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A Feed-Forward Circuit Controlling Inducible NF-κB Target Gene Activation by Promoter Histone Demethylation

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SUMMARY

Activation of transcription from a silenced state is crucial to achieve specific gene expression in many biological contexts. Methylation of lysine 9 on histone H3 (H3K9) is widely associated with transcriptional silencing, and its disappearance is linked to the activation of several inflammatory genes by NF-κB. Here we describe that this event is controlled by a feed-forward circuit catalyzed by the activity of the histone demethylase Aof1 (also known as Lsd2/Kdm1b). We find that Aof1 is required for removal of dimethyl H3K9 at specific promoters, and thereby it controls stimulus-induced recruitment of NF-κB and gene expression. However, Aof1 is itself recruited by interaction with the c-Rel subunit of NF-κB, which is found at low levels associated with promoters in unstimulated cells. Thus, at these tightly regulated genes, NF-κB functions both as a transcriptional activator and as an upstream targeting signal that marks promoters to be derepressed by histone demethylation.

INTRODUCTION

A central challenge to understanding gene expression is to explain how the activity of transcription factors can be regulated in a promoter-specific fashion. This is exemplified by the activation of specific sets of target genes by the NF-κB family of transcription factors. The NF-κB family is conserved in most metazoan organisms, and critically regulates the expression of genes involved in numerous biological processes, including development, signaling, apoptosis, and immunity (Hoffmann and Baltimore, 2006). Many NF-κB-dependent genes are broadly expressed in multiple cell types and in response to diverse stimuli, mirroring the widespread activity of the NF-κB pathway (Hoffmann and Baltimore, 2006; Pahl, 1999; also see http://www.nf-kb.org/). Others, however, show a tightly regulated pattern of expression, with stimulus-dependent expression restricted to a particular biological setting (Hoffmann et al., 2006; Natoli and De Santa, 2006). This is the case for a subset of inflammatory genes including *Il12b* and *Mdc* (*Abcd1*, *Ccl22*), whose expression is strictly limited to hematopoietic cells of the immune system, and particularly dendritic cells (DCs) and macrophages (Saccani and Natoli, 2002; Trinchieri, 2003; Yamashita and Kuroda, 2002). Elucidating the molecular details of how such genes are activated in the correct context constitutes an essential step toward understanding the specificity of gene expression.

Covalent modifications to histone tails have the capacity to mark genetic loci for activation or repression, through their recognition by specific binding proteins (Kouzarides, 2007; Strahl and Allis, 2000). Methylation of lysine 9 of histone H3 (H3K9) is involved in long-term transcriptional silencing through the formation of heterochromatin, and is also implicated in transcriptional repression of euchromatic genes (Boggs et al., 2002; Kouzarides, 2002; Martin and Zhang, 2005; Peters et al., 2002; Schultz et al., 2002; Tachibana et al., 2002). However, when genes controlled in this way are later to be activated, the repressive state must be reversed. One of the first examples that this can occur came from the finding in human DCs that inflammatory gene expression is accompanied by loss of methylated H3K9 at specific promoters (Saccani and Natoli, 2002). However, the mechanism by which this methylation is removed has remained elusive (despite numerous studies [Chan et al., 2005; El Gazzar et al., 2007, 2008; Foster and Medzhitov, 2009; Ma et al., 2004; Villeneuve et al., 2008] and attempts to identify the activity [De Santa et al., 2007]), and it has not been established how (or even whether) it is causally linked to the gene activation process.

Enzymes capable of catalyzing the demethylation of lysine residues on histone tails have recently been identified (Cloos et al., 2008; Shi and Whetstine, 2007). As with other chromatin-modifying enzymes, histone demethylases have been found as part of coactivator and corepressor complexes (Lee et al., 2007; Pasini et al., 2008; Shi et al., 2004), and their removal has been shown to result in transcriptional misregulation of specific subsets of genes (Agger et al., 2007, 2009; Christensen et al., 2007; De Santa et al., 2007; Garcia-Bassets et al., 2007; Lan et al., 2007; Lee et al., 2007; Okada et al., 2007; Pasini et al., 2008; Saleque et al., 2007; Tahiliani et al., 2007; Tateishi et al., 2009; Wang et al., 2007). However, the mechanistic basis for their targeting to particular gene loci, and the downstream effects of their catalytic activities on transcription are far from clear (Lan et al., 2008). In particular, conflicting models describe the relative roles of transcription factors and demethylase-containing coactivator complexes: in some instances, it appears that transcription factors act upstream of demethylase activity,

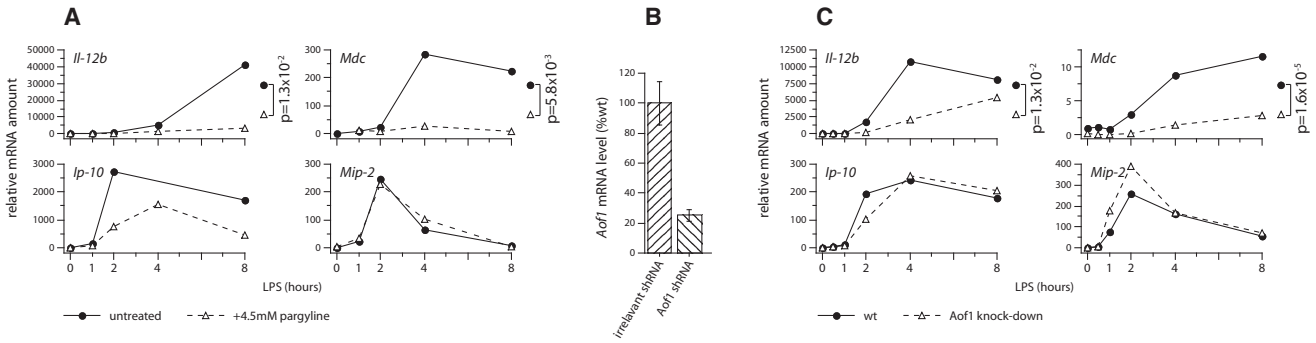

Figure 1. Expression of *Mdc* and *Il12b* by Dendritic Cells Requires the Activity of Aof1

(A) Expression of *Il12b*, *Mdc*, *Ip10*, and *Mip-2* mRNA in wild-type mouse DCs stimulated with LPS. Circles, untreated; triangles, pretreated for 18 hr with 4.5 mM pargyline. mRNA levels are expressed relative to unstimulated, untreated cells.

(B) *Aof1* mRNA levels (expressed as a percentage of the level in control cells) in mouse DCs transduced with retroviruses expressing an irrelevant shRNA (hereafter referred to as wild-type [WT]) or an shRNA targeting *Aof1* (Aof1 knockdown).

(C) Expression of *Il12b*, *Mdc*, *Ip10*, and *Mip-2* mRNA in wild-type (circles) or Aof1 knockdown (triangles) DCs upon stimulation with LPS. Error bars indicate standard errors. The results presented here are representative of more than ten experiments.

by mediating target gene choice (Wang et al., 2007; Yamane et al., 2006); however, in other cases, demethylase activity appears somehow to precede and regulate the downstream binding and activity of transcription factors at their target sequences (Metzger et al., 2005; Tateishi et al., 2009).

To try to uncover mechanisms underlying promoter-specific transcriptional activation of NF-κB target genes, we focused on the stimulus-dependent expression of *Mdc* and *Il12b* in mouse DCs. We identified Aof1 as an H3K9 demethylase, which is targeted to these promoters by interacting with the initially weakly bound c-Rel subunit of NF-κB, and yet whose activity is also required to enable subsequent stimulus-induced high-level recruitment of NF-κB, responsible for transcriptional activation.

RESULTS

Activation of *Mdc* and *Il12b* Expression Is Dependent on Aof1 Activity

In unstimulated DCs, the promoters of several tightly regulated inflammatory genes including *Mdc* and *Il12b* are associated with high levels of dimethylated H3K9 (H3K9me2), while those of other, more widely expressed NF-κB target genes such as *Ip-10* and *Mip-2* are not (Saccani and Natoli, 2002; see Figure S1A available online). Upon exposure to bacterial lipopolysaccharide (LPS), a potent inflammatory stimulus, transcription of all of these genes is strongly induced (Figure 1A), and this coincides with a rapid disappearance of H3K9me2 from the *Mdc* and *Il12b* promoters (Figure S1A), closely resembling the situation in human cells (Saccani and Natoli, 2002). Methylation of H3K9 is generally associated with transcriptionally inactive promoters, suggesting that the erasure of this mark could be responsible for controlling the expression of these genes.

Two classes of lysine demethylase enzymes have been described, belonging to the JmjC domain-containing and amine oxidase families (Shi and Whetstine, 2007). To investigate whether an enzyme belonging to the amine oxidase family might be required for *Mdc* and *Il12b* expression, we treated DCs before stimulation with the irreversible monoamine oxidase inhibitor pargyline. Pargyline treatment completely abolished stimulus-induced transcription of both *Mdc* and *Il12b*, while expression of several other NF-κB target genes including *Ip10* and *Mip2* was only slightly reduced or unaffected (Figure 1A and data not shown).

To try to identify the molecular target of pargyline inhibition, we focused on Lsd1, a histone demethylase belonging to the amine oxidase family (Shi et al., 2004), and its close paralogue Aof1 (also known as Lsd2/Kdm1b; Karytinos et al., 2009). Knockdown of *Lsd1* mRNA using RNA interference by more than ≈50% in a number of mouse hematopoietic cell lines was lethal, precluding functional analysis, and by chromatin immunoprecipitation (ChIP) we found that although Lsd1 binds to the promoters of many NF-κB target genes, these include those which are not highly methylated on H3K9, and whose expression is not inhibited by pargyline treatment (Figure S1B). This suggested that Lsd1 may not be involved in the specific acivation of *Mdc* and *Il12b*. In contrast, hematopoietic progenitor cells in which *Aof1* mRNA and protein levels were reduced by 70%–80% or more were viable (Figure 1B and Figure S1C) and could be differentiated in vitro into macrophages and DCs. Strikingly, we found that the effect of pargyline treatment was closely mimicked in Aof1 knockdown DCs: expression of *Il12b* and *Mdc* in response to LPS stimulation was strongly reduced, but expression of other NF-κB target genes including *Ip10* and *Mip2* was not (Figure 1C).

Macrophages showed a similar behavior to DCs (Figure S1D), and we obtained the same results with short hairpin RNAs (shRNAs) targeting two independent regions of the Aof1 coding sequence, ruling out an off-target effect of the RNA interference strategy. We used the J774 macrophage cell line in which Aof1 was stably knocked down to analyze expression of a panel of NF-κB target genes. The impairment of *Mdc* and *Il12b* expression was highly specific, as expression of most other genes tested was unchanged or only modestly affected (Figure S1E). Interestingly, though, we observed that the *Tarc* (*CCL17*) and

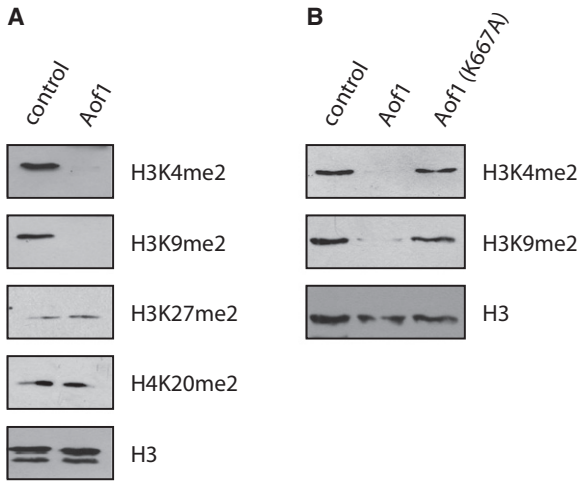

Figure 2. Aof1 Demethylates Dimethyl H3K9 and H3K4 In Vitro

(A) Aof1 was immunoprecipitated from LPS-stimulated DCs transduced with a tagged, truncated form of Aof1 and used to demethylate total histones in vitro. Control lanes contain an identical reaction using material from untransduced DCs. Histone methylation levels were assayed by western blotting with antibodies specific for the indicated modified residues.

(B) A catalytically inactive mutant of Aof1 (K667A), expressed at similar levels (Figure S2A) and isolated in the same way shows no demethylase activity. The results presented here are representative of three experiments.

Fractalkine (*CX3CL1*) genes, which are located adjacent to the *Mdc* locus, were also strongly inhibited by Aof1 knockdown, and their Aof1 dependence was also verified in DCs (Figure S1E).

Retroviral overexpression of an shRNA-resistant truncated form of Aof1 (including its putative catalytic domain) completely rescued the knockdown phenotype and even resulted in a stimulus-dependent increase in *Mdc* and *Il12b* expression (by up to 10- and 3-fold, respectively; Figure S1F).

Together, these results indicate that Aof1 activity is required for the expression of a subset of inflammatory genes including *Mdc* and *Il12b* in LPS-stimulated DCs.

Aof1 Is a Histone Demethylase with Activity against H3K9 and H3K4 In Vitro

Aof1 was recently reported to exhibit catalytic activity in vitro against H3K4me2 (Ciccone et al., 2009; Karytinos et al., 2009). However, the genes affected by Aof1 knockdown in our experiments suggested that Aof1 might instead function as a H3K9 demethylase. To test this, we isolated a tagged, truncated form of Aof1 from retrovirally transduced, stimulated DCs and measured its demethylation activity on purified histones in vitro. Aof1 showed demethylase activity against dimethyl H3K4, as reported, but also against dimethyl H3K9 (Figure 2A). In contrast, Aof1 had no activity against dimethyl H3K27 or H4K20 (Figure 2A). A point mutant of Aof1 (K667A) predicted to be catalytically inert showed no demethylase activity against either dimethyl H3K4 or H3K9 (Figure 2B), indicating that it is Aof1 itself which is catalytically active against both substrates. To rigorously prove this, we expressed Aof1 in *E. coli* and measured the activity of the purified recombinant protein in vitro. Again, Aof1 was able to demethylate both dimethyl H3K4 and H3K9, and not H3K27 (Figure S2B). Complete demethylation of dimethyl H3K9 required around 3- to 5-fold higher amounts of Aof1 than for H3K4 (in line with the relative abundances of these modifications on bulk histones [Thomas et al., 2006]), which may explain its initial identification only as an H3K4 demethylase (Ciccone et al., 2009; Karytinos et al., 2009; see Figure S2C and legend). As a control for specificity, recombinant Lsd1 showed no activity against H3K9me2 under the same conditions (Figure S2C). Interestingly, the in vitro activity of Aof1 against both monomethylated H3K4 and H3K9 was much less robust than that against the same dimethylated residues, in contrast to the known activity of Lsd1 (Shi et al., 2004). Recombinant Aof1 activity could be inhibited by pargyline in vitro (Figure S2D), corroborating the notion that Aof1 is an in vivo target of pargyline in DCs (Figure 1A). Thus, the in vitro activity of Aof1 against H3K9me2 made it a good candidate as an enzyme responsible for demethylation of this mark at the *Mdc* and *Il12b* promoters.

Aof1 Is Recruited to the *Mdc* and *Il12b* Promoters, and Is Required for Promoter H3K9 Demethylation In Vivo

We used ChIP to analyze the presence of Aof1 at the *Il12b*, *Mdc*, and *Ip10* promoters in DCs. Before stimulation, endogenous Aof1 could not be detected at any promoters studied. After LPS stimulation, however, Aof1 was readily found at the promoters of the *Il12b* and *Mdc* genes, but not that of *Ip10* (Figure 3A and Figure S3A). Since Aof1 is constitutively expressed in DCs and macrophages (data not shown), these results suggest that Aof1 is actively recruited to target promoters in response to stimulation. In support of this, a tagged, truncated form of Aof1 expressed from a retroviral vector was also inducibly recruited to the *Il12b* and *Mdc* promoters upon LPS treatment (Figure 3A).

We next examined the effect of removing Aof1 on the changes in histone lysine methylation that take place at these promoters upon LPS stimulation. The depletion of Aof1 by shRNA knockdown was sufficient to reduce stimulus-induced recruitment of Aof1 to promoters to background levels (Figure S3B). Total levels of histone H3, reflecting nucleosome occupancy at promoters, were not affected by Aof1 knockdown (Figure S3C), nor was there any consistent change in the low levels of H3K9me2 at the *Ip10* and *Mip2* promoters, to which Aof1 is not recruited ($p > 0.11$ in the experiment depicted in Figure 3B). Critically, however, the sharp drop in H3K9me2 levels which occurs at the *Mdc* and *Il12b* promoters in stimulated wild-type cells was completely prevented by Aof1 knockdown (Figure 3B). Thus, Aof1 is required for H3K9 demethylation at the *Mdc* and *Il12b* promoters in vivo.

The expression levels of other, known H3K9 demethylases were not appreciably reduced in Aof1 knockdown cells (Figure S3D), arguing that Aof1 is the directly responsible enzyme. To exclude possible indirect effects of Aof1 deficiency during DC differentiation in vitro, we generated cells in which endogenous Aof1 is knocked down, but can be complemented by a retrovirally encoded Aof1 fragment fused to the ligand-binding domain of the estrogen receptor (ER^{T2}-Aof1). In these cells, Aof1 activity is inducible by addition of the estrogen analog 4-hydroxy-tamoxifen (HO-Tam). Addition of HO-Tam only 24 hr

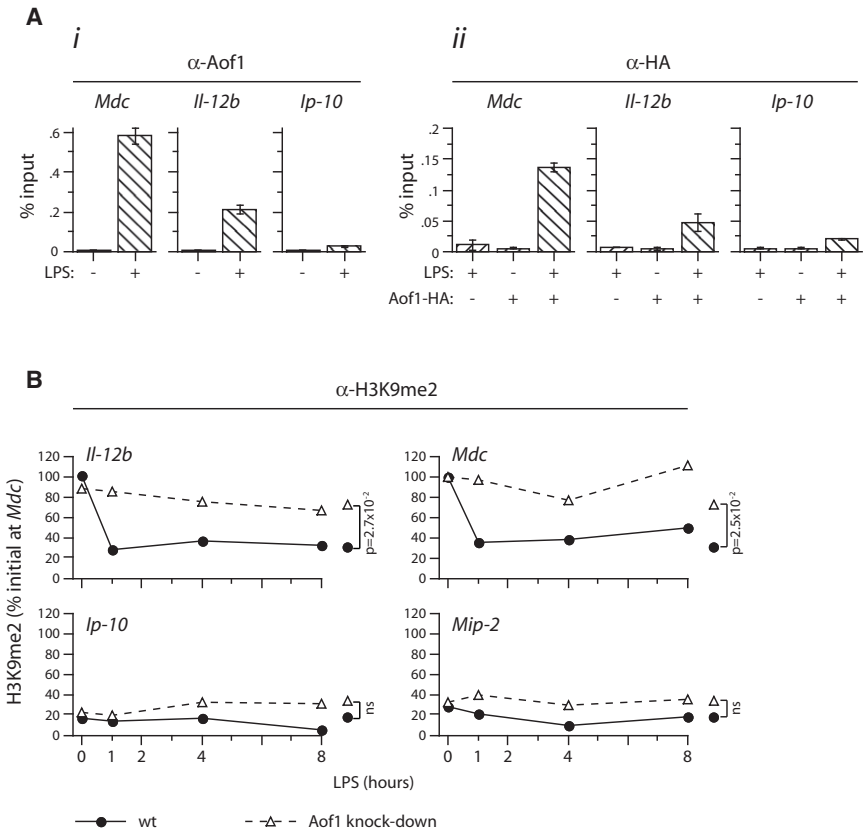

Figure 3. Aof1 Is Recruited to the *Mdc* and *Il12b* Promoters and Is Required for Demethylation of Dimethyl H3K9 In Vivo

(A) LPS-induced recruitment of Aof1 to the *Mdc* and *Il12b* promoters measured by ChIP using antibodies against (i) endogenous Aof1 in wild-type DCs, and (ii) HA-tagged, truncated Aof1 in retrovirally transduced DCs, before and after 1 hr LPS stimulation. Recruitment to the *Ip10* promoter was also examined as an Aof1-independent control gene.

(B) Changes in the relative levels of dimethyl H3K9 at the *Il12b*, *Mdc*, *Ip10*, and *Mip-2* promoters upon LPS stimulation of wild-type (circles) and Aof1 knockdown (triangles) DCs. H3K9me2 levels at each promoter were determined by ChIP and are expressed as a percentage of the level at the *Mdc* promoter in the same cells before stimulation. Note that in several experiments absolute global H3K9me2 recoveries were slightly lower in unstimulated Aof1 knockdown DCs (mean, 61% ± 13% of wild-type recovery), although we cannot rule out the possibility of saturation of the immunoprecipitating antibody. (A) Aof1 in unstimulated versus stimulated cells: *Mdc* $p = 4.6 \times 10^{-2}$, *Il12b* $p = 4.3 \times 10^{-2}$; Aof1-HA in transduced versus untransduced cells: *Mdc* $p = 1.9 \times 10^{-2}$, *Il12b* $p = 2.8 \times 10^{-2}$; (B) Wild-type H3K9me2 levels before versus after stimulation: *Il12b* $p = 1.2 \times 10^{-2}$, *Mdc* $p = 1.4 \times 10^{-2}$. Error bars indicate standard errors. The results presented here are representative of three to five experiments (the slight increase in H3K9me2 levels seen here at the *Ip10* and *Mip-2* promoters in Aof1 knockdown cells is not significant [ns; $p > 0.11$] and was not consistently observed in other experiments).

before LPS stimulation was able to completely rescue *Mdc* expression, indicating that Aof1 activity at any earlier stage is not necessary for target gene expression in DCs (Figure S3E). Conversely, differentiation of DCs in the continuous presence of HO-Tam, followed by its washout 48 hr before LPS stimulation, resulted in a notable reduction in *Mdc* expression, and impaired H3K9 demethylation (although the washout was not sufficient to completely ablate *Mdc* expression; see Figure S3F). In a separate experiment to rule out Aof1-dependent recruitment of a different demethylase enzyme to target promoters, we overexpressed tagged Aof1 or its catalytic point mutant (K667A) in wild-type DCs. Both proteins were efficiently recruited to the *Mdc* and *Il12b* promoters in response to LPS stimulation, but H3K9me2 demethylation of these promoters was impaired in cells overexpressing the catalytic mutant protein, and the expression of these genes was prevented (Figure S3G). Overexpression of the catalytic mutant may also have some indirect effects on DC function, since expression of *Ip10* was also diminished (but to a lesser extent; data not shown). However, taking these results together, we conclude that Aof1 itself demethylates dimethyl H3K9 at the *Mdc* and *Il12b* promoters in LPS-stimulated DCs.

In contrast to H3K9, the levels of dimethyl H3K4 displayed a transient decrease upon stimulation at both Aof1-dependent (*Il12b*) and -independent (*Ip10* and *Mip2*) genes, and this behavior was unaffected by knockdown of Aof1 (Figure S3C). Hence, despite its measurable in vitro activity against H3K4me2, Aof1 does not appear to demethylate this residue in vivo at the promoters studied here.

Aof1 Is Required for Stimulus-Driven Recruitment of NF-κB to the *Mdc* and *Il12b* Promoters

Methylation of H3K9 at promoter regions strongly correlates with transcriptional repression, but the level at which it acts is not well understood (Kouzarides, 2002; Martin and Zhang, 2005). The most straightforward notion would be that changes to promoters mediated by methylation of H3K9 render them inaccessible for stable binding by transcription factors. To investigate this, we examined recruitment of the NF-κB family members p65 and c-Rel to the *Mdc* and *Il12b* promoters in LPS-stimulated Aof1-deficient DCs, where H3K9 demethylation does not occur (Figure 3B). Remarkably, stimulus-driven recruitment of both p65 and c-Rel to these promoters was almost completely blocked in the absence of Aof1, whereas recruitment to the *Ip10* and *Mip2* promoters was unimpaired (and even somewhat augmented; Figures 4A and 4B). Since p65 is recruited only at a modest level in DCs to the *Il12b* and *Mdc* promoters, we also measured recruitment of p65 in Aof1 knocked down J774 macrophages (in which p65 recruitment is generally more pronounced). Again, without Aof1, p65 recruitment to the *Il12b*

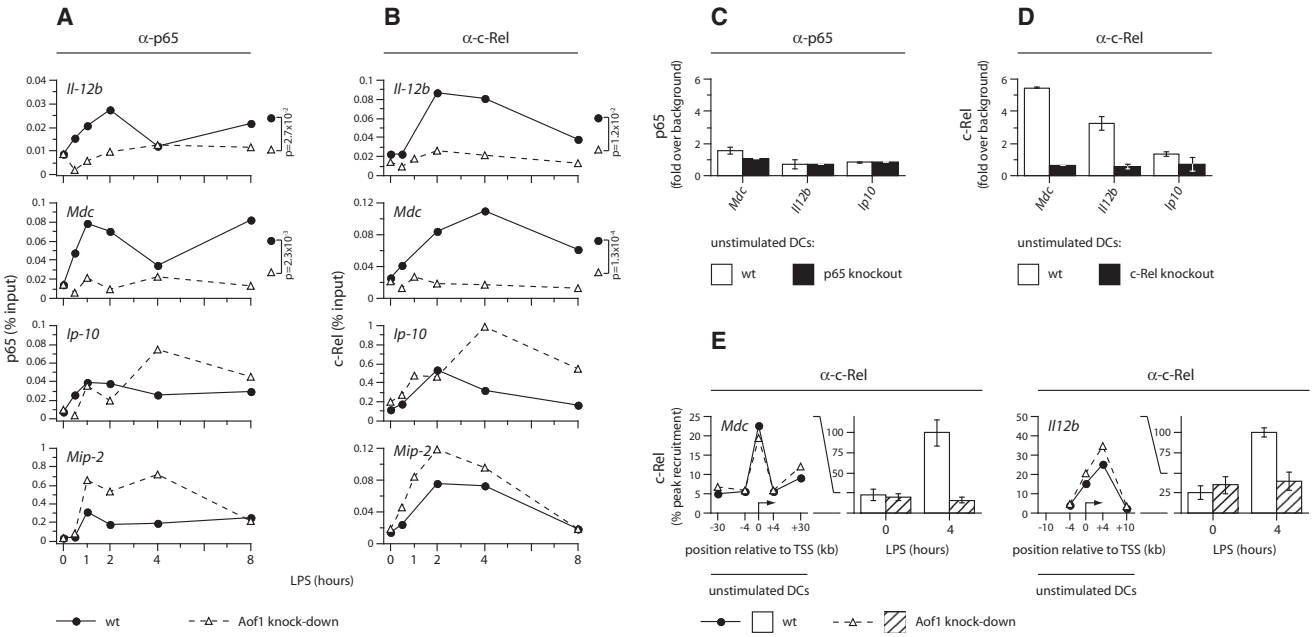

Figure 4. Aof1 Is Required for Stimulus-Driven Recruitment of NF-κB to the *Mdc* and *Il12b* Promoters, but Not for Low-Level Prestimulus Binding of c-Rel

(A and B) Recruitment of p65 (A) and c-Rel (B) to the *Il12b*, *Mdc*, *Ip10*, and *Mip-2* promoters upon LPS stimulation of wild-type (circles) and Aof1 knockdown (triangles) DCs.

(C and D) Binding of p65 (C) and c-Rel (D) to the *Mdc*, *Il12b*, and *Ip10* promoters in unstimulated cells, determined by ChIP using DCs derived from wild-type (white bars) and p65- (black bars, C) or c-Rel knockout (black bars, D) mice. Yields are expressed as the fold enrichment over the mean background value obtained in the respective knockout cells.

(E) Binding of c-Rel to the *Mdc* and *Il12b* promoters in wild-type (circles and white bars) and Aof1 knockdown (triangles and striped bars) DCs. The left panels show the levels of recovery after ChIP at locations surrounding the transcriptional start sites (TSS) in unstimulated cells; the right panels indicate the levels of recovery at the peak location before and after stimulation for 4 hr with LPS. Yields are expressed as a percentage of the peak recruitment in wild-type cells after LPS stimulation. (D) Wild-type versus knockout c-Rel recovery at *Mdc* $p = 1.2 \times 10^{-2}$, *Il12b* $p = 2.3 \times 10^{-3}$. Error bars indicate standard errors. The results presented here are representative of two to four experiments (note that the increase in p65 recruitment to the *Il12b* and *Mdc* promoters between 4 and 8 hr in A was not consistently observed in other experiments).

and *Mdc* promoters was completely prevented (Figure S4A). Thus, Aof1 is required at specific promoters to demethylate H3K9, and controls their activity by enabling stimulus-driven recruitment of NF-κB.

Aof1 Is Not Required for Weak Prestimulus c-Rel Binding to the *Mdc* and *Il12b* Promoters

In unstimulated cells, the majority of transcriptionally competent NF-κB dimers are retained in the cytoplasm by inhibitory IκB molecules. Upon release, they enter the nucleus and account for the high-level stimulus-driven recruitment to target gene promoters and the induction of transcriptional activation (Hoffmann and Baltimore, 2006). In contrast, it is unclear whether low levels of these dimers in the nucleus of unstimulated DCs can also engage in promoter binding, and whether this is linked to subsequent gene activation.

We analyzed the presence of p65 and of c-Rel at target promoters in unstimulated wild-type DCs. Since the amount of material recovered by ChIP under these conditions was low, we used DCs derived from the respective knockout mice as a control for specificity. While we could not detect binding of p65 above background to any promoters in unstimulated cells, we consistently found low levels of c-Rel bound near the *Mdc* and *Il12b* promoters (5- and 3-fold above background, respectively; Figures 4C and 4D and Figure S4B). The highest recoveries corresponded to around 20%–30% of the peak levels of recruitment after LPS stimulation (Figure 4E). At the *Il12b* promoter, the location of highest recovery in unstimulated cells was slightly downstream of the transcriptional start site (TSS), whereas upon LPS stimulation both c-Rel and p65 bound close to the TSS itself, suggesting that c-Rel may utilize distinct binding sites before and after stimulation (Figure S4B). To identify the c-Rel-containing dimer bound to the *Mdc* and *Il12b* promoters, we also used ChIP to analyze the presence of the p50 and p52 NF-κB subunits in unstimulated cells. Neither of these subunits could be detected at either promoter (although p50 was already present at the *Ip10* and *Mip2* promoters; Figure S4C and not shown), suggesting that before stimulation, c-Rel most likely binds as a homodimer. Notably, the magnitude and location of prestimulus c-Rel binding was unaffected in Aof1 knockdown cells (Figure 4E), in stark contrast to the complete prevention of the strong, stimulus-induced c-Rel recruitment in the same cells (Figure 4B). Therefore, Aof1 is not required for the weaker c-Rel association with the *Mdc* and *Il12b*

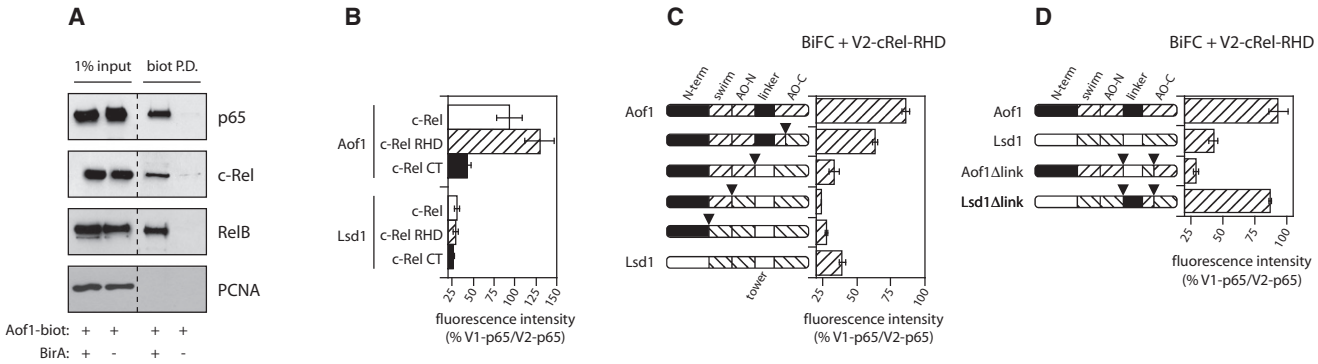

Figure 5. Aof1 Interacts with the Rel Homology Domain of NF-κB

(A) Pull-down of biotin-tagged Aof1 (biot P.D.) from transfected HEK293 cells, with and without coexpression of the biotin ligase BirA. Interacting proteins were assayed by western blotting with antibodies specific for p65 (top), c-Rel (upper middle), RelB (lower middle), and PCNA (which does not interact; bottom). Dotted lines indicate where nonrelevant lanes have been cropped from the figure.

(B–D) In vivo interaction of Aof1 with the c-Rel RHD, revealed by BiFC in transfected HEK293 cells. Geometric mean fluorescence of cells cotransfected with V2-c-Rel (white), V2-c-Rel RHD (striped), or V2-c-Rel C terminus (black), together with V1-Aof1 (B, top) or V1-Lsd1 (B, bottom); or together with proteins bearing sequential exchanges between Aof1- and Lsd1-derived regions (indicated by arrowheads; C); or together with Aof1 and Lsd1 proteins containing swapped linker regions (indicated by flanking arrowheads; D). Fluorescence intensities are expressed as the percentage of the level in cells coexpressing V1-p65 and V2-p65. All V1 fusion proteins were expressed at comparable levels, as determined by intracellular staining (Figure S7B and data not shown). (B) Fluorescence of Aof1/c-Rel RHD versus Aof1/c-Rel CT $p = 1.1 \times 10^{-2}$, or versus Lsd1/c-Rel RHD $p = 8.7 \times 10^{-3}$; (C) Aof1 up to AO-C (second row) versus Aof1 up to linker (third row) $p = 1.3 \times 10^{-3}$; (D) Aof1 versus Aof1Δlink $p = 2.8 \times 10^{-3}$; Lsd1 versus Lsd1Δlink $p = 7.9 \times 10^{-4}$. Error bars indicate standard errors. The results presented here are representative of three to six experiments.

promoters in unstimulated cells. This is consistent with the observation that Aof1 itself is not found at these promoters at this point (Figure 3A).

Aof1 Interacts with the Rel Homology Domain of NF-κB

The activity of Aof1 at NF-κB-dependent target genes, and the parallels between the stimulus-dependent recruitment of Aof1 and of NF-κB itself, suggested that promoter binding of Aof1 and of NF-κB might be mechanistically linked. To investigate this, we tested whether Aof1 could biochemically associate with members of the NF-κB family.

A tagged form of Aof1 was able to coprecipitate the NF-κB subunits c-Rel, p65, and RelB from nuclear extracts of transfected cells (Figure 5A), indicating that they are able to interact together. To verify this interaction in vivo, we used bimolecular fluorescence complementation (BiFC; Hu et al., 2002). We transfected cells with plasmids encoding fusion proteins of Aof1 joined to an N-terminal fragment of the fluorescent protein Venus (V1), and of NF-κB subunits joined to a fragment from the Venus C terminus (V2). Cells expressing fusion proteins individually were nonfluorescent (Figure S5A), but coexpression of Aof1 together with either c-Rel or p65, fused to complementary Venus fragments, resulted in strong fluorescence, indicative of an in vivo interaction (Figure 5B and Figures S5A and S5B). In contrast, Lsd1 displayed no interaction with either NF-κB subunit, despite its similarity to Aof1. The interaction with Aof1 mapped to the Rel homology domain (RHD) of NF-κB, which is conserved between subunits, in agreement with their shared ability to coprecipitate with Aof1 (Figures 5A and 5B and Figure S5B).

To identify the region of Aof1 responsible for interacting with NF-κB, we generated chimaeric proteins by exchanging reciprocal portions of Aof1 and Lsd1. These proteins share an organization consisting of unrelated N-terminal regions followed by adjacent swirm (Pfam, pf044339) and amino oxidase (Pfam, pf01593) domains. The amino oxidase domain itself comprises two highly conserved subdomains (which we term AO-N and AO-C; Figure S5C) separated by a less-conserved linker region. The linker region has very little similarity between Aof1 and Lsd1 (Figure S5C), adopts diverse structural conformations among other family members (Chen et al., 2006; Forneris et al., 2008; Yang et al., 2006), and is the site of insertion of the tower domain in Lsd1 (Shi et al., 2004). Exchange of the Lsd1 N-terminal, swirm, and AO-N regions into Aof1 had little effect on its interaction with the RHDs of c-Rel or p65 (Figure S5D), or with the full-length NF-κB proteins (data not shown), but additional exchange of the linker region drastically diminished the interaction. Likewise, the interaction with NF-κB RHDs was only slightly reduced by switching the AO-C region of Aof1 for that of Lsd1, but it was abolished by any further substitutions that involved the linker region (Figure 5C and Figure S5E). These results implicated the linker region of Aof1 to be required for its interaction with NF-κB. To confirm this, we generated mutant forms of Aof1 and Lsd1 in which their respective linker regions were swapped. Aof1 containing the Lsd1 linker region (including the inserted tower domain; Aof1Δlink) was expressed at equal levels to wild-type Aof1 and was localized similarly to the cytoplasm and nucleus of transfected cells (Figure S5G). However, it was completely unable to interact with the RHDs of c-Rel or p65 (Figure 5D and Figures S5F and S5H). Conversely, incorporation of the Aof1 linker region into Lsd1 was sufficient to promote an interaction with NF-κB comparable to that of wild-type Aof1 (Figure 5D and Figure S5F). Together, these data indicate that Aof1 can physically interact with the RHDs of NF-κB proteins, through the linker region between its AO-N and AO-C subdomains.

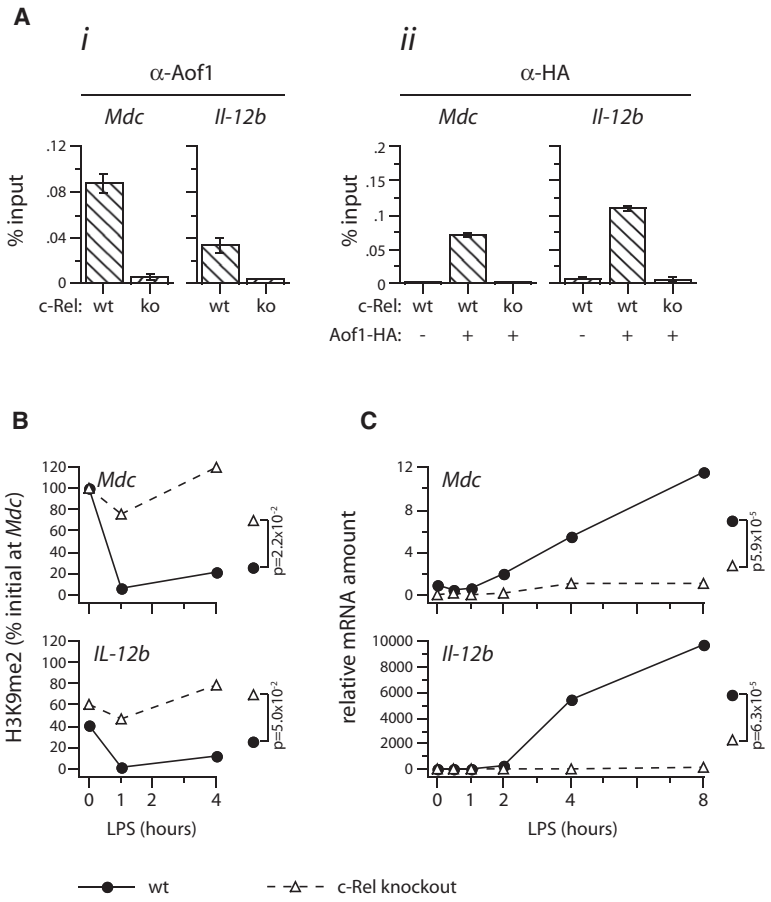

Figure 6. c-Rel Is Required for Recruitment and Activity of Aof1 at Target Promoters

(A) Recruitment of Aof1 to the *Mdc* and *Il12b* promoters in wild-type and c-Rel knockout DCs stimulated for 1 hr with LPS, measured by ChIP using antibodies against (i) endogenous Aof1, and (ii) HA-tagged, truncated Aof1 in retrovirally transduced cells.

(B) Changes in the relative levels of dimethyl H3K9 at the *Il12b* and *Mdc* promoters upon LPS stimulation of wild-type (circles) and c-Rel knockout (triangles) DCs. H3K9me2 levels at each promoter were determined by ChIP and are expressed as a percentage of the level at the *Mdc* promoter in the same cells before stimulation.

(C) Expression of *Il12b* and *Mdc* mRNA in wild-type (circles) or c-Rel knockout (triangles) DCs upon stimulation with LPS. mRNA levels are expressed relative to unstimulated, wild-type cells. (A) Wild-type versus c-Rel knockout Aof1 recruitment: *Mdc* $p = 2.1 \times 10^{-2}$, *Il12b* $p = 3.7 \times 10^{-2}$; Aof1-HA, *Mdc* $p = 5.5 \times 10^{-2}$, *Il12b* $p = 3.2 \times 10^{-2}$. Error bars indicate standard errors. The results presented here are representative of two to six experiments.

c-Rel Is Required for Recruitment of Aof1 to Target Promoters, and for Their Subsequent H3K9 Demethylation and Expression

Although NF-κB can interact with Aof1, the stimulus-dependent recruitment of NF-κB to the *Mdc* and *Il12b* promoters is itself dependent on the activity of Aof1 (Figures 4A and 4B) and so is unlikely to act upstream of Aof1 recruitment. However, the low level of prestimulus c-Rel detectable at the *Mdc* and *Il12b* promoters is independent of Aof1 (Figures 4D and 4E). We therefore explored the possibility that c-Rel could be responsible for targeting Aof1 to promoters. According to this scenario, H3K9 demethylation by Aof1 would act as the catalytic step in a feed-forward circuit, linking the initial low levels of c-Rel found at promoters before stimulation to the subsequent high-level, stimulus-induced recruitment of NF-κB, including c-Rel itself, responsible for gene activation.

To test this, we examined Aof1 recruitment in the absence of c-Rel. Using LPS-stimulated DCs derived from c-Rel knockout mice, we found that recruitment of endogenous Aof1 to the *Mdc* and *Il12b* promoters was completely prevented (Figure 6A). *Aof1* mRNA levels were unaffected in c-Rel knockout cells (data not shown), and recruitment of a retrovirally expressed, tagged, truncated form of Aof1 to these promoters was also c-Rel dependent (Figure 6A). In keeping with their inability to recruit Aof1, the promoters of *Mdc* and *Il12b* did not undergo demethylation of H3K9me2 in c-Rel knockout cells (Figure 6B), and recruitment of p65 was prevented (data not shown), resulting in a complete block in transcriptional activation of these genes (Figure 6C). The block was specific for c-Rel, since DCs derived from p65-knockout mice, or from cells in which RelB had been knocked down by RNA interference, were still able to express *Mdc* and *Il12b* (Figures S6A and S6B). Reconstitution of c-Rel knockout cells with a retrovirally expressed form of c-Rel restored Aof1 recruitment to the *Mdc* and *Il12b* promoters and expression of these genes (Figures S6D and S6E). Thus, c-Rel is required for Aof1 recruitment and activity at target promoters.

Since Aof1 interacts with the conserved RHD of NF-κB, the selectivity for c-Rel is likely to arise from its particular capacity to bind specific promoters in unstimulated DCs (Figures 4C and 4D). Sanjabi et al. (2005) previously studied the ability of c-Rel to selectively activate the *Il12b* promoter in macrophages, and identified the region responsible within the DNA-binding portion of the RHD. We therefore tested whether this selectivity region also conferred the ability to activate *Mdc* and *Il12b* in DCs. Retroviral expression of wild-type p65 in c-Rel knockout DCs was unable to rescue activation of either gene, as expected, whereas expression of a mutant form of p65 with the binding selectivity of c-Rel restored activation of both *Mdc* and *Il12b* to almost wild-type levels (p65N/3,4; Figure S6C; this protein contains 46 c-Rel-specific residues substituted into the RHD of p65 [Sanjabi et al., 2005]; see the Experimental Procedures).

A Mutant Form of Aof1 that Cannot Interact with NF-κB Is Not Recruited to Target Promoters

These data are consistent with a model in which interaction with the c-Rel found at low levels at the *Mdc* and *Il12b* promoters in resting DCs is responsible for targeting Aof1 to these promoters upon LPS stimulation. To directly address this, we used the

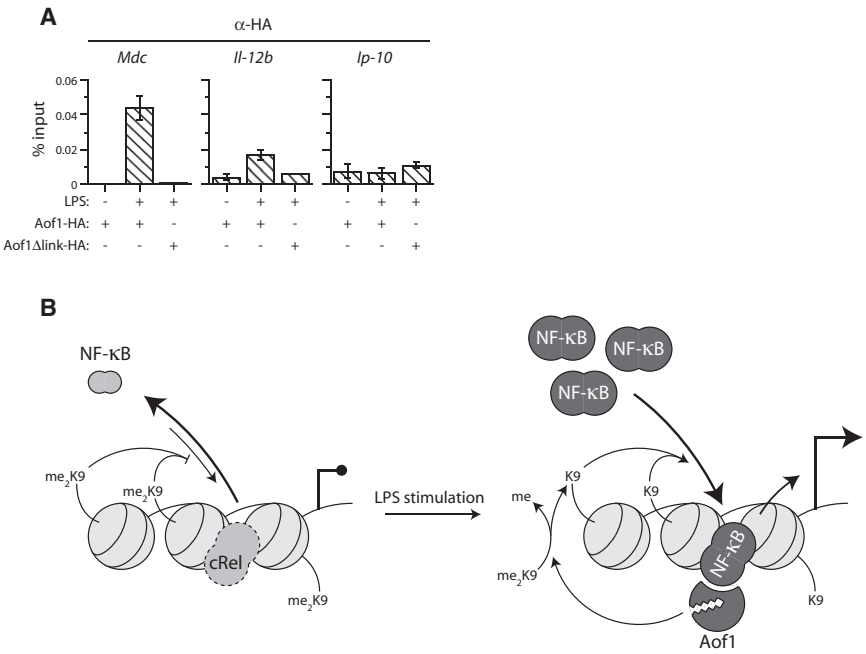

Figure 7. Interaction with c-Rel Is Required for Aof1 Targeting to Promoters

(A) LPS-induced recruitment of HA-tagged, truncated Aof1, or Aof1Δlink to the *Mdc*, *Il12b*, and *Ip10* promoters in retrovirally transduced DCs, measured by ChIP.

(B) Illustration of the events regulating NF-κB recruitment to the *Mdc* and *Il12b* promoters in DCs. (Left) In unstimulated DCs, the low level of nuclear NF-κB allows weak binding of c-Rel to the *Mdc* and *Il12b* promoters, without gene activation; binding of other NF-κB dimers is inhibited by promoter H3K9 methylation. (Right) Upon LPS stimulation, bound c-Rel acts as a targeting signal for Aof1, which is recruited to these promoters through interaction with the NF-κB RHD, and demethylates H3K9me2. Removal of dimethyl H3K9 from promoter regions releases the inhibition of high-level NF-κB recruitment, allowing the strong binding of NF-κB dimers required to drive transcription. (A) Recovery of Aof1 versus Aof1Δlink at *Mdc* promoter $p = 6.1 \times 10^{-7}$, *Il12b* $p = 3.6 \times 10^{-3}$. Error bars indicate standard errors. The results presented here are representative of two experiments.

mutant form of Aof1, which can no longer interact with NF-κB (Aof1Δlink; Figure 5D). Wild-type DCs transduced with retroviruses encoding a tagged, truncated form of Aof1, or of the mutant Aof1Δlink, expressed either protein at similar levels (Figure S7A). However, only wild-type Aof1 was recruited to the *Mdc* and *Il12b* promoters upon LPS stimulation (Figure 7A). Hence, disruption of the interaction between Aof1 and NF-κB prevents its recruitment to target promoters.

DISCUSSION

Here, we have identified Aof1 as an H3K9 demethylase responsible for expression of the strictly regulated *Mdc* and *Il12b* genes in DCs. In resting cells, the promoters of these genes are associated with high levels of H3K9me2, and removal of this mark by the catalytic activity of Aof1 is required for stimulus-induced recruitment of NF-κB transcription factors and the activation of transcription. Intriguingly, recruitment of Aof1 is itself dependent on c-Rel, a member of the NF-κB family. We find c-Rel associated at a low level with the *Mdc* and *Il12b* promoters in unstimulated cells, and its physical interaction with Aof1 is required for Aof1 targeting. Together, these results indicate that DCs activate a subset of tightly controlled genes via a feed-forward circuit, illustrated in Figure 7B, in which NF-κB serves dual functions: initially as a targeting signal to specify promoters for Aof1-driven demethylation, and subsequently as a transcriptional activator at the same promoters.

The key features of this circuit are as follows: (1) initially, in unstimulated cells, c-Rel-containing dimers of NF-κB are weakly associated with specific promoter regions; this is not sufficient to drive gene expression, and binding of other NF-κB dimer species is inhibited by promoter H3K9 methylation. (2) Upon stimulation, recruitment of Aof1 is mediated by interaction with this promoter-associated c-Rel, which functions as its specific targeting signal. (3) Aof1 catalyzes demethylation of H3K9me2 found at promoter regions. (4) Removal of H3K9me2 enables high-level recruitment of NF-κB, including both the c-Rel and p65 subunits, which can now stably bind and drive transcription. We have tested this model by experimentally interfering with each step of the circuit, respectively by using c-Rel knockout cells, by analyzing an interaction-deficient mutant of Aof1 (Aof1-Δlink), by knocking down Aof1, and by treatment with an inhibitor of Aof1 catalytic activity (pargyline).

Mdc and interleukin-12 both signal to T lymphocytes and thereby play a pivotal role in immune responses (Trinchieri, 2003; Yamashita and Kuroda, 2002); thus, their expression must be tightly restricted to both the appropriate stimuli and cell types. This is achieved by repression of the *Mdc* and *Il12b* loci in nonexpressing contexts via methylation of H3K9 (Saccani and Natoli, 2002), and the events described above reveal the mechanism used by LPS-stimulated DCs and macrophages to activate these genes from this transcriptionally silenced state. However, Aof1 itself is broadly expressed in adult mice (Su et al., 2004; and data not shown), indicating that additional controls must contribute to limiting Aof1-dependent expression of *Mdc* and *Il12b* to these particular cell types.

One of our key findings is that the c-Rel subunit of NF-κB is essential to target Aof1, but that it is detectable at only a low level at the *Mdc* and *Il12b* promoters in unstimulated cells. However, at any instant, a sequence-specific transcription factor like NF-κB must either be bound to its particular target sequence in a promoter, or be unbound. Accordingly, a low recovery by ChIP indicates that only a small fraction of promoters have been efficiently crosslinked to c-Rel. Since knockout of c-Rel completely prevents downstream recruitment of Aof1 and p65, and transcription of *Mdc* and *Il12b* (Figure 6), the reduced level

of c-Rel detectable before stimulation must reflect uniformly weaker association with these promoters, in *all* cells. It remains to be determined whether this corresponds to a qualitatively distinct mode of binding, or is simply due to the less-favored equilibrium level of occupancy due to the lower concentrations of nuclear NF-κB. Evidently, the weak association of c-Rel with promoters in unstimulated cells is insufficient to drive transcription, which is induced by more than 10^4-fold in the case of *Il12b* upon high-level recruitment of NF-κB after LPS treatment (Figure 1).

A related issue concerns the mechanism by which LPS stimulation triggers Aof1 recruitment to c-Rel-targeted promoters. The simplest scenario would be that the increased nuclear concentration of c-Rel after stimulation promotes a more sustained promoter occupancy, and that this is required by Aof1 for productive binding. However, numerous parallel signaling pathways are activated downstream of the LPS receptor TLR4 (Beutler, 2000), and an alternative possibility would be that these may induce regulatory modifications to either Aof1 or c-Rel. Moreover, since Aof1 is not recruited to *all* promoters bound by c-Rel (e.g., *Ip10*; Figure 3A), there are likely to be additional regulatory mechanisms that influence the precise choice of promoters targeted. The *Il12b* promoter has been described to undergo stimulus-dependent nucleosome remodeling, which does not require c-Rel (although c-Rel may still play a nonessential role; Weinmann et al., 1999, 2001). While this may occur in parallel to H3K9me2 demethylation by Aof1, it also might promote the independent promoter binding of other factors which could coregulate Aof1 recruitment in concert with c-Rel.

We find that Aof1 exhibits in vitro activity against dimethyl H3K4 in addition to H3K9 (Figure 2; see also Karytinos et al., 2009). This result was unexpected, since methylated H3K4 and H3K9 generally correlate with opposite biological outcomes—transcriptional activation and repression, respectively (Martin and Zhang, 2005). It is likely that in some contexts, demethylation of H3K4 may represent an alternative, biologically relevant activity of Aof1—and indeed Ciccone et al. (2009) find that H3K4me2 levels are increased in Aof1 null oocytes (where wild-type Aof1 expression levels are highest; Su et al., 2004). However, in our experiments, we did not observe any evidence for H3K4 demethylation by Aof1 during gene activation in vivo; whether Aof1 may have a distinct role during gene shutoff, or whether additional factors control its substrate specificity in DCs (analogous to the situation described for Lsd1 [Metzger et al., 2005]), remains to be determined.

A number of lysine demethylase enzymes have been discovered in the past few years; however, although biological roles for some have been described (Agger et al., 2007; Garcia-Bassets et al., 2007; Katz et al., 2009; Lan et al., 2007; Metzger et al., 2005; Okada et al., 2007; Saleque et al., 2007; Tateishi et al., 2009; Wang et al., 2007; Yamane et al., 2006; and reviewed in Cloos et al., 2008), the molecular bases of their in vivo activity are not yet well characterized. A particularly unresolved issue for most histone demethylases (and indeed often for other chromatin-modifying enzymes in general) is whether their recruitment to individual genetic loci requires targeting by sequence-specific transcription factors, or whether, conversely, it is their activity which regulates transcription factor binding and function (Cloos et al., 2008; Lan et al., 2008). These appear to be mutually exclusive alternatives, and some evidence from various systems favors of each of them (see, respectively, Saleque et al., 2007; Tahiliani et al., 2007; Yamane et al., 2006; and Metzger et al., 2005; Tateishi et al., 2009; Wissmann et al., 2007). The molecular events controlling the activation of the *Mdc* and *Il12b* genes which we have described here reconcile these models, in that recruitment of the demethylase Aof1 is targeted by NF-κB, but its activity in turn regulates further NF-κB recruitment. This feed-forward model has the conceptually pleasing property of explaining not only the promoter specificity of Aof1 activity, but also its downstream functional role in transcriptional activation, and we expect that this mode of action will have parallels in many other biological settings.

EXPERIMENTAL PROCEDURES

Cell Culture

Progenitor cells from mouse bone marrow (from wild-type or c-Rel knockout mice) or d14.5 fetal liver (from p65 knockout mice) were retrovirally infected and differentiated in vitro into DCs for 8–10 days in GM-CSF, or into macrophages for 5 days in L929 supernatant. DCs were stimulated with 100 ngml^{-1} LPS; macrophages and J774 cells were stimulated with LPS plus 5 ngml^{-1} IFN-γ. HEK293 cells were transfected with calcium phosphate. See the Supplemental Experimental Procedures for additional details.

Plasmids

Endogenous Aof1 was stably knocked down using shRNAs directed against the coding sequence. For retroviral expression, truncated Aof1 (from Met 271 until the endogenous stop codon, including the swirm and catalytic domains) was HA tagged at the C terminus and cloned into pMY-ires-Tomato. The inducible form was additionally fused at the N terminus to ER^{T2}. For pull-down and BiFC experiments, full-length Aof1 was biotin tagged at the C terminus or fused to V1 and cloned into pCDNA-3. See the Supplemental Experimental Procedures for full details of plasmids used.

Antibodies

Antibodies used are listed in the Supplemental Experimental Procedures.

ChIP and RT-PCR

ChIP was performed as described (Saccani et al., 2002). All PCR was performed using quantitative analysis with gene-specific fluorescent probes. Primer sequences are available on request. mRNA levels are normalized with respect to *Tbp* and expressed relative to unstimulated, wild-type cells.

Demethylation Assays

Aof1-HA was immunoprecipitated from nuclear extracts of LPS-stimulated DCs using monoclonal anti-HA and eluted using 200 mgml^{-1} HA peptide. Full-length Aof1-his$_6$, Gfp-his$_6$, and his$_6$-Lsd1 were expressed in Rosetta *E. coli* and purified by Ni^{2+} chromatography. Eluates from immunoprecipitations, or up to 2 μg of purified proteins, were incubated overnight at 37°C with 500 ng calf histones in a 15 μl reaction volume, according to Lee et al. (2005). Histone modifications were detected by western blotting.

Pull-Down of Biotinylated Aof1

Transfected HEK293 cells were lysed in L1 buffer (50 mM Tris, 2 mM EDTA, 0.1% NP-40, 10% glycerol [pH 8]) and nuclear proteins extracted using L1 +250mM NaCl for 10 min. After centrifugation, the salt in the supernatant was diluted to 100 mM, and extracts were incubated for 2 hr with 20 μl slurry of streptavidin-M280 magnetic beads (Dynal; slurry is 10 mgml^{-1}) per 1 mg total protein. Bound material was washed extensively at room temperature in L1 +150 mM KCl and analyzed by western blotting.

Intracellular Staining

Transduced DCs or trypsinized HEK293 cells were fixed at room temperature for 10 min with 2% formaldehyde and stained in PBS + 0.3% saponin with high-affinity monoclonal anti-HA or anti-V1, followed by fluorescently conjugated secondary antibodies. Fluorescence was measured by flow cytometry.

Statistical Analysis

Statistical differences between experimental groups were analyzed using two-tailed Student's t tests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2010.08.010.

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