Distinct Epigenetic Signatures Delineate Transcriptional Programs during Virus-Specific CD8+ T Cell Differentiation

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SUMMARY

The molecular mechanisms that regulate the rapid transcriptional changes that occur during cytotoxic T lymphocyte (CTL) proliferation and differentiation in response to infection are poorly understood. We have utilized ChIP-seq to assess histone H3 methylation dynamics within naive, effector, and memory virus-specific T cells isolated directly ex vivo after influenza A virus infection. Our results show that within naive T cells, codeposition of the permissive H3K4me3 and repressive H3K27me3 modifications is a signature of gene loci associated with gene transcription, replication, and cellular differentiation. Upon differentiation into effector and/or memory CTLs, the majority of these gene loci lose repressive H3K27me3 while retaining the permissive H3K4me3 modification. In contrast, immune-related effector gene promoters within naive T cells lacked the permissive H3K4me3 modification, with acquisition of this modification occurring upon differentiation into effector/memory CTLs. Thus, coordinate transcriptional regulation of CTL genes with related functions is achieved via distinct epigenetic mechanisms.

INTRODUCTION

Antigen-dependent activation of naive cytotoxic T lymphocytes (CTLs) initiates a program of differentiation that has been shown to be largely autonomous (van Stipdonk et al., 2003) and results in the acquisition of effector mechanisms including the production of proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) (La Gruta et al., 2004) and the expression of cytolytic effector molecules including perforin (PFP) (Kägi et al., 1994) and the granule enzymes granzymes (GZM) A, B, and K (Jenkins et al., 2007; Peixoto et al., 2007).

Once an infection is cleared, the expanded effector CTL population contracts, leaving a pool of long-lived, pathogen-specific memory T cells. In contrast to naive CD8+ T cells, virus-specific memory CTLs are able to respond more readily and rapidly to subsequent infections, and without the need for further differentiation (Kaech et al., 2002; La Gruta et al., 2004; Veiga-Fernandes et al., 2000). Although naive, effector, and memory CD8+ T cells have distinct molecular, phenotypic, and functional characteristics, the molecular mechanisms that underpin the initiation and maintenance of CD8+ T cell differentiation in response to infection are not well understood.

Posttranslational modification (PTM) of histones is an important mechanism for regulation of gene transcriptional where both the combination and extent of histone PTMs at specific genomic locations can be an indicator of gene transcriptional activity (Kouzarides, 2007). For example, trimethylation of histone 3 at lysine 4 (H3K4me3) is typically enriched within gene promoters and correlates with active transcription (Santos-Rosa et al., 2002). In contrast, deposition of trimethylated H3K27 (H3K27me3) within gene loci typically correlates with transcriptional repression (Wang et al., 2008). Further, studies in embryonic stem cells (ESCs) have identified a subset of gene loci that exhibit coenrichment of both active (H3K4me3) and repressive (H3K27me3) modifications (termed bivalent loci) (Bernstein et al., 2006; Hawkins et al., 2010). This particular epigenetic signature marks developmentally important gene loci that lose either the active or repressive PTM contributing upon differentiation. Hence, within ESCs, bivalency is an epigenetic state from which a gene can be rapidly activated or repressed depending on the differentiation pathway initiated and ensures appropriate cell lineage gene transcription. However, it remains to be determined whether these findings extend beyond embryonic development and represent a general mechanism for regulating cell lineage commitment.

It is now apparent that CD4+ T cell lineage commitment correlates with dynamic changes in histone PTM deposition at gene loci associated with directing subset-specific CD4+ T cell effector function (Allan et al., 2012; Ansel et al., 2003; Wei et al., 2009). Whether changes in histone PTMs also play a role
in directing naive CD8+ T cell fate is less clear. Analysis of histone PTMs at single effector CTL loci has demonstrated that changes in H3K4me3 and H3K27me3 deposition correlate with dynamic changes in effector function during naive to effector and memory differentiation (Denton et al., 2011; Zedkias et al., 2011). Consistent with these studies, genome-wide analysis of H3K4me3 and H3K27me3 deposition within human polyclonal, naive, and memory CTLs showed that specific methylation patterns correlated with subset-specific gene expression (Araki et al., 2009). However, it is difficult to link changes in the global epigenetic patterns within polyclonal CTL populations to those induced specifically by infection.

Here we report genome-wide mapping of histone H3 methylation patterns within antigen-specific naive, effector, and memory CTLs elicited by an acute viral infection. This, combined with global transcriptional analyses of resting and stimulated CTLs, demonstrated that establishment of permissive chromatin domains at CTL-lineage-defining genes correlates with observed phenotypic and functional differences between virus-specific CTLs at each phase of the immune response. Surprisingly, rather than a broad increase in the activating H3K4me3 histone PTM, effector and memory CTLs could be defined on the basis of a large-scale but focused reduction in the repressive H3K27me3 PTM, suggesting that naive CTLs are maintained in a state of restraint until activation. Strikingly, the particular dynamics of PTM loss and gain during differentiation identified functionally distinct classes of genes, providing a mechanistic basis for the coordinate regulation of each group. These data suggest that upon naïve CTL activation, distinct epigenetic histone methylation patterns are established to tightly coregulate transcription of gene modules that in turn ensure programmed CTL differentiation and establishment of effective cellular immunity.

RESULTS

Epigenetic PTM Patterns Associate with Distinct Cellular Functions during CTL Differentiation

To investigate the dynamics of H3K4me3 and H3K27me3 histone modifications during CTL differentiation, we utilized an infection model where naive (CD44hiCD62Lhi) OT-I TCR transgenic CD8+ T cells, specific for the ovalbumin peptide (OVA257-264), were adoptively transferred into congenic C57BL/6J (B6) hosts, followed by intranasal (i.n.) infection with the A/HKx31-OVA virus (Jenkins et al., 2006). We then carried out ChiP-seq analysis on nuclei isolated from infected OT-I CTLs after immunoprecipitation by using antibodies specific for either H3K4me3 or H3K27me3 modifications. We were able to map a total of 28.9 × 10^6, 44.5 × 10^6, and 48.5 × 10^6 H3K4me3-associated sequence tags for naive, effector, and memory CTLs, respectively. For H3K27me3-associated sequences, a total of 18.9 × 10^6, 42.8 × 10^6, and 26 × 10^6 sequence tags were mapped from naive, effector, and memory CTLs. To validate our data, we examined sequences mapping to the constitutively expressed housekeeping gene, Oaz1, and the constitutively repressed Krt8 (keratin protein) gene (Figure S1 available online). As expected, there was a permissive epigenetic signature around the promoter and transcriptional start site (TSS) of Oaz1 with high H3K4me3 deposition (red histogram) and little or no H3K27me3 (blue histogram) (Figure S1A). This contrasted with the Krt8 locus that had a repressive epigenetic signature with little or no H3K4me3 but extensive H3K27me3 deposition (Figure S1B). Thus, these data were suitable for identifying patterns of genome-wide methylation in virus-specific naive, effector, and memory CD8+ T cells.

We next determined the proportion of H3K4me3 and H3K27me3 sequence tags associated with the promoter (defined as −3 kb and +1 kb of the TSS of genes, Table S1), gene body (+1 kb to 3’ UTR of genes), and intergenic regions. As expected, the majority of H3K4me3 sequence tags were associated within gene promoters and the gene bodies of loci within naive, effector, and memory CTLs (Figure 1A). There was an increase in the proportion of H3K4me3 sequence tags associated with intergenic regions within effector and memory CTLs (Figure 1A), demonstrating that there is modulation of H3K4me3 at noncoding genomic regions upon naive CTL activation. We observed that the majority of H3K27me3 sequence tags were predominantly associated with intergenic regions and gene bodies within naive, effector, and memory CTLs (Figure 1A), with a small proportion of sequence tags (approx. 10%) associated with our defined promoter region of 4 kb. There was little change in the proportionality of H3K27me3 deposition within these regions between naive, effector, and memory CTLs.

Whereas H3K4me3 is primarily associated with short domains of enrichment, typically associated with promoter regions (Wang et al., 2008), H3K27me3 typically associates with broad domains of enrichment that are associated with transcriptional repression (Jung et al., 2014; Young et al., 2011). Further, recent analyses have demonstrated that H3K27me3 also associates with promoter regions where it coincides with H3K4me3 and transcriptional activation (Bernstein et al., 2006; Roh et al., 2006; Young et al., 2011). We observed that H3K4me3 and H3K27me3 deposition was associated with a bimodal peak of enrichment around the TSS of gene loci (Figures S1C and S1D). As such, we were interested in the dynamics of H3K4me3 and H3K27me3 enrichment within gene promoters during T cell differentiation in response to infection. We first established the distribution of gene loci that had high (hi), intermediate (int), and low (lo) amounts of H3K4me3 and H3K27me3 enrichment within gene promoters (Figures S2A–S2D) and then determined the number of gene promoters with distinct patterns of H3K4me3 or H3K27me3 enrichment within naive, effector, and memory CTLs (Figures S2E and S2F). At a genome-wide level, there was little change in the total amount of H3K4me3 enrichment within the gene promoters between naive, effector, and memory CTLs (Figure S2E) but clear changes in H3K27me3 deposition between naive, effector, and memory CTLs (Figure S2F).

We next identified genes for which the presence or absence of peaks for one or both PTMs changed across the phases of differentiation, again focusing on the promoter region (−3 kb to +1 kb around the TSS). Overall, we identified 4,516 genes that exhibited changes in peaks of either H3K4me3 or H3K27me3 between naive, effector, and memory CTLs (Figure 1B). We then determined the proportion and number of genes that shared distinct patterns of H3K4me3 or H3K27me3 deposition within the naive, effector, and memory CTL subsets (Figures 1C and 1D). Strikingly, of these, 3,616 gene promoters (81%) exhibited modulation of the repressive H3K27me3 modification, with
only 900 (19%) exhibiting modulation of H3K4me3 (Figures 1C and 1D). To further validate our approach of identifying H3K27me3 peaks within gene promoters, we selected six gene loci that were identified as having an H3K27me3 peak within naive CTLs that was then lost upon differentiation into effector and memory CTLs but had a range of sequence tags that mapped to the promoter (Figures S3A and S3B). The change in peak patterns within the promoters of these six gene loci between naive and effector CTLs (N27+E27 or N27/C0E27+) correlated with changes in the normalized count of H3K27me3 sequence tags (Table S1). To further validate that changes in H3K27me3 deposition determined by changes in H3K27me3 peaks reflected actual changes in H3K27me3 enrichment, we performed H3K27me3 ChIP on naive and in vitro activated OT-I CTLs (Figures S3A and S3B). ChIP analysis on the promoters of the selected genes largely reflected the differences in H3K27me3 tag counts observed between naive and effector CTLs. Hence, we concluded that our peak was a valid means of examining how changes in histone methylation within promoter regions influence virus-specific CTL differentiation in response to infection.

The loss of H3K27me3 or gain of H3K4me3 represent two distinct epigenetic mechanisms that could lead to transcriptional activation upon naive CD8+ T cell differentiation. Therefore, we investigated whether these alternative mechanisms regulated functionally distinct gene groupings. Gene ontology (GO) analysis (Huang et al., 2009) identified that the majority of genes that were activated by gaining H3K4me3 were involved specifically in T cell immune function and regulation (Table 1). In contrast, genes that were activated by loss of
Table 1. GO Analysis of Genes Regulated via Gain of H3K4me3 or Loss of H3K27me3 Modifications

<table>
<thead>
<tr>
<th>H3K4me3 N− E+ M+</th>
<th>Number of Genes</th>
<th>Fisher Exact</th>
</tr>
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<tbody>
<tr>
<td>chemotaxis</td>
<td>10</td>
<td>6.1 × 10⁻⁷</td>
</tr>
<tr>
<td>immune response</td>
<td>19</td>
<td>2.8 × 10⁻⁶</td>
</tr>
<tr>
<td>locomotory behavior</td>
<td>13</td>
<td>5.0 × 10⁻⁶</td>
</tr>
<tr>
<td>positive regulation of immune system process</td>
<td>11</td>
<td>3.2 × 10⁻⁵</td>
</tr>
<tr>
<td>regulation of lymphocyte activation</td>
<td>9</td>
<td>5.1 × 10⁻⁵</td>
</tr>
<tr>
<td>positive regulation of leukocyte activation</td>
<td>9</td>
<td>8.6 × 10⁻⁵</td>
</tr>
<tr>
<td>positive regulation of cell proliferation</td>
<td>12</td>
<td>1.4 × 10⁻⁵</td>
</tr>
<tr>
<td>regulation of immune effector process</td>
<td>7</td>
<td>7.9 × 10⁻⁵</td>
</tr>
<tr>
<td>regulation of lymphocyte mediated immunity</td>
<td>6</td>
<td>1.1 × 10⁻⁴</td>
</tr>
<tr>
<td>positive regulation of cell activation</td>
<td>7</td>
<td>1.9 × 10⁻⁴</td>
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<tr>
<td>regulation of T cell activation</td>
<td>7</td>
<td>2.8 × 10⁻⁴</td>
</tr>
<tr>
<td>positive regulation of lymphocyte proliferation</td>
<td>6</td>
<td>2.9 × 10⁻⁴</td>
</tr>
<tr>
<td>positive regulation of cell communication</td>
<td>9</td>
<td>4.0 × 10⁻⁴</td>
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<td>positive regulation of signal transduction</td>
<td>9</td>
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</tr>
<tr>
<td>defense response</td>
<td>13</td>
<td>2.5 × 10⁻³</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>H3K27me3 N− E− M−</th>
<th>Number of Genes</th>
<th>Fisher Exact</th>
</tr>
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<tbody>
<tr>
<td>peptide metabolic process</td>
<td>8</td>
<td>1.3 × 10⁻⁵</td>
</tr>
<tr>
<td>glutathione metabolic process</td>
<td>6</td>
<td>5.8 × 10⁻⁵</td>
</tr>
<tr>
<td>cellular amino acid derivative metabolic process</td>
<td>13</td>
<td>3.1 × 10⁻⁴</td>
</tr>
<tr>
<td>carbohydrate phosphorylation</td>
<td>3</td>
<td>4.1 × 10⁻⁴</td>
</tr>
<tr>
<td>positive regulation of macromolecule metabolic process</td>
<td>33</td>
<td>1.4 × 10⁻³</td>
</tr>
<tr>
<td>DNA metabolic process</td>
<td>24</td>
<td>1.9 × 10⁻³</td>
</tr>
<tr>
<td>superoxide metabolic process</td>
<td>4</td>
<td>2.1 × 10⁻³</td>
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<tr>
<td>regulation of transcriptional preinitiation complex assembly</td>
<td>2</td>
<td>2.6 × 10⁻³</td>
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<tr>
<td>cellular macromolecule catabolic process</td>
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<tr>
<td>transcription</td>
<td>72</td>
<td>3.7 × 10⁻³</td>
</tr>
<tr>
<td>coenzyme metabolic process</td>
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<td>3.8 × 10⁻³</td>
</tr>
<tr>
<td>establishment of protein localization</td>
<td>32</td>
<td>4.4 × 10⁻³</td>
</tr>
<tr>
<td>positive regulation of macromolecule biosynthetic process</td>
<td>27</td>
<td>4.9 × 10⁻³</td>
</tr>
<tr>
<td>cellular response to stress</td>
<td>22</td>
<td>5.1 × 10⁻³</td>
</tr>
<tr>
<td>cell cycle</td>
<td>29</td>
<td>9.4 × 10⁻³</td>
</tr>
</tbody>
</table>

H3K27me3 had broader biological functions, being related to cellular differentiation, transcription, and proliferation (Table 1). Thus, these data suggest that distinct histone methylation patterns are used to promote transcriptional activity of gene modules that have particular roles to play during naive CD8⁺ T cell differentiation.

Distinct Transcriptional Signatures Define CTL Subsets Both before and after Stimulation

To understand the association between the distribution of histone PTMs in resting state CTLs and gene transcription in recently activated cells, we performed RNA-seq on naive, effector (day 10), and memory (day 60) OT-I CTLs, before and after a brief (5 hr) peptide stimulation (Table S2 and Figure 1E, total number of reads listed in Supplemental Experimental Procedures). We identified 8,219 genes that were differentially expressed (DE) after stimulation, with 3,321 genes upregulated and 4,898 genes downregulated, with the number of specific DE genes between each subset outlined in Figure 1E. Next, we performed multidimensional scaling on the RNA-seq data to determine the extent of similarity in gene transcription between each condition (Figure S3C). To summarize these data, each CTL subset could be distinguished by this approach, regardless of their activation state (Figure S3C); however, when the data were viewed as a whole, effector and memory CTLs were more similar to each other than either was to the naive, both before and after stimulation.

We then sought to determine the correlation between differential gene expression and the extent of histone methylation patterns within gene promoters (Figures 1F–1H). We correlated the difference in log₂ fold change in mRNA expression after activation (as measured by RNA-seq) between effector and naive (Figure 1F), memory and naive (Figure 1G), or memory and effector (Figure 1H) CTL subsets, with the difference in the number of normalized tag counts for H3K4me3 (Figures 1F–1H, green plots) and H3K27me3 (Figures 1F–1H, red plots) for each pair. As expected, we observed increased mRNA in effector and memory CTLs, compared to naive CTLs, correlated with higher H3K4me3 enrichment within the promoter of effector and memory CTLs (Figures 1F and 1G). Moreover, given the similarity in the transcriptional signatures identified between memory and effector CTLs by multidimensional scaling (Figure S3C), it was not surprising that the difference in H3K4me3 and H3K27me3 enrichment was less apparent between memory and effector CTL (Figure 1H). As expected, we observed that greater amounts of H3K27me3 within the promoter of effector and memory CTLs was associated with decreased mounts of mRNA compared to naive CTLs (Figures 1F and 1G). Rather than a direct correlation, differences in mRNA expression between naive and effector and between
naive and memory CTLs was associated with either the presence (Figures 1F and 1G, top left quadrant) or absence (Figures 1F and 1G, bottom right quadrant) of H3K27me3 at the promoter. Thus, although increased H3K4me3 deposition correlates with increased transcriptional activation, it is more the presence or absence of H3K27me3 within the promoter that correlates with transcriptional repression and activation, rather than any differences in the degree of K27me3 enrichment.

Distinct Epigenetic Methylation Signatures Employed during CTL Differentiation

To gain a deeper understanding of how the genomic location and degree of H3K4me3 or H3K27me3 deposition might influence gene regulation during CTL differentiation, we generated heat maps showing the location and degree of enrichment of H3K4me3 and H3K27me3 PTMs within our defined promoter region (−3 kb to +1 kb around the TSS) of genes known to regulate various aspects of virus-specific CTL differentiation and effector function (listed in Table S3). The heat maps were then organized by hierarchical clustering, with the clusters rooted to the naive CTL subset (Figure 2A).

Three major clusters were identified. The first cluster included genes such as Sell (CD62L), Bcl11b, Gzma, GzmK, and Bcl2, each characterized by a permissive methylation signature (H3K4me3↑H3K27me3↓) in naive and memory CTLs but with increased H3K27me3 deposition in the effector subset (Figure 2A). The second cluster of genes was characterized by a repressive signature (H3K4me3↓H3K27me3↑) in naive CTLs that was altered to a permissive signature (H3K4me3↑H3K27me3↑) in the memory and effector populations (Figure 2A). The majority of these genes encode effector molecules such as CCL3, CCL5, XCL1, IFNG, or GZMB or markers of effector CTL differentiation such as KLRG1, IL-2Rα (encoded by Cd25), and Blimp-1 (encoded by Prdm1). The third and final cluster was characterized by colocalization of H3K4me3 and H3K27me3 deposition at the same genomic location in the naive subset. This cluster included transcription factors known to be important regulators of CTL differentiation such as T-bet (encoded by Tbx21), IRF4, BMII, GATA3, and EOMES (Figure 2A). Strikingly, virus-specific CTL differentiation was associated with loss of the repressive H3K27me3 from these bivalent loci and resolution to a permissive histone methylation pattern (H3K4me3+H3K27me3+) (Figure 2A). Thus, it appears that distinct epigenetic regulatory mechanisms are utilized to coregulate the transcriptional activity of distinct gene modules that serve to underpin CTL differentiation and effector function.

To better define the relationship between histone PTMs and transcriptional activation, we utilized a similar approach as described for Figures 1G–1I and enumerated the number of H3K4me3 (Figures 2B–2D) and H3K27me3 (Figures 2E–2G) sequence tags within the promoters of the genes listed in Figure 2A, and then determined the ratios of normalized tag counts between effector versus naive, memory versus naive, and memory versus effector CTL populations. We then correlated this with the log2FC in mRNA expression 5 hr after peptide stimulation for the same comparisons. Similar to our global analysis of histone PTMs and differences between naive, effector, and memory CTLs in the resting state (Figures 1G–1I), transcriptional upregulation of lineage-specific CTL gene expression after peptide stimulation was associated with a greater ratio of H3K4me3

Figure 2. Histone Methylation Patterns Identify Gene Cohorts with Distinct Functional Roles during CTL Differentiation

(A) The number of H3K4me3 or H3K27me3 sequences tags from −3 kb to +1 kb around the TSS for genes known to have roles in CTL differentiation (listed in Table S3) was transformed (log2) and converted into a heatmap (described in the Experimental Procedures). Hierarchical clustering was then used to determine the relationship of histone methylation patterns observed in the promoter regions between the listed genes. Shown is the order of genes after clustering.

(B–G) The ratio of sequence tags for H3K4me3 (B–D) or H3K27me3 (E–G) were correlated with the log2 fold change in transcriptional mRNA expression between naive, effector, and memory CTL sublists as described in Figure 1 for the gene listed in Table S3.
and lower ratio of H3K27me3 in the promoters of effector and memory CTLs compared to naive CTLs (Figures 2B, 2C, 2E, and 2F). In contrast, lower mRNA transcription after 5 hr of peptide stimulation by effector and memory CTLs when compared to naive CTLs was associated with a low ratio of H3K4me3 and high ratio of H3K27me3 associated with gene promoters (Figures 2B, 2C, 2E, and 2F). There was little difference between effector and memory cell mRNA expression and either H3K4me3 (Figure 2D) and H3K27me3 (Figure 2G) ratios, supporting the notion that both of these CTL subsets respond similarly to peptide stimulation. Overall, these data demonstrate that despite distinct patterns of histone PTM deposition within the promoter of naive, CTL lineage-specific gene loci, transcriptional activation is associated with establishment of a permissive histone signature upon differentiation in response to infection.

Changes in Histone Methylation and Acquisition of Lineage-Specific Effector Gene Expression

To gain a better insight into the dynamics of epigenetic remodeling across the gene body of key CTL gene loci, we mapped the methylation signatures across the gene body for selected effector genes in naive, effector, and memory CTLs (Figures 3A–3H and S4A–S4E). Extending previous observations, naive OT-I CD8+ T cells were H3K4me3hiH3K27me3lo at the Tnfa locus, consistent with the ability of naive T cells to express TNF-α rapidly after stimulation (Brehm et al., 2005; Denton et al., 2011). Upon differentiation, H3K4me3 at the Tnfa locus was enriched in the middle of the gene body in effector and memory CTLs (Figure 3A). A similar pattern of H3K4me3 enrichment was observed for Cd44 at the TSS, suggesting that transcribed genes might undergo further H3K4me3 methylation upon naive CTL activation to reinforce active transcription (Figures S4A–S4E). Other effector genes, such as Ifng and Fasl, had a repressive signature (H3K4me3loH3K27me3hi) in naive OT-I CD8+ T cells and acquired an active signature (H3K4me3hiH3K27me3lo) at the TSS and 5′ region of the gene body upon differentiation into effector and memory CTLs (Figures 3B and 3C). Although a similar pattern was observed for the Gzm loci (Figures 3D–3F), the increased H3K4me3 deposition seen in effector CTLs was not as evident in memory CTLs (Figures 3D–3F). This diminished permissive histone methylation pattern correlates with the lower amounts of Gzm expression observed in memory than in effector CTLs (Jenkins et al., 2007; Moffat et al., 2009; Peixoto et al., 2007). Thus, the idea that permissive histone PTM signatures acquired upon memory CTL differentiation are maintained in the resting state at effector gene loci does not extend to all lineage-specific effector gene loci. Naive CD8+ T cells can exhibit rapid changes in cell surface marker expression and effector function upon activation and prior to division. This includes upregulation of activation markers such as CD69 and IL-2Rx and expression of TNF-α, CCL3, CCL4, and XCL1 (Figures S5A–S5D). In agreement with previous studies, naive CD8+ T cells did not demonstrate significant expression of IFN-γ (Figure S5C), IL-4, IL-17, or GZMB after short-term in vitro stimulation (data not shown). We have previously demonstrated that the Tnfa locus of naive CD8+ T cells exhibits a permissive H3K4me3hiH3K27me3lo signature (Denton et al., 2011), an observation confirmed by our ChIP-seq analysis (Figure 3A). It was therefore of interest to observe that despite being strongly transcribed within 5 hr of stimulation, there was little H3K4me3 detected at the Ccl3, Xci1, and Il2ra promoters of naive CD8+ T cells (Figures S4B–S4D). In contrast, Cd69 exhibited H3K4me3 deposition (Figure S4E) and was highly expressed within 5 hr of naive CTL activation (Figure S5B).

To determine whether H3K4me3 deposition occurred rapidly after naive T cell activation, we performed ChIP analysis for H3K4me3 (Figure 3G) and H3K27me3 (Figure 3H) at the proximal promoter region of selected effector gene loci shown to be rapidly transcribed after naive CTL activation. Deposition of the permissive H3K4me3 PTM was apparent at the Ccl3 and Xci1 promoters at 5 hr after peptide stimulation, with further increases at 24 hr postactivation (Figure 3G). This coincided with loss of the repressive H3K27me3 PTM within these same gene promoters at these time points (Figure 3H). This rapid change in the histone signature correlated with rapid transcriptional upregulation of Ccl3 and Xci1 upon naive CTL activation (Figure S5A). Despite loss of the repressive H3K27me3 modification at the Ifng promoter, there was little gain of H3K4me3 (Figures 3G and 3H). These data are consistent with the observation that IFN-γ is not expressed rapidly upon activation of naive CD8+ T cells (Figure S5C) or indeed prior to cellular division (Denton et al., 2011;
Figure 4. H3K4me2 Marks a Subset of Rapidly Transcribed Effector Gene Loci in Naive CTLs
(A) Shown are the patterns of H3K4me2 (red, above line) and H3K4me3 (blue, below line) peaks within the promoter, gene body, and 3’ UTR of genes within naive and effector OT-I CD8+ T cells.
(B) ChiP for either H3K4me3, H3K27me3, or H3K4me2 was performed on naive CD8+ T cells (white bars) or OT-I CD8+ T cells stimulated with OVA 257-264 after 5 (gray bars) or 24 (black bars) hr. Enrichment for H3K4me3, H3K27me3, or H3K4me2 was determined by qPCR, via primers that targeted the proximal promoter just upstream of the TSS (inset) of the specific genes. Error bars show ± standard deviation of triplicate samples.

Lawrence and Braciale, 2004). Thus, the loss of the repressive H3K27me3 modification is not sufficient to enable Ifng gene expression. Thus, these data demonstrate that rapid epigenetic reprogramming occurs within naive CD8+ T cells at selected gene loci upon activation and this correlates with rapid effector gene transcription before onset of cell division.

H3K4me2 Patterns Are Predictive of Rapid Effector Function upon Naive CD8+ T Cell Activation
In multipotent hematopoietic stem cells, dimethylation of H3K4 (H3K4me2) has been reported to mark genes that become transcriptionally active upon lineage commitment (Orford et al., 2008). These lineage-specific genes, maintained as H3K4me2 + H3K4me3 in the undifferentiated state, acquire H3K4me3+ after differentiation, suggesting that H3K4me2 might act as a substrate enabling rapid transition to a permissive H3K4me3 PTM (Orford et al., 2008). We performed ChiP-seq to determine the distribution of H3K4me2 in naive OT-I CD8+ T cells and effector OT-I CTLs and examined the extent of H3K4me2 and H3K4me3 deposition at effector genes rapidly upregulated upon stimulation (Figure 4A). H3K4me2 was observed in naive CD8+ T cells at the Tnfa, Ccl3, Xcl1, and Il2ra loci, all of which were rapidly transcribed and/or expressed after stimulation. The Tnfa locus was enriched for both H3K4me2 and H3K4me3 (me2+/me3+) in both naive and effector CTLs, whereas Il2ra and Ccl3 were me2+/me3- in naive CTLs, becoming me2+/me3- only upon differentiation into effector CTLs. Xcl1 remained me2+/me3- in both naive and effector CTLs (Figure 4A). Genes not expressed by naive or effector CTLs (e.g., Ift4 and Krts) lacked H3K4me2 and H3K4me3 in both differentiation states (Figure 4A).

Given that antigen stimulation of naive CD8+ T cells resulted in transcription of Ccl3, Xcl1, and Il2ra within 5 hr despite the absence of the permissive H3K4me3, it was of interest to determine how rapidly these loci acquire H3K4me3. We stimulated naive OT-I CD8+ T cells for 5 and 24 hr and carried out ChiP to determine the amount of H3K4me2, H3K4me3, and H3K27me3 at the TSSs of Ilng, Il2ra, Ccl3, and Xcl1 (Figure 4B). The Il2ra, Ccl3, and Xcl1 gene promoters showed changes in epigenetic marks at 5 and 24 hr after activation (Figure 4B). All three loci exhibited loss of H3K27me3 and increases in H3K4me3 deposition, particularly at 24 hr after stimulation, with the H3K4me3 deposition greatest at the Il2ra promoter. H3K4me2 deposition also increased at both 5 and 24 hr after activation (Figure 4B), in particular at the Ccl3 and Il2ra promoters. There was also a trend for increased H3K4me2 at the Xcl1 promoter with little change observed at the Ilng promoter. Overall, these data demonstrate that within naive OT-I CD8+ T cells, a subset of effector gene loci that are marked with H3K4me2 are differentially regulated via dynamic changes in H3K4 methylation status, consistent with a role for H3K4me2 enrichment in maintaining selected gene loci in a transcriptionally ready state (Orford et al., 2008).

Identification and Resolution of Bivalent Loci during Virus-Specific CTL Differentiation
Previous studies have shown that genes involved in human ESC pluripotency and cell fate decisions upon differentiation exhibit colocalization of the permissive H3K4me3 and the repressive H3K27me3 modifications (Araki et al., 2009; Bernstein et al., 2006). Upon ESC differentiation, the majority of these “bivalent” loci become heritably silenced, losing H3K4me3 while maintaining H3K27me3, ensuring that only lineage-specific genes are expressed. Our hierarchical clustering analysis of CTL lineage-associated gene loci (Figure 2A) and quantitation of H3K4me3 and H3K27me3 read counts (Table S1) found that genes such as Tbx21, Irf4, and Eomes, TFs known to play a key role in virus-specific CTL differentiation (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005; Man et al., 2013), were bivalent for both H3K4me3 and H3K27me3 histone methylation modifications in the naive state but resolved to H3K4me3+-H3K27me3+ upon differentiation. To further explore the role of promoter bivalency in regulating virus-specific CTL differentiation, we identified genes that were bivalent in naive CTLs and performed hierarchical clustering based on their enrichment patterns around the TSS (–3 kb to +1 kb) (Figure 5A). We limited our analysis to H3K4me3 and H3K27me3 peaks that occurred within 400 bp of each other, reasoning that these modifications occurred on either the same or proximal nucleosomes. From this analysis we identified 2,254 genes that were bivalent in naive CTLs. Upon naive CTL differentiation, 43% of bivalent loci lost one or both modifications in effector and/or memory CTLs (Figure 5B). However, in contrast to what is observed upon differentiation of ESCs, of those bivalent genes that resolved from naive to effector or memory states, approximately 80% of gene loci that had resolved either in memory or in effector and memory had lost
Figure 5. Bivalent Loci within Naive CTLs Rapidly Resolve to a Permissive H3K4me3\(^+\)H3K27me3\(^–\) Methylation Signature

(A) Bivalent loci were identified in naive OT-I as having overlapping H3K4me3 and H3K27me3 peaks within 400 bp of each other. Clustering analysis, based on log\(_2\) enrichment of histone methylation patterns within the promoter region, was performed to generate a hierarchical list and the histone methylation patterns in effector and memory OT-I determined.

(B) Methylation patterns of genes that were bivalent in naive cells were determined in effector and memory CTL populations. Shown is the proportion of genes that lost H3K27me3 (K4\(^+\)K27\(^–\), red), lost H3K4me3 (K4\(^–\)K27\(^+\), green), or lost both methylation marks (K4\(^–\)K27\(^–\), purple) in effector (E), memory (M), or both effector and memory (E+M) subsets.

(C–F) Shown is the pattern of H3K4me3 (red, above line) and H3K27me3 (blue, below line) enrichment within the promoter, gene body, and 3' UTR of bivalent genes within naive, effector, and memory OT-I CD8\(^+\) T cells.

(legend continued on next page)
H3K27me3 while maintaining H3K4me3 (Figure 5B). Many of these genes were found to encode transcription factors including Tbx21, Eomes, Irf4, and Irf8 (Figures 5C–5F; Prdm1, Socs7, Zbtb7b (ThPOK), and Nr4A3; as well as other genes such as Nrp1 and Sema7a (Figures S4F–S4K). Thus, analysis of bivalent histone methylation patterns can be used to identify gene loci that are likely key for naive CD8+ T cell lineage commitment into the effector and memory CTL subsets.

It was possible that the presence of overlapping H3K4me3 and H3K27me3 peaks identified by ChiP-seq represented heterogeneity within the CTL populations analyzed. Thus, to determine whether the peaks we observed in our ChiP-seq data represented the presence of both modifications on the same nucleosome, we performed sequential ChiP (Figures 5G–5J). We purified naive (CD44loCD62Lhi) OT-Is and initially immunoprecipitated chromatin with H3K4me3 antibody and were able to detect H3K4me3 deposition within the Actin, Eomes, Irf4, Tbx21, and Sema7a gene promoters (Figure 5G). When we did a second immunoprecipitation with H3K27me3 antibodies on the material from the initial H3K4me3 pull-down, we were able to demonstrate that the H3K27me3 modification was enriched at the Eomes, Irf4, Tbx21, and Sema7a, but not the Actin, promoter (Figure 5I). Moreover, when we reversed the order of immunoprecipitation (H3K27me3, then H3K4me3) to validate our initial observations (Figures 5H and 5J), we observed after the initial pull-down enrichment of H3K27me3 at the Eomes, Irf4, Tbx21, and Sema7a, but not Actin, gene promoter. As expected from our earlier observations, we observed evidence of H3K4me3 deposition at the Eomes, Irf4, Tbx21, and Sema7a promoters upon secondary immunoprecipitation with H3K4me3 antibody (Figure 5J). There was no signal in the Actin control because it would not have been pulled down with the initial H3K27me3 immunoprecipitation. Together, these data suggest that identification of bivalent gene loci via ChiP-seq data accurately reflects the epigenetic state within naive CTLs.

To examine the dynamics of resolution of bivalent gene loci, we used ChiP to examine the amount of H3K4me3 and H3K27me3 deposition within gene promoter regions for Tbx21, Eomes, Irf4, and Irf8 in naive OT-I CD8+ T cells stimulated with cognate peptide for 0, 5, and 24 hr (Figures 5K–5M). All gene loci had measurable H3K4me3 and H3K27me3 within naive CD8+ T cells (Figure 5K). Upon T cell activation, there was loss of H3K27me3, with a concomitant increase in H3K4me3 deposition, within the Tbx21, Irf4, and Irf8 promoters at the 5 hr time point, and this was exacerbated at 24 hr (Figures 5L and 5M). There was little change in the amount of H3K27me3 at the Eomes promoter (Figure 5L), correlating with its reported expression late after T cell activation (Cruz-Guilloty et al., 2009). The resolution to an active methylation signature (H3K4me3+H3K27me3−) correlated with the rapid upregulation of Tbx21, Irf4, and Irf8 mRNA 5 hr after stimulation (Table S2). These data suggest that within naive CD8+ T cells, bivalent loci such as Tbx21, Irf8, and Irf4 are poised for activation and rapidly acquire a permissive histone methylation signature upon receipt of TCR signals. Thus, in a manner similar to ESCs, maintenance of bivalency at key gene loci in naive CTLs is an epigenetic mechanism that helps ensure rapid transcriptional activation of factors that are known early drivers of CTL lineage commitment and is a key step in initiating programmed CTL differentiation.

DISCUSSION

Our genome-wide analysis of H3K4me3 and H3K27me3 patterns in naive, effector, and memory CTLs supported previous single-gene analyses (Araki et al., 2008; Denton et al., 2011; Northrop et al., 2006; Zediak et al., 2011), demonstrating that acquisition and maintenance of CTL-specific effector function upon naive T cell differentiation correlated with establishment of a permissive epigenetic signature at gene promoters. Interestingly, given the large number of gene loci that underwent transcriptional activation upon stimulation, it was surprising that deposition of H3K4me3 and subsequent establishment of a permissive histone methylation signature accounted for only approximately 20% of the changes in histone methylation patterns observed between naive, effector, and memory CTLs. Strikingly, this mechanism for enabling transcriptional activation was most prominent for genes associated with lineage-specific T cell function.

In contrast to this, our data demonstrated that modulation of H3K27me3 deposition was the primary regulatory mechanism for activation of genes within naive, effector, and memory CTLs. In particular, a large proportion of genes that possessed H3K27me3 peaks in naive OT-I CD8+ T cells also had H3K4me3 peaks within the same promoter region. This finding is consistent with our observations that data clearly show that loss of H3K27me3 occurred rapidly after T cell activation (5–24 hr), suggesting that transcriptional activation can be initiated after loss of the repressive H3K27me3 modification without the need for de novo deposition of H3K4me3 and that this occurs prior to initiation of cell division. We hypothesize that H3K27me3 deposition within naive CD8+ T cells acts as a brake on transcription at specific genes already marked with the permissive H3K4me3 modification. We speculate that this pattern of histone methylation within naive CD8+ T cells represents an epigenetic state whereby rapid removal of the repressive signature enables rapid transcriptional activation of genes that underpin the reported instructional differentiation program induced by TCR signals (Kaech and Ahmed, 2001; van Stipdonk et al., 2003). To that end, gene ontology analysis demonstrated that genes regulated in this manner played roles in cellular processes such as transcriptional activation, cellular division, and cellular metabolism, all processes required for sustaining a rapid...
proliferative cellular response, a cardinal feature of adaptive T cell immunity in response to infection. Thus, the data suggest that, upon T cell activation, distinct histone methylation regulatory mechanisms are rapidly engaged to regulate distinct transcriptional signatures during CTL differentiation and help ensure the establishment of effective cellular immunity.

An important consideration for this study is what represents a biologically relevant difference in H3K4me3 or H3K27me3 sequence tag deposition between naive, effector, and memory CTLs. To that end, we validated that change in peaks correlated with changes in tag density and that such changes could be validated by ChIP analysis (Figure S4). From this we were able to determine that whereas increased H3K4me3 correlated with increased transcriptional activation, it is more the presence or absence of H3K27me3 within the promoter that correlated with transcriptional repression or activation, rather than any differences in the degree of H3K27me3 enrichment. Further, we were able to determine that specific changes in histone PTMs during CTL differentiation identified distinct functional gene groupings (Table 1). Moreover, the observation that genuine epigenetic bivalency for H3K4me3 and H3K27me3 within the promoters identified a subset of gene loci key for CD8+ T cell lineage commitment and that this bivalent state resolved quickly upon activation prior to cellular division also provides biological insights into how histone PTMs can coordinate regulated gene transcription upon T cell activation.

The identification of gene promoters that exhibited overlapping peaks of H3K4me3 and H3K27me3 deposition, termed bivalent genes (Bernstein et al., 2006), was of particular interest. H3K4me3 and H3K27me3 bivalency is present within the promoters of developmentally important genes within ESCs, whereby the majority of bivalent loci resolve to a repressive methylation signature by losing the active H3K4me3 modification. In this way, bivalent loci within ESCs are rapidly shut down, ensuring appropriate cell lineage specific patterns of gene expression (Bernstein et al., 2006; Hawkins et al., 2010). Within naive CD8+ T cells, bivalency was observed at the gene promoters of transcription factors known to be critical for effector and memory CTL differentiation, such as Tbx21, Eomes (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005; Joshi et al., 2007), Prdm1 (Kallies et al., 2009), and Irf4 (Man et al., 2013; Nayar et al., 2012). However, in contrast to ESC differentiation, the majority of bivalent loci within naive CD8+ T cells resolved to a permissive histone methylation pattern upon effector or memory CTL differentiation. Moreover, we demonstrated that resolution of bivalent domains within Tbx21, Irf4, and Irf8 promoter regions occurred within 24 hr, prior to cellular division, and coinciding with rapid transcription of these genes. Importantly, rapid resolution of the bivalent Prdm1 and Eomes gene promoters was not observed. Together this suggests that commitment of naive CD8+ T cells to effector and memory CTL subsets is underpinned by staged transcription of key factors that ensures appropriate gene transcription at distinct stages of the differentiation process. Our data also identified a number of bivalent gene loci that, despite having been implicated in immunological processes, do not have any ascribed role in effector or memory CTL differentiation. Thus, the data generated from this study have the potential to be a resource for the further discovery of additional genes that might play a key role in the commitment and establishment of effector and memory CTL subsets.

Within naive CD8+ T cells, we also identified a number of genes, such as Il2ra, Ccl3, and Xcr1, that lacked H3K4me3 but were rapidly transcribed and expressed upon TCR activation. Genome-wide analysis of H3K4 methylation patterns within pluripotent erythroid progenitor cells demonstrated that although there is strong concordance with H3K4me2 and H3K3me3 at deposition most genes, a subset of hematopoietic-specific genes were H3K4me2 †H3K4me3 † in the undifferentiated state (Orford et al., 2008). Upon receipt of differentiation signals, these genes became H3K4me2 †H3K4me3 † transcriptionally active. Thus, this specific histone methylation signature appears to mark genes that are poised for expression in a cell lineage-specific manner. A similar pattern emerges upon genome-wide analysis of H3K4 methylation patterns between naive CD8+ T cells and effector CTLs. The Il2ra and Ccl3 loci become concordant for both H3K4 di- and trimethylation only upon differentiation into effector CTLs. As has been consistently observed for the other dynamic changes in histone methylation patterns in this study, the acquisition of H3K4me3 at these genes occurred rapidly after naive CD8+ T cell activation. H3K4me2 deposition within promoters of genes may therefore represent a substrate allowing for rapid transition to a permissive histone methylation pattern upon receipt of appropriate signals, enabling rapid gene transcription. However, we noted that the number of gene loci that exhibited this transition from H3K4me2 † H3K4me3 † in naive to H3K4me2 † H3K4me3 † was small (308 genes, data not shown). Nevertheless, a preliminary analysis demonstrated enrichment for genes involved in cell adhesion, immune response, and immune defense (data not shown). Thus, this might represent yet another epigenetic regulatory mechanism that ensures appropriate gene expression to promote effective T cell activation and function in response to infection.

This study adds to a growing appreciation of how specific histone PTMs underpin regulation of gene transcription within T cells. By using a CD4+ Th1 versus Th2 cell model system, Allan et al. (2012) recently demonstrated that epigenetic silencing of the Th1-cell-associated Tbx21 locus was dependent on deposition of H3K9me3, a process mediated by the histone methyltransferase, Suv39H1. This process of Suv39H1 mediated trimethylation of H3K9 is an initial step that triggers histone deacetylation and binding of transcriptional repressor protein complexes that stably silence targeted loci (Fujita et al., 2003). Suv39H1-deficient T cells were unable to stably repress Th1 cell gene expression, even after Th2 cell differentiation, demonstrating that this particular histone modification plays a key role in gene silencing during T cell differentiation, and this is a mechanism that ensures lineage-specific effector gene expression. Another study examined the dynamics of genome-wide modulation of H3 acetylation, H3K4me2 and H3K27me3 during T cell development and concluded that diverse epigenetic mechanisms were utilized to regulate gene transcriptional activation or repression at distinct stages of T cell development (Zhang et al., 2012). For example, acetylated histones appeared to rate-limit transcription, probably by directly regulating promoter accessibility, whereas H3K27me3 appeared to contribute to gene repression by operating “after the fact” by stabilizing a
transcriptionally silent signature, rather than directly repressing transcription. Moreover, H3K4me2 functioned as an intermediate between unmethylated H3K4 and the activating trimethylated state at gene promoters, thus allowing rapid transcriptional change after differentiation signals. This latter finding in developing T cells aligns with our observations that a subset of rapidly transcribed genes within naive OT-I CD8⁺ T cells are enriched for H3K4me2 within their promoters and that this is rapidly converted to H3K4me3 upon activation. Other genome-wide studies have demonstrated that histone PTMs such as H3K27Ac and H3K4me1 can be used to identify enhancer regulatory elements within noncoding regions of the genome. It will be of particular interest to examine the dynamics of these specific epigenetic marks within intergenic regions because these may identify enhancers that are differentially regulated during CD8⁺ T cell differentiation.

An intriguing question arising from this study is why there is a need for distinct histone methylation patterns to regulate distinct transcriptional signatures upon naive CTL activation. One hypothesis is that each histone methylation pattern forms a unique platform that allows the coordinated regulation of a cohort of gene loci. This might involve the recruitment of protein complexes that facilitate rapid changes in transcriptional activity (Kouzarides, 2007). For example, 7bx21 determines CD4⁺ Th1 cell differentiation via recruitment of H3K27 demethylases to the Ifng gene promoter, resulting in establishment of a permissive histone methylation signature (Miller et al., 2010). Thus, a similar mechanism might also be involved in CTL differentiation, and this is currently being investigated. Identification of such pathways and molecular targets might in the future lead to a better understanding of the molecular mechanisms that underpin acquisition and establishment of effective CTL immunity. This in turn has the potential to provide specific targets that can be modulated to alter the dynamics of histone PTMs, thus stably promoting or repressing CTL immune function where appropriate.

**EXPERIMENTAL PROCEDURES**

**Mice, Viruses, and Infection**

Ly5.2 C57BL/6J (B6) and congenic Ly5.1 OT-I mice were bred and housed under specific-pathogen-free conditions in the Department of Microbiology and Immunology Animal Facility at the University of Melbourne. For infection, mice were anesthetized and injected i.n. with 10⁴ p.f.u. of recombinant A/HKx31 virus engineered to express the OVA 257–264 peptide (x31-OVA) in Staining preparations (10⁷/ml) were resuspended in PBS/0.1% FCS and stained with anti-CD8α-FITC and anti-CD45.1-allophycocyanin (to detect OT-I cells). Naive cells were sorted with a FACS Aria (BD Biosciences). For measurement of cytokine production, lymphocytes preparations were stimulated for 5 hr with 1 μM (final concentration) of SIINFEKL in the presence of GolgiPlug (BD Biosciences). Samples were run on a Bio-Rad iQ5 real-time PCR detection system, and Ct values were calculated with the iQ5 real-time PCR analysis software. Data were analyzed with the 2⁻ΔΔCt method (Livak and Schmittgen, 2001), with expression values normalized to L32.

**RNA Extraction and Real-Time PCR**

RNA was extracted from sorted naive, effector, or memory OT-I cells (~10⁶) with 1 ml TRizol reagent (Invitrogen) and isopropanol precipitation. RNA (200–500 ng) was reverse transcribed to cDNA with Omniscript RT (QIAGEN) according to the manufacturer’s instructions. The equivalent of 50 ng RNA was then assayed with Sybr Green with custom-designed primers (see Table S1). Samples were run on a Bio-Rad iQ5 real-time PCR detection system, and Ct values were calculated with the iQ5 real-time PCR analysis software.

**ChIP-Seq**

ChIP was performed as previously described (Juelich et al., 2009) with the monoclonal antibodies 04–745 (Millipore) and Ab4729 (Abcam) and to precipitate H3K4me3 and H3K27me3 histones, respectively. After validating the specificity of each precipitation by real-time PCR via primers targeting characterized loci (Denton et al., 2011) (primers are described in Table S1), DNA fragments of ~200 bp were purified from 10 ng of template and ligated to Illumina oligonucleotide adapters. Samples were then sequenced with an Illumina GAII or Hi-Seq 2000 sequencer and mapped to the mouse genome (build mm10) with the Bowtie software (Langmead and Salzberg, 2012). Processing of ChIP–seq data is described in Supplemental Experimental Procedures.

**RNA-Seq**

RNA-seq was carried out as described in Supplemental Experimental Procedures and libraries run on an Illumina HiSeq 2000. Processing of RNA-seq data was performed as described in Supplemental Experimental Procedures.

**ACCESSION NUMBERS**

The RNA-seq data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession numbers GSEXXXX and GSEXXX, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.11.001.

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