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BRD4 Coordinates Recruitment of Pause Release Factor P-TEFb and the Pausing Complex NELF/DSIF To Regulate Transcription Elongation of Interferon-Stimulated Genes

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RNA polymerase II (Pol II) and the pausing complex, NELF and DSIF, are detected near the transcription start site (TSS) of many active and silent genes. Active transcription starts when the pause release factor P-TEFb is recruited to initiate productive elongation. However, the mechanism of P-TEFb recruitment and regulation of NELF/DSIF during transcription is not fully understood. We investigated this question in interferon (IFN)-stimulated transcription, focusing on BRD4, a BET family protein that interacts with P-TEFb. Besides P-TEFb, BRD4 binds to acetylated histones through the bromodomain. We found that BRD4 and P-TEFb, although not present prior to IFN treatment, were robustly recruited to IFN-stimulated genes (ISGs) after stimulation. Likewise, NELF and DSIF prior to stimulation were hardly detectable on ISGs, which were strongly recruited after IFN treatment. A shRNA-based knockdown assay of NELF revealed that it negatively regulates the passage of Pol II and DSIF across the ISGs during elongation, reducing total ISG transcript output. Analyses with a BRD4 small-molecule inhibitor showed that IFN-induced recruitment of P-TEFb and NELF/DSIF was under the control of BRD4. We suggest a model where BRD4 coordinates both positive and negative regulation of ISG elongation.

Genome-wide analyses of *Drosophila melanogaster* and mammalian cells have established that the majority of active and inactive genes are occupied by polymerase II (Pol II), paused at the promoter proximal region, and associated with the DRB sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) (1–7). Productive elongation begins with the recruitment of P-TEFb, a positive elongation factor that phosphorylates NELF and DSIF, causing pause release (3, 8, 9). P-TEFb also phosphorylates serine 2 of the Pol II C-terminal domain (CTD), launching mRNA elongation (8, 10–13). *In vitro* studies have shown that this phosphorylation prompts dissociation of NELF and conversion of DSIF from a negative to a positive elongation factor (11). Accordingly, genome-wide studies have shown that whereas NELF occupancy is mostly restricted to promoter proximal regions, DSIF is distributed over the coding regions (4). However, the modes of NELF occupancy and its functional significance have remained elusive, and the literature on this issue is varied and inconsistent. In *Drosophila* cells, NELF is released from the *hsp70* gene upon heat shock (14, 15). NELF has been shown to be transiently released from the *TNF*-α gene in response to inflammatory stimulation by lipopolysaccharides (LPS), although other LPS-induced genes are not associated with NELF (16). On the other hand, NELF is continuously bound to *JunB* before and after interleukin-6 stimulation (17). Moreover, NELF functions as a negative regulator in some cases; it has been reported to act as a positive regulator for other genes (13, 17–20). Another critical issue concerning the activity of the NELF/DSIF that has remained poorly understood is how P-TEFb is recruited upon activation. Although P-TEFb is recruited by DNA binding transcription factors in some genes, other genes do not seem to rely on DNA-specific factors to recruit P-TEFb (3, 4, 8). Rather, accumulating evidence indicates that the bromodomain protein BRD4 acts as a broad P-TEFb recruiting factor for many cellular and viral genes (21–25). BRD4, through the C-terminal extra terminal domain region, binds to the core complex of P-TEFb, cyclinT/CDK9 (24). At the same time, BRD4 binds to acetylated histones through its double bromodomain (26). We previously showed that BRD4 recruitment is triggered by increased histone acetylation at and near the transcription start site (TSS), which leads to transcription activation of many cell growth-controlling genes (27, 28). Others have shown that BRD4 plays a pivotal role in LPS-induced transcription of inflammatory genes, a conclusion supported by a recent study that utilized a small-molecule inhibitor specific for the bromodomain (23, 29). However, it is uncertain whether BRD4 plays a role in elongation itself, nor it is clear whether BRD4 affects the activity of NELF and DSIF. We studied these questions for interferon (IFN)-stimulated genes (ISGs), genes that are important for innate immunity and are activated through the JAK/STAT pathway (30–33). Upon IFN stimulation, ISG transcription starts rapidly without relying on new protein synthesis, although ISG induction is often slower than that of some LPS-induced proinflammatory genes (33, 34). Collectively, ISGs confer timely antiviral and antipathogen resistance upon the host. We show here that IFN stimulation triggers robust recruitment of BRD4, initiating a cascade of elongation factor recruitment involving P-TEFb, NELF, and DSIF. Our anal-

Received 29 August 2012 Returned for modification 5 December 2012
Accepted 10 April 2013
Published ahead of print 15 April 2013
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doi:10.1128/MCB.01180-12

ysis shows that the NELF/DSIF complex is recruited only after IFN stimulation and that NELF negatively regulates ISG transcription elongation. Together, BRD4 coordinates positive and negative regulation of ISG elongation, thereby fine-tuning the quantity and quality of ISG transcripts.

MATERIALS AND METHODS

Cells and treatment. NIH 3T3 cells were treated with 100 U/ml of murine recombinant IFN-β (PBL) for the indicated periods, with slight modifications (35). The small-molecule inhibitor for the BET bromodomains, (+)-JQ1, its stereoisomer, (−)-JQ1, 5, 6-dichlorobenzimidazone-1-β-D-ribofuranoside (DRB), or flavopiridol (the last two compounds from Sigma-Aldrich) was added 1 h before IFN treatment (36). IFN-induced ISG transcripts were detected for the nascent and mature RNA by quantitative reverse transcription-PCR (qRT-PCR) by using primers covering the exon/intron boundary or exons only, respectively (27, 35). RNA levels were normalized by *Gapdh* or *Hprt*. Information on the primers used for qRT-PCR is available upon request.

ChIP. Chromatin immunoprecipitation (ChIP) was performed essentially as described earlier (27). Briefly, antibodies (1 to 2 μg) for RNA polymerase II (8WG16 [Covance] and N-20 [Santa Cruz Biotechnology]), CDK9, pSTAT1, STAT2, and NELF-A (Santa Cruz Biotechnology), and H4Ac, H3K9Ac, H3K14Ac, and H3K4me3 (Millipore), histone H3 (Abcam), and SPT5 (BD Bioscience) were conjugated to 10 μl of Dynabeads bound to protein G (Invitrogen) for 4 h at 4°C. Anti-S2P Pol II IgM antibody (1 μg) was incubated with Dynabeads conjugated to rat anti-mouse IgM. Normal rabbit and mouse IgG or IgM (Millipore) were used as controls. Antibody-conjugated beads were incubated with chromatin preparations corresponding to 0.2×10^6 cells overnight at 4°C. Immunoprecipitated chromatin was washed extensively, treated with proteinase K, and de-cross-linked at 68°C. Recovered DNA was extracted by using phenol-chloroform and precipitated with ethanol and glycogen. Input DNA for individual samples was prepared from 2% of respective chromatin before precipitation. Immunoprecipitated DNA and input DNA were amplified with gene-specific primers by qPCR. The ChIP signal was calculated as follows: $2^{-CT(\mathrm{IP})}/2^{-CT(\mathrm{input})} \times 2$. Samples with control IgG were tested for each primer set, all of which gave values at least 10-fold lower than the values with specific antibody, with standard deviations (SD) of less than 0.016. Background signals by control IgG were reproducible between different primer sets, among samples, and in replicate experiments. For each experiment, ChIP signals by control IgG with the respective primer set for all the time points were averaged and used to normalize ChIP signals by a specific antibody. The normalized ChIP signals are expressed as the relative ChIP signal, unless stated otherwise (37). The information on the primers used for the ChIP assay is available on request.

shRNA for NELF-A and BRD4. Cells were tranfected with the SureSilencing small hairpin RNA (shRNA) plasmid for mouse Whsc2 (SABiosciences) or with a negative-control shRNA vector, according to the manufacturer's instructions. Cells were selected with puromycin for 36 h and used for experiments immediately thereafter. A retroviral BRD4 shRNA vector and viral transduction procedures have been described previously (28).

FRAP. NIH 3T3 cells were transfected with the indicated green fluorescent protein (GFP) fusion constructs using Lipofectamine LTX Plus (Invitrogen) for 16 to 20 h, followed by treatment with 1 μM (−)-JQ1 or (+)-JQ1 and 50 ng/ml of trichostatin A (TSA) for 4 h (26). Fluorescence recovery after photobleaching (FRAP) was performed using a Zeiss confocal LSM510 microscope attached to 488-nm laser and using a 100× oil immersion objective with a 1.3 numerical aperture (26). Moderately bright cells were selected for the FRAP assay. A circle of 25 pixels in diameter in the nucleus was bleached at 100% of argon laser power with 1 iteration, which retained about 20% of the initial fluorescence intensity. Fluorescence recovery was monitored every 44 ms for the next 33 s. Prebleach and recovery images were taken at 0.5% of laser power.

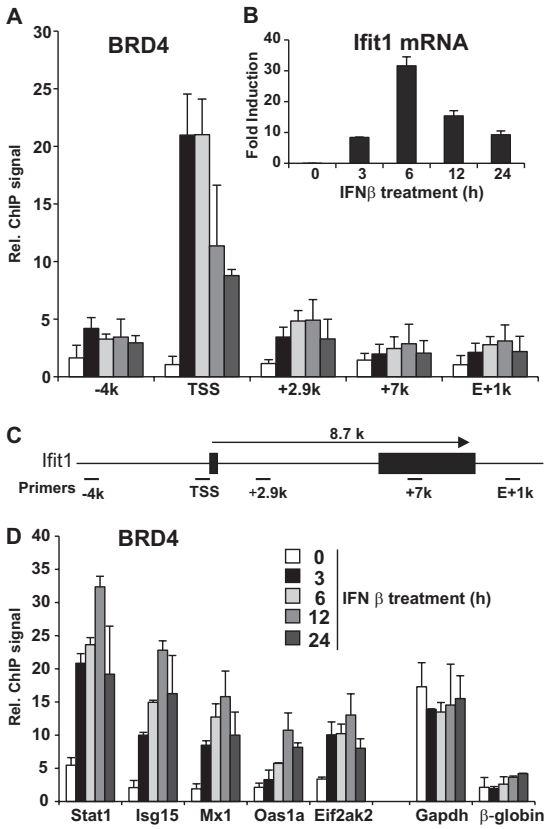

FIG 1 IFN-induced recruitment of BRD4 to ISGs. (A) NIH 3T3 cells were treated with 100 U/ml of IFN for the indicated times (in hours; see key in panel D). BRD4 binding to the indicated regions of *Ifit1* was analyzed by ChIP with an anti-BRD4 antibody. BRD4 binding was normalized against values obtained for control IgG. Values for all ChIP assays in this study represent the averages of three independent experiments ± SD. (B) The time course of *Ifit1* mRNA induction was assessed by qRT-PCR. Values for all mRNA data shown are averages of three independent experiments ± SD. (C) The exon-intron organization of *Ifit1*. Positions of the primers (in kb) used for ChIP are shown underneath. (D) Cells were treated with IFN as described for panel A, and recruitment of BRD4 to the TSS of the indicated ISGs was analyzed by ChIP. The *Gapdh* and β-*globin* genes were tested as examples of housekeeping genes and silent genes, respectively.

RESULTS

IFN-β triggers recruitment of BRD4 and P-TEFb to ISGs. To study whether BRD4 was recruited to ISGs, ChIP assays were performed with NIH 3T3 cells stimulated with IFN-β (referred to as IFN). BRD4 recruitment was tested at five different regions in the *Ifit1* gene, a typical ISG (Fig. 1C [gene map and location of ChIP primers]). The data in Fig. 1A showed that while BRD4 was undetectable before stimulation, it was recruited to the TSS of *Ifit1* gene within 3 h after IFN addition. BRD4 recruitment peaked at 3 h and 6 h and then declined at 12 h and 24 h. This kinetic profile mirrored that of *Ifit1* mRNA induction (Fig. 1B). IFN-induced BRD4 recruitment was mostly restricted to the TSS, although slight enrichment was also found in the kb −4 and kb +2.9 regions of the gene. Similar to *Ifit1*, other ISGs, including *Stat1*, *Isg15*, *Mx1*, *Oas1a*, and *Eif2ak2* also showed clear IFN-induced BRD4 recruitment in a TSS-restricted manner (Fig. 1D). The TSS-enriched BRD4 recruitment was also observed in cell cycle-regulated genes, although BRD4 is distributed intragenically in some

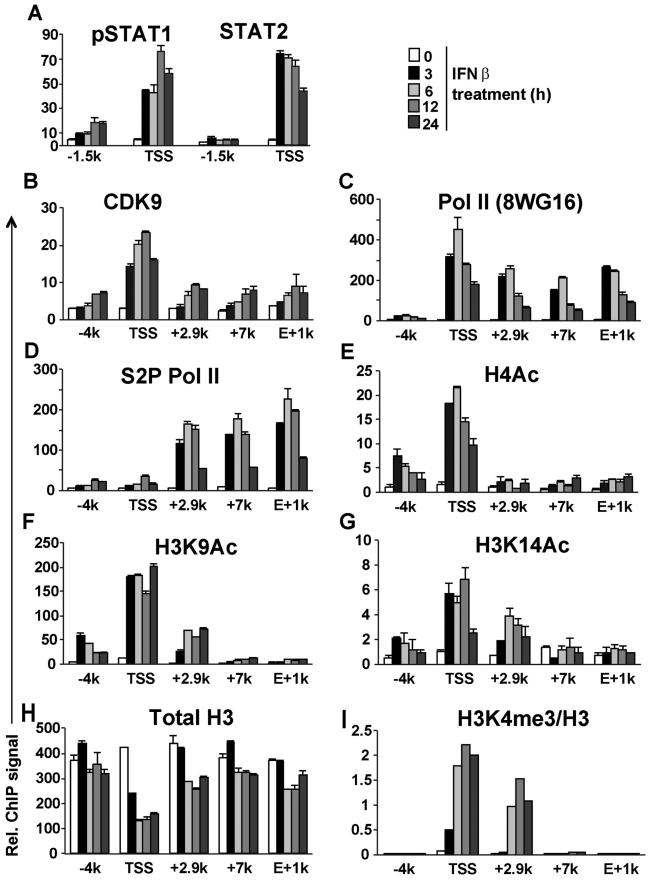

FIG 2 Recruitment of STAT1, P-TEFb, Pol II, and histone modifications on the *Ifit1* gene. (A to D) ChIP analysis was performed to detect binding of pSTAT1 and STAT2 (A), CDK9 (B), Pol II (8WG16) (C), and S2P Pol II (D) at the indicated site of the *Ifit1* gene in cells treated with IFN for the indicated times. (E to I) ChIP analysis was performed to detect acetylation of histone H4 (E), H3K9 (F), H3K14 (G), total H3 (H), and trimethylation of H3K4 (I) by using corresponding antibodies in cells treated with IFN as described above. Antibody for tetra-acetyl H4 was used to detect histone H4 acetylation. Trimethylation of H3K4 was normalized based on total H3 levels.

constitutively expressed genes (27). In contrast, BRD4 binding was essentially absent in the *Hbb-b1* (β-globin) gene, which is silent in NIH 3T3 cells, while BRD4 was constitutively bound to the housekeeping gene *Gapdh* (Fig. 1D).

BRD4 recruitment to *Ifit1* was concurrent with the binding of phosphorylated STAT1 (pSTAT1) and STAT2, indicating that BRD4 was recruited as a result of JAK/STAT pathway activation (30, 32, 33) (Fig. 2A). Pol II and CDK9, a core component of P-TEFb, were also recruited to *Ifit1* following IFN stimulation (Fig. 2B and C). ChIP assays for Pol II were performed with the 8WG16 antibody, which has been reported to detect unphosphorylated or hypophosphorylated Pol II and is referred to as Pol II (8WG16) (38, 39). Binding of Pol II (8WG16) was highest at the TSS. On the other hand, the elongating form of Pol II, phosphorylated at serine 2 in the CTD (S2P Pol II), showed increasingly greater binding in the coding region, peaking near the transcription end site, illustrating that Pol II traveled through the *Ifit1* gene body after IFN stimulation (Fig. 2D). Together, IFN triggered recruitment of BRD4 and P-TEFb, leading to productive ISG elongation. We found that upon IFN stimulation, acetylation of histones H3 and H4 increased predominantly at the TSS in the *Ifit1* gene, supporting the idea that induced binding of the BRD4 bromodomain to newly acetylated histones accounts for IFN-induced BRD4 recruitment to ISGs (Fig. 2E to G) (23, 27). On the other hand, the amount of total H3 at the *Ifit1* TSS was reduced by 50 to 60% following IFN stimulation, suggesting partial chromatin depletion, as noted in some active promoters (Fig. 2H) (40). Nevertheless, H3K4 trimethylation, a mark for active gene expression, was increased at the TSS and 5′-coding region after IFN stimulation (Fig. 2I). It is of note here that *Gapdh* not induced by IFN was used as a representative of housekeeping genes in all ChIP assays in this work. Figure 8C, below, shows that binding of BRD4, CDK9, Pol II (8WG16), S2P Pol II, NELF, DSIF, and acetyl histone levels were unchanged on *Gapdh* before and after IFN treatment.

NELF and DSIF are recruited to ISGs following IFN stimulation. It has been shown that NELF and DSIF are bound to Pol II paused at the promoter proximal region in many active and inactive genes (2–4, 7). Although the association of NELF/DSIF with Pol II prior to activation is well documented, the fate of NELF after transcriptional activation is less well understood. NELF is a complex of four subunits, NELF-A, -B, -C/D, and -E, while DSIF is a heterodimeric protein complex consisting of SPT4 and SPT5 subunits (5, 6). We examined binding of NELF and DSIF on ISGs by using antibodies for NELF-A and SPT5, respectively. Results for *Ifit1* and *Isg15* are shown in Fig. 3A to D. NELF binding was negligible prior to IFN stimulation but dramatically increased following IFN treatment at the TSS. A high level of NELF binding continued up to 24 h, even when ISG transcription declined (Fig. 3A and C). Likewise, binding of DSIF to ISGs was very low to undetectable before IFN treatment but greatly increased after IFN stimulation, and it remained high for 24 h (Fig. 3B and D). Unlike NELF, whose binding was confined to the TSS, DSIF binding was seen throughout the coding regions and at around the transcription end site. For Fig. 3A to D, the reported NELF/DSIF recruitment level is expressed after normalization with control IgG. To further investigate IFN-induced NELF/DSIF binding, we checked their recruitment to four additional ISGs. In Fig. 3E and F, the binding of specific antibodies is compared with that of control IgG. Similar to *Ifit1* and *Isg15*, we observed IFN-stimulated NELF/DSIF binding to these ISGs but not to *Gapdh*. Thus, induced recruitment is the major feature of NELF/DSIF for IFN-stimulated transcription, suggesting that this complex, defined as the pausing factor, may have a role beyond pausing prior to elongation.

NELF negatively regulates ISG transcription. In light of the IFN induced NELF recruitment observed above, we next investigated its functional significance by testing cells stably expressing an shRNA for NELF-A. This subunit is thought to anchor the NELF complex to Pol II (41). It has been shown that the stability of NELF subunits is dependent on the presence of other subunits, and knockdown of one subunit reduces the expression of other subunits (19, 42). We confirmed that expression of NELF-A shRNA reduced levels of NELF-A transcripts by about 80%, while control shRNA had no effect (Fig. 4A). Immunoblot analysis of extracts prepared with 180 and 420 mM salt concentrations showed that the NELF-A protein levels were significantly reduced in NELF knockdown cells compared to control cells (Fig. 4A). Data in Fig. 4B show that nascent mRNA levels of all ISGs tested were consistently higher in NELF knockdown cells than in control cells throughout the IFN treatment, including the initial activa-

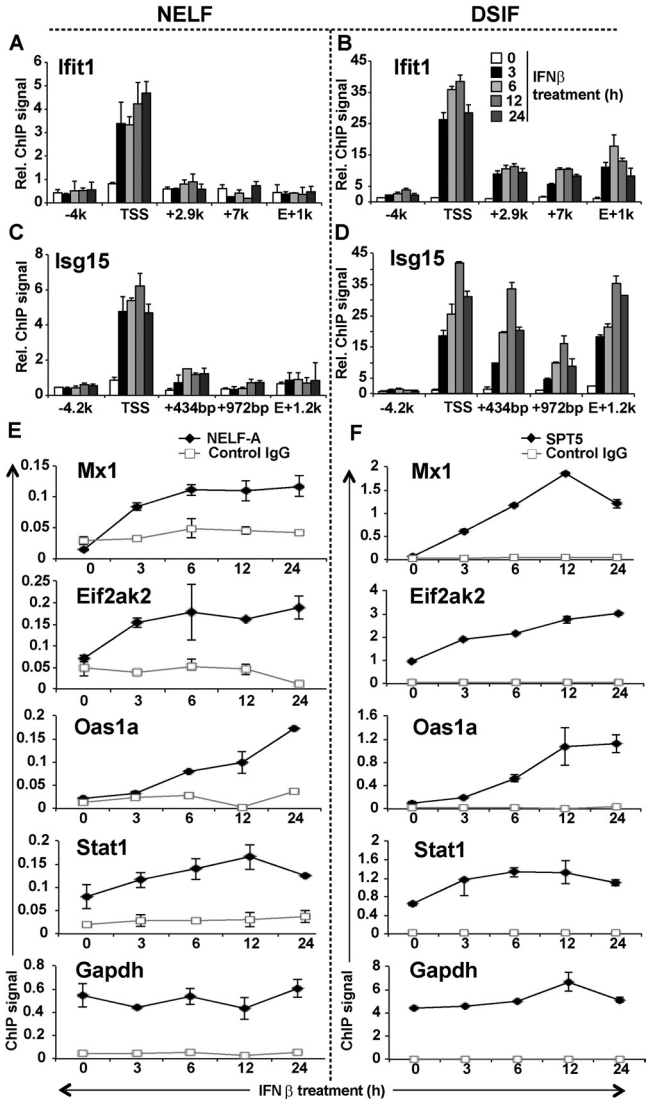

FIG 3 IFN-induced recruitment of NELF and DSIF to ISGs. (A to D) Recruitment of NELF and DSIF at the indicated regions of the *Ifit1* gene (A and B) and *Isg15* (C and D) in cells stimulated with IFN for the indicated times was analyzed by ChIP with antibodies against NELF-A and SPT5. Signals produced by NELF-A and SPT5 antibodies were normalized against those of the control IgG. (E and F) ChIP analysis was performed for the indicated ISGs and *Gapdh* at the TSS by using antibodies against NELF-A (E), SPT5 (F), and control IgG in cells treated with IFN for the indicated periods.

tion stage, the subsequent amplification phase, and the postactivation phase. It should be stated here that the kinetics and the pattern of ISG induction were virtually identical between the nascent and mature RNA following IFN stimulation (data not shown). In contrast, NELF knockdown had virtually no effect on *Gtf2b* and *Gapdh* transcript levels, indicating that it does not significantly impact expression of housekeeping genes. ChIP analysis (Fig. 4C) showed that IFN-stimulated NELF-A recruitment tested for *Ifit1*, *Isg15*, and *Mx1* was inhibited in NELF knockdown cells by 40 to 60% compared to control cells. These results strongly indicate that NELF negatively regulates a process in elongation during the course of transcription, resulting in increased ISG transcript output in NELF knockdown cells.

NELF inhibits binding of S2P Pol II and DSIF on the ISG coding regions. To test whether NELF knockdown impacted the recruitment of elongation factors, ChIP analysis was performed for DSIF, Pol II (8WG16), and S2P Pol II. As shown in Fig. 5A and B, binding levels of Pol II (8WG16) to the TSS and that of S2P Pol II to the coding region were consistently higher in NELF knockdown cells relative to control cells for the *Ifit1*, *Isg15*, and *Mx1* genes. These results suggested that NELF knockdown affected elongation and Pol II recruitment. To assess whether NELF knockdown affected binding of the elongating Pol II more significantly than Pol II, we normalized binding of S2P Pol II to that of total Pol II at the coding region. As shown in Fig. 5C, normalization of S2P Pol II signals by total Pol II confirmed that NELF knockdown increased binding of S2P Pol II to a greater extent than total Pol II at the coding region. Increased levels of Pol II (8WG16) at the TSS under the NELF knockdown condition may represent heightened reinitiation (43). Consistent with the increased S2Pol II binding on the gene body, DSIF binding was distinctly higher in NELF knockdown cells than in control cells at the coding region (Fig. 5D). Together, these data indicate that NELF impedes the movement of elongating Pol II and DSIF, thereby inhibiting binding of incoming Pol II to the TSS. The relatively modest effects seen with NELF knockdown may be attributed to the relatively modest inhibition of NELF-A recruitment achieved by the shRNA approach used here (Fig. 4C).

A small-molecule BRD4 inhibitor suppresses ISG induction. The data in Fig. 1 and 2 pointed to the idea that BRD4, recruited to the ISGs by virtue of binding to acetylated histones, plays a critical role in IFN-stimulated transcription. We thus investigated the role of BRD4 in ISG induction by testing a bromodomain inhibitor, JQ1 (36). This cell-permeable inhibitor interferes with the binding of acetylated histone tails to BET family bromodomains and has been shown to inhibit growth of several cancer cells (36, 44). A related compound, I-BET, has been shown to inhibit expression of LPS-induced inflammatory genes (29). To confirm that JQ1 inhibits BRD4-acetyl histone binding in live NIH 3T3 cells, we performed FRAP, in which binding of BRD4 to acetylated chromatin can be quantitatively measured (26, 45). Cells expressing GFP-tagged wild-type BRD4 or mutant BRD4 defective in acetyl histone binding (Y139A and Y433A) were treated with the active inhibitor (+)-JQ1, an inactive stereoisomer (−)-JQ1, or vehicle alone (dimethyl sulfoxide [DMSO]) in the presence of TSA for 4 h and tested for FRAP (Fig. 6A). In the presence of vehicle alone, GFP-BRD4 recovered slowly, showing only 50% recovery 30 s after photobleaching. FRAP in the presence of (−)-JQ1 gave virtually the identical recovery pattern. In contrast, GFP-BRD4 recovered much faster in the presence of (+)-JQ1, reaching 80% recovery within 10 s, consistent with previous observations in U2OS cells (36). The deletion of either of the bromodomain or specific point mutation in two bromodomains (Y139A and Y433A) showed a very rapid recovery irrespective of the inhibitor (Fig. 6A). The recovery pattern of wild-type BRD4 in the presence of (+)-JQ1 was very similar to that of the mutant. These results confirmed that (+)-JQ1, but not (−)-JQ1, inhibits bromodomain-dependent binding of BRD4 to acetylated chromatin *in vivo*. (+)-JQ1 also inhibited recovery of another BET family protein, BRD2, but it did not inhibit binding of bromodomain proteins from different subfamilies, such as BRG1, p300, and PCAF (Fig. 6A), consistent with the selectivity profile determined previously in purified protein assays (36).

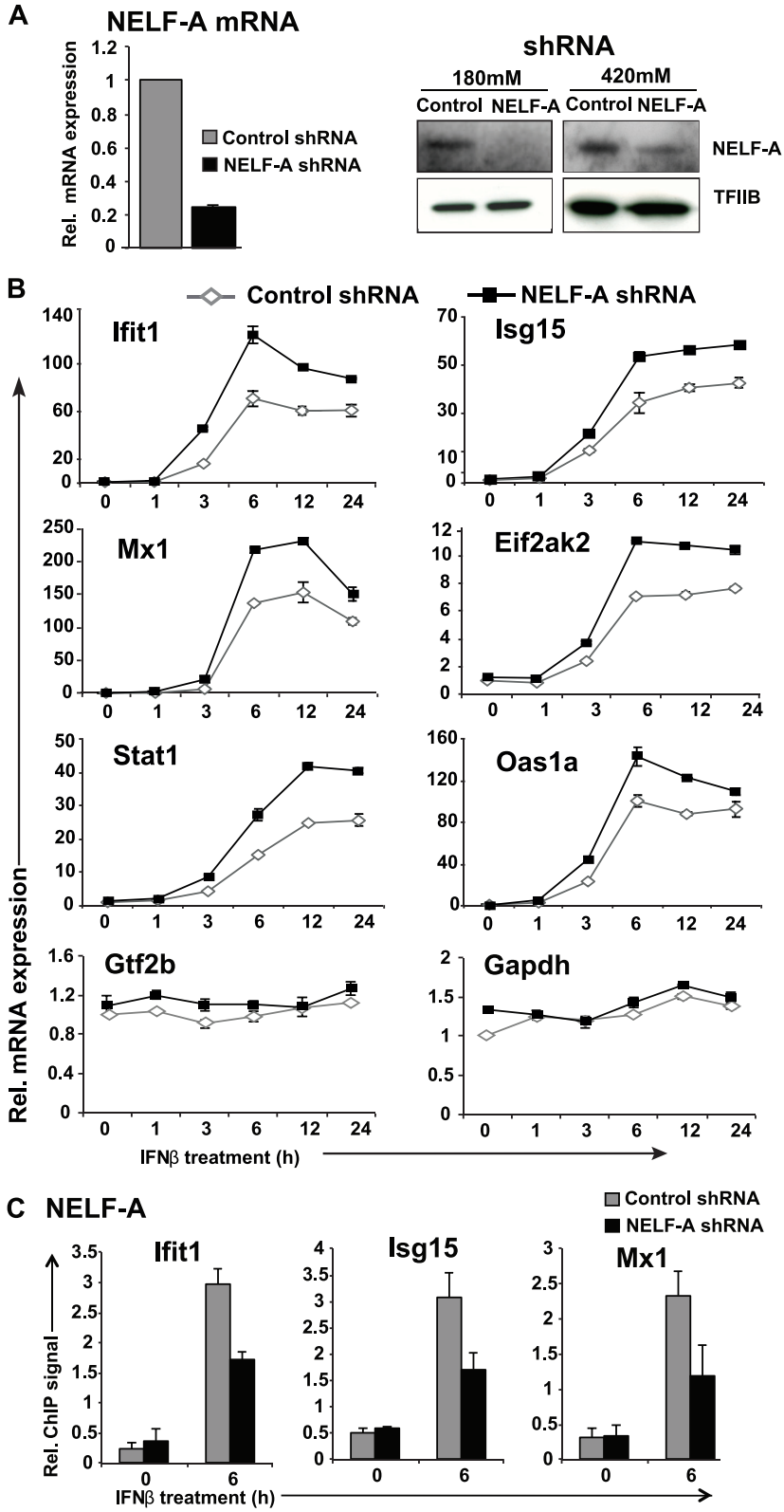

FIG 4 NELF knockdown increases ISG transcription. (A) Reduction of *NELF-A* mRNA and the protein by NELF-A shRNA. *NELF-A* transcripts in cells with control shRNA or NELF-A shRNA were quantified by qRT-PCR (left). Nuclear extracts from the above cells were extracted with buffers containing 180 mM or 420 mM NaCl. Extracts were immunoblotted with the indicated antibodies to detect NELF-A and TFIIB, used as a loading control (right). (B) Nascent mRNA levels of ISGs and *Gtf2b* were normalized against *Gadph*. Levels of *Gapdh* mRNA were normalized against *Hprt*. (C) ChIP analysis was performed for cells with control shRNA or NELF-A shRNA treated with IFN to detect binding of NELF-A at the TSS of the indicated ISGs.

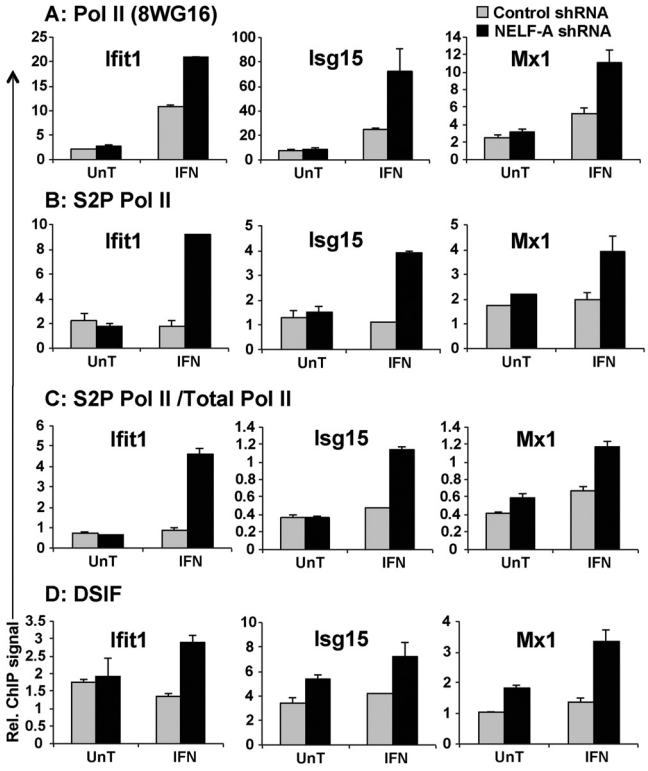

FIG 5 NELF knockdown increases binding of DSIF and S2P Pol II in the ISG gene body. Cells expressing NELF-A shRNA or control shRNA were either kept untreated (UnT) or treated with IFN for 1 h and analyzed for binding of Pol II (8WG16) to the TSS (A), S2P Pol II (B), S2P Pol II/total Pol II (C), and DSIF (D) to the coding region (kb +2.9, bp +434, and kb +3 for *Ifit1*, *Isg15*, and *Mx1*, respectively) of the indicated ISGs by ChIP. The ratios of S2P Pol II/total Pol II were calculated based on ChIP binding obtained with total Pol II (N-20 Ab) and S2P Pol II on the coding region.

Titration experiments were performed to determine the concentrations of JQ1 required for optimal inhibition of ISG mRNA induction and BRD4 recruitment. As shown in Fig. 6B and C, 10 μM (+)-JQ1 inhibited induction of all six ISG mRNAs by 70 to 80%, while the same concentration of (−)-JQ1 showed no inhibition. This was the case for both nascent and mature ISG mRNAs (data not shown). Increasing the (+)-JQ1 concentration to 30 μM did not change the levels of specific inhibition (data not shown). On the other hand, 1 μM (+)-JQ1 inhibited ISG induction by only approximately 40 to 50%, and 0.1 μM (+)-JQ1 gave no inhibition. (+)-JQ1 at the concentrations tested above did not inhibit expression of housekeeping genes, such as *Gtf2b*, *Gapdh*, *Brd4*, *Fbl10*, *HDAC1*, and *Hira.* Similarly, BRD4 recruitment was inhibited at 10 μM (+)-JQ1 by approximately 75%, while inhibition was only 30 to 50% at 1 μM and negligible at a 0.1 μM inhibitor concentration (Fig. 6D). Based on these results, (+)-JQ1 at 10 μM was judged to be an optimal inhibitor concentration for the above parameters and was used in all subsequent experiments. Because (−)-JQ1 gave essentially the same results as those obtained with vehicle alone, values obtained with (+)-JQ1 were compared with those for (−)-JQ1 for Fig. 6, 7, and 8.

We confirmed that (+)-JQ1 did not affect BRD4 protein expression in these cells, with or without IFN treatment (Fig. 6E). Furthermore, induction of pSTAT1 was unaffected by (+)-JQ1, indicating that the inhibitor did not affect the JAK-STAT signaling pathway (Fig. 6F). In sum, (+)-JQ1, by inhibiting the binding of BRD4 to acetylated histones, potently inhibits ISG induction. Because JQ1 can also inhibit binding of acetylated histones to other BET family members, it was of importance to further assess the potential role of other BET bromodomain proteins in ISG transcription. To this end, we tested BRD4 knockdown cells, where BRD4 protein expression was reduced by more than 85% (27, 28). The results in Fig. 6G show that BRD4 knockdown led to marked inhibition of ISG mRNA induction.

JQ1 blocks the factor recruitment cascade. In view of multiple factors recruited to ISGs upon IFN stimulation, we next sought to delineate the hierarchical order of the recruitment of these factors. The ChIP analysis results in Fig. 7A show that IFN-induced recruitment of BRD4 was almost completely eliminated by (+)-JQ1 but not by (−)-JQ1 in *Ifit1*, *Isg15*, and *Mx1*. In addition, JQ1 strongly inhibited recruitment of P-TEFb, as evidenced by reduced CDK9 binding (Fig. 7B). JQ1 also inhibited binding of Pol II (8WG16) at the TSS (Fig. 7C). In agreement with our results, I-BET has been reported to inhibit recruitment of Pol II to the promoter of LPS-induced genes (29). Even more importantly, JQ1 was also effective in inhibiting binding of S2P Pol II to the coding region of ISGs but not to *Gapdh* (Fig. 7D; see also Fig. 8C below). The degree of inhibition appeared greater for S2P Pol II than Pol II (8WG16). This differential inhibition was more clearly observed when binding of S2P Pol II was normalized to that of total Pol II (detected with the N-20 Ab) (Fig. 7E). Moreover, JQ1, when tested at a lower concentration (1 μM), selectively inhibited binding of Pol II (8WG16) in the coding region, without changing binding of Pol II (8WG16) at the TSS (Fig. 7H and data not shown). This concentration of JQ1 was still active in inhibiting ISG mRNA induction (Fig. 6B). However, at a higher JQ1 concentration (10 μM), at which ISG transcription is more robustly inhibited, binding levels of S2P Pol II and Pol II (8WG16) were both inhibited (Fig. 7C and D). We observed very similar dose-dependent differential inhibition by flavopiridol, a CDK9 inhibitor. A 100 nM concentration of flavopiridol selectively inhibited binding of Pol II (8WG16) in the coding region with no change in binding of Pol II (8WG16) at the TSS (Fig. 7I and data not shown). This concentration of flavopiridol was capable of inhibiting *Isg15* transcription (Fig. 7J). Mimicking the effect of JQ1 at a higher concentration, flavopiridol at 300 nM greatly inhibited binding of Pol II (8WG16) at the TSS (Fig. 7K). These results indicate that both JQ1 and flavopiridol target the elongation step for inhibition, and at higher concentrations, where transcription is more potently inhibited, they inhibit binding of Pol II (8WG16) at the TSS. This may be because at higher concentrations the inhibitors inhibit elongation more strongly, leading to a secondary inhibition of transcription reinitiation (43, 46). Together, these results support a model in which JQ1 inhibits primarily the elongation step. In keeping with these results, BRD4 knockdown strongly inhibited recruitment of P-TEFb and binding of S2P Pol II to the *Ifit1* gene (Fig. 7L). Thus, BRD4, among other BET family proteins, likely plays a major role in ISG elongation, which is attributed to its ability to recruit P-TEFb.

We then asked whether JQ1 inhibits BRD4 recruitment by altering ISG histone acetylation states. I-BET has been reported to alter the status of histone acetylation for some LPS-stimulated genes (29). As shown in Fig. 7F and G, acetylation of H3 and H4 on ISGs was unaffected by JQ1 treatment under these settings. Our results are in agreement with previous findings, in which BRD4

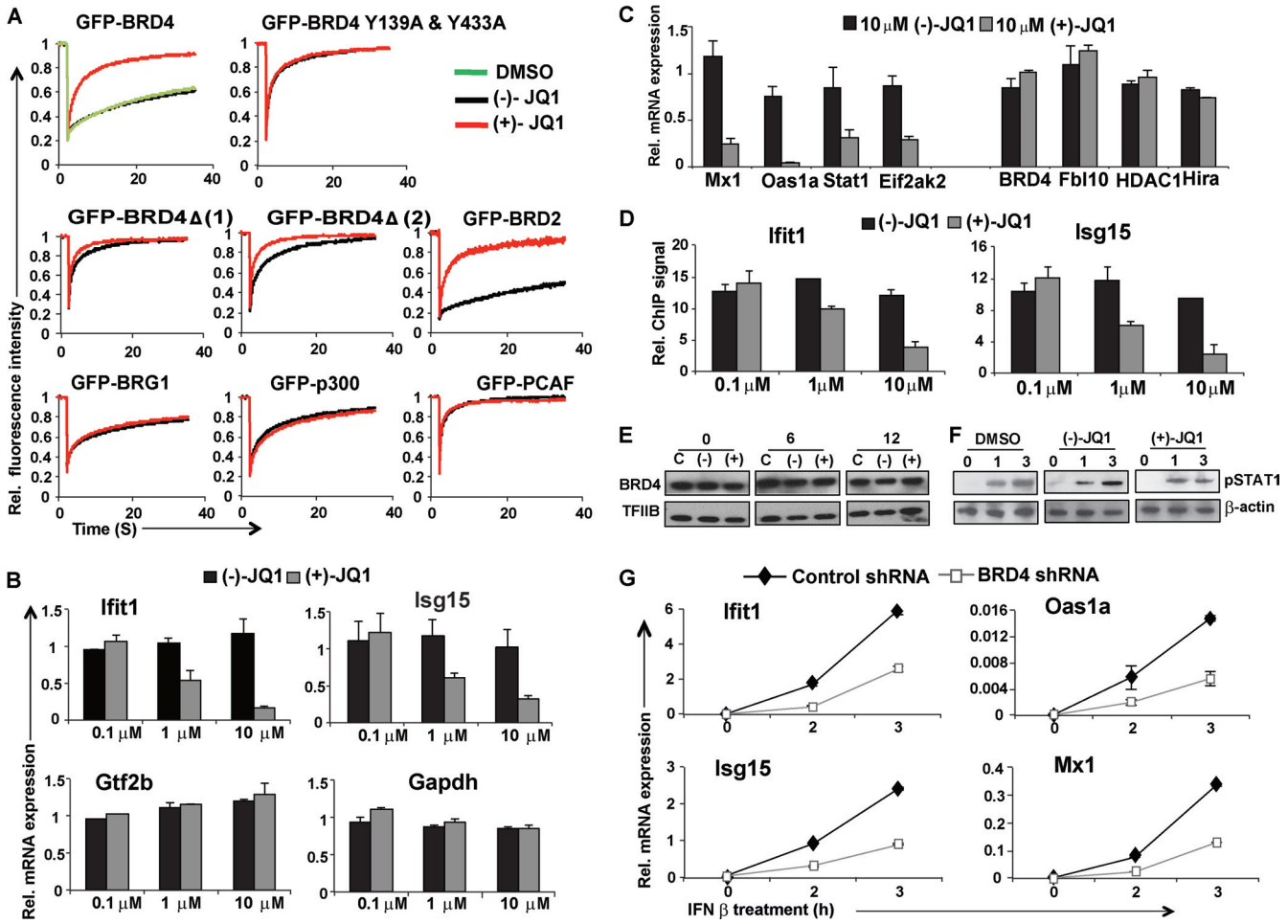

FIG 6 Specificity and effect of JQ1 on ISG induction. (A) FRAP was performed for cells expressing GFP-tagged BRD4 constructs, BRD4 deletions, PCAF, BRG1, p300, and BRD2, which were treated with 1 μM (−)-JQ1, (+)-JQ1, or vehicle (DMSO) in the presence of TSA for 4 h. The graphs represent values averaged from 12 to 15 independent measurements of different cells. (B and C) Cells were treated with 0.1 μM, 1 μM, or 10 μM (−)-JQ1, (+)-JQ1, or DMSO (B) or with 10 μM (−)-JQ1, (+)-JQ1, or DMSO (C) for 1 h, followed by stimulation with IFN for 6 h. mRNA levels of the indicated ISGs and housekeeping genes were normalized against *Gapdh*, while *Gapdh* mRNA levels were normalized against *Hprt* values. Values for (−)-JQ1 and (+)-JQ1 were normalized based on the value for the DMSO control data set. (D) Binding of BRD4 to the TSS of *Ifit1* and *Isg15* was analyzed by ChIP for cells treated with 0.1 μM, 1 μM or 10 μM (−)-JQ1, or (+)-JQ1 for 1 h followed by IFN treatment for 6 h. (E and F) Cells were treated with 10 μM (−)-JQ1, (+)-JQ1, or DMSO for 1 h, followed by stimulation with IFN for the indicated times. Nuclear extracts from these cells were immunoblotted with antibody against BRD4 or TFIIB (E) or antibody against STAT1 phosphorylated at tyrosine 701 or against β-actin (F). (G) Effect of BRD4 knockdown on ISG induction. Cells were transduced with control or BRD4 shRNA vectors and treated with IFN. Induction of ISG mRNA was measured by qRT-PCR. Transcripts levels were normalized against *Gapdh* values.

knockdown had no effect on histone acetylation of cell cycle-regulated genes (27, 28).

Given the established role of NELF/DSIF to hold Pol II near the TSS before the arrival of P-TEFb, our observation that NELF and DSIF were recruited to ISGs only after IFN stimulation was somewhat unexpected. We examined the possibility that recruitment of NELF/DSIF to ISGs is under the control of BRD4. As shown in Fig. 8A and B, recruitment of both factors to ISGs was greatly inhibited by JQ1, although JQ1 did not inhibit constitutive binding of NELF/DSIF to *Gapdh* (Fig. 8C). Consistent with this result, JQ1 did not inhibit binding of BRD4, CDK9, or either form of Pol II to *Gapdh* (Fig. 8C). These results indicate that inducible transcription is more susceptible to JQ1 than constitutive transcription. This may be because inducible transcription often relies on rapid changes in BRD4-acetyl histone interactions.

Furthermore, IFN-induced binding of NELF and DSIF was potently inhibited by the kinase inhibitors DRB and flavopiridol, both of which target P-TEFb (Fig. 8A and B and data not shown) (47, 48). These inhibitors had no effects on binding of NELF and DSIF to *Gapdh*, as reported before (49). To delineate the order of factor recruitment, we examined binding of BRD4, CDK9, NELF, and DSIF at an early time point, 1.5 h after IFN treatment. As shown in Fig. 8D, all factors were recruited to the ISGs even at this early time point, making it difficult to dissect the sequence of factor binding. However, when early factor recruitment was tested in the presence of flavopiridol, binding of CDK9, NELF, and DSIF was substantially lower than that without the inhibitor. In contrast, BRD4 recruitment was not significantly affected by flavopiridol. These results illustrate an IFN-triggered binding cascade by which recruitment of BRD4 initiates binding of P-TEFb, which in turn leads to the subsequent binding of NELF and DSIF (Fig. 8E, model).

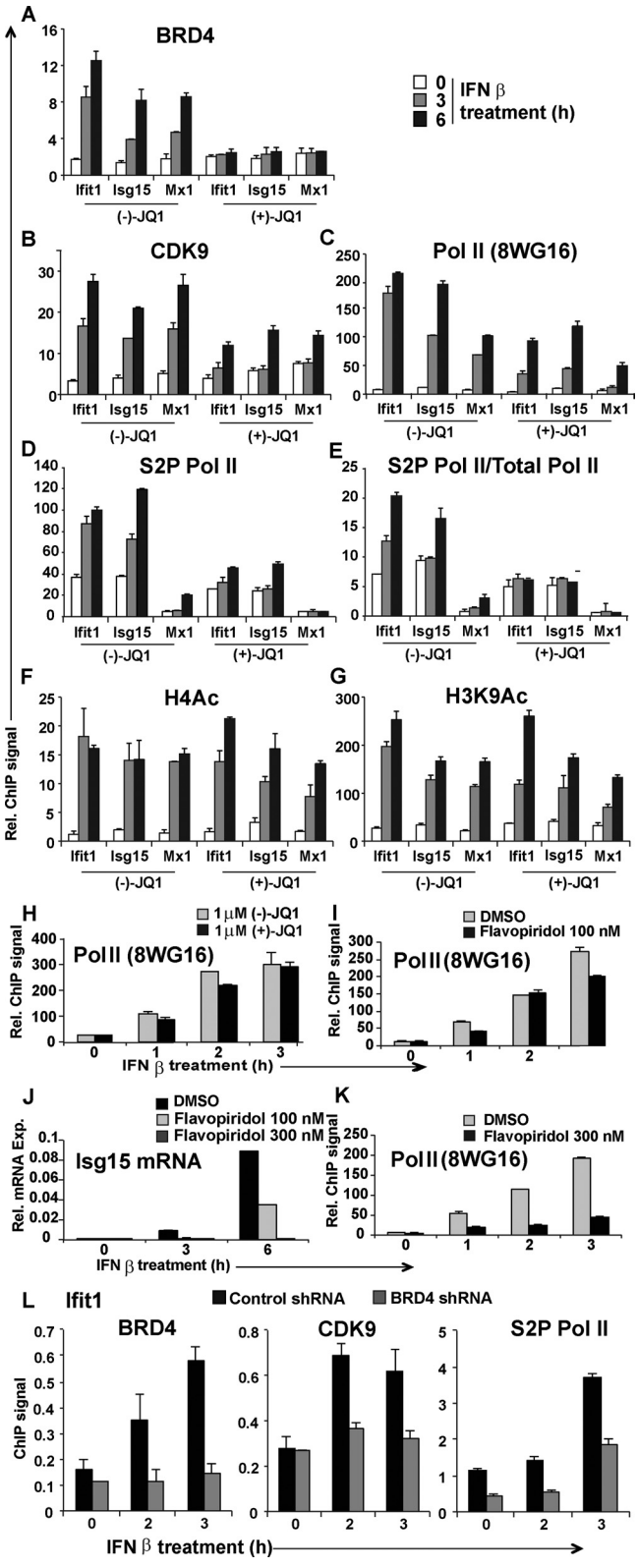

FIG 7 JQ1 inhibits recruitment of BRD4 and P-TEFb and impedes ISG transcription elongation. (A to G) Binding of BRD4 (A), CDK9 (B), and Pol II (8WG16) (C) to the TSS and binding of S2P Pol II (D) and the S2P Pol II/total Pol II ratio (E) to the coding region (kb +7, bp +972, and kb +15 of *Ifit1*, *Isg15*, and *Mx1*, respectively) of the indicated ISGs was analyzed by ChIP for cells treated with 10 μM (−)-JQ1 or (+)-JQ1 and IFN. Acetylation of histoneH3 (F) and H4 (G) at the TSS of the indicated ISGs was analyzed by ChIP with antibody against tetra-acetyl H4 or H3K9Ac in cells treated with (−)-JQ1 or (+)-JQ1 and IFN, as described above. (H to K) Concentration-dependent inhibition of *Isg15* mRNA and Pol II (8WG16) binding by JQ1 and flavopiridol. (H, I, and K) Cells were treated with the indicated concentrations of JQ1 (H) or flavopiridol (I and K), followed by IFN treatment. ChIP analysis was performed for Pol II (8WG16) at the TSS of *Isg15*. (J) Cells were treated with the indicated concentrations of flavopiridol, followed by IFN stimulation for 3 and 6 h. Induction of *Isg15* mRNA was measured by qRT-PCR. Transcript levels were normalized against *Gapdh* values. (L) ChIP analysis was performed for BRD4, CDK9 at the TSS, and S2P Pol II at the coding region of *Ifit1* (kb +7) for cells expressing control shRNA or the BRD4 shRNA vector.

DISCUSSION

Upon IFN stimulation, BRD4 was recruited to the TSS of ISG. We showed that BRD4 recruitment is a causal event that initiates the recruitment of P-TEFb and NELF/DSIF to ISGs to direct ISG transcription elongation (Fig. 8E). An interesting observation made in this study is that the pausing complex NELF/DSIF was hardly present on the ISGs prior to stimulation, but both factors were robustly recruited after IFN treatment. These results differ from many earlier reports that described that NELF is engaged prior to activation and in some cases released after activation (14, 16, 17). Our observations unravel a previously less-appreciated mode of NELF/DSIF action, where this complex, absent prior to stimulation, is recruited after transcription activation. These results point to a role for NELF during elongation, rather than a role solely for Pol II pausing prior to activation. Supporting this view, NELF and DSIF were shown to remain on *JunB* after transcriptional activation (17). NELF has also been shown to be recruited to the estrogen-responsive genes upon estrogen treatment, although it was reported to be present on the genes prior to activation as well (18, 50). The mode of NELF/DSIF binding may be dependent on how transcription is activated. Unlike "poised" genes, such as *hsp70* and *TNF*-α, that are transcribed immediately after stimulation, the majority of ISGs are transcribed with somewhat slower kinetics. Nevertheless, ISG transcription activated by JAK/STAT signaling occurs without requiring *de novo* protein synthesis (34). Given that a wide range of genes are stimulated by JAK/STAT pathways in addition to ISGs, regulating cell growth, development, and responses to various external signals, it is reasonable to assume that the pattern of NELF/DSIF occupancy revealed in this study may represent a fairly common feature (30, 31, 33, 51).

Analysis with NELF shRNA showed that NELF is a negative regulator of ISG transcription, since larger amounts of ISG transcripts were produced in NELF knockdown cells than in control shRNA cells. Indeed, NELF knockdown caused a sustained increase in ISG mRNA levels, affecting the course of ISG transcription, from the early induction phase to the middle amplification stage and the late declining phase. In line with this result, ChIP analysis showed that NELF persisted on ISGs even after transcription ceased. Moreover, NELF knockdown increased binding of DSIF and S2P Pol II to the ISG coding regions, indicating that NELF, while stationed at the TSS, may restrict the movement of elongating Pol II and DSIF across the gene body, restraining transcription elongation. NELF may negatively regulate elongation by imposing a short-lived Pol II pausing at the 5′-promoter-proximal region and inhibiting recruitment of the RNA capping enzyme (52). By so doing, NELF may also limit transcription reini-

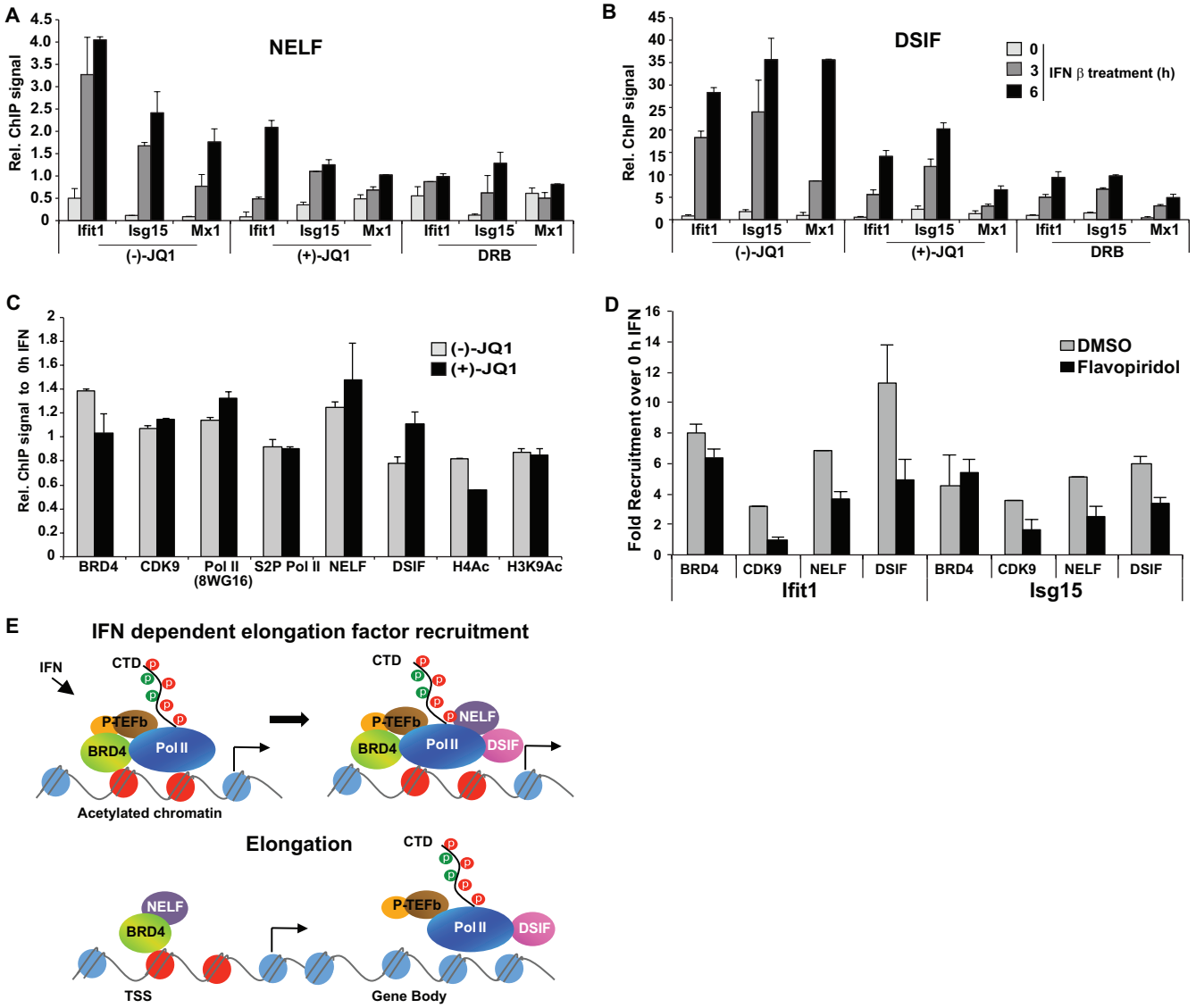

FIG 8 Recruitment of the NELF/DSIF complex to the ISGs requires BRD4 and P-TEFb. (A and B) Recruitment of NELF (A) and DSIF (B) to the TSS of the indicated ISGs was analyzed by ChIP with antibodies against NELF-A and SPT5 for cells treated with 10 μM (−)-JQ1, (+)-JQ1, or 100 μM DRB for 1 h, followed by IFN stimulation. (C) JQ1 does not affect factor recruitment to the *Gapdh* gene. Cells were treated with 10 μM (−)-JQ1 or (+)-JQ1 for 1 h, followed by IFN stimulation for 6 h. ChIP analysis was performed to test the binding of BRD4, CDK9, Pol II (8WG16), NELF, DSIF, H4Ac, and H3K9Ac at the TSS and S2P Pol II at the coding region (kb +3.7) of *Gapdh*. ChIP binding was normalized against the values obtained from 0 h of IFN treatment. (D) Cells were treated with IFN for 1.5 h in the presence or absence of 300 nM flavopiridol, and binding of BRD4, CDK9, NELF, and DSIF to the TSS of *Ifit1* and *Isg15* was tested by ChIP. ChIP binding is represented as the fold recruitment over the 0-h IFN treatment. (E) A model for the IFN-triggered cascade of factor recruitment. Upon the IFN-triggered increase in histone acetylation, BRD4 is recruited to the TSS of ISGs, bringing P-TEFb along. This leads to the recruitment of NELF/DSIF, to proceed through elongation. During this period, BRD4 and NELF remain at the TSS, while other factors travel through the ISG gene body along with S2P Pol II.

tiation and reduce cycles of ISG transcription, the view supported by increased binding of Pol II (8WG16) at the TSS in NELF knockdown cells. Apart from 5′-promoter-proximal pausing during transcription elongation, it is possible that NELF works by an additional mechanism to repress transcription. It is possible that NELF-mediated negative regulation contributes to the processing of newly synthesized transcripts, including splicing and degradation, to help ensure the quality of mature ISG transcripts.

Analysis with JQ1, a small molecule inhibitor for bromodomains of the BET family, allowed us to delineate a chain of events that govern ISG transcription. JQ1 blocked not only BRD4 recruitment but also the entire cascade of elongation factor recruitment to ISGs, including that of NELF/DSIF, causing a potent inhibition of ISG transcription. Because JQ1 targets binding of acetylated histones to the BET bromodomains, it was evident that BRD4 recruitment took place as a result of increase in histone acetylation in ISGs, which then prompted the recruitment of downstream factors. Given that P-TEFb recruitment was also inhibited by JQ1, it is clear that P-TEFb recruitment followed that of BRD4. BRD4 facilitated P-TEFb recruitment most likely through a direct physical interaction, as BRD4 stably binds to the P-TEFb core subunits, CDK9 and cyclin T (24, 25). Since this BRD4–P-

TEFb interaction is mediated by the C-terminal motif unique to BRD4 among other BET members, it is safe to conclude that JQ1 inhibition of P-TEFb recruitment is, for the most part, accounted for by inhibition of BRD4 binding to ISGs (21). The proposition that BRD4, but not other BET members, plays a predominant role in ISG transcription was further supported by the marked inhibition of ISG transcription observed in BRD4 knockdown cells.

The observation that JQ1 inhibited binding of not only S2P Pol II but also Pol II (8WG16) suggests that BRD4 has a role in the initial recruitment of Pol II. However, results from a number of additional experiments contrast with this possibility. Instead, they indicate that BRD4 primarily regulates elongation and that the inhibition of Pol II binding by JQ1 is due to secondary inhibition of reinitiation, rather than inhibition of initial activation, since (i) JQ1 inhibited S2P Pol II binding to a greater extent than that of Pol II (8WG16) (Fig. 7C to E); (ii) at a lower concentration of JQ1 (1 μM), where ISG transcription is partially inhibited, only the binding of Pol II (8WG16) to the coding region, and not at the TSS, was inhibited (Fig. 7H); (iii) the CDK9 inhibitor that blocks elongation gave very similar inhibition profiles as JQ1 (Fig. 7I and K); and conversely, (iv) NELF knockdown led to the opposite results (Fig. 5A to C). Further, the results that IFN-induced binding of NELF/DSIF was inhibited by JQ1 as well as CDK9 inhibitors place NELF/DSIF recruitment downstream of BRD4 and P-TEFb recruitment. The results that flavopiridol inhibited early binding of CDK9, NELF, and DSIF, but not BRD4 recruitment, further support the above-suggested chain of binding events. It should be noted, however, that P-TEFb may be recruited to some genes through specific transcription factors, such as c-Myc, where activity of the NELF/DSIF complex may be regulated in a BRD4-independent manner (4, 8, 53). Nevertheless, in view of the ubiquitous and highly abundant expression of BRD4 observed in cells from early embryos to many specialized cells, a BRD4-initiated elongation cascade may have a broad regulatory role in diverse physiological and pathological processes.

In summary, BRD4, bound to ISGs through acetylated histones, recruits P-TEFb and NELF/DSIF and orchestrates positive and negative transcription elongation of ISGs. The chain of events outlined in this work may control signal-dependent transcription of many other genes.

ACKNOWLEDGMENTS

We thank D. Singer and T. Kanno for critical reading of the manuscript. We thank J. McNally and T. Karpova from Center for Cancer Research, LRBGE, Fluorescence Imaging Facility, NIH, for their assistance with FRAP experiments.

This work was supported by the Intramural Program of NICHD and the NIH Intramural AIDS Targeted Antiviral Program, National Institutes of Health.

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