ER stress protects from retinal degeneration

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The unfolded protein response (UPR) is a specific cellular process that allows the cell to cope with the overload of unfolded/misfolded proteins in the endoplasmic reticulum (ER). ER stress is commonly associated with degenerative pathologies, but its role in disease progression is still a matter for debate. Here, we found that mutations in the ER-resident chaperone, *neither inactivation nor afterpotential A* (*NinaA*), lead to mild ER stress, protecting photoreceptor neurons from various death stimuli in adult *Drosophila*. In addition, *Drosophila* S2 cultured cells, when pre-exposed to mild ER stress, are protected from H₂O₂, cycloheximide- or ultraviolet-induced cell death. We show that a specific ER-mediated signal promotes antioxidant defences and inhibits caspase-dependent cell death. We propose that an immediate consequence of the UPR not only limits the accumulation of misfolded proteins but also protects tissues from harmful exogenous stresses.

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Introduction

The unfolded protein response (UPR) is an evolutionary conserved adaptive response to perturbations of normal endoplasmic reticulum (ER) physiology. Strikingly, the UPR is implicated in several human pathologies, such as neurodegeneration, diabetes and cancer (Marciniak and Ron, 2006; Lin *et al*, 2008). The UPR engages several responses, including transcriptional upregulation of ER-resident chaperones, selective inhibition of translation and activation of ER-associated degradation (ERAD) (reviewed in Ron and Walter,

2007). Despite a large body of work in this field, little is known about the cellular consequences of the UPR upon physiological levels of ER stress *in vivo*.

Genetic and cell culture studies have unveiled the core of the UPR pathway, linking the detection of ER stress to the effector responses. In unstressed cells, the ER stress sensor and chaperone, Bip/Hsc3, binds and restricts the molecular components of the UPR. In stressed cells, Bip/Hsc3 is titrated away by unfolded proteins, relieving its inhibition on the UPR. This leads to trans-autophosphorylation of the PKR-like ER kinase (Perk) (Harding *et al*, 1999). Active Perk phosphorylates eIF2 α , inhibiting protein synthesis and limiting the flux of misfolded proteins to the ER. In addition, the UPR promotes the phosphorylation of Inositol-requiring enzyme 1 (Ire1). Activated Ire1 splices an intron from the mRNA of *x-box binding protein-1* (*xbp1*), creating a translational frameshift (Yoshida *et al*, 2001; Calton *et al*, 2002). The spliced form of *xbp1* acts as a transcriptional activator, resulting in the expression of ER-stress target genes, including protein chaperones and components of the ERAD pathway (Travers *et al*, 2000). In parallel, the membrane-bound protein Atf6 translocates to the golgi, where it is cleaved by SP1 and SP2. The cleaved peptide translocates to the nucleus, where it acts as a transcription factor (Ye *et al*, 2000).

If UPR activation cannot overcome ER stress, then its target pathways induce the activation of the apoptotic program (reviewed by Szegezdi *et al*, 2006). Apoptosis is executed by specific caspases and regulated both by pro- and anti-apoptotic Bcl-2 proteins. However, the UPR has also been shown to induce an antioxidant response, limiting the deleterious effect of prolonged ER stress (Cullinan and Diehl, 2004). How the UPR switches between inducing two such opposite programs is not clear.

Drosophila melanogaster has recently emerged as a useful *in vivo* model system to study the UPR pathway (Pomar *et al*, 2003; Hollien and Weissman, 2006; Ryoo and Steller, 2007; Souid *et al*, 2007). Most UPR components found in yeast and mammalian cells, such as Atf4 (Crc), Perk and eIF2 α , have *Drosophila* homologs (Pomar *et al*, 2003 and reviewed in Ryoo and Steller, 2007). In the *Drosophila* model of autosomal dominant retinitis pigmentosa (ADRP), the misfolding rhodopsin-1 (Rh1) mutation (*ninaE*^{G69D}, termed *rh1*^{G69D} for clarity) induces UPR activation by increasing *xbp1* splicing and Ire1-dependent expression of the *heat shock cognate protein 3* (Hsc3, the *Drosophila* Bip homolog; Ryoo *et al*, 2007). The reduction of *xbp1* expression leads to increased retinal degeneration, indicating that *xbp1* and its transcriptional targets are required to reduce the accumulation of misfolded Rh1 in the ER. Although the UPR pathway is conserved in *Drosophila*, the relationship between ER stress and apoptosis remains to be explored.

Searching for genes that protect adult photoreceptor cells (PRCs) against apoptosis, we identified mutations that disrupt the maturation and folding of the Rh1 protein as strong suppressors of neurodegeneration. These mutations lead to the activation of the UPR, which promotes neuroprotection

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against cellular insults by inducing an antioxidant response while inhibiting caspase activation. These results are not restricted to photoreceptor neurons, as cultured S2 cells become more resistant to cell death stimuli if pre-treated with ER stress-inducing chemical agents.

Results

PRCs exposed to ER stress exhibit death resistance

In a dominant modifier screen to identify cell death regulators in differentiated *Drosophila* PRCs (CSM, HS and BM, unpublished data), we have found that mutations in the *Drosophila* cyclophilin B homolog *ninaA* suppress *dp53*-induced cell death in PRCs (Figure 1; Table I). *NinaA* is a membrane-bound chaperone localized in the ER devoted exclusively to the biogenesis of the light sensing Rh1 protein. It has been shown that the peptidyl-prolyl *cis-trans* isomerase activity of *NinaA* allows proper Rh1 folding. In addition, *NinaA* functions as a chaperone, escorting Rh1 through the secretory pathway from the ER to the rhabdomeres (microvillar membrane in which rhodopsins accumulate) of outer PRCs (the rods-like PRCs of *Drosophila*) (Colley *et al*, 1991; Baker *et al*,

1994). *NinaA* *amorphs* lead to the accumulation of misfolded Rh1 protein in the ER, likely resulting in ER stress (Colley *et al*, 1991).

We analysed the protective effect induced by the loss of *ninaA* against several apoptotic stimuli. The expression of the pro-apoptotic gene *reaper* (*rpr*) induces cell death by a caspase-dependent mechanism through the inactivation of the *Drosophila* inhibitor of apoptosis (Diap1) (Hay *et al*, 1995; Goyal *et al*, 2000). The ectopic expression of *rpr*, under the control of the *rh1* promoter, induces a progressive loss of outer PRCs that can be visualized in tangential plastic retina sections of 2-day-old flies (Figure 1D). Loss of one genomic copy of the *ninaA* gene, using the amorphic allele *ninaA*^{E110V}, protects the outer PRCs from *rpr*-induced apoptosis (Figure 1D and E). This anti-apoptotic effect is dose dependent, as complete loss of *ninaA* lead to further protection (Figure 1D–F, K; Supplementary Table 1). To determine whether resistance to PRC death observed in *ninaA* mutants is specific to *rpr*-induced cell death, *ninaA* mutant retinas were submitted to the ectopic expression of *Drosophila* *p53* (*dp53*) or *death caspase-1* (*dcp-1*, a *Drosophila* homolog of caspase-3) (Brodsky *et al*, 2000; Laundrie *et al*, 2003). Similarly to *rpr*,

Figure 1 Loss of *ninaA* function protects PRCs from cell death. PRC integrity is visualized in outer PRCs submitted to the ectopic expression of *rpr*, *dp53* and *dcp-1*, in a wild-type or *ninaA* mutant background. (A–J) PRC rhabdomeres are visualized in semi-thin tangential retinal sections. Scale bar, 10 μ m. Outer PRC rhabdomeres of a representative ommatidium are circled in yellow. (A–C) PRC rhabdomeres are organized in a trapezoidal pattern in control retinas (5-day-old flies). (A) Wild type. (B) *ninaA*^{E110V/+}. (C) *ninaA*^{E110V}. (D–F) Loss of *ninaA* protects from *rpr*-induced cell death (1-day-old flies). (D) Ectopic expression of *rpr* in outer PRCs with *rh1-Gal4;uas-rpr* (*rh1>rpr*) induces a massive loss of PRC integrity. (E) Reduced PRC killing in *rh1>rpr*; *ninaA*^{E110V/+}. (F) Reduced PRC killing in *rh1>rpr*; *ninaA*^{E110V}. (G, H) Loss of *ninaA* protects from *dp53*-induced cell death (8-day-old flies). (G) Ectopic expression of *dp53* in outer PRCs with *rh1-Gal4;uas-dp53* (*rh1>dp53*) induces a massive PRC killing. (H) Reduced PRC killing in *rh1>dp53*; *ninaA*^{E110V/+}. (I, J) Loss of *ninaA* protects from *dcp-1*-induced cell death (1-day-old flies). (I) Loss of PRCs in *rh1-Gal4;uas-dcp-1* (*rh1>dcp-1*). (J) Reduced killing in *rh1-Gal4;uas-dcp-1*; *ninaA*^{E110V/+}. (K) Quantitative analysis of photoreceptor survival seen in (A, D–J). The results are expressed as mean percentage of wild-type control $\pm$ s.e.m. of three animals (**P* < 0.05 in Student's *t*-test).

Table 1 *ninaA* mutant alleles protect from *dp53*-induced cell death

Genotype	NinaA levels ^a [null (●) to WT (●●●●●)]	Amount of outer PRC/ommatidium ± s.e.m.
<i>Wild type</i>	●●●●●	6.0 ± 0
<i>rh1-Gal4;uas-dp53</i>	●●●●●	2.18 ± 0.41 [–]
<i>rh1-Gal4;uas-dp53/ninaA^{E110V}</i>	●●●●●	3.99 ± 0.30*
<i>rh1-Gal4;uas-dp53/ninaA^{Q137L}</i>	●●●●●	3.56 ± 0.16*
<i>rh1-Gal4;uas-dp53/ninaA^{G98D}</i>	●●●●●	3.29 ± 0.21*
<i>rh1-Gal4;uas-dp53/ninaA^{W208@}</i>	●●●●●	3.44 ± 0.17*
<i>rh1-Gal4;uas-dp53/ninaA^{R120K}</i>	●●●●●	2.44 ± 0.33 ^{NS}

PRC death was induced by *dp53* ectopic expression under the control of *rh1* promoter (*rh1 > dp53*). Viable PRCs expressing GFP (*rh1-GFP*) were visualized by the immersion technique (Pichaud and Desplan, 2001) in wild-type or *ninaA* mutant retinas.

^aNinaA protein levels obtained from western blot analysis (from Ondek *et al*, 1992). *ninaA^{E110V}/+*, *ninaA^{Q137L}/+*, *ninaA^{G98D}/+* and *ninaA^{W208@}/+* mutant retinas are protected from *dp53*-induced PRC death. The results are expressed as amount of outer PRC/ommatidium ± s.e.m. of three animals (**P* < 0.05 in Student's *t*-test compared with *rh1-Gal4;uas-dp53* in a wild-type background (–); NS, non significant).

retinas mutant for *ninaA* were resistant to cell death induced by the ectopic expression of *dp53* as well as *dcp-1* (Figure 1, G–J, K; Supplementary Table 1). Similar protective effects were also observed with several *ninaA* alleles, with the strongest effects associated with strong loss-of-function alleles (*ninaA^{E110V}*, *ninaA^{Q137L}* in Table I and Supplementary Figure 1). These results support the hypothesis that PRCs with reduced levels of *ninaA* are protected from apoptosis, either downstream or in parallel to caspase activation.

We next tested whether the resistance to PRC death, induced by loss of *ninaA*, is due to the accumulation of misfolded Rh1 in the ER. If true, reducing the amount of Rh1 in *ninaA* mutant retinas should reduce resistance to PRC death. Using a *rh1* null allele (*ninaE¹¹⁷*, here termed *rh1¹¹⁷*), we found that double heterozygous mutants for *ninaA* and *rh1* exhibited similar death sensitivity as wild-type PRCs (Figure 2B–E and G). In addition, PRCs carrying a heterozygous *rh1* null mutation in an otherwise wild-type background were not protected from cell death (Figure 2D). This indicates that it is the accumulation of unfolded Rh1 in the *ninaA* mutant retina responsible for the death resistance of PRCs and not the loss of Rh1 function. We then tested whether cells exposed to unfolded Rh1 in the ER could phenocopy the protective effect seen in *ninaA* mutant retinas. There are several dominant *rh1* mutations leading to Rh1 misfolding and accumulation in the ER (Colley *et al*, 1995). PRCs maintain their integrity for up to 45 days under normal light and temperature conditions, but dominant *rh1* mutations cause a slow age-dependent retinal degeneration when exposed to constant light. (Kurada and O'Tousa, 1995, and data not shown). We examined PRC death sensitivity in *rh1^{G69D}* retina in 2-day-old adults submitted to *dp53* or *rpr* expression. As in *ninaA* mutant retina, PRCs exhibited strong resistance to *dp53* (Figure 2F and G) and *rpr* expression (data not shown).

To circumvent possible issues relating to the ectopic expression of apoptotic genes under the control of the UAS/GAL4 system, we tested whether *ninaA* mutations could inhibit cell death in another PRC degeneration paradigm. Loss of *apc1* triggers ectopic *armadillo* expression and PRC degeneration (Ahmed *et al*, 1998). We found that while *apc1^{Q8}* adult retina exhibited complete PRC loss (Figure 2H), heterozygous or homozygous *ninaA* mutations were sufficient to induce a partial rescue of PRCs (Figure 2I and J). Taken together, these results favour a model in which

accumulation of unfolded Rh1 in the ER is responsible for the increased resistance to death in PRCs.

Misfolded Rh1 induces an UPR

We next investigated whether the accumulation of misfolded Rh1 in the ER causes UPR activation in *Drosophila*. During UPR, Ire1 catalyses the unconventional splicing of a small intron from the mRNA of *xbp1* (Yoshida *et al*, 2001). This splicing creates a frameshift in the *xbp1* mRNA, creating an active transcription factor. A stress indicator was engineered by fusing *Drosophila* Xbp1 with GFP. Upon ER stress, the spliced mRNA is translated into a Xbp1:GFP fusion that can be detected by fluorescence or with antibodies against GFP (Ryoo *et al*, 2007). To detect the UPR in *ninaA* and *rh1* mutants, we first used the Xbp1:GFP sensor as readout for UPR activation. *xbp1* splicing was detected by the presence of GFP, revealed using an antibody against GFP in horizontal cryosections (Figure 3; Supplementary Figure 2). Expression of the Xbp1:GFP sensor in a wild-type background showed a low level of GFP immunoreactivity (Figure 3A), suggesting that PRCs are exposed to basal UPR activation due to the high levels of rhabdomere-targeted Rh1 proteins that transit through the ER. We observed high levels of Xbp1:GFP in *rh1^{G69D}* mutant retina (Figure 3C; Ryoo *et al*, 2007). Strikingly, the loss of one genomic copy of *ninaA* was sufficient to cause UPR activation, as indicated by the significant increase of GFP immunoreactivity (Figure 3E; Supplementary Figure 2). This is in accordance with the fact that reducing the dose of *ninaA* by 50% is sufficient to cause the accumulation of misfolded Rh1 in the ER (Baker *et al*, 1994). Complete inactivation of *ninaA* exhibited widespread GFP staining (Figure 3G). These results indicate that Ire1-dependent *xbp1* splicing is activated in response to the accumulation of misfolded Rh1 in the ER of *ninaA* and *rh1^{G69D}* mutant PRCs.

To further characterize the extent of UPR activation, we evaluated the levels of Hsc3 expression in *ninaA* and *rh1* mutant retinas (Figure 3). Similarly to Xbp1:GFP, *rh1^{G69D}* mutant retinas show a robust increase of Hsc3 expression as seen in horizontal cryosections and western blots (Figure 3, D, I, J; Ryoo *et al*, 2007). The loss of *ninaA* also led to an increase in the levels of Hsc3 (Figure 3F and H). Interestingly, western blot quantification shows that the increase in Hsc3 protein is dependent on the dosage of *ninaA* gene expression, as flies heterozygous for *ninaA* exhibit less of an increase

Figure 2 The protective effect induced by the loss of *ninaA* is dependent on Rh1 levels. (A–F) Semi-thin tangential retina sections of 2-day-old flies. Scale bar, 10 μ m. Outer PRC rhabdomeres of representative ommatidium are surrounded by a yellow circle. (A) Wild type. (B) Ectopic expression of *dp53* in outer PRCs (*rh1>dp53*) induces PRC killing. (C) PRC killing is reduced in *rh1>dp53; ninaA^{E110V/+}*. (D) Reduced *rh1* dosage does not protect from *dp53*-induced PRC killing (*rh1>dp53; rh1^{I17/+}*). (E) Reduced *rh1* dosage suppresses *ninaA^{E110V/+}* protective effect in PRCs submitted to *dp53*-induced killing (*rh1>dp53; ninaA^{E110V/+}; rh1^{I17/+}*). (F) A missense mutation in *rh1* protects from *dp53*-induced PRC killing (*rh1>dp53; rh1^{G69D/+}*). (G) Quantitative analysis of photoreceptor survival seen in (A–F). Results are expressed as mean percentage of survival $\pm$ s.e.m. of three animals (* P <0.05; *** P <0.001 in Student's *t*-test). (H–J) Loss of *ninaA* protects from *apc^{Q8}*-induced cell death (1-day-old flies). Scale bar, 10 μ m. (H) *apc^{Q8}* flies display no PRC. (I, J) Loss of *ninaA* reduces cell death induced by *apc^{Q8}* seen by the presence of rhabdomeres (yellow circles). (I) *apc^{Q8}; ninaA^{E110V/+}*. (J) *apc^{Q8}; ninaA^{E110V}*. Representative images of three animals.

when compared with *ninaA* amorphs (Figure 3I and J). This is supported by the fact that the amount of unfolded Rh1 is dependent on the genomic dosage of *ninaA* (Baker *et al*, 1994). For the rest of the study, we will consider that heterozygous *ninaA* mutants induce moderate ER stress, while homozygous *ninaA* or *rh1^{G69D}* mutants trigger strong ER stress. Taken together, the intensity of UPR activation, visualized by *xbp1* splicing and Hsc3 expression, depends on the amount of misfolded Rh1 proteins that accumulate in the ER.

Prolonged and moderate ER stress does not lead to neurodegeneration

Accumulation of misfolded proteins and UPR activation protect PRCs from death in young adult retina (Figures 1 and 2). We next asked whether prolonged ER stress and long-term UPR activation under normal physiological conditions can affect long-term PRC survival. PRC integrity was determined in aged flies (60 days old) carrying *ninaA^{E110V}* or *rh1^{G69D}* mutations, reared under normal temperature (25°C) and light:dark cycle (12:12 h) conditions. In 60-day-old adults, we found that both homozygous *ninaA^{E110V}* and *rh1^{G69D}* mutant retinas (strong ER stress) display defects in the ommatidia trapezoidal arrangement and occasional PRC loss (Figure 4C and D, and data not shown). These results correspond with an earlier study showing that long-term PRC

degeneration occurs in homozygous *ninaA* mutant retinas (Rosenbaum *et al*, 2006). Conversely, heterozygous *ninaA^{E110V}* retinas (moderate ER stress) remained intact, showing normal morphology (Figure 4B) despite displaying hallmarks of ER stress and UPR activation (Figure 3E and F, and data not shown). This result indicates that a prolonged moderate ER stress does not cause PRC death.

An ER-mediated signal inhibits caspase activation

Mutations leading to the accumulation of unfolded Rh1 and activation of the UPR can protect PRCs from cell death induced by *dp53*, *rpr* or the caspase *dcp-1* (Figures 1–3). We first tested whether the UPR activation could lead to a reduction of the *rh1-GAL4/UAS* system and a subsequent decrease of the ectopic expression of the apoptotic proteins. To address this question, we compared GFP expression levels (ectopically expressed using GAL4/UAS system), in *ninaA*, *rh1* mutant and wild-type retinas (Figure 5). Although GFP levels were decreased in homozygous *ninaA^{E110V}* or *rh1^{G69D}* mutant retinas (strong ER stress), no reduction of GFP was observed in heterozygous *ninaA^{E110V}* mutants (moderate ER stress) compared with the β -tubulin loading control (Figure 5A and B). These results suggest that while attenuation of the *rh1-GAL4/UAS* system can contribute to cell death inhibition under strong ER stress conditions, it does not play a role in moderate ER stress situations. In addition, we tested whether

Figure 3 *xbp1* splicing and Hsc3 expression are increased in *ninaA* mutant retinas. Horizontal retina cryosections are stained with an antibody raised against GFP (A, C, E, G) or against Hsc3 (B, D, F, H) in 7-day-old flies. *xbp1:GFP* is expressed in PRCs under the control of *rh1* promoter (*rh1*>*xbp1:GFP*). Scale bar, 100 μm. (A) Wild-type PRCs expressing *xbp1:GFP* show weak GFP staining. (C) *rh1^{G69D}/+*, (E) *ninaA^{E110V}/+* or (G) *ninaA^{E110V}* retina expressing *xbp1:GFP* display an increased GFP staining in PRC nuclei (yellow arrowheads). (B) Wild-type retinas do not exhibit Hsc3 staining. (D) *rh1^{G69D}/+* retinas show a marked Hsc3 staining. (F) *ninaA^{E110V}/+* and (H) *ninaA^{E110V}* retinas show weak Hsc3 staining. (I) Western blot analysis performed on fly eye tissue. *ninaA^{E110V}/+* or *ninaA¹/+* exhibit a clear Hsc3 increase compared with wild-type retina. Hsc3 protein level is further increased in *ninaA¹* compared with *ninaA^{E110V}/+*. The Hsc3 level in *rh1^{G69D}/+* is increased to a level similar to that in *ninaA* amorphs. Representative blot of four independent experiments. (J) Quantification of Hsc3 immunoreactivity. The results are expressed as mean percentage of wild-type control ± s.e.m. of four independent experiments (**P*<0.05; ***P*<0.01; ****P*<0.001 in Student's *t*-test).

the UPR could regulate *rh1* promoter activity. We measured *rh1* promoter activity (using a *rh1-LacZ* transgenic line) by quantifying X-Gal staining of retinal sections and measuring

Figure 4 Mild and long-term accumulation of unfolded Rh1 protein does not promote age-related retinal degeneration. (A–D) Semi-thin tangential retina sections in 60-day-old flies raised at 25°C in a 12:12 light–dark cycle. Outer PRC rhabdomeres of representative ommatidium are circled in yellow. Scale bar, 10 μm. (A) Wild type and (B) *ninaA^{E110V}/+* retinas exhibit a preserved retina structure with intact PRC rhabdomeres (100% ± 0.0 PRC survival). (C) *ninaA^{E110V}* (98.37% ± 0.74 PRC survival) and (D) *rh1^{G69D}/+* (95.94% ± 1.88 PRC survival) retinas show occasional signs of degeneration.

Figure 5 Effect of ER stress on the GAL4/UAS ectopic expression system. (A) Western blot analysis against GFP on head extracts expressing GFP under the control of the *rh1-Gal4* driver in wild-type, *ninaA^{E110V}/+*, *ninaA^{E110V}* or *rh1^{G69D}/+* backgrounds. Representative blot from eight independent experiments. (B) Quantification of the amount of GFP detected in (A), normalized to the loading control and to the amount of GFP expressed in a wild-type background. There is no reduction of GFP protein levels in *ninaA^{E110V}/+* compared with wild type. *ninaA^{E110V}* or *rh1^{G69D}/+* exhibit reproducible reductions in GFP levels compared with wild-type tissues. Reprobing with β-tubulin antibody serves as a loading control. The results are expressed as a mean percentage of wild-type control ± s.e.m. of eight independent experiments (***P*<0.01; ****P*<0.001 in Student's *t*-test).

β-galactosidase activity with an ONPG assay in *ninaA^{E110V}* and *rh1^{G69D}* mutants (Supplementary Figure 3). No difference was observed between the different genotypes, indicating

that *rh1* promoter activity functions normally in PRCs under ER stress. This result corroborates an earlier study showing that *rh1* transcription is unaffected in *ninaA* mutants (Zuker *et al*, 1988). Although we cannot exclude that subtle translation attenuation can push the balance in favour of anti-apoptotic genes in *ninaA*^{E110V} heterozygous retina, it suggests that another ER-mediated mechanism inhibits cell death.

Next, we examined whether ER stress inhibits caspase activation. If *ninaA* mutants block primarily caspases sensitive to the baculovirus caspase inhibitor p35, then *ninaA* mutations should not show an additive effect on PRC protection mediated by p35 expression. We took advantage that *p35* only partially

suppresses *dp53*-induced death (Figure 6 and data not shown). We found that *dp53*-induced PRC death was equally inhibited by the expression of *p35* or by *ninaA* mutation. Moreover, *p35*-mediated caspase inhibition was not enhanced by *ninaA* mutations (Figure 6A–E). These results suggest that *ninaA* mutation blocks primarily *p35*-inhibitable caspases in PRCs submitted to *dp53*-mediated PRC death.

We then examined the effector caspase activation levels in PRCs submitted to ER stress by measuring PARP cleavage and using a DEVD-based caspase activity assay. We first assayed cleavage of the genetically encoded caspase probe CD8:PARP:Venus as a read-out for effector caspase activation (Williams *et al*, 2006). We found that loss of *ninaA* strongly

Figure 6 Caspase activation is inhibited in the *ninaA* mutant retina. (A–D) Semi-thin tangential retinal sections of 13-day-old flies expressing *dp53* in outer photoreceptor cells. Outer PRC rhabdomeres of representative ommatidium are circled in yellow. Scale bar, 10 μm. (A) Ectopic expression of *dp53* (*rh1>dp53*) induces outer PRC, but not inner PRC death that do not express *dp53* (orange arrowhead). (B) *dp53*-induced PRC death is reduced by p35 concomitant expression (*rh1>dp53; gmr-p35*). (C) *dp53*-induced PRC death is reduced in *ninaA* mutants (*rh1>dp53; ninaA^{E110V/+}*). (D) *dp53*-induced PRC death is reduced by p35 expression in the *ninaA* mutant (*rh1>dp53; ninaA^{E110V/+}; gmr-p35*). (E) Quantitative analysis of photoreceptor survival seen in (A–D). The results are expressed as mean percentage of survival ± s.e.m. of three animals. (F) Caspase activity measured by the appearance of a cleaved PARP (cPARP) epitope by western blot using an anti-cPARP antibody. cPARP is present in tissue expressing *dp53* in wild type but strongly reduced in *ninaA^{E110V/+}* background. Reprobing with β-tubulin antibody serves as loading control. Representative blot of three independent experiments. (G) Quantification of cPARP immunoreactivity. The results are expressed as mean percentage of total ± s.e.m. of three independent experiments. (H) Caspase activity measured by luminescence activity. The results are expressed as luminescence signal relative to the amount of protein ± s.e.m. of three independent experiments (***P* < 0.01; ****P* < 0.001 in Student's *t*-test).

suppressed the *dp53*-induced cleavage of CD8:PARP:Venus in PRCs as visualized by western blot analysis (Figure 6F and G). Next, we measured effector caspase activity in retina extracts using a caspase activity assay (Caspase Glo[®]). DEVD cleavage is strongly impaired in heterozygous *ninaA*^{E110V} mutants compared with wild-type retinas (Figure 6H), suggesting an inhibition of *Drosophila* interleukin-1 converting enzyme (Drice) activity (Fraser *et al*, 1997). Together with the observation that *ninaA* mutants inhibit *dcp-1*-induced apoptosis (Figure 1I and J), our results indicate that an ER-mediated protective signal inhibits the activation of effector caspases.

An antioxidant response is induced upon ER stress

We found that a moderate ER stress protects PRC from apoptosis (Figures 1, 3 and 6). It has been proposed that the UPR contributes to redox homeostasis after ER stress in mouse fibroblasts (Cullinan and Diehl, 2004). We asked whether an antioxidant response is induced in *Drosophila* PRCs under ER stress. We have observed that the antioxidant genes *ferritins* (*fer*) protect PRCs from death induced by ectopic expression of apoptotic genes or by light-mediated photo-oxidation (AG, CSM, HS and BM, unpublished). *Fer* are iron storage proteins found in all animal species, and thought to sequester ferric iron (Fe³⁺) in a nonreactive form that cannot promote redox reactions (Harrison and Arosio, 1996). We thus evaluated expression of the antioxidant *fer* in ER-stressed retinas. The *Drosophila* multimeric *ferritin* complex is composed of *fer* light chain (*fer2lch*) and *fer* heavy chain (*fer1hch*). As a means to assess transcription activation of *fer* genes, we used an enhancer trap transposable P-element, carrying β -galactosidase inserted in *fer1hch* or *fer2lch* gene locus. We found increased *fer2lch* expression in nuclei of PRCs mutant for *ninaA*^{E110V} or *rh1*^{G69D} (Figure 7A–D; Supplementary Figure 4A). However, no *Fer2lch* protein variation was detected by western blots analysis of dissected *ninaA*^{E110V} or *rh1*^{G69D} retinas compared with control retinas (data not shown). This could be due to strong *fer2lch* levels in the optic lobe, masking any increase of levels in the PRCs due to ER stress (Figure 7A–D). In contrast to *fer2lch*, *fer1hch* remained constant (data not shown). The increase of *fer2lch* transcripts suggests that an antioxidant response could contribute to the protective effect in ER-stressed retina.

The *Drosophila* eIF4E binding protein (d4E-BP) is induced in response to oxidative stress (Tettweiler *et al*, 2005). Although d4E-BP is not an antioxidant protein *per se*, it downregulates translation of cap-dependent mRNA and mediates survival in animals exposed to oxidative stress. Whether ER stress is capable of inducing *d4E-BP* expression is not known. We examined *d4E-BP* levels in retinas submitted to ER stress. We found that PRCs under ER stress exhibit an increase of *d4E-BP* transcriptional activity and protein expression compared with wild-type conditions (Figure 7E–I; Supplementary Figure 4B). We used the enhancer trap line *Pz* [*d4E-BP*⁰⁶²⁷⁰] to evaluate *d4E-BP* transcriptional activity in *ninaA* and *rh1* mutant retinas. We observed that the *rh1*^{G69D} mutant exhibited a marked increase in β -galactosidase activity staining compared with the wild-type retina (Figure 7H). Increased staining in *ninaA*^{E110V} and *ninaA*¹ heterozygotes was modest, which is consistent with the fact that *ninaA* heterozygous mutants exhibit a weaker UPR than in *rh1*^{G69D}

retinas (Figure 3J). We next tested whether the upregulation of *d4E-BP* transcriptional activity is associated with an increase of d4E-BP protein expression in western blots of *ninaA* or *rh1* mutant retinas (Figure 7I). Retinas submitted to ER stress (*ninaA*¹, *ninaA*^{E110V} and *rh1*^{G69D} alleles) exhibited an increase of d4E-BP protein compared with wild type (Figure 7I, and data not shown). This result shows that PRCs under ER stress trigger a stress response marked by the induction of *ferritin* and *d4E-BP* genes.

To evaluate redox status in PRCs submitted to ER stress, we measured oxidative stress levels by immunodetection of carbonyl groups in oxidized proteins (Levine *et al*, 1994). We found an overall reduction of carbonylated proteins in ER-stressed mutant retina, which is most evident in homozygous *ninaA* retina (*ninaA*¹ and *ninaA*^{E110V}) (Supplementary Figure 5). This suggests that retinas submitted to ER stress exhibit reduced oxidative stress levels.

We then explored whether an ER-mediated stress response is capable of protecting from reactive oxygen species (ROS)-induced cell death. We used an *in vitro* approach in which pharmacologically induced ER stress protects cultured cells from apoptosis. *Drosophila* S2 cells can activate an UPR when submitted to tunicamycin (Tm, a glycosylation inhibitor) or thapsigargin treatment (Tg, an ER calcium storage inhibitor) (Plongthongkum *et al*, 2007; Ryoo *et al*, 2007). When pre-treated with a sub-lethal dose of Tm or Tg, S2 cells exhibit oxidative stress resistance to H₂O₂ (Figure 8A, and data not shown). This result suggests that moderate ER stress can protect cells from oxidative stress. We did not observe an increase in *ferritin* expression in S2 cells, suggesting that these cells may induce the expression of other antioxidant genes to promote an antioxidant response. In contrast, we found that Tm or Tg treatments induced a dose dependent increase of d4E-BP proteins (Figure 8B). This result is consistent with the fact that PRCs submitted to ER stress exhibit an increase of d4E-BP protein expression.

Next, we tested whether moderate ER stress could also protect from other apoptotic stimuli, such as cycloheximide (CHX) and ultraviolet (UV). We found that Tm and Tg also protected S2 cells from apoptosis induced by CHX and UV. As moderate ER stress is capable of inhibiting caspase activation in the fly retina (Figure 6), we tested whether Tm can limit caspase activation induced by CHX in S2 cells. We found that a pre-treatment with Tm mitigates caspase activation (Figure 8C).

To test whether Tm-mediated protection requires the UPR, we inactivated *xbp1* gene using RNAi. Double-stranded RNA (dsRNA) against *xbp1* was efficient as it totally abrogated *xbp1* and strongly reduced *hsc3* transcript expression (Figure 8D). Tm-mediated protection is *xbp1*-dependent as dsRNA against *xbp1* restored UV-induced cell death (Figure 8E). This suggests that the UPR mediates the Tm survival response in S2 cells. All together, these results argue that moderate ER stress protects from both ROS- and caspase-dependent cell death *in vitro* and *in vivo*.

Discussion

In pathological conditions such as diabetes and neurodegenerative diseases, a massive accumulation of misfolded

Figure 7 ER stress triggers upregulation of the *fer2lch* and *d4E-BP* genes. (A–H) Horizontal retinal cryosections stained for nuclear β-galactosidase activity from flies carrying the transposable P-element line inserted in *fer2lch* (Pz[ry, lacZ]^{*fer2lch035*}) (A–D) or *d4E-BP* (Pz[ry, lacZ]^{*thort(2)06270*}) (E–H). Arrows point to nuclear β-galactosidase activity staining in PRC nuclei (B–H). (A) Wild-type retinas exhibit a *fer2lch* basal background staining in the optic lobe but no apparent staining in the retina. (B) *ninaA^{E110V}/+*, (C) *ninaA^{E110V}* and (D) *rh1^{G69D}/+* retinas exhibit increased β-galactosidase activity staining at the level of outer PRC nuclei. (E) Wild-type retina exhibit some *d4E-BP* staining in nuclei. *d4E-BP* expression is merely increased in *ninaA¹/+* (F) and *ninaA^{E110V}/+* (G) mutant retinas, but a marked increase is observed in *rh1^{G69D}/+* (H). Scale bar, 100 μm. (I) Western blot analysis of d4E-BP protein levels in *rh1^{G69D}/+* and *ninaA¹* compared with wild-type fly eye tissues. Representative blot of three independent experiments.

proteins in the ER induces a UPR, which in turn triggers apoptosis (Marciniak and Ron, 2006). Here we show that a more moderate activation of the UPR (*ninaA* and *rh1* mutants) can mediate a survival response. The UPR protects PRCs from apoptosis induced by the expression of pro-apoptotic genes, *rpr*, *dp53* and *dcp-1*, as well as the degeneration observed in *apc* mutants (Figure 1). We found that the ER inhibits caspase activation and triggers an antioxidant response (Figures 6 and 7). In addition, we showed that a pharmacological pre-treatment with sub-lethal dose of the ER inducers (Tm or Tg) protects S2 cells from H₂O₂, CHX or UV exposure (Figure 8, and data not shown). Our results indicate that moderate ER stress mediates a

cellular response that inhibits additional external insults and allows the cells to have a survival response to the initial ER stress.

The ER stress-mediated protective effect can be assimilated as pre-conditioning or an adaptative stress response, also termed hormesis. Hormesis is a cellular protective signal induced by exposure to a low dose (or mild) stress-inducing agent that allows the cell to better respond to a second insult (for review Mattson, 2008). Our work is an example of ER-mediated hormesis (or ER-hormesis), because low levels of ER stress cause the UPR to protect the cells against cell death, whereas high levels of ER stress cause the UPR to trigger the apoptosis pathway.

Figure 8 Mild ER stress protects S2 cells from oxidative and apoptotic insults. (A) Percentage of cell death was evaluated by trypan blue uptake in untreated (control, gray columns) and pre-treated S2 cells with 1 μg/ml of Tm (black columns), which were then submitted to H₂O₂ (500 nM), CHX (1 μM) or UVC exposure (300 mJ/cm²). The results are expressed as mean percentage of cell death ± s.e.m. of three independent experiments. (B) Tm or Tg treatment increased d4E-BP expression in a dose-dependent manner. d4E-BP expression is visualized by western blot analysis on S2 cells treated with Tm or Tg for 6 h. (C) Tm pre-treatment reduces caspase activity induced by CHX treatment. Cells were either untreated (control, gray columns) or pre-treated with 1 μg/ml of Tm (black columns) then submitted to CHX (1 and 10 μM). The results are expressed as RLU (relative luminescent unit) ± s.e.m. of one experiment (triplicates) representative of three independent experiments. (D) *xbp1* knock down by dsRNA strongly reduced the amount of *xbp1* and *hsc3* transcripts. The expression of the ribosomal *rp49* gene was used as internal control for RNA extraction and RT-PCR efficiency. Its expression is constant in control and treated cells. (E) Knock down of *xbp1* reverts the protective effect of Tm. The results are expressed as mean percentage of cell death ± s.e.m. of three independent experiments. (in A, B, C and E, **P* < 0.05; ***P* < 0.01; ****P* < 0.001 in Student's *t*-test).

What makes ER stress lethal?

The current view is that prolonged intense ER stress promotes apoptosis. UPR-regulating proteins have been implicated in the activation of death factors that trigger caspase activation (Nakagawa *et al*, 2000; Hetz *et al*, 2006; Puthalakath *et al*, 2007). Here, we have shown that prolonged but mild ER stress can promote a long-term survival response, allowing the cells to cope with additional oxidative or apoptotic insults. So what is the cellular switch that pushes the cell from survival to death? Our data suggest that both intensity and duration of the ER stress play a part in this decision. We show that a strong ER has short-term protective effects but leads to PRC degeneration in the long term (homozygous *ninaA* mutants and *rh1*^{G69D}, Figures 1, 2 and 4). In contrast, more moderate ER stress is protective in the

short term and remains harmless even if it is maintained the entire life of the animal (heterozygous *ninaA* mutant tested in 60-day-old animals, Figure 4).

Using an ADRP rat model, Peter Walter and colleagues have recently brought some insight to a molecular switch that pushes PRCs towards death pathway (Lin *et al*, 2007). They have shown that rat PRCs carrying the misfolding mutation *rh1* (*rh1*^{P23H}) degenerate at early post-natal stages. In contrast with *ninaA* mutant flies, 'low level' ER stress leads to rat PRC demise within a few weeks (Transgenic rat TgP23H3). This may be due to the fact that the transgenic expression of misfolded Rh1 still generates too much ER stress. Nevertheless, the same group has shown that sustained Ire1 does not promote death, whereas sustained Perk mediates cell death (Lin *et al*, 2007, 2009). Our results support the

hypothesis that Ire1 has a protective effect, as inactivation of *xbp1* is sufficient to abolish the Tm protective effect in S2 cells (Figure 8E).

Is a moderate activation of the Ire1 pathway mandatory for long-term *Drosophila* PRC survival? Upon ER stress, we have shown that Ire1-mediated *xbp1* splicing is induced in PRCs, as demonstrated by the appearance of spliced *xbp1* mRNA and Hsc3/Bip protein (Figure 3). A moderate increase of Hsc3 expression is associated with long-term resistance to cell death in heterozygous *ninaA* mutants. In contrast, a more robust Hsc3 expression is associated with short-term protection and long-term neurodegeneration in homozygous *ninaA* mutants (Figures 3 and 4). Thus, a moderate activation of the Ire1 pathway is correlated with a long-term PRC survival.

Although all UPR branches are activated upon ER stress, the immediate response is Perk/eIF2 α -mediated translation attenuation (Wu and Kaufman, 2006). We found that when the ER stress is intense (homozygous *ninaA* or *rh1*^{G69D} mutant retinas), the reduction of protein synthesis limits the expression of proteins in PRCs through the UAS/GAL4 system, hence contributing to attenuated PRC death (Figure 5). In contrast, when ER stress is moderate (heterozygous *ninaA* mutant retina), there is no apparent reduction of protein synthesis and no long-term deleterious effects. This suggests that Perk/eIF2 α is not activated or does not mediate the translation attenuation in heterozygous *ninaA* mutant retinas. One possible explanation is that limited activation of the Perk/eIF2 α pathway favours survival. In support of this hypothesis, attenuation of Perk-mediated eIF2 α phosphorylation by the compound salubrinal protects cells from the deleterious consequences of prolonged ER stress (Boyce *et al*, 2005). In addition, the genetic reduction of eIF2 α protects from oxidative stress (Tan *et al*, 2001). Thus, low level of Perk/eIF2 α pathway activation is correlated with enhanced cell survival.

What are the signals that inhibit PRC death in ER-stressed retinas?

We found that ER stress mediates caspase inhibition and apoptosis resistance in PRCs (Figure 6). In addition, ER stress induces an antioxidant response as shown by the increase of *fer2lch* and *d4E-BP* expression (Figures 7 and 8B). This antioxidant response is associated with a reduction of basal oxidative stress levels as visualized by the detection of protein oxidation in the retina (Supplementary Figure 5). We propose that the inhibition of basal levels of oxidative stress limits caspase activation and contributes to the protection of PRC submitted to ER stress.

In support of an ER-mediated antioxidant response, we show S2 cells, pre-treated with Tm or Tg, are resistant to ROS exposure (H₂O₂) (Figure 8A, and data not shown). Similarly, it has been shown that UPR activation counteracts ROS accumulation and cell death induced by tumour necrosis factor alpha in mouse embryonic fibroblasts (Xue *et al*, 2005). Together, these data argue that the UPR can limit ROS accumulation, inhibiting apoptosis in a conserved process.

Activation of the UPR, as well as the increase of d4E-BP upon ER stress, suggests that selective changes in protein levels regulate cell death. In support of this hypothesis, it was proposed that 4E-BP favours the translation of proteins

through their internal ribosome-entry site (IRES), contributing to survival (for review Holcik and Sonenberg, 2005). The translation of genes, such as Bip, that contain IRES in their mRNAs can limit the UPR and protect the cell from excessive ER stress.

Although it has been shown that ER stress can induce the IRES-dependent translation of the human inhibitory of apoptosis protein 2 and reduce cell death (Warnakulasuriyachchi *et al*, 2004), we failed to detect an increase of the *Drosophila* inhibitory of apoptosis protein 1 (DIAP1), measured by western blot or immunofluorescence of *Drosophila* retinas exposed to ER stress (data not shown). It is possible that other IRES-dependent proteins contribute to an increased antioxidant response and the inhibition of caspase activation in PRCs. It has been shown that an antioxidant treatment can prevent JNK-mediated caspase activation in NF- κ B deficient mouse fibroblasts (Kamata *et al*, 2005). Whether an ER-mediated signal can inhibit JNK-induced cell death remains to be demonstrated.

Relevance to pathology

Increasing evidence has linked ER stress and the UPR to pathologies such as neurodegenerative diseases, cancer and diabetes (Marciniak and Ron, 2006). ER stress markers have been observed in degenerating tissues, and it has been proposed that an overloaded ER promotes cell death. Our data tackle a new and important issue, in that moderate ER stress is not only protective against unfolded protein accumulation but also against external apoptotic insults. We also show that moderate and protective ER stress remains harmless in the long term. Whether moderate ER stress in a healthy mammalian brain could protect against or delay the onset of neurodegenerative diseases remains to be demonstrated.

Materials and methods

Fly stocks

The following genotypes were used: Canton S (CS) and *cnbr* as wild-type strains. The *ninaA*¹ (*ninaA*^{W208@}), *pzfer1hch*⁰⁴⁵¹, *pzfer2lch*⁰³⁵, *pzthor*¹⁽²⁾⁰⁶²⁷⁰ alleles were obtained from the Bloomington Stock Center. The *ninaA* alleles *ninaA*^{E110V}, *ninaA*^{Q137L}, *ninaA*^{R120K}, and *ninaA*^{G98D} were a gift from Charles Zuker (Ondek *et al*, 1992). The *apc*^{Q8} allele was a gift of Yashi Ahmed. The *rh1* alleles, *ninaE*¹¹⁷ and *ninaE*^{G69D} (termed *rh1*¹¹⁷ and *rh1*^{G69D}, respectively in the text) were a gift from Joseph O'Tousa (Kurada and O'Tousa, 1995). The *rh1-Gal4* driver, a gift from Jessica Treisman, was used for ectopic expression in the outer PRCs (Mollereau *et al*, 2000). The *uas-CD8:PARP:venus* stock was a gift from Darren Williams (Williams *et al*, 2006). The following genetic combinations were used to express transgenes in adult outer PRCs: (1) *rh1-Gal4; uas-rpr*, (2) *rh1-Gal4; uas-dp53*, (3) *rh1-Gal4; uas-dcp-1*, (4) *rh1-Gal4; uas-xbp1:GFP*, (5) *rh1-Gal4; GMR-p35*, (6) *rh1-Gal4; uas-GFP* and (7) *rh1-Gal4; uas-CD8:PARP:venus*. Flies were maintained at 25°C and a 12:12 h light cycle.

Caspase assay

S2 cells were seeded in 96-well culture plates at 15 × 10³ cells per wells. After a 4-h Tm treatment, cells were submitted to CHX for 5 h and 30 min. Caspase-Glo[®] 3/7 reagent (Promega, France) was added (V/V) to wells and incubated for 1 h 30 min. Luminescence was measured by a luminometer (VeritasTM microplate luminometer).

RT-PCR

The effect of *xbp1* knock down on S2 cells was monitored by RT-PCR. mRNA was extracted from treated S2 cells using RNeasy Mini Kit (Qiagen). cDNA was produced using the Enhanced Avian

RT First Strand Synthesis Kit (Sigma). *xbp1* mRNA expression was visualized after 22 cycles and melting temperature of 58°C.

A more detailed Material and methods section can be found in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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