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Anti-tumorigenic effects of Type 1 interferon are subdued by integrated stress responses

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Abstract

Viral and pharmacologic inducers of PKR-like ER kinase (PERK) were shown to accelerate the phosphorylation-dependent degradation of the IFNAR1 chain of the type 1 interferon (IFN) receptor and to limit cell sensitivity to IFN. Here we report that hypoxia can elicit these effects in a PERK-dependent manner. The altered fate of IFNAR1 affected by signaling downstream of PERK depends on phosphorylation of eIF2 α and ensuing activation of p38 α kinase. Activators of other eIF2 α kinases such as PKR or GCN2 are also capable of eliminating IFNAR1 and blunting IFN responses. Modulation of constitutive PKR activity in human breast cancer cells stabilizes IFNAR1 and sensitizes these cells to IFNAR1-dependent anti-tumorigenic effects. Whereas downregulation of IFNAR1 and impaired IFNAR1 signaling can be elicited in response to amino acid deficit, the knockdown of GCN2 in melanoma cells reverses these phenotypes. We propose that, in cancer cells and the tumor microenvironment, activation of diverse eIF2 α kinases followed by IFNAR1 downregulation enables multiple cellular components of tumor tissue to evade the direct and indirect anti-tumorigenic effects of Type 1 IFN.

Keywords

interferon; tumor microenvironment; integrated stress response; PERK; PKR; GCN2; IFNAR1

Introduction

Tumors are sculpted toward a more malignant phenotype while selecting for highly aggressive and resilient clones under limitations of nutrients and oxygen (1–3) and attack by the host immune system (4). The harsh conditions of the tumor microenvironment may

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CONFLICT OF INTEREST:

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activate mechanisms that enable tumor cells to increase their proliferative, survival and invasive potential (5). Given that successful execution of these mechanisms encourage the cells to outgrow/outlive these conditions or move away from the primary tumor site (i.e., metastasize), delineation of these mechanisms is paramount to understanding tumor progression and devising the means for cancer treatment.

Insufficient availability of essential amino acids triggers the activation of GCN2 (General Control Nonrepressed-2, GCN2) protein kinase, which phosphorylates the translational regulator eIF2 α on Ser51. This phosphorylation can be also mediated by PERK-like ER kinase (PERK, induced by an unfolded protein response to an insufficient supply of oxygen or/and glucose), or by protein kinase RNA-activated (PKR). These signaling pathways, known under an overarching term of integrative signaling response (ISR; reviewed in Ref (6)), converge on eIF2 α phosphorylation resulting in temporal stalling of global translation while activating ATF4-mediated transcription of genes that replenish amino acids, deal with accumulated unfolded proteins and restore the oxidative-reductive balance (5, 7).

Intriguingly, various branches of ISR have been implicated in the mechanisms of tumor development and progression. For example, despite well documented pro-apoptotic activity of PKR under conditions of viral infection, this kinase is overexpressed and constitutively activated in some hematologic malignancies (8) as well as in breast cancers (9–11). Furthermore, previous reports demonstrated that the functions of GCN2 (2) and PERK (12–14) are essential for efficient tumorigenesis and tumor progression. We have also found that activation of PERK within the unfolded protein response triggered either by viral infection or by the Ca²⁺ channel inhibitor thapsigargin (TG), stimulated phosphorylation-dependent ubiquitination and degradation of the IFNAR1 chain of the Type 1 interferon (IFN) receptor rendering cells insensitive to the effects of IFN α / β (15, 16). These cytokines signal through the IFNAR1/IFNAR2c chains of the Type 1 IFN receptor to activate Janus kinases and signal transducers and activators of transcription (STAT) to elicit the anti-tumorigenic effects that include inhibition of tumor cell motility, growth, and survival (17–19). Here we report that activation of diverse branches of ISR downregulates IFNAR1 and desensitizes cells to the anti-tumorigenic effects of IFN.

Results

Integrated stress signals converging on phosphorylation of eIF2 α mediate phosphorylation and downregulation of IFNAR1

PERK-dependent acceleration of IFNAR1 proteolysis in cells undergoing unfolded protein response evoked by viral infection or TG treatment prompted us to investigate the effects of tumor microenvironment-relevant inducers of PERK such as hypoxia (13). As expected, the exposure of WM266-4 human melanoma cells to hypoxia (0.5% oxygen) induced phosphorylation of eIF2 α (Figure 1A). Knockdown of PERK attenuated this phosphorylation but not the accumulation of HIF1 α protein (Figure S1). Intriguingly, hypoxia triggered a robust IFNAR1 downregulation that could be partially reversed upon the knockdown of PERK (Figure 1A). We next tested sensitivity of cells to Type 1 IFN using a cell viability assay that combines assessment of anti-proliferative and anti-survival effects of these cytokines. Consistent with the importance of IFNAR1 levels for responsiveness of cells to its ligands (15, 16, 20–26), hypoxic conditions also attenuated the inhibition of cell viability by various doses of IFN α (Figure 1B). Importantly, this attenuation could be reversed by knocking down PERK (Figure 1B). Under hypoxic conditions, the IC₅₀ of IFN α for cells that received PERK shRNA was 125 U/mL (versus that of 207 U/mL for control shRNA-treated cells, Figure S2). Collectively these data suggest that activation of PERK by hypoxia triggers downregulation of IFNAR1 and ensuing desensitization of cells to IFN α .

Previous studies suggested that activation of PERK mediates IFNAR1 degradation through stimulation of the stress activated p38 α protein kinase (27) that mediates phosphorylation of a priming site (Ser532 within human IFNAR1 or Ser523 within the mouse receptor (15)). In turn, this priming phosphorylation increases the efficacy of IFNAR1 degron phosphorylation by casein kinase 1 α (28) leading to the recruitment of the β -Trcp E3 ubiquitin ligase, IFNAR1 ubiquitination, internalization and lysosomal degradation (reviewed in (29, 30)). Besides phosphorylating eIF2 α , PERK has been also implicated in directly activating other phosphorylation cascades (31, 32). Given that, we sought to investigate how PERK signals towards IFNAR1 phosphorylation. A short term treatment of mouse embryo fibroblasts (MEF) from wild type mice with thapsigargin (TG) led to a robust activation of p38 kinase as well as priming phosphorylation and ubiquitination of IFNAR1 (Figure 1C). Consistent with earlier reports (15, 16, 27), a longer exposure to TG decreased the levels of IFNAR1 in these cells (Figure 1D). Intriguingly, the effects of either short term or prolonged treatment with TG were not evident in MEFs from eIF2 α ^{S51A} knock-in mice (Figures 1C–1D). These results suggest that inducers of PERK require phosphorylation of eIF2 α for the ubiquitination and downregulation of IFNAR1.

To further test this hypothesis, we used isogenic human HT29-derived cells that were stably transduced with empty retrovirus or made to express the constitutively active fragment of eIF2 α phosphatase, GADD34 (33). Consistent with previous reports, basal or TG-induced phosphorylation of eIF2 α on Ser51 in these HT29A1 cells was attenuated (Figure 1E). In response to TG these cells neither activated p38 kinase nor phosphorylated IFNAR1 at its priming site (Figure 1E). Furthermore, a prolonged TG treatment did not efficiently downregulate IFNAR1 in cells expressing active GADD34 (Figure 1F). These results collectively suggest that phosphorylation of eIF2 α downstream of PERK is required for IFNAR1 phosphorylation, ubiquitination and downregulation in response to PERK inducers.

Phosphorylation of eIF2 α on Ser51 can also be stimulated by inducers of the ISR such as amino acid deficit acting via GCN2 or activators of PKR (7, 30). Thus we sought to investigate whether these pathways converge and affect phosphorylation and levels of IFNAR1. To this end, we used a panel of MEFs derived from wild type mice or mice lacking PERK, GCN2, or PKR. This panel was subjected to either TG treatment to induce PERK, or heparin (HE) treatment to induce PKR, or leucine starvation (LS) to activate GCN2. All of these inducers stimulated phosphorylation of Ser51 of eIF2 α , activation of p38 kinase and priming phosphorylation of IFNAR1 in wild type MEFs (Figure 2A). Furthermore, a prolonged exposure of wild type MEFs to these inducers noticeably downregulated IFNAR1 (Figure 2B). As expected the phosphorylation of eIF2 α and activation of p38 α kinase in response to TG was impaired in PERK-null cells. Similarly, knockout of GCN2 impaired eIF2 α /p38 α phosphorylation in response to leucine starvation, and knockout of PKR had the same effect in cells treated with heparin (Figure 2A). Remarkably, the patterns of either priming phosphorylation of IFNAR1 (Figure 2A) or its downregulation (Figure 2B) followed an analogous trend.

Furthermore, the results of additional experiments using MEFs from eIF2 α ^{S51A} knock-in and p38 α knockout mice suggested that the integrity of eIF2 α Ser51 phosphorylation and the availability of p38 α protein kinase were required for IFNAR1 priming phosphorylation and downregulation elicited in response to either heparin (Figure 2C–2D) or leucine starvation (Figure 2E–2F). In all, these results suggest that, in addition to established PERK-dependent regulation, the inducers of the ISR can utilize activation of either GCN2 or PKR to trigger eIF2 α /p38 α -dependent phosphorylation and ensuing downregulation of IFNAR1. A key role of the stress-activated p38 α protein kinase in regulating IFNAR1 levels is consistent with a previous report that activation of this kinase was inversely correlated with levels of endogenous IFNAR1 in clinical malignant melanoma samples (34).

Constitutive PKR activity downregulates IFNAR1 levels and signaling

We further sought to corroborate the role of PKR in regulating IFNAR1 levels and activities. Although linked to induction of apoptosis (35, 36), a high level of constitutively active PKR has been reported in clinical human breast cancer tissues and cell lines including T47D cells (9–11). Whereas priming phosphorylation of endogenous IFNAR1 in these cells could be detected upon treatment with a lysosomal inhibitor (to prevent IFNAR1 degradation), further addition of PKR inhibitor C16 readily decreased this phosphorylation (Figure 3A). Furthermore, merely adding C16 to T47D cells led to an increase in IFNAR1 levels in a dose-dependent manner (Figure 3B). Pre-treatment of T47D cells with this PKR inhibitor also increased the IFN α -induced phosphorylation of STAT1 (Figure 3C).

This pharmacologic analysis was supplemented with genetic experiments using a RNAi approach. Knockdown of PKR in T47D cells increased the levels of endogenous IFNAR1 (Figure 3D) and augmented STAT1 activation assessed by its phosphorylation on Tyr701 (Figure 3E) or Ser727 (Figure S3). Importantly, while viability of T47D cells did not robustly change in response to IFN α , these cells could be significantly sensitized to this treatment by adding PKR inhibitor C16 (Figure 3F). Knockdown of PKR noticeably decreased the ability of T47D cells to invade matrigel and/or to migrate through a membrane in a Boyden chamber invasion/motility assay (Figure 3G). In line with the well-established role of Type 1 IFN in restricting cell motility (37), knockdown of IFNAR1 (Figure S4) noticeably stimulated the breast cancer cells movement through the Matrigel/membrane (Figure 3G). Remarkably, in cells that received shRNA targeting IFNAR1, knockdown of PKR did not significantly decrease cell migration/invasion (Figure 3G) indicating that inhibition of invasive phenotype can at least in part be attributed to IFNAR1 stabilization. These results collectively suggest that high levels of constitutive PKR activity in breast cancer cells may restrict IFNAR1 expression and signaling as well as IFNAR1-mediated anti-tumorigenic effects.

Induction of GCN2-dependent pathways promotes IFNAR1 downregulation to attenuate IFN signaling

Culturing MEFs in medium deficient in the essential amino acid leucine triggered IFNAR1 phosphorylation and downregulation that was dependent on GCN2, eIF2 α phosphorylation and p38 α kinase (Figures 2A, E, F). Leucine starvation also stimulated priming phosphorylation, ubiquitination and downregulation of endogenous IFNAR1 in WM266-4 melanoma cells (Figure 4A). Similar effects were seen in 1205Lu human melanoma cells (Figure S5). We next investigated the role of activated GCN2 in cellular responses to Type 1 IFN. When WM266-4 cells were cultured in the absence of leucine, the activation of STAT1 in response to IFN α was attenuated (Figure 4B and S6). A similar attenuation was seen in 1205Lu cells treated with IFN α but not with IFN (Figure S7) that signals through a different receptor, IFNGR (19). Furthermore, leucine deficit noticeably blunted the sensitivity of WM266-4 cells to the anti-proliferative effects of IFN α (Figure 4C). Importantly, knockdown of GCN2 in these cells at least partially restored their responsiveness to IFN α as evident from analyses of STAT1 phosphorylation (Figures 4B, S6) and cell viability (Figure 4C). In addition, migration and/or invasion of WM266-4 cells grown without leucine was markedly inhibited by knockdown of GCN2 unless IFNAR1 also was downregulated (Figures 4D and S8). These results collectively suggest that activation of GCN2 triggered by amino acid deficit leads to downregulation of IFNAR1 and attenuation of its signaling and of ensuing anti-tumorigenic effects.

Discussion

Numerous studies define the robust anti-tumorigenic effects of IFN in vitro (38). Perplexingly, human malignancy manifestation occurs despite the presence of endogenous Type 1 IFN. Furthermore, while recombinant IFN α/β has been used extensively as anti-cancer agent, the efficacy of this treatment is limited (38–40). Why are these cytokines ineffective in protecting the hosts from developing tumors and in treating already developed malignancies? A plausible answer to this paradox is that tumors may evolve to develop adaptive mechanisms that help them to evade the effects of endogenous Type 1 IFN. Our current work contributes to the delineation of such mechanisms.

Previous findings suggested that induction of PERK by pharmacological agents or viruses attenuates the cellular responses to Type 1 IFN via phosphorylation-dependent ubiquitination and degradation of IFNAR1. Here we report that hypoxia, a stress stimulus that can induce PERK within the tumor microenvironment, is capable of a similar effect. Delineation of a signaling pathway downstream of PERK identified phosphorylation of eIF2 α as a requisite mediator of p38 α protein kinase activation and subsequent phosphorylation and downregulation of IFNAR1 in response to PERK activation (Figure 1). Remarkably, activation of other eIF2 α kinases (GCN2 and PKR) that function within the complex signaling pathway termed the ISR are also capable of phosphorylation and downregulation of IFNAR1 in an eIF2 α /p38 α -dependent manner (Figure 2). Furthermore, human breast cancer cells harboring constitutively active PKR display low IFNAR1 levels and attenuated signaling as well as limited sensitivity to the anti-tumorigenic effects of IFN α . These phenotypical characteristics can be ameliorated by either pharmacologic or genetic inhibition of PKR (Figure 3). Conversely, human melanoma cells that are intrinsically sensitive to IFN α partially forfeit their ability to respond to this cytokine upon essential amino acid starvation-induced GCN2 activation and ensuing IFNAR1 downregulation. Knockdown of GCN2 restores the sensitivity of these cells to IFN α signaling and IFNAR1-dependent anti-tumorigenic effects (Figure 4).

Constitutive activity of PKR in human breast cancer cells is likely to suppress the effects of IFN in a tumor cell autonomous manner. Activities of GCN2 or PERK in tumor cells were implicated in supporting tumor growth and progression (2, 12, 13). Furthermore, it is also plausible that downregulation of IFNAR1 and ensuing deficiency in Type 1 IFN signaling may also occur in non-tumor cells within the tumor microenvironment. Accordingly, we propose a model wherein activation of the pathway encompassed into the ISR in the tumor microenvironment may summarily downregulate IFNAR1 and reduce overall Type 1 IFN responses in all cell types constituting the tumor (Figure 4E). Given that IFN α/β can act via numerous direct and indirect mechanisms, the overall decrease in sensitivity of tumor tissue to these cytokines may encourage tumor development and progression in a number of ways.

Within this model (Figure 4E), activation of various branches of the ISR can alleviate the direct anti-proliferative, anti-migratory, or pro-apoptotic effects of Type 1 IFN on tumor cells. In addition, accelerated degradation of IFNAR1 in the endothelial cells and their precursors may alleviate the suppressive effects of these cytokines on angiogenesis (41). Lack of IFNAR1-mediated suppression of growth and proliferation of tumor-associated fibroblasts may not only change tumor architecture but also hypothetically stimulate and optimize the ability of these fibroblasts to provide a niche for putative cancer stem cells (42). Finally, downregulation of IFNAR1 in infiltrating immune cells is expected to dramatically impair anti-tumor immunity.

It has been suggested that the immune system follows the “elimination-equilibrium-escape” mode to suppresses viability/growth of cancer cells while also selecting for resistant sub-

clones that subsequently invade and metastasize (43). Given a paramount role for Type 1 IFN in the tumor-specific dendritic cell-mediated recruitment and priming of infiltrating T cells and subsequent tumor elimination (44, 45), it is plausible that downregulation of IFNAR1 in the tumor microenvironment represents an essential mechanism that enables the switch from the elimination to the equilibrium to the escape phases of cancer immunoediting. It has been indeed suggested that hypoxic conditions favor tumor immunosuppression (46). Future studies will determine the role of IFNAR1 degradation in tumor stroma and immune compartments of tumors in the processes of tumor development, progression and metastases.

Furthermore, given the limited efficacy of IFN therapy, investigation of potential role of mechanisms uncovered here in the innate or acquired resistance of tumors to exogenously administered Type 1 IFN is also of substantial merit. As diverse branches of ISR signaling converge on the activation of the stress-activated p38 protein kinase, it is worth noting that activation of this kinase was inversely correlated with IFNAR1 levels in clinical malignant melanoma samples and that inhibition of this kinase sensitized human melanoma cells to the anti-tumorigenic effects of IFN *in vitro* and in a xenograft tumor model (34). Further delineation of these pathways may lead to identification of important therapeutic targets, the inhibitors of which may enable stabilization of IFNAR1 and could be combined with pharmacologic IFN α/β modalities. Conversely, it is hypothetically possible to exploit accelerated IFNAR1 degradation in hypoxic tumors to target these regions with oncolytic viruses that would be otherwise counteracted by Type 1 IFN pathway.

Materials and Methods

Plasmids, Chemicals and Cells

Short hairpin RNA (shRNA) against GCN2 (2), IFNAR1 (21), or PERK (16) have been described previously. PKR inducer heparin sodium salt (HE, used at 1 μ g/mL), thapsigargin (TG, used at 1 μ g/mL) PKR inhibitor Imidazolo-oxindole (C16), and MCDB 153 medium without leucine were purchased from Sigma. ShRNA for knockdown of PKR were purchased from Santa Cruz Biotechnology (sc-36263-SH). Dulbecco's Modification of Eagle's medium without leucine was obtained from MP Biomedicals. Human IFN α 2 was purchased from Bio-Sidius S.A. Murine IFN β was expressed in Chinese Hamster Ovary cells, and purified to homogeneity from conditioned culture medium using successive Blue Sepharose, copper chelating Sepharose, lentil lectin Sepharose, and Uno S (Biorad) cationic ion exchange chromatographies. The specific activity of the protein was 2 \times 10⁸ units/mg in an *in vitro* antiviral cytopathic effect assay using murine L929 cells challenged with encephalomyocarditis virus. The purified protein had an endotoxin level of <0.05 EU/mg.

Human breast cancer T47D cells (from Ze'ev Ronai, Burnham Institute, San Diego, CA) and melanoma WM266-4 and 1205Lu cells (from Meenhard Herlyn, Wistar Institute, Philadelphia, PA) were cultured as described previously (47, 48). WM266-4 cells were maintained in MCDB 153 medium supplemented with bovine insulin (5 μ g/mL). Other human cells as well as mouse embryo fibroblasts (MEFs) from knockout mice lacking p38 α (from Angelo Nebreda, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain), PKR (from Antonis Koromilas, McGill University, Montreal, Quebec), PERK or GCN2 (from David Ron, New York University, New York, NY) or knock-in mice harboring the eIF2 α ^{S51A} allele (from Randal Kaufman, University of Michigan, Ann Arbor, MI) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Hyclone), non-essential amino acids and β -mercaptoethanol (55 μ M). For knockdown experiments in human cells, T47D or WM266-4 cells were transduced with lentiviral particles encoding shRNA against GFP, PKR or GCN2 and selected in 2 μ g/mL of puromycin for 2 weeks. The human colon adenocarcinoma HT-29 cell line and its derivative

stably expressing the constitutively active GADD34 C-terminal fragment (HT29-A1) were cultured in the presence of puromycin (1 $\mu\text{g}/\text{mL}$) as described previously (33). Cells were exposed to the hypoxia condition of 0.5% oxygen, 5% carbon dioxide and 94.5% nitrogen for the indicated times through the use of the Invivo2–400 Hypoxia Workstation (Ruskin Technology Ltd).

Antibodies and Immunotechniques

Analyses of phosphorylated (on Ser532 in human IFNAR1 or Ser523 on mouse IFNAR1) and total levels of human and mouse IFNAR1 were carried out as described elsewhere (22, 49). Commercially available antibodies against pSTAT1, p-eIF2 α , p-p38 kinase, STAT1, PKR, (Cell Signaling), eIF2 α (Biosources), β -actin (Sigma), HIF1 α (BD Biosciences), p38 α , and ubiquitin (Santa Cruz Biotechnology) were purchased. Immunoprecipitations, immunoblotting, and in vivo ubiquitination assay using denaturing immunoprecipitation were carried out as described previously (23).

Cell invasion assays

BD Biocoat chambers coated with Matrigel matrix (BD Matrigel, 354234) were overlaid with 0.5 mL of cell suspension (2.5×10^4 cells) to each 24-well invasion chamber in media without serum. Medium with 10% serum (0.75 mL) was then added to the outside chamber and cells were incubated at 37°C with 5% CO₂ for either 24 hours (WM266–4 cells) or for 5 days (T47D cells). Cells were stained with crystal violet and images were obtained using a BX-51 Olympus microscope. The images were quantified using Image J.

Viability assays

Cells transfected with the indicated plasmids were selected with puromycin for 3 days and then seeded at 5×10^4 cells per well in a 96 well plate. The cells were treated with indicated doses of IFN α for 24–48 h and were analyzed using a cell proliferation colorimetric assay kit, CellTiter 96 (Promega). The Student *t* test was used for analysis of statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

GCN2	general control nonrepressed 2
eIF2α	eukaryotic translation initiation factor 2-alpha
HE	heparin
IFN	interferon
IFNAR1	IFN α/β receptor chain 1

ISR	integrated stress response
LS	leucine starvation
MEF	mouse embryo fibroblasts
PERK	pancreatic ER kinase
PKR	Protein kinase RNA-activated
STAT	Signal Transducer and Activator of Transcription
TG	thapsigargin

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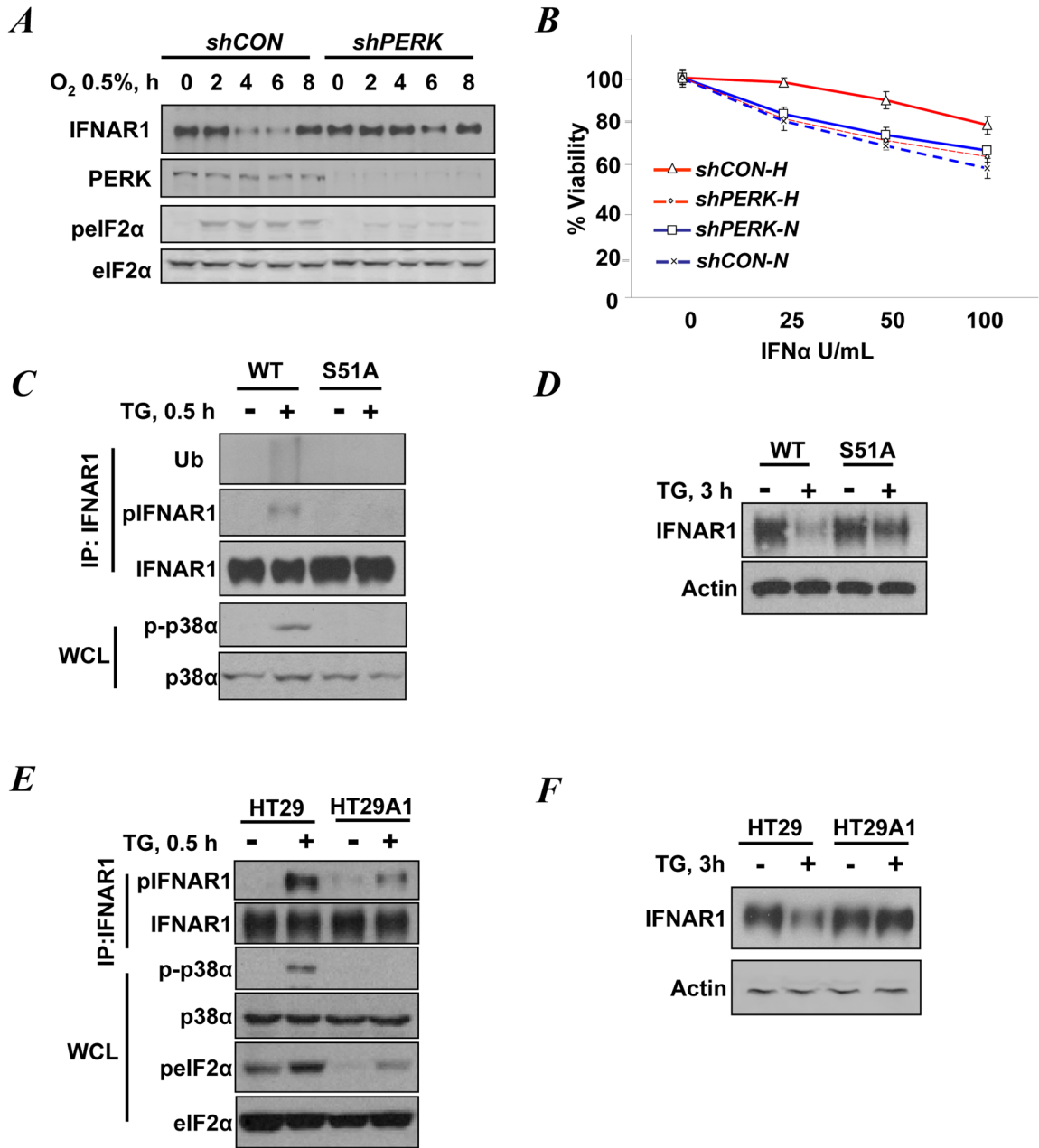


Figure 1. PERK induces phosphorylation and downregulation of IFNAR1 in a manner that depends on phosphorylation of eIF2α.

A. WM266-4 cells that received shRNA against PERK or GFP (control, shCON) were exposed to 0.5% O₂ for the indicated times. Levels of IFNAR1, PERK, eIF2α, and the phosphorylation of eIF2α on S51 were detected by immunoblotting.

B. WM266-4 cells transduced with the indicated shRNA and incubated under normoxic (N) or hypoxic (H) conditions for 4h were treated with the indicated doses of IFNα. The extent of PERK knockdown in these cells is shown in panel A. Cell viability was assessed 24 h later.

C. Phosphorylation, ubiquitination, and levels of IFNAR1 immunopurified from untreated or TG-treated (0.5 h) MEFs were analyzed by immunoblotting using the indicated antibodies. Levels and activation of p38α in the whole cell lysates were also analyzed.

D. Immunoblotting analyses of the levels of IFNAR1 and β -actin (used as a loading control) in untreated or TG-treated (3 h) MEFs.

E. Immunoblotting analysis of phosphorylation and levels of IFNAR1, p38 α , and eIF2 α in untreated or TG-treated (0.5 h) HT29-derived cells treated.

F. Immunoblotting analyses of the levels of IFNAR1 and β -actin (used as a loading control) in untreated or TG-treated (3 h) HT29-derived cells.

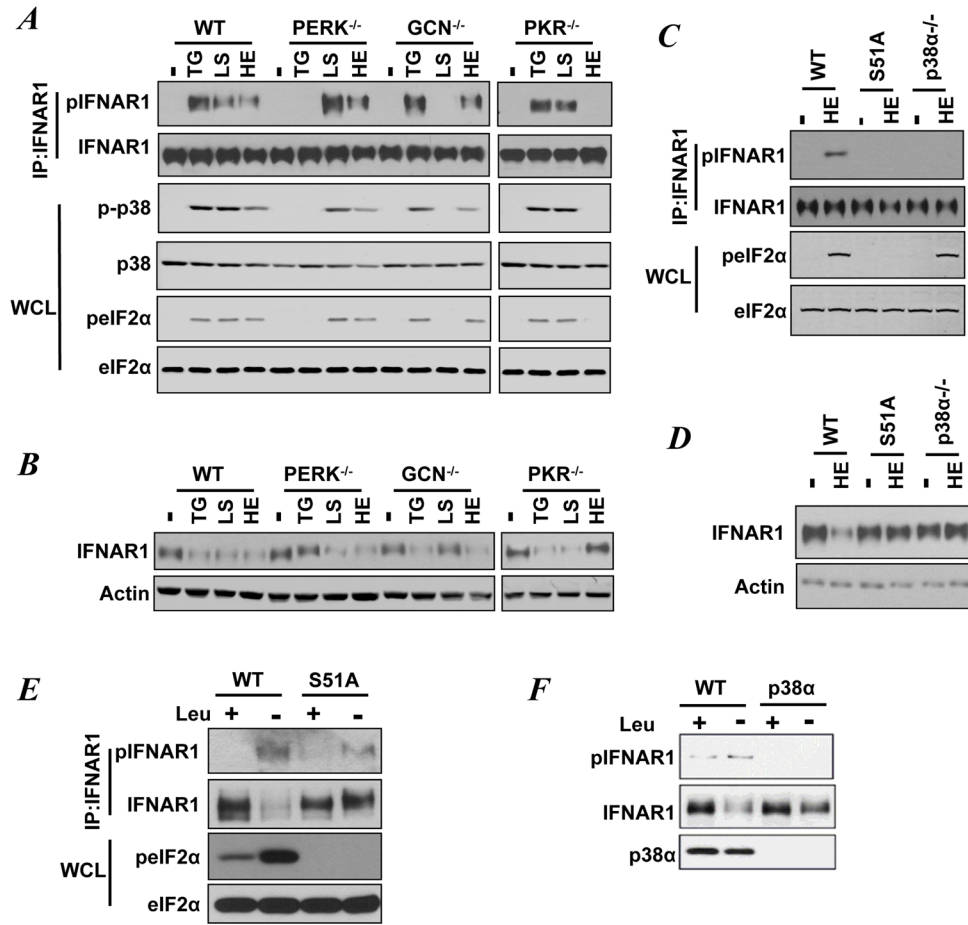


Figure 2. Diverse stress stimuli induce eIF2α phosphorylation to mediate phosphorylation and downregulation of IFNAR1

A. Immunoblotting analyses of phosphorylation and levels of IFNAR1, p38α, and eIF2α in MEFs exposed to TG (for 30 min) or heparin (HE, for 40 min), or leucine starvation (LS, for 24 h).

B. Immunoblotting analyses of the levels of IFNAR1 and β-actin in MEFs treated with TG (for 3 h), or HE (for 7 h), or incubated with media without leucine (LS, 48 h).

C. Immunoblotting analyses of phosphorylation and levels of IFNAR1 and eIF2α in MEFs exposed to heparin (HE) for 40 min.

D. Immunoblotting analyses of phosphorylation and levels of IFNAR1 and eIF2α in MEFs exposed to HE for 7 h.

E. Immunoblotting analyses of phosphorylation and levels of IFNAR1 and eIF2α in MEFs cultured in the presence or absence of leucine for 48 h.

F. Immunoblotting analyses of phosphorylation and levels of IFNAR1 and p38α kinase in MEFs cultured in the presence or absence of leucine for 48 h.

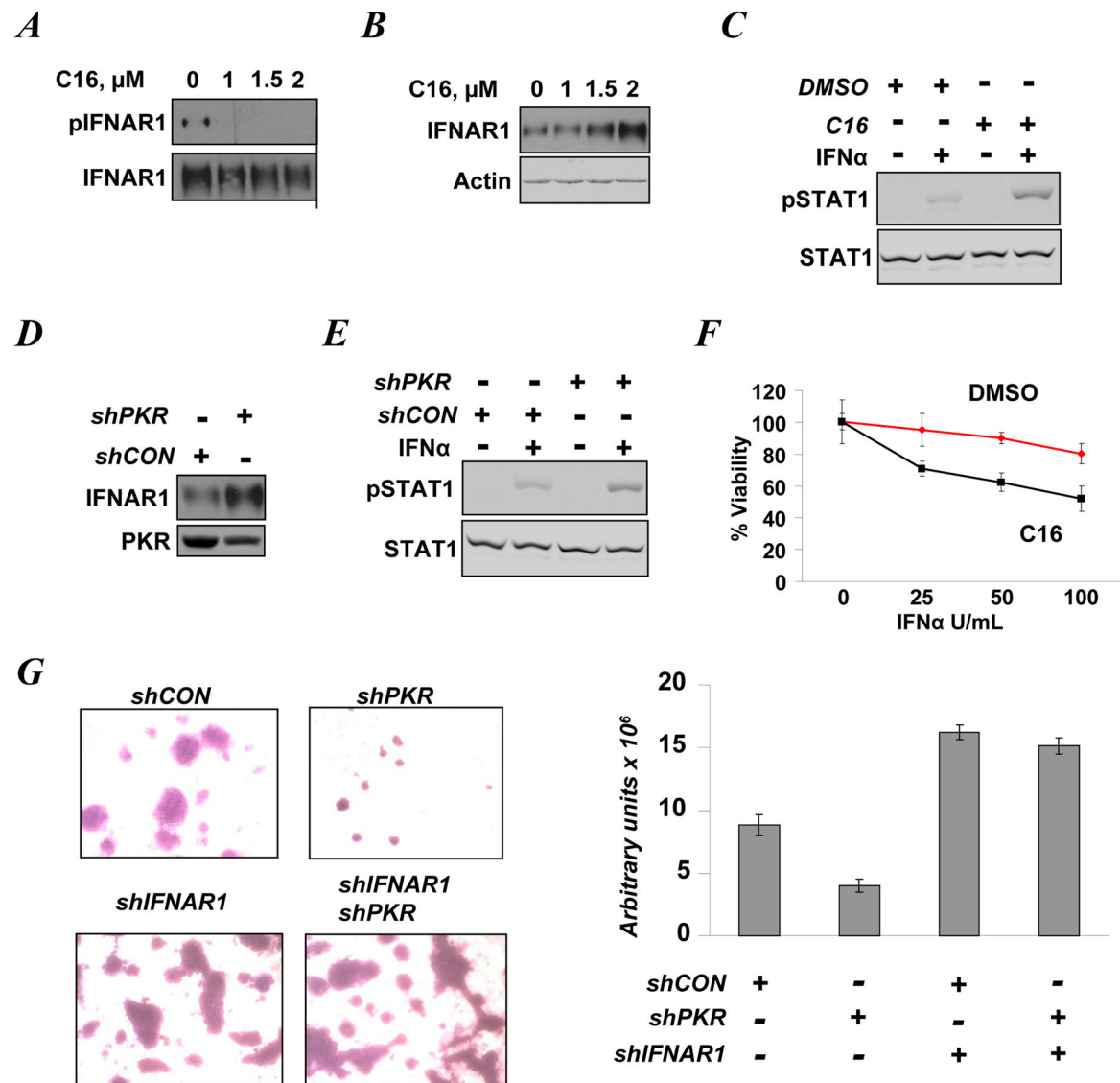


Figure 3. Constitutively active PKR suppresses IFNAR1 levels and signaling in T47D human breast cancer cells

- A. T47D cells treated with indicated doses of C16 (for 24 h) were incubated with the lysosomal inhibitor methyl amine chloride (40 mM) for 4 h prior to harvesting. Phosphorylation and levels of IFNAR1 were analyzed by immunoblotting.
- B. Immunoblotting analysis of IFNAR1 and β -actin levels in T47D cells treated with C16 for 24 h.
- C. Immunoblotting analyses of levels and phosphorylation of STAT1 in T47D cells treated with vehicle (DMSO) or C16 (2 μ M) for 24 h prior to treatment with 250 U/mL of IFN α (30 min).
- D. Levels of IFNAR1 and PKR in T47D cells that received control or PKR shRNA.
- E. Immunoblot analysis of levels and phosphorylation of STAT1 on Tyr701 in T47D cells that received control or PKR shRNA 48 h prior to treatment with of IFN α (250 U/mL for 30 min). The extent of PKR knockdown in the cells before treatment is shown in panel D.
- F. Viability of T47D cells treated with vehicle (DMSO) or C16 (2 μ M) for 24 h prior to treatment with the indicated amounts of IFN α (for 48 h) was analyzed using a MTT assay.

G. Images and quantification of T47D cells that received indicated shRNA and migrated through Matrigel/membrane in Boyden chambers. Density of migrating cells was assessed by densitometry and presented in arbitrary units of absorbance. The extent of PKR and IFNAR1 knockdown is shown in Figure S4.

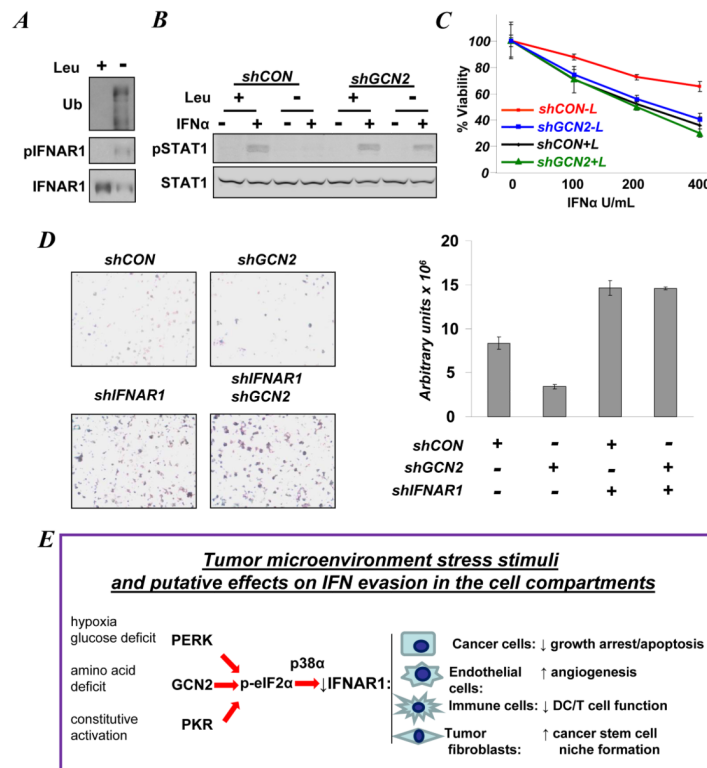


Figure 4. Activation of GCN2 by amino acid deficit promotes downregulation of IFNAR1 and inhibits its signaling

A. Levels, phosphorylation, and ubiquitination of IFNAR1 immunopurified from WM266-4 cells grown in the presence or absence of leucine for 48 h were analyzed by immunoblotting using the indicated antibodies.

B. STAT1 phosphorylation and levels in WM266-4 cells that received the indicated shRNAs and were incubated in medium in the presence or absence of leucine for 48 h prior to treatment with IFN α (250 U/mL for 30 min). Extent of protein knockdown is shown in Figure S6.

C. Viability of WM266-4 cells that received the indicated shRNAs and incubated in the presence (+L) or absence (-L) of leucine for 48 h prior to treatment with the indicated doses of IFN α (for 24 h) and subsequent analysis using a MTT assay.

D. Images and quantification of WM266-4 cells (that received indicated shRNA and were incubated in media lacking leucine) and migrated through Matrigel/membrane in Boyden chambers. Density of migrating cells was assessed by densitometry and presented in arbitrary units of absorbance. Extent of protein knockdown is shown in Figure S8.

E. Hypothetical model in which various types of ISR signaling converge on phosphorylation of eIF2 α and activation of p38 α kinase to downregulate IFNAR1 and inhibit IFN α responses in various compartments of the tumor. This leads to alleviation of growth inhibition, stimulation of angiogenesis, and niche formation and impairment of anti-tumorigenic immune responses.