Am J Cancer Res 2019;9(8):1766-1775
www.ajcr.us /ISSN:2156-6976/ajcr0094897

Original Article

Methionine restriction activates the integrated stress response in triple-negative breast cancer cells by a GCN2- and PERK-independent mechanism

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Received April 4, 2019; Accepted July 8, 2019; Epub August 1, 2019; Published August 15, 2019

Abstract: Transformed cells are often selectively susceptible to depletion of the amino acid methionine, which induces growth arrest and/or apoptosis. In non-transformed cells, amino acid deficiency is sensed by two stress-activated kinases, general control nonderepressible 2 (GCN2) and protein kinase R-like endoplasmic reticulum kinase (PERK), which phosphorylate and inactivate elongation initiation factor 2 α (eIF2α), thereby suppressing global mRNA translation and inducing activated transcription factor (ATF4). ATF4 and its downstream transcriptional targets including Sestrin-2 constitute an adaptive integrated stress response. We postulated that methionine depletion activates the integrated stress response in breast cancer cells by a GCN2- and/or PERK-dependent mechanism and that selective disruption of one or both of these kinases would enhance the therapeutic activity of methionine restriction. Here we demonstrate that methionine restriction induces eIF2α phosphorylation and enhances ATF4 gene expression and protein levels of ATF4 and Sestrin-2 in triple (ER/PR/HER2)-negative breast cancer (TNBC) cells. However, knockdown of GCN2, PERK or both in TNBC cells did not prevent induction of ATF4 or Sestrin-2 by methionine restriction. In contrast, deletion of *GCN2* in murine embryonic fibroblasts abrogated ATF4 and Sestrin-2 induction in response to methionine restriction. Moreover, knockdown of GCN2, PERK or both did not affect TNBC cell growth or apoptosis in response to methionine restriction. Overall, our findings point to a GCN2- and PERK-independent mechanism(s) by which methionine restriction activates the integrated stress response in TNBC cells. Elucidation of this pathway(s) could lead to strategies to enhance the therapeutic response of methionine restriction.

Keywords: Methionine restriction, cancer, integrated stress response, nutrition, metabolism

Introduction

Methionine is an essential amino acid required for protein and polyamine synthesis as well as for generating S-adenosylmethionine, the universal methyl-donor utilized by histone and DNA methyltransferases to maintain the epigenome [1]. Many tumor cells are more vulnerable to depletion of methionine than normal cells by mechanisms that are poorly understood [2]. Methionine restriction or replacement of methionine with homocysteine selectively induces cell cycle blockade or apoptosis in cancer cells [2-5]. Methionine depletion has also been shown to inhibit tumor growth in diverse murine models of cancer [6, 7]. We recently demonstrated that methionine restriction primes breast tumors to respond to TRAIL receptor agonists by increasing cell surface expression of TRAIL receptor 2/DR5 (TRAIL-R2) [8]. Dietary methionine restriction also enhanced the activity of TRAIL-R2 agonists against primary mammary tumors and lung metastases in a murine model of clinically aggressive triple (ER/PR/HER2)-negative breast cancer (TNBC). However, the underlying mechanisms by which TNBC cells sense methionine deficiency and activate downstream stress signaling pathways is entirely unknown. Clearly, the elucidation of these methionine-sensing mechanism could lead to the identification of novel molecular targets to enhance the therapeutic efficacy of methionine restriction.

Protein translation is an energy intensive process that is strictly regulated to be active only

when amino acids are abundant to conserve cellular energetics. The ability to sense and respond to amino acid deficiency and shut down protein translation is therefore an essential mechanism common to all mammalian cells [9]. General control nonderepressible 2 (GCN2) is a cytoplasmic kinase and a direct amino acid sensor in mammals. With a high affinity for uncharged tRNAs, GCN2 senses amino acid starvation by binding to uncharged tRNAs and undergoing dimerization and autophosphorylation. GCN2 activation leads to suppression of global protein translation through inhibitory phosphorylation of elongation Initiation Factor 2α (eIF2α) on Ser^{51} [10]. In addition, methionine restriction activates protein kinase R-like endoplasmic reticulum kinase (PERK), which also directly phosphorylates and inactivates eIF2α [11, 12]. The GCN2- or PERK-mediated inhibitory phosphorylation of eIF2α initiates induction of the activated transcription factor (ATF4) via its two upstream untranslated open reading frames (uORFs) [13]. The activation of ATF4 by stress-induced eIF2α kinases is a highly conserved adaptive response known as the integrated stress response. ATF4 is a basic leucine zipper transcription factor that activates amino acid biosynthesis pathways such as asparagine synthetase and amino acid transporters to replenish nutrient levels and restore cellular homeostasis [10]. Another downstream target of ATF4 is Sestrin-2, the recently described leucine-sensor that directly attenuates activity of the mechanistic target of rapamycin complex 1 (mTORC1) [14, 15]. The serine/threonine kinase mTORC1 is activated by amino acids under nutrient rich conditions to induce a panoply of anabolic metabolic alterations including protein translation and cell growth [9]. Hence, induction of Sestrin-2 in response to amino acid deprivation directly links the integrated stress response to suppression of mTORC1 activity [14].

We postulated that methionine restriction activates the integrated stress response by a GCN2- and/or PERK-dependent mechanism. Here we report that methionine restriction induces ATF4 gene expression in a panel of human TNBC cells and activates the integrated stress response as defined by eIF2α phosphorylation and induction of ATF4 and Sestrin-2. However, knockdown of GCN2, PERK or both did not prevent activation of the integrated stress response by methionine restriction in TNBC cells. In contrast, genetic deletion of *GCN2* in murine embryonic fibroblasts (MEFs) abrogated ATF4 and Sestrin-2 induction in response to methionine restriction, suggesting that transformed and/or human cells may acquire additional adaptive mechanisms to activate the integrated stress response in the setting of metabolic stress such as amino acid deprivation. Consistent with our observation that knockdown of GCN2, PERK or both did not prevent activation of the integrated stress response by methionine restriction in TNBC cells, these interventions also did not affect TNBC cell growth or apoptosis in response to methionine restriction. Overall, our findings point to a GCN2- and PERK-independent mechanism(s) by which methionine restriction activates the integrated stress response in TNBC cells. Elucidation of this pathway(s) could lead to strategies to enhance the therapeutic response of methionine restriction by abrogating activation of the adaptive integrated stress response.

Materials and methods

Cell culture

Human MDA-MB-231-mCherry (hereafter referred to as MDA-MB-231), MDA-MB-468 and GILM2 triple-negative breast cancer (TNBC) cells were cultured as previously described [16, 17]. Cell lines were authenticated by STR analyses and tested periodically for mycoplasma contamination. Immortalized $GCN2^{-/-}$ and WT MEFs [14] were kindly provided by Drs. Jiangbin Ye and Craig Thompson and were cultured in DMEM/F12 supplemented 10% FBS, 1 × non-essential amino acids, 100 IU/mL penicillin/streptomycin (Thermo Fisher Scientific) and 55 μM β-mercaptoethanol. For methionine restriction assays, cells were cultured in control RPMI-1640 media supplemented with additional nutrients to match the original media for each cell line or the corresponding methionine-free RPMI-1640 media.

Real-time PCR

Total RNA was harvested using SpinSmart™ Total RNA Mini Purification Kits (Denville Scientific) and cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. Primers for ATF4 (forward: 5'-CTTACAACCTCTTCCCCTTTCC-3', reverse: 5'-GGCTTCCTATCTCCTTCAGTG-3') and

GAPDH (forward: 5'-GAAGGTGAAGGTCGGAGTC 3', reverse: 5' GAAGATGGTGATGGGATTTC-3') were purchased from Integrated DNA Technologies (IDT). PCR was performed with iQ SYBR™ Green Supermix (Bio-Rad) and the products were analyzed with the CFX96™ Real Time PCR Detection System (Bio-Rad). RNA expression in samples was compared to the control in each experiment using a comparative C_t method. Experiments were performed twice in triplicate.

Immunoblotting

Whole cell lysates were immunoblotted as described [18] using primary Abs for GCN2 (#3302), ATF4 (#11815), p-eIF2α (#3597), eIF2α (#9722), PERK (#5683), Vinculin (#13901) (Cell Signaling Technology) and Sestrin-2 (#10795-1-AP) (Protein Tech).

siRNA experiments

Mission siRNAs including GCN2 (SASI_Hs01 00097889), PERK (SASI_Hs01_00096844) and negative control (SIC-001) were purchased from Sigma-Aldrich. Cells were transfected with control or target siRNAs using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol. Gene silencing was verified by immunolobtting.

Trypan blue exclusion assay

At the designated time point, floating cells were collected and the adherent cells were detached by trypsinization. Floating and trypsinized cells were pooled in DPBS (Thermo Fisher Scientific). 10 µl cell suspension was combined with 10 µl Trypan Blue solution (Thermo Fisher Scientific) and trypan blue-negative cells were scored using a hemocytometer (Thermo Fischer Scientific). Trypan blue negative cells were counted and noted at 10^4 cells/ml.

Annexin V assay

TNBC cells were transiently transfected with siRNAs targeting *GCN2*, *PERK*, both (*GCN2* + *PERK*) or non-silencing control (NC) siRNAs. Twelve hours later, TNBC cells were grown in control or methionine-free media for 48 hours and labelled with Annexin V-FITC (BD BioSciences) and DAPI (Sigma Aldrich). Samples were analyzed using a BD™ LSRII flow cytometer and gated using Flow Jo analysis software.

Statistics

Statistical significance was assessed by ANOVA with Bonferroni post tests using GraphPad Prism 4 software.

Results

Methionine deprivation activates the integrated stress response in TNBC cells

To investigate the effect of methionine restriction on the integrated stress response in TNBC cells, TNBC cells were incubated in control or methionine-free media and *ATF4* mRNA levels were determined by real-time PCR. Methionine restriction resulted in rapid and robust induction of *ATF4* gene expression in a panel of TNBC cell lines (**Figure 1A**). Moreover, methionine deprivation resulted in inhibitory phosphorylation of eIF2α followed by activation of ATF4 in a time-dependent manner in TNBC cells (**Figure 1B**). The levels of Sestrin-2, a known transcriptional target of ATF4 [14], also increased following ATF induction. Collectively, these findings demonstrate that methionine restriction activates the conserved integrated stress response in TNBC cells.

Methionine restriction activates the integrated stress response in TNBC cells by a GCN2-independent mechanism

To examine the potential role of the amino acid-sensing kinase GCN2 in the activation of the integrated stress response by methionine restriction, *GCN2* was knocked down in TNBC cells using siRNAs targeting *GCN2* and then these cells were grown in control or methionine-free media. Knockdown of GCN2 in TNBC cells did not prevent ATF4 or Sestrin-2 induction in response to methionine deprivation (**Figure 2A**) compared to TNBC cells incubated with non-silencing control (NC) siRNAs. The levels of phosphorylated eIF2α were also not affected by GCN2 knockdown, although there was little or no increase in phosphorylated eIF2α in response to methionine deprivation in the TNBC cells at the time points studied. In contrast, deletion of GCN2 in MEFs, dramatically suppressed eIF2α phosphorylation and induction of ATF4 and Sestrin-2 (**Figure 2B**). These results suggest that transformed and/or human cells may acquire additional adaptive mechanisms to activate the integrated stress response in

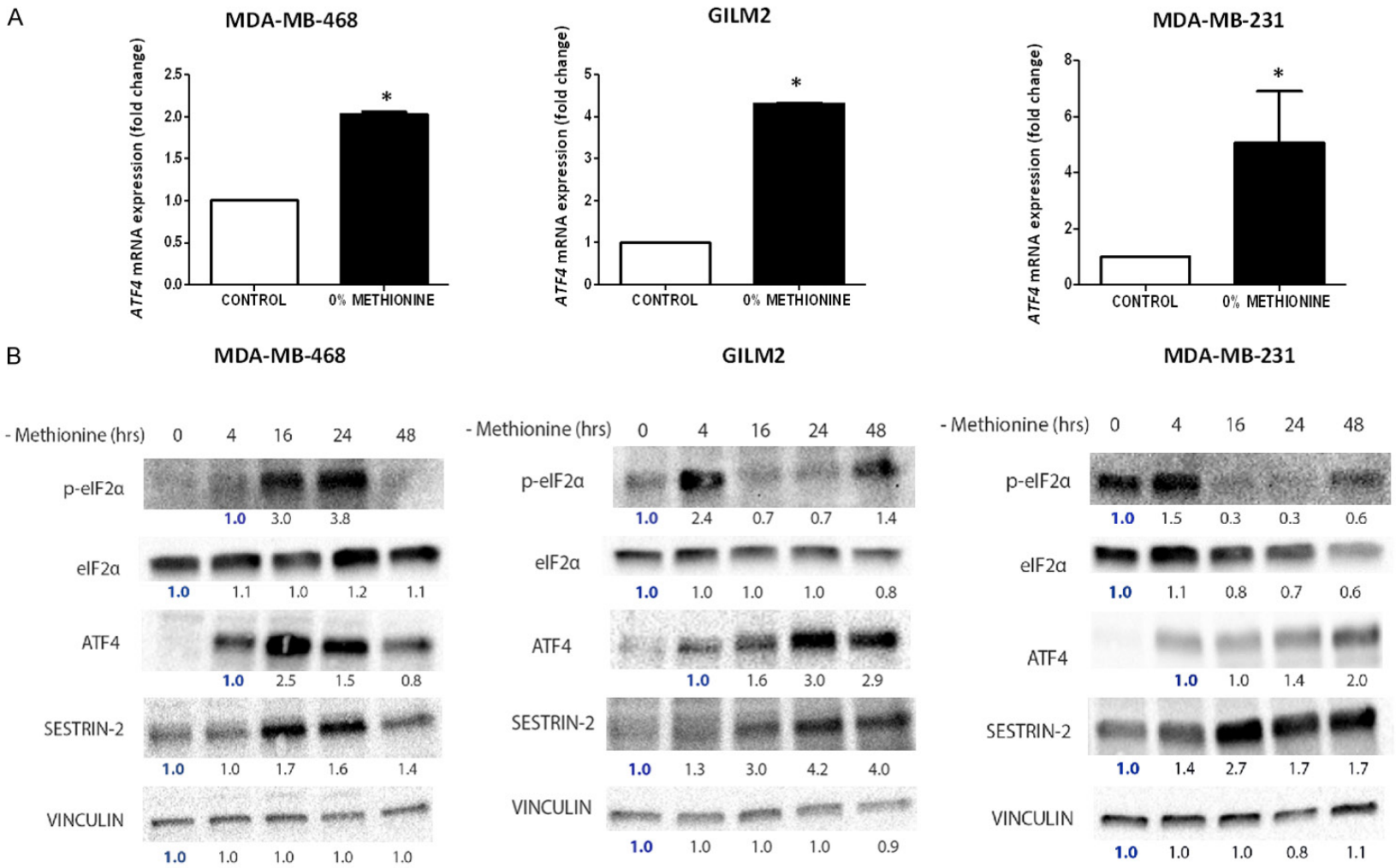


Figure 1. Methionine restriction activates the integrated stress response in TNBC cells. A. Induction of *ATF4* gene expression by methionine restriction. Real-time PCR analysis of *ATF4* mRNA levels in TNBC cells grown in control or methionine-free media for 6 hours. *ATF4* mRNA levels in each assay were normalized to *GAPDH* mRNA levels. $*P < 0.05$. B. Immunoblot analysis of TNBC cells grown in methionine-free media for the indicated number of hours. The intensity of immunoblot bands in each panel was measured using NIH ImageJ and normalized to the intensity of bands indicated as 1.0 (labeled in blue) in each panel.

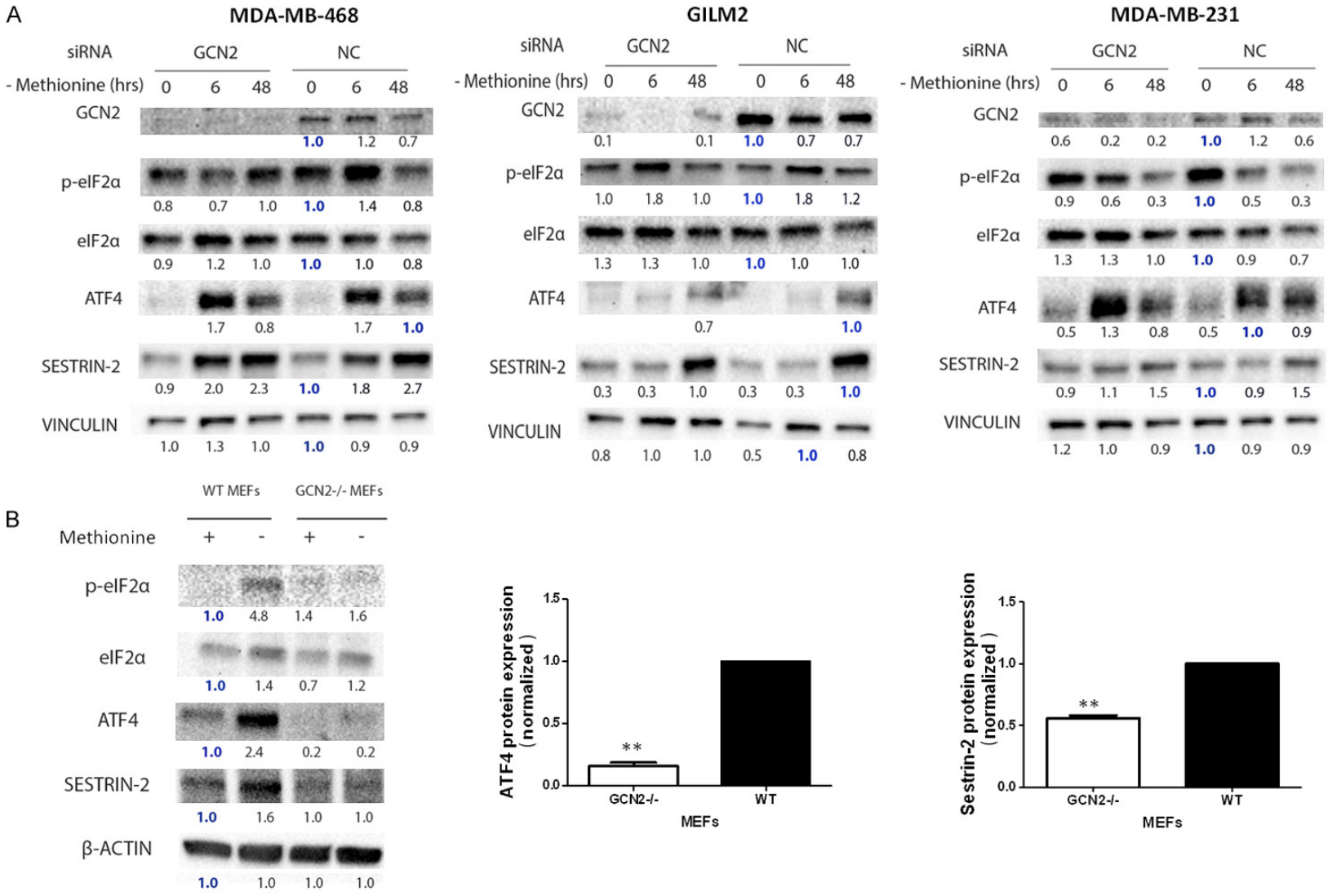


Figure 2. Methionine restriction activates the integrated stress response in TNBC cells by GCN2-independent mechanism. A. Knock down of *GCN2* does not inhibit induction of the integrated stress response by methionine restriction in TNBC cells. TNBC cells were transiently transfected with siRNAs targeting GCN2 or non-silencing control (NC) siRNAs. Twelve hours later, TNBC cells were grown in methionine-free media for 0-48 hours and analyzed by immunoblotting. B. Immunoblot of *GCN2*$^{-/-}$ or WT MEFs cultured in methionine-free media for 8 hours. The quantitation of three independent immunoblots is shown (right panels) $^{**}P < 0.01$. The intensity of immunoblot bands in each panel was measured using Image Lab software (Bio-Rad) and normalized to the intensity of bands indicated as 1.0 (labeled in blue) in each panel.

the setting of metabolic stress such as amino acid deprivation.

Methionine restriction activates the integrated stress response in TNBC cells by a GCN2- and PERK-independent mechanism

Since PERK has been implicated in the metabolic effects of methionine restriction [11], we knocked down PERK alone or in combination with GCN2 in TNBC cells and then subjected them to methionine deprivation. Knockdown of PERK, GCN2 or both did not prevent the induction of ATF4 or Sestrin-2 in TNBC cells subjected to methionine deprivation (**Figure 3A**). However, knock down of PERK did robustly inhibit CHOP induction by tunicamycin (**Figure 3B**), confirming that the observed PERK silencing inhibits its function. These results indicate that methionine restriction activates the integrated stress response independently of the eIF2α kinases GCN2 and PERK.

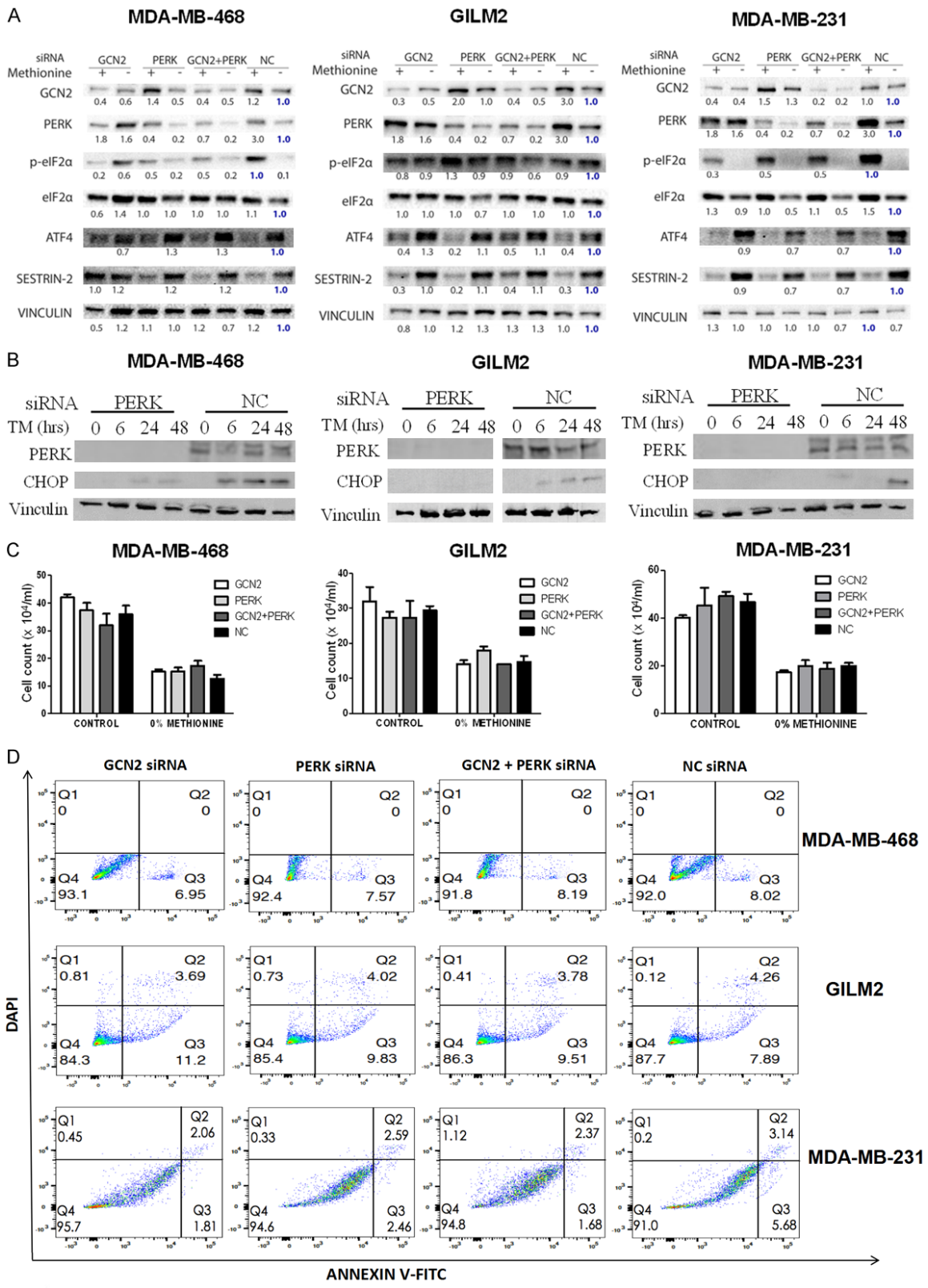
Methionine restriction inhibits cell growth and induces apoptosis by a GCN2- and PERK-independent mechanism

Methionine deprivation inhibits cancer cell growth by inducing cell cycle blockade and/or apoptosis [2]. Growth of each of the TNBC cell lines was robustly suppressed in methionine-free media compared to control media as determined by trypan blue exclusion to determine the number of viable cells (**Figure 3C**). Consistent with the inability of knockdown of GCN2, PERK or both to suppress the integrated stress response in TNBC cells subjected to methionine deprivation, none of these interventions affected growth inhibition (**Figure 3C**) or apoptosis induction (**Figure 3D**) by methionine restriction. Consistent with these results, the integrated stress response inhibitor ISRIB (IC_{50} 5 nM) that blocks the effects of eIF2α phosphorylation [19] did not enhance growth inhibition by methionine restriction (**Figure 3E**). Collectively, these results underscore that growth inhibition of TNBC cells in response to methionine restriction does not require GCN2, PERK or activation of the canonical integrated stress response.

Discussion

We have demonstrated that restriction of the essential amino acid methionine induces *ATF4* gene expression, increases phosphorylation of eIF2α, and induces ATF4 and Sestrin-2 in TNBC cells consistent with activation of the canonical integrated stress response. To our knowledge this is the first demonstration that methionine restriction induces the integrated stress response in cancer cells. Given the central role of the eIF2α kinase GCN2 in sensing uncharged tRNAs [10], we postulated that silencing GCN2 would suppress induction of the integrated stress response in TNBCs subjected to methionine restriction. However, we observed that silencing GCN2 did not abrogate ATF4 or Sestrin-2 induction by methionine restriction in TNBC cells, although we cannot exclude a minor component of GCN2-regulated ATF4/Sestrin-2 induction. These findings are consistent with prior reports indicating that GCN2 deletion did not consistently alter p-eIF2α levels in sarcomas in murine models [20]. In contrast, GCN2$^{-/-}$ MEFs failed to activate ATF4 or Sestrin-2 in response to methionine deprivation compared to WT MEFs, suggesting fundamentally different responses to methionine restriction in non-transformed and/or murine cells. Intriguingly, PERK has recently been demonstrated to be activated by methionine restriction and mediate activation of the integrated stress response in the liver of mice subjected to methionine deprivation [11]. Moreover, PERK is often aberrantly activated in the absence of GCN2 and can compensate for its loss, while GCN2 is induced in response to PERK loss [10, 20, 21]. Nevertheless, we observed that knockdown of PERK or combined knockdown of PERK and GCN2 did not alter ATF4 or Sestrin-2 induction by methionine restriction in TNBC cells. Collectively, our findings underscore that methionine restriction activates the integrated stress response in TNBC cells by a GCN2- and PERK-independent mechanism.

How then do TNBC cells sense methionine deficiency and activate the integrated stress response? One possibility is that methionine deficiency is sensed by other eIF2α kinases, such as double-stranded RNA-dependent protein kinase (PKR) or heme-regulated eIF2a kinase (HRI), which can be activated by oxidative and endoplasmic reticulum stress in addition to their prototypical stressors, double-stranded RNA and heme, respectively [10, 22]. Another possible explanation is the recently reported GCN2/eIF2α phosphorylation-inde-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eIF2α
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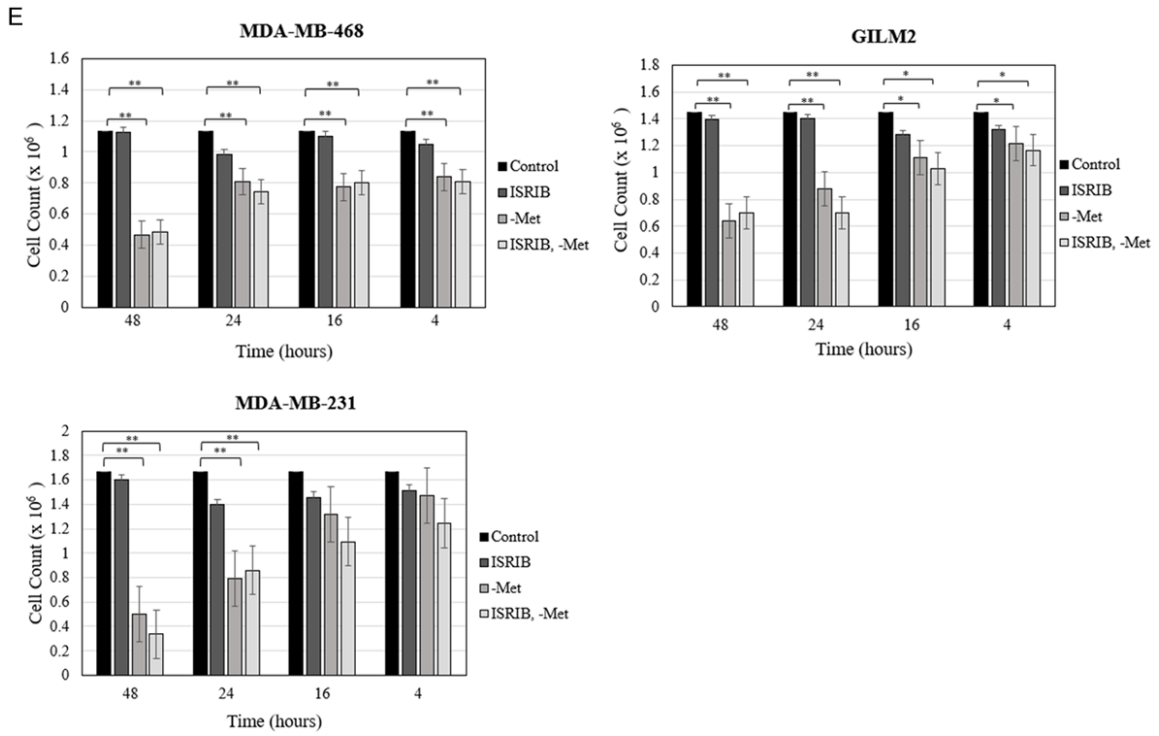

Figure 3. Methionine restriction activates the integrated stress response and inhibits cell growth in TNBC cells by a GCN2- and PERK-independent mechanism. A. Knockdown of *GCN2*, *PERK* or both does not inhibit induction of the integrated stress response by methionine restriction in TNBC cells. TNBC cells were transiently transfected with siRNAs targeting *GCN2*, *PERK*, both (*GCN2* + *PERK*) or non-silencing control (NC) siRNAs. Twelve hours later, TNBC cells were grown in control or methionine-free media for 48 hours and analyzed by immunoblotting. The intensity of immunoblot bands in each panel was measured using Image Lab software and normalized to the intensity of bands indicated as 1.0 (labeled in blue) in each panel. B. Silencing PERK inhibits stress-induced CHOP expression. TNBC cells were transiently transfected with siRNAs targeting PERK or non-silencing control (NC) siRNAs. Twelve hours later, the media was replaced and the TNBC cells were treated with 5 μg/mL of tunicamycin (TM) for 0-48 hours. The cells were then harvested for immunoblotting. C. Methionine restriction inhibits cell growth by a GCN2- and PERK-independent mechanism. TNBC cells were transiently transfected with siRNAs targeting *GCN2*, *PERK*, both (*GCN2* + *PERK*) or non-silencing control (NC) siRNAs. Twelve hours later, TNBC cells were grown in control or methionine-free media for 48 hours and the number of viable cells was determined by trypan blue exclusion. Each experiment was performed in triplicate (mean ± SEM, n=3). D. Methionine restriction induces apoptosis in a GCN2- and PERK-independent mechanism. Cells were transfected with the indicated siRNA and incubated in control or methionine-free media for 48 hours. Cells were then harvested and analyzed by Annexin-V assay using a LSRII flow cytometer. The percent of cells in each quadrant is indicated. E. ISRIB does not enhance growth inhibition by methionine restriction. TNBC cells were treated with control media, the integrated stress response inhibitor ISRIB (100 nM), methionine-free media or the combination of ISRIB (100 nM) and methionine-free media for 4-48 hours. The number of viable cells was determined by trypan blue exclusion. Each experiment was performed in triplicate (mean ± SEM, n=3). **P* < 0.05 and ***P* < 0.01.

pendent activation of the integrated stress response by methionine deprivation [23, 24]. For example, the GTPase OLA1 binds to eIF2, hydrolyzes GTP and disrupts the eIF2-GTP-initiator methionyl-tRNA (tRNAi), thereby shutting down global mRNA translation and activating the integrated stress response independently of eIF2α phosphorylation [25]. Similarly, depletion of methionine-loaded initiator methionyl-tRNA levels by methionine restriction would also likely reduce assembly of this ternary complex and activate ATF4 expression independently of eIF2α phosphorylation. Clearly, the potential contribution of one or more of these pathways to the observed GCN2/PERK-independent activation of the integrated stress response by methionine deprivation will require additional studies.

Consistent with the inability of GCN2, PERK or combined GCN2 and PERK knockdown to prevent activation of the integrated stress res-

ponse by methionine restriction, none of these interventions altered growth suppression of TNBC cells by methionine restriction. Consistent with these findings, ISRIB, a potent inhibitor of the effects of eIF2α phosphorylation [19], did not affect growth inhibition by methionine restriction. These findings have profound therapeutic implications because they indicate that inhibitors of GCN2 or PERK which are being actively developed will not enhance the activity of methionine restriction. Moreover, elucidation of the pathway(s) by which methionine restriction activates the integrative stress response or mediates its prosurvival effects could lead to novel drug targets to enhance the therapeutic response of methionine restriction by abrogating this adaptive stress response. In support of this concept, we have demonstrated that methionine restriction primes TNBC cells to respond to TRAIL-R2 agonists by selectively enhancing cell surface expression of TRAIL-R2, an approach we have termed "metabolic priming" [8]. We postulate that activation of the adaptive stress response by methionine restriction will prime tumors to respond to targeted drugs that selectively disrupt this response.

Acknowledgements

We thank Dr. Craig Thompson (Memorial Sloan Kettering Cancer Center) and Dr. Jiangbin Ye (Stanford University) for providing MEFs. These studies were funded in part by grants from the V Foundation for Cancer Research (to VLC), the Wisconsin Partnership Program (VLC), and the Breast Cancer Research Foundation (VLC) and P30CA14520 University of Wisconsin Comprehensive Cancer Center core facility support.

Disclosure of conflict of interest

None.

Abbreviations

ATF4, activated transcription factor 4; eIF2α, elongation initiation factor 2α; GCN2, general control nonderepressible 2; mTORC1, mechanistic target of rapamycin complex 1; MEFs, murine embryonic fibroblasts; PERK, protein kinase R-like endoplasmic reticulum kinase; TNBC, triple-negative breast cancer; TRAIL-R2, TRAIL receptor 2; WT, wild-type.

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