000), binds to a conserved variant cyclic AMP response element (CRE) in the *TNF* proximal promoter, which mediates activation of *TNF* gene expression in many cell types and in response to multiple stimuli (Barthel et al., 2003; Brinkman et al., 1999; Diaz & Lopez-Berestein, 2000; Falvo, Brinkman, et al., 2000; Falvo, Uglialoro, et al., 2000; Newell, Deisseroth, & Lopez-Berestein, 1994; Steer, Kroeger, Abraham, & Joyce, 2000; Tsai et al., 2000; Tsai, Jain, et al., 1996; Tsai, Yie, et al., 1996; Tsytsykova & Goldfeld, 2002). The HATs PCAF and Gcn5 are also critical for *TNF* gene expression in Jurkat T cells in response to phytohemagglutinin (PHA)/phorbol 12-myristate 13-acetate (PMA) stimulation (Ranjbar et al., 2006). PCAF has also been implicated in *TNF* expression in THP-1 cells in response to high glucose conditions (Miao, Gonzalo, Lanting, & Natarajan, 2004). By contrast, HDAC1 and HDAC3, as well as the HDAC-recruiting corepressors NCoR and CoREST, associate with the *Tnf* promoter in unstimulated bone marrow-derived macrophages (BMDMs), and this association is dramatically reduced within an hour of stimulation with LPS (Hargreaves, Horng, & Medzhitov, 2009).

Epigenetic modifications have been characterized at a number of HSs across the *TNF/LT* locus in human and murine primary cells and cell lines (Biglione et al., 2011; Ranjbar et al., 2006; Tsytsykova et al., 2007; Fig. 2.2A). At the *TNF* promoter, for example, in Jurkat T cells it was initially shown that acetylation of histone H3 is induced by PHA/PMA, while histone H4 is constitutively acetylated (Ranjbar et al., 2006). Furthermore, in murine primary CD4⁺ T cells, anti-CD3/CD28 stimulation resulted in increased acetylation of histones H3 and H4 at the *Tnf* promoter as well as distal enhancers HSS-9 and HSS+3 (sites 9 kb upstream and 3 kb downstream of the *Tnf* TSS, respectively; Tsytsykova, Rajsbaum, et al., 2007). H3ac and H4ac marks are also enriched at both the *Tnf* promoter and a novel monocyte-specific matrix attachment region (MAR) at HSS-7 of the *Tnf/ Lt* locus in the murine J774 monocytic cell line (Biglione et al., 2011). PHA/ PMA stimulation also results in recruitment of the HATs PCAF and Gcn5 to the *TNF* promoter in Jurkat cells (Ranjbar et al., 2006). As was reported in the same study, the transactivator of transcription (Tat) protein from HIV-1 subtype E (HIV-1_{93TH64}Tat) suppresses *TNF* transcription by, at least in part, inhibiting PCAF and Gcn5 recruitment to the *TNF* promoter, with concomitant reduction in histone H3 and H4 acetylation (Ranjbar et al., 2006). These studies were confirmed and extended in an analysis of histone modifications at the *TNF/LT* locus in unstimulated and PMA/ionomycin-stimulated Jurkat cells, which showed a peak of histone H3 and H4 acetylation, as well as H3K4 trimethylation, at an HS within exon 4 of *LTB*, with other

peaks at HSs 3.4 kb upstream of *LTA* (corresponding to the murine HSS-9 distal enhancer described by Tsytsykova, Rajsbaum, et al., 2007) and at the *LTA* and *TNF* promoter regions, as well as at a group of HSs near the 3' end of the *NFKB1* gene (Taylor et al., 2008).

These observations of epigenetic modifications at the *TNF/LT* locus were subsequently supported by a number of other studies of histone acetylation at the *TNF* promoter. An increase in acetylation of histones H3 and H4 at the *TNF* promoter correlates with LPS-induced *TNF* transcription in primary human monocytes and THP-1 cells (Garrett, Dietzmann-Maurer, Song, & Sullivan, 2008; Sullivan, Reddy, et al., 2007) and high glucose-induced *TNF* gene expression in THP-1 cells (Miao et al., 2004). Enriched H3ac and H4ac levels at the *TNF* promoter are also associated with maturation of monocytes into macrophages (Lee, Kim, Sanford, & Sullivan, 2003), and with the disease states of diabetes (Miao et al., 2004) and systemic lupus erythematosus (SLE; Sullivan, Suriano, et al., 2007) in primary monocytes. Moreover, IFN- γ treatment of primary human monocytes leads to persistent histone H4 acetylation at the *TNF* promoter, along with recruitment of ATF-2 and RNA Pol II; this “poised” pre-transcription state results in enhanced histone H3/H4 acetylation and *TNF* transcription in response to LPS stimulation (Garrett et al., 2008). In addition, the BRD protein Brg1, which interacts with acetylated histones and is an ATPase component of the SWI/SNF chromatin remodeling complex (Euskirchen, Auerbach, & Snyder, 2012) binds to the *Tnf* promoter in unstimulated murine J774 monocytic cells and BMDMs; however, expression of dominant-negative Brg1, or RNAi-mediated knockdown of Brg1 or the SWI/SNF ATPase Brm, does not impair LPS-induced *Tnf* transcription in these cells, suggesting that SWI/SNF complexes are dispensable for activation of *Tnf* gene expression in myeloid cells, and may act in some other capacity (Ramirez-Carrozzi et al., 2006, 2009).

The activating histone marks H3K4me1, H3K4me2, and H3K4me3 are enriched at the *TNF* promoter following LPS or TNF stimulation of THP-1 cells and PMA/ionomycin stimulation of Jurkat cells (Li et al., 2008; Sullivan, Reddy, et al., 2007; Taylor et al., 2008). In unstimulated murine BMDMs, high levels of H3K4me3 and H3ac (but not H4ac), along with RNA Pol II, TBP, and CBP/p300, are present at the *Tnf* promoter, consistent with a primary response gene poised for transcription (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). Assembly of this transcriptional complex at the *Tnf* promoter does not depend on signals mediated through Toll-like receptors (TLRs), as it was observed in macrophages from mice that are deficient in the essential TLR signaling components MyD88 and TRIF (Hargreaves et al., 2009). By contrast, LPS activation of wild-type BMDMs via TLR4 leads to enhanced association of the *Tnf* promoter with acetylated histone H4, the HATs Gcn5 and PCAF, the p-TEFb components cyclin 11 and cdk9, and the BRD protein Brd4 (Hargreaves et al., 2009). Furthermore, H3K4me2, which is enriched at the *TNF* promoter and 5' coding region prior to cellular activation, is lost in response to LPS stimulation of THP-1 cells, while trimethylation of H3K4 increases at the promoter after LPS treatment (Sullivan, Reddy, et al., 2007). By contrast, in LPS-tolerant THP-1 cells, LPS stimulation fails to induce H3K4 methylation, H3K9 demethylation, and HP1 loss at the *TNF* promoter, which are all events that occur in LPS-responsive cells (El Gazzar et al., 2008; El Gazzar, Yoza, Hu, Cousart, & McCall, 2007). Consistent with the effects of these histone marks upon

transcription, inhibition of H3K4 methylation through RNAi-mediated knockdown of either the histone methyltransferase SET7/9 or components of the mixed-lineage leukemia (MLL) histone methyl-transferase complex suppresses *TNF* transcription (Li et al., 2008; Sullivan, Reddy, et al., 2007), while inhibition of H3K9 methylation through RNAi of the histone methyltransferase G9a in LPS-tolerant cells decreases HP1 binding to the *TNF* promoter and restores *TNF* transcription (El Gazzar et al., 2008).

The activating histone mark H3S10p has also been observed at the *TNF* promoter in THP-1 cells, but not in primary human dendritic cells, following LPS stimulation (El Gazzar et al., 2007; Saccani et al., 2002). Infection of murine BMDMs with *Toxoplasma gondii*, which inhibits LPS-induced TNF expression, results in decreased LPS-mediated H3S10 phosphorylation and histone H3 acetylation at the *Tnf* promoter (Leng, Butcher, Egan, Abi Abdallah, & Denkers, 2009), while in LPS-tolerant THP-1 cells, H3S10 phosphorylation is reduced at the *TNF* promoter in comparison to LPS-responsive THP-1 cells (El Gazzar et al., 2007).

Thus, regulation of the *TNF* gene at its native locus involves a range of specific histone modifications and chromatin-modifying proteins associated with activation and repression. In the T cell lineage, activating histone marks strongly correlate with HSs present at promoter and enhancer regions, including distal enhancers that stimulate *TNF* transcription. In cells of the monocyte/macrophage lineage, activating histone marks are present at a monocyte-specific HS, and a range of stimuli correlate with the appearance of activating histone marks at the *TNF* promoter. Furthermore, the *TNF* promoter exhibits histone marks characteristic of a transcriptionally poised gene prior to activation, while under conditions of LPS tolerance the promoter is associated with histone marks and chromatin-binding proteins that typify a repressed transcriptional state. Taken together, these findings show that HSs at conserved noncoding sequences strongly correspond to the presence of distinct histone modifications, and strongly indicate that epigenetic modification of histones that are associated with *TNF* regulatory elements play a key role in inducible expression of the gene in the monocyte and T cell lineages.

2.1.1 DNA methylation at the *TNF/LT* locus—Methylation of DNA at the *TNF* proximal promoter has also been correlated with regulation of *TNF* gene transcription. For example, in primary granulocytes, which express TNF but not LT- α , the *TNF* proximal promoter is unmethylated and the *LTA* promoter is methylated, while in primary lymphocytes, which express both genes, both promoters are hypomethylated, and in sperm, where neither gene is expressed, both promoters are methylated (Kochanek, Toth, Dehmel, Renz, & Doerfler, 1990). In addition, the *TNF* coding sequence is hypomethylated in HL-60 (promyelocytic) cells, which actively produce TNF in response to PMA stimulation, and it is also hypomethylated in the RPMI 1788 (B-lymphoblastoid) human cell line, in which PMA induces modest *TNF* gene expression. By contrast, in the Jurkat human T cell line, which fails to produce TNF in response to PMA treatment, the *TNF* promoter is heavily methylated (Kochanek et al., 1990). It should be noted that PMA alone is not sufficient to induce TNF expression in a range of cell types (reviewed in Falvo et al., 2010) and selectively induces TNF in T and B cell lines (Goldfeld et al., 1991). In other experiments with primary human monocytes and lymphocytes, where TNF is expressed, the *TNF* coding sequence and

proximal *TNF* promoter are unmethylated, while in non-TNF-expressing HeLa cells these regions are highly methylated (Kochanek, Radbruch, Tesch, Renz, & Doerfler, 1991). Similarly, in the murine RAW 264.7 macrophage cell line, in which *Tnf* transcription can be induced by LPS or cycloheximide, the *Tnf* coding region and 3' and 5' UTRs are unmethylated, while in the murine 3T3 fibroblast line, in which *Tnf* transcription is not activated by either stimulus, these areas of the locus are highly methylated. Moreover, the *Tnf* gene is highly methylated in hybrid cells created from the fusion of RAW 264.7 and 3T3 cells, and these cells do not express TNF when treated with LPS or cycloheximide (Kruys, Thompson, & Beutler, 1993). More recently, analysis of the *TNF* promoter region and exon 1 in three human cell lines revealed high levels of DNA methylation in non-TNF-expressing K562 cells and clones of the THP-1 cell line that were selected for lack of TNF expression, and low levels of methylation, especially at the proximal *TNF* promoter (–200 nt upstream of the TSS), in TNF-expressing HL-60 cells and THP-1 clones (Sullivan, Reddy, et al., 2007). Taken together, these studies indicate that methylation at a number of *TNF* regions, including the promoter, is associated with transcriptional repression.

Lending further support to this conclusion, demethylation of the *TNF* gene correlates with cellular differentiation status and increasing competence to express TNF. One study reported that the *TNF* proximal promoter and first exon are highly methylated in human embryonic stem cells and embryoid bodies, exon 1 is demethylated in hematopoietic stem cells and liver cells, and both the *TNF* proximal promoter and exon 1 are demethylated in primary monocytes and macrophages, where the gene is readily expressed (Sullivan, Reddy, et al., 2007). Indeed, methylation status at the *TNF* gene also changes during myeloid commitment, as methylation at two CpG sites flanking the *TNF* promoter is lower in HL-60 cells than in the more differentiated THP-1 cells (Takei, Fernandez, Redford, & Toyoda, 1996). The *TNF* proximal promoter is also highly methylated in unrestricted somatic stem cells (USSCs) and in human bone marrow mesenchymal stem cells (BM-MSCs), and after TLR activation of these cells the methylation status of *TNF* remains unchanged and the gene is not activated to any extent (van den Berk et al., 2009, 2010).

Additional data in support of an important role for DNA methylation in the repression of TNF expression under certain circumstances comes from studies showing that inhibition of DNA methylation at the *TNF* gene can enhance its transcription. For example, treatment of THP-1 cells with 5-azacytidine, a DNA methyltransferase inhibitor, results in decreased levels of methylation at the *TNF* promoter and enhanced LPS-mediated TNF expression (Sullivan, Reddy, et al., 2007). In LPS-tolerant THP-1 cells, RNAi-mediated knockdown of the histone methyltransferase G9a inhibits recruitment of DNMT3A and DNMT3B methyltransferases to the *TNF* gene, and restores *TNF* transcription (El Gazzar et al., 2008).

The binding of Sp1 to its GC-rich cognate DNA motifs in the *TNF* promoter is required for *TNF* gene expression in cells of the monocyte/ macrophage lineage in response to LPS stimulation, Sendai virus infection, or MTb infection (Barthel et al., 2003; Falvo, Uglialoro, et al., 2000; Tsai et al., 2000; Tsytsykova & Goldfeld, 2002). Notably, the relatively high percentage of CpG dinucleotides in the *TNF* promoter places this gene in a category of primary response genes that are independent of SWI/SWF remodeling after TLR-induced activation, consistent with the findings described above (Ramirez-Carrozzi et al., 2006,

2009). In murine BMDMs for example, the promoters of genes with a similarly high density of CpG islands tend to have a lower affinity for nucleosomes and are usually associated with acetylated histone H3, H3K4me3, RNA Pol II, and TBP in the resting state, poising these genes for transcription (Ramirez-Carrozzi et al., 2009). Furthermore, binding of Sp1 to promoters of this class of primary response genes tends to be essential for RNA Pol II recruitment under basal conditions (Ramirez-Carrozzi et al., 2009). Thus, the cell type- and stimulus-specific binding of Sp1 at the *TNF* promoter may function in concert with epigenetic modifications that regulate the gene.

2.1.2 The role of intrachromosomal interactions at the *TNF/LT* locus—In T cells, *TNF* is one of the first genes expressed upon cellular activation (Goldfeld et al., 1991, 1993). Analysis of the murine *Tnf/Lt* locus by 3C revealed that, upon T cell activation, intrachromosomal interactions form between the *Tnf* promoter and two novel, DNase-hypersensitive elements. Specifically, intrachromosomal interactions form between the *Tnf* promoter and the HSS-9 distal enhancer, between the *Tnf* promoter and the HSS+3 distal enhancer, and between HSS-9 and HSS+3 (Tsytsykova, Rajsbaum, et al., 2007; Fig. 2.2A). These three pairs of interactions result in a double-loop configuration at the *Tnf/Lt* locus that brings regulatory regions bound by NFATp into close proximity, creating a higher local concentration of active nucleoprotein complexes (Fig. 2.2B). This higher-order structure is reminiscent of the active chromatin hub observed at the β -globin locus, although instead of directing alternative enhancer-promoter interactions it positions the *Tnf* gene for optimal transcriptional activation. Specifically, the interaction between the *Tnf* promoter and HSS+3 circularizes the *Tnf* gene, potentially facilitating reinitiation of transcription by juxtaposing the transcription initiation and termination sites. In addition, the interaction between the *Tnf* promoter and HSS-9 sequesters the *Lta* gene into a discrete loop, placing this gene in a distinct transcriptional environment relative to *Tnf* and *Ltb* (Tsytsykova, Rajsbaum, et al., 2007).

Notably, the AT-rich HS 7 kb upstream of the *Tnf* TSS in the murine *Tnf/Lt* locus, HSS-7, acts as a MAR. HSS-7 serves as a substrate for topoisomerase II, and treatment of murine monocytic and T cell lines with the topoisomerase II inhibitor etoposide attenuates *Tnf* mRNA synthesis (Biglione et al., 2011). This presents another level of structural organization of the *Tnf/Lt* locus. Notably, HSS-7 is only accessible to interact with the nuclear matrix in murine monocytes, suggesting that the *Tnf/Lt* locus is associated with the matrix in structurally distinct fashions based on cell type (Biglione et al., 2011). It is generally thought that interactions between the nuclear lamina and MARs, along with inter- and intrachromosomal interactions, are major contributing factors to the three-dimensional arrangement of chromosomes in the nucleus (van Steensel & Dekker, 2010). Topoisomerase II may dock at the *Tnf/Lt* HSS-7 MAR and act to relax positive supercoiling at the locus, which results from transcription of the constitutively expressed upstream *Nfkb1l* gene; this ensures efficient transcriptional output at the highly inducible *Tnf* gene (Biglione et al., 2011). Thus, this finding of cell type-specific epigenetic control of chromatin structure at the *Tnf/Lt* locus extends previous observations that regulation of *TNF* gene expression is controlled in a cell type-specific manner at the *TNF* promoter via distinct factors and regulatory elements (Barthel et al., 2003; Falvo, Uglialoro, et al., 2000; Goldfeld et al.,

1993; Tsai et al., 2000; Tsai, Jain, et al., 1996; Tsai, Yie, et al., 1996; Tsytsykova & Goldfeld, 2000, 2002). In summary, these studies indicate that the *TNF/LT* locus is subject to dynamic structural reconfiguration in response to various stimuli and in a manner that varies based on cell type.

In support of this model, another study found that intrachromosomal interactions among exon 4 of *LTB*, the *LTB* promoter, the *LTA* promoter, and the *TNF* 3'-UTR occur in unstimulated Jurkat cells and decrease upon PMA/ionomycin stimulation; the intrachromosomal interactions are thus associated with repression of *LTB* gene transcription (Wicks & Knight, 2011; Fig. 2.2A). This study also found that CTCF binds to *LTB* exon 4, indicating that CTCF may contribute to the formation of a repressive loop structure (Wicks & Knight, 2011). By contrast, another study in hepatocellular carcinoma cells implicated the formation of an enhancer-containing chromosomal loop, dependent upon CTCF and the cohesin RAD21, in the activation of *LTB* transcription (Watanabe et al., 2012). CTCF/RAD21 binding sites were characterized within *LTB* exon 4 (the site being designated TC3), upstream of and within the *NFKB1* gene (TC1 and TC2, which lie 29.5 and 34.2 kb from TC3, respectively), and upstream of the *LST1* gene (TC4, which lies 4.7 kb from TC3; Fig. 2.2A). In the early phase of gene expression following TNF stimulation in these cells, in which expression of both *TNF* and *LTB* is favored, TC1–TC4 are physically associated with the *TNF*, *LTA*, and *LTB* promoters and with an NF- κ B-dependent enhancer region, TE2, in the 3'-UTR of *TNF*. By contrast, in the late phase of gene expression, in which *LTB* transcription is favored, TC3, TE2, and the *LTB* promoter remain associated, as do TC2 and the *TNF* and *LTA* promoters (Watanabe et al., 2012; Fig. 2.2A). Taken together, all these data support a model in which dynamic changes in intrachromosomal interactions within the *TNF/LT* locus correlate with both activation and repression of specific genes within the locus, most likely through a combination of bringing enhancer regions into close proximity with specific promoters, and by sequestering genes into subnuclear regions of active or inactive transcription.

2.2. CD4⁺ T cell differentiation: The *IFNG* locus, Th2 locus, and *IL17A/IL17F* locus

A central cytokine-regulated process in the establishment of the immune response is the differentiation of naïve CD4⁺ T cells to helper T cell subsets (Fig. 2.3). Cytokines present in the local environment during antigen presentation strongly influence the differentiation pathway taken by a naïve CD4⁺ T cell. Th1 cells are primarily involved in host defense to intracellular pathogens, and differentiation of naïve CD4⁺ T cells to a Th1 phenotype requires the transcription factor T-bet (Szabo et al., 2000). In an elegant model proposed by Schulz et al., Th1 differentiation involves several steps: (i) exposure of the naïve CD4⁺ T cell to autocrine and/or paracrine IFN- γ during TCR engagement, which induces T-bet expression; (ii) T-bet-mediated expression of the IL-12 receptor subunit β 2 once TCR signaling ceases; and (iii) signals transduced by APC-derived IL-12, which drive STAT4 expression and sustained IFN- γ and T-bet synthesis (Schulz, Mariani, Radbruch, & Hofer, 2009). IL-2 is also required for both differentiation and subsequent expansion of the *de novo* Th1 population (Liao, Lin, Wang, Li, & Leonard, 2011).

Th2 cells are primarily involved in the immune response to extracellular parasites. The master regulator of Th2 differentiation is the transcription factor GATA3 (Zheng & Flavell, 1997). Th2 differentiation is usually considered to rely on IL-4 in the context of TCR ligation and exposure to IL-2, with IL-4 inducing STAT6 and GATA3 expression (Cote-Sierra et al., 2004). However, *in vivo* mouse studies have found that IL-4 is dispensable for Th2 differentiation, suggesting that IL-4-independent pathways play a role in Th2 development (van Panhuys et al., 2008). As Th2 differentiation proceeds, GATA3 mediates the expression of IL-5 and IL-13, classical Th2 cytokines whose genes share a locus with the *IL-4* gene (Kishikawa, Sun, Choi, Miaw, & Ho, 2001; Lavenu-Bombled, Trainor, Makeh, Romeo, & Max-Audit, 2002; Siegel, Zhang, Ray, & Ray, 1995; Yamashita et al., 2002; Zhang, Yang, & Ray, 1998). Initial strength of TCR engagement on naïve CD4⁺ T cells has also been linked to Th1 versus Th2 differentiation, with weak TCR signaling pushing the cell to a Th2 phenotype and strong TCR signaling pushing the cell to a Th1 phenotype (Constant, Pfeiffer, Woodard, Pasqualini, & Bottomly, 1995; Tao, Constant, Jorritsma, & Bottomly, 1997).

Th17 cells were first described as a distinct CD4⁺ T helper lineage in 2005 (Harrington et al., 2005). Th17 cells are involved in host defense against extracellular bacteria and fungi, and differentiation of a naïve CD4⁺ T cell to a Th17 cell is regulated by the transcription factor ROR γ t (Ivanov et al., 2006). The differentiation of Th17 cells is complex, and a clear picture of the cytokines required for Th17 lineage commitment has not emerged. Both IL-6 and TGF- β have been linked to Th17 differentiation (Bettelli et al., 2006; Gutmacher et al., 2011; Li, Wan, & Flavell, 2007; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006; Veldhoen, Hocking, Flavell, & Stockinger, 2006), although a recent report has suggested that TGF- β is not required for *in vivo* generation of at least some Th17 cells in mice (Ghoreschi et al., 2010). During TCR engagement by MHC Class II/antigen, induction of STAT3 and, in turn, ROR γ t by IL-6 and TGF- β , as well as other cytokines including IL-1 β and IL-23, drives the production of Th17-associated cytokines. These include IL-17A, IL-17F, IL-21, IL-22, and (in humans) IL-26 (Langrish et al., 2005; McGeachy et al., 2009; Wilson et al., 2007). IL-21 may act in an autocrine manner to potentiate Th17 differentiation (Korn et al., 2007; Nurieva et al., 2007; Zhou, Ivanov, et al., 2007).

Dysregulation of Th1 and Th17 responses leads to autoimmune disease states, while dysregulation of Th2 responses leads to atopic conditions (Kanno, Vahedi, Hirahara, Singleton, & O'Shea, 2012; Maddur, Miossec, Kaveri, & Bayry, 2012; Mills, 2011; Oliphant, Barlow, & McKenzie, 2011; Wilson et al., 2009). Below, we discuss epigenetic mechanisms involved in the regulation of gene expression at three specific loci that are associated with the differentiation of Th1, Th2, and Th17 cells, respectively: the *IFNG* locus, the Th2 locus (which contains the *IL4*, *IL5*, and *IL13* genes), and the *IL17A/IL17F* locus.

2.2.1 The *IFNG* locus—The *IFNG* gene is quite isolated in the context of its native locus: the nearest downstream gene coding region lies ~420 and ~500 kb away in mice and humans, respectively, and the nearest upstream gene, which encodes IL-26 in humans and IL-22 in mice, lies ~245 kb away (Kanno et al., 2012). An early study reported that 8.6 kb of the human *IFNG* locus, containing the coding sequence, 2.3 kb upstream and 1 kb

downstream of the *IFNG* TSS, is sufficient for T cell-specific expression of the gene when integrated into the genome of a transgenic mouse (Young et al., 1989). However, sites outside the *IFNG* promoter and coding region are essential for proper regulation of the gene's expression, as transgenic mice bearing the extended human *IFNG* locus, but not the murine *Ifng* locus with only the upstream promoter region, exhibited normal *IFNG* regulation during T helper cell differentiation (Soutto, Zhang, et al., 2002; Soutto, Zhou, & Aune, 2002; Zhu et al., 2001). Indeed, conserved sequences as distant as ~70 kb upstream and ~66 kb downstream of the murine *Ifng* gene (corresponding to sequences ~63 kb upstream and ~119 kb downstream of the human *IFNG* gene) contribute to its regulation during T cell lineage commitment (Hadjur et al., 2009; Sekimata et al., 2009). Conserved CNSs that coincide with HSs are spread across the *IFNG* locus and function as enhancers and/or mediate higher-order chromatin structure (Amsen et al., 2009; Balasubramani, Mukasa, Hatton, & Weaver, 2010; Hatton et al., 2006; Kanno et al., 2012; Lee, Avni, Chen, & Rao, 2004; Shnyreva et al., 2004; Wilson et al., 2009). In the murine *Ifng* locus, these CNSs include (from 5' to 3' with respect to *Ifng*) CNS-70, CNS-54, CNS-34, CNS-22, CNS-6 (also known as CNS1), *Ifng* intron 1, CNS+17-19 (also known as CNS2 or CNS+18/20), and CNS+30 (also known as CNS+29), CNS+46, and CNS+66 (Balasubramani, Shibata, et al., 2010; Mukasa et al., 2010; Wilson et al., 2009). The corresponding regions in the human *IFNG* locus are CNS-63, CNS-31, CNS-22, CNS-18, CNS-4, *IFNG* intron 1, CNS+22, CNS+40, CNS+80, and CNS+119 (Amsen et al., 2009; Balasubramani, Mukasa, et al., 2010; Barski et al., 2007; Boyle et al., 2008; Rowell et al., 2008; Wang et al., 2008; Wilson et al., 2009; Fig. 2.4).

A role for histone acetylation in the regulation of the *IFNG* locus was initially suggested by studies with the HDAC inhibitor sodium butyrate, which enhanced expression of IFN- γ in murine CD4⁺ T cells (Bird et al., 1998). In naïve murine CD4⁺ T cells, H4ac is enriched at CNS-34 and CNS-22 of the *Ifng* locus (Hatton et al., 2006). With respect to histone methylation, one study has reported that, in naïve murine CD4⁺ T cells, low levels of H3K4me1 are present at CNS-34 and CNS-22 (Mukasa et al., 2010), while a second study found that low levels of H3K4me2 are only found at CNS-22 (Schoenborn et al., 2007) and a third study reported that H3K4me2 is present at low levels at CNS-6, the *Ifng* promoter, and at a region ~13 kb downstream of the *Ifng* TSS (Hamalainen-Laanaya, Kobie, Chang, & Zeng, 2007). On the other hand, H3K27me3 is modestly enriched at the 3' end of the *Ifng* gene and at distal sites ~30-50 kb downstream in naïve murine CD4⁺ T cells (Mukasa et al., 2010; Schoenborn et al., 2007; Wei et al., 2009), indicating that this repressive mark may counteract any positive activity induced by the methylated H3K4 histones upstream.

Upon differentiation into Th1 cells, there is a marked increase in H3K4me2, H3ac, and H4ac across the *IFNG* locus, with concomitant removal of H3K27me3 (Agarwal & Rao, 1998a, 1998b; Hatton et al., 2006; Lee et al., 2004; Schoenborn et al., 2007; Shnyreva et al., 2004). In addition, at the early stages of Th1 development the Brg1-containing SWI/SNF remodeling complex is recruited to the murine *Ifng* promoter in a STAT4-dependent manner and promotes accessibility of the promoter to nuclease digestion (Zhang & Boothby, 2006). Furthermore, the binding of T-bet at multiple enhancers in the locus displaces HDAC-containing complexes (Chen, Osada, Santamaria-Babi, & Kannagi, 2006); T-bet is able to

recruit JMJD3 and SET7 to demethylate H3K27me3 and induce dimethylation of H3K4, respectively (Miller, Huang, Miazgowiec, Brassil, & Weinmann, 2008). By contrast, as Th2 differentiation proceeds, H3K27me3 is deposited throughout the *Ifng* locus (Agarwal & Rao, 1998a; Chang & Aune, 2007; Jones & Chen, 2006; Schoenborn et al., 2007). Notably, some repressive H3K9me2 marks persist at the locus in murine Th1 cells, which may modulate normal expression of the *Ifng* gene (Berger, 2007; Chang & Aune, 2007). Thus, repressive histone modifications accumulate to silence the *IFNG* locus in Th2 cells, while primarily activating histone modifications are deposited at enhancers and other conserved regions of the locus as Th1 differentiation proceeds.

Early studies reported that hypomethylation of a CpG island between the CAAAT and TATA boxes in the human *IFNG* promoter (conserved in mice) corresponds to transcriptional competence for IFN- γ production in human B cells (Pang, Norihisa, Benjamin, Kantor, & Young, 1992), murine Th1 clones, and human CD4⁺ Th0 clones, but that hypermethylation of this site is found in murine Th2 clones (Young et al., 1994). Furthermore, treatment of a murine Th2 T cell clone with the DNA methylation inhibitor 5-azacytidine promotes IFN- γ production (Young et al., 1994), reminiscent of earlier studies demonstrating that 5-azacytidine restored IFN- γ production in response to IL-2 in a cytotoxic T cell line (Farrar, Ruscetti, & Young, 1985). Progressive demethylation of ten CpG dinucleotides in an HS near *Ifng* intron 1 occurs as primary murine CD4⁺ T cells develop into Th1, but not Th2, cells. Moreover, demethylation of DNA at this site ensues after T-bet-mediated chromatin remodeling of the locus and maximal IFN- γ expression occurs, suggesting that the primary function of this modification is to poise the *Ifng* locus for rapid activation in response to cellular stimulation (Mullen et al., 2002). Similarly, in the human *IFNG* locus, six CpG sites in the *IFNG* promoter and seven CpG sites in CNS1 (CNS-4) are hypermethylated in naïve CD4⁺ cells, and become progressively demethylated during differentiation into Th1 cells, but not Th2 cells (Janson, Marits, Thörn, Ohlsson, & Winqvist, 2008; White et al., 2006).

Detailed analysis of DNA methylation at HSs in the murine *Ifng* locus revealed that the *Ifng* promoter and CNS-34, CNS-22, CNS+29, and CNS+46 are demethylated in naïve T cells, and that upon Th2 differentiation CpG methylation is induced at these sites, with a concomitant loss of NFAT binding at the *Ifng* promoter (Jones & Chen, 2006; Lee et al., 2004; Schoenborn et al., 2007). Notably, methylation of the *Ifng* promoter does not inhibit the binding and function of T-bet (Tong, Aune, & Boothby, 2005). However, it is interesting to speculate that the limited availability of T-bet in developing Th2 cells most likely precludes any functional impact of T-bet binding to a methylated region of the *Ifng* locus. The increase of CpG methylation at the *Ifng* promoter during Th2 differentiation, as well in Th1 cells from Stat4-deficient mice, also correlates with recruitment of DNMT3a (Jones & Chen, 2006; Yu, Thieu, & Kaplan, 2007). Furthermore, in mice lacking DNMT1 or the DNA-binding domain of MBD2 there is an increase in IFN- γ expression not only in naïve and Th1 cells, but also in Th2 cells (Hutchins et al., 2002; Lee, Fitzpatrick, et al., 2001; Makar & Wilson, 2004). Thus, at both the *Ifng* promoter and at multiple widely separated HSs in the *Ifng* locus, DNA hypomethylation correlates with the competence of a given T cell subset to express IFN- γ .

Both intra- and interchromosomal interactions have been characterized at the *Ifng* locus (Amsen et al., 2009; Lee et al., 2006; Williams, Spilianakis, & Flavell, 2010). In primary murine naïve CD4⁺ T cells, Th1 cells, and Th2 cells, the *Ifng* gene coding region associates with CNS-6, located ~5 kb upstream of the gene. However, in Th1 cells but not naïve or Th2 cells, the *Ifng* gene coding region also associates with CNS+18/20, located ~18 to 20 kb downstream of the gene (Spilianakis, Lalioti, Town, Lee, & Flavell, 2005; Fig. 2.4). Another study examined intrachromosomal interactions among fragments generated by *EcoRI* digestion at positions (relative to the *Ifng* TSS) -5048 (near CNS1/CNS-6), -1248, +229 (*Ifng* first intron), +6891, and +10,264, designated sites 1 through 5, respectively (Eivazova & Aune, 2004). Sites 1, 3, and 4 were found to be in close proximity in naïve, Th1, and Th2 cells. Sites 2 and 5 formed relatively weaker associations with sites 1, 3, and 4 in naïve cells, and these interactions were strengthened in Th1 and Th2 cells 5 days after primary stimulation. However, 2 days after secondary stimulation, the interactions with sites 2 and 5 were greatly reduced in Th1 cells (Fig. 2.4). This suggested a more tightly packed, “closed” conformation at the *Ifng* locus in naïve and Th2 cells, and an “open” conformation in Th1 cells (Eivazova & Aune, 2004). A number of MARs have also been identified in the *Ifng* locus in unstimulated naïve CD4⁺ cells as well as in CD4⁺ cells polarized under neutral, Th1, and Th2 conditions (Eivazova et al., 2007). In CD4⁺ cells cultured under neutral conditions (Th0) and the murine T cell line EL4, a MAR ~7 kb upstream of the *Ifng* TSS was observed to form intrachromosomal interactions with a MAR located ~10.5 kb downstream of the *Ifng* TSS, creating a ~18 kb loop (Eivazova et al., 2009; Eivazova et al., 2007; Fig. 2.4). Thus, distinct intrachromosomal interactions form at the *ifng* locus depending upon the stage of T cell differentiation.

CTCF and cohesins bind constitutively to the *Ifng* locus at intron 1 in Th1 cells, in which the CpG motifs are hypomethylated, but they do not bind to the locus in Th2 cells (Parelho et al., 2008; Sekimata et al., 2009). A Th1-specific CTCF/cohesin binding region is also present at CNS+66, located ~66 kb downstream of the murine *Ifng* gene (corresponding to CNS+119, ~119 kb downstream of the human *IFNG* gene; Sekimata et al., 2009). Functional consequences of CTCF/cohesin binding at the *Ifng* locus have been characterized. These studies have shown that a highly conserved hypomethylated element in the mouse locus, CNS-70, which corresponds to CNS-63 of the human *IFNG* locus, binds to CTCF and cohesin in naïve CD4⁺, Th1, and Th2 cells; however, it is only in Th1 cells that this element forms intrachromosomal interactions with the *Ifng* gene and with CNS+66, both of which are able to recruit T-bet (Sekimata et al., 2009). These interactions, as well as IFN- γ expression, are inhibited by shRNA-mediated ablation of CTCF or in a T-bet-deficient genetic background. In addition, Th1-specific CTCF/ T-bet-dependent interactions also occur between the *Ifng* gene and the CNS-34, CNS-29, and CNS+18/20 regions (Sekimata et al., 2009). Other chromosomal proteins may also play a role in the establishment of intrachromosomal interactions at the *Ifng* locus: by extending 3C assays using additional immunoprecipitation steps (“ChIP-loop” assays), topoisomerase II α and MeCP2, but not CTCF and HP1, were implicated as possible mediators of the interaction between the MARs at CNS1/CNS-6 and at -11 kb relative to the *Ifng* TSS in naïve cells (Eivazova et al., 2009). Notably, MeCP2, implicated as a mediator of structure in naïve cells, binds to methylated CpG sites that correspond with inactive transcription, while CTCF

binds to hypomethylated sites associated with active transcription (Eivazova et al., 2009; Sekimata et al., 2009). Thus, CTCF and other mediators of long-range chromosomal interactions direct the higher-order chromatin structure of the *IFNG* locus, which is further correlated with the level of gene activity and stage of T cell differentiation.

In murine naïve CD4⁺ T cells, but not Th1 or Th2 cells, the *Ifng* gene (located on chromosome 10) interacts with the *Il5* promoter, the *Rad50* promoter, and an HS in the 3' region of the *Rad50* gene (RHS6), all at the Th2 locus on chromosome 11 (Spilianakis et al., 2005). Furthermore, FISH analysis revealed that the *Ifng* and Th2 loci colocalize in nonheterochromatic regions in naïve CD4⁺ T cells, and that this interaction is diminished in Th1 and Th2 cells. Moreover, deletion of an HS (RHS7) that lies downstream of RHS6 in the *Rad50* gene, which is critical for Th2 locus function and for intrachromosomal interactions between RHS6 and elements within *Il4*, results in weakened association of the two loci in FISH analysis and in delayed, decreased levels of *Ifng* transcription in response to anti-CD3/CD28 stimulation (Spilianakis et al., 2005). As Flavell and colleagues proposed based on these structural findings, the *Ifng* and Th2 loci may participate in the formation of a chromatin hub in naïve CD4⁺ T cells, primed for rapid initiation of cytokine expression in response to TCR engagement. Only once Th1 or Th2 polarization signals are fully transmitted and one locus is shut down with concomitant upregulation of the other locus does polarization occur, in part due to a transition from interchromosomal to intrachromosomal interactions (Amsen et al., 2009; Lee et al., 2006; Williams, Spilianakis, & Flavell, 2010).

2.2.2 The Th2 cytokine locus: *IL4*, *IL5*, and *IL13*—The Th2 cytokine locus includes the genes that encode the canonical Th2 effector cytokines IL-4, IL-5, and IL-13. In mice, these cytokine genes occupy a ~120 kb region in chromosome 11, and are flanked by the gene encoding the transcription factor IRF-1 at one end and the genes encoding the constitutively expressed kinesin-2 subunit Kif3A and septin 8 (Sept8) at the other (Fig. 2.5; Frazer et al., 1997; Gorham et al., 1996; Lee & Rao, 2004; Loots et al., 2000). The Th2 locus is on the q arm of chromosome 5 in humans. The *IL13* and *IL4* genes lie in tandem at one end of the locus, downstream and in the same orientation as the gene encoding the DNA repair enzyme *Rad50*, while *IL5* resides ~120 kb telomeric to the *IL4* and *IL13* genes and in the opposite transcriptional orientation, an arrangement that is conserved in mammals (Frazer et al., 1997; Gorham et al., 1996; Lee & Rao, 2004; Loots et al., 2000). An array of CNSs, constitutive and inducible HSs, and binding sites for the architectural protein special AT-rich sequence binding protein 1 (SATB1) are scattered across the Th2 locus in mice (Fig. 2.5). Specifically, *Rad50* hypersensitive sites (RHSs) 1 through 7, each of which contains one to three discrete HSs, lie between *Il5* and the 3' end of *Rad50*. RHS4, RHS5, RHS6, and RHS7 are clustered near the 3' end of *Rad50*, forming the Th2 LCR. This region was classified as an LCR because it drives *Il4* and *Il13* transcription in Th2 effector cells in transgenic mice in a copy number-dependent fashion and irrespective of integration location (Fields, Lee, Kim, Bartsevich, & Flavell, 2004; Lee, Fields, Griffin, & Flavell, 2003). In addition, three HSs lie within *Il13*: HS1 (which coincides with a CG-rich element, CGRE), HS2, and HS3. A conserved region between *Il13* and *Il4* contains HSs as well (named Hss1, Hss2, and Hss3; Hss1 and Hss2 lie within a highly conserved sequence, CNS-1; Loots et al.,

2000). The *Il4* gene contains HS-I (at the promoter), HS-II (an enhancer in the second intron), and HS-III (Lee, Fields, & Flavell, 2001; Takemoto et al., 1998). Downstream of *Il4* lies HS-IV, which coincides with a conserved silencer region, and HS-V_A and HS-V, which coincide with a second CNS, CNS-2 (Agarwal, Avni, & Rao, 2000; Tanaka et al., 2006; Fig. 2.5). *Il4* transcription is enhanced by the Th2 LCR, Hss1, Hss2, HS-I, HS-II, HS-V_A, and HS-V, while *Il13* transcription is enhanced by the Th2 LCR, HS1, Hss1, and Hss2; however, these elements do not drive *Il5* transcription (Lee, Fields, et al., 2003). Several SATB1-binding sequences, designated SBS-C1 to SBS-C9, lie between *Il5* and the Th2 LCR and downstream of *Il4*. As described below, these are involved in establishing long-range intrachromosomal interactions (Cai et al., 2006; Fig. 2.5).

2.2.2.1 Histone modifications: As CD4⁺ T helper cell differentiation proceeds, DNase I accessibility at HSs in the promoter, intronic, intergenic, and 3' regions of *Il13* and *Il4* increases or diminishes depending on the external signals that are received (Agarwal & Rao, 1998b; Takemoto et al., 1998). These findings correspond well with early data that supported an important role for histone acetylation in the regulation of the Th2 locus, which initially came from observations that two pharmacological inhibitors of the HDACs, sodium butyrate and trichostatin A, can derepress *IL4* expression in naïve murine CD4⁺ T cells and in activated human peripheral blood CD4⁺ T cells (Bird et al., 1998; Valapour et al., 2002). Indeed, mice with T cell-specific loss of HDAC1 exhibit increased inflammatory responses in an *in vivo* allergic airway inflammation model (Grausenburger et al., 2010). These mice show enhanced production of IL-5, IL-13, and (with PMA/ionomycin stimulation) IL-4 by peripheral lung CD4⁺ T cells during disease, enhanced production of IL-4, IL-5, IL-13, and IL-10 in Th2-polarized peripheral CD4⁺ T cells, and higher proliferation of and IL-4 expression by naïve CD4⁺ T cells that are activated in Th2 conditions. Furthermore, binding of HDAC1 is detected in unstimulated CD4⁺ T cells at multiple sites in the Th2 cytokine locus, including the *Il4* intron 2 enhancer (HS-II), HS-V and HS-V_A downstream of *Il4*, CNS-1/Hss2 in the *Il13/Il4* intergenic region, and HS2 in the *Il13* promoter (Grausenburger et al., 2010).

In naïve murine CD4⁺ T cells, low to undetectable levels of H3ac and H4ac are found at the *Il5* promoter, the *Il13* promoter and first intron, the *Il13/Il4* intergenic region CNS-1 (Hss1 and Hss2), the *Il4* promoter, the *Il4* intron 2 enhancer (HS-II), the *Il4* 3' enhancer (HS-V_A), the Th2-specific constitutive HS site HS-V (CNS-2), and the common naïve/Th1/Th2 HS site HS-IV, while in Th2 cells all of these regions become persistently hyperacetylated (Avni et al., 2002; Baguet & Bix, 2004; Fields, Kim, & Flavell, 2002; Fields et al., 2004; Grogan et al., 2003; Hatton et al., 2006; Wurster & Pazin, 2008; Yamashita et al., 2002). Th2-specific acetylation of histone H3 also occurs at the murine Th2 LCR (Fields et al., 2004; Wurster & Pazin, 2008). Similarly, in peripheral blood naïve human CD4⁺ T cells H3ac is not detected at the *IL4* promoter, but becomes enriched in Th2-polarized cells relative to Th1-polarized cells (Messi et al., 2003). Histone H3 acetylation at CNS-1, HS-II, HS-III, and HS-V_A also correlates with high levels of *Il4* expression in murine Th2 clones (Guo et al., 2002). By contrast, histone H3 and H4 are acetylated at roughly equal levels at the murine *Rad50* promoter and at two *Rad50* intronic regions in Th1 and Th2 cells, albeit at higher levels than at these sites in naïve CD4⁺ T cells (Fields et al., 2004; Yamashita et al.,

2002). Thus, as is found at the *IFNG* locus, histone acetylation at the Th2 cytokine locus strongly corresponds to the ability of a differentiated CD4⁺ T helper cell to rapidly synthesize the appropriate array of cytokines in response to activation.

Acetylation during CD4⁺ T helper cell differentiation appears to be biphasic. After TCR stimulation of murine naïve CD4⁺ T cells, hyper-acetylation is rapidly induced at the *Il4* promoter, HS-II, CNS-1, HS-V_A, CNS -2, HS-IV, and the Th2 LCR in both Th1- and Th2-polarized cells; however, with continual exposure to Th2-polarizing conditions, hyperacetylation is sustained at *Il4* and gradually lost at *Ifng* (Avni et al., 2002; Fields et al., 2002). This corresponds to the finding that both IL-4 and IFN- γ are rapidly synthesized after anti-CD3/CD28 stimulation of murine naïve CD4⁺ T cells under Th2-polarizing conditions, with *Ifng* expression decreasing as differentiation proceeds (Grogan et al., 2001). The persistence of histone acetylation at the Th2 cytokine locus in mature Th2 cells provides a contrast to the transient histone hyperacetylation that occurs at an innate immune gene like *IFNB1* at the time of transcriptional activation (Agalioti, Chen, & Thanos, 2002; Parekh & Maniatis, 1999).

Specific transcription factors, in particular those that regulate Th1/Th2 differentiation, have been linked to histone acetylation at the Th2 locus. Histone H3 and H4 hyperacetylation at the *Il4* promoter, the *Il4* intron 2 enhancer (HS-II), CNS-1, HS-IV, HS-V_A, and HS-V/CNS -2 are decreased under Th2-polarizing conditions and increased under Th1-polarizing conditions in cells from mice lacking STAT6 or STAT4, respectively (Avni et al., 2002; Fields et al., 2002; Yamashita et al., 2002). GATA3, which binds to the *Il4* 3' HS-V_A enhancer site upon cell stimulation via interaction with NFATp, can induce hyperacetylation at these sites when overexpressed (Avni et al., 2002; Yamashita et al., 2002, 2004), even in STAT6-deficient murine Th2 cells (Fields et al., 2002). Moreover, conditional ablation of GATA3 expression in fully differentiated Th2 cells, which reduces IL-4, IL-5 and IL-13 production, also selectively decreases histone acetylation at the *Il5* promoter (Yamashita et al., 2004). Deletion of CNS-1 from the endogenous murine Th2 locus abrogates basal levels of H3ac associated with the *Il4* and *Il13* promoters, and inhibits partitioning of the *Il4* gene to heterochromatin in lymph node CD4⁺ T cells polarized to a Th1 phenotype by *Leishmania major* infection. Notably, CNS-1 binds to Ikaros, which can recruit histone modifying complexes, and deletion of the cognate Ikaros binding motifs in CNS-1 attenuates CNS-1-mediated enhancement of SV40 promoter-driven reporter gene expression in Jurkat cells (Grogan et al., 2003).

In addition to acetylases and deacetylases, a number of other chromatin-modifying proteins have been linked to Th2 lineage commitment. Mice deficient in the Polycomb group (PcG) ring fingerprotein Mel-18 display impaired Th2 differentiation and expression of IL-4, IL-5, and IL-13 (Kimura et al., 2001). While Mel-18 binds to the mouse *Il4* promoter, as well as the *Ifng* promoter, in an NFAT-dependent manner (Jacob, Hod-Dvorai, Schif-Zuck, & Avni, 2008; Jacob, Hod-Dvorai, Ben-Mordechai, Boyko, & Avni, 2011) its role in mediating CD4⁺ T helper cell differentiation remains unclear. Another PcG family member, the H3K27-specific histone methyltransferase EZH2, binds to HS-IV (the *Il4* 3' silencer) and Hss3 (in the *Il13/Il4* intergenic region) in naïve murine CD4⁺ T cells, Th1 cells, and Th2 cells. As described below, in the case of naïve and Th1 cells, the binding of EZH2

coincides with H3K27 methylation in the *Il13/Il4* region of the locus (Koyanagi et al., 2005). Mice haploinsufficient for the H3K4 methyltransferase MLL exhibit wild-type Th1 differentiation but develop memory Th2 cells that are defective in *Il4*, *Il5*, and *Il13* gene expression in response to activation, in part due to reduced GATA3 activity; MLL binds to the Th2 locus and *Gata3* locus in memory Th2 cells but not in memory Th1 cells or naïve CD4⁺ T cells (Yamashita et al., 2006). The SWI/SNF ATPase Brg1 also functions at the Th2 cytokine locus; the SWI/SNF complex binds to the Th2 LCR, HS-V (CNS-2), and the *Il4* and *Il13* proximal promoter regions in Th2 cells, and siRNA-mediated Brg1 knockdown impairs expression of IL-4, IL-5, IL-13, and IL-10 during Th2 differentiation, as well as IL-4, IL-13, and IL-10 expression in differentiated Th2 cells (Wurster & Pazin, 2008).

Both permissive and repressive histone methyl modifications have been characterized at the Th2 cytokine locus. H3K4me2 is present at low levels at CNS-1, the *Il4* promoter, and the *Il4* intron 2 enhancer in naïve murine CD4⁺ T cells, is enriched at these sites in Th2 cells, and is absent at these sites in Th1 cells (Ansel et al., 2004; Makar et al., 2003). In comparison, HS-V, which lies within the *Il4* 3' enhancer region, is marked by relatively high levels of H3K4me2 in naïve murine CD4⁺ T cells; H3K4me2 levels at HS-V are dramatically reduced in response to Th1 polarization but only decline modestly in response to Th2 polarization, suggesting that this modification might be important for both early IL-4 expression following TCR engagement and then sustained Th2 locus activity under Th2-polarizing conditions (Baguet & Bix, 2004). Bivalent histone modifications have also been associated with CD4⁺ helper T cell differentiation. These modifications, which consist of a combination of permissive and repressive marks, have been associated with genes that are either expressed at low levels or that are poised for activation (Bernstein et al., 2006). This is illustrated by HS-IV at the Th2 locus, which is enriched in both H3K4me2 and H3K27me3 in naïve, Th1, and Th2 cells; HS-IV is essential for suppression of IL-4 expression during Th1 lineage commitment, and its deletion skews the differentiation of naïve murine CD4⁺ T cells to a Th2 phenotype after TCR stimulation under neutral polarizing conditions (Ansel et al., 2004).

The activating mark H3S10p is also enriched at HS-IV in murine Th1 and Th2 clones. However, H3S10p is also enriched at HS-I and CNS-1 at the Th2 locus in the Th2 clone, providing further evidence of distinct histone modification patterns at the Th2 locus in Th1 versus Th2 cells (Baguet & Bix, 2004).

As noted above, the repressive H3K27me3 mark is enriched at HS-IV of the Th2 locus. H3K27me2 is also present at HS-IV, as well as Hss3, in murine naïve CD4⁺ T cells and Th1-primed cells, but is almost absent in Th2-primed cells (Koyanagi et al., 2005). Little or no enrichment of H3K9me2 or H3K9me3 is found throughout the *Il4/Il13* section of the locus in murine Th1 cells, nor at CNS-1, the *Il4* promoter, or the *Il4* intron 2 enhancer in murine naïve CD4⁺ T cells (Makar et al., 2003), although an earlier study detected the presence of H3K9me2 at the *Il4* and *Il13* promoters in a murine Th1 clone (Grogan et al., 2003), and a second study reported that H3K9me2 is enriched in Th1 cells at the Th2 LCR HS sites RHS6 and RHS7, while H3K4me2 levels at these sites are higher in Th2 cells than in Th1 cells, similar to the *Il4* promoter (Lee & Rao, 2004). A genome-wide screen also detected H3K27me3 in murine Th1 cells, and H3K4me3 in murine Th2 cells, at the Th2

cytokine locus (Wei et al., 2009). Furthermore, the level of H3K27 methylation at HS-IV correlates with *Il4* and *Il13* silencing in murine Th1-primed cells; after a second round of Th1 priming H3K27 methylation spreads to neighboring regions of the locus (Koyanagi et al., 2005). Thus, specific regulatory elements in the Th2 cytokine locus are major targets for repressive histone modifications, and maintenance of these silencing marks is critical for proper CD4⁺ T helper cell differentiation upon antigen recognition.

2.2.2.2 DNA methylation: The first indication that CpG methylation plays a prominent role in regulation of the Th2 locus came from observations that 5-aza-2'-deoxycytidine treatment markedly increases *Il4* gene expression by naïve murine CD4⁺ T cells, and that the *Il4* intronic enhancer region (HS-II) and the *Il5* promoter are demethylated in murine Th2, but not Th1, clones (Agarwal & Rao, 1998b; Bird et al., 1998). The *Il4* proximal promoter and HS-V/ CNS-2 regions are also hypomethylated in naïve murine CD4⁺ T cells; these regions remain demethylated in mature Th2 cells, where hypomethylation at CNS-1 and Hss3 in the *Il13/Il4* intergenic region and extension of demethylation throughout the *Il4* gene are also found (Lee, Agarwal, & Rao, 2002; Tykocinski et al., 2005). Demethylation of the GATA3-binding first intron of the *Il4* gene, the *Il13* promoter, and HS-V_A, and partial demethylation of the *Il5* promoter, directly correlates with competence for Th2 cytokine secretion in Th2 cells (Guo et al., 2002; Kim, Fields, & Flavell, 2007; Tykocinski et al., 2005). Conversely, HS-V/CNS-2, which is hypomethylated in naïve CD4⁺ T cells, becomes hypermethylated in Th1 cells (Lee et al., 2002). The Th2 LCR is also subject to DNA methylation-mediated control: RHS7 undergoes Th2-specific demethylation following acetylation of the LCR; however, RHS4, RHS5, and RHS6 remain methylated (Kim et al., 2007).

At the human Th2 locus, while demethylation is clearly evident at the *Il13* promoter, *Il4* intron 2, and at a CpG motif near the human CNS-1 element in Th2 cells relative to naïve CD4⁺ T cells and Th1 cells, little difference in methylation state at the *Il4* promoter is apparent in these cell types, unlike what is found in the mouse (Santangelo, Cousins, Winkelmann, & Staynov, 2002). Hypomethylation of the *Il13* promoter is also modestly enhanced in human Th2 cells as compared to naïve CD4⁺ T cells, Th1 cells, and Th17 cells (Janson et al., 2011). Further dissection of the human Th2 locus is necessary to determine whether DNA methylation is of greater importance for regulation of Th2 lineage commitment in mice than it is in humans.

Specific DNA methyltransferases have also been implicated in the regulation of cytokine gene expression at the Th2 locus. In single-positive murine CD4⁺ thymocytes, but not single-positive CD8⁺ thymocytes, *Il4* gene expression is strongly induced in response to TCR stimulation, suggesting that some level of programming at the Th2 locus has already taken place by the time of CD8⁺ versus CD4⁺ fate decision in the thymus (Makar et al., 2003). DNMT3B binds to CNS-1 in single-positive CD4⁺ thymocytes, but is lost in response to TCR stimulation, while DNMT1 is constitutively bound to *Il4/Il13* both prior to and after TCR engagement (Makar et al., 2003). DNMT1 recruitment to *Il4/Il13* is sustained in naïve CD4⁺ T cells, even after TCR activation under nonpolarizing conditions; however, under Th2-polarizing conditions DNMT1 presence at *Il4/Il13* wanes, followed by demethylation at the *Il4* intronic enhancer. Indeed, DNMT1 is actively required for repression of *Il4* expression in naïve murine CD4⁺ T cells, as knockout of DNMT1 results in

significantly increased IL-4 production even under nonpolarizing activation conditions (Makar et al., 2003). DNMT1 is also required for suppression of *Il10* and, to a lesser extent, *Il5* and *Il13* expression after TCR engagement under nonpolarizing conditions in both murine CD8⁺ and murine CD4⁺ T cells, although exposure to polarizing conditions can partially reverse this skewed profile, consistent with a broad but not exclusive role for DNA methylation in silencing Th2 cytokine expression (Makar & Wilson, 2004). MBD2 also associates with the murine Th2 locus at CNS-1 and the second intron of *Il4* in Th1 cells, and Th1 cells from MBD2-deficient mice are competent to express IL-4, suggesting that, upon binding to CpG sites in the locus, MBD2 recruits chromatin modifying factors that are essential for sustained silencing of *Il4* expression in Th1 cells (Hutchins et al., 2002). GATA3 overexpression displaces MBD2 from methylated DNA and is critical for demethylation at *Il4* intron 2 during Th2 differentiation (Makar & Wilson, 2004; Yamashita et al., 2004). By contrast, GATA3 is not required for demethylation of RHS7 in the murine Th2 LCR (Kim et al., 2007).

2.2.2.3 Higher-order chromatin interactions: The higher-order chromatin structure of the murine Th2 cytokine locus has been extensively investigated in a number of primary cell and cell line systems, revealing several intrachromosomal interactions and, as noted in the previous section, interchromosomal interactions with the *Ifng* locus (Amsen et al., 2009; Lee et al., 2006; Williams, Spilianakis, & Flavell, 2010). The Th2 locus exhibits constitutive intrachromosomal interactions in T cells, NK cells, B cells, and fibroblasts; these interactions place the *Il4*, *Il5*, and *Il13* promoters into close proximity and result in looping out of the intervening ~60 kb *Rad50* coding region (Spilianakis & Flavell, 2004; Fig. 2.5). The *Rad50* gene is constitutively expressed in all of these cell types, and this core configuration of the locus is present regardless of whether the cell expresses IL-4, IL-5, and/or IL-13. It represents a “pre-poised” chromatin conformation that, upon subsequent cell type-specific intrachromosomal and protein-DNA interactions, can become competent for expression of Th2 cytokines. Such interactions are apparent in naïve T cells, Th1, Th2, and NK cells, where the *Il4* and *Il13* promoters associate with both the Th2 LCR (with the strongest interaction observed at RHS7), and HSs at the 3' end of the *Il4* gene. RHS7 also interacts with several HSs within and adjacent to *Il4* and *Il13*, while the *Il5* promoter interacts with these HSs as well, but does not directly interact with the Th2 LCR. A further layer of conformational structure is evident only in Th2 and NK cells, where the *Il4* promoter and RHS3 (near the 5' end of the *Rad50* gene) interact (Spilianakis & Flavell, 2004; Fig. 2.5). These data indicate that robust, direct interactions between the Th2 LCR and the *Il4* and *Il13* genes, along with interactions between the *Il5* promoter and sequences within *Il4/Il13* that bring the *Il5* promoter into close proximity to the Th2 LCR, result in a “poised” conformation at the Th2 locus in CD4⁺ T cells that enables optimal access for Th2-associated transcription factors as CD4⁺ T helper cell differentiation proceeds. In addition, because the *Rad50* promoter fails to interact with any region of the Th2 locus, regardless of cell type, this suggests that regulation of this constitutively expressed gene involves mechanisms that are distinct from those at play at the Th2 locus (Spilianakis & Flavell, 2004).

The functional importance of RHS7 in the higher-order conformation of the Th2 locus has been clearly demonstrated by experiments with mice in which the site is deleted. In these animals, intrachromosomal interactions between the *Il4* promoter and sites RHS4 and RHS6 in the Th2 LCR are abolished, and interaction between the *Il4* promoter and the *Il5* and *Il13* promoters is reduced (Lee, Spilianakis, & Flavell, 2005). Notably, in mice lacking STAT6, interactions between RHS7 and other sites in the Th2 cytokine locus (and, reciprocally, between the *Il4* promoter and sites in the Th2 LCR) are modestly impaired in naïve CD4⁺ cells, and more markedly impaired in Th1 and Th2 cells (Spilianakis & Flavell, 2004). GATA3 and NFAT proteins are sufficient to induce interactions between the Th2 LCR and the rest of the Th2 locus, as combined ionomycin treatment and ectopic expression of GATA3 in a murine fibroblast line induces interactions between the Th2 LCR and the rest of the locus (Spilianakis & Flavell, 2004). Thus, GATA3 and NFAT proteins participate in both the formation of the “poised” chromosomal conformation at the Th2 locus and transcriptional activation of the locus during Th2 differentiation. However, as the “poised” conformation of the Th2 locus is also present in Th1 cells, where GATA3 expression is very low, another factor, in combination with NFAT, is most likely capable of mediating this structural configuration.

The higher-order configuration of the murine Th2 cytokine locus is further organized into a dense series of chromatin loops mediated by the architectural protein SATB1. SATB1 expression is rapidly induced upon Th2 cell activation, and the protein binds to ATC-rich DNA sequences that readily separate into single strands upon superhelical strain, termed base-unpairing regions (BURs; Cai et al., 2006). SATB1 is recruited to nine sites within a ~200 kb region of chromosome 11 (consisting of the Th2 locus and flanking *Kif3a* and *Sept8* genes) upon activation of a murine Th2 clone (Fig. 2.5). CNS-1 and CNS-2 were also identified as SATB1-binding regions, although interaction between SATB1 and these sites may be indirect given the lack of cognate SATB1-binding motifs in these sequences (Cai et al., 2006). By combining 3C and the ChIP-loop assay, which detects DNA loops in chromatin that are anchored by a specific protein (Horike, Cai, Miyano, Cheng, & Kohwi-Shigematsu, 2005), Cai et al. found several looped structures at the Th2 locus prior to activation: SBS-C1 (near the *Il5* promoter) is spatially juxtaposed with CNS-2, SBS-C7 (which lies near CNS-2), and, weakly, SBS-C9 (downstream of *Sept8*), while SBS-C9 is spatially juxtaposed with the *Il5* promoter, SBS-C2 (upstream of *Rad50*), and the 3' end of the Th2 LCR near RHS7 (Cai et al., 2006). After activation of the Th2 clone, additional intrachromosomal loops rapidly form at the Th2 cytokine locus. Specifically, SBS-C1 forms additional contacts with SBS-C3 through C6 (in introns at the center of the *Rad50* gene), the *Il13* promoter, the *Il4* promoter, and CNS-1, and its interaction with SBS-C9 is dramatically strengthened. In turn, SBS-C9 makes additional contacts with SBS-C3 through C6 and the *Il13* and *Il4* promoters. Direct analysis of promoter juxtapositions revealed weak interactions between the *Il4* and *Il13* promoters, and the *Il4* and *Il5* promoters, prior to stimulation; after cellular activation new interactions form between the *Il5* and *Il13* promoters, and the interaction between the *Il4* and *Il5* promoters is enhanced. The *Rad50* promoter is excluded from this *Il4/Il5/Il13* promoter assembly. Ablation of SATB1 expression inhibits *Il5*, *Il13*, and, especially, *Il4* expression in response to Th2 activation, and disrupts formation of the *de novo* SBS-C1 and SBS-C9 interactions that occur after cell

stimulation (Cai et al., 2006). Expression of the transcription factor c-Maf, which is a critical mediator of *Il4* transcriptional induction (Ho, Hodge, Rooney, & Glimcher, 1996), is also markedly diminished in stimulated SATB1-negative cells (Cai et al., 2006). Together, these findings suggest that activation of Th2 cells results in SATB1 binding to numerous sites throughout the Th2 locus, followed by condensation of the Th2 cytokine promoters at a core node formed by SATB1 association with the *Rad50* intronic region and sites near the 5' and 3' ends of the locus. Intriguingly, SATB1 binding to the *IL5* promoter during early human Th2 differentiation appears to suppress *IL5* gene expression, in part via competition with GATA3 (Ahlfors et al., 2010). This suggests that the functions carried out by SATB1 may evolve over the course of full Th2 lineage commitment.

As discussed above, the murine Th2 cytokine locus on chromosome 11 engages in highly stable interchromosomal interactions with the *Ifng* locus on chromosome 10, and these loci colocalize to nonheterochromatic regions in naïve CD4⁺ T cells and NK cells, but not in B cells or fibroblasts (Spilianakis et al., 2005). In an interesting contrast to the intrachromosomal interactions at the Th2 locus, the *Rad50* promoter participates in these inter-chromosomal interactions with the *Ifng* gene, along with the *Il5* promoter and RHS6; while the crosslinking frequency of these interchromosomal interactions is reduced in both Th1 and Th2 cells relative to naïve CD4⁺ T cells, this reduction in overall cross-chromosomal interactions is most modest for the *Rad50* promoter in Th2 cells (Spilianakis et al., 2005). In naïve CD4⁺ T cells (as well as Th1 and Th2 cells) from the aforementioned RHS7-deficient mice (Lee, Spilianakis, et al., 2005), interchromosomal interactions are diminished based on both 3C and FISH analysis, and *Ifng* and *Il5* gene transcription is delayed and attenuated (Spilianakis et al., 2005), indicating that these cross-chromosomal interactions influence stimulation-induced gene expression at loci associated with both helper cell types. In addition, in murine Th1 and Th2 cells, CTCF binds strongly to sites upstream of *Il5*, a site in the *Il13/Il4* intergenic region, and a site within *Kif3a*, and knockout of CTCF markedly reduces IL-4, IL-5, and IL-13 production in Th2 cells, marginally reduces IFN- γ production in Th1 cells, and has no effect on IL-17 production in Th17 cells (Ribeiro de Almeida et al., 2009). Thus, CTCF also contributes to the formation and/or stability of long-range chromosomal interactions at the Th2 locus.

In summary, a series of interchromosomal and intrachromosomal interactions at the Th2 locus controls gene expression during Th2 lineage commitment. Based on these findings, a preliminary model of Th2 locus regulation can be proposed. In murine naïve CD4⁺ T cells, interchromosomal interactions place the *Ifng* and Th2 loci into close proximity, potentially placing the genes in a transcriptional hub sufficient to encourage their low level synthesis prior to cellular activation. A number of intrachromosomal interactions are also already present at the Th2 locus. Once TCR engagement occurs, Th2 polarizing conditions result in physical separation of the loci and, in turn, the establishment of GATA3/NFAT-mediated intrachromosomal interactions within the Th2 locus that place the Th2 LCR in close proximity to the *Il4* and *Il13* promoters. SATB1 is also recruited to the locus at this time, where it binds to several elements throughout the locus and condenses the locus into a node of transcriptionally competent loops. Finally, additional chromatin binding proteins and transcription factors, such as c-Maf, are recruited to the “primed” locus, and strong

activation of Th2 cytokine synthesis proceeds (Amsen et al., 2009; Cai et al., 2006; Lee et al., 2006; Williams, Spilianakis, & Flavell, 2010).

2.2.3 The *IL17A/IL17F* locus—Due to its recent identification as a distinct CD4⁺ T helper lineage, fewer investigations have been performed of the epigenetic mechanisms involved in (i) the differentiation of Th17 cells from naïve precursors, and (ii) the regulation of the signature cytokines expressed by this helper subtype, including IL-17A, IL-17F, IL-21, IL-22 and, in humans, IL-26. A physiological role for pathogen-infected apoptotic APCs in directing Th17 differentiation has been identified (Torchinsky, Garaude, Martin, & Blander, 2009). However, as described in the introduction to this section, the precise set of factors required for Th17 lineage commitment, particularly in humans, remains a subject of debate.

In mammals the genes encoding IL-17A and IL-17F are oriented tail-to-tail and are separated by ~44 kb of sequence; the genes lie on chromosome 6 in mice and chromosome 1 in humans. In the murine *Il17a/Il17f* locus, a total of eight CNSs were initially characterized and designated CNS-1 through CNS-8; Th17-specific HSs have been found to correspond to several of these CNSs (Akimzhanov, Yang, & Dong, 2007; Mukasa et al., 2010; Fig. 2.6). Relative to naïve CD4⁺, Th1, and Th2 cells, hyper-acetylation of histone H3 is Th17-specific at all of these sites apart from CNS-5, and H3ac is also uniquely enriched at both the *Il17a* promoter and *Il17f* promoter in Th17 cells (Akimzhanov et al., 2007). In humans, anti-CD3/CD28 stimulation of naïve CD4⁺ T cells results in H3K18ac enrichment at CNS1, CNS2, CNS4, CNS5, CNS6 and the *IL17A* proximal promoter, and peripheral blood T cells isolated from patients with SLE, in whom circulating IL-17A levels are elevated, exhibit higher levels of H3K18ac at these sites than do CD4⁺ T cells from healthy controls (Hedrich, Rauen, Kis-Toth, Kytтарыs, & Tsokos, 2012; Rauen, Hedrich, Juang, Tenbrock, & Tsokos, 2011).

With respect to histone methyl modifications at the *Il17a/Il17f* locus, one study reported that in murine Th17 cells, H3K4me3 is strongly enriched at the *Il17a* and *Il17f* promoters, present at low levels at the *Ifng* promoter, and absent from the *Il4* promoter, while a second study confirmed H3K4me3 enrichment at the *Il17a* and *Il17f* promoters in murine Th17 cells (Akimzhanov et al., 2007; Mukasa et al., 2010). In addition, H3K4me3 is enriched at several other sites (excepting a site ~97 kb upstream of the *Il17a* TSS) throughout the *Il17a/Il17f* locus in Th17 cells as compared to murine naïve CD4⁺ T cells, while H3K4me3 is only elevated at a site ~10 kb downstream of the *Il17a* TSS in Th1 cells as compared to naïve cells (Mukasa et al., 2010). On the other hand, H3K27me3 is enriched at several sites throughout the *Il17a/Il17f* locus in murine Th1 cells as compared to naïve CD4⁺ T lymphocytes, but present at similar or lower levels at these sites in Th17 cells as compared to naïve CD4⁺ T cells (Fig. 2.6). A recent study also reported that H3K27me3 levels are similarly enriched at seven distinct CNSs in the *Il17a/Il17f* locus in naïve CD4⁺ T cells and Th1 cells from mice, but strongly enhanced at these sites in Th2 cells; in Th17 cells H3K27me3 is lost, and H3K4me3 is deposited, at most of these sites (Thomas, Sai, & Wells, 2012; Fig. 2.6). Several Th17-associated cytokines have been found to promote changes in H3K4me3 levels at the *Il17a* and *Il17f* promoters in murine Th17 cells, with TGF- β driving increased H3K4me3 presence at both promoters, and IL-23 driving reduced H3K4me3

presence at the *Il17a* promoter; under the latter conditions Th17 cells primarily secrete IL17F (Mukasa et al., 2010; Thomas et al., 2012).

Th17 cells exhibit a surprising level of epigenetic plasticity. Treatment with IL-12 or, to a lesser extent, IL-23, in the absence of TGF- β drives murine Th17 cells to a Th1 phenotype characterized by loss of IL-17 expression and gain of IFN- γ expression (Lee, Turner, et al., 2009; Lexberg et al., 2008). IL-12 treatment of murine Th17 cells also induces H3K4me accumulation at the *Ifng* locus and concomitant H3K27me3 enrichment throughout the *Il17a/Il17f* locus, further emphasizing the *in vitro* epigenetic plasticity characteristic of murine Th17 cells (Mukasa et al., 2010). Several studies have found CD4⁺ T cells that express both IFN- γ and IL-17 at sites of inflammation in autoimmune disease (Aarvak, Chabaud, Miossec, & Natvig, 1999; Annunziato et al., 2007; Nistala et al., 2010). Comparative epigenetic profiling of these “Th1/Th17” cells, along with Th1 and Th17 cells isolated from peripheral human blood, revealed that H3K4me3 is enriched near the TSS of *IFNG* in both Th1 and Th1/Th17 cells, and near the TSS of *IL17A* in Th17 cells, while H3K27me3 is deposited at the *IL17A* TSS in Th1 and Th1/Th17 cells (Cohen et al., 2011). Furthermore, culturing human Th17 cells in Th1-promoting conditions or culturing human Th1 cells in Th17-promoting conditions (which induces IL-17A expression), leads to bivalent deposition of H3K4me3 and H3K27me3 at the *IL17A* promoter (Cohen et al., 2011). Intriguingly, the *Rorc* gene, which encodes the Th17 master regulator ROR γ t, is strongly marked in its 5' region by H3K4me3 in murine Th17 cells and, conversely, by H3K27me3 throughout the coding region in Th1 cells; the *Tbx21* gene, however, which encodes T-bet, is decorated at its 5' end by high levels of H3K4me3 in Th1 cells, but by bivalent H3K4me3/H3K27me3 in murine Th17 cells, indicating that T-bet may be poised for induction under the right conditions (e.g. IL-12 exposure) in Th17 cells (Wei et al., 2009). The physiological importance of Th17 plasticity is underscored by findings that conversion of Th17 cells to Th1/Th17 and/or Th1 cells under certain stimulatory conditions *in vivo* is sufficient to drive inflammatory disease (Hirota et al., 2011; Lee, Turner, et al., 2009; Martin-Orozco, Chung, Chang, Wang, & Dong, 2009).

The roles of several histone-modifying proteins in the transcriptional regulation of the *IL17A/IL17F* locus have also been analyzed. At the murine locus, p300 associates with CNS-2 (~2 kb upstream of *Il17a*), the *Il17a* promoter, and the *Il17f* promoter in Th17 cells but not in Th1 cells; in reporter assays CNS-2 functions as a Th17-restricted enhancer not only for the *Il17a* and *Il17f* promoters, but also for the *Ifng* and *Il4* promoters (Wang et al., 2012). The H3K27me3-specific histone demethylase JMJD3 is also recruited to CNS-2 in Th17 cells, consistent with earlier observations that this repressive histone mark is removed at the *Il17a/Il17f* locus during Th17 lineage commitment (Thomas et al., 2012). Deletion of CNS-2 in mice results in dramatically reduced secretion of IL-17A and IL-17F by *in vitro*-polarized Th17 cells, diminished recruitment of RNA Pol II and p300 to the *Il17a* and *Il17f* promoters, and increased H3K27 trimethylation at the two promoters; in all cases the observed effects were more modest at the *Il17f* promoter relative to the *Il17a* promoter (Wang et al., 2012). The CNS-2-deficient mice are also resistant to experimental autoimmune encephalomyelitis, a Th17 cell-dependent autoimmune disease model resembling human multiple sclerosis (Wang et al., 2012). The histone methyltransferase G9a

has also been linked to regulation of the *Il17a/Il17f* locus, as CD4⁺ T cells isolated from mice deficient in T cell-specific expression of G9a produce elevated levels of IL-17A under neutral or Th2-polarizing conditions (Lehnertz et al., 2010). Increased IL-17A mRNA and/or protein expression in the mesenteric lymph nodes and intestines of these mice is found in response to infection with the helminth *Trichuris muris*, which primarily drives a Th2 response in wild-type animals (Lehnertz et al., 2010). Stimulation of CD4⁺ T cells under neutral and Th2-polarizing conditions in the presence of BIX-01294, a specific inhibitor of G9a methyl-transferase, also results in increased expression of IL-17A (Lehnertz et al., 2010). Consistent with this, when naïve CD4⁺ T cells from mice lacking hematopoietic-specific G9a expression are stimulated under neutral, Th1, and Th2 conditions, a strong reduction in H3K9me2 is found at several sites in the *Il17a/Il17f* locus, including the *Il17a* promoter, *Il17a* CNS-2, and *Il17a* CNS-3 (Lehnertz et al., 2010). Lysine-specific demethylase 1 (LSD1), which targets H3K4me1 and H3K4me2, has also been implicated in modulation of the *Il17a/Il17f* locus. In CD4⁺ T cells deficient in transcriptional repressor growth factor independent 1 (Gfi-1), which associates with LSD1 (Saleque, Kim, Rooke, & Orkin, 2007), Th2 polarization results in enrichment of H3K4me3 at the 5' end of *Rorc*, while in wild-type Th2 cells this mark is almost completely absent at the *Rorc* locus. *Il17a* and *Il17f* gene expression can be inhibited by ectopic expression Gfi-1 in murine Th17 cells, while these cytokines are expressed by murine Th2 cells lacking Gfi-1 upon a shift to Th17-polarizing conditions, unlike wild-type Th2 cells (Zhu et al., 2009). Gfi-1 and LSD1 are also recruited to the *Il17a/Il17f* intergenic region, and association of LSD1 with the locus is erased in the absence of Gfi-1. As TGF- β treatment directly inhibits Gfi-1 synthesis, a mechanistic link between Th17 polarizing conditions and derepression of the *Il17a/Il17f* locus can be made (Zhu et al., 2009).

The relationship between gene transcription and DNA methylation at the CNSs of the murine *Il17a/Il17f* and human *IL17A/IL17F* loci has also been investigated. In the human locus, CNS1, CNS2 (proximal *IL17A* promoter), CNS3, CNS4, CNS5 (proximal *IL17F* promoter) and CNS6 display increased CpG methylation in non-IL-17A-secreting CD4⁺ T cells, and decreased CpG methylation in T cells from SLE patients (Hedrich et al., 2012; Rauen et al., 2011). Undifferentiated human naïve CD4⁺ cells exhibit high levels of CpG methylation in the *IL17A* proximal promoter, and culture of human CD4⁺ T cells in the presence of 5-aza-2'-deoxycytidine promotes *IL17A* expression (Janson et al., 2011). Furthermore, T cells from SLE patients show decreased recruitment of DNMT3A to a CRE site in the *IL17A* proximal promoter that is positioned at nucleotides -111 to -104 relative to the TSS and is recognized by cAMP-response element modulator α (CREM α), which interacts with DNMT3A (Rauen et al., 2011). Ectopic overexpression of DNMT3A in activated Jurkat T cells inhibits *IL17A* mRNA expression, and in these cells gene reporters driven by 195 bp of the *IL17A* proximal promoter are inhibited by increased methylation of the reporter plasmid (Rauen et al., 2011). CpG dinucleotides in the vicinity of the human *IL17A* TSS are strongly methylated in Th0 and Th1 cells, hypomethylated in Th17 cells, and partially demethylated (at sites downstream of the TSS) in Th1/Th17 cells; notably, culture of Th17 cells in Th1-polarizing conditions, or Th1 cells in Th17-polarizing conditions, has little effect on the methylation status of the TSS region (Cohen et al., 2011). In the case of the murine locus, the *Il17a* and *Il17f* promoters, as well as an enhancer 28 kb downstream of

the *Il17a* TSS that promotes transcription of both genes, undergo Th17 lineage-specific DNA demethylation, which correlates with demethylation of H3K27 and increased H3K4 methylation in these regions. This CpG demethylation tends to coincide with STAT3 binding sites, and hypermethylation at one site in the *Il17a* proximal promoter blocks STAT3 binding and full promoter activity (Thomas et al., 2012). Furthermore, in Th17 cells cultured in the presence of IL-23, the *Il17f* promoter becomes preferentially demethylated, consistent with the aforementioned finding that exposure of murine Th17 cells to IL-23 shifts their cytokine secretion profile to one dominated by IL-17F instead of IL-17A (Thomas et al., 2012). Thus, as is found at Th1 and Th2 loci, CpG methylation at promoter and enhancer regions of *IL17A/IL17F* inversely correlates with activation of the locus.

Recently, 3C assays in murine Th17 and Th1 cells have provided a first glimpse into the higher-order conformation of the *Il17a/Il17f* locus (Wang et al., 2012). The CNS-2 enhancer, which interacts with p300 and JMJD3, makes Th17-specific intrachromosomal interactions with the *Il17a* and *Il17f* promoters (Wang et al., 2012; Fig. 2.6). As additional Th17-specific HS sites are present throughout the *IL17a/IL17f* locus, additional long-range chromosomal interactions may contribute to the epigenetic regulation of Th17-specific differentiation and cytokine synthesis.

2.3. Other loci

Examination of the *TNF/LT*, *IFNG*, Th2, and *IL17A/IL17F* loci has provided a wealth of data on the epigenetic regulation of critically important factors for both innate and adaptive immune responses. In addition, these loci can serve as models for epigenetic modulation of other genes that are expressed in a cell-type and/or stimulus-specific manner in the immune system, as well as other tissues. For example, another key locus involved in CD4⁺ T helper cell differentiation is the locus that encodes the forkhead box p3 (Foxp3) master regulator, which is critical for regulatory CD4⁺ T cell (Treg) differentiation (Fontenot, Gavin, & Rudensky, 2003; Hori, Nomura, & Sakaguchi, 2003; Khattri, Cox, Yasayko, & Ramsdell, 2003). Several CNS elements in the locus, including the *FOXP3* promoter, a TGF- β -sensitive element, and the Treg cell-specific demethylated region (TDSR), are regulated through histone modifications and changes in CpG methylation (Cavassani et al., 2010; Floess et al., 2007; Janson, Winerdal, et al., 2008; Kim & Leonard, 2007; Liu, Tahk, Yee, Fan, & Shuai, 2010; Mantel et al., 2006; Polansky et al., 2008).

Expression of the antiinflammatory cytokine IL-10 is also regulated epigenetically during CD4⁺ T helper cell differentiation. Unlike IFN- γ and IL-4, whose expression is tightly restricted to the Th1 and Th2 lineages, respectively, IL-10 is expressed by both subsets, albeit at a much higher level in Th2 cells than in Th1 cells (Fiorentino, Bond, & Mosmann, 1989; Jankovic et al., 2007). Relative to what is found in Th1 cells, high levels of histone H3 and H4 acetylation are observed at the *Il10* locus in macrophages, which also produce high levels of IL-10 in response to activation, in naïve CD4⁺ T cells, and in Th2 cells (Chang, Helbig, et al., 2007; Lee, Sahoo, et al., 2009; Motomura et al., 2011; Saraiva et al., 2005; Shoemaker, Saraiva, & O'Garra, 2006; Villagra et al., 2009). H3K4 methylation is also higher at the *Il10* locus in murine Th2 cells as compared to Th1 cells (Chang, Helbig, et al., 2007; Motomura et al., 2011), and phosphorylation at H3S10 is observed at the locus in

murine macrophages stimulated by LPS treatment or FcγR ligation (Lucas, Zhang, Prasanna, & Mosser, 2005; Villagra et al., 2009; Zhang, Edwards, & Mosser, 2006). The SWI/SNF chromatin remodeling complex components Brg1 and Brm also associate with multiple HSs in the *IL10* locus in murine Th1, Th2, and Th17 cells; furthermore, CBP and acetylated histone H3 are enriched at the locus in Th2 cells lacking the repressive SWI/SNF component BAF180 as compared to wild-type Th2 cells, and this correlates with an increase in *IL10* gene expression (Wurster et al., 2012). Conversely, HDAC11 interacts with the distal region of the *IL10* promoter, induces deacetylation of histones H3 and H4, and represses *IL10* gene expression in human and murine APCs (Villagra et al., 2009). This was the first physiological role discovered for HDAC11, and revealed a mechanism that may be important for the establishment of immune tolerance. Repression of *IL10* expression in Th1 cells has also been linked to Ets-1-dependent recruitment of HDAC1 (Lee et al., 2012). Finally, CpG dinucleotides in the human *IL10* promoter are hypomethylated in PBMC, where the gene is active, but highly methylated in primary keratinocytes and HeLa cells, where the gene is silent (Szalmás et al., 2008). While HSs and histone modifications have been characterized in the *IL10* locus, the epigenetic mechanisms that regulate *IL10* gene expression are still being elucidated (Lee, Sahoo, et al., 2009; Saraiva & O'Garra, 2010). In an intriguing recent study, histone marks associated with transcriptional competence, including H3K27ac, H3K4me3, and H3K4me1, were found to be enriched at the *IL10* locus in human monocytes and mouse neutrophils, which both express IL-10, but not in human neutrophils, which are unable to express IL-10 even after mitogenic stimulation (Tamassia et al., 2013). This provides the first evidence of a species-specific difference in epigenetic regulation of the *IL10* gene in a shared cell type. It is anticipated that characterization of intrachromosomal interactions at the *IL10* locus will shed further light on the epigenetic regulation of this cytokine's expression in an array of immune cells.

Macrophage differentiation is another process that is closely linked to differential cytokine expression, and for which there is strong evidence of epigenetic regulation. Macrophages can be polarized towards two major subtypes: M1 and M2. M1 macrophages develop in response to bacterial and viral infection and express high levels of TNF and other proinflammatory cytokines. M1 macrophages can be induced *in vitro* by treatment of primary monocytes with a combination of IFN-γ and TLR ligands or with granulocyte macrophage-colony stimulation factor (GM-CSF). By contrast, M2 macrophages are involved in the response to parasitic infection and other "alternative" activation signals. M2 macrophages can be induced *in vitro* by treatment of primary monocytes with macrophage-colony stimulation factor (M-CSF) and by IL-4 or IL-13 (Fleetwood, Lawrence, Hamilton, & Cook, 2007; Martinez, Gordon, Locati, & Mantovani, 2006; Verreck et al., 2004). The H3K27 demethylase JMJD3 is a critical player in M2 macrophage polarization, as JMJD3 is induced in a STAT6-dependent manner after IL-4 treatment of unpolarized murine macrophages, is recruited to the promoters of several M2 marker genes, and acts to demethylate H3K27 at these genes (Ishii et al., 2009). JMJD3 is also recruited at higher levels to M2 macrophage marker genes in peritoneal macrophages isolated from mice challenged with *Schistosoma mansoni* as compared to unchallenged mice (Ishii et al., 2009). A second study found that JMJD3 is also required for M2 macrophage polarization in mice in response to infection by the helminth *Nippostrongylus brasiliensis* or chitin

administration, as JMJD3-deficient mice exhibit significantly reduced M2 macrophage activity when subjected to these conditions in comparison to wild-type mice (Sato et al., 2010). In addition, JMJD3-deficient BMDMs demonstrate impaired M2 development in response to M-CSF, but not to IL-4. The expression of IRF-4, a key transcription factor in M2 macrophage polarization, is inhibited in macrophages lacking JMJD3, most likely due to loss of JMJD3-dependent H3K27 demethylation at the *Irf4* promoter, which correlates with *Irf4* transcriptional induction (Sato et al., 2010).

3. PERSPECTIVES AND FUTURE DIRECTIONS

Here we have described the roles of a range of post-translational histone modifications, DNA methylation states, and higher-order chromatin interactions that control regulation of cytokine gene transcription. Epigenetic regulation strongly correlates with evolutionarily conserved regions of the genome where DNA is accessible to regulatory factors at DNase hypersensitive sites, often occurring in noncoding sequences separated by kilobases from the gene they regulate. These sites, in turn, associate with histones bearing specific, reversible covalent modifications and, in some cases, regions of hypo- or hypermethylation of cytosine at CpG dinucleotide motifs in DNA.

Histone modifications can promote gene transcription not only by weakening the nucleosome-DNA interaction and making DNA motifs more accessible to their cognate transcription factors and the general transcription machinery, but also by serving as docking sites for chromatin-modifying factors, which can exert permissive or repressive effects upon gene transcription. Furthermore, regulatory factors that interact with HMs can drive the formation of long-range intra- and interchromosomal interactions, which in turn place gene loci into regions of active transcription or into regions of transcriptional repression. In turn, these epigenetic regulatory mechanisms are profoundly influenced by cell type and stimulus, as well as developmental stage, which stimulate the epigenetic changes that control the transcriptional program.

The components of epigenetic cytokine gene regulation thus present potential targets for the manipulation of cytokine transcription in disease states that arise from, or are strongly influenced by, the dysregulation of cytokine gene expression occurring in specific cell or tissue types or stimulated by specific signaling pathways. Indeed, specific histone and DNA modifications have been associated with disease states, and in some cases compounds that reverse these epigenetic changes have been shown to be clinically effective. In particular, some HDAC inhibitors have been approved for use in cancer therapy. As more information becomes available about cell type- and stimulus-specific epigenetic modifications at key gene loci, it can be imagined that therapies can be designed to manipulate an individual gene's expression in its native chromatin context and in tissues uniquely affected by its inappropriate expression by targeting these epigenetic control processes.

DNA methylation has been implicated in a number of autoimmune disease states, various cancers, chronic obstructive pulmonary disease, neurodegenerative diseases, and neurological disorders driven by chronic inflammation (Shanmugam & Sethi, 2012; Strickland & Richardson, 2008; Villagra et al., 2010). This is well illustrated in the case of

SLE, in which strong phenotypic and functional similarities were observed between CD4⁺ cells isolated from patients with active SLE and experimentally manipulated mature human CD4⁺ T cells treated with agents that induce hypomethylation. Treatment of CD4⁺ T cells with 5-azacytidine results in an increase in transcription of *ITGAL* (integrin, alpha L), which codes for a subunit of the adhesion molecule lymphocyte function-associated antigen 1 (LFA-1), in expression of LFA-1, and in demethylation of *alu* elements 5' of the *ITGAL* promoter. This treatment also results in autoreactive T cells, which respond to APCs without added antigen in a class II MHC-specific fashion (Lu et al., 2002; Richardson, 1986; Richardson et al., 1992). Coculture of 5-azacytidine-treated hypomethylated T cells with autologous B cells results in hypersecretion of IgG, partially mediated by IFN- γ , IL-4, and IL-6 (Quddus et al., 1993; Richardson, Liebling, & Hudson, 1990). Consistent with this observation, transcription of *IL6*, like that of *IFNG* and *IL4*, is repressed by DNA methylation (Reiner, 2005). Another intriguing example is provided by the lupus-like disease state that can result from drug therapy with the DNA methyltransferase inhibitors procainamide and hydralazine, an antiarrhythmic and anti-hypertensive respectively (Deng et al., 2003; Lee, Yegnasubramanian, Lin, & Nelson, 2005; Mazari, Ouarzane, & Zouali, 2007; Yung & Richardson, 1994). This is of particular interest since T cells from patients with active SLE have decreased m⁵C content and *DNMT1* mRNA relative to patients with inactive SLE and healthy controls (Richardson, Scheinbart, et al., 1990). Furthermore, T cells from SLE patients exhibit a range of similarities to experimentally demethylated T cells: for example, they induce hypersecretion of IgG in autologous B cells, and a subset of SLE T cells overexpress LFA-1, similar to 5-azacytidine-treated T cells (Oelke et al., 2004; Richardson et al., 1992). Moreover, demethylation of the *alu* elements upstream of the *ITGAL* promoter correlates with the activity of lupus disease (Lu et al., 2002). DNA hypomethylation has also been implicated in gene regulation underlying other autoimmune disease states. For example, studies with PBMCs from patients with rheumatoid arthritis revealed that hypomethylation at a single CpG site in the *IL6* promoter correlates with both increased IL-6 expression and sustained inflammation (Nile, Read, Akil, Duff, & Wilson, 2008).

By contrast, a hallmark of cancer is selective hypermethylation and, as a result, persistent repression of the promoter regions of a wide range of genes, especially genes that encode for tumor suppressor proteins and proteins involved in DNA repair and the cell cycle (Heyn & Esteller, 2012; Shanmugam & Sethi, 2012). Although compounds that broadly inhibit DNA methylation can induce autoimmune disease-like states, they have proven to be clinically effective for patients in certain clinical entities: for example, 5-azacytidine (Vidaza) and 5-aza-2'-deoxycytidine (Dacogen) have been used as relatively low-toxicity therapies for myelodysplastic syndrome (MDS) and secondary acute myeloid leukemia (AML; Griffiths & Gore, 2013). Although our understanding of the role of DNA demethylation in these diseases is still emerging, it is anticipated that novel small molecules able to specifically modulate DNA methylation or demethylation at particular genomic regions may be of great benefit in treatment strategies for certain autoimmune and neoplastic disorders.

Considerable progress has been made in the design and the clinical application of compounds that interact specifically with histone modifying enzymes. As of the writing of

this review, three second-generation HDAC inhibitors are in Phase III clinical trials or used in treatment (Arrowsmith et al., 2012). Panobinostat (LBH589), which targets HDAC1, HDAC2, HDAC3, and HDAC6, is in Phase III clinical trials for treatment of Hodgkin's lymphoma and multiple myeloma (Arrowsmith et al., 2012; Zhou, Atadja, & Davidson, 2007). Suberoylanilide hydroxamic acid (SAHA), also called vorinostat (Zolinza), which targets HDAC1, HDAC2, HDAC3, and HDAC6, and romidepsin (Istodax), which targets HDAC1, HDAC2, HDAC3, and HDAC8 were approved for the treatment of cutaneous T-cell lymphoma (CTCL) in 2006 and 2009, respectively (Arrowsmith et al., 2012; Bertino & Otterson, 2011; Marks & Breslow, 2007; Prince, Bishton, & Harrison, 2009).

Notably, a novel indication for HDAC inhibition is treatment for HIV infection. A major obstacle to cure of HIV infection is the residual, latent viral reservoir that persists even after sustained and highly effective antiretroviral therapy (ART). Approaches have been attempted using HDACs to reactivate the viral reservoir, which then allows the combination of ART and the reconstituting T cell compartment to eliminate residual virus as it exits from its sequestered state. A number of HDAC inhibitors are capable of reactivating latent HIV that is integrated into the host genome (Hakre, Chavez, Shirakawa, & Verdin, 2011), and several have been tested in humans. For example, the HDAC1 inhibitor valproic acid (VPA) caused a decline in HIV-1 infection of resting CD4⁺ T cells *in vivo* in four patients when it was added to an intensified ART regimen (Lehrman et al., 2005). VPA is a weak HDAC inhibitor, however, and later clinical studies found no significant benefit for VPA in HIV treatment (Archin et al., 2008). A recent report found panobinostat to be superior to several other HDAC inhibitors, including VPA and SAHA, at inducing viral reactivation in both cell line and primary CD4⁺ T cell latency models (Rasmussen et al., 2013).

Other than HDAC inhibitors, no small-molecule inhibitors of other histone modifying enzymes are currently in clinical use; however, a number of the factors described in this review that are important in epigenetic cytokine gene regulation present attractive therapeutic targets. For example, aberrantly expressed mutant forms of the histone lysine methyltransferase EZH2 have been found in various types of leukemia and solid tumors (Simon & Lange, 2008), and certain cancers are marked by fusions of the bromodomain proteins BRD3 and BRD4 (Filippakopoulos et al., 2010) and the histone lysine methyltransferase MLL (Daigle et al., 2011; Okada et al., 2005). In some cases, *in vitro* studies have shown that the function of these histone modifying enzymes can be selectively inhibited by small-molecule drugs, including compounds with low nanomolar affinity for the bromodomains of members of the BET protein family (BRD2, BRD3, BRD4, and BRDT) (Chung et al., 2011; Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010; Nicodeme et al., 2010). For example, the compound (+)-JQ1, which selectively interacts with BRD3 and BRD4, has been shown to promote terminal differentiation and inhibit proliferation of squamous carcinoma cells expressing the BRD4-NUT (nuclear protein in testis) fusion protein, displacing BRD4-NUT from acetylated chromatin through competitive binding (Filippakopoulos et al., 2010). Notably, BRD4 is a regulatory cofactor of the oncoprotein Myc, which has proven to be refractory to direct inhibition by therapeutic compounds (Arrowsmith et al., 2012). Inhibition of BRD4 activity, which has been shown to have an anti-proliferative effect in several models of AML, multiple myeloma, and mixed

lineage leukemia, may thus allow for indirect inhibition of Myc (Dawson et al., 2011; Delmore et al., 2011; Zuber et al., 2011).

In addition to histone deacetylase “erasers” and bromodomain “readers,” histone acetyltransferase and histone methyltransferase “writers” and histone demethylase “erasers” present potential therapeutic targets. For example, specific histone acetyltransferases, deacetylases, and methyltransferases have all been linked to neuropsychiatric disorders: haploinsufficiency of CBP and HDAC4 lead to Rubinstein-Taybi syndrome and brachydactyly mental retardation syndrome, respectively (Petrij et al., 1995; Williams et al., 2010), and mutations in the histone lysine methyltransferase GLP1 result in a complex intellectual disability syndrome (Kleefstra et al., 2009; Kramer & van Bokhoven, 2009; Schaefer et al., 2009). Although appropriately selective HAT inhibitors have proven elusive, inhibitors have been developed for the histone lysine methyltransferases G9a and GLP-1. These include BIX-01294, which, as noted in the previous section, can increase *Il17a* transcription in cell-based assays (Kubicek et al., 2007; Lehnertz et al., 2010), and the more potent and selective second-generation inhibitor UNC638 (Vedadi et al., 2011). Moreover, the compound EPZ004777, a specific inhibitor of the histone methyltransferase DOT1-like (DOT1L), selectively induces apoptosis in cells containing MLL fusion proteins that directly or indirectly interact with DOT1L (Daigle et al., 2011). A selective inhibitor has also been successfully designed to block activity of the H3K27me3-specific demethylases JMJD3 and UTX; this compound, GSK-J4, was shown to inhibit LPS-induced expression of proinflammatory cytokines, including TNF, in human primary macrophages *in vitro* (Kruidenier et al., 2012).

Thus, new treatments for cancer, autoimmune diseases, neurodegenerative diseases, and chronic infections such as HIV will benefit from an improved understanding of the role of histone modifications, DNA methylation, and protein-DNA interactions involved in establishing functional intra- and interchromosomal interactions in gene expression. Moreover, such studies of epigenetic regulation will greatly enhance our general knowledge of how eukaryotic genes are regulated in a cell type- and inducer-specific manner.

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Figure 2.1.

The structure of the nucleosome. The histone octamer viewed down the superhelical axis of the DNA, illustrating the position of N-terminal histone tails that are targets of posttranslational modifications. Histones H3, H4, H2A, and H2B are shown in blue, green, gold, and red, respectively. Diagram of 2.8 Å resolution structure (Luger, Mader, Richmond, Sargent, & Richmond, 1997) (Protein Data Bank code 1AOI) kindly provided by Karolin Luger.

Figure 2.2.

The *TNF/LT* locus. A. Positions of the *LTB*, *TNF*, and *LTA* genes (numbered exons in dark gray; transcriptional orientation indicated by white arrows) in the murine (top) and human (bottom) *TNF/LT* loci. Murine HS sites are labeled as in Tsytsykova, Rajsbaum, et al. (2007) and Biglione, Tsytsykova, and Goldfeld (2011) and human HS sites are labeled as in Taylor, Wicks, Vandiedonck, and Knight (2008). The *TNF* promoter is indicated in yellow, the murine HSS-9/human DHS44500 enhancers in green, the murine enhancer HSS +3 in magenta, and the murine monocyte-specific MAR HSS-7 in cyan. Red bars indicate the position of permissive histone modifications: H3 and H4 histone acetylation, mono-, di-, or trimethylation (1, 2, or 3) at H3K4, and phosphorylation at H3S10, in T cells or monocytes

as indicated. Green bars indicate the position of repressive histone modifications: di- or trimethylation (2 or 3) H3K9. Blue bars indicate positions where DNA methylation inversely correlates with *TNF* gene expression. Arrows between sites in the locus indicate intrachromosomal interactions in the murine (top) and human (bottom) locus (Tsytsykova, Rajsbaum, et al., 2007; Watanabe et al., 2012; Wicks & Knight, 2011). B. Diagram of the higher-order structure of the murine *Tnf/Lt* locus following T cell activation, adapted from Tsytsykova, Rajsbaum, et al. (2007). Simultaneous interactions between the *Tnf* promoter and HSS+3, between the *Tnf* promoter and HSS-9, and between HSS +3 and HSS-9, are depicted, illustrating the facilitation of *Tnf* transcription by juxtaposition of NFAT-containing nucleoprotein complexes and circularization of the gene to promote reinitiation of transcription.

Figure 2.3. CD4⁺ T helper cell differentiation. Cytokines that polarize a naïve CD4⁺ T cell to the Th1, Th2, or Th17 lineage; transcription factors that serve as master regulators for the differentiation of each T helper cell lineage; and the effector cytokines expressed by each T helper cell lineage are shown.

Figure 2.4.

The *IFNG* locus. Comparative histone posttranslational modifications, DNA methylation status, and intra- and interchromosomal interactions are shown for the murine *Ifng* locus in naïve CD4⁺ T, Th1, Th2, and Th17 cells. Position and transcriptional orientation of the *Ifng* gene (gray box, arrow) and regulatory elements (black boxes) in the murine *Ifng* locus are shown, with names of the murine sequences indicated in black at the bottom, and of corresponding elements in the human *IFNG* locus in gray. Red bars indicate the position of permissive histone modifications: H3 and H4 histone acetylation, and di- or trimethylation (2 or 3) at H3K4. Green bars indicate regions associated with the repressive histone mark H3K27me3. Regions of CpG hypomethylation and methylation are indicated by open and filled blue boxes, respectively. At the bottom, arrows denote intrachromosomal interactions that form within the murine *Ifng* locus between the *Ifng* gene and distal CNSs in a Th1-specific fashion or that are present in naïve, Th1, and Th2 cells (Sekimata et al., 2009; Spilianakis, Lalioti, Town, Lee, & Flavell, 2005) as well as intrachromosomal interactions detected among fragments centered at EcoRI sites at positions (relative to the *Ifng* TSS)

–5048, –1248, +229; +6,891, +10,264 and between MARs at positions –7000 and –10,500 in naïve, neutral (Th0), Th1, and Th2 cells (Eivazova & Aune, 2004; Eivazova, Vassetzky, & Aune, 2007; Eivazova et al., 2009). Interchromosomal interactions present in naïve T cells between the *Ifng* gene and the indicated sites in the Th2 cytokine locus (Spilianakis et al., 2005) are shown by arrows at the top.

Figure 2.5.

The Th2 cytokine locus. Comparative histone posttranslational modifications, DNA methylation status, and intra- and interchromosomal interactions are shown for the murine Th2 cytokine locus in naïve CD4⁺ T, Th1, and Th2 cells. Position and transcriptional orientation of the *Il4*, *Il5*, *Il13*, *Rad50*, *Kif3a*, and *Sept8* genes (gray boxes, arrows) and regulatory elements (black boxes) are shown; the length of the region containing *Kif3a* and *Sept8* (to the right of the vertical double line) is compressed approximately twofold relative to the rest of the locus. Regulatory sequences and binding sites of the Th2-specific architectural factor SATB1 are labeled with thick and thin vertical arrows, respectively. Red bars indicate the position of permissive histone modifications: H3 and H4 histone acetylation, di- or trimethylation (2 or 3) at H3K4, and phosphorylation at H3S10. Green bars indicate the position of repressive histone modifications: di- or trimethylation (2 or 3) at H3K27 or dimethylation (2) at H3K9. Regions of CpG hypomethylation and methylation are indicated by open and filled blue boxes, respectively. Interchromosomal interactions that form in naïve T cells between sites in the Th2 locus and the *Ifng* gene are shown at the top. Intrachromosomal interactions within the locus involving the *Il5* promoter, the Th2 LCR,

the *Il13* promoter, and the *Il4* promoter, that are present in T cells, B cells, NK cells, and fibroblasts, or only in T cells and NK cells, are shown by thick arrows at the bottom, along with intrachromosomal interactions among SATB1 binding sites in unstimulated Th2 cells, which are shown by thin arrows. Additional SATB1-mediated intrachromosomal interactions that form in activated Th2 cells are described in Cai, Lee, and Kohwi-Shigematsu (2006). ND, not determined in a given cell type; question mark indicates conflicting results in separate studies.

Figure 2.6.

The *IL17A/IL17F* locus. Comparative histone posttranslational modifications, DNA methylation status, and intrachromosomal interactions are shown for the murine *Il17a/Il17f* locus in naïve CD4⁺ T, Th1, Th2, and Th17 cells. Position and transcriptional orientation of the *Il17a*, *IL17f*, *Mcm3*, and *Phkd1* genes (gray boxes, arrows) and regulatory elements (black boxes) are shown, with sequence names indicated at the bottom. Red bars indicate the position of permissive histone modifications: H3 and H4 histone acetylation and trimethylation (3) at H3K4. Green bars indicate regions associated with the repressive histone mark H3K27me3. Regions of CpG hypomethylation and methylation are indicated by open and filled blue boxes, respectively. Intrachromosomal interactions that form in a Th17-specific fashion are shown by arrows at the bottom. ND, not determined in a given cell type.

Histone acetylation, methylation, and phosphorylation marks that are of particular importance in cytokine gene regulation, with an overview of their effects on transcriptional activation/repression. Principal associated gene positions are indicated in bold type.

Table 2.1

Histone net charge affected?	Histone mark abbreviation	Representative targeted residues	Associated gene activity	Associated gene position	Protein interacting domain
Acetylation	Yes	Histone H3: lysines 9, 14, 18, 27	Active	Enhancer, promoter, coding region	Bromodomain, PHD domain
		Histone H4: lysines 5, 8, 12, 16	Active	Enhancer, promoter, coding region	
Methylation	No	Histone H3: lysine 4 (monomethylated)	Active, poised	Enhancer , coding region, boundary element	Chromodomain, PHD domain, Tudor domain, MBT domain
		Histone H3: lysine 4 (dimethylated)	Active	Downstream of promoter , enhancer, promoter, coding region, boundary element	
		Histone H3: lysine 4 (trimethylated)	Active, poised	TSS , enhancer, promoter	
		Histone H3: lysine 27 (trimethylated)	Inactive/ repressed	Enhancer, promoter, coding region, adjacent region	
		Histone H3: lysine 27 (dimethylated)	Inactive/ repressed	Enhancer, promoter, coding region, adjacent region	
		Histone H3: lysine 9 (trimethylated)	Inactive/ repressed	Enhancer, promoter, coding region, adjacent region	
Phosphorylation	Yes	Histone H3: lysine 10	Active, poised	Enhancer, promoter, coding region	14-3-3 domain

Table 2.2

Histone marks and the modifying enzymes that act to “write” or “erase” these marks

Histone modification	Histone mark(s)	“Writer”	“Eraser”
Acetylation (H3, H4)	H3K9ac, H3K14ac, H3K18ac	Gen5, PCAF	HDAC1, HDAC2
	H3K14ac, H3K18ac, H4K5ac, H4K8ac	CBP, p300	HDAC1, HDAC2
Methylation	H3K4me1	SET7	LSD1, JARID1B
	H3K4me2, H3K4me3	MLL	LSD1, JARID1A-D
	H3K27me2, H3K27me3	EZH2	JMJD3, UTX
	H3K9me2, H3K9me3	G9a, SUV39H	LSD1, JMJD2A-D
Phosphorylation	H3S10p	MSK1, MSK2?	unknown