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# miR-211 is a pro-survival micro-RNA that regulates *chop* expression in a PERK-dependent manner

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## Abstract

Micro-RNAs typically function at the level of post-transcriptional gene silencing within the cytoplasm; however increasing evidence suggests that they may also function in nuclear, Argonaut containing complexes, to directly repress target gene transcription. We have investigated the role of micro-RNAs in mediating endoplasmic reticulum (ER) stress responses. ER stress triggers the activation of three signaling molecules: Ire-1α/β, PERK and ATF6 whose function is to facilitate adaption to the ensuing stress. We demonstrate that PERK induces miR-211, which in turn attenuates stress-dependent expression of the pro-apoptotic transcription factor *chop/gadd153*. MiR-211 directly targets the proximal *chop/gadd153* promoter where it increases histone methylation and represses *chop* expression. Maximal *chop* accumulation ultimately correlates with miR-211 down regulation. Our data suggests a model where PERK-dependent miR-211 induction prevents premature *chop* accumulation and thereby provides a window of opportunity for the cell to re-establish homeostasis prior to apoptotic commitment.

### Keywords

miR-211; histone methylation; PERK; CHOP



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## Introduction

Folding and maturation of all secretory pathway proteins occurs in the endoplasmic reticulum (ER). Perturbation of protein biogenesis within the ER initiates a stress response termed the Unfolded Protein Response (UPR). The UPR is an adaptive response that mediates expression of genes whose protein products function to counter the accumulation of misfolded proteins in the ER; enigmatically, the UPR is also characterized by the transcriptional induction of the pro-apoptotic transcription factor CHOP/GADD153 (C/EBP homologous protein/Growth Arrest and DNA Damage 153)) raising questions regarding how survival and death are balanced.

Mammalian cells contain three ER transmembrane proteins that function as proximal effectors of the UPR. Ire1α/β isoforms are composed of a luminal domain that senses stress, a single transmembrane domain, and a cytosolic tail that contains both a protein kinase domain and a RNase domain (Tirasophon et al., 1998; Yoshida et al., 1998). Ire1 isoforms trigger increased expression of numerous ER chaperones through activation of the X-box binding protein 1 (Xbp1) transcription factor. PERK, another ER transmembrane protein kinase activated in a manner analogous to Ire1 (Liu et al., 2000), catalyzes serine 51 phosphorylation of eIF2α resulting in the suppression of protein synthesis (Harding et al., 1999; Shi et al., 1998). The third signaling component is ATF6α/β, transcription factors that contribute to the transcriptional induction of ER chaperones (Haze et al., 1999; Li et al., 2000; Yoshida et al., 2000).

Among these UPR signal transducers, PERK functions as a key mediator of tumor and normal cell survival in response to stress stimuli (Bi et al., 2005; Bobrovnikova-Marjon et al., 2010; Gupta et al., 2009; Harding et al., 2000b; Zhang et al., 2002). Key to the pro-survival activity of PERK is its function as a regulator of protein translation. While PERK-dependent phosphorylation of eIF2α reduces the synthesis of a majority of cellular proteins, the translation of mRNAs containing small upstream open reading frames (uORFs) within their 5'UTR is selectively increased (Harding et al., 2000a; Miller and Hinnebusch, 1990). The ATF4 transcription factor is an example of the latter and is a key mediator PERK-dependent pro-survival functions (Blais et al., 2004; Harding et al., 2000a; Lu et al., 2004; Vattem and Wek, 2004). ATF4 signaling promotes two divergent responses: an increase in the transcription of both pro-survival and pro-apoptotic genes. ATF4 functions as an essential regulator of many genes involved in redox homeostasis, demonstrating a protective role for ATF4 signaling (Harding et al., 2003). However, ATF4 also promotes the expression of the pro-apoptotic transcription factor, *chop* (Fawcett et al., 1999; Harding et al., 2000a; Ma et al., 2002). *Chop*-deficient cells are modestly resistant to ER stress-inducing agents compared to their wild type counterparts (Zinszner et al., 1998), while enforced *chop* expression sensitizes cells to ER stress (McCullough et al., 2001).

How PERK-dependent pro-survival signals are balanced with the induction of *chop* remains poorly understood. We now provide evidence that a PERK-ATF4-dependent micro-RNA, miR-211, mediates temporal accumulation of *chop* and thereby functions as a key regulator of PERK-dependent pro-survival signaling.

## Results

### Identification of ER stress responsive miRNAs

Because PERK elicits its primary cell survival effects via translational regulation, we conducted a micro-array to identify miRNAs whose induction was PERK-dependent following exposure of cells to ER stress. For this experiment, we utilized tunicamycin, an inhibitor of N-linked glycosylation, as it is a relatively specific and rapid UPR inducer.

Embryonic fibroblasts derived from mice harboring PERK alleles flanked by loxP sites, permitting excision of PERK via expression of the Cre recombinase, were used. Following PERK excision with retroviral Cre (Fig S1A), cells were treated with tunicamycin, for 0–2 hours and RNA was analyzed by micro-array using Exiqon Chip. We reasoned that 2h of stress would facilitate the identification of proximal PERK-dependent targets while miRNAs induced at later time-points might reflect a more generalized cell adaptive response rather than acute PERK signaling. We observed a significant increase in the expression of seven miRNAs and a downregulation of an additional ten miRNAs (Fig 1A; Supplementary tables 1 and 2; >1.5 fold). Of these, miR-211 expression was the most robustly induced, with no apparent induction evident in PERK−/− MEFs (Fig 1A–B). To corroborate this, wild type or PERK−/− MEFs were exposed to thapsigargin (ER calcium ATPase inhibitor) or cultured in media without glucose for various intervals and expression of miR-211 was assessed by quantitative RT-PCR (Q-PCR). Accumulation of miR-211 was evident in PERK wild type cells while no induction was apparent in PERK−/− cells (Fig 1B). We also noted PERK-dependent induction of both CHOP mRNA and protein (Fig 1C; S1B).

Mir-211 is embedded in intron 2 of the *trpm1* calcium channel gene, suggesting that it might be expressed coordinately with the host gene. Indeed both *trpm1* and pri-211 expression were induced following ER stress (Fig 1D–E). Conversely, we failed to detect promoter-like activity when 1000bp of intronic sequence was cloned upstream of a luciferase reporter gene (data not shown) consistent with miR-211 expression as an intrinsic feature of the *trpm1* parent transcript. MiR-204, which is embedded within intronic sequence in the related calcium channel *trpm3* and contains the same seed sequence as miR-211 was also strongly induced by ER stress in a PERK-dependent manner (Fig S1D). MiR-449 expression was down regulated in PERKdependent manner demonstrating that miRNA induction is not a general feature of ER stress signaling (Fig S1C).

## PERK induces miR-211 through eIF2α phosphorylation and ATF4 induction

While PERK-dependent phosphorylation of eIF2α inhibits translation of many proteins, it paradoxically increases translation of ATF4 (Harding et al., 2003), which ultimately contributes to the transcriptional activation of key stress responsive genes. To address whether miR-211 regulation is eIF2α− or ATF4-dependent, we utilized MEFs isolated from *eIF2S51A* knockin embryos (Scheuner et al., 2001) or from *atf4*−/− embryos (Harding et al., 2003). MiR-211 induction was absent in both genetic backgrounds, but was induced in wild type MEFs (Fig 2A). To address ATF4 sufficiency, either ATF4 or a second PERK-responsive transcription factor, Nrf2 (Cullinan et al., 2003), was overexpressed in mouse fibroblasts. ATF4 but not Nrf2 induced miR-211 expression (Fig 2B). As expected, overexpression of ATF4 also induced *chop* (a direct transcriptional target of ATF4) expression in same samples (Fig 2C). Thus, ATF4 induction is necessary and sufficient for miR-211 expression.

## MiR-211 regulates *chop* accumulation

To address miR-211 function, we generated a luciferase reporter gene containing a complement to the miR-211 seed sequence in its 3'UTR. Thapsigargin treatment dramatically reduced luciferase expression in wild type MEFs (Fig 3A; 211-Luc). Repression of this reporter was significantly attenuated in PERK−/− MEFs and no repression was detected in wild type MEFs expressing luciferase reporter lacking a miR-211 responsive element (Fig 3A, first 4 columns).

To identify miR-211 targets, we searched the 3'UTR region of annotated cDNAs for the presence of matches to the miR-211 seed sequence. We also examined proximal promoter regions for potential miR-211 matches, given accumulating evidence for the involvement of

miRNAs in transcriptional repression (Gonzalez et al., 2008). While a large number of potential targets were identified, the relevance of most putative targets to ER stress signaling was not immediately apparent, as they were not differentially regulated by ER stress (negative data not shown). However, one target containing two potential miR-211 sites in the proximal promoter and a third low relevance site in its 3'UTR was of particular interest, *gadd153/chop* (Excel Table S3; Ddit3=chop).

To test a potential relationship between *chop* and miR-211, we transfected NIH3T3 cells with an antagomir designed to oppose miR-211 (A211) and subsequently challenged cells with thapsigargin. Exposure of cells expressing A211 to thapsigargin resulted in increased kinetics of CHOP accumulation relative to those expressing a scrambled control (Fig 3B). Conversely, expression of miR-211 resulted in a significant decrease in CHOP accumulation following exposure of cells to ER stress (Fig 3C). Because the potential high relevance regulatory sequences are located in the promoter, we determined whether miR-211 could regulate *chop* mRNA accumulation. Introduction of the A211 significantly increased *chop* mRNA accumulation following exposure of cells to ER stress (Fig 3D) while expression of the miR-211 completely ablated ER stress-dependent *chop* expression (Fig 3E). These results reveal a relationship between miR-211 expression and CHOP accumulation following ER stress.

## MiR-211-dependent silencing of *chop*

While the above results suggest that miR-211 can regulate *chop* expression during an ER stress response, they do not address whether regulation is direct. Given the strong affect on *chop* mRNA accumulation by both the antagomir or miR-211 expression along with the absence of high relevance seed matches for miR-211 in the 3'UTR of *chop*, we anticipated that translational regulation through the 3'UTR might not contribute significantly. Indeed, a luciferase reporter fused with the 3'UTR of *chop* failed to respond to thapsigargin or miR-211 (Fig S2A–B). Therefore, we focused on potential nuclear regulation of *chop* by miR-211. If miR-211-dependent regulation of *chop* is direct, we reasoned that we should be able to detect miRNA-promoter RNA complexes. NIH3T3 cells transfected with biotin-fused miR-211 or a scramble control were subjected to precipitation using streptavidin beads. Primers spanning regions from −619 to −503 or −34 to −135 were used to quantify *chop* promoter (RNA) levels in the pull-down. *Chop* RNA was enriched in biotin-211 precipitations relative to mutant 211 (Fig 4A). Equivalent amounts of biotin labeled mut-211 and miR-211 were pulled down as determined by Q-PCR of precipitates (Fig 4A). We surmised that if miR-211 is pulling down promoter RNA, it should be able to precipitate nascent *chop* RNA as well. To evaluate this hypothesis, we designed primers, which either span the first (nucleotide 118 of exon 1 to nucleotide 2820 of the adjacent) or second exon-intron (nucleotide 46 of exon 2 to 1561 of the adjacent intron) junctions. In unstressed cells, no nascent transcript was detected using either primer set (data not shown). In contrast, following ER stress, RNA spanning the first exon-intron junction of *chop* nascent transcript was precipitated (Fig 4B).

The proximal *chop* promoter has one perfect miR-211 seed (site 2) and a second (site 1) with two mis-matches (Fig S2C). Critically, these sites are conserved in *chop* sequences from multiple species (human, mouse, hamster). To evaluate the potential role of these sequences, we utilized a luciferase reporter, wherein the hamster *chop* promoter region drives expression of the luciferase reporter gene; this hamster *chop*-reporter has been widely used to dissect regulatory elements in the *chop* promoter (Fawcett et al., 1999; Luethy et al., 1990; Ma et al., 2002; Yoshida et al., 2000). Co-transfection of miR-211 reduced *chop*-luciferase ~6-fold but had no effect on the luciferase lacking the *chop* promoter (Fig 4C). To assess the role of the putative 211 seed sites, we generated constructs where site 1 or site 2 was deleted. Deletion of either site abolished miR-211-dependent repression suggesting

direct regulation (Fig 4C). Importantly, mutant luciferase constructs showed similar expression as that of wild type indicating that seed matches do not fall within key regulatory regions.

We next determined the impact of mutation of these sequences on ER stress-dependent *chop* expression. Consistent with site 1 and 2 mediating expression, disruption of site 1 increased stress-dependent expression 2-fold while mutation of site 2 increased expression nearly 7-fold (Fig 4D). To further address whether regulation is direct, we generated a reporter wherein either miR-211 site 1 or 2 was replaced with seed matches for let-7, which like miR-211 has nuclear functions (Benhamed et al., 2012). Consistent with direct regulation, co-expression of let-7 repressed *chop* expression in a site-specific manner (Fig 4E). As an independent measure, we also generated a *chop* reporter construct wherein site 1 or site 2 was replaced with the sequence matching the scrambled sequence of our miR-211 control. The scrambled miR-211 repressed *chop*-luciferase expression in a site-specific manner (Fig S2D).

## MiR-211 induces chromatin-modifications at the *chop* locus

MiRNA-dependent transcriptional silencing in mammalian cells has been associated with stalled RNA polymerase elongation (Kim et al., 2006), increased trimethylation of Lys27 of Histone 3 (H3K27me3; Kim et al., 2006), and Argonaut recruitment to the promoter (Kim et al., 2006; Verdel et al., 2004). We therefore evaluated the effect of miR-211 on each of these processes by chromatin immunoprecipitation, ChIP. Expression of A211 increased RNA polymerase occupancy following exposure of cells to ER stress for either 5 or 8 hours (Fig 5A) relative to scramble control. In contrast, expression of the miR-211 suppressed RNA polymerase occupancy by ~4 fold compared to scrambled RNA and ~6 fold compared to A211 after 8 hours of ER stress (Fig 5A). The absence of significant suppression at 5 hours by miR-211 presumably reflects low RNA polymerase occupancy at that time point.

We next determined whether ER stress or miR-211 expression was associated with increased deposition of the heterochromatin mark, H3K27me3, as this modification is induced by other miRNAs that have nuclear functions (Kim et al., 2006). Transfection of miR-211 enhanced thapsigargin-induced H3K27me3 at the *chop* promoter (Fig 5B). Conversely, H3K27me3 deposition at the *chop* promoter in response to ER stress was suppressed by A211 (Fig 5B). In contrast, no specific increase in H3K9me2 at the *chop* promoter was detected following introduction of miR-211 (Fig S3). The H3K27 methylation of the *chop* promoter is specific as no such modification was induced by miR-211 at the promoters of neighboring genes, *dctn2* or *gli1*, which lack potential miR-211 interacting sites (Fig 5C).

Finally, we assessed whether Argonaut proteins were recruited to the *chop* promoter by ChIP analysis. Because suitable antisera could not be identified for endogenous Ago proteins, HeLa cells were transfected with myc-tagged Ago1 along with either miR-211 or A211. A 3-fold increase in Ago1 recruitment to the *chop* promoter was observed following induction of miR-211. Conversely, we observed a 2-fold suppression of Ago1 recruitment in A211 transfected cells (Fig 5D). We were unable to detect Ago1 at neighboring promoters such as *dctn2* or *gli1* (negative data not shown). Collectively, these data are consistent with a direct mode of miR-211-dependent regulation of *chop* expression.

## Suppression of miR-211 regulates ER-stress-dependent apoptosis

If *chop* is a significant and primary target of miR-211, then antagonizing its function should be associated not only with increased CHOP accumulation, but also with accelerated cell death following exposure of cells to ER stressing agents. Indeed expression of the miR-211

antagomir (A211) resulted in accelerated accumulation of cleaved caspase 3 (Fig 6A). Consistent with increased caspase 3 cleavage cells expressing A211 exhibited an increase in the kinetics of cell death (Fig 6B). To evaluate whether *chop* is a significant target we compared the sensitivity of wild type versus *chop*−/− MEFs to A211 expression and low dose thapsigargin treatment. A211 transfected wild type cells exhibited accelerated kinetics of caspase activation and cell death compared to scramble RNA-transfected cells (Fig 6C–D). In contrast, *chop*−/− cells were refractory to the effects of A211 expression with or without thapsigargin consistent with *chop* being a primary target in cells exposed to ER stress.

### MiR-211 is overexpressed in mammary carcinoma in a PERK-dependent manner

Recent work has demonstrated that PERK provides critical pro-survival signals that permit tumor cells to evade a hostile microenvironment. Because miR-211 functions to mediate PERK-dependent cell survival in cultured cells, we reasoned that miR-211 accumulation would likely be apparent in primary tumors. We focused on mammary tumors given that PERK potentiates mammary tumorigenesis (Bobrovnikova-Marjon et al., 2010). We assessed miR-211 levels in a panel of nine tumors isolated from mice wherein mammary carcinoma was driven by MMTV-D1T286A (Lin et al., 2008). This analysis revealed accumulation of miR-211 in tumor tissue relative to normal mammary epithelium (Fig 7A). Because miR-211 regulates *chop* accumulation, we expected an inverse correlation between miR-211 and *chop* expression. In tumors 149, 179, 266, 1846, 1886 and 355, which exhibited the highest expression of miR-211, we noted a significant inverse correlation with *chop* expression that reached significance (Fig 7B; p=0.03; pearson coefficient=-0.71). In the remaining three tumors, (197, 1848, 1967) miR-211 and *chop* expression were low. We also assessed miR-211 expression in a panel of MMTV-Neu tumors that were derived in mice with either wild type PERK or from mice where PERK was excised from mammary epithelium with Cre recombinase (Bobrovnikova-Marjon et al., 2010). In this cohort, miR-211 expression was significantly reduced in tumors that developed in the absence of PERK, implicating PERK in the induction of miR-211 during tumorigenesis (Fig 7C). We also noted that expression of miR-211 was reduced in MMTV-Neu tumors relative to MMTV-D1T286A tumors (data not shown) suggesting cell intrinsic effects of a particular oncogene on miR-211 expression. These data provide evidence for PERK-dependent regulation of miR-211 in vivo and support the antagonistic relationship between miR-211 and *chop*. To determine whether increased expression of miR-211 to murine tumor models, we analyzed miR expression in 5 available primary human lymphomas and two independent isolates of normal B-lymphocytes. All tumors had higher miR-211 expression than normal tissue (Fig 7D). We were unable to assess *chop* expression in the latter due to insufficient material. Collectively, these data reveal increased expression of miR-211 in both mammary tumors and B-cell lymphoma. Importantly, expression of miR-211 in mammary tumors correlates with PERK expression and reduced *chop* expression.

## Discussion

MiRNAs contribute to the regulation of cellular regulatory processes via their capacity to alter gene expression at both post-transcriptional and transcriptional levels. We have interrogated the contribution of miRNAs to the cellular response to ER stress. In this work, we have identified miR-211, and the related miR-204, as miRNAs that respond to ER stress in a PERK-dependent fashion. While PERK activation is associated with a generalized inhibition of protein translation (Harding et al., 1999; Shi et al., 1998; Sood et al., 2000), it also triggers activation of two transcriptional programs: one directed by ATF4, which is translated more efficiently following phosphorylation of eIF2α (Harding et al., 2003) and the second directed by Nrf2, a direct PERK substrate (Cullinan et al., 2003). Our analysis

revealed a requisite role for only eIF2α and ATF4. With regard to miR-211 function, bio-informatic and biochemical approaches revealed a multitude of potential mRNA targets for miR-211-containing RISC complexes; however, we were unable to confirm that a majority of these putative targets were responsive to ER stress and thus were likely not relevant to UPR signaling. The one relevant target revealed by our analysis is the pro-apoptotic transcription factor *chop*. Based upon its antagonistic relationship with *chop*, we expected that miR-211 function should be pro-survival. Indeed expression of a miR-211 antagomir sensitized cells to ER-stress induced cell death. This was associated with increased *chop* expression and was exquisitely dependent upon the presence of CHOP as *chop*−/− cells were refractory to antagomir expression. Thus, our results identify a PERK-responsive micro-RNA that elicits pro-survival influence by antagonizing pro-apoptotic *chop* expression.

The contribution of miRNAs to signaling is apparent in most biological processes. They contribute to various aspects of development, stem cell biology and tumor progression as well as tumor suppression (Garzon et al., 2009). MiRNAs typically function at the level of posttranscriptional gene silencing within the cytoplasm. MiRNAs are processed from stem-loop structures, resulting in 22 nucleotide small interfering RNAs (siRNA) which then direct an effector complex known as RISC to target mRNAs through seed sequence based, base-pairing (Garzon et al., 2009). RISC contains additional proteins including an Argonaut family member, which will either degrade the target mRNA or inhibit its translation.

More recently, miRNAs have been demonstrated to exhibit nuclear functions where they regulate gene expression directly via chromatin modification; RNAi-induced transcriptional silencing or RITS (Verdel et al., 2004). Here again, specificity is determined by seed sequences within the miRNA itself (Gu et al., 2012). Like cytoplasmic RISC, RITS formation requires Dicer-dependent generation of the 22nt siRNAs that are loaded into Ago1 or Ago2 containing complexes (Janowski et al., 2006; Kim et al., 2006). RITS depends upon miRNA targeting proximal transcripts that initiate upstream of primary start sites; binding and transcriptional silencing may also be mediated by the accumulation of histone modifications that reduce gene transcription (Noma et al., 2004; Pal-Bhadra et al., 2004; Verdel et al., 2004). Within the context of this nuclear function, small RNA containing complexes direct histone-modifying enzymes such as Ezh2 in mammalian cells (Kim et al., 2006) and Clr4 in fission yeast (Motamedi et al., 2004; Sugiyama et al., 2005), to chromatin which, in turn, generates heterochromatic chromatin marks such as H3K27me3 to effect gene silencing.

Regarding the regulation of *chop*, miR-211 interacts directly with the *chop* promoter and *chop* nascent transcript. MiR-211 also directs Ago1 to the *chop* promoter, triggering an enrichment of polymerase II, consistent with induced polymerase stalling. Induction of miR-211 is also associated with an increased H3K27 tri-methylation at the *chop* promoter, but not at the promoters of adjacent genes. In final support of a direct regulatory relationship, the *chop* promoter has two high relevance seed matches identified through an unbiased bioinformatics search. Mutation of either of these two sites increases stress dependent *chop* promoter activity and eliminates miR-211-dependent repression. Regulation can be restored through introduction of nucleotide bases that match either an artificial seed, based upon our scrambled miR-211 sequence or bona fide *let7*. Thus, our data support a model where miR-211 functions in the context of ER stress signaling to regulate post-transcriptional gene silencing via a chromatin-modification-dependent mechanism to moderate induction of pro-apoptotic CHOP and potentiate cell survival.

MicroRNAs are generally considered stable entities. However, miR-211 has evolved to respond quickly to environmental cues such as dark-light cycles (Krol et al., 2010). While

mechanisms that determine stability of specific miRNAs remain to be clearly defined, the stability of certain micro-RNAs can be influenced by GLD-2 (Burns et al., 2011; Katoh et al., 2009). GLD-2 is a regulatory poly(A) polymerase which catalyzes 3' adenylation of miR-122. In the absence of 3' adenylation, miR-122 exhibits decreased stability. The mechanisms that contribute to decreased miR-211 stability following ER stress remain to be determined. It should be noted, that in addition that *trpm1* mRNA is also unstable. Whether the two are linked and what determines the decline in their accumulation remains an issue of interest.

While the mechanism remains unclear, the importance of regulated miR-211 stability is perhaps more apparent. First, the transient accumulation of miR-211 is necessary for temporal regulation of *chop* expression. Maintenance of miR-211 would inhibit maximal accumulation of *chop* and in so doing prevent execution of damaged cells. Second, miR-211 has also been implicated in neoplastic growth via regulation of the CHD5 tumor suppressor (Cai et al., 2012). This recent observation of potential oncomir activity further emphasizes the importance of temporal regulation of miR-211.

Tumors frequently engage the UPR to facilitate growth and survival; not surprisingly both PERK and ATF4 have been demonstrated to contribute significant survival advantages to tumors (Avivar-Valderas et al., 2011; Bi et al., 2005; Bobrovnikova-Marjon et al., 2010; Ye et al.). Given the functional significance of miR-211 to PERK-dependent pro-survival signaling and additional published data implicating miR-211 in the regulation of CHD5 (Cai et al., 2012), it seems logical that tumors might exhibit increased expression of miR-211. Indeed, we observed elevated expression of miR-211 in two mouse models of mammary carcinoma. Furthermore, in Neu-driven tumors, expression of miR-211 was found to be PERK-dependent emphasizing a direct regulatory relationship. Importantly, expression of miR-211 was not restricted to murine mammary tumors as we also observed elevated expression in human B-cell lymphoma. In the former, elevated miR-211 levels inversely correlated with *chop* consistent with miR-211 antagonizing *chop* expression.

Collectively, our data suggest a model wherein miR-211 functions as a rheostat that mediates the temporal accumulation of the pro-apoptotic transcription factor CHOP. While accumulation of CHOP is dependent upon PERK-dependent increases in the ATF4 transcription factor, which in turn directly regulates *chop* expression (Harding et al., 2003; Ma et al., 2002), PERK is simultaneously inducing a miRNA (miR-211) that functions to attenuate *chop* expression at early points during the ER stress response. Thus, while PERK mediates cell survival, it is also setting the stage for cellular demise by establishing the temporal onset of CHOP accumulation. MiR-211/204 is in turn silenced if stress is sustained permitting maximal CHOP accumulation upon prolonged stress.

## Experimental procedures

### Cell culture

All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), β-mercapotethanol, and nonessential amino acids (Hamanaka et al., 2005; Vaites et al., 2011).

### MicroRNA microarray and Q-PCR

PERK+/+ and PERK−/− Mouse Embryonic Fibroblasts were treated with either tunicamycin (2μg/mL) or DMSO for 2 hrs and total RNA was purified using Ambion (Austin TX) micro-RNA purification kit mirVANA. Microarray was carried out using mouse Exiqon micro-RNA microarray chip (Vedbaek, Denmark). The quality of small RNA was determined using Agilent bioanalyzer (Santa Clara, CA). Results were analyzed using GENESPRING

software. ANOVA and T-tests were used to calculate fold change and p-values. Spotfire (Somerville, MA) software was used to generate heat maps. For Q-PCR, total RNA was purified after appropriate treatments using Qiagen (Valencia, CA, USA) miRNeasy kit as per manufacture's instructions.

For Taqman Q-PCR, RNA was reverse transcribed using appropriate specific primers with microRNA reverse transcription kit (Applied Biosystems). Q-PCR was performed on an Applied Biosystems (Carlsbad, CA) 7900 Q-PCR machine. Pri-211 levels were determined using SYBR green (SuperArray, Frederick, MD). Primers used were, forward pri-211 5'-CTGCTTGGACCTGTGACCTGT-3' and reverse 5'-TCTGCAGTAGAGGTGACCA-3'. Human B-cell lymphoma and normal B-cells were procured from Penn human immunology core. Total RNA was purified using miRNeasy kit from Qiagen (Valencia, CA, USA). MicroRNA was reverse transcribed using TaqMan MicroRNA Reverse transcription kit with specific Taqman RT primers for hsa-miR-211 and U6 RNA (Applied Biosystems, MA) as per manufacturer's protocol. Q-PCR was performed with hsa-miR-211 and U6 Taqman primers using no AmpErase® UNG Master mix (Applied Biosystems, MA).

### Luciferase assays

Oligonucleotides containing two copies of the miR-211 seed sequences were cloned into the 3'UTR of the firefly luciferase gene in pRLTK. For luciferase assays, cells were transfected with pGL4.11 and pRLTK vector with Lipofectamine plus method and treated with thapsigargin (500nM) for 8 hrs. The hamster *chop* promoter was amplified by PCR and cloned in pGL4.11 using primers: Forward 5'CAAACAACAGGTACCACAGGAATTC TGGCGTGC 3'; Reverse 5'CAA AAACCTCGAGGACCGAAGTGTGAGACTC 3'. The mutant *chop* promoter miR-211-binding site mutant was generated using the QuikChange kit (Stratagene, Santa Clara, California). Mutagenic primers used were: Site 1 primer 1, 5'-CCTTTCATGGAGGAGTAAGTTTGGGAGGTGGG-3' primer 2, 5'-CCCACCTCCCAAACTTACTCCTCCATGAAAGG-3'; Site 2 primer 1, 5'-GAGGGGCCGGACAGCATTCGCTCTCC-3'; primer 2 5'-GGAGAGCGAATGCTGTCCGGCCCCTC 3'. Wild type or mutant *chop* promoter-reporter constructs were co-transfected with the pRLTK vector, which served as an internal control.

### Anti-miR-211 (A211) and miR-211 transfections and analysis

NIH3T3 cells were transfected with of miR-211 mimic or control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were treated with 500nM thapsigargin unless otherwise noted, 48h post-transfection, lysed in EBC buffer (50mM Tris pH 8.0; 120mM NaCl; 0.5% NP-40), resolved by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were then probed with the indicated antibodies.

### Chromatin immunoprecipitation assays and Biotin-211 chromatin precipitation

MiR-211 or mutant miR-211 was introduced into NIH3T3 cells. ChIP assays were performed 24 hrs post-transfection using Imagenex (Port Coquitlam, Canada) Quikchip kit as per manufacturer's protocol. Histone methylation was analyzed using H3K9me2 and H3K27me3 antibodies (1μg) (Abcam). RNA polymerase was analyzed using ChIP grade CTD repeat antibody (Abcam). QPCR was performed using SYBR green on Applied Biosystems (Carlsbad, CA) 7900.

To assess miR-211 promoter association, pull-down was performed as described previously (Orom and Lund, 2007) with some modifications. Biotin-labeled wild type miR-211 and scramble, mutant miR-211 were prepared: MicroRNA-211 5'-GCAAAGGAUGACAAAGGGGAAG-3'; 3'-UCCGUUUCCUACUGUUUCCCUU-5' Mutant MicroRNA-211 5'-GCAAAGGAUGACCCGCUGAAG-3' 3'-

UCCGUUUCCUACUGGGCGACGU-5'. NIH 3T3 cells were transfected with 100μM biotin labeled miR-211 or biotin-labeled scrambled miR-211 using lipofectamine 2000 for 36 hrs. Prior to fixation with 1% formaldehyde for 10 min at 37°C, cells were treated with thapsigargin (500nM) for 2 hours. Fixing was terminated by addition of Glycine and cells were washed with ice-cold PBS twice and pelted at 3000×g for 5 min. Cell pellets were resuspended in lysis buffer (20mM Tris; pH7.5, 200mM NaCl, 2.5mM MgCl2, 0.05% Igepal, 60U Superase-In/ml (Ambion), 1mM DTT, 1 × Pefabloc (Roche). Cells were sonicated for 10 min and pelleted at 12000×g for 15 min at 4°C. Input (50μl) was removed for later analysis. Samples were rotated with streptavidin-coated agarose beads at 4°C for 1 hr following which, beads were washed with lysis buffer and crosslinking was reversed with 5M NaCl at 65°C for 4 hrs. Trizol (200μl) was added to input as well as beads. Chloroform (40μl) was added and samples were centrifuged at 12000Xg for 15 min. Supernatant was removed and RNA was precipitated with 1μl Glycoblue, 6 μl 5M NaCl and 95% ethanol (360μl) for 1 hr. RNA was pelleted at 12000 g for 15 min, washed with 70% ethanol and dried. RNA was dissolved in 15μl water. Reverse transcription was carried out using Superscript II kit (Invitrogen, CA) as per manufacturer's protocol using promoter specific primers (2μM) covering the miR-211-specific site positions: Forward 5'-TCTGATTGGTAGGCTCCTGGAC-3'; Reverse 5'-TCAAGAGCGGAGAGCGAGTATCCTT-3'.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

- MiR-211 is a pro-survival micro-RNA
- MiR-211 is induced by ER stress in a PERK-dependent manner
- MiR-211 expression induces histone H3K27 methylation of the *chop* promoter
- MiR-211 represses *chop* expression and thereby mediates threshold CHOP accumulation

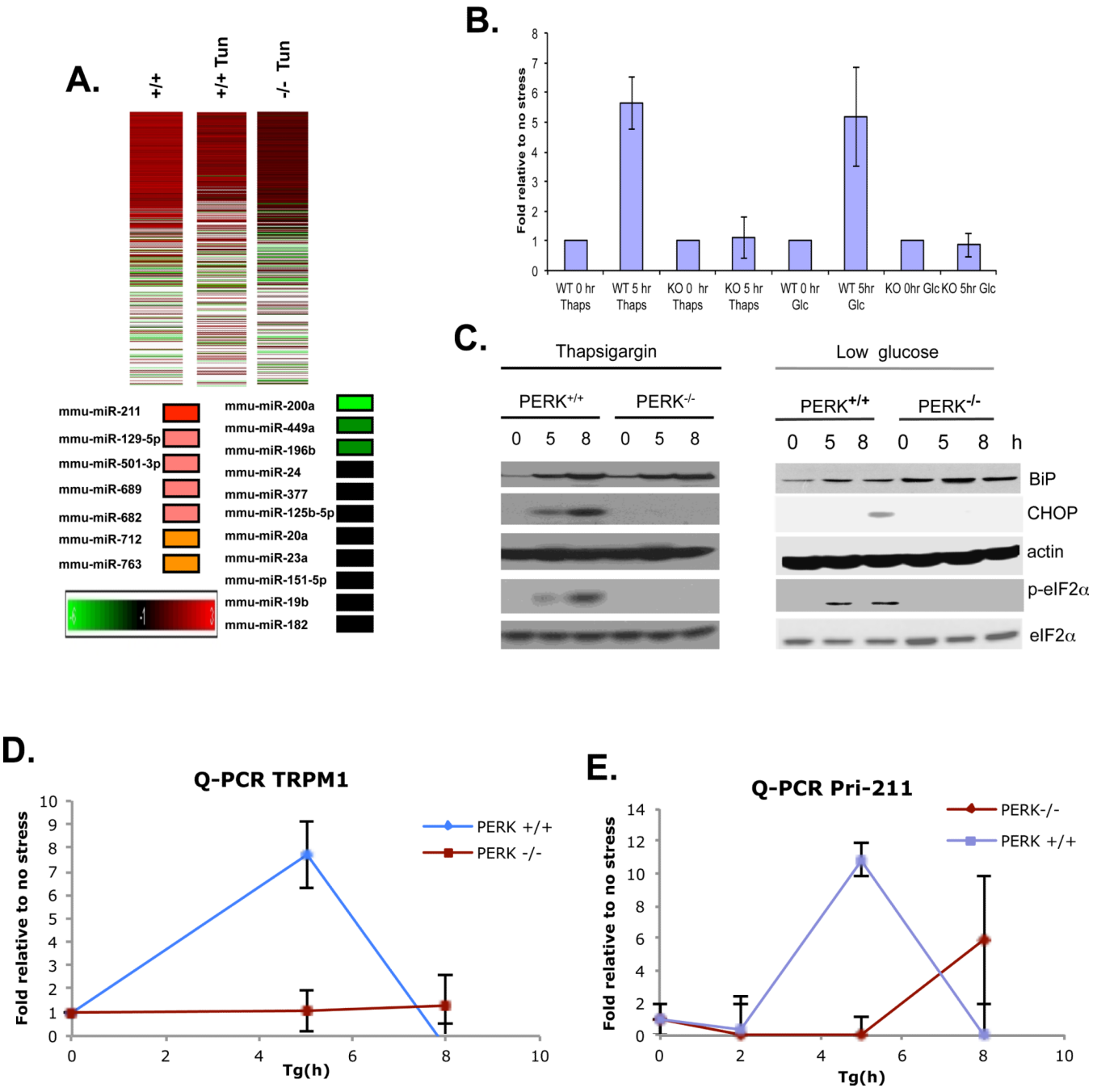


**Figure 1. PERK-dependent expression of miR-211**

**A)** Heat map representation of ER-stress dependent micro-RNA expression. **B)** Q-PCR Analysis of PERK+/+ (WT), and PERK−/− (KO) treated with thapsigargin (500nM) or starved for glucose. Values are the average of at least 3-independent experiments and error bars indicate standard deviation between experimental replicates. **C)** Induction of CHOP and eIF2α phosphorylation in response to thapsigargin and glucose starvation. **D–E)** Q-PCR analysis of *trpm1* or pri-211 in wild type or PERK−/− MEFs treated with thapsigargin (500nM) for the indicated intervals. Values are the average of at least 3-independent experiments and error bars indicate standard deviation between experimental replicates.

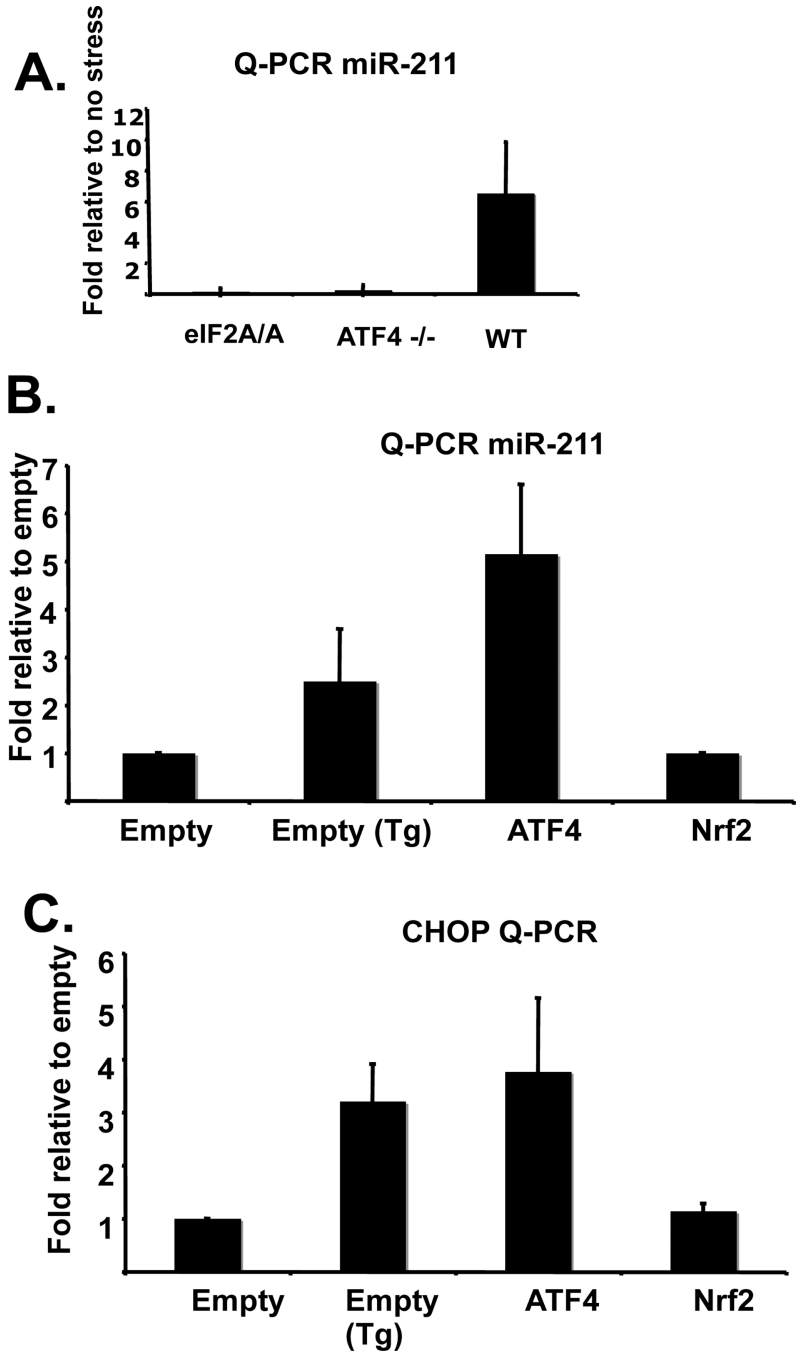


**Figure 2. MiR-211 induction depends on eIF2α phosphorylation and expression of ATF4**

**A)** Q-PCR analysis of *PERK*+/+ (WT), eIF2αA/A, and *ATF4−/−* (KO) MEFs treated with thapsigargin (500nM) for 5 hours. **B**) HeLa cells were either transfected with either empty, ATF4 or Nrf2 encoding vectors. Cells transfected with empty vector were treated with thapsigargin (500nM) as a positive control or not treated (negative control). After 24 hours, total RNA was isolated and miR-211 levels were assessed by Q-PCR. **C)** Cells in B were also assessed for *chop* expression by Q-PCR to confirm functional ATF4. All panels provide values that are the average of at least 3-independent experiments and error bars indicate standard deviation between experimental replicates.

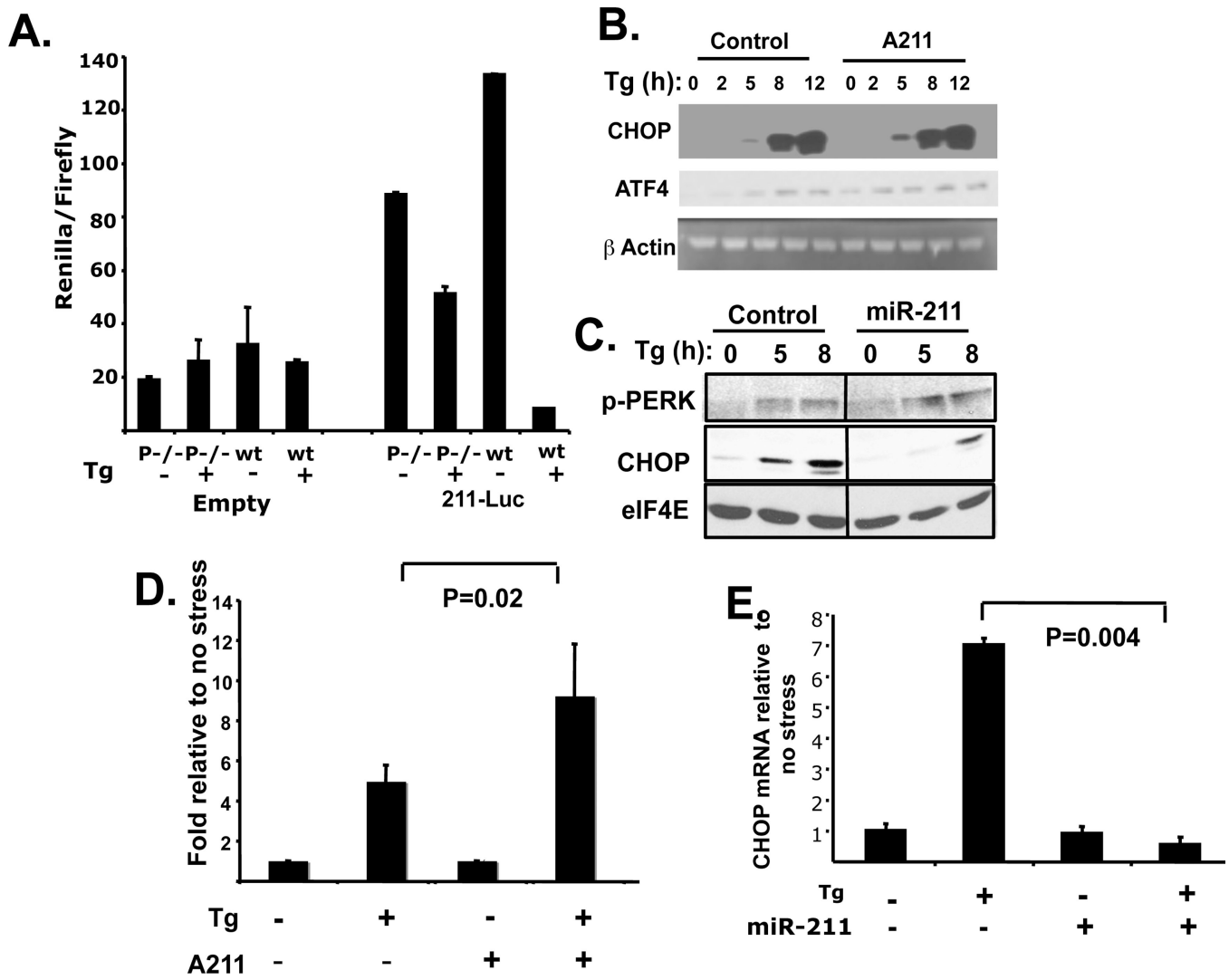


**Figure 3. MiR-211 regulates stress-dependent CHOP expression**

**A)** The recognition site of miR-211 was cloned at the 3'UTR of luciferase gene (211-Luc); empty vector or 211-Luc were expressed in PERK+/+ or −/− MEFs and treated with thapsigargin. Error bars represent standard deviation for 3 independent experiments. **B)** Scrambled (control) or A211 were introduced into NIH3T3 cells, treated with thapsigargin for the indicated intervals and levels of CHOP and ATF4 were assessed by western. **C)** Scramble (control) or a miR-211 were introduced into NIH3T3, treated with thapsigargin for the indicated intervals and levels of phospho-PERK, CHOP and eIF4E were assessed by immunoblot. **D)** Q-PCR analysis of *chop* mRNA in NIH3T3 cells transfected with A211 and challenged with thapsigargin. The difference between untransfected and A211 transfected cells challenged with thapsigargin is significant (P=0.023). **E)** Q-PCR assessment of *chop* mRNA in cells challenged with thapsigargin +/− miR-211. Values are the average of at least 3-independent experiments and error bars indicate standard deviation.

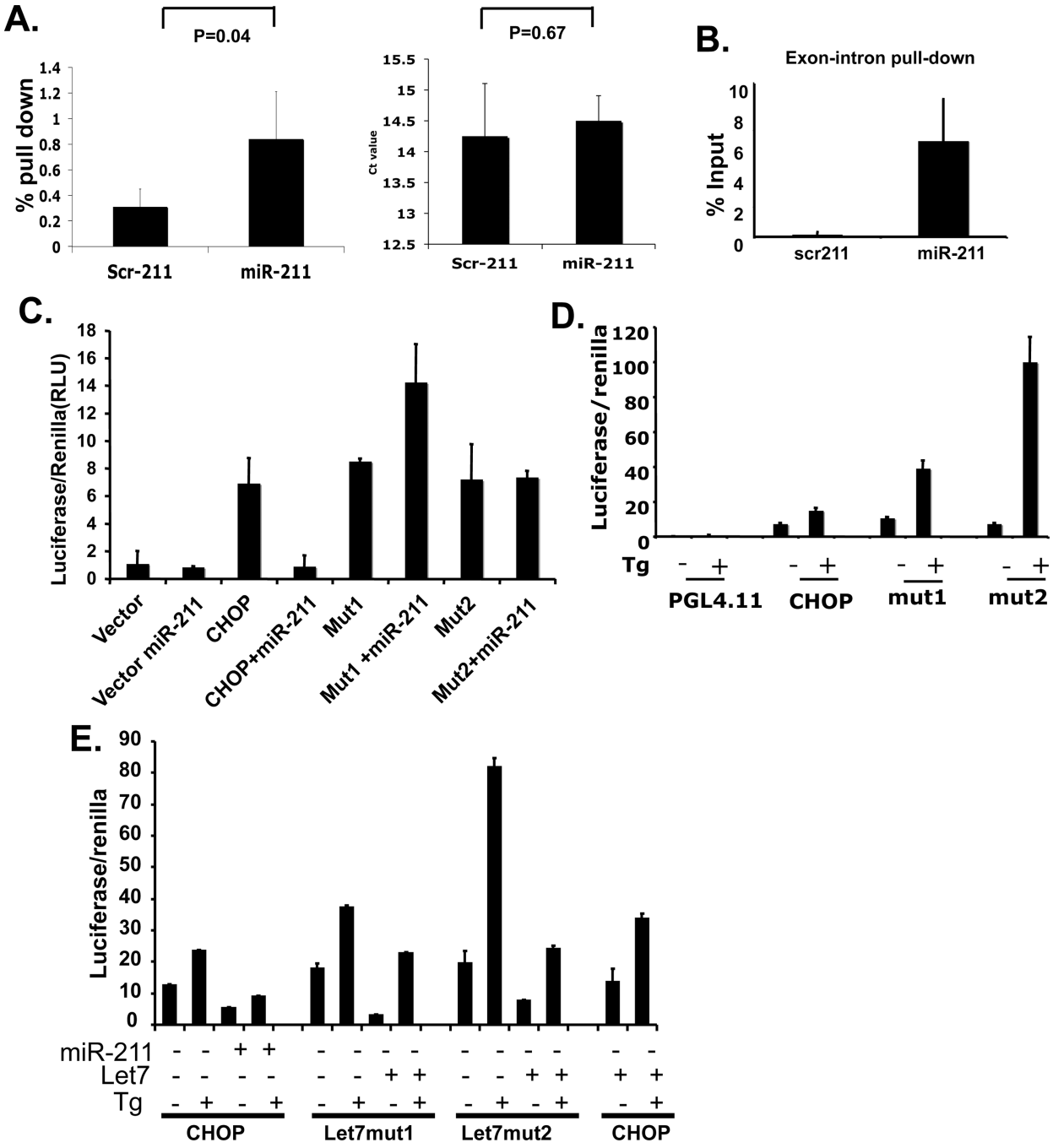


**Figure 4. MiR-211 regulates stress-dependent CHOP accumulation through binding sites in promoter**

**A)** MicroRNA-211 pulls down Chop promoter RNA. Biotinylated wild type miR-211 or control scramble were introduced into NIH3T3 cells (mut-211). Cells were processed essentially for chromatin IP, except that RNA:RNA duplexes were collected on streptavidin beads. Precipitation of *chop* with miR-211 was analyzed by Q-PCR using primers scanning region −619 to −503. (Right) Equivalent amounts of biotinylated microRNAs were present in precipitates. **B)** NIH3T3 cells transfected with biotinylated microRNAs were stressed for 8 hours and then precipitated with streptavidin beads. Precipitates were analyzed for presence of RNA spanning first exon-intron junction (118 exonic-2820 intronic). Data

represents the average of 3 experiments. **C)** MiR-211 regulates CHOP promoter activity through its recognition sites at nucleotide 121(site 1) and 141(site 2). NIH3T3 cells were transfected with either a promoter-less luciferase reporter, with the wild type or indicated mutant CHOP promoter (mut1=site1; mut2=site2). In addition, each transfected construct was supplemented with or without mimic miR-211. Error bars show values for 3 independent experiments. **D)** MiR-211 site deletion mutants in the CHOP promoter abrogates regulation by ER stress. Cells expressing the wild type *chop*-luc or the indicated mutants were challenged with thapsigargin for 8 hours. Values are the average of at least 3-independent experiments. **E)** NIH3T3 cells expressing *chop*-luc reporter constructs containing let-7 seed matches at either site 1 or 2 were challenged with thapsigargin following expression of a let-7 miR where indicated. The error bars provided in each panel indicate standard deviation.

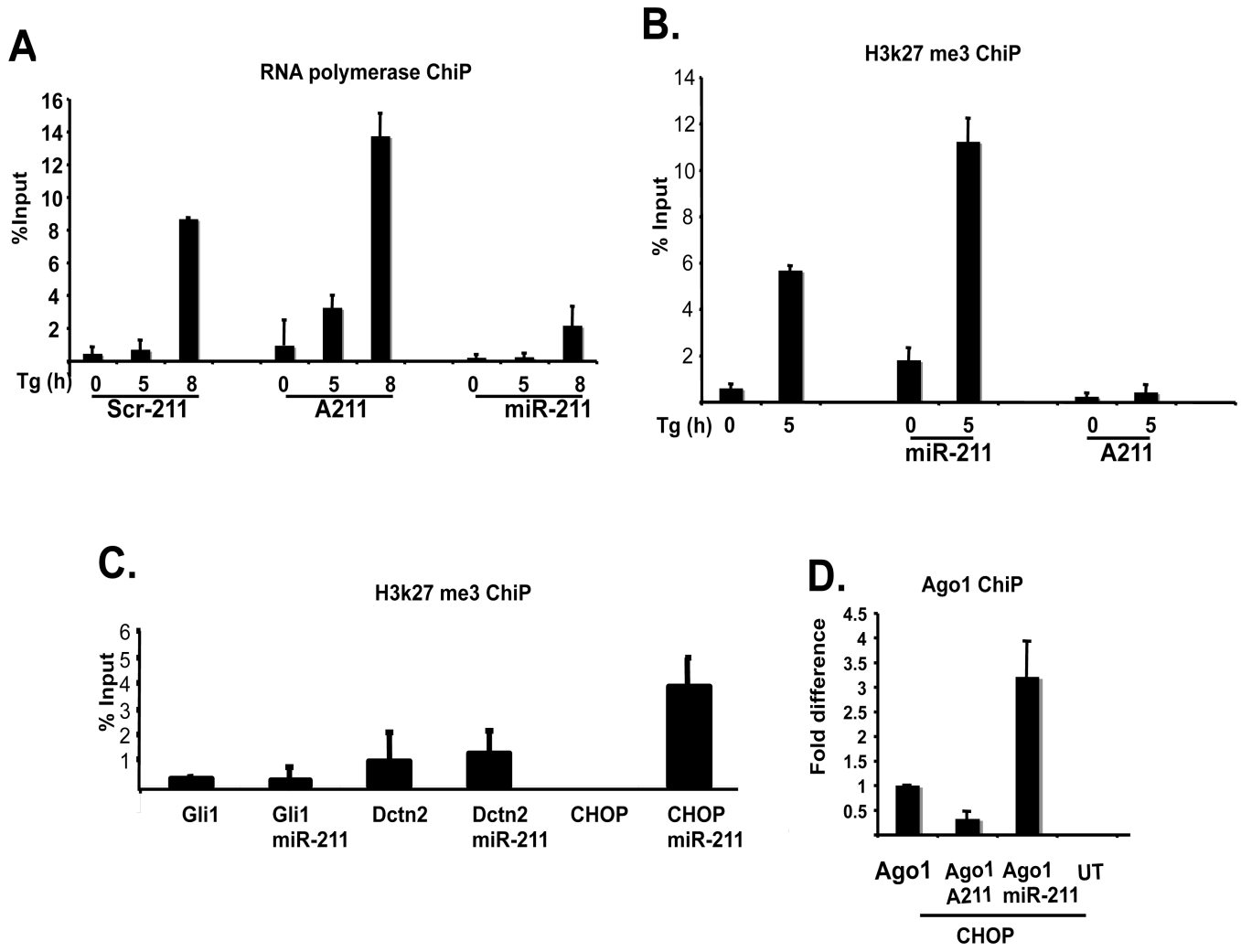


**Figure 5. MiR-211 induces histone modifications at the *chop* promoter**

**A)** MiR-211 or A211 were introduced into NIH3T3 cells after which cells were stressed with thapsigargin for indicated intervals. Subsequently, association of RNA polymerase with the *chop* promoter was assessed by ChIP. **B–C)** H3K27me3 ChIP was performed to assess modification of either the *chop* (B) or neighboring *Dctn2* and *Gl1* promoters (C) following 5 hours of thapsigargin treatment. **D)** To determine Ago1 occupancy on the *chop* promoter, myc-tagged Ago1 construct along with either miR-211 or A211 oligos were introduced into HeLa cells and processed for ChIP using myc-beads. Error bars reflect standard deviation between 3 independent experiments.

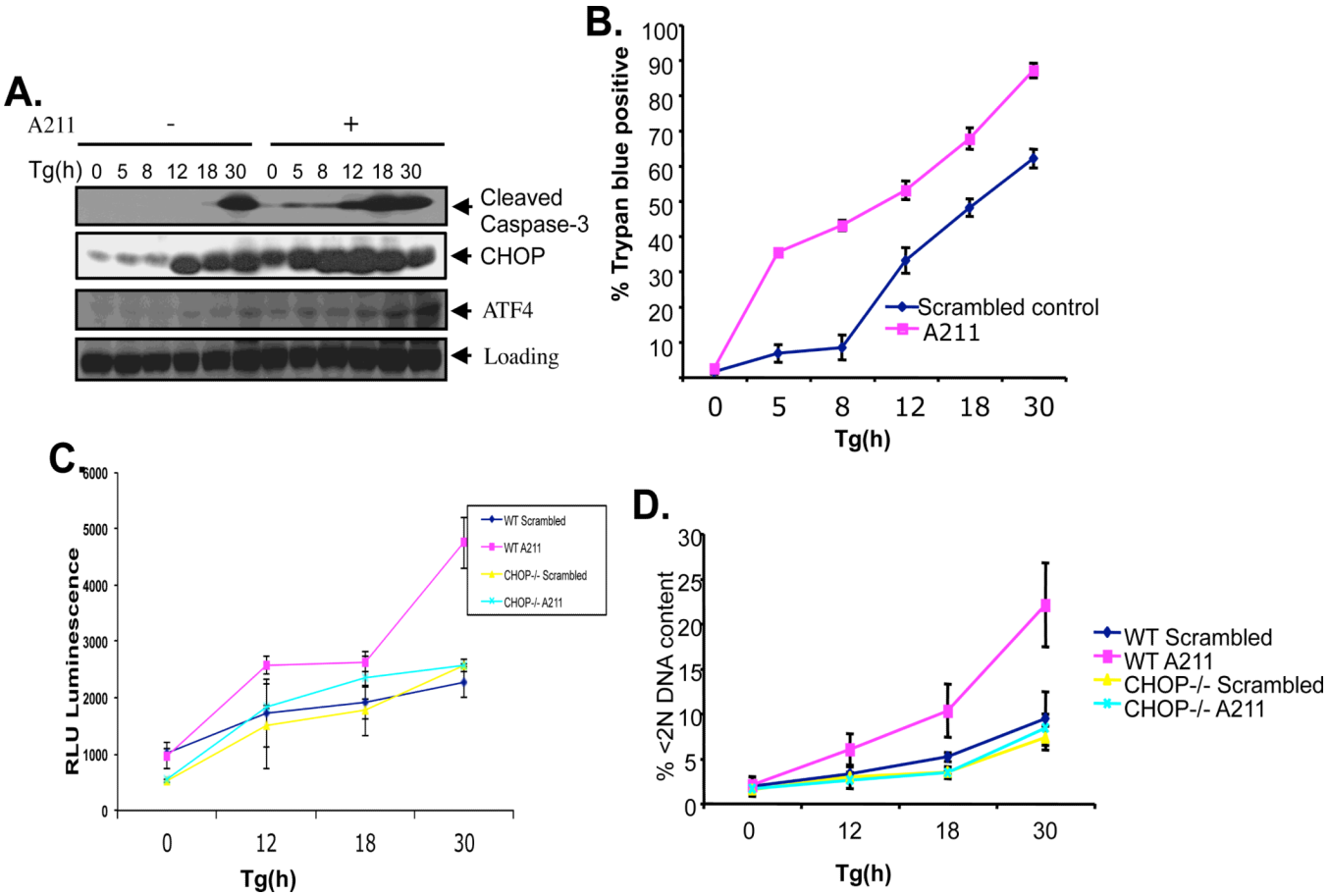


**Figure 6. Suppression of miR-211 accelerates apoptosis**

**A)** NIH3T3 cells were transfected with control (−) or A211(+) for 48 hours and then treated with thapsigargin at indicated time-points. Cell lysates were probed for cleaved caspase 3, CHOP, ATF4 as indicated. **B)** Viability of NIH3T3 cells transfected with A211 or scrambled control and treated with 500nM thapsigargin for the indicated intervals. Error bars indicate values for 3-independent experiments. **C)** Wild-type or *chop*−/− MEFs were transfected with either scrambled or A211for 48 hours and then treated with thapsigargin at indicated time-points. Caspase-Glo reagent was added and caspase activation was assessed by luminescence. **D)** Following transfection of wild type or *chop*−/− MEFs with scramble or A211 and exposure to 50nM thapsigargin, viability was assessed by propidium iodide staining and cells containing less than 2N DNA content were quantified by FACS. Error bars indicate standard deviation between 3 independent experiments.

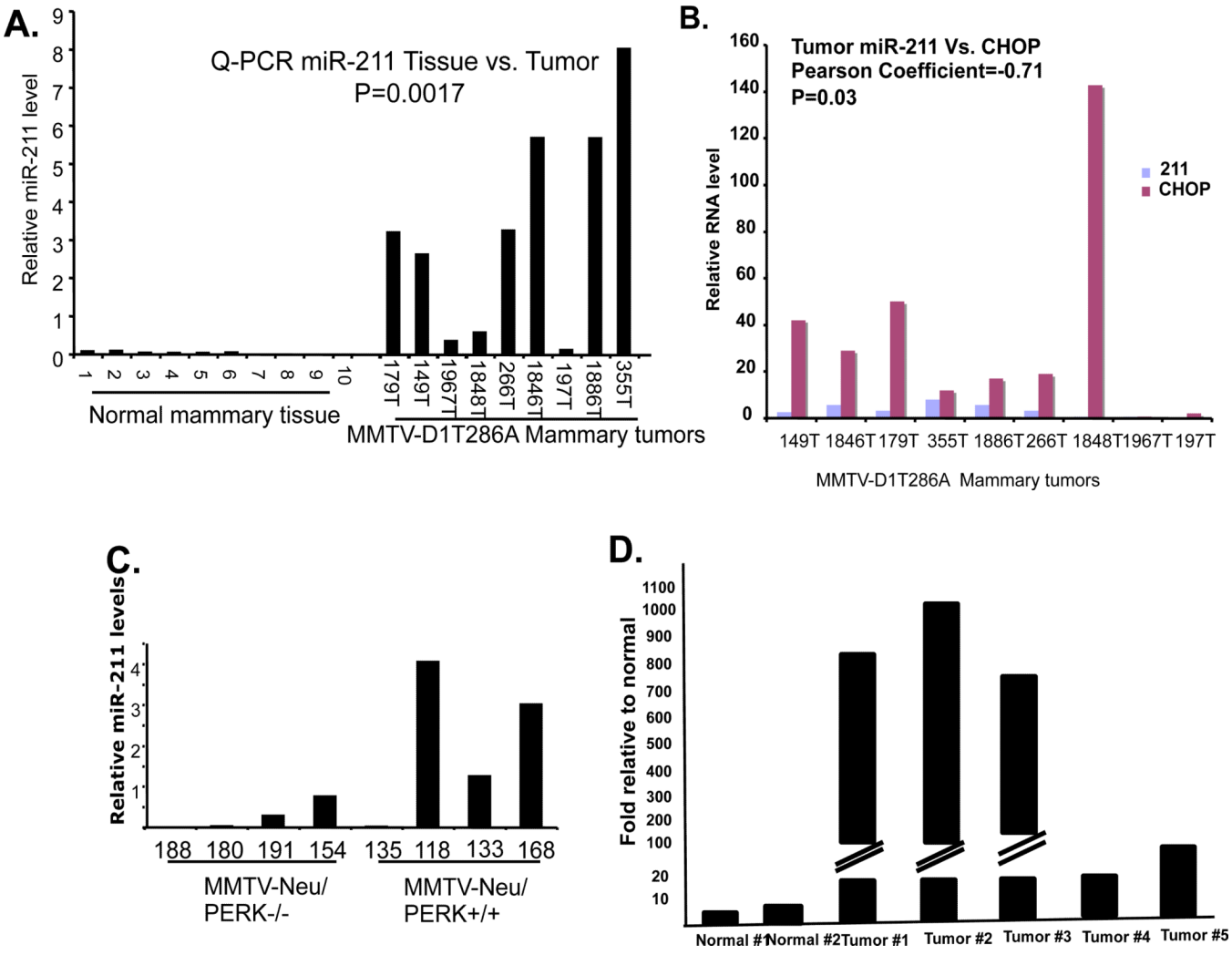


**Figure 7. PERK-dependent regulation of miR-211 in tumors**

Total RNA from tumors was purified and analyzed by Q-PCR. Sno202 served as an internal control for mouse samples and U6 for human samples. **A)** Analysis of miR-211 levels in MMTV-D1T286A mammary tumors. **B)** Accumulation of miR-211 and *chop* are inversely correlated in mammary carcinomas. **C)** MiR-211 expression was analyzed in MMTV-Neu tumors on either PERK+/+ or −/− background. **D)** Assessment of miR-211 levels in primary human lymphoma.