The Integrated Stress Response in Hypoxia-Induced Diffuse White Matter Injury

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Currently no treatments exist for preterm infants with diffuse white matter injury (DWMI) caused by hypoxia. Due to the improved care of preterm neonates and increased recognition by advanced imaging techniques, the prevalence of DWMI is increasing. A better understanding of the pathophysiology of DWMI is therefore of critical importance. The integrated stress response (ISR), a conserved eukaryotic response to myriad stressors including hypoxia, may play a role in hypoxia-induced DWMI and may represent a novel target for much needed therapies. In this study, we use *in vitro* and *in vivo* hypoxic models of DWMI to investigate whether the ISR is involved in DWMI. We demonstrate that hypoxia activates the ISR in primary mouse oligodendrocyte precursor cells (OPCs) *in vitro* and that genetically inhibiting the ISR in differentiating OPCs increases their susceptibility to *in vitro* hypoxia. We also show that a well established *in vivo* mild chronic hypoxia (MCH) mouse model and a new severe acute hypoxia (SAH) mouse model of DWMI activates the initial step of the ISR. Nonetheless, genetic inhibition of the ISR has no detectable effect on either MCH- or SAH-induced DWMI. In addition, we demonstrate that genetic enhancement of the ISR does not ameliorate MCH- or SAH-induced DWMI. These studies suggest that, while the ISR protects OPCs from hypoxia *in vitro*, it does not appear to play a major role in either MCH- or SAH-induced DWMI and is therefore not a likely target for therapies aimed at improving neurological outcome in preterm neonates with hypoxia-induced DWMI.

Key words: diffuse white matter injury; hypoxia; integrated stress response; oligodendrocytes

Significance Statement

Diffuse white matter injury (DWMI) caused by hypoxia is a leading cause of neurological deficits following premature birth. An increased understanding of the pathogenesis of this disease is critical. The integrated stress response (ISR) is activated by hypoxia and protects oligodendrocyte lineage cells in other disease models. This has led to an interest in the potential role of the ISR in DWMI. Here we examine the ISR in hypoxia-induced DWMI and show that while the ISR protects oligodendrocyte lineage cells from hypoxia *in vitro*, genetic inhibition or enhancement of the ISR has no effect on hypoxia-induced DWMI *in vivo*, suggesting that the ISR does not play a major role in and is not a likely therapeutic target for DWMI.

Introduction

Diffuse white matter injury (DWMI), also known as perinatal white matter injury, is a white matter disorder affecting low-birth weight premature infants born between 23 and 32 weeks of ges-

tation. Importantly, with improved care of premature neonates, the prevalence of DWMI is increasing as more low-birth weight premature infants survive, approximately half of whom manifest cognitive and learning disabilities by school age (Wilson-Costello et al., 2005; Deng, 2010; Back, 2015). Although the cellular and molecular mechanisms that cause DWMI are unknown, hypoxia, caused by underdeveloped neural vasculature and inefficient oxygenation from immature lungs, is thought to play a major role (Volpe, 2001, 2009; Scafidi et al., 2014). Currently, there are no approved therapies for DWMI.

Susceptibility to DWMI occurs before the onset of myelination when the most prevalent member of the oligodendrocyte lineage is the oligodendrocyte progenitor cell (OPC; Back et al., 2001, 2002, 2005, 2007). The hypoxic insults damage OPCs, causing death and inhibiting their maturation, leading to decreased white matter (Back and Miller, 2014). This coupled with the known increased susceptibility of OPCs to oxidative stress is

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thought to be the underlying cause of OPC-specific damage in individuals with DWMI (Back et al., 2007; Deng, 2010).

Hypoxia, through protein kinase RNA-activated (PKR)-like endoplasmic reticulum (ER) kinase (PERK; also known as *EIF2AK3*), is also a known activator of the integrated stress response (ISR), a conserved eukaryotic stress response that signals through phosphorylation of the α -subunit of eukaryotic translation initiation factor 2 α (eIF2 α ; Koumenis et al., 2002). PERK is also a component of the unfolded protein response (UPR) and phosphorylated eIF2 α (p-eIF2 α) decreases global protein translation and upregulates cytoprotective gene expression through activating transcription factor 4 (ATF4; Donnelly et al., 2013). ATF4 also increases the expression of the transcription factor CAAT enhancer binding protein homologous protein (CHOP; also known as *DDIT3*; Harding et al., 2003). CHOP increases the expression of growth and arrest DNA damage 34 (GADD34; also known as *PPP1R15A*), which forms a complex with protein phosphatase 1 to dephosphorylate eIF2 α forming a negative feedback loop that dampens the ISR (Novoa et al., 2001). The ISR, especially via PERK, is protective in multiple disorders of oligodendrocytes and myelin, and enhancement of the ISR by inactivating GADD34 or CHOP is known to protect oligodendrocytes in multiple mouse models of disease. (Clayton and Popko, 2016; Way and Popko, 2016). In addition, the ISR responds to inflammation and excitotoxicity, both of which are insults known to contribute to DWMI (Deng, 2010; Hetz et al., 2013). This ability of the ISR to respond to multiple stresses involved in DWMI along with the known protective role of the ISR in other white matter disorders positions the ISR as an intriguing potential player and novel therapeutic target in DWMI (Bueter et al., 2009).

In this study, we explored the role of the ISR in DWMI with the goal of better understanding the mechanism and potential therapeutic targets of DWMI. To study this, we have examined primary isolated OPCs exposed to hypoxia and used two hypoxic mouse models of DWMI, a well established model of mild chronic hypoxia (MCH; Scafidi et al., 2009, 2014; Fancy et al., 2011; Yuen et al., 2014) and a new alternative model of severe acute hypoxia (SAH). We demonstrate that both *in vitro* and *in vivo* hypoxia increase the phosphorylation of eIF2 α , without induction of downstream ISR components. Moreover, oligodendrocyte-specific genetic inhibition of the ISR via *Perk* deletion exacerbates *in vitro* hypoxic damage to differentiating OPCs but does not affect either MCH- or SAH-induced DWMI. Together, these results suggest that while neonatal hypoxia leads to increased phosphorylation of eIF2 α , it does not activate a full ISR response and that PERK-mediated ISR activation does not likely play a major role in hypoxia-induced DWMI. In addition, the genetic enhancement of the ISR in mice exposed to MCH or SAH by inactivating GADD34 and CHOP did not provide increased protection, suggesting that the ISR is an unlikely therapeutic target for DWMI.

Materials and Methods

Animals. All animals were housed under pathogen-free conditions, and all animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Chicago. All mice were on the C57BL/6 background, and, unless otherwise stated, male and female mice were used.

C57BL/6 mice were obtained from The Jackson Laboratory (catalog #000664). *Olig2-Cre* mice were provided by Dr. David Rowitch (University of Cambridge, Cambridge, UK) (Schüller et al., 2008) and floxed *Perk* mice were provided by Dr. Douglas Cavener (Pennsylvania State University, University Park, PA) (Zhang et al., 2002). *Olig2-Cre;Perk FL/FL* (OL-*Perk*-null) and littermate control *Perk FL/FL* mice lacking *Cre* expression (OL-*Perk*-FL) mice were used for experiments. Recombination

efficiency with *Olig2-Cre* mice is reported to be >90% (Kucharova and Stallcup, 2015). To determine the recombination efficiency *Olig2-Cre* mice were crossed to a *ROSA26-YFP* reporter line (stock #006148, The Jackson Laboratory). Brain tissue collected and stained for *Olig2* and yellow fluorescent protein (YFP) exhibited $79.6 \pm 11.6\%$ of *Olig2*⁺ cells that were also YFP⁺ (data not shown).

Gadd34-null mice were provided by Dr. David Ron (University of Cambridge, Cambridge, UK), and *Chop*-null mice (stock #005530, The Jackson Laboratory) have been previously described and were bred in-house (Zinszner et al., 1998; Novoa et al., 2001).

OPC isolation and culture. OPCs were isolated from postnatal day 5 (P5) to P7 mouse brains following the immunopanning protocol described by Barres et al. (1992) and subsequently modified and described previously (Dugas and Emery, 2013a; Emery and Dugas, 2013; Way et al., 2015). Cells were maintained as OPCs or differentiated into mature oligodendrocytes in media as previously described under control conditions as previously described (Dugas and Emery, 2013b; Emery and Dugas, 2013; Way et al., 2015). Wild-type (WT) OPCs were generated from WT mice while *Perk* knock-out (KO) OPCs were generated from OL-*Perk*-null mice.

Propidium iodide survival assay. Isolated OPCs were plated onto glass coverslips in proliferation media, and after 24 h of recovery cells were transferred to differentiation media with 500 ng/ml tunicamycin (catalog #T7765, Sigma-Aldrich) for 24 h. Following tunicamycin treatment, cells were stained with propidium iodide (catalog #P4170, Sigma-Aldrich) to label dead cells and with fluorescein acetate (catalog #F7378, Sigma-Aldrich) to label living cells. Three coverslips were counted per group, and the percentage of survival was presented.

In vitro hypoxia. For *in vitro* hypoxia experiments, OPCs isolated from P5–P7 mouse brains were transferred from control proliferation media to identical proliferation media that was deoxygenated under 100% nitrogen flow and then equilibrated to 0.1% O₂ for at least 2 h in a ProOx C21 carbon dioxide and oxygen controller (BioSpherix). Cells were then cultured at 0.1% O₂ and 10% CO₂ for various time periods based on the experiment.

Immunocytochemistry and cell counts. Immediately following 48 h of differentiation in either 0.1% O₂ or normoxia, cells were fixed with ice-cold 4% paraformaldehyde for 15 min at room temperature, washed with PBS, dried, and stored at -80°C .

Cells were blocked with 10% FCS/0.3% Triton in PBS and then incubated overnight in primary antibody in blocking solution. Cells were then washed with PBS and incubated with Alexa Fluor-conjugated secondary antibodies and mounted with Vectashield with DAPI mounting medium. The following primary antibodies were used: 1:250 myelin basic protein (MBP; catalog #808402, BioLegend; RRID: AB_2314771) and 1:250 *Olig2* (catalog #MABN50, Millipore; RRID: AB_10807410).

Stained cells were imaged with an Olympus IX81 Inverted Microscope with a Hamamatsu Orca Flash 4.0 Camera. Five nonoverlapping fields of view were taken with a 20 $\times$ objective, and the percentage of *Olig2*⁺ cells that were also MBP⁺ were counted.

In vivo MCH and SAH models of DWMI. MCH is a well described model of DWMI (Scafidi et al., 2009, 2014; Fancy et al., 2011; Yuen et al., 2014). Mouse pups, both male and female, were genotyped and fostered to lactating CD1 dams at P2 and designated for either MCH or room air (RA) control. Fostering is required for this protocol since C57BL/6 dams do not care for their litters under hypoxic conditions (Scafidi et al., 2009, 2014). At P3, pups assigned to MCH were placed into a BioSpherix glove box maintained at $10 \pm 0.5\%$ O₂ by displacement with nitrogen and controlled by a ProOx 360 oxygen controller from BioSpherix. Pups were exposed to MCH for 8 d from P3 to P11, after which they were returned to room air until the end of the experiment. Room air control mice were also fostered to CD1 dams and continued to breathe room air for the duration of the experiment.

To our knowledge, SAH as a model of DWMI has not been previously described. Male and female mouse pups were fostered to lactating CD1 dams at P2 and designated for either SAH or RA control. Fostering was determined to be necessary experimentally. At P3, pups with acute severe hypoxia were placed into a BioSpherix glove box that was maintained at $7 \pm 0.5\%$ O₂ by displacement with nitrogen under the control of a ProOx 360 oxygen controller from BioSpherix. Pups were exposed to SAH for

24 h from P3 to P4; after that, they were returned to room air until the end of the experiment. The duration of SAH was determined experimentally and was the maximum duration that lactating CD1 foster females could tolerate. Control mice breathing RA were also fostered to lactating CD1 dams and continued breathing room air for the duration of the experiment.

Total protein and RNA isolation. Protein was isolated from cells and snap frozen half-brain or frontal cortex rostral to the hippocampus using RIPA lysis buffer (catalog #R0278, Sigma-Aldrich) supplemented with protease inhibitor pills (cOmplete Mini Protease Inhibitor Cocktail, catalog #11836170001, Roche), Phosphatase Inhibitor Cocktail 2 (catalog #P2850, Sigma-Aldrich), Phosphatase Inhibitor Cocktail 3 (catalog #P5726, Sigma-Aldrich), and 17.5 mM β -glycerophosphate (catalog #G9422, Sigma-Aldrich). Protein lysates were then clarified by centrifugation and stored at -80°C . Protein concentration was determined using a BCA Protein Assay Kit (catalog #23255, Thermo Fisher Scientific). The region of the frontal cortex rostral to the hippocampus was isolated to study myelin protein levels in subcortical white matter that is susceptible to hypoxia-induced DWM (Ment et al., 1998; Fagel et al., 2006; Jablonska et al., 2012; Scafidi et al., 2014; Yuen et al., 2014).

RNA was isolated from cells and snap-frozen half-brain as previously described (Way et al., 2015). RNA quality was confirmed on a model 2100 Bioanalyzer using a model 6000 Nano Kit (catalog #5067-1511, Agilent Technologies) according to the manufacturer instructions. Only samples with an RNA integrity number >7 were used.

Western blot. Western blot analysis was performed as previously described (Way et al., 2015). The following primary antibodies were used: 1:500 p-eIF2 α (catalog #AB32157, Abcam; RRID: AB_732117), 1:1000 eIF2 α (catalog #9722S, Cell Signaling Technology; RRID: AB_1069509), 1:250 ATF4 (catalog #ARP37017, Aviva Systems Biology; RRID: AB_593104), 1:250 myelin-associated glycoprotein (MAG; catalog #346200, Thermo Fisher Scientific; RRID: AB_2533179), 1:250 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP; catalog #836401, BioLegend; RRID: AB_510037), 1:1000 MBP (catalog #808402, BioLegend; RRID: AB_2314771), 1:500 ATF6 (catalog #BAM-73-505, Cosmo Bio; RRID: AB_10709801), 1:500 pPKR (catalog #PA5-37704, Thermo Fisher Scientific; RRID: AB_254312), 1:500 PKR (catalog #AB45427, Abcam; RRID: AB_777309), and 1:2000 actin (catalog #A2066, Sigma-Aldrich; RRID: AB_476693). For the presentation of representative blots, bands from the same membrane are presented in the same window and are separated by a solid line when cropped. Bands that are presented in separate windows are from different membranes.

Quantitative real-time PCR and real-time PCR *Xbp1* splicing assay. Quantitative real-time PCR was performed as previously described (Way et al., 2015). Results were analyzed using the $\Delta\Delta\text{Ct}$ method with the Pfaffl correction for primer set-specific PCR efficiency (Pfaffl, 2001) on the Bio-Rad CFX Manager software. RPL13A was used as the reference gene. The efficiency of each primer set was determined by running reactions with known dilutions of cDNA at 60°C followed by calculation on the Bio-Rad CFX Manager software. Primers and efficiencies can be found in Table 1).

A real-time PCR *Xbp1* splicing assay was performed as previously described (Hussien et al., 2015). The primers for *Xbp1* splicing assay were as follows: forward primer, 5'-A AAC AGA GTA GCA GCG CAG ACT GC-3'; and reverse primer, 5'-TC CTT CTG GGT AGA CCT CTG GGA G-3'. Unspliced *Xbp1* product size is 480bp and spliced *Xbp1* product size is 454 bp. Positive and negative controls for the assay were from 3T3 cells treated with thapsigargin or untreated, respectively.

Immunohistochemistry and cell counts. Immunohistochemistry and cell counts were performed as previously described (Way et al., 2015). The following primary antibodies were used: 1:250 MBP (catalog #808402, BioLegend), 1:50 Olig2 (catalog #AB9610, Millipore), and 1:50 CC1 (catalog #OP80, Calbiochem), 1:100 Sox10 (catalog #AF2864, R&D Systems), 1:100 p-eIF2 α (catalog #AB32157, Abcam; RRID: AB_732117), 1:500 ATF4 (catalog #ARP37017, Aviva Systems Biology; RRID: AB_593104), 1:250 CHOP (catalog #MA1-250, Thermo Fisher Scientific; RRID: AB_2292611), 1:100 ATF6 (catalog #BAM-73-505, Cosmo Bio; RRID: AB_10709801). Images were acquired on an Olympus IX81 Inverted Microscope with a Hamamatsu Orca Flash 4.0 Camera. Images were ac-

Table 1. Primer sequences and PCR efficiencies

Gene of interest	Primers	PCR efficiency at 60°C
<i>Bip</i>	Fwd: 5'-ACT CCG GCG TGA GGT AGA AA-3' Rev: 5'-AGA GCG GAA CAG GTC CAT GT-3'	102.0%
<i>Atf4</i>	Fwd: 5'-TGG ATG ATG GCT TGG CCA GTG-3' Rev: 5'-GAG CTC ATC TGG CAT GGT TTC-3'	114.0%
<i>Chop</i>	Fwd: 5'-CCA CCA CAC CTG AAA GCA GAA-3' Rev: 5'-AGG TGC CCC CAA TTT CAT CT-3'	106.8%
<i>Gadd34</i>	Fwd: 5'-CCC TCC AAC TCT CCT TCT TCA G-3' Rev: 5'-CAG CCT CAG CAT TCC GAC AA-3'	87.4%
<i>Mbp</i>	Fwd: 5'-GCT CCC TGC CCC AGA AGT-3' Rev: 5'-TGT CAC AAT GTT CTT GAA GAA ATG G-3'	102.7%
<i>Plp</i>	Fwd: 5'-CAC TTA CAA CTT CGC CGT CCT-3' Rev: 5'-GGG AGT TTC TAT GGG AGC TCA GA-3'	115.8%
<i>Mag</i>	Fwd: 5'-CTG CTC TGT GGG GCT GAC AG-3' Rev: 5'-AGG TAC AGG CTC TTG GCA ACT G-3'	109.5%
<i>Rpl13a</i>	Fwd: 5'-TTC TCC TCC AGA GTC GCT GT-3' Rev: 5'-GGC TGA AGC CTA GGA GAA AG-3'	98.9%

Fwd, Forward; Rev, reverse. List of primers used for quantitative real-time PCR analysis. Efficiency was determined by running standard dilutions at 60°C and calculated on Bio-Rad CFX Manager Software.

quired from the subcortical white matter at the level of the corpus callosum and cingulum in the frontal cortex rostral to the hippocampus and of anatomically similar sections. Approximately 100 Olig2 $^{+}$ cells were counted per mouse to calculate the percentage of CC1 $^{+}$ /Olig2 $^{+}$ cells.

Statistics. Data are presented as mean $\pm$ SEM unless otherwise noted. Multiple comparisons were made using ANOVA with Tukey's post-test. Comparisons of two data points were made using a two-sided unpaired *t* test. A *p* value of <0.05 was considered significant, and all statistical analysis was run with GraphPad Prism software.

Results

Hypoxic activation of the ISR in primary OPCs is PERK dependent

It has been shown in mouse embryonic fibroblasts that *in vitro* hypoxia activates the ISR, as indicated by increased phosphorylation of eIF2 α (Koumenis et al., 2007; Liu et al., 2010). Nevertheless, it is not known whether *in vitro* hypoxia increases eIF2 α phosphorylation in OPCs. In addition, the kinase PERK has been shown to be responsible for eIF2 α phosphorylation in response to hypoxia, since *Perk* knock-out mouse embryonic fibroblasts have decreased the phosphorylation of eIF2 α in response to hypoxia compared with WT mouse embryonic fibroblasts (Koumenis et al., 2002; Blais et al., 2006; Liu et al., 2010). To investigate whether *in vitro* hypoxia increases the phosphorylation of eIF2 α and whether PERK plays a role in this activation, we generated WT and *Perk* KO OPCs. We first confirmed the decrease in PERK protein levels in *Perk* KO OPCs (Fig. 1A) and that *Perk* KO OPCs were more susceptible to the ER stressor tunicamycin (Fig. 1B,C). We then exposed primary isolated mouse OPCs to 0.1% O $_2$ for 0, 3, and 6 h. Following hypoxic exposure, the levels of p-eIF2 α were measured. We found that the exposure of WT OPCs to 3 and 6 h of hypoxia resulted in significantly increased levels of phosphorylated eIF2 α compared with WT OPCs exposed to 0 h hypoxia (Fig. 1D,E). Moreover, when compared with time-matched *Perk* KO OPCs, we found that WT OPCs had significantly higher p-eIF2 α levels at 3 and 6 h (Fig. 1D,E). This showed that hypoxia increases p-eIF2 α levels in isolated OPCs and that PERK plays a role in these increased levels of p-eIF2 α . Nevertheless, *Perk* KO OPCs after 6 h of hypoxia still had significantly higher levels of p-eIF2 α compared with control *Perk* KO OPCs (Fig. 1D,E). This suggests that PERK is not the sole kinase responsible for phosphorylating eIF2 α in response to hypoxia.

Figure 1. PERK signaling protects oligodendrocytes from hypoxia *in vitro*. **A**, Representative blot and quantification of PERK protein levels in WT and *Perk* KO OPCs. Quantification represents three individual OPC isolations with * $p < 0.05$ by unpaired two-tailed *t* test. **B**, Representative images of WT and *Perk* KO OPCs exposed to 500 ng/ml ER stressor tunicamycin for 24 h and stained with fluorescein diacetate (green) and propidium iodide (red) to label living and dead cells, respectively. **C**, Quantification of the percentage of survival of WT and *Perk* KO OPCs exposed to 500 ng/ml tunicamycin for 24 h with * $p < 0.05$ by unpaired two-tailed *t* test. **D**, Representative p-eIF2 α and eIF2 α blots of WT and *Perk* KO OPCs exposed to 0, 3, and 6 h of 0.1% O₂. **E**, Quantification of p-eIF2 α and eIF2 α blots, with data presented as the p-eIF2 α /eIF2 α ratio normalized to genotype-matched 0 h 0.1% O₂ control. Data represent three individual OPC isolations each with three replicates. *** $p < 0.005$ and **** $p < 0.0001$ vs time-matched WT controls by paired *t* test. † $p < 0.05$ vs 0 h *Perk* KO control by ANOVA. **F**, Representative images of ATF4 blots of WT OPCs exposed to 0.1% O₂ for 0, 3, and 6 h. **G**, Quantification of ATF4 protein levels in 0.1% O₂ exposed WT OPCs. **H**, Representative images of WT and *Perk* KO OPCs differentiated for 48 h under normoxia (NORM) or 0.1% O₂ and stained for the mature oligodendrocyte marker MBP (green) and the pan-oligodendrocyte lineage cell marker Olig2 (red). **I**, Quantification of the percentage of MBP⁺ cells in WT and *Perk* KO cultures exposed to 0.1% O₂ and NORM. Data represent four repeat experiments with ** $p < 0.01$ by ANOVA with Tukey's post-test. All data are presented as the mean $\pm$ SEM. Scale bars, 50 μ m. Norm. Dens., Normalized density.

Figure 2. Mild chronic hypoxia causes diffuse white matter injury. **A**, Top panels are representative images of MBP-stained (green) subcortical white matter (SCWM) from P18 mice exposed to MCH or RA control. Bottom panels are representative images of mature CC1⁺ (green) oligodendrocytes and OLIG2⁺ (red) oligodendrocyte lineage cells from P18 mice exposed to MCH or RA. **B**, Quantification of the percentage of OLIG2⁺ oligodendrocyte lineage cells that are CC1⁺ mature oligodendrocytes and density of OLIG2⁺ oligodendrocyte lineage cells in P18 MCH- and RA-exposed mice. Data represent $N \geq 3$ mice/group with * $p < 0.05$ by unpaired two-tailed t test. **C**, Analysis of myelin-enriched mRNA *Mag*, *Plp*, and *Mbp* in P11 mice exposed to MCH or RA. Data represent $N \geq 3$ with *** $p < 0.005$ and **** $p < 0.0001$. **D**, Representative blots of myelin-enriched proteins MAG, CNP, and MBP from P18 mice exposed to MCH or RA. **E**, Quantification of MAG, CNP, and MBP protein levels from P18 mice exposed to MCH or RA. Data represent $N \geq 3$ mice/group with ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.0001$ by unpaired two-tailed t test. All data are presented as the mean $\pm$ SEM. Scale bar, 50 μ m. Norm. Exp., Normalized expression; Norm. Dens., normalized density.

We also examined the levels of ATF4 protein in WT OPCs exposed to 0.1% O₂ and found that there is no significant difference in ATF4 protein levels (Fig. 1*H,G*).

To determine whether the diminished capacity of *Perk* KO OPCs to phosphorylate eIF2 α in response to hypoxia results in an enhanced susceptibility to hypoxia, we exposed differentiating WT and *Perk* KO OPCs to 0.1% oxygen for 48 h. We found that, as previously reported (Yuen et al., 2014), hypoxia decreased the percentage of MBP⁺ mature oligodendrocytes compared with cells differentiated under normoxic conditions (Fig. 1*H,I*). In addition, we discovered that *Perk* KO cells were more vulnerable to *in vitro* hypoxia and displayed significantly fewer MBP⁺ cells compared with WT cells exposed to hypoxia (Fig. 1*H,I*). This demonstrates that in WT oligodendrocyte lineage cells, PERK activation provides protection against hypoxia since the lack of PERK leads to increased loss of MBP⁺ cells in hypoxia-exposed cultures.

MCH causes DWMI in neonatal mice

Brain injury from chronic hypoxia caused by immature lung development has been modeled by MCH in neonatal mice (Scafidi et al., 2009). We first examined the brains of MCH-exposed mice to validate that MCH causes DWMI. MCH caused decreased

MBP immunostaining and decreased numbers of mature CC1⁺ oligodendrocytes in the subcortical white matter (Fig. 2*A,B*). Importantly, the total number of oligodendrocyte lineage cells, marked by oligodendrocyte transcription factor OLIG-2, was not significantly altered, suggesting that MCH caused decreased maturation of OPCs into mature CC1⁺ oligodendrocytes (Fig. 2*B*). In addition, MCH led to decreased levels of the mature myelin-specific proteins and/or mRNA MAG, CNP, myelin proteolipid protein (PLP), and MBP (Fig. 2*C–E*). This confirms that, as previously described, MCH is a valid model of DWMI.

MCH increases phosphorylation of eIF2 α in brain without increasing downstream ISR components or activating other arms of the UPR

As shown *in vitro*, hypoxia increases the phosphorylation of eIF2 α , which is indicative of the activation of the ISR. To determine whether *in vivo* MCH activates the ISR in neonatal mouse brains, we collected brain tissue at various durations of MCH exposure. We found that at 4 and 6 d of MCH, levels of p-eIF2 α in total brain lysates were significantly higher compared with age-matched room air controls (Fig. 3*A,B*). Nevertheless, there was no detectable increase in the percentage of p-eIF2 α /Sox10⁺ oligodendrocyte lineage cells in sections from pups exposed to 4 d

Figure 3. Mild chronic hypoxia increases the phosphorylation of eIF2 α in the brain but decreases ATF4 protein levels and does not induce downstream ISR components CHOP and GADD34. **A**, Representative p-eIF2 α , eIF2 α , and ATF4 blots from mice exposed to 1, 2, 4, 6, or 8 d of MCH or RA control. **B**, Quantification of p-eIF2 α , eIF2 α , and ATF4 protein levels. Data are presented as the p-eIF2 α /eIF2 α ratio normalized to time-matched RA-exposed control mice. **C**, Expression of *Chop*, *Gadd34*, and *Bip* mRNA levels normalized to time-matched RA-exposed controls. **D**, Representative image of P7 mouse brain exposed to 4 d of MCH and RA stained with Sox10, a pan-oligodendrocyte lineage cell marker, and p-eIF2 α . Scale bar, 100 μ m. **E**, Quantification of Sox10 $^{+}$ cells that are p-eIF2 α $^{+}$. **F**, Representative images of P7 mouse brain exposed to MCH and RA for 4 d and stained for Sox10 and ATF4. Scale bar, 50 μ m. **G**, Quantification of Sox10 $^{+}$ cells that are ATF4 $^{+}$. **H**, Representative images of Sox10 and CHOP staining from P7 mouse brain exposed to 4 d of MCH and RA. Scale bar, 50 μ m. **I**, Quantification of Sox10 $^{+}$ cells that are CHOP $^{+}$. All data are presented as the mean $\pm$ SEM for $N \geq 3$ mice/group with $*p < 0.05$ compared with time-matched controls (red dashed line) by unpaired two-tailed *t* test.

of MCH versus RA controls (Fig. 3D,E). Interestingly, the hypoxia-induced increase of p-eIF2 α (Fig. 3A,B) was not accompanied by an increase in protein levels of the downstream ISR transcription factor ATF4. Instead, ATF4 protein levels in total brain at 4 and 6 d of MCH were significantly decreased compared with age-matched room air controls (Fig. 3A,B). Consistent with the lack of an oligodendrocyte lineage-specific increase in p-eIF2 α staining (Fig. 3D,E), there was no difference in the number of ATF4 $^{+}$ /Sox10 $^{+}$ cells in MCH versus control brains (Fig. 3F,G). We also saw no evidence of increased expression of the downstream ISR factors *Chop*, *Gadd34*, or *Bip* (Fig. 3C), and no evidence of increased CHOP $^{+}$ /Sox10 $^{+}$ cells in MCH brains (Fig. 3H,I). Our results suggest that while increased phosphorylation of eIF2 α occurs in the brain in response to MCH, this increase does not result in the activation of downstream aspects of the ISR.

As mentioned, the PERK pathway plays a role in both the ISR and the UPR, and phosphorylation of eIF2 α occurs in both stress response pathways. Consequently, when studying the PERK pathway, it is important to determine whether increased p-eIF2 α is occurring with, or independent of, activation of the other two UPR sensors IRE1 and ATF6. To determine whether phosphorylation of eIF2 α in MCH is occurring in concert with the activation of the UPR or independently as a component of the ISR, we measured *Xbp1* splicing in brains exposed to either MCH or RA control. MCH did not induce splicing of *Xbp1* at any time point examined, suggesting that the IRE1 arm of the UPR is not activated (Fig. 4A). Moreover, the levels of activated cleaved ATF6 were not significantly affected by MCH (Fig. 4B,C), and no increase in Sox10 $^{+}$ oligodendrocyte lineage cells with nuclear ATF6 staining was observed (Fig. 4D,E). These data suggest that phosphorylation of eIF2 α by MCH is not associated with ER stress and activation of the unfolded protein response.

Effects of genetic inhibition of the ISR on MCH-induced DWMI

We next examined whether the PERK arm of the ISR protects oligodendrocytes and myelin from MCH-induced DWMI, similar to what we observed *in vitro* (Fig. 1). To determine whether oligodendrocyte-specific deletion of *Perk* exacerbated MCH-induced DWMI, we crossed *Olig2/Cre* and *Perk*^{FL/FL} mice to generate OL-*Perk*-FL controls and OL-*Perk*-null mice that were then exposed to MCH. *Olig2/Cre* mice express *Cre* in all cells of the oligodendrocyte lineage; therefore, *Perk* is deleted specifically from oligodendrocyte lineage cells generating OL-*Perk*-null mice.

We first determined that oligodendrocyte-specific deletion of *Perk* has no effect on developmental myelination. The expression of myelin-specific proteins MAG and MBP was not significantly different between untreated OL-*Perk*-FL and OL-*Perk*-null mice (Fig. 5A,B). Moreover, oligodendrocyte lineage cell-specific deletion of *Perk* had no effect on the number of mature CC1 $^{+}$ oligodendrocytes (Fig. 5C,D). These data are consistent with our previous demonstration that PERK activity is not required for oligodendrocyte development or myelination (Hussien et al., 2014).

We next found that, compared with OL-*Perk*-FL mice exposed to MCH, OL-*Perk*-null mice exposed to MCH displayed no significant difference in expression levels of myelin-specific proteins MAG, CNP, and MBP by Western blot analysis (Fig. 6A,B), levels of MBP staining on tissue sections (Fig. 6C), or the percentage of mature oligodendrocytes stained with CC1 (Fig. 6C,D). In addition, oligodendrocyte-specific deletion of *Perk* had no effect on the increased levels of p-eIF2 α caused by MCH (Fig. 6E,F). These data suggest that PERK signaling within oligodendrocyte lineage cells does not play a protective role under mild chronic hypoxic stress *in vivo*.

Figure 4. Mild chronic hypoxia does not activate the IRE-1 or ATF6 arms of the unfolded protein response. **A**, Representative *Xbp1* PCR gel image of mice exposed to MCH or RA. Unspliced *Xbp1* product size of 480 bp and spliced *Xbp1* product size of 454 bp. Lines represent where images from different gels have been cropped and spliced together. **B**, Representative ATF6 blots from mice exposed to 1, 2, 4, 6, or 8 d of MCH or RA. **C**, Quantification of ATF6 protein levels normalized to time-matched RA-exposed controls. **D**, Representative images from P7 mice exposed to MCH for 4 d and stained with ATF6 versus the oligodendrocyte lineage marker Sox10. **E**, Quantification of the percentage of Sox10⁺ cells that are nuclear ATF6⁺. All data are presented as the mean $\pm$ SEM for $N \geq 3$ mice/group. Scale bar, 50 μ m.

In addition to PERK, other eIF2 α kinases, including the eIF2 α kinase PKR (also called *Eif2ak2*) are activated by cytotoxic stress (Liu et al., 2010; Taniuchi et al., 2016). Therefore, we measured PKR activation by MCH and found that hypoxia exposure increased PKR phosphorylation in total brain lysate compared with room air controls (Fig. 6G,H). These results may explain the lack of effect caused by oligodendrocyte-specific excision of PERK.

Severe acute hypoxia is an alternative model of hypoxia-induced DWMI in neonatal mice

In addition to chronic hypoxic insults, the premature neonate is also exposed to brief episodes of severe hypoxia (Martin et al., 2011). Since oligodendrocyte-specific *Perk* ablation did not have a measurable effect on MCH-induced DWMI, we examined whether the ISR plays a role in DWMI caused by severe acute hypoxia. To address this question, we determined whether SAH leads to DWMI in neonatal mice. Neonatal mice were exposed to $7 \pm 0.5\%$ O₂ for 24 h from P3 to P4. Similar to the MCH model, the SAH model leads to decreased MBP staining in the subcortical white matter and decreased numbers of mature CC1⁺ oligodendrocytes with no change in the number of total OLIG2⁺ cells (Fig. 7A,B). Moreover, SAH-exposed pups exhibited decreased levels of the mature myelin proteins and/or mRNA of MAG, CNP, PLP, and MBP (Fig. 7C,E). These data establish that SAH is a valid model of DWMI in which we could examine the role of the ISR.

Severe acute hypoxia increases phosphorylation of eIF2 α in neonatal mouse brains without increasing downstream ISR components or activating other arms of the UPR

Having established SAH as a model of DWMI, we next examined whether SAH activates the ISR in the neonatal brain. Similar to

MCH, SAH leads to increased phosphorylation of eIF2 α in whole-brain lysates from mice exposed to the hypoxic insult (Fig. 8A,B). SAH increased p-eIF2 α levels within 12 h, more rapidly than under conditions of mild hypoxic stress, suggesting that activation of the ISR is tuned to the severity of hypoxic insult. Phosphorylation of eIF2 α was also transient in SAH, returning to baseline level by the end of the 24 h hypoxic exposure. Similar to MCH, there was no significant increase in the percentage of p-eIF2 α ⁺/Sox10⁺ oligodendrocyte lineage cells in the subcortical white matter of SAH-exposed pups (Fig. 8D,E). Moreover, SAH did not increase the levels of downstream ISR components. Like MCH, SAH caused a decrease in ATF4 protein levels (Fig. 8A,B), which likely explains the subsequent lack of *Chop*, *Gadd34*, and *Bip* mRNA induction (Fig. 8C). There was also no difference in the number of ATF4⁺/Sox10⁺ or CHOP⁺/Sox10⁺ cells in SAH-exposed pups compared with controls (Fig. 8F–I). Together, these experiments suggest that the transcriptional response driven by ATF4 does not play a significant role in the response to SAH.

SAH also did not induce the splicing of *Xbp1* at any time point, suggesting that the IRE1 arm of the UPR is not activated by SAH (Fig. 9A). Moreover, levels of activated cleaved ATF6 were not significantly affected by any duration of SAH (Fig. 9B,C), and there was no measurable increase in the number of Sox10⁺ cells with nuclear ATF6 positivity (Fig. 9D,E). These data again suggest that phosphorylation of eIF2 α by SAH is not associated with ER stress and activation of the unfolded protein response.

Effects of genetic inhibition of the ISR on SAH-induced DWMI

We next examined the effect that genetic manipulation of the ISR would have on SAH-induced DWMI. We exposed OL-*Perk*-null

Figure 5. Excision of *Perk* from oligodendrocyte lineage cells has no effect on developmental myelination. **A**, Representative MAG and MBP blots from untreated P11 OL-*Perk*-FL and OL-*Perk*-null mice. **B**, Quantification of MAG and MBP protein levels. Data are presented as the mean $\pm$ SEM for $N \geq 3$ mice/group. **C**, Representative images of subcortical white matter from untreated P11 OL-*Perk*-FL and OL-*Perk*-null mice stained for OLIG2 (red) and CC1 (green). **D**, Quantification of the percentage of CC1⁺/OLIG2⁺ cells and OLIG2⁺ cell density. Data are presented as the mean $\pm$ SEM for $N = 3$ mice/group. Scale bar, 50 μ m. Norm. Dens, Normoxia density.

and littermate OL-*Perk*-FL controls to SAH and examined myelin protein levels after 7 d of recovery at P11. There was no difference in the level of myelin-enriched proteins MAG, CNP, and MBP between OL-*Perk*-null and OL-*Perk*-FL pups exposed to SAH (Fig. 10*A,B*). These results were validated by MBP immunohistochemistry and cell counts of the percentage of mature CC1⁺ oligodendrocytes. No significant difference was found in the percentage of mature CC1⁺ oligodendrocytes in OL-*Perk*-null and OL-*Perk*-FL pups exposed to SAH (Fig. 10*C,D*). In addition, the levels of p-eIF2 α were not decreased in total brain lysate from OL-*Perk*-null animals (Fig. 10*E,F*). These results suggest that, similar to MCH-induced DWMI, the PERK arm of the ISR does not play a significant role in SAH-induced DWMI.

We also examined whether PKR activation might be responsible for the increase in p-eIF2 α levels in SAH-exposed brains in the absence of PERK. Similar to our MCH results, we found that the level of phosphorylated PKR was higher in total brain lysate from SAH-exposed pups compared with controls (Fig. 10*G,H*).

Effects of genetic enhancement of the ISR on MCH- and SAH-induced DWMI

Inhibition of *Gadd34* has been shown to protect oligodendrocytes in mouse models of multiple sclerosis (Lin et al., 2008; Way et al., 2015). Therefore, we examined whether enhancing the ISR through global *Gadd34* deletion protects oligodendrocytes and white matter from MCH- and SAH-induced DWMI. We found that myelin protein levels were significantly lower in *Gadd34*-null mice exposed to MCH compared with MCH-exposed controls (Fig. 11*A,B*), while myelin protein levels were similar between *Gadd34*-null and control mice exposed to SAH (Fig. 11*D,E*). We also discovered that while *Gadd34* deletion had no effect on p-eIF2 α levels in mice exposed to MCH for 6 d (Fig. 11*C*), *Gadd34* deletion increased p-eIF2 α levels in mouse brains exposed to SAH for 24 h (Fig. 11*F*). In addition to decreased levels of myelin-specific proteins, *Gadd34*-null mice exposed to MCH exhibited decreased body weight and increased mortality compared with MCH-exposed *Gadd34* WT mice. *Gadd34* WT mice exposed to MCH weighed 7.8 ± 0.3 g compared with 5.0 ± 0.3 g

Figure 7. Severe acute hypoxia is an alternative model of diffuse white matter injury. **A**, Top panels, Representative images of MBP-stained (green) subcortical white matter (SCWM) from P11 mice exposed to SAH or RA control. **A**, Bottom panels, Representative images of mature CC1⁺ (green) oligodendrocytes and Olig2⁺ (red) oligodendrocyte lineage cells from P11 mice exposed to SAH or RA. **B**, Quantification of the percentage of Olig2⁺ oligodendrocyte lineage cells that are CC1⁺ mature oligodendrocytes and density of Olig2⁺ oligodendrocyte lineage cells in P11 SAH- and RA-exposed mice. Data represent $N \geq 3$ mice/group with $*p < 0.05$ by unpaired two-tailed t test. **C**, Analysis of myelin-enriched proteins MAG, CNP, and MBP from P11 mice exposed to SAH or RA. Data represent $N \geq 3$ with $*p < 0.05$. **D**, Representative blots of myelin enriched proteins MAG, CNP, and MBP from P11 mice exposed to SAH or RA. **E**, Quantification of MAG, CNP, and MBP protein levels from P11 mice exposed to SAH or RA. Data represent $N \geq 3$ mice/group with $**p < 0.01$ and $****p < 0.0001$ by unpaired two-tailed t test. All data are presented as the mean $\pm$ SEM. Scale bar, 50 μ m.

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Figure 6. Oligodendrocyte-specific deletion of *Perk* has no effect on mild chronic hypoxia-induced diffuse white matter injury. **A**, Representative blots of myelin-enriched proteins MAG, CNP, and MBP from P18 OL-*Perk*-FL and OL-*Perk*-null mice exposed to MCH and RA control. **B**, Quantification of MAG, CNP, and MBP protein levels from P18 OL-*Perk*-FL and OL-*Perk*-null mice exposed to MCH and RA. Data represent $N \geq 3$ mice/group with $**p < 0.01$, $***p < 0.005$, and $****p < 0.0001$ by ANOVA with Tukey's post-test. **C**, Representative images of subcortical white matter (SCWM) from P18 OL-*Perk*-FL and OL-*Perk*-null mice exposed to MCH and RA and stained with MBP (green, top panels) or CC1 (green, bottom panels) and Olig2 (red, bottom panels). **D**, Quantification of the percentage of Olig2⁺ oligodendrocyte lineage cells that are CC1⁺ mature oligodendrocytes and density of Olig2⁺ oligodendrocyte lineage cells in P18 OL-*Perk*-FL and OL-*Perk*-null mice exposed to MCH and RA. **E**, Representative blots of p-eIF2 α and eIF2 α blots from P7 OL-*Perk*-FL RA, OL-*Perk*-FL MCH, and OL-*Perk*-null MCH-exposed mice. **F**, Quantification of p-eIF2 α and eIF2 α blots. **G**, Representative blots of pPKR and PKR from wild-type mice exposed to 1, 2, 4, 6, or 8 d of MCH or RA. **H**, Quantification of pPKR and PKR blots. Data represent $N \geq 3$ mice/group with $**p < 0.01$ and $*p < 0.05$ by ANOVA with Tukey's post-test. Scale bar, 50 μ m. Norm. Dens., Normoxia density.

for *Gadd34*-null mice exposed to MCH, and while 85% of *Gadd34* WT mice survived MCH exposure, only 31% of *Gadd34*-null mice survived. These data raise the possibility that the exacerbated effects of MCH on *Gadd34*-null mice are systemic and not CNS specific.

CHOP is known to play a proapoptotic role in response to sustained activation of the ISR (Zinszner et al., 1998), and global *Chop* deletion protects myelinating Schwann cells in mouse models of Charcot-Marie-Tooth disease (Pennuto et al., 2008). Nevertheless, the role of CHOP in myelinating glia is controversial, and there is evidence to suggest that CHOP expression can be protective in oligodendrocytes (Southwood et al., 2002; Gow and Wrabetz, 2009). To examine whether CHOP plays a role in either MCH- or SAH-induced DWMI, we exposed *Chop*-null and control mice to MCH and SAH. We found that *Chop* deletion had no effect on decreased myelin protein levels caused by either MCH or SAH (Fig. 11G–J). These results are in agreement with our findings that *Chop* is not upregulated by either *in vivo* model.

Figure 8. Severe acute hypoxia increases the phosphorylation of eIF2 α in the brain but decreases ATF4 protein levels and does not induce downstream ISR components CHOP or GADD34. **A**, Representative p-eIF2 α , eIF2 α , and ATF4 blots from mice exposed to either 12 or 24 h of SAH or RA control. **B**, Quantification of p-eIF2 α , eIF2 α , and ATF4 protein levels. Data are presented as the p-eIF2 α /eIF2 α ratio normalized to time-matched RA-exposed control mice. **C**, Expression of *Chop*, *Gadd34*, and *Bip* mRNA levels normalized to time-matched RA-exposed controls. **D**, Representative image of SAH- and RA-exposed brains stained with Sox10, a pan-oligodendrocyte lineage cell marker, and p-eIF2 α . Scale bar, 100 μ m. **E**, Quantification of Sox10 $^{+}$ cells that are p-eIF2 α $^{+}$. **F**, Representative images of Sox10 and ATF4 staining. Scale bar, 50 μ m. **G**, Quantification of Sox10 $^{+}$ cells that are ATF4 $^{+}$. **H**, Representative images of Sox10 and CHOP staining. Scale bar, 50 μ m. **I**, Quantification of Sox10 $^{+}$ cells that are CHOP $^{+}$. All data are presented as the mean $\pm$ SEM for $N \geq 3$ mice/group with $*p < 0.05$ compared with time-matched controls (red dashed line) by unpaired two-tailed t test.

Discussion

The PERK arm of the ISR responds to and protects cells from hypoxia, the main cause of DWMI in premature infants (Liu et al., 2010; Scafidi et al., 2014). PERK is also known to play a protective role in other white matter disorders (Clayton and Popko,

2016; Way and Popko, 2016). This has led to an interest in whether the ISR might play a role in DWMI and whether this cytoprotective pathway could be a therapeutic target for this disorder (Bueter et al., 2009). In the current study, we demonstrate that hypoxia increases phosphorylation of eIF2 α in isolated

Figure 9. Mild chronic hypoxia does not activate the IRE-1 or ATF6 arms of the unfolded protein response. **A**, Representative *Xbp1* PCR gel image of mice exposed to SAH or RA. Unspliced *Xbp1* product size of 480 bp and spliced *Xbp1* product size of 454 bp. Lines represent where images from different gels have been cropped and spliced together. **B**, Representative ATF6 blots from mice exposed to either 12 or 24 h of SAH or RA. **C**, Quantification of ATF6 protein levels normalized to time-matched RA-exposed controls. **D**, Representative images from mouse subcortical white matter stained with ATF6 versus the oligodendrocyte lineage marker Sox10. **E**, Quantification of the percentage of Sox10⁺ cells that are nuclear ATF6⁺. All data are presented as the mean $\pm$ SEM for $N \geq 3$ mice/group. Scale bar, 50 μ m. Norm. Dens., Normoxia density.

OPCs *in vitro* and that this is diminished in *Perk* KO OPCs, leading to increased susceptibility to *in vitro* hypoxia. Moreover, we show that both MCH and SAH increase the levels of p-eIF2 α in the brain of neonatal mice, without activation of downstream ISR components or the other arms of the UPR. In fact, there were decreased protein levels of the ISR transcription factor ATF4 in the CNS in response to MCH and SAH. Finally, we demonstrate that oligodendrocyte-specific deletion of *Perk* had no detectable effect on MCH- or SAH-induced DWMI. It is possible that the loss of PERK is compensated for by the activation of other hypoxia-responsive eIF2 α kinases (Taniuchi et al., 2016). In fact, we detected increased levels of phosphorylated PKR in brains exposed to either MCH or SAH. Together, these studies show that although PERK plays a role in oligodendrocyte lineage cells exposed to *in vitro* hypoxia, PERK signaling within oligodendrocytes does not likely play a crucial role in either *in vivo* model of DWMI.

PERK-mediated increased phosphorylation of eIF2 α in response to hypoxia occurs in cell lines *in vitro* (Koumenis et al., 2002). We have demonstrated that in primary isolated OPCs, *in vitro* hypoxia also increases the phosphorylation of eIF2 α in a mostly PERK-dependent manner. Nonetheless, some increase in phosphorylation of eIF2 α was still seen in *Perk* KO OPCs exposed to *in vitro* hypoxia. This may be due to the overlap among the stress-sensing eIF2 α kinases. For example, GCN2 has also been shown to respond to *in vitro* hypoxia (Liu et al., 2010; Donnelly et al., 2013). Regardless of the potential activation of other eIF2 α kinases, *Perk* KO OPCs still showed a significantly increased susceptibility to *in vitro* hypoxia, demonstrating that PERK activity is critical for OPC survival in the face of hypoxia.

In an inflammatory context where PERK activation is shown to increase p-eIF2 α levels, it has also been shown that oligodendrocyte-specific PERK insufficiency increases the susceptibility of these cells to inflammation (Lin et al., 2007, 2013, 2014; Hussien et al., 2014). Nonetheless, we found that although *in vivo* MCH increased eIF2 α phosphorylation in neonatal mouse brains, oligodendrocyte-

specific deletion of *Perk* did not have a detectable effect on MCH-induced DWMI. The disparate results generated by *in vitro* and *in vivo* hypoxia could be due to the severity of the hypoxic stress. Although we were unable to directly measure the percentage of oxygen available to oligodendrocyte lineage cells during MCH *in vivo*, it is unlikely to reach the level of oxygen deficiency experienced by oligodendrocyte lineage cells *in vitro*. *In vitro* hypoxia allows for a much more severe level of hypoxia without the confounding factor of the systemic effects of hypoxia on survival *in vivo*. To address this, we developed the SAH model of DWMI where we exposed pups to $7 \pm 0.5\%$ oxygen for 24 h. The 7% oxygen set point was used as it was the lowest percentage of oxygen tolerated by the lactating females over a 24 h period. Even with the increased severity of hypoxia, oligodendrocyte-specific deletion of *Perk* had no effect on the DWMI caused by SAH. While our studies were performed in neonatal mice, it is possible that in the adult mouse the ISR may play a more significant role in response to hypoxic insult. Together, these results suggest that PERK signaling specifically within the oligodendrocyte lineage does not play a pivotal role in neonatal hypoxia-induced DWMI.

It is interesting that while both MCH and SAH lead to a transient increase in p-eIF2 α levels in whole-brain lysate, neither hypoxic insult causes the induction of a full ISR response. We discovered that the lack of activation of downstream ISR signaling was correlated with hypoxia-driven decreased ATF4 protein levels in whole brain. The decrease in ATF4 protein levels corresponded to the duration of hypoxia in which p-eIF2 α levels were increased. Activation of a truncated ISR is not unprecedented: cells exposed to UV irradiation have been shown to phosphorylate eIF2 α without increasing levels of downstream ISR components (Dey et al., 2010, 2012). It is possible that while the survival response to hypoxia requires phosphorylation of eIF2 α and energy conservation via decreased protein translation, an adaptive transcriptional response driven by ATF4 is expendable.

The major transcriptional regulator in response to hypoxia is hypoxia-inducible factor 1- α (HIF1 α), which is stabilized under

Figure 10. Oligodendrocyte-specific deletion of *Perk* has no effect on severe acute hypoxia-induced diffuse white matter injury. **A**, Representative blots of MAG, CNP, and MBP from P11 OL-Perk-FL and OL-Perk-null mice exposed to SAH and RA control. **B**, Quantification of MAG, CNP, and MBP protein levels from P11 OL-Perk-FL and OL-Perk-NUL mice exposed to SAH and RA control. **C**, Representative immunofluorescence images of MBP (green) and OLIG2 (red) in OL-Perk-FL and OL-Perk-NUL mice exposed to SAH and RA control. **D**, Quantification of OLIG2+ cells/mm and the percentage of OLIG2+ cells that are CC1+ from P11 OL-Perk-FL and OL-Perk-NUL mice exposed to SAH and RA control. **E**, Representative blots of pEIF2α and eIF2α from P11 OL-Perk-FL and OL-Perk-NUL mice exposed to SAH and RA control. **F**, Quantification of pEIF2α/eIF2α protein levels from P11 OL-Perk-FL and OL-Perk-NUL mice exposed to SAH and RA control. **G**, Representative blots of pPKR and PKR from P11 OL-Perk-FL and OL-Perk-NUL mice exposed to SAH and RA control at 12hr and 24hr. **H**, Quantification of pPKR/PKR protein levels from P11 OL-Perk-FL and OL-Perk-NUL mice exposed to SAH and RA control at 12hr and 24hr. Error bars represent SEM. Statistical significance is indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, *****p < 0.00001, N.S. = not significant).

Figure 11. Genetic deletion of *Gadd34* or *Chop* does not protect mice from diffuse white matter injury caused by mild chronic hypoxia or severe acute hypoxia. **A**, Representative blots of myelin proteins MAG, CNP, and MBP from P18 *Gadd34*-null and *Gadd34* WT mice exposed to MCH or RA. **B**, Quantification of MAG, CNP, and MBP protein levels from P18 *Gadd34*-null and *Gadd34* WT mice exposed to MCH or RA. Data represent $N \geq 3$ mice/group with $**p < 0.01$, $***p < 0.005$, and $****p < 0.0001$ by ANOVA with Tukey's post-test. **C**, Representative blots and corresponding quantification of p-eIF2 α and eIF2 α protein in *Gadd34* WT and *Gadd34*-null mice exposed to 6 d of MCH. **D**, Representative blots of myelin proteins from P11 *Gadd34*-null and *Gadd34* WT mice exposed to SAH or RA. **E**, Quantification of myelin protein levels from P11 *Gadd34*-null and *Gadd34* WT mice exposed to SAH or RA. **F**, Representative blots and corresponding quantification of p-eIF2 α and eIF2 α protein in *Gadd34* WT and *Gadd34*-null mice exposed to 24 h SAH. **G**, Representative blots of myelin proteins MAG and MBP from P18 *Chop*-null and *Chop* WT mice exposed to MCH or RA. **H**, Quantification of myelin protein levels from P18 *Chop*-null and *Chop* WT mice exposed to MCH or RA. **I**, Representative blots of myelin proteins from P11 *Chop*-null and *Chop* WT mice exposed to SAH or RA. **J**, Quantification of myelin protein levels from P11 *Chop*-null and *Chop* WT mice exposed to SAH or RA. Data represent $N \geq 3$ mice/group with $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$ by ANOVA with Tukey's post-test.

hypoxic conditions and induces the expression of genes with hypoxic response elements like vascular endothelial growth factor (VEGF; Sharp and Bernaudin, 2004). It is possible that under hypoxic conditions ATF4 is expendable due to an overlap with

HIF1 α targets. In fact, it has been shown that the ISR increases expression of the HIF1 α target gene *Vegf* (Ghosh et al., 2010). In addition, genetic removal of *Hif1 α* increases baseline expression of ATF4, suggesting that HIF1 α is a negative regulator of ATF4 expression (Guimarães-Camboa et al., 2015). Therefore, the truncated activation of the ISR by hypoxia may be the result of ATF4 inhibition by HIF1 α .

Our results also show that phosphorylation of eIF2 α in response to hypoxia is transient. It has been shown that translational inhibition in response to hypoxia exhibits a biphasic response, with the initial inhibition of protein translation caused by the phosphorylation of eIF2 α (Wouters et al., 2005; van den Beucken et al., 2006). Perhaps the transient nature of hypoxia-induced eIF2 α phosphorylation is due to activation of the negative feedback loop culminating in dephosphorylation of eIF2 α . Our data support this possibility in that *Gadd34*-null mice have higher levels of p-eIF2 α after 24 h of SAH than controls. The lack of an apparent effect of the *Gadd34* mutation on MCH-induced

(Figure legend continued.) OL-Perk-null mice exposed to SAH and RA. Data represent $N \geq 3$ mice/group with $**p < 0.01$, $***p < 0.005$, and $****p < 0.0001$ by ANOVA with Tukey's post-test. **C**, Representative images of subcortical white matter (SCWM) from P11 OL-Perk-FL and OL-Perk-null mice exposed to SAH and RA and stained with MBP (green, top panels) or CC1 (green, bottom panels) and Olig2 (red, bottom panels). **D**, Quantification of the percentage of Olig2 $^{+}$ oligodendrocyte lineage cells that are CC1 $^{+}$ mature oligodendrocytes and the density of Olig2 $^{+}$ oligodendrocyte lineage cells in P11 OL-Perk-FL and OL-Perk-null mice exposed to SAH and RA. **E**, Representative blots of p-eIF2 α and eIF2 α from OL-Perk-FL RA, OL-Perk-FL MCH, and OL-Perk-null mice exposed to 12 h of SAH. **F**, Quantification of p-eIF2 α and eIF2 α blots. **G**, Representative blots of pPKR and PKR from wild-type mice exposed to 1, 2, 4, 6, or 8 d of MCH or RA. **H**, Quantification of pPKR and PKR blots. Data represent $N \geq 3$ mice/group with $**p < 0.01$ and $*p < 0.05$ by ANOVA with Tukey's post-test. Scale bars, 50 μ m. Norm. Dens., Normoxia density.

p-eIF2 α levels is potentially due to the possibility that we did not assess an appropriate time point.

Inhibition of GADD34 is known to be protective in mouse models of multiple sclerosis, and *Chop* deletion protects myelinating Schwann cells in mouse models of Charcot-Marie-Tooth disease (Pennuto et al., 2008; Way et al., 2015). We found that, in agreement with the lack of increased *Chop* expression, *Chop* deletion had no detectable effect on DWMI, while the effect of global *Gadd34* deletion was dependent on the hypoxic insult. GADD34 acts as a regulatory subunit for protein phosphatase 1 and is responsible for dephosphorylation of eIF2 α . Therefore, inhibition of GADD34 enhances the ISR by prolonging the phosphorylation of eIF2 α (Novoa et al., 2001). Multiple genetic and pharmacological approaches have shown that *Gadd34* inhibition is protective in disorders of myelinating glia, including oligodendrocytes (Lin et al., 2008; D'Antonio et al., 2013; Way et al., 2015). Using a global *Gadd34*-null mutant, we found that GADD34 inactivation is detrimental in the case of MCH. *Gadd34*-null mice exposed to MCH had decreased levels of myelin proteins, decreased total body weight, and increased mortality compared with *Gadd34* WT mice exposed to MCH. Surprisingly, *Gadd34*-null mice exposed to SAH showed no measurable difference when compared with *Gadd34* WT mice exposed to SAH. This suggests that the combined effects of global *Gadd34* deletion and a chronic hypoxic stress is detrimental, perhaps due to an impaired ability to adapt to the hypoxic environment. In support of this possibility, *Gadd34* mutant mice were shown to have impaired hemoglobin production (Patterson et al., 2006). Impaired hemoglobin synthesis could explain the increased susceptibility of the *Gadd34*-null mice to hypoxia. Preliminary data from our laboratory support this possibility: we have found that, when exposed to MCH, *Gadd34*-null mice have a decreased hematopoietic response that is characterized by decreased hemoglobin and red blood cell production when compared with *Gadd34* WT mice exposed to MCH (data not shown).

In summary, we have investigated the role of the ISR, a protective pathway that responds to a variety of cytotoxic insults, in both *in vitro* and *in vivo* hypoxia-induced models of DWMI. We found that while PERK is critical to protect oligodendrocyte lineage cells from hypoxia *in vitro*, its removal had no measurable effect on hypoxia-induced DWMI *in vivo*. Moreover, the enhancement of the ISR via GADD34 or CHOP inactivation did not provide increased protection in models of DWMI. These results suggest that targeting the PERK arm of the ISR would not likely be an effective strategy for DWMI therapy.

References

- Back SA (2015) Brain injury in the preterm infant: new horizons for pathogenesis and prevention. *Pediatr Neurol* 53:185–192. [CrossRef Medline](#)
- Back SA, Miller SP (2014) Brain injury in premature neonates: a primary cerebral dysmaturation disorder? *Ann Neurol* 75:469–486. [CrossRef Medline](#)
- Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, Kinney HC (2001) Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci* 21:1302–1312. [Medline](#)
- Back SA, Han BH, Luo NL, Chricton CA, Xanthoudakis S, Tam J, Arvin KL, Holtzman DM (2002) Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. *J Neurosci* 22:455–463. [Medline](#)
- Back SA, Luo NL, Mallinson RA, O'Malley JP, Wallen LD, Frei B, Morrow JD, Petito CK, Roberts CT Jr, Murdoch GH, Montine TJ (2005) Selective vulnerability of preterm white matter to oxidative damage defined by F2-isoprostanes. *Ann Neurol* 58:108–120. [CrossRef Medline](#)
- Back SA, Riddle A, McClure MM (2007) Maturation-dependent vulnerability of perinatal white matter in premature birth. *Stroke* 38:724–730. [CrossRef Medline](#)
- Barres BA, Hart IK, Coles HS, Burne JF, Voyvodic JT, Richardson WD, Raff MC (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70:31–46. [CrossRef Medline](#)
- Blais JD, Addison CL, Edge R, Falls T, Zhao H, Wary K, Koumenis C, Harding HP, Ron D, Holcik M, Bell JC (2006) Perk-dependent translational regulation promotes tumor cell adaptation and angiogenesis in response to hypoxic stress. *Mol Cell Biol* 26:9517–9532. [CrossRef Medline](#)
- Bueter W, Dammann O, Leviton A (2009) Endoplasmic reticulum stress, inflammation, and perinatal brain damage. *Pediatr Res* 66:487–494. [CrossRef Medline](#)
- Clayton BL, Popko B (2016) Endoplasmic reticulum stress and the unfolded protein response in disorders of myelinating glia. *Brain Res* 1648:594–602. [CrossRef Medline](#)
- D'Antonio M, Musner N, Scapin C, Ungaro D, Del Carro U, Ron D, Feltri ML, Wrabetz L (2013) Resetting translational homeostasis restores myelination in Charcot-Marie-Tooth disease type 1B mice. *J Exp Med* 210:821–838. [CrossRef Medline](#)
- Deng W (2010) Neurobiology of injury to the developing brain. *Nat Rev Neurol* 6:328–336. [CrossRef Medline](#)
- Dey S, Baird TD, Zhou D, Palam LR, Spandau DF, Wek RC (2010) Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response. *J Biol Chem* 285:33165–33174. [CrossRef Medline](#)
- Dey S, Savant S, Teske BF, Hatzoglou M, Calkhoven CF, Wek RC (2012) Transcriptional repression of ATF4 gene by CCAAT/enhancer-binding protein β (C/EBP β) differentially regulates integrated stress response. *J Biol Chem* 287:21936–21949. [CrossRef Medline](#)
- Donnelly N, Gorman AM, Gupta S, Samali A (2013) The eIF2 α kinases: their structures and functions. *Cell Mol Life Sci* 70:3493–3511. [CrossRef Medline](#)
- Dugas JC, Emery B (2013a) Purification of oligodendrocyte precursor cells from rat cortices by immunopanning. *Cold Spring Harb Protoc* 2013:745–758. [CrossRef Medline](#)
- Dugas JC, Emery B (2013b) Purification and culture of oligodendrocyte lineage cells. *Cold Spring Harb Protoc* 2013:810–814. [CrossRef Medline](#)
- Emery B, Dugas JC (2013) Purification of oligodendrocyte lineage cells from mouse cortices by immunopanning. *Cold Spring Harb Protoc* 2013:854–868. [CrossRef Medline](#)
- Fagel DM, Ganat Y, Silbereis J, Ebbitt T, Stewart W, Zhang H, Ment LR, Vaccarino FM (2006) Cortical neurogenesis enhanced by chronic perinatal hypoxia. *Exp Neurol* 199:77–91. [CrossRef Medline](#)
- Fancy SP, Harrington EP, Yuen TJ, Silbereis JC, Zhao C, Baranzini SE, Bruce CC, Otero JJ, Huang EJ, Nusse R, Franklin RJ, Rowitch DH (2011) Axin2 as regulatory and therapeutic target in newborn brain injury and remyelination. *Nat Neurosci* 14:1009–1016. [CrossRef Medline](#)
- Ghosh R, Lipson KL, Sargent KE, Mercurio AM, Hunt JS, Ron D, Urano F (2010) Transcriptional regulation of VEGF-A by the unfolded protein response pathway. *PLoS One* 5:e9575. [CrossRef Medline](#)
- Gow A, Wrabetz L (2009) CHOP and the endoplasmic reticulum stress response in myelinating glia. *Curr Opin Neurobiol* 19:505–510. [CrossRef Medline](#)
- Guimarães-Camboa N, Stowe J, Aneas I, Sakabe N, Cattaneo P, Henderson L, Kilberg MS, Johnson RS, Chen J, McCulloch AD, Nobrega MA, Evans SM, Zamboni AC (2015) HIF1 α represses cell stress pathways to allow proliferation of hypoxic fetal cardiomyocytes. *Dev Cell* 33:507–521. [CrossRef Medline](#)
- Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 11:619–633. [CrossRef Medline](#)
- Hetz C, Chevet E, Harding HP (2013) Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* 12:703–719. [CrossRef Medline](#)
- Hussien Y, Cavener DR, Popko B (2014) Genetic inactivation of PERK signaling in mouse oligodendrocytes: normal developmental myelination with increased susceptibility to inflammatory demyelination. *Glia* 62:680–691. [CrossRef Medline](#)
- Hussien Y, Podojil JR, Robinson AP, Lee AS, Miller SD, Popko B (2015) ER chaperone BiP/GRP78 is required for myelinating cell survival and provides protection during experimental autoimmune encephalomyelitis. *J Neurosci* 35:15921–15933. [CrossRef Medline](#)
- Jablonska B, Scafidi J, Aguirre A, Vaccarino F, Nguyen V, Borok E, Horvath TL, Rowitch DH, Gallo V (2012) Oligodendrocyte regeneration after

- neonatal hypoxia requires FoxO1-mediated p27Kip1 expression. *J Neurosci* 32:14775–14793. [CrossRef Medline](#)
- Koumenis C, Naczki C, Koritzinsky M, Rastani S, Diehl A, Sonenberg N, Koromilas A, Wouters BG (2002) Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2 α . *Mol Cell Biol* 22:7405–7416. [CrossRef Medline](#)
- Koumenis C, Bi M, Ye J, Feldman D, Koong AC (2007) Hypoxia and the unfolded protein response. *Meth Enzymol* 435:275–293. [CrossRef Medline](#)
- Kucharova K, Stallcup WB (2015) NG2-proteoglycan-dependent contributions of oligodendrocyte progenitors and myeloid cells to myelin damage and repair. *J Neuroinflammation* 12:161. [CrossRef Medline](#)
- Lin W, Bailey SL, Ho H, Harding HP, Ron D, Miller SD, Popko B (2007) The integrated stress response prevents demyelination by protecting oligodendrocytes against immune-mediated damage. *J Clin Invest* 117:448–456. [CrossRef Medline](#)
- Lin W, Kunkler PE, Harding HP, Ron D, Kraig RP, Popko B (2008) Enhanced integrated stress response promotes myelinating oligodendrocyte survival in response to interferon- γ . *Am J Pathol* 173:1508–1517. [CrossRef Medline](#)
- Lin W, Lin Y, Li J, Fenstermaker AG, Way SW, Clayton B, Jamison S, Harding HP, Ron D, Popko B (2013) Oligodendrocyte-specific activation of PERK signaling protects mice against experimental autoimmune encephalomyelitis. *J Neurosci* 33:5980–5991. [CrossRef Medline](#)
- Lin Y, Huang G, Jamison S, Li J, Harding HP, Ron D, Lin W (2014) PERK activation preserves the viability and function of remyelinating oligodendrocytes in immune-mediated demyelinating diseases. *Am J Pathol* 184:507–519. [CrossRef Medline](#)
- Liu Y, László C, Liu Y, Liu W, Chen X, Evans SC, Wu S (2010) Regulation of G1 arrest and apoptosis in hypoxia by PERK and GCN2-mediated eIF2 α phosphorylation. *Neoplasia* 12:61–68. [CrossRef Medline](#)
- Martin RJ, Wang K, Köröglu O, Di Fiore J, Kc P (2011) Intermittent hypoxic episodes in preterm infants: do they matter? *Neonatology* 100:303–310. [CrossRef Medline](#)
- Ment LR, Schwartz M, Makuch RW, Stewart WB (1998) Association of chronic sublethal hypoxia with ventriculomegaly in the developing rat brain. *Dev Brain Res* 111:197–203. [CrossRef](#)
- Novoa I, Zeng H, Harding HP, Ron D (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 α . *J Cell Biol* 153:1011–1022. [CrossRef Medline](#)
- Patterson AD, Hollander MC, Miller GF, Fornace AJ Jr (2006) Gadd34 requirement for normal hemoglobin synthesis. *Mol Cell Biol* 26:1644–1653. [CrossRef Medline](#)
- Pennuto M, Tinelli E, Malaguti M, Del Carro U, D'Antonio M, Ron D, Quattrini A, Feltri ML, Wrabetz L (2008) Ablation of the UPR-mediator CHOP restores motor function and reduces demyelination in Charcot-Marie-Tooth 1B mice. *Neuron* 57:393–405. [CrossRef Medline](#)
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45. [CrossRef Medline](#)
- Scafidi J, Fagel DM, Ment LR, Vaccarino FM (2009) Modeling premature brain injury and recovery. *Int J Dev Neurosci* 27:863–871. [CrossRef Medline](#)
- Scafidi J, Hammond TR, Scafidi S, Ritter J, Jablonska B, Roncal M, Szigeti-Buck K, Coman D, Huang Y, McCarter RJ Jr, Hyder F, Horvath TL, Gallo V (2014) Intranasal epidermal growth factor treatment rescues neonatal brain injury. *Nature* 506:230–234. [CrossRef Medline](#)
- Schüller U, Heine VM, Mao J, Kho AT, Dillon AK, Han YG, Huillard E, Sun T, Ligon AH, Qian Y, Ma Q, Alvarez-Buylla A, McMahon AP, Rowitch DH, Ligon KL (2008) Acquisition of granule neuron precursor identity is a critical determinant of progenitor cell competence to form Shh-induced medulloblastoma. *Cancer Cell* 14:123–134. [CrossRef Medline](#)
- Sharp FR, Bernaudin M (2004) HIF1 and oxygen sensing in the brain. *Nat Rev Neurosci* 5:437–448. [CrossRef Medline](#)
- Southwood CM, Garbern J, Jiang W, Gow A (2002) The unfolded protein response modulates disease severity in Pelizaeus-Merzbacher disease. *Neuron* 36:585–596. [CrossRef Medline](#)
- Taniuchi S, Miyake M, Tsugawa K, Oyadomari M, Oyadomari S (2016) Integrated stress response of vertebrates is regulated by four eIF2 α kinases. *Sci Rep* 6:32886. [CrossRef Medline](#)
- van den Beucken T, Koritzinsky M, Wouters BG (2006) Translational control of gene expression during hypoxia. *Cancer Biol Ther* 5:749–755. [CrossRef Medline](#)
- Volpe JJ (2001) Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* 50:553–562. [CrossRef Medline](#)
- Volpe JJ (2009) Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol* 8:110–124. [CrossRef Medline](#)
- Way SW, Popko B (2016) Harnessing the integrated stress response for the treatment of multiple sclerosis. *Lancet Neurol* 15:434–443. [CrossRef Medline](#)
- Way SW, Podojil JR, Clayton BL, Zaremba A, Collins TL, Kunjamra RB, Robinson AP, Brugarolas P, Miller RH, Miller SD, Popko B (2015) Pharmaceutical integrated stress response enhancement protects oligodendrocytes and provides a potential multiple sclerosis therapeutic. *Nat Commun* 6:6532. [CrossRef Medline](#)
- Wilson-Costello D, Friedman H, Minich N, Fanaroff AA, Hack M (2005) Improved survival rates with increased neurodevelopmental disability for extremely low birth weight infants in the 1990s. *Pediatrics* 115:997–1003. [CrossRef Medline](#)
- Wouters BG, van den Beucken T, Magagnin MG, Koritzinsky M, Fels D, Koumenis C (2005) Control of the hypoxic response through regulation of mRNA translation. *Semin Cell Dev Biol* 16:487–501. [CrossRef Medline](#)
- Yuen TJ, Silbereis JC, Griveau A, Chang SM, Daneman R, Fancy SP, Zahed H, Maltepe E, Rowitch DH (2014) Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. *Cell* 158:383–396. [CrossRef Medline](#)
- Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR (2002) The PERK eukaryotic initiation factor 2 α kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* 22:3864–3874. [CrossRef Medline](#)
- Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 12:982–995. [CrossRef Medline](#)