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# ER Stress-Mediated Upregulation of miR-29a Enhances Sensitivity to Neuronal Apoptosis\*

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## **Abstract**

Disturbance of homeostasis within the ER lumen leads to the accumulation of unfolded and misfolded proteins. This results in the activation of an evolutionary conserved stress response termed ER stress that, if unresolved, induces apoptosis. We previously identified the Bcl-2 homology domain 3 (BH3)-Only Protein Puma as a mediator of ER stress-induced apoptosis in neurons. In the search of alternative contributors to ER stress-induced apoptosis, we noted a downregulation of the anti-apoptotic Bcl-2 family protein Mcl-1 during ER stress in both mouse cortical neurons and human SH-SY5Y neuroblastoma cells. Downregulation of Mcl-1 was associated with an upregulation of microRNA-29a (miR-29a) expression, and subsequent experiments showed that miR-29a targeted the 3' UTR of the anti-apoptotic Bcl-2 family protein, Mcl-1. Inhibition of miR-29a expression using sequence specific antagomirs or the overexpression of Mcl-1 decreased cell death following tunicamycin treatment, while gene silencing of Mcl-1 increased cell death. miR-29a did not alter the signalling branches of the ER stress response, rather its expression was controlled by the ER stress-induced transcription factor activating-transcription-factor-4 (ATF4). Our data demonstrate that the ATF4-mediated upregulation of miR-29a enhances the sensitivity of neurons to ER stress-induced apoptosis.

## Introduction

Disturbance of homeostasis within the lumen of the endoplasmic reticulum (ER) often results in the accumulation of unfolded and misfolded proteins and activation of an evolutionary conserved stress response termed the unfolded protein response (Wang & Kaufman, 2012). The primary role of this response is to limit damage to the cells, however, under periods of extended or prolonged ER stress where irreparable damage has ensued, cell death via apoptosis is executed (Tabas & Ron, 2011). ER stress has been implicated in a number of pathophysiological conditions most notably those associated with disorders of the nervous system including amyotrophic lateral sclerosis (Kikuchi *et al.*, 2006; Kieran *et al.*, 2007; Saxena *et al.*, 2009), Alzheimer's disease (Kudo *et al.*, 2002; Scheper *et al.*, 2011) and Parkinson's disease (Imai *et al.*, 2000; Ryu *et al.*, 2002). Therefore, further understandings of the role of ER stress in disease pathology are required and in particular the mechanisms involved in mediating cell death following prolonged ER stress.

Central to the initiation of apoptotic signalling pathways are a family of proteins, termed the Bcl-2 protein family. These proteins have been linked to the regulation of the intrinsic pathway of apoptosis which is executed following the release of cytochrome c and other pro-apoptotic factors from mitochondria (Llambi & Green, 2011). Members of the Bcl-2 family are classified on the basis of their function as either pro- or anti-apoptotic proteins. The pro-apoptotic Bcl-2 family protein Bax and Bak are believed to constitute the release channel in the outer mitochondrial membrane through which pro-apoptotic factors are released, and are essential for the induction of apoptosis (Wei *et al.*, 2001). Other pro-apoptotic members of the Bcl-2 family contain only a single Bcl-2 homology domain, the BH3-only proteins (Strasser, 2005). Under stress conditions these BH3-only proteins are activated either via

transcription or via post-translational means. They serve to link the stress event to the initiation of apoptosis signalling cascade by activating Bax and Bak. By employing a transcriptome analysis of human SH-SY5Y neuroblastoma cells, we have previously investigated the genes involved in ER stress induced apoptosis in neural cells and identified the BH3-only protein, Puma, as an essential, stress-induced mediator of apoptosis within this paradigm (Reimertz *et al.*, 2003; Concannon *et al.*, 2008). ATF4, an ER stress-inducible transcription factor has been shown to activate *puma* gene expression in cortical neurons undergoing ER stress induced-apoptosis through upregulation of the transcription factor Chop (Galehdar *et al.*, 2010). Of note, in neurons although loss of *puma* has a significant impact on ER stress mediated apoptosis residual levels of apoptosis are still evident (Reimertz *et al.*, 2003; Concannon *et al.*, 2008; Galehdar *et al.*, 2010). A previous study performed in non-neuronal cells has also suggested a contribution of the BH3-only protein Bim during ER stress induced apoptosis (Puthalakath *et al.*, 2007). Mouse embryonic fibroblasts deficient for the BH3-only protein Noxa have also been shown to be protected against ER stress-induced cell death (Li *et al.*, 2006). Noxa induction during ER stress has been identified in parallel with Puma induction (Pagliarini *et al.*, 2015). Equally important for the sensitization of cells to ER stress-induced apoptosis may be a down-regulation of anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-xL, Mcl-1, and Bcl-w. These function to inhibit the activity of Bax, Bak and BH3-only proteins. Indeed previous studies have demonstrated an important role for Bcl-2 and Bcl-xl in ER stress-induced apoptosis (Hacki *et al.*, 2000; Kaufmann *et al.*, 2003).

In recent years much research has eluded to the key role for micro RNAs, small non coding RNAs, as potent modulators of gene expression downstream of transcription (Bartel, 2009) . Micro RNAs (miRNAs) regulate the expression of proteins post-transcriptionally by their

ability to bind to the 3' untranslated region (UTR) of mRNAs resulting in either mRNA degradation or disruption of protein translation (Baek *et al.*, 2008). We therefore sought to identify novel regulators of ER stress-induced apoptosis, and investigated the potential role of microRNA-29a (miR-29a) in mediating apoptosis.



## **Materials and Methods**

### **Cell culture**

Human SH-SY5Y neuroblastoma cells were maintained in DMEM/F12 Ham (Sigma-Aldrich, Wicklow, Ireland) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. The motor neuron-like NSC34 cells were maintained in DMEM high glucose (Lonza, Dublin, Ireland) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. HeLa cells were maintained in RPMI1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Cells were seeded at  $3 \times 10^5$  cells per 6-well plate and  $1.5 \times 10^5$  cells per 24-well plate. After cells reached 70-80% confluence, experiments were initiated.

### **Gene targeted mice**

*puma*<sup>-/-</sup> mice were generated as previously described (Bouillet *et al.*, 1999; Villunger *et al.*, 2003). The *puma*<sup>-/-</sup> mice were generated on an inbred C57BL/6 background using C57BL/6-derived embryonic stem (ES) cells. The *bax*<sup>-/-</sup> mice were obtained from Jackson Laboratory and maintained in house. The *bax*<sup>-/-</sup> mice were originally generated on a mixed C57BL/6\_129SV genetic background, using 129SV-derived ES cells but had been backcrossed for >12 generations onto the C57BL/6 background. The genotype of *bax*<sup>-/-</sup> mice was confirmed by PCR as described by The Jackson Laboratory.

### **Preparation of primary cortical neurons**

Mouse cortical neurons were isolated from C57BL/6J embryonic day 16 pups and prepared as described previously (Concannon *et al.*, 2008). Briefly embryos (E15–16) were isolated

from inbred C57BL6/J mouse by hysterectomy of the uterus following an abdominal injection of 0.2 ml of pentobarbital as lethal anaesthesia. The embryos were transferred to a dissection medium on ice (PBS with 0.25% glucose and 0.3% bovine serum albumin). The cerebral cortices from each of the embryos were isolated, the surrounding meninges removed and the tissue pooled in dissection media on ice. The tissue was incubated with trypsin (0.125 mg/ml) at 37°C for 15 min. After the incubation, the trypsinization was stopped by addition of medium containing sera. The neurons were dissociated from tissue by gentle pipetting, centrifuged (200 x g for 3 min) and the medium was carefully aspirated. The neurons were resuspended in plating media (MEM containing 5% foetal calf serum, 5% horse serum, 100 U/ml penicillin/streptomycin, 0.5 mM L-glutamine and 6% glucose) and plated at  $7 \times 10^5$  cells/ml on poly-lysine coated plates. Cells were incubated at 37°C, 5% CO<sub>2</sub>. The plating medium was exchanged with 50% feeding medium (Neurobasal medium (Invitrogen, Paisley, United Kingdom) - containing 100 U/ml of penicillin/streptomycin, 1% B27 supplement and 0.5 mM L-glutamine) and 50% plating medium with additional 600 nM cytosine arabinofuranoside (Sigma) at DIV 2 and again exchanged to complete feeding medium at DIV 4. Experiments were performed from DIV 6 to DIV 9.

### **Plasmids and transfection**

The shRNA vector targeting human Mcl-1 (pGFP-V-RS, TG 303313), the pCMV6-Mcl-1 overexpression vector and the pCMV-pre-miRNA29a vector were purchased from Origene (Rockville, Maryland, USA) and the pcDNA3.1 control empty vector was purchased from Invitrogen (Paisley, United Kingdom). The shRNA vector targeting mouse Mcl-1 and the pLKO.1 control empty vector were obtained from Sigma-Aldrich. For inhibition of miR-29a expression a LNA oligonucleotide antagomir specific for miR-29a (Exiqon, Vedbaek, Denmark) or scrambled sequence (Exiqon) was used. In some experiments the pEGFP-N1

plasmid (Clontech) was used to identify transfected cells as indicated. SH-SY5Y cells were transfected using Metafectene (Biontex, Martinsried, Germany) using the manufacturer's protocol. NSC 34 cells and neocortical neurons were transfected using Lipofectamine 2000 (Invitrogen, Paisley, United Kingdom) as per manufacturer's instruction.

### **Stem-loop reverse transcription and real-time qPCR using TaqMan low-density arrays**

Reverse transcription of 50 ng of RNA from Tunicamycin-treated SH-SY5Y cells at 4 h, 8 h and 24 h was carried out using the stem-loop Multiplex primer pools (Applied Biosystems), allowing reverse transcription of 48 different miRNA in each of eight RT pools. Reverse transcription for individual qPCRs was carried out using 50 ng of total RNA and the High-Capacity Reverse Transcription Kit (Applied Biosystems). The microRNA qPCR screen was carried out on the 7900HT Fast Realtime System using TaqMan Low-Density Arrays (TLDA) (TaqMan TLDA MicroRNA Assays AIF v5 containing 364 human microRNAs assays; Applied Biosystems). RNU6 was used for normalization in microRNA expression studies. A relative fold change in expression of the target gene transcript was determined using the comparative cycle threshold method ( $2^{-\Delta\Delta CT}$ ).

### **Real time qPCR analysis of stem loop miRNAs**

Following treatment cells were harvested and the miRNA fraction extracted using the miRNeasy mini kit (Qiagen, Hilden, Germany). For miRNA cDNA synthesis, 100 ng of RNA was reverse transcribed using the Taqman miRNA reverse transcription kit (Applied Biosystems, Paisley, UK) in combination with miRNA specific primers for miR-29a or the control RNU19 (Applied Biosystems) as per manufacturer's protocol. miRNA primer sequences were 5'-UAGCACCAUCUGAAAUCGGUU-3' for miR-29a and 5'-UUGCACCUCUGAGAGUGGAAUGACUC

CUGUGGAGUUGAUCCUAGUCUGGGUGCAAACAAUU-3' for RNU19 control.

PCR amplification reactions were carried out in a total volume of 20 µl, containing 10 µl of TaqMan Master Mix (Applied Biosystems), 1.33 µl of cDNA and 1 µl of miRNA TaqMan probe and primers (Applied Biosystems). Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All PCR reactions were performed on the 7500 FAST RT-qPCR system (Applied Biosystems). Ct values were calculated using the SDS software v.2.1 using automatic baseline and threshold settings.

### **RT-qPCR**

Total RNA was extracted using the Qiagen RNeasy minikit (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed using 1 µg of total RNA as the template and reverse transcribed using Superscript II (Invitrogen) primed with 50 pmol of random hexamers. Specific primers for each gene analysed were designed using Primer3 software. The sense and antisense primers were

CAACACAAACCCCAAGCCT and CATTGCAAACACCCTCCTT for *bim*,  
TCTCAGGAAAGGCTGCTGGT and GCTGGGCACTGGGTTAAGAA for *puma*,  
TCAGGAAGATCGGAGACAAA and TGAGCACACTCGTCCTTCAA for *nox4*,  
CTCATGGCCTTGTAGTTGAGA and AGGGCATTGGAAGAACATGAC for *xbp1*,  
ACAGCAAGGAGGATGCCTTCT and GGCTGCTTATTAGTCTCCTGGAC for *atf4*  
AACCTCAGCCACTTTCTCCAG and GGCTCCGCTGAAGAGAGACTATT for *atf6* and  
GGTGTGATGGTGGGAATGG and GGTTGGCCTTAGGGTTCAGG for *β-actin*.

Quantitative real-time PCR (RT-qPCR) was performed using the LightCycler 4.0 (Roche Diagnostics, Basel, Switzerland) and the QuantiTech SYBR green PCR kit (Qiagen) as per manufacturer's protocol. The PCRs were performed in 20 µl volumes with the following parameters – 95°C for 15 min, followed by 40 cycles of 94°C for 20 s, 58°C for 20 s and 72°C

for 20 s. The generation of specific PCR products confirmed by melting curve analysis and gel electrophoresis. The data were analysed using the LightCycler software 4.0 and samples were normalised to  $\beta$ -actin.

### **ER-stress reporter cell lines**

SH-SY5Y cell lines stably expressing YFP fluorescent labelled reporters indicating translation of ATF4 (PERK-reporter) or splicing of XBP1 (IRE1-reporter) were generated as described in (Walter *et al.*, 2015). The ATF6-reporter construct was generated through amplifying the 5xATF6-binding site and adjacent *c-fos* promoter from the 5xATF6-GL3 plasmid (Wang *et al.*, 2000) using primer 5'-TAGATATTAATCTCGACCTGCAGCCC-3' and 5'-AATATTGAGCTCGGAGATCCTCTAGAG-3' and cloning into pEYFP-N1 (Clontech) using *AseI* and *SacI* restriction sites, thus replacing the CMV-promoter. The 5xATF6-EYFP construct was transfected into SH-SY5Y cells and stable cells were selected in medium containing 0.5 mg/ml G418 (Sigma, Arklow, Ireland).

### **High content live cell imaging**

High content live cell imaging and data processing was performed as described in (Walter *et al.*, 2015). Briefly, ER-stress reporter cell lines were cultivated for 24 h in Nunc Micro Well 96 well optical bottom 96 well plates. Prior to imaging the cells were stained with 100 ng/ml Hoechst 33588 and 2  $\mu$ g/ml Propidium Iodide and treated with Tunicamycin, Thapsigargin or the same volume DMSO. The plate was incubated on stage at 37°C, 5% CO<sub>2</sub> and imaged at 1 h increments for 48 h using a Cellomics ArrayScan VTi instrument (Fisher Scientific) equipped with a  $\times 10$  PlanApo objective lens (NA 0.45), a 120 W Hg arc illumination source (EXFO, Chandlers Ford, UK) and a monochrome CCD camera

(Orca Hamamatsu Photonics, Hertfordshire, UK). CellProfiler 2.0 cell image analysis software (Carpenter *et al.*, 2006) was used to identify individual objects and measure their fluorescence intensity. Intensities of all images of the same channel were. A purpose built Matlab script (Matlab; MathWorks Inc., Cambridge, UK) was used to calculate and plot the fraction of dying cells and the mean of the mean YFP fluorescence intensity values of all cells per treatment group and time point as well as standard errors of these values.

### **shRNA and lentivirus production/transduction**

MISSION shRNA vectors for gene silencing of ATF6 (clone number TRCN0000017855), XBP1 (TRCN0000019804) or ATF4 (TRCN0000013573) in mammalian cells and scrambled control containing plasmids were obtained from Sigma. Lentiviral particles were produced and transduction with lentiviral particles was conducted as described in (Walter *et al.*, 2015).

### **ER stress induction**

ER stress was induced by addition of 3  $\mu$ M tunicamycin (Sigma-Aldrich) directly to the culture medium for the indicated time periods. DNA damage was induced by addition of 100  $\mu$ M etoposide (Sigma-Aldrich) directly to the culture medium for the indicated time periods. Control cells were treated with an equivalent volume of DMSO vehicle (Sigma-Aldrich).

### **Annexin V/PI staining**

Cells were stained with Annexin V/Propidium iodide (BioVision, Mountain View, CA, USA) as per manufacturer's instructions and analyzed using a CyFlow ML flow cytometer (Partec, Munster, Germany) and FloMax software. A minimum of  $10^4$  events were recorded for all samples and analyzed using FlowMax software (Partec).

### **Determination of caspase-3-like protease activity**

DEVDase activity was determined fluorometrically using N-benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (DEVD-AMC) as the substrate (10  $\mu$ M). Cleavage of DEVD-AMC to liberate free AMC was monitored in live cells by measuring fluorescence after 1- and 2 h intervals. Protein content was determined using the Pierce Coomassie Plus protein assay reagent (Perbio, Northumberland, United Kingdom). Caspase activity was expressed as change in fluorescent units per hour per microgram of protein.

### **Quantification of cell death in neurons**

Neocortical neurons were stained with Hoechst 33342 (1  $\mu$ g/ml) (Invitrogen) and propidium iodide (1  $\mu$ g/ml) (Sigma-Aldrich) for 20 min in medium. Fluorescence was observed using a Nikon Eclipse TE 300 inverted microscope (Nikon, Düsseldorf, Germany) with a 20x 0.43 NA phase-contrast objective using the appropriate filter for Hoechst and a charge-coupled device camera (SPOT RT SE 6; Diagnostics Instruments, Sterling Heights, MI). All experiments were performed at least three times from independent cultures. For each timepoint images of nuclei were captured in three subfields containing 300-400 neurons each and repeated in triplicate. Condensed and/or fragmented nuclei were scored as percent nuclear condensation and expressed as a percent of total population. Resultant images were processed using ImageJ (Micron-Optica; National Institute of Health) and AlphaEase software (Genetic Technologies, Miami, FL).

### **Western blotting**

Preparation of cell lysates and western blotting was performed as described previously (Reimertz *et al.*, 2003). The resulting blots were probed with a mouse monoclonal anti-Grp78 antibody (BD Biosciences, New Jersey, USA) diluted 1:1000, a rabbit polyclonal anti-Chop

antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:250, a rabbit polyclonal anti-Mcl-1 antibody (Rockland, Pennsylvania, USA) diluted 1:1,000 (specific for mouse Mcl-1, capable of detecting Mcl-1 on the outer mitochondrial membrane (OM) (40 kDa) and the inner mitochondrial membrane (IM) (36 kDa) of mitochondria (Perciavalle *et al.*, 2012)), a mouse monoclonal anti-Mcl-1 antibody (BD Pharmingen, New Jersey, USA) diluted 1:1000 (specific for human Mcl-1, detects Mcl-1 on the OM (40 kDa) of mitochondria), a rabbit polyclonal anti-Bim antibody (Stressgen, Victoria, Canada) diluted 1:1000, a mouse monoclonal anti-Xbp1 antibody (BioLegend, San Diego, CA, USA) diluted 1:500, a rabbit polyclonal anti-Atf6 antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:100, a mouse monoclonal anti-tubulin antibody (Sigma-Aldrich, Tallaght, Dublin, Ireland) diluted 1:8,000, a mouse monoclonal anti- $\beta$ -actin antibody (clone DM 1A; Sigma-Aldrich) diluted 1:8,000. Horseradish peroxidase-conjugated secondary antibodies diluted 1:5,000 (Thermo Fisher Scientific, Dublin, Ireland) were detected using Immobilon western chemiluminescent HRP substrate (Millipore, Cork, Ireland) and imaged using an imaging system (LAS-4000; Fujifilm, Sheffield, UK).

### **Statistical Analysis**

For statistical comparison, when comparing two treatments, two-tailed Student's t-test was used. Data involving comparisons of more than two groups were analysed using one-way ANOVA followed by the Tukey's *post hoc* analysis to determine differences amongst treatment groups. Values of  $p < 0.05$  were considered to be statistically significant. All the data reported in the text and figures represent the mean  $\pm$  SEM.



## Results

### **ER stress in cortical neurons is Bax-dependent but only partially PUMA-dependent -**

We assessed the ability of primary cortical neurons to undergo cell death following treatment with the inhibitor of N-glycosylation, tunicamycin. As demonstrated in Figure 1A, tunicamycin treatment of primary cortical neurons resulted in a time dependent increase in the number of neurons which displayed pyknotic nuclei and were propidium iodide (PI) positive (n=4 cultures per treatment, ANOVA post-hoc Tukey, pyknotic nuclei:  $F_{3,32}=203.54$ ,  $*p = 0.001$  compared to control treated cultures, propidium iodide:  $F_{3,12}=107.33$ ,  $*p=0.001$  compared to control treated culture). This cell death was associated with increased DEVDase activity indicative of the activation of effector caspases-3 and-7 (n=4 cultures per treatment, ANOVA post-hoc Tukey,  $F_{3,16}=6.15$ ,  $*p=0.005$  compared to control treated culture, Figure 1C). Increased expression of known ER stress markers including the ER resident chaperone, Grp78, and the transcription factor Chop at both the mRNA and protein level were evident within this paradigm (n=3 cultures per treatment, ANOVA post-hoc Tukey, Grp78:  $F_{3,8}=67.43$ ,  $*p=0.001$  compared to control treated culture, Chop: $F_{3,8}=9.02$ ,  $*p=0.006$  compared to control treated culture, Figure 1D-G).

We investigated the changes in the expression levels of several BH3-only proteins by qPCR during tunicamycin-induced ER stress. Tunicamycin treatment induced a prominent and time dependent up-regulation of *puma* mRNA (n=3 cultures per treatment, ANOVA post-hoc Tukey,  $F_{3,8}=17.26$ ,  $*p=0.0007$  compared to control treated culture, Figure 2A). The expression of *nox*a mRNA was much more moderately affected (n=3 cultures per treatment, ANOVA post-hoc Tukey,  $F_{3,8}=14.55$ ,  $*p=0.0001$  compared to control treated culture, Figure 2B). Despite induction of *bim* mRNA (n=3 cultures per treatment, ANOVA post-hoc Tukey,

$F_{3,8}=8.15$ ,  $*p=0.0008$  compared to control treated culture, Figure 2C), this was not reflected on a protein level with Bim protein expression unchanged or slightly decreased at 16 and 24 h (n=2 independent experiments, Figure 2D). In order to investigate the role of increased Puma expression in ER stress-induced apoptosis (Figure 2A), we utilized neurons from *puma*<sup>-/-</sup> mice and assessed the levels of cell death in response to tunicamycin treatment. Loss of Puma expression significantly protected cortical neurons from tunicamycin-induced cell death as assessed by reduced levels of nuclear apoptosis (n=4 cultures per treatment, ANOVA post-hoc Tukey,  $F_{7,63}=109.12$ ,  $*p=0.001$  compared to WT treated culture, Figure 2E). However, the protection seen in the absence of *puma* expression was not complete and suggested the involvement of other mechanisms in the activation of apoptosis in this setting. To assess whether cortical neuronal ER stress induced cell death was indeed dependent on the mitochondrial apoptosis pathway we treated *bax*<sup>-/-</sup> neurons with tunicamycin. As cortical neurons only express a non-functional form of Bak, N-Bak (Uo *et al.*, 2005) loss of *bax* expression has been shown to be sufficient to prevent neuronal apoptosis induction by a variety of stimuli (Cregan *et al.*, 1999). Loss of *bax* expression completely protected cortical neurons from cell death compared to wildtype (WT) neurons (n=3 independent samples, ANOVA post-hoc Tukey,  $F_{3,20}=180$ ,  $*p=0.003$  compared to control treated culture, Figure 2F) suggesting that *bax*-independent cell death did not play a significant role in this paradigm.

**Identification of miR-29a as an ER stress-induced miRNA** - Previously, we have utilized high density microarrays to identify genes which are differentially regulated in SH-SY5Y neuroblastoma cells following treatment with the ER stressor, tunicamycin (Reimertz *et al.*, 2003). In order to investigate novel regulators of ER stress mediated apoptosis we chose to examine miRNA regulation in the same setting of SH-SY5Y cells which had the advantage of

having been previously profiled for mRNA expression data as well as being a more homogenous cell composition compared to cortical neurons. To this end we treated SH-SY5Y neuroblastoma cells with 3  $\mu$ M tunicamycin for 4, 8 and 12 h as described previously (Reimertz *et al.*, 2003) after which we performed real time qPCR using Taqman low density arrays (TLDA) to investigate changes in miRNA expression. Induction of ER stress within this setting was re-confirmed by the time dependent increase in the ER stress markers, *Grp78* and *Chop* on mRNA level. The arrays were performed in duplicate with biological replicates for each time point. The cycle threshold value was calculated for each miRNA and normalised to RNU6 levels. The relative fold changes were calculated using the comparative cycle threshold method, and miRNAs defined as differentially expressed if their levels were changed by more than 1.5 fold on both arrays. Using these criteria, we identified hsa-miR-29a to be 1.71 and 1.70 fold upregulated after 8 and 12 h of tunicamycin treatment.

In order to validate the results of the TLDA we performed real time qPCR analysis of miR-29a expression in both SH-SY5Y neuroblastoma cells and primary cortical neurons. miR-29a demonstrated a time dependent increase in expression up to 24 h of tunicamycin treatment in the SH-SY5Y cells (n=3 independent samples, ANOVA post-hoc Tukey,  $F_4=9.94$ ,  $*p=0.0016$  compared to control treated culture, Figure 3A). In cortical neurons, miR-29a was significantly upregulated at 8 h, 16 h and 24 h tunicamycin treatment (n=3 independent samples, ANOVA post-hoc Tukey,  $F_3=0.16$ ,  $*p<0.05$  compared to control treated culture, Figure 3B). Taken together, these data suggested that miR-29a was robustly increased during ER stress.

**Inhibition of miR-29a attenuates ER stress induced apoptosis** - Previous work has identified members of the miR-29 family as proficient in targeting the 3' UTR of the anti-apoptotic Bcl-2 family protein Mcl-1 (Mott *et al.*, 2007). We therefore next assessed whether

modulation of miR-29a levels could affect cellular outcome following tunicamycin treatment. Initially we examined the constitutive overexpression of miR-29a by transfecting cells with a miR-29a expression plasmid driven by a CMV promoter and investigated the effects on tunicamycin-induced cell death. Increased expression of miR-29a resulted in increased levels of apoptosis under control conditions in SH-SY5Y cells. Furthermore, the increased expression of miR-29a resulted in enhanced sensitivity to ER stress induced apoptosis (n=3 independent samples, ANOVA post-hoc Tukey,  $*p < 0.05$  compared to tunicamycin treated control antagomir transfected cultures, Figure 3C). In order to further investigate the functional significance of miR-29a upregulation during ER stress we investigated the effect of inhibiting miR-29a using sequence specific antagomirs. SH-SY5Y cells were transfected with either miR-29a antagomir or a control sequence and subsequently treated with tunicamycin to induce ER stress. Inhibition of miR-29a significantly decreased the levels of tunicamycin induced cell death (n=3 independent samples, ANOVA post-hoc Tukey,  $F_3=15.45$ ,  $*p=0.01$  compared to tunicamycin treated control antagomir transfected cultures, Figure 3D). This result was paralleled in cortical neurons, where miR-29a inhibition provided significant protection against tunicamycin-induced apoptosis (n=3 cultures per treatment, ANOVA post-hoc Tukey,  $F_3=12.73$ ,  $*p=0.002$  compared to control treated culture, Figure 3E). Taken together these data suggest a role for miR-29a in mediating tunicamycin-induced, ER stress mediated apoptosis in neurons.

**Mcl-1 expression is decreased during ER stress and is sufficient to enhance susceptibility to ER stress** - Using several miRNA target prediction algorithms (TargetScan, PicTar) we identified a previously characterized region within the 3' UTR of Mcl-1 where miR-29a could potentially bind and was evolutionarily conserved across several species (Figure 4A). As demonstrated in Figure 4B and 4C, Western blot analysis demonstrated a

time-dependent decrease in Mcl-1 expression in SH-SY5Y neuroblastoma cells and in primary cortical neurons during tunicamycin-induced ER stress using two distinct antibodies raised against Mcl-1 (n=3 independent experiments, Figure 4C. tunicamycin 2 h & 4 h n=2 independent experiments).

To investigate whether miR-29a targets the 3'UTR of Mcl-1 in neural cells, we generated luciferase expression plasmids whereby the 3' UTR of Mcl-1 containing the putative miR-29a binding site was cloned downstream of the luciferase coding sequence. Using this construct we could demonstrate decreased luciferase expression following tunicamycin treatment suggesting that the 3' UTR of Mcl-1 was being targeted during ER stress (n=4 independent samples, ANOVA post-hoc Tukey,  $F_2=1.51$ ,  $*p<0.05$  compared to control treated cultures, Figure 4D). Furthermore, transfection with pre-miR-29a sequences significantly decreased luciferase expression compared to control pre-miR sequences again suggesting that miR-29a targeted the 3' UTR of Mcl-1 (n=4 independent samples, ANOVA post-hoc Tukey,  $F_1=33$ ,  $*p=0.0012$  compared to control pre-miR transfected cultures, Figure 4E). In contrast, treatment with etoposide, a DNA damage inducing agent, failed to modulate luciferase expression levels suggesting that this was not an unspecific response to apoptosis induction (n=4 independent samples, Figure 4F).

### **Modulation of Mcl-1 levels affects cellular sensitivity to ER stress induced cell death -**

To address whether the modulation of Mcl-1 levels may affect cellular outcome to ER stress induced cell death we first examined the effects of shRNA-mediated gene silencing of Mcl-1 on apoptosis using a shRNA vector targeting human Mcl-1 (pGFP-V-RS, TG 303313) previously described by our group (Anilkumar *et al.*, 2013). We confirmed shRNA-mediated knockdown of Mcl-1 in human HeLa cells (Figure 5A) and motoneuron-like NSC34 cells

(Figure 5B). shRNA mediated silencing of Mcl-1 in SH-SY5Y cells greatly enhanced the number of cells undergoing apoptosis following tunicamycin treatment (n=4 independent samples, ANOVA post-hoc Tukey,  $*p<0.05$  compared to control treated cultures,  $\#p<0.05$  between indicated groups, Figure 5D). Next we quantified the effects of *mcl-1* gene silencing in mouse neocortical neurons. Here we observed that shRNA silencing of Mcl-1 expression alone induced significant levels of apoptosis under control conditions and tunicamycin treatment suggesting the central role of Mcl-1 in regulating cell death in neurons (n=3 independent samples, ANOVA post-hoc Tukey,  $F_3=25.56$ ,  $*p=0.0013$  compared to control shRNA treated cultures, Figure 5C and 5E). To address whether overexpression of Mcl-1 could protect from tunicamycin induced cell death we transfected cortical neurons from wild type mice with a Mcl-1 expression plasmid prior to tunicamycin treatment. As shown in Figure 5F, Mcl-1 overexpression significantly reduced the levels of tunicamycin-induced cell death in wildtype neurons, demonstrating that Mcl-1 played a key role in regulating ER stress induced cell death (n=3 independent samples, ANOVA post-hoc Tukey,  $F_3=30.01$ ,  $*p=0.0001$  compared to control treated cultures,  $\#p<0.05$  between indicated groups, Figure 5F).

### **miR-29a inhibition does not alter ER stress responses**

To explore whether miR-29a also acted by modulating the ER stress response, we employed the previously described IRE1- and PERK-reporter cell lines (Walter *et al.*, 2015). IRE1-reporter cells stably expressed a full-length *Xbp1-yfp* construct. Upon ER stress activated IRE1 splices the 26 bp intron from *Xbp1-yfp* which leads to the translation of full length XBP1-YFP. The PERK-reporter construct contained three regulatory upstream ORF of human ATF4 in frame with YFP, which is sufficient for translation initiation following activation of PERK and phosphorylation of eIF2 $\alpha$  under ER-stress conditions. Additionally

we also generated an ATF6-reporter cell line, which stably expressed the ATF6-binding sequence, described by Wang et al, (Wang *et al.*, 2000), which under ER-stress induced the transcription of *yfp*. To investigate whether miR-29a is involved in the regulation of ER-stress responses PERK-, IRE1- or ATF6-reporter cells were transfected with antagomirs targeting human miR-29a or control. 48 h after transfection the cells were treated with 1  $\mu$ M tunicamycin, thapsigargin or control. The rate of fluorescent reporter activation in response to ER-stress over time was imaged for up to 48 h using high content live cell imaging (Figure 6). Comparing the fluorescent intensities indicating ATF4-translation in response to tunicamycin or thapsigargin induced ER stress in the PERK-reporter cells transfected with antagomirs or control, we did not find any difference in the response between the two groups (n= 7 wells from two independent experiments, independent t-test, no significance between cells transfected with miR-29a antagomirs and cells transfected with control antagomirs, Figure 6A). Similarly, transfection with antagomirs did not appear to influence ER stress dependent splicing of XBP1 in the IRE1-reporter cells (n= 7 wells from two independent experiments, independent t-test, no significance between cells transfected with miR-29a antagomirs and cells transfected with control antagomirs, Figure 6B) nor ATF6 driven transcription of YFP in the ATF6-reporter cells (n= 7 wells from two independent experiments, independent t-test, no significance between cells transfected with miR-29a antagomirs and cells transfected with control antagomirs, Figure 6C). These experiments suggested that the cytoprotection afforded by miR-29a inhibition was not a result of altered ER stress response modulation.

### **miR29a upregulation during ER stress**

Finally, we wished to explore which signalling arm of the ER stress response was responsible for the induction of miR-29a during ER stress. To this end we employed lentiviral delivered

shRNA constructs to effectively silence *atf6*, *xbp1* and *atf4* expression, respectively. The shRNA mediated loss of expression was confirmed by qPCR (n=3 independent samples, independent t-test,  $*p < 0.05$  compared control and knockdown groups, Atf6 DMSO:  $t_4 = 4.56, *p = 0.012$ , Atf6 Tuni:  $t_4 = 30.77, *p = 0.001$ , Xbp1 DMSO:  $t_4 = 9.83, *p = 0.001$ , Xbp1 Tuni:  $t_2 = 7.34, *p = 0.002$ , Atf4 DMSO:  $t_4 = 16.03, *p = 0.0001$ , Atf4 Tuni:  $t_4 = 10.66, *p = 0.0004$ , Figure 7A-C). We found expression levels of Atf6-, Xbp1- and Atf4 significantly reduced in response to 3  $\mu$ M tunicamycin as well as in control treated cells (n=3 independent samples, independent t-test,  $*p < 0.05$  compared control and knockdown groups, Atf4-kd DMSO:  $t_4 = 8.70, *p = 0.001$ , Atf4-kd Tuni:  $t_4 = 4.64, *p = 0.009$ , Figure 7D-E).

Comparing miR-29a levels between cells deficient for Atf6, - Xbp1- or Atf4 expression and control cells we found no significant changes in the miR-29a levels in the cells silenced for Atf6 or Xbp1 compared cells transduced with scrambled control. However in cells transduced with shRNA targeting Atf4 we observed that miR-29a levels were lower than in control cells, suggesting a role for ATF4 or a downstream target of ATF4 in the regulation of miR-29a expression.



## Discussion

In the present study we identified miR-29a to be robustly induced during ER stress in an ATF4-dependent manner. We also found miR-29a to target and inhibit expression of the anti-apoptotic Bcl-2 protein, Mcl-1, and demonstrated a role for miR-29a in sensitizing cells to ER stress induced apoptosis.

In our initial experiments we found ER stress induced apoptosis in cortical neurons to be dependent on *bax* expression. Activation of *bax* was likely to be mediated in part by induction of the BH3-only protein, Puma, with loss of *puma* expression protecting from cell death similar to what have been observed in other settings of ER stress induced death (Reimertz *et al.*, 2003; Kieran *et al.*, 2007; Concannon *et al.*, 2008; Galehdar *et al.*, 2010). In line with a previous report, we could not find a prominent role for *bim* in ER stress-induced apoptosis (Galehdar *et al.*, 2010). We did not observe Bim protein induction in the presence of mildly increased *bim* mRNA under conditions of tunicamycin treatment. Activation of PERK and phosphorylation of eIF2 $\alpha$  following tunicamycin treatment could inhibit the cap-dependent translation of Bim. At present, we cannot fully exclude the contribution of other pro-apoptotic BH3-only proteins to ER stress-induced neuronal apoptosis in our system (Wong *et al.*, 2005, Concannon *et al.*, 2010, Li *et al.*, 2006). Nevertheless, we also sought to investigate other, “BH3-only protein-independent” mechanisms which could be controlling the activation of Bax. One such means could be the modulation of protein levels of anti-apoptotic Bcl-2 family proteins by miRNA as demonstrated in previous studies (Mott *et al.*, 2007; Shimizu *et al.*, 2010; Singh & Saini, 2012). Here, we identified an increased expression of miR-29a during ER stress, a miRNA that targets the expression of the anti-apoptotic Mcl-1. Decreased expression of miR-29a has been associated with a number of malignancies

including lung cancer (Fabbri *et al.*, 2007) and anaplastic large cell lymphomas (Desjobert *et al.*, 2011). Indeed, downregulation of miR-29a expression in anaplastic large cell lymphomas has been associated with blockage of apoptotic pathways with forced increased expression of miR-29a mediating increased levels of apoptosis and decreased expression of Mcl-1 (Desjobert *et al.*, 2011). Enforced expression of miR-29a has been associated with inducing apoptosis and growth arrest in hepatic cancer cells (Meng *et al.*, 2010), while overexpression of miR-29a in pancreatic  $\beta$  cells decreased insulin production and increased apoptosis through targeting of Mcl-1 (Roggli *et al.*, 2012). We demonstrate that ER stress triggers miR-29a upregulation and cell death in SH-SY5Y cells and primary cortical neurons and subsequently targets Mcl-1 for translational repression and thereby decreases Mcl-1 protein levels. We also provide direct evidence that miR-29a targets the 3' UTR of Mcl-1 (Mott *et al.*, 2007). Indeed, we could demonstrate that shRNA silencing of Mcl-1 enhanced cell death induced by ER stress. Furthermore, overexpression of Mcl-1 was sufficient to protect neurons from ER stress induced cell death. Interestingly, inhibition of Mcl-1 expression in SH-SY5Y cells did not significantly alter the basal levels of apoptosis, but had much more dramatic effects in cortical neurons. Previous studies have demonstrated the central role of Mcl-1 expression in developing neurons with loss of Mcl-1 expression associated with increased levels of apoptosis and sensitivity to stressing agents such as DNA damage (Arbour *et al.*, 2008; Germain *et al.*, 2011). Because Mcl-1 mediates an anti-apoptotic response in a variety of cell types and settings (Opferman *et al.*, 2003; Opferman *et al.*, 2005; Arbour *et al.*, 2008), the identification of a miRNA that regulates Mcl-1 expression may suggest a possible therapeutic role for miR-29a modulation. Interestingly, downregulation of miR-29a expression has been demonstrated to be protective in a cardiac ischemia-reperfusion model (Ye *et al.*, 2010) and has been shown to affect insulin secretion in mouse pancreatic  $\beta$  cells through selective gene targeting (Pullen *et al.*, 2011), two settings where ER stress mediated

cell death has been implicated (Toth *et al.*, 2007; Lemaire & Schuit, 2012). In the latter paradigm, decreased expression of Mcl-1 has also been observed (Allagnat *et al.*, 2011). We also identified that the ER stress-induced activation of the transcription factor ATF-4 was required for miR-29a expression. PERK activation and ATF4 signalling have been described as regulators of neurodegeneration and ER stress-induced apoptosis in disease models of Alzheimer's disease (Devi & Ohno, 2014), Parkinson's Disease (Wu *et al.*, 2014) and ALS (Matus *et al.*, 2013) suggesting that inhibition of miR-29a could represent a novel therapeutic target for neurodegenerative disorders. ATF4 has previously been demonstrated to be an important 'switch' in ER stress-induced activation of apoptosis by upregulating the expression of *puma* (Galehdar *et al.*, 2010). ATF4 therefore may mediate ER stress-induced apoptosis and neurodegeneration by a dual action: activation of BH3-only protein expression and miR-induced downregulation of anti-apoptotic Mcl-1 expression.

While our study and other studies demonstrated a pro-apoptotic role for miR-29a in neurons similar to previous studies in non-neuronal cells (Mott *et al.*, 2007), evidence has also been presented that members of the miR-29 family, specifically miR-29b, may inhibit apoptosis through targeting of BH3-only proteins in other settings. miR-29b, a closely linked member of the miR-29 family has been shown to decrease expression of Bim and Puma and promote survival in mature sympathetic neurons following nutrient growth factor deprivation or etoposide treatment (Kole *et al.*, 2011). miR-29a and miR-29b are co-expressed from the same chromosome/cluster and owing to their sequence similarity there is an expected redundancy in their target mRNAs. This suggests that the effects of the miR-29 family members must be highly context dependent. Effects may depend on the type of stress induced, or the type of cells in which the stress is induced. Therefore it is possible, in apoptosis paradigms where Mcl-1 is required for survival, miR-29a may mediate a pro-

apoptotic effect but in paradigms or cell types where there is less reliance on Mcl-1, miR-29a or miR-29b may mediate an anti-apoptotic effect. Clearly, miR-29a may also have important functions in neurons outside the control of cell death. miR-29a has been implicated in mediating synaptogenesis in psychoactive drug-treated hippocampal neurons through modulating dendritic spine morphology (Lippi *et al.*, 2011). Furthermore, in Alzheimer's disease, increased expression of BACE1 protein, a pathological event in the disease, was shown to correlate with decreased expression of miR-29a (Hebert *et al.*, 2008). Interestingly, miR-29a/b-1 knockout mice presented with an ataxic phenotype and altered cerebellar morphology but unchanged levels of BACE-1 mRNA and protein, suggesting that targets other than BACE-1 mediated this phenotype (Papadopoulou *et al.*, 2015). miR-29a and downregulation of Mcl-1 may also serve as a signal for the induction of autophagy (Germain *et al.*, 2011) to aid with the increased levels of misfolded and aggregated proteins seen during ER stress. As microRNAs have been shown to be quite promiscuous in their ability to target multiple sites and pathways in many cell types, Mcl-1 is clearly not the only major target of miR-29a. MiR-29a has been shown to have multiple targets across a range of non-neuronal systems (Fabbri *et al.*, 2007). In HeLa cells, miR-29a was shown to upregulate p53 levels and enhance apoptosis in a p53 dependent manner (Desjobert *et al.*, 2011). miR-29a has also been shown to target Voltage dependent anion channels, VDAC1 and VDAC2 (Bargaje *et al.*, 2012), which have been identified as playing a role in mediating ER stress induced apoptosis (Deniaud *et al.*, 2008).

In conclusion, our work shows a pro-apoptotic role for miR-29a involving Mcl-1 regulation in the setting of ER stress, and demonstrates that miR-29a expression during ER stress is controlled by the transcription factor ATF4.

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## **Abbreviations**

Activating-transcription-factor-4 – ATF4, ER - endoplasmic reticulum, IM - inner mitochondrial membrane, miRNA – microRNA, OM - outer mitochondrial membrane, PI - propidium iodide, RT-qPCR - quantitative real-time polymerase chain reaction, UTR - untranslated region, WT - wildtype

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## FIGURE LEGENDS

FIGURE 1: ER stress induced apoptosis in primary cortical neurons is mediated in part by BH3-only protein Puma. (A) Significant increase in cell death in mouse cortical neurons treated with 3  $\mu$ M tunicamycin observed in a time-dependent manner. Cell death was assessed by Hoechst and propidium iodide staining. (B) Representative images of primary cortical neurons treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for 24 h. Cells were stained with Hoechst 33358 or propidium iodide. (C) Significant changes in caspase-3 like (DEVDase) activity were observed at 16 h and 24 h in cortical neurons treated as described above and expressed relative to non-treated controls. (D) Treatment of mouse cortical neurons with 3  $\mu$ M tunicamycin significantly alters *grp78* mRNA expression across time. *grp78* mRNA expression were normalised to  $\beta$ -actin and expressed relative to control treated cultures. (E) Treatment of mouse cortical neurons with 3  $\mu$ M tunicamycin significantly alters *chop* mRNA expression across time. *chop* mRNA expression were normalised to  $\beta$ -actin and expressed relative to control treated cultures. (F-G) Cortical neurons were treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for the indicated time periods. Increased expression of Grp78 and Chop were analyzed by Western blotting. Probing for  $\beta$ -actin served as the loading control. Scale bar:=20  $\mu$ M. Error bars indicate SEM. \*indicates statistical significant increase ( $p<0.05$ )

FIGURE 2: Regulation of BH3-only proteins is altered during ER stress in cortical neurons. (A-C) Significant time-dependent increases in *puma*, *nox4* and *bim* mRNA were observed in cortical neurons from wildtype (WT) mice treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for indicated time points. Expression levels were normalised to  $\beta$ -actin and

expressed relative to control treated cultures.(D) Densitometry analysis showing Bim levels in cortical neurons treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for the indicated time periods (upper panel). Western blot analysis of cortical neurons treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for the indicated time periods.. Probing for  $\beta$ -actin served as the loading control (lower panel). (E) Significant decrease in nuclear apoptosis was observed in cortical neurons from wild type (WT) and *puma*<sup>-/-</sup> mice were treated with 3  $\mu$ M tunicamycin. Cell death assessed using Hoechst staining. (F) Significant decrease in nuclear apoptosis was observed in cortical neurons from wild type (WT) and *bax*<sup>-/-</sup> mice were treated with 3  $\mu$ M tunicamycin for 24 h. Cell death was assessed by Hoechst staining and counting of apoptotic nuclei. Error bars indicate SEM. \*indicates statistical significant increase ( $p < 0.05$ )

FIGURE 3: Modulation of miR-29a expression during ER stress is associated with altered levels of cell death. (A) SH-SY5Y neuroblastoma cells were treated with 3  $\mu$ M tunicamycin for varying time periods (4-24 h) or control (0.1% DMSO). Significant changes in expression levels of miR-29a were examined using real time qPCR. (B) Primary cortical neurons were treated with 3  $\mu$ M tunicamycin for varying time periods (8-24 h) or control (0.1% DMSO). Significant changes in expression levels of miR-29a were examined using real time qPCR. (C) Increased miR-29a expression increases % nuclear apoptosis in SH-SY5Y cells treated with 3  $\mu$ M tunicamycin or DMSO (control) for 30 h. Cell death was assessed by Hoechst staining and counting of apoptotic nuclei. (D) Reduced expression of miR-29a using 30 nM miR-29a antagomir decreased cell death in SH-SY5Y cells compared to control antagomir. Cell death was assessed by annexin V and propidium iodide staining and subsequent flow cytometry analysis. (E) Significant decrease in nuclear apoptosis was achieved in primary cortical neurons transfected with 50 nM of control or miR-29a specific antagomir and treated with 3  $\mu$ M tunicamycin for 24 h. Nuclei were stained with Hoechst33358 and morphology of

pyknotic nuclei was assessed using Image J software (National Institute of Health). Error bars indicate SEM. \*indicates statistical significant increase ( $p < 0.05$ ), (# $p < 0.05$ ) between indicated groups.

FIGURE 4: miR-29a targeting of Mcl-1 3'UTR reduces Mcl-1 expression during ER stress (A) Schematic shows 3' UTR of Mcl-1 where miR-29a can bind is shown in red box (B) Densitometry analysis showing time course of Mcl-1 expression in SH-SY5Y cells treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for indicated time periods (upper panel). Western blot analysis of Mcl-1 expression in SHSY5Y cells treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for indicated time periods (lower panel). Anti-Mcl-1 antibody specific for human Mcl-1 was used (BD Pharmingen). Probing with  $\beta$ -actin served as a loading control. (C) Densitometry analysis showing Mcl-1 levels in cortical neurons treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for indicated time periods (upper panel). Western blot analysis of Mcl-1 expression in primary cortical neurons treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for indicated time periods. Anti-Mcl-1 antibody specific for mouse Mcl-1 was used (Rockland). Probing with  $\beta$ -actin served as a loading control (lower panel). (D) Tunicamycin treatment significantly alters expression of Mcl-1 3' UTR. SH-SY5Y cells were transfected with a plasmid containing the 3' UTR of Mcl-1. After 24 h the cells were treated with 3  $\mu$ M tunicamycin for 24 h or control (0.1% DMSO). (E) Increased miR-29a expression significantly alters Mcl-1 3' UTR expression. Cells were transfected with a plasmid containing the 3' UTR of Mcl-1 and either pre-miR-29a sequence or a control pre-miR sequence. (F) Etoposide treatment did not significantly alter Mcl-1 3' UTR expression. SH-SY5Y cells were transfected with a plasmid containing the 3' UTR of Mcl-1. After 24 h the cells were treated with 100  $\mu$ M etoposide for 24 h or control (0.1%

DMSO). All luciferase assays were performed and normalized for the co-transfected Renilla expression. Error bars indicate SEM. \*indicates statistical significant increase ( $p < 0.05$ )

FIGURE 5: Modulation of Mcl-1 levels affects cellular sensitivity to ER stress induced cell death. (A-B) Representative Western blots of Mcl-1 levels after transfection with indicated shRNA plasmid and probing with Mcl-1 human or mouse specific antibodies in HeLa cells (A). or NSC34 cells (B). Probing with  $\beta$ -actin served as a loading control. (C) Representative images of primary cortical neurons transfected with Mcl-1 shRNA or control shRNA and treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for 24 h. Cells were stained with Hoechst 33358 and imaged using Image J software (National Institute of Health). (D) Tunicamycin-induced cell death was significantly increased with shRNA knockdown of Mcl-1 expression. SH-SY5Y cells were transfected with a shRNA targeting Mcl-1. After 24 h transfection cells were treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for a further 24 h. Cell death was assessed by means of PI staining/ GFP expression and subsequent flow cytometry analysis. (E) Significant changes in % nuclear apoptosis were seen with decreased Mcl-1 expression irrespective ER stress. Mouse neocortical neurons were transfected with a shRNA targeting Mcl-1. After 24 h transfection cells were treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for a further 24 h. Cell death was assessed by Hoechst staining and counting of apoptotic nuclei. (F) Overexpression of Mcl-1 significantly decreased tunicamycin induced cell death. Mouse neocortical neurons from wildtype (WT) mice were transfected with an Mcl-1 overexpression vector. After 24 h transfection cells were treated with 3  $\mu$ M tunicamycin or control (0.1% DMSO) for a further 24 h. % nuclear apoptosis was assessed by Hoechst staining and counting of apoptotic nuclei. Scale bar = 10  $\mu$ m. Error bars indicate SEM. \*indicates statistical significant increase ( $p < 0.05$ ), (# $p < 0.05$ ) between indicated groups.

FIGURE 6: MiR-29a inhibition does not alter ER-stress responses. SH-SY5Y cells stably expressing fluorescent protein reporter constructs for the activation of (A) PERK (B) activation and splicing activity of IRE1 or (C) cleavage and transcriptional activity of ATF6 were transfected with antagomirs targeting miR-29a or control. 48 h after transfection the cells were treated with 1  $\mu$ M Tuni or 0.1 % DMSO stained with Hoechst and PI. (A, C) Mean fluorescence intensity per cell or (B) amount of YFP positive cells over time were monitored over time employing live-cell high content imaging (left panel). Images were taken at 1 h intervals starting immediately after treatment for up to 48 h. Error bars indicate S.E.M. of all cells per time point and treatment. The experiments were repeated with similar results. Right panel: YFP mean fluorescence intensity after 30 h (A,C) or 16 h (B) after treatment.

FIGURE 7: miR-29a regulation during ER-stress. SH-SY5Y cells were transduced with shRNA targeting *atf6*, *xbp1*, *atf4* or scrambled control. 72 h after transduction cells were treated for 24 h with 3  $\mu$ M Tuni or 0.1 % DMSO (A-C) Expression levels of *atf6*-, *xbp1*- and *atf4* were analysed using qPCR. Expression levels were normalised to  $\beta$ -actin and expressed relative to DMSO or Tuni treated scrambled control. miR-29a expression levels in untreated (D) or Tuni (E) treated cell *atf6*-, *xbp1*- and *atf4*-silenced cells were assessed using qPCR. Expression levels were normalised to RNU19 control and expressed as n-fold over DMSO or Tuni treated scrambled control. Error bars indicate SEM. \*indicates statistical significant increase ( $p < 0.05$ ).