Scientific Report

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EMBO reports

Gcn5 and PCAF negatively regulate interferon-β production through HAT-independent inhibition of TBK1

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Abstract

Viral infection triggers innate immune signaling, which in turn induces interferon-β (IFN-β) production to establish innate antiviral immunity. Previous studies showed that Gcn5 (Kat2a), a histone acetyltransferase (HAT) with partial functional redundancy with PCAF (Kat2b), and Gcn5/PCAF-mediated histone H3K9 acetylation (H3K9ac) are enriched on the active *IFNB* gene promoter. However, whether Gcn5/PCAF and H3K9ac regulate IFN-β production is unknown. Here, we show that Gcn5/PCAF-mediated H3K9ac correlates well with, but is surprisingly dispensable for, the expression of endogenous *IFNB* and the vast majority of active genes in fibroblasts. Instead, Gcn5/PCAF repress IFN-β production and innate antiviral immunity in several cell types in a HAT-independent and non-transcriptional manner: by inhibiting the innate immune signaling kinase TBK1 in the cytoplasm. Our results thus identify Gcn5 and PCAF as negative regulators of IFN-β production and innate immune signaling.

Keywords Gcn5/PCAF; H3K9ac; innate immune signaling; interferon-β; TBK1
Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction; Transcription
DOI 10.15252/embr.201438990 | Received 30 April 2014 | Revised 2 September 2014 | Accepted 4 September 2014 | Published online 30 September 2014
EMBO Reports (2014) 15: 1192–1201

Introduction

Type-I interferons (hereafter referred to as IFNs), encoded by one *IFNB* gene and multiple *IFNA* genes, are crucial for innate immunity against viral infection [1,2]. IFNs are activated upon virus infection by TANK-binding kinase 1 (TBK1), which is activated by a signaling pathway triggered by interactions between pattern recognition receptors (PRRs) within the host cells, such as RIG-I, MDA5, and TLR3, and viral double-stranded RNA (dsRNA). Activated TBK1 phosphorylates latent transcription factors interferon regulatory factor 3 (IRF3) and IRF7. Phosphorylated IRF3 and IRF7 translocate to the nucleus and directly induce low-level expression of IFN-β. Secreted IFN-β acts in an autocrine and paracrine manner to bind and stimulate the IFN receptor (a heterodimer of IFNAR1 and IFNAR2), which activates transcription factor ISGF3 to strongly induce *IRF7* gene transcription. The newly synthesized IRF7 protein is activated by TBK1-mediated phosphorylation and subsequently induces high level production of IFNs, which in turn amplify the innate immune response in a positive-feedback manner to establish an antiviral state [1]. Activated TBK1 is phosphorylated at Ser172 (S172), and this modification is critical for TBK1 kinase activity [3,4]. Suppression of TBK1 kinase activity prevents inappropriate activation of innate immune signaling and IFN production in the absence of virus infection.

During transcriptional activation of the *IFNB* gene, Gcn5 and a highly related HAT, PCAF, are enriched on the *IFNB* promoter, correlating with increased H3K9ac on this promoter [5]. Gcn5 is mainly localized in the nucleus but can also be found in the cytoplasm [6], and it has both histone and non-histone substrates [7]. We previously reported that Gcn5 and PCAF functions appear to be redundant in mouse fibroblasts, and deletion of both of these HATs profoundly reduces global levels of H3K9ac [8]. H3K9ac is enriched on numerous active gene promoters [9], but its role in gene activation is not clear. For example, although Gcn5/PCAF-mediated H3K9ac correlates well with ligand-induced nuclear receptor target gene activation, this modification is dispensable for expression of these genes [8].

Here, we determine that Gcn5 and PCAF loss only affects expression of about 6% of active genes in fibroblasts, indicating these HATs are gene specific, rather than global, activators of transcription. We also find that even though Gcn5/PCAF-mediated H3K9ac

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correlates well with *IFNB* expression, loss of this mark does not compromise activation of this gene. Instead, our data indicate that Gcn5 and PCAF have a HAT-independent role in repression of IFN-β production that is mediated by inhibition of the innate immune signaling.

Results

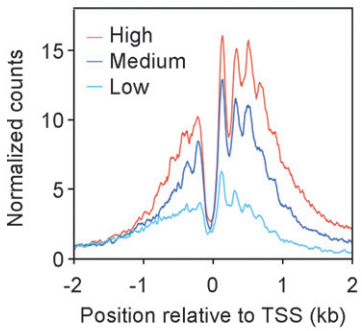
Deletion of *Gcn5* and *PCAF* activates expression of IFN-stimulated genes (ISGs)

Our previous work established that loss of PCAF alone has minimal effects on H3K9ac levels in MEFs, but that loss of both Gcn5 and PCAF leads to approximately 19-fold decrease of this modification [8]. To further investigate the genome-wide role of H3K9ac in gene activation, we profiled the distribution of H3K9ac in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs by ChIP-Seq. ChIP-Seq data sets were deposited in GEO under accession no. GSE60969. Our results indicate that H3K9ac is enriched globally around transcription start sites (TSSs) and that the signal intensities of H3K9ac enrichment correlate positively with gene expression levels (Fig 1A). These results in MEFs are highly consistent with the previously reported genome-wide distribution of H3K9ac in human CD4$^+$ T cells [9].

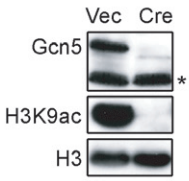
Expression of Cre in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs leads to deletion of *Gcn5*, generating *Gcn5/PCAF* double KO (dKO) cells with substantially reduced global levels of H3K9ac, as expected (Fig 1B) [8]. To determine how this loss affected gene expression profiles in the dKO cells, we performed RNA-Seq using "spike-in controls" [10]. RNA-Seq data sets were deposited in GEO under accession no. GSE60969. Loss of Gcn5 in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs led to over twofold down-regulation of 844 genes (Fig 1C), which constitutes 6.4% of the total number of expressed genes (13,265) in MEFs. Among these 844 genes, expression of 482 genes was decreased more than threefold (Supplementary Table S1). These data are consistent with functions for Gcn5 and PCAF as gene specific, rather than general, transcriptional co-activators. In addition, loss of Gcn5 in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs led to more than twofold up-regulation of 224 genes (Fig 1C and Supplementary Table S2, 1.7% of total number of active genes), indicating that these two HATs also function to directly or indirectly repress some genes. In general, genes with lower levels of promoter H3K9ac, which associated with lower expression levels in the control cells, were more sensitive to the loss of Gcn5/PCAF and showed more significant changes of expression in the dKO cells

PCAF$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs

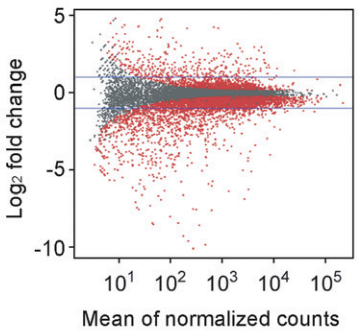
A ChIP-Seq: H3K9ac



B



C Spike-in RNA-Seq (Cre vs. Vec)



>2-fold changed genes

FDR	Down-regulated	Up-regulated
0.00001	844	224

D GO analysis

>2-fold down-regulated genes

Term	FDR
Cell adhesion	9.0E-09
Biological adhesion	4.9E-09
Wnt receptor signaling pathway	6.5E-09
Tube development	2.2E-07
Morphogenesis of a branching structure	2.4E-07
Respiratory system development	.4.3E-06
Lung development	9.6E-06
Respiratory tube development	1.2E-05
Epithelium development	2.5E-05
Tube morphogenesis	2.8E-05
Branching morphogenesis of a tube	3.3E-05

>2-fold up-regulated genes

Term	FDR
Immune response	5.4E-16
Response to virus	1.6E-05

Figure 1. H3K9ac correlates well with gene activation, and H3K9ac loss by *Gcn5/PCAF* dKO affects the expression of only a small number of genes.

A ChIP-Seq of H3K9ac in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs. Normalized tag counts of H3K9ac signals surrounding the TSS are indicated for genes with high, medium, or low levels of expression. Gene expression levels were obtained from RNA-Seq data (C). Data are representative of two independent ChIP-Seq experiments.

B Immortalized *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs were infected with retroviral vector (Vec) or Cre. Whole-cell extracts were analyzed by immunoblotting. The asterisk indicates a non-specific band.

C MA plot for gene expression changes responding to *Gcn5* deletion in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs (upper panel). The mRNA levels were analyzed by spike-in RNA-Seq. Red dots indicate gene expression with statistically significant changes (False Discovery Rate (FDR) < 0.05). Blue lines indicate twofold change threshold. The numbers of genes with over twofold changes (FDR < 0.00001) are shown in the lower panel.

D GO analysis of down-regulated or up-regulated genes in *Gcn5/PCAF* dKO MEFs.

(Supplementary Fig S1A and B). By ChIP-Seq of H3K9ac, we confirmed the depletion of H3K9ac on the TSSs of unaffected, down-regulated, and up-regulated genes in the dKO cells (Supplementary Fig S1C–I).

Gene Ontology (GO) analysis revealed that the down-regulated genes relate to multiple biological functions, including many developmental processes and cell adhesion. In contrast, genes up-regulated upon loss of these HATs are strongly related to immune response or response to viruses (Fig 1D). Indeed, 69 of the 224 up-regulated genes were functionally linked to immune response (Fig 2A), and more than half of these 69 genes are ISGs involved in innate antiviral immunity [1] (Supplementary Table S2 and Fig 2B–D). These genes encode such factors as PRRs RIG-I, MDA5, DAI, and TLR3 (Fig 2B), as well as IRF7 and the IRF9, STAT1, and STAT2 subunits of the hetero-trimeric transcription factor ISGF3 (Fig 2C) along with other antiviral ISGs (Fig 2D).

Deletion of *Gcn5* and *PCAF* activates innate immune response and IFN production

To determine whether the increased mRNA levels of ISGs led to antiviral activity, we infected *Gcn5/PCAF* dKO MEFs, as well as MEFs with single KO of either *Gcn5* or *PCAF*, with a vesicular stomatitis virus (VSV) expressing GFP. VSV replicated efficiently in cells with either single KO, as indicated by strong GFP signals and severe cytopathic effects (Fig 2E). However, deletion of both *Gcn5* and *PCAF* rendered cells refractory to VSV infection, leading to approximately 33,000-fold decrease of viral titers, while deletion of either *Gcn5* or *PCAF* alone resulted in only approximately four-fold decrease (Fig 2E and F). These effects are not cell type specific as deletion of both *Gcn5* and *PCAF* in brown preadipocytes also activated expression of ISGs and rendered cells resistant to VSV infection (Supplementary Fig S2). In addition, MEFs lacking

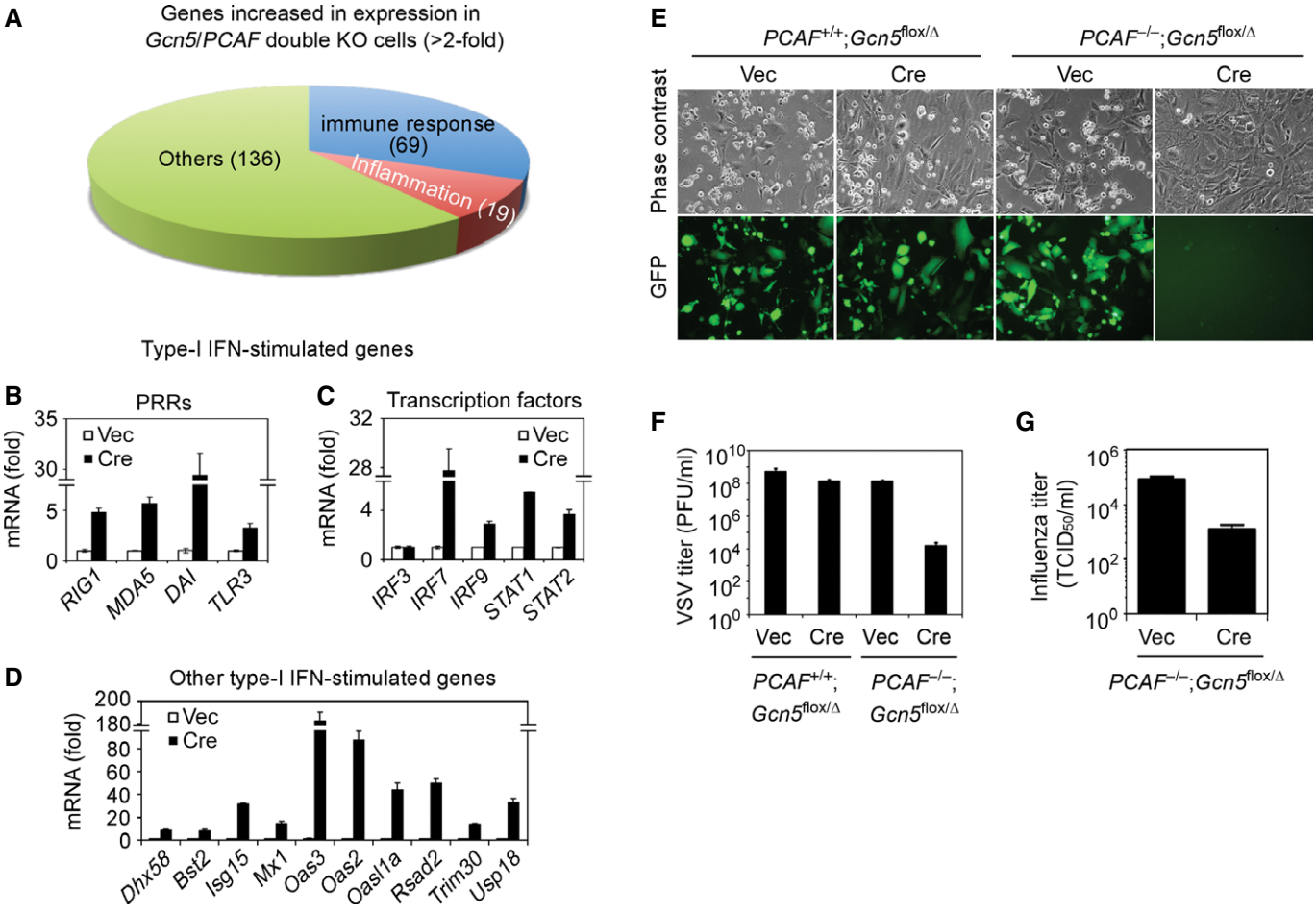


Figure 2. Deletion of *Gcn5* and *PCAF* activates IFN-mediated innate immune response.

A Classification of the 224 genes with over twofold increase of expression in retroviral Cre-infected $PCAF^{-/-};Gcn5^{flox/\Delta}$ cells identified in RNA-Seq.

B–D qRT–PCR confirmation of ISGs identified in RNA-Seq. (B) PRRs. (C) Transcription factors. *IRF3* is not an ISG and serves as a negative control. (D) Other antiviral ISGs. qPCR data are presented as mean values ± SD (n = 3).

E–G Deletion of *Gcn5* in $PCAF^{-/-};Gcn5^{flox/\Delta}$ MEFs renders cells resistant to virus infection. Cells were infected with 5 MOI VSV expressing GFP for 24 h (E and F) or with 0.5 MOI of influenza virus for 48 h (G). (E) Upper panels: the cytopathic effects of VSV infection; lower panels: VSV replication indicated by the green GFP signals. (F, G) VSV and influenza virus titers in the media. Virus titers are presented as mean values ± SD (n = 3).

Data information: All data are representative of 2–4 independent experiments.

both Gcn5 and PCAF were resistant to influenza virus infection (Fig 2G). The C-terminal region (amino acids 400–830) of mouse Gcn5 (Gcn5-C) contains HAT and Bromo domains and shares sequence homology with yeast Gcn5 (Supplementary Fig S3A). Ectopic expression of Gcn5-C, but not an N-terminal fragment (amino acids 1–500; Gcn5-N), restored repression of ISGs and susceptibility to VSV infection in the dKO cells (Supplementary Fig S3B–D). Together, these results indicate that deletion of *Gcn5* and *PCAF* activates expression of ISGs in the absence of viral infection and that Gcn5 and PCAF are redundant in repressing innate antiviral immunity.

The simultaneous up-regulation of a large number of ISGs suggests that IFNs may be secreted from the dKO cells. Indeed, conditioned media collected from the dKO cells not only induced expression of ISGs such as *IRF7* and *Isg15* in the wild-type (WT) MEFs, but also protected the WT MEFs from VSV infection (Supplementary Fig S4). Although IFN levels in conditioned media from unexposed cells were below limits of detection, we were able to detect approximately 400 pg/ml IFN-α and approximately 55 pg/ml IFN-β after 30-fold concentration of conditioned media from the dKO cells (Fig 3A). IFN mRNAs are highly unstable, but they can be stabilized by cycloheximide (CHX) [11]. Without CHX treatment, IFN mRNA levels were very low. CHX treatment elevated IFN mRNA levels as expected, and we observed approximately fourfold higher levels of IFN mRNAs in the dKO cells than in the control cells (Fig 3B). These data indicate that deletion of *Gcn5* from $PCAF^{-/-}$;$Gcn5^{flox/\Delta}$ cells activates low-level production of IFNs in the absence of virus infection.

Next, we treated cells with poly(I:C), which mimics viral dsRNA and serves as a potent inducer of IFNs. Deletion of *Gcn5* from $PCAF^{-/-}$;$Gcn5^{flox/\Delta}$ MEFs led to a marked increase of poly(I:C)-induced IFN-β production at both protein and mRNA levels (Fig 3C), as well as increased recruitment of RNA polymerase II (Pol II) to the *IFNB* promoter (Fig 3D). Deletion of *Gcn5* from the $PCAF^{-/-}$;$Gcn5^{flox/\Delta}$ cells blocked induction of H3K9ac, but not H4K8ac, on the *IFNB* promoter following poly(I:C) treatment (Fig 3D). These data further indicate that Gcn5 and PCAF suppress expression of *IFN* genes, even though these HATs induce H3K9ac at *IFN* promoters.

Macrophages play an important role in host defense against virus infection. We observed that Gcn5 and PCAF repressed poly(I:C)-induced IFN production in peritoneal (Fig 3E) and bone marrow-derived macrophages (Supplementary Fig S5). These results further demonstrate that Gcn5/PCAF repress IFN production.

Repression of IFN production by Gcn5 and PCAF does not require HAT activity

The above results indicate that suppression of IFN production by Gcn5 and PCAF occurs in spite of induction of H3K9ac by these HATs at *IFN* gene promoters. To directly determine whether Gcn5 HAT activity is required for repressing IFN production, $PCAF^{-/-}$;$Gcn5^{flox/\Delta}$ MEFs were infected with retroviral vectors expressing either WT Gcn5 or an enzymatically inactive Gcn5 mutant, D608A [12], and then, the endogenous *Gcn5* gene was deleted by retroviral Cre. Expression of WT Gcn5, but not the D608A mutant, prevented the loss of global H3K9ac in the dKO cells, as expected (Fig 3F, lanes 4–6; Supplementary Fig S6A). The D608A Gcn5 mutant behaved as a dominant-negative form of the protein, causing depletion of H3K9ac in cells carrying one WT *Gcn5* allele (Fig 3F, lane 3). Surprisingly, both expression of WT and D608A Gcn5 blocked basal and poly(I:C)-induced IFN production, up-regulation of ISGs, as well as resistance to VSV infection in the dKO cells (Fig 3G and H; Supplementary Fig S6B–D). Similar results were obtained when WT or enzymatically inactive PCAF was expressed in the dKO cells (Supplementary Fig S6E–H). These data indicate that repression of IFN production by Gcn5 and PCAF does not require HAT activity.

Gcn5 and PCAF repress IFN production by inhibiting innate immune signaling

Since IFN production is under the control of innate immune signaling [1] (see also Fig 5L), we knocked down individual components of this pathway in the dKO cells to determine steps required for Gcn5 repression of IFNs (see Supplementary Fig S7A for a summary). Consistent with IRF7 being the master regulator of IFN-dependent immune responses, knockdown of IRF7 in the dKO cells not only blocked IFN-β production but also prevented up-regulation of ISGs and cell resistance to VSV infection (Supplementary Fig S7B–D). Depletion of the IFNAR1 subunit of the IFN receptor in the dKO cells did not affect IFN-β production but did prevent up-regulation of ISGs, resulting in loss of cell resistance to VSV infection (Fig 4A–C). These findings confirm that up-regulation of ISGs and VSV resistance is due to IFN production from the dKO cells, and they indicate that Gcn5 and PCAF work upstream of the IFN receptor to repress IFN production.

IRF3, TBK1, and TRAF3 work upstream of the IFN receptor [1]. Knockdown of either IRF3 or TBK1 in the dKO cells not only blocked IFN-β production but also prevented up-regulation of ISGs and VSV resistance (Fig 4D–F and G–I, respectively). In contrast, depletion of TRAF3, which works upstream of TBK1 [1], in the dKO cells had no effect on up-regulation of ISGs or VSV resistance (Fig 4J–L and Supplementary Fig S7E). In luciferase reporter assays, Gcn5 inhibited the transcriptional activity of the WT IRF3, but it had little effect on the activity of a constitutively active, nuclear mutant form of IRF3, IRF3(5D), which mimics the phosphorylated state [13] (Supplementary Fig S8). Finally, treating the dKO cells with BX795, a TBK1 kinase inhibitor that blocks IRF3 but not NF-κB signaling [14], was functionally equivalent to TBK1 depletion in blocking IFN-β production and up-regulation of ISGs (Fig 4M and N). These data suggest that Gcn5 and PCAF repress innate immune signaling by affecting the activity or expression of the TBK1 kinase.

Gcn5 inhibits TBK1 kinase activity

Deletion of *Gcn5* and *PCAF* does not affect endogenous TBK1 protein levels (Fig 4O). Therefore, we next investigated whether Gcn5 physically interacts with TBK1 to regulate TBK1 kinase activity. Gcn5 interacted with TBK1 *in vitro* and in 293T cells (Fig 5A and B). Overexpression of Gcn5 in 293T cells almost completely blocked the ability of TBK1 to activate the *IFNB* promoter (Fig 5C). Gcn5-C but not Gcn5-N also interacted with TBK1 and inhibited TBK1-stimulated *IFNB* promoter activity in 293T cells (Supplementary Fig S9). Conversely, deletion of *Gcn5* from $PCAF^{-/-}$;$Gcn5^{flox/\Delta}$ MEFs strongly enhanced TBK1-stimulated *IFNB* promoter activity (Fig 5D).

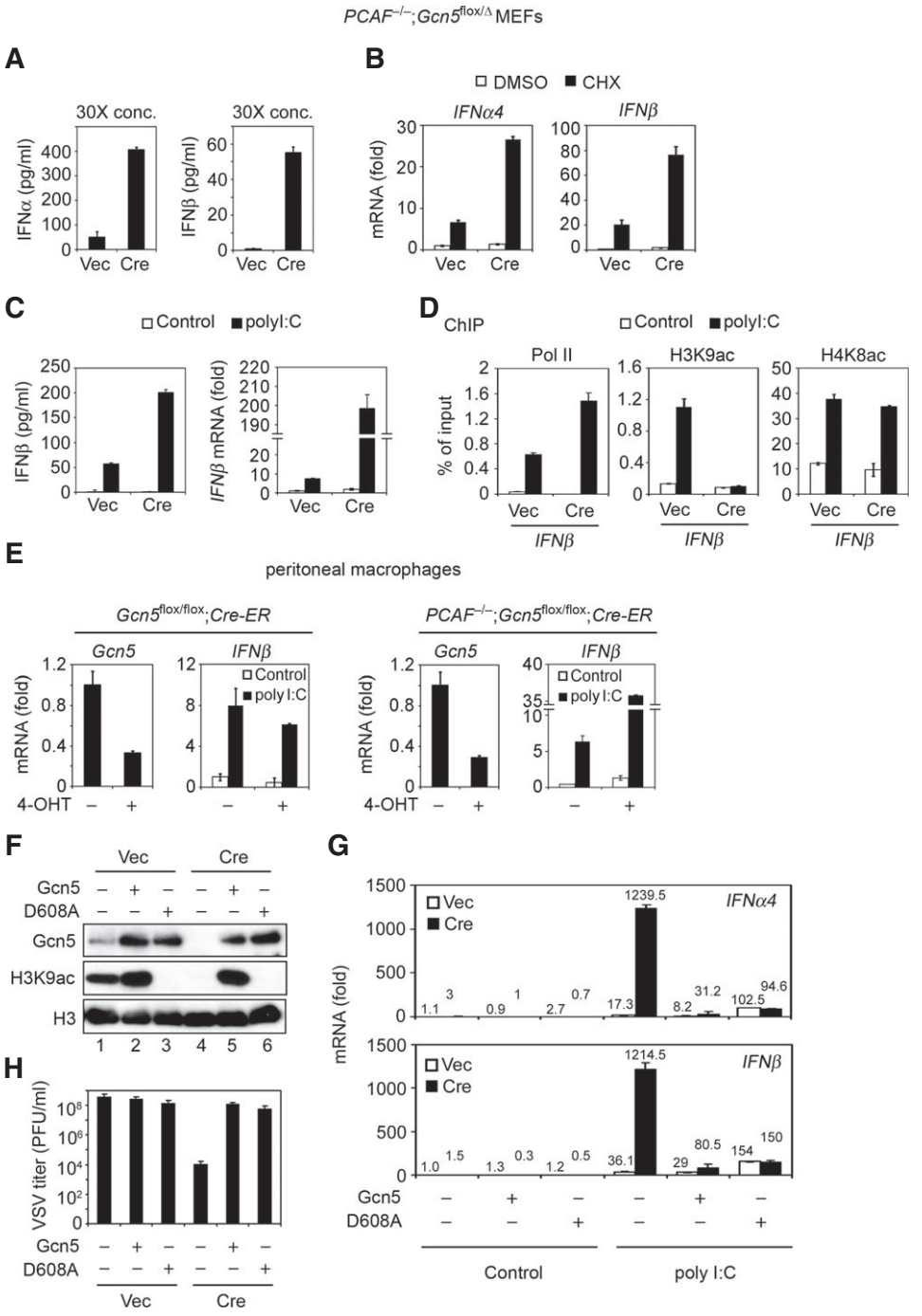


Figure 3. Gcn5 and PCAF repress both basal and poly(I:C)-induced IFN production independent of HAT activities.

A, B Deletion of *Gcn5* in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs activates low-level production of IFN-α and IFN-β. (A) Conditioned media was concentrated 30-fold, followed by ELISA. (B) Cells were treated with 100 μg/ml cycloheximide (CHX) or DMSO for 6 h, followed by qRT–PCR analysis.

C, D Gcn5/PCAF-mediated H3K9ac correlates with, but is dispensable for, *IFNB* gene transcription. Cells were transfected with 2 μg/ml poly(I:C) for 6 h, followed by ELISA of IFN-β levels in the conditioned media (C, left panel), qRT–PCR of *IFNB* levels (C, right panel), and ChIP of Pol II, H3K9ac, and H4K8ac on the *IFNB* promoter (D).

E Gcn5 and PCAF repress *IFNB* production in macrophages. Peritoneal macrophages of the indicated genotypes were treated with 4-hydroxytamoxifen (4-OHT) for 2 days to delete *Gcn5*. 24 h later, cells were transfected with 0.01 μg/ml poly(I:C) for 6 h, followed by qRT–PCR of *Gcn5* and *IFNB* levels.

F–H Gcn5 repression of IFN production does not require HAT activity. *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs were infected with retroviral vectors expressing WT Gcn5 or enzymatically inactive mutant (D608A), followed by infection with retroviral Cre to delete endogenous *Gcn5*. (F) Immunoblotting. (G) Cells were transfected with 2 μg/ml poly(I:C) for 6 h, followed by qRT–PCR of *IFNA4* and *IFNB* levels. (H) VSV titers after cells were infected with 5 MOI VSV-GFP for 24 h. ELISA, qPCR data, and VSV titers are presented as means ± SD (n = 3).

Data information: All data are representative of 2–4 independent experiments.

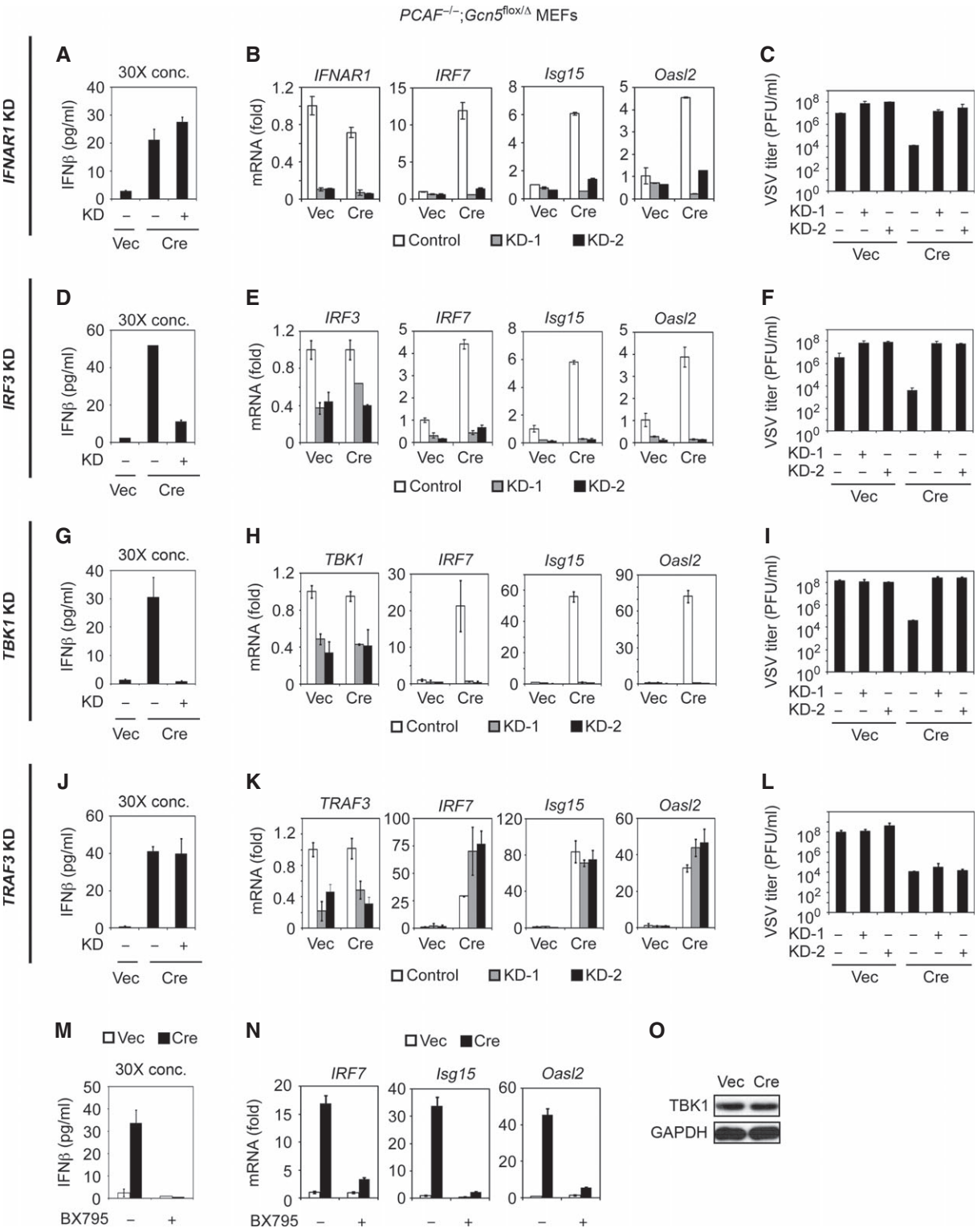


Figure 4. Gcn5 represses IFN production by targeting TBK1.

A–L Retroviral Vec- or Cre-infected *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs were infected with lentiviral shRNAs to specifically knockdown IFN receptor gene *IFNAR1* (A–C), *IRF3* (D–F), *TBK1* (G–I) or *TRAF3* (J–L). Conditioned media were concentrated 30-fold for analysis of IFN-β levels by ELISA (A, D, G, J). Expression of representative ISGs *IRF7*, *Isg15*, and *Oasl2* was determined by qRT–PCR (B, E, H, K). Cells were infected with 5 MOI of VSV for 24 h, and virus titers were determined (C, F, I, L).

M, N Inhibiting TBK1 kinase activity blocks production of IFN-β protein and up-regulation of ISGs in *Gcn5/PCAF* dKO cells. Cells were treated with 1 μM TBK1 inhibitor BX795, followed by ELISA of IFN-β levels (M) and qRT–PCR of *ISG* expression (N).

O Immunoblotting of endogenous TBK1.

Data information: ELISA, qPCR data, and VSV titers are presented as means ± SD (n = 3). All data are representative of 2–4 independent experiments.

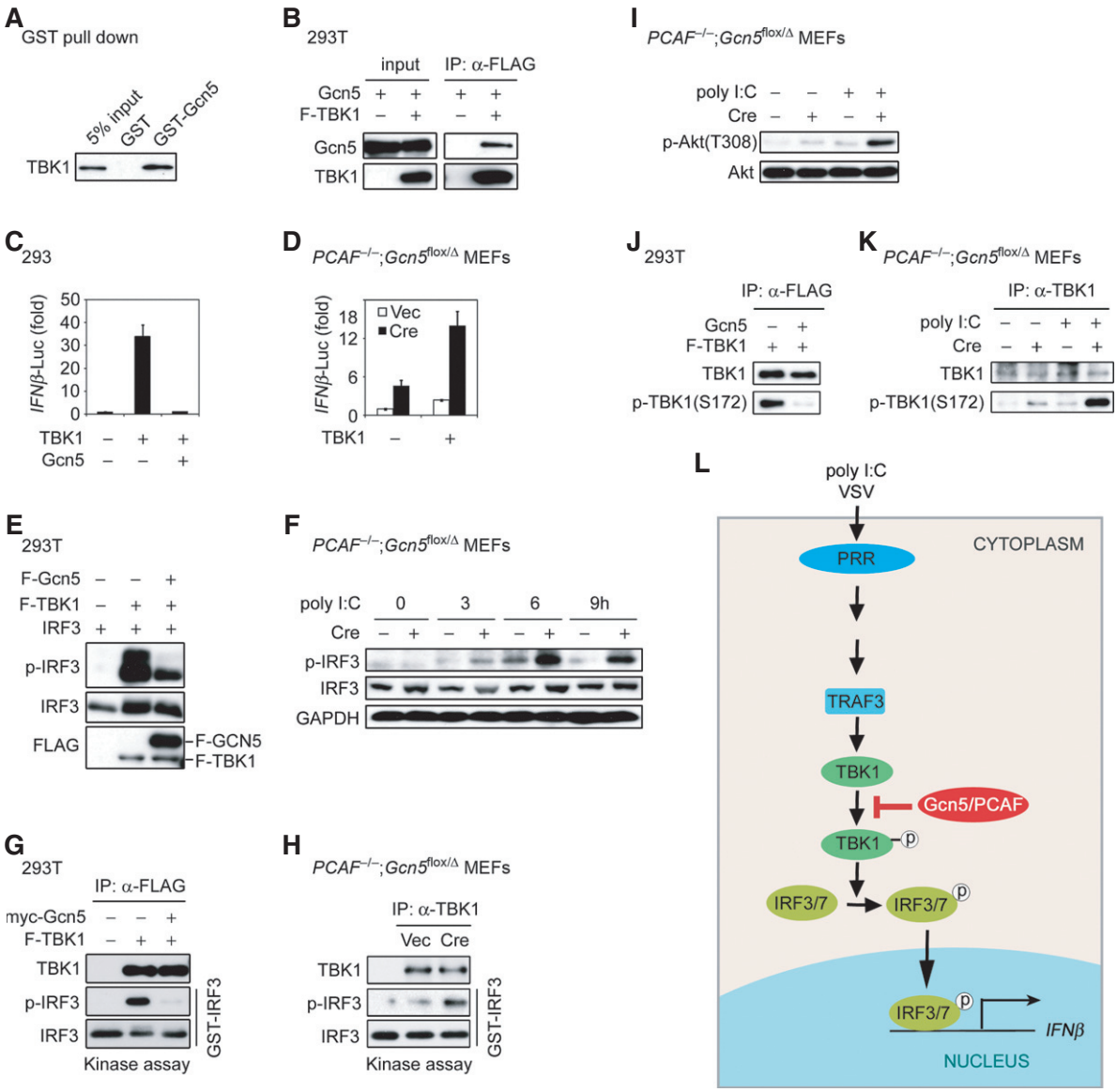


Figure 5. Gcn5 inhibits TBK1 kinase activity.

A Gcn5 interacts directly with TBK1 *in vitro*. GST-Gcn5 was incubated with recombinant TBK1. Bound proteins were monitored by immunoblotting with anti-TBK1 antibody.

B Gcn5 interacts with TBK1 in cells. 293T cells were transfected with plasmids expressing Gcn5 and FLAG-tagged TBK1 (F-TBK1). 48 h later, whole-cell extracts were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting.

C, D Gcn5 inhibits TBK1-induced activation of *IFNB* promoter. (C) 293 cells were transfected with reporter plasmid *IFNβ*-Luc and plasmids expressing TBK1 and Gcn5. (D) MEFs were transfected with *IFNβ*-Luc and plasmid expressing TBK1. Two days later, *IFNβ*-Luc activities were determined by luciferase assay. The data are presented as means ± SD (n = 3).

E, F Gcn5 inhibits TBK1-induced IRF3 phosphorylation in cells. (E) 293T cells were transfected with plasmids expressing FLAG-tagged Gcn5 (F-Gcn5), F-TBK1, and IRF3 for 48 h. (F) MEFs were transfected with 2 μg/ml poly(I:C) for indicated hours. Whole-cell lysates were prepared for immunoblotting. p-IRF3, phospho-IRF3.

G, H Gcn5 inhibits TBK1 kinase activity. (G) 293T cells were transfected with plasmids expressing F-TBK1 and myc-tagged Gcn5. Whole-cell extracts were mixed with anti-FLAG antibody. (H) Whole-cell extracts were mixed with anti-TBK1 antibody. TBK1 kinase activities of these immunoprecipitates were analyzed in the *in vitro* kinase assays using GST-IRF3 as the substrate.

I Deletion of *Gcn5* in *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs increases basal and poly(I:C)-induced Akt phosphorylation on Thr308. Cells were transfected with 2 μg/ml poly(I:C) for 6 h, followed by immunoblotting.

J, K Gcn5 inhibits TBK1 S172 phosphorylation. (J) 293T cells were transfected with plasmids expressing F-TBK1 and Gcn5. Whole-cell extracts were mixed with anti-FLAG antibody. (K) MEFs were transfected with 2 μg/ml poly(I:C). 6 h later, whole-cell extracts were mixed with anti-TBK1 antibody. Immunoprecipitates were analyzed by immunoblotting.

L Model. Gcn5 and PCAF repress IFN-β production in cells by inhibiting innate immune signaling.

Data information: All data are representative of 2–4 independent experiments.

Accordingly, Gcn5 strongly inhibited TBK1-mediated phosphorylation of IRF3 in 293T cells and MEFs (Fig 5E and F). Furthermore, using GST-IRF3 as the substrate and FLAG-TBK1 isolated from 293T cells as an enzyme in *in vitro* kinase assays [15], we found that Gcn5 strongly inhibited TBK1 kinase activity (Fig 5G). Conversely, deletion of *Gcn5* from *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs increased the kinase activity of endogenous TBK1 (Fig 5H). TBK1 directly phosphorylates AKT on Thr308 [16], and this phosphorylation event was markedly increased upon deletion of *Gcn5* from *PCAF*$^{-/-}$;*Gcn5*$^{flox/\Delta}$ MEFs under both basal and poly(I:C)-induced conditions (Fig 5I). Collectively, these results indicate that physical association of Gcn5 with TBK1 inhibits TBK1 kinase activity.

S172 phosphorylation is critical for TBK1 kinase activity [3,4]. Overexpression of Gcn5 in 293T cells strongly inhibited TBK1 S172 phosphorylation (Fig 5J). In contrast, deletion of *Gcn5* from *PCAF*$^{-/-}$; *Gcn5*$^{flox/\Delta}$ MEFs markedly increased both basal and poly(I:C)-induced levels of endogenous TBK1 S172 phosphorylation (Fig 5K). These data indicate that Gcn5 limits TBK1 kinase activity by inhibiting TBK1 S172 phosphorylation.

It has been reported that mutating multiple Lys (K) residues in the nuclear localization signal (NLS) of PCAF to Arg (R) leads to cytoplasmic localization of PCAF [17]. We mutated K425, K427, and K438 in the Gcn5 NLS, which are conserved between PCAF and Gcn5, to R to generate Gcn5-NLSm. Unlike WT Gcn5, Gcn5-NLSm was mainly localized in the cytoplasm, as expected (Supplementary Fig S10A and B). However, cytoplasmic Gcn5-NLSm is functionally equivalent with WT Gcn5 in repressing innate immune signaling and antiviral response (Supplementary Fig S10C and D), and in inhibiting TBK1 kinase activity in cells (Supplementary Fig S10E). These data suggest that Gcn5 mainly functions in the cytoplasm to inhibit TBK1 kinase activity.

Discussion

H3K9ac, mediated by Gcn5 and PCAF, is enriched on active promoters and is a hallmark for active genes. Gcn5/PCAF have been proposed to promote *IFNB* gene transcription through histone acetylation [5]. We demonstrate here that although H3K9ac correlates well with *IFNB* expression, it is not required for activation of the *IFNB* gene. Further, we demonstrate that Gcn5 and PCAF repress, rather than activate, both basal and poly(I:C)-induced production of IFN-β and other IFNs independently of their HAT activities. Gcn5 represses IFN production by targeting the innate immune signaling kinase TBK1. Altogether, our data suggest the following model (Fig 5L). Viral infection or poly(I:C) treatment triggers innate immune signaling, leading to S172 phosphorylation and activation of TBK1 [1]. Gcn5 prevents inappropriate activation of this signaling pathway by physically binding to TBK1 to inhibit S172 phosphorylation, subsequently limiting phosphorylation and activation of IRF3/7, and ultimately inhibiting transcriptional activation of the *IFNB* gene. Our data thus identify a HAT activity-independent function of Gcn5 as a novel negative regulator of IFN-β production and innate immune signaling. Our data also suggest a functional redundancy between PCAF and Gcn5 both in mediating H3K9ac and in repressing IFN-β production and innate immunity. Although HAT activity-independent functions of Gcn5 have been reported previously [12,18], our study is the first to identify Gcn5, better known as a transcriptional coactivator and histone-modifying enzyme, as a negative regulator of signal transduction.

While the IFN-α/β levels in the supernatant of dKO MEFs are low, they are sufficient to induce significant ISG expression in WT MEFs (Supplementary Fig S4). The detected IFN concentrations likely underestimate the actual IFN production from the dKO cells, because the high-affinity IFN receptors on the cell surface would bind and deplete IFN-α/β from the supernatant. Importantly, depletion of the IFN receptor in the dKO cells blocks the up-regulation of ISGs and reverses cell resistance to VSV infection (Fig 4A–C). While we cannot rule out the possibility that something in addition to IFN is produced and contributes to the observed results, our data indicate that the observed phenotype of the dKO cells is mainly due to IFN production.

We identified TBK1 as the target of Gcn5 inhibition by depleting individual components of innate immune signaling pathway that controls IFN-β production. Additional lines of evidence also support negative regulation of innate immune signaling and IFN-β production by Gcn5 through targeting TBK1. First, Gcn5 inhibits transcriptional activity of wild-type IRF3 but does not affect the activity of a nuclear-localized, constitutively active mutant of IRF3. These findings suggest that Gcn5 does not interfere with IRF3-dependent transcriptional activation of *IFNB* gene in the nucleus and that Gcn5 works upstream of IRF3. Second, inhibiting TBK1 kinase activity is functionally equivalent to TBK1 depletion in blocking IFN production and reversing ISG up-regulation in *Gcn5/PCAF* dKO cells. In addition, Gcn5 directly binds to TBK1 *in vitro* and interacts with TBK1 in cells. Gcn5 also inhibits TBK1-induced *IFNB* promoter activity. Gcn5 inhibits TBK1 S172 phosphorylation, a modification critical for TBK1 kinase activity. Lastly, Gcn5 inhibits phosphorylation on Thr308 of AKT, an endogenous substrate of TBK1.

We do not rule out the possibility that additional mechanisms may contribute to the observed results. For example, Gcn5 has been shown to repress the transcriptional activity of NF-κB [19]. Loss of Gcn5/PCAF in cells would increase the transcriptional activity of NF-κB and may lead to the production of not only IFN-β but also pro-inflammatory cytokines [20]. Such a mechanism may contribute to the observed phenotype in the dKO cells.

IFN expression is tightly regulated to prevent excessive harmful immune responses. Aberrant activation of IFN expression is related to autoimmune diseases, such as the systemic lupus erythematosus [21]. Our discovery that double deletion of *Gcn5* and *PCAF* causes aberrant activation of IFN expression, even in the absence of virus infection, indicates that Gcn5/PCAF may also play an important role in preventing systemic innate immune activation that ultimately leads to autoimmune disease.

Materials and Methods

Detailed information can be found in Supplementary Materials and Methods.

Plasmids, antibodies, cell culture, retroviral gene transfer, and viruses

Plasmids, shRNA constructs (Supplementary Table S3), and antibodies (Supplementary Table S4) are described in Supplementary

Materials and Methods. Unless indicated, cells were cultured in DMEM plus 10% FBS. Retroviral infection of cells was done as described [8]. VSV expressing GFP (VSV-GFP) and influenza virus A/WSN/33 (WSN) were used to test cell sensitivity to virus infection.

ELISA and luciferase assay

IFN-α/β levels in media were determined by ELISA. Luciferase assays were done using a dual-luciferase reporter assay system.

qRT–PCR, ChIP, ChIP-Seq, Spike-in RNA-Seq, and bioinformatics

See Supplementary Materials and Methods for details. qPCR primers are listed in Supplementary Table S5. Data are presented as mean values ± SD. ChIP-Seq was done as described [22]. From ChIP-Seq data, the ChIP-enriched regions were identified using SICER [23]. ChIP-Seq and RNA-Seq data sets were deposited in GEO database (accession no. GSE60969).

GST pull-down, co-immunoprecipitation, immunoblotting, and kinase assays

GST pull-down, co-immunoprecipitation, and immunoblotting were done as described [8]. Protein kinase assays were done as described [24].

Supplementary information for this article is available online: http://embor.embopress.org

Acknowledgements
We thank Kate Fitzgerald for Gal4-IRF3, pcDNA3-TBK1, and *IFNβ*-Luc plasmids, Rongtuan Lin and John Hiscott for Gal4-IRF3(5D) and GST-IRF3(380-427aa) plasmids, Jonathan Ashwell for VSV-GFP, Sarah Park for proof-reading the manuscript. This work was supported by the Intramural Research Program of the NIDDK, NIH to KG, and by NIH RO1GM067718 to SYRD. RNA-Seq was made possible by CPRIT (#RP120348).

Author contributions
QJ, SYRD, and KG designed research; QJ, LZ, CW, BD, WL, ZW, and KG performed research; QJ, LZ, CW, BD, KZ, WP, YL, BL, SYRD, and KG analyzed data; and QJ, SYRD, and KG wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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