

# Molecular basis of the regulation of the double-stranded RNA-activated protein kinase PKR by the Src homology domain-containing adaptor protein Nck1

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## ABSTRACT

Tight regulation of the double-stranded RNA-activated protein kinase PKR is critical for the maintenance of cellular homeostasis due to its potent inhibitory role on general translation. However, the endogenous molecular mechanisms that regulate PKR are still largely unknown. We have previously identified the Src homology domain-containing adaptor protein Nck1 as a novel regulator of PKR through its interaction with PKR. In this study, we report that the negative control of PKR by Nck1 is reversible by showing that significant levels of dsRNA override Nck1 binding and inhibition of PKR. Biochemical approaches reveal that Nck1 must be in its full length form to interact with and modulate PKR activation. However, both binding and regulation appear to occur independently of the classical binding function of Nck1 Src-homology domains and the interaction between the two proteins involves two binding sites for Nck1 on PKR. Furthermore, we found that the dissociation of Nck1 from PKR in the presence of significant levels of dsRNA results from the activation of PKR catalytic activity rather than competition by dsRNA or change in PKR conformation. Finally, we provide strong evidence that Nck1 is phosphorylated by PKR in a cellular context. Hence, Nck1 not only buffers PKR activation, but it also appears to be a substrate of PKR. Therefore, we propose that PKR-mediated Nck1 phosphorylation is part of the mechanism that

promotes Nck1 dissociation from PKR and leads to efficient activation of PKR.

**KEYWORDS:** PKR, Nck adaptor proteins, phosphorylation

## INTRODUCTION

Cells recognize and process diverse signals to cope with various types of stress. Cellular defense mechanisms contributing to these processes involve global downregulation of protein synthesis and concomitantly, initiation of signaling pathways that control the expression of specific genes to overcome different types of stress. In mammals, members of the eIF2 $\alpha$  kinase family are known to regulate protein synthesis at the level of translation in response to various stress conditions by phosphorylating the  $\alpha$ -subunit of the eukaryotic initiation factor 2 (eIF2) on an inhibitory site (Ser<sup>51</sup>) [1]. This family includes HRI (heme-regulated inhibitor, EIF2AK1), activated by heme deficiency, oxidative and heat stresses in erythroid cells [2]; GCN2 (general control non-repressible-2, EIF2AK4), activated in response to amino acid deprivation [3]; PERK (PKR-like endoplasmic reticulum kinase, EIF2AK3), a type 1 transmembrane protein that resides at the endoplasmic reticulum (ER) and is activated upon accumulation of improperly folded secretory proteins in the ER [4]; and PKR (double stranded (ds)RNA-activated protein kinase, EIF2AK2), a component of the antiviral response induced by interferons and activated by viral dsRNA [5, 6]. Of note, all eIF2 $\alpha$

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kinases consist of a conserved kinase domain linked to different regulatory domains that mediate stress-specific activation [7].

PKR is a 551 amino acid protein that consists of two RNA-binding motifs (RBMs) at the N-terminus linked to a Ser/Thr protein kinase domain by a central region of unknown function [8]. It has been proposed that in unstressed mammalian cells, PKR is typically in a monomeric latent state due to the auto-inhibitory function of the second RBM, which prevents adenosine triphosphate (ATP) and substrate access by occluding the kinase domain [9]. This model has been challenged and recently, a small-angle scattering structural analysis revealed that PKR belongs to the family of proteins that contain intrinsically disordered regions [10]. This model does not exclude the fact that PKR folds into an ordered structure upon binding of dsRNA or its interaction with its endogenous activator PACT/RAX [11]. In agreement, activation of PKR involves dimerization and autophosphorylation upon binding of viral or synthetic dsRNA [12]. Previously, we identified a novel role for the Src homology (SH) domain-containing adaptor protein Nck1 in limiting PKR activation and signaling, through its interaction with PKR [13]. Nck1, a small 47 kDa protein composed exclusively of three N-terminal SH3 and one C-terminal SH2 domains, is devoid of catalytic activity [14]. We then proposed a model in which constitutive association of Nck1 with PKR maintains PKR in an inactive state in unstressed conditions. In the presence of significant levels of dsRNA, PKR activation initiated by dsRNA binding promotes Nck1 dissociation from PKR [13]. In this study, we further examine the molecular basis of the Nck1-PKR interaction as well as the regulatory role of Nck1 in limiting PKR activation. Our findings are consistent with a model indicating that the interaction of Nck1 and PKR occurs independently of any functional Nck1 SH domains, while involving full length Nck1 interacting with two distinct binding regions on PKR.

## MATERIALS AND METHODS

### Cell culture and transfection

HeLa and Cos-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with antibiotic/antimycotic (Invitrogen)

and 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) at 37 °C in 5% CO<sub>2</sub>/95% O<sub>2</sub>. Cells (80% confluent) were transfected with a plasmid encoding HA-tagged-Nck1 (pRK5-HA-hNck1), HA-tagged-Nck1 mutated in all SH3 domains (SH3M, W38, 143, 229R) or in the SH2 domain (SH2M, R308K). In addition, Flag-tagged PKR wild-type or mutant that lacks six invariant amino acids (PKR $\Delta$ 6: residues 361-366) between the kinase subdomain V and VI [15] and has been reported to function in a dominant-negative fashion [16] were also transiently expressed. Transfections were performed using Lipofectamine-Plus reagent (Invitrogen) according to the manufacturer's instructions and as reported previously [17].

### PKR activation

Twenty-four hours after transfection, PKR was activated by transfecting cells for two hours with a synthetic form of dsRNA (poly IC (InvivoGen), amount indicated in the figure legends) using Lipofectamine-Plus as reported above.

### Cell lysate preparation and immunoblot analysis

Cells were washed once with cold phosphate buffered saline (PBS) and lysed in ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM NaPP<sub>i</sub>, 10 mM NaF) supplemented with protease inhibitors (10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, 200  $\mu$ M activated sodium orthovanadate). Cell lysates were centrifuged at 13,000 g for 10 minutes at 4 °C and protein levels were quantified using a Bradford assay (Bio-Rad). Protein concentrations were normalized using lysis buffer, and subsequent to the addition of 6X Laemmli buffer, samples were heated at 95 °C for 5 minutes and stored at -20 °C until analyzed.

### Western blot and antibodies

Equal amounts of total cell lysate proteins (10-50  $\mu$ g) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad), which were subsequently immunoblotted with antibodies as indicated in the figures. We detected phosphorylation of PKR on its activation site by using a pT<sup>446</sup>-PKR antibody (E120, Abcam) and

eIF2 $\alpha$  Ser<sup>51</sup> phosphorylation by a phosphospecific antibody directed against this site (Invitrogen). Total eIF2 $\alpha$  (FL-315), HA-probe (F-7, for immunoprecipitation and Y-11, for western blotting) and PKR (K-17) were purchased from Santa Cruz. To detect Flag-tagged PKR, an anti-FLAG antibody (M2, Sigma) was used. Anti-Myc antibody (clone 9E10) was purchased from Upstate. Nck1- and Nck2-specific polyclonal antibodies and pan-Nck, which recognizes both Nck proteins, were generated as previously reported [18, 19]. Secondary antibodies used were goat anti-rabbit, goat anti-mouse or Protein A-conjugated horseradish peroxidase (GAR-HRP, GAM-HRP and Prot A-HRP) (Bio-Rad). Signal detection was performed using ECL Plus (Enhanced Chemiluminescence, GE Healthcare) according to the manufacturer's instructions.

### Phospho-affinity polyacrylamide gel electrophoresis (Phos-tag)

A stock solution of Phos-tag<sup>TM</sup> acrylamide (AAL-107; NARD Institute, Japan) was prepared in water (5 mM). The composition of the resolving phospho-affinity SDS-polyacrylamide mini-gels containing acrylamide-pendant Mn<sup>2+</sup>-Phos-tag ligand was the following: 8.25% acrylamide, 0.375 M Tris-HCl (pH 8.8), 50  $\mu$ M Phos-tag, 100  $\mu$ M MnCl<sub>2</sub>, 0.15% ammonium persulfate and 0.003% tetramethylethylenediamine. Gels were run at 90 V for 3 hours and then soaked in transfer buffer supplemented with 1 mM of EDTA for 10 minutes. Subsequently, gels were washed in transfer buffer devoid of EDTA for 10 minutes at room temperature before the proteins were transferred to PVDF membranes and then immunoblotted with pan-Nck antibody.

### Immunoprecipitation

Cos-7 cells were treated with 2 mM of the cross linker Dithiobis(succinimidyl propionate) (DSP) (Thermo Scientific) in PBS for 30 minutes at room temperature. Cells were then washed with a stop buffer (50 mM Tris, pH 7.4) and lysed in radioimmunoprecipitation (RIPA) buffer (50 mM HEPES (pH 7.4), 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM NaPP<sub>i</sub>, 100 mM NaF) supplemented with protease inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mM dithiothreitol (DTT), 10  $\mu$ g/ml Pefabloc<sup>®</sup> SC). HA-Nck1 was

immunoprecipitated from 1.3 mg of protein extracts using lysates normalized at 1  $\mu$ g/ $\mu$ l with RIPA buffer and following overnight incubation at 4 °C with 3  $\mu$ g of anti-HA antibody. Protein A-agarose beads (80  $\mu$ l of 25% slurry solution) were added to the samples and further incubated with agitation for 1 hour at 4 °C. Following three washes with RIPA buffer, final bead pellets were resuspended in 2X Laemmli buffer and heated for 5 minutes at 95 °C before being loaded on a 10% acrylamide gel for SDS-PAGE and western blotting.

### GST pull-down assay

Cos-7 cell lysates were prepared in pull-down lysis buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1% Triton X-100, 10 mM NaPP<sub>i</sub>, 100 mM NaF, 1 mM DTT, 17.5 mM  $\beta$ -glycerophosphate, 4  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine). Clarified lysates (4.5 mg protein) were incubated at 4 °C for 2 hours with recombinant glutathione S-transferase (GST) (20  $\mu$ g) or GST-Nck1 proteins (20  $\mu$ g) previously expressed in bacteria and immobilized on glutathione-agarose beads. Bound proteins were submitted to SDS-PAGE, transferred on nitrocellulose and processed for western blotting with antibodies as indicated in the figures. Equivalent amounts of recombinant proteins used in the assays were revealed by Ponceau S staining of the membrane after western blotting.

### PKR plasmids

C-terminal segments of PKR (amino acid 249 to 551), both wild type (WT) and K296R mutant, were amplified by polymerase chain reaction (PCR) using a forward primer containing a start codon and a HindIII restriction site (5' TTTTAAGC TTATGGCACCCAGATTTGACCTTC 3'), and a reverse primer that lacks a stop codon, but includes a XbaI restriction site (5' TTTTCTC TAGACATGTGTGTCGTTTCATTTTCT 3') from PKR WT and PKR K296R cDNAs. PKR WT N-terminal segment (amino acid 1 to 248) was amplified by PCR using a forward primer containing a start codon and a HindIII restriction site (5' TTTTA AGCTTATGGCTGGTGATCTTTCAGC 3'), and a reverse primer lacking a stop codon, but containing a XbaI restriction site (5' TTTTCTCTAGAGAT CTTTTGCCTTCCTTTG 3'). PCR products,

following HindIII-XbaI digestion, were subcloned into pcDNA 3.1(+) Myc-His version B (Invitrogen) previously digested by the same restriction enzymes and electroporated into *E. coli* DH5 $\alpha$ . Corresponding recombinant plasmids were amplified and purified using QIAGEN Plasmid Maxi Kit. All PKR constructs were sequenced to confirm the absence of unwanted mutations.

### Statistical analyses

Statistical significance was determined using unpaired *Student's t-test* with *P*-values  $\leq 0.05$  considered as significant. In all tests, two groups with one changed parameter were compared.

## RESULTS

### Nck1 mutants with non-functional SH3 or SH2 domains attenuate dsRNA-mediated PKR activation and phosphorylation of eIF2 $\alpha$ Ser<sup>51</sup>

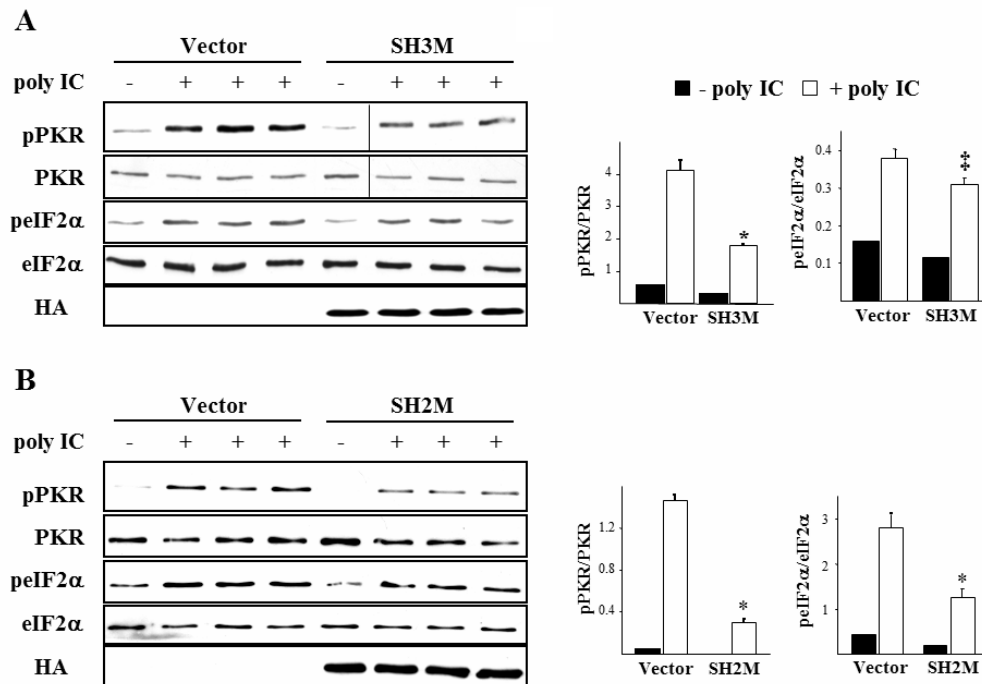
We previously reported that overexpression of Nck1 reduces dsRNA-mediated PKR activation and signaling in various cell lines [13, 20]. Furthermore, we identified PKR as a novel interacting partner of Nck1 and demonstrated that Nck1 variants with point mutations that inactivate either all three SH3 domains or the phosphotyrosine binding function of the SH2 domain [21] still interact with PKR [13]. This suggests that the Nck1 and PKR interaction proceeds through a mechanism that is apparently independent of any classical SH domain-binding function. It is therefore reasonable to propose that these Nck1 variants will still attenuate PKR activation and signaling if the regulation of PKR by Nck1 is solely dependent on the interaction. To test this hypothesis, we overexpressed the same Nck1 variants functionally mutated in all SH3 domains or in the SH2 domain in Cos-7 cells and assessed PKR activation and eIF2 $\alpha$  Ser<sup>51</sup> phosphorylation upon poly IC transfection. Nck1 variants mutated in all three SH3 domains (SH3M: W38, 143, 229R; Fig. 1A) or in the SH2 domain (SH2M: R308K; Fig. 1B) significantly reduced PKR activation and phosphorylation of eIF2 $\alpha$  at Ser<sup>51</sup> compared with control (empty vector) transfected cells. These results reveal that Nck1-mediated regulation of PKR proceeds independently of any classical Nck1 SH domain binding function.

### Nck1 full length is required to interact with PKR

To determine which segment(s) of Nck1 mediate(s) the interaction with PKR, we performed *in vitro* pull-down assays using Cos-7 cell lysates to assess the ability of the bacterial recombinant fusion proteins of GST-Nck1 full length (FL), GST-Nck1 only SH3 domains or only SH2 domain to interact with endogenous PKR (Fig. 2). As expected, we found that GST-Nck1 FL efficiently pulled down endogenous PKR from Cos-7 cell lysate. The specificity of this interaction was supported by the finding that GST alone failed to interact with PKR. Of note, neither GST-Nck1 SH3 domains nor GST-Nck1 SH2 domain fusion proteins succeeded in binding PKR, supporting the notion that full length Nck1 is required to efficiently interact with PKR. In mammals, the Nck family of adaptor proteins includes Nck1 and Nck2 that are encoded from different genes, but share overall 68% amino acid identity with variations essentially only in the interval sequences between the SH domains [22]. To determine whether Nck2 also interacts with PKR, HA-Nck1 and HA-Nck2 were transiently overexpressed in Cos-7 cells and respective HA immunoprecipitates were tested for PKR co-immunoprecipitation by western blotting. Our findings showed that like Nck1, Nck2 interacts with PKR in cultured cells (Fig. 3).

### Nck1 interacts only with inactive PKR

Using *in vitro* pull-down assays, we previously reported that Nck1 interacts only with inactive PKR. To confirm this finding in a cellular context, Cos-7 cells transiently transfected with a control plasmid (vector) or a plasmid encoding HA-Nck1 were treated with or without a high concentration of poly IC (3.0  $\mu$ g/ml) to induce robust activation of PKR. HA-Nck1 interaction with PKR was subsequently assessed by co-immunoprecipitation and western blotting (Fig. 4). As predicted, in the absence of poly IC treatment, endogenous PKR co-immunoprecipitated with HA-Nck1 (lane 3). In contrast, PKR was not detected in the HA-Nck1 immunoprecipitates upon poly IC treatment (lane 4). As anticipated, levels of eIF2 $\alpha$  phosphorylated at Ser<sup>51</sup> were increased in response to poly IC treatment in control and HA-Nck1 overexpressing cells (compare lanes 2 to 1 and 4 to 3). However, as previously reported [13, 20], eIF2 $\alpha$  Ser<sup>51</sup>

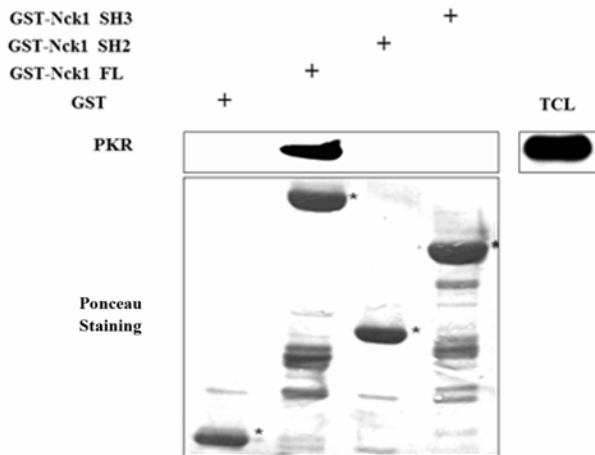


**Fig. 1.** Nck1 variants with point mutations that abolish the classical binding function of the SH domains reduce dsRNA-mediated PKR activation and phosphorylation of eIF2 $\alpha$  Ser<sup>51</sup>. HEK-293 cells were transfected with 1  $\mu$ g of pRK5 (Vector) or pRK5 encoding HA-tagged Nck1 variants mutated in either **A**) all three SH3 domains (SH3M: W38, 143, 229R) or **B**) the SH2 domain (SH2M: R308K). Twenty-four hours after transfection, PKR was activated by transfecting cells again for 2 hours with a synthetic form of dsRNA (poly IC, 0.8  $\mu$ g). Total cell lysates normalized for protein content (30  $\mu$ g) were analyzed by western blotting with antibodies specific for phosphorylated PKR (pPKR) and eIF2 $\alpha$  Ser<sup>51</sup> (peIF2 $\alpha$ ), and HA tag epitope. The blots were then stripped and re-probed with antibodies that recognize PKR and eIF2 $\alpha$  to determine total levels of these proteins in each sample. The ratios of phosphorylated/total protein for PKR and eIF2 $\alpha$  were determined following quantification of their respective band intensities and represent the mean  $\pm$  SEM of 3 independent experiments. In all cell conditions, the white bars represent cells treated with poly IC while the black bars represent cells transfected in the absence of poly IC. In panel A, p-PKR and PKR include images that were obtained with samples simultaneously processed but that needed to be spliced to align samples for the representative final figure. Statistical analyses using unpaired Student's *t*-test revealed \* $p < 0.005$  and ‡ $p < 0.05$  compared to poly IC-treated vector transfected cells.

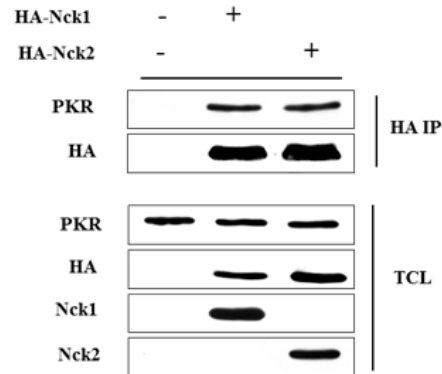
phosphorylation was lower in HA-Nck1 overexpressing cells compared to control cells treated or not with poly IC (compare lanes 3 to 1 and 4 to 2). Altogether, these results confirm our previous observations that Nck1 interacts only with inactive PKR and reduces PKR signaling induced by dsRNA. In addition, they also support the concept that Nck1 dissociates from PKR during PKR activation.

To determine whether increased PKR catalytic activity is responsible for the loss of interaction with HA-Nck1 in poly IC treated cells, we co-expressed HA-Nck1 with either Flag-tagged PKR WT or the dominant kinase dead PKR $\Delta$ 6 [16] in

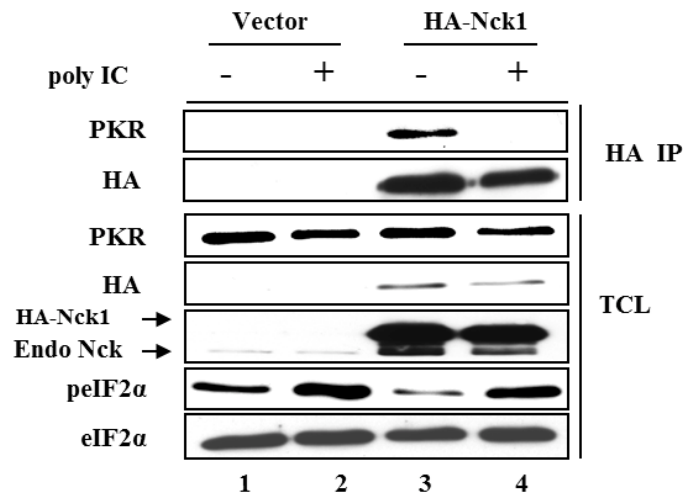
Cos-7 cells and monitored Nck1-PKR interaction (Fig. 5). Consistent with this, we found that overexpression of PKR WT, but not PKR $\Delta$ 6, lead to constitutive activation of PKR as demonstrated by increased PKR phosphorylation at Thr<sup>446</sup> (pPKR) in total lysates (TCL) from cells overexpressing PKR WT compared to kinase dead PKR $\Delta$ 6 (compare lanes 3 and 2). Of note, we found that only PKR $\Delta$ 6 co-immunoprecipitated with HA-Nck1, further supporting the concept that Nck1 interacts only with inactive PKR. As expected, endogenous PKR was detected in immunoprecipitated HA-Nck1 in control cells. However, this interaction was lost upon



**Fig. 2. Nck1 full length is required to bind PKR.** Cos-7 cell lysates (4.5 mg of protein) were incubated with 20  $\mu$ g of GST, GST-Nck1 full length (FL), GST-SH2 or GST-all three SH3 domains of Nck1. Proteins bound to GST fusion proteins were analyzed for PKR by western blotting using a PKR-specific antibody. TCL: total cell lysate (30  $\mu$ g protein). Levels of GST fusion proteins in the assays were revealed by Ponceau staining upon western blotting (indicated by \*). Shown is a typical of three independent experiments.



**Fig. 3. Nck1 and Nck2 bind PKR.** Cos-7 cells were transfected with 1  $\mu$ g of pRK5 encoding HA-Nck1 or HA-Nck2. HA-tagged Nck proteins were immunoprecipitated using anti-HA antibody and lysates from transfected cells previously exposed to the crosslinker DSP. HA immunoprecipitates (HA IP) were analyzed by western blotting with PKR antibody to reveal the presence of PKR and with HA antibody to ascertain that equal amount of HA proteins were immunoprecipitated. HA IP from pRK5 transfected cells was used as control. Total cell lysates (TCL: 30  $\mu$ g protein) were analyzed by western blotting using antibodies as indicated in the figure. Shown is a typical of three independent experiments.



**Fig. 4. dsRNA-induced activation of PKR disrupts Nck1-PKR interaction.** Cos-7 cells transiently transfected with 1  $\mu$ g of pRK5 (Vector) or pRK5 encoding wild type HA-Nck1 were subjected 24 hours later to a second transfection with 5  $\mu$ g of synthetic dsRNA (poly IC) for 2 hours. HA immunoprecipitates were then prepared using lysates from cells previously exposed to the crosslinker DSP. PKR and HA-Nck1 proteins in the immunoprecipitated samples (HA IP) and PKR, HA-Nck1, endogenous Nck1, peIF2 $\alpha$ <sup>Ser51</sup> (peIF2 $\alpha$ ) and total eIF2 $\alpha$  proteins in the total cell lysates (TCL) were detected by western blotting using specific antibodies as indicated in the figure. Shown is a typical of three independent experiments.

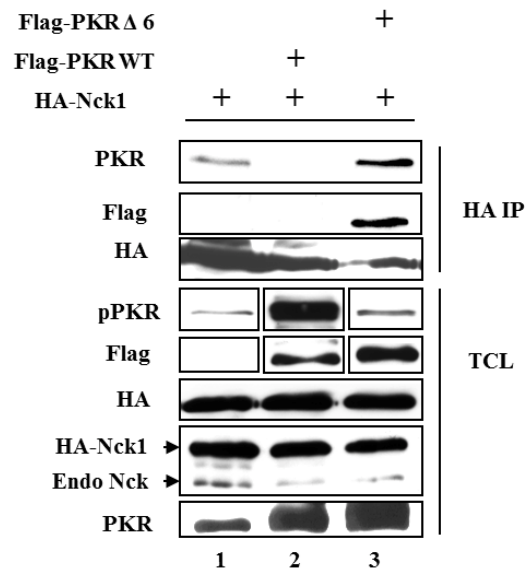
overexpression of PKR WT. We believe that this can be potentially explained by exogenous PKR dimerization and activation of endogenous PKR. Altogether these results confirm that Nck1 only interacts with inactive PKR and support our hypothesis that PKR activation leads to dissociation of Nck1.

### PKR kinase activation induces Nck1 dissociation

Activation of PKR is a multi-step process that involves dsRNA binding, dimerization and autophosphorylation leading to increased PKR kinase activity [23, 24]. PKR $\Delta$ 6, while being characterized as an inactive kinase due to the deletion of 6 amino acids (Leu-Phe-Ile-Gln-Met-Glu) between catalytic domains V and VI, has been shown to maintain dsRNA binding [16]. Therefore, we used PKR $\Delta$ 6 to provide insight about the mechanism by which Nck1 dissociates from activated PKR. Lysates of Cos-7 cells co-transfected with HA-Nck1 and either Flag-PKR $\Delta$ 6 or control vector, and treated with or without poly IC, were analyzed to assess the Nck1 and PKR $\Delta$ 6 interaction by co-immunoprecipitation (Fig. 6). While we confirmed that Flag-PKR $\Delta$ 6 co-immunoprecipitates with HA-Nck1 in the absence of poly IC (lane 3), we discovered that co-immunoprecipitation of Flag-PKR $\Delta$ 6 with HA-Nck1 persisted upon poly IC treatment (lane 4). This contrasts with endogenous PKR, which loses the ability to co-immunoprecipitate with HA-Nck1 upon poly IC treatment (Fig. 6 lanes 1 and 2, Fig. 4). Of note, phosphorylation of PKR (pPKR) and eIF2 $\alpha$ Ser<sup>51</sup> (peIF2 $\alpha$ ) that typically increase upon poly IC treatment in HA-Nck1 cells (compare lane 2 to 1 and lane 4 to 3) is lower in cells overexpressing PKR $\Delta$ 6 (compare lane 4 to 2). This is in agreement with the ability of PKR $\Delta$ 6 to act in a dominant negative manner by sequestering endogenous PKR in an inactive complex [16]. Altogether, these results strongly suggest that dissociation of Nck1 upon PKR activation is dependent on the PKR kinase activity rather than binding of dsRNA.

### Nck1 interacts with PKR through two independent regions

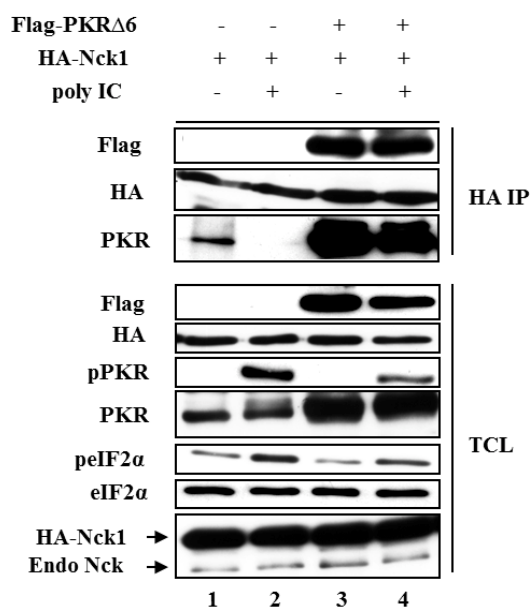
To identify which domain(s) of PKR interact(s) with Nck1, we generated Myc-tagged PKR constructs encompassing the N- and C-terminal moieties of PKR, respectively encoding the RBMs and the kinase domain. We also produced the



**Fig. 5. Nck1 interacts with the kinase-dead PKR $\Delta$ 6.**

Cos-7 cells were transiently co-transfected with plasmids encoding wild type HA-Nck1 (1  $\mu$ g) and either wild type Flag-PKR (5  $\mu$ g) or kinase dead Flag-PKR $\Delta$ 6 (5  $\mu$ g). Twenty-four hours later, cells were exposed to the crosslinker DSP prior to subjecting cell lysates to HA immunoprecipitation. Flag-PKR, PKR and HA-Nck1 proteins in the immunoprecipitated samples (HA IP) and Flag-PKR, PKR, HA-Nck1, endogenous Nck and pPKR in the total cell lysates (TCL) were detected by western blotting using specific antibodies as indicated in the figure. TCL p-PKR and Flag western blotting include images obtained with samples simultaneously processed but that needed to be spliced to align samples for the representative final figure. Shown is a typical of three independent experiments.

Myc-PKR C-terminal segment inactivated by a point mutation in the ATP binding site (K296R) to further address the importance of the kinase activity of PKR in regulating its binding to Nck1 (Fig. 7A). Cos-7 cells transiently expressing HA-Nck1 and individual Myc-PKR variants were used to assess Nck1 binding to truncated PKR-related proteins (Fig. 7B). We observed that both the PKR N- (lane 3) and inactive C-terminus (K296R) (lane 5), but not the PKR C-terminus WT (lane 4), co-immunoprecipitated with HA-Nck1. Activation of endogenous PKR in cells overexpressing the WT active PKR C-terminal segment (lane 4, pPKR) could explain why it failed to interact with Nck1. As a positive control, Flag-PKR $\Delta$ 6 co-immunoprecipitated with HA-Nck1 as detected by



**Fig. 6. Nck1-PKR $\Delta$ 6 interaction is insensitive to dsRNA.** Cos-7 cells were co-transfected with plasmids encoding HA-Nck1 wild type (1  $\mu$ g) and either kinase dead Flag-PKR $\Delta$ 6 (5  $\mu$ g) or control empty plasmid (5  $\mu$ g). Twenty-four hours later, cells were subjected to a second transfection with 5  $\mu$ g of synthetic dsRNA (poly IC) for 2 hours. At the end of the poly IC transfection, cells were exposed to the crosslinker DSP and HA immunoprecipitation was performed on total cell lysates. Flag-PKR, PKR and HA-Nck1 proteins in the immunoprecipitated samples (HA IP) and Flag-PKR, PKR, HA-Nck1, endogenous Nck, peIF2 $\alpha$ Ser<sup>51</sup>, total eIF2 $\alpha$  and pPKR in the total cell lysates (TCL) were detected by western blotting using specific antibodies as indicated in the figure. Shown is a typical of three independent experiments.

anti-Flag immunoblotting of HA immunoprecipitates (lane 2). Altogether, these results confirm that Nck1 binds to the catalytically inactive PKR while also revealing that Nck1 interacts with PKR through two distinct regions.

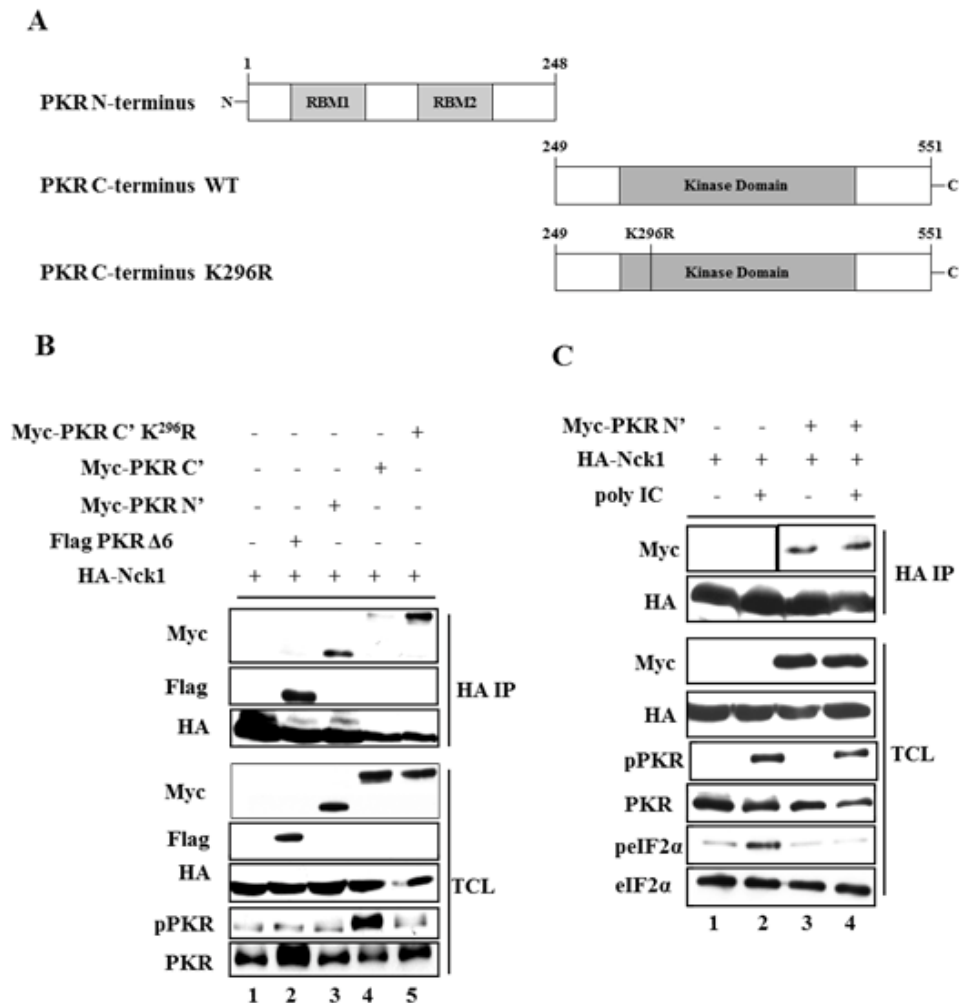
dsRNA promotes PKR activation following its direct binding to RNA binding motifs 1 and 2 in the N-terminal segment of PKR [12]. To confirm our previous finding that dsRNA and Nck1 do not compete for binding to PKR and to exclude that dsRNA promotes Nck1 dissociation during the process of PKR activation, we investigated Nck1 binding to the PKR N-terminal segment in poly IC-treated cells. Cos-7 cells transiently expressing HA-Nck1 and the PKR N-terminal segment were

treated with or without poly IC and the interaction of Nck1 with the PKR N-terminal segment was evaluated in co-immunoprecipitation assays (Fig. 7C). We found that poly IC treatment did not affect the interaction of HA-Nck1 with the Myc-PKR N-terminal segment (compare lanes 4 and 3), suggesting that the binding of dsRNA to PKR does not directly contribute to the dissociation of Nck1 following PKR activation. Of note, PKR activation (phosphorylation) and consequently, phosphorylation of eIF2 $\alpha$ Ser<sup>51</sup> were increased in poly IC-treated, control-transfected cells (compare lanes 2 and 1), while both were decreased in poly IC-treated cells expressing the N-terminal segment of PKR (compare lanes 4 and 2). This might be explained by the fact that expression of the PKR N-terminus significantly reduces the amount of poly IC available to bind and activate endogenous PKR.

#### Nck is phosphorylated by PKR in cultured cells

Using recombinant proteins *in vitro*, we previously reported that Nck1 is phosphorylated by PKR [13]. To assess whether endogenous Nck is phosphorylated by PKR, we compared Nck phosphorylation in poly IC-treated PKR<sup>+/+</sup> and PKR<sup>-/-</sup> mouse embryonic fibroblasts (MEFs). Phosphorylation of Nck was assessed using a Phos-tag-based SDS-PAGE approach that allows sensitive detection of phosphorylated from non-phosphorylated proteins by western blotting [25]. We observed that the amount of Nck which showed gel retardation is increased in poly IC-treated PKR<sup>+/+</sup> compared to PKR<sup>-/-</sup> MEFs (Fig. 8A), suggesting that Nck is phosphorylated by PKR *in vivo*. As expected, eIF2 $\alpha$ Ser<sup>51</sup> phosphorylation was also increased in poly IC-treated PKR<sup>+/+</sup> MEFs compared to PKR<sup>-/-</sup> MEFs. To further demonstrate that Nck is phosphorylated by PKR, we used HEK293 cells transiently overexpressing PKR wild type (Flag-PKR) and HA-Nck1. Consistent with our previous results, overexpression of PKR leading to spontaneous PKR activation results in an increase in eIF2 $\alpha$ Ser<sup>51</sup> phosphorylation as well as an increase in Nck1 phosphorylation as detected by HA western blotting using Phos-tag SDS-PAGE (Fig. 8B), further suggesting that Nck is a substrate of PKR. Finally, to provide further evidence that Nck1 is phosphorylated following PKR activation, HA-Nck1-overexpressing HEK293 cells were treated with or without poly IC and HA immunoprecipitates

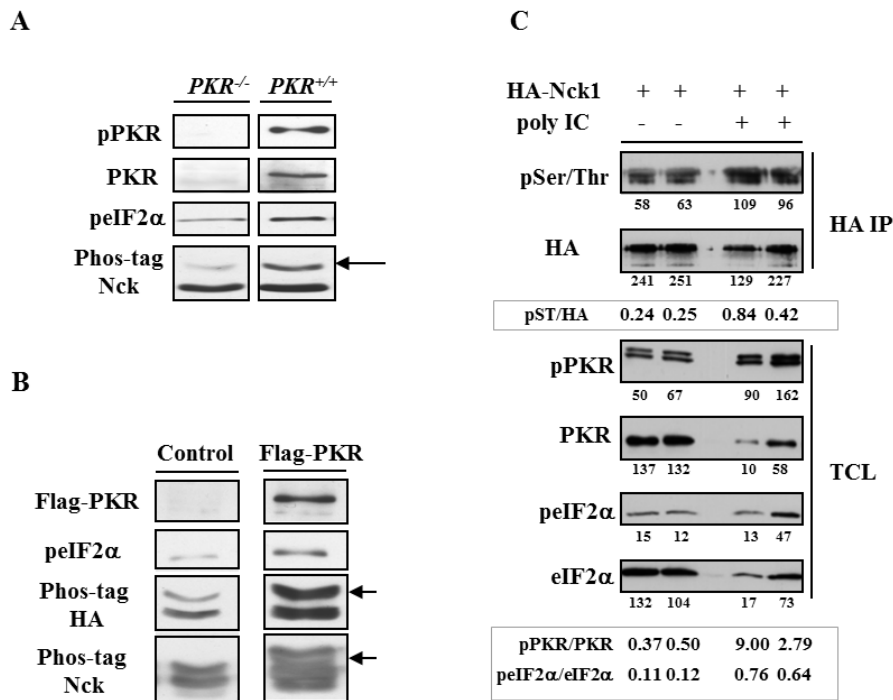




**Fig. 7. Nck1 binds both the PKR N-terminus and the inactive C-terminus.** **A)** Indicated PKR domains were amplified from PKR wild type (WT) or kinase dead cDNA and subcloned into pcDNA 3.1(+) Myc-His version B vector. PKR N-terminus encompasses the N-terminal PKR amino acids 1-248. PKR WT C-terminus (aa 249-551) includes the kinase domain of PKR that is constitutively active when expressed in mammalian cells. PKR C-terminal K296R is the inactive C-terminal domain of PKR for which lysine 296 in the kinase domain has been replaced by an arginine. **B)** Cos-7 cells were co-transfected with a plasmid encoding HA-Nck-1 (1  $\mu$ g) and 5  $\mu$ g of a plasmid encoding full length Flag-PKR $\Delta$ 6, PKR C-terminus wild-type (Myc-PKR C'), PKR C-terminus K296R (Myc-PKR C' K296R) or PKR N-terminus (Myc-PKR N'). Twenty-four hours after transfection, HA immunoprecipitation was performed using lysates from transfected cells previously exposed to the crosslinker DSP. Myc-PKR, Flag-PKR and HA-Nck1 proteins in the immunoprecipitated samples (HA IP) and Myc-PKR, Flag-PKR, HA-Nck1, pPKR and PKR in total cell lysates (TCL) were detected by western blotting using specific antibodies as indicated in the figure. **C)** Cos-7 cells transiently expressing the Myc-PKR N-terminus and HA-Nck1 were transfected with or without poly IC for 2 hours prior to being subjected to HA IP and western blotting with antibodies as indicated in the figure. Anti-Myc western blotting in HA IP includes images obtained with samples simultaneously processed but that needed to be spliced to align samples for the representative final figure. Shown is a typical of three independent experiments.

were subjected to western blot analysis using anti-phospho Ser/Thr antibodies. Our findings show that poly IC treatment induces an increase in phospho Ser/Thr immunoblot signal associated

with immunoprecipitated HA-Nck1 (Fig. 8C). As expected, poly IC treatment also results in an increase in the phosphorylation of PKR (pPKR) and eIF2 $\alpha$  Ser<sup>51</sup>. Collectively, these results provide



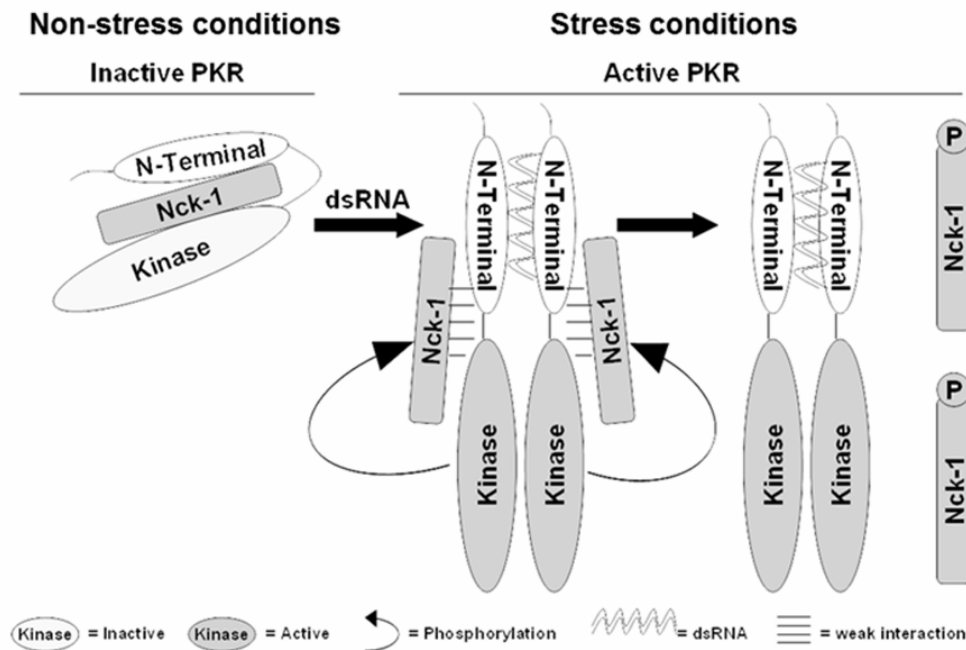
**Fig. 8. PKR phosphorylates Nck in cultured cells.** **A)** Mouse embryonic fibroblasts from *PKR*<sup>-/-</sup> and *PKR*<sup>+/+</sup> mice were transfected with or without synthetic dsRNA (poly IC, 10  $\mu$ g) for 2 hours. Cell lysates were then collected and analyzed by SDS-PAGE and western blotting for pPKR, PKR and peIF2 $\alpha$ . Ser<sup>51</sup> and Phos-tag SDS-PAGE for Nck. Proteins were detected using specific antibodies as indicated in the figure. Arrows indicate phosphorylated Nck with decreased mobility in Phos-tag gels. Shown is a typical of 3 independent experiments. **B)** HEK 293 cells were cotransfected with pRK5 encoding HA-Nck1 and either pcDNA3.1 or pcDNA3.1 encoding wild type Flag-PKR (5  $\mu$ g) and 24 hours later subjected to a second transfection with synthetic dsRNA (poly IC, 10  $\mu$ g) for 2 hours. Total cell lysates were then collected and analyzed by SDS-PAGE or Phos-tag SDS-PAGE. Proteins were detected by western blot using specific antibodies as indicated in the figure. Arrows indicate phosphorylated HA-Nck1 (HA WB) or Nck (Nck WB) with decreased mobility in Phos-tag gels. Shown is a typical of 3 independent experiments. **C)** HEK 293 cells were transiently transfected with a plasmid encoding wild-type HA-Nck1 (1  $\mu$ g) and 24 hours later subjected to a second transfection with or without synthetic dsRNA (poly IC, 10  $\mu$ g) for 2 hours. HA immunoprecipitation was performed using total cell lysates (HA IP) and subjected to western blotting analysis using pSer/Thr and HA antibodies. In parallel, total cell lysates (TCL) were subjected to western blotting analysis to determine pPKR, PKR, peIF2 $\alpha$ . Ser<sup>51</sup> and total eIF2 $\alpha$ . Densitometry value of signal intensity is reported below each band and the ratios of specific phosphorylated/total protein for each condition are indicated under their respective western blots. Presented is one of two independent experiments showing similar observations.

strong evidence that Nck1 is phosphorylated by PKR *in vivo*.

## DISCUSSION

In agreement with our previous findings [13, 20], this study further supports the concept that the adaptor protein Nck1 interacts with and limits the activation of the eIF2 $\alpha$  kinase PKR. We have shown previously that Nck1 interacts with PKR independently of the binding function of its SH

domains [13]. Herein, we demonstrate that Nck1 attenuates dsRNA-mediated PKR activation also independently of the classical binding function of its SH domains. Furthermore, we have demonstrated that Nck1 interacts with both the N- and C-terminal moieties of PKR. Thus, we propose an updated model wherein Nck1 limits PKR activation by maintaining PKR in a closed conformation that prevents ATP and substrate access to the PKR catalytic site (Fig. 9). Alternatively, binding of Nck1 to PKR



**Fig. 9. Model of PKR regulation by Nck1.** Under normal conditions, PKR interacts constitutively with Nck1 through its N- and C-terminal segments, keeping PKR in a closed inactive conformation. In stress conditions created by accumulation of dsRNA, PKR binding of dsRNA induces PKR dimerization resulting in PKR activation through autophosphorylation. During this process, PKR phosphorylates Nck1, destabilizing the interaction of Nck1 with PKR. This leads to the dissociation of Nck1 from PKR, promoting PKR activation.

might prevent spontaneous PKR dimerization that would lead to unsolicited PKR activation in the absence of significant stress. Notwithstanding how Nck1 interacts with PKR, dissociation of Nck1 from PKR is needed to allow PKR activation in response to accumulation of significant intracellular levels of dsRNA. Supporting this, we found that activated PKR does not bind Nck1 while a kinase dead PKR interacts with Nck1 even in the presence of a high concentration of dsRNA that would normally maximally induce activation of wild type PKR, suggesting that the PKR kinase activity determines PKR-Nck1 dissociation. In agreement, we provide evidence that Nck1 is phosphorylated by PKR *in vivo*.

At this point, considering all our results, we postulate that under non-stress conditions, the interaction of Nck1 with PKR buffers PKR activation, whereas under dsRNA accumulation over a threshold level in virally-infected cells, PKR binds dsRNA, dimerizes, changes conformation, and undergoes autophosphorylation that leads to activation of its intrinsic kinase activity. During this process, phosphorylation of Nck1 by PKR would promote

Nck1 dissociation, allowing optimal PKR activation. In this model, dissociation of Nck1 from PKR would result from charge repulsion that lowers its affinity for activated PKR. At the moment, we cannot exclude that PKR autophosphorylation rather than Nck1 phosphorylation triggers Nck1 release. Interestingly, this model parallels the interaction of Nck1 with the p21-activated protein kinase Pak1 [26]. In fact, through its direct interaction with inactive Pak1, Nck1 is believed to translocate Pak1 from the cytosol to the plasma membrane where Pak1 is activated. Plasma membrane-activated Pak1 then leads to Pak1-mediated phosphorylation of Nck1 and subsequent dissociation of Nck1 from Pak1 [27]. Therefore, as for Pak1, Nck1 only binds the inactive form of PKR, and PKR-induced phosphorylation of Nck1 during the process of PKR activation could promote Nck1 dissociation from PKR. However, whether the interaction of Nck1 with PKR plays a role in PKR subcellular localization and appropriate activation has to be further investigated.

PKR, a major mediator of the host antiviral defense, is known to be activated by direct binding of viral

dsRNA that induces PKR dimerization and autophosphorylation. In these conditions, activation of PKR causes transient inhibition of protein synthesis [28, 29], expression of multiple genes [30] and apoptosis [31, 32], all of which contribute to limit viral production. On the other hand, it is well known that some viruses have developed specific mechanisms to counteract PKR activation at different levels and using different mechanisms. Indeed, there are viral proteins that sequester dsRNA away from PKR, inhibit PKR dimerization, behave as PKR pseudo-substrates, activate protein phosphatases that inactivate PKR or contribute to PKR degradation [33]. Despite all that is known of the viral mechanisms regulating PKR activation, very few endogenous proteins have been identified as PKR regulators. These include the inhibitors p58<sup>IPK</sup> [34] and the glycoprotein p67 [35], and the activator PACT/RAX [36]. However, a role for p58<sup>IPK</sup> as a negative regulator of PKR has been recently challenged due to its exclusive localization in the lumen of the endoplasmic reticulum [37].

In this study, we further provide strong evidence that Nck1 limits PKR activation. The exact mechanism by which Nck1 regulates PKR activity has not yet been completely elucidated. However, the fact that Nck1 variants with impaired SH3 or SH2 domain binding activity still interact with and limit PKR activation strongly argues in favor of Nck1 self-sufficiently regulating PKR. Nevertheless, we cannot totally rule out the possibility that these Nck1 variants still control PKR activation by recruiting PKR regulators in close proximity of PKR. Candidates for such PKR regulators could include the serine/threonine protein phosphatase PP1c. Indeed, we have previously shown that Nck1 assembles a molecular complex that includes PP1c [38] and others have demonstrated that PP1c down-regulates PKR activity through its interaction with PKR [39]. PP1 is controlled by its interaction with a variety of regulatory subunits that target the catalytic subunit (PP1c) to specific subcellular localizations or protein substrates. Therefore, Nck1 may contribute in targeting PP1c to PKR.

The biological relevance of the interaction of Nck1 with PKR and its regulation of PKR activation has yet to be demonstrated. Interestingly, PKR has recently been reported to play an important role in the control of metabolic homeostasis and insulin

signaling by directly phosphorylating IRS-1 on an inhibitory site [40], and Nck1 has been shown to directly associate with IRS-1 [41]. Therefore, it would be of interest to determine whether Nck1 plays a role in targeting PKR in close proximity of IRS-1 in pathological conditions or whether Nck1, by increasing the threshold of PKR activation, prevents PKR-mediated inhibitory phosphorylation of IRS-1, thus protecting insulin signaling. Furthermore, Nck1 control of PKR activation could also be significant in the pathogen-sensing function of PKR by allowing PKR activation only when a significant amount of dsRNA accumulates upon a viral infection. Considering that Nck1 interacts with inactive PKR and limits its activation, it would be of interest to investigate whether viruses could use Nck1 as a mean to inhibit PKR activation.

Finally, during the process of PKR activation, Nck1 dissociates from PKR, a process which can be promoted by PKR-mediated phosphorylation of Nck1. In this model, Nck1 can be considered as a modulator of PKR activation, as the interaction of Nck1 with PKR establishes the threshold at which PKR is activated, while their re-association would influence the timeframe of PKR activation.

## CONCLUSION

Through the analysis of the Nck1 and PKR interaction, we have determined that Nck1 binding and regulation of PKR requires Nck1 in its full length form, but is independent of any classical Nck1 SH domain function. Surprisingly, this strongly suggests that Nck1 displays additional protein interaction activity outside of its classical SH domain-mediated protein interactions. Further investigation is required to elucidate the mechanism involved and whether Nck1 regulation of PKR is significant in modulating the PKR's ability to sense and respond to pathogens.

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**CONFLICT OF INTEREST STATEMENT**

The authors declare no conflict of interest.

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