Control of Proinflammatory Gene Programs by Regulated Trimethylation and Demethylation of Histone H4K20

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SUMMARY

Regulation of genes that initiate and amplify inflammatory programs of gene expression is achieved by signal-dependent exchange of coregulator complexes that function to read, write, and erase specific histone modifications linked to transcriptional activation or repression. Here, we provide evidence for the role of trimethylated histone H4 lysine 20 (H4K20me3) as a repression checkpoint that restricts expression of toll-like receptor 4 (TLR4) target genes in macrophages. H4K20me3 is deposited at the promoters of a subset of these genes by the SMYD5 histone methyltransferase through its association with NCoR corepressor complexes. Signal-dependent erasure of H4K20me3 is required for effective gene activation and is achieved by NF-κB-dependent delivery of the histone demethylase PHF2. Liver X receptors antagonize TLR4-dependent gene activation by maintaining NCoR/SMYD5-mediated repression. These findings reveal a histone H4K20 trimethylation/demethylation strategy that integrates positive and negative signaling inputs that control immunity and homeostasis.

INTRODUCTION

The survival of vertebrate organisms relies on innate and adaptive immune mechanisms to detect, combat, and eliminate foreign pathogens. Recognition of foreign pathogens by pattern recognition receptors (PRRs) triggers transcriptional activation of genes that amplify inflammatory responses, enable antimicrobial activities, and initiate the development of acquired immunity (Kawai and Akira, 2010; Takeuchi and Akira, 2010). The conservation of key signaling pathways required for these responses, including the Toll, JAK/STAT, and Immune Deficiency (Imd) pathways, throughout metazoan evolution underscores their importance in effectively defending against bacterial and viral infection (Lemaitre and Hoffmann, 2007; Martinelli and Reichhart, 2005). Macrophages represent a crucial cell type for initial recognition of pathogens based on their expression of numerous PRRs, including the toll-like receptors (TLRs). The TLR family is comprised of a highly conserved set of membrane receptors that recognize specific components of bacterial and viral pathogens including lipoproteins (TLR1/2/6), lipopolysaccharide (TLR4), flagellin (TLR5), single-stranded RNA (TLR7/8), double-stranded RNA (TLR3), and double stranded DNA (TLR9) (Kawai and Akira, 2010; Martinelli and Reichhart, 2005). Upon TLR ligation by their respective ligands, adaptor proteins are recruited to the cytoplasmic domains of TLRs and initiate signaling cascades, which ultimately result in the activation of signal-dependent transcription factors such as nuclear factor-κ B (NF-κB), activator protein 1 (AP1), and interferon regulatory factor (IRFs). These factors function in a combinatorial manner to promote the induction of inflammatory cytokines, type I interferons, and numerous other regulatory and effector molecules required for host defense (Kawai and Akira, 2010).

Although required for effective immunity, persistent or inappropriate activation of inflammatory gene programs can contribute to chronic diseases that include type II diabetes (Hotamisligil, 2006), atherosclerosis (Tedgui and Mallat, 2006), cancer (Karin et al., 2006), and neurodegenerative diseases (Glass et al., 2010). Precise control of innate and adaptive immune responses is therefore critical for maintaining proper tissue homeostasis. Multiple mechanisms have been identified that function to prevent signal-independent activation of inflammatory responses and to mediate their resolution upon eradication of the inciting stimulus. Several studies have suggested that many highly inducible inflammatory response genes are maintained in a repressed state under basal conditions by corepressor complexes containing nuclear receptor corepressor 1 (NCoR), silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) complexes, and/or the corepressor of REST.
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Identification of SMYD5 as a Negative Regulator of Inflammatory Response Genes

Nuclear receptors exert potent anti-inflammatory effects as important aspects of their biological functions, but these mechanisms remain poorly understood. As Drosophila melanogaster utilizes a conserved pathway involving signal-dependent activation of the NF-κB homolog Relish (Rel) to initiate inflammatory responses (Lazzaro, 2008; Lemaitre and Hoffmann, 2007; Figure S1A) and also expresses nuclear receptors (King-Jones and Thummel, 2005), we explored the use of Drosophila Schneider (S2) cells as a model to identify genes involved in nuclear receptor-mediated repression of inflammatory gene expression. A screen of Drosophila nuclear receptors identified Tailless (Tll) as an orphan nuclear receptor capable of repressing LPS-dependent activation of an Attacin A promoter-luciferase reporter gene (Figure S1B). Using this assay, we evaluated a dsRNA library targeting 21,300 genes for silencing RNAs (Boutros et al., 2004) that could reverse Tll-repression of Attacin A promoter activity. In addition to known suppressors of inflammatory responses, this screen led to the identification of CG3353 (Figure S1C), encoding a 393 amino acid protein containing a SET domain. CG3353 is most closely related to mammalian SMYD5, a member of the SMYD family of SET and MYND domain-containing proteins (Figure 1A). Using an independent dsRNA, we confirmed that knockdown of CG3353 abolished Tll transrepression, as well as resulted in an increase in basal expression of the Attacin A reporter gene (Figure 1B).

Primary mouse macrophages express all five members of the mammalian SMYD subfamily (Figure S1D), providing a biologically relevant model system to evaluate their potential roles in innate immune responses. Using specific siRNAs to deplete expression of each SMYD family member (Figure S1E), we observed that knockdown of SMYD5 resulted in exaggerated transcriptional responses of a subset of TLR4-responsive genes following stimulation with a purified LPS, Kdo2 lipid A (KLA) (Figures 1C, 1D, and S1F). In contrast, siRNAs directed against the other members of the mammalian SMYD family (Smyd1–4), resulted in suppressed responses (Figures 1C and S1F), indicating distinct molecular functions. Overexpression of wild-type SMYD5 markedly repressed KLA induction of a TNFα-luciferase reporter gene, while overexpression of SMYD5, containing a mutation predicted to abolish methyltransferase activity (H315L; SMYD5-mut), did not (Figure 1E). Knockdown of SMYD5 expression also abolished or compromised the ability of the LXR-specific agonist (GW3965) to inhibit LPS-induced expression of Ccl4 (Figure 1F) and other LXR-sensitive genes such as Il1a (Figure S1G), suggesting a conserved function in Drosophila and mammalian cells. In contrast, knockdown of SMYD5 did not alter inhibitory effects of the glucocorticoid receptor (data not shown), suggesting that SMYD5 contributes to receptor-specific repression mechanisms.

SMYD5 Trimethylates H4K20 at TLR4-Responsive Promoters

Next, we investigated the ability of bacterially expressed SMYD5 to methylate recombinant histones. Although SMYD1–3 have been shown to methylate histone H3 (Albert and Helin, 2010), a GST-SMYD5 fusion protein purified from bacteria exhibited methyltransferase activity on recombinant histone H4, but not histone H3 (Figure 2A). Mutations in the catalytic domain of SMYD5 (H315L or C317A) abrogated this activity (Figure 2A). To date there are only two identified methylation sites on histone H4: arginine at position 3 and lysine at position 20. Since SET domains typically methylate only lysine residues, our data suggested that SMYD5 most likely functions as an H4K20 methyltransferase. Consistent with this, methylation-specific antibodies detected trimethylation of lysine 20 on histone H4 incubated with wild-type, bacterially expressed SMYD5, but no methylation was observed with mutant SMYD5 (Figure 2B),...
Immunoprecipitates of FLAG-tagged wild-type and mutant SMYD5 expressed in mammalian cells also catalyzed trimethylation of H4K20, while no methylation was observed for H3K4me3, H3K9me3, H3K27me3, or H3K36me3 (Figure 2C). The specificity of the H4K20me3 antibody was confirmed using recombinant H4 proteins chemically mono-, di-, or trimethylated at position 20 (Figure S2A).

To determine whether H4K20 is the only residue in histone H4 modified by SMYD5 methylation, we performed methyltransferase reactions for SET8, a SET domain protein that monomethylates H4K20, or SMYD5, using recombinant unmodified histone H4 or recombinant H4 chemically trimethylated at position 20. SMYD5 and SET8 methylated unmodified histone H4, but not chemically trimethylated H4K20 (Figure 2D). In addition, FLAG-tagged SMYD5 and SET8 methylated recombinant histone H4, but not recombinant histone H4 in which lysine 20 was mutated to alanine (H4K20A, Figure S2B). Collectively, these results indicate that SMYD5 functions to catalyze trimethylation of H4K20.

H4K20me3 has been established as a mark for transcriptional repression that is primarily associated with heterochromatin (Gonzalo et al., 2005; Kourmouli et al., 2005; Schotta et al., 2004). However, genome-wide ChIP with parallel high-throughput sequencing (ChIP-Seq) studies have identified specific promoters that are enriched for H4K20me3 (Barski et al., 2007; Gonzalo et al., 2005; Mikkelsen et al., 2007). Therefore, we hypothesized that SMYD5 functions as a corepressor of inflammatory genes by catalyzing local trimethylation of H4K20. Consistent with this, chromatin immunoprecipitation (ChIP) experiments detected H4K20me3 near the transcriptional start sites of several proinflammatory promoters, exemplified by the Tnf (Figure 2E) and Cxcl10 (Figure 2F) promoters, under basal conditions. Notably, there was a marked reduction in this mark upon stimulation of TLR4 with KLA (Figures 2E and 2F). Furthermore, knockdown of SMYD5 by siRNA treatment resulted in an almost complete loss of H4K20me3 on the Tnf and Cxcl10 promoters (Figures 2E and 2F), while not affecting total H4 occupancy (Figures S2C and S2D). SMydd siRNA did not reduce mRNA expression of the previously identified H4K20 methyltransferases: Set8, Nsd1, Suv420H1, or Suv420H2, indicating that the observed loss of H4K20me3 on proinflammatory promoters is most likely dependent on reduced SMYD5 expression (Figure S2E). Furthermore, siRNA mediated
knockdown of the previously identified H4K20me3 methyltransferases SUV420H1 and SUV420H2 did not alter the H4K20me3 status of the Tnf (Figure S2F) or Cxcl10 (Figure S2G) promoters, and knockdown of SUV420H1 or SUV420H2 reduced, rather than enhanced, KLA-dependent activation of Tnf (Figure S2H) or Cxcl10 mRNA (Figure S2I).

**SMYD5 Is a Component of NCoR Corepressor Complexes**

Next, we sought to determine whether SMYD5 resides at inflammatory promoters. Since available antibodies were not suitable for ChIP, we implemented a strategy to biotin-tag SMYD5 in RAW 264.7 macrophages (de Boer et al., 2003). A biotin ligase recognition peptide (BLRP) was fused to the amino terminus of SMYD5 and the resulting protein was coexpressed with the bacterial biotin ligase BirA in RAW 264.7 macrophages. This strategy enabled streptavidin-based ChIP assays to detect enrichment for SMYD5 on the Tnf and Cxcl10 promoters in the absence of inflammatory signals, whereas activation of TLR4 by KLA resulted in the dismissal of SMYD5 from these promoters (Figures 3A and 3B).

The amino terminus of SMYD5 contains a MYND domain, which was previously demonstrated to mediate interactions with corepressor proteins including NCoR and SMRT (Liu et al., 2007). Gel filtration analysis of nuclear extracts from HEK293 cells transfected with GFP-SMYD5 and FLAG-NCoR indicated that GFP-SMYD5 eluted with NCoR and HDAC3 in high molecular weight fractions (Figure 4). Coimmunoprecipitation assays in 293T cells detected interaction between full-length SMYD5 and NCoR (Figure 3D). This interaction suggested that the NCoR complex could possess H4K20 histone methyltransferase activity. To test this hypothesis, we expressed FLAG-NCoR in 293T cells detected interaction between full-length SMYD5 and NCoR (Figure 3D). This interaction suggested that the NCoR complex could possess H4K20 histone methyltransferase activity. To test this hypothesis, we expressed FLAG-NCoR in 293T cells, purified the complex using an anti-FLAG affinity
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Figure 3. The NCoR Complex Directs SMYD5 to TLR4-Responsive Promoters

(A) Chromatin immunoprecipitation assays assessing BLRP-SMYD5 occupancy of the Tnf promoter before and after KLA stimulation for 2 hr. Values represent the average of three experiments ± SEM, *p < 0.05. (B) Chromatin immunoprecipitation assays assessing BLRP-SMYD5 occupancy of the Cxcl10 promoter before and after KLA stimulation for 2 hr. Values represent the average of three experiments ± SEM, *p < 0.05. (C) Gel filtration experiments for HEK293 cell lysates transfected with FLAG-NCoR and GFP-SMYD5. The lysates were then separated using SDS-PAGE analysis and immunoblotted with anti-NCoR, HDAC3, and GFP antibodies. (D) Coimmunoprecipitation of BLRP-SMYD5 with FLAG-NCoR in BLRP-SMYD5 RAW 264.7 cells. (E) Methyltransferase reaction for SMYD5 and NCoR. HEK293 cells were transfected with vectors containing FLAG-Empty, FLAG-SMYD5, or FLAG-NCoR and immunoprecipitated using FLAG M2 beads. Lysates were subjected to a methyltransferase reaction and immunoblotted using a specific H4K20me3 antibody. (F) Chromatin immunoprecipitation assays assessing the H4K20me3 levels on the Tnf and Cxcl10 promoters in WT and NCoR knockout RAW 264.7 macrophages. Values represent the average of three experiments ± SEM, *p < 0.05. (G) Chromatin immunoprecipitation assays assessing the BLRP-SMYD5 recruitment to the Ccl4, Tnf, and Il6 promoters following shCtrl and shNCoR transfection. Values represent the average of three experiments ± SEM, *p < 0.05.

matrix, and subjected the precipitate to histone methyltransferase assays using recombinant histone H4 as a substrate. Interestingly, both the SMYD5 and NCoR complexes methylated H4K20me3 (Figure 3E). Genetic deletion of NCoR in bone marrow-derived macrophages resulted in the loss of H4K20me3 on proinflammatory promoters, including Tnf and Cxcl10, as compared to WT macrophages (Figure 3F). Furthermore, SMYD5 occupancy on proinflammatory promoters was significantly reduced in BLRP-SMYD5 expressing RAW 264.7 cells following shRNA-mediated knockdown of NCoR (Figure 3G).

PHF2 Is Required for Removal of H4K20me3 and Activation of TLR4-Responsive Promoters

The observation that the H4K20me3 mark at inducible promoters was markedly reduced shortly after TLR4 ligation suggested the possibility of signal-dependent demethylation. Although no H4K20me3 demethylases have been described, recent studies have shown that PHF8 demethylates H4K20me1 (Liu et al., 2010; Qi et al., 2010), raising the possibility that two closely related proteins previously shown to possess demethylase activity, PHF2 (Wen et al., 2010) and KIAA1718 (Horton et al., 2010), may target H4K20me3 on inflammatory gene promoters. Knockdown of Phf2, but not Kiaa1718 or Phf8 (Figures S3A–S3C), significantly diminished KLA induction of the SMYD5-sensitive Tnf and Cxcl10 genes (Figures 4A and 4B).

To investigate PHF2 enzymatic function, wild-type PHF2 and a mutant PHF2-A/A (H248A, D250A), which alters conserved residues required for iron binding in the active site of the Jumonji domain, were purified from bacteria cell extracts and subjected to an in vitro histone demethylation assay using purified core histones and mononucleosomes. PHF2 effectively removed H4K20me3 methylation on mononucleosomes, while the mutant PHF2-A/A was much less active (Figures 4C and 4D). PHF2 also demethylated H3K9me1 on mononucleosomes as well as on core histones, consistent with recent observations (Wen et al., 2010) (Figure 4C).

ChiP experiments were next performed to investigate whether PHF2 was involved in the removal of the H4K20me3 mark at TLR4-responsive promoters. Primary bone marrow-derived macrophages were transfected with control siRNAs or siRNAs directed against Phf2, and the cells were then treated with vehicle or KLA for 4 hr. The H4K20me3 mark was depleted from the Tnf and Cxcl10 promoters in siCtrl-treated cells upon KLA treatment, as previously seen, but not in PHF2 knockdown macrophages (Figures 4E and 4F). Next, we sought to determine whether PHF2 is recruited to promoters exhibiting loss of H4K20me3 in response to LPS signaling. Because available antibodies were not suitable for the ChiP studies of PHF2, we implemented the above described biotin-tagging strategy to perform ChiP studies of PHF2 in RAW 264.7 macrophages. These studies demonstrated that PHF2 is recruited to the proinflammatory promoters Tnf and Cxcl10, but not β-actin, only upon activation of TLR4 signaling, correlating with the removal of H4K20me3 (Figure 4G).

Exchange of SMYD5 and PHF2 Controls the Regulation of TLR4-Dependent Gene Expression

To globally evaluate the roles of PHF2 and SMYD5 in the regulation of TLR4-dependent gene activation, we performed RNA sequencing of polyA RNA isolated from primary macrophages following transfections with Ctrl, Smyd5, or Phf2 siRNAs for...
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63) of mRNAs that were hyperinduced by knockdown of SMYD5 required PHF2 for activation (p < 0.005). However, the observation that the PHF2-dependent subset of TLR4-responsive genes was substantially larger than the subset of genes hyperinduced following SMYD5 knockdown raised the question of whether the actual set of H4K20me3-marked promoters are more broadly distributed than that defined by hyperinduction. Consistent with this possibility, ChIP experiments indicated that H4K20me3 was present on the promoters of H4K20me3-marked genes hyperinduced following SMYD5 depletion (Figure 5B). These results suggest that SMYD5 and PHF2 function in a reciprocal manner to regulate the transcriptional response to KLA. Consistent with this possibility, knockdown of Smyd5 mRNA, which depletes SMYD5-dependent promoters of H4K20me3, circumvents the
necessity for PHF2 in LPS-dependent gene activation of the Tnf and Cxcl10 mRNAs (Figure 5C).

To gain insights into potential proteins involved in the signal-dependent recruitment of PHF2 to these promoters, we analyzed the promoters of PHF2-dependent target genes and found NF-kB and IRF recognition motifs to be highly enriched (Figure 5D). Therefore, we performed coimmunoprecipitation assays to determine whether PHF2 interacts with p65, a member of the NF-kB family, which is required for activation of a large subset of TLR4-dependent genes (Lim et al., 2007; Medzhitov and Horng, 2009). We detected p65 interaction between p65 and PHF2 (Figure 5E). Notably, knockdown of p65 resulted in diminished recruitment of PHF2 to both the Cxcl10 and Tnf promoters, demonstrating that p65 is required for full recruitment of PHF2 (Figure 5F).

LXRαs exert repressive effects on TLR4 target genes by preventing signal-dependent NCoR clearance (Ghisletti et al., 2009). Since SMYD5 is delivered to inflammatory promoters by NCoR complexes and is required for LXR-mediated transrepression, we tested whether LXR agonists result in retention of H4K20me3 following TLR4 ligation. Consistent with this possibility, the LXR agonist GW3965 inhibited KLA-induced erasure of H4K20me3 on the Ccl4 promoter (Figure 5G).

**DISCUSSION**

In concert, these findings reveal remarkable specificity in the growing spectrum of biological processes controlled by regulated methylation/demethylation of H4K20, previously established to play roles in cell cycle (H4K20me1) (Liu et al., 2010; Wu et al., 2010), DNA damage repair (H4K20me2) (Pei et al., 2011; Sanders et al., 2004), and Wnt signaling (Li et al., 2011). Here, we provide evidence for an H4K20me3 methylation/demethylation mechanism, catalyzed by SMYD5 and PHF2, respectively, which is required for signal-dependent regulation of a subset of inflammatory response genes. We propose that under resting conditions, SMYD5 is recruited to a subset of TLR4-responsive promoters through its association with NCoR
corepressor complexes, where it trimethylates H4K20 (Figure 6).
Thus, in addition to mediating repression through the removal of acetylation by associated histone deacetylases, such as HDAC3, NCoR complexes also contribute to repression through the histone methyltransferase activity of associated SMYD5. The basis for the repressive function of H4K20me3 remains to be established; however, recent studies have suggested that H4K20me3 inhibits acetylation of H4K16, resulting in the pausing of RNA polymerase II (Kapoor-Vazirani et al., 2011). Consistent with this, paused Pol II is a characteristic feature of many genes that are rapidly and highly induced by TLR4 signaling (Escoubet-Lozach et al., 2011; Hargreaves et al., 2009).

The present studies expand on the emerging recognition of distinct biochemical activities and biological functions of members of the SMYD family of histone methyltransferases. SMYD1–3 have been reported to mediate trimethylation of histone H3K4, and reduced deposition of this activation mark could potentially account for why siRNA knockdown of Smyd1, 2, and 3 resulted in reduced responses to TLR4 activation (Albert and Helin, 2010). It will thus be of interest to define the molecular mechanisms that dictate distinct substrate specificities. The preference of SMYD5 for H4K20 appears to be an intrinsic property of the enzyme based on the observed activity of the purified GST-SMYD5 expressed in bacteria. In addition to SMYD5 and SUV420H1/2, SMYD3 has also recently been reported to trimethylate H4K20 (Foreman et al., 2011). However, the observations that knockdown of each of these enzymes resulted in reduced activation of TLR4 target genes, and that the presence of the H4K20me3 mark at TLR4-responsive promoters was largely dependent on SMYD5, strongly argue that SMYD3 and SUV420H1/2 primarily act on different substrates and/or are directed to H4K20 at different genomic locations by alternative protein complexes. Of note, we find that knockdown of SUV420H1/2 results in significant reduction in the expression of Phf2, which could at least partly explain the effect of their knockdown on TLR4-dependent gene activation (J.D.S., unpublished data).

We were initially led to SMYD5 by an RNAi screen that identified the Drosophila homolog CG3353 as being required for Tailless repression of LPS signaling. The observation that SMYD5 is required for transrepression activities of LXRs on at least a subset of its target genes suggests evolutionarily conserved molecular functions of SMYD5 that link nuclear receptor signaling to antagonism of innate immune responses. The finding that SMYD5 is required for LXR-dependent, but not glucocorticoid receptor-dependent, antagonism of TLR4-induced gene expression is also consistent with prior observations that LXR, but not glucocorticoid receptor, transrepression requires NCoR (Blaschke et al., 2006; Ghisletti et al., 2009; Venteclef et al., 2010; Ogawa et al., 2005).

We further show that TLR4-induced removal of H4K20me3 is catalyzed by PHF2, which is delivered to responsive promoters by the p65 subunit of NF-κB (Figure 6). The observation that more genes require PHF2 for optimal responses to TLR4 signaling than are hyperinduced in the setting of SMYD5 knockdown has several potential explanations. One is that while removal of H4K20me3 may be required for signal-dependent activation, lack of this mark may not be sufficient for hyperactivation, suggesting that it serves a repression “checkpoint” function. Consistent with this possibility, many PHF2-dependent promoters that were not hyperresponsive in response to SMYD5 knockdown were nevertheless marked by H4K20me3 under basal conditions. It is also possible that PHF2 targets additional methylated residues, such as H3K9me1, on proinflammatory promoters that are deposited by other histone methyltransferases. It is of interest that PHF2 was capable of catalyzing removal of H3K9me1, but not H4K20me3, on core histones. We speculate that the requirement for a nucleosome substrate for demethylation of H4K20me3 may be due to the ability of the plant homeodomain in PHF2 to recognize H3K4me3 (Wen et al., 2010). This interaction may anchor the enzyme in the correct conformation or allosterically regulate substrate specificity, as previously suggested for H4K20me1 demethylation by PHF8 (Liu et al., 2010). As our findings suggest that demethylation of H4K20me3 is a critical step in gene activation of inflammatory response genes, such as Tnf, modulation of the expression or activity of PHF2 may provide novel approaches for treatment of inflammatory diseases.

**EXPERIMENTAL PROCEDURES**

**Drosophila RNAi Screen**
Schneider S2 cells were plated in 6 cm dishes and transfected with 1.5 μg Attacin A reporter and 1.5 μg Tailless expression vector using Effectene (QIAGEN) transfection reagent according to the manufacturer’s guidelines. The cells were then plated in 384-well plates containing 250 ng of dsRNA and incubated for 48 hr. Cells were then treated with 100 ng/ml LPS for 8 hr and lysed, and luciferase activity was monitored using a luminometer.

**Gei Filtration**
HEK293 cells were transfected with FLAG-tagged NCoR WT and GFP-SMYDSWT using Lipofectamine 2000. Forty-eight hours post transfection, HEK293 cultured cells were harvested and washed twice with ice-cold PBS, and cytoplasmic extracts were prepared using a low-salt buffer (10 mM HEPES [pH 7.9], 50 mM NaCl, 1 mM DTT). Nuclei were spun down and extracted in 20 mM HEPES (pH 7.9), 20% glycerol, 350 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and protease inhibitors. A 1 mg sample was then applied onto a 250 ml column of Sephacryl S-300 (Amersham Biosciences, Inc.) previously equilibrated with gel filtration buffer (20 mM HEPES [pH 7.9], 120 mM NaCl, 0.2 mM EDTA, 1 mM DTT) and 60 1 ml fractions were collected. Fractions were analyzed by western blot.
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Cell Culture and Western Blots
Raw 264.7 cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. Raw 264.7 cells were transfected using Superfect (Qiagen) according to the manufacturer’s guidelines and as previously published (Ghiatti et al., 2009). Primary thyroglobulin-electrified macrophages and bone marrow-derived macrophage cells were generated as previously described (Ogawa et al., 2005). BLRP stables for SMYD5 and PHF2 were generated as previously reported (Heinz et al., 2010). LPS (Sigma) and KLA (Avanti) were used at a concentration of 100 ng/ml unless otherwise stated. The siRNA constructs were constructed using pSilencer 3.0 H1 (Ambion). Antibodies used are as follows: FLAG (Sigma, F3165), GFP (Abcam, Ab290), p65 (Santa Cruz, sc-6008), BLRP (Genscript, A00674), HDAC3 (Santa Cruz, sc-11417), H4 (Active Motif, 39269), H4K20me1 (Abcam, Ab9051), H4K20me2 (Abcam, Ab9052), H4K20me3 (Abcam, Ab9053), H3K9me1 (Abcam, Ab8896), H3K9me2 (Abcam, Ab1220), H3K9me3 (Abcam, Ab8898), H3K27me2 (Abcam, Ab24684), H3K27me3 (Millipore, 07–449) H3K4me3 (Abcam, Ab8898), H3K36me3 (Abcam, Ab9050), H3 (Active Motif, 9163), and His (Santa Cruz, SC-803). Chemically methylated H4 proteins were purchased from Active Motif.

RNA Isolation, Quantification, and Sequencing
RNA was purified using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s guidelines and as previously published previously (Sullivan et al., 2011). Promoters of regulated genes were detected using western blot analysis. Cells were lysed and sonicated in lysis buffer (10 mM Tris [pH 8.0], 0.5% NP40, 1.0 mM EDTA, Protease Inhibitors [Roche]). Samples were then diluted with dilution buffer (10 mM Tris [pH 8.0], 0.5% NP40, 1.0 mM EDTA) and protein complexes were purified using FLAG M2 beads (Sigma). Beads were washed with dilution buffer five times and eluted with 3X FLAG peptide. Lysates were then separated using SDS-PAGE and interactions were detected using western blot analysis.

ACCESSION NUMBERS
Sequencing data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.goc/geo) under the accession number GSE39113.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.07.020.

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