Published in final edited form as:
Cell. 2009 July 10; 138(1): 129–145. doi:10.1016/j.cell.2009.05.047.

Control of inducible gene expression by signal-dependent transcriptional elongation

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Abstract

Most inducible transcriptional programs consist of primary and secondary response genes (PRGs and SRGs) which differ in their kinetics of expression and in their requirements for new protein synthesis and chromatin remodeling. Here we show that many PRGs, in contrast to SRGs, have pre-assembled RNA polymerase II (Pol II) and positive histone modifications at their promoters in the basal state. Pol II at PRGs generates low levels of full-length unspliced transcripts, but fails to make mature, protein-coding transcripts in the absence of stimulation. Induction of PRGs is controlled at the level of transcriptional elongation and mRNA processing, through the signal-dependent recruitment of P-TEFb. P-TEFb is in turn recruited by the bromodomain-containing protein Brd4, which detects H4K5/8/12Ac inducibly acquired at PRG promoters. Finally, the permissive structure of PRGs both stipulates their unique regulation in the basal state by co-repressor complexes and enables their rapid induction in multiple cell types.

Introduction

Inducible transcription is triggered by signal-dependent activation of inducible DNA-binding transcription factors, which account for the specificity of gene expression in response to external stimuli. Following their activation and binding to recognition sequences present in target genes, DNA-binding transcription factors can recruit a variety of proteins that enable gene expression, including Polymerase II (Pol II) and chromatin modifiers (Kadonaga, 2004). Recruitment of chromatin remodeling complexes (CRCs) results in remodeling of the nucleosome:DNA template in order to reveal critical regulatory regions, including transcription factor binding sites or the transcription start site (TSS) (Chi, 2004). In addition, transcription factors can recruit histone modifying enzymes, such as histone acetyl transferases (HATs) and histone deacetylases (HDACs), which add or remove covalent modifications on histone tails (Kouzarides, 2007). These histone modifications in turn create binding sites for additional components of the transcriptional machinery. In this way, signal-induced activation of DNA-binding transcription factors couples target gene selection to recruitment of the transcription machinery necessary for gene expression.

Transcription by Pol II consists of two phases: initiation and elongation (Sims et al., 2004). Following recruitment of Pol II to a gene promoter, TFIIH phosphorylates serine 5 (S5) of

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the heptapeptide repeats in the C-terminal domain (CTD) of the Rbp1 subunit of Pol II, allowing initiation to occur (Sims et al., 2004). During initiation, Pol II makes short transcripts, but pauses ~40bp downstream of the TSS prior to elongation (Rasmussen and Lis, 1993). The release of Pol II from pausing occurs following a second phosphorylation event on serine 2 (S2) of the CTD mediated by P-TEFb, a complex composed of the kinase cdk9 and a cyclin T, predominantly cyclin T1 (Sims et al., 2004). P-TEFb also phosphorylates two proteins that regulate elongation, DSIF and NELF, to relieve their repression (Sims et al., 2004). Although phosphorylations of S5 and S2 are thought to happen in rapid succession during most inducible transcription events, this transition may in fact be an important regulatory step.

Indeed, while signal-dependent Pol II recruitment and transcription initiation have been the paradigm for inducible gene expression, several studies suggest that induction of some genes may be regulated post transcription initiation. Early studies of the heat-shock genes in *Drosophila melanogaster* demonstrated that Pol II is pre-loaded at their promoters prior to their induction (Saunders et al., 2006); transcription of these genes is regulated at the transition from initiation to elongation by the signal-induced recruitment of P-TEFb and subsequent phosphorylation of DSIF, NELF, and S2 of Pol II (Lis et al., 2000; Wu et al., 2003). Therefore, in this case the signal-dependent step is not Pol II recruitment, but the transition from Pol II initiation to Pol II elongation. Another well-studied example of signal-dependent elongation of pre-associated Pol II is the induction of *c-myc* expression (Bentley and Groudine, 1986). Furthermore, genome-wide analyses of Pol II binding in murine embryonic stem cells and *Drosophila* cells suggest that Pol II is bound to the promoters of many genes that are not actively transcribed (Guenther et al., 2007; Muse et al., 2007; Zeitlinger et al., 2007). These studies indicate that Pol II recruitment at inactive genes may be more prevalent than previously appreciated. Moreover, they raise several important questions regarding Pol II occupancy at inactive gene promoters, including the nature of the genes that display this feature; the mechanisms for recruiting Pol II in the absence of the signals that induce transcription; the role of inducible transcription factors in regulating transcription at the post-initiation step; and the mechanisms of signal-dependent P-TEFb recruitment and S2 phosphorylation at these genes.

We addressed these questions using Toll-like receptor (TLR)-inducible gene expression in macrophages as a model system. Lipopolysaccharide (LPS) signaling through TLR4 induces several hundred genes, which can be divided into two categories, primary response genes (PRGs) and secondary response genes (SRGs), based on their requirement for new protein synthesis. PRGs are generally induced within an hour of stimulation, whereas the induction of SRGs is delayed due to the requirement for new protein synthesis and chromatin remodeling at their promoters (Ramirez-Carrozzi et al., 2006).

Here we find that transcription of PRGs and SRGs is regulated by distinct mechanisms. Specifically, most PRG promoters have high basal levels of H3K4me3 and H3K9Ac and are pre-associated with S5-phosphorylated (S5-P) Pol II even prior to LPS stimulation. However, unlike *Drosophila* heat-shock genes, S5-P Pol II at PRGs generates full-length unspliced transcripts that are quickly degraded. In response to LPS stimulation, Pol II is S2 phosphorylated by recruited P-TEFb and generates mature full-length transcripts. Furthermore, inducible acetylation of H4K5/8/12 is responsible for P-TEFb recruitment through the adaptor protein Brd4. Finally, we show that PRGs and SRGs utilize distinct regulatory mechanisms in the basal state to ensure their robust, stimulus-dependent transcription.

Results

Chromatin at PRGs is permissive at basal state

The expression kinetics of two representative PRGs (*tnfa*, *tnfaip3*) and SRGs (*il-6*, *lipg*) and their sensitivity to cycloheximide (CHX), a protein synthesis inhibitor, are shown in Figure 1A. Some PRGs are super-inducible in the presence of CHX due to stabilization of their mRNAs in the absence of translation (Shaw and Kamen, 1986). In the basal state (in the absence of stimulation) the majority of LPS-inducible genes are not expressed (Figure 1B). Some PRGs, however, are expressed constitutively (e.g., *nfkbia* and *irf1*) but are further induced upon stimulation with LPS (Figure 1B and data not shown). We refer to these genes as housekeeping PRGs (HK/PRG) to distinguish them from PRGs that are not expressed in unstimulated cells.

Analysis of histone modifications at the two classes of genes revealed that PRGs and SRGs differ markedly in H3K4me3 levels in unstimulated macrophages: while PRGs had high levels of H3K4me3, SRGs lacked this histone mark at the basal state (Figure 1C). As expected (Kayama et al., 2008), H3K4me3 levels were robustly induced at SRG promoters following stimulation, suggesting that this modification correlates with transcriptional activity at SRGs but marks transcriptionally inactive PRGs (Figure 1C). We then analyzed an extensive list of genes including constitutively active housekeeping genes (HKGs), PRGs, and SRGs for levels of H3K4me3 and H3Ac at their promoters. As expected, HKGs had high levels of H3Ac and H3K4me3 at their promoters (Figure 1D, 1E). Many PRGs also had high basal levels of H3K4me3 and H3Ac; in contrast, SRGs were largely negative for these modifications (Figure 1D, 1E). Notably, H3K4me3 and H3Ac levels at PRG promoters ranged from very high, comparable to that of HKGs, to very low, comparable to SRGs (Figure 1D, 1E). The abundance of these marks correlated with the GC content in PRG promoters, such that high levels of the histone modifications were present at GC-rich promoters, and conversely low levels at GC-poor promoters (S. Smale, accompanying manuscript). We thus divided PRGs into two classes according to their GC content, those that are GC-rich (PRG-I) and those that are GC-poor (PRG-II).

Importantly, the high levels of H3K4me3 and H3Ac found at PRG-I promoters were not due to basal TLR signaling, because the levels of these histone marks were similar between wild type and MyD88/TRIF double deficient (MyD88/TRIF−/−) macrophages where TLR signaling is abolished (Figure 1F, 1G).

Signal-independent transcription initiation of PRGs

The presence of the high levels of H3K4me3 and H3Ac modifications at many PRG-I promoters prompted us to examine whether Pol II was associated with these promoters in the basal state. We found high levels of Pol II at the promoters of many inactive PRG-Is in the absence of stimulation. At many PRG-Is, Pol II levels were comparable to those of constitutively active HKGs (Figure 2A); in addition, there was a striking correlation between the levels of Pol II and the GC content of a given PRG promoter (S. Smale, accompanying manuscript). In general, the amounts of pre-associated Pol II correlated with the levels of H3K4me3 and H3Ac histone modifications at PRG-I promoters (Figure 2B). Notably, Pol II was absent from the promoters of SRGs at the basal state, but was inducibly acquired following LPS stimulation (Figure 2A and data not shown).

The presence of Pol II at the promoters of inactive PRG-Is raised the possibility that Pol II may be stalled at these promoters in an elongation-incompetent state. Comparison of S5-P Pol II and S2-P Pol II levels in unstimulated macrophages demonstrated that while S5-P Pol II was present at PRG-Is, S2-P Pol II was very low at these genes (Figure 2C, Supplemental Figure 1A, 1B). However, at HK/PRGs, such as *nfkbia*, S2-P Pol II was clearly detectable.

In addition, the levels of S5-P Pol II and S2-P Pol II at the PRG-I promoters were similar in WT and MyD88/TRIF−/− macrophages, indicating that the S5-P Pol II present at these promoters was not due to basal signaling through the TLR pathway (Figure 2D). Importantly, while the levels of total and S5-P Pol II were comparable between many transcriptionally inactive PRG-I and HKGs, both were further increased at PRG-Is following LPS stimulation (Figure 2E, 2F). Thus, additional recruitment of Pol II also contributes to PRG-I expression, presumably because the amount of pre-associated Pol II is insufficient to sustain multiple transcription rounds at highly inducible genes.

The open chromatin structure of PRG-I promoters could be acquired in a gene-specific manner during macrophage differentiation or it could be an intrinsic property of these genes. Interestingly, a comparison of our data to published data suggested that most PRG-I promoters have high levels of H3K4me3 and Pol II even in ES cells (Guenther et al., 2007). Furthermore, similar to macrophages, Pol II recruitment correlated with the presence of CpG islands in ES cells (Guenther et al., 2007). These data indicate that the permissive state of PRG-Is is established prior to differentiation, possibly by constitutively active transcription factors that bind GC-rich sequences. Sp1 is a particularly good candidate; it is constitutively expressed and active in most cell types, binds GC-rich regions, and can recruit Pol II (Wierstra, 2008). We found that Sp1 was indeed associated with PRG-I promoters that bound Pol II at basal state, but was absent from the promoters of Pol II-negative PRG-IIs and SRGs (Figure 2G). Furthermore, Sp1 knockdown resulted in a dramatic reduction of S5-P Pol II levels at PRG-I promoters, indicating that Sp1 is responsible for Pol II recruitment to these promoters in the basal state (Figure 2H).

PRG transcription is regulated by CTD S2 phosphorylation

The presence of the initiating (S5-P) but not elongating (S2-P) form of Pol II at basal state suggested that PRG transcription is controlled at the level of elongation. Indeed, the S2-P form of Pol II was robustly induced following LPS stimulation of WT but not MyD88/TRIF −/− macrophages (Figure 3A). Moreover, cdk9 and cyclin T1, components of the P-TEFb complex, were recruited following LPS stimulation in WT, but not MyD88/TRIF−/− macrophages (Figure 3B and data not shown). To demonstrate that S2 phosphorylation was mediated by cdk9, we used a cdk9 inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB), and found that it blocked the induction of S2-P Pol II by LPS (Figure 3C). Together, these results suggest that inducible transcription of PRG-Is is regulated, in part, by the inducible recruitment of P-TEFb and subsequent phosphorylation of S2 of the Pol II CTD.

S2-P independent generation of full-length unspliced PRG transcripts

One hallmark of S5-P Pol II is the production of short, initiating transcripts, whereas S2-P Pol II produces full-length transcripts that can undergo further processing, including splicing (Sims et al., 2004). We measured total precursor transcripts from PRGs in unstimulated cells using primers specific to the last exon and compared these to spliced transcripts (Figure 3D). Surprisingly, we detected full-length precursor transcripts generated from many PRG-Is, indicating that S5-P Pol II is competent for processive transcription in the absence of S2 phosphorylation. The levels of these transcripts were low to undetectable for PRG-IIs and SRGs (Figure 3D). HK/PRGs, for example *nfkbia* and *irf1*, generated similar levels of spliced transcripts and total precursor transcripts (Figure 3D), consistent with the presence of S2-P Pol II at these genes (Figure 2C). However, for the majority of PRG-Is, precursor transcripts were detectable whereas spliced transcripts were not. Precursor transcripts corresponded to the sense, not the anti-sense strand, and were inhibited by Actinomycin D (ActD) (data not shown). The amount of precursor transcripts was similar in WT and MyD88/TRIF−/− macrophages, and as expected, spliced transcripts were dramatically

induced at all PRGs following stimulation of WT, but not MyD88/TRIF−/− macrophages (Figure 3E, 3F).

The presence of full-length transcripts from PRG-Is suggested that S5-P Pol II does not pause at these promoters, as it does at heat-shock genes in *Drosophila* (Saunders et al., 2006). However, because Pol II processivity is thought to require S2 phosphorylation (Marshall and Price, 1992), we wondered whether these PRG-I unspliced transcripts are indeed made in the absence of S2 phosphorylation. We treated cells with DRB to inhibit S2 phosphorylation and measured full-length unspliced transcripts from PRG-Is (Figure 3G). We found that the generation of unspliced PRG-I transcripts in unstimulated cells was DRB insensitive, whereas signal-induced generation of spliced transcripts from these genes was DRB sensitive (Figure 3G and data not shown). Consistent with this result, we detected S5-P Pol II, but not S2-P Pol II, at the 3′ ends of PRG-Is (Figure 3H and data not shown). Collectively, these data indicate that PRG-Is are ‘preloaded’ with S5-P Pol II that is competent for full-length transcription, but not co-transcriptional mRNA processing. The signal-dependent recruitment of P-TEFb and phosphorylation of CTD S2 thus results in a switch from nonproductive to productive elongation at PRG-Is.

Constitutively generated PRG-I transcripts were extremely unstable compared to transcripts of active genes (Figure 3I), suggesting that they may not have a function of their own. Their generation could be a consequence of the open chromatin structure of PRG-Is, or they may be responsible for the maintenance of the unique chromatin structure of PRG-Is. To address the latter possibility, we treated macrophages with ActD for 12 hours to inhibit constitutive PRG-I transcription and then assessed the levels of H3K4me3, H3K9Ac, and S5-P Pol II at PRG-Is. We found that both H3K4me3 and H3K9Ac were lost at PRG-Is in the absence of PRG-I transcription, while H3K27me3 at developmental genes was unchanged (Figure 3J–L). In addition, S5-P Pol II was lost from PRG-I promoters over a 12 hour ActD treatment, but not a 2 hour treatment that is sufficient to abolish PRG-I transcription, suggesting that H3K4me3 may maintain Pol II association following its recruitment by Sp1 (Figure 3M, 3N) (Vermeulen et al., 2007). These results suggest that low-level constitutive transcription from PRG-Is is responsible for the maintenance of the permissive chromatin structure at these genes.

Brd4 is recruited to PRG promoters following inducible acetylation of H4K5, H4K8, and H4K12

We next examined the mechanism of inducible P-TEFb recruitment to PRG promoters. P-TEFb can associate with the double bromodomain-containing protein Brd4, suggesting a role of Brd4 in P-TEFb recruitment (Jang et al., 2005; Yang et al., 2005). We found that Brd4 was absent (or present at very low levels) at PRG promoters in unstimulated macrophages, but was inducibly recruited within one hour (Figure 4A). Because Brd4 was reported to bind acetylated H3 (H3K9/14) and H4 (H4K5/12) peptides *in vitro* (Dey et al., 2003), we next asked whether Brd4 recruitment is regulated by signal dependent H3K9/14 and/or H4K5/8/12 acetylation. High basal levels of total acetylated H3 (Figure 1E) and acetylated H3K9 (Figure 4B) at PRG promoters suggested that H3K9/14Ac could not account for inducible recruitment of Brd4. We then tested the levels of H4K5Ac, H4K8Ac, and H4K12Ac in unstimulated macrophages and found that in contrast to H3K9Ac and H3K4me3, these modifications were absent from both PRG and SRG promoters but present at HKGs (Figure 4C). HK/PRGs, such as *nfkbia*, had moderate levels of these marks (Figure 4C). Notably, H4K5, H4K8 and H4K12 were inducibly acetylated following LPS stimulation (Figure 4D), suggesting that H4K5/K8/K12Ac may be a binding platform for the recruitment of Brd4. In contrast, H3K9Ac is not sufficient, and may even be dispensable, for the recruitment of Brd4. Indeed, using *in vitro* histone peptide binding assays, we found that bromodomains 1 and 2 of Brd4 interact with acetylated H4K5/8/12 peptides, but not

H3K9/14 acetylated peptides (Figure 4E and data not shown). Together, these results suggest that signal-dependent acetylation of H4K5/8/12 is responsible for the inducible recruitment of Brd4.

We reasoned that constitutive or inducible recruitment of different HATs to PRG promoters might account for the acetylation of H3K9 and H4K5/8/12, respectively. We analyzed the recruitment of p300/CBP, GCN5, and PCAF, which acetylate residues in H3 (K9, K14, K18) and H4 (K5, K8, K12) *in vitro* and have been implicated in inducible transcription (Kouzarides, 2007). We found that p300/CBP were present at many PRG promoters at the basal state, suggesting that they maybe responsible for constitutive acetylation of H3K9 at these promoters (Figure 4F). Indeed, there was a strong correlation between the levels of H3K9Ac and p300/CBP binding at many PRG-Is (data not shown). In contrast, GCN5 and its close homolog, PCAF, were inducibly recruited to PRG promoters following stimulation (Figure 4F), suggesting that they might be responsible for the inducible acetylation of H4K5/8/12 at PRGs, although other HATs might also participate. The possibility that H4K5/8/12 are coordinately acetylated by the related HATs GCN5 and PCAF is consistent with the fact that H4 K5, K8, and K12, are all embedded within the same ‘GKG’ sequence, while H4K16 and lysines in the H3 tail do not share this sequence motif (Figure 4G). The GKG motif may constitute a recognition platform for GCN5 and PCAF, allowing the acetylation of all three residues by the same or closely related HATs. In contrast, H4K16Ac was not uniformly induced at all PRGs (data not shown), while H3K9 was constitutively acetylated.

Collectively, these results suggest that signal-dependent acetylation of H4K5/K8/K12, possibly mediated by PCAF and/or GCN5, leads to the recruitment of Brd4 to PRG promoters.

Brd4 is required for the recruitment of P-TEFb to PRG promoters

We next asked whether Brd4 is required for the recruitment of P-TEFb to PRG promoters, S2 phosphorylation of Pol II CTD, and subsequent gene expression. We performed siRNA-mediated knockdown of Brd4 in the RAW 264.7 macrophage cell line (RAW), which was similar to primary bone marrow-derived macrophages for all the properties described thus far (data not shown). We confirmed that expression of Brd4 was successfully reduced in cells that had been transfected with two different duplexes complementary to Brd4 (d1 or d2), but not by a scrambled control oligo (sc) (Figure 5A, 5B). We then analyzed the recruitment of P-TEFb to PRG promoters and found that recruitment of cyclin T1 and cdk9 to PRG promoters was significantly inhibited following stimulation in Brd4 knockdown cells (Figure 5C, Supplemental Figure 2). Similarly, S2-P Pol II levels and PRG transcription were reduced in Brd4 knockdown cells (Figure 5D, 5E). We performed additional experiments in mouse embryonic fibroblasts (MEFs), in which we achieved more efficient knockdown, to confirm that Brd4 is required for P-TEFb recruitment to PRGs (Figure 5F–J).

We next tested the role of acetylated H4K5/8/12 in Brd4 recruitment and Pol II elongation. To this end, we used cell permeable peptides corresponding to the H4 tail that were either unmodified, or acetylated at K5 and K12 (Nishiyama et al., 2008). We incubated MEFs with either peptide and found that while the unacetylated H4 peptide had no effect, the H4K5/12Ac peptide inhibited signal-dependent Brd4 recruitment, S2 phosphorylation of Pol II, and PRG transcription (Figure 5K-M). These results underscore the essential function of inducible H4K5/8/12 acetylation in recruiting Brd4, which in turn is necessary for P-TEFb recruitment and subsequent S2 phosphorylation.

NF-κB controls PRG induction post transcription initiation

NF-κB is robustly induced by TLR signaling and majority of TLR-induced genes are NF-κB-dependent (Ghosh and Karin, 2002). NF-κB, like Brd4, has been shown to recruit P-TEFb to artificial promoters to promote transcription (Barboric et al., 2001). However, because Brd4 is required for P-TEFb recruitment to PRG promoters, NF-κB and Brd4 presumably play non-redundant roles in PRG transcription. To test the distinct role of NF-κB in the regulation of PRG transcription, we first confirmed that NF-κB (RelA/p65) was inducibly recruited to PRGs and SRGs following LPS stimulation of macrophages (Figure 6A). In addition, inhibition of NF-κB with the NF-κB inhibitor, BAY 11-7082 (BAY), abolished transcription of PRGs in macrophages (Figure 6B). Using this inhibitor, we further probed the events leading to P-TEFb recruitment and inducible transcription.

We first assayed for the levels of Pol II in the coding regions of PRGs after LPS stimulation, and found it to be significantly reduced in the presence of the NF-κB inhibitor (Figure 6C). We then tested whether the acetylation of H4K5/8/12 and subsequent recruitment of Brd4 were dependent on NF-κB activation. Indeed, we found that recruitment of GCN5, acetylation of H4K5 and H4K12, and recruitment of Brd4 were inhibited in LPS stimulated cells treated with BAY (Figure 6D–F). Lastly, due to the inhibition of each of these upstream events, P-TEFb recruitment to PRG promoters was also blocked by treatment with BAY (Figure 6G). Though inhibition of NF-κB blocked all of the signal-induced events, it did not affect the basal levels of Pol II, S5-P Pol II, H3K9Ac and H3K4me3 (Figure 6H, 6I).

Similar results were obtained using macrophages harboring floxed NEMO alleles, a critical component of NF-κB activating complex (Schmidt-Supprian et al., 2000). Deletion of NEMO by retroviral transduction of Cre resulted in reduced levels of S2-P Pol II at PRGs and inhibition of PRG transcription (Figure 6J, 6K). These findings demonstrate that while NF-κB is not involved in the initial Pol II recruitment and transcription initiation, it controls post-initiation events and is required for productive elongation at PRG promoters.

PRGs are uniquely associated with corepressor complexes

In the basal state, PRG-Is have many of the same features that enable transcription of constitutively active genes, including Sp1 and S5-P Pol II recruitment, but these are insufficient for PRG-I expression. This suggested that constitutive expression of PRG-Is might be actively repressed by HDACs that maintain H4K5/8/12 in an unacetylated form and prevent P-TEFb recruitment in unstimulated cells. Several corepressors, including NCoR, SMRT, CoREST and mSin3A, can recruit HDACs to gene promoters (Cunliffe, 2008). In particular, NCoR associates with gene promoters in the basal state and is subsequently dismissed following stimulation in a process known as “derepression” (Baek et al., 2002; Ogawa et al., 2004; Perissi et al., 2004). Therefore, we tested the levels of NCoR and CoREST at HKG, PRG, and SRG promoters, and found that these proteins were specifically present at PRGs in the basal state, and dismissed following LPS stimulation (Figure 7A, 7B). HDAC1 and HDAC3 had a similar pattern of recruitment to PRGs in the basal state, with loss of these proteins following stimulation (Figure 7C, 7D).

NCoR and CoREST are recruited to promoters via their association with DNA binding transcription factors. In particular, NCoR is recruited to NF-κB-dependent and AP-1-dependent gene targets by p50/p50 and c-Jun/corepressor dimers, respectively, which bind promoters but cannot trans-activate (Baek et al., 2002; Perissi et al., 2004). We therefore tested whether p50 was present at PRG, HKG or SRG promoters and found that it was most abundant at PRG promoters in the absence of p65, indicating the presence of p50/p50 homodimers (Figure 7E, 6A). Thus, productive elongation of PRG-Is by constitutive transcription factors may be prevented by the p50- (or c-Jun-) dependent recruitment of

corepressor complexes, which maintain H4K5/8/12 in an unacetylated state. In contrast, most SRGs have very little or undetectable amounts of these negative regulators, presumably because their transcription is prevented by regulatory nucleosomes, which may occlude NF-κB binding sites.

Cell type and signal specificity of PRG-I induction

Because Sp1 is known to control ubiquitous expression of HKGs, we hypothesized that Sp1-bound PRG-Is may be ubiquitously inducible, whereas the induction of PRG-IIs and SRGs may be cell type specific. We analyzed published gene expression arrays performed on fibroblasts, endothelial cells and keratinocytes stimulated with NF-κB-inducing stimuli (Kodama T; Winsauer et al., 2008; Yano et al., 2008), and found that while PRG-Is were generally induced by all stimuli in all cell types, the induction of PRG-IIs and SRGs tended to be restricted to macrophages (Figure 7F). We performed additional studies on MEFs stimulated with IL-1 and found that 25 of 25 PRG-I genes (100%) were induced in MEFs while only 11 of 23 PRG-IIs and SRGs (48%) were induced (Figure 7F). In addition, there was an almost complete correlation between macrophages and MEFs of PRG-Is that had H3K4me3, H3K9Ac, S5-P Pol II, and Sp1 binding and PRG-II/SRGs that did not (Figure 7G, Supplemental Figure 3A–D). Moreover, PRG-Is are similarly devoid of H4K5Ac and H4K12Ac marks in unstimulated MEFs (Supplemental Figure 3E, 3F). Thus, CpG-rich sequences, active chromatin, and Sp1 binding seem to enable ubiquitous inducibility of PRG-Is by multiple stimuli. In contrast, PRG-IIs and SRGs most likely require lineage-specific transcription factors to establish a permissive chromatin structure, which ensures their cell type specific expression.

Discussion

Signal-dependent recruitment of Pol II to promoters of target genes is one of the key regulated steps in inducible gene expression. However, detailed analyses of several model genes and genome-wide studies of Pol II occupancy have demonstrated signal-independent Pol II recruitment in the absence of gene expression. Given the prevalence of this phenomenon, occurring at many genes and in at least a few cell types, several fundamental questions regarding signal-dependent gene expression emerge: What is the nature of the genes that are pre-associated with Pol II prior to expression? How is inducible transcription of these genes regulated? What are the roles of inducible transcription factors in the induction of these genes? We addressed these questions using LPS-inducible inflammatory gene expression in macrophages, to make the following findings. First, we find that genes pre-associated with Pol II are induced uniquely in the primary response. Second, we show that the induction of these genes is regulated at post-initiation steps, specifically by signal-dependent P-TEFb recruitment via Brd4 binding to H4K5/8/12Ac. We demonstrate that S5-P Pol II at PRG-Is constitutively produces unspliced transcripts, while signal-induced S2 Pol II phosphorylation results in productive elongation that generates mature, protein coding transcripts. Finally, we show that PRGs are uniquely associated with corepressor complexes that presumably prevent their constitutive, signal-independent expression.

We find a dramatic difference in the chromatin configuration of PRG-I and PRG-II/SRG promoters with respect to basal levels of H3K4me3, H3Ac, and promoter-bound Pol II. Interestingly, the status of PRGs correlated closely with the GC content of their promoters, PRG-Is having abundant levels of pre-associated Pol II, H3K4me3, and H3K9Ac and PRG-II/SRGs having little to none. In addition, the levels of positive histone modifications and Pol II at PRG-Is ranged from very high, comparable to that of transcriptionally active HKGs, to very low levels, comparable to PRG-II/SRGs. Thus, GC content may account for the qualitative differences between GC-rich PRG-Is and GC-poor PRG-IIs/SRGs, as well as the quantitative differences between different PRG-Is. Importantly, GC-rich PRG-I

promoters have intrinsically lower affinity for nucleosomes, a property that contributes to their inducible expression in the absence of remodeling (S. Smale, accompanying manuscript). Moreover, we found that Sp1 was required for Pol II recruitment to PRG-I promoters in the basal state (Figure 2G, 2H). Constitutive transcription driven by Sp1-recruited Pol II was required to maintain the permissive status of PRG-I promoters (Figure 3J–N). Thus, the number and distribution of constitutive transcription factor binding sites within promoters presumably also contributes to the quantitative differences between individual PRG-Is.

Multiple lines of evidence suggest that PRG-I transcription is regulated at post-initiation steps. For example, despite similar levels of S5-P Pol II at HKGs and PRG-Is in unstimulated macrophages, PRG-Is were not expressed while HKGs were. This is reminiscent of *Drosophila* heat-shock genes, which undergo abortive initiation due to Pol II pausing (Saunders et al., 2006). However, PRG-Is are regulated differently from heat-shock genes. Specifically, full length, unspliced transcripts of many PRG-Is were detectable at basal state, while the production of mature, processed transcripts was strictly signal-dependent (Figure 3D, 3F). Unspliced transcripts were not generated by low levels of S2-P Pol II, undetectable in our assay, as they were insensitive to DRB treatment (Figure 3G). Thus, although S2 phosphorylation is required for productive elongation and mRNA processing, S5-P Pol II can elongate in the absence of S2 phosphorylation, albeit with low efficiency, to generate unspliced transcripts. These data are in agreement with isolated examples of DRB-insensitive transcription of intron-less genes and histone genes, which are processed by a distinct mechanism (Medlin et al., 2005). Moreover, they are consistent with the role of S2 phosphorylation in the recruitment of splicing factors to Pol II (Sims et al., 2004).

Our data suggest a critical post-initiation checkpoint in the induction of PRG-Is. This is in contrast to SRGs, for which the key regulatory step is the recruitment of Pol II prior to initiation. LPS stimulation most likely amplifies both pre- and post-initiation steps at PRG-Is, as exemplified by the additional recruitment of Pol II following stimulation (Figure 2E, 2F), to allow for multiple rounds of transcription at these highly inducible genes. However, Pol II complexes recruited by constitutive (Sp1) versus inducible (NF-κB) transcription factors play distinct roles in PRG-I regulation: the former generates unspliced transcripts and maintains PRG-I chromatin in an active state, while the latter results in gene expression.

We demonstrate that P-TEFb engagement is a key regulatory step in PRG induction, and that Brd4 is essential for P-TEFb recruitment and CTD S2 phosphorylation at PRGs. These results are consistent with a recent report showing a requirement for Brd4 in the recruitment of P-TEFb to NF-κB-inducible genes following stimulation with LPS or TNFα(Huang et al., 2009). However, these authors describe a gene-specific requirement for Brd4 based on the recruitment of Brd4 to acetylated p65, while our study suggests that Brd4 is likely to be a general regulator of inducible gene expression through binding to H4K5/8/12Ac. Consistent with an essential role for these histone modifications in gene induction, a prior study showed that acetylation of H4K5/8 correlates strongly with gene expression genome-wide (Wang et al., 2008). Interestingly, mutation of any one of the lysines 5, 8, or 12 of H4 to arginines resulted in a similar change in gene expression in yeast, suggesting that these residues are interchangeable (at least in the context of transcription) (Dion et al., 2005). This is consistent with our finding that H4K5, K8 and K12 are all involved in Brd4 recruitment and thus individual mutations at these residues should have the same effect on transcription.

Other histone modifications have been associated with transcriptional elongation, including H2BK123Ub (K120 in humans), H3K36me3, H3K79me3, H2AK119Ub, and H3S10P, either because they map to coding regions, and/or because they are associated with gene

expression (Kouzarides, 2007; Pokholok et al., 2005; Schubeler et al., 2004). However, for some of the modifications (H3K36me3, H3K79me3), there is little evidence to suggest a causal role in transcription elongation, especially because they occur downstream of Pol II S2 phosphorylation (Kouzarides, 2007). Other modifications (H2BK123Ub, H2AK119Ub, and H3S10) may be permissive for, or enhance the rate and efficiency of transcriptional elongation, but have not been directly linked to the recruitment of the essential elongation factor P-TEFb (Ivaldi et al., 2007; Pavri et al., 2006; Stock et al., 2007). Unlike these histone modifications, H4K5/8/12Ac has a unique role in inducible recruitment of Brd4 and P-TEFb and thus appears to be a key switch regulating productive elongation and subsequent transcript processing.

To address this possibility further, we examined the constitutive and LPS-induced recruitment of several HATs. We found that p300/CBP were present at many PRG-Is in unstimulated cells, while PCAF and GCN5 were inducibly recruited to PRGs, suggesting that they may be responsible for the signal-dependent acetylation of H4K5/8/12 (Figure 4F). Consistent with this model, the acetylation of H4K8 at the IFN-β promoter was found to be inhibited by the depletion of PCAF/GCN5, and not p300/CBP (Agalioti et al., 2002). In addition, p300 interacts with S5-P Pol II, consistent with its constitutive recruitment to PRG-Is in unstimulated macrophages, while PCAF associates with S2-P Pol II (Cho et al., 1998).

To account for promoter specificity of PRG-I induction, we hypothesized that inducible DNA-binding transcription factors must contribute, directly or indirectly, to the recruitment of P-TEFb. We found that NF-κB initiates a cascade of events that ultimately leads to the signal-dependent and promoter-specific recruitment of P-TEFb. These data are consistent with reports showing that p65 knockdown inhibits the recruitment of PCAF and cdk9 to the initiation-competent CD80 promoter following stimulation with anti-CD40 (Sharma et al., 2007). Whether NF-κB directly recruits P-TEFb to PRGs is not clear. Addressing this question may require the generation of NF-κB mutants deficient in P-TEFb binding but not in any other function. However, it should be noted that any role for NF-κB in P-TEFb recruitment is clearly not redundant with the essential activity of Brd4. An important difference between NF-κB-mediated and Brd4-mediated recruitment of P-TEFb is that the former can only recruit P-TEFb to promoters of target genes, whereas the latter may function to recruit and maintain P-TEFb throughout the transcribed region, in proximity to elongating Pol II.

Sp1 recruits Pol II to both PRG-Is and HKGs, yet expression of HKGs is constitutive, while expression of PRG-Is is signal-dependent. What keeps PRG-Is inactive in unstimulated cells? We hypothesized that HDAC-containing corepressors would be constitutively present at the promoters of PRG-Is, but not HKGs, to maintain H4K5/8/12 in a deacetylated form thus preventing PRG-I transcription driven by Sp1. We found that NCoR/HDAC3 and CoREST/HDAC1 complexes are bound to PRGs, but not HKGs, in unstimulated cells and dismissed following stimulation (Figure 7A–D). These corepressors are most likely recruited by p50 homodimers (Figure 7E) or c-Jun/corepressors (Ogawa et al., 2004), which may serve as ‘placeholders’ in the absence of stimulation to ensure the inducible expression of PRGs following exchange with active p65:p50 and AP-1 heterodimers. Thus, PRGs may have evolved from constitutive genes by acquiring binding sites for inducible transcription factors, which account for both their signal-dependent expression and basal repression. Previous studies have identified several NF-κB-dependent genes that are regulated by NCoR derepression, but the features that stipulate this regulation were unknown (Baek et al., 2002; Perissi et al., 2004). Here we show that many PRGs are uniquely regulated by corepressor/HDAC complexes, while most SRGs employ other mechanisms, such as the requirement for nucleosome remodeling, to limit their transcription in the basal state. These findings also

emphasize the very distinct roles of constitutive and inducible transcription factors, represented by Sp1 and NF-κB in our system, in controlling PRG induction.

The permissive features of PRG-Is appear to be largely independent of cell-type given that they are shared between macrophages, MEFs, and ES cells (Guenther et al., 2007). We hypothesized that this would enable their inducibility in a variety of cell types, and indeed, PRG-Is were more likely than PRG-IIs or SRGs to be induced in different cell-types by NF-κB-inducing stimuli (Figure 7F). This is consistent with the role of the ubiquitous transcription factor Sp1 in the regulation of PRG-I expression. Interestingly, PRG-Is were also generally inducible by a range of stimuli, including TLR ligands, TNFα, serum, and TPA (S. Smale, accompanying manuscript). In contrast to PRGs, expression of SRGs is cell type specific, consistent with the fact that cell type specific genes are commonly regulated by lineage specific transcription factors, such as PU.1 and C/EBP in myeloid cells (Feng et al., 2008). Thus, the distinct regulation of inducible transcription at PRG-Is and SRGs has important implications for their cell type- and signal-specific expression.

Collectively, our results suggest the following model of inducible transcription (Figure 7H). We propose that the model presented here is not restricted to LPS-inducible gene expression; rather, PRGs in a variety of signal-dependent transcriptional programs may be maintained in a permissive state by constitutive transcription factors and regulated by Brd4- and H4K5/8/12Ac-mediated recruitment of P-TEFb initiated by inducible transcription factors. The utilization of this step allows inducibility in multiple cell types by a variety of signals that converge on the signal-dependent transcription factors utilized by a particular gene. Collectively, these data highlight the biological rationale for the regulatory design of inducible transcription.

Methods

Mice and cells

C57BL/6 (Jackson Laboratory) and F10 MyD88/TRIF−/− mice were maintained at Yale University School of Medicine. Bone marrow-derived macrophages (BMMΦs) were cultured as previously described (Foster et al., 2007). RAW 264.7 macrophages (RAWs) and 293T cells were obtained from ATCC. SV40-transduced mouse embryonic fibroblasts (MEFs) and NEMOflox/flox mice were kind gifts from Sankar Ghosh and Klaus Rajewsky, respectively.

Reagents and Cell Stimulations

LPS (10ng/ml), cycloheximide (CHX, 100μg/ml), 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB, 50μM), and actinomycin D (ActD, 5μg/ml) were purchased from Sigma, BAY 11-7082 (BAY, 30μM) and NF-κB inhibitor II (10μM) from Calbiochem, and mouse IL-1β (IL-1, 10ng/ml) from R&D systems. Antibodies are listed in Supplemental Table 2.

Reverse transcription and Quantitative PCR (RT-qPCR)

As described by (Foster et al., 2007) with additions (Supplemental Data).

ChIP

As described previously (Foster et al., 2007).

siRNA

Cells were transfected with siRNA oligos (25nM; Integrated DNA Technologies) using HiPerfect Reagent (Qiagen) on two consecutive days and used at 72 hours.

Preparation of nuclear lysates

Cells were lysed by resuspension (10mM HEPES pH 7.6, 1.5mM $MgCl_2$, 10mM KCl) and repeated passage through a 22-gauge needle. The lysate was spun at 1000xg and pelleted nuclei were resuspended (20mM HEPES pH 7.6, 2.5% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$) and rotated for 1 hour at 4 degrees. Nuclear lysates were spun at 100,000×g for 30 minutes at 4 degrees to clear the remaining membrane and quantified by BCA analysis (Pierce).

Recombinant proteins

Bromodomain1 (aa55-168) and Bromodomain2 (aa355-457) of Brd4 were cloned and inserted into the pGEX-4T-1 vector (GE Healthcare). Recombinant proteins were purified from lysates of BL21(DE3)pLysS cells induced with IPTG for 4 hours over a column of glutathione sepharose 4B (GE Healthcare). Proteins were eluted with glutathione and dialyzed into 20mM Tris pH 7.5.

Histone peptide binding assays

0.5 ug of biotinylated acetylated H4 (12-379) or unmodified H4 (12-372) peptide (Millipore) was incubated with 0.5ug of recombinant protein for 1 hour in binding buffer (50mM Tris pH 7.9, 150mM NaCl, 0.1% TX-100) and precipitated with NeutrAvidin Sepharose beads (ThermoScientific). Reactions were analyzed by Western blotting with anti-GST.

Histone peptide inhibition assays

MEFs were treated with H4K5/12 acetylated (RRRRRRRRRGGGSGRG[AcK]GGKGLG[AcK]GGAKRH) or unmodified H4 (RRRRRRRRRGGGSGRGKGGKGLGKGGAKRH) peptides (13μM; W.M.Keck Biotechnology Resource Center) for 6–8 hours, stimulated, and analyzed.

Retroviral transduction of BMMΦs

293T cells were transfected with pMSCV.hCD2 (mock) or Cre.pMSCV.hCD2 (Cre) and pCL-eco using Lipofectamine2000 (Invitrogen). 24 hours later, the media was changed and cells were moved to 32 degrees. 48 hours later, viral supernatant was collected, clarified, and incubated with Lipofectamine2000 (4μl/1ml viral sup) for 10 minutes. Bone marrow was prepared, resuspended in viral supernatant, and spun at 2500rpm for 90 minutes at 32 degrees. MCSF-supplemented media was added and cells were plated. The next day, the process was repeated. Five days later, the cells were sorted for hCD2 expression by AutoMACS and used in experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Klaus Rajewsky for NEMOflox/flox cells, Dr. Steven Smale for discussions and communication of unpublished results, and Drs. Tian Chi, David Schatz and Sankar Ghosh for helpful discussions and suggestions. Supported by the Howard Hughes Medical Institute and the NIH (UO-1A161360-01 and AI46688).

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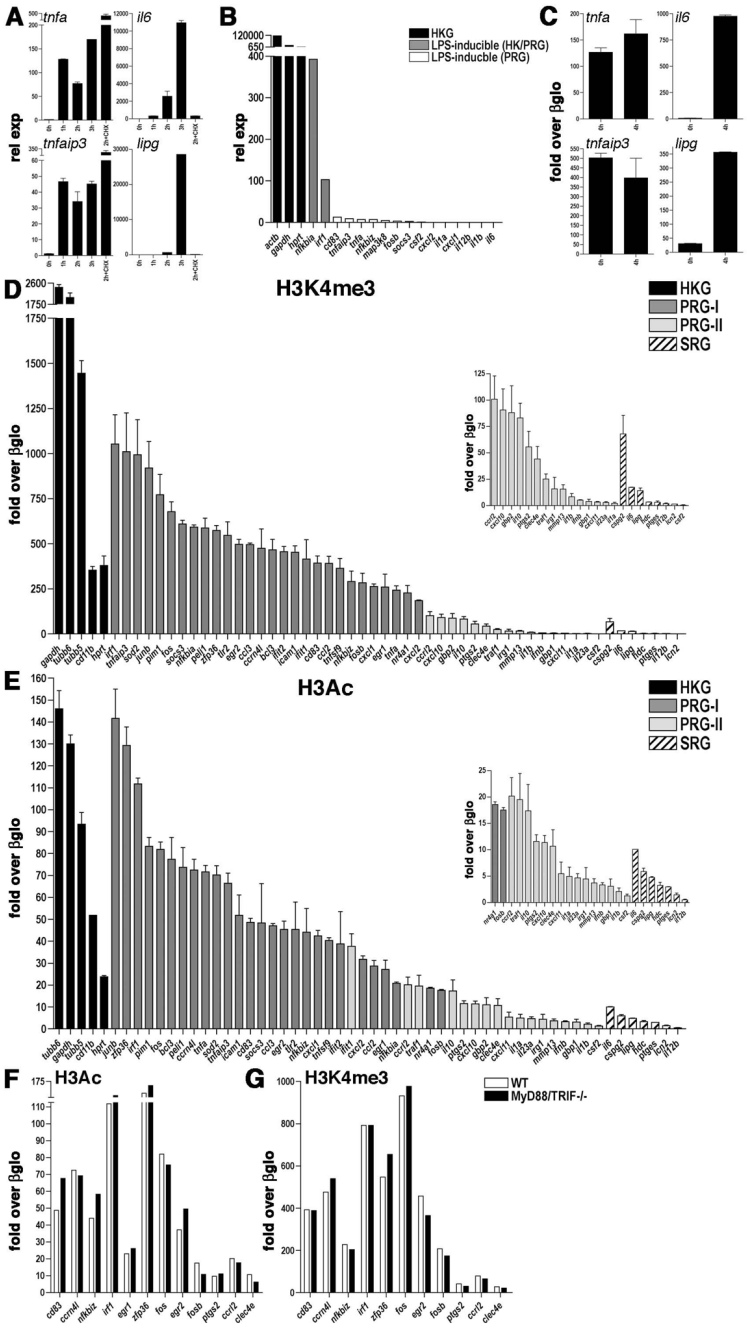

Figure 1. Primary response genes are permissive at the basal state

(A) Bone marrow-derived macrophages (BMMΦs) were treated for 1, 2, or 3 hours with LPS, or 2 hours with LPS+CHX, and analyzed by RT-qPCR. (B) Expression of housekeeping (HKG) and LPS-inducible genes in unstimulated BMMΦs was analyzed by RT-qPCR. (C) BMMΦs were stimulated for 4 hours and analyzed by ChIP (H3K4me3). (D, E) BMMΦs were analyzed by ChIP (H3K4me3, H3Ac). Genes are shaded and ranked as housekeeping (HKG), CpG-rich primary (PRG-I), CpG-poor primary (PRG-II), or secondary (SRG). Graphs magnifying the lower end of the spectrum are shown to the right. (F, G) WT and MyD88/TRIF−/− BMMΦs were analyzed by ChIP (H3Ac, H3K4me3). (A–

G) Data are representative of 3 or more independent experiments. Error bars represent mean +/−SEM.

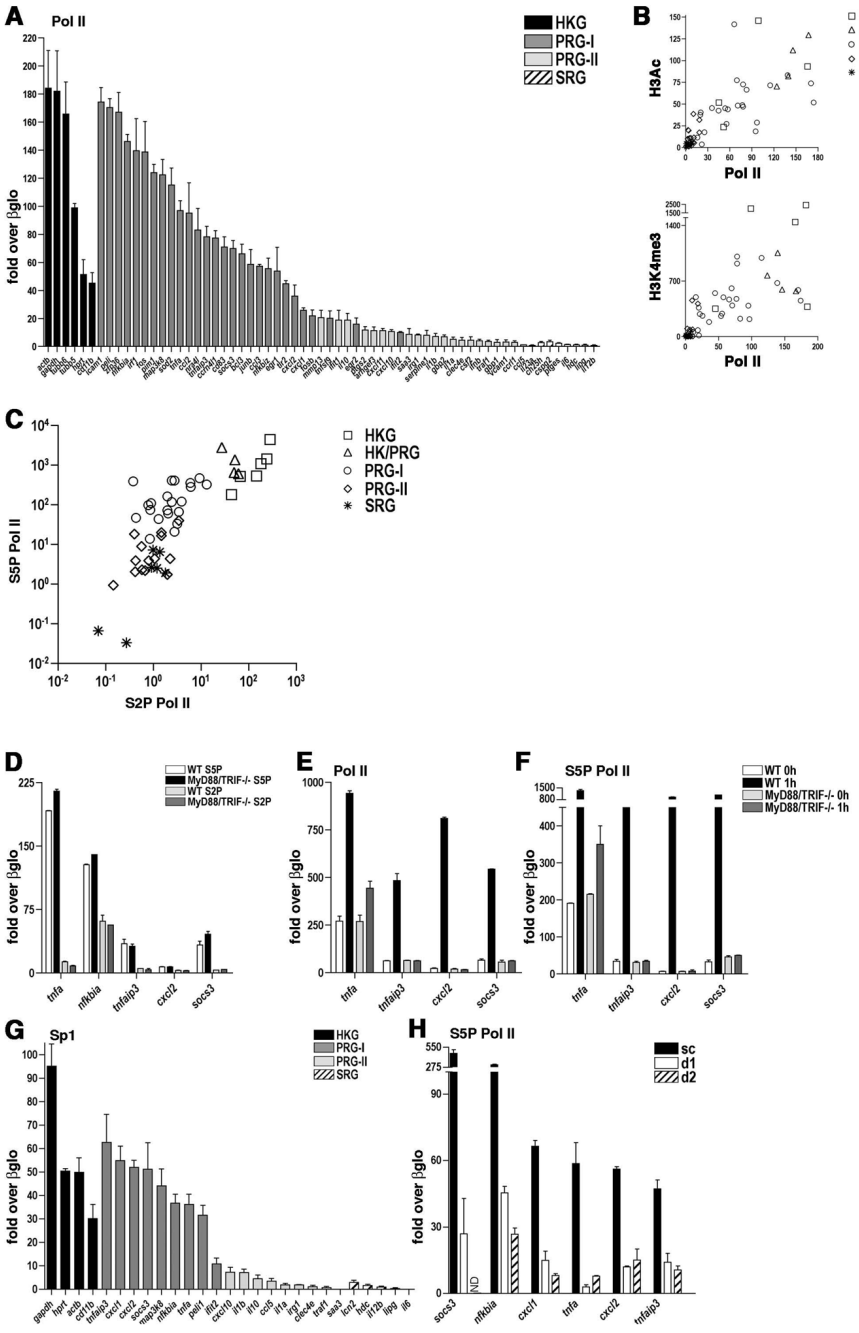

Figure 2. Signal-independent transcription initiation of PRG-Is

(A) BMMΦs were analyzed by ChIP (Pol II). (B) Pol II values (Figure 2A) were graphed against H3Ac or H3K4me3 values (Figure 1D, E). (C) BMMΦs were analyzed by ChIP (S5P Pol II, S2P Pol II). (D) WT and MyD88/TRIF−/− BMMΦs were analyzed by ChIP (S5P Pol II, S2P Pol II). (E, F) WT and MyD88/TRIF−/− BMMΦs were stimulated for 1 hour with LPS and analyzed by ChIP (Pol II, S5P Pol II). (G) BMMΦs were analyzed by ChIP (Sp1). (H) Mouse embryonic fibroblasts (MEFs) were transfected with siRNA oligos targeted to Sp1 (d1,d2) or a scrambled control oligo (sc) and analyzed by ChIP (S5P Pol II). (A–H) Data are representative of 3 or more independent experiments. Error bars represent mean+/−SEM.

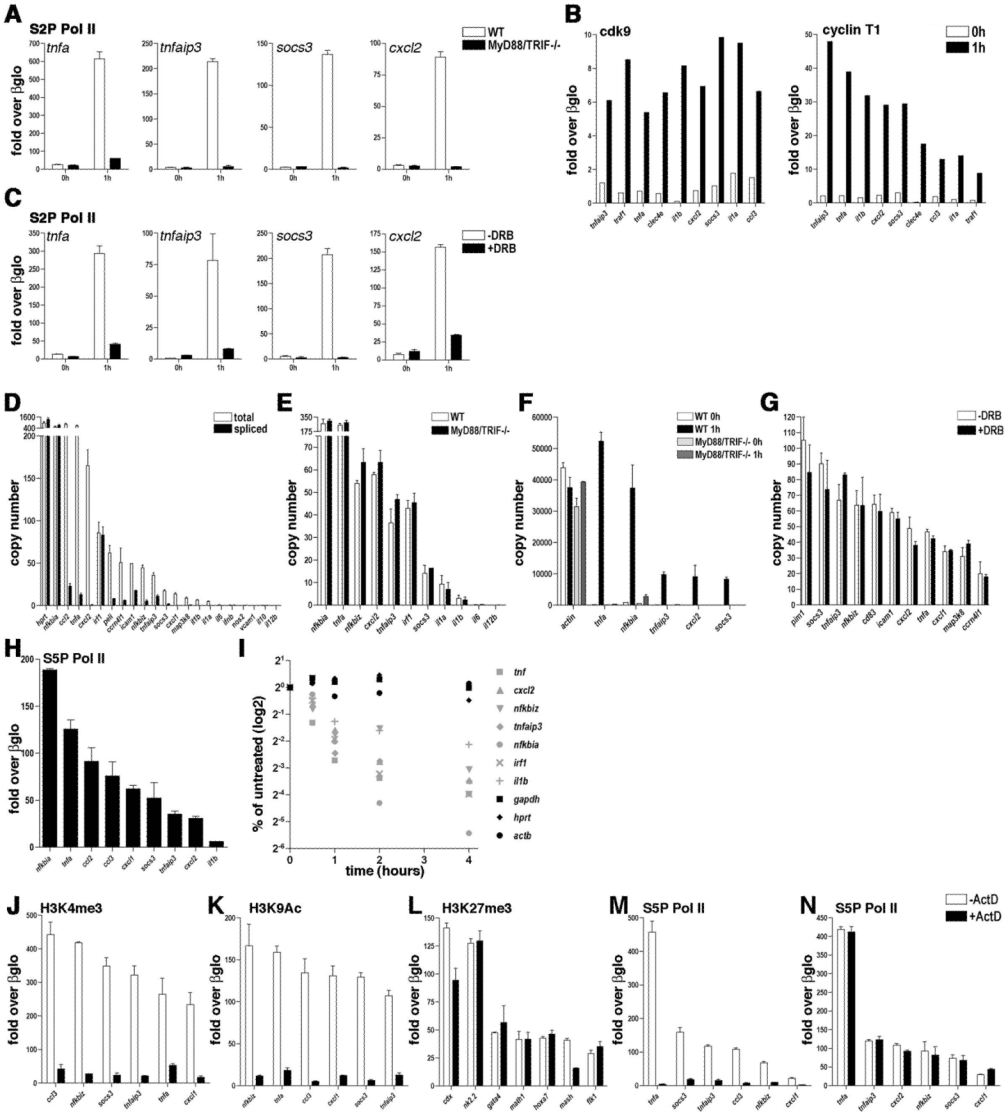

Figure 3. PRG-I transcription is regulated at the level of transcriptional elongation and processing

(A) WT and MyD88/TRIF−/− BMMΦs were stimulated for 1 hour with LPS and analyzed by ChIP at the 3′ end of the gene (S2P Pol II). (B) BMMΦs were stimulated for 1 hour with LPS and analyzed by ChIP (cdk9, cyclin T1). (C) BMMΦs were left untreated (white bars) or treated with DRB (black bars) and then stimulated with LPS for 1 hour and analyzed by ChIP at the 3′ end of the gene (S2P Pol II). (D) BMMΦs were analyzed by RT-qPCR for precursor (within an exon) and spliced (crosses an intron) transcripts. (E) WT and MyD88/TRIF−/− BMMΦs were analyzed by RT-qPCR for precursor transcripts. (F) WT and MyD88/TRIF−/− BMMΦs were stimulated for 1 hour with LPS and analyzed by RT-qPCR for spliced transcripts. (G) BMMΦs were left untreated (white bars) or treated with DRB (black bars) and then analyzed by RT-qPCR for unspliced transcripts (exon to intron). (H) BMMΦs were analyzed by ChIP (S5P Pol II) at the 3′ end of the gene. (I) BMMΦs were left untreated or treated with ActD for the indicated times and analyzed by RT-qPCR for precursor transcripts. (J–M) BMMΦs were treated with or without ActD for 12 hours and analyzed by ChIP (H3K4me3, H3K9Ac, H3K27me3, S5P Pol II). (N) BMMΦs were treated

with or without ActD for 2 hours and analyzed by ChIP (S5P Pol II). (A–N) Data are representative of 3 or more independent experiments. Error bars represent mean+/−SEM.

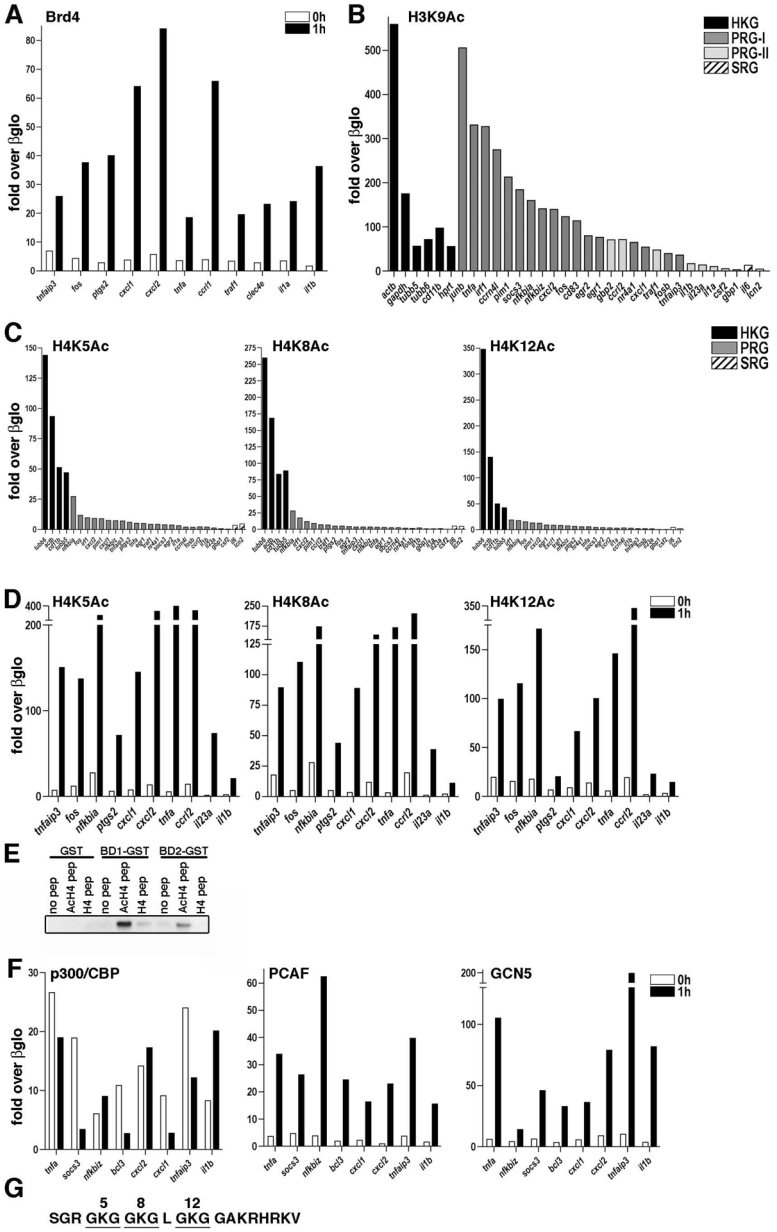

Figure 4. Brd4 is recruited to PRG promoters following inducible acetylation of H4K5/8/12

(A) BMMΦs were stimulated for 1 hour with LPS and analyzed by ChIP (Brd4). (B, C) BMMΦs were analyzed by ChIP (H3K9Ac, H4K5Ac, H4K8Ac, H4K12Ac). (D) BMMΦs were stimulated for 1 hour with LPS and analyzed by ChIP (H4K5Ac, H4K8Ac, H4K12Ac). (E) Histone peptide binding assays were performed with GST fusion proteins of bromodomain 1 or 2 of Brd4 (BD1, BD2) and either no peptide, AcH4 peptide, or unmodified H4 peptide. Reactions were analyzed by Western blotting with anti-GST. (F) BMMΦs were stimulated for 1 hour with LPS and analyzed by ChIP (p300/CBP, PCAF, GCN5). (G) Schematic of the H4 tail. (A–F) Data are representative of 3 or more independent experiments. Error bars represent mean+/−SEM.

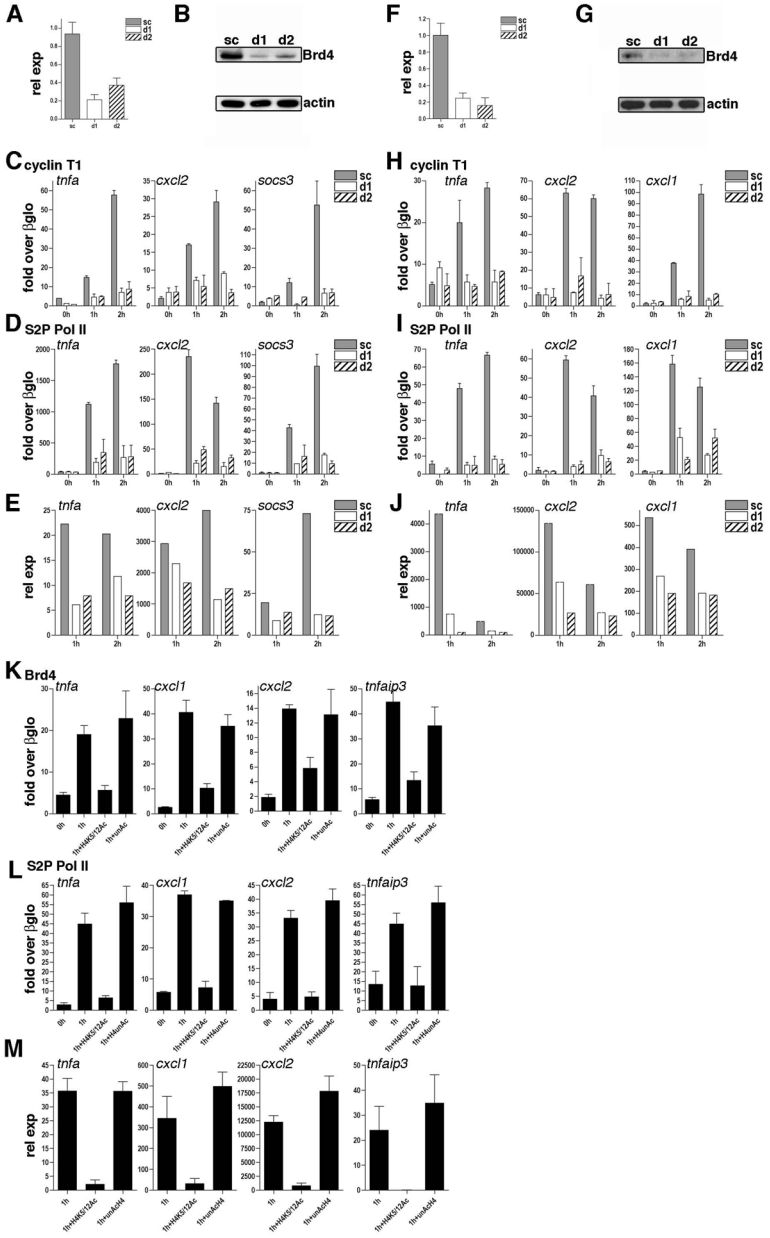

Figure 5. Brd4 is required for P-TEFb recruitment to PRGs

(A, F) BMMΦs (A) or MEFs (F) were transfected with siRNA oligos targeted to Brd4 (d1,d2) or a scrambled control oligo (sc), and analyzed by RT-qPCR for Brd4 expression. (B, G) BMMΦs (F) or MEFs (G) were transfected as in (A) and nuclear lysates were analyzed by Western blotting for Brd4 or actin. (C–J) BMMΦs (C–E) or MEFs (H–J) were transfected as in (A), stimulated for 1 or 2 hours with LPS (BMMΦs) or IL-1 (MEFs) and analyzed by (C, D and H, I) ChIP (cyclin T1, S2P Pol II) or (E, J) RT-qPCR. (K–M) MEFs were treated with H4K5/12Ac or unacetylated H4 (unAcH4) peptides, stimulated for 1 hour with IL-1 and analyzed by (K, L) ChIP (Brd4, S2P Pol II) or (M) RT-qPCR. (A–M) Data are representative of 3 or more independent experiments. Error bars represent mean+/−SEM.

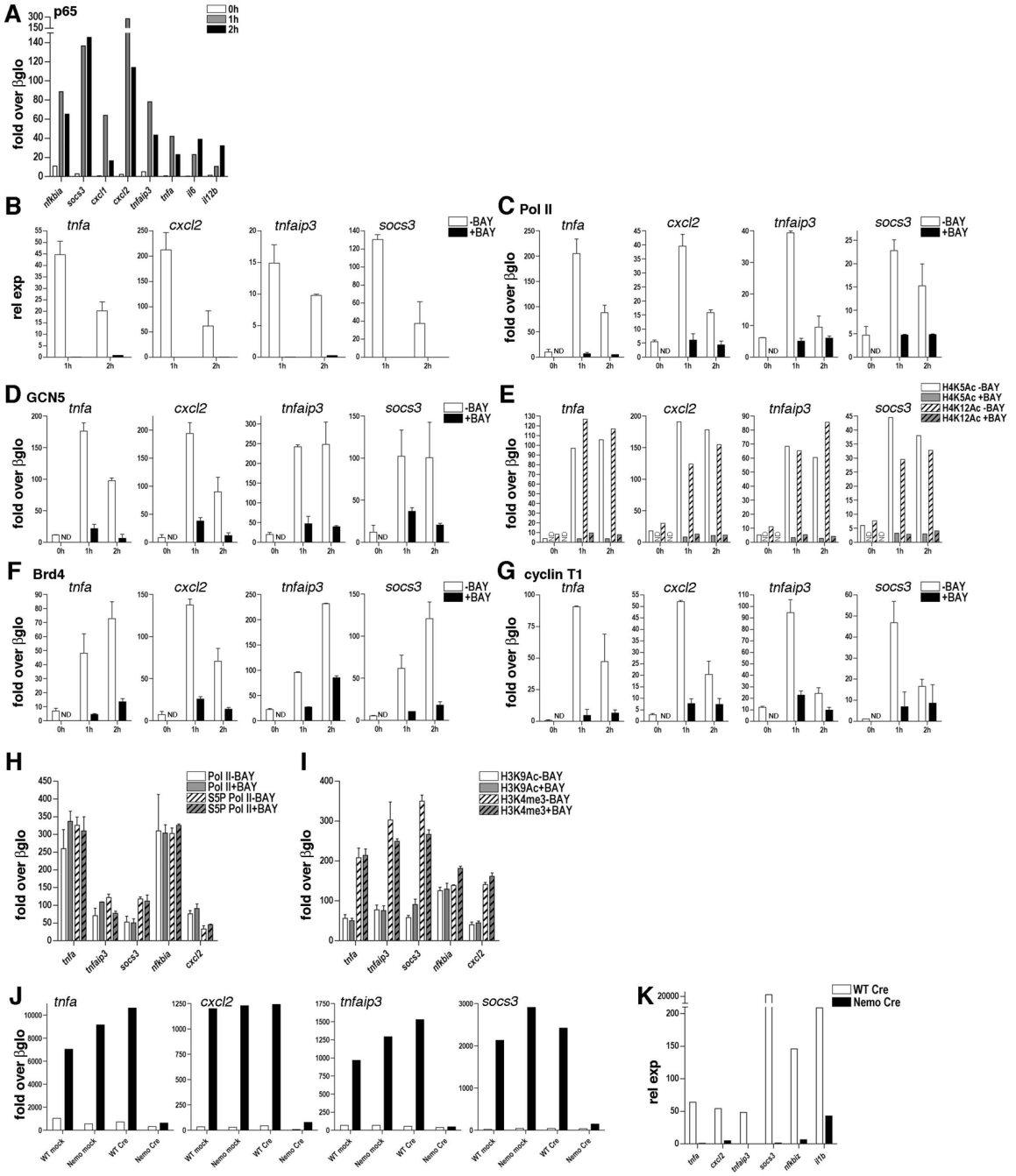

Figure 6. Signal-dependent NF-κB activation is required for inducible H4K5/8/12 acetylation, Brd4 recruitment and P-TEFb recruitment

(A) BMMΦs were stimulated for 1 or 2 hours with LPS and analyzed by ChIP (p65). (B–G) BMMΦs were left untreated (white bars) or treated with BAY-11087 (black bars), stimulated with LPS for 1 or 2 hours, and analyzed by (B) RT-qPCR or by (C–G) ChIP at the 3′ end (total Pol II) or at the TSS (GCN5, H4K5Ac, H4K12Ac, Brd4, cyclin T1). . ND indicates not done. (H, I) BMMΦs were treated with or without BAY-11087 and NF-κB inhibitor II and analyzed by ChIP (total Pol II, S5P Pol II, H3K9Ac, H3K4me3). (J) BMMΦs from wild-type and NEMOflox/flox mice were transduced with mock retrovirus or retrovirus expressing Cre. Cre-expressing (WT Cre, Nemo Cre) and non-expressing (WT

mock, Nemo mock) cells were stimulated for 1 hour with LPS and analyzed by ChIP (S2P Pol II). (K) Wild-type and NEMOflox/flox BMMΦs were transduced as in (J) and Cre-expressing cells were stimulated for 1 hour with LPS and analyzed by RT-qPCR. (A–K) Data are representative of 3 or more independent experiments. Error bars represent mean+/−SEM.

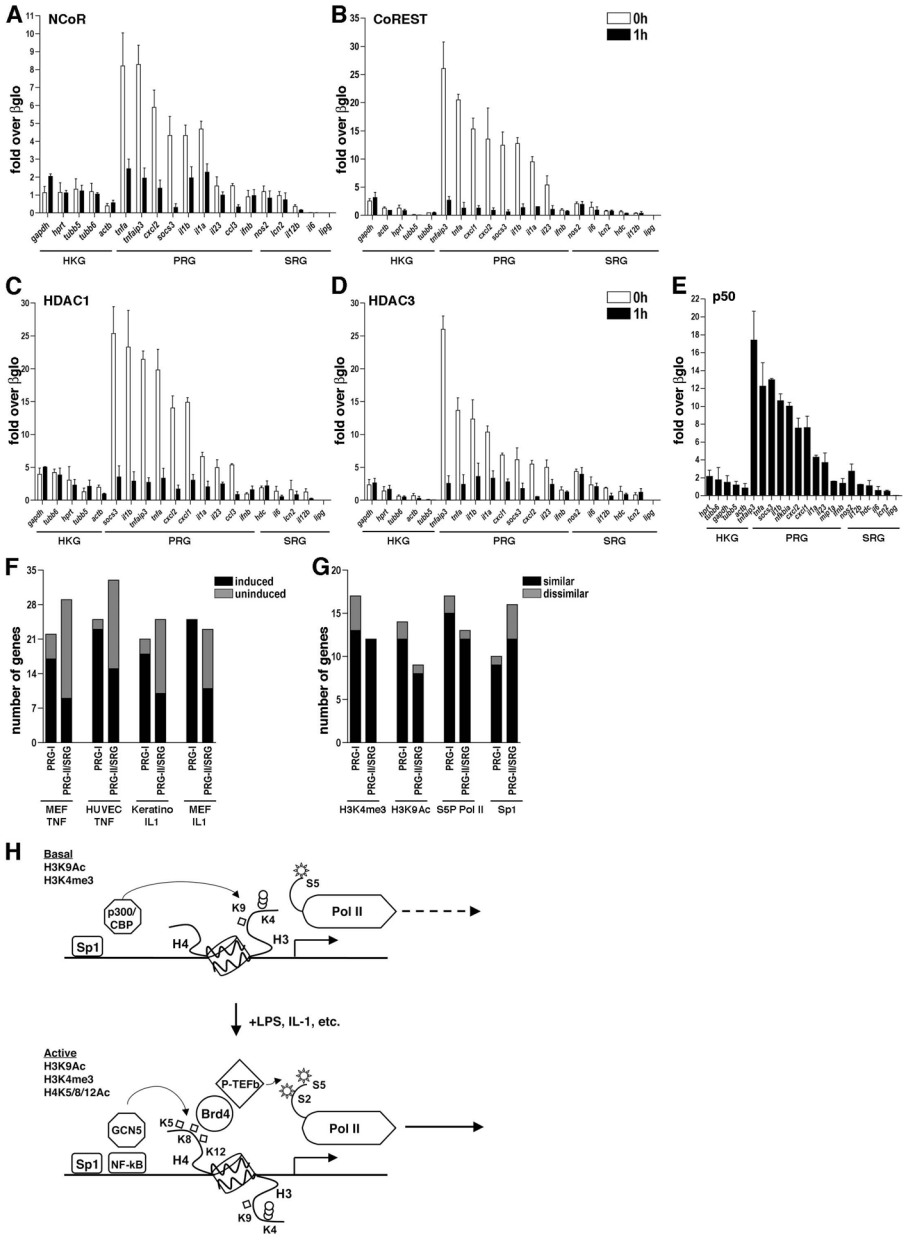

Figure 7. Implications for the inducible regulation of PRG-1 expression

(A–E) BMMΦs were stimulated for 1 hour with LPS and analyzed by ChIP (NCoR, CoREST, HDAC1, HDAC3, p50). Error bars represent mean of triplicate values+/−SEM. (F) MEFs were stimulated with IL-1 for 1 hour and analyzed by RT-qPCR. This data and data from three published microarrays was graphed as the number of genes induced following stimulation in MEFs, HUVECs, or Keratinocytes of macrophage LPS-inducible PRG-I and PRG-II/SRGs. A list of analyzed genes appears in Supplemental Table 1. (G) MEFs were analyzed by ChIP (H3K4me3, H3K9Ac, S5P Pol II, Sp1). Data is graphed as the number of genes in MEFs that are similar in H3K4me3, H3K9Ac, S5P Pol II, or Sp1 status to those genes in macrophages for either PRG-I or PRG-II genes. (H) Model of LPS-induced PRG-I transcription (see text for description). Squares indicate acetylated residues, circles indicate methylated residues, and stars indicate phosphorylated residues.