The human DEAD box protein 3 (DDX3) has been implicated in different processes contributing to gene expression. Interestingly, DDX3 is required as an essential host factor for the replication of HIV and hepatitis C virus (HCV) and is therefore considered a potential drug target. On the other hand, DDX3 interacts with IκB kinase ε (IKKe) and TANK-binding kinase 1 (TBK1) and contributes to the induction of antiviral type I interferons (IFNs). However, the molecular mechanism by which DDX3 contributes to IFN induction remains unclear. Here we show that DDX3 mediates phosphorylation of interferon regulatory factor 3 (IRF3) by the kinase IKKe. DDX3 directly interacts with IKKe and enhances its autophosphorylation and activation. IKKe then phosphorylates several serine residues in the N terminus of DDX3. Phosphorylation of DDX3 at serine 102 (S102) is required for recruitment of IRF3 to DDX3, facilitating its phosphorylation by IKKe. Mutation of S102 to alanine disrupted the interaction between DDX3 and IRF3 but not that between DDX3 and IKKe. The S102A mutation failed to enhance ifnb promoter activation, suggesting that the DDX3-IRF3 interaction is crucial for this effect. Our data implicates DDX3 as a scaffolding adaptor that directly facilitates phosphorylation of IRF3 by IKKe. DDX3 might thus be involved in pathway-specific activation of IRF3.

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Human DEAD Box Helicase 3 Couples IκB Kinase ε to Interferon Regulatory Factor 3 Activation

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The human DEAD box RNA helicase DDX3 is a multifunctional cellular protein which has been implicated in various processes linked to gene expression (1). Its RNA helicase activity is coopted by viruses that require DDX3 as an essential host factor for their replication, such as HIV and HCV (2–4). In contrast, we and others have previously demonstrated that DDX3 contributes to antiviral innate immune signaling pathways leading to ifnb induction (5–9). This function of DDX3 is independent of its ATPase and helicase activity (6, 7). ifnb promoter induction requires activation of the transcription factors IRF3 and IRF7, which occurs only downstream of certain, mostly antiviral, pattern recognition receptors (PRRs). Antiviral PRRs comprise the endosomal Toll-like receptors TLR3, TLR7, TLR8, and TLR9, cytosolic RIG-like helicases (RLHs), and several newly discovered cytosolic DNA receptors (10). These receptors recognize different species of viral nucleic acids and induce type I IFNs in response (11). TLR3 and TLR4 engage the TRIF (TIR domain-containing adaptor inducing IFN-β)-dependent pathway for activation of IRF3 (12). In this pathway, phosphorylation-dependent activation of IRF3 is mediated by the IKK-related kinases TBK1 and IKKe (13, 14). The RLHs also utilize TBK1 and IKKe for IRF3 activation after engaging the adaptor molecule MAVS (mitochondrial antiviral signaling) (15, 16). DNA receptors activate IRF3 through TBK1 and the adaptor molecule STING (stimulator of IFN genes).

While these signaling pathways converge on IKKe and TBK1 for IRF3 activation, not all receptors that activate IKKe and/or TBK1 lead to IRF3 activation (17). This suggests that additional factors are required for linking the activated kinases to their substrate IRF3.

We previously showed that DDX3 interacts with IKKe following activation of the RIG-I (retinoic acid-inducible) pathway and that it contributes to ifnb promoter induction downstream of PRRs that utilize IKKe and TBK1 for IRF3 activation (6). Soulat et al. published similar results regarding a role for DDX3 in ifnb induction and demonstrated that DDX3 interacts with and is phosphorylated by TBK1 (7). They also showed binding of DDX3 to the ifnb promoter and hypothesized that phosphorylated DDX3 directly mediates ifnb transcription (7). Interestingly, the function of DDX3 in ifnb induction is targeted by viral immune evasion strategies that inhibit the induction of this potent antiviral cytokine. For example, we previously showed that the vaccinia virus (VACV) protein K7 antagonizes DDX3 by binding to its N-terminal tail region, which is required for ifnb promoter induction (6, 18, 19). The hepatitis B virus (HBV) polymerase (Pol) is another viral protein that interacts with DDX3. Initially it was shown that DDX3 gets incorporated into the viral nucleocapsid and blocks viral replication (20). More recently, it was demonstrated that HBV Pol targets DDX3 in much the same way as K7: it blocks its interaction with IKKe/TBK1 and consequently inhibits IRF3 activation and ifnb induction (8, 9). Another recent study also confirmed a role for DDX3 in ifnb induction but placed DDX3 further upstream in the signaling pathway. Oshiumi et al. suggested that DDX3 directly senses HCV RNA and then triggers MAVS-dependent signaling (5). In a follow-up study, those authors also suggested that the HCV core protein, which had previously been shown to interact with DDX3 (21–23), blocks the interaction between DDX3 and MAVS and thereby prevents initiation of this pathway (24). In summary, there is now strong evidence that human DDX3 contributes to ifnb promoter induction, in particular with respect to the RIG-I pathway. However, its exact placement in the signaling pathway and its mechanism of action are still unclear. Several studies, including ours, placed DDX3 downstream of TBK1/IκKe (6–9). However, Oshiumi et al. placed it upstream of MAVS, as a viral RNA sensor that functions in conjunction with RIG-I/MAVS (5, 24). It is also unclear whether the...
effect of DDX3 is located upstream of IRF3 activation and/or whether it binds directly to the ifnb promoter to enhance transcription (6, 7). The present study therefore aimed at placing DDX3 more firmly in the signaling pathway(s) leading to ifnb transcription (6, 7). The present study therefore aimed at placing the functional consequences of this interaction. We show here that DDX3 mediates IKKe activation and then couples the active kinase to its substrate, the transcription factor IRF3. Mechanistically, DDX3 therefore acts as a downstream scaffolding adaptor that links IKKe and IRF3.

MATERIALS AND METHODS

Plasmids, recombinant proteins, and antibodies. The expression constructs pCMV-HA-DDX3, pCMV-Myc-DDX3, pHis-DDX3, HA-K7, and His-K7 were described in our previous study (6). pCMV-HA-DDX3 and pHis-Parallel2-DDX3 point mutations were introduced by following the instructions for the QuikChange site-directed mutagenesis kit (Agilent), except that Phusion HiFi polymerase was used (New England Biolabs). TBK1-Flag, IKKe-Flag, IKKe KD-Flag (kinase dead, K48A), and IRF3-Flag were kind gifts from Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA). IKKe-Flag truncation constructs were generated by amplifying the relevant sequences by PCR from IKKe-Flag and inserting them into pFlag-CMV2 (Sigma-Aldrich).

DDX3(1–408) (a DDX3 construct consisting of amino acids [aa] 1 to 408), DDX3(409–662), DDX3(139–408), and DDX3(110–408), DDX3(120–408), and DDX3(130–408) were constructed by amplifying the relevant sequences by PCR from pCMV-HA-DDX3 and inserting them into pHisParallel2 or pCMV-HA vectors. Full-length DDX3 was subcloned from pHis-DDX3 into pGSTParallel2 using the EcoRI and SalI restriction sites to generate the bacterial expression construct pGST-DDX3, pHis-Rab14 and DDX3(5–172) constructs were kind gifts from Amir Khan (Trinity College Dublin). Glutathione S-transferase (GST)–IRF3 (aa 380 to 427) peptides and constructs were kindly provided by John Hiscott and Qiang Sun (McGill University, Montreal, Canada). A construct for full-length His-IRF3 was provided from Marion Butler (NUI Maynooth). Purified recombinant protein kinase GST-IKKε was purchased from Proinase (Freiburg, Germany). The antibodies used were anti-Flag M2 monoclonal antibody (MAb) (Sigma-Aldrich), anti-Myc MAb clone 9E10 (Sigma-Aldrich), antithamagglutinin (anti-HA) MAb (Covance), anti-DDX3 (Santa Cruz or Bethyl Laboratories), anti-IKKε (Abcam, Cambridge, United Kingdom), anti-IRF3 (IBL), anti-phospho-Ser396-IRF3, anti-phospho-Ser172-TBK1/TBK2, anti-IRF3-Flag were kind gifts from Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA). IKKe-Flag truncation constructs were generated by amplifying the relevant sequences by PCR from IKKe-Flag and inserting them into pFlag-CMV2 (Sigma-Aldrich).

Cell culture and transfection. HEK293T and A549 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) with Glutamax ( Gibco), supplemented with 10% fetal calf serum and 50 ng/ml gentamicin (Sigma-Aldrich). Transient DNA transfections for immunoprecipitation and Western blot analysis. Data are expressed as mean fold induction ± standard deviations (SD) relative to control levels for an individual experi-
DDX3 enhances activation of IKKe and IRF3. First, we wanted to confirm whether DDX3 affects activation of IRF3 or whether it exerts its effect downstream of or in parallel with IKF3. We had previously shown that DDX3 knockdown affects an IKKe transactivation assay based on a GAL4-IRF3 fusion protein used in conjunction with a GAL4-dependent reporter construct (6). VACV K7 and HBV Pol, both of which target DDX3, have been shown to inhibit IRF3 activation, which provides indirect evidence for involvement of DDX3 in IKF3 activation (6, 8, 9). However, it has also been suggested that DDX3 binds directly to the ifnb promoter and has no interaction with IRF3 (7). In order to test for an effect of DDX3 on IKKe and IRF3 activation, we used small interfering RNA (siRNA) to knock down endogenous human DDX3. Flag-IKKε was then expressed in these cells, followed by immunoprecipitation and *in vitro* kinase assays with recombinant glutathione- S-transferase (GST)-IRF3 (aa 380 to 427). DDX3 knockdown with two different siRNA oligonucleotides led to a clear reduction of IKKe-induced phosphorylation of IRF3 at serine 396 (Fig. 1A). S396 is one of the key phosphorylation sites for IKKe/TBK1 and is required for IRF3 activation (25). This finding suggested that IKKe activation or activity is decreased in the absence of DDX3. Consistent with this, the addition of recombinant DDX3 enhanced IKKe-mediated S396 phosphorylation of IRF3 in kinase assays with recombinant IKKe (Fig. 1B). We also noticed that the presence of DDX3 induced a higher-molecular-weight form of IKKe (Fig. 1B, asterisk), which likely represents a phosphorylated form of IKKe. We therefore tested whether DDX3 enhances phosphorylation of IKKe at serine 172. S172 is located in the activation loop of IKKe and required for its activation (26). Indeed, S172 phosphorylation of IKKe was enhanced in the presence of DDX3 (Fig. 1C, top, lanes 2 and 4). DDX3 also enhanced IKKe-mediated IRF3 phosphorylation in the same experiment (Fig. 1C, third panel, lane 4), likely as a consequence of increased IKKe activity. When DDX3 was coexpressed in HEK293 cells with small amounts of exogenous IKKe, DDX3 also enhanced IKKe and IRF3 phosphorylation (Fig. 1D). Furthermore, exogenous expression of DDX3 enhanced Sendai virus (SeV)-induced phosphorylation of IRF3 (Fig. 1E). Finally, we wanted to confirm that endogenous DDX3 is required for activation of endogenous IKKe and IRF3 following SeV stimulation. HEK293 cells have low levels of endogenous IKKe, and we therefore knocked down DDX3 expression in the human lung epithelial cell line A549. In A549 cells with DDX3 knockdown, S396 phosphorylation of IRF3 was reduced compared to that in cells treated with a control siRNA oligonucleotide (NC) (Fig. 1F, fifth panel). In experiments with recombinant or overexpressed IKKe (Fig. 1A to E), we used an antibody that recognizes pS172 in both TBK1 and IKKe. With the same antibody, we observed a clear reduction of endogenous pS172 TBK1/IKKe phosphorylation in cells treated with DDX3 siRNA (Fig. 1F, second panel). In addition, we used a newer antibody that is more specific for phosphorylated IKKe and saw a similar reduction in pS172 IKKe levels in DDX3 knockdown cells (Fig. 1F, third panel). As DDX3 also interacts with TBK1 (7), it is possible that DDX3 affects both TBK1 and IKKe activation. Our study focused on IKKe activation (because in our hands it is the stronger binding partner for DDX3), and we carried out a combination of experiments with recombinant, overexpressed, and endogenous IKKe. Our results clearly demonstrate that DDX3 acts at the level of IKKe and IRF3 by enhancing autophosphorylation of IKKe and (consequently) IRF3 phosphorylation.

**RESULTS**

**DDX3 directly interacts with the scaffolding/dimerization domain of IKKe.** The *in vitro* kinase assays whose results are shown in Fig. 1B and C were carried out with recombinant IKKe and DDX3, suggesting that DDX3 directly interacts with IKKe. We next carried out pulldown assays to confirm this and to map their interaction. The recombinant His-tagged DDX3 proteins we used for this analysis were the full-length protein (aa 1 to 662), the N-terminal domain (aa 1 to 408), the C-terminal domain (aa 409 to 662), and the N-terminal domain with a truncated N-terminal tail (aa 139 to 408). The 1–408 and 409–662 constructs split DDX3 at the linker region between its two RecA-like globular domains (27). We have previously shown that the flexible N-terminal tail region (aa 1 to 139) is required for the effect of DDX3 on the ifnb promoter (6), which is why we included the 139–408 truncation mutant.

Full-length recombinant His-DDX3, as well as its N-terminal (aa 1 to 408) and C-terminal (aa 409 to 662) domains, pulled down GST-tagged recombinant IKKe, confirming the direct interaction and suggesting that DDX3 contains at least two distinct interaction sites for IKKe (Fig. 2A). Interestingly, the 139–408 truncation of DDX3 did not interact with IKKe (Fig. 2A), indicating that the IKKe interaction is at least partially mediated by residues in the flexible N-terminal region of DDX3 that is required for ifnb promoter induction.

In Fig. 1, we have demonstrated that DDX3 enhances IKKe autoactivation. Other adapters proteins that contribute to IKKe and TBK1 activation in cells are NAP1 (NAK-associated protein 1), TANK (TRAF family member-associated NF-κB activator) and Sintbad (similar to NAP1 TBK1 adaptor) (28), which have recently been shown to bind to the C-terminal region of IKKe (aa 684 to 716) and the corresponding region in TBK1 (29, 30). We therefore asked whether DDX3 interacts with IKKe in a similar manner. We transfected HEK293T cells with constructs for Myc- tagged DDX3 and a range of different Flag-tagged IKKe truncation mutants, followed by immunoprecipitation with an anti-Flag antibody. DDX3 interacted strongly with a truncated IKKe construct lacking the kinase and ubiquitin-like domains (aa 383 to 716), as well as constructs lacking the adapter-binding region (aa 1 to 684 and 1 to 647) (Fig. 2B). DDX3 weakly interacted with a truncated IKKe containing the kinase and ubiquitin-like domains only (aa 1 to 383). We also performed a semidogenous co-IP by transfecting HEK293T cells with truncated IKKe constructs, followed by immunoprecipitation of endogenous DDX3. Again, DDX3 interacted with all truncated IKKe constructs but only weakly with the 1-383 construct (Fig. 2C). We did not observe consistent differences in binding between the other IKKe truncations, suggesting that DDX3 binds mainly to a region between aa 383 and 647 in IKKe, which corresponds to its scaffolding/dimerization domain (SDD). The adapter binding region (aa 684 to 716) was not required for DDX3 binding.
While the kinase and ubiquitin-like domains of IKKε are also not required for the interaction, DDX3 binds to this region weakly, presumably as a substrate for IKKε’s kinase activity.

The N terminus of DDX3 is phosphorylated by IKKε. There is evidence that interaction with IKKε leads to phosphorylation of DDX3. Soulat et al. described DDX3 as a phosphorylation target of TBK1 (7), and we also observed the appearance of higher-molecular-weight forms of DDX3 after coexpression of DDX3 with IKKε (6) (Fig. 3A). We carried out in vitro kinase assays with IKKε and recombinant DDX3 in order to confirm that DDX3 is a substrate of IKKε. Recombinant DDX3 was phosphorylated in vitro by recombinant IKKε (Fig. 3B). This phosphorylation was comparable in intensity to that of the bona fide IKKε substrate IRF3 in the same experiment (Fig. 3B). Because the N- and C-terminal domains of DDX3 both directly interacted with IKKε (Fig. 2A), we asked whether both domains are phosphorylated by IKKε. While we observed a very weak phosphorylation signal for the C-terminal domain of DDX3 (aa 409 to 662), the N-terminal domain (aa 1 to 408) was phosphorylated much more strongly (Fig. 3C).
truncation mutant DDX3(139–408) failed to interact with IKKε (Fig. 2A), which suggested that the phosphorylation sites for IKKε might be contained in the N-terminal tail region. Therefore, we next used the isolated N-terminal region of DDX3 as a substrate. Polypeptides comprising aa 1 to 139 or aa 5 to 172 of DDX3 were strongly phosphorylated by IKKε in kinase assays, suggesting that this region indeed contains phosphorylation sites for IKKε, as evidenced by the shift of DDX3(5–172) after nonradioactive kinase assays with immunoprecipitated IKKε or TBK1 (Fig. 3E).

Mapping of IKKε phosphorylation sites in DDX3. We next aimed at identifying the exact residues in DDX3 that are phosphorylation sites for IKKε. This was not a trivial task, considering that the N terminus of DDX3 (aa 1 to 172) contains 29 serine residues and the C-terminal SG-rich domain (aa 580 to 662) contains 20 serine residues, many of them arranged in clusters that are potential targets for IKKε phosphorylation.

Hutti et al. recently defined an IKKε consensus phosphorylation motif, which they generated using a positional scanning peptide library assay (31). Those authors kindly provided us with their Scansite matrix (31), which we used to scan the DDX3 protein sequence for IKKε consensus motifs (32). This revealed a large number of potential, if suboptimal, IKKε phosphorylation sites, mainly clustered in the N- and C-terminal serine-rich regions of DDX3. Due to our experimental evidence that the N-terminal tail region of DDX3 (aa 5 to 172) is strongly phosphorylated by IKKε, we focused on this region. Within this region, serine S71 had the best Scansite score (0.7571), followed by S82, S57, S86, S61, S152, S74, S28, S131, S62, S83, and S102 (1.1486). Of these, S82, S57, S86, S61, S152, S74, S28, S131, S62, S83, and S102 were also predicted to be phosphorylated by NetPhos 2.0 (score 0.75) (33). Due to this large number of potential phosphorylation sites, we carried out phosphorylation site mapping by mass spectrometry. For this analysis, we used full-length DDX3 and DDX3(1–408) that had been phosphorylated in vitro using recombinant IKKε. We separated the phosphorylated protein by SDS-PAGE and carried out a tryptic digest of the protein band, followed by analysis of the tryptic peptides by ion trap mass spectrometry (IT-MS). For full-length DDX3, the only significant hit we obtained was a phosphorylated peptide containing S70, S71, and S74. For DDX3(1–408), we obtained hits that matched phosphorylation sites at S152, at S70, S71, or S74, at S102 or S109, and at S82, S83, or S86 (Table 1). Based on these data and the Scansite predictions, we selected S71, S82, S83, S102, and S152 for serine-to-alanine substitution mutations. We generated single-alanine substitution mutants for S71, S102, and S152, a double-alanine mutant for S82 and S83 (2A mutant), a triple-alanine mutant for S82, S83, and S102 (3A mutant), and a quadruple-alanine mutant for S71, S82, S83, and S102 (4A mutant). In kinase assays with IKKε, our single-alanine mutants displayed reductions in phosphorylation between ~32% for the S102A mutant and ~60% for the S71A and S152A mutants. For the 2A mutant, phosphorylation was reduced by ~50%, and the 3A and 4A mutants retained only ~35% of phosphorylation compared to the wild-type 1–408 truncation construct (Fig. 4A). These data suggest that the serine residues we identified are indeed phosphorylated by IKKε; however,
none of our alanine substitution mutants displayed a complete loss of phosphorylation. We therefore also generated additional truncation mutants to narrow down the region containing the main phosphorylation site(s) for IKKε. In kinase assays with IKKε, the 80-408 and 100-408 truncation mutants retained strong phosphorylation (even though their phosphorylation was reduced by 11% and 37% compared to the 1-408 mutant), while constructs consisting of aa 110 onwards were not phosphorylated by IKKε (Fig. 4B). These data pinpointed the region between aa 100 and 110 as a major target site for IKKε. This region of DDX3 contains two serine residues, S102, and S109. Interestingly, S102 was predicted to be phosphorylated by both the IKKε Scansite matrix and Netphos. Our mass spectrometry results and the alanine substitution mutants also confirmed that S102 is likely an IKKε phosphorylation target.

We therefore decided to mutate S102 to alanine in the context of the 80-408 truncation mutant. This 80-408 S102A mutant nearly completely lost phosphorylation by IKKε (Fig. 4C), suggesting that S102 accounts for the majority of the strong phosphorylation displayed by the 80-408 mutant.

Serine 102 is required for the effect of DDX3 on the ifnb promoter. Next, we tested the interaction of our truncation and alanine mutants with IKKε and their functionality in ifnb reporter gene assays. Phosphorylation of the truncation mutants correlated with their ability to bind to IKKε. While DDX3(80–408) and DDX3(100–408) interacted with IKKε in His pull-down assays, the DDX3(110–408) mutant failed to bind to IKKε (Fig. 5A). We hypothesized that the interaction with IKKε would be required for DDX3 to induce ifnb and therefore tested our truncation mutants in ifnb promoter reporter gene assays. DDX3(1–408) enhanced IKKε-induced ifnb promoter activation similarly to full-length DDX3 (6) (Fig. 5B). As expected from the interaction data, constructs consisting of aa

![Image of FIG 3](image_url)

**FIG 3** The N terminus of DDX3 is a direct phosphorylation target for IKKε. (A) HEK293T cells were transfected with constructs for Myc-DDX3 and 100 ng (+) or 250 ng (+) of a Flag-IKKε construct or 1,000 ng (+) or 1,800 ng (+) of a construct for a kinase-dead (KD) Flag-IKKε mutant (K38A). Cell lysates were subjected to SDS-PAGE and WB analysis. (B to D) For in vitro kinase assays, recombinant GST-IKKε was incubated with recombinant substrate in the presence of [γ-32P]ATP. Samples were then subjected to SDS-PAGE and autoradiograph analysis showing incorporation of [γ-32P]ATP. Total amounts of recombinant proteins were visualized by Coomassie staining. Substrates for IKKε were full-length DDX3 or full-length IRF3 (B), full-length His-DDX3 or the indicated His-DDX3 truncation constructs (C), or recombinant His-DDX3(1–139) or His-DDX3(5–172) or the unrelated control protein His-Rab14 (D). (E) In vitro kinase assays were carried out as described above but in the presence of unlabeled ATP. This was followed by SDS-PAGE and WB analysis with an anti-His antibody.
110 onwards failed to enhance IKKε-induced ifnb promoter activation, while DDX3(80–408) and DDX3(100–408) still enhanced ifnb induction, albeit with reduced potency (Fig. 5B).

Next, we asked whether mutation of S102 in DDX3 to alanine disrupts binding to IKKε. However, all our alanine mutants, including the S102A mutant, interacted with IKKε in pulldown assays (Fig. 5C). Interestingly, a phosphomimetic S102D mutant, in which serine 102 was replaced by negatively charged aspartic acid, enhanced IKKε-induced ifnb promoter activation similarly to wild-type DDX3 (Fig. 5D), suggesting that phosphorylation of this residue is indeed part of the mechanism. Because the S102A mutant of DDX3 retained its functional interaction with IKKε (Fig. 5C and E) but was impaired with respect to IRF3 phosphorylation and ifnb promoter induction (Fig. 5D and E), we next tested its interaction with IRF3. We used His-tagged recombinant IRF3 in pulldown assays with ectopically expressed wild-type DDX3 or S102A DDX3 from cell lysates. As seen before, wild-type DDX3 interacted with IRF3, while, interestingly, the S102A mutant failed to interact with IRF3 (Fig. 6C). This suggested that the defect of the DDX3 S102A mutant might lie in an inability to interact with IRF3. It also suggested that IRF3 and IKKε bind to a similar region in DDX3, and we therefore used our DDX3 truncation mutants to map its IRF3 interaction site. IRF3 failed to interact with the DDX3(100–408) mutant but not the DDX3(110–408) mutant (Fig. 6D). This indicates that IRF3 and IKKε have adjacent and potentially overlapping, but not identical, binding sites in the N terminus of DDX3. Next, we tested whether phosphorylation of S102 is required to mediate IRF3 binding. To this end, we transfected HEK293T cells with constructs for either wild-type DDX3, the phosphodeficient S102A mutant, or the phosphomimetic S102D mu-
tant, followed by stimulation with SeV. Cell lysates were tested for DDX3 binding to recombinant His-DDX3 or His-DDX3 truncation mutants were incubated with cell lysates containing Flag-IKKε. Following pulldown, interacting proteins were subjected to SDS-PAGE and Western blot (WB) analysis. The black border indicates images originate from the same autoradiography film and can be compared in intensity. (B) HEK293 cells were transfected with an ifnb promoter reporter gene construct and expression constructs for Flag-IKKε and HA-DDX3 or HA-DDX3 truncations. Expression of HA-DDX3 and Flag-IKKε was confirmed by WB analysis. Data for reporter gene assays are expressed as mean fold induction relative to control levels and standard deviations. Shown are results of one representative experiment out of four, performed in triplicate. (C) The assay was performed as described for panel A but using His-tagged alanine mutants of DDX3(1–408). Black borders indicate images that originated from the same autoradiography film and can be compared in intensity. (D) The assay was performed as described for panel B but testing full-length HA-tagged alanine mutants of DDX3. (E) Recombinant GST-IKKε was incubated with ATP, GST-IRF3 (aa 380 to 427), and His-DDX3(1–408) or the S102A mutant of His-DDX3(1–408). Samples were then subjected to SDS-PAGE and WB analysis. Phosphorylation of IKKε and IRF3 was detected with phospho-specific antibodies against pS172 (IKKε) and pS396 (IRF3).

FIG 5 IKKε binding and serine 102 are required for the effect of DDX3 on the ifnb promoter. (A) Recombinant His-DDX3 or His-DDX3 truncation mutants were incubated with cell lysates containing Flag-IKKε. Following pulldown, interacting proteins were subjected to SDS-PAGE and Western blot (WB) analysis. The black border indicates images originate from the same autoradiography film and can be compared in intensity. (B) HEK293 cells were transfected with an ifnb promoter reporter gene construct and expression constructs for Flag-IKKε and HA-DDX3 or HA-DDX3 truncations. Expression of HA-DDX3 and Flag-IKKε was confirmed by WB analysis. Data for reporter gene assays are expressed as mean fold induction relative to control levels and standard deviations. Shown are results of one representative experiment out of four, performed in triplicate. (C) The assay was performed as described for panel A but using His-tagged alanine mutants of DDX3(1–408). Black borders indicate images that originated from the same autoradiography film and can be compared in intensity. (D) The assay was performed as described for panel B but testing full-length HA-tagged alanine mutants of DDX3. (E) Recombinant GST-IKKε was incubated with ATP, GST-IRF3 (aa 380 to 427), and His-DDX3(1–408) or the S102A mutant of His-DDX3(1–408). Samples were then subjected to SDS-PAGE and WB analysis. Phosphorylation of IKKε and IRF3 was detected with phospho-specific antibodies against pS172 (IKKε) and pS396 (IRF3).

Our data therefore implicate DDX3 as a scaffolding adaptor that links IKKε and IRF3 and coordinates their activation.

DISCUSSION

While we and others previously showed that the human DEAD box protein DDX3 contributes to innate immune signaling path-
ways leading to IFN-β induction, its exact placement in the signaling pathways and mechanism of action was controversial. Our present study elucidated the functional relevance of the interaction between DDX3 and IKKε, and its role in IRF3 activation. We show that DDX3 binds directly to IKKε and to IRF3 and contributes to their activation. In fact, strong IRF3 phosphorylation occurred in an in vitro system comprising only recombinant IRF3, IKKε, and DDX3 in the presence of ATP. The N-terminal domain of DDX3(1–408) was sufficient for this and its effect on the IFNβ promoter. While DDX3 enhanced IKKε autoactivation (as evidenced by increased phosphorylation of S172 in the activation loop of the kinase), this was only partially responsible for the increased phosphorylation of IRF3 observed in the presence of DDX3. In this context, it is important to note that activation of...
IKKe or TBK1 is not sufficient for activation of IRF3 in cells but that an additional adaptor is required for linking the activated kinases to IRF3 (17, 35). TRAF3 (TNF receptor-associated factor 3) was suggested as a candidate for this role, as it is required for IRF3 but not TBK1/IKKe activation (35). Also, in a recent study, the adaptor molecule STING, which facilitates signaling downstream of DNA receptors, was shown to link TBK1 to IRF3 (36). Interestingly, the effects of STING on TBK1 and IRF3 strongly resemble the effects we describe here for DDX3, IKKe, and IRF3. STING also mediated TBK1 and IRF3 activation in an *in vitro* reconstitution system that is essentially identical to our experimental setup (36). Interestingly, both DDX3 and STING had distinct effects on IKKe and IRF3 activation.

**Effect of DDX3 on IKKe activation.** With respect to its mechanism for enhancing IKKe activation, we have shown that DDX3 interacts with the scaffolding/dimerization domain (SDD) of IKKe (aa 383 to 647) and not with the C-terminal region (aa 684 to 716) that mediates binding to NAP1, TANK, and Sin3beta. These adaptor proteins have also been shown to be involved in activation of TBK1 and IKKe downstream of PRRs (28). However, as they interact with a different region of IKKe, they appear to have a distinct mode of action compared to DDX3 (29, 30). It was suggested that they might recruit TBK1/IKKe to distinct subcellular locations for activation (29).

In the recently solved crystal structure of IKKβ, a kinase that is closely related to IKKe, the SDD forms an elongated α-helical region (37). The SDD mediates dimerization of the kinase and might contribute to substrate specificity. Based on their structure, the authors concluded that autophosphorylation of the activation loop could not occur within a single IKKβ dimer but was more likely to be mediated by a second IKKβ dimer in a transautophosphorylation event. Thus, higher-order arrangements of IKKβ might be required for autophosphorylation (37). It is expected that TBK1 and IKKe display a domain structure similar to that of IKKβ. Indeed, recent biochemical evidence suggests that TBK1 is activated in a similar transautophosphorylation event requiring the juxtaposition of two TBK1 dimers for phosphorylation of S172 (38). These studies suggest that a critical event for activation of the IKKs and IKK-related kinases is the positioning of kinase dimers in a conformation that allows for their transautophosphorylation.

Through its interaction with the SDD of IKKe, DDX3 could potentially stabilize such arrangements of IKKe dimers and thereby support efficient transautophosphorylation. Further biochemical studies will be required to confirm whether DDX3 indeed supports the formation of such higher-order IKKe complexes.

DDX3 also weakly interacted with the kinase domain of IKKe. This is likely because DDX3 is also a phosphorylation substrate of IKKe and therefore has to transiently interact with its kinase domain.

**Phosphorylation of DDX3 by IKKe.** Our data provide strong evidence that the N-terminal tail region of DDX3 is phosphorylated directly by IKKe. DDX3 has previously been identified as an interaction partner and phosphorylation target of TBK1 (7). Several TBK1 phosphorylation sites were identified within DDX3; however, most of these were located within the two RecA-like globular domains of DDX3, and none mapped to the N-terminal tail of DDX3 (7). In our hands, both the N-terminal (aa 1 to 408) and C-terminal (aa 409 to 662) domains of DDX3 interacted with IKKe, but only the N-terminal domain was strongly phosphorylated (Fig. 3C). It is likely that TBK1 and IKKe phosphorylate the same or at least overlapping residues in DDX3, and we showed that TBK1 can phosphorylate the isolated N terminus of DDX3 (aa 5 to 172) to a degree similar to that exhibited by IKKe (Fig. 3E). The published TBK1 phosphorylation sites were determined using a peptide array (7), and it is possible that specificity of phosphorylation differs in the context of larger protein domains. However, we also detected an interaction of IKKe with the C-terminal domain of DDX3 (aa 409 to 662) as well as weak phosphorylation of this domain by IKKe. It is therefore possible that the C terminus of DDX3 also contains veritable IKKe phosphorylation sites.

Here, we show that the N-terminal tail region of DDX3 that is required for *ifnb* induction undergoes strong phosphorylation by IKKe. Our N-terminal truncation mutants revealed the region between aa 100 and 110 of DDX3 as an IKKe binding site and serine 102 as a functionally important IKKe phosphorylation site. In addition, our mass spectrometry analysis and alanine substitution mutants suggested that there are multiple other phosphorylation sites for IKKe within the N terminus of DDX3, including S71, S82 or S83, and S152. It is not unusual for IKKe to phosphorylate several clustered serine residues. In IRF3, it phosphorylates at least seven serine residues within two C-terminal clusters (39). It is possible that some of these phosphorylation events are interdependent in that phosphorylated serine residues prime for subsequent phosphorylation of nearby serine residues. This interdependency could explain the significant
reductions in phosphorylation we observed with our single-alanine-substitution mutants. On the other hand, we were unable to fully abrogate phosphorylation of DDX3 by IKKε with our quadruple-alanine mutant (4A 1–408 mutant), suggesting that there might be further IKKε phosphorylation sites in the N-terminus of DDX3.

In order to identify functionally important IKKε phosphorylation sites, we tested our alanine substitution mutants in ifnb reporter gene assays. S71 most closely matches the IKKε consensus sequence determined by Hutti et al. and was also the only phosphopeptide we identified in our mass spectrometry analysis with full-length DDX3. However, phosphorylation of the 80–408 truncation mutant was only slightly reduced compared to that of full-length DDX3, and mutation of S71 to alanine did not affect the ability of DDX3 to enhance IKKε-mediated ifnb promoter activation. We were initially also particularly interested in S82 and S83, as these are in the region of DDX3 that is targeted by its VACV antagonist K7. We had previously shown that mutation of the adjacent double phenylalanine motif (F84 and F85) abrogated the effect of DDX3 on the ifnb promoter (19). And while the S82,83A (2A) mutant displayed reduced phosphorylation by IKKε and phosphorylation of the 100–408 construct was also reduced compared to the 80–408 construct, the 2A mutant had no apparent defect with respect to ifnb promoter induction. It is possible that the phosphorylation of S71, S83, S82, and S152 by IKKε has subtle effects on ifnb induction that we failed to detect, or that there is functional redundancy between these residues. It is also possible that the phosphorylation of these residues in DDX3 affects cellular processes unrelated to ifnb induction. We observed increased DDX3 protein levels in the presence of IKKε (Fig. 3A); thus, it is possible that DDX3 phosphorylation by IKKε increases its stability.

In this study, we focused on serine 102 of DDX3, because the phosphodefective S102A mutant, but not a phosphomimetic S102D mutant, failed to mediate ifnb promoter activation. This demonstrated that phosphorylation of S102 in DDX3 by IKKε is required for ifnb induction. The S102A mutant still interacted with IKKε and enhanced its autophosphorylation, but its ability to enhance IRF3 phosphorylation was reduced compared to that of wild-type DDX3 (Fig. 5E). This suggested to us that DDX3 might affect IRF3 activation more directly.

**Effect of DDX3 on IRF3 activation.** We show here that DDX3 directly interacts with IRF3. Interestingly, phosphorylation of DDX3 at S102 by IKKε appeared to increase the affinity of DDX3 for IRF3: the phosphodefective S102A mutant did not interact with IRF3, while the phosphomimetic S102D mutant did (Fig. 6E). Stimulation with SeV increased the interaction between DDX3 and IRF3, which was prevented by the IKKε/TBK1 inhibitor BX795 (Fig. 6E). This provided evidence that DDX3 has to be phosphorylated by IKKε before it can bind to IRF3. Results from our communoprecipitation experiments support this sequence of events: after SeV stimulation, IKKε was recruited to DDX3 first, followed by delayed IRF3 binding (Fig. 6F and G).

The failure of the S102A mutant to induce ifnb promoter activation suggests that the phosphorylation-induced interaction between DDX3 and IRF3 is critical for ifnb induction. We also demonstrate here that IKKε and IRF3 have immediately adjacent binding sites in the N-terminal region of DDX3 (Fig. 6D). We therefore propose a model whereby IKKε-mediated phosphorylation of serine 102 in DDX3 facilitates recruitment of IRF3 to DDX3. This recruitment is required for efficient IRF3 phosphorylation by IKKε, which is possibly facilitated by their juxtaposition when bound to DDX3 (Fig. 7).

**DDX3 and STING act as adaptors that link activated IKK-related kinases to IRF3.** As mentioned above, the protein STING has also recently been shown to facilitate IRF3 phosphorylation by TBK1. STING is required for IRF3 activation and ifnb induction downstream of cytosolic DNA receptors (40). There are intriguing parallels between the effects of STING on TBK1 and IRF3, as reported by Tanaka and Chen (36), and the effects of DDX3 on IKKε and IRF3 we demonstrate here. STING also enhanced both TBK1 autophosphorylation and IRF3 phosphorylation in an in vitro reconstitution system. Intriguingly, for both STING and DDX3, the effects on TBK1/IKKε and IRF3 activation could be uncoupled by a serine-to-alanine mutation. Mutations to Ser366 (or Leu374) in STING disrupted its effect on IRF3 activation but not on TBK1 activation (36). This is strongly reminiscent of our data for the DDX3 S102A mutant. While Tanaka and Chen found no direct evidence that S366 of STING is phosphorylated by TBK1 but suggested that STING phosphorylation by TBK1 strengthens its interaction with IRF3 (36), we demonstrate here that S102 of DDX3 is directly phosphorylated by IKKε and that this phosphorylation mediates IRF3 binding.

It thus appears that STING and DDX3 employ very similar mechanisms for coordinating activation of the IKK-related kinases and IRF3. As mentioned above, it recently emerged that an additional adaptor is required for linking the activated IKK-related kinases to IRF3 in cells (34). Both STING and DDX3 appear to be able to carry out this function. In addition to this, both of them also enhance the upstream activation of the IKK-related kinases. These two functions are distinct, because they can be uncoupled by point mutations in DDX3 (S102A) and STING. This suggests that it might be possible to target the DDX3-IRF3 interaction interface surrounding S102 therapeutically for blocking excess IFN production while leaving other IKKε- and DDX3-mediated cellular functions intact.

In summary, our study has identified a novel direct interaction between DDX3 and IRF3 that is regulated by IKKε phosphorylation of DDX3 and critical for ifnb induction. Our data implicate DDX3 as a bridging adaptor for IKKε and IRF3 in the RLF pathway, reminiscent of the suggested role for STING in DNA receptor pathways. It is therefore possible that STING and DDX3 belong to a group of functionally related scaffolding adaptors that coordinate activation of the IKK-related kinases and IRF3 in different signaling pathways. It will be interesting to see whether more proteins are identified that employ a similar sequential mechanism for the coordinated activation of IKKε/TBK1 and IRF3.

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