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Bromodomain-containing protein 4–independent transcriptional activation by autoimmune regulator (AIRE) and NF-κB

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Autoimmune regulator (AIRE) and nuclear factor-κB (NF-κB) are transcription factors (TFs) that direct the expression of individual genes and gene clusters. Bromodomain-containing protein 4 (BRD4) is an epigenetic regulator that recognizes and binds to acetylated histones. BRD4 also has been reported to promote interactions between the positive transcription elongation factor b (P-TEFb) and AIRE or P-TEFb and NF-κB subunit p65. Here, we report that AIRE and p65 bind to P-TEFb independently of BRD4. JQ1, a compound that disrupts interactions between BRD4 and acetylated proteins, does not decrease transcriptional activities of AIRE or p65. Moreover, siRNA-mediated inactivation of BRD4 alone or in combination with JQ1 had no effects on AIRE- and NF-κB–targeted genes on plasmids and in chromatin and on interactions between P-TEFb and AIRE or NF-κB. Finally, ChIP experiments revealed that recruitment of P-TEFb to AIRE or p65 to transcription complexes was independent of BRD4. We conclude that direct interactions between AIRE, NF-κB, and P-TEFb result in efficient transcription of their target genes.

Transcription is the fundamental step for gene expression. Generally, mammalian RNA polymerase II (RNAP II)[2] is engaged at most promoters, and promoter clearance occurs when the C-terminal domain of RNAP II is phosphorylated on serines at position 5 (Ser-5) in the heptapeptide repeats by the cyclin-dependent kinase 7 (CDK7) from the general transcription factor TFIIH (1, 2). At the majority of these genes, the negative elongation factor (NELF) and DRB sensitivity–inducing factor (DSIF) pause the RNAP II after transcribing 20–100 nucleotides (3, 4). To release RNAP II for productive elongation, the positive transcription elongation factor b (P-TEFb) is required. P-TEFb consists of cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CycT1) or CycT2 (5). CDK9 phosphorylates subunits of NELF and DSIF and serines at position 2 (Ser-2) in the C-terminal domain of RNAP II (6). Thus, the recruitment of P-TEFb is critical for the transition of RNAP II from promoter-proximal pausing to productive elongation (7).

The autoimmune regulator (AIRE) plays a critical role in regulating central tolerance in the thymus. It directs the expression of otherwise tissue-specific antigens (TSAs) in medullary thymic epithelial cells (mTECs) (8). The absence of or mutations of AIRE lead to the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), also called autoimmune polyglandular syndrome type 1 (APS1) (9). Detailed molecular mechanisms of AIRE-directed transcription have been explored extensively in the past few years. AIRE is recruited to the stalled RNAP II on TSA promoters by binding to unmodified histone H3 Lys-4 and DNA-dependent protein kinase (10, 11). AIRE then binds to P-TEFb. Phosphorylation and ubiquitylation of AIRE can increase its transcriptional activities (12). Furthermore, the ectopic expression of AIRE in human embryonic kidney (HEK) 293T cells and in mouse 1C6 mTEC cells also activates TSA genes (13).

Nuclear factor-κB (NF-κB) is a transcription factor that is activated by a variety of stimuli and plays critical roles in cell survival and proliferation (14). p65, which forms a heterodimer with p50, is the most active member of the mammalian NF-κB family (15). Phosphorylation and acetylation of p65 are required for its nuclear translocation and transcriptional activation (16, 17). After binding to the indicated promoters, p65 also recruits P-TEFb to activate the transcription of its target genes (18).

Bromodomain-containing protein 4 (BRD4) belongs to the BET family proteins with two N-terminal bromodomains and an extraterminal (ET) domain. BRD4 is distributed on chromatin by binding to acetylated histones H3 and H4 (19). BRD4 also helps to maintain the chromatin structure and facilitates communications between distal elements (20, 21). Depletion of BRD4 or its inactivation causes profound effects on cellular viability (22). Additionally, BRD4 binds to P-TEFb mainly through its C-terminal domain (23). The chemical inhibitor JQ1 interrupts interactions between BRD4 and chromatin, especially acetylated histones. JQ1 is a bromodomain inhibitor that has direct anti-tumor and anti-angiogenic properties

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[2] The abbreviations used are: RNAP II, RNA polymerase II; NELF, negative elongation factor; DSIF, DRB sensitivity–inducing factor; AIRE, autoimmune regulator; BRD4, bromodomain-containing protein 4; P-TEFb, positive transcription elongation factor b; TSA, tissue-specific antigen; mTEC, medullary thymic epithelial cell; HEK, human embryonic kidney; WB, Western blot; UAS, upstream activating sequence; Dox, doxycycline; TNFα, tumor necrosis factor α; TF, transcription factor; RIPA, radioimmune precipitation assay; qPCR, quantitative PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

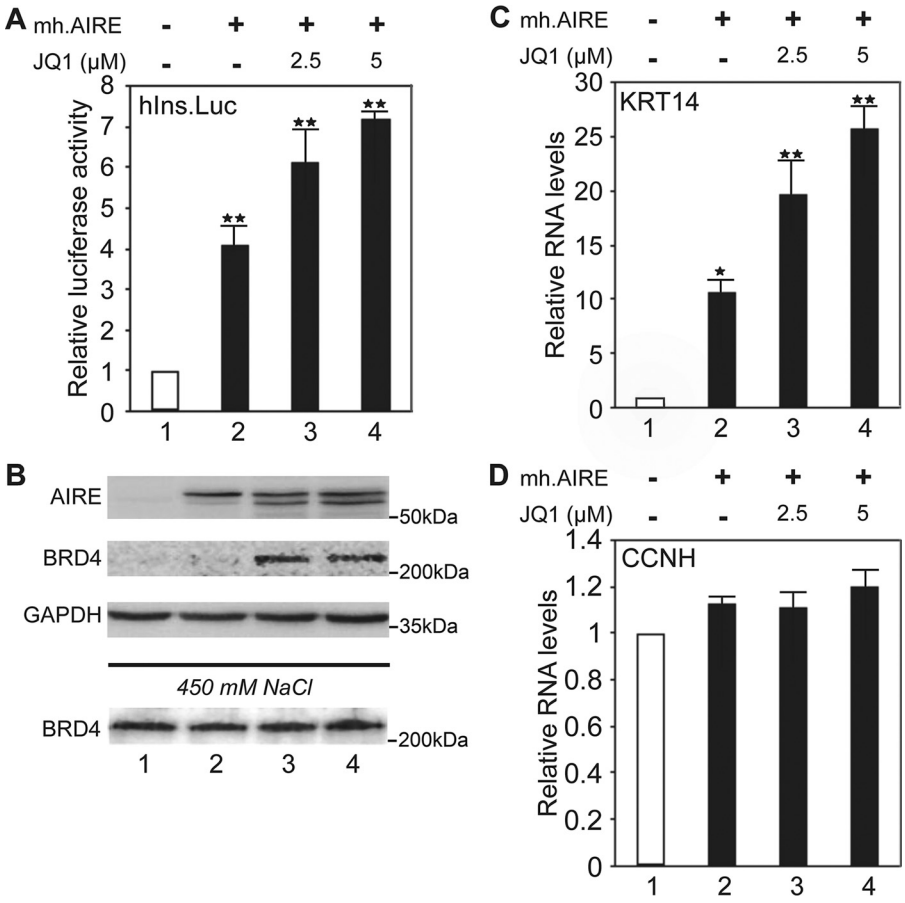


Figure 1. Inhibition of BRD4 by JQ1 does not affect transcriptional activity of AIRE. *A*, AIRE activates the human insulin promoter in the presence of JQ1. Relative luciferase activity of hIns.Luc in the presence of AIRE only (*bar 2*) or with JQ1 (*bars 3* and *4*) is presented as -fold activation above levels of the empty vector alone (*bar 1*). *B*, levels of proteins are presented in WBs. Note that free BRD4 is released from chromatin only after the addition of JQ1 (RIPA buffer, *second panel*, *lanes 3* and *4*). Levels of total BRD4 (released with 450 mM NaCl) are found in the *bottom panel below* the *black line*. GAPDH was used as the loading control (*third panel*). *C*, JQ1 does not affect the induction of an AIRE-responsive gene in chromatin. Relative RNA levels of *KRT14* induced by AIRE without (*bar 2*) or with increasing amounts of JQ1 (*bars 3* and *4*) are presented as -fold activation above levels of the empty vector alone (*bar 1*). *D*, neither AIRE nor JQ1 affect the expression of an AIRE-nonresponsive gene. Relative RNA levels of *CCNH* are presented as in *C*. For *A*, *C*, and *D*, *error bars* represent S.E., $n = 3$. Statistical significance is indicated by *asterisks* (paired *t* test; *, $p < 0.05$; **, $p < 0.01$).

(24). As reported, JQ1 blocks interactions between BRD4 and *C-MYC* enhancer and promoter, which leads to the reduction of *C-MYC* gene expression (25).

Although BRD4 binds to P-TEFb, its exact roles in regulating transcription are still controversial. Nevertheless, it has been suggested that the transcriptional activation by AIRE or p65 is dependent on BRD4, which bridges interactions between P-TEFb and acetylated AIRE or p65 proteins (26, 27). However, a recent study revealed that the removal of BRD4 does not affect the recruitment of CycT1 or CDK9 to DNA (28). In this study, we found that BRD4 is also dispensable for effects of AIRE and p65 on a variety of target genes. Furthermore, depletion of BRD4 did not disrupt interactions between AIRE or p65 and P-TEFb. Finally, the recruitment of P-TEFb to AIRE or p65 on DNA was independent of BRD4. Thus, transcriptional activities by AIRE and p65 on promoters are independent of BRD4.

Results

Inhibition of BRD4 does not decrease transcriptional activities of AIRE

Primary mTECs are difficult to isolate and exist in small numbers (<50,000) in thymi of neonatal mice. They grow slowly in culture, where they lose rapidly the expression of AIRE. Thus, not only do surviving populations lack AIRE; many have differentiated into other cell types (9). Importantly, studies of transcriptional effects of AIRE have relied on other cell types (*e.g.* 293T cells), where the ectopic expression of AIRE also activates TSA genes. Although it also impairs their growth, effects of AIRE can be studied by transient expression assays or inducibly in these and other cell lines (29).

JQ1 is an inhibitor of bromodomain proteins that dissociates BRD4 from chromatin. To determine the exact role of BRD4 in the transcriptional activity of AIRE, we treated cells transiently expressing AIRE (Myc, His epitope–tagged AIRE, mh.AIRE) or the empty vector (pcDNA3.1) with JQ1. These 293T cells also co-expressed the human insulin promoter linked to the luciferase reporter gene (hIns.Luc). The insulin gene is a *bona fide* target of AIRE. Cells were treated with increasing amounts of JQ1 (0, 2.5, and 5 μM). Compared with the empty vector, AIRE increased levels of luciferase 4.1-fold (Fig. 1*A*, *bars 1* and *2*). Furthermore, JQ1 did not decrease this AIRE-mediated activation of the insulin promoter (Fig. 1*A*, *bars 3* and *4*). Levels of AIRE are presented in the Western blot (WB) in Fig. 1*B* (*top panel*). The distribution of free BRD4 could be correlated to the

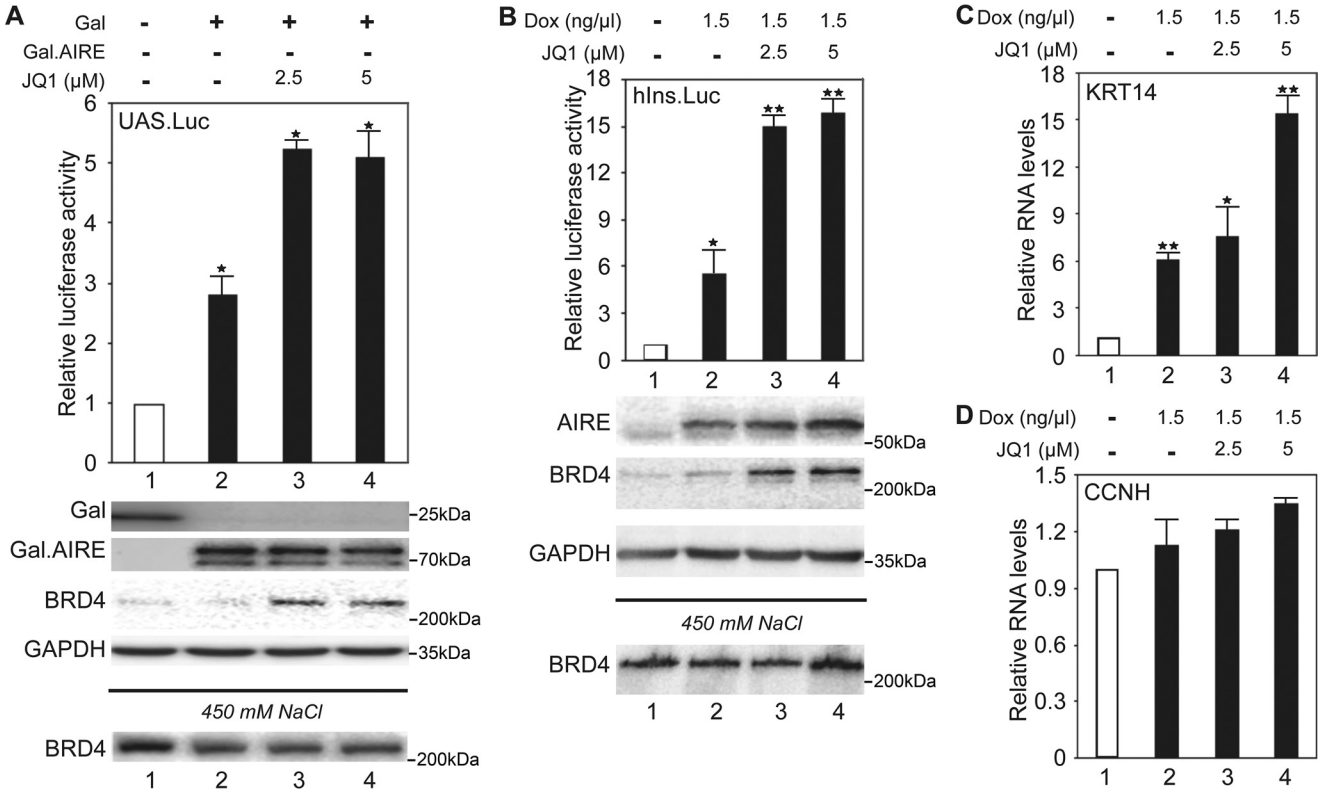


Figure 2. Gal.AIRE and Dox-induced AIRE function independently of BRD4. *A*, Gal.AIRE activates ectopic UAS.Luc in the absence and presence of JQ1. Relative luciferase activity of UAS.Luc for Gal.AIRE without (*bar 2*) or with increasing amounts of JQ1 (*bars 3* and *4*) is presented as -fold activation above levels of Gal alone (*bar 1*). Levels of Gal (*top panel*), Gal.AIRE (*second panel*), BRD4, and GAPDH are presented in WBs *below* the *bar graph*. Levels of released (*third panel*, *lanes 3* and *4*), and total BRD4 (*bottom panel*, *below* the *black line*) are presented. *B*, Dox-induced AIRE also activates hIns.Luc independently of JQ1 (*bars 2–4*). Levels of AIRE (*lanes 2–4*), BRD4, and GAPDH are presented *below* the *bar graph*. Levels of released and total BRD4 are as in *A*. *C*, Dox-induced AIRE also activates the expression of the *KRT14* gene (AIRE-responsive) irrespective of JQ1 (*bars 2–4*). *D*, Dox-induced AIRE has no effect on *CCNH* gene (AIRE-nonresponsive). Relative RNA levels of *CCNH* are presented by the same analyses and conditions as in *C*. For *A–D*, *error bars* represent S.E., $n = 3$. Statistical significance is indicated by *asterisks* (paired *t* test; *, $p < 0.05$; **, $p < 0.01$).

JQ1 treatment, which released BRD4 from chromatin (Fig. 1*B*, *second panel*, low-salt extraction, 150 mM NaCl). Levels of total BRD4 remained unchanged (Fig. 1*B*, *bottom panel below* the *black line*, high-salt extraction, 450 mM NaCl). These WBs reveal that most if not all BRD4 is tightly bound to chromatin, which is not the case with AIRE or GAPDH. In addition to the ectopic insulin promoter, AIRE also induced endogenous target gene expression in cells. AIRE activated the *KRT14* gene (AIRE-responsive) 11-fold compared with the empty vector (Fig. 1*C*, *bars 1* and *2*), and JQ1 slightly enhanced this induction (Fig. 1*C*, *bars 3* and *4*). AIRE and JQ1 had no effect on the *CCNH* gene (AIRE-nonresponsive) (Fig. 1*D*). Thus, despite profound effects on the distribution of BRD4, its inhibition caused no effect on AIRE-activated transcription.

To demonstrate further that AIRE activates transcription independently of BRD4, we used the Gal-UAS.Luc system. As Gal binds to upstream activating sequences (UASs), chimeric proteins containing Gal and target proteins can be used to study their transcriptional activation domains independently of their cognate DNA-binding activities (30, 31). Gal.AIRE or Gal (containing just the DNA-binding domain of Gal) and UAS.Luc were co-expressed in 293T cells in the absence or presence of JQ1. Similar to Fig. 1*A*, Gal.AIRE increased the luciferase activity 2.8-fold compared with Gal, which was also not affected by JQ1 (Fig. 2*A*, *top bar graph*). As in Fig. 1*B*, protein levels are presented in the *bottom panels* (Fig. 2*A*, *WB*). Taken together, the inhibition of BRD4 by JQ1 also does not inhibit the function of Gal.AIRE.

In addition to transient transfection assays, we also examined the 293.iAIRE cell line, where the expression of AIRE is induced by doxycycline (Dox). Upon the addition of Dox to these cells, AIRE increased the luciferase activity of hIns.Luc 5.8-fold, and the addition of JQ1 even increased this induction (Fig. 2*B*, *top bar graph*). As in Fig. 1*B*, protein levels are presented in the *bottom panels* (Fig. 2*B*, *WB*). The *KRT14* gene (AIRE-responsive) was also activated by AIRE independently of JQ1 (Fig. 2*C*). The *CCNH* gene (AIRE-nonresponsive) did not respond to AIRE or JQ1 (Fig. 2*D*). Taken together, in all systems, the transcriptional activity of AIRE is independent of BRD4.

BRD4 knockdown does not affect the function of AIRE

To further confirm the dispensability of BRD4 for transcriptional activity of AIRE, BRD4-targeting siRNAs (siBRD4) were used to deplete levels of endogenous BRD4 protein, with scrambled siRNAs (siScr) as the control. AIRE's function was measured by studying AIRE-dependent promoters with siBRD4 alone or in combination with JQ1. After introducing siBRD4 or siScr, mh.AIRE or empty vector and hIns.Luc plasmids were co-expressed in 293T cells. JQ1 was added at increasing amounts (0, 2, and 5 μM). siBRD4 effectively knocked down endogenous BRD4 levels compared with siScr (Fig. 3*B*, *second*

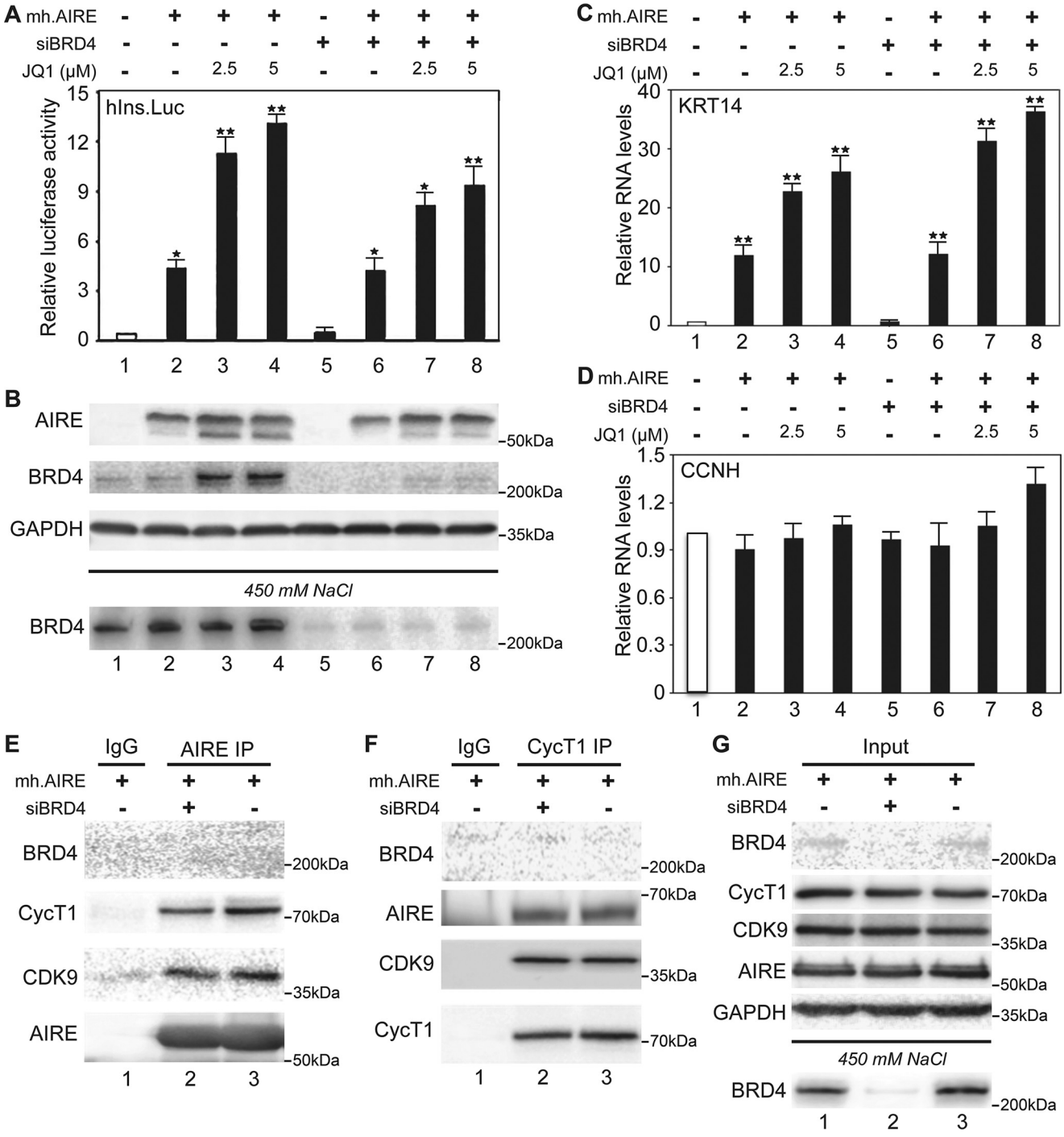


Figure 3. Depletion of BRD4 via siBRD4 and JQ1 does not affect the transcriptional activity of AIRE. *A*, AIRE activates the human insulin promoter despite the depletion and inhibition of BRD4. Relative luciferase activities from hIns.Luc in the presence of mh.AIRE alone (*bars 1–4*) and with depleted BRD4 (*bars 5–8*), absent JQ1 (*bars 1*, *2*, *5*, and *6*), and increasing amounts of JQ1 (*bars 3*, *4*, *7*, and *8*) are presented as -fold activation above levels of the empty vector alone (*bar 1*). *B*, levels of informative proteins are presented in WBs. Levels of mh.AIRE (*top panel*), released BRD4 (*second panel*), total BRD4 (*bottom panel*, *below* the *black line*), and GAPDH (*third panel*) are presented. *C*, AIRE activates the *KRT14* gene (AIRE-responsive) independently of BRD4. Relative RNA levels of *KRT14* in the presence of mh.AIRE alone (*bars 1–4*) or with siBRD4 (*bars 5–8*) and increasing amounts of JQ1 (*bars 3*, *4*, *7*, and *8*) or in the absence of JQ1 (*bars 1*, *2*, *5*, and *6*) are presented as -fold activation above levels of the empty vector alone (*bar 1*). *D*, the *CCNH* gene (AIRE-nonresponsive) is not affected by AIRE or BRD4. Relative RNA levels are presented by the same analyses and conditions as in *C*. *E–G*, AIRE interacts with P-TEFb directly under physiological conditions. For AIRE immunoprecipitations (*E*), interactions between the ectopically expressed AIRE (*bottom panel*) and endogenous BRD4, CycT1, and CDK9 proteins (*top* to *third panels*) are presented in the presence or absence of siBRD4, compared with the IgG control (*lane 1*). CycT1 immunoprecipitations (*F*) were performed under the same conditions. Interactions between endogenous CycT1 (*bottom panel*) and BRD4, AIRE, and CDK9 (*top* to *third panels*) proteins are presented. *G*, WBs reveal levels of input proteins for *E* and *F*. Levels of total BRD4 (450 mM NaCl extraction) are presented in the *bottom panel below* the *black line*. For *A*, *C*, and *D*, *error bars* represent S.E., $n = 3$. Statistical significance is indicated by *asterisks* (paired *t* test; *, $p < 0.05$; **, $p < 0.01$).

and *bottom panels*). The induction of hIns.Luc by AIRE was not affected by depletion of BRD4 (Fig. 3*A*, compare *bars 2* and *6* with *bars 1* and *5*). Furthermore, the addition of JQ1 to siScr or siBRD4 did not affect AIRE-mediated activation of hIns.Luc (Fig. 3*A*, *bars 3*, *4*, *7*, and *8*). Also, the expression of AIRE did not change (Fig. 3*B*, *top panel*). In addition to hIns.Luc activation, AIRE also induced *KRT14* gene (AIRE-responsive) expression 13-fold (Fig. 3*C*, compare *bars 2* and *6* with *bars 1* and *5*), and JQ1 did not decrease this induction in the presence of siBRD4 (Fig. 3*C*, *bars 3*, *4*, *7*, and *8*). Again, levels of the *CCNH* gene (AIRE-nonresponsive) did not change under the same conditions (Fig. 3*D*). Thus, the combined depletion and inhibition of BRD4 by siBRD4 and JQ1 also did not affect the transcriptional activity of AIRE.

Under physiological conditions, BRD4 is tightly bound to chromatin. Thus, lysing cells in the presence of low salt (150 mM NaCl) does not liberate any BRD4 into solution. Nevertheless, interactions between AIRE and P-TEFb were unchanged in our co-immunoprecipitations (Fig. 3, *E* and *F*, *bottom three panels*, *lanes 2* and *3*). In addition, neither AIRE nor P-TEFb interacted with BRD4 under the same conditions (Fig. 3, *E* and *F*, *top panel*, *lanes 2* and *3*). Inputs for these two co-immunoprecipitations are also presented (Fig. 3*G*). This finding confirms our previous studies, where AIRE interacted directly with P-TEFb in co-immunoprecipitations under low-salt conditions in 1C6 cells and GST pulldown *in vitro* (10).

Depletion of BRD4 does not decrease transcriptional activity of p65

Similar conditions were used to study effects of BRD4 depletion on the function of p65. We co-expressed Gal.p65 or Gal and UAS.Luc in 293T cells and treated cells with JQ1 as above. Gal.p65 increased luciferase activity 6-fold compared with Gal (Fig. 4*A*, *bars 1* and *2*). JQ1 did not decrease this activation by Gal.p65 (Fig. 4*A*, *bars 3* and *4*). Levels of Gal and Gal.p65 are presented in Fig. 4*A* (*WB*, *top two panels*). Again, JQ1 released BRD4 from chromatin and levels of total BRD4 remained constant (Fig. 4*A*, *WB*, *third* and *bottom panels below* the *bar graph*).

Additionally, siBRD4 was also introduced into these cells. After expressing siBRD4 or siScr, Gal.p65 or Gal and UAS.Luc were co-expressed in 293T cells. Cells were treated with JQ1 as above. The depletion of BRD4 did not decrease the induction of UAS.Luc by Gal.p65 (Fig. 4*B*, *bars 2* and *6*). Consistently, JQ1 did not decrease transcriptional activation of UAS.Luc by Gal.p65 in the presence of siBRD4 (Fig. 4*A*, *top bar graph*, *bars 3*, *4*, *7*, and *8*). Levels of Gal and Gal.p65 are presented in Fig. 4*B* (*WB*, *top two panels*), and siBRD4 effectively depleted BRD4 (Fig. 4*B*, *WB*, *third* and *bottom panels below* the *bar graph*). Furthermore, under the same conditions, ectopic p65 protein induced tumor necrosis factor α *(TNFα*, p65-responsive) gene 5-fold, irrespective of BRD4 (Fig. 4*C*, compare *bars 2* and *6* with *bars 1* and *5*). JQ1 did not decrease this induction by p65 in the presence of siBRD4 (Fig. 4*C*, *bars 3*, *4*, *7*, and *8*). The *CCNH* gene (p65-nonresponsive) remained unchanged under the same conditions (Fig. 4*D*). Thus, the depletion of BRD4 does not affect the transcriptional activity of p65.

In addition to the ectopic expression of p65, we also investigated whether the depletion of BRD4 could decrease the expression of TNFα-induced NF-κB–responsive genes. After depleting BRD4 by siBRD4, cells were treated with 15 ng/ml TNFα for 2 h. The induction level of *IL-8* gene (NF-κB–responsive) and another two genes (*A20* and *TNFα*; data not shown) was not decreased by BRD4 depletion (Fig. 4*E*, compare *bars 2* and *4* with *bars 1* and *3*). Levels of the *CCNH* gene (NF-κB–nonresponsive) did not change under the same conditions (Fig. 4*F*). Taken together, BRD4 depletion does not affect the transcriptional activity of NF-κB.

Like AIRE and P-TEFb, p65 also interacts directly with P-TEFb under low-salt conditions in cells and GST pulldown *in vitro* (18). We repeated these studies with identical results (data not shown). We conclude that AIRE and NF-κB do not require BRD4 for their interactions with P-TEFb.

Complete inactivation of BRD4 does not disrupt interactions between P-TEFb and AIRE or p65

To investigate whether BRD4 mediates interactions between P-TEFb and AIRE under nonphysiological conditions, co-immunoprecipitations were performed in the presence or absence of siBRD4 and JQ1. After lysing cells in high salt (450 mM NaCl), these lysates were diluted to low-salt conditions (150 mM NaCl). Again, in the presence or absence of BRD4, AIRE co-immunoprecipitated equivalently with endogenous CycT1 and CDK9, but not with BRD4 (Fig. 5*A*, *top three panels*, *lanes 2–5*), compared with the IgG control (Fig. 5*A*, *lane 1*). Under the same conditions, CycT1 co-immunoprecipitated with CDK9 and AIRE compared with IgG control (Fig. 5*B*, *top three panels*, *lanes 2–5*). Consistent with previous reports (32), CycT1 interacted with BRD4, and JQ1 enhanced this interaction (Fig. 5*B*, *top*, *lanes 3* and *5*). Inputs for these two co-immunoprecipitations are also presented (Fig. 5*C*). Similar studies were performed for interactions between P-TEFb and p65. p65 also co-immunoprecipitated with endogenous CycT1 and CDK9, but not with BRD4 (Fig. 5*D*, *top three panels*, *lanes 2–5*), compared with the IgG control (Fig. 5*D*, *lane 1*). CycT1 co-immunoprecipitated with CDK9 and p65 (Fig. 5*E*, *top three panels*, *lanes 2–5*), compared with the IgG control (Fig. 5*E*, *lane 1*). As above, CycT1 also interacted with BRD4, which was enhanced by JQ1 (Fig. 5*E*, *top*, compare *lanes 3* and *5* with *lanes 2* and *4*). Inputs for these two co-immunoprecipitations are also presented (Fig. 5*F*). Thus, the complete inactivation of BRD4 does not disrupt interactions between P-TEFb and AIRE or p65.

Recruitment of P-TEFb to AIRE- or p65-responsive promoters is independent of BRD4

To determine whether AIRE and p65 could recruit P-TEFb to promoters of target genes independently of BRD4, ChIPs of P-TEFb, AIRE, and p65 were performed. To examine the recruitment of P-TEFb to AIRE-responsive genes, AIRE was expressed transiently after siBRD4 or siScr had been introduced into 293T cells. ChIPs were performed on *KRT14* and *S100A8* promoters (AIRE-responsive) (Fig. 6, *A* and *B*, *bars 1–8*) as well as on the *IL-8* promoter (AIRE-nonresponsive) (Fig. 6, *A* and *B*, *bars 9–12*) with appropriate antibodies. CycT1 and AIRE binding was enriched in the same position of *KRT14* and *S100A8*

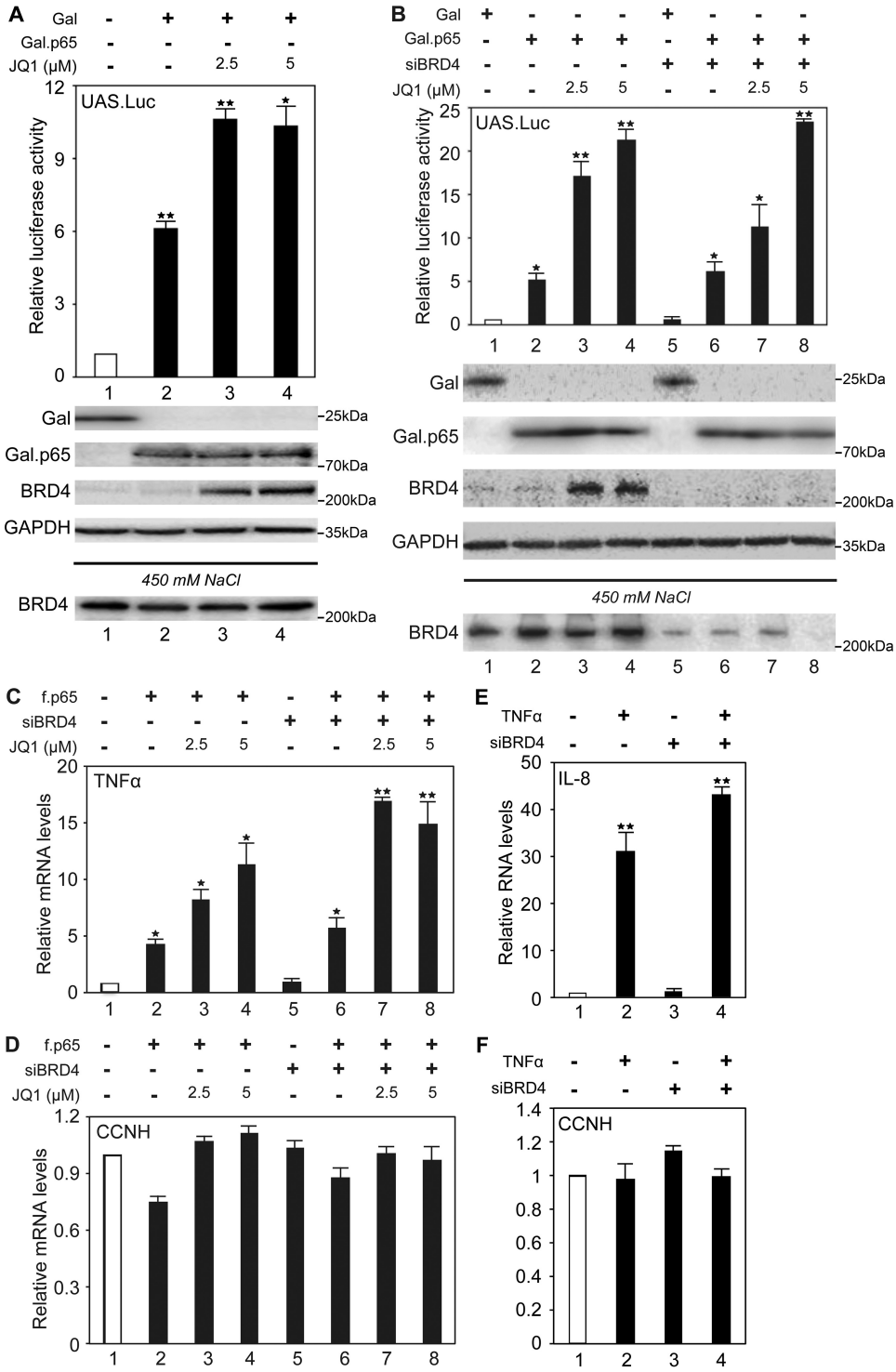


Figure 4. Depletion of BRD4 has no effect on transcriptional activity of p65. *A*, JQ1 does not decrease the ability of Gal.p65 chimera to activate the ectopic UAS.Luc. Similar conditions and analyses as for Gal.AIRE (Fig. 2) are presented. Levels of Gal, Gal.p65, BRD4, and GAPDH are given in WBs *below* the *bar graph*. *B*, depleting and inhibiting BRD4 by siBRD4 and JQ1 do not interfere with transcriptional activation of UAS.Luc by Gal.p65. Relative luciferase activities of UAS.Luc with Gal.p65 alone (*bars 1–4*) or with BRD4 depletion (*bars 5–8*) in the absence (*bars 3* and *4*) or presence (*bars 7* and *8*) of JQ1 are presented as -fold activation above levels of the empty vector alone (*bar 1*). Levels of proteins are presented in WBs *below* the *bar graph*. *C*, removal of BRD4 does not affect the activation of the *TNFα* gene (NF-κB–responsive) by p65. Relative RNA levels of TNFα in the presence of p65 alone (*bars 1-4*) or with depletion of BRD4 (*bars 5–8*) and increasing amounts of JQ1 (*bars 3*, *4*, *7*, and *8*) or in the absence of JQ1 (*bars 1*, *2*, *5*, and *6*), are presented as -fold activation above levels of the empty vector alone (*bar 1*). *D*, the *CCNH* gene (NF-κB–nonresponsive) is not affected by p65 or BRD4. Relative RNA levels of *CCNH* are presented by the same analyses and conditions as in *C*. *E* and *F*, depletion of BRD4 by siBRD4 has no effect on TNFα responses of an endogenous gene. Relative RNA levels of *IL-8* (*E*, NF-κB–responsive) and *CCNH* (*F*, NF-κB–nonresponsive) are presented as in *C* and *D*. For *A–F*, *error bars* represent S.E., $n = 3$. Statistical significance is indicated by *asterisks* (paired *t* test; *, $p < 0.05$; **, $p < 0.01$).

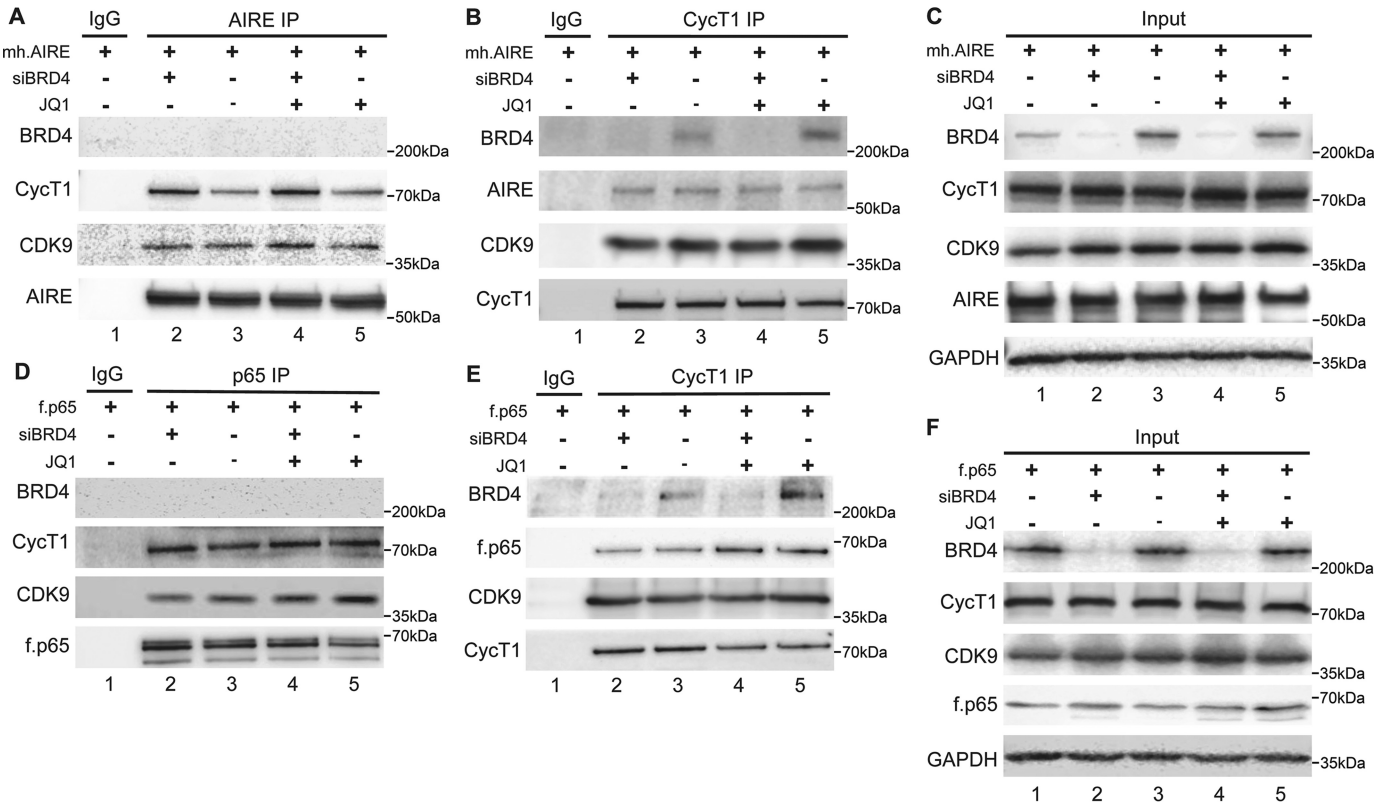


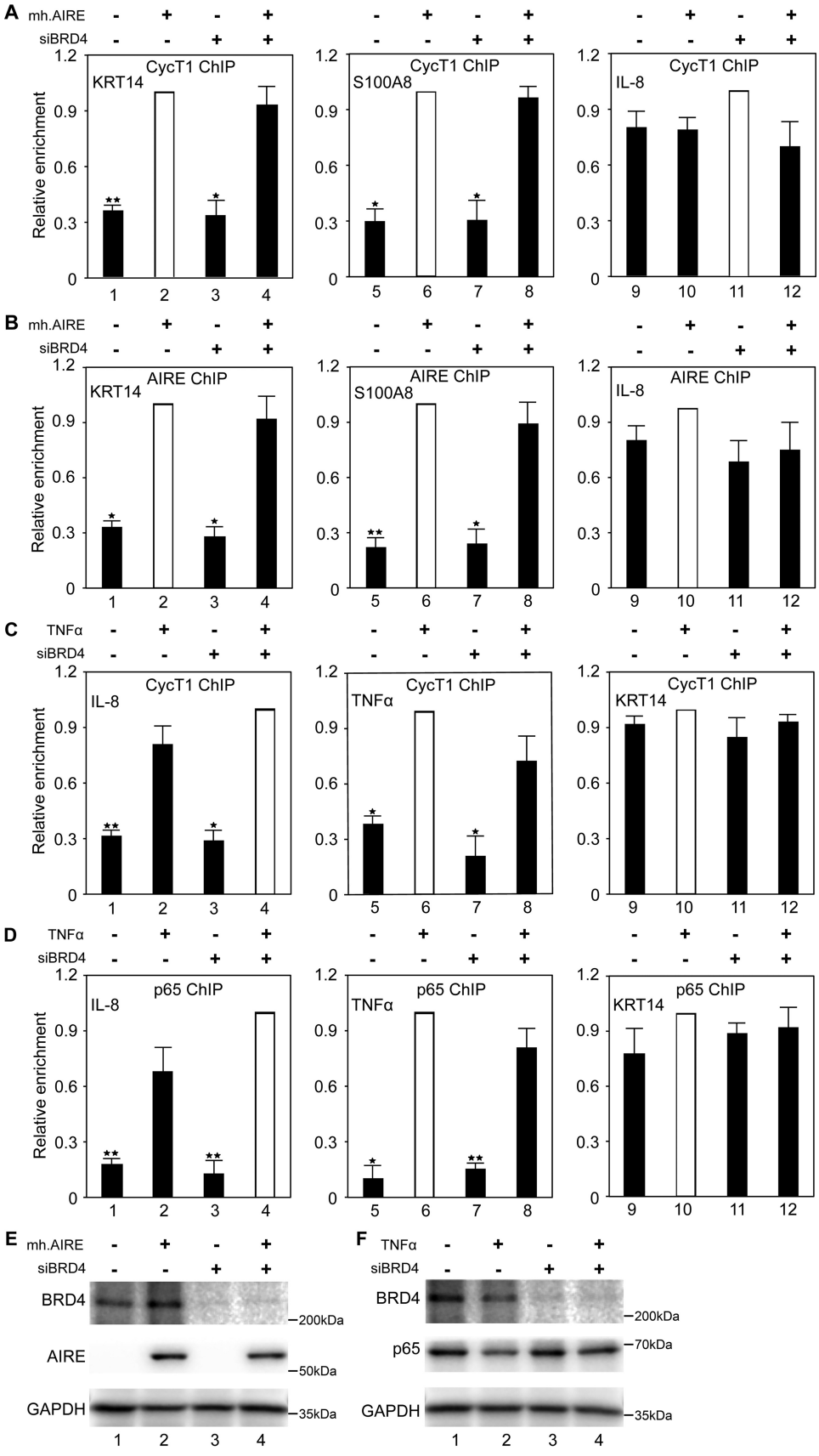
Figure 5. Depletion of BRD4 by siBRD4 and inactivation by JQ1 do not disrupt interactions between P-TEFb and AIRE or p65. *A–C*, for AIRE immunoprecipitations (*A*), interactions between the ectopically expressed AIRE (*bottom*) and endogenous BRD4, CycT1, and CDK9 proteins (*top* to *third panels*) are presented, in the absence or presence of siBRD4 and JQ1, compared with the IgG control (*lane 1*). For CycT1 immunoprecipitations (*B*), under the same conditions, compared with IgG (*lane 1*), interactions between endogenous CycT1 (*fourth panel*) and BRD4, AIRE, and CDK9 (*top* to *third panels*) are presented. *C*, WBs reveal levels of input proteins for *A* and *B*. Under the same conditions, compared with the IgG control (*lane 1*), for p65 immunoprecipitations (*D*), interactions between ectopically expressed p65 (*bottom panel*) and endogenous BRD4, CycT1, and CDK9 proteins (*top* to *third panels*) are presented. For CycT1 immunoprecipitations (*E*), interactions between endogenous CycT1 (*bottom panel*) and BRD4, p65, and CDK9 (*top* to *third panels*) are presented. *F*, WBs reveal levels of input proteins for *D* and *E*.

promoters compared with the empty vector (Fig. 6, *A* and *B*, *bars 2* and *6* and *bars 1* and *5*). Depletion of BRD4 did not decrease this enrichment of CycT1 and AIRE (Fig. 6, *A* and *B*, *bars 4* and *8*). In addition, the enrichment of AIRE and CycT1 on the *IL-8* promoter remained unchanged under the same conditions (Fig. 6, *A* and *B*, *bars 9–12*). Similar studies were performed on NF-κB–responsive promoters. After introducing siBRD4 or siScr, 293T cells were treated with or without 15 ng/ml TNFα for 1 h. ChIPs were performed on the *IL-8* and *TNFα* promoters (p65-responsive) (Fig. 6, *C* and *D*, *bars 1–8*) as well as on the *KRT14* promoter (p65-nonresponsive) (Fig. 6, *C* and *D*, *bars 9–12*). CycT1 and p65 binding were enriched in the same position of *IL-8* and *TNFα* promoters compared with the empty vector (Fig. 6, *C* and *D*, *bars 2* and *6* and *bars 1* and *5*), and depletion of BRD4 had no effect (Fig. 6, *C* and *D*, *bars 4* and *8*). Consistently, enrichment of CycT1 and p65 on the *IL-8* promoter remained unchanged under the same conditions (Fig. 6, *C* and *D*, *bars 9–12*). Levels of BRD4 and AIRE or p65 are also presented (Fig. 6, *E* and *F*, *top two panels*). Taken together, the recruitment of P-TEFb to AIRE- or p65-responsive promoters is independent of BRD4.

Discussion

In this study, the removal and depletion of BRD4 did not decrease the ability of AIRE to activate its responsive promoters on plasmids and in chromatin. Likewise, BRD4 was also not required for the effects of p65 (NF-κB) on its target genes. Moreover, complete inactivation of BRD4 did not disrupt interactions between P-TEFb and AIRE or p65 in cells. Finally, recruitment of P-TEFb to the AIRE- or p65-responsive promoters in chromatin was equivalent in the presence or absence of BRD4. We conclude that transcriptional effects of AIRE and NF-κB can occur independently of BRD4.

BRD4 is very important for maintaining the stability of chromatin structure, and the complete genetic inactivation of BRD4 is lethal to cells (33). Thus, we used combinations of BET bromodomain inhibitors like JQ1 and siBRD4 not only to remove BRD4 from chromatin but to deplete it from cells. Functional and structural studies of effects of BRD4 could follow. Functional studies revealed that AIRE and NF-κB activate transcription from promoters independently of BRD4. However, direct binding studies are difficult, as post-translational modifications direct interactions between these TFs and P-TEFb. They include phosphorylation, acetylation, and ubiquitylation (26, 34, 35). Nevertheless, co-immunoprecipitations revealed that interactions between AIRE or NF-κB and P-TEFb are BRD4-independent in cells. AIRE and p65 can also activate individual genes and gene clusters. Because the depletion and/or degradation of BRD4 has additional off-target effects, probing these two


A
mh.AIRE
siBRD4
CycT1 ChIP
KRT14
S100A8
IL-8
Relative enrichment
B
AIRE ChIP
C
TNFα
D
p65 ChIP
E
BRD4
AIRE
GAPDH
200kDa
50kDa
35kDa
F
p65
70kDa

TFs by more global analyses, such as RNA-seq and ChIP-seq, in the presence or absence of BRD4 would not be informative (28). Finally, we studied AIRE in transformed cell lines rather than primary mTECs from the thymus. mTECs grow slowly in culture, and surviving populations differentiate and lose their expression of AIRE, which makes them prohibitive for detailed transcriptional studies (9, 13). Importantly, ectopic AIRE protein activates TSA genes in all cells examined to date, which includes our cell lines (34). Taken together, neither AIRE nor NF-κB requires BRD4 to mediate their transcriptional activities.

Previous reports suggested that BRD4 mediates interactions between P-TEFb and acetylated AIRE or p65 proteins and that these acetylated TFs bind to bromodomains of BRD4 (26, 27). However, BRD4 prefers acetylated histones and is required to maintain chromatin structure (21, 36). Thus, it is difficult to envision how the same bromodomains could serve both functions, maintenance of chromatin structure and interactions between TFs and P-TEFb. To this end, it is of some interest that most if not all BRD4 is tightly bound to chromatin and not accessible for other proteins under physiological conditions (Figs. 1–4). Importantly, BRD4 has to be released first from chromatin under high-salt conditions for this binding to occur. That is not the case with interactions between AIRE or p65 and P-TEFb. In addition, free P-TEFb is required for effects of AIRE and NF-κB (10, 37). JQ1 leads to chromatin stress, the release of free P-TEFb and the subsequent increased synthesis of HEXIM1, which inactivates P-TEFb in the 7SK small nuclear RNP (32). Thus, activating or inhibitory effects of this compound can be indirect, depending on when transcriptional profiles are examined (38, 39). Of note, the acetylation of Lys-310 in p65 is also required for its optimal binding to DNA (40). Thus, the recruitment of P-TEFb to NF-κB–responsive genes could also be affected by the lower affinity of the nonacetylated p65 protein for DNA. We conclude that at least for promoters, BRD4 is dispensable for interactions between P-TEFb and AIRE or NF-κB. Our findings are also in agreement with a recent report where the complete and rapid degradation of BRD4 by dBET6 did not affect the recruitment of P-TEFb to DNA and RNAP II (28). Nevertheless, unlike the depletion of BRD4 by JQ1, its degradation led to the loss of global transcriptional elongation. Importantly, the assembly of critical elongation factors, DSIF and NELF, was found to be defective. Because caspases and DNA repair enzymes were also cleaved, this finding could reflect global degradation of proteins. Thus, the rapid degradation rather than depletion of BRD4 has more deleterious effects on cells. Consistent with our study, the depletion of BRD4 had only small effects on overall transcription and transcriptional elongation in cells (28). AIRE and NF-κB can also activate transcription from distal sites (*e.g.* enhancers) (41, 42). In that context, DNA looping could play a more important role, and these interactions (chromatin looping) could well depend on BRD4, but for totally different reasons. Future studies of distal effects of BRD4 on enhancers and other chromatin interactions should further elucidate these distinct but complementary roles of TFs and chromatin remodeling factors, such as BRD4.

Experimental procedures

Plasmids, antibodies, and reagents

The Myc-His-AIRE (mh.AIRE) and FLAG-p65 (f.p65) were constructed by cloning PCR fragments containing the coding sequences of AIRE and p65 into pcDNA3.1 vector with the indicated epitope tags. Gal.p65, Gal.AIRE, Gal, UAS.Luc, and hIns.Luc plasmids were described previously (18, 34). The antibodies for WB, co-immunoprecipitation, and ChIP were as follows: AIRE (H300, sc-33188, Santa Cruz Biotechnology), FLAG (F3165, Sigma-Aldrich), Myc (9E10, ab32, Abcam), CycT1 (D1B6G, catalog no. 81464, Cell Signaling), CDK9 (C-20, sc-484, Santa Cruz Biotechnology), GAL4 (1–147, sc-4050, Santa Cruz Biotechnology), BRD4 (E2A7X, catalog no. 13440, Cell Signaling), GAPDH (GA1R, catalog no. MA5-15738, Invitrogen), RelA/p65 (D12E12, catalog no. 8242, Cell Signaling), ECL mouse IgG HRP-linked whole Ab (NA9310, GE Healthcare), ECL rabbit IgG HRP-linked whole Ab (NA9340, GE Healthcare), and rabbit and mouse control IgG (sc-2070 and sc-2050, Santa Cruz Biotechnology). Amersham Biosciences ECL Prime Western blotting detection reagent (RPN2232, GE Healthcare) was used for WB detection. Cells were treated with the indicated concentration of TNFα (T6674, Sigma-Aldrich), JQ1 (A1910, Apexbio), Dox (631311, Clontech), and DMSO (D8418, Sigma-Aldrich).

Cell culture and transfection

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Corning, Inc.) with 10% fetal bovine serum (Sigma-Aldrich) at 37 °C and 5% CO_2. An inducible AIRE expression cell line (293.iAIRE), based on the HEK 293 cell line that stably expresses Tet-on transactivator, was derived from single cell clone, which was selected by puromycin after transfection of pFlip.AIRE plasmid into cells. For inducing AIRE expression in 293.iAIRE cells, Dox was added into medium at a final concentration of 1.5 ng/ml and maintained for 16 h.

Transfection of plasmid DNA was conducted using Lipofectamine 3000 (Life Technologies, Inc.), where the mixture of transfection reagents and plasmid DNA was added before seeding the cells for maintaining the high transfection efficiency. For JQ1 experiments, 6 h after transfection, medium was changed, and JQ1 was added with final concentration of 2.5 or 5 μM. Every 21 h, medium was changed, and JQ1 was added as above, and the same amount of DMSO was used as control.

Luciferase assay and protein detection

The luciferase assay was performed as described previously (31). For the AIRE- and p65-induced luciferase activity assay,

Figure 6. BRD4 depletion has no effect on the recruitment of P-TEFb to AIRE or p65 in chromatin. *A* and *B*, for CycT1 (*A*) and AIRE (*B*) ChIPs, relative enrichment of target proteins at indicated promoters is presented: *KRT14* (*bars 1–4*), *S100A8* (*bars 5–8*), and *IL-8* (*bars 9–12*) promoters. *C* and *D*, for CycT1 (*C*) and p65 (*D*) ChIPs, relative enrichment of target proteins at indicated promoters is presented: *IL-8* (*bars 1–4*), *TNFα* (*bars 5–8*), and *KRT14* (*bars 9–12*) promoters. *E* and *F*, WBs reveal levels of the indicated proteins for ChIPs. Relative enrichment of target proteins at chosen promoters is presented as -fold change compared with the sample with the highest value, which was set to 1. For *A–D*, *error bars* represent S.E., $n = 3$. Statistical significance is indicated by *asterisks* (paired *t* test; *, $p < 0.05$; **, $p < 0.01$).

293T cells were transfected with mh.AIRE, Gal.AIRE, or Gal.p65 and the indicated luciferase plasmids and then were treated with JQ1 or DMSO as above. For 293.iAIRE cells, cells were transfected with hIns.Luc plasmid and treated with JQ1 or DMSO as above for 21 h and then treated with Dox (plus JQ1 or DMSO) as above for another 16 h. Cells were lysed using passive lysis buffer (Promega), and firefly luciferase activities were measured according to the manufacturer's instructions. The final data of each sample were normalized to the concentration of whole-cell extract, which was measured by Pierce BCA protein assay kit (Thermo Scientific).

For protein detection, cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% SDS, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitor mixture (catalog no. 78430, Thermo Fisher Scientific). This buffer was used to detect the free BRD4 (not chromatin-bound), and high-salt RIPA buffer (450 mM NaCl) was used to detect total BRD4 in cells. SDS-polyacrylamide gel was used with 5% stacking gel and 10% resolving gel. Then WB was performed as described previously (31).

siRNA knockdown

siRNA knockdown was performed in 293T cells using Lipofectamine RNAiMAX (Life Technologies) as above. siRNAs against BRD4 (siBRD4) (HSS140720 and HSS141059, Thermo Fisher Scientific) were mixed at a ratio of 1:1 and then used for BRD4 knockdown; silencer negative control siRNAs (siScr) (AM4611, Thermo Fisher Scientific) were used as control. siRNAs were left in culture for 72 h.

Co-immunoprecipitation

One batch of 293T cells was lysed on ice using high-salt RIPA buffer supplemented with the protease inhibitor mixture for 30 min. The lysed mixture was then diluted to the final concentration of 150 mM NaCl. Another batch of these cells was also lysed on ice using the physiological RIPA buffer (150 mM NaCl). Lysate was incubated on ice for 15 min, followed by a 10-min centrifugation (14,000 × *g*). The supernatant was then precleared by incubating with protein G-Sepharose beads (Life Technologies) for 2 h at 4 °C. The lysate was then centrifuged for 2 min (10,000 × *g*), and the precleared lysate was incubated with 4 μg of the indicated primary antibody or control IgG overnight at 4 °C. The mixture was then incubated with protein G-Sepharose beads for 2 h at 4 °C. The beads were washed five times with RIPA buffer at 4 °C, 5 min each wash. Finally, the beads were boiled with 2× SDS Laemmli sample buffer (Bio-Rad) containing 2-mercaptoethanol (Sigma-Aldrich), beads and lysates were centrifuged for 1 min (10,000 × *g*), and the resulting supernatant was used as a co-immunoprecipitation sample. Detection of proteins in supernatant and input (1% of supernatant from the first centrifugation) were performed by WB as above.

Chromatin immunoprecipitation with quantitative PCR (ChIP-qPCR)

The ChIP assay was performed as described previously with slight modifications. 293T cells were treated with 1% formaldehyde (Sigma-Aldrich) for 10 min to cross-link proteins and then quenched with 250 mM glycine (Sigma-Aldrich) for another 5 min. Cells were lysed with RIPA buffer supplemented with protease inhibitor mixture and 1 mM DTT (Invitrogen) for 30 min on ice, and then the mixture was sonicated at power level 4 12 times for 8 s with a 52-s interval by using a Sonic Dismembrator model 100 (Fisher). Lysate obtained after a 15-min centrifugation (20,000 × *g*) was precleared with protein G-Sepharose beads for 2 h at 4 °C, and the precleared lysate was incubated with 5 μg of the indicated antibodies or control IgG overnight at 4 °C (same steps as co-immunoprecipitation). The mixture was then incubated with precoated protein G-Sepharose beads (coated with 1 mg/ml BSA, 0.5 mg/ml salmon testis DNA, and 0.5 mg/ml tRNA) for another 2 h at 4 °C. After washing with RIPA buffer, TSE-500 buffer, LiCl detergent buffer, and TE buffer (buffers and washing steps are the same as in previous reports) (13), the immunoprecipitated DNA was eluted from beads. After reversing the crosslinking overnight at 65 °C, purification of DNA fragments was performed using the QIAquick PCR purification kit (Qiagen). Then the precipitated DNA was detected by qPCR with the indicated primers using a SensiFAST SYBR Lo-ROX kit (Bioline) on the Mx3005p thermocycler (Stratagene). The result for each set of primers was quantified relative to input and was presented as -fold over IgG control. The following primers were used: *KRT14* (−35/+78) and *S100A8* (−130/−228) promoters, based on our previous studies (34); *IL-8* (−84/+97) promoter, forward (5′-GGGCCATCAGTTGCAAATC) and reverse (5′-TTCCTTCCGGTGGTTTCTTC); *TNFα* (−167/+55) promoter, forward (5′-CGATGGAGAAGAAACCGAGACAGAAGGTG) and reverse (5′-AGTTGCTTCTCTCCCTCTTAGCTGGTCCTC).

RNA extraction and reverse transcription and qPCR (RT-qPCR)

Cells were washed with cold PBS and collected using TRIzol reagent (Life Technologies). Total RNA was extracted and treated with a Turbo DNA kit (Life Technologies) for genomic DNA removal. Purified RNA was reverse-transcribed using SuperScript III reverse transcriptase (Life Technologies). The indicated mRNA expression was measured by qPCR using the SensiFAST SYBR Lo-ROX Kit on the Mx3005p thermocycler and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for *KRT14*, *S100A8*, *CCNH*, *GAPDH*, *IL-8*, and *TNFα* were based our previous studies (18, 29).

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