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Mechanism of gene regulation by STATs and interacting transcriptionfactors

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Abstract

According to recent studies PRGs (primary response genes) that are expressed rapidly after providing the activating stimulus are characterized by the initial presence of paused polymerases and high levels of H3K4me3 at their promoter. In contrast, SRGs (secondary response genes) are regulated by the active recruitment of RNA Pol II (RNA polymerase II), requiring the interplay of transcription factors and co-factors to induce transcription, and are therefore delayed in their expression, compared to PRGs. To examine the mechanisms driving the expression of SRGs, we focused on the regulation of STAT-regulated genes, stimulated by IFNγ (Interferon) or IFNβ. Effects of the latter were studied in macrophages infected with the Gram+, facultative intracellular bacterium *Listeria monocytogenes*. Infected cells produce IFNβ to synergize with additional pathways in the upregulation of host-defense genes.

To examine the role of STAT1 (signal transducer and activator of transcription) in the regulation of IFNγ-induced SRGs, we focused on the regulation of the *Gbp2* (guanylate binding protein) gene. Previous work in the lab had shown that *Gbp2* belongs to a subgroup of genes that require both STAT1 and IRF1 (interferon regulatory factor) for transcriptional induction upon IFNγ treatment and that recruitment of HDACs (histone deacetylase) and CBP/p300 (CREB – cyclic AMP response element binding protein – binding protein) to the *Gbp2* promoter directly depends on the presence of STAT1 and its phosphorylation on Ser727. We could further demonstrate that IRF1 binding was needed for the recruitment of RNA Pol II. Moreover, IRF7 was involved in the regulation of *Gbp2* and other ISRE (interferon stimulated response element)-driven genes after IFNγ treatment.

This finding is novel, since IRF7 was not known to be expressed upon IFNγ treatment and to participate in the regulation of interferon-induced genes. Contrasting IRF1, IRF7 function in this context relied on the presence and constitutive activity of the S/T kinase TBK1 (TANK - TRAF family member associated NFκB – nuclear factor of kappa light polypeptide gene enhancer in B-cells - activator - binding kinase). TBK1-mediated IRF7 phosophorylation was required for GBP2 expression, but did not alter IRF7 binding to the *Gbp2* promoter. Expression of the *Socs1* gene did require the presence of IRF7, but not its phosphorylation by TBK1. In contrast to TBK1, the related S/T kinase IKKε (IκB – inhibitor of NFκB - kinase) repressed IRF7 activity to induce GBP2 expression. These findings indicate that phosphorylated, aswell as unphosphorylated IRF7 participates in the regulation of ISRE-driven gene expression after IFNγ treatment. A distinct subset of SRGs requires the cooperation of signals derived from IFNs as well as additional signals derived from the infecting pathogen to acquire full-fledged transcriptional activity. Paradigmatic for this group is the *Nos2* gene, encoding iNOS (inducible nitric oxide synthase), which is highly upregulated in macrophages during infections with various pathogens. In this study we show that the transcription factors NFκB and ISGF3 (interferon stimulated gene factor - a complex of STAT1, STAT2, and IRF9 which is formed and activated in response to type I IFNs, like IFNβ) cooperate in the induction of iNOS and iNOS like genes in macrophages, infected with *Listeria monocytogenes*.

We were able to demonstrate that, NFκB preceded ISGF3 at the *Nos2* promoter and generated a transcriptional memory effect by depositing basal transcription factor TFIIH with the associated CDK7 (cyclin dependent kinase) kinase for serine 5 phosphorylation of the RNA Pol II - CTD (carboxyterminal domain). Moreover, p-TEFb (positive transcription elongation factor), a complex containing the kinase CDK9, which is required to release the Pol II enzyme from the NELF (negative elongation factor) dependent elongation block, was similarly deposited in an NFκB dependent manner. Deposition of CDK7-TFIIH at the proximal *Nos2* promoter, was followed by TBP (TATA binding protein) and RNA Pol II binding, which were found to be recruited in an ISGF3 dependent manner. Hence, the two transcription factors cooperate in our infection model by assembling different components of the PIC (pre-initiation complex), and the RNA-Pol II enzyme itself, in order to induce iNOS transcription. Taken together our results indicate that at least for some SRGs not only the recruitment of RNA Pol II is a limiting step to induce gene expression, but also the recruitment of co-factors follows strict rules and results from the division of labor between different transcription factors. According to our findings this hypothesis is valid for transcription factors that are activated within a single pathway, such as STAT1 and IRF1, but also those activated through different signaling pathways, like NFκB and ISGF3. Collectively these proteins converge at the promoters of a subset of infection-induced genes to assemble the PIC – Pol II complex, and engage the Pol II enzyme in active transcription.

Einleitung

Auf der Basis existierender Publikationen, werden zwei große Gruppen von Genen, nach dem Prinzip ihrer Regulation, voneinander unterschieden. Eine spezielle Kathegorie von Genen wird im Zuge einer ersten/primären Antwort auf einen Stimulus gebildet und kann als PRGs (primary response genes) zusammengefasst werden. Als Unterscheidungskriterium von jenen Genen die zur Gruppe der sekundär exprimierten Gene (SRGs – secondary response genes) gerechnet werden, wird das Vorhandensein der RNA Polymerase II und die Histonmodifikation H3K4me3 am Promoter der betreffenden Gene, bereits vor dem Eintreffen des Stimulus, herangezogen. PRGs zeichnen sich durch einen sehr hohen Anteil, sowohl von RNA Pol II als auch H3K4me3 aus, wohingegen SRGs erst durch einen aktiven Prozeß im Verlauf des Stimulierens, das Binden der RNA Pol II am Promoter des zu regulierenden Gens gewährleisten müßen, einhergehend mit gesteigerter H3K4me3. Dieser zusätzliche Aufwand und das hierfür benötigte Vorhandensein von mehreren aktiven Transkriptionsfaktoren, ist in weiterer Folge, für die späte Expression dieser Gruppe von Genen, im Vergleich zu der Gruppe der PRGs, verantwortlich. Um die Mechanismen der Regulation dieser spät-exprimierten Gene zu analysieren konzentrierten wir uns auf jene Gene, die als Antwort auf Interferone exprimiert werden, und somit unter der Kontrolle der STAT (Signal transducers and activators of transcription) Familie von Transkriptionsfaktoren liegen. Zum Einen behandelten wir Zellen in unseren Untersuchungen mit IFNγ (Interferon), zum anderen analysierten wir die Effekte einer weiteren Klasse von Interferonen, der Typ I Interferone am Beispiel IFNβ, im Zuge der Infektion von Makrophagen mit dem Gram+, fakultativ intrazellulärem Bakterium *Listeria monocytogenes*. Makrophagen produzieren als Antwort auf eine Infektion mit Listerien große Mengen IFNβ, welches wiederum, im Zusammenwirken mit anderen Signalen, für die Regulation von Genen benötigt wird, die an der Abwehr der Infektion beteiligt sind.

Als Paradebeispiel für die große Gruppe der IFNγ regulierten Gene betrachteten wir die Regulation von *Gbp2* (guanylate binding protein). Aufbauend auf bereits bestehende Arbeiten im Labor, welche STAT1 und IRF1 (interferon regulatory factor) als die wichtigsten Transkriptionsfaktoren identifizierten, und darüber hinaus die Bedeutung der Ser 727 Phosphorilierung von STAT1, für die Rekrutierung von HDACs (histone deacetylases) und CPB/p300 (CREB – cyclic AMP response element binding protein – binding protein) an den *Gbp2* promoter beschrieben, entdeckten wir die Abhängigkeit der RNA Pol II Rekrutierung von der Anwesenheit von IRF1 am Promoter. Zusätzlich zu IRF1 identifizierten wir IRF7 als noch nicht beschriebenen Schlüßelfaktor für die Regulation von *Gbp2*, wie auch andere über einen ISRE (interferon stimulated response element) Konsensus regulierte Gene, im Zuge der IFNγ Antwort. Im Gegensatz zu IRF1, benötigt IRF7 für seine Aktivierung, auch im Kontext der IFNγ Antwort, die Aktivität der S/T Kinase TBK1 (TANK - TRAF family member associated NFκB – nuclear factor of kappa light polypeptide gene enhancer in B-cells - activator - binding kinase). IRF7 Phosphorylierung durch TBK1 zeigte sich als notwendig für die Regulation von *Gbp2* und anderen Genen, deren Abwesenheit führte jedoch nicht zu einem Verlust der IRF7 Promoterbindung. SOCS1 Expression zeigte sich im Gegensatz zu GBP2, unabhängig von der IRF7 Phosphorylierung durch TBK1. Die verwandte S/T Kinase IKKε (IκB – inhibitor of NFκB - kinase) hatte im Gegensatz zu TBK1, einen reprimierenden Effekt auf die GBP2 Expression. Zusammengenommen zeigen diese Daten, dass phosphoryliertes wie unphosphoryliertes IRF7 an der Genregulation von ISRE regulierten Genen, nach Behandlung mit IFNγ, beteiligt ist.

Eine weitere Gruppe von Genen innerhalb der SRGs benötigt zusätzlich zu den Faktoren, welche über Interferone aktiviert werden, noch die Aktivierung von Transkriptionsfaktoren über andere Signaltransduktionswege, und deren Zusammenspiel um eine effiziente Expression zu erfahren. Ein Beispiel für diese speziell regulierten Gene ist das *Nos2* Gen, welches für die Bildung der iNOS (inducible nitric oxide synthase) verantwortlich ist und nach Infektion durch verschiedenste intrazelluläre Pathogene, wie Listerien, gebildet wird. Es benötigt die Aktivierung von NFκB im selben Ausmaß wie die IFN abhängige Aktivierung von ISGF3 (interferon stimulated gene factor – ein Komplex aus STAT1, STAT2, und IRF9 welcher im Zuge der Typ I IFN Signaltransduktion aktiviert wird, zum Beispiel IFNβ).

Im Zuge der detailierten Analyse des Mechanismus der iNOS Regulation, konnten wir zeigen, dass die Bindung von NFκB an den *Nos2* Promoter jener von ISGF3 vorangeht. NFκB zeichnet dafür verantwortlich, dass der basale Transkriptionsfaktor TFIIH mit der assoziierten Kinase CDK7 (cyclin dependent kinase), welcher die RNA Pol II am Ser5 des CTD (C-terminal domain) phosphoriliert, an den Promoter rekrutiert, und dort verankert wird. Dies erzeugt einen transkriptionellen Gedächtniszustand, der bestehen bleibt, auch wenn NFκB den Promoter wieder verlassen hat. Neben CDK7-TFIIH konnte auch das Rekrutieren von CDK9 p-TEFb (positive transcription elongation factor) als NFκB abhängig gezeigt werden. CDK9 aktivität ist notwendig um die NELF (negative elongation factor) abhängigen Blockade der Polymerase am Übergang zur Elongation aufzuheben. Dem Binden von CDK7 an den Promoter folgt die Bindung von TBP (TATA binding protein), und der Polymerase selbst, welche beide auf das Interferonsignal und die daraus resultierende Aktivierung von ISGF3 angewiesen sind. Die so geschaffene RNA Pol II Bindung kann schließlich die iNOS Transkription auslösen. Die beiden Transkriptionsfaktoren kooperieren in der Regulation des *Nos2* Gens durch das kombinierte Rekrutieren von verschiedenen Faktoren des PIC (pre initiation complex) und der RNA Pol II selbst.

Zusammenfassend zeigen unsere Ergebnisse, dass nicht nur das Rekrutieren der Polymerase selbst ein limitierender Schritt zur Transkription ist, sondern auch das Rekrutieren von Kofaktoren einer strengen Kontrolle unterliegt, und von verschiedenen Transkriptionsfaktoren gesteuert wird. Diese Hypothese findet, anhand unserer Daten, nicht nur Anwendung bei Genen die im Zuge eines einzigen Stimulus exprimiert werden, wie im Fall von IFNγ aktiviertem STAT1 und IRF1, sondern gilt im selben Maße auch für Gene deren Expression die Zusammenarbeit mehrerer Signaltransduktionswege bedingt, wie am Beispiel der iNOS für NFκB und ISGF3 gezeigt. Die getesteten Transkriptionsfaktoren vermitteln die Bindung von Faktoren des PIC und der Polymerase selbst um diese dann zur Expression zu veranlassen

INTRODUCTION

Transcription and RNA-Pol II function

Every transcriptional process leading to the expression of protein coding genes needs the activity of RNA-Pol II enzyme. In prokaryotes, one DNA dependent RNA polymerase is sufficient to transcribe all genes into RNA molecules. The labor of transcription in eukaryotes, however, is divided between three distinct multi-subunit enzymes. Each one of these three is dedicated to specific gene types: Pol I (polymerase I) transcribes exclusively genes encoding 18S and 28S rRNA; Pol III transcribes short genes including tRNA and 5S rRNA genes; and Pol II is the one responsible for the transcription of protein coding genes and snRNAs (small nucleolar RNAs). How the activity of mainly Pol II is regulated and what factors are involved in the different steps of transcription will be the focus of the following chapter.

The Pol II - CTD (carboxy terminal domain)

Pol II is made up of 12 different subunits named Rbp1-12 that together comprise a ~0,5 MDa functional machinery. The different subunits are structurally and functionally conserved from yeast to humans. It was first noted in 1985 that the largest subunit of yeast Pol II, Rbp1, has a very unusual domain at the C-terminus which comprises tandem repeats of the heptapeptide sequence $Y_1S_2P_3T_4S_5P_6S_7$, flanked by a linker sequence at the more N-terminal end of the CTD and a divergent C-terminal sequence (Allison et al., 1985; Chapman et al., 2008). The increased number of repeats corresponds to a larger complexity in its regulation and varies from 26 in yeast, 32 in nematodes, 45 in Drosophila to 52 in mammals.

The repeats in yeast Pol II are perfectly conserved throughout the CTD. The heptads of mammalian CTDs can be further divided into a domain containing identical heptads (proximal domain) and heterogeneous heptads (distal domain), which contain a lysine instead of the serine at position 7 of the consensus repeat (Chapman et al., 2008).

Possible modifications at the Pol II CTD

Phosphorylation has been detected on tyrosine, threonine and all three serines of the CTD repeat in vivo. Rbp1 exists in two forms that migrate differently in SDS PAGE: in the form designated IIa, the CTD is hypophosphorylated, whereas the form designated IIo has a hyperphosphorylated CTD. IIa preferentially associates with the PIC (preinitiation complex) at the promoter, and any phosphorylation of the CTD at this point prevents recruitment and initiation. For that reason early experiments revealed a role for glycosylation of the serines, as well as the threonine residues, which prevents CTD phosphorylation before onset of transcription. The glycosylation has to be removed from the residues in order to become phosphorylated (Kelly et al., 1993). In addition, peptidyl-prolyl bonds, which exist in either *cis* or *trans* conformation, can be converted to all-*trans* by PPIases (peptidyl-prolyl *cis*/*trans* isomerases). The function of CTD modifications, especially phosphorylation of the serine residues, is to provide a binding platform for factors, engaged in mRNA processing and in mediating or directly inducing chromatin modifications. Each serine is phosphorylated in different steps of the transcription cycle and can be used as a read-out for transcriptional progress (Figure 1).

DNA-Binding, Initiation, Elongation and Termination

Transcription can be subdivided into 4 steps that are categorized based on the progress the RNA-Pol II enzyme has made in transcribing the mRNA (Figure1). Each step can be monitored by the presence or absence of signals that are generated during transcription on the Pol II CTD itself, at the promoter-chromatin, or on chromatin within the coding region of the transcribed gene (see below).

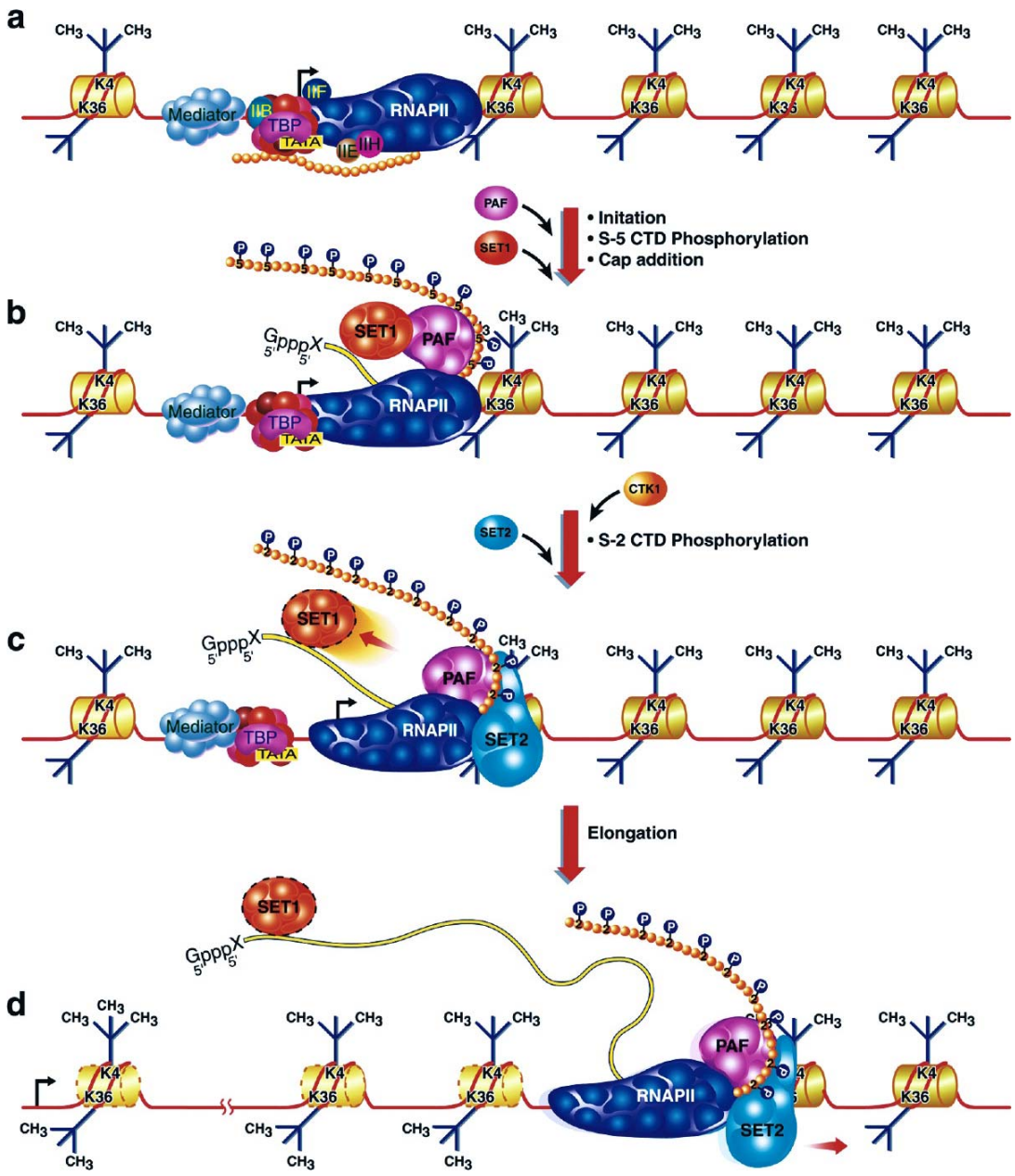

Figure 1: Poised, Paused and elongated Polymerases. **a**) Pol II can be poised for activation after recruitment and awaits phosphorylation of its CTD on S5 to initiate transcription. **b**) After CTD Ser5 phosphorylation by the TFIIH kinase CDK7 Pol II initiates transcription and pauses after the nascent transcript reaches a length of 30-50bp. The SET1 methyltransferase is recruited to the Pol II CTD via interactions with the PAF complex in a P-S5 dependent manner. SET1 methyltransferase interacts with capping enzymes and tri-methylates histone H3K4. **c/d**) Upon recruitment of P-TEFb, CDK9 (CTK1 in yeast) phosphorylates Ser2 of the CTD, which leads to PAF mediated SET2 binding and release from the NELF dependent elongation block (Hampsey and Reinberg, 2003).

DNA-Binding and the establishment of Pol II in a poised state

In prokaryotes, promoter binding of the polymerase is mediated by the binding of σ-factors to the target promoters. Different σ-factors control the expression of different subsets of genes. In eukaryotes the σ-factor has evolved to become a multisubunit complex, called PIC (pre-initiation complex), comprised of the so called basal transcription factors, which facilitates binding of RNA-Pol II (including all 12 subunits). Direct contact to DNA in case of TATA-driven genes is mediated by TBP (TATA-binding protein), whereas in case of TATA-less genes DNA-binding is mediated via TAF (TBP associated factor), TBP like subunits of the basal transcription factor TFIID. TFIID binds to the minor groove of the DNA. TAF-proteins share close structural homology to histone proteins, that build up the nucleosomes forming a histone octamer (see below) (Maldonado and Reinberg, 1995). Nearly all of the TBP population is highly mobile in vivo, displaying FRAP-recovery rates of <15s for inducible genes (Sprouse et al., 2008). In contrast, turnover rates of TBP at constitutively expressed genes range between 10-20 minutes (Tora and Timmers, 2010).

Two models have been discussed in the last years, for the binding of Pol II to the promoter of a protein coding gene. One model suggested Pol II to be recruited as a holoenzyme complex together with all the factors of the PIC and its release from the PIC after initiation of transcription. The second model describes Pol II binding subsequent to an ordered recruitment of the PIC factors starting with the TFIID complex as described above, closely followed by, TFIIA, TFIIB and TFIIF enabling Pol II binding (Figure1). TFIIE, TFIIH and TFIIJ thereafter join the bound Pol II enzyme and provide helicase and kinase activity to generate the transcription initiation bubble and initiate transcription (see below). In vitro binding studies to promoter DNA revealed that the second model is more likely to reflect the in vivo situation.

However, in the last years both models have been proven to be partially right, since Pol II has been found to be stably associated with both TFIIF and TFIIB before recruitment in vivo (Elsby et al., 2006; Liu et al., 2010). Nevertheless, with the notable exception of TFIIF and TFIIB, the ordered recruitment of PIC factors is still the general accepted model for Pol II recruitment (Roeder, 1996).

Following promoter binding Pol II remains bound to the promoter in a poised state and awaits initiation to occur. Some genes are generally poised for transcription even in the absence of activating stimuli, by a promoter bound but not yet initiated Pol II. Upon signaling and subsequent transcription factor binding to the promoter or to enhancer sequences, Pol II is permitted to initiate transcription. Gene expression of poised genes is faster than expression of genes containing no polymerase at all or are even actively repressed by various mechanisms. In this regard, poised polymerases very often mark the promoters of PRGs (primary response genes) as described below (Saunders et al., 2006).

Initiation of transcription

As soon as Pol II and TFIIH are bound together to the promoter, Pol II CTD undergoes phosphorylation at serine 5 of the conserved heptameric repeats. TFIIH is comprised of several core proteins providing ATP'ase and helicase activity, of CDK7 (cyclin dependent kinase 7) and its regulatory subunit cyclin H. CDK7 is responsible for Pol II S5 phosphorylation, predominantly in the distal domain of Pol II CTD, clearly favoring a YSPTSPK motif (Pinhero et al., 2004). Ser5 phosphorylation is greatest near the 5'-end of genes, where initiation takes place (Sims et al., 2004). Moreover, CDK7 was recently found to be able to provide kinase function for the phosphorylation of Ser7 in addition to Ser5 (Akhtar et al., 2009; Glover-Cutter et al., 2009).

Phosphorylation of Ser7 by DNA-PK is known to be important in the integrator dependent 3'-end processing of mammalian snRNA genes (Egloff et al., 2007). Whether or not CDK7 driven Ser7 phosphorylation plays a different role than Ser5 phosphorylation still remains enigmatic. CDK7 dependent Ser7 phosphorylation leading to integrator binding at the 3'end of genes would rely on CDK7 activity at the 3'end of the nascent transcript where integrator complexes should form. Since CDK7 activity is restricted to the 5'end of genes a possible explanation of how this might happen is provided by Nick Proudfood and colleagues showing that many genes depend on DNA-bendind of the 3'end towards the promoter for fast expression upon reactivation (Tan-Wong et al., 2009). Thereby CDK7 would be able to contact Pol II at the 3'end of genes, enabling faster integrator formation and mRNA processing. Dephosphorylation of Pol II at Ser5, for example, is mediated in mammals by the phosphatase SCP1 which is crucial for Pol II recycling (Meinhart et al., 2005).

Recent findings by Graziano Lolli revealed the mechanism of Pol II retention in the unphosphorylated state and its release after Ser5 phosphorylation. Early studies showed that heptad repeats in the CTD can directly contact and intercalate into DNA (Suzuki, 1990). Lolli now demonstrated that the unphosphorylated Pol II which is linked to DNA via CTD-DNA intercalation, is released after Ser5 phosphorylation. Phosphorylation introduces negative charges that disturb the CTD interaction with the negatively charged DNA backbone. Moreover, he concluded, that DNA-CTD interaction is needed to prevent Ser2 phosphorylation, which would permit Pol II to enter the phase of productive elongation (see below) (Lolli, 2009).

An additional function for CTD Ser5 phosphorylation has been described in yeast very early on. The modified Ser5 serves to bind, enzymes that add a methylguanosine cap to the 5' end of nascent RNAs, enzymes of the splicing machinery, and the histone methyltransferase SET1 containing complex (Orphanides and Reinberg, 2002; Hampsey and Reinberg, 2003). To ensure that Pol II only proceeds to productive elongation after the successful addition of the 5'cap, nature has installed a checkpoint at the transition from initiation to elongation where Pol II pauses after transcription of the first 30-50bp (Rasmussen and Lis, 1993). This checkpoint is controlled by the factors NELF (negative elongation factor) and DSIF. NELF recruitment depends on the presence of CDK7 (Glover-Cutter et al., 2009).

Transcriptional elongation

To proceed to productive elongation Pol II has to be released from the NELF mediated elongation block. This is achieved by the katalytic activity of the P-TEFb (positive transcription elongation factor b) subunit CDK9. Ser2 of the Pol II CTD as well as NELF and DSIF themselves are substrates for CDK9. Negative effects of NELF and DSIF on transcription are relieved by P-TEFb-mediated phosphorylation of NELF. However NELF can also support transcription when it is associated with GAGA factor (a trx – trithorax - family member)(Lee et al., 2008).

Phosphorylation of Ser2 increases towards the 3'-end of genes. Interestingly, expression of genes containing no intronic sequences, like genes encoding histone proteins or mammalian snRNA genes, does not rely on Ser2 phosphorylation, indicating that this mark is involved in targeting the spliceosome to the nascent mRNA transcript (Medlin et al., 2005). Indeed it was shown in vitro and in vivo that recruitment of splicing factors depends on the presence of S2– phosphorylation (Hirose et al., 1999; Misteli and Spector, 1999). CDK9 predominantly phosphorylates the proximal domain of mammalian Pol II CTD. Recent observations show that phosphorylation of Ser2 by CDK9 was greatest after initial phosphorylation of Ser5 in the distal CTD-domain by CDK7, thereby reflecting the basic principal of sequential transcriptional activation (Pinhero et al., 2004).

Pol II can be transcriptionally arrested right after recruitment or at the transition from initiation to elongation, by the lack of CDK7 or CDK9 activity, respectively (Margaritis and Holstege, 2008). A third form of arrested polymerase can occur within the coding region of the gene leading to polymerase stalling. Sequences next to alternative splice sites have been proven to slow down the elongation rate and processivity of Pol II, which leads to possible stalling of the polymerase. This in turn ensures the formation of alternative exons (Kornblihtt et al., 2004).

Thus Pol II stalling can occur at regions where stalling is required for subsequent RNA processing events, or it takes place accidently. To ensure that a defective polymerase does not block the transcription of a gene, defective Pol II is ubiqutinated and proteasomally degraded (Leung et al., 2008; Daulny et al., 2008). Nature has installed a unique mechanism to distinguish a defective Pol II from a stalled Pol II enzyme. A classically stalled Pol II that awaits the recruitment of factors required for alternative splicing, is unable to be reengaged in the transcriptional process unless the transcription factor TFIIS is recruited (Reines et al., 1989). Very recent findings of Adelman and colleagues indicate that Pol II backtracks on the nascent transcript and generates an 3' overhang of 8-12 bp in length, which needs to be cleaved by TFIIS in order to reengage the Pol II enzyme in transcription (Adelman et al., 2005).

Pol II CTD and transcriptional termination

Pol II CTD has lost most of the Ser5 phosphorylation by the time it reaches the polyadenylation signal. Ser2 and Ser7 (in case of snRNA genes) phosphorylation increases towards the 3'-end of the mRNA (Hirose and Ohkuma, 2007). Egloff and colleagues demonstrated in case of snRNA genes, that both residues need to be phosphorylated to enable binding of integrator complexes (Egloff et al., 2010). Whether both marks are required for the binding of CPSF (cleavage and polyadenylation specificity factor) to the mRNA of protein coding genes remains to be determined. However the role of CTD modifications in transcriptional termination is not yet fully understood.

Where the CTD code meets the histone code

Like the CTD code, the histone code comprises reversible modifications of the histone tails, that form binding platforms for factors associated with both active and repressed transcription. Phosphorylation, acetylation, ubiquitination and methylation of histone tails play fundamental roles in orchestrating transcription (see below). Interestingly, the chromatin state of the transcribed DNA template is closely related to the phosphorylation state of RNA Pol II CTD (Hampsey and Reinberg, 2003; Berger, 2007).

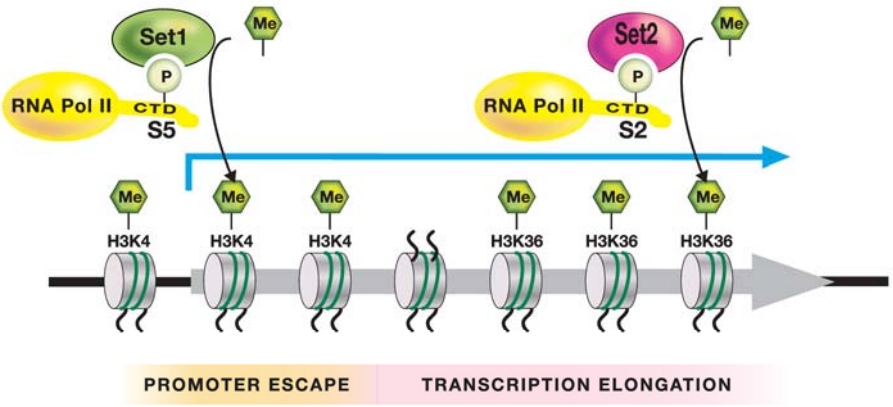

Figure 5. Role of Histone Lysine Methylation in Transcriptional Elongation

RNA polymerase II recruits distinct types of HKMTs, depending on the phosphorylation state of its carboxy-terminal domain (CTD). RNA pol II is activated for transcriptional initiation in the vicinity of the promoter, when Ser-5 is phosphorylated. This recruits the Set1 HKMT to methylate H3K4. Phosphorylation of Ser-2 occurs during transcriptional elongation, prompting H3K36 methylation as a result of Set2 HKMT recruitment to the chromatin template.

Figure 2: Role of Histone Lysine Methylation by HKMTs (histone lysine methyltransferase) in Tanscription Elongation.

The yeast SET1/Compass methyltransferase complex is recruited to the Pol II CTD in a P-Ser5 dependent manner. SET1 introduces the H3K4me3 mark, which peaks in line with the P-Ser5 pattern, at the 5'-end of genes (Figure2). SET1 contacts the CTD via interaction with the PAF1 complex. SET2 on the other hand is responsible for H3K36me3 which peaks at the 3'-end of transcribed genes and directly binds the Ser2 and Ser5 phosphorylated CTD (Kizer et al., 2005).

In addition, two histone acetyltransferases, p300 and PCAF, have been found to associate with the IIa form and the IIo form of the polymerase, respectively. p300 is thereby recruited directly to the TSS to maintain the chromatin/DNA template in a loose and accessible state, and PCAF is travelling along with the elongation competent polymerase to facilitate effective transcription (Cho et al., 1998).

Chromatin and Transcription

Chromatin is the physiological template of all eukaryotic genetic information, and undergoes a series of PTMs (posttranslational modifications) that largely impinge on histone amino termini to regulate access to the underlying DNA template. Recently an ancient role for a chromatin-like structure was identified in *Mycobacteria* (Colangeli et al., 2009). However, chromatin is still a feature of eukaryotic cells. Chromatin modifications have emerged to become highly dynamic binding platforms for an increasing number of chromatin associated factors. These factors themselves are "writers" and/or "readers" of chromatin modifications, in other words they can de novo introduce modifications and/or are capable of binding chromatin modifications to regulate the onset or repression of transcription. All steps of transcription including the binding of transcription factors, are subject to the chromatin-state at the promoter or within the coding region of genes. Large and defined regions of chromatin have been found initially in liverwort mosses to be either highly condensed and transcriptionally silent, termed heterochromatic regions, or have an open chromatin structure and are transcriptionally active, termed euchromatic regions. The initial hypothesis, called the "Histone code hypothesis", of Strahl and Allis implies that not a single chromatin mark facilitates activation or repression, but a combination of marks is needed to regulate gene-expression, and one established mark can stimulate the introduction of other marks (Strahl and Allis, 2000).

The identification and exploration of chromatin modifications accelerated the research field of epigenetics. The findings that these marks can be transmitted to daughter cells upon cell divison, leading both to the formation of transcriptional active or repressive chromatin and also to the maintenance of the chromatin-state through cell-generations, provided new insight into the maintenance of cell identity. In line with this, chromatin marks, and factors responsible for introducing these marks, have been identified to be responsible for cell differentiation and lineage commitment by the regulation of cell type specific gene expression. The following chapter will not deal with the epigenetic consequences of chromatin modifications in the context of ES-cell differentiation or lineage specific gene expression, but will focus on the direct influence of mainly activating chromatin marks on the regulation of the different steps of transcription.

The Nucleosome

In eukaryotic cells, genes are complexed with core histones and other chromosomal proteins in the form of chromatin. The basic repeating unit of chromatin, the nucleosome, includes two copies of each of the four core histones H2A, H2B, H3 and H4 wrapped by 146 bp of DNA. The connection from one nucleosome to the other by the DNA template is called “spacer” and generates a ~20bp long part of DNA that is accessible to DNA binding factors. With the aid of additional proteins including histone H1 the nucleosomes are further packaged into 30nm fibers with six nucleosomes in a spiral or solenoid arrangement (Zhang and Reinberg, 2001). To become transcriptionally active the 30nm fiber has to unfold into a 11nm structure also called “beads-on-a-string”. Still the 11nm structure is not permissive for transcription. Remodelling of nucleosomes has to take place to enable access to DNA.

Chromatin remodeling and histone variant exchange

Pol II activity on target genes frequently requires the ATP-dependent multi-subunit chromatin remodeling complex Swi/Snf or histone acetyltransferases such as SAGA (Spt-Ada-Gcn5-acetyltransferase). It was long questioned whether the binding of transcription factors to the promoter DNA leads to promoter proximal chromatin remodelling, or whether the action of chromatin remodelling complexes is required to facilitate binding of transcription factors to DNA. Evidence for the latter hypothesis was provided in 1999 by Kim Nasmyth and colleagues who demonstrated that the action of chromatin remodelling complexes is needed for sequential binding of transcription factors in yeast (Cosma et al., 1999).

With some exceptions this dogma has been strenghened by numerous studies during recent years (Drobic et al., 2010). Relevant for this thesis, recent work of Rod Bremners group also supports this model for STAT (signal tranducers and activators of transcription) transcription factors by showing that STAT1 dependent activation of the CIITA promoter requires the activity of BRG1 (Brahma-related gene 1), the ATP'ase subunit of Swi/Snf (or BAF complex in humans)(Ni et al., 2008). Moreover they could show that chromatin remodeling at the CIITA promoter is not restricted to defined regions at the proximal promoter but can spread over 100kb (Ni et al., 2005). These events in turn link BRG1 to the phenomenon of DNA bending, since long range interactions are very often required for transcription factors to get in contact with the Polymerase to affect transcription. At the CIITA promoter DNA bending is required for factors bound to different distal enhancers to communicate with the promoter bound RNA Pol II (Ni et al., 2008).

ATP dependent chromatin remodeling can result in the deposition of nucleosomes on the DNA to make DNA-binding elements accessible, or it leads to the eviction of some or all components of the histone octamer. H3 and H4 are mainly depleted from nucleosomes during remodeling. Since the interaction of the two H3 molecules is critical for nucleosomal stability this disturbs the nucleosomal structure, whereas H2A and H2B can remain bound to the DNA (Talbert and Henikoff, 2010). In yeast, NDR (nucleosome depleted region) formation for most promoters is dependent on the RSC remodelling complex, which displaces nucleosomes from the NDR (Hartley and Madhani, 2009).

Interestingly eviction is limited to the nucleosomes upstream of the TSS. The first nucleosome downstream of the TSS, the +1 nucleosome, has proven to be crucial in the process of transcriptional initiation. It is found to be the most highly positioned nucleosome and very often contains a histone variant of H2A, H2A.Z. A role for the displacement of H2A to H2A.Z in Pol II pausing, has been shown recently in Drosophila (Mavrich et al., 2008). In human cells positioning of H2A.Z by the ATP dependent factor Swr1, in upstream promoter elements contributes to both activation and repression (Gévry et al., 2007; Gévry et al., 2009).

FACT (facilitates chromatin transcription) mediated exchange of H2A-H2B dimers by the H2A.Z-H2B dimer-variant during transcriptional elongation can reveal cryptic promoters within the coding region of the transcribed gene (Talbert and Henikoff, 2010). FACT recruitment and activity depend on H3K4 mono-methylation and is ATP independent in in-vitro experiments, but it associates with CHD1 in vivo, a protein that hydrolizes ATP and binds H3K4me to remodel nucleosomes (Reinberg and Sims, 2006).

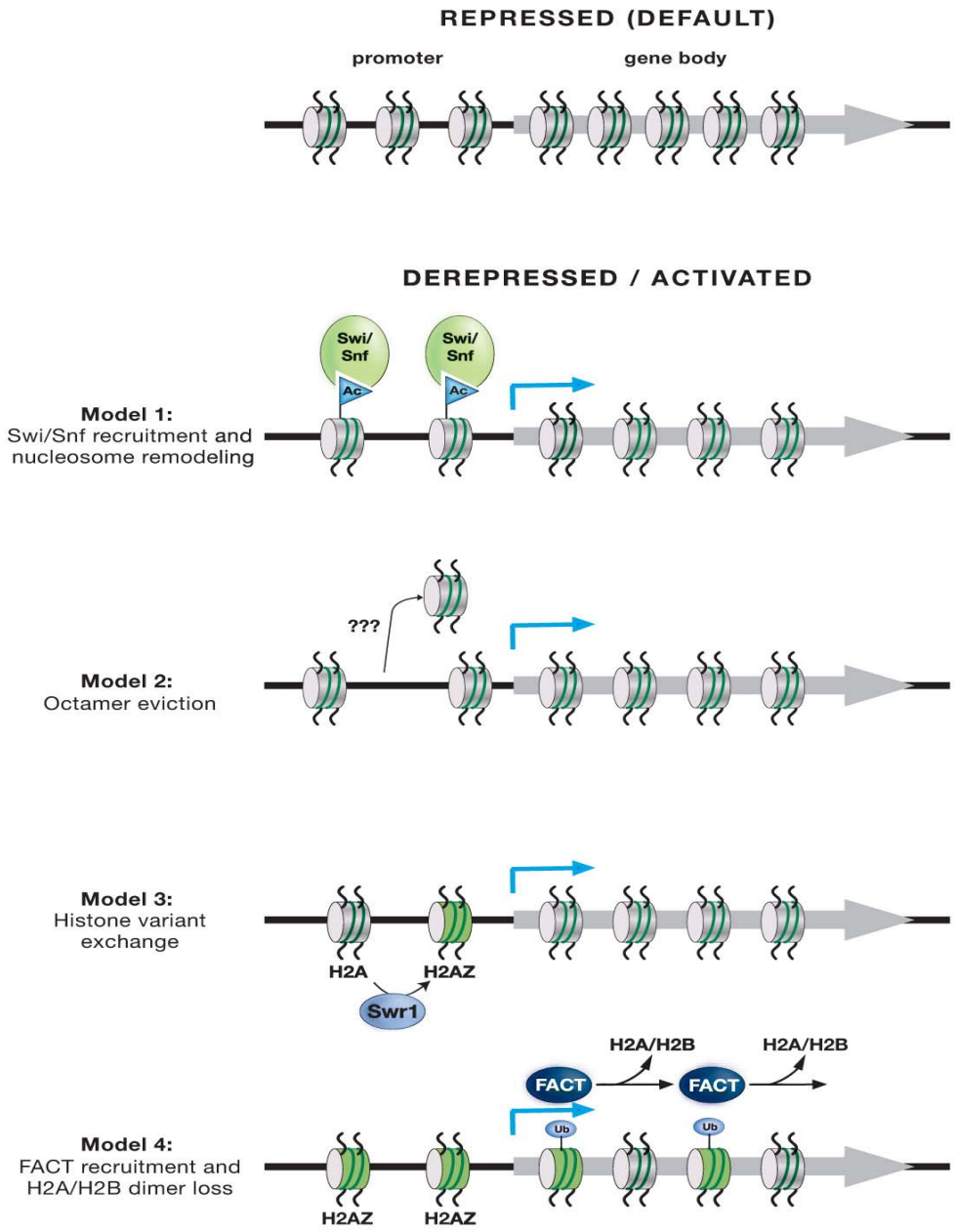

Figure 8. Models for the Involvement of Chromatin Remodeling and Histone Exchange in Transcriptional Processes

In Model 1, the Swi/Snf family of ATPase binds chromatin through bromodomain recognition of acetylated histones and acts to alter the local chromatin structure. Model 2 depicts the reported octamer eviction that occurs at certain loci such as *PHO5* by an unknown mechanism. In Model 3, the ATPase SWR1 catalyzes the replacement of histone H2A with H2AZ, which poises chromatin for transcription. Model 4 focuses on the involvement of FACT in transcriptional elongation, assisting in nucleosome unraveling by the displacement of an H2A/H2B dimer. Concomitantly, histone H3 may be exchanged with H3.3 during the process.

Figure 3: Models for the Involvement of Chromatin Remodeling and Histone Exchange in Transcriptional Processes

Moreover, canonical histones can be exchanged by histone variants during certain processes in the cell that require the subsequent recruitment of specialized factors. One well studied example is the exchange of histone H3 by the variant H3.3 during DNA-DSB (double strand break) – repair. The hallmark of variant histones is that they contain additional and modified residues on their amino-terminal tail, prior to incorporation, that provide binding platforms for proteins involved for example in DNA-DSB repair, as shown in case of H3.3 (Talbert and Henikoff, 2010).

In addition to repositioning and eviction of nucleosomes from chromatin, covalent modifications of aminoacid-residues in the histone amino-termini are crucial for chromatin reorganisation and transcription. Known HPTMs (histone post transcriptional modification) are: acetylation, ubiquitination, methylation and phosphorylation.

Histone acetylation

The first HAT (histone acetyl transferase) that has been identified as an homolog of the yeast transcriptional co-activator GCN5, immediately linked histone acetylation to gene activation (Brownell et al., 1996). Several transcriptional co-activators contain intrinsic HAT activity: GCN5, p300/CBP, PCAF, TAF250 (TFIID subunit) and the p160 family of nuclear receptor coactivators. On the other hand transcriptional repressors like SIN3 or NcoR/SMRT are associated with HDACs leading to the removal of the mark.

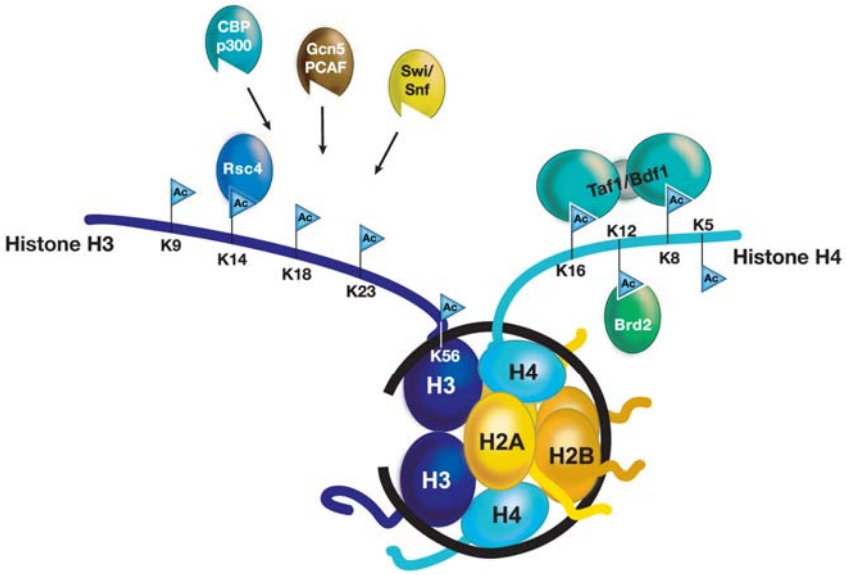

Figure 3. Characterized Sites of Histone Acetylation

Histones are mostly acetylated at lysine residues located in the amino termini of H3 and H4, with the exception of H3K56 localized in the globular domain. The proteins that express binding specificity to acetylated histones are shown.

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Figure 4: Characterized Sites of Histone Acetylation

The mechanisms linking histone acetylation to transcriptional activation are divergent. Acetylation takes place on lysine residues predominantly described at the N-terminal tails of H3 and H4 and leads in the first place to changes in structure and charge. In the case of acetylation, the neutralization of positively charged lysines reduces the strength of binding of the strongly basic histones or histone tails to negatively charged DNA, thus opens DNA binding sites (Vettese-Dadey et al., 1996). This is also relevant to open up higher chromatin order structures, like the 30nm fiber.

Several HPTMs in combination can form binding surfaces for DNA/Chromatin binding factors that associate with chromatin compaction or are engaged in the transcriptional process. This mode of action for HPTMs was first shown for acetylation. Proteins that contain a bromodomain can bind to acetylated lysines (Dhalluin et al., 1999). Examples for proteins containing a bromodomain are GCN5 and CBP/p300, which are themselves HATs ("writer" and "reader" in one molecule) and can therefore initiate the spreading of an existing acetylation mark (Figure 3). Further examples are TAF1, BDF1 of the TFIID complex, RSC4 in the RSC remodeling complex, and BRD2, member of a large family of bromodomain proteins. Whereas chromatin remodeling can affect large regions as in the case of the CIITA enhancers, hyperacetylation of histones frequently occurs on very defined regions in proximity to the binding sites of the transcription factors responsible for HAT recruitment (Parekh and Maniatis, 1999).

Histone phosphorylation and phospho-acetylation

Phosphorylation of histones was discovered very early on. PRGs, in former times also called "immediate-early genes", showed increased expression in correlation with the phosphorylation of H3 (Mahadevan et al., 1991). H3S10 has been identified as a very important residue in all eukaryotic species, and is closely associated with active transcription. Moreover, H3S28 and H3T11 have been identified to become phosphorylated (Figure 4). Many kinases have been described that target these sites: SNF1 in *S. cerevisiae*, MSK1/2 and the related RSK2, and IKKα (IkB - Inhibitor of NFkB – Nuclear factor of kappa light polypeptide gene enhancer in B Cells - kinase) in mammals (Sassone-Corsi et al., 1999; Lo et al., 2001; Soloaga et al., 2003; Yamamoto et al., 2003; Anest et al., 2003). Phosphorylation, like acetylation, causes a change in the charge of the phosphorylated residues, resulting in lose affinity of the histone tails to the underlying DNA.

In vitro experiments for several acetylating enzymes show a strong binding preference for histone H3 phosphorylated at Ser10. Indeed, recent studies identified a phosphorylation- acetylation link, where both marks cooperate in the recruitment of proteins of the 14-3-3 family, ζ and ε. The binding preference for H3S10 phosphorylation of 14-3-3 proteins was greatest with the nearby presence of H3K9ac. In case of H3S28p the nearby H3K27ac mark was not required, although it shares the sequence motif with H3S10 (Macdonald et al., 2005; Winter et al., 2008). Recent work of Kristian Helin and colleagues shows that MSK mediated H3S28p induces the displacement of PcG (Polycomb group) proteins from the neighbouring H3K27me3 mark, thereby inducing gene expression (Gehani et al., 2010).

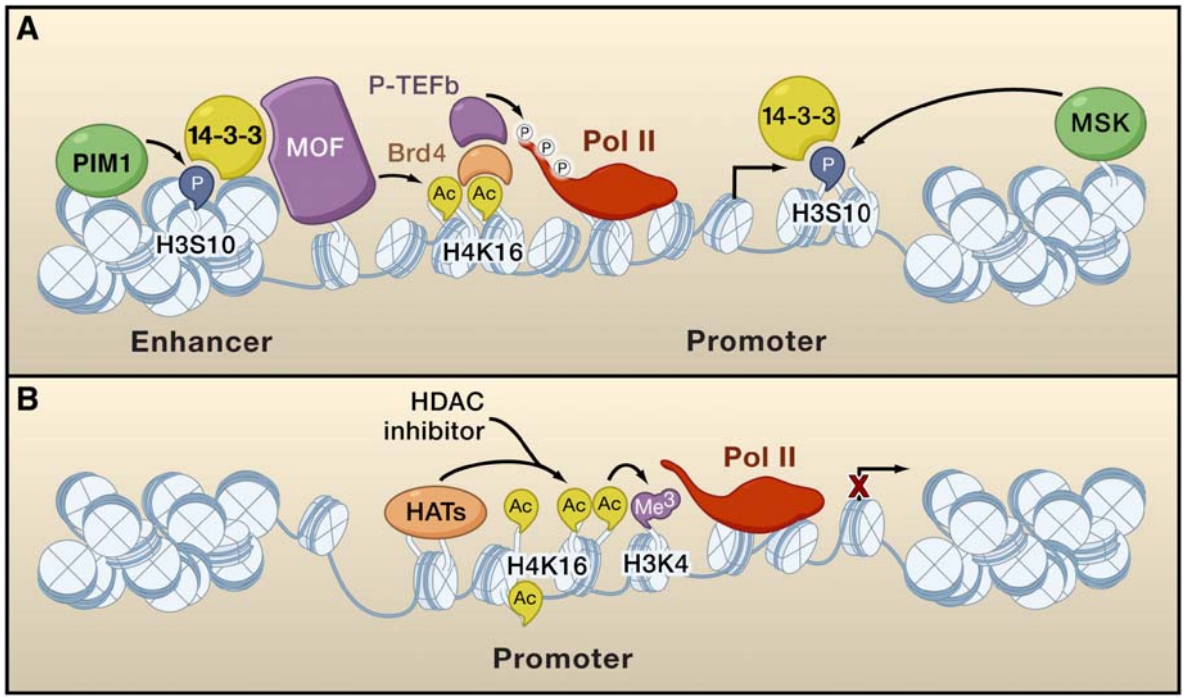

Figure 5: Examples for recently identified histone modification crosstalk. (A) Histone H3S10 phosphorylation leads to the binding of 14-3-3 protein family members which in turn serve to recruit BRD1 or MOF. MOF mediates the subsequent binding of Brd4 by acetylation of H4K16. Brd4 was found to be able to recruit P-TEFb to induce gene expression of PRGs. (B) The simultaneous presence of H3K4me3 and H4K16ac, when the latter is induced by the repression of HDAC activity, is not always associated with transcriptional activation, pointing towards a more complex role of modification crosstalk (Lee et al., 2010).

Recent findings point out at a series of functions for P-Ac (phosphoacetylation), serving as a binding platform for 14-3-3 proteins. Studying PRG promoters, James Davie and colleagues revealed the dependency of BRG1 recruitment to 14-3-3 proteins on MSK1 mediated P-Ac (Figure 5A). Transcription factors Elk-1 or NF-κB serve to recruit BRG1 via this mechanism, in turn enabling subsequent chromatin remodelling and binding of transcription factors like c-Jun that initiate transcription (Drobic et al., 2010). Findings of Oliviero and colleagues add another level of complexity to the issue of histone cross-talk, in favor of the histone code hypothesis. Based on the observation that H3S10ph enhances the recruitment of GCN5, which in turn acetylates H3K14 (Figure 7A) (Cheung et al., 2000; Agalioti et al., 2002), this study demonstrated that H3S10 phosphorylation by the kinase PIM1 on a serum responsive gene, recruits 14-3-3 proteins, thereby providing a binding platform for the HAT MOF. MOF induces acetylation of H4K16 which forms a recognition site for the bromodomain containing BRD4 protein. BRD4 subsequently recruits P-TEFb leading to the release of the promoter proximal paused Pol II (Figure 5A) (Zippo et al., 2009).

Histone ubiquitination

Ubiquitination can occur on Histone H2A and H2B and increase the size of the histone by approximately two-thirds. In contrast to K48-linked ubiquitylation, which leads to degradation of the protein via the 26S proteasomal pathway, histones undergo K63-linked ubiquitylation. This adds just one ubiquitin molecule to a lysine residue of the histone tail of H2A and H2B at residues K119 and K120, respectively. In yeast residue K123 in H2B is homologous to vertebrate H2BK120. In contrast to other histone modifications, which are localized in unstructured C-termini of the respective histones, these residues are located in the highly structured C-terminus of H2A and H2B, thereby enabling limited access of binding factors (Weake and Workman, 2008).

H2Aub is associated with transcriptional repression via Polycomb group BMI1/RING1A proteins. However there is no evidence for evolutionary conservation of H2Aub in yeast (Wang et al., 2004).

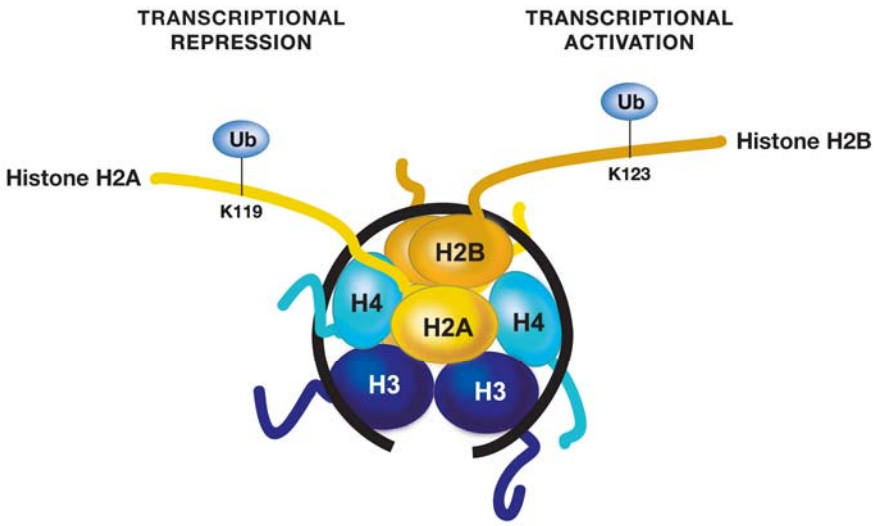

Figure 7. Sites of Histone Ubiquitylation and Their Consequence for Transcriptional Regulation

Ubiquitylation of H2A at Lys-119 is correlated with transcriptional repression. H2BK123 ubiquitylation is conversely associated with transcriptional activation.

Figure 6: Sites of Histone Ubiquitylation and Their Consequence for Transcriptional Regulation

H2Bub has been extensively studied in the last years and was found to provide a direct link between histone modifications and the transcriptional activity of RNA-Pol II. In yeast the ubiquitin E2 ligase RAD6 was shown to be recruited in an activator and BRE-1 dependent manner. BRE-1 acts as the ubiquitin conjugating E3 ligase. Consistently, H2Bub colocalizes with RNA-Pol II in dependence on BRE-1 and the PAF complex. Importantly, requirements of the PAF complex and the Pol II C-terminal domain (CTD) serine 5 kinase Kin28/CDK7 for H2B ubiquitylation, have implicated H2B ubiquitylation in transcription elongation (Ng et al., 2003a; Wood et al., 2003; Xiao et al., 2005). Moreover, H2Bub has been identified in yeast as the critical determinant for H3K4 and H3K79 trimethylation (see below) by COMPASS and Dot1 methyltransferases (Figure 7B) (Nakanishi et al., 2009).

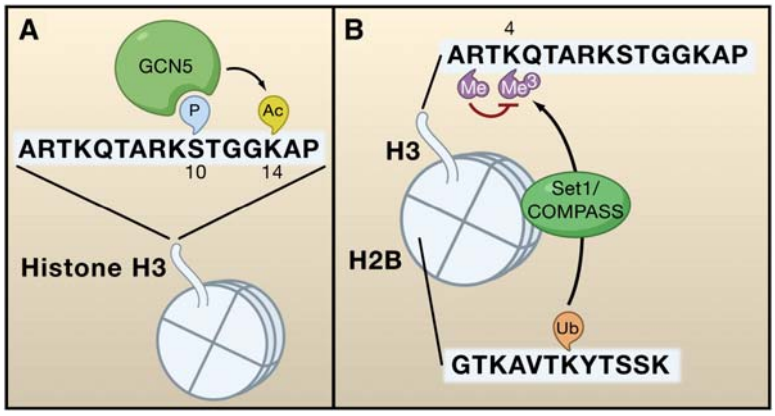

Figure 7: Examples of histone crosstalk. Phosphoacetylation mediated by GCN5 binding to H3S10-Ph (A). H2Bub mediates H3K4me3 by the recruitment of SET1/COMPASS (B) (Lee et al., 2010).

A histone H3 trimethylation independent function of H2Bub has been reported recently by showing that it directly facilitates FACT dependent chromatin transcription in vitro. Furthermore, FACT activity and H2Bub are connected during transcription elongation in vivo, and the modification has a selective role in regulating FACT mediated chromatin reassembly following RNA-Pol II transcription (Fleming et al., 2008). These findings are supported in the recent publication of Roeder and colleagues reporting the afore mentioned RAD6/BRE-1/PAF connection and the role of H2Bub in FACT-mediated chromatin reassembly in the wake of elongating Pol II in human cells (Kim et al., 2009). A contrasting result was published showing in budding yeast that H2B deubiquitination by Ubp8 is crucial to allow Ctk1/CDK9 to phosphorylate Pol II at Ser2 (Wyce et al., 2007). These conflicting data may result from the use of different genetic model systems.

Histone methylation

Methylation of histones is the most intensively studied HPTM. It occurs on either lysines or arginines and is therefore mediated by two distinct classes of enzymes. Lysines can become mono-, di-, or tri-methylated. The number of identified HKMTs (histone lysine methyltransferases) has increased and they have all been linked to the methylation of several known lysine residues (Martin and Zhang, 2005). Six of these residues have been well characterized to date: five on H3 (K4, K9, K27, K36, K79) and one on H4 (K20). Methylation at H3K4, H3K36, and H3K79 has in general been linked to transcriptional activation, and the rest to transcriptional repression (Figure 8). H3K79 and H4K20 methylation have been found to play a key role in DNA repair processes (Martin and Zhang, 2005). In contrast to acetylation or phosphorylation, methylation does not significantly change the charge of the N-terminal histone tail. Therefore the main regulatory function of methylation is to directly recruit effector proteins, or adaptors, that regulate transcription or other cellular processes. Proteins that contact methylated lysines have to contain either a tudor, chromo or PHD repeat domain.

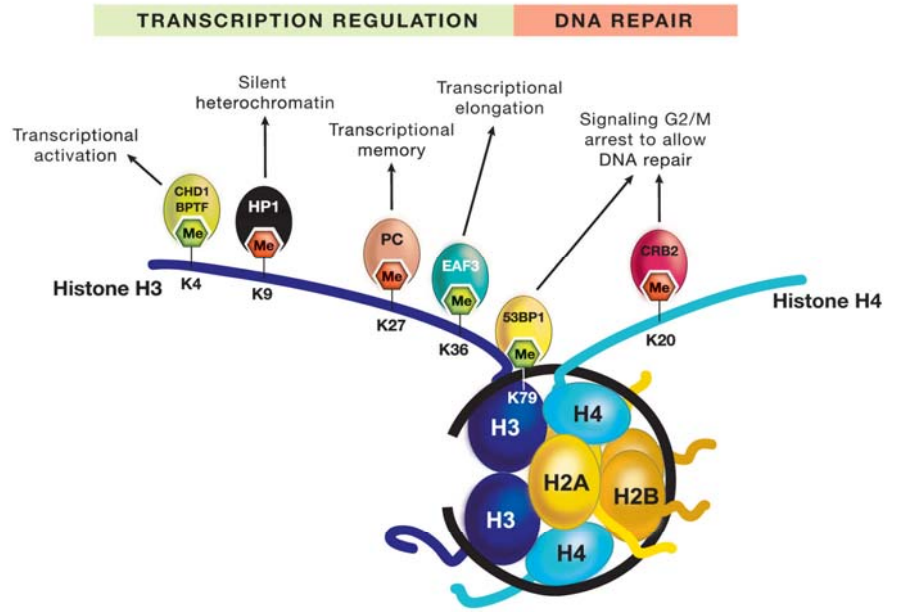

Figure 4. Sites of Histone Methylation, Their Protein Binders, and Functional Role in Genomic Processes

Methylation of histones occurs at lysine residues in histones H3 and H4. Certain methylated lysine residues are associated with activating transcription (*green Me flag*), whereas others are involved in repressive processes (*red Me flag*). Proteins that bind particular methylated lysine residues are indicated.

Figure 8: Sites of Histone Lysine Methylation, Their Protein Binders, and Functional Role

H3K4 methylation has been extensively studied, since it has been found to closely correlate with active transcription (see above). H3K4me3 appears at transcriptionally active genes as defined islands located at the 5'end of genes. The first H3K4 methylase complex to be identified, COMPASS, was purified from yeast and contains SET1 and seven other polypeptides (Miller et al., 2001). SET1 belongs to the trithorax group of transcriptional regulators and comprises a SET-domain, which is responsible for catalyzing mono-, di-, and trimethylation of H3K4. SET1/COMPASS in yeast has six mammalian homologs, MLL1 to MLL4 (mixed-lineage leukemia gene), SET1a and SET1b. The last two are responsible for methylating the bulk of H3K4 methylation marks in mammals (Wu et al., 2008). The MLL family of SET containing methylases is responsible for methylating only a small proportion of H3K4 residues. MLL1/MLL2 activity has recently been found to act together and rely on the binding partner Menin, that mediates MLL1/MLL2 by binding both of the molecules (Caslini et al., 2007). MLL1/MLL2 regulated H3K4 methylation was found to be present in only 3% of genes in MEFs within a gene transcriptional profiling array. Most of these genes lack the TATA-Box and are responsible for developmental regulation and differentiation, like part of the genes encoded within the hox-gene cluster (Wang et al., 2009).

The primary function of H3K4me3 is thought to be exerted after Pol II recruitment and depends on Ser5 phosphorylation of Pol II CTD (Ng et al., 2003b). SET1/COMPASS in yeast and also the SET1 homolog in mammals were found to be recruited to genes by the ubiquitination of histone H2B, requiring prior recruitment of Pol II and the PAF1 complex (Krogan et al., 2003; Shilatifard, 2006; Kim et al., 2009). However, recent findings also indicate a role for H3K4me3 prior to Pol II recruitment at least for the MLL family of SET domain-containing proteins in mammals (Wang et al., 2009). This result is strengthened by the findings that H3K4me3 can serve to recruit CHD1 protein and the NURF complex, known to mobilize nucleosomes at active genes in *Drosophila*. The domains that mediate association with H3K4 are a tandem set of chromodomains in CHD1 and a PHD finger within NURF (Sims et al., 2005; Li et al., 2006). In addition, the PIC subunit TFIID was recently found to directly recognize the H3K4me3 mark via the PHD finger of TAF3. In the same study binding of the TAF3 - PHD finger was shown to be further enhanced by the additional presence of acetylation at H3K9 and H3K14, providing a link between methylation and (phospho-) acetylation in the activation of transcription (Vermeulen et al., 2007).

However, introduction of H3K4me3 is closely followed by the di-methylation of H3K4 on the neighbouring nucleosome close to the transcription initiation site, by SET1 or its mammalian homologs. Stephen Buratowski recently showed that H3K4me2 is bound by the PHD finger of SET3C in yeast. SET3 contains a SET domain, but no methyltransferase activity has yet been reported. SET3C interacts physically with two HDACs, HOS2, and HST1, leading to the subsequent deacetylation of nucleosomes downstream of the promoter. This may in turn be necessary to prevent transcription initiation from cryptic promoters next to the TSS. Supportive evidence was provided by the finding that yeast mutants for the SET1 – SET3 complex pathway affect Pol II association with a target gene (Kim and Buratowski, 2009).

H3K4 mono-methylation by i.e. human SET9 is highly dispersed throughout genes. It appears to be predominantly localized at enhancer regions (Ghisletti et al., 2010). For as yet unknown reasons some enhancers contain both H3K4me and H3K4me3 marks at the same time (Robertson et al., 2008; Wilson et al., 2009).

In the elongation phase of transcription, phosphorylation of CTD serine 2 by CDK9, together with the phosphorylated Ser 5, creates a binding site for SET2, resulting in H3K36me2 and H3K36me3. Both marks can be detected throughout transcribed regions but clearly peak towards the 3'end of genes. SET2 has been shown to interact directly with the PAF complex and CHD1 that can in turn bind to H3K36me2. CHD1 plays a role in transcriptional termination. Like in the case of H3K4me2, H3K36me2 recruits the Rpd3S HDAC complex which removes the acetylation of histones in the wake of Pol II transcription in order to block transcription initiation from cryptic promoters (Carrozza et al., 2005).

Methylation at H3K79 is very unusual because it is located within the core of the histone rather than in the tail, like all the other known modifications. The only methylase capable of introducing this mark known so far, is the human hDOT1L which has been linked to leukemogenesis. Since there is no protein to date that binds this mark, the link to transcriptional regulation is still enigmatic. The only described function links it to DNA repair processes (Martin and Zhang, 2005).

H3K9, H3K27, and H4K20 methylation, are linked to transcriptional repression. For example, H3K9me3 is a target for the protein HP1, which induces and maintains the formation of pericentric heterochromatin thereby participating in gene silencing. H3K9me2 and phosphorylation on the nearby H3S10 counteract each other and result in the demethylation of H3K9 once H3S10 is phosphorylated. The methyltransferase SUV39H1 was first described to be able to methylate H3K9. H3K27-methylation has largely the same function. It binds Polycomb proteins and is predominantly located near PREs (Polycomb responsive elements). H4K20 methylation appears to be dependent on H3K9 methylation. The exact function is still unclear, but it was linked to DNA – repair processes via the binding of DNA damage check point protein CrB2 in budding yeast (Martin and Zhang, 2005).

Arginine methylation has been implicated in both active and repressive chromatin. PRMT1 (protein arginine methyltransferase) and PRMT4/CARM1 have both been linked to transcriptional activation. PRMT1 targets the residue H4R3, whereas PRMT4 is known to target the methylation of residues on histone H3, H3R2, H3R17, and H3R26. Both were shown to be specifically recruited by transcription factors like p53 and NFκB, and closely correlate with the appearance of RNA-Pol II at target genes. Moreover, arginine methylation is linked to histone acetylation by CBP (Strahl et al., 2001; Bauer et al., 2002; Daujat et al., 2002; Cuthbert et al., 2004). PRMT1 was found to participate in negative regulation of type I IFN signaling, thereby linking known histone modifyers to modification events in the cytoplasm (Weber et al., 2009).

Class I, II and III transcription factors

The rate at which mammalian protein-encoding genes are transcribed depends on the frequency at which RNA polymerase II is recruited into an initiation complex and subsequently rendered competent for transcriptional elongation. These processes require the binding of transcription factors and are intimately linked with the structure and modification of the surrounding chromatin.

DNA binding transcription factors like STATs (signaltransducers and activators of transcription) can recruit a variety of proteins that enable gene expression, including Pol II (RNA polymerase II) and chromatin modifiers (Kadonaga, 2004). Recruitment of CRCs (chromatin-remodeling complexes) results in the remodeling of the nucleosome:DNA template in order to reveal critical regulatory regions, including transcription factor-binding sites or the TSS (transcription start site) (Chi, 2004). Transcription factors in general can be divided into three different categories on the basis of their mode of activation and function (Figure 9).

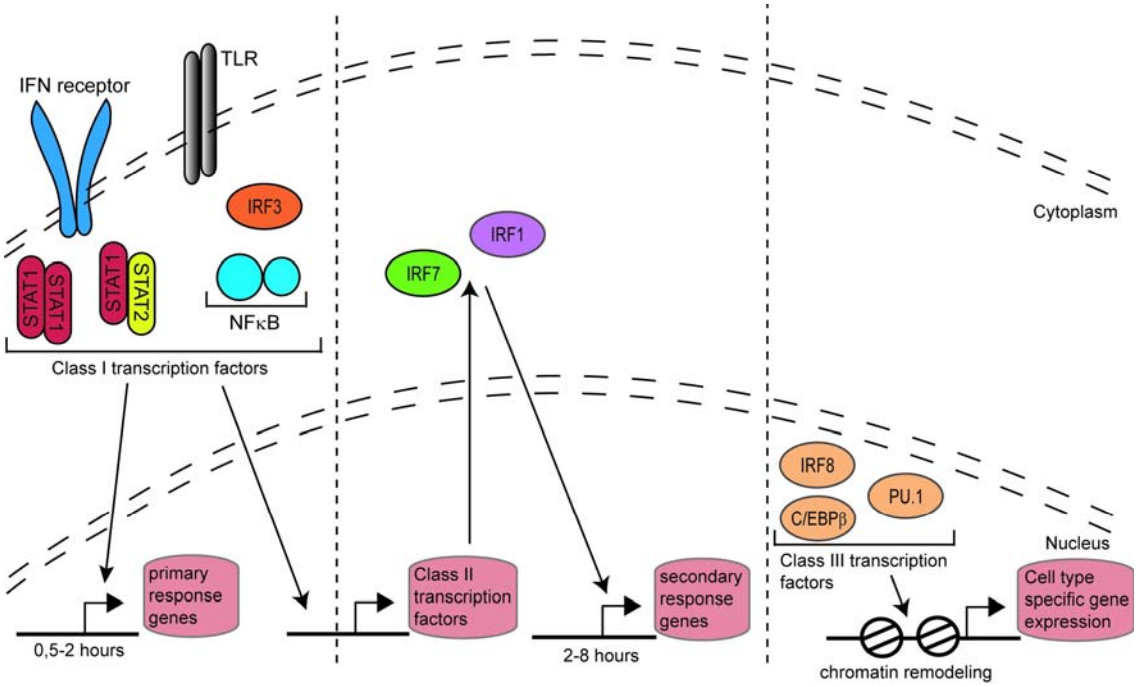

Figure 9: Model of Class I, II and III transcription factor relationship. Class I transcription factors are constitutively expressed and, when activated, regulate the expression of PRGs (primary response genes) within 2hrs after treatment, STATs, NFκB or IRF3 belong to this group. Class II transcription factors like IRF1 or IRF7, are synthesized de novo, within two to eight hours after treatment, and therefore PRGs. They control the expression of SRGs (secondary response genes), either on their own or in conjunction with Class I transcription factors. Class III transcription factors are defined as lineage specific factors expressed upon differentiation. In the case of macrophages PU.1, C/EBPβ, or IRF8 belong to this group.

Class I transcription factors

The first category consists of transcription factors that are constitutively expressed. This kind of transcription factors are mostly retained in the cytoplasm in an inactive state and translocate to the nucleus subsequent to their signal-dependent activation. Activation is achieved by either modifying enzymes (e.g. by phosphorylation, K63-linked ubiquitination, methylation, acetylation, …) as in the case of STATs, or by sequestration/ degradation of an inhibitory molecule. The best studied example of this latter possibility is the canonical pathway of NFκB activation (nuclear factor for kappa light chain enhancement in B-cells) causing the degradation of IκBα (inhibitor of NFκB alpha). This class of transcription factors is the best characterized of the three in the literature and contains a significant amount of the key players relevant for inducing an immune-response. Relevant for immne responses this group contains some but not all IRF family members, like IRF3 and IRF9, in addition to STATs and NFκB. A hallmark of this class of transcription factors is their ability to be rapidly (within minutes) activated upon stimulation and to be therefore responsible for regulating the transcriptional induction of PRGs.

PRGs (primary response genes)

The classical PRG is actively transcribed within a time frame of 2 hours after stimulation and contains conserved binding sites for class I transcription factors. Medzhitov and colleagues recently sub-divided this group of genes according to their GC-content in the promoter proximal region into PRG-I and PRG-II genes, with high or low GC-content, respectively. PRG-I genes can, based on their findings, be distinguished from PRG-II genes by the initial presence of activating chromatin marks, like H3K4me3 and H3K9Ac, and the presence of a paused polymerase at their promoters. Recruitment of the paused polymerase at these promoters is dependent on the constitutive factor SP1 that has the ability to bind GC-rich sequences. Some PRGs (i.e. *Nfkbia*, *Irf1*) are basally expressed but further induced upon treatment (Hargreaves et al., 2009). PRG-II genes lack these activating marks and are very often occupied by a polymease in a poised state. PRG-II genes are therefore only expressed after the recruitment of histone and polymerase modifying co-factors (see below), subsequent to class-I transcription factor binding. Additionally the promoters of PRGs are in most cases depleted of histones at the transcription factor binding sites and the TSS, thereby enabeling the fast access to DNA by DNA binding factors.

Pol II can be preloaded on the promoters of many genes that are not actively transcribed (Guenther et al., 2007; Muse et al., 2007; Zeitlinger et al., 2007). This was further demonstrated in regard to immediate mediators of the inflammatory response, i.e. TNFα gene expression (Adelman et al., 2009). Medzhitov and colleagues recently added another line of understanding to the observed phenomenon of NELF dependent polymerase stalling (see below), demonstrating that PRGs are very often occupied by paused polymerases waiting for the release of the elongation block. Moreover they could show that several PRGs do produce full-length but unmature and therefore unstable, transcripts even in the absence of an activating signal, due to the lack of Ser2 phosphorylation (Hargreaves et al., 2009).

As a consequence of their capability to induce rapid target gene expression, negative regulation of class I transcription factors is an important step to terminate expression of PRGs. Interestingly, most class I transcription factors downregulate their own activity by creating a negative "feedback loop" on the basis of their own PRGs. For example, STAT activity is antagonized by the expression of SOCS (suppressor of cytokine signaling) proteins, which are under the control of STAT itself (Alexander and Hilton, 2004). NFκB activity is antagonized by the NFκB driven expression of IκBα, which binds and exports NFκB from the nucleus and or facilitates its removal from the promoters of target genes (Hayden and Ghosh, 2008). STAT activation can, for example, occur downstream of IFN (interferon) signaling which leads to the binding of STAT1 homodimers or ISGF3 (interferon stimulated gene factor 3), a heterotrimeric factor comprised of STAT1, STAT2, and IRF9, to GAS (γ-IFN activated sequence) or ISRE (IFN stimulated response element), respectively (see below for detailed description). In particular STATs, IRFs and NFκB are relevant factors in the context of the work below.

Additional examples for PRGs relevant in the context of inflammation and anti-viral responses are: *Mx1/2* and *Irf7* genes, as examples for ISRE only driven genes downstream of IFN-I signaling (signaling initiated by binding of IFNα species or IFNβ to the IFNAR – IFNα receptor); or *Irf1* which is like SOCS genes, a GAS driven gene. The *Hif1α* (hypoxia induced factor) gene, which is an NFκB responsive gene and was shown to be expressed after TLR4 (Toll like receptor) stimulation (Ramanathan et al., 2009).

Class II transcription factors

Transcription factors of this category are synthesized de novo after a given stimulus and depend on the function of class I transcription factors. Therefore they all belong to the group of PRGs described above. These factors control the expression of SRGs (secondary response genes) subsequent to PRG expression over a prolonged period of time. Some class II transcription factors act, once expressed, on their own accord to drive target gene expression and some act in concert with Class-I transcription factors leading to transcriptional autoregulation and amplification of the response (Medzhitov and Horng, 2009). Examples of class II transcription factors in the context of an interferon mediated response after infection are: IRF1 which is expressed after activation of STAT1 homodimers in response to IFNγ and to a minor extent in response to IFN-I; IRF7, the master regulator of IFN-I responses, has proven to be crucial for the expression of all IFN-I genes, leading to the amplification of IFN-I signaling during sustained infections (Honda et al., 2005). Moreover HIF1α was shown to assist NFκB to drive the expression of a subset of NFκB target genes (Ramanathan et al., 2009).

SRGs (secondary response genes)

This sub-group of genes is expressed within 2-8 hours after treatment, according to the categorization of Medzhitov and colleagues. Most genes that participate in the generation of an antiviral-state belong to the group of SRGs. Prime examples for well known SRGs in the context of immunity against pathogens are: *Gbp1/2*, *Nos2* and *Ifnβ*. *Gbp1/2* (IFNγ inducible members of the p65 GTPase gene family with putative roles in the resistance to intracellular pathogens) are genes controlled via both GAS and ISREs. In the context of an IFNγ response STAT1 homodimers, as class I transcription factor, and IRF1, as class II transcription factor interact to induce their expression (Lew et al., 1991; Briken et al., 1995).

Nos2 encodes the protein iNOS (inducible nitric oxide synthase), an enzyme responsible for the production of NO (nitric oxide) radicals and their derivatives that directly lead to killing or reduced replication of infectious agents by mutation of DNA and inhibition of DNA repair and protein synthesis (MacMicking et al., 1997). Expression of the *Nos2* gene depends on signals emanating from IFN-receptors and on the activation of NFκB (i.e. by TNFα - tumor necrosis factor alpha - or TLR ligands). iNOS regulation has been extensively studied in the context of IFNγ and the TLR4 ligand LPS, leading to the conclusion that *Nos2* expression follows the same rules as any other IFN-II target gene, including the dependency on IRF1 (Kamijo et al., 1994; Meraz et al., 1996).

During bacterial infection, iNOS expression requires synthesis of IFN-β and signalling through the type I IFN receptor (IFNAR). IFN-β can itself be categorised as a SRG because its transcription depends on the formation of an enhanceosome complex (see below) comprised of class I and class II transcription factors, namely AP1 (activator protein1), NFκB, IRF3 and IRF7 (Panne et al., 2007). In keeping with this notion, IFN-β mRNA is not strongly expressed before 4hrs after treatment.

In contrast to PRGs, SRGs harbor no poised or paused polymerase, nor any activating marks on the core-histone tails (see below). The delayed expression of SRGs compared to PRGs is due to their dependence on de novo gene expression of class II transcription factors and the active recruitment of chromatin remodellers, histone modifying enzymes and Pol II complexes.

Class III transcription factors

This category of transcription factors consists of lineage specific factors that are upregulated during cell differentiation. In the case of macrophages PU.1 (also known as SPI1) and C/EBPβ (CCAAT/enhancer-binding-protein-β), as well as RUNX1 (runt-related transcription factor 1) and IRF8 are prime examples. They play important roles in maintaining the constitutive expression of genes, the preparatory chromatin-remodeling at inducible genes and the silencing of genes associated with alternative cell-fates (Medzhitov and Horng, 2009). RUNX1 for example has been shown to 'anchor' specific genomic loci to the nuclear matrix (the architectural scaffold of the nucleus) to assemble domains of active or inactive chromatin (Zeng et al., 1997).

Furthermore, Ghisletti and colleagues have generated new insight in the macrophage specific regulation of inducible genes. They monitored PU.1 binding at gene enhancers which was found to be colocalized with H3K4me1. Enhancers containing both PU.1 and H3K4me1 were primed to become activated by subsequent binding of stimulus dependent factors like NFkB, IRFs or AP.1 (activator protein1). This study, for the first time, showed a mechanism how tissue specific factors together with stimulus dependent factors together orchestrate the onset of macrophage specific gene-expression (Ghisletti et al., 2010).

Interferons and STATs

Since their discovery more than 50 years ago by Isaacs and Lindenmann (ISAACS and LINDENMANN, 1957), interferons (IFNs) have been subject to numerous studies leading to the identification of various IFN types, their receptors and the proteins involved in transmitting the signal from the receptor to the nucleus. Over 50 members of four-helix bundle cytokines like IFNs have been characterized so far resulting in an increased number of proteins involved in IFN and IFN-like signaling. The key-players of these signaling pathways are the members of the JAK (Janus kinase) family of protein tyrosine kinases and the STAT (signal transducers and activators of transcription) transcription factors. The following chapters will deal with the molecular principles of the JAK-STAT signaling and give an overview on STAT function as transcription factor.

Events leading to STAT activation

Subsequent to dimerization of the respective receptor, JAKs are activated. Contrasting RTK (receptor tyrosine kinases) the receptors recognizing IFNs (IFNAR, interferon alpha receptor, IFNGR, interferon gamma receptor or IFNλR, interferon λ receptor, for the recognition of type I IFNs, IFNγ or IFNλ, respectively) are not capable of autophosphorylation. Therefore they rely on the kinase function of the permanently associated JAKs to guarantee signal transduction to STATs. Four members of JAKs have been identified, namely TYK2 and JAK1/2/3.

The role and function of JAKs

JAKs range in size from 120 to 140 kDa and bear two critical C-terminal domains, one kinase (Ki) and one pseudokinase domain (ΨKi). Critical for JAK activation is the phosphorylation of two tyrosine residues in the activation loop that results in accessibility of the substrate binding site. Association with receptors is mediated by the N-terminal FERM (four point one, ezrin, radixin, moesin) domain that faciliates contact to proline-rich, membrane-proximal box1/box2 domains on cytokine receptors. In addition, JAKs contain a conserved SH2- related domain with unknown function (Decker et al., 2005; Schindler et al., 2007).

TYK2 was found to be involved in signaling downstream of IFN-I (type I IFNs), IL-6 (interleukin 6), IL-10, IL12 and IL23, thereby participating in allergic and antimicrobial responses. JAK1 is engaged in signaling downstream of IFN-I and IFN-II (IFNγ), IL-2/4, IL10 and IL6. JAK2 associates with single-chain receptors (i.e. Epo-R, GH-R, Prl-R) but also with IL-3R, IL-5R, GM-CSFR and IFNGR. JAK3 is expressed mainly in leukocytes. It exclusively associates with the common γ chain and therefore mediates signaling in response to IL-2 and additionally to IL-4, IL-7, IL-9, IL-15, and IL-21. All respective receptors share the common γ-chain for signaling (Schindler et al., 2007).

Upon receptor dimerization subsequent to ligand binding, JAKs become activated by transphosphorylation, as shown initially for JAK2 (Remy et al., 1999). This leads to the phosphorylation of the receptor on tyrosines that in turn can be recognized by the SH2 domain of STATs. Receptor bound STATs are thereby brought in close proximity to the JAKs leading to STAT phosphorylation. Thus, JAK kinase function is essential for signal transduction by the receptor as well as the STAT.

The STAT dimer

In mammals 7 STATs are known to receive the signal from the upstream JAKs as described above (STATs 1, 2, 3, 4, 5a, 5b and 6). The nature of STAT activation has remained enigmatic for a long time. Initial experiments focused mainly on STAT-effector function, clarifying their role as transcription factors thereby identifying STAT binding sites on promoters of hundreds of target genes.

Little was known about the physiological principal of STAT activation until Darnell and colleagues critically questioned whether the current model of STAT dimer formation only upon phosphorylation, reflected the situation in cells (Mertens et al., 2006). Becker and colleagues and Chen and colleagues (Mao et al., 2005; Neculai et al., 2005), who first crystalized STATs in the inactive /unphosphorylated form, demonstrated an anti-parallel structure of the inactive dimer. Based on these findings, Darnell and colleagues provided a novel model for STAT activation and their recycling after dephosphorylation in the cell nucleus (Mertens et al., 2006) (Figure 8).

Accordingly, STATs reside in the cytoplasm as preformed homo- or heterodimers (Braunstein et al., 2003) in an anti-parallel confirmation, where the N-terminus of one dimer partner lies next to the C-terminus of the other. STATs in general comprise several conserved domains: a CC (Coiled-Coil) domain is located next to the short N-terminal domain followed by a conserved DBD (DNA binding domain). The so called linker-domain connects the DBD with the SH2 domain. At the C-terminus transcription activation competent STATs contain the TAD (trans-activation – domain). The antiparallel STAT dimer is held together in the inactive state via direct contact of the N-terminal domains and reciprocal interactions of the CC domains with the DBDs (Figure 8).

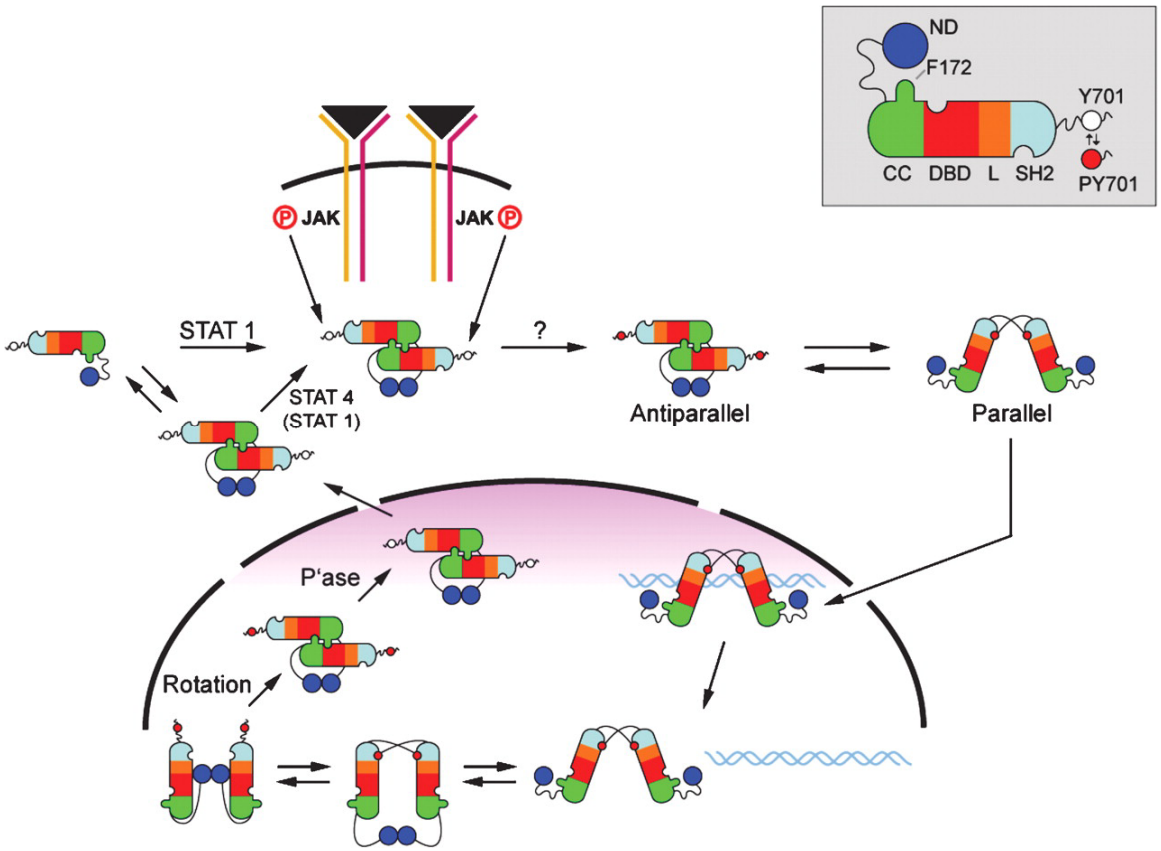

Figure 10: Model for STAT1 activation and recycling. Upon ligand binding and subsequent phosphorylation by JAKs, preformed STAT dimers undergo a conformational change from an anti-parallel to a parallel structure. The NDs (N-terminal domain), CC (coiled-coil) and DBDs (DNA-binding-domain) associate only in the anti-parallel and inactive dimer. Upon activation and rotation the SH2 domains, located at the C-terminal end, reciprocally bind the phosphotyrosine 701 of the dimer-partner. The active dimer translocates into the nucleus and binds at promoters of ISGs (interferon stimulated genes) to drive their expression. Inactivation of the STAT dimer is initiated by de novo interaction of NDs which might contact the body of the pSTAT. This in turn leads to rotation and CC-DBD interactions can occur. Dephosphorylation of Y701 by phosphatases takes place in the nucleus leading to subsequent nuclear export (Mertens et al., 2006).

Essential modifications for STAT function

Two amino acid residues were proven to be crucial for dimerization and activation of STAT proteins, residue F172 located in the CC-domain and a conserved tyrosine next to the SH2 domain (Y701 in case of STAT1). Phophorylation at the tyrosine residue by JAKs upon ligand binding is needed for the reciprocal pY-SH2 (tyrosine-SH2) interaction that initiates the rotation of the dimer into a parallel and transcriptionally active form. According to Darnell and colleagues this rotation enables the STATs to form a structure capable of binding DNA and at the same time hiding the tyrosine residue from becoming dephopshorylated by phosphatases. The N-terminal interaction between the two dimer partners is lost upon rotation into the active form. In addition, this event is crucial for liberating the NLS (nuclear localization sequence) for subsequent nuclear import by importin α, thereby shifting the balance for continuous nuclear export/import toward nuclear accumulation (Schindler et al., 2007).

Nevertheless, the N-terminal domain has been shown to be essential also in the process of inactivating the STAT dimer (Mertens et al., 2006). De novo interaction of the STAT N-terminus in the parallel conformation induces rotation into the antiparallel state resulting in the protrusion of the SH2 domains out of both ends of the now anti-parallel dimer. This in turn results in the dephosphorylation of the tyrosine residue, which is in this conformation no longer protected from phosphatases, and in the nuclear export of the now inactive STAT dimer (Figure 1).

Besides tyrosine phophorylation, STATs in general undergo further modifications including serine phosphorylation, acetylation and O-glycosylation. Serine phosphorylation takes place in the TAD of all STATs with STAT2 as notable exception (Decker and Kovarik, 2000). For STAT1 the phosphorylation occurs within a conserved PMS*P motif at position 727. STAT1 and STAT5, at least, contain a second serine-residue in the TAD at position 708 and 779, respectively, which can be phosphorylated (Tenoever et al., 2007). The phosphorylation of serine residues in the TAD domain of STATs has been shown to be needed for transcriptional activity (Varinou et al., 2003). The kinase responsible for STAT1 serine phosphorylation is still unknown though recent data suggest that in the context of IFN responses STAT1 has to be localized in the nucleus to become S^{727} phosphorylated (Sadzak et al., 2008). Furthermore, recent findings indicate a role for non-tyrosine phosphorylated STATs in regulating transcription following stimulation with TLR- (toll like receptor), IL1R- or TNFR (tumor necrosis factor) ligands, or in the context of IFN responses (Decker and Kovarik, 2000; Yang et al., 2005; Cheon and Stark, 2009).

The role of STAT lysine-acetylation has been subject to numerous studies. Reportedly, acetylation of STAT3 positively regulates transcriptional activity and homodimer stability (Yuan et al., 2005). STAT1 acetylation at lysines K410 and K413 was suggested to regulate dephosphorylation. Because dephosphorylation is essential for STAT1 recycling this finding may explain the repression of STAT1 target genes following pretreatment with the HDAC (histone deacetylase) inhibitor TSA (trichostatin A) (Zupkovitz et al., 2006; Krämer et al., 2009; Krämer and Heinzel, 2010).

O-glycosylation has been shown to be essential for STAT5 interaction with its co-activator CBP (cAMP responsive element binding protein – CREB binding protein). The glycosylated residue of STAT5 is conserved also in STAT1, STAT3 and STAT6 (Gewinner et al., 2004).

The biological role of STAT-family members

The seven members of the STAT family of transcription factors are embedded in a series of signaling events ranging from signal transduction downstream of hormone receptors (prolactin, growth hormone), hematopoietin receptors (e.g. GM-CSFR (granulocyte/macrophage – colony stimulating factor receptor, IL-3, 5, 7-R, EpoR (erythropoietin receptor), or receptors for various interleukines and IFN that regulate immune responses.

STAT1 as the first STAT described in literature, is activated subsequent to IFN-II (Type II IFN - IFNγ) stimulation, thereby initiating the formation of STAT1 homodimers, also called GAF (GAS – γ-IFN activated site - binding transcription factor), the key factor for IFNGR signaling. The GAS consensus binding sequence for STAT1 homodimers is a palendromic sequence: TTCNNNGAA (N = any base). STAT1 target genes promote inflammation and antagonize proliferation thereby antagonizing the effects of STAT3 target genes. Studies in gene targeted mice and humans expressing STAT1 mutants underline the importance of STAT1 in the immune response against viral and bacterial infections. Many genes stimulated by STAT1, in context of an IFN-γ response, require cooperative effects by other transcription factors like IRF1, SP1, USF-1 or C/EBPβ (Decker and Kovarik, 1999).

STAT1 like STAT2 was originally identified as part of the ISGF3 (interferon stimulated gene factor 3) complex downstream of IFN-I signaling, which is comprised of a STAT1/2 heterodimer in complex with IRF9 (interferon regulatory factor 9). This heterotrimeric factor binds to ISREs (interferon stimulated response elements) following the consensus sequence: PuPuTTTCNNTTTPyPy (Pu=Purine; N=any base; Py=Pyrimidine). Recent data indicate that ISGF3 is also activated downstream of the IFN-III (IFNλ) receptor. In the context of an IFNγ response the ISRE mediates transcriptional effects of IRF1 and non-canonical STAT1 complexes e.g., STAT1 homodimer associated with IRF9 (Bluyssen et al., 1995; Kimura et al., 1996). Many ISGs (interferon stimulated genes) can be expressed after treatment with both IFN-I or IFN-II, pointing towards a similar role of IFN-I and IFN-II in inducing an anti-viral response. Still the two IFN classes play different roles in immunity to infection. IFNγ, is produced by NK- or NKT-cells very early after infection in a STAT4 dependent manner or by T_H-1 or $CD8^+$ T-cells of the adaptive immune-system at a later stage of the immune response. IFNγ is the main macrophage activating cytokine, thus participating in the defence against invading bacterial pathogens (see below) (Schroder et al., 2004). Studies in STAT1 deficient mice confirm the essential role of STAT1 for both ISGF3 complex-mediated IFN-I responses and for STAT1 homodimer-mediated antibacterial responses. Such animals are exceedingly sensitive to both viral and bacterial pathogens (Durbin et al.1996 Meraz et al.1996) Despite the predominant role of IFN-I and IFN-γ in antiviral and antibacterial immune responses, respectively, there are significant contributions of both IFN types to immune responses against all classes of pathogens (Decker et al., 2002; Decker et al., 2005).

The main role of STAT3 is to mediate signalling by cytokines of the IL-6 family. However, this family member is engaged also downstream of the receptors for IL-10 as well as for (G)-CSF, leptin, IL-21 and IL-27. STAT3 often counteracts the proinflammatory role of STAT1. Tissue specific knock-out in mice confirmed the anti-inflammatory role of STAT3 (Kisseleva et al., 2002; Levy and Darnell, 2002). Finally, STAT3 was found to promote tumor growth through non-canonical mechanisms, i.e. in the absence of tyrosine phosphorylation and/or DNA binding (Yang et al., 2005; Schindler et al., 2007).

STAT4 and STAT6 are non-redundant antagonistic players, essential for the development of either a type-I or type-II immune-responses. Both were extensively studied in the field of T-Cell development. STAT4 directs the IL-12 dependent lineage commitment of $CD4^+$ T-cells to T_H-1 cells (Lee et al., 2006; Wilson et al., 2009). As mentioned above STAT4 induces IFNγ production by T_H1 as well as NK- or NKT-cells and is involved in the IL-23 dependent expansion of T_H-17 cells (Hunter, 2005). In contrast, STAT6 mediates T_H2 differentiation and the production of T_H2 signiture cytokines IL-4, IL-5 and IL-13. In line with this, STAT4 is essential for the clearence of infection with viral, intracellular bacterial or protozoan pathogens via cellular mechanisms, whereas STAT6 enhances IL-4 mediated isotype switching in B-Cells, predominatly leading to IgE production. Therefore STAT6 mainly contributes to mast-cell activation dependent immunity against helminths, such as gastrointestinal nematodes (Finkelman et al., 2004). The molecular principles how these two STATs orchestrate differential gene expression will be further highlighted (see below).

Two tandem genes encode STAT5a and STAT5b which share extremely high sequence identity (~ 96% aa). STAT5a/b are activated downstream of IL-3 (IL-3, IL-5, and GM-CSF), single chain and γ_c receptor families. The main function of STAT5a/b was revealed by recent gene targeting studies demonstrating a role for STAT5(s), among others, in erythropoiesis and lymphopoiesis (Yao et al., 2006).

STATs and chromatin

STATs, upon activation and binding to their consensus binding sites at promoter or enhancer regions, make contact to co-factors needed to remodel and modify histones at the promoter as a pre-requisite for Pol II binding and/or transcriptional initiation. To date, a limited set of experiments provide an idea how STATs interact with co-factors to induce transcription of both PRGs and SRGs. With the development of ChIP (chromatin immune precipitation) -sequencing, as a method to analyze ChIP data quantitatively on a genome wide basis, it was possible to compare histone modification patterns with sites of STAT recruitment to find correlations and interdependencies (Robertson et al., 2007).

STATs and chromatin remodeling

The STAT1 driven expression of the transcription factor CIITA upon IFNγ treatment dependens on BRG1. Interestingly, detailed analyses of the CIITA enhancers conferred only partial dependency on BRG1 (Ni et al., 2008). In line with the reports by the Nasmyth laboratory (see above), STAT1 binding itself relies on BRG1-dependent chromatin remodeling at the CIITA promoter and a series of other STAT1 and ISGF3 target genes, like *Gbp1/2*. Constitutive BRG1 association to IFN-γ target genes is STAT1 independent (Ni et al., 2005). Nevertheless, BRG1 specificity and effects on the promoters of different STAT target genes can be highly divergent, as illustrated by the requirement for BRG1 activity at the *Irf1* promoter for the recruitment of STAT3 but not for STAT1 homodimers (Ni and Bremner, 2007). In contrast, BRG1 recruitment to the *Ifnγ* promoter in T_H1-cells is STAT4 dependent (Zhang and Boothby, 2006).

STATs and histone modifications

Recent data demonstrate that chromatin modification and remodeling events have to precede STAT1 binding to the promoters of IFNγ driven genes (Robertson et al., 2007; Robertson et al., 2008). To date the *Ifnγ* promoter itself is one of the most intensively studied promoters, in the context of STAT-orchestrated chromatin dynamics. STAT5 was shown to be responsible not only for inducing remodelling at the *Ifnγ* promoter but also for increased histone acetylation (Shi et al., 2008). STAT4 binds to the *Ifnγ* promoter and and enhancer regions leading to the induction of permissive genetic modifications and the activation of gene expression. The *Ifnγ* locus comprises bivalent chromatin modifications, a combination of the activating H3K4me3 and the repressive H3K27me3 mark, which makes it poised for either activation or silencing. Upon activation H3K4 methylation is enhanced and H3K27 methylation is reduced (Wilson et al., 2009).

Recently, Lei Wei and colleagues studied the chromatin status of *Ifnγ, Il4* and *Il17* gene loci in T_H1, T_H2, iTreg and nTreg cells. STAT4- and STAT5-dependent increases of H3K4me3 marks were found in T_H1 and iTreg cells, respectively. Observed H3K27me3 patterns in these cells support the notion that, once differenciated, STATs and other transcription factors regulate the cell fait of these cells by the introduction of active or repressive chromatin marks (Wei et al., 2009). In a different approach, Lei Wei and colleagues monitored the distribution of H3K4me3, H3K27me3, and H3K36me3 along with STAT4 and STAT6 binding to reveal the interdependency of the chromatin modifications with either of the STATs in the context of T_H1/T_H2 – differential gene regulation. Only a small proportion of STAT dependent genes showed also STAT dependent changes in histone modifications. STAT4 was in this regard found to mainly promote accessible marks (H3K4me3), whereas STAT6 had a prominent role in antagonizing repressive marks (H3K27me3), to induce transcription. Genes whose transcriptional and epigenetic regulation depend on STATs in T_H1/T_H2-cells, include phenotype-defining cytokines (*Ifnγ, Il4* and *Il24*), receptors (*Il18rap, Il18r1, Lag3,* and *Il4ra*), transcription factores (*Gata3, Tbx21*), transcriptional repressors (*Id2* and *Zbtb32*), and to date unrecognized STAT dependent genes, upregulated in T_H1/T_H2 –cells. These included *Hipk2*, *Plcd1*, *Skap2*, and *Gbp2* (Wei et al., 2010).

STAT cooperation with Class I transcription factors

As described above STAT-mediated gene expression very often relies on the concerted interplay of STATs with other factors. In context of STAT1 during an IFNγ response it is very well established that IRF1 is required for gene induction of SRGs (Decker and Kovarik, 1999). Interestingly, STATs can also share labor with partners of their own family, for example when STAT1 and STAT2 are combined in the ISGF3 complex downstream of IFNAR signaling. A very recent and surprising relationship is the interdependent action of STAT4 and STAT6 to ensure differential expression of lineage-specifying genes, like *Il-18r1-l18rap*. Both STATs can bind the promoter to either suppress or activate transcription (Wei et al., 2010).

The mechanism of STAT3 and NFκB co-requirement for the induction of the *Il1rn* (IL1 receptor antagonist) gene has recently been clarified. In case of the human *Il1rn* promoter STAT3, upon IL-10 and LPS cotreatment, precedes NFκB recruitment, and leads to chromatin rearrangements in order for NFκB to get access to its promoter binding site, and to initiate transcription (Tamassia et al., 2010).

NFκB and STAT interactions have been subject to numerous studies in recent years, since both factors are crucial mediators of an immunological response to pathogens. Paradigmatic for the group of genes, induced after pathogen exposure, that requires both STAT and NFκB to get expressed, is the *Nos2* gene, encoding iNOS.

iNOS as a prime example for STAT-NFκB convergence

Upon innate immune responses iNOS is expressed and produces NO (nitric oxide) radicals, which exert direct antimicrobial activity and regulate cell survival (Bogdan, 2001; Zwaferink et al., 2008). The regulation of iNOS expression was mainly studied using, on the one hand, LPS (lipopolysaccharid) as TLR4 ligand to activate NFκB, and IFNγ for active STAT1 homodimer formation, on the other hand. Activation of the NFκB, p65/p50 heterodimer, is induced downstream of the canonical NFκB pathway involving the activation of the IKK-complex, leading to the subsequent phosphorylation, and thereafter ubiquitination of IκBα (Hayden and Ghosh, 2008).

The *Nos2* promoter contains among others, GAS, ISRE and NFκB-BS (NFκB- binding sites). It was shown to require NFκB, STAT1 and, like other ISGs, IRF1 for full expression, after LPS and IFNγ treatment (Kamijo et al., 1994; Meraz et al., 1996). However, to date little effort was made in understanding the molecular events leading to iNOS regulation in response to infections with intracellular pathogens. A well-known example of these is the Gram-positive, facultatively intracellular bacterium *Listeria monocytogenes*. Following uptake, this pathogen escapes to and replicates in the host cell cytoplasm, owing to its ability to disrupt endosomal or phagosomal compartments with the help of its encoded hemolysin, listeriolysin O (LLO). In murine BMDMs (bone marrow derived macrophages) a hitherto unknown cytoplasmic receptor senses infection and induces signaling to the IFN-I genes. The first IFN-I to be produced by infected cells is IFN-β. Formation of an enhancosome at the IFN-β promoter and subsequent transcription requires members of the IRF family, particularly IRF3 (Panne et al., 2007).

The activation of IRF3 was shown to depend on the two kinases TBK1 (TANK - TRAF family member associated NFκB activator - binding kinase) and IKKε, but not on the adaptor protein MAVS (mitochondrial anti-viral signaling protein) (Stockinger et al., 2004; Stetson and Medzhitov, 2006; Soulat et al., 2006). Importantly, both IRF3, IFN-β and the IFNAR were essential for iNOS expression in macrophages infected with *L. monocytogenes* (Stockinger 2004), suggesting iNOS to belong with the large category of antimicrobial SRG that are co-regulated by primary infection-derived signals such as NFκB and by IFN-I (Figure 11). The molecular mechanism or biological significance underlying this two-signal requirement for iNOS expression have not been explored.

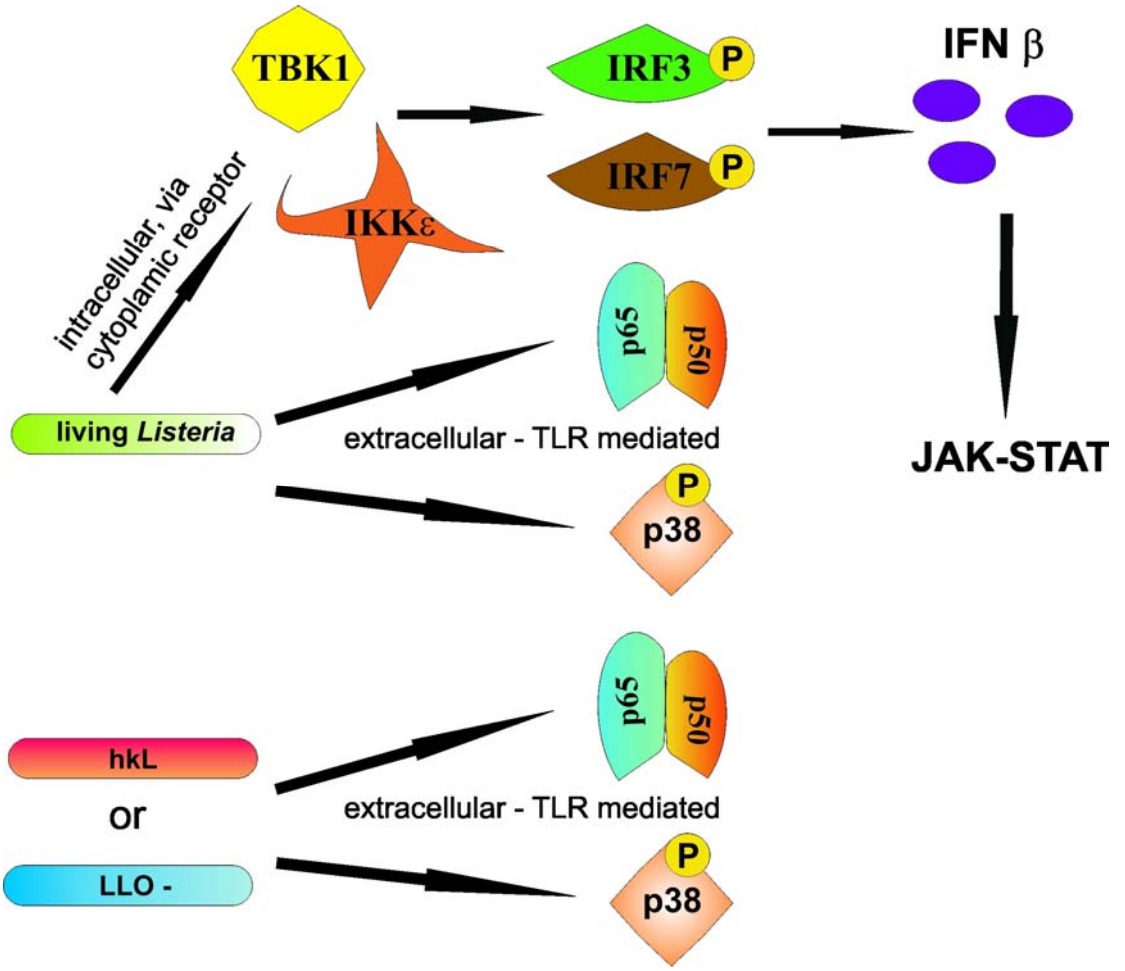

Figure 11: Pathways activated after infection with LL (living *Listeria*) ot treatment with hkL (heat killed *Listeria*) or LLO – *Listeria* (*Listeria* deficient for the poreforming protein Listeriolysin O). Among other factors, LL induce early after their recognition by host cells, the activation of the heterodimers p65/p50, forming the transcription factor NFκB, and the MAPK p38 via TLR mediated signaltransduction. In addition, a hitherto unknown receptor senses *Listeria* as soon as they escape, in an LLO dependent manner, from the phagolysosome to the cytoplasm. Once recognized in the cytoplasm the signaltransduction in turn activates the two kinases TBK1 and IKKε, which are required for the activation of the class I transcription factor IRF3 and the class II transcription factor IRF7. Activation of both lead, together with acticated NFκB and AP1, to the production of type I IFNs, in particular IFNβ and IFNα4, in a first wave of type I IFN production. IFNβ production and subsequent signaling leads to the activation of ISGF3 and to the expression of ISGs. Notably, signaling cascades emanating from the treatment with hkL or LLO- *Listeria*, activate the TLR dependent extracellular signaltransduction but fail to induce endogenous IFNβ production, due to their inability to reach the host cell cytoplasm.

Aims

In recent publications STATs have been found to cooperate with many other factors to induce gene expression of ISGs. The major goal of this study was to identify and characterize the role of STAT1, in cooperation with IRF1 or NFκB, in the processes leading to active gene expression. For this purpose two different situations were chosen: first, the interdependency of STAT1 and IRF1 in the regulation of genes after treatment with IFNγ represented an example of transcription factor cooperation within the same signalling pathway. Second, the cooperation of STAT1 and NFκB in the regulation of genes after infection with *Listeria monocytogenes* represented the cooperation of transcription factors activated by distinct signalling pathways. The main focus of these studies was to examine the requirement of transcription factors for co-factor recruitment and for induced changes in chromatin structure and/or composition, which in turn enables the binding of factors of the PIC and Pol II.

Results

Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-g inducible *Gbp2* gene

Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-γ-inducible *gbp2* gene

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A subgroup of genes induced by IFN-γ requires both STAT1 and IRF1 for transcriptional activation. Using WT, *stat1*$^{-/-}$, or *irf1*$^{-/-}$ cells, we analyzed the changes induced by IFN-γ in *gbp2* promoter chromatin. STAT1 associated with the promoter independently of IRF1 and played an essential role in the ordered recruitment of the coactivator/histone acetyl transferase CREB-binding protein (CBP) and the histone deacetylase HDAC1. Hyperacetylation of histone 4 also required STAT1. Phosphorylation at S727 in the transactivating domain increased transcriptional activity of STAT1. In cells expressing a STAT1S727A-mutant CBP recruitment, histone 4 hyperacetylation and RNA polymerase II association with the *gbp2* promoter were strongly reduced. IRF1 association with the *gbp2* promoter followed that of STAT1, but STAT1 association with DNA or histone hyperacetylation were not necessary for IRF1 binding. RNA polymerase II association with the *gbp2* promoter required both STAT1 and IRF1, suggesting that both proteins mediate essential steps in transcriptional activation. IRF1, but not STAT1, was found to coimmunoprecipitate with RNA polymerase II. Together, the data support the assumption that the main role of STAT1 in activating *gbp2* transcription is to provide transcriptionally competent chromatin, whereas the function of IRF1 may lie in directly contacting RNA polymerase II-containing transcriptional complexes.

chromatin | interferon | signal transduction | interferon regulatory factor

IFN-γ enhances cell-mediated immunity against both nonviral pathogens and viruses (1). STAT1, the central mediator of IFN-γ-induced gene expression is phosphorylated at Y701 by the IFN-γ receptor-associated Janus kinases Jak1 and Jak2, an essential prerequisite for dimerization and nuclear translocation (2). In addition, a serine/threonine kinase phosphorylates the STAT1 transactivating domain at S727 and increases transcriptional competence (3–5). Promoter sequences found in IFN response regions are the γ-IFN activated site (6) recognized by STAT1 dimers and the IFN-stimulated response element (ISRE) (7). ISRE sequences bind STAT complexes and also IFN regulatory factors (IRFs) (8, 9). In the context of the IFN-γ response, the ISRE mediates transcriptional effects of IRF1 and noncanonical STAT1 complexes, e.g., STAT1 dimers associated with IRF9 (10–12).

STAT1 has been linked predominantly to positive gene regulation, but some genes are repressed by STAT1 (13–15). Many genes stimulated by STAT1 in the context of an IFN-γ response require cooperative effects with other transcription factors, such as IRF-1, USF-1, SP1, or C/EBPβ (16). In most cases both STAT1 and the cooperating transcription factor bind to their cognate promoter sequences, although the cooperation with C/EBPβ is mediated by a sequence designated GATE, which binds C/EBPβ but not STAT1 (17).

The *gbp1* and *gbp2* genes are IFN-γ-inducible members of the p65 GTPase gene family with putative roles in the resistance to intracellular pathogens (18). *gbp2* transcription in both humans and mice requires promoter binding sites for both STAT1 dimers and IRF transcription factors (6, 19). Guanylate-binding protein (GBP) expression in response to IFN-γ is virtually absent in cells from *irf1* knockout mice (10, 11), The promoter of the *irf1* gene contains a binding site for STAT1 dimers. Therefore, IRF1 accumulates in cells treated with IFN-γ (20).

Transcriptional activation of the *gbp* genes is accompanied by promoter acetylation (5). Consistently, STAT1 interacts with the coactivator/histone acetyl transferase (HAT) CREB-binding protein (CBP) that is required for STAT1-dependent transcription of chromatin templates *in vitro* (5, 21, 22). Moreover, microarray analysis of HDAC1-deficient cells identified *gbp* genes as belonging within a group of genes requiring histone deacetylase 1 (HDAC1) for IFN-γ-induced expression (23). STAT1 also directly binds a complex of MCM proteins that enhance *gbp* transcription most likely by providing helicase activity for strand separation in the initiation and elongation steps (24). BRG1, an ATPase subunit of the SWI/SNF chromatin remodeling complex, binds the human *gbp* promoter as a prerequisite for the association of STAT1 (25, 26).

Serving as a paradigm for the group of genes coregulated by STAT1 and IRF1, the *gbp2* gene allows us to address two important open questions. (*i*) What is the relative importance of STAT1 for IFN-γ-induced *gbp2* transcription as a transcriptional activator of the *irf1* gene on the one hand and as a cognate binding factor of the *gbp2* promoter on the other? (*ii*) What is the nature of the molecular mechanisms mediating cooperative stimulation of *gbp* transcription by STAT1 and IRF1? Our studies show that both STAT1 and IRF1 are required at the *gbp2* promoter to recruit RNA polymerase II (RNA pol II) to the transcription initiation site. STAT1 and its phosphorylation at S727 are essential for CBP recruitment, and STAT1 also mediates the association of the promoter with HDAC1. By contrast, the binding of IRF1 to the *gbp2* promoter occurs independently of STAT1 binding and histone acetylation, but it cooperates with STAT1's activities in recruiting RNA pol II.

Results

Organization of the Murine *gbp1*/*gbp2* Promoters and Regulation of Their Activity by STAT1 and IRF1. A cluster of five GBP genes maps to mouse chromosome 3 (www.ensembl.org). Previously, three groups described murine GBP promoters. The first report assigned

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The authors declare no conflict of interest.

Abbreviations: BMDM, bone marrow-derived macrophage; CBP, CREB-binding protein; dox, doxycyclin; IP, immunoprecipitation; GAS, IFN-γ-activated site; GBP, guanylate-binding protein; HAT, histone acetyl transferase; HDAC, histone deacetylase; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element.

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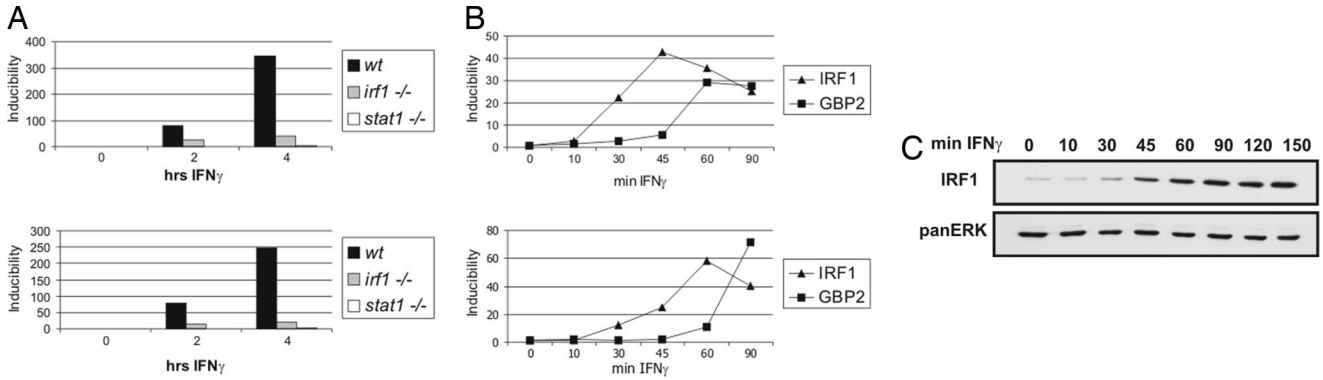

Fig. 1. Regulation of the *gbp1* and *gbp2* genes by IFN-γ dependence on the presence of STAT1 and IRF1. (*A*) *gbp1* (*Upper*) and *gbp2* (*Lower*) expression in *irf1*- and *stat1*-deficient fibroblasts. Immortalized WT, *irf1*$^{-/-}$, and *stat1*$^{-/-}$ fibroblasts were treated with 10 ng/ml (240 units/ml) IFN-γ for the indicated times and analyzed for expression of *gbp1* and *gbp2* by real-time PCR. Inducibility was calculated after normalizing to GAPDH mRNA levels. (*B*) Kinetics of *irf1* and *gbp2* expression. Primary BMDMs were treated with 10 ng/ml (240 units/ml) IFN-γ for the indicated times. Nuclear RNA (hnRNA), isolated from purified nuclei, and mRNA from whole-cell extracts were isolated and reverse transcribed. Inducibility of *irf1* and *gbp2* hnRNA (*Upper*) and mRNA (*Lower*) expression were analyzed by real-time PCR and normalized to endogenous GAPDH. (*C*) Western blot analysis of IRF1 protein expression was performed with lysates from WT BMDM treated with 10 ng/ml (240 units/ml) IFN-γ for the indicated time. Equal loading was determined by probing the membrane with anti-panERK Abs.

the cloned promoter to the *gbp1* gene (27). Two further groups subsequently cloned the same stretch of DNA and a further one differing by only a few base pairs but with an identical IFN response region (19, 28). Those two groups concurred in their interpretation that the cloned DNAs contained highly homologous promoters of the *gbp1* and *gbp2* genes. The ensembl database shows the *gbp1* and *gbp2* genes juxtaposed and in the same orientation, spaced by a short intergenic region of 1,466 bp. The previously described promoters are all highly homologous to the intergenic region containing the *gbp2* promoter, but none shows significant homology to the region upstream of *gbp1*, suggesting that all of them represent allelic variations of the *gbp2* promoter (see Fig. 2*A*). Quantitative real-time PCR analysis showed that the *gbp1* and *gbp2* genes are strictly coregulated, as would be expected from the use of common promoter elements (Fig. 1*A*). Both showed an identical requirement for the presence of STAT1 and IRF1, with the need for STAT1 being more stringent than that for IRF1. Inspection of the *gbp1* upstream sequence revealed the presence of bona fide IFN-γ-activated site (GAS) and ISRE sequences at positions −253/−245 (TTCATAGAA) and −139/−127 (AATTTCACTTTCT), respectively. These *gbp1* upstream sequences most likely constitute an IFN-γ response region, but functional analysis will be required to ascertain this assumption.

The IFN-response region of the *gbp2* promoter is divided into an ISRE proximal to the cap site and more distal GAS and ISRE sequences (Fig. 2*A*). Our recent ChIP experiments showed that the proximal *gbp2* promoter ISRE associates with a noncanonical, IFN-γ-activated STAT1 complex (5). The distal GAS element is a canonical, although imperfect, binding site for STAT1 dimers (19).

To determine the temporal sequence of IRF1 accumulation and *gbp2* expression, we determined IFN-γ-induced accumulation of *irf1* nuclear RNA (hnRNA), mRNA (Fig. 1*B*), and protein (Fig. 1*C*). Unspliced *irf1* hnRNA was already 50% maximal 30 min after addition of cytokine. The accumulation of cytoplasmic mRNA was delayed by ≈15 min and protein synthesis by yet another 15 min. Nuclear *gbp2* mRNA closely correlated with amounts of IRF1 protein, and the delay between hnRNA synthesis and cytoplasmic mRNA accumulation was similar as in the case of *irf1*. The data thus confirm that *gbp2* mRNA transcription is mostly a secondary response to IFN-γ and that in the presence of STAT1 dimers, IRF1 availability limits the rate of *gbp2* nuclear RNA synthesis.

Recruitment of Transcriptional Regulators to the *gbp2* Promoter. To address the changes of *gbp2* promoter chromatin after IFN-γ stimulation, we performed antibody (Ab)-mediated ChIP. Consistent with our previous results (5), STAT1 was recruited rapidly to the proximal and distal response elements (Fig. 2*B*). Recruitment of the HAT CBP and histone 4 hyperacetylation closely paralleled STAT1 binding. Recruitment of HDAC1 also paralleled that of

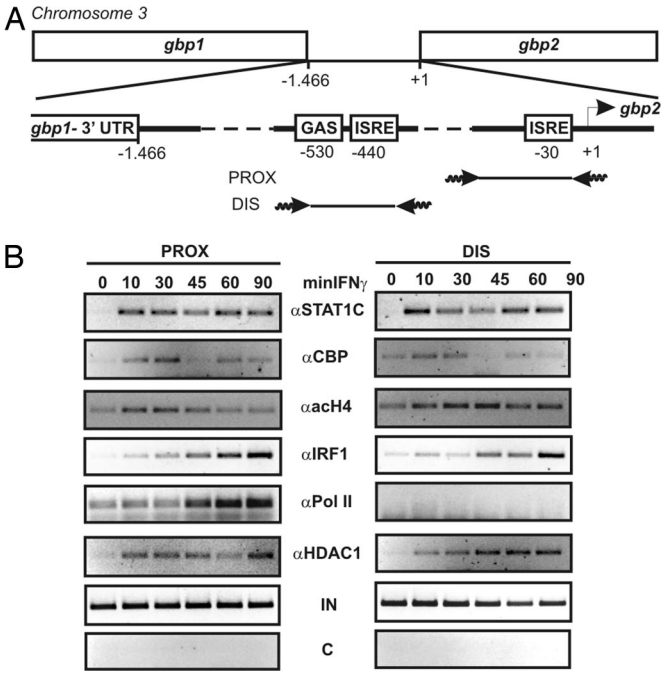

Fig. 2. Recruitment of transcriptional regulators to the *gbp2* promoter chromatin. (*A*) Graphic representation of the murine *gbp2* promoter region. *gbp1* and *gbp2* are located on chromosome 3 and are separated by 1.466 bp of intergenic region. This region contains the regulatory GAS and ISRE elements. Primer pairs used for ChIP assays are depicted. (*B*) Recruitment of STAT1 (αS1C), IRF1 (αIRF1), RNA pol II (αPol II), CBP (αCBP), and HDAC1 (αHDAC1) to the distal (DIS) and proximal (PROX) regions of the *gbp2* promoter, and hyperacetylation of histone 4 (αacH4) of the respective promoter regions, as analyzed by ChIP. Primary BMDM were treated with 10 ng/ml (240 units/ml) IFN-γ for the indicated time points (minIFN-γ), and formaldehyde-cross-linked chromatin was isolated and subjected to IP with the indicated Abs. The promoter elements were analyzed by amplification of the distal and proximal *gbp2* promoter regions by PCR. The specificity for the IP was determined by using preimmune serum (C) as negative control and amplification of input DNA (IN) by PCR.

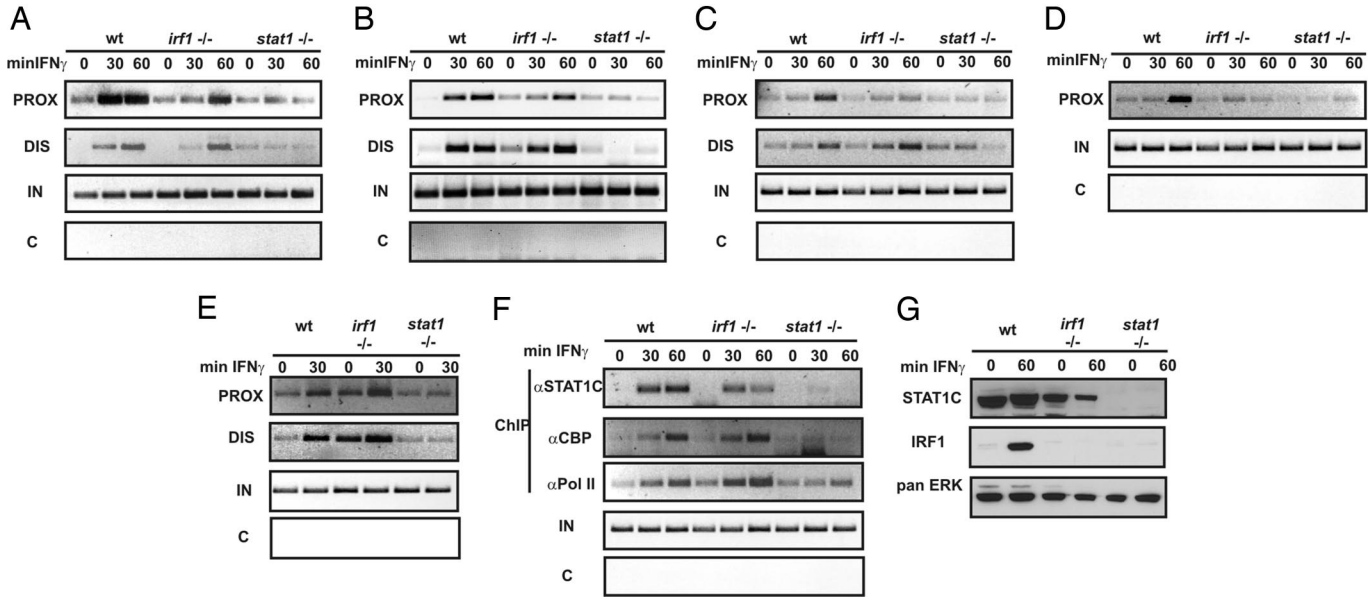

Fig. 3. Impact of *irf1* or *stat1* deficiency on the IFN-γ-induced alterations of *gbp2* promoter chromatin. (*A–E*) ChIP assays were performed with immortalized WT, *stat1* (*stat1*$^{-/-}$)-, and *irf1* (*irf1*$^{-/-}$)-deficient fibroblasts for binding of STAT1 (αS1C) (*A*), hyperacetylation of histone 4 (αacH4) (*B*), recruitment of CBP (αCBP) (*C*), RNA pol II (αPol II) (*D*), and HDAC1 (αHDAC1) (*E*) to the proximal (PROX) and distal (DIS) regions of the *gbp2* promoter. The cells were treated for 30 or 60 min with 10 ng/ml (240 units/ml) IFN-γ, and formaldehyde-cross-linked chromatin was immunoprecipitated with the indicated Abs. (*F*) IFN-γ-dependent factor recruitment to the *irf1* promoter. DNA isolated from *A–E* was subjected to PCR by using primers recognizing the *irf1* promoter. (*G*) Western blot analysis of whole-cell extracts from WT, *irf1*-, and *stat1*-deficient fibroblast for protein expression of STAT1 and IRF1. The cells were treated for 60 min with 10 ng/ml (240 units/ml) IFN-γ or left untreated. The membrane was probed with a STAT1 C-terminal Ab, α-IRF1 Ab, and with anti-panERK Abs for equal loading.

STAT1. This finding is consistent with earlier reports showing that STAT1 interacts with these proteins when coimmunoprecipitated from cell extracts or in pull-down assays (29). IRF1 also bound both the proximal and distal promoter regions (Fig. 2*B*). Promoter binding was delayed by ≈20 min compared with STAT1. The kinetics of RNA pol II recruitment were similar to those of IRF1 binding, which in turn closely paralleled the accumulation of IRF1 protein and nuclear *gbp2* mRNA (Fig. 1).

Further investigation of the *gbp2* promoter was performed in gene-disrupted fibroblasts. The kinetics of *gbp2* expression and transcription factor recruitment in this cell type were not significantly different from those observed in macrophages (data not shown). IFN-γ-induced STAT1 binding was virtually unaffected by the absence of IRF1 (Fig. 3*A*). Slightly reduced association particularly with the proximal site reflects reduced STAT1 expression in *irf1*$^{-/-}$ cells (Fig. 3*G*), which is most likely due to the role of IRF1 in maintaining STAT1 expression through autocrine type I IFN production (30). IRF1 binding to the *gbp2* promoter was completely abolished in *stat1*$^{-/-}$ cells (data not shown) because of the complete lack of IFN-γ-induced IRF1 synthesis (Fig. 3*G*).

STAT1 deficiency caused an almost complete absence of CBP and HDAC1 recruitment, histone 4 hyperacetylation, and RNA pol II binding (Fig. 3 *B–F*). By contrast, IRF1 deficiency had little impact on the association of HDAC1. CBP recruitment or the hyperacetylation of H4 particularly at the proximal promoter were reduced, but an IFN-γ-stimulated increase was clearly detectable. Strikingly, however, RNA pol II recruitment to the *gbp2* cap site was highly dependent on the presence of IRF1. To demonstrate specificity of these findings, IFN-γ-dependent factor recruitment to the *irf1* promoter was examined. Consistent with the lack of an IRF1-binding site in its IFN-γ-response region, CBP and, importantly, RNA pol II association were found to be unaffected by the absence of IRF1 protein (Fig. 3*F*). In contrast, neither protein was found to be associated with the *irf1* promoter in absence of STAT1.

Effect of Mutating the S727 Phosphorylation Site in the STAT1 Transactivating Domain. STAT1 S727 phosphorylation is essential for IFN-γ-induced, *gbp2* promoter histone 4 hyperacetylation. Moreover, CBP does not efficiently bind STAT1S727A in IFN-γ-treated cells (5). Consistent with these earlier findings, the recruitment of CBP to *gbp2* promoter chromatin was virtually absent in cells expressing a STAT1S727A phosphorylation site mutant, and absence of CBP coincided with strongly reduced H4 acetylation (Fig. 4 *A* and *B*). Accumulation of IRF1 protein in response to IFN-γ is reduced by >50% in cells expressing STAT1S727A (Fig. 4*E*). Despite this reduction and the lack of histone acetylation, particularly at the proximal *gbp2* promoter, IRF1 association with the IFN response region was not very different from that found in cells expressing wild-type (WT) STAT1 (Fig. 4*C*). The specificity of IRF1 binding was confirmed by the lack of amplification of the *irf1* promoter in the same ChIP DNA samples (Fig. 4*C*). The binding of RNA pol II was significantly decreased in IFN-γ-treated cells expressing STAT1S727A (Fig. 4*D*), which is in line with the strong effect of the STAT1S727A mutation on *gbp* transcription (5, 31).

Analysis of *gbp2* Expression and *gbp2* Promoter Chromatin in Cells Expressing IRF1 in Absence of Stat1 Activity. *Irf1* being a STAT1-regulated gene, STAT1-independent effects of IRF1 on IFN-γ-regulated genes cannot be studied in *stat1*$^{-/-}$ cells. Therefore, we resorted to two different strategies to study IRF1 in the absence of STAT1 activity. First, a doxycyclin (dox)-repressed IRF1 gene was introduced into STAT1-deficient fibroblasts. ChIP analysis of IRF1 showed a strong increase of chromatin-associated IRF1 in transfected cells after dox withdrawal (Fig. 5*A*) and a concomitant expression of endogenous *gbp2* mRNA. *gbp2* expression caused by IRF1 alone was much lower than that noted in IFN-γ-treated WT cells (Fig. 5*B*). These results are consistent with previous reports (32) showing transcriptional effects of IRF1 overexpression. They demonstrate that IRF1 alone is able to bind *gbp2* chromatin and stimulate target gene transcription but that expression is low compared with cytokine-treated cells. Therefore, the data also stress the important role of STAT1 dimer association with chromatin for *gbp2* promoter activity. Similar conclusions could be drawn from experiments with a recently established line of murine

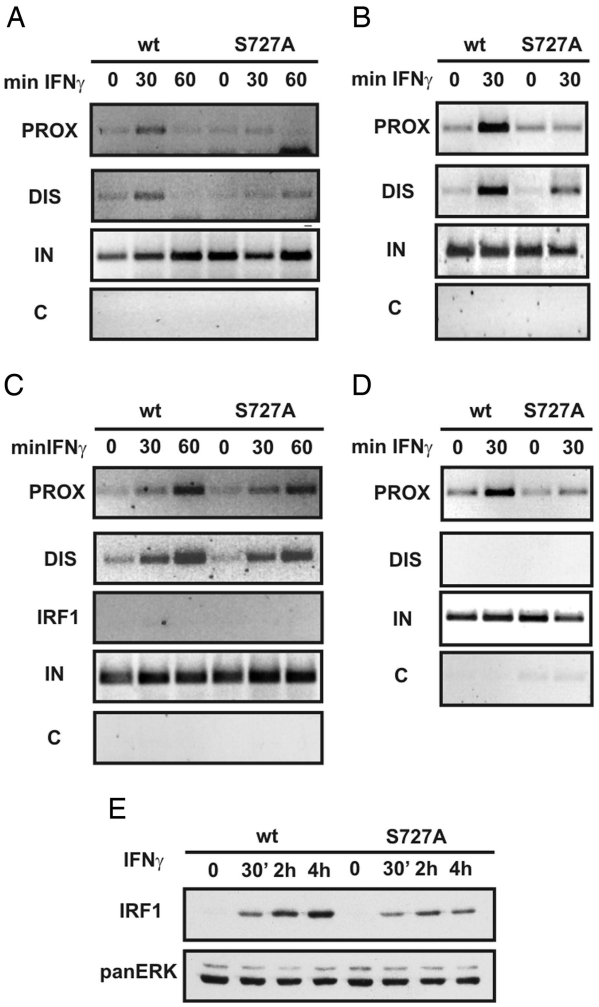

Fig. 4. Role of STAT1 S727 phosphorylation in the activation of the *gbp2* promoter by IFN-γ. BMDM, obtained from WT and STAT1 S727A mice, were treated with 10 ng/ml (240 units/ml) IFN-γ for the indicated time points, and formaldehyde-cross-linked chromatin was isolated. (*A–D*) IP of sonicated fragments was performed overnight with polyclonal Abs against CBP (*A*), hyperacetylated histone 4 (acH4) (*B*), IRF1 (*C*), and RNA Pol II (*D*). (*E*) Western blot analysis of whole-cell extracts from WT and S727A macrophages. The cells were treated with IFN-γ and analyzed for IRF1 protein levels. Equal loading was determined by reprobing the membrane with anti-panERK Abs.

fibroblasts expressing a fusion protein between IRF1 and the ligand-binding domain of the human estrogen receptor (IRF1-hER) (33, 34). In this cell line, IRF1 is constitutively expressed, but its transcription factor activity is strictly controlled by estrogen [see supporting information (SI) Fig. 7]. IRF1-ER bound the *gbp2* promoter in absence of exogenous stimuli and binding was increased after treatment with estrogen, IFN-γ, or both. Induction of *gbp2* expression by estrogen alone was low compared with that by IFN-γ.

IRF1, but Not STAT1, Is Found in Complexes with RNA Pol II in IFN-γ-Treated Cells. STAT1 being the major player in *gbp2* promoter histone hyperacetylation, we tested whether the role of IRF1 might be to directly contact protein complexes containing RNA pol II. Extracts from IFN-γ-treated macrophages were precipitated with Abs to either IRF1 or STAT1, and the precipitates were analyzed by Western blot for the presence of RNA pol II. Fig. 6 shows that RNA pol II was associated with IRF1 60 min after IFN-γ treatment. By contrast, association of

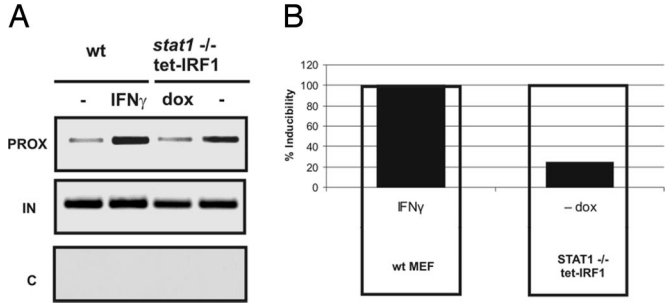

Fig. 5. Analysis of *gbp2* promoter chromatin and of *gbp2* in Stat1$^{-/-}$ cells. (*A*) STAT1-deficient fibroblasts were pretreated with dox for 6 h and then transiently transfected with pRETRO-tet-OFF-FLAG-IRF1. Twenty-four hours after transfection, dox was removed from the cells (−) or left on the cells (dox) for an additional 24 h. Control cells were treated with 10 ng/ml (240 units/ml) IFN-γ for 1 h. Formaldehyde-cross-linked chromatin was isolated and subjected to IP with IRF1 Abs. The proximal *gbp2* promoter was analyzed by PCR. The specificity for the IP was determined by using preimmune serum (C) as negative control, and amplification of input DNA (IN) by PCR. (*B*) RNA was isolated, reverse-transcribed, and analyzed for endogenous *gbp2* expression by real-time PCR.

RNA pol II with STAT1 was not detected at this or earlier time points. Control blots demonstrated the expected increase in STAT1 tyrosine phosphorylation and IRF1 protein expression.

Discussion

The STAT and IRF protein families contribute in numerous ways to the development and regulation of innate and adaptive immune responses. As an example, STATs and IRFs interact functionally both in the synthesis of and response to type I IFN (35, 36). For the subgroup of genes represented by *gbp2*, the functional interaction does not appear to require tight association or cooperative binding of the two proteins, but rather results from a requirement for both STAT1 and IRF1 in the process of transcriptional activation. The main goal of this study was to gain insight into the IFN-γ-induced chromatin changes requiring, or resulting from, the STAT1–IRF1 interaction.

Both the distal and proximal IFN-γ response regions of the *gbp2* promoter contain a canonical IRF binding site. Our data as well as

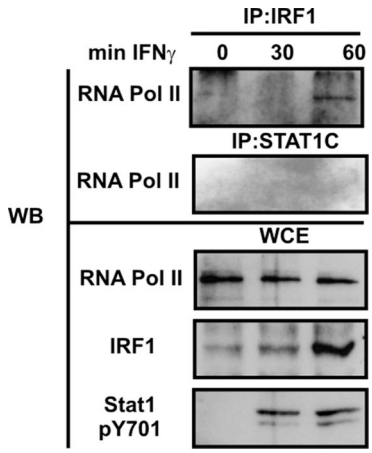

Fig. 6. IRF1 associates with RNA pol II complexes in IFN-γ-treated cells. BMDM obtained from WT mice were treated with 10 ng/ml (240 units/ml) IFN-γ for 30 or 60 min. Nuclei were isolated, and IP with the indicated Abs was performed. Western blot membranes were probed with an Ab to RNA pol II. Aliquots from the lysates were recovered before the IP (WCE) and analyzed by Western blot for the input of RNA pol II, Y701-phosphorylated STAT1 (pY701), and IRF1.

those from previous studies (11, 19) show that these sites are occupied by IRF1 during an IFN-γ response. Residual *gbp2* transcription still occurred in absence of IRF1 and may reflect exclusive STAT1 action or result from the activity of a different IRF family member. Distinguishing these possibilities requires further investigation. STAT1 binds the distal part of the IFN-γ response region using an imperfect GAS, whereas the proximal site represents one of the rather rare cases (15) where STAT1 binds a different promoter sequence as a consequence of IFN-γ signaling. In an analogous situation, the human 9/27 gene is rendered IFN-γ-inducible by a STAT1/IRF9 complex associating with an ISRE (12). The presence of IRF9 at the *gbp2* promoter in IFN-γ-treated cells has not been tested.

Consistent with recently reported immunoprecipitation (IP) experiments (5, 29), our study demonstrates that CBP and HDAC1 are recruited to promoter chromatin in a STAT1-dependent manner. The almost identical kinetics of recruitment after IFN-γ treatment suggest that complexes of STAT1 dimers with CBP and/or HDAC1 are either formed before binding to *gbp2* chromatin or that their assembly at the promoter is extremely rapid. Both the HAT function of CBP and HDAC1 activity are needed to efficiently activate *gbp2* transcription (5, 23). CBP function closely correlates with promoter histone hyperacetylation, but the target of HDAC1 is still unknown. The concomitant recruitment of CBP and HDAC1 favors the assumption that their target proteins are different. Alternatively, they might be regulated to act on the same targets in different phases of the transcriptional cycle. The highly transient nature of histone 4 hyperacetylation at the *gbp2* promoter is in good agreement with the assumption that deacetylase activity must closely follow that of the HAT.

Absence of IRF1 reduced, but did not abrogate CBP recruitment and *gbp2* promoter hyperacetylation. The rather small decrease was closely correlated with, and most likely due to, the reduced association of the promoter with STAT1 dimers in *irf1*$^{-/-}$ cells. Reduced levels of STAT1 in gene-targeted cells result from IRF1's role in regulating constitutive STAT1 expression downstream of autocrine type I IFN activity (30). This IRF1 activity and its delayed binding to *gbp2* promoter chromatin with respect to that of STAT1 binding argue against a helper function of IRF1 for Stat1 association. More likely the reduction of CBP recruitment and histone acetylation in *irf1*-deficient cells results from a combination of low STAT1 amounts and low affinity of the protein for the *gbp2* IFN response region.

Using cells expressing IRF1 in absence of STAT1 dimers, we were able to show that IRF1 associates with the *gbp2* promoter in the absence of prebound STAT1. Hence, STAT1 must neither directly contact IRF1 nor modify chromatin as a prerequisite for IRF1 binding. Furthermore, IRF1 association with the *gbp2* promoter chromatin was unaffected by the absence of STAT1 TAD phosphorylation at S727 and the concomitant decrease in CBP association to STAT1 dimers and histone 4 hyperacetylation (5). Unperturbed association of IRF1 with the *gbp2* promoter under these conditions shows that the reduction of IFN-γ-induced *gbp2* expression in cells expressing STAT1S727A is correlated with an absence of histone hyperacetylation, not a lack of IRF1 binding. Our findings also suggest a very limited potential of IRF1 to recruit HATs to the *gbp2* promoter. Otherwise, defective histone acetylation in STAT1S727A cells should be rescued by IRF1. *gbp2* belongs with a group of IFN-γ-induced genes that strongly require STAT1 serine phosphorylation. It is tempting to speculate that IFN-γ-induced genes that are less dependent on the STAT1 serine phosphorylation require STAT1 interaction with transcriptional proteins that either reduce the need for histone hyperacetylation, or that, unlike IRF1, significantly contribute to HAT recruitment.

RNA pol II binding shows a virtually complete dependence on IRF1. Gain-of-function analysis in cells expressing IRF1 in absence of active STAT1 shows its limited intrinsic ability to stimulate gene expression, and IPs demonstrate that IRF1 and RNA pol II are parts of the same transcriptional complex. Therefore, our data are consistent with a division of labor between STAT1 and IRF1 in stimulating *gbp2* expression. IRF1 plays an essential role in directing RNA pol II to the CAP site through its ability to contact either the enzyme itself or associated proteins, one of which might be TFIIB (37). The important role of IRF1 in RNA pol II recruitment is suggested not only by the coimmunoprecipitation experiment, but also by the nearly identical kinetics of IRF1 and RNA pol II association with *gbp2* chromatin and the onset of nuclear *gbp2* RNA accumulation. For STAT1, our findings support (at least) two different tasks in activating *gbp2* gene transcription. First, it must stimulate *irf1* mRNA transcription. Second, it must directly contribute to *gbp2* promoter activation by creating a more permissive chromatin environment for RNA pol II through the recruitment of CBP and possibly other HATs. STAT1 is also essentially required for HDAC1 association with the *gbp2* promoter chromatin, and HDAC1 is important for *gbp2* expression (23). Identifying the relevant targets for this enzyme and determining whether they are identical to those associated with type I IFN-induced transcription (29, 38, 39) will be an important future task. Besides CBP STAT1 is instrumental in directing MCM proteins and possibly also other chromatin remodeling factors to target promoters (24). The question of whether IRF1 additionally contributes to the remodeling of promoter chromatin and structure will need to be answered. Several events appear to be parallel between the *gbp*2 promoter and the pIV promoter of the *cIIta* gene, encoding the master regulator of MHC II genes. Both promoters recruit STAT1 and IRF1 with similar kinetics and mediate a secondary, delayed response to IFN-γ (this work and refs. 26 and 40). Both employ the HAT activity of P300/CBP and the chromatin remodeling activity of the SWI/SNF subunit BRG1 (25, 26, 41). On the other hand, HDAC1 has so far not been implicated in CIITA regulation, and, conversely, the E box binding protein USF1, which is needed for cIIta gene stimulation (42), has not been linked to *gbp*2 mRNA expression. It will be interesting to see whether these differences really distinguish the two promoters or whether they reflect incomplete knowledge of their regulation. In the latter situation, the identical mechanisms of induction may represent a molecular paradigm for secondary response promoters stimulated by IFN-γ.

Materials and Methods

Antibodies. Antiserum to the STAT1 C terminus used for Western blot analysis and ChIP assays was as described (43). STAT1 phospho-Y701 Ab was purchased from New England Biolabs (Beverly, MA). Monoclonal panERK Abs were purchased from Transduction Laboratories (Lexington, KY). Abs for IRF1 (M-20) and RNA Pol II (N-20) and a polyclonal antiserum to the N terminus of CBP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ab against acetylated H4 was purchased from Upstate Biotechnology (Lake Placid, NY). Affinity-purified rabbit Abs were used to analyze HDAC1 by ChIP (23).

Cytokines and Reagents. Recombinant mouse IFN-γ was used at a final concentration of 10 ng/ml (240 units/ml). Dox was purchased from Sigma–Aldrich (St. Louis, MO) and used at a final concentration of 1 μg/ml.

Cells. Bone marrow-derived macrophages (BMDM) were obtained by culture of bone marrow in L-cell derived CSF-1 as described (44). Immortalized fibroblasts from WT, *stat1*$^{-/-}$ (45), and *irf1*$^{-/-}$ mice (46) (kindly provided by J. Pavlovic, University of Zurich, Zurich, Switzerland) were cultured in DMEM containing 10% FCS.

Plasmids and Transfections. *irf1* cDNA was generated by using RNA from IFN-γ-treated macrophages and inserted into

pRetro-Off (Clontech, Palo Alto, CA). STAT1-deficient cells were pretreated with dox for 6 h before transfection by using ExGen (Fermentas) reagent according to the manufacturer's instructions. Cells were analyzed 48 h after transfection. Expression of IRF1 from the transfected plasmid was induced by removal of dox for 24 h.

ChIP. A total of 10^7 cells (primary macrophages or fibroblasts with identical results) were used per time point. ChIP assays were performed as recently described (23) with the following Abs: αSTAT1-C (1:100), αIRF1 (3 μg), αRNA Pol II (3 μg), αCBP (3 μg), αacH4 (4 μg), and αHDAC1 (1:100). Primers used for the analysis of the proximal (PROX) and distal (DIS) *gbp2* promoter were as described (5). For the *irf1* promoter the following primers were used: forward, 5′-AGCACAGCTGCCTTGTACTTCC-3′, and reverse, 5′-CTTAGACTGTGAAAGCACGTCC-3′. All ChIP data presented in this work represent a minimum of three independent experiments.

RNA Preparation, cDNA Synthesis, and Quantitative RT-PCR. Total RNA was isolated from 1×10^6 macrophages or fibroblasts by using the NucleoSpin RNA II kit (Macherey & Nagel, Düren, Germany) as described (47). Nuclear RNA was isolated from nuclear extracts, prepared as described (48). The cDNAs were reverse-transcribed from 5 μg of total or nuclear RNA. Real-time PCR experiments were normalized to the GAPDH housekeeping gene. Primers for real-time PCR of the GAPDH, *gbp2*, and *irf1* genes were used as described (31, 47). Unspliced hnRNA was analyzed by real-time PCR using primer pairs recognizing exon–intron borders. hn-*irf1*: forward, 5′-ACATCGATGGCAAGGGATAC-3′, and reverse, 5′-GCATGCTGGGATGCTTTAAT-3′; hn-*gbp2*: forward, 5′-TCCAAGGCAGATGTTGTT-3′, and reverse, 5′-CTCCACAAC TGAGGACTCCA-3′.

Coimmunoprecipitation. A total of 10^7 cells were harvested in PBS and resuspended in 300 μl of sucrose buffer (0.32 M sucrose/10 mM Tris·HCl, pH 8/3 mM $CaCl_2$/2 mM MgOAc/0.1 mM EDTA/0.5% Nonidet P-40/1 mM DTT/0.5 M PMSF). The nuclei were pelleted and washed twice in sucrose buffer (without Nonidet P-40) and resuspended in 100 μl of low-salt buffer (20 mM Hepes/1.5 mM $MgCl_2$/20 mM KCl/0.2 mM EDTA/25% glycerol/0.5 mM DTT/0.5 mM PMSF). Then, 100 μl of high-salt buffer (20 mM Hepes/1.5 mM $MgCl_2$/800 mM KCl/0.2 mM EDTA/25% glycerol/1% Nonidet P-40/0.5 mM DTT/0.5 mM PMSF) was added slowly to the suspension. The lysates were incubated for 45 min at 4°C on a rotating wheel and centrifuged for 15 min at $14{,}000 \times g$. One-tenth of the supernatant was used as IP-input control and boiled with Laemmli buffer for 10 min. Binding of STAT1 C-terminal Ab (1:100 dilution) or with 3 μg of IRF1 Ab was performed overnight at 4°C. Protein A beads were added for 2 h, and the immunocomplexes were washed three times in low-salt buffer. The beads were boiled in 50 μl of Laemmli buffer and subjected to SDS gel electrophoresis.

Western Blot. A protocol for this procedure was recently described (31).

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1. Schroder K, Hertzog PJ, Ravasi T, Hume DA (2004) *J Leukoc Biol* 75:163–189.
2. Levy DE, Darnell JE, Jr (2002) *Nat Rev Mol Cell Biol* 3:651–662.
3. Wen Z, Zhong Z, Darnell JE, Jr (1995) *Cell* 82:241–250.
4. Decker T, Kovarik P (2000) *Oncogene* 19:2628–2637.
5. Varinou L, Ramsauer K, Karaghiosoff M, Kolbe T, Pfeffer K, Muller M, Decker T (2003) *Immunity* 19:793–802.
6. Lew DJ, Decker T, Strehlow I, Darnell JE (1991) *Mol Cell Biol* 11:182–191.
7. Levy DE, Kessler DS, Pine R, Reich N, Darnell JE, Jr (1988) *Genes Dev* 2:383–393.
8. Pine R, Decker T, Kessler DS, Levy DE, Darnell JE, Jr (1990) *Mol Cell Biol* 10:2448–2457.
9. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N (2001) *Annu Rev Immunol* 19:623–655.
10. Kimura T, Nakayama K, Penninger JM, Kitagawa M, Harada H, Matsuyama T, Tanaka N, Kamijo R, Vilcek J, Mak TW, Taniguchi T (1994) *Science* 264:1921–1924.
11. Kimura T, Kadokawa Y, Harada H, Matsumoto M, Sato M, Kashiwazaki Y, Tarutani M, Tan RS, Takasugi T, Matsuyama T, *et al.* (1996) *Genes Cells* 1:115–124.
12. Bluyssen HA, Muzaffar R, Vlieststra RJ, van der Made AC, Leung S, Stark GR, Kerr IM, Trapman J, Levy DE (1995) *Proc Natl Acad Sci USA* 92:5645–5649.
13. Ramana CV, Grammatikakis N, Chernov M, Nguyen H, Goh KC, Williams BR, Stark GR (2000) *EMBO J* 19:263–272.
14. Ramana CV, Gil MP, Han Y, Ransohoff RM, Schreiber RD, Stark GR (2001) *Proc Natl Acad Sci USA* 98:6674–6679.
15. Hartman SE, Bertone P, Nath AK, Royce TE, Gerstein M, Weissman S, Snyder M (2005) *Genes Dev* 19:2953–2968.
16. Decker T, Kovarik P (1999) *Cell Mol Life Sci* 55:1535–1546.
17. Weihua X, Kolla V, Kalvakolanu DV (1997) *Proc Natl Acad Sci USA* 94:103–108.
18. MacMicking JD (2004) *Trends Immunol* 25:601–609.
19. Briken V, Ruffner H, Schultz U, Schwarz A, Reis LF, Strehlow I, Decker T, Staeheli P (1995) *Mol Cell Biol* 15:975–982.
20. Pine R, Canova A, Schindler C (1994) *EMBO J* 13:158–167.
21. Zhang JJ, Vinkemeyer U, Gu W, Chakravarti D, Horvath CM, Darnell JE, Jr (1996) *Proc Natl Acad Sci USA* 93:15092–15096.
22. Zakharova N, Lymar ES, Yang E, Malik S, Zhang JJ, Roeder RG, Darnell JE, Jr (2003) *J Biol Chem* 278:43067–43073.
23. Zupkovitz G, Tischler J, Posch M, Sadzak I, Ramsauer K, Egger G, Grausenburger R, Schweifer N, Chiocca S, Decker T, Seiser C (2006) *Mol Cell Biol* 26:7913–7928.
24. Snyder M, He W, Zhang JJ (2005) *Proc Natl Acad Sci USA* 102:14539–14544.
25. Pattenden SG, Klose R, Karaskov E, Bremner R (2002) *EMBO J* 21:1978–1986.
26. Ni Z, Karaskov E, Yu T, Callaghan SM, Der S, Park DS, Xu Z, Pattenden SG, Bremner R (2005) *Proc Natl Acad Sci USA* 102:14611–14616.
27. Nicolet CM, Paulnock DM (1994) *J Immunol* 152:153–162.
28. Anderson SL, Carton JM, Zhang X, Rubin BY (1999) *J Interferon Cytokine Res* 19:487–494.
29. Nusinzon I, Horvath CM (2003) *Proc Natl Acad Sci USA* 100:14742–14747.
30. Taniguchi T, Takaoka A (2001) *Nat Rev Mol Cell Biol* 2:378–386.
31. Kovarik P, Mangold M, Ramsauer K, Heidari H, Steinborn R, Zotter A, Levy DE, Muller M, Decker T (2001) *EMBO J* 20:91–100.
32. Pine R (1992) *J Virol* 66:4470–4478.
33. Kirchhoff S, Schaper F, Hauser H (1993) *Nucleic Acids Res* 21:2881–2889.
34. Kroger A, Dallugge A, Kirchhoff S, Hauser H (2003) *Oncogene* 22:1045–1056.
35. Levy DE, Marie I, Prakash A (2003) *Curr Opin Immunol* 15:52–58.
36. Honda K, Yanai H, Takaoka A, Taniguchi T (2005) *Int Immunol* 17:1367–1378.
37. Wang IM, Blanco JC, Tsai SY, Tsai MJ, Ozato K (1996) *Mol Cell Biol* 16:6313–6324.
38. Chang HM, Paulson M, Holko M, Rice CM, Williams BR, Marie I, Levy DE (2004) *Proc Natl Acad Sci USA* 101:9578–9583.
39. Sakamoto S, Potla R, Larner AC (2004) *J Biol Chem* 279:40362–40367.
40. Morris AC, Beresford GW, Mooney MR, Boss JM (2002) *Mol Cell Biol* 22:4781–4791.
41. Kretsovali A, Agalioti T, Spilianakis C, Tzortzakaki E, Merika M, Papamatheakis J (1998) *Mol Cell Biol* 18:6777–6783.
42. Muhlethaler-Mottet A, Di Berardino W, Otten LA, Mach B (1998) *Immunity* 8:157–166.
43. Kovarik P, Stoiber D, Novy M, Decker T (1998) *EMBO J* 17:3660–3668.
44. Baccarini M, Bistoni F, Lohmann Matthes ML (1985) *J Immunol* 134:2658–2665.
45. Durbin JE, Hackenmiller R, Simon MC, Levy DE (1996) *Cell* 84:443–450.
46. Reis LF, Ruffner H, Stark G, Aguet M, Weissmann C (1994) *EMBO J* 13:4798–4806.
47. Stockinger S, Reutterer B, Schaljo B, Schellack C, Brunner S, Materna T, Yamamoto M, Akira S, Taniguchi T, Murray PJ, *et al.* (2004) *J Immunol* 173:7416–7425.
48. Solan NJ, Miyoshi H, Carmona EM, Bren GD, Paya CV (2002) *J Biol Chem* 277:1405–1418.

A Role for IRF7 and TBK1 in the Regulation of IFN-γ Induced Genes

IFNγ driven IRF7 expression is mediated via GAS and/or ISRE bound STAT1

As discussed in Ramsauer et al., GBP2 expression is absent in *Stat1* -/- MEFs whereas *Irf1* -/- MEFs still show residual expression of GBP2 mRNA after treatment with IFNγ (Ramsauer et al., 2007). Residual GBP2 transcription may reflect exclusive STAT1 action or result from the activity of a different IRF family member, like IRF8 (ICSBP), which has been shown to contribute to IFNγ mediated gene expression under various conditions. Additional IRF family members that have been linked to contribute in the regulation of ISGs are IRF3 and IRF7 (Savitsky et al., 2010). IRF3 belongs to the Class I family of transcription factors and is therefore constitutively expressed. In contrast, IRF7 is a Class II transcription factor. Binding of ISGF3 to two defined ISREs in the 5'UTR of the gene, confers IRF7 gene responsiveness exclusively to type I IFNs. IRF3 and IRF7 have both been shown to be crucial regulators of responses to viral or bacterial infections, since gene expression of IFNβ and IFNα4 strictly depends on the activation of IRF3 and IRF7 (Honda et al., 2005; Panne et al., 2007). Moreover, IRF7 expression and activation is needed for the second wave of type I IFN production during prolonged infections by upregulating the expression of all IFNα genes, independently of IRF3, with IFNα4 as notable exception (Marié et al., 1998; Honda et al., 2005; Caillaud et al., 2005; Génin et al., 2009).

To study a possible contribution of IRF7 in the regulation of IFNγ driven gene expression, we tested whether IRF7 is expressed after treatment of WT MEFs with IFNγ. Indeed we could show an increase of IRF7 mRNA after IFNγ treatment (Figure 12B). Since IFNγ signaling in most situations occurs not through the formation of an active ISGF3 complex, which can bind to the two known ISRE sites, this finding suggests two possible explanations. First, IRF7 expression after IFNγ treatment can be mediated by the presence of a GAS in the promoter or an enhancer region of the *Irf7* gene. Or, second, it may indicate a contribution of a STAT1-IRF9 complex. Such complexes are known to form in vitro (Bluyssen et al., 1995) and previous studies addressing the regulation of the *Gbp2* gene by IFNγ suggest they may play a role in this situation (Varinou et al., 2003).

Sequence analysis of the murine *Irf7* gene promoter and upstream sequences revealed a GAS, which follows the perfect consensus sequence TTCTCTGAA, located in an enhancer region 1117bp upstream of the TSS (Figure 12A). Moreover, the human *Irf7* gene promoter, contains a GAS directly at the promoter ranging from 35bp to 27bp upstream of the TSS and confers IFNγ responsiveness in recent microarray experiments (Saha et al., 2010). To test whether the GAS of the murine *Irf7* enhancer binds STAT1 homodimers in response to IFNγ, we performed ChIP assay analysis for STAT1 and STAT2 binding in WT MEFs. STAT1 but not STAT2 was found to bind the GAS enhancer in response to IFNγ within 30 minutes after treatment. In addition, very little STAT1 but again no STAT2 was found at the GAS enhancer after IFNβ treatment. Interestingly, STAT1 was found to bind the ISRE containing promoter in addition to the enhancer after 30 minutes of treatment with IFNγ. As expected both STAT1 and STAT2 bind the ISRE sites after treatment with IFNβ.

Our data suggests that IRF7 mRNA expression in response to IFNγ is regulated via the binding of STAT1 to both the enhancer and promoter region. Promoter binding of STAT1 appears to be much more transient compared to the recruitment to the enhancer region, pointing at a stronger effect of STAT1 homodimers at the newly defined enhancer. Although STAT1 binding to both regions might be needed for full expression of IRF7 mRNA, the sustained IRF7 expression after 4hrs of IFNγ treatment (Figure 12B) might depend predominantly on the enhancer bound STATs.

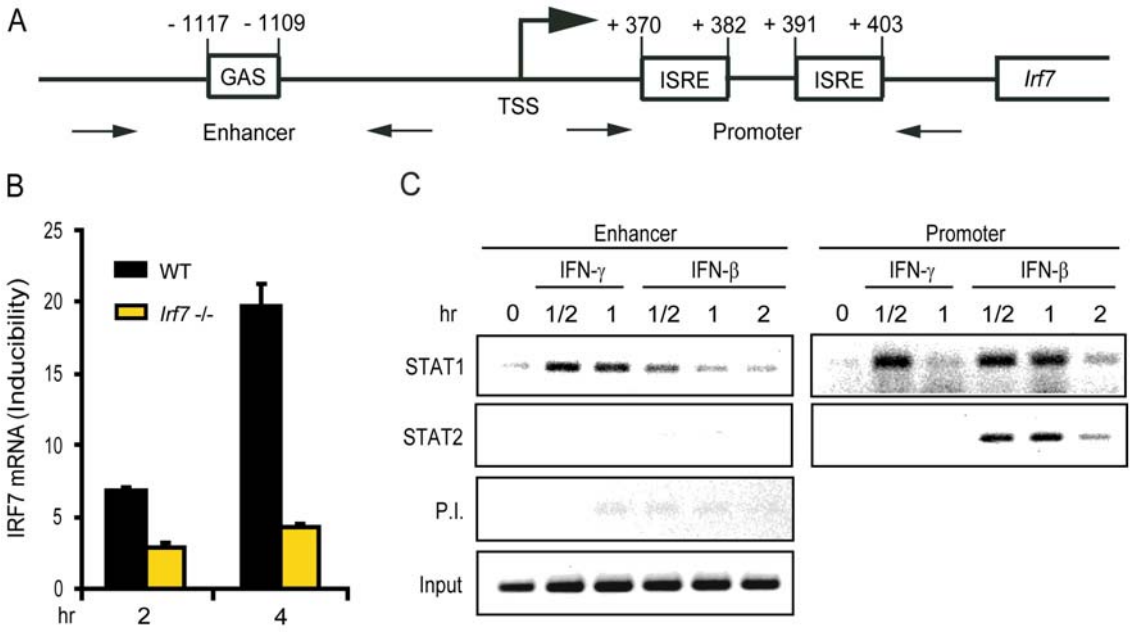


Figure 12: Kinetics of IRF7 mRNA expression and STAT1 recruitment to the *Irf7* promoter, determined by qPCR and ChIP, respectively. (A) Schematic drawing of the promoter ISRE sites located in the 5'UTR of the *Irf7* gene, and the newly defined GAS located in the enhancer region 1.1kb upstream of the TSS. (B) IRF7 mRNA expression after treatment of WT and *Irf7* -/- MEFs with IFNγ for the indicated time points, was determined by qPCR. (C) WT MEFS were stimulated with IFNγ or IFNβ and processed for ChIP at the indicated time points. Antibodies for ChIP are shown on the left, P.I. indicates controls performed with preimmune sera. The precipitates were amplified by PCR with primers flanking the enhancer (GAS) or promoter (ISRE) region as indicated in (A) and analyzed by gel-electrophoresis.

ISGF3 driven gene-expression after IFNγ treatment depends on IRF7

Next we asked whether IRF7 and/or IRF3 might contribute to IFNγ-induced target gene expression and in turn be responsible for the residual expression of GBP2 mRNA, observed in the previous section (Ramsauer et al. 2007). We monitored the mRNA expression profile of several ISGs by qPCR analysis. WT MEFs or MEFs deficient for IRF7 or IRF3 were analyzed after treatment with IFNγ. mRNA expression of ISRE-driven genes, like *Gbp2* or *Tap1*, depended on the presence of IRF7, which appeared to be strongest at later time points after treatment. Expression of SOCS1, was also found to be IRF7 dependent. Interestingly, the *Socs1* 5' flanking region contains a bonafide IRF binding site in an enhancer at position -1772 to -1764, following the consensus TTTCTTTTT, which is conserved in humans (Schlüter et al., 2000). mRNA expression of *Irf1*, which contains only a GAS in its promoter, showed very little IRF7 dependency. ISG mRNA expression in *Irf3 -/-* MEFs was strongly diminished in all tested cases, including IRF1 (Figure 13A-D). This finding points to a more general and maybe indirect effect of IRF3 in the regulation of ISGs and is consistent with the reported role of IRF3 in maintaining STAT1 expression through autocrine type I IFN production (Taniguchi and Takaoka, 2001; Stockinger et al., 2004). To examine STAT1 levels in *Irf3 -/-* or *Irf7 -/-* MEFs we performed Western blot analysis using a STAT1 C-terminal antibody. The activation status of STAT1 was revealed by the use of a STAT1 phosphotyrosine 701 specific antibody, since small differences in STAT1 levels might not alter STAT1 activation under the tested conditions. In line with the published work on IRF3-dependent autocrine type I IFN signaling, *Irf3 -/-* MEFs displayed severely reduced STAT1 levels compared to WT MEFs, which altered STAT1 activation capacity, leading to drastically reduced levels of tyrosine-phosphorylated STAT1. Reduced IRF1 mRNA expression in *Irf3 -/-* MEFs was supported by the finding of reduced IRF1 protein levels in these cells. In contrast, *Irf7 -/-* MEFs displayed normal levels of STAT1 and a normal STAT1 phosphotyrosine pattern upon IFNγ treatment, comparable with WT MEFs. In line with mRNA expression, IRF1 protein expression was, not altered in *Irf7 -/-* MEFs (Figure 13E).

Taken together our results indicate a direct role for IRF7 in regulating ISG expression in response to IFNγ stimulation. However, the gene regulatory function of IRF7 in this context appears to be confined to ISRE-driven genes. IRFs in general have the capability to bind directly the core sequence within the ISRE consensus repeat, with little or no variation within the different IRF species (Savitsky et al., 2010). Moreover, the results monitoring the mRNA expression of identified IRF7-driven genes, including *Gbp2*, show that target gene expression is reduced, but not completely abrogated, in *Irf7* -/- MEFs. This in turn points to a role for IRF7 in enhancing target gene expression, to achieve more prolonged expression profiles.

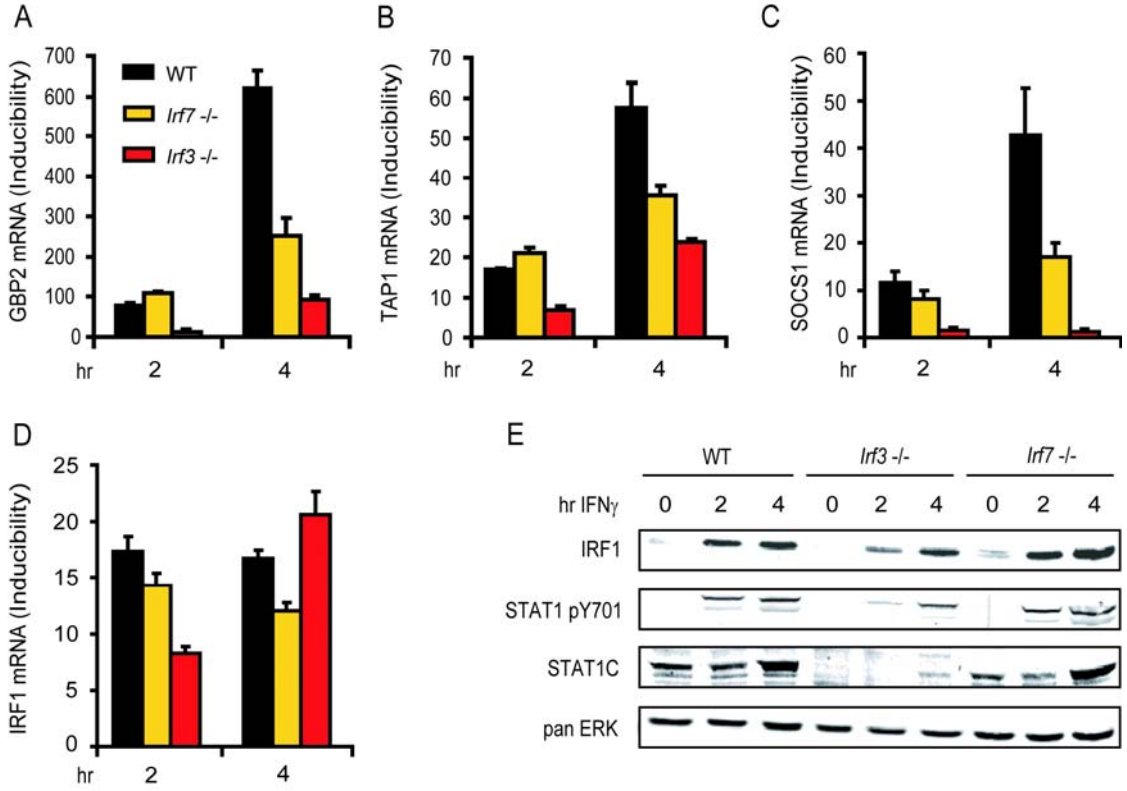


Figure 13: Kinetics of ISRE and GAS-driven genes, after IFNγ treatment, determined by qPCR and Western-blot. WT, *Irf3-/-* and *Irf7 -/-* MEFs were treated with IFNγ for the indicated time points. (A-D) GBP2, TAP1, SOCS1 and IRF1 mRNA expression was determined by q-PCR after normalization to GAPDH levels. (E) IRF1 protein expression and STAT1 tyrosine phosphorylation were detected in Western-blot analysis. Differences in STAT1 expression levels between WT MEFs and MEFs deficient for *Irf3* or *Irf7*, were analyzed by reprobing the blot with an antibody against STAT1 C-terminus. Pan ERK levels were analyzed as a normalization control.

IRF1 and IRF7 synergistically drive GBP2 expression; Differential requirement for TBK1 and IKKε for IRF7 mediated, IFNγ driven gene expression

IRF7 is known to form either homo- or heterodimers with other IRF family members, mainly IRF3 in the context of IFNβ and IFNα4 gene expression after pathogen exposure (Lin et al., 2000; Au et al., 2001; Au and Pitha, 2001). IRF1 is so far known to physically and functionally interact with IRF8 among the IRF family (Laricchia-Robbio et al., 2005), and participates in the IRF3 and IRF7 mediated regulation of human IFNalpha genes (Au and Pitha, 2001). Since all of the IFNγ-inducible genes identified above as IRF7 target genes are known targets for IRF1, we wondered whether IRF7 has the capability to functionally interact with IRF1 to drive ISG expression. To test for a possible interplay of IRF7 with IRF1 in the regulation of GBP2 mRNA expression, we performed reporter gene assays with IRF7 and IRF1 expressing constructs. Transfection of equal copy numbers of *Irf1* or *Irf7* genes in the context of an otherwise identical expression plasmid demonstrated a nearly identical ability to stimulate the *Gbp2* promoter-luciferase reporte gene. Cotransfection of the same copy number of combined *Irf1* and *Irf7* genes produced a two-fold higher activity of the *Gbp2* promoter (Figure 14A). In this experiment, *Stat1 -/-* MEFs were transfected to avoid indirect stimulation of the *Gbp2* reporter through IRF7-driven IFNα expression.

IRF7, like IRF1, was able to induce the *Gbp2* reporter on its own. In the context of infection, the transcriptional activity of IRF7 has been shown in numerous studies to rely on the activity of the kinases TBK1 and IKKε. The two enzymes were shown to act redundantly for the activation of IRF3 and IRF7 and the production of type I IFN in anti-viral responses (Perry et al., 2004)(Stockinger et al., 2004; Stetson and Medzhitov, 2006; Soulat et al., 2006), although recent reports show differences at least in the mode of their activation (Honda et al., 2005; Chau et al., 2008). Challenging the view of functional redundany, antagonistic activity of TBK1 and IKKε, based on competition for a common adapter protein, was recently suggested by the group of John Hiscott (Paz et al., 2009).

To determine whether TBK1 and IKKε kinase function is needed for the transcriptional activity of IRF7 in connection with IFNγ induced genes, we performed qPCR analysis for the expression of IRF7 target genes after treatment of either WT or double-deficient *Tbk1/Ikbke* -/- MEFs with IFNγ. mRNA expression of the previousely identified IRF7 targets, depended differentially on the presence of the two kinases. We observed the strongest effect on gene expression in *Tbk1/Ikbke* -/- MEFs for *Gbp2* and *Tap1*, showing a decrease in mRNA expression levels by more than 50% (Figure 14B,C). Conversely, like the IRF7 independent *Irf1* gene, SOCS1 mRNA expression did not display a significant reduction in *Tbk1/Ikbke* -/- MEFs (Figure 14D,E).

To further examine the role of particularly TBK1 in the regulation of the transcriptional activity of IRF7 on the *Gbp2* gene, we performed co-transfection experiments with IRF7 and TBK1 in *Tbk1/Ikbke* -/- MEFs. In contrast to the transfection of IRF7 in *Stat1* -/- MEFs (Figure 14A), which still express TBK1 and IKKε, IRF7 transfection into *Tbk1/Ikbke* -/- MEFs was not able to induce *Gbp2* reporter gene expression (Figure 14F). By contrast, cotransfection of IRF7 and TBK1 strongly increased *Gbp2* reporter gene expression. Addition of an antibody blocking the accessibility of the IFNAR for its ligands (Sheehan et al., 2006), was without effect on expression of the reporter gene in this experiment. Hence we can rule out a contribution of type I IFN signalling to the ability of transfected TBK1/IRF7 to activate the *Gbp2* promoter under our experimental conditions. (Figure 14F).

Given the ability of IFNγ to induce IRF7 mRNA expression and the TBK1 requirement for IRF7 activity, we wondered whether IFNγ treatment causes activation of TBK1. To test this hypothesis, we performed cotransfection experiments introducing IRF7 and TBK1 in *Stat1 -/-* MEFs. The cells were subsequently treated with IFNγ or left without treatment. *Stat1 -/-* MEFs express the kinases TBK1 and IKKε, but are not able to stimulate the *Gbp2* reporter via activation of STAT1. Treatment of IRF7-transfected *Stat1 -/-* MEFs with IFNγ did not significantly enhance the reporter gene activity. Moreover, IRF7 synergized with TBK1 in these cells to enhance *Gbp2* reporter gene expression in *Stat1 -/-* MEFs, irrespective of prior treatment with IFNγ (Figure 14G). The synergism suggests that TBK1 directly acts on the large amounts of IRF7 present in transfected cells. Unlike IRF7, IRF1's transcriptional activity is thought to occur without phosphorylation-mediated activation (Savitsky et al., 2010). In keeping with this notion, IRF1 and TBK1 cotransfection resulted in a modest increase of *Gbp2* reporter gene expression. This increase is likely to reflect the activity of TBK1 on endogenous IRF7 substrates rather than on the cotransfected IRF1 (Figure 14H).

The results from IRF7 and TBK1 cotransfection experiments in *Tbk1/Ikbke -/-* MEFs indicates that IRF7 must be phosphorylated to efficiently drive the expression of GBP2 and that the phosphorylating activity is present in WT or Stat1-/-, but not in TBK1/IKKe-deficient fibroblasts (Figure 14F). To examine the IRF7 phosphorylation status we performed 2D gel electrophoresis with nuclear extracts of WT and *Tbk1/Ikbke -/-* MEFs after treatment with IFNγ for 4hrs to induce IRF7 expression, and probed the membrane with polyclonal IRF7 antibody (Caillaud et al., 2005). Preliminary results showed the formation of at least one TBK1/IKKε-dependent IRF7 phosphoisoform (Figure 14I).

Thus, our results indicate, that IRF1 and IRF7 synergistically regulate the expression of ISRE- driven genes upon stimulation with IFNγ. IRF7 in this context appears to be phosphorylated in an TBK1/IKKε dependent manner. IRF7 is phosphorylated at least on one serine residue by constitutively active TBK1 and/or IKKε, since IFNγ treatment has no enhancing effect on *Gbp2* reporter gene expression, and the expression levels of the *Gbp2* reporter observed after transfection of only IRF7 in *Stat1 -/-* MEFs, is completely abrogated in *Tbk1/Ikbke -/-* MEFs.

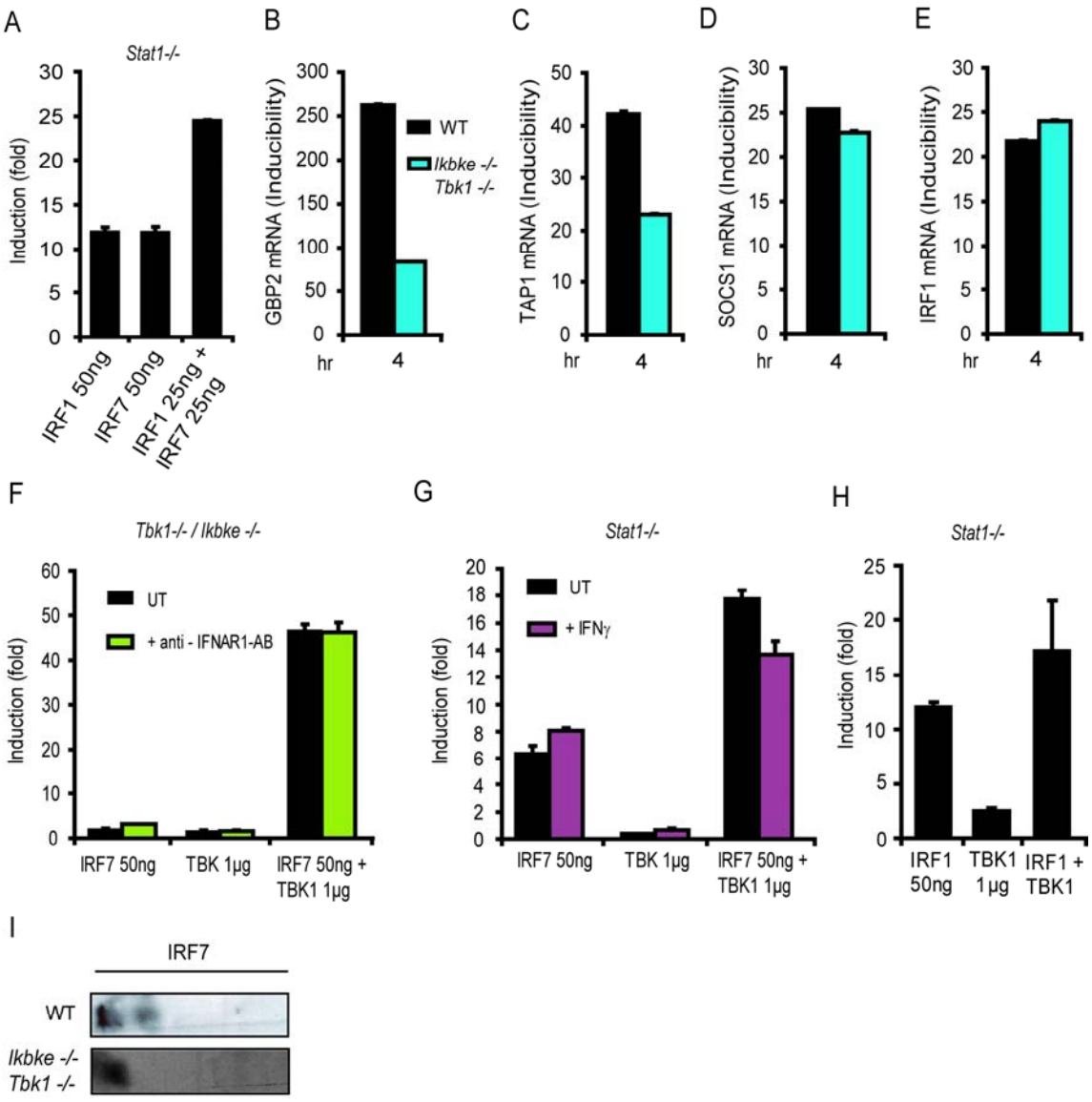

A
Stat1-/-
Induction (fold)
IRF1 50ng
IRF7 50ng
IRF1 25ng + IRF7 25ng
B
GBP2 mRNA (Inducibility)
WT
Ikbke -/- Tbk1 -/-
hr
4
C
TAP1 mRNA (Inducibility)
D
SOCS1 mRNA (Inducibility)
E
IRF1 mRNA (Inducibility)
F
Tbk1-/- / Ikbke -/-
UT
+ anti - IFNAR1-AB
IRF7 50ng
TBK 1µg
IRF7 50ng + TBK1 1µg
G
Stat1-/-
UT
+ IFNγ
H
Stat1-/-
IRF1 50ng
TBK1 1µg
IRF1 + TBK1
I
IRF7
WT
Ikbke -/- Tbk1 -/-

Figure 14: Regulation of IFNg-induced genes by IRF1 and IRF7; requirement for TBK1-mediated phosphorylation. , (A) *Stat1 -/-* MEFs were transfected with either IRF1 or IRF7 and *Gbp2* – luciferase reporter activity was measured. The values are expressed as -fold induction relative to cells transfected only with reporter construct after normalization to a co-transfected Renilla luciferase reporter. (B-E) Expression of GBP2, TAP1, SOCS1 and IRF1 mRNA in WT and *Tbk1/Ikbke -/-* MEFs after IFNγ treatment for the times indicated, was analyzed by qPCR and normalized to GAPDH mRNA levels. (F) *Tbk1/Ikbke -/-* MEFs were transfected with IRF7 or TBK1 alone, or a combination of both. MEFs were treated with IFNAR1 blocking antibody for the whole period of transfection or left untreated. (G) *Stat1 -/-* MEFs were transfected with IRF7 or TBK1 alone or in combination. Trnafected cells were stimulated over night with IFNγ or left untreated. (H) *Stat1 -/-* MEFs were transfected with IRF1 or TBK1 alone or in combination .In panels F-H *Gbp2* luciferase reporter activity was measured as decribed for (A). (I) Requirement for TBK1/IKKε mediated IRF7 phosphorylation determined by 2D gel electrophoresis. Nuclear extracts of WT or *Tbk1/Ikbke -/-* MEFs were subjected to 2D gel electrophoresis. IRF7 isoforms were analyzed by Western blotting using an antibody against IRF7.

Critical role of serines (S425 - S426 and S437 - S438) of the IRF7 regulation domain as phosphoacceptors for both the stimulation of IRF7 activity by TBK1 and its repression by IKKε.

IRF7 can be activated by phosphorylation of several serine residues, located in the carboxy terminal regulatory domain (Marié et al., 2000; Caillaud et al., 2005). For in depth analyzes of the serine residues required for IRF7 transcriptional activity at the *Gbp2* promoter we performed transfection experiments in *Stat1 -/-* MEFs and *Tbk1/Ikbke -/-* MEFs with a set of IRF7 phosphomutants, generated by Isabelle Marié and David Levy (Figure 15A; (Caillaud et al., 2005)).

Transfection of WT IRF7 as well as the serine to alanine mutants M1 (S425A and S426A) and M5 (S437A and S438A) in *Tbk1/Ikbke -/-* MEFs failed to induce *Gbp2* reporter gene expression, whereas transfection of the serine to aspartate mutant M15 (all serines except S425 and Ser426 are mutated to aspartate), upregulated *Gbp2* reporter gene activity very efficiently (Figure 15B). Co-transfection of TBK1, to reconstitute kinase activity, increased reporter gene activity drastically in the case of both WT IRF7 and the M15 mutant. TBK1 co-transfection with M1 and M5 mutants led to a moderate increase of *Gbp2* reporter gene expression.

Since the two pairs of serines (S425 - S426 and S437 - S438) mutated in the IRF7 M1 and M5 mutants proved crucial in our experiments we next transfected the IRF7 M12 and M18 mutant in our setup with *Tbk1/Ikbke* -/- MEFs, to further examine the effect of aspartate substitutions of the most N-terminal serines (S425 and S426) as well as serines in the middle (S429, S430, and S431) and at the C-terminal end (S441) of the regulatory region (Figure 15A). The IRF7 M18 mutant did not increase reporter gene expression when transfected alone into *Tbk1/Ikbke* -/- MEFs, but produced an enhanced reporter gene activity when co-transfected with TBK1. The M12 mutant exerted a similar pattern, but stimulation of reporter gene expression by this mutant in absence of TBK1 was higher compared to M18. In spite of containing aspartates in all except the most C-terminally located position, the IRF7 M12 mutant was not as efficient as the IRF7 M15 mutant in inducing reporter gene expression, neither when transfected alone, nor together with TBK1 (Figure 15D).

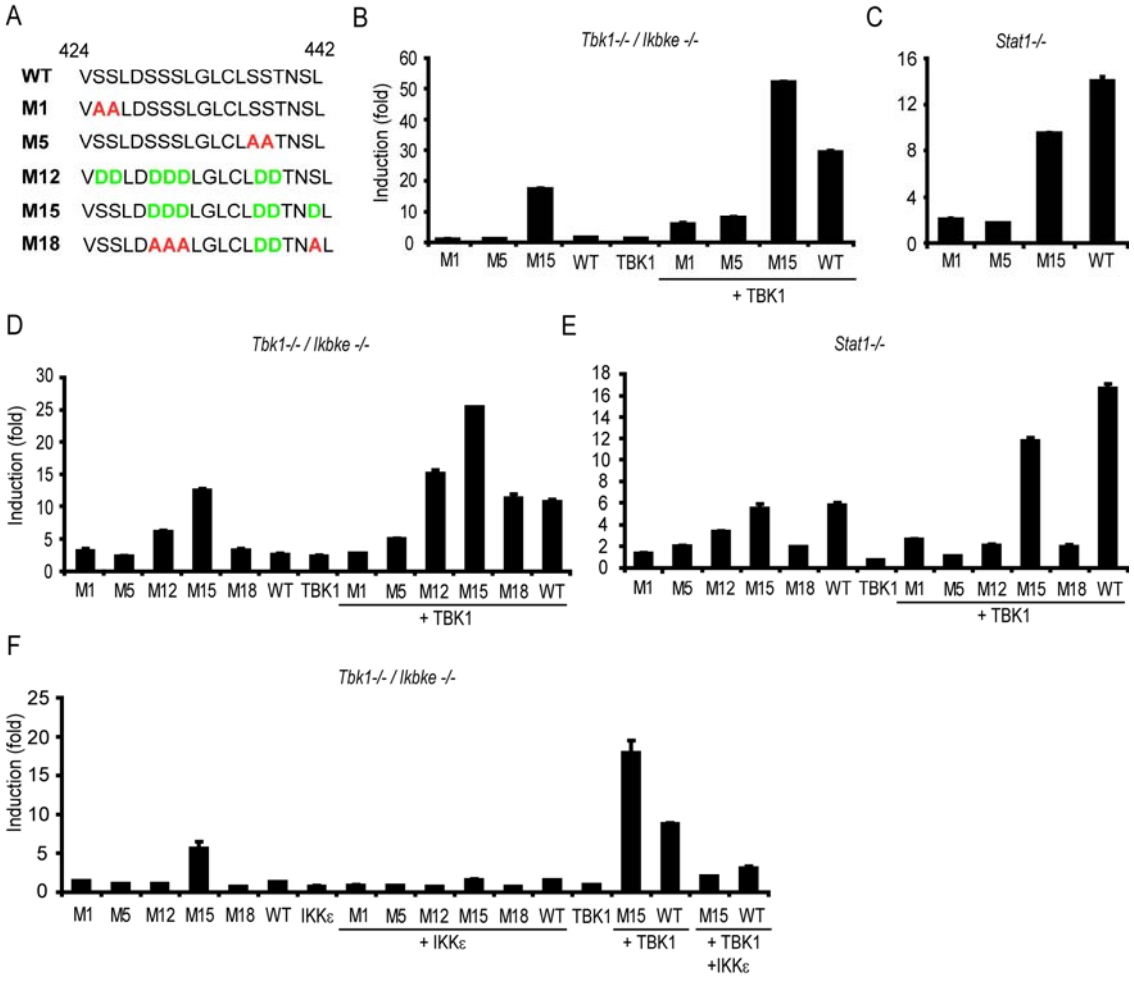
Compared with the results obtained in *Tbk1/Ikbke* -/- MEFs, all mutants behaved similar when transfected into *Stat1*-/- MEFs, with two notable exceptions: first, reporter gene expression stimulated by the M15 mutant did not exceed the levels obtained with WT IRF7; second, the overall inducibility of the reporter gene by transfected IRF7 was much lower in *Stat1* -/- MEFs than in *Tbk1/Ikbke* -/- MEFs (Figure 15C).

Extending the setup in *Stat1* -/- MEFs with the additional transfection of IRF7 M12 and M18 mutants revealed that in contrast to the picture seen in *Tbk1/Ikbke* -/- MEFs, IRF7 M18 mutant was not able to drive reporter gene expression, neither when transfected alone nor when cotransfected with TBK1. Transfection of IRF7 M12 mutant still induced *Gbp2* reporter gene expression in *Stat1* -/- MEFs when transfected alone, but, unlike *Tbk1/Ikbke* -/- MEFs, co-transfection with TBK1 did not enhance the reporter gene expression (Figure 15E).

Transfection of the IRF7 M15 mutant, alone or together with TBK1 in *Tbk1/Ikbke -/-* MEFs, was more efficient in driving *Gbp2* reporter gene expression than WT IRF7 under the same conditions. In contrast, transfection of IRF7 M15 mutant in *Stat1 -/-* MEFs, alone or together with TBK1, never induced the *Gbp2* reporter gene to the level, or even beyond the level seen after transfection of WT IRF7 (compare Figure 15D with 15E). This finding prompted us to to examine whether the damped reporter gene expression levels in *Stat1 -/-* MEFs could be an effect of IKKε, the most prominent player in IRF regulation differing between Stat1-/- MEFs and *Tbk1/Ikbke -/-* MEFs after reconstitution with TBK1. To directly assess whether effects of TBK1 and IKKε differ with regard to GBP2 promoter activation, WT or mutant IRF7 was introduced to *Tbk1/Ikbke -/-* MEFs by transfection and IKKε was co-introduced instead of TBK1. Interestingly, we found that unlike TBK1, IKKε did not activate either WT or mutant IRF7 for increased activity on the *Gbp2*-reporter gene. In addition, the phosphorylation-independent activity of the IRF7 M15 mutant was suppressed by IKKε. Importantly, the increase in reporter gene expression seen after cotransfection of TBK1 with either WT IRF7 or IRF7 M15 was strongly suppressed by the additional presence of IKKε. (Figure 15F).

Taken together our results indicate that a combination of phosphorylated serines is needed for full transcriptional activity of IRF7 to induce GBP2 expression. Compared to WT IRF7 the M15 mutant exhibited higher activity in *Tbk1/Ikbke-/-* MEFs, after cotransfection with TBK1. This suggests that not all of the phosphomimetic sites in the M15 mutant are phosphorylated by TBK1 on WT IRF7. In addition, the two most N-terminal serines in the regulatory domain are crucial for IRF7 activity on the GBP2 promoter. These residues are the only ones not mutated to aspartate in the M15 mutant and must, therefore, be responsible for further activation of this mutant after TBK1 cotransfection. The reduced activation of the M1 mutant with the two N-terminal serines mutated to Ala corroborates this interpretation. Our findings are very much in line with the data generated by Marié and colleagues in previous studies (Caillaud et al., 2005).

Co-transfection of IKKε instead of TBK1 resulted in decreased transcriptional activity of the M15 mutant, indicating that one of the two N-terminal serines is a target for IKKε and results in repression of IRF7 transcriptional activity. In line with our findings, Marié and colleagues identified in previous studies the first serine residue to be a possible IKKε target and assumed a role for IKKε in negative regulation of IRF7 activity (Caillaud et al., 2005).



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Name	Mutant (aminoacids)	*Stat1 -/-*	*Stat1 -/-* + TBK1	*Tbk1-Ikbke -/-*	*Tbk1-Ikbke -/-* + TBK1	*Tbk1-Ikbke -/-* + IKKε	*Tbk1-Ikbke -/-* + TBK1 + IKKε
WT	VSSLDSSSLGLCLSSTNSL	++	+++	-	+++	-	+
M1	VAALDSSSLGLCLSSTNSL	-	+	-	-	-	not tested
M5	VSSLDSSSLGLCLAATNSL	+/-	-	-	+/-	-	not tested
M12	VDDLDDDDLGLCLDDTNSL	+	+/-	+	+++	-	not tested
M15	VSSLDDDDLGLCLDDTNDL	++	++	++	+++++	-	+/-
M18	VSSLDAAALGLCLDDTNAL	+/-	+/-	-	+++	-	not tested

Figure 15: Transcriptional activity of IRF7 mutants. (B-G) Transactivation of the Gbp2 promoter by different mutants of IRF7. *Stat1* -/- MEFs (C,E) or *Tbk1/Ikbke* -/- MEFs (B,D,F) were transfected with WT IRF7 or the IRF7 mutants indicated in (A). 50ng of IRF7 constructs were transfected alone or co-transfected with either 1µg TBK1 (B,D,E,F), 1µg IKKε (F) or the combination of 0,5µg TBK1 and 0,5µg IKKε (F) as indicated. *Gbp2* reporter gene expression is indicated as -fold induction relative to cells transfected only with reporter construct after normalization to co-transfected, constitutively expressed and Renilla luciferase reporter. (G) Summerizing Table of the results with the transfected IRF7 mutants in *Stat1-/-* MEFs or *Tbk1-Ikbke* -/- MEFs, alone or together with TBK1 and/or IKKε.

Simultaneous recruitment of both IRF1 and IRF7 to the Gbp2 promoter of IFNγ-treated cells

According to our findings, IRF1 and IRF7 interacted at least functionally to drive the expression of ISRE driven genes in the context of an IFNγ response. Next, we asked whether IRF1 and IRF7 colocalize on the promoter of the *Gbp2* gene following treatment of cells with IFNγ. For this purpose we performed ChIP and reChIP assay analysis and monitored the binding of IRF1 and IRF7 by qPCR to the *Gbp2* gene promoter. This protocol was chosen to reduce the background obtained in single-round ChIPs with our antibody to IRF7. The *Gbp2* promoter can be devided into a distal and proximal region, containing either a GAS and ISRE site or only one ISRE site, respectively (Figure 16A). Both regions were shown to bind IRF1 efficiently after IFNγ treatment (Ramsauer et al., 2007). Repeatedly, we observed an increased binding of IRF1 to both promoter regions, with virtually indistinguishable kinetics (Figure 16B,E). ChIP-reChIP experiments with an IRF7 antibody recently generated by our lab revealed IRF7 to be associated with both regions of the *Gbp2* promoter. In contrast to IRF1 binding, kinetics of IRF7 association was different comparing the distal and the proximal region (Figure 16C,F). In general IRF7 occupancy of the *Gbp2* promoter, followed the IRF7 mRNA expression profile (Figure 12). IRF7 association with the distal *Gbp2* promoter region appeared to be more sustained and increased towards the end of the measurement period, whereas IRF7 binding to the proximal promoter clearly peaked after 3 hrs of IFNγ treatment. Sequential ChIP with antibody against IRF7 and re-ChIP with an IRF1 specific antibody, displayed a pattern for IRF1 and IRF7 co-occupancy at both promoter regions similar to the one observed for IRF7 association, (Figure 16D,G).

To test whether phosphorylation of IRF7 is important for its association with the *Gbp2* promoter we assessed IRF7 and IRF1 recruitment to the proximal *Gbp2* promoter in WT and *Tbk1/Ikbke -/-* MEFs by sequential ChIP assays. ChIP for IRF1 and reChIP for IRF1 revealed that IRF1 binding to the *Gbp2* promoter is similar in both genotypes. IRF7 recruitment was unimpeded in *Tbk1/Ikbke -/-* MEFs, as revealed by ChIP of IRF1 and reChIP of IRF7 (Figure 16H,I).

To test the specificity of the newly generated IRF7 antibody, we performed ChIP-reChIP analysis in WT and *Irf7 -/-* MEFs, stimulated with IFNγ. Our results show that, IRF7 does not bind to the *Gbp2* promoter of *Irf7 -/-* MEFs. Furthermore, the background of IRF7 binding was similar between untreated WT MEFs and untreated *Irf7 -/-* MEFs, suggesting that IRF7 is not constitutively associated with the *Gbp2* promoter (Figure 16J).

Taken together our results indicate that IRF1 and IRF7 co-occupy the distal and proximal *Gbp2* promoter, but binding of IRF1 precedes that of IRF7, since the IRF prototype binds to the *Gbp2* promoter already 1 hour after IFNγ treatment (Ramsauer et al., 2007), reaching its maximum after 2 hrs. In contrast, little IRF7 binding was detected after 2 hours, followed by a peak of IRF7 binding after 3 hrs of treatment. IRF7 binding was not reduced in *Tbk1/Ikbke -/-* MEFs, indicating that phosphorylation of IRF7 by either kinase is not required for nuclear translocation and DNA binding.

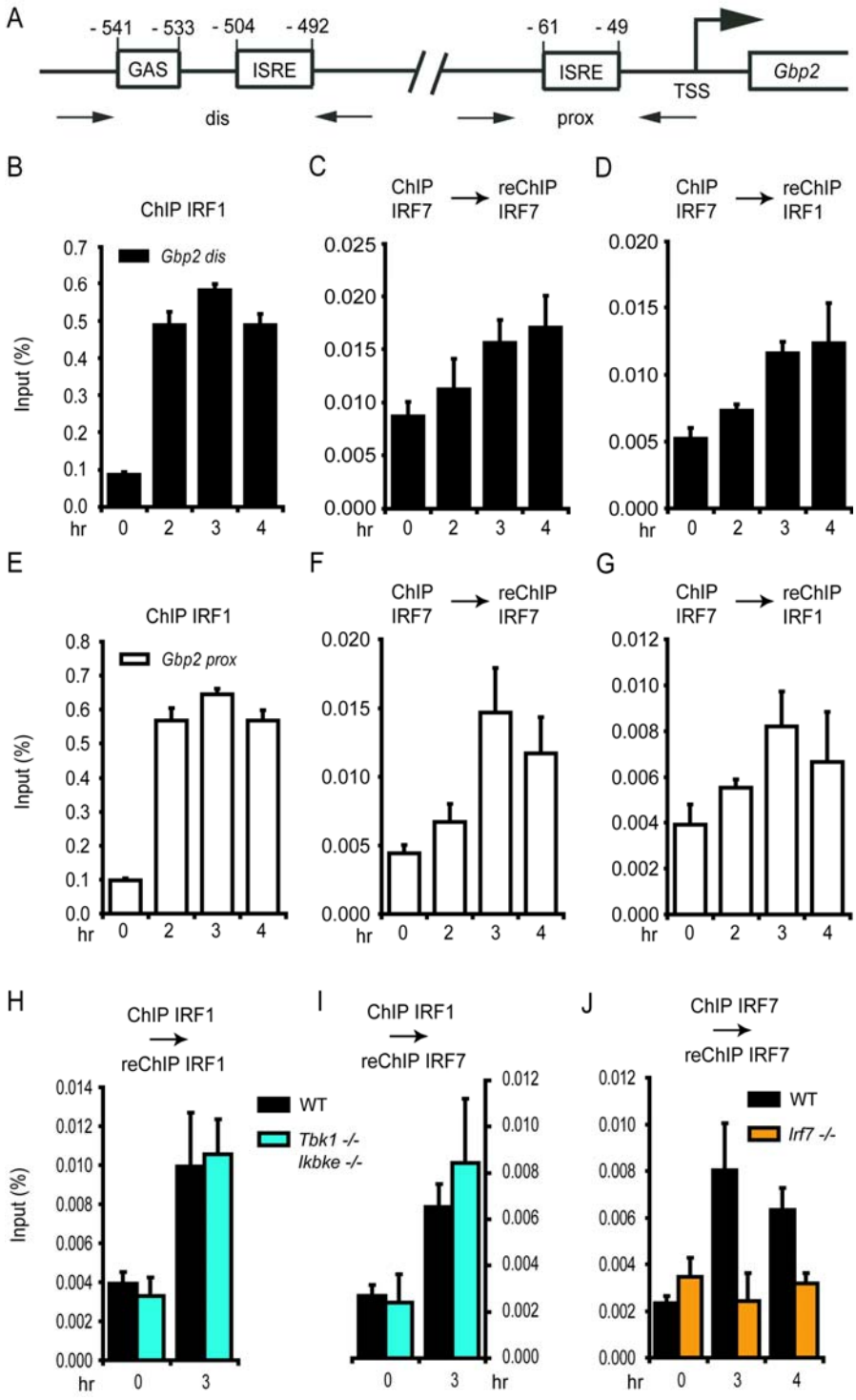

Figure 16: IRF1 and IRF7 recruitment to the *Gbp2* promoter after treatment with IFNγ. (A) Schematic drawing of the GAS located in the distal region and ISRE sites located in both, the distal and proximal promoter region of the *Gbp2* gene. (B-J) WT MEFs (B-J), *Tbk1/Ikbke -/-* MEFs (H,I), or *Irf7 -/-* MEFs were treated with IFNγ for the times indicated. The cells were processed for ChIP (B,E) or ChIP-reChIP (C,D,F,G-J). Antibodies used are shown on top of the panels. The precipitates were amplified with primers flanking the distal (B-D) or proximal (E-J) *Gbp2* promoter and analyzed by qPCR. Data are expressed as % of input DNA.

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Immunity
Article

Nonconventional Initiation Complex Assembly by STAT and NF-κB Transcription Factors Regulates Nitric Oxide Synthase Expression

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SUMMARY

Transcriptional regulation of the *Nos2* gene encoding inducible nitric oxide synthase (iNOS) requires type I interferon (IFN-I) signaling and additional signals emanating from pattern recognition receptors. Here we showed sequential and cooperative contributions of the transcription factors ISGF3 (a complex containing STAT1, STAT2, and IRF9 subunits) and NF-κB to the transcriptional induction of the *Nos2* gene in macrophages infected with the intracellular bacterial pathogen *Listeria monocytogenes*. NF-κB preceded ISGF3 at the *Nos2* promoter and generated a transcriptional memory effect by depositing basal transcription factor TFIIH with the associated CDK7 kinase for serine 5 phosphorylation of the RNA polymerase II (pol II) carboxyterminal domain (CTD). Subsequent to TFIIH deposition by NF-κB, ISGF3 attracted the pol II enzyme and phosphorylation at CTD S5 occurred. Thus, STATs and NF-κB cooperate through pol II promoter recruitment and the phosphorylation of its CTD, respectively, as a prerequisite for productive elongation of iNOS mRNA.

INTRODUCTION

The production of nitric oxide (NO) occurs during innate immune responses to all classes of pathogens (Bogdan, 2001). The molecule has direct antimicrobial activity, contributes to cell signaling, and regulates cell survival (Bogdan, 2001; Zwaferink et al., 2008). Inducible nitric oxide synthase (iNOS), the enzyme encoded by the *Nos2* gene and responsible for NO production during infection, is synthesized de novo as a response to the recognition of microbial molecular patterns. Studies with bacterial lipopolysacharide (LPS) or with pathogen-infected murine cells showed that full transcriptional induction of *Nos2* and of NO production occurs only after synthesis of type I interferons (IFN-I) and signaling through the Janus kinase (JAK)-STAT pathway (Bogdan, 2001; Gao et al., 1998). Type II IFN (IFN-γ), produced by natural killer (NK) and T cells, also enhances mouse *Nos2* induction by LPS in a manner requiring STAT1 activation by the IFN-γ receptor complex (IFNGR [Meraz et al., 1996]). Together the published work suggests that IFN receptor-activated STATs cooperate with non-IFN signals in the transcriptional regulation of *Nos2*.

Previous analyses of the murine *Nos2* promoter revealed an IFN response region and binding sites for NF-κB (Kleinert et al., 2003). The IFN response region contains binding sites for STAT1 dimer (gamma IFN-activated site, GAS [Xie et al., 1993]) and interferon regulatory factors (IRF [Kamijo et al., 1994; Spink and Evans, 1997]). IFN-γ signaling leads to the formation of STAT1 homodimers and IRF1, both of which were shown to be essential for *Nos2* induction by IFN-γ/LPS (Kamijo et al., 1994; Meraz et al., 1996). IFN-I causes formation of both STAT1 dimers and the ISGF3 complex, which comprise a STAT1/STAT2/IRF9 heterotrimer (Darnell, 1997; Schindler et al., 2007). It is unclear which of these complexes contributes to iNOS regulation by IFN-I and whether IFN-I, like IFN-γ, stimulate *Nos2* transcription with strong dependence on IRF1 or other IRF family members.

The analysis of signals received by the *Nos2* promoter directly from pattern recognition receptors emphasizes the role of NF-κB. Two sites for the transcription factor were identified (Kleinert et al., 2003; Lowenstein et al., 1993; Xie et al., 1994). Particularly the binding element proximal to the transcription start proved essential for the activity of the transfected promoter.

Listeria monocytogenes is a Gram-positive bacterial pathogen replicating in the cytoplasm of mammalian host cells. It is recognized by a variety of different pattern recognition receptors including toll-like receptors and NOD-like receptors (TLR and NLR, respectively) (Edelson and Unanue, 2002; Herskovits et al., 2007). In murine bone marrow-derived macrophages, a hitherto unknown cytoplasmic receptor initiates signaling to the IFN-I genes and subsequent release of IFN-I from the infected cells (Stetson and Medzhitov, 2006; Stockinger et al., 2004). Exclusion of *L. monocytogenes* from the cytoplasm, e.g., by mutation of its major virulence factor Listeriolysin O, completely abrogates the ability to stimulate IFN-I production (Stockinger et al., 2002). As with LPS, transcriptional induction of the *Nos2* promoter was strongly diminished when either IFN-I production

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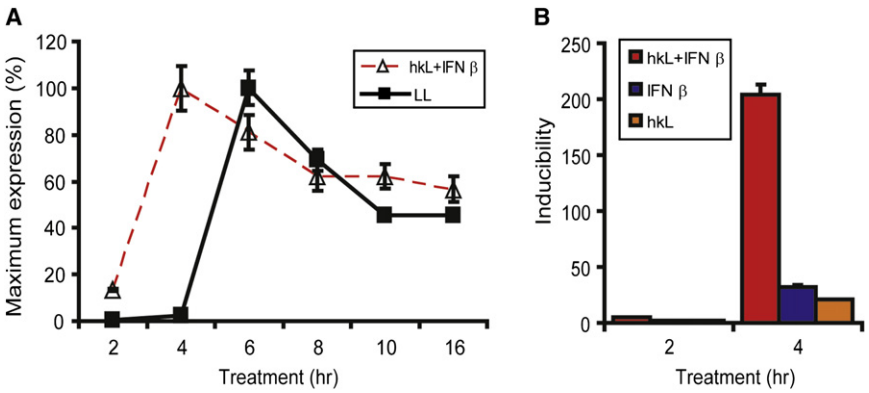

Figure 1. Kinetics of iNOS Induction Determined by q-PCR

(A) Exposure of bone marrow-derived macrophages to living *L. monocytogenes* (LL) or to cotreatment with *heat-killed Listeria* (hkL) and IFN-β.

(B) Bone marrow-derived macrophages were treated with hkL, IFN-β, or a combination of both. Error bars represent standard deviations from triplicate samples. The experiments were repeated at least three times.

or signaling were disrupted (Stockinger et al., 2004). To continue this work, we now asked the question why the *Nos2* gene, unlike classical IFN-I-stimulated genes (ISGs) or NF-κB target genes, requires input from both STATs and signals derived directly from pattern recognition receptors for maximal transcriptional induction. Combining an examination of transcription factor and signaling requirements for transcriptional induction with an analysis of transcription factor binding to the *Nos2* promoter in situ, we conclude that NF-κB enhances carboxy-terminal domain (CTD) phosphorylation of RNA pol II, after recruitment of the enzyme by STATs.

RESULTS

Cytoplasmic and Precytoplasmic Signals Synergize in *Nos2* Induction

As discussed above, the innate immune response to *L. monocytogenes* results initially from plasma membrane and endosomal pattern recognition during entry and from cytoplasmic sensing after cytoplasmic escape. The *Nos2* gene is paradigmatic for a large group of genes coregulated by pattern recognition receptors and IFN-I (Doyle et al., 2002; Toshchakov et al., 2002). To test whether IFN-I synthesis was the only essential signal for *Nos2* induction derived from the cytoplasmic signaling, the two recognition phases were separated by treating macrophages with heat-killed *L. monocytogenes* (hkL) and with IFN-β either separately or together. Heat-killed *Listeria* are confined to phagosomes and cannot stimulate the cytoplasmic signal required for IFN-I production. hkL and IFN-β alone were poor inducers of iNOS mRNA synthesis (Figure 1). By contrast, both signals together synergized to produce the full-blown iNOS synthesis seen with viable *L. monocytogenes*. This result suggests that cytoplasmic signaling can indeed be recapitulated by providing IFN-I. In addition, it provides a valuable experimental tool to separate effects of non-IFN-I and IFN-I signals on the *Nos2* promoter and to study each independently from the other. In agreement with IFN-I synthesis preceding *Nos2* transcription, the kinetics of mRNA synthesis after infection with viable *L. monocytogenes* were delayed compared to the simultaneous treatment with hkL and IFN-β.

Many genes expressed in macrophages infected with *L. monocytogenes* were found in a microarray experiment to display a pattern of regulation resembling that of the *Nos2* gene. 38 genes showing the strongest synergy effect between IFN-β alone and the additional presence of *L. monocytogenes*-derived signals are shown in Figure S1 available online.

Signals and Transcription Factors Required for iNOS Regulation by *L. monocytogenes*

To examine transcription factor requirements for transcriptional induction of the *Nos2* gene, we used bone marrow-derived macrophages from either wild-type or gene-targeted mice and infected them with *L. monocytogenes* (Figure 2). As expected, *Nos2* expression required signaling through both the IFN and

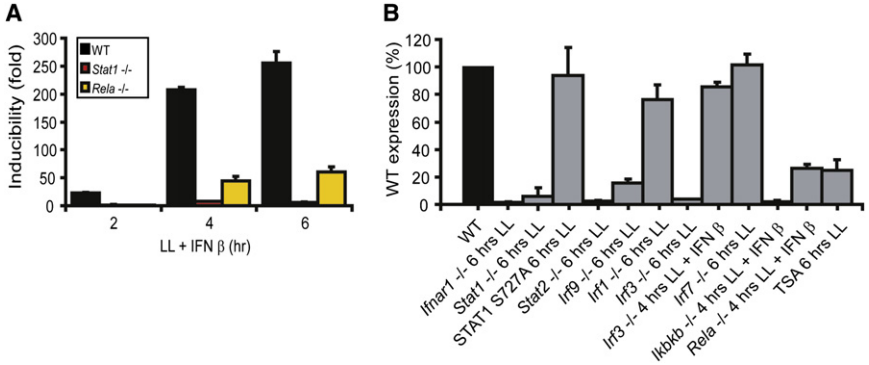

Figure 2. iNOS mRNA Induction by *L. monocytogenes* Requires Stat1, Stat2, IRF9, and NF-κB Signaling

(A) Bone marrow-derived macrophages of WT, *Stat1*$^{-/-}$, and *Rela*$^{-/-}$ mice were infected with living *L. monocytogenes* (LL) for the times indicated. IFN-β was additionally present to compensate for potential defects in IFN-I production. iNOS mRNA expression was determined by q-PCR.

(B) Bone marrow-derived macrophages with the indicated genotypes were infected with living *L. monocytogenes* (LL) for 6 hr or a combination of LL and IFN-β (*Ikbkb*$^{-/-}$ + IFN-β; *Rela*$^{-/-}$ + IFNβ; *Irf3*$^{-/-}$ + IFN-β) for 4 hr. iNOS mRNA expression was determined by q-PCR.

To be able to compare data between individual experiments, genotype-specific expression is shown as percent induction found in wild-type macrophages. Error bars represent standard deviations from triplicate samples. The mentioned experiments were repeated at least three times.

NF-κB pathways as deletion of either the *Stat1* or *Rela* (NF-κB p65) genes strongly suppressed iNOS mRNA induction in infected macrophages (Figure 2A). More refined analyses confirmed the importance of the IFN-I receptor (*Ifnar1*$^{-/-}$ mice) and the NF-κB pathway (*Rela*$^{-/-}$ and *Ikbkb* $^{-/-}$ mice, deficient for NF-κB p65 and the IKKβ kinase, respectively) and established the importance of the ISGF3 subunits STAT1, STAT2, and IRF9 (Figure 2B). The diminished *Nos2* expression observed upon interference with NF-kB signaling was not due to reduced IFN-I production as shown by the fact that addition of exogenous IFN-β did not rescue this effect. Use of macrophages derived from mice expressing STAT1 mutated at its S727 phosphorylation site (STAT1S727A) showed that phosphorylation of STAT1 at S727, important for full transcriptional induction of some IFN-γ-induced genes (Varinou et al., 2003), was not required for *Nos2* expression. This contrasts with the reduced induction of *Nos2* by IFN-γ early after treatment in STAT1S727A-expressing macrophages (Varinou et al., 2003). In further distinction from the IFN-γ response, the decrease resulting from IRF1 deficiency was marginal. Two additional members of the IRF family, IRF3 and IRF7, are active in *L. monocytogenes*-infected macrophages (Stockinger et al., 2009). IRF7 deficiency did not affect *Nos2* expression. IRF3 deficiency reduced *Nos2* induction, but the defect could be rescued by the addition of IFN-β, suggesting that it resulted from reduced IFN-β synthesis, but not from a direct effect on the *Nos2* gene. The data suggest that IFN-I participate in *Nos2* regulation during *L. monocytogenes* infection by deploying the ISGF3 complex, but not the ancillary activity of IRFs. The low levels of iNOS expression seen after treatment up to 6 hr with IFN-β alone (Figure 1) were strongly reduced in mice unable to form ISGF3 (data not shown). Interestingly, this differs from the regulation of *Nos2* mRNA during the late stage of the IFN-I response, which has been shown to be independent of STAT1 (Plumlee et al., 2009).

A distinguishing feature of typical IFN-I-induced genes is that a deacetylation step is required for transcriptional induction, which can be inhibited with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA [Nusinzon and Horvath, 2005]). Induced synthesis of *Nos2* mRNA was TSA sensitive, suggesting that the activity of STATs on the *Nos2* promoter abides by the same rules in this regard as the transcriptional activation of classical ISGs. MAP kinase pathways downstream of pattern recognition receptors (targeting ERK, JNK, and p38MAPK) were probed by pharmacological inhibition. None of the inhibitory drugs produced a significant reduction of *L. monocytogenes*-induced *Nos2* expression (data not shown). In summary, the data from Figures 1 and 2 suggest that ISGF3 is the main signal derived from cytoplasmic signaling, recapitulated by the addition of exogeneous IFN-β, and that NF-κB is the major signal stimulated by hkL, provided by plasma membrane and/or endosomal pattern recognition receptors for *Nos2* induction. Our further investigations therefore concentrated on the interaction between these two pathways.

Binding of STATs and NF-κB to *Nos2* Promoter Chromatin in Macrophages Infected with *L. monocytogenes*

Association of the ISGF3 complex with *Nos2* chromatin was examined with antibodies against STAT1 and STAT2 for ChIP. Likewise, NF-κB binding was determined with antibodies to its p50 and p65 subunits. Amplification by polymerase chain reaction (PCR) was performed to reveal binding to the promoter-proximal region containing the essential NF-κB site as well as the more distal promoter containing the IFN response region and a second potential binding site for NF-κB (Figure 3A). Treating macrophages simultaneously with hkL and IFN-β stimulated binding of the ISGF3 subunits STAT1 and STAT2 with indistinguishable kinetics (Figure 3B). The same observation was made for the NF-κB subunits p50 and p65 with the notable exception that a reduction of constitutive p50 binding at the earliest time point after stimulation and preceding the phase of increased promoter binding was reproducibly observed. This finding is consistent with the reported negative regulation of NF-κB target genes by p50 homodimers in resting cells (Zhong et al., 2002). NF-κB association was found exclusively with the promoter-proximal, essential site, whereas no evidence for binding to the distal site was obtained. As expected, STAT binding was caused by treatment with IFN-I alone, whereas NF-κB binding occurred after exposure to hkL (data not shown). No evidence for interdependent binding of the two transcription factors was obtained. Consistently, infection with viable *L. monocytogenes* resulted in similar kinetics of NF-κB p65 binding, but STAT1 association now required prior IFN-I synthesis and was therefore delayed by about 2 hr compared to direct stimulation with IFN-I (Figure 3C). Thus, during infection, binding of NF-κB precedes that of STAT1 and STAT2. The simultaneous presence of these proteins was further examined via a ChIP-re-ChIP procedure (Figure 3D). It confirmed that after both IFN-β treatment and infection with *L. monocytogenes*, STAT1 could be reprecipitated from a STAT2 ChIP with the expected difference in binding kinetics (see Figure 1).

Acetylation of Histones in Proximity to the IFN Response Region and to the Promoter-Proximal NF-κB Site

Synergistic enhancement of transcriptional activation by ISGF3 and NF-κB might result from an interaction in the recruitment of histone acetylases to the *Nos2* promoter. Acetylation of histone H4 at the proximal and distal promoter elements was assessed. To correct for histone eviction, data were normalized to the levels of total H3. Increases of histone acetylation are often rather subtle, so we verified significance and quantified our data by using a q-PCR protocol. All experiments were repeated at least five times. Black bars represent amplification of the distal promoter and white bars amplification of the proximal promoter. This convention is maintained through all subsequent figures.

Combined treatment of macrophages with IFN-I and hkL produced an increase of histone acetylation at both the proximal and distal promoter locations (Figure 4A). Treatment with IFN-I alone led to an increase of H4 acetylation almost exclusively at the distal IFN response region (Figure 4B). Conversely, hkL treatment alone caused an increase in H4 acetylation predominantly at the proximal NF-κB element (Figure 4C). Our findings suggest that ISGF3 and NF-κB indeed cooperate in producing hyperacetylated *Nos2* promoter chromatin, but that their histone acetyl transferase (HAT)-recruiting activities show no signs of functional interdependence.

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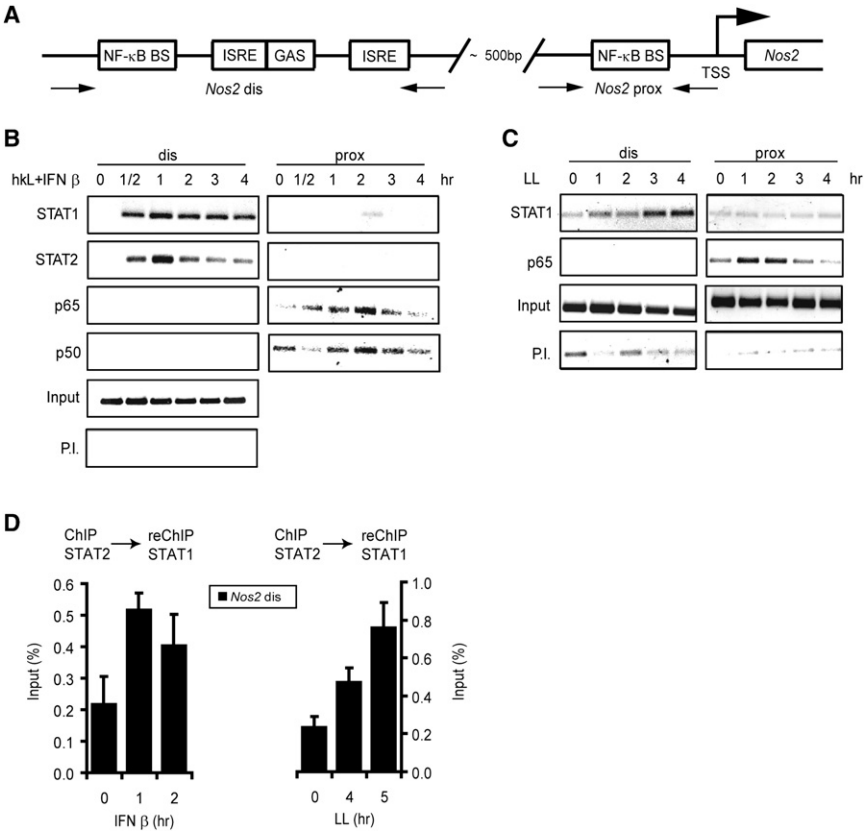

Figure 3. Binding of STATs and NF-κB to the *Nos2* Promoter

(A) Schematic drawing of the IFN response region and the NF-κB sites (NF-κB BS) in the *Nos2* promoter (Kleinert et al., 2003). Binding of STATs and NF-κB to the *Nos2* promoter in response to signals stimulated by exposure to *L. monocytogenes*.

(B) Bone marrow-derived macrophages were stimulated with hkL and IFN-β and the cells were processed for ChIP at the indicated time points. Antibodies used for ChIP are shown on the left, P.I. indicates controls performed with preimmune sera. The precipitates were amplified with primers flanking the proximal (NF-κB) or distal (STAT1, IRF) promoter regions as depicted in (A) and analyzed by gel electrophoresis.

(C) Bone marrow-derived macrophages were infected with viable *L. monocytogenes* and processed as described in (B).

(D) Bone marrow-derived macrophages were either treated with IFN-β or infected with living *L. monocytogenes* (LL) for the times indicated and processed for ChIP-Re-ChIP.

Antibodies used for ChIP and Re-ChIP are shown on top of the panels. The precipitates were amplified with primers flanking the distal *Nos2* promoter region and analyzed by q-PCR. Error bars represent standard deviations from triplicate samples. The experiments were repeated at least three times.

Recruitment of RNA Polymerase II to the *Nos2* Transcription Start Site

Pol II can be bound to transcription start sites in a poised state (Adelman et al., 2009; Koch et al., 2008; Margaritis and Holstege, 2008). Alternatively, the enzyme is recruited in response to the stimulus of gene activation (Adelman et al., 2009). To determine which situation applies to the macrophage *Nos2* gene, we analyzed pol II association by ChIP. As shown in Figure 5A, infection with *L. monocytogenes* strongly increased pol II binding, suggesting that it occurs by regulated recruitment. Surprisingly, treatment with IFN-I alone also stimulated binding of pol II (Figure 5B). Association was somewhat, but not much, weaker than after the additional presence of hkL. In contrast to IFN-I, hkL alone did not stimulate pol II binding (Figure 5C). This result indicates (1) that the histone acetylation caused by NF-κB is not an absolute requirement for pol II binding and (2) that there is

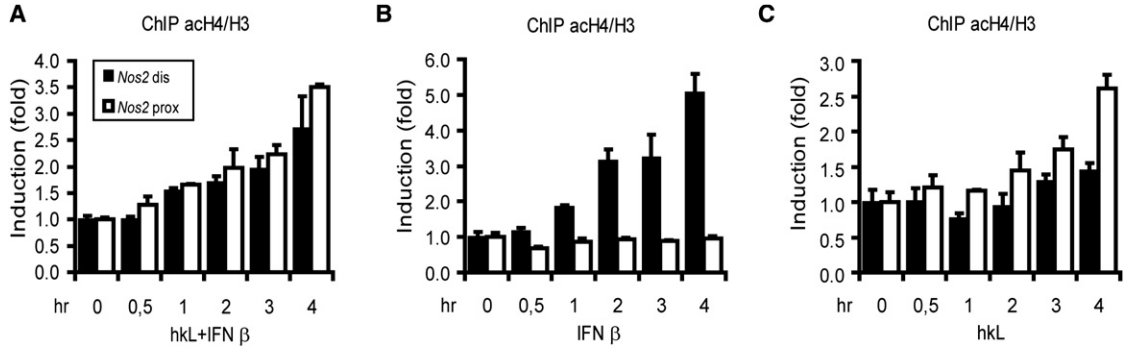

Figure 4. Histone 4 Acetylation at the *Nos2* Promoter

Bone marrow-derived macrophages were treated with hkL and IFN-β (A), IFN-β alone (B), or hkL alone (C) as indicated. ChIP was performed with antibodies to acetyl-histone 4 (acH4) and with antibodies to histone 3 (H3). The presence of distal (black) or proximal (white) *Nos2* promoter fragments was determined by q-PCR. Data are expressed as increase of acH4 signals normalized to H3 signals to correct for histone eviction. The histograms thus denote the ratio of acetyl-histone 4 binding as a function of total histone 3 (acH4/H3). Error bars represent standard deviations from triplicate samples. All experiments were repeated at least five times.

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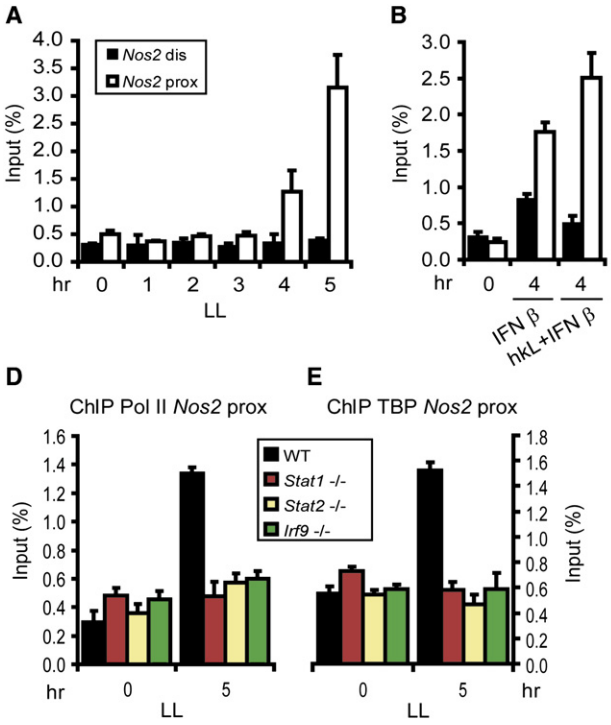

Figure 5. Recruitment of RNA Polymerase II to the *Nos2* Promoter by *L. monocytogenes*-Derived Signals

Bone marrow-derived macrophages from wild-type mice (A–E) or *Stat1*$^{-/-}$, *Stat2*$^{-/-}$, and *Irf9*$^{-/-}$ mice (D, E) were infected with living *L. monocytogenes* (LL [A, D, E]), with IFN-β alone (B), heat-killed *Listeria* alone (hkL [C]), or with a combination of IFN-β and hkL (B, C) for the times indicated. The cells were processed for ChIP with antibodies against pol II (A–D) or TBP (E). The precipitated DNA was analyzed by q-PCR with primers amplifying the distal (black) and proximal (white) promoter regions. Panels (D) and (E) show a comparison of proximal promoter fragments in ChIP from WT (black), *Stat1*$^{-/-}$ (red), *Stat2*$^{-/-}$ (yellow), and *Irf9*$^{-/-}$ (green) macrophages. Error bars represent standard deviations from triplicate samples. The experiments were repeated at least three times.

a mechanistic difference between ISGF3 and NF-κB in their mode of activating the *Nos2* promoter. IFN and STAT-dependent recruitment of pol II predicts that binding of TFIID and its TBP subunit displays the same requirement. Figures 5D and 5E indeed show that both pol II and TBP binding was completely abrogated when *Stat1*$^{-/-}$, *Stat2*$^{-/-}$, or *Irf9*$^{-/-}$ macrophages were infected with *L. monocytogenes*.

Recruitment of TFIIH-CDK7 and Phosphorylation of the Pol II CTD

Pol II, once stably bound to the initiation site, must be phosphorylated at its CTD to associate with proteins required for promoter clearance, capping of the mRNA, and elongation (Chapman et al., 2008; Hirose and Ohkuma, 2007). Serine 5 (S5) of the CTD amino acid heptarepeat becomes phosphorylated first, followed by S2, to proceed to productive elongation. With NF-κB playing only a minor role in pol II recruitment, we wondered whether it might play a role in distinct steps of transcriptional initiation. We investigated CTD phosphorylation at S5 by using phosphospecific antibodies for ChIP. S5-phosphorylated pol II was precipitated from the *Nos2* initiation site only after treatment with both hkL and IFN-I, but not after treatment with IFN-I alone (Figure 6A). This confirms our notion that NF-κB might be involved in regulating CTD phosphorylation. CTD S5 kinase activity is associated with the general transcription factor TFIIH. TFIIH usually joins the initiation complex only after pol II binding. It is a multiprotein transcription factor containing the CTD S5 kinase CDK7 and a number of additional subunits including p62 (Egly, 2001). As in the case of S5-phosphorylated pol II, CDK7 was associated with the *Nos2* initiation site after stimulation with hkL and IFN-I, but not after treatment with IFN-I alone (Figure 6B). In contrast to IFN-I, hkL treatment alone produced as much CDK7 binding as the combined IFN-I-hkL treatment (Figure 6C). The kinetics of CDK7 binding as induced by *L. monocytogenes* demonstrated association with the *Nos2* promoter at 2 hr postinfection (Figure 6D). At this time, NF-κB is associated with *Nos2* chromatin, but no or very little ISGF3 is present (Figure 3C). Binding of CDK7 as well as that of TFIIH p62 was abrogated by both NF-κB p65 and IKKβ deficiency (Figures 6E–6H). Together, these data confirm the hypothesis that a TFIIH complex is recruited by NF-κB, providing kinase activity for the pol II CTD at S5. Comparing the kinetics of NF-κB and TFIIH binding in the course of infection suggested that TFIIH remains bound at the promoter even after dissociation of NF-κB (Figures 3, 6G, and 6H; Figure S2). We tested the possibility that NF-κB, by depositing TFIIH, primes the *Nos2* promoter for subsequent ISGF3 activity, thus providing a "transcriptional memory" effect. To this end, macrophages were given a 2 hr pulse of hkL treatment, a period sufficient for CDK7 recruitment (Figure 6D). The pulsed cells were left without further stimulation for various intervals, followed by a 4 hr treatment with either IFN-β alone, hkL alone, or a combination of IFN-β and hkL. The data show that for at least 24 hr, the level achieved by IFN-β treatment of pulsed cells exceeded the level achieved by IFN-β treatment of unpulsed cells (Figure 6I). This result is in agreement with the notion of a transcriptional memory or priming effect of NF-κB-recruited CDK7.

Pol II and CDK7 Recruitment by Interferon-Stimulated Genes or Classical NF-κB Target Genes

The results obtained by studying *Nos2* regulation raise the question why the *Nos2* gene requires both ISGF3 and NF-κB to achieve elongation competence. They also predict that genes induced by either IFN-I or the NF-κB pathway alone should demonstrate promoter binding of both CDK7 and pol II after single treatment with IFN-I or hkL. This assumption was tested by analyzing pol II and CDK7 binding to the promoters of the

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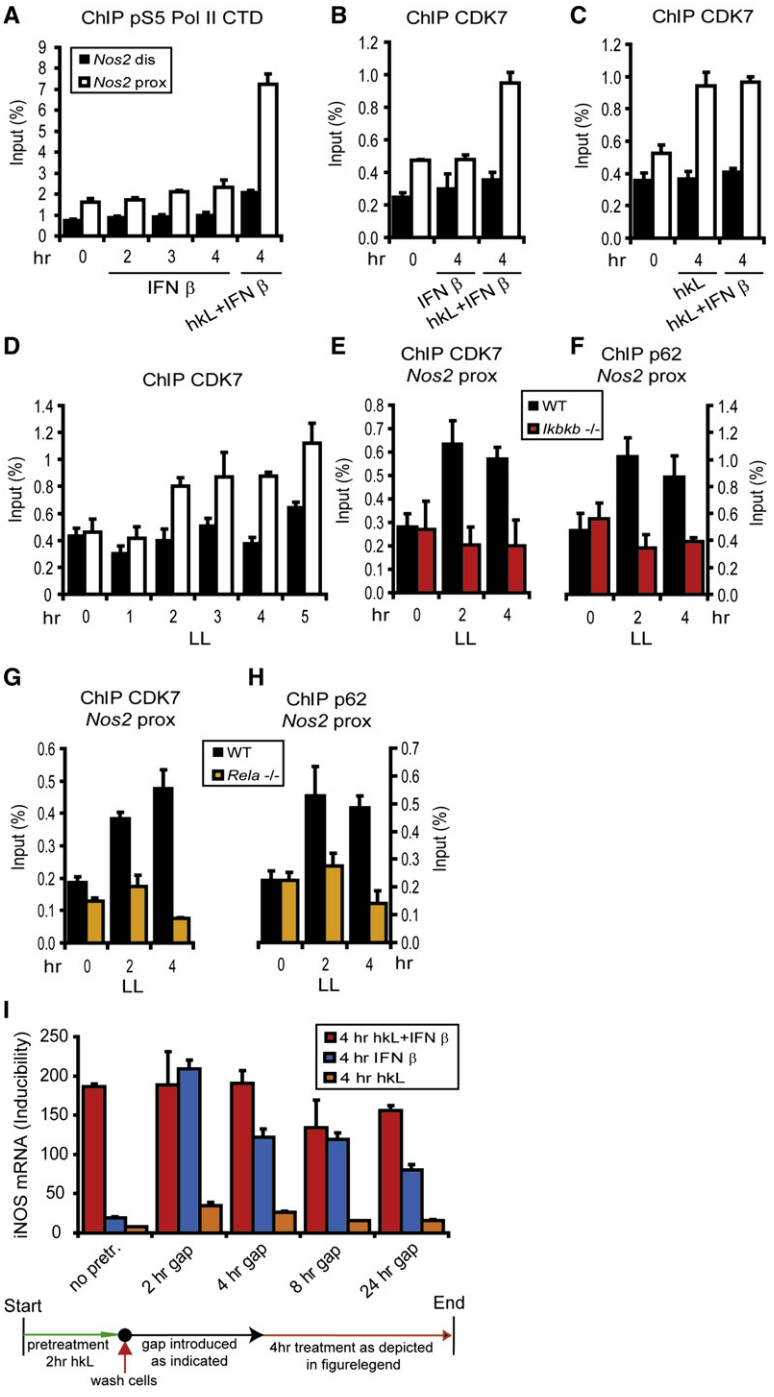

Figure 6. TFIIH-CDK7 Recruitment to the *Nos2* Promoter and S5 Phosphorylation of the RNA Polymerase II CTD by *L. monocytogenes*-Derived Signals; Analysis of *Nos2* Promoter Priming by hkL

(A–H) Bone marrow-derived macrophages from WT mice (A–H), *Ikbkb*$^{-/-}$ mice (E, F), or *Rela*$^{-/-}$ mice (G, H) were infected with living *L. monocytogenes* (LL [D–H]), with IFN-β alone (A, B), with hkL alone (C), or with a combination of IFN-β and hkL (A–C) for the times indicated. The cells were processed for ChIP with antibodies against S5-phosphorylated pol II (A), CDK7 (B–E, G), or the TFIIH subunit p62 (F, H). The precipitated DNA was analyzed by q-PCR with primers amplifying the distal (black) and proximal (white) promoter regions. Panels (E)–(H) show a comparison of proximal promoter fragments in ChIP from WT (black), *Ikbkb*$^{-/-}$ (red, E, F), and *Rela*$^{-/-}$ (orange, G, H) macrophages.

(I) Bone marrow-derived macrophages were pretreated with hkL for 2 hr or left without pretreatment followed by extensive washing of the cells. The cells were then left without treatment for different periods of time (indicated as hours gap). Thereafter cells were stimulated with hkL + IFN-β, IFN-β alone, or hkL alone for 4 hr. iNOS mRNA expression was determined by q-PCR.

Error bars represent standard deviations from triplicate samples. The experiments were repeated at least three times.

IFN-I-induced *Mx2* gene or of the *Nfkbia* gene, which is activated by NF-κB for the production of IκBα to establish a feedback inhibition loop. The data summarized in Figures 7A–7D show that IFN-β treatment for 2 or 4 hr caused association of both pol II and CDK7 with the *Mx2* but not the *Nfkbia* promoter. Conversely, treatment with hkL to activate the NF-κB pathway increased association of pol II and CDK7 with the *Nfkbia* but not the *Mx2* promoter. Both stimuli provided together did not increase promoter binding beyond the level observed with single treatments. Consistent with this, transcriptional priming by the NF-κB pathway was not observed with either the *Mx2* or the *Nfkbia* gene (Figures 7E and 7F). The data are consistent with our notion that the establishment of elongation competence by cooperative signals is a gene-specific attribute and a major contribution to the regulation of the *Nos2* promoter by the transcription factors NF-κB and ISGF3.

DISCUSSION

NO production is a hallmark of innate immune responses, but its influence on infected cells or organisms varies. For some pathogens, NO is an important clearance mechanism (Bogdan, 2001). By contrast, *L. monocytogenes* stimulates macrophages to synthesize large quantities of NO, but appears to be relatively insensitive to its toxic effects under our experimental conditions (Zwaferink et al., 2008). The main effect of NO is to promote the death of *Listeria*-infected macrophages. Our studies of *Nos2* regulation were prompted by the findings of several labs that the gene expression signature of cells infected with pathogens, or exposed to their pathogen-associated molecular patterns (PAMPs), results to a significant extent from cooperative signaling by pattern recognition and IFN-I receptors (Doyle et al., 2002; Toshchakov et al., 2002). By using the *Nos2* gene as a well-studied example, we show that the need for cooperation between the ISGF3 complex and NF-κB arises from the inability of the former to provide CTD kinase activity and the lack of pol II recruitment by the latter. The prevalent mode of rendering a gene competent for transcription is to assemble a TFIID-TFIIB-pol II complex prior to the association

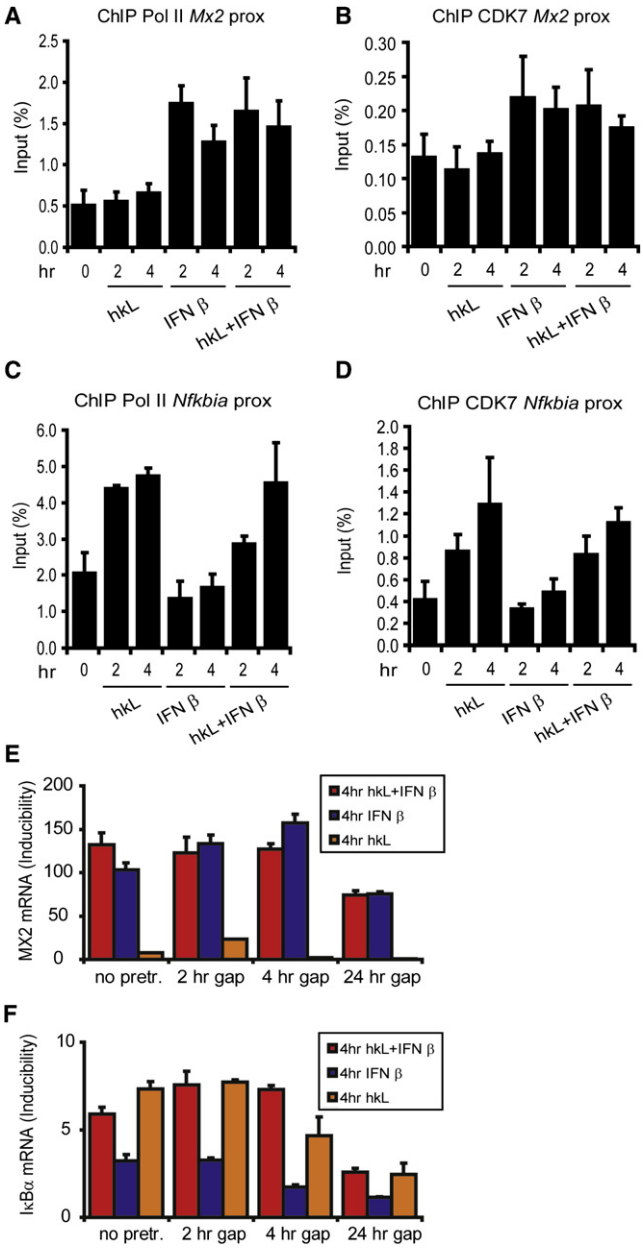

Figure 7. RNA Pol II and CDK7 Recruitment to the Proximal Promoter Regions of the IFN-Inducible *Mx2* Gene and the Gene Encoding IκB; Analysis of *Mx2* and *Nfkbia* Promoter Priming by hkL

(A–D) Bone marrow-derived macrophages from wild-type mice were treated with hkL + IFN-β, hkL alone, or IFN-β alone for the times indicated. The cells were processed for ChIP with antibodies against pol II (A, C) or CDK7 (B, D). The precipitated DNA was analyzed by q-PCR with primers amplifying the proximal promoter regions of the *Mx2* gene (A, B) and the *Nfkbia* gene (the gene encoding IκBα) (C, D).

(E and F) Bone marrow-derived macrophages were pretreated with hkL for 2 hr or left without pretreatment followed by extensive washing of the cells. The cells were then left without treatment for different periods of time (indicated as hours gap). Thereafter cells were stimulated with hkL + IFN-β, IFN-β alone, or hkL alone for 4 hr. Mx2 (E) and IκBα (F) mRNA expression was determined by q-PCR. Error bars represent standard deviations from triplicate samples. The experiments were repeated at least three times.

with TFIIH (Roeder, 1996). The combined ISGF3 and NF-κB activity at the *Nos2* promoter results in an unconventional transcription initiation complex assembly where TFIIH binds the promoter first to provide kinase activity for the subsequent recruitment of pol II. Three lines of evidence led us to this conclusion: (1) the kinetics of transcription factor and CDK7 binding during *L. monocytogenes* infection show that TFIIH-CDK7 recruitment occurs before pol II binding, (2) IFN-I alone is able to bring about the recruitment of pol II and hkL alone are able to stimulate CDK7 binding, and (3) CDK7 binding is abrogated in absence of the NF-κB pathway and TBP-pol II binding is abrogated in absence of ISGF3. CDK7 binding trails that of NF-κB by about 1 hr, suggesting that a complex forms at the promoter that is not preassembled and may require intermediate steps and partner proteins. Similarly, pol II binding occurs roughly 1 hr after the observed increase in STAT1 association. CDK7 remains associated with *Nos2* chromatin once NF-κB p65 leaves the promoter (best seen in Figure S2), suggesting that the function of NF-κB is to load the promoter with CDK7-TFIIH, but not to maintain this association once it has been established.

To our knowledge this is the first time this mode of initiation complex assembly is shown for a gene in the context of the cellular genome and as a result of ISGF3-NF-κB interaction, although several recent studies are in line with our findings (Spilianakis et al., 2003). The most compelling evidence that TFIIH-CDK7 recruitment by NF-κB may be more widely used was provided in studies on the activation of the HIV LTR in response to TNF (Kim et al., 2006). Contrasting the situation with *Nos2*, an initiation complex including a hyperphosphorylated RNA pol II was preassembled at the LTR, but, similar to our findings with *Nos2*, elongation competence required TNF and NF-κB to attract TFIIH-CDK7. The authors propose that NF-κB both associates with TFIIH and stimulates release of the inhibitory CDK8 from the mediator complex. Genes induced by LPS differ concerning the rate-limiting regulatory step for the onset of transcription, consisting either in the release of an elongation block to a paused polymerase or the pol II recruitment step (Adelman et al., 2009). Whether and how NF-κB-mediated TFIIH recruitment contributes in both situations is not known. Therefore it will be of interest to determine to what extent this mechanism contributes to the large impact of the NF-κB pathway on infection-related gene expression and in how far *Nos2* represents a paradigm valid for the many genes synergistically induced by STATs and NF-κB. The mechanism of pol II recruitment to the many genes regulated by NF-κB in absence of ISGF3 requires further investigation and, conversely, the mode of TFIIH recruitment to ISGF3 target genes in the absence of NF-κB remains to be clarified. This may generally be determined by cooperative transcription factors bound to their target promoters and/or by differences in the preexisting chromatin structure and composition.

Our studies allow some conclusions about the mechanism of ISGF3 action beyond the functional division of labor with NF-κB. At the *Nos2* promoter, ISGF3 stimulated binding of RNA pol II without requiring STAT1 phosphorylation at S727 or the helper function of IRF1. This differs from the STAT1 dimer, which is transcriptionally more active with its transactivating domain phosphorylated and requires IRF1 to induce the expression of *Nos2* and other genes in response to IFN-γ (Kamijo et al., 1994;

Ramsauer et al., 2007; Varinou et al., 2003). Data from our lab addressing IFN-γ induction of the *Gbp2* promoter showed that the STAT1 dimer alone cannot recruit RNA pol II (Ramsauer et al., 2007). This allows speculation that the STAT2 transactivating domain may generally supersede the requirement for STAT1 S727 phosphorylation and the ancillary activity of IRF1. In accordance with our findings about acetylation of the *Nos2* promoter, STAT2-dependent transcriptional initiation via mediator and TFIID subunits correlates with the ability of the STAT2 TAD to contact the HATs GCN5 and PCAF (Lau et al., 2003; Paulson et al., 2002). Histone acetylation is an important regulatory step for both NF-κB and STAT target genes (Chen and Greene, 2004; Ramsauer et al., 2007). NF-κB as well as ISGF3-dependent acetylation of *Nos2* promoter chromatin was restricted to the nucleosomes adjacent to their binding sites. This resembles virus-induced histone acetylation at the IFN-β promoter or the promoter of the IFN-I-induced *Ifi-56K* gene that was similarly restricted to a region around the transcription factor binding sites and the transcription start (Parekh and Maniatis, 1999).

Reviewing our findings and corroborating studies in the perspective of *L. monocytogenes* infection or pathogen infection in general raises the question why some, but not all, ISGs are coupled to the NF-κB pathway. IFN-I synthesis during infection occurs in response to nucleic acid PAMPs in the cytoplasm, when endosomal TLRs are stimulated, or when TLRs resident at the plasma membrane travel to late endosomes in the process of pathogen uptake (Kagan et al., 2008). With the notable exception of the cytoplasmic DNA receptor (Stetson and Medzhitov, 2006), all PRRs stimulating IFN-I synthesis will also stimulate the NF-κB pathway, thus providing both signals necessary for *Nos2* induction. Vice versa, some PRRs capable of activating NF-κB are not normally coupled to IFN-I synthesis. Examples of these are TLR2, TLR5, and the NLR family receptors NOD1 and NOD2, which have been associated with IFN-I synthesis only in a limited number of cell types or under specific circumstances (Barbalat et al., 2009; Pandey et al., 2009). Furthermore, a large number of stress or inflammatory signals, most notably those emanating from the TNF receptor family, provide NF-κB activity without concomitant IFN-I production and signaling (Dempsey et al., 2003). We hypothesize that such receptors and their signals provide a TFIIH-dependent transcriptional memory effect for *Nos2* expression, independently of pathogen uptake. Vigorous iNOS expression and NO production are limited, however, to situations where a pathogen is engulfed and processed by host cells, and when PAMPs appear in the late endosome and cytoplasm. This mechanism is consistent with our results in Figure 6 showing that the hkL-stimulated NF-κB pathway can provide the *Nos2* promoter with transcriptional memory for a subsequent treatment with IFN-I. It ensures that large amounts of NO are made only when its antipathogen activity is needed inside cells. Continuing along these lines, the reason why classical ISGs do not require this prime-and-trigger mechanism may be that their products are less harmful and cells can afford to prepare for pathogen entry without running the risk of inflicting damage upon themselves (Zwaferink et al., 2008). Although our study provides a mechanism for signal integration and a potential paradigm for cooperativity between the STAT and NF-κB pathways during infection, further experiments must reveal the biological impact of STAT-NF-κB convergence.

EXPERIMENTAL PROCEDURES

Reagents

Recombinant IFN-β was purchased from Biomedica (Nova Scotia, Canada) and added to culture medium to a final concentration of 250 U/ml. The inhibitors Trichostatin A (TSA) (WAKO Biochemicals, Osaka, Japan), SP600125 for c-JUN kinase inhibition (Sigma-Aldrich, St Louis, MO), SB203580 for p38MAPK inhibition (Sigma-Aldrich), and U0126 for MEK inhibition (Calbiochem, Nottingham, UK) were used in a final concentration of 150 nM, 25 nM, 4 nM, and 10 nM, respectively.

Bacteria and Infection

The *Listeria monocytogenes* strain LO28 was cultured in brain heart infusion broth overnight at 37°C. Infection of cells at MOI 10 was performed as described (Stockinger et al., 2002). Heat-killed *Listeria* (hkL) were generated by incubation of an overnight culture of LO28 in a waterbath at 70°C for 20 min.

Mice and Cells

Animal experiments were discussed and approved by the University of Veterinary Medicine, Vienna, institutional ethics committee and carried out in accordance with protocols approved by the Austrian law (GZ 680 205/67-BrGt/2003). Mice (WT C57BL/6, *Ifnar1*$^{-/-}$ [Muller et al., 1994], *Stat1*$^{-/-}$ [Durbin et al., 1996], STAT1S727A [Varinou et al., 2003], *Stat2*$^{-/-}$ [Park et al., 2000], *Irf1*$^{-/-}$ [Reis et al., 1994], *Irf3*$^{-/-}$ [Sato et al., 2000], *Irf7*$^{-/-}$ [Honda et al., 2005], *Irf9*$^{-/-}$ [Harada et al., 1996], and *Ikbkb*$^{\Delta}$ and *Rela*$^{\Delta}$ [Greten et al., 2007]) were sacrificed for bone marrow between 7 and 10 weeks of age. All animals were in a C57BL/6 genetic background. The mice were housed under specific-pathogen-free conditions. Poly I:C-mediated deletion of IKKβ and NF-κB p65 in bone marrow cells was performed as described (Greten et al., 2007). Bone marrow-derived macrophages (BMDM) were obtained by culture of bone marrow in L-cell-derived colony-stimulating factor 1 as described previously (Baccarini et al., 1985).

RNA Preperation and qRT-PCR

RNA preparation was performed with NucleoSpin RNA II Kit purchased from Macherey-Nagel (Düren, Germany) according to the manufacturer's protocol. Quantitative real-time PCR was performed on Mastercycler ep realplex S, purchased from Eppendorf (Vienna, Austria). Primer for iNOS mRNA expression and qRT-PCR were described previously (Stockinger et al., 2004). Primer for MX2 and IκBα mRNA expression were as follows: MX2 fwd 5′-CCAGTTCCTCTCAGTCCCAAGATT-3′; MX2 rev 5′-TACTGGATGATCAA GGGAACGTGG-3′; IκBα fwd 5′-GCAATTTCTGGCTGGTGGG-3′; IκBα rev 5′-GATCCGCCAGGTGAAGGG-3′.

Chromatin Immunoprecipitation and Re-ChIP

Chromatin immunoprecipitation (ChIPs) were performed according to the protocol described in Nissen and Yamamoto (2000). Antibodies used were described recently (anti-STAT1C [Kovarik et al., 1998], anti-STAT2 [Park et al., 2000]), purchased from Santa Cruz (Santa Cruz, CA) and used at a 1:20 dilution (anti-NF-κB p65, anti-NF-κB p50, anti-RNA Pol II, anti-CDK7, and anti-p62-TFIIH) purchased from Bethyl (Montogomery, TX) and used in a dilution of 1:100 (anti-pS5 CTD Pol II), purchased from Abcam (Cambridgeshire, UK) and used in a dilution of 1:100 (anti-histone 3 and anti-TBP), or purchased from Upstate and used in a dilution of 1:100 (anti-acetyl histone 4). ChIP data were normalized to input and, in case of histone acetylation, further normalized to total H3 and to the untreated sample to correct for histone eviction. In the re-ChIP experiments, the immunecomplexes were eluted by adding 10 mM DTT and incubation for 30 min at 37°C. The samples were diluted 40-fold in RIPA-buffer and reimmunoprecipitated.

Primers used for PCR and q-PCR of the *Nos2* promoter were as follows: iNOS dis fwd 5′-CCAACTATTGAGGCCACACAC-3′; iNOS dis rev 5′-GCT TCCAATAAAGCATTCACA-3′; iNOS prox fwd 5′-GTCCCAGTTTTGAAGTG ACTACG-3′; iNOS prox rev 5′-GTTGTGACCCTGGCAGCAG-3′; Mx2 prox fwd 5′-ACCCAGCCAAGGCCCCCTTA-3′; Mx2 prox rev 5′-GCAGCTGCCAG GGCTCAGAC; IκBα prox fwd 3′-GGACCCCAAACCAAAATCG-5′; IκBα prox rev 3′-TCAGGCGCGGGGAATTTCC-5′.

Microarray Analysis

Macrophages were infected with an overnight culture of *L. monocytogenes* for 8 hr (MOI10) or treated 4 hr with IFN-β. RNA was extracted with Trizol and QIAGEN RNeasy Kit according to the manufacturers' protocol. 1 μg of RNA per sample was used for cDNA synthesis. cDNA syntheses and array-hybridizations were performed according to the manufacturers' protocol (Amersham-BioSciences; GE Healthcare).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.immuni.2010.07.001.

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REFERENCES

Adelman, K., Kennedy, M.A., Nechaev, S., Gilchrist, D.A., Muse, G.W., Chinenov, Y., and Rogatsky, I. (2009). Immediate mediators of the inflammatory response are poised for gene activation through RNA polymerase II stalling. Proc. Natl. Acad. Sci. USA *106*, 18207–18212.

Baccarini, M., Bistoni, F., and Lohmann Matthes, M.L. (1985). In vitro natural cell-mediated cytotoxicity against *Candida albicans*: Macrophage precursors as effector cells. J. Immunol. *134*, 2658–2665.

Barbalat, R., Lau, L., Locksley, R.M., and Barton, G.M. (2009). Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat. Immunol. *10*, 1200–1207.

Bogdan, C. (2001). Nitric oxide and the immune response. Nat. Immunol. *2*, 907–916.

Chapman, R.D., Heidemann, M., Hintermair, C., and Eick, D. (2008). Molecular evolution of the RNA polymerase II CTD. Trends Genet. *24*, 289–296.

Chen, L.F., and Greene, W.C. (2004). Shaping the nuclear action of NF-kappaB. Nat. Rev. Mol. Cell Biol. *5*, 392–401.

Darnell, J.E., Jr. (1997). STATs and gene regulation. Science *277*, 1630–1635.

Dempsey, P.W., Doyle, S.E., He, J.Q., and Cheng, G. (2003). The signaling adaptors and pathways activated by TNF superfamily. Cytokine Growth Factor Rev. *14*, 193–209.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R., and Cheng, G. (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. Immunity *17*, 251–263.

Durbin, J.E., Hackenmiller, R., Simon, M.C., and Levy, D.E. (1996). Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell *84*, 443–450.

Edelson, B.T., and Unanue, E.R. (2002). MyD88-dependent but Toll-like Receptor 2-independent innate immunity to *Listeria*: No role for either in macrophage listericidal activity. J. Immunol. *169*, 3869–3875.

Egly, J.M. (2001). The 14th Datta Lecture. TFIIH: From transcription to clinic. FEBS Lett. *498*, 124–128.

Gao, J.J., Filla, M.B., Fultz, M.J., Vogel, S.N., Russell, S.W., and Murphy, W.J. (1998). Autocrine/paracrine IFN-alphabeta mediates the lipopolysaccharide-induced activation of transcription factor Stat1alpha in mouse macrophages: pivotal role of Stat1alpha in induction of the inducible nitric oxide synthase gene. J. Immunol. *161*, 4803–4810.

Greten, F.R., Arkan, M.C., Bollrath, J., Hsu, L.C., Goode, J., Miething, C., Goktuna, S.I., Neuenhahn, M., Fierer, J., Paxian, S., et al. (2007). NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. Cell *130*, 918–931.

Harada, H., Matsumoto, M., Sato, M., Kashiwazaki, Y., Kimura, T., Kitagawa, M., Yokochi, T., Tan, R.S., Takasugi, T., Kadokawa, Y., et al. (1996). Regulation of IFN-alpha/beta genes: evidence for a dual function of the transcription factor complex ISGF3 in the production and action of IFN-alpha/beta. Genes Cells *1*, 995–1005.

Herskovits, A.A., Auerbuch, V., and Portnoy, D.A. (2007). Bacterial ligands generated in a phagosome are targets of the cytosolic innate immune system. PLoS Pathog. *3*, e51.

Hirose, Y., and Ohkuma, Y. (2007). Phosphorylation of the C-terminal domain of RNA polymerase II plays central roles in the integrated events of eucaryotic gene expression. J. Biochem. *141*, 601–608.

Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., and Taniguchi, T. (2005). IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature *434*, 772–777.

Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat. Immunol. *9*, 361–368.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S.I., Kimura, T., Green, S.J., et al. (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science *263*, 1612–1615.

Kim, Y.K., Bourgeois, C.F., Pearson, R., Tyagi, M., West, M.J., Wong, J., Wu, S.Y., Chiang, C.M., and Karn, J. (2006). Recruitment of TFIIH to the HIV LTR is a rate-limiting step in the emergence of HIV from latency. EMBO J. *25*, 3596–3604.

Kleinert, H., Schwarz, P.M., and Forstermann, U. (2003). Regulation of the expression of inducible nitric oxide synthase. Biol. Chem. *384*, 1343–1364.

Koch, F., Jourquin, F., Ferrier, P., and Andrau, J.C. (2008). Genome-wide RNA polymerase II: Not genes only! Trends Biochem. Sci. *33*, 265–273.

Kovarik, P., Stoiber, D., Novy, M., and Decker, T. (1998). Stat1 combines signals derived from IFN-gamma and LPS receptors during macrophage activation. EMBO J. *17*, 3660–3668.

Lau, J.F., Nusinzon, I., Burakov, D., Freedman, L.P., and Horvath, C.M. (2003). Role of metazoan mediator proteins in interferon-responsive transcription. Mol. Cell. Biol. *23*, 620–628.

Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., and Murphy, W.J. (1993). Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proc. Natl. Acad. Sci. USA *90*, 9730–9734.

Margaritis, T., and Holstege, F.C. (2008). Poised RNA polymerase II gives pause for thought. Cell *133*, 581–584.

Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., et al. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell *84*, 431–442.

Muller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. Science *264*, 1918–1921.

Nissen, R.M., and Yamamoto, K.R. (2000). The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev. *14*, 2314–2329.

Nusinzon, I., and Horvath, C.M. (2005). Histone deacetylases as transcriptional activators? Role reversal in inducible gene regulation. Sci. STKE *2005*, re11.

Pandey, A.K., Yang, Y., Jiang, Z., Fortune, S.M., Coulombe, F., Behr, M.A., Fitzgerald, K.A., Sassetti, C.M., and Kelliher, M.A. (2009). NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to *Mycobacterium tuberculosis*. PLoS Pathog. *5*, e1000500.

Parekh, B.S., and Maniatis, T. (1999). Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter. Mol. Cell *3*, 125–129.

Park, C., Li, S., Cha, E., and Schindler, C. (2000). Immune response in Stat2 knockout mice. Immunity *13*, 795–804.

Paulson, M., Press, C., Smith, E., Tanese, N., and Levy, D.E. (2002). IFN-Stimulated transcription through a TBP-free acetyltransferase complex escapes viral shutoff. Nat. Cell Biol. *4*, 140–147.

Plumlee, C.R., Lee, C., Beg, A.A., Decker, T., Shuman, H.A., and Schindler, C. (2009). Interferons direct an effective innate response to *Legionella pneumophila* infection. J. Biol. Chem. *284*, 30058–30066.

Ramsauer, K., Farlik, M., Zupkovitz, G., Seiser, C., Kroger, A., Hauser, H., and Decker, T. (2007). Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-gamma-inducible gbp2 gene. Proc. Natl. Acad. Sci. USA *104*, 2849–2854.

Reis, L.F., Ruffner, H., Stark, G., Aguet, M., and Weissmann, C. (1994). Mice devoid of interferon regulatory factor 1 (IRF-1) show normal expression of type I interferon genes. EMBO J. *13*, 4798–4806.

Roeder, R.G. (1996). The role of general initiation factors in transcription by RNA polymerase II. Trends Biochem. Sci. *21*, 327–335.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity *13*, 539–548.

Schindler, C., Levy, D.E., and Decker, T. (2007). JAK-STAT signaling: From interferons to cytokines. J. Biol. Chem. *282*, 20059–20063.

Spilianakis, C., Kretsovali, A., Agalioti, T., Makatounakis, T., Thanos, D., and Papamatheakis, J. (2003). CIITA regulates transcription onset via Ser5-phosphorylation of RNA Pol II. EMBO J. *22*, 5125–5136.

Spink, J., and Evans, T. (1997). Binding of the transcription factor interferon regulatory factor 1 to the inducible nitric oxide synthase promoter. J. Biol. Chem. *272*, 24417–24425.

Stetson, D.B., and Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity *24*, 93–103.

Stockinger, S., Materna, T., Stoiber, D., Bayr, L., Steinborn, R., Kolbe, T., Unger, H., Chakraborty, T., Levy, D.E., Muller, M., and Decker, T. (2002). Production of type I IFN sensitizes macrophages to cell death induced by *Listeria monocytogenes*. J. Immunol. *169*, 6522–6529.

Stockinger, S., Reutterer, B., Schaljo, B., Schellack, C., Brunner, S., Materna, T., Yamamoto, M., Akira, S., Taniguchi, T., Murray, P.J., et al. (2004). IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. J. Immunol. *173*, 7416–7425.

Stockinger, S., Kastner, R., Kernbauer, E., Pilz, A., Westermayer, S., Reutterer, B., Soulat, D., Stengl, G., Vogl, C., Frenz, T., et al. (2009). Characterization of the interferon-producing cell in mice infected with *Listeria monocytogenes*. PLoS Pathog. *5*, e1000355.

Toshchakov, V., Jones, B.W., Perera, P.Y., Thomas, K., Cody, M.J., Zhang, S., Williams, B.R., Major, J., Hamilton, T.A., Fenton, M.J., and Vogel, S.N. (2002). TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat. Immunol. *3*, 392–398.

Varinou, L., Ramsauer, K., Karaghiosoff, M., Kolbe, T., Pfeffer, K., Muller, M., and Decker, T. (2003). Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity. Immunity *19*, 793–802.

Xie, Q.W., Whisnant, R., and Nathan, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. J. Exp. Med. *177*, 1779–1784.

Xie, Q.W., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J. Biol. Chem. *269*, 4705–4708.

Zhong, H., May, M.J., Jimi, E., and Ghosh, S. (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol. Cell *9*, 625–636.

Zwaferink, H., Stockinger, S., Reipert, S., and Decker, T. (2008). Stimulation of inducible nitric oxide synthase expression by beta interferon increases necrotic death of macrophages upon *Listeria monocytogenes* infection. Infect. Immun. *76*, 1649–1656.

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Supplemental Information

Nonconventional Initiation Complex Assembly by STAT and NF-κB Transcription Factors Regulates Host Defense Genes

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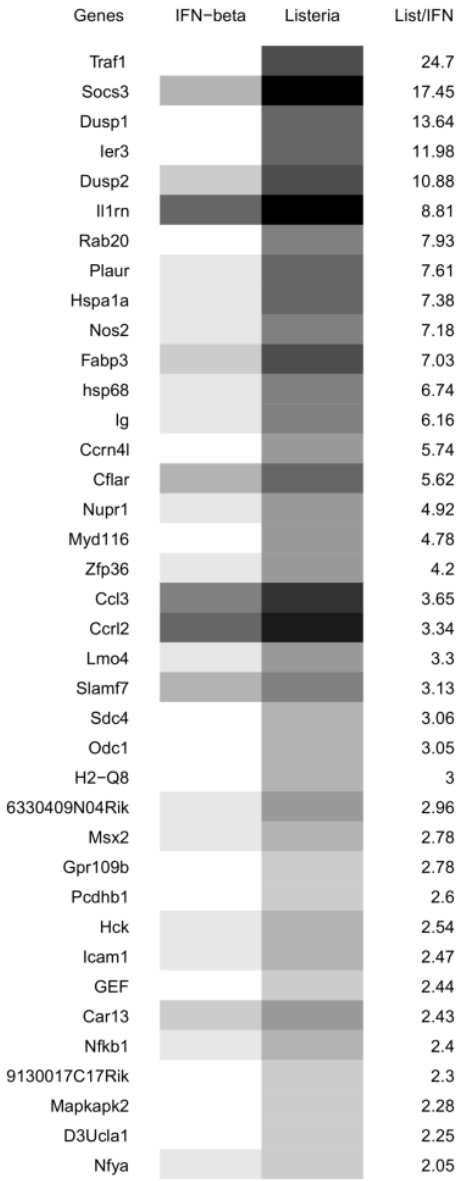

Figure S1. Microarray analysis of genes induced in macrophages by IFN-β treatment or infection with *Listeria monocytogenes*. Infection produces the IFN-β signal as well as additional signals from pattern recognition receptors. IFN-β -induced genes were further examined for increased expression in Listeria-infected cells. The 38 genes showing the highest increase of Listeria-induced expression over IFN-β treatment alone are shown. First column: gene symbol; second column: gray-scale indicating induction after IFN-β treatment; third column: gray-scale indicating induction after Listeria treatment; fourth column: ratio of induction after Listeria infection to induction after IFN-β treatment. The gray-scale intensity corresponds to values equally spaced on the log-scale from white (lowest induction of absolute ratio of 1.40) to black (highest induction of absolute ratio of 207.11).

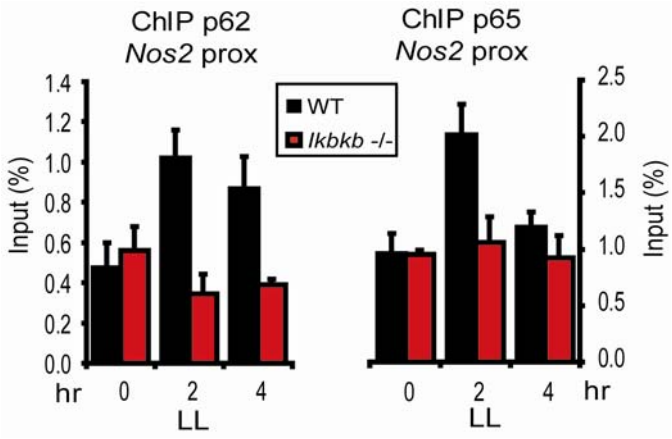

Figure S2. TFIIH-p62 remains at the *Nos2* promoter after binding of NF-κB-p65 decreases. Bone marrow-derived macrophages from wild-type mice (black bars) or *Rela*-/- mice (red bars) were infected with living *L. monocytogenes* (LL) for 4hr or 6hr. The cells were processed for ChIP using antibodies against TFIIH-p62 (left panel) or NF-κB-p65 (right panel). The precipitated DNA was analyzed by q-PCR with primers amplifying the proximal *Nos2* promoter region. Error bars represent standard deviations from triplicate samples. The experiment was repeated at least three times.

Additional experiments addressing the mechanism of gene induction in macrophages after infection with *Listeria monocytogenes*

iNOS and IL1ra mRNA expression underlies the same transcriptional initiation mechanism

Our microarray experiment revealed 37 genes resembling *Nos2* with regard to the stimulus requirements, for full transcriptional expression. To determine whether the mechanism characterized for *Nos2* gene regulation applies also to the additional genes identified in the microarray, mRNA expression profiles of DUSP1, DUSP2, and IL1ra were analyzed by qPCR. In keeping with the microarray, all genes displayed a synergistic enhancement of mRNA expression by hkL and IFNβ. Particularly the expression pattern of the gene encoding the IL1 receptor antagonist (Il1ra) was highly similar to that of *Nos2* (Figure 17A-C).

CDK7 and Pol II recruitment to the *Nos2* promoter was found to depend on hkL and IFNβ derived signals, respectively (Farlik et al., 2010). Based on these findings we wanted to know whether the mechanism observed at the *Nos2* promoter also applied at the IL1ra gene. In support of the findings with *Nos2*, ChIP assay analysis revealed that both CDK7 and Pol II recruitment to the *Il1ra* proximal promoter depended on hkL and IFNβ, respectively (Figure 17D,E). Furthermore, the priming effect on the transcriptional activation of the *Nos2* gene, caused by NFκB-mediated deposition of TFIIH-CDK7, also applied for the *Il1ra* gene (Figure 17F). The level achieved by IFNβ treatment of hkL pulsed cells exceeded the level achieved by IFNβ treatment of unpulsed cells for at least 8hrs. The transcriptional memory effect in case of *Il1ra* was less stable than with *Nos2*, where the hkL pulse enhanced IFNβ treatment for at least 24hrs (Farlik et al., 2010).

Taken together our results indicate, that the regulation of *Il1ra* and, possibly, other genes co-regulated by STAT1 and NFκB, follows the same mechanism as *Nos2* regulation, under our conditions, with some differences in the binding kinetics of the crucial components to the *Il1ra* promoter. hkL treatment causes the recruitment of CDK7-TFIIH to the *Nos2* promoter within 2hrs, whereas CDK7 binding at the *Il1ra* promoter was observed only after 4 hrs. Vice versa, Pol II recruitment to the *Il1ra* gene occurred earlier than binding to the *Nos2* promoter. Consistently, Pol II precedes the binding of CDK7 at the *Il1ra* promoter after cotreatment with IFNβ and hkL.

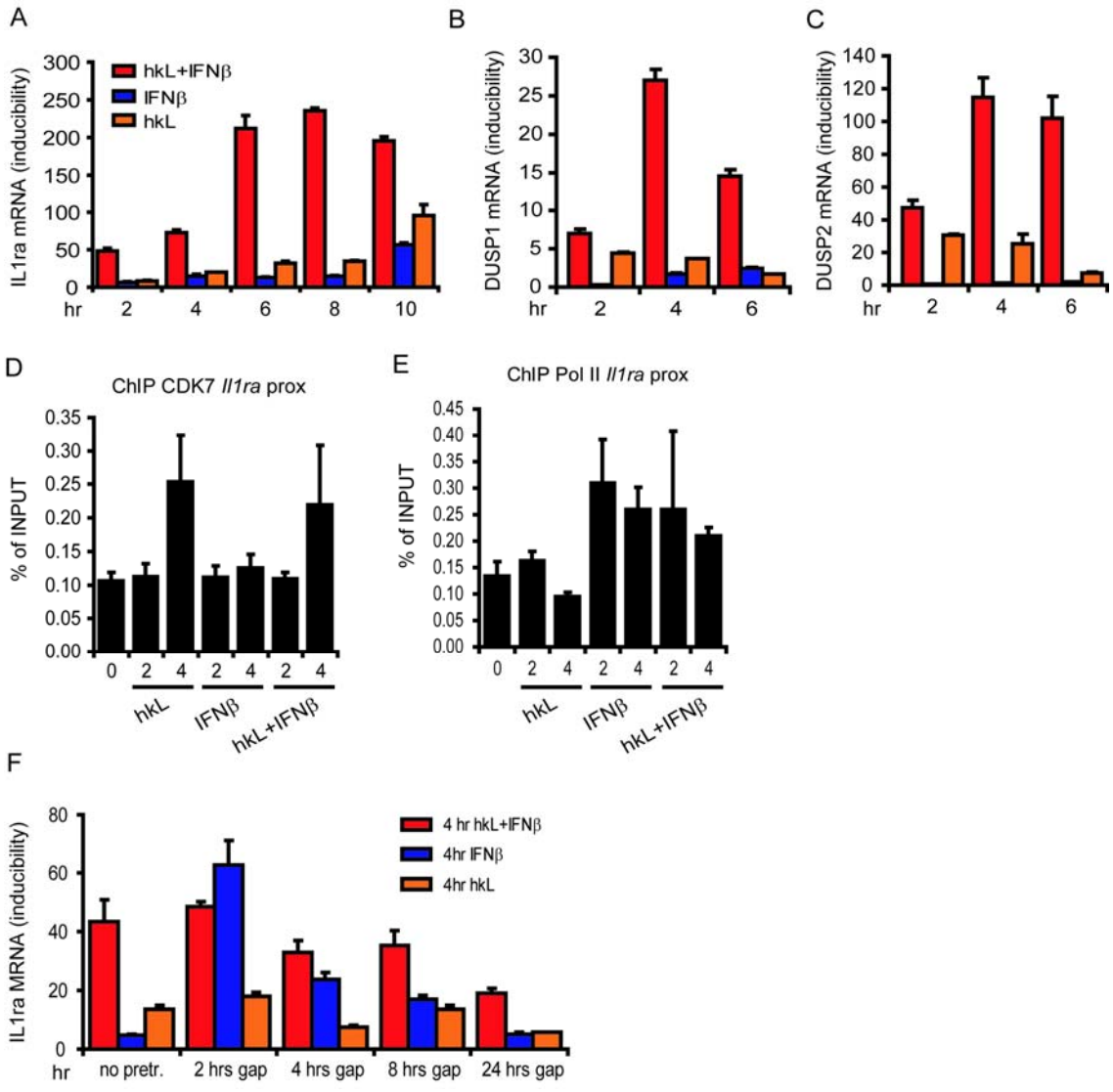

Figure 17: Gene regulation by heat-killed Listeria (hkL) and IFNb). (A-C). mRNA expression of IL1ra (A), DUSP1 (B), and DUSP2 (C) was determined by qPCR. (D,E) Bone marrow derived macrophages from WT mice were treated with hkL alone, IFNβ alone or a combination of both for the times indicated. Macrophages were processed for ChIP with antibodies against CDK7 (D), or Pol II (E). The precipitated DNA was analyzed by qPCR with primers amplifying the proximal promoter region of the *Il1ra* gene. (F) Analysis of *Il1ra* promoter priming by hkL. Bone marrow-derived macrophages were pretreated with hkL for 2 hr or left without pretreatment, followed by extensive washing of the cells. The cells were then left without treatment for different periods of time (indicated as hours gap). Thereafter cells were stimulated with hkL+IFNβ, IFN-β alone, or hkL alone for 4 hr. IL1ra mRNA expression was determined by q-PCR.

Recruitment of RNA POLII and ISGF3 to the Nos2 promoter, correlation with the H3K4me3 chromatin mark

Since type I IFN induced recruitment of ISGF3 to the distal *Nos2* gene promoter was shown to be required for subsequent RNA Pol II recruitment (Farlik et al., 2010), we addressed the question whether Pol II recruitment is mediated by a direct interaction between ISGF3 and Pol II. Therefore we performed detailed binding analysis of Pol II and ISGF3 by ChIP and compared their binding kinetics. IFNβ treatment resulted in the recruitment of Pol II enzyme within 2-3 hrs (Figure 18A). This finding was supported by the data generated with a ChIP against phosphorylated Ser5 at RNA Pol II CTD, which revealed small amounts of initiated Pol II enzyme at the proximal *Nos2* promoter after 2 hrs of treatment with hkL and IFNβ (Figure 18B). Differences in the output of the ChIPs monitoring RNA Pol II and phS5 Pol II CTD binding are likely to be due to different sensitivity of the respective antibodies, the latter being able to bind to more than one phosphorylated serine/epitope on the same Pol II molecule. Nevertheless, a contribution of NFκB, provided by the hkL co-treatment, resulting in faster recruitment of Pol II to the *Nos2* promoter, can at this point not be excluded. As expected, the binding of Pol II was delayed by about 2hrs after infection with *Listeria monocytogenes*, and appeared at the proximal *Nos2* promoter 4hrs after infection (Figure 18C).

Recruitment of Pol II after infection with *Listeria monocytogenes* appears to occur concomitant with the association of ISGF3 revealed by ChIP-reChIP analysis (compare Figure 18C and Figure 18D right panel), suggesting that Pol II might indeed be recruited via direct interaction with ISGF3. Of note, ISGF3 recruitment to the distal *Nos2* promoter after exposure to IFNβ takes place within 0,5 to 1 hr (Figure 18D left panel)(Farlik et al., 2010), and precedes Pol II recruitment by more than 1 hour (compare Figure 18D left panel and Figure 18 A and B). This finding suggests that Pol II requires the recruitment of a co-factor in order to bind the *Nos2* promoter. Furthermore, this result points to a contribution of NFκB in Pol II recruitment, since ISGF3 is able to recruit the Pol II enzyme faster when NFκB is active. NFκB is activated in response to hkL and recruited to the proximal *Nos2* promoter prior to Pol II recruitment after 1hr of treatment (Farlik et al., 2010).

The tri-methylation of H3K4 is a hallmark of active genes and a marker for recent transcriptional activity. It is closely correlated with the step of transcriptional initiation since the recruitment of the H3K4 methylase, SET1, has been shown, in yeast, to depend on the phosphorylation of Pol II CTD at Ser5 (Ng et al., 2003b). However, recent studies in mammalian cells indicate that H3K4me3 can occur independent of Pol II phosphorylation and can even serve to bind factors of the Pol II PIC as a possible prerequisite for Pol II binding (Vermeulen et al., 2007; Wang et al., 2009).

We wondered whether the recruitment of a H3K4 methylase to the *Nos2* promoter could be the rate-limiting step for efficient recruitment of RNA-Pol II. Therefore we monitored the H3K4me3 pattern by ChIP assay analysis after treatment of WT bone marrow derived macrophages with IFNβ alone and the combination of IFNβ and hkL. The H3K4me3 level was indeed increased upon treatment with IFNβ alone, but could be slightly enhanced upon co-treatment with hkL, a picture similar to the recruitment pattern observed for Pol II, which was similarly enhanced upon co-treatment with hkL (compare Figure 18E with 18A). Moreover, enrichment of H3K4me3 at the proximal *Nos2* promoter directly follows the recruitment of ISGF3 (compare Figure 18E with Figure 18D left panel). Additionally, we tested whether H3K4me3 is a feature unique for IFNβ treatment, or whether the proximal *Nos2* promoter is also enriched for the H3K4me3 mark in response to hkL treatment. Again we performed ChIP assay analysis after treatment of the cells for 4 hrs with hkL or IFNβ alone, or a combination of both. In all three tested situations the proximal *Nos2* promoter showed an enrichment of H3K4me3, with slightly enhanced values when both stimuli where present simultaneously (Figure 18F).

Together our results indicate that the ISGF3 mediated recruitment of a yet still unknown H3K4 methylase might be required for the subsequent, stable recruitment of RNA Pol II to the *Nos2* promoter. Further experiments adressing the mechanism behind Pol II recruitment and H3K4me3 introduction in reponse to IFNβ, need to be performed in future studies. Furthermore, our data suggest that in response to an infection with *Listeria monocytogenes,* NFκB can assist in the recruitment of Pol II by introducing the H3K4me3 mark prior to recruitment of ISGF3, thereby enabeling a faster recruitment of RNA Pol II. Also this hypothesis has to be strenghened by additional experiments to display the interdependency of Pol II and H3K4me3 on both ISGF3 and NFκB transcription factors. Still the existing data clearly indicate that H3K4me3 in bone marrow derived macrophages, is introduced to *Nos2* promoter chromatin independent of RNA Pol II phosphorylation on Ser5 as shown after treatment with IFNβ, and can even take place in the absence of RNA Pol II, after treatment with hkL alone.

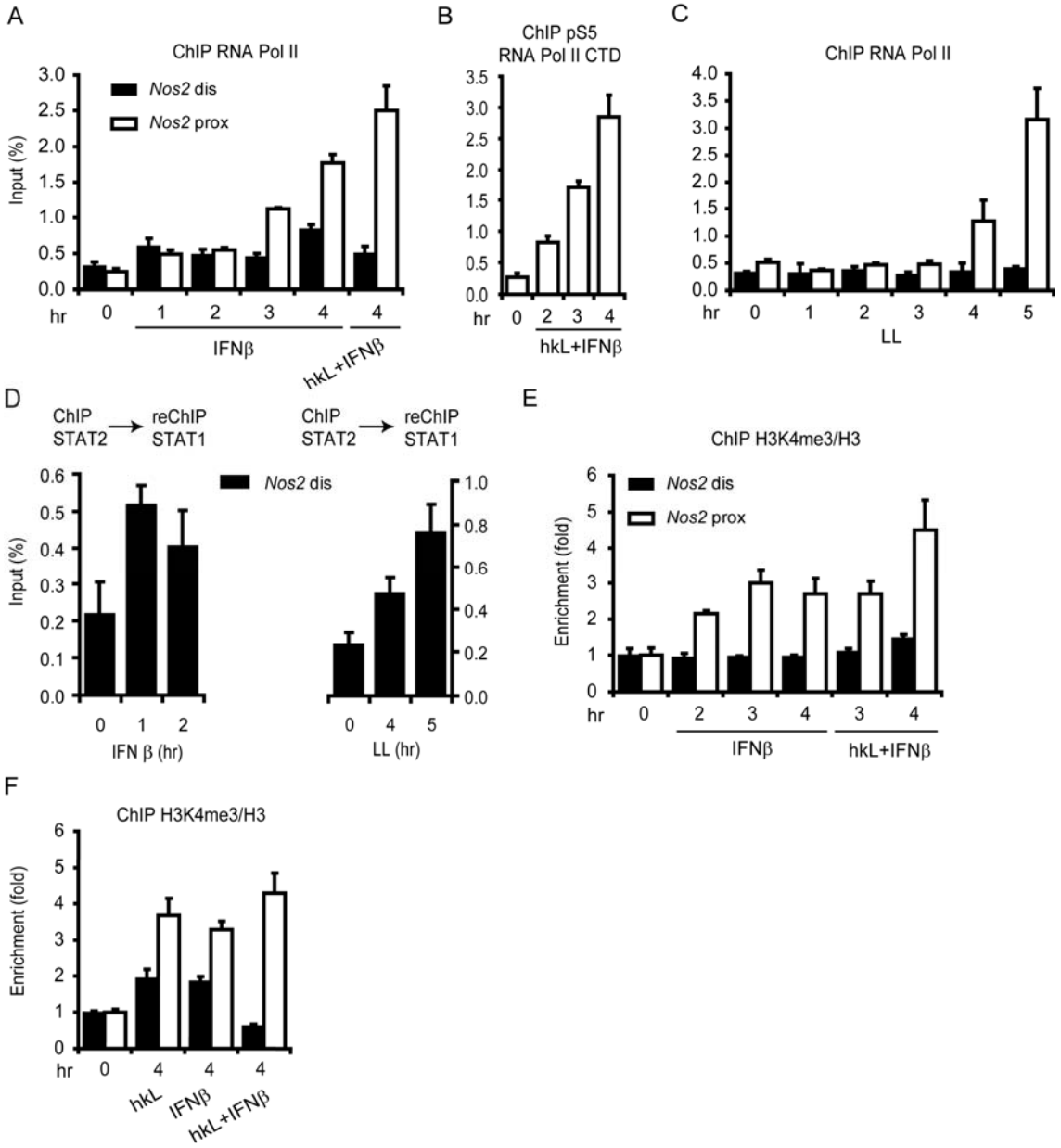

Figure 18: RNA PolII and ISGF3 recruitment to the *Nos2* promoter.. (A-F) Bone marrow derived macrophages were treated with IFNβ alone, heat-killed Listeria (hkL) alone or the combination of hkL and IFNβ for the times indicated (A,B,D,F). In panelsC and D) the cells were infected with living Listeria monocytogenes (LL). ChIP was performed with antibodies against Pol II (A,C), phosphoS5 RNA PolII CTD (B), and H3K4me3 (E,F). In panel D ChIP-reChIP was performed with antbodies against STAT1 and STAT2 as indicated . . The precipitates were amplified with primers flanking the distal or proximal *Nos2* promoter and analyzed by qPCR. Data are expressed as % of Input (A-D). Data of H3K4me3 ChIP (E, F) are normalized to total H3 to correct for histone eviction and expressed as relative enrichment compared to untreated cells.

CDK7 and CDK9 recruitment to the Nos2 promoter, link to the H3S10phK14ac chromatin mark

Based on the recent findings in Farlik et al. 2010 and Figure 19A,B showing that CDK7/TFIIH recruitment to the *Nos2* promoter and phosphorylation of the pol II CTD at S5 requires the presence of NFκB, we next asked the question whether NFκB is necessary also for the recruitment of CDK9/p-TEFb to the *Nos2* promoter. CDK9 activity is required for the phosphorylation of RNA Pol II at Ser2, which in turn leads to the release of the NELF mediated elongation block. To test this hypothesis, we performed ChIP assay analysis with extracts from WT bone marrow derived macrophages and monitored CDK9 recruitment after treatment with either hkL or IFNβ alone or a combination of both. As with CDK7 we observed a clear dependency of CDK9 recruitment on the presence of hkL, whereas IFNβ alone was not able to recruit detectable amounts of CDK9 to the *Nos2* promoter (Figure 19C). Thus, NFκB serves to recruit both CDK7/TFIIH and CDK9/p-TEFb to the proximal *Nos2* promoter.

Next we examined the molecular mechanism for CDK7/TFIIH retention at the *Nos2* gene promoter, a prerequisite for the maintainance of transcriptional memory (Farlik et al., 2010). Recent findings show that CDK9/p-TEFb recruitment can be mediated via BRD4 to the promoter of actively transcribed genes. BRD4 recruitment depends on the formation of a nucleosomal recognition code, comprised of H3K9acS10ph/H4K16ac (Zippo et al., 2009). This report further highlights that H4K16ac, required for BRD4 association, is mediated by the recruitment of the HAT MOF, which itself is bound to 14-3-3 proteins. 14-3-3 proteins were shown to recognize a phosphoacetylated motif on histone H3 either including Ser10 or Ser28 (Winter et al., 2008; Zippo et al., 2009).

Since our data clearly demonstrate that CDK9 recruitment follows the same rules as CDK7 recruitment in our system, we asked the question whether the same or a similar mechanism as the one described by Zippo et al. applies to CDK7/TFIIH recruitment and positioning in the context of iNOS regulation. At least some NFκB-responsive genes rely on the activity of IKKα as a H3S10 kinase (Yamamoto et al., 2003). To test whether the histones at the *Nos2* promoter are enriched for the phosphoacetylation motiv H3S10phK14ac, we performed ChIP assays with a phosphoacetylation specific antibody. Surprisingly, we could detect an enrichment for this mark predominantly at the distal promoter following treatment with hkL and IFNβ, displaying kinetics of enrichment similar to the recruitment of NFκB and ISGF3 under these conditions. By contrast only a marginal increase of this mark was detected at the proximal *Nos2* promoter, the site of NFκB recruitment (Figure 19D)(Farlik et al., 2010).

Since NFκB is recruited exclusively to the proximal promoter region, we hypothesized, that the distally bound ISGF3 complex might have an impact on the introduction of this mark. To test this, we examined the phosphoacetylation pattern after treatment with either hkL alone, IFNβ alone, or a combination of both. Interestingly, only hkL treatment, hence the NFkB signal, was able to enrich the distal *Nos2* promoter for the presence of the phosphoacetylation mark (Figure 19E). The hkL-mediated introduction of this mark to the distal promoter chromatin appears counterintuitive to both the NFkB site in the proximal promoter and the idea of CDK9 recruitment to the site of Pol II pausing. To further examine the pattern of H3S10phK14ac we asked whether the lack of signal-dependent increase results from a high constitutive presence of this mark at the proximal promoter. We therefore normalized the data of the uninduced sample for the H3S10phK14ac mark at the proximal *Nos2* promoter to the data generated for the distal promoter. In line with our hypothesis, we detected an up to seven fold enrichment of the mark at the proximal promoter, compared to the distal region (Figure 19F).

The up to seven fold difference in the uninduced sample suggests that even after the signal-dependent enrichment of H3S10phK14ac in the distal region, absolute levels of this mark still remain higher at the proximal *Nos2* promoter. Since the enrichment of the H3S10phK14ac mark in the distal region is dependent on signalling by hkL, a possible explanation for the observed increase at the distal region could be provided by hkL-dependent reorganization of nucleosomes. In line with this assumption, an alternative mechanistic explanation to the link between H3S10phK14ac, the binding of 14-3-3 proteins and the recruitment of HATs like MOF (Zippo et al.), is provided by the group of James Davie (Drobic et al., 2010). These authors linked H3S10ph-mediated 14-3-3 binding at the promoters of PRGs to the recruitment of BRG1. BRG1 mediated mobilisation of nucleosomes has been shown in many recent studies to be a feature of ISGs, necessary for long range interactions between the promoter and enhancer regions (Zhang and Boothby, 2006; Ni and Bremner, 2007; Ni et al., 2008). To test whether chromatin remodeling and the sliding of nucleosomes from the proximal to the distal promoter could be an explanation for the enriched H3S10phK14ac mark at the distal promoter, we analyzed H3 levels after treatment with hkL by ChIP. Interestingly, both promoter regions showed a strong reduction of total H3, very early after stimulation, in both regions. However, the drop in H3 levels at the proximal promoter has been reproducibly shown to remain low throughout the stimulation with hkL, whereas the level of H3 at the distal region increases, concomitant with the binding of NFκB and the observed increase in H3S10phK14ac at this region of the *Nos2* promoter (compare black bars in Figure 19G with black bars in Figure 19D).

Taken together these results show that both CDK7/TFIIH and CDK9/p-TEFb are recruited in response to hkL, independent of the recruitment of Pol II. The H3S10phK14ac mark was shown to be present already in untreated cells at the proximal region, but increases dependent on hkL derived signals at the distal region of the *Nos2* gene promoter. Chromatin remodelling at the distal *Nos2* promoter region appears to follow the same pattern as the enrichment of distal H3S10phK14ac and proximal NFκB binding, pointing to a possible link between these events.

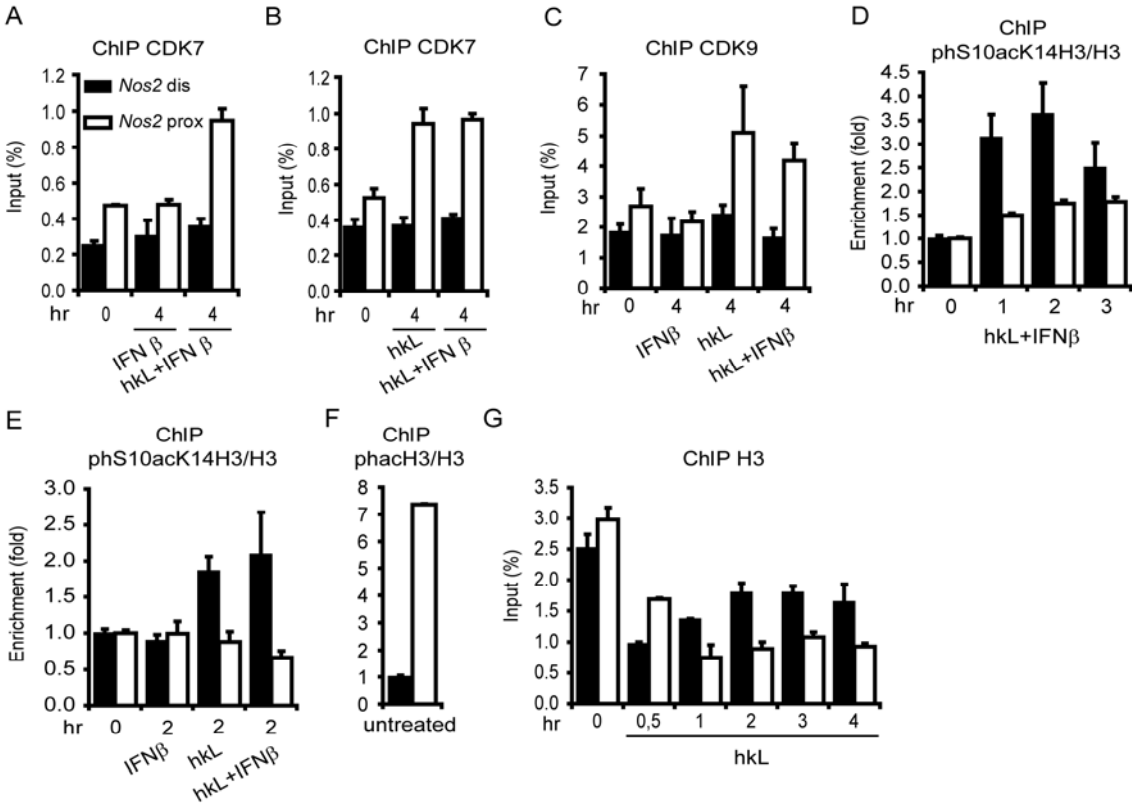

Figure 19: CDK7 and CDK9 recruitment to the *Nos2* promoter after treatment with IFNβ and/or hkL (heat killed *Listeria*). (A-G) WT bone marrow derived macrophages were treated with IFNβ alone, hkL alone or the combination of hkL and IFNβ for the times indicated. The cells were processed for ChIP (A-G) with antibodies against CDK7 (A,B), CDK9 (C), H3S10phK14ac (D-F), and H3 (G). The precipitates were amplified with primers flanking the distal or proximal *Nos2* promoter and analyzed by qPCR. Data are expressed as % of Input (A-C,G). Data of H3S10phK14ac ChIP are expressed as relative enrichment, compared to untreated cells and further normalized to total H3 to correct for histone eviction (D,E). The data obtained for the proximal promoter were further normalized to the values obtained for the distal *Nos2* promoter region to investigate the basal presence of the H3S10phK14ac mark (F).

NFκB-deficient macrophages retain IFN-β production and low levels of Stat1-dependent Nos2 expression during infection with Listeria monocytogenes

Recently we identified ISGF3 and NFκB as the main transcription factors involved in iNOS regulation upon infection with *Listeria monocytogenes* (Farlik et al., 2010). Detailed analysis of iNOS mRNA expression after infection revealed that iNOS mRNA expression in bone marrow derived macrophages isolated from *Stat1 -/-* mice was hardly detectable. In contrast, *Rela -/-* BMDMs retain some iNOS mRNA expression (Figure 20A). Since iNOS has been shown to rely on NFκB and ISGF3 activity, the remaining iNOS expression in *Rela -/-* macrophages might be due to type I IFN signaling resulting from endogenous production of IFNβ upon infection with *Listeria monocytogenes*. Intriguingly, expression of IFNβ has been shown to depend on the formation of an enhanceosome comprised of AP1, IRF3, IRF7, and NFκB (Panne et al., 2007). To test our hypothesis we monitored IFNβ mRNA expression in *L. monocytogenes* infected WT, *Stat1 -/-*, and *Rela -/-* BMDMs by qPCR analysis. Unexpectedly, we could detect IFNβ mRNA expression in *Rela-/-* macrophages that appears to be even higher than in WT or *Stat1-/-* macrophages (Figure 20B). This suggests that NFkB-independent production of type I IFNs and subsequent ISGF3 activation are responsible for the residual *Nos2* expression in infected macrophages.

While NFκB is the major signal for *Nos2* induction stimulated by hkL through pattern recognition receptors (Farlik et al., 2010), additional transcription factors may contribute to the regulation of iNOS expression. One example is HIF-1α (hypoxia induced factor) that regulates iNOS expression in response to hypoxic stress in concert with NFκB (Lu et al., 2006). The group of Samuel Leibovich recently demonstrated a function for HIF-1α downstream of TLR-4 signaling (Ramanathan et al., 2009). Since NFκB activation in the early phase of *Listeria* infection is TLR-dependent we examined HIF-1α expression upon treament with hkL or infection with living *Listeria*. qPCR analysis of macrophages treated with hkL and IFNβ, hkL alone, LPS and IFNγ or infected with living *Listeria* revealed that HIF-1α is indeed expressed in all situations albeit at low levels compared to antimicrobial genes such as iNOS or IFNβ (Figure 20C).

Additionally to NFκB, iNOS expression depends on the activation of STATs and the subsequent formation of the ISGF3 complex. Crucial for STAT1 activation is the phosphorylation on Y701, although there is an increasing amount of evidence that non-tyrosine phosphorylated STAT1 still exerts some functions (Cheon and Stark, 2009). To test whether the phosphorylation at Y701 of STAT1 is required for iNOS mRNA expression, macrophages were obtained from mice expressing a STAT1Y701F mutant. qPCR analysis of iNOS mRNA from infected macrophages shows that iNOS mRNA expression in STAT1Y701F macrophages is reduced to the level found in *Stat1 -/-* macrophages after 6hrs of infection.

Together our results indicate that the expression of IFNβ mRNA, after infection with *Listeria monocytogenes* does not require the presence of the p65 subunit of NFκB. Moreover we could show that HIF-1α is expressed under infection conditions as well as in response to hkL and IFNβ. Whether HIF-1α plays a role in the regulation of iNOS mRNA expression remains to be determined. Analysis of the STAT1Y701F mutant reveals that STAT1 phosphorylation at Y701 is required for ISGF3 mediated iNOS expression in the early phase of infection.

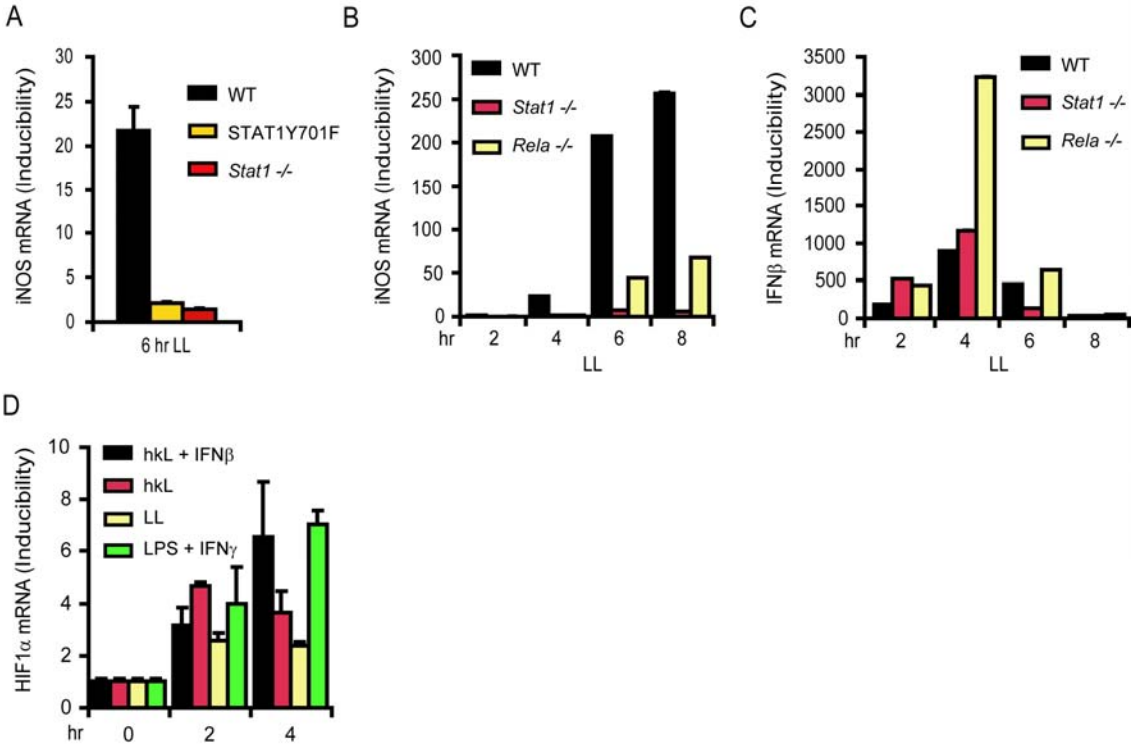

Figure 20: Kinetics of iNOS, IFNβ and HIF1α mRNA expression, after infection with *Listeria monocytogenes*, determined by qPCR analysis. Bone marrow derived macrophages from WT, STAT1Y701F, *Stat1* -/- (A) WT, *Stat1* -/-, and *Rela* -/- (B,C) and WT (D) mice, were infected with LL (living *Listeria*) (A-D), or treated with hkL (heat killed *Listeria*), a combination of hkL and IFNβ, or a combination of LPS and IFNγ (D) for the times indicated. iNOS (A,B), IFNβ (C), and HIF1α (D) mRNA expression was determined by q-PCR after normalization to GAPDH levels.

Discussion

The induction of gene expression is a complex process involving transcription factor recruitment and the introduction of chromatin modifications which together orchestrate the recruitment of a transcription-competent RNA Pol II enzyme. PRGs rely mainly on one stimulus and class I transcription factors for the regulation of their expression. However, beside the de novo recruitment of transcription factors in response to a certain stimulus, a hallmark of PRGs is the initial presence of factors like SP2 and chromatin marks, like H3K4me3, which render the promoter poised for activation, prior to stimulation. Moreover, RNA Pol II itself is present in a paused state at the promoters of most PRGs, ahead of stimulation, leading to basal gene expression and the generation of unmature transcripts. The stimulus-dependent regulation of PRGs lies in the transcription factor mediated recruitment of P-TEFb, which is required to release RNA Pol II from the NELF mediated elongation block, and to induce Ser2 phosphorylation-dependent maturation of the mRNA (Medzhitov and Horng, 2009).

Until recently, it was thought that the need for CDK7-TFIIH mediated initiation, and the requirement of CDK9-P-TEFb for the release from the NELF mediated elongation block, resulted from the monitoring function of these two checkpoints of transcription for the recruitment of capping enzymes and factors of the spliceosome to the nascent transcript. Taken together our results and the results from the groups of Papamatheakis (Spilianakis et al., 2003) and Medzhitov (Hargreaves et al., 2009) indicate that CDK7-TFIIH and/or CDK9 P-TEFb recruitment additionally serve to restrict and actively regulate the onset of productive transcription.

Here we have shown that at least for some SRGs not only the recruitment of RNA Pol II is a limiting step to induce gene expression, but also the recruitment of co-factors follows strict rules and results from the division of labor between different transcription factors. According to our findings this hypothesis is valid for transcription factors that are activated within a single pathway, such as STAT1 and IRF1, but also those activated through different signaling pathways, like NFκB and ISGF3. Collectively these proteins converge at the promoters of a subset of infection-induced genes to introduce active chromatin, to assemble the PIC – Pol II complex, and engage the Pol II enzyme in active transcription.

Using IFNγ as a potent inducer of ISGs we could show that the formation of active chromatin was largly mediated by the promoter presence of STAT1, which in turn proved responsible for the recruitment of CPB/p300 to the *Gbp2* promoter. However, STAT binding was not sufficient to induce gene expression, due to it's failure to recruit RNA-Pol II. Interestingly, IRF1, which has been shown in numerous studies to act in concert with STAT1 to induce IFNγ target gene expression, was found to be able to recruit Pol II even in the absence of STAT1 (Ramsauer et al., 2007). Addressing the role of IRFs in the regulation of the IFNγ-driven *Gbp2* gene, we uncovered a new role for IRF7 in the regulation of IFNγ-responsive, ISRE-containing genes. Our finding that IRF7 participates in IFNγ signalling is consistent with recent findings that IRF7 expression is detected after IFNγ administration to various cells of different organisms, ranging from a monocyte/macrophage cell line of rainbow trouts, to murine astrocytes and human fetal microglial cells or NIH3T3 cells (Holland et al., 2008; Saha et al., 2010).

We could reproducibly show that MEFs induce IRF7 expression in response to IFNγ, and identified a novel GAS in the 5'flanking region of the *Irf7* gene. The IRF7 GAS bound STAT1 after treatment with IFNγ, and was conserved in the human *Irf7* gene promoter. We were able to demonstrate that, due to the role of IRF7 as a class II transcription factor, late but not initial gene expression of IFNγ target genes in MEFs depended on the presence of IRF7. As expected, IRF7 activity, was confined to ISRE driven genes. Our data are supported by recent results from the group of Tenoever, showing that ISRE sites in the promoters of ISGs can be a targeted by IRF7 and induce an antiviral response in absence of ISGF3 (Schmid et al., 2010).

Both IRF7 and IRF1 were able to stimulate GBP2 expression individually when transfected into WT or *Stat1* -/- MEFs. The added value of having both present simultaneously lay in a synergistic induction of the *Gbp2* promoter. Unlike IRF1, IRF7 activity on the *Gbp2* promoter depended on the presence of the kinases TBK1 and/or IKKε. Absence of both kinases reduced expression of the ISRE-driven genes *Gbp2* and *Tap1* to an equal extent as the disruption of the *Irf7* gene. This led us to conclude that IRF7 needs to be phosphorylated to drive ISG expression. This notion was further supported by the finding that IRF7 was unable to stimulate the expression of the *Gbp2* promoter-luciferase construct in *Tbk1* and *Ikbke* double-deficient MEFs. Contrasting the data with *Gbp2*, SOCS1 expression was reduced in *Irf7* -/- MEFs but still fully responsive to IFNγ in *Tbk1/Ikbke* -/- MEFs. Hence, IRF7 presence is required for full expression of ISRE-driven genes in response to IFNγ, but its phosphorylation is required only for a subset of IFNγ-inducible genes. We speculate that IRF7 function at the promoters of ISRE driven genes may require phosphorylated IRF7 to recruit co-factors and/or to introduce activating chromatin marks. These prerequisites for transcriptional activation may be implemented at promoters of SOCS1-like genes independently of IRF7 recruitment, either by other IFN-regulated factors, or present ahead of stimulation. In support for the first hypothesis the *Socs1* promoter does contain a bonafide IRF binding site at an enhancer but no IRF binding site was found directly at the promoter. Therefore, one can conclude that promoter rearrangements leading to SOCS1 expression is likely to be regulated by promoter bound STAT1 homodimers, and the contribution of IRF7 might be to orchestrate events required to maintain and support productive trancription of SOCS1 mRNA.

Since treatment with IFNγ in *Stat1*-/- MEFs did not further enhance the transcriptional activity of IRF7 we were able to conclude that TBK1/IKKε are not activated downstream of the IFNGR. Concomitant with the finding that IRF7-mediated GBP2 expression was absent in *Tbk1/Ikbke* -/- MEFs, but present after transfection of IRF7 in *Stat1*-/- MEFs, which still express the two kinases, we were attracted to the idea that constitutive activity of TBK1/IKKε might suffice to bring about the necessary IRF7 phosphorylation. Support for this hypothesis was obtained by 2D PAGE analysis suggesting the presence of at least one IRF7 phosphoisoform in WT, but not in *Tbk1/Ikbke* -/- MEFs. This is, to our knowledge, the first report of a constitutive activity of the TBK1/IKKε kinase modul with a clear biological impact not only on the pattern recognition pathways through which the kinases are usually activated, but on an independent pathway. This adds a new line of understanding the mechanisms of crosstalk between pathways regulating innate immunity.

The IRF7 regulatory region contains eight serines as possible targets for TBK1/IKKε. Detailed functional analysis by the lab of David Levy revealed that a series of IRF7 phosphoisoforms is generated upon viral infection and the resulting activation of the two kinases (Caillaud et al., 2005). Since only one isoform could be detected in our system, we were interested to find the serine residue or the combination of serine residues phosphorylated by constitutively active TBK1/IKKε. Analysis of several different IRF7 mutants revealed that a combination of phosphorylated serines is required for IRF7 transcriptional activity on ISRE driven genes like *Gbp2*. Especially S425 - S426 and S437 - S438 proved essential in our system and were of particular interest. The serine to alanine mutation of both renders IRF7 inactive. An aspartate mutant of all serines of the regulatory domain except S425 and S426 was active in the absence of TBK/IKKε and this activity could be enhanced upon selective introduction of TBK1 to a greater extent than that of WT IRF7. In contrast, transfection of the same mutant into *Stat1* -/- MEFs expressing both TBK1 and IKKε resulted in GBP2 expression levels comparable to those initiated by WT IRF7. This phenomenon can best be explained by a negative effect of IKKε in *Stat1* -/- MEFs, due to either competition for required adaptor proteins, as proposed by the group of John Hiscott (Paz et al., 2009), or by a direct inhibitory phosphorylation of IRF7 by IKKε (Caillaud et al., 2005). In favour of the latter assumption, Ser425 was shown to be phosphorylated in vitro by IKKε.

Cotransfection of IKKε together with TBK1 clearly established a repressive effect of IKKε on the transcriptional activity of IRF7. This effect was observed with every mutant analysed, including the phosphomimetic M15 mutant mentioned above. This indicates that IKKε indeed serves to exert negative control over the transcriptional activation of IRF7 by TBK1. This interpretation is in line with our observation that the inducibility of the *Gbp2* reporter was generally lower in *Stat1 -/-* MEFs than in *Tbk1/Ikbke -/-* MEFs (compare Figures 15B,D with Figures 15 C,E). In accordance with the data shown in Caillaud et al., our result that the inherent ability of the M15 mutant, which has all serines, except S425 and S426, mutated to aspartate, to activate the *Gbp2* promoter was suppressed by IKKε in absence of TBK1, suggests that the antagonistic effect of IKKε is due to phosphorylation of Ser425 rather than competition for binding sites in a signaling complex. For the first time our report clearly demonstrates a non-redundant, antagonistic role of IKKε on TBK1, which is required to limit the effect of TBK1 on IRF7 activation and possibly directs IRF7 specificity towards target gene recognition. Whether this antagonistic effect still occurs when TBK1 and IKKε are activated by PAMP signalling will be subject to future studies.

IRF1 is expressed very early in the response to IFNγ and binds the *Gbp2* promoter within 1 hour after the onset of signaling. Compared to IRF1, IRF7 binding kinetics to the *Gbp2* promoter confirmed a function later in GBP2 expression, since IRF1 precedes IRF7 at the *Gbp2* promoter. This is in line with the effects on *Gbp2* mRNA expression observed in *Irf7 -/-* MEFs. The difference in recruitment kinetics suggests that IRF1 binds the *Gbp2* promoter independent of IRF7. Similar to IRF1, IRF7 binds both the distal and proximal *Gbp2* promoter. Sequential ChIP analysis revealed that the promoter regions are simultaneously occupied by both IRF1 and IRF7, which therefore have to share the same ISRE site. The transfection experiments show that IRF7 can regulate GBP2 expression independently of IRF1, which suggests that IRF7 binding to the *Gbp2* promoter does not require previous promoter association by IRF1. Surprisingly, IRF7 leaves the proximal *Gbp2* promoter after 4 hrs of IFNγ treatment, whereas it remains bound at the distal region, indicating that IRF7 presence at the proximal promoter is not required for the maintenance of *Gbp2* transcription.

This sequential mode of transcription factor assembly at the *Gbp2* promoter, starting with STAT1, which introduces H4 acetylation by the recruitment of CBP/p300, followed by IRF1, which could be linked to the recruitment of RNA Pol II, and finally IRF7, gives rise to a number of questions addressing the function of IRF7 at the promoter of IFNγ target genes. One possible function for IRF7 would be to assist IRF1 in the subsequent recruitment of Pol II, to enable sustained target gene expression. Whether, this results from direct association of IRF7 with RNA Pol II, leading to the recruitment of more RNA Pol II, or from the recruitment of GTFs, such as TFIID, is subject to ongoing experiments. A further possible function for IRF7 could be the introduction of activating chromatin marks, which in turn would lead to the stable association of GTFs or the RNA Pol II itself. Finally, IRF7 might mediate the recruitment of factors needed to efficiently engage RNA Pol II into active transcription. In this situation CDK7-TFIIH or CDK9-P-TEFb, which are both required to generate mature, full length transcripts would be potential IRF7 targets. Whether STAT1, IRF1 or IRF7 is the factor responsible for CDK7-TFIIH recruitment remains to be solved.

Which of the above mentioned possibilities resembles the actual situation at the *Gbp2* promoter will be revealed by future experiments. Interesting to note at this point is that the input of IRF7 to transcriptional initiation of the *Gbp2* promoter, but not its nuclear translocation or the recruitment to target promoters, requires TBK1-mediated phosphorylation. This is demonstrated by the TBK1-independent effect of IRF7 on SOCS1 expression and points to a difference in the mode of transcriptional activation by IRF7 when comparing the *Gbp2* or *Tap1* promoters with the *Socs1* promoter.

In addition to these mechanistic statements our data allow some conclusions about the consequences of IFNγ-induced IRF7 expression and its role in the regulation of IFNγ–responsive genes. Most cells in an organism do not express IRF7 constitutively, consistent with its classification as a class II transcription factor. A notable exception from this rule is a subset of DCs, pDCs (plasmacytoid dendritc cells), often refered to as IPCs (interferon producing cells) due to their capability to produce high amounts of type I IFNs very soon after their exposure to a pathogen. A hallmark of pDCs, which is linked to their ability to produce type I IFNs very rapidly, is the constitutive expression of IRF7, the 'master regulator' of type I IFN production. Since type I IFNs are mainly produced in response to intracellular pathogens, pDCs play a fundamental role in the defence against various viral infections (Honda et al., 2005; McCullough et al., 2009). Our results, together with recent work by others, indicates that IRF7 is expressed in a variety of cell types upon IFNγ treatment, and may participate in the induction of an antiviral state independently of type I IFN (Holland et al., 2008; Saha et al., 2010; Schmid et al., 2010). By elevating the basal levels of IRF7 in non-pDCs IFNγ may play a prominent role in enhancing the innate immune response against invading viral pathogens.

After IFNγ treatment STAT1 was shown to recruit CBP/p300 and to stimulate the hyperacetylation of histone 4 (H4). Likewise, the ISGF3 complex proved essential for the enrichment of acetylated histone H4 at the *Nos2* promoter in the context of a response to type I IFNs. Interestingly, increased acetylation was confined to distal *Nos2* promoter regions adjacent to the ISRE consensus, whereas acetylation of the proximal *Nos2* promoter required the binding of NFκB to its proximal consensus binding sequence. H4 acetylation at promoters closely correlates with transcriptional activation, and several H4 lysine residues, including K5/8/12 and 16, have been linked to the sequential binding of BRD4 and CDK9, which are required for the onset of transcription (Hargreaves et al., 2009; Zippo et al., 2009). Given that IFNβ-induced histone acetylation at the *Nos2* promoter is limited to the distal region, one can speculate that the absence of activating histone modifications at the proximal *Nos2* promoter might be responsible for the inability of ISGF3 to recruit CDK7-TFIIH and CDK9 - P-TEFb, to the site of RNA Pol II recruitment in order to induce transcription. In further support for this hypothesis, the binding of CDK9 was also shown to be dependent on hkL derived signals. Whether or not the binding and retention of CDK7 and CDK9 to the proximal *Nos2* promoter is mediated by BRD4 in the context of *Listeria monocytogenes* infection will be subject to future investigations.

Continuing on the idea of promoter proximal H4 acetylation as a prerequisite for CDK7 and CDK9 deposition, one has to ask why classical ISGs, like *Gbp2* or *Mx2* are expressed upon IFN treatment without the need for NFκB assistance in recruiting CDK7/9. One possible explanation is that the promoter of classical ISGs is preloaded with factors required for CDK7/9 recruitment and/or RNA Pol II itself. This notion is in line with the above-mentioned findings of the groups of Papamatheakis and Medzhitov (Spilianakis et al., 2003; Hargreaves et al., 2009). A second possibility is given by the idea that the structure and composition of the promoter itself, i.e. different or differently positioned transcription factor binding sites, determine whether a gene responds to STATs alone or whether it requires the information input from a second signalling pathway as shown for iNOS. Interesting to note, at this point, is that, in contrast to *Nos2*, STATs bind the promoters of most classical ISGs very close to the transcriptional start site. Even in the case of IFNγ-driven *Gbp2* and *Irf7* expression STAT1 was bound to ISRE sites close to the TSS, most likely as part of an unconventional, ISGF3-like complex formed by STAT1 homodimers together with IRF9 (Ramsauer et al., 2007). STAT1 binding at the *Gbp2* promoter was shown to be required for H4 acetylation but is not able to stimulate the recruitment of RNA Pol II.

In contrast to IFNγ driven *Gbp2* expression, type I IFN mediated ISGF3 activation and subsequent iNOS expression did not depend on the phosphorylation of STAT1 at Ser727. In case of IFNγ-induced *Gbp2* expression STAT1 Ser727 phosphorylation was required for the recruitment of CBP/p300. This finding suggests that the TAD of STAT2 might be used to facilitate contact to HATs, as described for GCN5 and PCAF (Paulson et al., 2002; Lau et al., 2003). Another difference between IFNγ and type I IFN mediated gene induction is the differential requirement for IRFs to initiate gene expression. IFNγ-regulated gene expression was found to rely on both IRF1 and IRF7, whereas *Nos2* expression after infection with *Listeria monocytogenes*, was unaltered in macrophages deficient for IRF1 or IRF7. This invites the speculation that STAT1 homodimers require serine 727 phosphorylation to contact HATs and need the assistance of IRF1 to recruit the RNA Pol II enzyme, whereas the TAD of STAT2, embedded in the ISGF3 complex, seems to perform both functions.

Despite the fact that ISGF3 fails to induce H4 acetylation at the proximal *Nos2* promoter, it efficiently introduces the H3K4me3 mark at the site of RNA Pol II recruitment, independent of the Pol II phosphorylation status. Together with the observation that ISGF3 is responsible for mediating the recruitment of TBP and RNA Pol II, this suggests that direct contact of the distally bound ISGF3 with the proximal *Nos2* promoter has to occur. This interaction might be enabled by DNA bending, a mechanism shown to contribute to the regulation of the *CIIta* promoter. Specifically, BRG1 dependent *CIIta* remodelling requires DNA bending to allow for long-range interactions in cis (Ni et al., 2008). Still the question remains, why the introduction of H3K4me3 at the proximal *Nos2* promoter is mediated by ISGF3, but H4 acetylation stays confined to the distal promoter. One possible explanation could be that HAT recruitment is directly mediated by ISGF3 and occurs very locally as shown for the *Ifnβ* promoter and the *Ifi56K* gene (Parekh and Maniatis, 1999), whereas the introduction of H3K4me3 at the proximal promoter might be a result of ISGF3 mediated H2Bub, which has been shown in recent reports to lead to RNA Pol II independent binding of SET domain containing proteins (Dover et al., 2002; Kim et al., 2009; Nakanishi et al., 2009). This mechanism would also explain the delayed kinetics of H3K4me3 enrichment compared to ISGF3 recruitment in response to IFNβ. Beside ISGF3, NFκB has also been shown to introduce the H3K4me3 mark after treatment with hkL alone, which therefore occurs completely independent of RNA Pol II recruitment. To our knowledge this is the first report demonstrating this in primary cells. Identifying the responsible H3K4 methylase at the *Nos2* promoter will be of immediate future interest, since the only report showing H3K4 tri-methylation independent of Pol II phosphorylation was performed in MEFs and resulted in the conclusion that this might be a feature unique for TATA less genes (Wang et al., 2009). Contrasting this notion, *Nos2* is clearly a TATA-containing gene. The consequences of H3K4me3 are of further interest since this mark has recently been found to be recognized by TAF proteins of the TFIID complex (Vermeulen et al., 2007). Whether or not the introduction of this mark can assist in, or is required for the deposition of TFIID and RNA Pol II at the *Nos2* promoter remains to be determined.

Phosphoacetylation of histone H3 at the iNOS promoter presents another task for future studies. Unexpectedly, the H3S10phK14ac mark was present at very high levels at the proximal *Nos2* promoter already before treatment and did not significantly increase upon treatment with hkL. IKKα has been shown in numerous studies to be able to serve as a H3S10 kinase in response to activation of the NFκB p50/p65 heterodimer (Anest et al., 2003; Yamamoto et al., 2003). However, we found the proximal NFκB site of the *Nos2* promoter to be occupied by p50 homodimers in unstimulated cells. p50 homodimers repress transcription by blocking the accessibility for the NFκB binding site and by the binding of HDAC repressor complexes. In more recent studies IKKα was shown to function not only as H3S10 kinase, but also to derepress genes from p50 homodimer–HDAC mediated repression (Hoberg et al., 2006; Gloire et al., 2007). The question whether or not basal H3S10 phosphorylation levels at the *Nos2* promoter are mediated by IKKα, and whether p50 homodimers play a role in targeting IKKα to the *Nos2* promoter in uninduced cells, to render the promoter poised for activation, will be addressed in future studies. Furthermore, recent data of the group of James Davie link the H3 phosphoacetylation mark to the association of 14-3-3 proteins, enabeling the binding of BRG1 to remodel chromatin (Drobic et al., 2010). Since hkL treatment induces rapid chromatin remodeling at the whole *Nos2* promoter, the same or a similar mechanism requiring BRG1 function might apply. A second possible function of this mark is provided by the recent finding that 14-3-3 proteins, after binding the phosphoacetylation mark, serve to bind HATs such as MOF, which in turn acetylate histone H4 at lysine 16. This mark can further be recognised by BRD4, which was shown to mediate CDK9 recruitment to gene promoters of PRGs (Zippo et al., 2009).

The recently identified mechanism of gene induction by NFκB shows that NFκB mediated CDK7-TFIIH recruitment and deposition is required to maintain transcriptional memory. Gene induction by NFκB alone did not take place due to its inability to recruit RNA-Pol II. Moreover, a group of interferon responsive genes was identified by microarray analysis, which was upregulated during infection with *Listeria monocytogenes* following an expression pattern similar to iNOS (Farlik et al., 2010). The question whether this mechanism is specific for the identified IFN responsive genes, or whether NFκB recruits and stably deposits CDK7 and CDK9 to NFκB target gene promoters at many genomic loci without rendering the genes transcriptionally active, will be examined using A ChIP-sequencing approach.

It is important at this point to note that the observed deposition of both CDK7 and CDK9 by NFκB independently of Pol II binding, is a novel finding that challenges the current view of gene regulation in many ways. Our data in (Farlik et al., 2010) show that this mechanism introduces transcriptional memory with a built-in integrator function that allows the separation of the NFκB and ISGF3 activating signals over time (Levy, 2010). CDK7 is required for transcriptional initiation and the recruitment of capping factors to the nascent transcript. On the other hand CDK9, and subsequent Ser2 phosphorylation at the RNA Pol II CTD, functions to recruit factors of the spliceosome and in the release of the NELF mediated elongation block, thereby enabeling RNA Pol II to proceed to productive elongation. Deposition of both, CDK7 and CDK9, now raises the question, whether the maturation of iNOS mRNA occurs faster when NFκB is active and recruited to the *Nos2* promoter before ISGF3. Comparing the expression of iNOS pre-mRNA transcripts and mature mRNA after infection will allow us to gain insight into the communication between NFκB and the factors governing the mRNA maturation process.

Several observations concerning the role of NFκB in *Listeria*-infected macrophages are worthy of mention. We identified the p65 subunit of NFκB as the most important factor in the regulation of *Nos2* gene expression downstream of hkL-induced signaling. This result was obtained by the use of mice after conditional deletion of p65. Reportedly, p65 is an essential component of the IFNβ enhanceosome (Panne et al., 2007), hence we expected to lose expression of IFNβ in bone marrow derived macrophages of these mice. Surprisingly, macrophages after infection with *Listeria monocytogenes* expressed significant amounts of iNOS mRNA even in the absence of exogenous IFNβ. Consistent with this, the *Nos2* expression remaining in p65-depleted macrophages resulted from residual production of endogenous IFNβ and ISGF3 activation. These findings support the conclusion that at least in context of *Listeria* infection, p65 is dispensable for a functional IFNβ enhanceosome. Although this finding contrasts the results of Panne et al., recent data from the labs of Ruslan Medzhitov and Amer Beg agree with the idea, that NFκB can be dispensible for IFNβ expression (Stetson and Medzhitov, 2006; Wang et al., 2010).

Separating *L. monocytogenes* signalling into signals provided by hkL and IFN-I has proven to be a successful experimental approach throughout this study and will be similarly useful to address some of the questions raised above. On the one hand, pretreatment by hkL for a prolonged period of time might allow us to study NFκB dependent effects on the expression and maturation of iNOS mRNA. On the other hand pretreatment with IFNβ before the addition of hkL will be helpful in determining whether IFNβ and its recruitment of ISGF3 to the *Nos2* promoter, is able to stably deposit RNA Pol II at the *Nos2* promoter, similar to what was shown for NFκB and CDK7/9. This poses an interesting problem because during viral infection IFNβ may impinge on tissue, surrounding an infected cell, prior to the second signal required for iNOS expression, hence a memory effect produced by Pol II deposition would be required. This mechanism would convert a SRG suddenly into a PRG, where *Nos2* mRNA is expressed as soon as the IFN-I-primed cell encounters a PAMP that activates NFκB and recruits CDK7 and CDK9 to the *Nos2* promoter. This scenario is highly reminiscent of the mode of transcriptional induction shown for PRGs after LPS treatment by the group of Medzhitov. In further agreement with the Medzhitov model, IFNβ restructures the *Nos2* promoter in a manner characteristic for LPS-induced PRGs, including the recruitment of Pol II and the presence of H3K4me3 (Hargreaves et al., 2009). IFNβ has also been shown to induce moderate levels of *Nos2* mRNA on its own. Whether this mRNA is mature or unprocessed due to the lack of CDK7 and CDK9 and therefore unstable, according to Hargreaves and Medzhitov another hallmark of PRGs, has yet to be deciphered.

In an entire organism, paracrine stimulation by IFNβ is very often a result of viral infections such as that of the airways by influenza. Elevated serum levels of IFNβ would therefore cause the proposed *Nos2* conversion to a "part-time PRG" in responding cells. The second signal, leading to NFκB activation, can be, but must not be provided by the same pathogen that caused the production of type I IFNs, and can also be the result of, for example, TNFα secretion and signaling in response to infections. As shown in numerous studies, secondary infections, which very often occur in the wake of influenza infection can cause severe damage in an organism due to an overreaction of the already highly active immune system (Jamieson et al., 2010). Part of this damage might be caused by the products of iNOS and/or similarly regulated genes.

Materials

Additional reagents not decribed in references Farlik et al (Farlik et al., 2010), or Ramsauer et al. (Ramsauer et al., 2007) were as follows:

Antibodies

Monoclonal antibody to H3K4me3 was purchased from Millipore (Billerica, MA, USA) (Cat. No.: 04-745) and used for ChIP in a dilution of 1:100.

Monoclonal antibody to H3S10phK14ac was purchased from Upstate Biotechnology (Lake Placid, NY, USA) and used for ChIP in a dilution of 1:100.

Monoclonal antibody to IFNAR1 was described recently (Sheehan et al., 2006) and used in a dilution of 1:1000

Polyclonal antibody specific for IRF7 was generated in the Lab by immunisation of a rabbit with a peptide containing aminoacids 207-452 of the IRF7 protein fused to GST and used for ChIP at a dilution of 1:20; and 1:100 for Western blot.

Secondary antibody for WB analyses by Odyssey Infrared Imaging System of Li-Cor Biosciences to mouse IgG (Cat. Nr. 610-132-121) and rabbit IgG (Cat. Nr. 611-132-122) were purchased by Rockland.

Primer

Mouse IL1ra - Forward: GCTCATTGCTGGGTACTTACAA
Reverse: CCAGACTTGGCACAAGACAGG
Mouse Dusp1 - Forward: GGATATGAAGCGTTTTCGGCT
Reverse: GGATTCTGCACTGTCAGGCA
Mouse Dusp2 - Forward: GATTCAGAGCCACCGGGTAC
Reverse: AGCTTCCCCCAGGAGTCAGT
Mouse IRF7 - Forward: CTGGAGCCATGGGTATGCA
Reverse: AAGCACAAGCCGAGACTGCT

Mouse IRF1 – Forward: CCGAAGACCTTATGAAGCTCTTTG
Reverse: GCAAGTATCCCTTGCCATCG

Mouse SOCS1 – Forward: ACTCCGTGACTACCTGAGTTCCTT
Reverse: GCATCTCACCCTCCACAACCACT
Mouse HIF1α – Forward: TCACCAGACAGAGCAGGAAA
Reverse: CTTGAAAAAGGGAGCCATCA
Mouse TAP1 – Forward: CTGGCAACCAGCTACGGGT
Reverse: TGAGAATGAGGATGTGGTGGG
Mouse IFNβ was used as described previousely (Stockinger et al., 2004)
ChIP mouse IRF7 enhancer – Forward: TCGAACTCAGAAATCTGCCTGC
Reverse: TCAAATCCCAGCAACCACAAGG
ChIP mouse IRF7 promoter – Forward: GGTCGGGTGTAGTTTGAGGA
Reverse: GCCAAGGTGGCTGTAGATGT
ChIP mouse IL1ra promoter – Forward: CACGCCTCTGGAAGCTGGGC
Reverse: AGGAGCACCCAGGCAGTGTC

Cells

Irf3 -/-, *Irf7 -/-*, were derived from recently published mice (Sato et al., 2000; Honda et al., 2005). *Tbk1-Ikbke -/-* MEFs were kindly provided by Akira S. and generated as described in (Hemmi et al., 2004). MEFs were cultured in DMEM containing 10% FCS.

ChIP buffers

ProtA- beads: Amersham (Nr:17-0780-01)
1.5g beads in 7,5ml TE
200 µg/ml Hering sperm
1mg/ml BSA
0.05%NaAz
Lysis buffer: 50mM HEPES-KOH
1mM EDTA
0.5mM EGTA
140mM NaCl

10% glycerol
0.5% NP-40
0.25% Triton X-100
(+protease inhibitor cocktail, +PMSF)

Wash buffer: 10mM Tris.HCl pH8
1mM EDTA
0.5mM EGTA
200mM NaCl
(+protease inhibitor cocktail, +PMSF)

RIPA buffer: 10mM Tris.HCl pH8
1mM EDTA
0.5mM EGTA
140mM NaCl
5% glycerol
0.1% sodium deoxycholate
0.1% SDS
1% Triton X-100
(+protease inhibitor cocktail, +PMSF)

Proteinase K buffer: 10mM Tris-HCl pH8
1mM EDTA
100mM NaCl
0.5% SDS
add 200µg/ml proteinase K

TE: 10mM Tris-HCl pH8
1mM EDTA

PMSF: if required add to final concentration of 1mM
protease inhibitor cocktail: complete EDTA-free tablets (Roche Nr:11873580001)

Nuclear extraction buffers

Buffer A: 10 mM HEPES (pH 7.9)
10 mM KCl
0.1 mM EDTA
0.1 mM EGTA
1 mM DTT
(+PMSF, + proteinase inhibitors)

Buffer C: 20 mM HEPES (pH 7.9)
0.4 M NaCl
1 mM EDTA
1 mM EGTA
1 mM DTT
(+PMSF, + proteinase inhibitors)

2D-PAGE-buffers and strip

Rehydration Buffer: 8M UREA (12g for 25ml)
2% CHAPS (0,5g for 25ml)
125µl (for 25ml) IPG buffer (optional, or add 2µl fresh before sample loading)
Bromphenolblue (a few grains)
ddH2O to 25ml
store in 1ml aliquots at -20°C
just before use add 18µl of 1M DTT per 1ml rehydration buffer

SDS equilibration buffer: 1,5M Tris pH 8,8 endconc. 50mM (6,7ml)

UREA	6M	(72,07g)
(87% v/v) Glycerol	30%	(69ml)
SDS	2%	(4g)
Bromphenolblue	a few grains	
ddH2O		ad 200ml

store 10ml aliquots at -20°C

just before use add 650µl 1M DTT per 10ml buffer

Agarose sealing solution: SDS electrophoresis buffer
0,5% Agarose
Bromphenolblue a few grains

Strips used were purchased from Amersham: Immobiline Dry Strip pH 4-7 (18cm).

Methods

Additional reagents not decribed in references Farlik et al (Farlik et al., 2010), or Ramsauer et al. (Ramsauer et al., 2007) were as follows:

Transfection and Luciferase detection

The day before transfection $2x10^5$ MEFs were seeded in each well of a 6 well plate, tissue culture treated, in the presence of 2ml DMEM + 10% FCS.
For transfection Turbofect of Fermentas was used according to manufacturers instructions. The medium containing transfection reagent and DNA was exchanged 4hours after transfection by fresh medium, DMEM + 10% FCS. 24 hours after transfection cells were lysed and luciferase was measured using the Dual Luciferase Reporter Assay System of Promega (Madison, WI, USA; Cat. Nr. E1910), according to the manufacturers protocol.
RLUs of the Firefly and Renilla Luciferase were measured with the FB 12 Luminometer – Berthold Detection Systems (Bad Wildbad, Germany).

Protein A Bead preperation for ChIP

Beads used: Protein A Sepharose CL-4B 17-0780-01; GE-Healthcare

- Wash/dissolve Beads in Falcon 50ml with 20ml sterile H_2O by shaking gently
- Put Beads on ice and let the Beads settle down by there own (or in urgent needs centrifuge at 4°C 1000rpm 3min.)
- Transfer supernatant to a new 50ml Falcon (on ice)
- Add another 20ml of sterile H_2O and shake gently
- Let the Beads settle down again on ice (or centrifuge again – see above)
- Collect the supernatant
- Add 10ml of TE (sterile filtered) and dissolve by shaking gently
- Let the Beads settle down again on ice (or centrifuge again – see above)
- Discard supernatant of both falcons (including the one with the collected supernatants) and dissolve the Beads in an equal amount (according to the amount of Beads) of fresh TE

- Aliquot the Beads TE mix to 1,5ml reaction tubes (~ 1ml per tube = 0,5ml Beads + 0,5ml TE)
- Centrifuge the aliquots at 3000rpm, 1min at 4°C
- Discard supernatant and keep tubes on ice!

Prepair the following Master Mix (for 100µl of Beads):

2µl Salmon Sperm DNA

10µl BSA (10% in H_2O)

5µl NaAzid (5%)

83µl TE

- Add 500µl of mastermix to each reaction tube and incubate the tubes 30min (better over night!) at 4°C on a rotating wheel (20rpm)
- Store the Beads at 4°C (expires within 4-5 months)

ChIP and Re-ChIP Procedure

ChIPs were performed according to the protocol described by Nissen and Yamamoto (Nissen and Yamamoto, 2000) with minor corrections.

- In case of MEFs use a 80% confluent 15cm tissue culture dish. For BMDMs 1.2-1.5 x 10^7 macrophages per dish are seeded the day before treatment.
- Treat the cells
- Crosslink cells 10min at 37°C with FA: add 540µl FA (33%) directly to 20ml medium
- add 125mM Glycin to stop crosslinking: add 1ml of 2,5M Glycin to 20ml medium and incubate for 5min at RT
- suck off medium
- wash with ice cold 1xPBS
- scrape cells in 5 to 10 ml ice cold 1x PBS
- centrifuge 600g for 10min at4°C
- lyse the cells 10min 4°C (wheel) with 5ml ice cold lysis buffer (+Pille, +PMSF)
- centrifuge 600g for 5min at 4°C
- wash cells 10min 4°C (wheel) with 10ml ice cold Wash Buffer (+Pille, +PMSF)
- centrifuge 600g for 5min at 4°C
- resuspend in 1,5ml ice cold RIPA buffer

Here extracts can be stored for 3-5 days at 4°C.

- Sonication for MEFs 90% duty cycle/ 40% output (7x-15sec) on ice (fragment size 200-800bp); for macrophages use 90% duty cycle/50% output (10-15x15sec) on ice; for other cells these settings have to be individually optimised
- Centrifuge 16000g for 15min at 4°C and transfer supernatant in new tube
> extracts can be stored at -80°C after shockfreezing in liquid nitrogen for several months
- Save 40µl (10% of the amount you plan to use for the IP) as Input at 4°C
- $OD_{260/280}$ measurement of a 1:50 dilution of the extract (if necessary adjust DNA concentration to an equal amount in all samples with RIPA containing PMSF+Pille)
- preclear samples with 40µl protein A sepharose beads (60min.)
- Centrifuge 5´ 2000rpm at 4°C
- transfer extract to 1,5ml tubes and add antibodies (one of them has to be control antibody for background – e.g. rabbit 0 serum) – antibodies of interest concentration depending on the reactivity of the antibody
- incubate samples over night on the rotating wheel at 4°C
- add 40µl beads and incubate the samples on the rotating wheel at 4°C for 1 hour
- centrifuge beads at 2000rpm for 3min at 4°C
- suck off supernatant and wash with 500µl RIPA
- incubate the samples on the rotating wheel at 4°C for 5 min
- repeat washing step 4 times with RIPA buffer + PMSF
- include Input again in the following steps!
- add 200µl protease K buffer + 4µl protease K (20mg/ml)
- incubate 2-3 hours at 55°C on the rotating block at 900rpm
- transfer tubes to 65°C over night for reverse crosslinking
- centrifuge beads at 2000rpm for 5min at RT
> possibility to store the samples at -20°C
- DNA – extraction: add 200µl phenol/chloroform/isoamylalkohol and centrifuge 15min at 14000rpm, RT (opt. Phase lock Tubes 1,5ml heavy – Eppendorf)
- transfer aqueous phase to a new tube and add 500µl Ethanol, 40µl NH_4oAc, 20µg Glycogen to each sample
- vortex samples
- precipitate DNA at -20°C (2 hours) over night or at -80°C for 45min
- centrifuge at 14000rpm for 15min at 4°C
- discard supernatant

- wash 1x with 500µl 70% EtOH (ice cold)
- discard supernatant and airdry the pellet for 15min at RT
- dissolve the pellet in 40-200µl TE and incubate samples at 55°C for 5min
- start analysis of DNA by PCR or qPCR, ChIP on Chip or ChIP Sequencing analysis

For Re-ChIP the immunecomplexes were eluted by adding 10 mM DTT and incubation for 30 min at 37°C. The samples were diluted 40-fold in RIPA-buffer (containing PMSF and proteinase inhibitors) and reimmunoprecipitated.

Nuclear extraction

Nuclear extracts were performed as described in Chan et al. (Chan et al., 2010).
- Use in case of MEFs a 80% dense 10cm dish
- add 2 ml of buffer A directly to the plate
- leave 15 min on ice
- add 125 µL of 10 % NP-40
- scrape the cells off
- vortex vigorously for 10 s
- centrifuge 30sec at 4 °C at 500 g
- supernatant is cytoplasmic (collect it)
- wash the pellet once again in buffer A without NP-40 (it may need two washings)
- resuspend pellet in 80 µL of buffer C (in case of 2D-gels one might need to decrease the volume to 20µl)
- incubate 15 min on ice with agitation
- centrifuge 5 min at 4 °C at maximum rpm
- collect the supernatant (nuclear fraction)
- prepare proteins for loading

2D – PAGE

After prepairing the nuclear extract to enrich for nuclear proteins and reduce concentration of detergent, 20µl of nuclear extract are taken up in 280µl rehydration buffer (freshly add 2µl IPG buffer with the pH corresponding to the IPG strips used in the next steps).

- incubate the nuclear extract together with the rehydration buffer at 95°C for 10 min. and load everything to the IPG – Strip, which is placed gelside down into the strip holder.
- Cover the strip with IPG Dry Strip Cover Fluid (Amersham)
- Attach the lid and place the holder into the IPGphor machine (plus = pH1 - minus = pH12)
- Program for the first dimension used was as follows: 200V (15min.); 300V (30min.); 400V (over night); 800V (1hr)
- Remove strip from holder and place it into 10ml equilibration buffer (freshly add DTT) into a glass tube (with the gel side open to the tube) and shake it 15 minutes at room temperature
- After equilibration place the strip by plastic side on the back to the larger gel glass plate, washed by electrophoresis buffer and move it down to touck the SDS gel
- Overlay the strip with agarose sealing solution (avoid bubbles) and run the gel (90 – 100 mA).
- After the gel is finished the proteins are blotted to a nitrocellulose membrane (blotting time can last up to 3-4 hrs) and further detected by Western blot.

References

1. Adelman, K., Kennedy, M.A., Nechaev, S., Gilchrist, D.A., Muse, G.W., Chinenov, Y., and Rogatsky, I. (2009). Immediate mediators of the inflammatory response are poised for gene activation through RNA polymerase II stalling. Proc. Natl. Acad. Sci. U.S.A *106*, 18207-18212

2. Adelman, K., Marr, M.T., Werner, J., Saunders, A., Ni, Z., Andrulis, E.D., and Lis, J.T. (2005). Efficient release from promoter-proximal stall sites requires transcript cleavage factor TFIIS. Mol. Cell *17*, 103-112

3. Agalioti, T., Chen, G., and Thanos, D. (2002). Deciphering the transcriptional histone acetylation code for a human gene. Cell *111*, 381-392

4. Akhtar, M.S., Heidemann, M., Tietjen, J.R., Zhang, D.W., Chapman, R.D., Eick, D., and Ansari, A.Z. (2009). TFIIH kinase places bivalent marks on the carboxy-terminal domain of RNA polymerase II. Mol. Cell *34*, 387-393

5. Alexander, W.S., and Hilton, D.J. (2004). The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu. Rev. Immunol *22*, 503-529

6. Allison, L.A., Moyle, M., Shales, M., and Ingles, C.J. (1985). Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell *42*, 599-610

7. Anest, V., Hanson, J.L., Cogswell, P.C., Steinbrecher, K.A., Strahl, B.D., and Baldwin, A.S. (2003). A nucleosomal function for IkappaB kinase-alpha in NF-kappaB-dependent gene expression. Nature *423*, 659-663

8. Au, W.C., and Pitha, P.M. (2001). Recruitment of multiple interferon regulatory factors and histone acetyltransferase to the transcriptionally active interferon a promoters. J. Biol. Chem *276*, 41629-41637

9. Au, W.C., Yeow, W.S., and Pitha, P.M. (2001). Analysis of functional domains of interferon regulatory factor 7 and its association with IRF-3. Virology *280*, 273-282

10. Bauer, U., Daujat, S., Nielsen, S.J., Nightingale, K., and Kouzarides, T. (2002). Methylation at arginine 17 of histone H3 is linked to gene activation. EMBO Rep *3*, 39-44

11. Berger, S.L. (2007). The complex language of chromatin regulation during transcription. Nature *447*, 407-412

12. Bluyssen, H.A., Muzaffar, R., Vlieststra, R.J., van der Made, A.C., Leung, S., Stark, G.R., Kerr, I.M., Trapman, J., and Levy, D.E. (1995). Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. Proc. Natl. Acad. Sci. U.S.A *92*, 5645-5649

13. Bogdan, C. (2001). Nitric oxide and the immune response. Nat. Immunol *2*, 907-916

14. Braunstein, J., Brutsaert, S., Olson, R., and Schindler, C. (2003). STATs dimerize in the absence of phosphorylation. J. Biol. Chem *278*, 34133-34140

15. Briken, V., Ruffner, H., Schultz, U., Schwarz, A., Reis, L.F., Strehlow, I., Decker, T., and Staeheli, P. (1995). Interferon regulatory factor 1 is required for mouse Gbp gene activation by gamma interferon. Mol. Cell. Biol *15*, 975-982

16. Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y., and Allis, C.D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell *84*, 843-851

17. Caillaud, A., Hovanessian, A.G., Levy, D.E., and Marié, I.J. (2005). Regulatory serine residues mediate phosphorylation-dependent and phosphorylation-independent activation of interferon regulatory factor 7. J. Biol. Chem *280*, 17671-17677

18. Carrozza, M.J., Li, B., Florens, L., Suganuma, T., Swanson, S.K., Lee, K.K., Shia, W., Anderson, S., Yates, J., Washburn, M.P., et al. (2005). Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell *123*, 581-592

19. Caslini, C., Yang, Z., El-Osta, M., Milne, T.A., Slany, R.K., and Hess, J.L. (2007). Interaction of MLL amino terminal sequences with menin is required for transformation. Cancer Res *67*, 7275-7283

20. Chan, L.L.Y., Cheung, B.K.W., Li, J.C.B., and Lau, A.S.Y. (2010). A role for STAT3 and cathepsin S in IL-10 down-regulation of IFN-gamma-induced MHC class II molecule on primary human blood macrophages. J. Leukoc. Biol *88*, 303-311

21. Chapman, R.D., Heidemann, M., Hintermair, C., and Eick, D. (2008). Molecular evolution of the RNA polymerase II CTD. Trends Genet *24*, 289-296

22. Chau, T., Gioia, R., Gatot, J., Patrascu, F., Carpentier, I., Chapelle, J., O'Neill, L., Beyaert, R., Piette, J., and Chariot, A. (2008). Are the IKKs and IKK-related kinases TBK1 and IKK-epsilon similarly activated? Trends Biochem. Sci *33*, 171-180

23. Cheon, H., and Stark, G.R. (2009). Unphosphorylated STAT1 prolongs the expression of interferon-induced immune regulatory genes. Proc. Natl. Acad. Sci. U.S.A *106*, 9373-9378

24. Cheung, P., Tanner, K.G., Cheung, W.L., Sassone-Corsi, P., Denu, J.M., and Allis, C.D. (2000). Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. Mol. Cell *5*, 905-915

25. Chi, T. (2004). A BAF-centred view of the immune system. Nat. Rev. Immunol *4*, 965-977

26. Cho, H., Orphanides, G., Sun, X., Yang, X.J., Ogryzko, V., Lees, E., Nakatani, Y., and Reinberg, D. (1998). A human RNA polymerase II complex containing factors that modify chromatin structure. Mol. Cell. Biol *18*, 5355-5363

27. Colangeli, R., Haq, A., Arcus, V.L., Summers, E., Magliozzo, R.S., McBride, A., Mitra, A.K., Radjainia, M., Khajo, A., Jacobs, W.R., et al. (2009). The multifunctional histone-like protein Lsr2 protects mycobacteria against reactive oxygen intermediates. Proc. Natl. Acad. Sci. U.S.A *106*, 4414-4418

28. Cosma, M.P., Tanaka, T., and Nasmyth, K. (1999). Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell *97*, 299-311

29. Cuthbert, G.L., Daujat, S., Snowden, A.W., Erdjument-Bromage, H., Hagiwara, T., Yamada, M., Schneider, R., Gregory, P.D., Tempst, P., Bannister, A.J., et al. (2004). Histone deimination antagonizes arginine methylation. Cell *118*, 545-553

30. Daujat, S., Bauer, U., Shah, V., Turner, B., Berger, S., and Kouzarides, T. (2002). Crosstalk between CARM1 methylation and CBP acetylation on histone H3. Curr. Biol *12*, 2090-2097

31. Daulny, A., Geng, F., Muratani, M., Geisinger, J.M., Salghetti, S.E., and Tansey, W.P. (2008). Modulation of RNA polymerase II subunit composition by ubiquitylation. Proc. Natl. Acad. Sci. U.S.A *105*, 19649-19654

32. Decker, T., and Kovarik, P. (1999). Transcription factor activity of STAT proteins: structural requirements and regulation by phosphorylation and interacting proteins. Cell. Mol. Life Sci *55*, 1535-1546

33. Decker, T., and Kovarik, P. (2000). Serine phosphorylation of STATs. Oncogene *19*, 2628-2637

34. Decker, T., Muller, M., and Stockinger, S. (2005). The Yin and Yang of type I interferon activity in bacterial infection. Nat Rev Immunol *5*, 675-687

35. Decker, T., Stockinger, S., Karaghiosoff, M., Müller, M., and Kovarik, P. (2002). IFNs and STATs in innate immunity to microorganisms. J. Clin. Invest *109*, 1271-1277

36. Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K., and Zhou, M.M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. Nature *399*, 491-496

37. Dover, J., Schneider, J., Tawiah-Boateng, M.A., Wood, A., Dean, K., Johnston, M., and Shilatifard, A. (2002). Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. J. Biol. Chem *277*, 28368-28371

38. Drobic, B., Pérez-Cadahía, B., Yu, J., Kung, S.K., and Davie, J.R. (2010). Promoter chromatin remodeling of immediate-early genes is mediated through H3 phosphorylation at either serine 28 or 10 by the MSK1 multi-protein complex. Nucleic Acids Res *38*, 3196-3208

39. Egloff, S., O'Reilly, D., Chapman, R.D., Taylor, A., Tanzhaus, K., Pitts, L., Eick, D., and Murphy, S. (2007). Serine-7 of the RNA polymerase II CTD is specifically required for snRNA gene expression. Science *318*, 1777-1779

40. Egloff, S., Szczepaniak, S.A., Dienstbier, M., Taylor, A., Knight, S., and Murphy, S. (2010). The integrator complex recognizes a new double mark on the RNA polymerase II carboxyl-terminal domain. J. Biol. Chem *285*, 20564-20569

41. Elsby, L.M., O'Donnell, A.J.M., Green, L.M., Sharrocks, A.D., and Roberts, S.G.E. (2006). Assembly of transcription factor IIB at a promoter in vivo requires contact with RNA polymerase II. EMBO Rep *7*, 898-903

42. Farlik, M., Reutterer, B., Schindler, C., Greten, F., Vogl, C., Müller, M., and Decker, T. (2010). Nonconventional Initiation Complex Assembly by STAT and NF-kappaB Transcription Factors Regulates Nitric Oxide Synthase Expression. Immunity *33*, 25-34

43. Finkelman, F.D., Shea-Donohue, T., Morris, S.C., Gildea, L., Strait, R., Madden, K.B., Schopf, L., and Urban, J.F. (2004). Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. Immunol. Rev *201*, 139-155

44. Fleming, A.B., Kao, C., Hillyer, C., Pikaart, M., and Osley, M.A. (2008). H2B ubiquitylation plays a role in nucleosome dynamics during transcription elongation. Mol. Cell *31*, 57-66

45. Gehani, S.S., Agrawal-Singh, S., Dietrich, N., Christophersen, N.S., Helin, K., and Hansen, K. (2010). Polycomb Group Protein Displacement and Gene Activation through MSK-Dependent H3K27me3S28 Phosphorylation. Mol. Cell *39*, 886-900

46. Génin, P., Vaccaro, A., and Civas, A. (2009). The role of differential expression of human interferon--a genes in antiviral immunity. Cytokine Growth Factor Rev *20*, 283-295

47. Gévry, N., Chan, H.M., Laflamme, L., Livingston, D.M., and Gaudreau, L. (2007). p21 transcription is regulated by differential localization of histone H2A.Z. Genes Dev *21*, 1869-1881

48. Gévry, N., Hardy, S., Jacques, P., Laflamme, L., Svotelis, A., Robert, F., and Gaudreau, L. (2009). Histone H2A.Z is essential for estrogen receptor signaling. Genes Dev *23*, 1522-1533

49. Gewinner, C., Hart, G., Zachara, N., Cole, R., Beisenherz-Huss, C., and Groner, B. (2004). The coactivator of transcription CREB-binding protein interacts preferentially with the glycosylated form of Stat5. J. Biol. Chem *279*, 3563-3572

50. Ghisletti, S., Barozzi, I., Mietton, F., Polletti, S., De Santa, F., Venturini, E., Gregory, L., Lonie, L., Chew, A., Wei, C., et al. (2010). Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. Immunity *32*, 317-328

51. Gloire, G., Horion, J., El Mjiyad, N., Bex, F., Chariot, A., Dejardin, E., and Piette, J. (2007). Promoter-dependent effect of IKKalpha on NF-kappaB/p65 DNA binding. J. Biol. Chem *282*, 21308-21318

52. Glover-Cutter, K., Larochelle, S., Erickson, B., Zhang, C., Shokat, K., Fisher, R.P., and Bentley, D.L. (2009). TFIIH-associated Cdk7 kinase functions in phosphorylation of C-terminal domain Ser7 residues, promoter-proximal pausing, and termination by RNA polymerase II. Mol. Cell. Biol *29*, 5455-5464

53. Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R., and Young, R.A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. Cell *130*, 77-88

54. Hampsey, M., and Reinberg, D. (2003). Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation. Cell *113*, 429-432

55. Hargreaves, D.C., Horng, T., and Medzhitov, R. (2009). Control of inducible gene expression by signal-dependent transcriptional elongation. Cell *138*, 129-145

56. Hartley, P.D., and Madhani, H.D. (2009). Mechanisms that specify promoter nucleosome location and identity. Cell *137*, 445-458

57. Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344-362

58. Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004). The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J. Exp. Med *199*, 1641-1650

59. Hirose, Y., Tacke, R., and Manley, J.L. (1999). Phosphorylated RNA polymerase II stimulates pre-mRNA splicing. Genes Dev *13*, 1234-1239

60. Hirose, Y., and Ohkuma, Y. (2007). Phosphorylation of the C-terminal domain of RNA polymerase II plays central roles in the integrated events of eucaryotic gene expression. J. Biochem *141*, 601-608

61. Hoberg, J.E., Popko, A.E., Ramsey, C.S., and Mayo, M.W. (2006). IkappaB kinase alpha-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300. Mol. Cell. Biol *26*, 457-471

62. Holland, J.W., Bird, S., Williamson, B., Woudstra, C., Mustafa, A., Wang, T., Zou, J., Blaney, S.C., Collet, B., and Secombes, C.J. (2008). Molecular characterization of IRF3 and IRF7 in rainbow trout, Oncorhynchus mykiss: functional analysis and transcriptional modulation. Mol. Immunol *46*, 269-285

63. Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., et al. (2005). IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature *434*, 772-777

64. Hunter, C.A. (2005). New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nat. Rev. Immunol *5*, 521-531

65. ISAACS, A., and LINDENMANN, J. (1957). Virus interference. I. The interferon. Proc. R. Soc. Lond., B, Biol. Sci *147*, 258-267

66. Jamieson, A.M., Yu, S., Annicelli, C.H., and Medzhitov, R. (2010). Influenza virus-induced glucocorticoids compromise innate host defense against a secondary bacterial infection. Cell Host Microbe *7*, 103-114

67. Kadonaga, J.T. (2004). Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. Cell *116*, 247-257

68. Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S.I., Kimura, T., and Green, S.J. (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science *263*, 1612-1615

69. Kelly, W.G., Dahmus, M.E., and Hart, G.W. (1993). RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc. J. Biol. Chem *268*, 10416-10424

70. Kim, J., Guermah, M., McGinty, R.K., Lee, J., Tang, Z., Milne, T.A., Shilatifard, A., Muir, T.W., and Roeder, R.G. (2009). RAD6-Mediated transcription-coupled H2B ubiquitylation directly stimulates H3K4 methylation in human cells. Cell *137*, 459-471

71. Kim, T., and Buratowski, S. (2009). Dimethylation of H3K4 by Set1 recruits the Set3 histone deacetylase complex to 5' transcribed regions. Cell *137*, 259-272

72. Kimura, T., Kadokawa, Y., Harada, H., Matsumoto, M., Sato, M., Kashiwazaki, Y., Tarutani, M., Tan, R.S., Takasugi, T., Matsuyama, T., et al. (1996). Essential and non-redundant roles of p48 (ISGF3 gamma) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies. Genes Cells *1*, 115-124

73. Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C.W. (2002). Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene *285*, 1-24

74. Kizer, K.O., Phatnani, H.P., Shibata, Y., Hall, H., Greenleaf, A.L., and Strahl, B.D. (2005). A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol. Cell. Biol *25*, 3305-3316

75. Kornblihtt, A.R., de la Mata, M., Fededa, J.P., Munoz, M.J., and Nogues, G. (2004). Multiple links between transcription and splicing. RNA *10*, 1489-1498

76. Krämer, O.H., Knauer, S.K., Greiner, G., Jandt, E., Reichardt, S., Gührs, K., Stauber, R.H., Böhmer, F.D., and Heinzel, T. (2009). A phosphorylation-acetylation switch regulates STAT1 signaling. Genes Dev *23*, 223-235

77. Krämer, O.H., and Heinzel, T. (2010). Phosphorylation-acetylation switch in the regulation of STAT1 signaling. Molecular and Cellular Endocrinology *315*, 40-48

78. Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M., et al. (2003). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol. Cell *11*, 721-729

79. Laricchia-Robbio, L., Tamura, T., Karpova, T., Sprague, B.L., McNally, J.G., and Ozato, K. (2005). Partner-regulated interaction of IFN regulatory factor 8 with chromatin visualized in live macrophages. Proc. Natl. Acad. Sci. U.S.A *102*, 14368-14373

80. Lau, J.F., Nusinzon, I., Burakov, D., Freedman, L.P., and Horvath, C.M. (2003). Role of metazoan mediator proteins in interferon-responsive transcription. Mol. Cell. Biol *23*, 620-628

81. Lee, C., Li, X., Hechmer, A., Eisen, M., Biggin, M.D., Venters, B.J., Jiang, C., Li, J., Pugh, B.F., and Gilmour, D.S. (2008). NELF and GAGA factor are linked to promoter-proximal pausing at many genes in Drosophila. Mol. Cell. Biol *28*, 3290-3300

82. Lee, G.R., Kim, S.T., Spilianakis, C.G., Fields, P.E., and Flavell, R.A. (2006). T helper cell differentiation: regulation by cis elements and epigenetics. Immunity *24*, 369-379

83. Lee, J., Smith, E., and Shilatifard, A. (2010). The language of histone crosstalk. Cell *142*, 682-685

84. Leung, A., Geng, F., Daulny, A., Collins, G., Guzzardo, P., and Tansey, W.P. (2008). Transcriptional control and the ubiquitin-proteasome system. Ernst Schering Found Symp Proc 75-97

85. Levy, D.E. (2010). NF-kappaB-ISGF3 transcription factor cooperation: coincidence detector or memory chip? Immunity *33*, 1-2

86. Levy, D.E., and Darnell, J.E. (2002). Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol *3*, 651-662

87. Lew, D.J., Decker, T., Strehlow, I., and Darnell, J.E. (1991). Overlapping elements in the guanylate-binding protein gene promoter mediate transcriptional induction by alpha and gamma interferons. Mol. Cell. Biol *11*, 182-191

88. Li, H., Ilin, S., Wang, W., Duncan, E.M., Wysocka, J., Allis, C.D., and Patel, D.J. (2006). Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. Nature *442*, 91-95

89. Lin, R., Mamane, Y., and Hiscott, J. (2000). Multiple regulatory domains control IRF-7 activity in response to virus infection. J. Biol. Chem *275*, 34320-34327

90. Liu, X., Bushnell, D.A., Wang, D., Calero, G., and Kornberg, R.D. (2010). Structure of an RNA polymerase II-TFIIB complex and the transcription initiation mechanism. Science *327*, 206-209

91. Lo, W.S., Duggan, L., Emre, N.C., Belotserkovskya, R., Lane, W.S., Shiekhattar, R., and Berger, S.L. (2001). Snf1--a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. Science *293*, 1142-1146

92. Lolli, G. (2009). Binding to DNA of the RNA-polymerase II C-terminal domain allows discrimination between Cdk7 and Cdk9 phosphorylation. Nucleic Acids Res *37*, 1260-1268

93. Lu, D., Liou, H., Tang, C., and Fu, W. (2006). Hypoxia-induced iNOS expression in microglia is regulated by the PI3-kinase/Akt/mTOR signaling pathway and activation of hypoxia inducible factor-1alpha. Biochem. Pharmacol *72*, 992-1000

94. Macdonald, N., Welburn, J.P.I., Noble, M.E.M., Nguyen, A., Yaffe, M.B., Clynes, D., Moggs, J.G., Orphanides, G., Thomson, S., Edmunds, J.W., et al. (2005). Molecular basis for the recognition of phosphorylated and phosphoacetylated histone h3 by 14-3-3. Mol. Cell *20*, 199-211

95. MacMicking, J., Xie, Q.W., and Nathan, C. (1997). Nitric oxide and macrophage function. Annu. Rev. Immunol *15*, 323-350

96. Mahadevan, L.C., Willis, A.C., and Barratt, M.J. (1991). Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. Cell *65*, 775-783

97. Maldonado, E., and Reinberg, D. (1995). News on initiation and elongation of transcription by RNA polymerase II. Curr. Opin. Cell Biol *7*, 352-361

98. Mao, X., Ren, Z., Parker, G.N., Sondermann, H., Pastorello, M.A., Wang, W., McMurray, J.S., Demeler, B., Darnell, J.E., and Chen, X. (2005). Structural bases of unphosphorylated STAT1 association and receptor binding. Mol. Cell *17*, 761-771

99. Margaritis, T., and Holstege, F.C.P. (2008). Poised RNA polymerase II gives pause for thought. Cell *133*, 581-584

100. Marié, I., Durbin, J.E., and Levy, D.E. (1998). Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. EMBO J *17*, 6660-6669

101. Marié, I., Smith, E., Prakash, A., and Levy, D.E. (2000). Phosphorylation-induced dimerization of interferon regulatory factor 7 unmasks DNA binding and a bipartite transactivation domain. Mol. Cell. Biol *20*, 8803-8814

102. Martin, C., and Zhang, Y. (2005). The diverse functions of histone lysine methylation. Nat. Rev. Mol. Cell Biol *6*, 838-849

103. Mavrich, T.N., Jiang, C., Ioshikhes, I.P., Li, X., Venters, B.J., Zanton, S.J., Tomsho, L.P., Qi, J., Glaser, R.L., Schuster, S.C., et al. (2008). Nucleosome organization in the Drosophila genome. Nature *453*, 358-362

104. McCullough, K.C., Ruggli, N., and Summerfield, A. (2009). Dendritic cells--at the front-line of pathogen attack. Vet. Immunol. Immunopathol *128*, 7-15

105. Medlin, J., Scurry, A., Taylor, A., Zhang, F., Peterlin, B.M., and Murphy, S. (2005). P-TEFb is not an essential elongation factor for the intronless human U2 snRNA and histone H2b genes. EMBO J *24*, 4154-4165

106. Medzhitov, R., and Horng, T. (2009). Transcriptional control of the inflammatory response. Nat. Rev. Immunol *9*, 692-703

107. Meinhart, A., Kamenski, T., Hoeppner, S., Baumli, S., and Cramer, P. (2005). A structural perspective of CTD function. Genes Dev *19*, 1401-1415

108. Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., et al. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell *84*, 431-442

109. Mertens, C., Zhong, M., Krishnaraj, R., Zou, W., Chen, X., and Darnell, J.E. (2006). Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain. Genes Dev *20*, 3372-3381

110. Miller, T., Krogan, N.J., Dover, J., Erdjument-Bromage, H., Tempst, P., Johnston, M., Greenblatt, J.F., and Shilatifard, A. (2001). COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. Proc. Natl. Acad. Sci. U.S.A *98*, 12902-12907

111. Misteli, T., and Spector, D.L. (1999). RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. Mol. Cell *3*, 697-705

112. Muse, G.W., Gilchrist, D.A., Nechaev, S., Shah, R., Parker, J.S., Grissom, S.F., Zeitlinger, J., and Adelman, K. (2007). RNA polymerase is poised for activation across the genome. Nat. Genet *39*, 1507-1511

113. Nakanishi, S., Lee, J.S., Gardner, K.E., Gardner, J.M., Takahashi, Y., Chandrasekharan, M.B., Sun, Z., Osley, M.A., Strahl, B.D., Jaspersen, S.L., et al. (2009). Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1. J. Cell Biol *186*, 371-377

114. Neculai, D., Neculai, A.M., Verrier, S., Straub, K., Klumpp, K., Pfitzner, E., and Becker, S. (2005). Structure of the unphosphorylated STAT5a dimer. J. Biol. Chem *280*, 40782-40787

115. Ng, H.H., Dole, S., and Struhl, K. (2003a). The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. J. Biol. Chem *278*, 33625-33628

116. Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003b). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol. Cell *11*, 709-719

117. Ni, Z., Abou El Hassan, M., Xu, Z., Yu, T., and Bremner, R. (2008). The chromatin-remodeling enzyme BRG1 coordinates CIITA induction through many interdependent distal enhancers. Nat. Immunol *9*, 785-793

118. Ni, Z., and Bremner, R. (2007). Brahma-related gene 1-dependent STAT3 recruitment at IL-6-inducible genes. J. Immunol *178*, 345-351

119. Ni, Z., Karaskov, E., Yu, T., Callaghan, S.M., Der, S., Park, D.S., Xu, Z., Pattenden, S.G., and Bremner, R. (2005). Apical role for BRG1 in cytokine-induced promoter assembly. Proc. Natl. Acad. Sci. U.S.A *102*, 14611-14616

120. Nissen, R.M., and Yamamoto, K.R. (2000). The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev *14*, 2314-2329

121. Orphanides, G., and Reinberg, D. (2002). A unified theory of gene expression. Cell *108*, 439-451

122. Panne, D., Maniatis, T., and Harrison, S.C. (2007). An atomic model of the interferon-beta enhanceosome. Cell *129*, 1111-1123

123. Parekh, B.S., and Maniatis, T. (1999). Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter. Mol. Cell *3*, 125-129

124. Paulson, M., Press, C., Smith, E., Tanese, N., and Levy, D.E. (2002). IFN-Stimulated transcription through a TBP-free acetyltransferase complex escapes viral shutoff. Nat. Cell Biol *4*, 140-147

125. Paz, S., Vilasco, M., Arguello, M., Sun, Q., Lacoste, J., Nguyen, T.L., Zhao, T., Shestakova, E.A., Zaari, S., Bibeau-Poirier, A., et al. (2009). Ubiquitin-regulated recruitment of IkappaB kinase epsilon to the MAVS interferon signaling adapter. Mol. Cell. Biol *29*, 3401-3412

126. Perry, A.K., Chow, E.K., Goodnough, J.B., Yeh, W., and Cheng, G. (2004). Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. J. Exp. Med *199*, 1651-1658

127. Pinhero, R., Liaw, P., Bertens, K., and Yankulov, K. (2004). Three cyclin-dependent kinases preferentially phosphorylate different parts of the C-terminal domain of the large subunit of RNA polymerase II. Eur. J. Biochem *271*, 1004-1014

128. Ramanathan, M., Luo, W., Csóka, B., Haskó, G., Lukashev, D., Sitkovsky, M.V., and Leibovich, S.J. (2009). Differential regulation of HIF-1alpha isoforms in murine macrophages by TLR4 and adenosine A(2A) receptor agonists. J. Leukoc. Biol *86*, 681-689

129. Ramsauer, K., Farlik, M., Zupkovitz, G., Seiser, C., Kröger, A., Hauser, H., and Decker, T. (2007). Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-gamma-inducible gbp2 gene. Proc. Natl. Acad. Sci. U.S.A *104*, 2849-2854

130. Rasmussen, E.B., and Lis, J.T. (1993). In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. Proc. Natl. Acad. Sci. U.S.A *90*, 7923-7927

131. Reinberg, D., and Sims, R.J. (2006). de FACTo nucleosome dynamics. J. Biol. Chem *281*, 23297-23301

132. Reines, D., Chamberlin, M.J., and Kane, C.M. (1989). Transcription elongation factor SII (TFIIS) enables RNA polymerase II to elongate through a block to transcription in a human gene in vitro. J. Biol. Chem *264*, 10799-10809

133. Remy, I., Wilson, I.A., and Michnick, S.W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. Science *283*, 990-993

134. Robertson, A.G., Bilenky, M., Tam, A., Zhao, Y., Zeng, T., Thiessen, N., Cezard, T., Fejes, A.P., Wederell, E.D., Cullum, R., et al. (2008). Genome-wide relationship between histone H3 lysine 4 mono- and tri-methylation and transcription factor binding. Genome Res *18*, 1906-1917

135. Robertson, G., Hirst, M., Bainbridge, M., Bilenky, M., Zhao, Y., Zeng, T., Euskirchen, G., Bernier, B., Varhol, R., Delaney, A., et al. (2007). Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. Nat. Methods *4*, 651-657

136. Roeder, R.G. (1996). The role of general initiation factors in transcription by RNA polymerase II. Trends Biochem. Sci *21*, 327-335

137. Sadzak, I., Schiff, M., Gattermeier, I., Glinitzer, R., Sauer, I., Saalmüller, A., Yang, E., Schaljo, B., and Kovarik, P. (2008). Recruitment of Stat1 to chromatin is required for interferon-induced serine phosphorylation of Stat1 transactivation domain. Proc. Natl. Acad. Sci. U.S.A *105*, 8944-8949

138. Saha, B., Jyothi Prasanna, S., Chandrasekar, B., and Nandi, D. (2010). Gene modulation and immunoregulatory roles of interferon gamma. Cytokine *50*, 1-14

139. Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A., and Allis, C.D. (1999). Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. Science *285*, 886-891

140. Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., et al. (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity *13*, 539-548

141. Saunders, A., Core, L.J., and Lis, J.T. (2006). Breaking barriers to transcription elongation. Nat. Rev. Mol. Cell Biol *7*, 557-567

142. Savitsky, D., Tamura, T., Yanai, H., and Taniguchi, T. (2010). Regulation of immunity and oncogenesis by the IRF transcription factor family. Cancer Immunol. Immunother *59*, 489-510

143. Schindler, C., Levy, D.E., and Decker, T. (2007). JAK-STAT signaling: from interferons to cytokines. J. Biol. Chem *282*, 20059-20063

144. Schlüter, G., Boinska, D., and Nieman-Seyde, S.C. (2000). Evidence for translational repression of the SOCS-1 major open reading frame by an upstream open reading frame. Biochem. Biophys. Res. Commun *268*, 255-261

145. Schmid, S., Mordstein, M., Kochs, G., Garcia-Sastre, A., and Tenoever, B.R. (2010). Transcription factor redundancy ensures induction of the antiviral state. J Biol Chem

146. Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. J. Leukoc. Biol *75*, 163-189

147. Sheehan, K.C.F., Lai, K.S., Dunn, G.P., Bruce, A.T., Diamond, M.S., Heutel, J.D., Dungo-Arthur, C., Carrero, J.A., White, J.M., Hertzog, P.J., et al. (2006). Blocking monoclonal antibodies specific for mouse IFN-alpha/beta receptor subunit 1 (IFNAR-1) from mice immunized by in vivo hydrodynamic transfection. J. Interferon Cytokine Res *26*, 804-819

148. Shi, M., Lin, T.H., Appell, K.C., and Berg, L.J. (2008). Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. Immunity *28*, 763-773

149. Shilatifard, A. (2006). Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu. Rev. Biochem *75*, 243-269

150. Sims, R.J., Belotserkovskaya, R., and Reinberg, D. (2004). Elongation by RNA polymerase II: the short and long of it. Genes Dev *18*, 2437-2468

151. Sims, R.J., Chen, C., Santos-Rosa, H., Kouzarides, T., Patel, S.S., and Reinberg, D. (2005). Human but not yeast CHD1 binds directly and selectively to histone H3 methylated at lysine 4 via its tandem chromodomains. J. Biol. Chem *280*, 41789-41792

152. Soloaga, A., Thomson, S., Wiggin, G.R., Rampersaud, N., Dyson, M.H., Hazzalin, C.A., Mahadevan, L.C., and Arthur, J.S.C. (2003). MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. EMBO J *22*, 2788-2797

153. Soulat, D., Bauch, A., Stockinger, S., Superti-Furga, G., and Decker, T. (2006). Cytoplasmic Listeria monocytogenes stimulates IFN-beta synthesis without requiring the adapter protein MAVS. FEBS Lett *580*, 2341-2346

154. Spilianakis, C., Kretsovali, A., Agalioti, T., Makatounakis, T., Thanos, D., and Papamatheakis, J. (2003). CIITA regulates transcription onset viaSer5-phosphorylation of RNA Pol II. EMBO J *22*, 5125-5136

155. Sprouse, R.O., Karpova, T.S., Mueller, F., Dasgupta, A., McNally, J.G., and Auble, D.T. (2008). Regulation of TATA-binding protein dynamics in living yeast cells. Proc. Natl. Acad. Sci. U.S.A *105*, 13304-13308

156. Stetson, D.B., and Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity *24*, 93-103

157. Stockinger, S., Reutterer, B., Schaljo, B., Schellack, C., Brunner, S., Materna, T., Yamamoto, M., Akira, S., Taniguchi, T., Murray, P.J., et al. (2004). IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. J. Immunol *173*, 7416-7425

158. Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45

159. Strahl, B.D., Briggs, S.D., Brame, C.J., Caldwell, J.A., Koh, S.S., Ma, H., Cook, R.G., Shabanowitz, J., Hunt, D.F., Stallcup, M.R., et al. (2001). Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. Curr. Biol *11*, 996-1000

160. Suzuki, M. (1990). The heptad repeat in the largest subunit of RNA polymerase II binds by intercalating into DNA. Nature *344*, 562-565

161. Talbert, P.B., and Henikoff, S. (2010). Histone variants--ancient wrap artists of the epigenome. Nat. Rev. Mol. Cell Biol *11*, 264-275

162. Tamassia, N., Castellucci, M., Rossato, M., Gasperini, S., Bosisio, D., Giacomelli, M., Badolato, R., Cassatella, M.A., and Bazzoni, F. (2010). Uncovering an IL-10-dependent NF-kappaB recruitment to the IL-1ra promoter that is impaired in STAT3 functionally defective patients. FASEB J *24*, 1365-1375

163. Taniguchi, T., and Takaoka, A. (2001). A weak signal for strong responses: interferon-alpha/beta revisited. Nat. Rev. Mol. Cell Biol *2*, 378-386

164. Tan-Wong, S.M., Wijayatilake, H.D., and Proudfoot, N.J. (2009). Gene loops function to maintain transcriptional memory through interaction with the nuclear pore complex. Genes Dev *23*, 2610-2624

165. Tenoever, B.R., Ng, S., Chua, M.A., McWhirter, S.M., García-Sastre, A., and Maniatis, T. (2007). Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. Science *315*, 1274-1278

166. Tora, L., and Timmers, H.T.M. (2010). The TATA box regulates TATA-binding protein (TBP) dynamics in vivo. Trends Biochem. Sci *35*, 309-314

167. Varinou, L., Ramsauer, K., Karaghiosoff, M., Kolbe, T., Pfeffer, K., Müller, M., and Decker, T. (2003). Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity. Immunity *19*, 793-802

168. Vermeulen, M., Mulder, K.W., Denissov, S., Pijnappel, W.W.M.P., van Schaik, F.M.A., Varier, R.A., Baltissen, M.P.A., Stunnenberg, H.G., Mann, M., and Timmers, H.T.M. (2007). Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. Cell *131*, 58-69

169. Vettese-Dadey, M., Grant, P.A., Hebbes, T.R., Crane- Robinson, C., Allis, C.D., and Workman, J.L. (1996). Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA in vitro. EMBO J *15*, 2508-2518

170. Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873-878

171. Wang, J., Basagoudanavar, S.H., Wang, X., Hopewell, E., Albrecht, R., García-Sastre, A., Balachandran, S., and Beg, A.A. (2010). NF-kappa B RelA subunit is crucial for early IFN-beta expression and resistance to RNA virus replication. J. Immunol *185*, 1720-1729

172. Wang, P., Lin, C., Smith, E.R., Guo, H., Sanderson, B.W., Wu, M., Gogol, M., Alexander, T., Seidel, C., Wiedemann, L.M., et al. (2009). Global analysis of H3K4 methylation defines MLL family member targets and points to a role for MLL1-mediated H3K4 methylation in the regulation of transcriptional initiation by RNA polymerase II. Mol. Cell. Biol *29*, 6074-6085

173. Weake, V.M., and Workman, J.L. (2008). Histone ubiquitination: triggering gene activity. Mol. Cell *29*, 653-663

174. Weber, S., Maass, F., Schuemann, M., Krause, E., Suske, G., and Bauer, U. (2009). PRMT1-mediated arginine methylation of PIAS1 regulates STAT1 signaling. Genes Dev *23*, 118-132

175. Wei, G., Wei, L., Zhu, J., Zang, C., Hu-Li, J., Yao, Z., Cui, K., Kanno, Y., Roh, T., Watford, W.T., et al. (2009). Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity *30*, 155-167

176. Wei, L., Vahedi, G., Sun, H., Watford, W.T., Takatori, H., Ramos, H.L., Takahashi, H., Liang, J., Gutierrez-Cruz, G., Zang, C., et al. (2010). Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. Immunity *32*, 840-851

177. Wilson, C.B., Rowell, E., and Sekimata, M. (2009). Epigenetic control of T-helper-cell differentiation. Nat. Rev. Immunol *9*, 91-105

178. Winter, S., Simboeck, E., Fischle, W., Zupkovitz, G., Dohnal, I., Mechtler, K., Ammerer, G., and Seiser, C. (2008). 14-3-3 proteins recognize a histone code at histone H3 and are required for transcriptional activation. EMBO J *27*, 88-99

179. Wood, A., Schneider, J., Dover, J., Johnston, M., and Shilatifard, A. (2003). The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. J. Biol. Chem *278*, 34739-34742

180. Wu, M., Wang, P.F., Lee, J.S., Martin-Brown, S., Florens, L., Washburn, M., and Shilatifard, A. (2008). Molecular regulation of H3K4 trimethylation by Wdr82, a component of human Set1/COMPASS. Mol. Cell. Biol *28*, 7337-7344

181. Wyce, A., Xiao, T., Whelan, K.A., Kosman, C., Walter, W., Eick, D., Hughes, T.R., Krogan, N.J., Strahl, B.D., and Berger, S.L. (2007). H2B ubiquitylation acts as a barrier to Ctk1 nucleosomal recruitment prior to removal by Ubp8 within a SAGA-related complex. Mol. Cell *27*, 275-288

182. Xiao, T., Kao, C., Krogan, N.J., Sun, Z., Greenblatt, J.F., Osley, M.A., and Strahl, B.D. (2005). Histone H2B ubiquitylation is associated with elongating RNA polymerase II. Mol. Cell. Biol *25*, 637-651

183. Yamamoto, Y., Verma, U.N., Prajapati, S., Kwak, Y., and Gaynor, R.B. (2003). Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. Nature *423*, 655-659

184. Yang, J., Chatterjee-Kishore, M., Staugaitis, S.M., Nguyen, H., Schlessinger, K., Levy, D.E., and Stark, G.R. (2005). Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. Cancer Res *65*, 939-947

185. Yao, Z., Cui, Y., Watford, W.T., Bream, J.H., Yamaoka, K., Hissong, B.D., Li, D., Durum, S.K., Jiang, Q., Bhandoola, A., et al. (2006). Stat5a/b are essential for normal lymphoid development and differentiation. Proc. Natl. Acad. Sci. U.S.A *103*, 1000-1005

186. Yuan, Z., Guan, Y., Chatterjee, D., and Chin, Y.E. (2005). Stat3 dimerization regulated by reversible acetylation of a single lysine residue. Science *307*, 269-273

187. Zeitlinger, J., Stark, A., Kellis, M., Hong, J., Nechaev, S., Adelman, K., Levine, M., and Young, R.A. (2007). RNA polymerase stalling at developmental control genes in the Drosophila melanogaster embryo. Nat. Genet *39*, 1512-1516

188. Zeng, C., van Wijnen, A.J., Stein, J.L., Meyers, S., Sun, W., Shopland, L., Lawrence, J.B., Penman, S., Lian, J.B., Stein, G.S., et al. (1997). Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors. Proc. Natl. Acad. Sci. U.S.A *94*, 6746-6751

189. Zhang, F., and Boothby, M. (2006). T helper type 1-specific Brg1 recruitment and remodeling of nucleosomes positioned at the IFN-gamma promoter are Stat4 dependent. J. Exp. Med *203*, 1493-1505

190. Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev *15*, 2343-2360

191. Zippo, A., Serafini, R., Rocchigiani, M., Pennacchini, S., Krepelova, A., and Oliviero, S. (2009). Histone crosstalk between H3S10ph and H4K16ac generates a histone code that mediates transcription elongation. Cell *138*, 1122-1136

192. Zupkovitz, G., Tischler, J., Posch, M., Sadzak, I., Ramsauer, K., Egger, G., Grausenburger, R., Schweifer, N., Chiocca, S., Decker, T., et al. (2006). Negative and positive regulation of gene expression by mouse histone deacetylase 1. Mol. Cell. Biol *26*, 7913-7928

193. Zwaferink, H., Stockinger, S., Reipert, S., and Decker, T. (2008). Stimulation of inducible nitric oxide synthase expression by beta interferon increases necrotic death of macrophages upon Listeria monocytogenes infection. Infect. Immun *76*, 1649-1656

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Farlik, M., Reutterer, B., Schindler, C., Greten, F., Vogl, C., Müller, M., and Decker, T. (2010). Nonconventional Initiation Complex Assembly by STAT and NF-kappaB Transcription Factors Regulates Nitric Oxide Synthase Expression. Immunity

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Farlik, M., Reutterer, B., Schindler, C., Greten, F., Vogl, C., Müller, M., and Decker, T. (2010). Nonconventional Initiation Complex Assembly by STAT and NF-kappaB Transcription Factors Regulates Nitric Oxide Synthase Expression. Immunity

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Farlik M., _Decker T._ (2009) COOPERATION OF STAT AND NFkB IN THE ASSEMBLY OF A TRANSCRIPTION COMPETENT INITIATION COMPLEX. Poster at the Cold Spring Harbor Meeting on Mechanisms of Eukaryotic gene expression 2009, New York, US.

Farlik M., _Decker T._ (2008) Chasing a murderer: Insights into the regulation of inducible nitric oxide synthase (iNOS) by Listeria monocytogenes. Poster at the SAB - Meeting at MFPL 2008, Vienna, AT.

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