

# IFI27 acts as a positive regulator of PACT-dependent PKR activation

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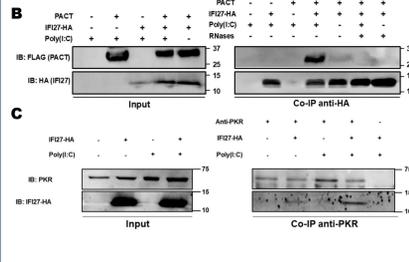
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## Summary

Protein kinase R (PKR) is an interferon (IFN)-induced protein, which is activated by double-stranded (ds) RNAs or RNAs containing duplex regions, which are produced after different stimuli, such as after infections of RNA viruses, leading to the phosphorylation of the eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), and subsequently inhibiting cellular and viral protein translation. This function may lead to different effects such as to impairing the replication of RNA viruses by inhibiting viral protein translation, and to modulating the innate immune responses after viral infections by affecting the translation of effector proteins. PKR regulation is highly complex and not completely elucidated yet. One of the most studied regulators of PKR is the protein kinase activator PACT, an RNA-binding protein that interacts with PKR, activating PKR under different types of stress. In this work, we identify, for the first time, an interaction of IFN alpha inducible protein 27 (IFI27) with PKR-activating protein (PACT or PRKRA) and with PKR, showing that the interaction of IFI27 with PACT is likely mediated by dsRNAs or RNAs containing duplex regions, and that the interaction of IFI27 with PKR is PACT-dependent. Interestingly, using IFI27 knock-down, knocked-out and overexpressing tumour-derived, established cells, we show that these interactions trigger a potentiation of the activity of PKR and, therefore, a decrease in protein translation. Also, we find that IFI27 increases PKR function in cells infected with different RNA viruses such as Severe Acute Respiratory Virus 2 (SARS-CoV-2), and Vesicular Stomatitis virus (VSV), and in cells transfected with poly(I:C), a dsRNA analog that simulates dsRNAs produced after viral infections, suggesting a broad effect of IFI27 on PKR activation. Moreover, we show that IFI27 expression increases the formation of stress granules (SGs) at the cell cytoplasm, correlating with the increased PKR activation mediated by IFI27, as it has been shown that the translational arrest induced by activated PKR leads to the formation of SGs. Mechanistically, we describe that this ability of IFI27 to activate PKR is dependent on its interaction with PACT. Further understanding of the regulation of PKR activity will allow us to develop new antiviral drugs to modulate this signalling axis, which is crucial in RNA virus infections.

## IFI27 interacts with PKR and PACT

Protein accession	Interferon-Inducible protein 27, IFI27	Protein score	Protein mass	Protein coverage	eIP/PA
A0A0B7WZ1R	Interferon-Inducible protein 27, IFI27	400	11233	67.2	17.15
Q75569	Interferon-Inducible double-stranded RNA-dependent protein kinase activator A, PRKRA, PACT	484	34750	44.4	1.92
P19525	Interferon-induced, double-stranded RNA-activated protein kinase, IFI2AK2	565	62424	26	0.71



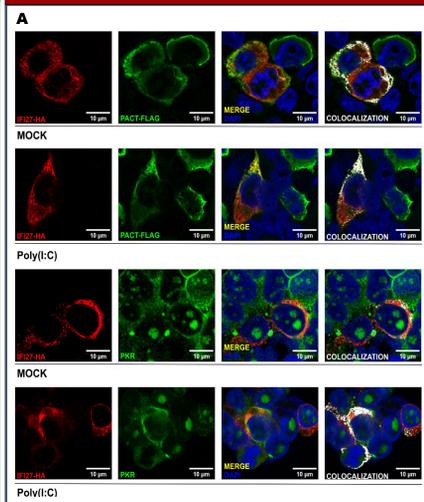
(A) Mass-spectrometry analysis of IFI27-protein interactome: HEK-293T cells were transfected with a pCAGGS-IFI27-HA plasmid, and later with poly(I:C). Protein extracts were obtained by lysis and were incubated with HA-bound agarose beads to retain IFI27-HA and all its associated proteins, which were then identified by MS.

Co-immunoprecipitations of PACT and PKR with IFI27-HA: (B) HEK-293T cells were transfected with pCAGGS-PACT-FLAG together with pCAGGS-IFI27-HA or empty pCAGGS plasmid. Then, cells were left mock-treated or were transfected with poly(I:C). Cellular extracts were treated with RNases or left untreated and a Co-IP with HA-bound agarose beads was performed. PACT and IFI27-HA were detected by Western blot both in the cellular lysates (Input) and after the Co-IP. (C) HEK-293T cells were transiently transfected with a pCAGGS-IFI27-HA expressing plasmid and 24 h later, cells were mock-transfected or transfected with 2  $\mu$ g/ml of poly(I:C) for 16 h. Cell lysates were incubated overnight at 4°C with the PKR specific antibody as well as with protein A-sepharose resin, except in the last Co-IP sample in which the cellular extract expressing IFI27-HA was incubated with the protein A-sepharose resin, without the anti-PKR antibody. PKR and IFI27-HA were detected by Western blot both in the cellular lysates (Input) and after the Co-IP.

These data indicate that IFI27 interacts with PACT and PKR, being these interactions facilitated in the presence of dsRNA.

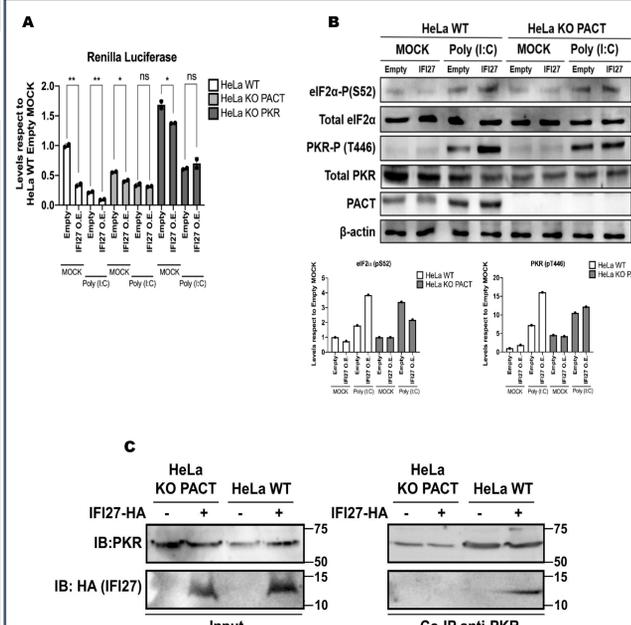
According to bioinformatic predictions using RNAbindRPlus, IFI27 contains 13 amino acids which are predicted to bind RNA, which are the amino acids 60-65, 69, 82-86. To further confirm that IFI27 binds PACT in an RNA-dependent manner, we generated two IFI27 variants encoding point mutations in two of these amino acids predicted to bind to RNAs (i.e. S63L and V82A). (D) Binding to poly(I:C) of IFI27 variants: HEK-293T cellular extracts overexpressing pCAGGS plasmids encoding GFP, IFI27-WT-HA, IFI27-S63L-HA, IFI27-V82A-HA, and PACT-FLAG, or an empty plasmid, were used to perform a pull-down (PD) using poly(I:C)-conjugated agarose beads. Proteins were detected by Western blotting using specific antibodies for GFP, the HA tag (to detect IFI27 variants) or the FLAG tag (to detect PACT), in the cellular lysates (Input) and after the pull-down (poly(I:C)-PD). (E) Binding to PACT of IFI27 variants: HEK-293T cells were transiently transfected with pCAGGS-PACT-FLAG in combination with pCAGGS plasmids encoding IFI27-WT or the IFI27 variants, or an empty pCAGGS plasmid. Then, cells were left mock-treated or were transfected with poly(I:C). A Co-IP with HA-bound agarose beads was performed. PACT and IFI27 variants were detected by Western blot employing anti-FLAG (to detect PACT) and anti-HA (to detect IFI27 variants) antibodies, both in the cellular lysates (Input) and after the Co-IP.

## IFI27 partially colocalizes with PKR and PACT



IFI27 partially colocalizes with PKR and PACT. To support the interaction of IFI27 with PACT and PKR, we studied whether IFI27 colocalizes intracellularly with PACT and/or PKR proteins. To this end, (A) HEK-293T cells were transfected with pCAGGS-IFI27-HA together with pCAGGS-PACT-FLAG (for PACT-IFI27 condition) or with pCAGGS-IFI27-HA only (for PKR-IFI27 condition), and then, the cells were either mock-transfected or poly(I:C)-transfected. After transfection, cells were fixed with paraformaldehyde and an immunofluorescence was performed to detect IFI27, PACT or endogenous PKR, using anti-HA, anti-FLAG, and anti-PKR antibodies, respectively. Cytoplasm colocalization was analysed and indicated in yellow (merge) in the third picture and white (co-localization) in the fourth picture. (B) The level of colocalization between PACT or PKR and IFI27 was analysed by dividing the area where PACT/PRK signal (Alexa Fluor 488, green) and IFI27 signal (Alexa Fluor 594, red) were colocalizing between the total cell area. This ratio was transformed into percentages that are represented on the Y-axis. For PACT-IFI27 mock-transfected and poly(I:C)-transfected conditions, 29 cells were analysed in each case. For PKR-IFI27 mock-transfected and poly(I:C)-transfected conditions, 40 and 30 cells, respectively, were analysed. Each cell result for each condition is represented with dots, and data is represented as the means and standard deviations of the different measures. Scale bar: 10  $\mu$ m.

## PACT is required for the activation of PKR by IFI27 protein, as IFI27 interacts with PKR in a PACT-dependent manner



PACT absence impairs IFI27-mediated PKR activation. To analyze whether PACT affects the ability of IFI27 to positively modulate PKR functions, (A) HeLa WT, PACT KO and PKR KO cells were transiently transfected with pCAGGS-IFI27-HA plasmid (IFI27 O.E.) or an empty pCAGGS plasmid (Empty) together with a pRL plasmid expressing RLuc luciferase, and later, cells were mock-transfected or transfected with poly(I:C). Protein extracts were obtained by lysis and the levels of RLuc luciferase were measured (A). (B) Same protein extracts from HeLa WT and HeLa PACT KO cells, either mock-transfected or transfected with poly(I:C) were employed to measure the protein levels of eIF2 $\alpha$ -P (S52), total eIF2 $\alpha$ , PKR-P (T446), total PKR and  $\beta$ -actin by Western blot employing their respective antibodies. Western blots were quantified by densitometry using ImageJ software. The amount of eIF2 $\alpha$ -P (S52) was normalised by total eIF2 $\alpha$  and  $\beta$ -actin. The amount of PKR-P (T446) was normalised by total PKR and  $\beta$ -actin. (C) IFI27 interacts with PKR in a PACT-dependent manner. HeLa WT and HeLa PACT KO cells were transiently transfected with a pCAGGS-IFI27-HA plasmid, and later, cells were transfected with poly(I:C). Cell lysates were incubated overnight at 4°C with the PKR specific antibody together with protein A-sepharose resin. Eluates were analysed by Western blot, to detect PKR and IFI27 by employing anti-PKR (to detect endogenous PKR, top panel) and anti-HA (to detect IFI27-HA, bottom panel) both in the cellular lysates (Input) and after the Co-IP.

## Conclusions

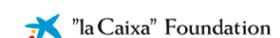
- IFI27 interacts with PACT and PKR, being these interactions facilitated in the presence of dsRNA
- IFI27 variants that do not bind to dsRNA (IFI27-S63L and IFI27-V82A, both with single point mutations) are impaired on their ability to bind PACT, further showing the importance of dsRNA on the interaction between IFI27 and PACT.
- IFI27 partially colocalizes with both PKR and PACT inside the cells, further supporting these interactions. dsRNA, although not essential, promotes the colocalization of IFI27 with PACT and the colocalization of IFI27 with PKR.
- IFI27 overexpression results in a stronger PKR activation, and IFI27 knock-out and knock-down results in a weaker PKR activation. Thus, IFI27 acts as a promoter of PKR function, positively affecting the inhibition of protein synthesis.
- The effect of IFI27 on promoting PKR activation and the inhibition of protein translation is PACT-dependent.
- The interaction of IFI27 with PKR is observed in WT but not in PACT KO cells, indicating that PACT mediates the interaction of IFI27 with PKR.
- Stress granules formation is a hallmark of PKR activation and host shut-off after the induction of stress such as in viral infections or treatments with dsRNA (poly(I:C) in this case). IFI27 overexpression promotes SGs formation in WT but not PACT KO HeLa cells, showing again the importance of PACT on this axis.

**Take home message:** IFI27 is a positive regulator of PKR function, promoting host shut-off after treatment with poly(I:C) and infection with different RNA viruses. IFI27 interacts with PACT, which further allows IFI27-PKR interaction and the positive modulation of PKR function.

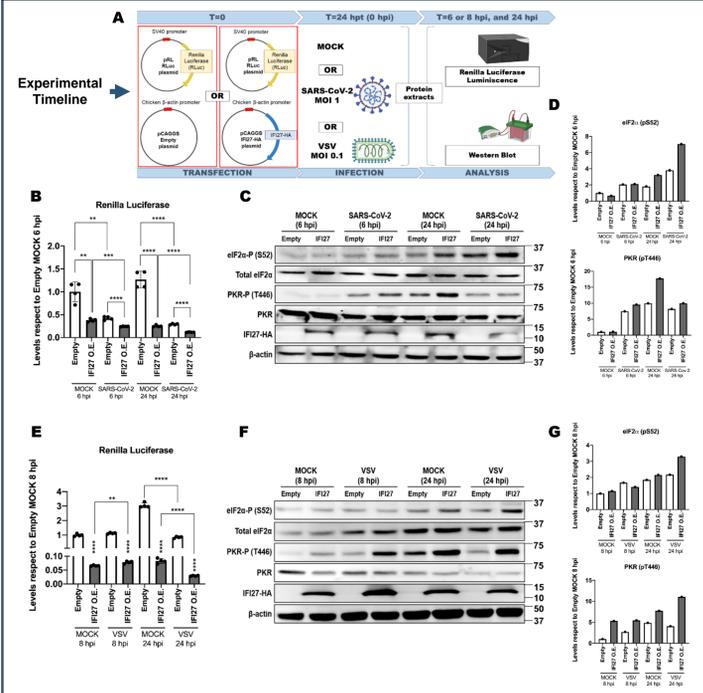
## Acknowledgements

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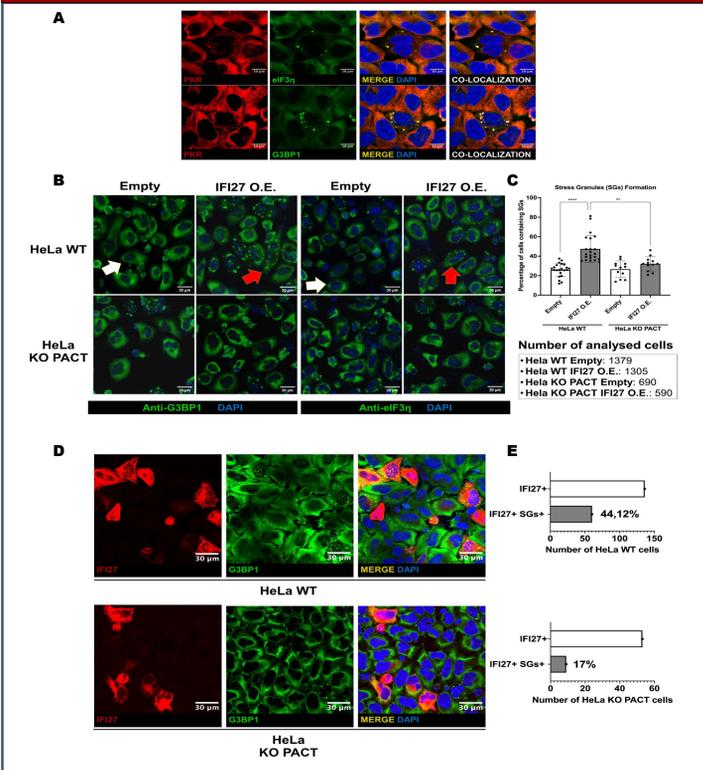


## IFI27 overexpression leads to a stronger PKR activation



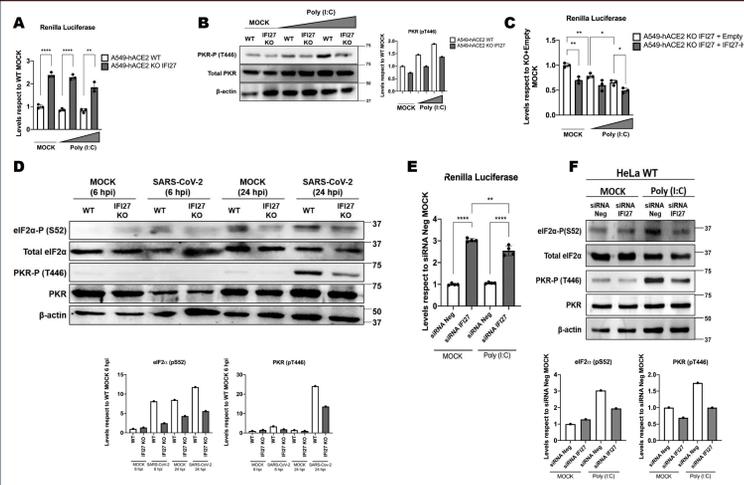
IFI27 overexpression leads to a stronger PKR activation. Given that IFI27 interacts with PKR and PACT, we studied whether IFI27 affects PKR functions. To this end, (B, C, D) HEK-293T-HACE2 cells were transiently transfected with a pCAGGS-IFI27-HA plasmid (IFI27 O.E.) or an empty pCAGGS plasmid (Empty) together with a pRL plasmid expressing RLuc luciferase, and transfected cells were left mock-infected or infected with SARS-CoV-2. Protein extracts were obtained and (B) the levels of RLuc luciferase were measured. (C) These protein extracts were also used to measure protein levels of eIF2 $\alpha$ -P (S52), total eIF2 $\alpha$ , PKR-P (T446), total PKR, IFI27-HA, and  $\beta$ -actin by Western blot employing their respective specific antibodies. Western blots were quantified by densitometry using ImageJ software. The amount of eIF2 $\alpha$ -P (S52) was normalised to the amount of total eIF2 $\alpha$  and  $\beta$ -actin. The amount of PKR-P (T446) was normalised to the amounts of total PKR and  $\beta$ -actin. (E, F, G) HEK-293T cells were transiently transfected with a pCAGGS-IFI27-HA plasmid (IFI27 O.E.) or an empty pCAGGS plasmid (Empty) together with a pRL plasmid expressing RLuc luciferase, and transfected cells were left mock-infected or infected with VSV. Protein extracts were obtained. (E) The levels of RLuc luciferase were measured. (F) These protein extracts were also used to measure protein levels of eIF2 $\alpha$ -P (S52), total eIF2 $\alpha$ , PKR-P (T446), total PKR, IFI27-HA, and  $\beta$ -actin by Western blot employing their respective antibodies. Western blots were quantified by densitometry using ImageJ software. The amount of eIF2 $\alpha$ -P (S52) was normalised to the amounts of total eIF2 $\alpha$  and  $\beta$ -actin. The amount of PKR-P (T446) was normalised to the amounts of total PKR and  $\beta$ -actin. The asterisks above each bar represent the comparison vs. the empty control at each time and condition.

## IFI27 promotes the formation of SGs in a PACT-dependent manner



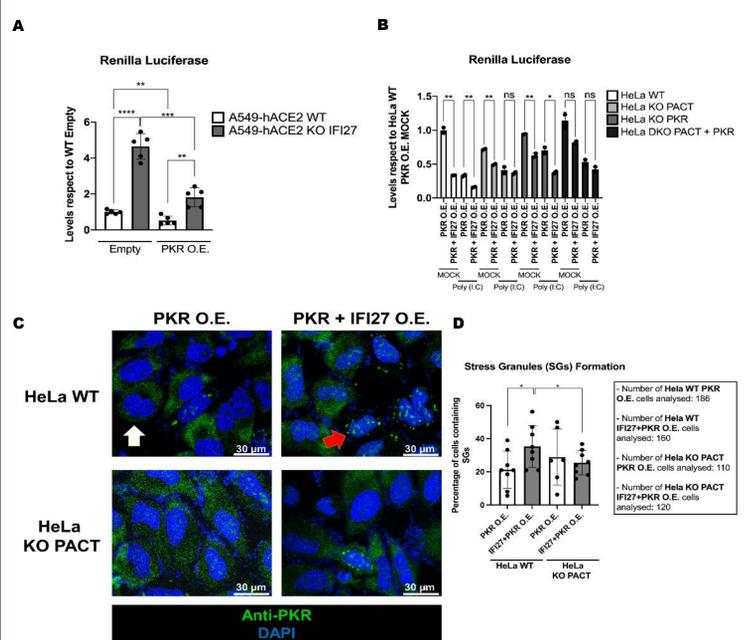
IFI27 promotes the formation of SGs in a PACT-dependent manner. The translational arrest induced by activated PKR leads to the formation of SGs, being these structures cytoplasmic aggregates that include stalled translation initiation complexes. As IFI27 can potentiate PKR activation, we determined whether IFI27 overexpression could indirectly increase the formation of SGs by positively affecting the activation of PKR. To this end, (A) HeLa WT cells were treated with poly(I:C) and then, cells were fixed with paraformaldehyde and permeabilized. PKR (red) and eIF3 $\alpha$  or G3BP1 (green) were labelled with specific antibodies for each protein. Nuclei were stained with DAPI and shown in blue. Areas of co-localization of both proteins appear in yellow in the third picture and in white in the fourth picture. Scale bar, 10  $\mu$ m. (B and C) HeLa WT and HeLa PACT KO cells were transiently transfected with either a pCAGGS-IFI27-HA plasmid or a pCAGGS empty plasmid, and later, treated with poly(I:C). Then, cells were fixed with paraformaldehyde and permeabilized. (B and C) An immunofluorescence was performed for both eIF3 $\alpha$  and G3BP1 as markers of SGs. White arrow (B) shows a cell with no presence of SGs, while the red arrow (B) shows a cell with positive presence of SGs. (C) Quantification of the percentage of SGs positive cells within the total number of counted cells for each condition is represented. Each dot indicates the percentage of SGs positive cells within a single region, having each region several cells. The total number of counted cells was 1379 and 1305 cells for WT cells transfected with the empty plasmid and the plasmid for IFI27 overexpression, and 690 and 590 cells for PACT KO cells transfected with the empty plasmid and the plasmid for IFI27 overexpression. Scale bar, 30  $\mu$ m. (D and E) HeLa WT and HeLa PACT KO cells were transiently transfected with a pCAGGS-IFI27-HA plasmid, and later, transfected with poly(I:C). Cells were fixed with paraformaldehyde and permeabilized. IFI27-HA (red) and G3BP1 (green) were labelled with specific antibodies for each protein. (D) Nuclei were stained with DAPI and shown in blue. Areas of co-localization of both proteins appear in yellow in the third picture. Scale bar, 30  $\mu$ m. (E) A quantification of the total IFI27-HA positive cells counted (white bar), and the number of SGs positive cells within the total IFI27-HA positive cells (grey bar) is represented. The number of counted cells is shown on the x-axis, being the counted cells 136 and 60 for WT cells overexpressing IFI27 and WT cells overexpressing IFI27 and containing SGs, respectively, and 53 and 9 for PACT KO cells overexpressing IFI27 and PACT KO cells overexpressing IFI27 and containing SGs, respectively. The percentage IFI27-HA positive cells containing SGs within the total IFI27-HA positive cells is showed next to the grey bar.

## IFI27 knock-out and knock-down results in a weaker PKR activation and higher de novo protein synthesis



IFI27 KO impairs PKR activation after poly(I:C) treatment. (A, B) WT or IFI27 KO A549-HACE2 cells were transiently transfected with a pRL plasmid expressing RLuc luciferase (pRL-RLuc), and later, cells were mock-transfected or transfected with increasing concentrations of poly(I:C). (A) Protein extracts were obtained and the levels of RLuc luciferase were measured. (B) Protein extracts obtained by lysis were also used to measure protein levels of PKR-P (T446), total PKR and  $\beta$ -actin (loading control) by Western blot. Western blots were quantified by densitometry using ImageJ software. The amount of PKR-P (T446) was normalised by total PKR and  $\beta$ -actin. (C) A549-HACE2 IFI27 KO cells stably transfected with an empty plasmid (KO+empty), and IFI27 KO cells stably expressing IFI27-HA (KO+IFI27), were transiently transfected with the plasmid pRL-RLuc and later, cells were mock-transfected or transfected with increasing concentrations of poly(I:C). Protein extracts were obtained and the levels of RLuc luciferase were measured. (D) WT or IFI27 KO A549-HACE2 cells were infected with SARS-CoV-2. Protein extracts were obtained and used to measure protein levels of eIF2 $\alpha$ -P (S52), total eIF2 $\alpha$ , PKR-P (T446), total PKR and  $\beta$ -actin by Western blot. Western blots were quantified by densitometry using ImageJ software. The amount of eIF2 $\alpha$ -P (S52) was normalised to the amounts of total eIF2 $\alpha$  and  $\beta$ -actin. The amount of PKR-P (T446) was normalised to the amounts of total PKR and  $\beta$ -actin. (E, F) IFI27 knock-down was performed on HeLa WT cells. Cells were transfected twice, with a 24-hour gap between each transfection, either with a negative control non-targeting siRNA (siRNA Neg) or with an IFI27 siRNA (siRNA IFI27). After the second siRNA transfection, cells were transfected with a pRL plasmid expressing RLuc luciferase, and later, cells were mock or poly(I:C)-transfected. Protein extracts were obtained by lysis and the levels of RLuc luciferase were measured (E) or (F) used to measure protein levels of eIF2 $\alpha$ -P (S52), total eIF2 $\alpha$ , PKR-P (T446), total PKR and  $\beta$ -actin by Western blot. Western blots were quantified by densitometry using ImageJ software. The amount of eIF2 $\alpha$ -P (S52) was normalised by total eIF2 $\alpha$  and  $\beta$ -actin. The amount of PKR-P (T446) was normalised by total PKR and  $\beta$ -actin.

## IFI27 promotes the activation after PKR overexpression in a PACT-dependent manner



IFI27 promotes the activation after PKR overexpression in a PACT-dependent manner. (A) A549-HACE2 WT and A549-HACE2 IFI27 KO cells were transiently transfected with a pCAGGS-PKR-myc plasmid (PKR O.E.) or an Empty pCAGGS plasmid (Empty) in combination with an RLuc expressing pRL plasmid. Protein extracts were obtained by lysis and the levels of RLuc luciferase were measured. (B) HeLa WT, PACT KO, PKR KO or double KO for PACT and PKR (DKO) were transiently transfected with a pCAGGS-PKR-myc plasmid (PKR O.E.) alone or in combination with a pCAGGS-IFI27-HA plasmid (PKR + IFI27 O.E.). These plasmids were co-transfected together with a pRL plasmid expressing RLuc luciferase, and later, the cells were left mock-transfected or transfected with poly(I:C). Protein extracts were obtained by lysis and the levels of RLuc luciferase were measured. (C) HeLa WT and HeLa PACT KO cells were transiently transfected with either a pCAGGS-PKR-myc plasmid alone (PKR O.E.) or in combination with a pCAGGS-IFI27-HA (PKR + IFI27 O.E.) plasmid, and later, transfected with poly(I:C). Cells were fixed with paraformaldehyde and permeabilized, and an immunofluorescence was performed for PKR (in green) and nuclei were stained with DAPI (in blue). White arrow (C) shows a cell with no presence of SGs, while the red arrow (C) shows a cell with positive presence of SGs. (D) Quantification of the percentage of SGs positive cells within the total number of counted cells for each condition is represented. Each dot indicates the percentage of SGs positive cells within a single region, having each region several cells. The total number of counted cells was 186 and 160 cells for WT cells transfected with the PKR plasmid alone, or with the PKR plasmid and the plasmid for IFI27 overexpression together, respectively; and 110 and 120 cells for PACT KO cells transfected with the PKR plasmid alone, or with the PKR plasmid and the plasmid for IFI27 overexpression together, respectively. Scale bar, 30  $\mu$ m.