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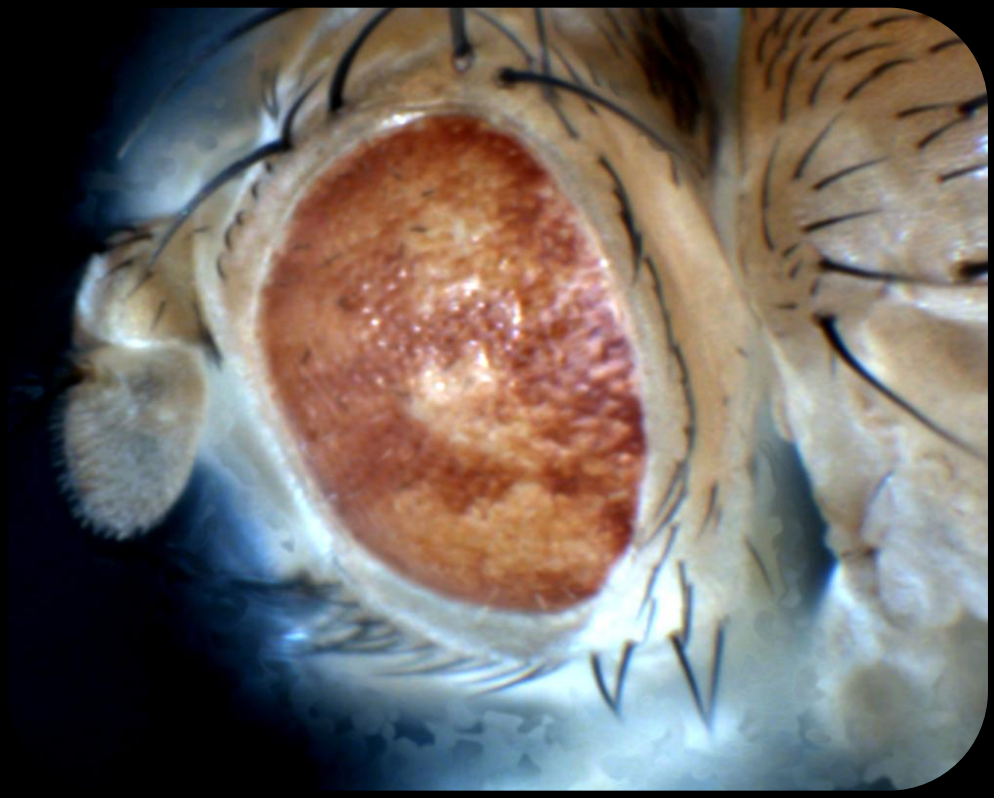
Nadine Simone Schweizer



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
June, 2015

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Summary

The Unfolded Protein Response (UPR) is a signaling pathway that is activated by an accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) that causes ER stress. The activation of the UPR aims to restore ER homeostasis by attenuation of ER client protein translation, increased transcription of ER chaperones and ER associated degradation (ERAD) factors. If ER stress is too long or too strong, cells may die. The main signaling branch of the UPR is mediated by the ER transmembrane protein IRE1 and the transcription factor Xbp1. The active, spliced form of Xbp1 (Xbp1spliced) acts as a transcription factor with protective function against toxic protein aggregation. However, over-expression of Xbp1spliced in the developing *Drosophila* eye causes degeneration of the eye ("glossy" eye phenotype).

In this work, we performed a mosaic genetic screen to identify downstream mediators of Xbp1spliced-induced cell death in the *Drosophila* eye and found that mutations in the gene CG6758, encoding an Fbox protein with unknown biological function, suppress Xbp1spliced-induced "glossy" eye phenotype. Fbox proteins form complexes with Skp, Cullin-1 and E2 ubiquitin ligases (SCF complexes) to mediate the ubiquitination of specific substrates, and leading to the degradation of these substrates by the proteasome. In these SCF complexes, the Fbox protein is responsible for substrate specificity, while Skp, Cullin-1 and the E2 ubiquitin ligases can associate with distinct Fbox proteins to ubiquitinate different substrates. We tested several CG6758 candidate substrates, such as Xbp1spliced, Xbp1unspliced, VCP, p53, CHK1 and Hrd1, but for all these we failed to see an accumulation of the protein levels in CG6758 mutant clones. We also found that CG6758 suppresses the "glossy" eye phenotype induced by over-expression of Rhodopsin-1 (Rh-1), which is an ER "client" protein and constitutes an alternative model for ER stress induced cell death. In this

model, CG6758 mutations suppress apoptosis by reducing the levels of Rh-1 in the ER, by a mechanism that is dependent on the activity of the proteasome. We conclude that it is likely that CG6758 substrates somehow regulate ERAD factors that are important for the proteasomal degradation of Rh-1.

Sumário

A *Unfolded Protein Response* (UPR) é uma via de sinalização que é ativada por uma acumulação de proteínas malformadas no retículo endoplasmático (RE), o que provoca stress no RE. A ativação da UPR visa restaurar a homeostase no RE pela atenuação da tradução de proteínas clients do ER, aumento da transcrição de chaperones do RE e fatores reguladores da degradação de proteínas associadas ao RE (ERAD). Se o stress no RE for muito longo ou forte, as células podem morrer. O principal ramo de sinalização da UPR é mediada pela proteína transmembranar residente no RE IRE1 e o fator de transcrição XBP1. Na sua forma ativa, Xbp1-spliced atua como um fator de transcrição com função protetora contra a agregação da proteína tóxica. No entanto, a sobre-expressão de Xbp1spliced no olho de Drosophila durante o desenvolvimento provoca degeneração dos olhos (fenótipo "glossy" no olho).

Neste trabalho, foi realizada um *screen* genético para identificar mediadores da morte celular induzida por Xbp1spliced no olho Drosophila. Descobrimos que mutações no gene CG6758, que codificam uma proteína Fbox com função biológica desconhecida, suprimem o olho "glossy" induzido por Xbp1spliced. Proteínas Fbox formam complexos com Skp, Cullin-1 e ligases E2 de ubiquitina (complexos SCF) para ubiquitinar substratos específicos, o que conduz à degradação destes substratos pelo proteassoma. Nestes complexos SCF, a proteína Fbox é responsável pela especificidade do substrato, enquanto Skp, as ligases E2 de ubiquitina e Cullin-1 podem associar-se com proteínas Fbox distintas para ubiquitinar substratos diferentes. Testamos várias proteinas como candidatos a substrato de CG6758, como Xbp1spliced, Xbp1unspliced, VCP, p53, CHK1 e Hrd1, mas para todos estas proteínas não conseguimos ver uma acumulação dos seus níveis em clones mutantes de CG6758. Descobrimos também que CG6758 suprime "glossy" fenótipo olho induzida

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for my family

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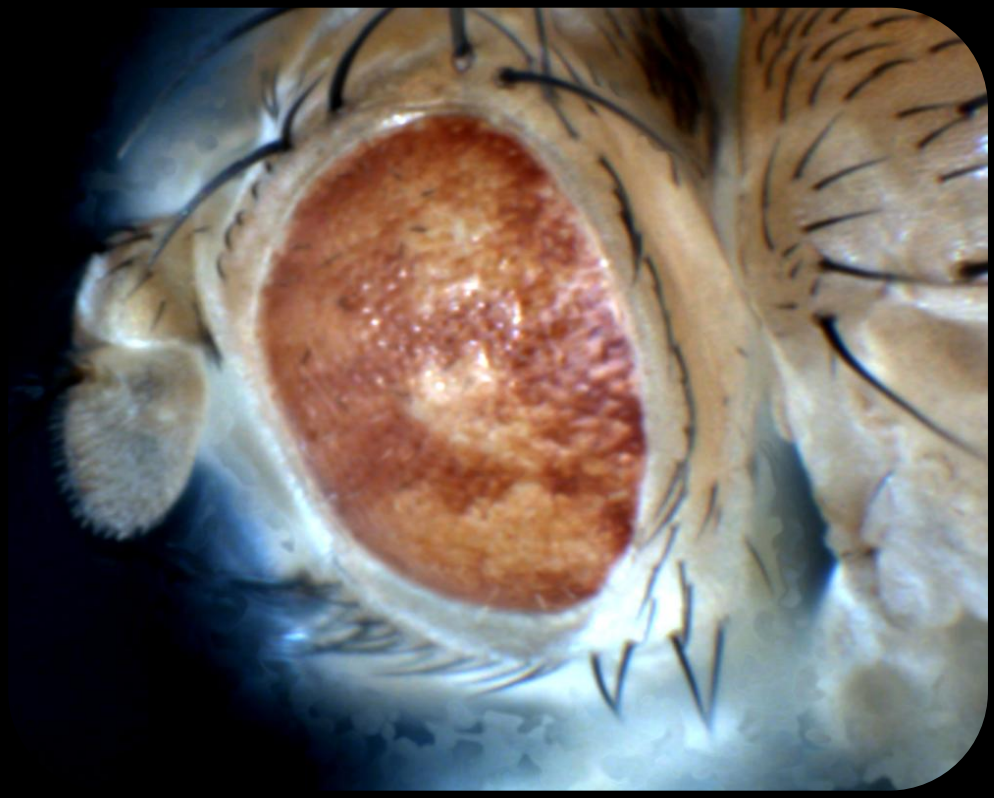

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Chaper I

Introduction

1.1. The Endoplasmic Reticulum

All eukaryotic cells contain an endoplasmic reticulum (ER), a cell organelle that consists of an interconnected network of flattened, membrane-enclosed sacs or tubes known as cisternae. The ER extends from the cell membrane to the cytoplasm, forming a continuous connection with the outer membrane of the nuclear envelope (Alberts et al., 2002).

There are two types of the ER, the smooth and the rough ER, which differ in their functions. Ribosomes sit on the rough ER, whereas the smooth ER is ribosome free.

At and in the ER, translation, protein folding, protein quality control, posttranslational modifications of proteins and protein transport of transmembrane and secretory proteins take place. The ER is also the place for lipid synthesis and the major calcium storage in the cell (Alberts et al., 2002).

Polypeptide chains produced by the ribosomes of the rough ER and in the cytosol are translocated via pore complexes composed of Sec61 and adaptor proteins into the lumen of the ER (Robson and Collinson, 2006), where they are processed and properly folded, a process in which chaperones and foldases take part. Translocation can be either co-translationally or post-translationally, whereby co-translational translocation is initiated when a signal recognition particle (SRP) detects a hydrophobic signal sequence in the nascent polypeptide chain and directs it to the ER membrane (Lütcke, 1995). In post-translational translocation the signal sequence is recognized by receptor proteins of the Sec62/63 complex associated with Sec61 in the ER membrane (Ng et al., 1996).

Most of the secreted proteins are glycosylated and leave the ER lumen in transport vesicles which merge with the Golgi apparatus.

Protein glycosylation includes various glycosidic linkages, including N-, O- and C-linked glycosylation, glypiation (GPI anchor attachment) and phospho-glycosylation. Glycosylation in the ER acts as a quality control

mechanism where it is used to monitor the status of protein folding. Only properly folded proteins are trafficked to the Golgi.

Misfolded or unfolded proteins activate the Unfolded Protein Response (UPR) (see 1.3.) and are degraded by a mechanism called ER associated degradation (ERAD), whereby the proteins are targeted, dislocated to the cytoplasm, ubiquitinated and subsequently degraded by a protein-degrading complex, called the proteasome (see 1.2.). Protein folding in the ER is depicted in **fig.1**.

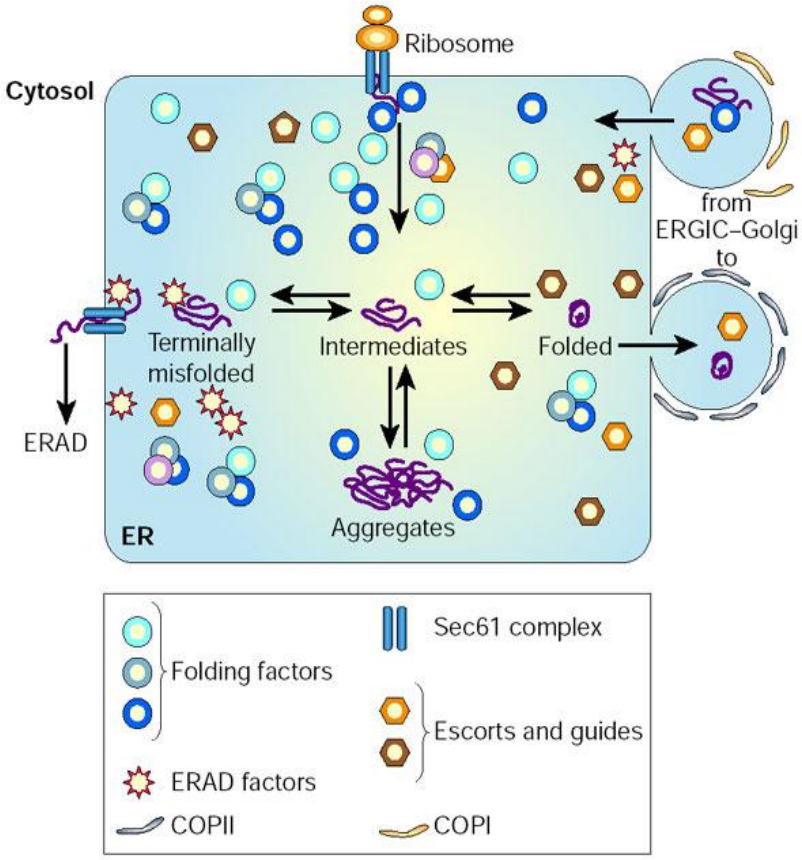

Fig.1: Protein folding in the ER. The port of entry for proteins destined for the secretory pathway is the ER. These proteins are synthesized by ER-associated ribosomes and co-translationally translocated across the membrane through the Sec61 complex. The ER is rich in chaperones and folding enzymes (folding factors), in molecules involved in mediating transport to the cytosol for proteasomal degradation (ERAD factors) or to the downstream stations of the secretory pathway (escort and guides). Some ER-resident proteins seem to form multi-molecular complexes, which can be excluded from transport by size selection, and provide a matrix that couples retention to folding. A distinctive feature of the ER is its ability to catalyse opposite reactions: folding and unfolding, oxidation and reduction, protein import and export through Sec61. It is debated whether a quality-control compartment, involved in the recognition and targeting of terminally misfolded proteins (alluded to in the picture by having ERAD factors concentrated on the left), exists. ERGIC, ER–Golgi intermediate compartment.

From the following article: Quality control in the endoplasmic reticulum protein factory. (2003). Roberto Sitia & Ineke Braakman. Nature 426, 891-894

1.2. ERAD

Endoplasmic Reticulum Associated Degradation (ERAD) is a process in which misfolded or unfolded proteins are cleared from the ER and are degraded by a protein-degrading complex, called the proteasome. The proteasome is a protein complex that serves to degrade most cellular proteins (Rock et al., 1994) and in eukaryotic cells it is located in the nucleus, the cytoplasm and is also associated with the ER (Rivett el al., 1992) and the cytoskeleton (Schemer and Bey, 1994). The proteasome is an essential component of the ATP-dependent proteolytic pathway to degrade rate-limiting enzymes, transcriptional regulators, regulatory proteins and abnormal proteins.

In eukaryotes, the proteasome consists of a big subunit (20S-proteasome) and two small subunits (19S-proteasome), which consist of different proteins (reviewed in McNaught et al., 2001). The 20S-proteasome has a hollow cylindric structure and acts as a multi-catalytic protease. It is built up of two outer rings consisting of 7 distinct α-subunits and two inner rings, consisting of 7 distinct ß-subunits (**fig.2**). The α-subunits are responsible for the substrate recognition and the entry of the unfolded proteins, the ß-subunits confer the proteolytic activity. The 19S-proteasome builds the cap on both sides of the cylindric 20S-proteasome. The 19S-proteasome is responsible for the recognition and unfolding of the delivered proteins. It consists of Rpn and Rpt proteins, whereby Rpn proteins recognize and bind ubiquitinated proteins fated to be degraded, Rpt proteins hydrolyse ATP to deliver the energy required for this process (**fig.2**).

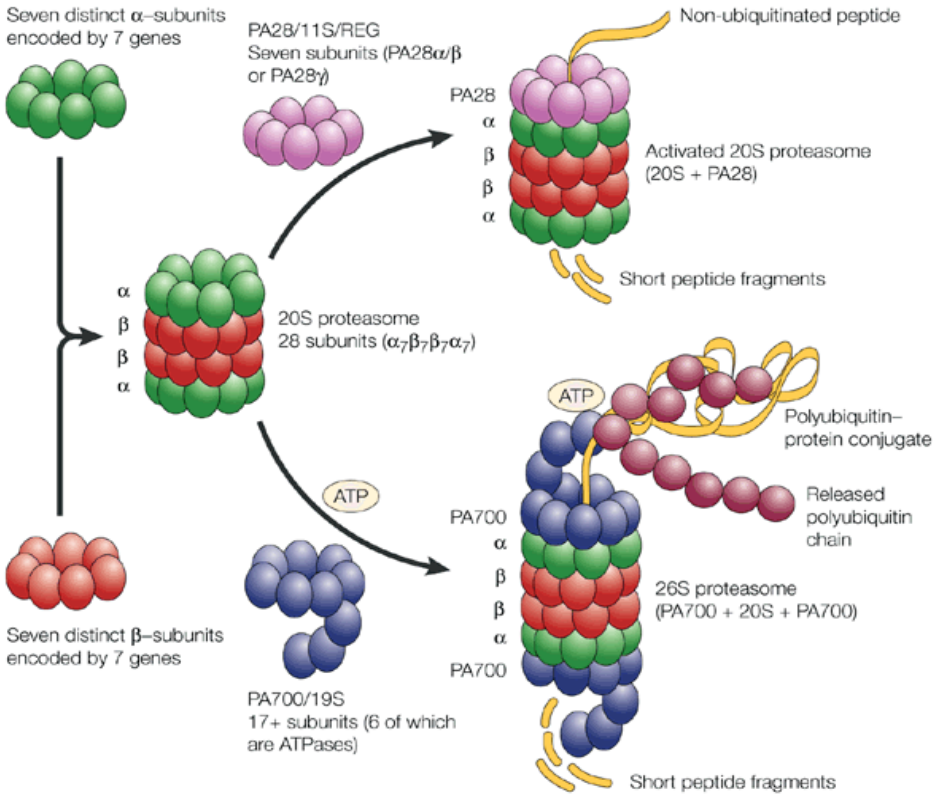

Fig.2: Composition of the 26S-proteasome. The 20S-proteasome is built up of seven a-subunits and seven ß-subunits, forming a cylindric complex in which proteolysis occurs. The two 19S-proteasome subunits form a cap on both sides of the cylinder and serve as ATPases to unfold the delivered proteins fated to be degraded and promote the entry of those unfolded proteins into the 20S-proteasome.

The PA28 (11S) regulatory (REG) complex (180 kDa) can also bind to the 20S proteasome and open the channel through the complex, but this process is ATP-independent, and mediates the degradation of non-ubiquitinated short peptides.

From the following article: Failure of the ubiquitin-proteasome system in Parkinson's disease. McNaught et al. (2001). Nature Reviews Neuroscience 2, 589-594

As mentioned above, ERAD serves to degrade unfolded or misfolded proteins from the ER.

Through studies in *Saccharomyces cerevisiae* three major ERAD sub-pathways are postulated, depending where the lesion that causes protein misfolding is subcellularily located. A misfolded protein with its lesion in the ER lumen is substrate of the ERAD-L sub-pathway, if the lesion is in a trans-membrane domain, degradation is targeted by the ERAD-M sub-pathway and proteins with lesions in the cytoplasmic domain are degraded via the ERAD-C sub-pathway (Vashist and Ng, 2004, Carvalho et al., 2006, Denic et al., 2006).

E3 ubiquitin ligase complexes (E3s) play a central role in all ERAD pathways, since they transfer ubiquitin chains to the misfolded or unfolded proteins, in order to direct them to the proteasome. E3s contain various numbers of trans-membrane domains and a RING domain at the cytosolic site of the ER membrane. E3s catalyze protein ubiquitination (Gardner, R. G. et al., 2000) and act in protein complexes, adaptor molecules facilitate substrate recognition, substrate delivery and regulate the activity of the E3s.

In yeast there are at least three E3 complexes involved in ERAD, Hrd1p (Bordallo et al., 1998), Doa10p (Swanson et al., 2001) and Asi1/2/3 (Ombretta et al., 2014), while in metazoans there are several E3s acting in ERAD, like HRD1, gp78, RMA1(RNF5), TRC8, and TEB4 (MARCH IV) (Kostova et al., 2007). The HRD ligases, which include Hrd1p in yeast as well as HRD1 and gp78 in metazoans are the most characterized E3s involved in ERAD. Adaptor molecules confer specificity to the E3s, Hrd3p in yeast or SEL1L in metazoans are the most characterized adaptor molecules (Gauss et al., 2006a). Hrd3p is a transmembrane protein and its luminal domain contains multiple tetratricopeptide repeats (TPRs) that are thought to facilitate protein-protein interaction. Hrd3p can directly bind to misfolded proteins, recruiting them to the E3 ligase (Denic et al., 2006; Gauss et al., 2006b). Furthermore, Hrd3p/SEL1L can bind to other adaptor

proteins, like the glycan-binding (lectin) protein Yos9p in yeast (Gauss et al., 2006) and OS-9 and XTP3 in mammals (Christianson 2008), recruiting them to the E3. Also house-keeping chaperones may act as adaptors, in mammals Bip was shown to interact with OS-9/XTP3-B-SEL1L (Hosokawa et al., 2008). Bip also interacts with other ERAD components (Ushioda et al., 2008) and substrates (Okuda-Shimizu et al., 2007), facilitating their localisation in proximity to the E3s.

E3s are regulated by their intrinsic stability, e.g. Hrd1p can confer its auto-ubiquitination (Gardner, R. G. et al., 2000), which is inhibited when it is bound to the adaptor protein Hrd3p, ensuring that Hrd1p is only active in the presence of controlled substrate delivery. In metazoans Hrd1 seems to stabilize SEL1L (Iida et al., 2011). Hrd1p forms oligomeres which modulate its activity, oligomerization is conferred by Usa1p (Carvalho et al., 2010; Horn et al., 2009).

The Unfolded Protein Response (see 1.3.) leads to the transcriptional activation of different ERAD components to increase ERAD activity and capacity (Yoshida et al., 2003; Bernasconi et al., 2008). Other central players of the ERAD machinery are the Derlins like Der1p which is a multi-pass trans-membrane protein and interacts with Hrd1p and Hrd3p (Gauss et al., 2006), having an adaptor function. Derlins contain a motif shared with rhomboid proteases, which cleave protein sequences within the membrane. Derlins lack the catalytic sites of rhomboid proteases and the shared motif is assumed to be involved in protein interaction.

Upstream of the E3s, oxidoreductases, like Pdi1p are required to dismantle the compact folds of disulfide-bonded substrates to make them accessible for the degradation pathway. ERAD may also require isomerization of trans-peptidyl-prolyl bonds to eliminate turns (Bernasconi et al., 2010).

Besides the E3s, Vcp (Valosin containing protein, also known as p97/Cdc48p), a cytosolic AAA-ATPase (ATPase associated with diverse cellular activities) also plays a central role in ERAD. Vcp is hexameric and physically interacts with ERAD substrates (Rabinovich et al., 2002), acting

as a chaperone with unfoldase activity and multiubiquitin binding capacity in order to present the ubiquitinated target proteins to the proteasome. Vcp physically interacts with its substrates at the ER membrane, where they become poly-ubiquitinated and are released into the cytosol (Ye, Y. et al., 2001). The Vcp complex is also associated with several deubiquitinating enzymes, including YOD1 and USP13 (Sowa et al., 2009). It was suggested that deubiquitination is required for protein dislocation from the ER (Ernst et al., 2009), with trimming of the poly-ubiquitin chain being necessary to allow the substrate to enter the central channel of the Vcp complex.

The most detailed mechanism of how substrates are delivered to the ERAD pathway is that for luminal glycoproteins. Prior to folding, most glycoproteins interact with the chaperone lectins calnexin (CNX) or calreticulin (CRT), or both; the interaction with these proteins is regulated by the glucosylation state of the oligosaccharide side chain (Helenius et al., 1997). The interaction between calnexin and the nascent proteins is enhanced by additional binding of Erp57, a calnexin-associated disulfide isomerase which stabilizes immature polypeptides and promotes folding (Oliver et al., 1997; Frenkel et al., 2004). Soon after entry into the ER, the gycoproteins enter the “calnexin/calreticulin cycle” (Ellgaard and Helenius, 2003), which is a cycle of binding to and release from these chaperones. CNX and CRT bind to monoglucosylated mannose, followed by removal of the innermost glucose of the glycoproteins due to the action of glucosidase II. The protein-linked glycan is then reglucosylated by the soluble ER enzyme UDP-Glc:glycoprotein glucosyltransferase, which acts as conformational sensor, only if the protein moiety displays non-native three-dimensional structures. When the glycoproteins attain their final native structure, they exit from the CNX/CRT cycle and are channeled to the secretory pathway. Besides the enzymes of the CNX/CRT cycle, mannosidases carry out competing reactions that remove mannose residues, decreasing the likelihood of entry into further folding cycles. The

action of EDEMs (Htm1p in yeast) e.g. lead to the exposure of a terminal α1,6–linked mannose that acts as a key signal for degradation (Molinari et al., 2003; Oda et al., 2003; Gauss et al. 2011). If EDEMs themselves are responsible for targeting the ERAD substrates to the E3 complex is unknown.

Autophagy (see 1.4.2.) is a catabolic process which can also be used to clear the ER from misfolded or unfolded proteins, ERAD and autophagy are coordinated and autophagy is for example activated when the proteasome is impaired (Nedelsky et al., 2008) or when misfolded proteins are resistant to ERAD (Houck et al., 2014). The events and components of ERAD are depicted in **fig.3**.

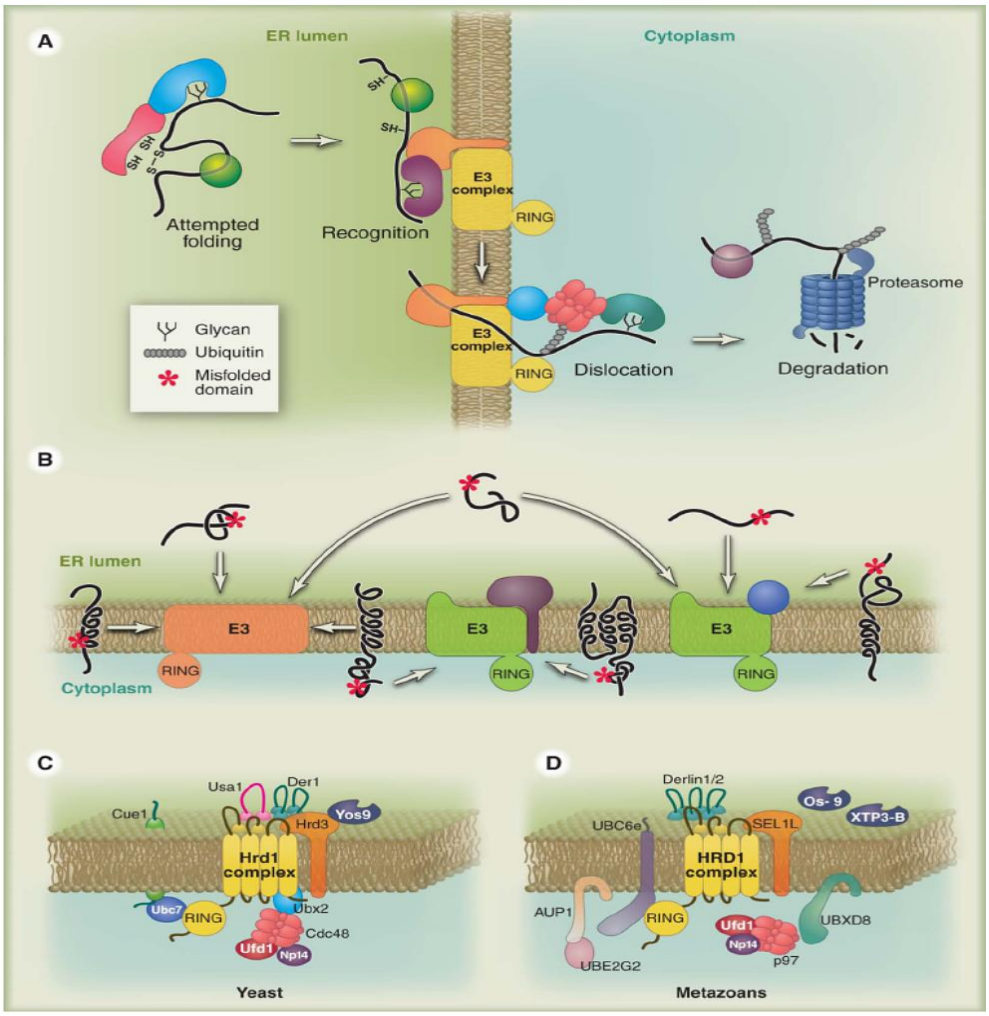

Fig.3: The events and components of ERAD. (A) The key steps of ERAD as shown for a luminal glycoprotein as applied to yeast or metazoans, and thus only the transmembrane E3 ubiquitin ligase complex is labeled along with its catalytic RING domain. **(B)** Overlapping substrate specificity of E3 ubiquitin ligases can be modulated by the presence of adaptors. Adaptors are depicted in purple and blue. **(C)** The core Hrd1p complex in yeast. **(D)** The core HRD1 complex in metazoans.

From the following article: Road to Ruin: Targeting Proteins for Degradation in the Endoplasmic Reticulum. Smith, M. H. et al., 2011. Science 334 (6059)

1.3. The Unfolded Protein Response

Perturbations in protein folding for example due to mutations in the coding sequence, mistakes in transcription or translation or oxidative stress can lead to an accumulataion of misfolded or unfolded proteins in the ER. Those unfolded or misfolded proteins lead to ER stress and cells cope with it by activating a diverse set of signaling mechanisms, collectively known as the Unfolded Protein Response (UPR).

The UPR aims to restore ER homeostasis by several mechanisms including the inhibition of protein translation to stop the load of newly synthesized proteins into the ER, upregulation of ER protein degradation (ERAD) and by increasing the folding capacity of the ER by the transcriptional activation of ER chaperones and foldases. Prolonged or severe ER stress, which can not be resolved by these mechanisms, may lead to apoptosis.

The UPR is composed of three ER stress sensors: Ire1 (Inositol-requiring Enzyme 1), PERK (Protein Kinase RNA-like Endoplasmic Reticulum Kinase) and ATF6 (Activating Transcription Factor 6), which trigger the interconnected pathways of the UPR.

Defective UPR signaling is associated with severe diseases like cancer, degenerative disorders and diabetes.

1.3.1. Ire1 signaling

Ire1 signaling is the most conserved arm of the UPR with homology in yeast, plants, worms, flies and vertebrates (Calfon et al., 2002; Yoshida et al., 1998; Ryoo et al., 2007; Souid et al., 2007). IRE1 is a type I trans-membrane protein, with Ser/Thr kinase and endoribonuclease activities. IRE1 is activated by direct binding of misfolded proteins to the luminal domain of IRE1 and dimerization is also required for activation (Liu, C. Y. et al., 2000; Papa et al., 2003; Shamu and Walter, 1996; Welihinda and

Kaufman, 1996). The cytosolic domain of IRE1 confers the kinase and endoribonuclease activities (Shamu and Walter, 1996; Tirasophon et al., 1998; Liu, C. Y. et al., 2002).

In budding yeast (*Saccharomyces cerevisiae)*, IRE1 signaling is linear. The only substrate to be cleaved by IRE1 endoribonuclease activity, is the mRNA of Hac1 (Cox and Walter, 1996; Mori et al., 1996; Nikawa et al., 1996). Hac1 mRNA is cleaved twice, removing a 252bp intron (Sidrauski and Walter, 1997) and both ends of the exons are joined by the tRNA ligase Trl1 (Sidrauski et al., 1996). This unconventional splicing event occurs in the cytoplasm leading to a translational frameshift and generating an active bZIP (basic Leucine zipper) transcription factor. The unspliced mRNA of Hac1 is not translated due to translational repression (Chapman and Walter, 1997; Cox and Walter, 1996). Xbp1 is the functional homologue of Hac1 in worms, flies and mammals (Calfon et al., 2002; Yoshida et al., 2001; Ryoo et al., 2007; Souid et al., 2007). In mammals the intron to be spliced out by IRE1 activity is 26bp and in flies 23bp. The RNA ligase which joins both ends of the Xbp1 mRNA has not been identified to date. Unspliced Xbp1 (Xbp1u) is translated with a C-terminal frameshift and depicts an inhibitory molecule for the active transcription factor Xbp1spliced (Xbp1s). Xbp1u shuttles between the cytoplasm and the nucleus where it interacts with Xbp1s, leading to the degradation of both proteins (Calfon et al., 2002; Yoshida et al., 2006). The bZIP transcription factor Xbp1s activates the transcription of UPR target genes, like ERAD factors and chaperones and also leads to its own transcription (Lee et al., 2003; Shaffer et al., 2004). In mammals, Xbp1s dependent transcription is linked to cell differentiation, signaling and DNA damage (Acosta-Alvear et al., 2007).

Hac1 mRNA is recruited to IRE1 clusters in the ER membrane, depending on translational repression of Hac1 mRNA and on a bipartite element (BE) at its 3' UTR (Aragón et al., 2009). In human cells, bringing Xbp1 mRNA in proximity to IRE1 depends on 2 hydrophobic regions (hydrophobic regions 1 and 2, HR1 and HR2) in the nascent chain of the Xbp1u polypeptide.

HR1 and HR2 interact with the ER membrane bringing the Xbp1 mRNA-ribosome-nascent chain (RNC) complex to the proximity of Ire1 (Yanagitani et al., 2009). The C-terminal region of the Xbp1u polypeptide is essential for translational elongation pausing, when the Xbp1u polypeptide protrudes from the ribosome exit tunnel. This translational pausing gives time for recruitment of HR1 and HR2 to the ER membrane and stabilizes the RNC complex (Yanagitani et al., 2011).

In unstressed conditions the ER chaperone Bip is bound to the luminal domain of IRE1 and keeps the enzyme inactive (Bertolotti et al., 2000; Okamura et al., 2000). The presence of unfolded or misfolded proteins in the ER titrates Bip away from IRE1, which in turn leads to its oligomerization and activation by auto-phosphorylation. However, it was shown that variants of IRE1 which can not bind to Bip confer IRE1 dependent activity, leading to the suggestion that the Bip titration model is more for inhibiting inappropriate IRE1 activation (Kimata et al., 2003). A study in yeast shows that IRE1 senses ER stress by directly binding to misfolded proteins (Gardner, B. M. and Walter, 2011). However, this mechanism does not seem to be conserved in mammals (Oikawa et al., 2009). In mammals, IRE1α is tissue-specific regulated by distinct regulatory protein complexes through binding of adaptor and modulator proteins (reviewed in Hetz and Glimcher, 2009), like the pro-apoptotic factor BAX and BAK, which modulate the unfolded protein response by a direct interaction with IRE1a (Hetz et al., 2006).

In addition to the transcriptional remodeling of the ER folding environment via IRE1/Xbp1 signaling, IRE1 in metazoans is also involved in the activation of cell death pathways in response to prolonged or strong ER stress, for example activated IRE1 associates with the tumor necrosis associated factor 2 (TRAF2), leading to the activation of the c-Jun N-terminal kinase (JNK) pathway via the apoptosis signaling-regulating kinase (ASK1) (Urano et al., 2000; Nishito et al., 2002). JNK activation then triggers an apoptotic response. Besides a link between IRE signaling and

apoptosis (see 1.4.1.), IRE1 signaling is also linked to autophagy (see 1.4.2.). Another mechanism in which activated IRE1 plays a role, is the degradation of mRNA localized to the ER membrane conferred by its RNAse activity (Hollien and Weissman, 2006; Hollien et al., 2009), leading to a global reduction in the load of newly synthesized proteins into the ER. This mRNA degradation is called RIDD for regulated Ire1 dependent decay. The different IRE1 signaling pathways are shown in **fig.4**.

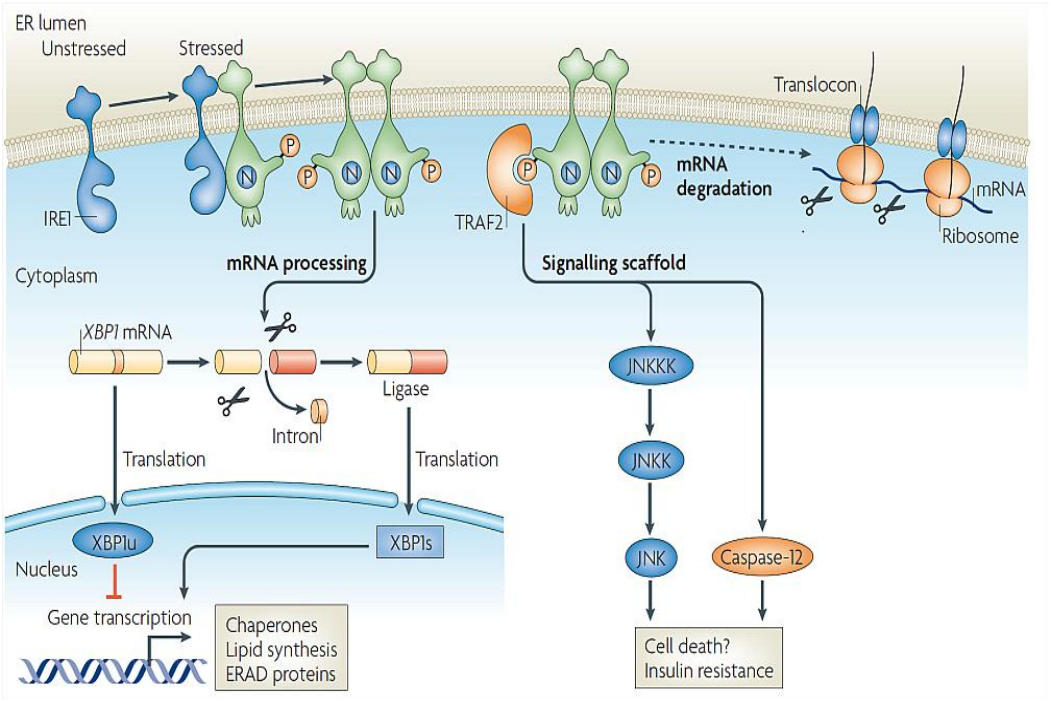

Fig.4: IRE1 signaling. Inositol-requiring protein-1 (IRE1) oligomerizes in the plane of the endoplasmic reticulum (ER) membrane in stressed cells. Trans-autophosphorylation in its cytosolic kinase domain increases the affinity for nucleotides (N), which allosterically activate IRE1 and unmask a dormant endoribonucleolytic activity. IRE1-mediated sequence-specific cleavage of a single known mRNA (X-box binding protein-1 (XBP1) in higher eukaryotes, HAC1 (homologous to ATF/CREB1) in yeast) excises a small RNA fragment (intron). The two ends of the mRNA are ligated (tRNA ligase (Trl1) has this role in yeast but the identity of the ligase is unknown in metazoans), which leads to a frame shift in the coding sequence (shown in the figure as a colour change from yellow to red after removal of the intron). Spliced XBP1 mRNA encodes a potent transcriptional activator (XBP1s), whereas the unspliced XBP1 mRNA encodes XBP1u, an inhibitor of the unfolded protein response (UPR). In yeast, the Hac1/XBP1 pathway activates most of the UPR, whereas in mammals, it appears that XBP1 regulates a subset of UPR genes that promote ER-associated degradation (ERAD) of misfolded proteins and ER biogenesis. IRE1 can also act by alternative means. In mammals, recruitment of TRAF2 (tumour necrosis factor receptor (TNFR)-associated factor-2) by phosphorylated IRE1 allows it to signal to Jun N-terminal kinase (JNK) and alter intracellular signaling (for example, resulting in insulin resistance). The IRE1–TRAF2 complex has also been linked to caspase-12 activation and cell death. In cultured Drosophila melanogaster cells, activated IRE1 can promote the cleavage of various ER-localized mRNAs, leading to their degradation called Regulated IRE1-Dependent Decay (RIDD). This reduces the load on the stressed ER and might facilitate reprogramming of the ER-associated protein synthesis and translocation machinery. It is unknown whether IRE1 cleaves these mRNAs directly or whether it promotes their degradation by activating or recruiting other RNases. JNKK, JNK kinase; JNKKK, JNKK kinase.

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1.3.2. PERK signaling

Like IRE1, also PERK is a type I trans-membrane protein spanning the ER membrane. It has an ER luminal stress-sensing domain and a cytoplasmic kinase domain which undergoes activation by oligomerization and trans-autophosphorylation upon ER stress (Bertolotti et al., 2000). Activated PERK phosphorylates eukaryotic translation initiation factor 2 α (eIF2α) at Ser51. This phosphorylation inhibits the guanine nucleotide exchange

factor eIF2ß**,** a pentameric complex that recycles eIF2 to its active GTP-bound form. Inhibition of eIF2 activity leads to a global decrease in protein translation initiation preventing the additional load of newly synthesized proteins into the stressed ER lumen (Harding et al., 1999). The phosphatase responsible for the dephosphorylation of eIF2α and restoration of normal protein translation when ER stress is resolved, is the protein phosphatase 1 (PP1) in association with CReP (constitutive repressor of eIF2α phosphorylation) and GADD34 (growth arrest and DNA-damage-inducible protein-34) (Jousseet al. 2003; Connor et al., 2001; Novoa et al., 2001).

Although PERK1 leads to translational repression, some proteins can avoid translation inhibition because they have inhibitory upstream open reading frames (uORFs) before the main ORF. Inhibition of eIF2 by phosphorylation of eIF2α leads to ribosomes skipping the inhibitory uORFs and the main ORF is then translated. One of such proteins which is translated upon PERK activation is the yeast transcription factor GCN4 (Hinnebusch et al., 2002) and its mammallian homologue ATF4 (Vattem et al., 2004; Lu, P. D. et al., 2004). ATF4 is a transcription factor that induces upregulation of genes encoding amino acid transporters, redox enzymes that promote protein folding within the ER lumen (e.g., ERO1), and a pro-apoptotic transcription factor called the CCAAT enhancer-binding homologous protein (CHOP) (Ma, Y. et al., 2002). It has been proposed that CHOP indirectly affects cell death by inducing the expression of ER oxireductin 1 (Ero-1, see 1.4.1.), an oxireductase enzyme that catalyses the formation of disulfide bonds in ER proteins (Marciniak et al., 2004). The PERK signaling pathway is shown in **fig.5**.

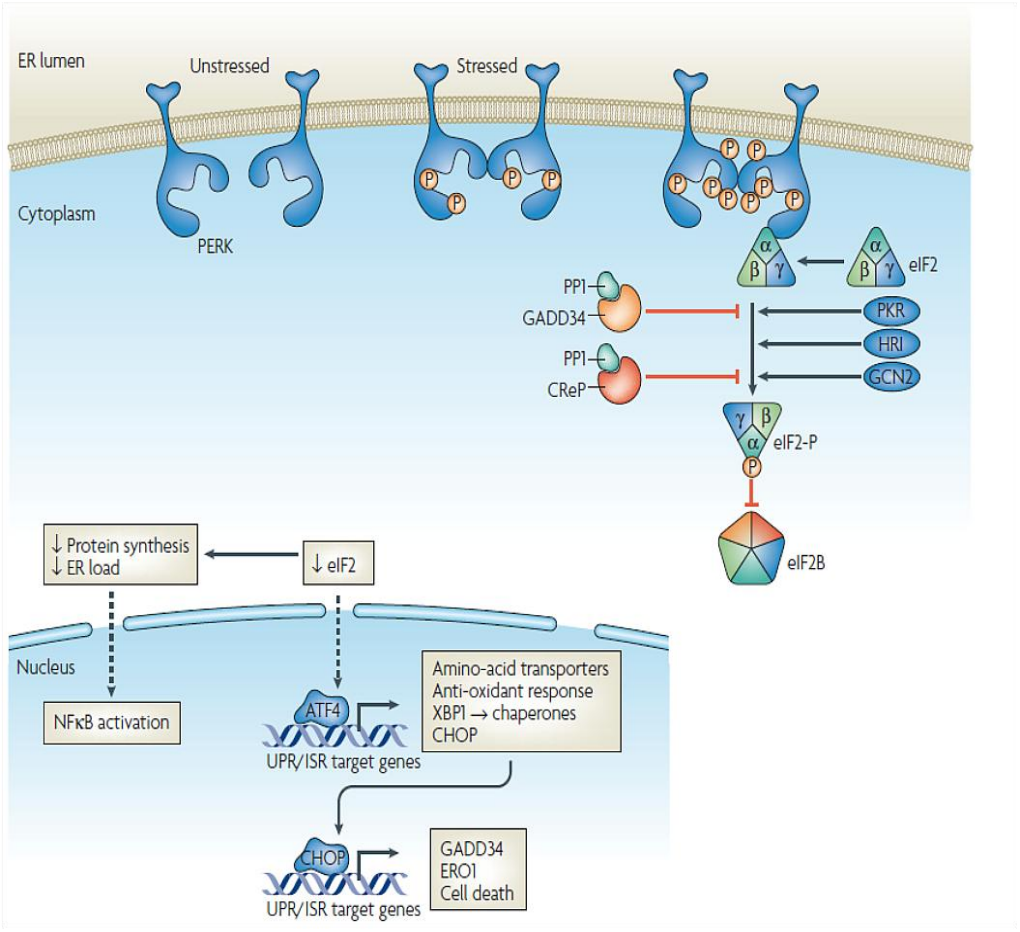
ER lumen
Unstressed
Stressed
Cytoplasm
PERK
eIF2
PP1
GADD34
PP1
CReP
PKR
HRI
GCN2
eIF2-P
eIF2B
↓ Protein synthesis
↓ ER load
↓ eIF2
Nucleus
NFκB activation
ATF4
UPR/ISR target genes
Amino-acid transporters
Anti-oxidant response
XBP1 → chaperones
CHOP
CHOP
UPR/ISR target genes
GADD34
ERO1
Cell death

Fig.5: Signaling by PERK to the translational machinery. In response to endoplasmic reticulum (ER) stress, protein kinase RNA (PKR)-like ER kinase (PERK), similar to inositol-requiring protein-1 (IRE1), oligomerizes in the plane of the membrane and is activated by trans-autophosphorylation of its activation loop. Extensive further phosphorylation of the large kinase insert loop facilitates substrate recruitment. Phosphorylation of a single known substrate, the α subunit of eukaryotic translation initiation factor-2 (eIF2) on Ser51, inhibits the pentameric guanine nucleotide exchange factor eIF2B from recycling eIF2 to its active GTP-bound form. The resulting reduced activities of eIF2B and the eIF2 complex account for all of the important consequences of PERK activity. Because other eIF2 kinases (PKR, haem-regulated inhibitor kinase (HRI) and general control non-derepressible-2 (GCN2)) can activate this pathway independently of ER stress, this portion of the unfolded protein response (UPR) is termed the integrated stress response (ISR). Lower global protein synthesis reduces ER unfolded protein load but also affects gene transcription. For example, translation of the activating transcription factor-4 (ATF4) is increased under conditions of limiting eIF2, whereas nuclear factor κB (NFκB) is activated post-translationally. The ISR activates genes that encode amino-acid transporters and genes that protect against oxidative stress, and it contributes to the transcriptional activation of XBP1. The transcription factor CHOP (C/EBP homologous protein) is also activated transcriptionally by ATF4 and its target genes include GADD34 (growth arrest and DNA damage-inducible protein-34), a regulatory subunit of phosphatase PP1 that dephosphorylates eIF2α and terminates signaling in the ISR77, and ER oxidase-1 (ERO1), which is required for disulphide bond formation in protein folding. A constitutive phosphatase CReP (constitutive repressor of eIF2α phosphorylation) assists GADD34 in this task.

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1.3.3. ATF6 signaling

ATF6 is an UPR transducer that is synthesized as an inactive precursor, which is tethered to the ER membrane by a transmembrane segment and has a stress-sensing portion that projects into the ER lumen. The cytosolic portion encodes a transcription factor (Haze et al., 1999; Wang et al., 2000; Yoshida et al., 2000).

Upon ER stress, ATF6 translocates to the Golgi apparatus where it is cleaved by Golgi-resident proteases, S1P (site 1 protease) and then in an intramembrane region by S2P (site 2 protease) to release the cytosolic DNA-binding portion, ATF6f ('f' for fragment) (Ye, J. et al., 2000). ATF6f then translocates to the nucleus where it is responsible for the transcription of target genes. ATF6 binds to the ATF/CRE element and the ER stress response elements I and II (ERSE), to induce the transcription of many UPR genes like ERAD factors and chaperones as well as Xbp1 (Yoshida et al., 2003; Kokame, 2000; Yoshida, 1998). ATF6 processing is shown in **fig.6**.

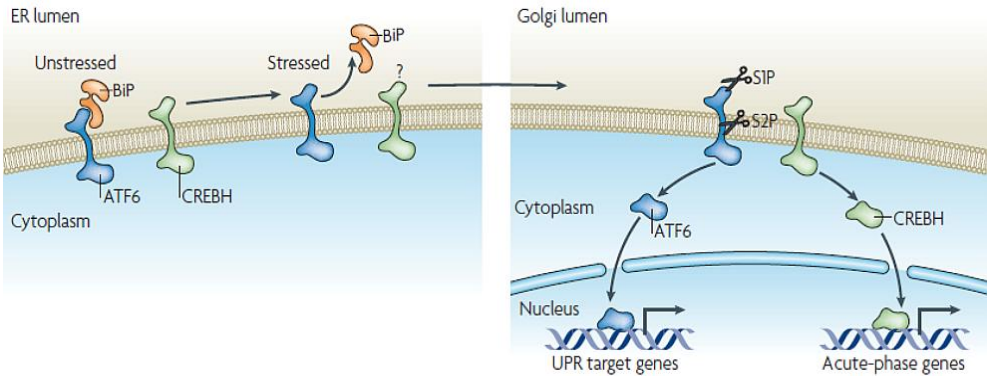


Fig.6: Signaling by ATF6. Activating transcription factor-6 (ATF6) and cyclic AMP response element binding protein hepatocyte (CREBH) are transmembrane proteins with a cytoplasmic portion that, when liberated from its transmembrane tether, can bind to DNA and activate target genes. In unstressed cells, ATF6 and CREBH reside in the endoplasmic reticulum (ER) membrane. ATF6 trafficking appears to be hindered by binding of the ER chaperone immunoglobulinbinding protein (BiP) to its lumenal domain. ER stress disrupts BiP binding and ATF6 (and CREBH) are delivered to the Golgi apparatus. The details of this vesicular transport event remain unknown. In the Golgi apparatus, these proteins are subject to consecutive cleavage, first by the lumenal site 1 protease (S1P) and then the intra-membrane site 2 protease (S2P), which liberates the cytosolic effector portions of the proteins from the membrane and allows their import into the nucleus. ATF6 probably activates a subset of UPR target genes, although these remain to be characterized, whereas CREBH activates acute-phase response genes that encode secreted proteins involved in inflammation.

From the following article: Signal integration in the endoplasmic reticulum unfolded protein response. David Ron & Peter Walter. Nature Reviews Molecular Cell Biology 8, 519-529 (2007).

1.4. Cell death and the UPR

1.4.1. Apoptosis

Apoptosis is a distinct form of programmed cell death. Apoptosis is executed by caspases, a unique group of cysteine proteases, which cleave their target proteins at a peptide bond C-terminally from aspartate and are expressed in the cytoplasm as inactive zymogens. Caspases are part of an enzymatic cascade, at the beginning of which there are the initiator caspases, which are activated first. The initiator caspases cleave the effector caspases, which become active and are responsible for the cleavage of target proteins, like actin and lamin (Degterev et al., 2003; Chowdhury et al., 2008). Additionally, the effector caspases activate nucleases, which in turn cleave DNA.

The apoptotic cells shrink and are eliminated by macrophages. Apoptosis is required for normal embryonic development as well as for the destruction of cells that may become a threat to the integrity of the adult organism.

There are three ways by which a cell commits apoptotic suicide, one generated by signals within the cell (intrinsic pathway), one triggered by death activators that bind to special receptors within the cell membrane (extrinsic pathway) and one which can be caused by dangerous reactive oxygen species.

In the extrinsic pathway, death activators bind to death receptors sitting in the cell membrane, for example the death activator FasL binds to the Fas receptor and TNF activators to the TNF receptor, respectively (Chen, G. and Goeddel, 2002; Wajant et al., 2003). This leads to the activation of caspase-8, which similar to caspase-9 triggers the apoptotic pathway leading to suicide of the cell.

In the intrinsic pathway, Bcl-2 family members like Bcl-2 and Bax/Bak play an important role (Cheng et al., 2001). Bcl-2 (anti-apoptotic) sits in the outer membrane of mitochondria and prevents apoptosis by inhibiting Bax/Bak (pro-apoptotic) which themselves are integrated into the outer mitochondrial

membrane. Upon apoptotic stimuli Bcl-2 is released from Bax/Bak leading to holes in the outer mitochondrial membrane and to the leaking of cytochrome-c to the cytoplasm, where it binds to Apaf-1 (apoptotic protease activating factor 1). Cytochrome-c and Apaf-1 form a large mulimeric complex - the apoptosome - which binds and activates caspase-9 (Riedl and Salvesen, 2007). Caspase-9 in turn cleaves and activates the executioner caspases caspase-3 and caspase-7, which in turn trigger the proteolytic cascade leading to apoptotic cell death.

In *Drosophila* the importance of cytochrome-c and Bcl-2 family members (debcl/Buffy) are still not clear, however the formation of an apoptosome is known to be involved in apoptosis (Rodriguez et al., 1999; Zhou et al., 1999; Kanuka et al., 1999a; Mills et al., 2006; Srivastava et al., 2007).

Ark (Apaf-1-related killer), the *Drosophila* homologue of mammalian Apaf-1 provides the structural backbone of the *Drosophila* apoptosome. Ark is an essential pro-apoptotic protein; most cell death is blocked in ark mutants (Zhou et al., 1999; Igaki et al., 2002; Srivastava et al., 2007). Ark contains a CARD (caspase activation and recruitment domain).

The *Drosophila* genome encodes seven caspase genes: dronc, dredd, strica, drICE, dcp-1, decay and damm. Dronc is similar to the initiator caspase-9 in humans, has a CARD domain and binds to Ark via a CARD/CARD interaction (Dorstyn et al., 1999; Rodriguez et al., 1999; Kanuka et al., 1999b). Dronc cleaves and activates the effector caspase drICE (Hawkins et al., 2000).

Inhibition of caspase activation is also part of the intrinsic pathway, where inhibitors of apoptosis proteins (IAPs) play a crucial role. Apotosis is induced by the expression of IAP antagonists like reaper, grim and head involution defective (hid) in *Drosophila* (Song and Steller, 1999) or Smac and Diablo in humans. Fly embryos lacking the IAP antagonists reaper, hid and grim display no apoptosis at all (White et al., 1994). The expression of reaper, hid and grim must somehow rely on the interpretation of the various pro-apoptotic stress signals and pathways. This interpretation is possible by

different regulation of the gene locus of those IAP antagonists, which spans more than 150kb and contains specific binding sites for mediators of apoptosis (Brodsky et al., 2000; Lohmann et al., 2002). Additionally, the locus is regulated epigenetically (Zhang, Y. et al., 2006). Furthermore, hid, for example is regulated by phosphorylation by MAP kinase (Bergmann et al., 1998; Bergmann et al., 2002).

In *Drosophila* there are at least four IAP proteins, namely Diap1, Diap2, bruce and deterin. The highly conserved baculovirus IAP repeats (BIRs) are essential for the function of IAPs. The BIR domains of IAPs bind to pro-apoptotic factors, such as caspases, inhibiting their function. Diap1, which is encoded by the gene *thread*, is the most important IAP in *Drosophila*, inhibiting pro-apoptotic factors, like caspases (Tenev et al., 2004). Diap1 also has a RING domain with E3 ubiquitin ligase activity, which is responsible for the ubiquitination of its substrates, leading either to their degradation by the proteasome or their inhibition, depending on the ubiquitin configuration. The binding of IAP antagonists to Diap1 leads to self-ubiquitination and degradation of Diap1, resulting in the activation of caspases (Chai et al., 2003; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002; Tenev et al., 2004; Yokokura et al., 2004).

It was suggested that the IAP antagonists help Diap1 to translocate to the mitochondrial membrane where factors for self-ubiquitination of Diap1 reside (Freel et al., 2008). Furthermore, it was suggested that IAP antagonist involved down-regulation of global protein synthesis regulates Diap1 levels (Yoo et al., 2002). Diap1 over-expression in the *Drosophila* eye with the GMR-GAL4 driver leads to suppression of reaper and hid induced cell death (Hay et al., 1995).

The UPR influences cell fate by promoting either cell adaptation or apoptosis when protein folding homeostasis is perturbed. Upon ER stress, pro-apoptotic BH3-only proteins are transcriptionally or post-translationally activated to stimulate pro-apoptotic Bax and Bak either directly or indirectly through antagonizing anti-apoptotic members, like Bcl-

2. The BH3-only family members, Puma, Noxa, Bid and Bim have been described to mediate apoptosis triggered by ER stress (Li, J. et al., 2006; Puthalakath et al., 2007; Upton et al., 2008). CHOP, which is expressed during ER stress, seems to be a link between BH3-only proteins and the UPR as CHOP was shown to induce bim expression (Puthalakath et al., 2007). Furthermore, CHOP antagonizes the expression of anti-apoptotic Bcl-2.

CHOP expression can also lead to the production of reactive oxygen species induced by the CHOP transcriptional target ER oxidase 1α (ERO1α) which oxidizes client proteins in the ER lumen, leading to hyper-oxidation and apoptosis (Marciniak et al., 2004). ERO1α is also linked to CHOP mediated apoptosis via activation the ER calcium release channel IP3R1, which leads to activation of pro-apoptotic cytoplasmic calcium signaling. Cytoplasmic calcium triggers apoptosis by activating the calcium sensing kinase CaMKII, which in turns phosphorylates downstream targets, leading to apoptosis (Seimon et al., 2006; Timmins et al., 2009; Li, G. et al., 2010).

There are other CHOP induced proteins that have been implicated in apoptosis, e.g. death receptor-5 (DR5) and Tribbles related protein 3 (TRB3). DR5 was shown to be involved in apoptosis in different cultured cancer cell lines (Yamaguchi et al., 2004). Persistent ER stress built up intracellular DR5 protein, driving ligand-independent DR5 activation and apoptosis engagement via caspase-8 (Lu, M. et al., 2014). It was shown that TRB3 is required for the full apoptotic response in cultured 293 and HeLa cells exposed to tunicamycin (Ohoka et al. 2005).

Ire1 signaling could also be associated with apoptosis by JNK activation via the association of IRE1 and tumor necrosis factor receptor-associated factor 2 (TRAF2) and activation of the MAPK kinase kinase (MAP3K) apoptosis signal-regulating kinase 1 (ASK1) (Nishitoh et al., 2002, Urano et al., 2000). JNK signaling can trigger protective or apoptotic mechanisms, depending on the context (Weston and Davis 2007).

Another link between IRE1 signaling and apoptosis may be conferred through the interaction of IRE1a with Bax and Bak which seems to be required for IRE1a activation (Hetz et al., 2006).

RIDD, which normally degrades mRNA to reduce the load of new proteins into the ER may also be a mechanism of apoptosis when ER stress is prolonged or too strong. Experiments in which IRE1 activity was manipulated towards RIDD showed ER stress induced apoptosis (Tabas and Ron, 2011). It was found that sustained IRE1α RNase activation caused rapid decay of selected microRNAs that normally repress translation of Caspase-2 mRNA, which encodes the initiator protease of the mitochondrial apoptotic pathway (Upton et al., 2012). Furthermore, allosteric inhibition of the IRE1α RNAse preserves cell viability and function during ER stress (Gosh et al., 2014). IRE1 has distinct catalytic mechanisms for Xbp1 splicing and RIDD, selective activation of RIDD promotes cell death (Tam et al., 2014).

In *Drosophila* a link of a special UPR branch to apoptosis has not been shown to date. Kang and Ryoo showed that the over-expression of Rhodopsin-1 in eye imaginal discs leads to ER stress and apoptosis which can be suppressed by ERAD (Kang et al., 2009). However, the involvement of a special UPR arm was not shown. It was also shown that Cdk5 and Mekk1 mediate a pro-apoptotic signaling response to ER stress in a *Drosophila* model for Autosomal Dominant Retinitis Pigmentosa (ADRP), whereby Cdk5 phosphorylates Mekk1, and together, activate the JNK pathway for apoptosis (Kang et al., 2012). However, also here, the involvement of a special UPR arm is not clear. In the *Drosophila* genome there are no obvious CHOP homologues, which could be expressed upon ER stress to trigger a pro-apoptotic response.

1.4.2. Autophagy

Autophagy which means „self-digestion“ is a catabolic process in which unnecessary or malfunctioning cellular components are degraded via the lysosomal pathway. The degradation of these components allows the recycling of cellular building blocks and promotes cellular survival during starvation by providing new energy (reviewed in Mizushima, 2014).

There are three forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Autophagy consists of the following steps: sequestration, transport to lysosomes, degradation, and utilization of degradation products.

Autophagy can mediate a variety of cellular processes like adaptation to starvation, intracellular protein and organelle clearance, differentiation, anti-aging, elimination of microorganisms, tumour suppression, antigen presentation and cell death (Mizushima, 2005). Thus autophagy can be pro-survival or pro-death. In macroautophagy, unique cell organelles, the autophagosomes engulf and fuse a portion of the cytoplasm with endosomes, which then fuse with the lysosomes, forming the so called autolysosomes. All these steps are mediated by the autophagy-related proteins (ATG proteins). The process is initiated when an isolation membrane is formed under the guidance of the class III PI3-kinase complex and ATG proteins. The expansion of the isolation membrane occurs by the translocation of Microtubule-associated protein light chain 3 II (LC3-II), which is formed by phosphatidylethanolamine conjugation of LC3-I, to the autophagosome membrane.

It was shown that under conditions of ER stress, the autophagy system is activated (Ogata et al., 2006). The first link between aggregated proteins in the ER and autophagy came from studies in mammalian cells, where the accumulation of a mutant form of a cell surface protein, decay accelerating factor, was associated with autophagy (Field et al., 1994).

The direct link between ER stress and autophagy was derived from studies in *Saccharomyces cerevisiae*, showing that ER stress inducing agents lead to the activation of autophagy (Yorimitsu et al., 2006). In these cases, it is unclear if activation of autophagy represents an attempt to survive, for example through the degradation of misfolded proteins, or a mechanism for induction of cell death.

The IRE1/Xbp1 pathway leads to the expansion of the ER and activation of autophagy could be required, as a regulatory feedback loop, to limit ER expansion and to maintain ER integrity (Schuck et al., 2014; Lee et al., 2005). The association of JNK signaling with IRE1α activity links autophagy to ER stress. JNK appears to phosphorylate Bcl-2, which no longer can repress Beclin and leads to the induction of autophagy mediated by Beclin (reviewed in Verfaillie et al., 2010).

PERK activity leads to the transcriptional upregulation of ATG genes by ATF4 (B'chir et al., 2014). ATF4 dependent transcription of CHOP can also lead to autophagy via calcium release from the ER into the cytoplasm, this is mediated by ERO1α and its activation of the ER calcium release channel IP3R1. The cytoplasmic calcium signals via stimulation of AMPK (reviewed in Verfaillie et al., 2010).

Also the ATF6 arm of the UPR seems to be involved in autophagy as depletion of ATF6 inhibits autophagy induced by S1P Phosphatase in human breast cancer cells (Lépine et al., 2010).

Furthermore, it was shown that Bip is required for ER stress induced autophagy in mammalian cells (Li, J. et al., 2008).

1.5. The *Drosophila* eye

The *Drosophila* compound eye is composed of about 800 units, called ommatidia, which are organized into a regular hexagonally packed array.

Each ommatidium consists of 20 cells: eight photoreceptors and 12 accessory cells, including the bristles, lens-secreting cone cells and pigment cells (Wolff and Ready 1993).

The rhabdomeres are microvillar membrane stacks in the photoreceptors that contain rhodopsin, the light sensitive protein. Rhabdomere morphogenesis starts around 67% of pupal development. Photoreceptors (R) R1-R6 express the blue sensitive opsin Rhodopsin-1 (Rh-1), R7 expresses two UV opsins (Rh-3 and Rh-4) and R8 is generally blue-green sensitive (Cook and Desplan, 2001; Mollereau and Domingos, 2005).

The eight photoreceptors are arranged in a stereotypical pattern and can be identified by their position in each ommatidium. Photoreceptor cells R1 to R6 of each ommatidium are placed radially around R7 and R8, forming an irregular trapezoid, while R7 sits on top of R8 (Wolff and Ready, 1993). The photoreceptors project their axons retinotopically to targets in the optic lobe of the brain, R1-R6 project their axons to the first optic ganglion, the lamina, while the axons of R7 and R8 terminate in the medulla, the second ganglion. Higher-order integration and image formation occurs at the lobula (Hardie, 1985; Braitenberg, 1967; Kunes and Steller, 1993). The structure of the compound *Drosophila* eye is depicted in **fig.7**.

The *Drosophila* eye develops during late larval stages from a monolayer epithelium, called the eye-antenna imaginal disc (Haynie and Bryant, 1986; Cohen, 1993). During development of the eye, a wave of differentiation sweeps from posterior to anterior across the eye imaginal disc, this wave of differentiation is called the morphogenetic furrow (MF) (Heberlein and Moses, 1995). The MF results from an apical constriction and apical-basal contraction of the cells. Anterior to the MF, cells are undifferentiated and divide asynchronously, whereas posterior to the MF, the photoreceptors are specified. The progression of the MF requires hedgehog (*hh*) function (Ma, C. et al.1993), and is the place where commitment to photoreceptor fate is initiated. Depending on hh activity, decapentaplegic and the proneural gene atonal (ato) are expressed in the furrow. Ato expression is required for R8

development. Posterior to the furrow, preclusters of R8, R2, R3, R4 and R5 are specified. R8 is the first photoreceptor to be specified in each ommatidium, followed by R2/5, R3/4, R1/6 and finally R7 is recruited to the ommatidial cluster (Wolff and Ready, 1993). These photoreceptors and accessory cells are recruited to each ommatidial cluster by waves of expression of the ligand *spitz* (*spi*), which binds to the EGF receptor (Freeman, 1996; Freeman, 1997; Kumar et al., 1998).

The developing ommatidia are organized in columns of regularly spaced clusters, with a new column of clusters appearing posterior to the MF approximately every two hours (Campos-Ortega and Hofbauer, 1977; Tomlinson and Ready, 1987). Immediately anterior to and in the MF, cells arrest in G1 phase of cell cycle. Cells that emerge posterior to the MF can be divided in two subpopulations, the cells of the ommatidial preclusters, which exit cell cycle and acquire a neuronal fate and undifferentiated cells surrounding the preclusters. The undifferentiated cells enter a terminal cell cycle called second mitotic wave (SMW). The cells of this second mitotic wave give rise to the photoreceptor cells R1, R6, R7, the cone cells, the pigment cells as well as the precursors of the mechanosensory bristles (Ready et al., 1976; Wolff and Ready, 1991a). The remaining undifferentiated cells undergo apoptosis (Wolff and Ready, 1991b).

The *Drosophila* retina exhibits planar polarity, there is a line of mirror symmetry, the “equator”, bisecting the retina horizontally. Within each adult ommatidium, the photoreceptors form an arranged trapezoid array. In the dorsal half of the eye the tip of the trapezoid (the R3/R4 pair) points up, while in the ventral half of the eye it points down (Ready et al., 1976; Wolff and Ready, 1991).

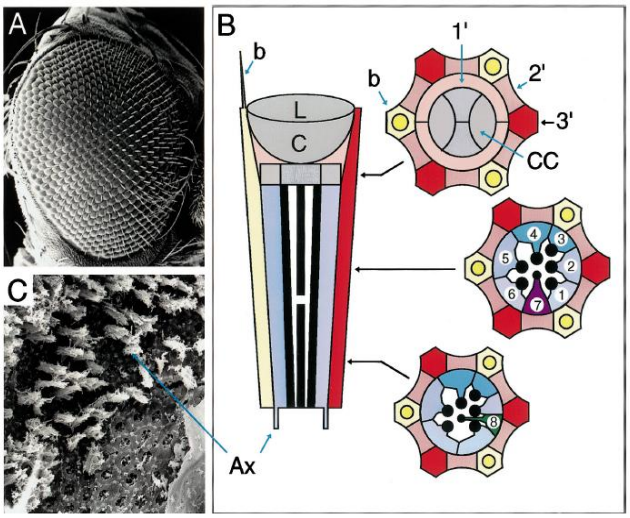


Fig.7: Structure of the compound eye. (A) A scanning electron micrograph of the compound eye. Anterior is to the right and dorsal is up. Note the regular array of lenses which overly each ommatidium and the mechanosensory bristles in each alternate vertex. The height of the eye is approximately 0.5 mm. **(B)** A diagram of the cells in one ommatidium. A longitudinal section is shown to the left, and three cross-sections to the right at the levels indicated. The cells of the ommatidium can be classified into four functional groups: (i) the light-sensitive photoreceptors (shown in blue, green, and violet); (ii) the screening pigment cells (red); (iii) the dioptic elements secreted by the cone cells (gray), and (iv) the mechanosensory bristle (yellow). (i) The photoreceptors: the six blue-sensitive outer photoreceptor cells are shown in shades of blue, and numbered in one of the two sections in which they can be seen. Their rhabdomeres are shown in black. Note the asymmetric trapezoidal arrangement of the rhabdomeres. The ultraviolet-sensitive apical central photoreceptor (R7) is shown in violet, and the blue/green-sensitive basal central cell (R8) is shown in green. Note the axons (labeled Ax) which project from the base of the ommatidium and innervate the optic lobes of the brain (not shown). (ii) The 1°, 2°, and 3° pigment cells are shown in shades of red, and one example of each class is labeled in the uppermost cross-section (1° pigment cells are in lightest red, and 3° in darkest). (iii) The cone cells (labeled CC in the uppermost section) secrete the lens (L) and the fluid-filled pseudocone **(C)**. (iv) The two cells that make up the mechanosensory bristle (b) are shown in yellow. Note that the bristle and 2° and 3° cells are shared among ommatidia. The convention is to count 20 cells per ommatidium: 8 photoreceptors, 4 cone cells, 2 1° pigment cells, 3 2° pigment cells, 1 3° pigment cell, and 2 bristle cells. **(C)** A scanning electron micrographsh owing ommatidial axons (Ax) emerging from the fenestrated membrane (formed by the feet of the 2° and 3° pigment cells) on the basal side of the retina.

From the following article: Determination of *Drosophila* photoreceptors: timing is everything. C. A. Brennan and K. Moses. CMLS, Cell. Mol. Life Sci. 57 (2000) 195–214.

1.6. Rhodopsin-1 mediated retinal degeneration

Rhodopsin-1 (Rh-1) is a seven transmembrane domain glycoprotein which is synthesized and processed in the ER. Rh-1 is highly conserved and undergoes several types of post-translational modifications such as binding of the chromophore 3-hydroxyretinal and glycosylation (Webel et al., 2000). The post-translational modifications of Rh-1 are essential to maintain the proteins structure as well as its proper function in the visual transduction cycle.

When properly folded and processed, Rh-1 is delivered via the secretory pathway to the rhabdomere membranes. Rh-1 requires the cyclophilin homolog NinaA as a chaperone for its exit from the ER and proper secretion (Colley et al., 1991; Ondek et al., 1992; Baker, E. K. et al., 1994). In addition, Xbp1-independent Ire1 signaling is required for proper rhabdomeric localization of Rh-1 and for the formation of the rhabdomere (Coelho et al, 2013).

Mutations that affect Rh-1 maturation or its delivery to the rhabdomere can cause retinal degeneration in flies and in humans. Rh-1 expression starts around 75-78% of pupal development and Rh-1 reaches the rhabdomere at around 84% of pupal development (Kumar and Ready, 1995). Rhodopsin is the most abundant membrane protein in the rhabdomere and plays a fundamental role in establishing and maintaining the rhabdomeres' architecture. Null mutations of the *Drosophila* gene encoding Rh-1, ninaE, result in severe defects in the formation of the rhabdomeres. By 90% of pupal development, a specialized catacomb-like membrane structure develops at the base of the rhabdomeres. In ninaE null mutants this membrane structure does not develop and rhabdomeres start to degenerate and disappear one day after eclosion (Kumar and Ready, 1995). It was shown that flies heterozygous for a wildtype rhodopsin gene and a deletion of Rh-1 display normal retinal morphology, demonstrating that one copy of the Rh-1 gene is sufficient to maintain a normal

photoreceptor cell structure. Whereas animals heterozygous for a wildtype gene and a mutated form show severe photoreceptor defects with missing rhabdomeres (Rh-1^{G69D}). Retinal degeneration was shown to result from interference of the wildtype protein with the mutated form (Colley et al., 1995).

Misfolded Rh-1 can cause Retinitis Pigmentosa (RP), which is a group of hereditary human diseases that cause retinal degeneration and eventually lead to blindness. More than 25% of all RP cases in humans appear to be caused by dominant mutations in the rhodopsin gene, which shows 22% amino acid identity with the fly Rh-1 (Zuker et al., 1985; O'Tousa et al., 1985). In *Drosophila*, mutations such as Rh-1^{G69D} (Colley et al., 1995) or other mutations (for example in ninaA or Ire1), that affect the maturation of Rh-1 or its delivery to the rhabdomere, cause retinal degeneration. Another example is the mutation (Rh-1N20I) in the N-linked glycolysation consensus motif, which causes the retention of the wildtype as well as mutated Rh-1 in the ER or Golgi (Webel 2000).

1.7. The cell cycle

The cell cycle consists of periodic events ultimately leading to cell duplication, which is fundamental for the development of multi-cellular organisms (reviewed in Vermeulen et al, 2003). Classically, the cell cycle is divided into four distinct phases: G(GAP)-1, S-(synthesis) phase, G(GAP)-2 and M-phase which consists of mitosis (nuclear division) and cytokinesis (division of the cytoplasm). G1, S-Phase and G2 together are also known as interphase. The crucial steps during cell proliferation are the duplication of genetic material and the equal separation of this material into two daughter cells. The first task is achieved during S-phase when DNA replication occurs. During mitosis, which is subdivided into five stages,

duplicated chromosomes are segregated to opposite sides of the cell where two daughter nuclei form, before the cytoplasm is cleaved (Pines and Rieder, 2001). G1 and G2 phases are characterized by cell growth and prepare the cell for S-phase and mitosis, respectively. Once a cell passed through the cell cycle it either prepares for a new round of cell division, or alternatively, it can leave the cell cycle to enter a dormant state, called the G (Gap)0 phase (Vermeulen et al., 2003). Some cells remain in this stage for ever, others might re-enter the cell cycle once growth factors are received.

Progression through the cell cycle is regulated by the activity of cyclin-dependent kinases (Cdks) and tightly controlled by several checkpoints. Cdks are proline-directed serine/threonine kinases that are only active when bound to cell cycle specific cyclins. However, Cdk activity is additionally regulated by Cdk inhibitors (CDKIs) and by its own phosphorylation status. Activated Cdks phosphorylate a wide range of substrates thereby orchestrating coordinated progression into the next cell cycle stage. While Cdks are expressed constitutively, levels of stage specific cyclins oscillate and thus modulate Cdk activity throughout the cell cycle.

In the MF of the *Drosophila* eye imaginal discs, cells are in G1 and either differentiate into a neuronal fate or stay in G1, possibly re-entering the cell cycle in the SMW, posterior to the MF (Ready et al, 1976; Thomas et al., 1994; Baker, 2001). The different cell cycle stages in *Drosophila* eye imaginal discs are shown in **fig.8**.

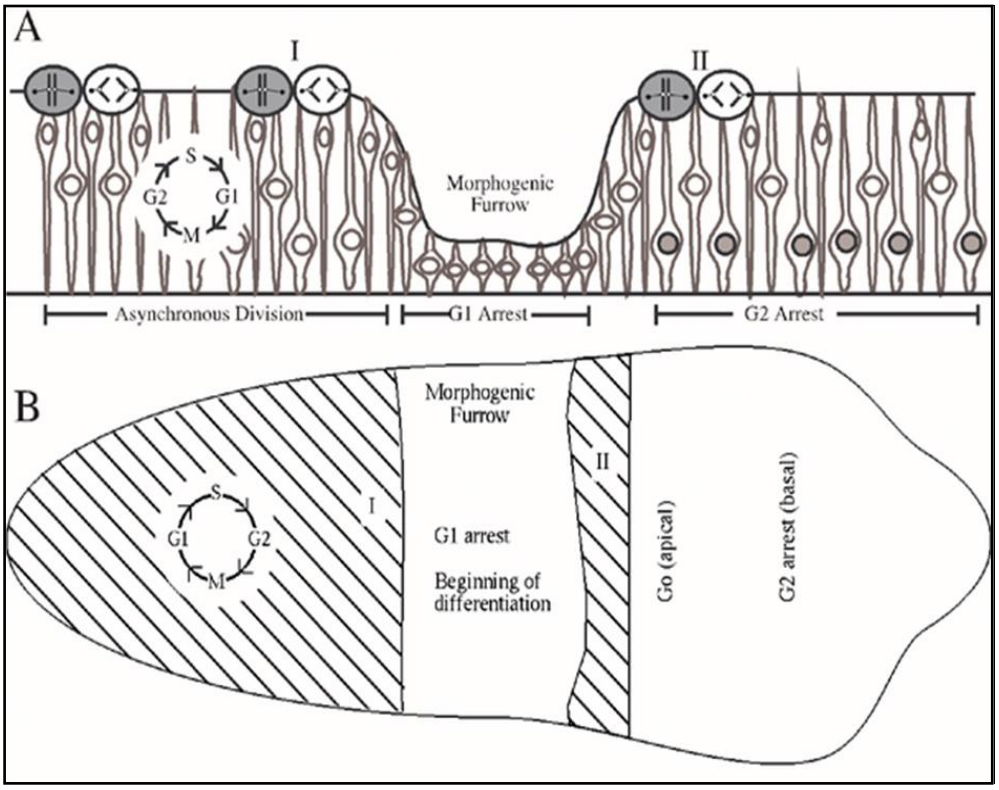


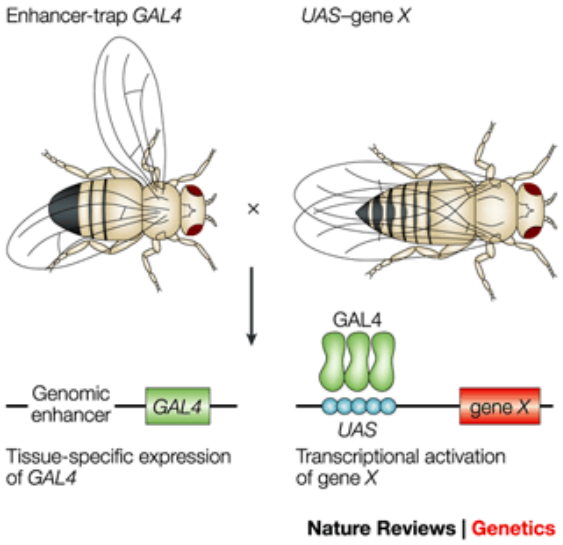
Fig.8: Cell cycle stages in *Drosophila* eye imaginal discs. **(A)** Cartoon of an eye disc that shows populations of dividing and cell-cycle-arrested cells. I, first mitotic wave; II, second mitotic wave. G2-arrested cells have filled nuclei and are located basally posterior to the furrow. **(B)** Cartoon of same disc viewed from above (apical side of disk). Anterior is to the left.

From the following article: Cell-by-Cell Dissection of Gene Expression and Chromosomal Interactions Reveals Consequences of Nuclear Reorganization. (2005). Brian Harmon, John Sedat. PLoS Biol 3 (3): e67

1.8. The GAL4-UAS System and mitotic recombination

The GAL4/UAS system allows the ectopic expression of a gene in a specific tissue or cell type. Two transgenic fly lines are crossed, one parent fly wich is the UAS line (Upstream Activating Sequence where the gene to be expressed is placed downstream of the UAS) to the other parent fly

which is the GAL-4 line (contains the yeast transcriptional activator GAL-4). GAL-4 is under the control of a tissue specific promoter. The gene under control of the UAS is only activated when these flies are crossed with the GAL-4 line, also known as “driver”.



The Gal4-UAS system for targeted gene expression. The yeast transcriptional activator Gal4 can be used to regulate gene expression in Drosophila by inserting the upstream activating sequence (UAS) to which it binds next to a gene of interest (gene X). The GAL4 gene has been inserted at random positions in the Drosophila genome to generate 'enhancer-trap' lines that express GAL4 under the control of nearby genomic enhancers, and there is now a large collection of lines that express GAL4 in a huge variety of cell-type and tissue-specific patterns. Therefore, the expression of gene X can be driven in any of these patterns by crossing the appropriate GAL4 enhancer-trap line to flies that carry the UAS–gene X transgene. This system has been adapted to carry out genetic screens for genes that give phenotypes when misexpressed in a particular tissue (modular misexpression screens). From: St. Johnston, 2002

A mitotically dividing cell normally gives rise to daughter cells that are genotypically identical. However, if exchange of sister chromatids (mitotic recombination) occurs, the resulting daughter cells can be genotypically different provided that the mother cell is heterozygous for the marker/ mutation under study. Mitotic recombination can be induced between FRTs by a site-specific recombinase (FLPase), and clones of cells that are homozygous for a mutation can be generated during development.

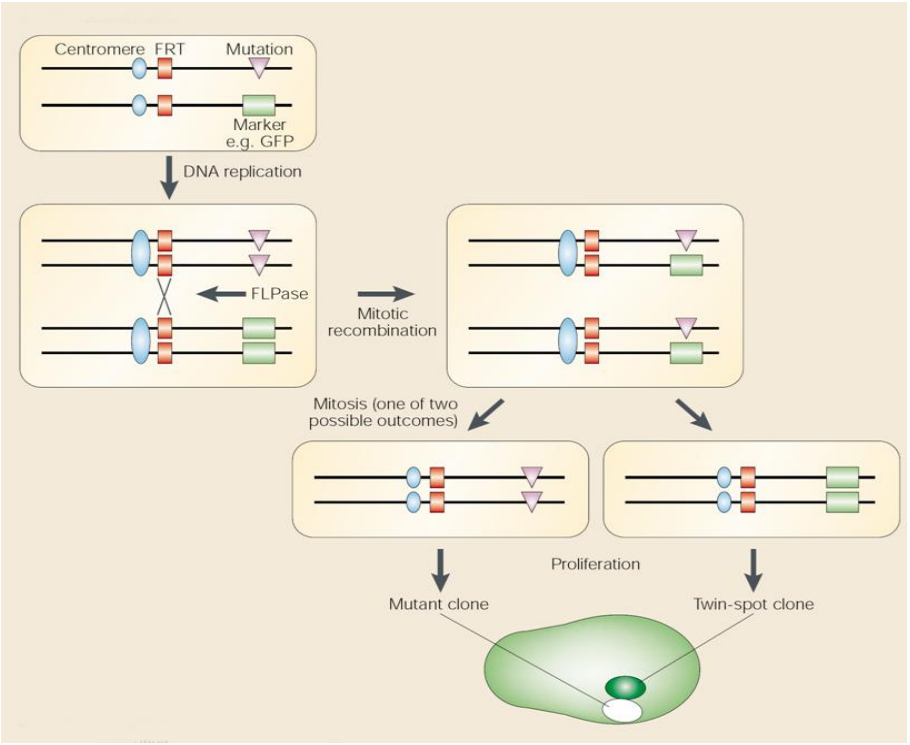


Fig.10: Induction of mitotic clones by the Flp/FRT system. A mitotically dividing cell normally gives rise to daughter cells that are genotypically identical to itself. However, if the exchange of sister chromatids (mitotic recombination) occurs, the resulting daughter cells can be genotypically different provided that the mother cell is heterozygous for the marker/mutation under study.
Mitotic recombination can be induced between FRTs by a site-specific recombinase (FLPase), and clones of cells that are homozygous for a mutation can be generated during development.
From: Tabata, T., 2001.

1.9. Aims of the work

The over-expression of the active transcription factor Xbp1s in the developing *Drosophila* eye leads to an eye degeneration phenotype. We wanted to investigate if this atrophic “glossy” eye phenotype is amenable for a modifier screen with EMS induced mutations making use of the Flp/FRT technique (Golic, 1991), in order to find downstream mediators of the Xbp1s induced cell death.

From this genetic screen, we could recover and identify suppressor mutations in the gene CG6758, encoding an Fbox protein with unkown biological role. We further investigated the role of CG6758 in Xbp1s induced cell death and in other models of ER stress induced cell death in the *Drosophila* eye. We aimed at identifying the contribution of apoptosis and autophagy in Xbp1s conferred cell death and the involvement of CG6758 mutations in these processes.

Chapter II

Identification of the SCF complex component Fbox^{CG6758} as a downstream mediator in Xbp1s induced “glossy” eye phenotype in *Drosophila*

Summary

We performed a mosaic genetic screen aimed to identify suppressors of the retinal degeneration phenotype caused by Xbp1spliced (Xbp1s) over-expression in the *Drosophila* eye. By screening around 80.000 mutagenized chromosome arms of the right arm of the second chromosome (2R), we could recover 32 lethal suppressor mutations, which form four different complementation groups. By mapping the mutations with the Bloomington deficiency kit and sequencing of candidate genes, we identified the molecular lesion for three of the complementation groups. To test if the complementation groups are real downstream mediators of Xbp1s conferred cell death, we tested our complementation groups in a secondary screen where we analysed whether GMR-GAL4>UAS-DsRed expression in eye imaginal discs or pupa is reduced in clones homozygous for the suppressor mutations. Only for CG6758, the transgene expression was equal in mutant and wildtype tissue. To test whether CG6758 is a downstream mediator of Xbp1s or modulates Xbp1s protein levels, we also tested Xbp1s protein levels in mosaic eye discs and pupal eyes. Xbp1s protein levels were equal in CG6758 mutant clones and control tissue, making CG6758 a real downstream mediator of GMR-GAL4>UAS-Xbp1s induced cell death. To be absolutely sure that the suppression effect of the mutant CG6758 alleles in the "glossy" eye is due to mutations in CG6758, we tested different rescue constructs for their ability to revert the suppression effect ([P]acman reagent CH322-12H15, UAS-CG6758-GFP, UAS-3xHA-3xFlag-CG6758) and found for all the constructs rescue.

To test whether CG6758 acts in an SCF E3 ubiquitin ligase complex in the context of the Xbp1s induced "glossy" eye and not independenty, we analysed RNA interference (RNAi) constructs of different SCF complex components for their ability to suppress the "glossy" eye.

phenotype. Most of the tested constructs showed a suppression of the “glossy” eye phenotype.

Introduction

We performed a mosaic genetic screen in the *Drosophila* eye to identify novel mediators of IRE1/Xbp1 signaling. Over-expression of Xbp1spliced (Xbp1s) in the *Drosophila* eye by the GMR-GAL4 driver leads to flies with "glossy" eyes (**fig.11**). The GMR-GAL4 driver is active in all cells posterior to the morphogenetic furrow except in the peripodial membrane.
Cells that emerge posterior to the MF can be divided in two subpopulations, the cells of the ommatidial preclusters, which acquire a neuronal fate (R8, R2/R5, R3/R4) and undifferentiated cells surrounding the preclusters. Subsequently, after a last round of cell division, R1 and R6, and, finally, R7 as well as the cone cells are recruited and begin differentiating within the newly formed ommatidium. Specification of the primary, secondary and terciary cells as well as the bristle cells only occur in the first half of pupal development. By mid pupal stages, after specification of all cell types, there is a wave of cell death that eliminates extra cells that do not fit in the lattice (Wolff and Ready, 1991b). GMR is active in all cells of the retina in the pupa and adult stages, meaning that we induced Xbp1s over-expression in all ommatidial cell types.
The external visible induced "glossy" eye phenotype is based on decreased lens and pigment deposition due to cell death induced by Xbp1s over-expression.

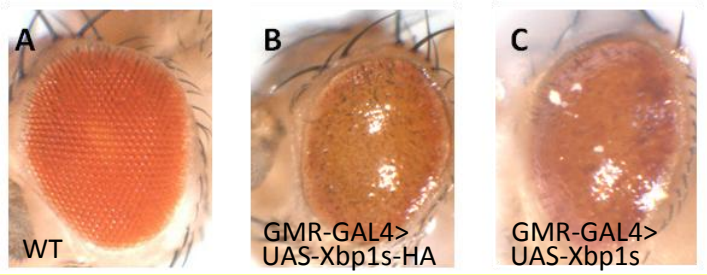


Fig.11: Over-expression of Xbp1s in the developing *Drosophila* eye leads to cell death. (A) Wildtype *Drosophila* eye. **(B, C)** Over-expression of UAS-Xbp1s with the GMR-GAL4 driver leads to a "glossy" eye phenotype. The external visible "glossy" eye phenotype is based on decreased lens and pigment deposition due to cell death induced by Xbp1s and is amenable to modifier genetic screens. **(B)** UAS-Xbp1s-HA construct. **(C)** UAS-Xbp1s construct with no tag.

In our screen, we induced mosaic eyes by the Flp/FRT technique (Golic 1991, **fig.10**) and searched for mutations that suppress the „glossy" eye phenotype caused by GMR-GAL4 driven over-expression of Xbp1s and could be defined by more pigmentation (and more structured ommatidial patterning) (**fig.11**). First, we tested the right chromosome arm of the second chromosome (2R) for downstream mediators of Xbp1s induced cell death.

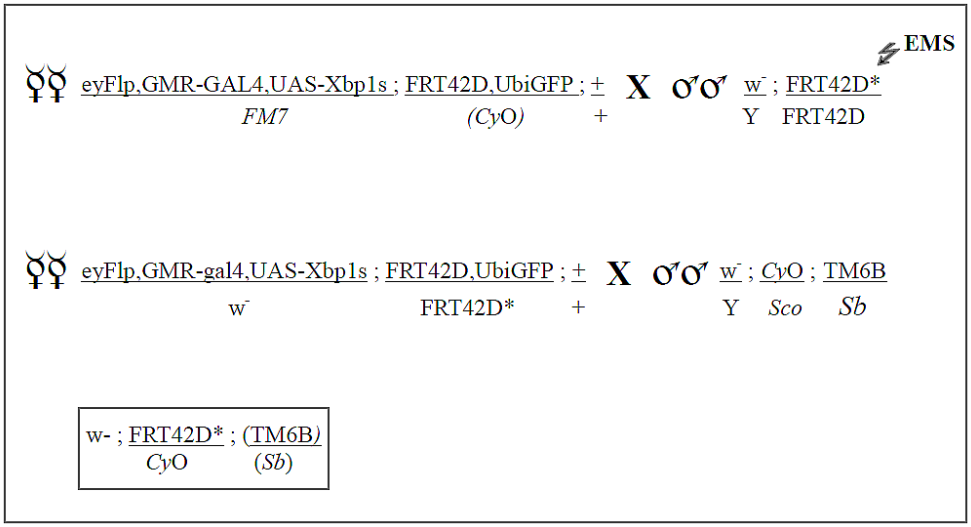


Fig. 12: Crossing scheme for the mosaic genetic screen. Virgin females over-expressing GMR-GAL4>UAS-Xbp1s with the FRT42D, UbiGFP chromosome were crossed to males treated with EMS, bearing the FRT42D chromosome. The F1 generation was screened for suppressors of the "glossy" eye phentotype. Such suppressors were crossed to double balancer flies to establish stable lines.

In our large-scale mutagenesis screen we used the mutagenic agent Ethyl methanesulfonate (EMS) to induce point mutations by alkylation of guanines. For mutagenesis, males with the appropriate FRT chromosome were starved for 8 hours and fed with a sugar-EMS solution (25mM EMS) overnight before being crossed with female virgins carrying eyFlp, GMR-Gal4, UAS-Xbp1s and the corresponding FRT, UbiGFP chromosomes. Progeny of this genetic cross with cell clones showing suppression of "glossyness" in the eye (more pigmentation and maybe more ommatidial patterning in the clones), potentially harboured a mutation in a gene required for Xbp1s induced cell death.

In our screen we could recover 32 suppressor mutations, meaning that those genes in which we obtained mutations could be required for cell death downstream of Xbp1s. It is also possible that the mutations somehow

affect the UAS-Xbp1s transgene expression. For example, some mutations could lead to decreased levels of UAS-Xbp1s transgene expression, due to a reduction in overall transcription levels, or to some detrimental modification of GMR-GAL4, which is responsible for driving the UAS-Xbp1s transgene expression.
It is also possible that the suppressor mutation is in a gene that is involved in Xbp1s protein levels (for example by conferring stability). In homozygous clones of such suppressor mutations, there would be less Xbp1s protein, leading to a suppression of the “glossy“ eye phenotype.
To distinguish between the possibilities of how a suppressor mutation can lead to reduced “glossyness” in the eye, we performed a secondary screen where we analyzed the expression of an unrelated GAL4/ UAS driven transgene (UAS-DsRed) in homozygous suppressor mutant clones. In addition, we analysed Xbp1s protein levels in homozygous suppressor mutant clones by immunofluorescent staining.

In our screen we identified CG6758, an Fbox protein with unknown function as a downstream mediator of Xbp1s induced cell death.

Phenotype driven genetic screens require an approach to identify the isolated mutations. Most of the mutations in CG6758, which suppress the “glossy” eye phenotype, are premature STOP codons that likely constitute strong loss of function alleles or even protein nulls. For these mutations, a rescue of the suppression effect leading to “normal glossyness” should be achieved by expressing CG6758 function in the background of homozygous CG6758 mutant suppressor clones. This can be done by using either a rescue construct which harbours the genomic region of CG6758, including its regulatory sequences, or by expression of UAS-CG6758 under the control of an eye specific GAL4 driver.

Fbox proteins are part of SCF ubiquitin ligase complexes which consist of three core units, an Fbox protein, SkpA and Cullin, whereby the Fbox

protein is linked to SkpA via its N-terminal Fbox domain. Besides being part of an SCF E3 ubiquitin ligase complex, Fbox proteins may also act independently of SCF complexes (reviewed in Hermand 2006).
To analyse the function of a newly discovered Fbox protein, it is necessary first to differentiate if it is acting as part of an SCF complex or independently of an E3 ubiquitin ligase complex.

Material and Methods

Genetic Screen:

The FRT42D stock was isogenized for the second chromosome and males were collected for EMS treatment. The males were starved for 8 hours in empty plastic vials and fed with a 25mM EMS solution overnight for 16-18 hours (paper tissue soaked with EMS solution on the bottom of the vials). After EMS treatment, around 60 males were given one hour for recovery on tissue paper and were then crossed *en masse* to around 150 virgins of the genotype eyFlp, GMR-GAL4, UAS-Xbp1spliced (**fig.10**). The crosses were settled in new bottles every day, for four days in total and the F1 generation was screened for suppressors of the “glossy” eye phenotype. Suppressors of the “glossy” eye phenotype were crossed to double balancer flies and the potential mutation was balanced over *Cy*O. After establishment of a stock, the flies were retested for a reproducible phenotype. Only suppressor stocks with a lethal mutation were kept, which was necessary for mapping of the mutations (see 2.2. and 2.3.).

Preparation of EMS solution:

0,18g sucrose

16ml H2O

2ml 1M Tris-HCL (pH 7,5)

48,7µl EMS (1.206 g/mL at 20 °C)

Complementation analysis:

Each suppressor line obtained in the screen was crossed to each other to bring the mutations in trans and the progeny was analysed if unbalanced flies or flies only with the balancer *Cy*O existed. If there were only *Cy*O balanced progeny, it means that the two suppressor mutations are lethal in trans and probably have a lethal hit in the same gene.

Mapping with the Bloomington 2R deficiency kit:

For mapping the suppressor mutations with the 2R deficiency kit (from the Bloomington Drosophila Stock Center), virgins of the suppressors balanced over *Cy*O were crossed to males of each of the stocks bearing the balanced deficiencies and the F1 generation was screened for trans-heterozygosity. When we observed in the F1 generation balanced flies only, we were sure that the lethal hit of our mutation is located within the region of the deficiency. When we observed lethality in the suppressor/deficiency cross, we then crossed our suppressor mutations to additional deficiencies covering the region, until we could define the smallest region harbouring the suppressor mutation. We then tested lethal mutations in candidate genes in the region for trans-lethality with our mutations. The molecular identification of the mutations was done by genomic DNA sequencing of candidate genes from larvae homozygous for the suppressor mutations.

2R General deficiency kit			
Deficiency	**Deficient region**	**Deficiency**	**Deficient region**
Df(2R)M41A10	41A;41A	Df(2R)BSC305	49A4;49A10
Df(2R)BSC630	41D3;41F11	Df(2R)Exel7121	49B5;49B12
Df(2R)ED1484	42A2;42A14	Df(2R)CX1	49C1--4;50C23--D2
Df(2R)ED1612	42A13;42E6	Df(2R)Exel6062	49E6;49F1
Df(2R)ED1673	42E1;43D3	Df(2R)Exel8057	49F1;49F10
Df(2R)ED1715	43A4;43F1	Df(2R)BSC361	50C3;50F1
Df(2R)ED1725	43E4;44B5	Df(2R)BSC383	50C6;50D2
Df(2R)BSC267	44A4;44C4	Df(2R)Exel7130	50D4;50E4
Df(2R)ED1770	44D5;45B4	Df(2R)Exel7131	50E4;50F6
Df(2R)ED1791	44F7;45F1	Df(2R)Jp6	52E--5;52F
Df(2R)BSC280	45C4;45F4	Df(2R)Exel9060	52E11;52F1
Df(2R)BSC132	45F6;46B4	Df(2R)Exel6063	52F6;53C4
Df(2R)BSC298	46B2;46C7	Df(2R)BSC550	53C1;53C6
Df(2R)X1, Mef2[X1]	46C;47A1	Df(2R)Exel7144	53C8;53D2
Df(2R)BSC152	46C1;46D6	Df(2R)Exel6064	53C11;53D11
Df(2R)BSC303	46E1;46F3	Df(2R)ED2747	53D11;53F8
Df(2R)BSC281	46F1;47A9	Df(2R)BSC331	53D14;54A1

Df(2R)BSC595	47A3;47F1	Df(2R)Exel6066	53F8;54B6
Df(2R)ED2219	47D6;48B6	Df(2R)BSC161	54B2;54B17
Df(2R)ED2247	48A3;48D5	Df(2R)BSC355	54B16;54C3
Df(2R)BSC199	48C5;48E4	Df(2R)Exel7149	54C10;54D5
Df(2R)BSC699	48D7;48E6	Df(2R)BSC347	54D2;54E9
Df(2R)BSC425	48F1;49A1	Df(2R)Kr10	60E10;60F5
Df(2R)Exel6061	48F1;49A6	Df(2R)ED50004	60F5;60F5
Df(2R)vg135	49A;49E1-2		

Additional deficiencies and lethal mutations tested for complementation group 1

Deficiency	**Deficient region**
Df(2R)PK1	57C5-57F5-6
Lethal mutation	**Affected gene**
Mi{ET1}Xpd[MB04535]	Xpd
Pu[W]	Punch
Pu[2]	Punch
tud[1]	Tudor
tud[1]	Tudor

P{GawB}NP4252	Glycogenin
dom[3]	Domino
dom[9]	Domino
PBac{y[+mDint]=3HPy[+]}dom[C281]	Domino
P{w[+mC]=lacW}Sdc[k10215]	Syndecan
P{w[+mGT]=GT1}Sdc[BG02774]	Syndecan
P{ry[+t7.2]=PZ}Fkbp14[00734]	CG9847
P{w[+mC]y[+mDint2]=EPgy2}TAF1C-like[EY20677]/	CG10496
P{w[+mC]=lacW}MESK2[k00119]	MESK2
P{ry[+t7.2]=PZ}MESK2[01467]	MESK2
P{y[+mDint2]w[BR.E.BR]=SUPor-P}MESK2[KG10185]	MESK2
Egfr[t1]	Egfr
Egfr[f2]	Egfr

Additional deficiencies and lethal mutations tested for complementation group 2

Deficiency	**Deficient region**
Df(2R)BSC160	[47F12-47F12];[48A5-48A5]
Df(2R)BSC259	[48A3-48A3];[48C4-48C4]

Df(2R)BSC263	[42F2-42F2];[43C1-43C1]
Df(2R)BSC264	[43B2-43B2];[43C5-43C5]
Df(2R)en[E]	47F17-48A1
Lethal mutation	**Affected gene**
P{w[+mC]=EP}Eaf[EP2475]	ELL-associated factor
P{w[+mC]=XP}so[d09734]	sine oculis
E(Pc)[1]	Enhancer of Polycomb
qvr[Delta40]	quiver
tou[2]	toutatis

Additional deficiencies and lethal mutations tested for complementation group 3

Deficiency	**Deficient region**
Df(2R)Pu-D17	57B4;58B1-2
Df(2R)2-65	57C2;58B2
Df(2R)XE3030	57C2;58C
Df(2R)BSC484/	57C3;57C7
Df(2R)Egfr5	57D--8;58D1
Df(2R)BSC664	57D12;58A3

Df(2R)BSC360	57E6;58A4
Df(2R)BSC813	57F5;58B3
Df(2R)BSC786	57F6;58B3
Df(2R)BSC424	57F8;58E3
Df(2R)01D01Y-R21	57F9--58A2;58D1-2
Df(2R)Exel6077	57F10;58A3
Df(2R)ED3943	57F10;58D4
Df(2R)BSC597	58A2;58F1
Df(2R)BSC802	58A3;58B3
Df(2R)Exel7170	58B1;58C1
Df(2R)Exel6078	58B1;58D1
Df(2R)X58-7	58B1-2;58E1--4
Df(2R)X58-8	58B3;59A1
Df(2R)01D01Y-R09	58B--10;58D1-2
Df(2R)ED3952	58B10;58E5
Df(2R)Exel7171	58C158D2
Df(2R)01D01W-L133	58D1-2;58D2
Df(2R)01D01W-L186	58D1-2;58D2--7
Df(2R)01D01W-L197	58D1-2;58D2--7
Df(2R)01D01W-L053	58D1-2;59A

Df(2R)01D01W-L149	58D2;58E1
Df(2R)02311	58D2;58E1
Df(2R)Exel7173	58D4;58E5
Df(2R)BSC598	58F3;59A1
Df(2R)BSC787	58F4;59B1
Lethal mutation	**Affected gene**
Df(2R)a[7]	arc
Df(2R)a[EX1]	arc
P{GawB}NP7371	CG3074
P{w[+mC] y[+mDint2]=EPgy2}Vps35 [EY16641]	Vps35
P{w[+mC] y[+mDint2]=EPgy2}Vps35 [EY14200]/	Vps35

Sequencing:

For sequencing we collected homozygous mutant larvae (in all cases L3 larvae could be observed) from apple agar plates and prepared genomic DNA for PCR. Homozygous larvae could be observed after replacing the *CyO* balancer with the *Cy*O-GFP balancer. The absence of the GFP signal in the larvae indicated homozygosity for the suppressor mutation. As control, we also sequenced the candidate genes in the parental isogenized FRT42D chromosome.

Genomic DNA preparation was done with the *High Pure PCR Template Preparation* kit from Roche. The DNA was eluted in H2O.

apple juice agar plates (for 1L):

20g agar-agar

750ml H2O

20g sucrose

250ml apple juice

12ml Nipagin (10% in EtOH)

PCR:

The PCR was performed with Phusion Polymerase from Fermentas/Thermo.

The primers for sequencing were chosen to include the region of the ORF.

Primers for sequencing Eaf in Su 280 and Su344:

forward primer 1: tccaagatgcacccatcttggc (Eaf1)

reverse primer 1: gacaaaccagcgctttcg (Eaf6)

Eaf1 + Eaf6 to PCR the ORF of Eaf

forward primer 2: cggattgccagtgctttcgca (Eaf2)

reverse primer 2: tgaacggcccaatactgttcc (Eaf3)

forward primer 3: ccaccgacctgctggccacc (Eaf4)

reverse primer 3: gcaggcagcttgccatcctgc (Eaf5)

Eaf1, Eaf2, Eaf3, Eaf4, Eaf5, Eaf6 for sequencing the ORF of Eaf.

Primers for sequencing Xpd in Su95-a, Su102a, Su373 and Su396:

forward primer 1: tgctcagctctaggaagaacatgtgc

forward primer 2: cctgtgtggtctttgatgaggcgc

forward primer 3: cacgcttgagatcagtgatttgacgg

forward primer 4: caacgaccaggtgaccatttcgtcc

forward primer 5: catgcatgtgcaaccagtctcaccg

forward primer 6: gatccaagagcatctggtggacagc

reverse primer 1: tgtgtgctagtgtacttcacgatcgc

forward primer 1 + reverse primer 1 to PCR the ORF of Xpd, all primers for sequencing reaction.

Primers for sequencing Med16 in Su218b and Su359:

forward primer 1: agtagttggcagacgccg

reverse primer 1: ctgggactcggatttgatggc

forward primer 2: ccagaccagcatatttctgggtcc

reverse primer 2: gctccactgtaaagtccttggcg

forward primer 3: gctgatggtcctgccacgagg

reverse primer 3: ccacgatgtctcggctgatatcg

Primers for sequencing CG6758 in Su212b, Su217c, Su218b, Su226, Su357 and Su359:

forward primer 1: gcacggtcacactgttcgcagc

reverse primer 1: gcaggaattgctgcgcttgc

forward primer 2: aaggggataccggataccag

reverse primer 2: ccttcaaagttcggcgtaag

Sequencing was done by Stabvida.

GMR-GAL4-UAS-DsRed expression

To test the suppressor mutations for GMR-GAL4>UAS-DsRed expression, flies with the corresponding genotype were generated after the following crossing scheme:

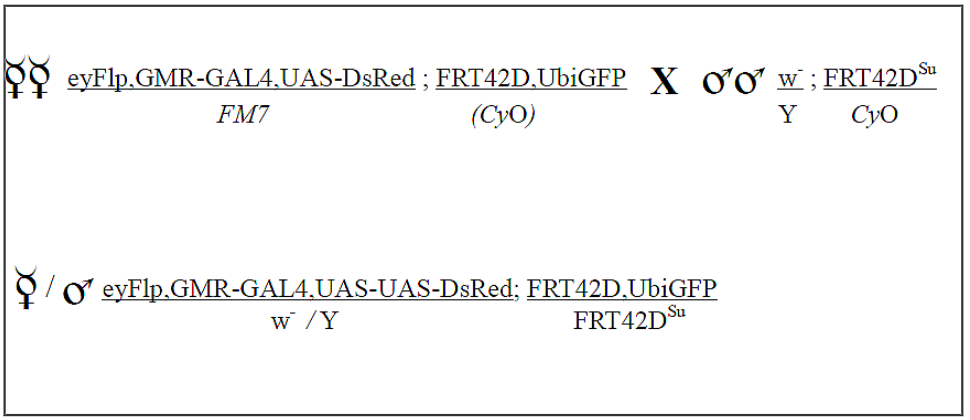


Fig.13: Crossing scheme to analyse GMR-GAL4>UAS-DsRed expression in the four complementation groups. Virgins expressing GMR-Gal4>UAS-DsRed and bear the corresponding FRT42D, UbiGFP chromosomes were crossed to the different suppressor lines. DsRed expression could be analysed in the F1 generation.

Immuno-staining of wing imaginal discs

Wing imaginal discs of L3 larvae of the genotype T155-Gal4, UAS-Flp; FRT42D, UbiGFP/ FRT42D, Su226 were dissected in PBS and immuno-stained with a guinea pig anti-senseless antibody (a gift from Hugo Bellen) as primary antibody and the appropriate secondary antibody (Jackson

ImmunoResearch), following the protocol for staining of eye imgainal discs (see below, p.56).

Immuno-staining of eye imaginal discs and pupal eyes:

Eye imaginal discs were dissected in PBS and incubated with 4% Formaldehyde in PBS for fixation for more than 20 minutes. After washing the discs 3x with PBT for 10 minutes, they were incubated with the primary antibodies at 4°C, overnight. Following 3x10min washes in PBT, the discs were incubated with the secondary antibodies (here: Cy5/ Phalloidin-FITC from Sigma) for one up to three hours at RT and then washed with PBT (3x10min). Primary antibodies were rat anti-Elav (DSHB, 7E8A10) and mouse anti-HA (Covance, MMS101P). Fluorescent conjugated secondary antibodies were obtained from Jackson ImmunoResearch. The final dissection took place in PBT and the discs were mounted on a slide in 90% glycerol and analysed under the confocal microscope (Zeiss LSM710).

Pupal eyes were dissected in 4% Formaldehyde and fixed for more than one hour, the staining procedure was the same as for the eye imaginal discs.

Cloning:

The CG6758 cDNA (cDNA clone GH08266, Drosophila Genome Resource Center) was cloned into the Gateway destination vectors pTWG and pTFHW following Invitrogen Gateway protocol.

Primers to PCR CG6758 and create an entry clone:

Entry clone for pTWG

Forward primer FP-GWS-C-Fbox:
ggggacaagtttgtacaaaaaagcaggcttcaccatggacgcgagcagatacaagg

Reverse primer RP-GWS-C-Fbox:
ggggaccactttgtacaagaaagctgggtcgataatatcacgtggcacactcagg

Entry clone for pTFHW

Forward primer FP-GWS-N-Fbox:
ggggacaagtttgtacaaaaaagcaggcttcgacgcgagcagatacaagg

Reverse primer RP-GWS-N-Fbox:
ggggaccactttgtacaagaaagctgggtcctagataatatcacgtggcacactcagg

The generated plasmids were amplified and purified using the nzytech Midi-prep kit and then sequenced by Stabvida.

The UAS-CG6758-GFP (recombination into pTWG) and UAS-3xHA-3xFlag-CG6758 (recombination into pTFHW) transgenic lines were generated by random P-element mediated transformation. The CH322-12H15 genomic construct from the P[acman] library was integrated into the genome by ϕC31 integrase-mediated transgenesis (acceptor strain #8622, attP2). The injection of DNA into embryos to establish *Drosophila* transgenic lines was performed by BestGene. *w*+ transformants were selected and stable transgenic stocks were established.

RNAi for SCF components in the adult "glossy" eye:

Flies with the following genotype were generated: eyFlp, GMR-GAL4, UAS-Xbp1s; [RNAi]/ *Cy*O or *Sco* or eyFlp, GMR-GAL4, UAS-Xbp1s; (); [RNAi]/ +. Flies were analysed under the dissecting microscope.

Results

2.1. The mosaic genetic screen

In order to do a saturation screen, we analysed around 80.000 progeny flies for the right arm of the second chromosome (2R) and we could recover 32 lethal mutations that suppress the Xbp1s induced “glossyness” and could be identified by more pigmentation (and a more defined ommatidial patterning) in the homozygous mutant clones (**fig.14**).

We recovered the following suppressor mutations (the number indicates the bottle in which the mutation was found): Su95-4, Su102a, Su110, Su138b, Su209b, Su212b, Su217c, Su218b, Su226a, Su233, Su273-1, Su274-1, Su280, Su282, Su287, Su289a2, Su289b, Su209-1, Su309-2, Su311, Su323, Su336, Su344, Su357-3; Su359, Su373, Su396, Su397, Su408, Su436, Su441, Su442.
Additionally we could recover 9 enhancer mutations, where the pigmentation in clones homozygous for the mutations was less than in the control “wildtype” and heterozygous tissue.

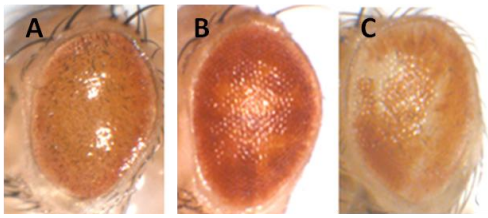


Fig.14: In the mosaic genetic screen, suppressors and enhancers were found. (A) “Glossy” eye phenotype due to the GMR-GAL4>UAS-Xbp1s over-expression. **(B)** Supressor of “glossy” eye phenotype (Su274), the eye pigmentation is restored as well as there is a more defined ommatidial patterning in the homozygous mutant cell clones. **(C)** Enhancer of “glossy” eye phenotype (E317), there is less pigmentation in the homozygous mutant cell clones.

2.2. Complementation analysis between the suppressor mutations

In our mosaic genetic screen we searched for mutations that suppressed the Xbp1s induced “glossy” eye phenotype, but for mapping we used lethality. In order to be sure that lethality and the suppression effect is due to a mutation in the same gene, and since EMS with a 25mM concentration can induce on average one mutation every 400kb (Cooper et al. 2008; Blumenstiel et al. 2009), we tried to find complementation groups among our suppressor mutations. If two suppressor mutations were lethal in trans, we could be sure that lethality and suppression in these mutants were due to hits in the same gene.

Therefore we crossed every lethal stock obtaind from the screen to each other, and among the 32 isolated suppressor mutations we could identify four different complementation groups.

The first complementation group consists of four alleles, Su95-4, Su102a, Su373 and Su396. The second complementation group consists of two alleles, Su 280 and Su344. In those two complementation groups, lethality was observed when bringing the alleles in trans. The third complementation group consists of 14 alleles, Su209b, Su212b, Su217c, Su218b, Su226a, Su273-1, Su274-1, Su311, Su323, Su336, Su357-3; Su359, Su397 and Su442. Not all of these alleles were lethal when in trans, for some combinations we could observe an out-held wing phenotype (**fig.15**), especially when the crosses were raised below 25°C.

Fig.15: Out-held wing phenotype observed in complementation group three. Some of the alleles of complementation group three are not lethal in trans, but give rise to an out-held wing phenotype. This wing phenotype is also observed with some deficiencies covering the mutated region (here: Su273).

	Su212	Su217	Su218	Su226	Su274	Su311	Su323	Su336	Su359
Su212		x	x	x	w	x	w	x	x
Su217			x	x	w	x	w	x	x
Su218				x	w	x	w	x	x
Su226					x	x	x	x	x
Su274						w	w	x	x
Su311							x	x	x
Su323								x	x
Su336									x
Su359									

Fig.16: Complementation analysis between some suppressors of complementation group three. Complementation between suppressor mutations of complementation group three leads to lethality (x) or the out-held wing phenotype (w).

The fourth complementation group consists of three alleles, Su309-2, Su434 and Su441. All these alleles were lethal when in trans.

2.3. Mapping of the complementation groups with deficiencies

Mapping was performed with the Bloomington deficiency kit. Lethality is necessary for mapping with deficiency kits, because mapping is done by complementation analysis with a collection of stocks containing different deletions in the genome. After mapping the mutations to a small region in the genome, we sequenced the candidate genes to identify the gene affected and the molecular nature of the mutations.

Xeroderma pigmentosum group D (Xpd)

For the first complementation group (Su95-4, Su102a, Su373, Su396) the deficiency with the common lethality when in trans over the suppressor mutations, was the deficiency Df(2R)ED3791 (57B1-57D4). After crossing

the mutations to the deficiency Df(2R)PK1 (57C5-57F5-6), we could render the mutation to 50 potential genes. We tested lethal mutations of those genes, when available, in trans over our mutations and lethality was found with a lethal transgenic element insertion in the gene Xeroderma pigmentosum group D (Xpd) (Mi{ET1}Xpd[MB04535]). After sequencing of the ORF of the four suppressor mutations, we found the following mutations: For Su95-4/Su396 we found C134Y, for Su102a C513Y and for Su373 we found the amino acid substitution R630W.

Xpd is a DNA helicase which is involved in three crucial cellular processes: DNA repair by nucleotide excision repair (NER), cell cycle regulation and transcription (reviewed in Cameroni et al, 2010).

Mutations in the human gene ERCC2 , which encodes the human Xpd lead to the inherited rare disease of Xerodermis pigmentosum type D, which makes patients very sensitive for skin cancer. Mutations in ERCC2 can also lead to Cockayne Syndrome type 2.

Xpd is part of the transcription factor IIH (TFIIH), which catalyses the opening of the DNA double strands during transcription after assembly of the pre-initiation complex. Xpd has a similar role during NER.

As part of TFIIH, Xpd links the core TFIIH to the CDK activating kinase (CAK) complex. The TFIIH complex which consists of 10 subunits possesses helicase activity and kinase activity, provided by the cyclin-dependent kinase 7 (Cdk7). Cdk7 is required to phosphorylate diverse transcription factors as well as the carboxy terminal domain (CTD) of RNA Polymerase II (RNA Pol II). Cdk7 in CAK also phosphorylates other Cdks as an essential step for Cdk activation during cell cycle. In *Drosophila* it was shown that CAK activity of Cdk7 is negatively regulated by Xpd (Chen, G. et al., 2003). Excess Xpd titrates CAK activity which results in less activation of Cdks, mitotic defects and lethality, whereas a decrease in Xpd leads to excess phosphorylation of Cdks leading to over-proliferation. Xpd is cell cycle dependently-expressed, its expression is downregulated at the beginning of mitosis which results in cell cycle progression due to increased

CAK activity. Since Xpd bridges the core TFIIH to the CAK complex, the downregulation of Xpd at the beginning of mitosis also leads to mitotic transcription silencing presumably due to diminished TFIIH/CAK-dependent phosphorylation of the RNA PolI CTD.

Ell-associated factor (Eaf)

The second complementation group (Su280, Su344) was lethal over the deficiencies Df(2R)Kr10, spanning from 60E10-60F5. Additionally it was lethal in trans with the deficiencies Df(2R)ED2219 (47D6-48B6), Df(2R)ED2222 (47F13-48B6) and the deficiencies Df(2R)ED1715 (43A4-43F1) and Df(2R)ED1673 (42E1-43D3).

Since Df(2R)Kr10 has more than 500kb deleted (flybase), which are replaced by known and unknown chromosomal material, we first focussed on the deficiencies Df(2R)ED2219 and Df(2R)ED2222, however, crossing the mutations to deficiencies that cover the region between 47F13 and 48B6 revealed no lethality. Then we crossed Su280 and Su344 to different deficiencies that cover the region 43A4-43D3 and found lethality with the deficiencies Df(2R)BSC263 (42F2-43C1) and Df(2R)BSC264 (43B2-43C5), suggesting a hit in the region 43B2-43C1. In the region covered by these deficiencies, there were 14 candidate genes. We tested available lethal mutations of those genes for lethality in trans and found a lethal EP element insertion in the gene Ell-associated factor (Eaf) (P{w[+mC]=EP}Eaf[EP2475]) to be lethal in trans with Su280 and Su344. To find the mutation in Su280 and Su344, we sequenced the ORF of these two mutations and found a hit in Su280 (C>T) leading to an early stop codon (CAG>TAG) at amino acid position 197. According to Flybase, Eaf protein has two isoforms which are 504 and 450 amino acids (aa) long.

The human homologues of the *Drosophila* Ell-associated factor, Eaf1 and Eaf2 are strong positive regulators of ELL elongation activity. Ell (RNA polymerase II elongation factor eleven-nineteen lysine-rich in leukemia) transcription factors stimulate the overall rate of elongation by RNA Pol II due to suppression of transient pausing of RNA Pol II along the transcript by physically interacting with RNA Pol II (Kong et al., 2005). In *Drosophila* Ell also interacts with RNA Pol II at transcriptionally active sites on polytene chromososmes. Knockdown of dELL and dEaf by RNA interference shows that both proteins are essenial for fly development. Upon induction, dEll is recruited to heat shock loci and its presence together with RNA Pol II is essential for proper heat shock gene expression, only after recruitment of Ell to the heat shock loci, there is proper phosphorylation of RNA Pol II (Smith, E. R. et al., 2008). It was also shown in a genetic screen in *Drosophila*, that Eaf is involved in wound healing (Campos et al, 2010).
In the mammalian system Xbp1s was shown to activate Ell transcription (Acosta-Alveolar et al., 2007).

CG6758

For the third and biggest complementation group (14 alleles) we found the non-overlapping deficiencies Df(2R)BSC19 (56F12-14-57A4) and Df(2R)BSC597 (58A2- 58F1) as common deficiencies which were lethal in trans over the tested alleles. Since the molecular lesion of Df(2R)BSC19 is not molecularly defined and we observed no lethality with the covering deficiencies Df(2R)Exel7162 (56F11-56F16) and Df(2R)BSC701 (56F15-57A9), we focussed on the region 58A2-58F1 and tested different deficiencies covering this region (**fig.17**). With some deficiencies we found the out-held wing phenotype (**fig.15**), with others lethality, also the same region was supposed to be deleted, this can be due to chromosomal material that was deleted and integrated somewhere else in the genome.

However we could render the position of our mutations to the region 58C1-58D2, harbouring 12 genes (**fig.18**).

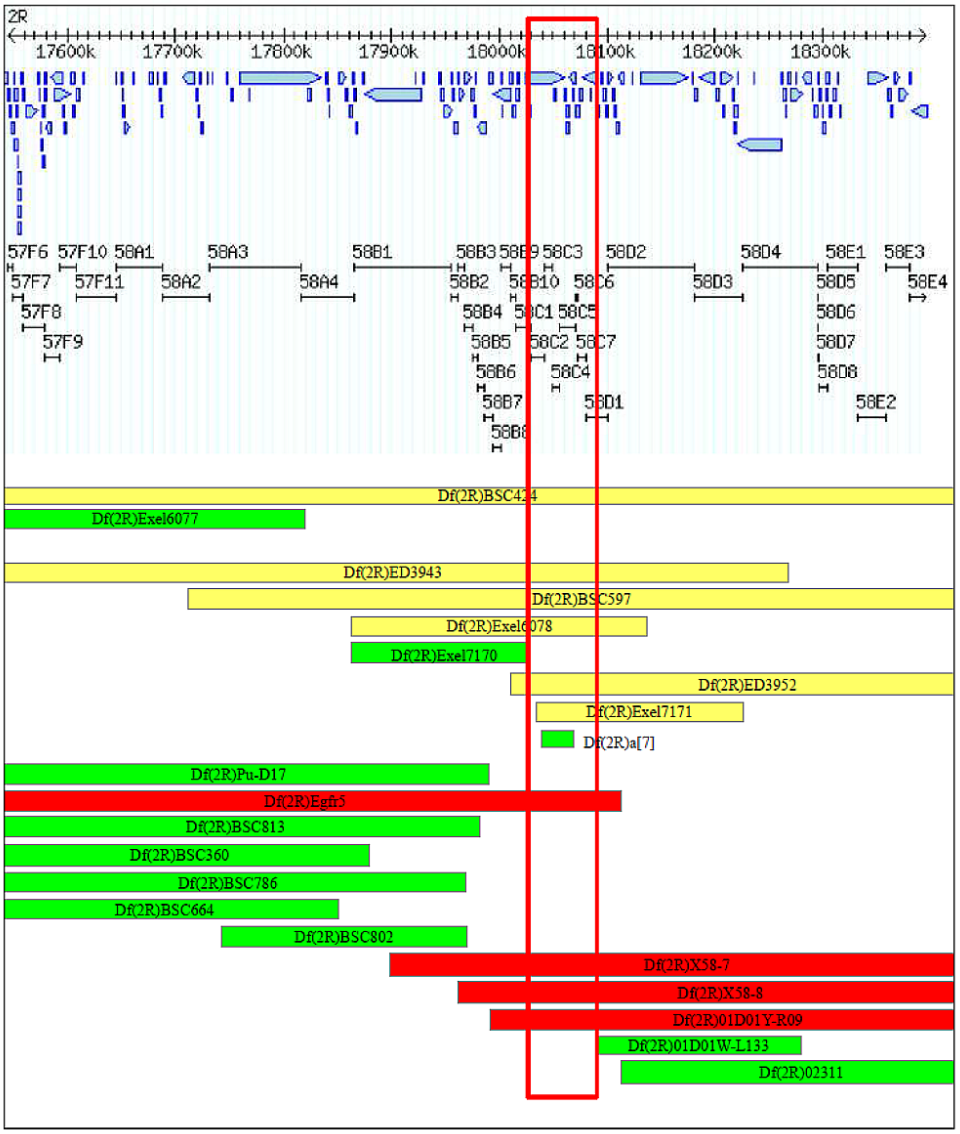


Fig.17: Mapping of complementation group three gave contradictory results. Additional deficiencies to the general deficiency kit were used to map complementation group three. Green bars represent deficiencies which were viable and red bars deficiencies that were lethal in trans over the suppressor mutations. Deficiencies represented by yellow bars gave rise to the outheld wing phenotype when crossed to suppressors of complementation group three.

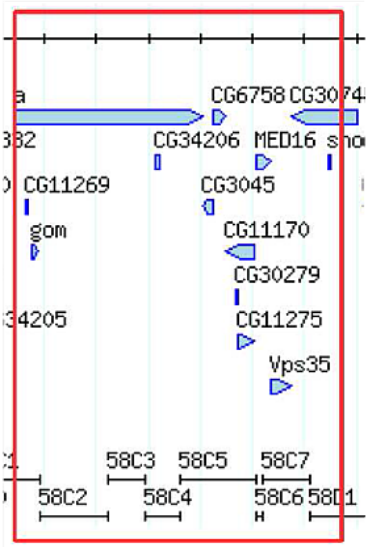


Fig.18: Zoom in of fig.16.

With the deficiency Df(2R)a^{EX1} we found lethality or the held-out wing phenotype for all our 14 alleles. Crossing our suppressor mutations to Df(2R)a[7], a lethal deletion of the arc gene, gave non-balanced offspring, suggesting that the hit was not in arc or the two intergenic genes CG11629 and gom. Since the 3 prime end of Df(2R)a^{EX1} is not defined molecularly (Liu, X. et al., 2000), we tested how far the deficiency reaches by crossing Df(2R)a^{EX1} to lethal P element insertions in Vps35 and CG3074. Since no lethality was observed, we could conclude that the deficiency does not reach the Vps35 gene. Additionally, our suppressor mutations were not lethal over the lethal P element insertion in Vps35. Nevertheless, we tested if Vps35 could be our gene of interest by checking senseless expression in clones of Su226 in wing imaginal discs (**fig.18**), since it was shown that senseless is not expressed in vps35 mutant clones (Franch-Marro et al., 2008). We observed normal senseless expression in homozygous mutant clones for Su226 (**fig.18**) and so we rendered our region of interest to six genes.

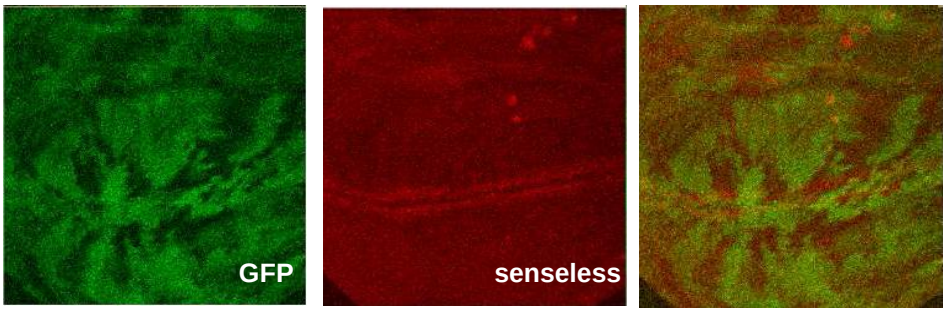


Fig.19: Senseless staining in complementation group three is normal. Mosaic wing imaginal discs of suppressor Su226 were stained with an antibody against senseless and analysed if in clones homozygous mutant for Su226 (absence of GFP) there is reduced senseless expression. Note: There is no difference in senseless expression in mutant and "wildtype" tissue and a mutation in Vsp35 was excluded.

Since no lethal alleles of these six genes exist, we started to sequence the ORF of Med16 in two of the alleles (Su218 and Su359), but found no hit. Our second attempt was to sequence CG6758 and we found several mutations for the sequenced alleles. In total we sequenced seven of the 14 alleles - Su209, Su212b, Su217c, Su218b, Su226, Su357 and Su359. The ORF of CG6758 encodes a 667 amino acid (aa) long protein. Su209 has a G>A mutation, leading to a premature STOP codon at position 130 aa. For Su212b, Su217c and Su218b we found the same 11bp deletion (aa 344-347), leading to a frameshift and an early stop codon at position 423 aa. For Su226a we found two mutations in the ORF (C>T) leading to an amino acid substitution (threonin to methionin at position 218) and an early stop codon at position 435 aa. Likely, these mutants with premature STOP codons encode protein nulls, as it is known that mRNA with premature STOP codons are degraded by nonsense-mediated RNA decay (reviewed in Brogna and Wen, 2009). For Su359 we found one mutation in the ORF

(C>T) leading to the exchange from glycine to glutamic acid at position 639 (**fig.20**). For Su357 we could not detect any mutation in the ORF, maybe the mutation lays within the promotor region of CG6758.

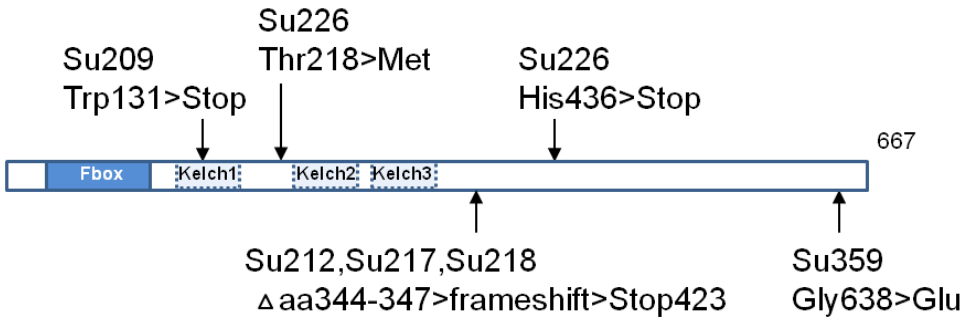


Fig.20: Scheme of the CG6758 protein with the obtained mutations. The domain structure of the CG6758 protein with its Fbox domain and the three Kelch repeats is depicted. The molecular lesions of the sequenced CG6758 alleles are shown.

In our screen we found 14 mutations in CG6758, from which we sequenced seven alleles. CG6758, with the cytological map location 58C5 encodes a protein with a Kelch repeat domain formed by three Kelch repeats and an Fbox domain (flybase).

The Kelch domain, first found in the *Drosophila* protein Kelch, is a protein-protein interaction domain. Proteins with an Fbox domain are known to be part of E3 ubiquitin ligase complexes (Skip-Cullin-Fbox complexes) catalyzing the ubiquitination of proteins destined for proteasomal degradation, whereby the Fbox domain of Fbox proteins bind SkpA/Skp1 in the complex.

The Fbox protein is responsible for specificity of the complex, since it binds specific substrates (mostly phosphorylated) bringing them in proximity of

the E2 Ubiquitin conjugating protein. There are three families of Fbox proteins named after their protein-protein interaction domains: FBXWs (WD40 repeats), FBXLs (leucine-rich repeats), or FBXOs (variable or no homology domains). The *Drosophila* genome contains 45 Fbox proteins (Dui et al., 2012), which are implicated in diverse cellular processes.
The CG6758 protein is 667 aa long and its biological function is unknown.

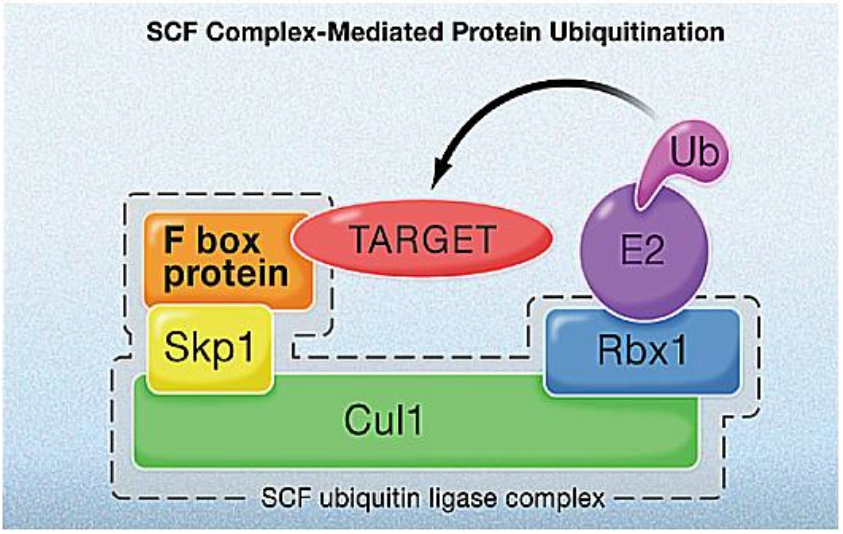


Fig.21: SCF complex-mediated protein ubiquitination. In the SCF ubiquitin ligase complex, Fbox proteins bind to Skp1 (SkpA, in *Drosophila*) via the Fbox domain and recruit a substrate/target that is ubiquitinated by the complex. Protein ubiquitination occurs via a linked E2 ubiquitin ligase.

(fig. from Skaar et al. (2009). SnapShot: Fbox Proteins I. Cell 137)

The fourth complementation group was not mapped so far.

2.4. Testing the suppressor mutations for an effect on GMR-GAL4>UAS-DsRed expression

All the recovered suppressor mutations were tested in a secondary screen, to confirm that the suppression effect is not just the result of reduced GMR-GAL4>UAS-Xbp1s transgene expression. Therefore, males bearing the suppressor mutation were crossed with female virgins carrying eyFlp, GMR-Gal4, UAS-DsRed and the corresponding FRT42D, UbiGFP chromosome. The eye imaginal discs of the offspring were analysed by confocal microscopy after dissection from 3rd instar larvae. The DsRed expression levels were compared between homozygous mutant clones, which were marked by the absence of GFP and “wildtype” GFP expressing control tissue. To confirm that the homozygous mutant clones are not just holes in the eye discs due to dissection errors, we also stained the discs with Elav which marks the photoreceptors.

For the first, second and fourth complementation group we found reduced levels of DsRed expression in the homozygous mutant clones (**fig.22**), meaning transcription from the UAS-DsRed transgene is reduced. For the third complementation group we found no difference in DsRed expression between homozygous clones and sorrounding control tissue (**fig.22**), indicating that those alleles are potential downstream targets of Xbp1s. To be sure that during later pupal stages of development there is no reduced transgene expression, we also analysed DsRed expression in homozygous CG6758 mutant clones in mid pupal stages (**fig.22**). Also in the pupa there is no difference in transgene expression in mutant, “wildtype” and heterozygous tissue.

Since mutations in Xpd and Eaf as well as mutations in the fourth complementation group lead to reduced expression of Xbp1s and thereby suppress the “glossy” eye phenotype, we could exclude that they are

downstream mediators of Xbp1s induced cell death. We further focussed on exploring the function of CG6758.

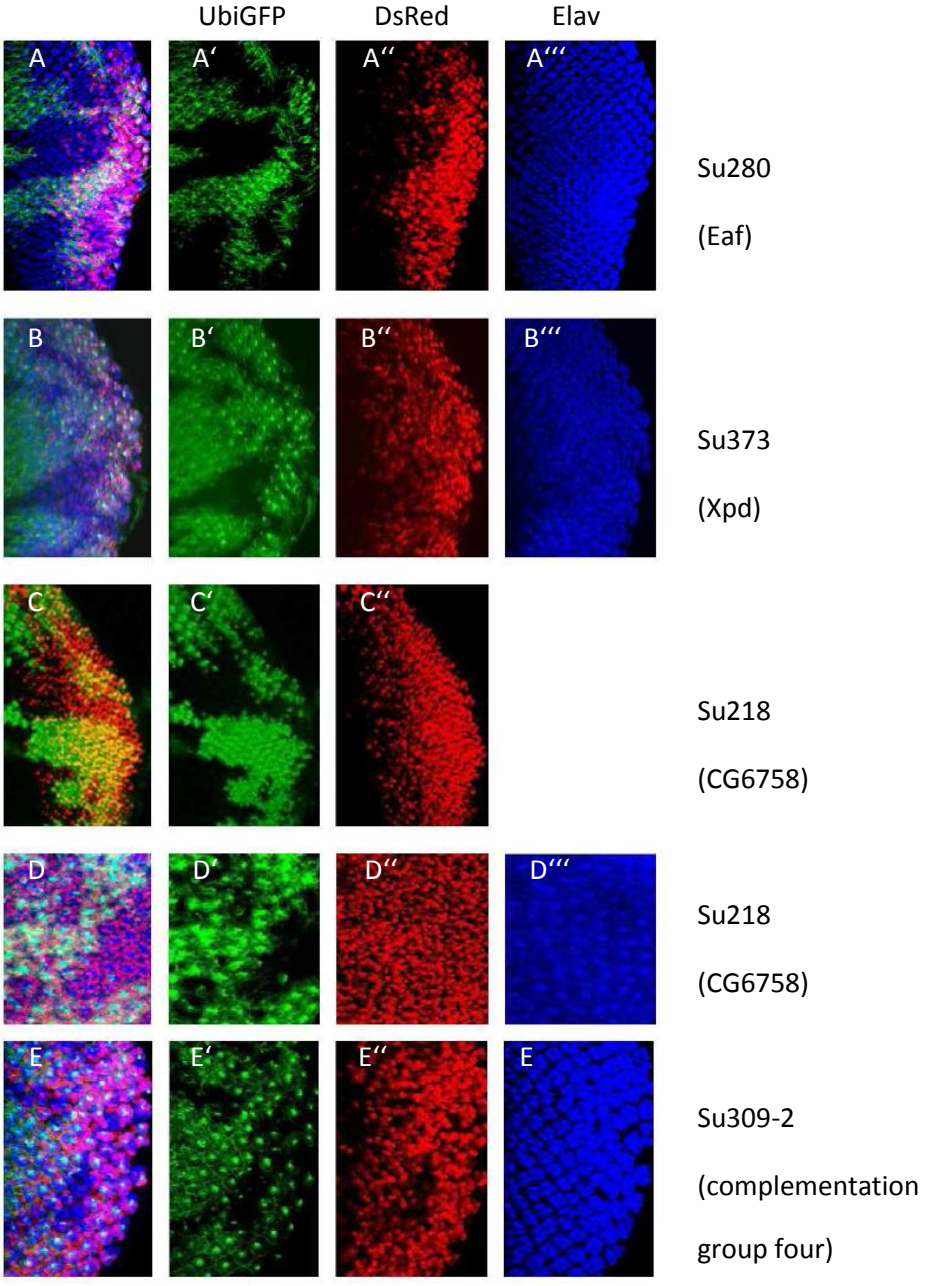


Fig.22: UAS-DsRed expression in the four complementation groups. GMR-Gal4 driven UAS-DsRed expression was analysed in homozygous mutant clones in mosaic eye imaginal discs and pupal eyes (Su218). Absence of GFP marks homozygous mutant clones, as control photoreceptors were stained with Elav (blue) to be sure that clones lacking GFP are no holes in the disc. Note: in complementation group one, two and four, there is reduced DsRed staining in the clones, whereas in complementation group three DsRed expression is the same in mutant clones and surrounding control tissue. Only complementation group three is a potential downstream mediator of Xbp1s induced cell death.

2.5. CG6758 acts downstream of Xbp1spliced

Although DsRed expression is not altered in homozygous mutant clones of CG6758, which indicates that UAS-transgene expression is not affected, it was still possible that CG6758 mutants cause a reduction in the protein levels of Xbp1s, thereby reducing the “glossyness” in the eye. To test this hypothesis, we analysed the Xbp1s protein levels in mosaic eye imaginal discs and in pupal eyes. The over-expressed Xbp1s protein is tagged with HA and therefore we could stain with an antibody against HA to detect protein levels. We observed no reduced levels of Xbp1s-HA staining in homozygous mutant clones compared to control tissue (**fig.23, 24**), neither in the eye imaginal discs nor in pupal eyes. We concluded that CG6758 is acting downstream of Xbp1s in the induced cell death signaling.

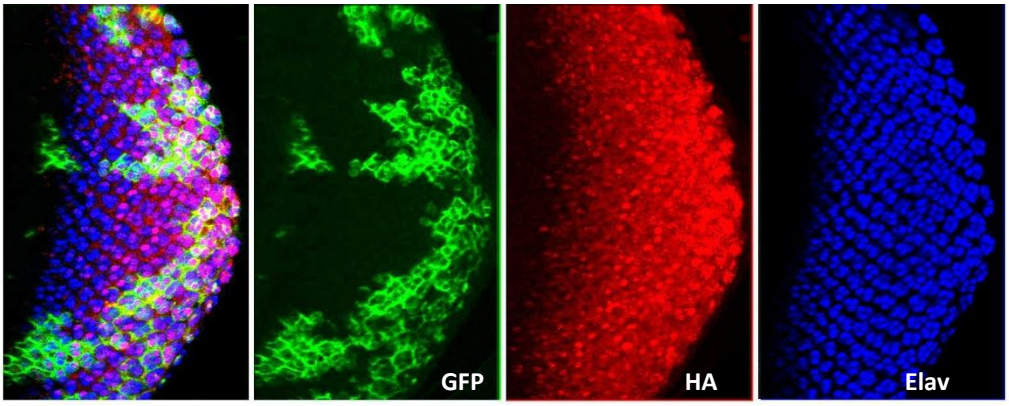


Fig.23: In eye discs, Xbp1s protein levels are not altered in CG6758 mutant clones. In clones homozygous mutant for Su218 (absence of GFP) HA levels (red) are equal to wildtype and heterozygous tissue (green). As a control, photoreceptors are stained with Elav (blue) to be sure that clones lacking GFP expression are no holes in the disc. CG6758 seems to be a downstream mediator of Xbp1s induced cell death.

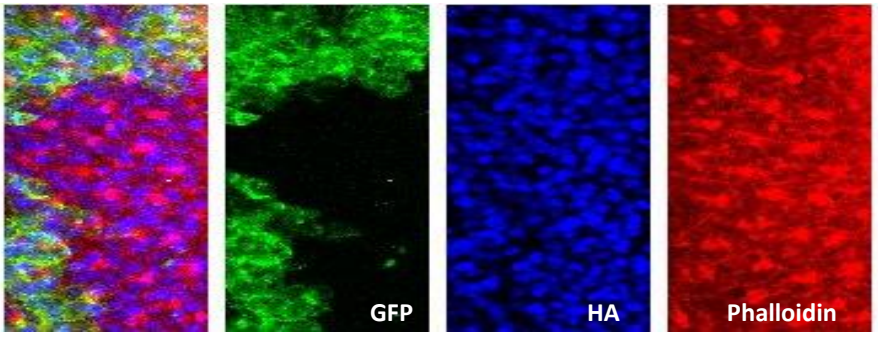


Fig.24: In pupal eyes, Xbp1s protein levels are not altered in CG6758 mutant clones. In clones homozygous mutant for Su218 (absence of GFP) HA levels (blue) are equal to wildtype and heterozygous tissue (green). Phalloidin stains Actin filaments to be sure that lack of GFP expression is not due to holes in the eye discs. CG6758 seems to be a downstream mediator of Xbp1s induced cell death.

2.6. Rescue of the suppression effect with [P]acman vectors

To ensure that the suppression effect in the “glossy” eye is really due to mutations in the Fbox protein CG6758, we tried to revert the suppression effect with the simultaneous expression of a genomic rescue construct. Therefore the genomic rescue constructs from the [P]acman library (BACs) CH322-12H15 and CH322-114L3 were injected into embryos for transformation. Since we were not able to balance the stock with CH322-114L3 for the second and third chromosomes, we only tested the genomic rescue construct CH322-12H15 for its rescue ability. The construct spans the CG6758 ORF and 20kb upstream as well as 2,7kb downstream of it. Flies with the genotype eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, Su357/ FRT42D, UbiGFP; CH322-12H15/ + were compared with their siblings with the genotype eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, Su357/ FRT42D, UbiGFP; TM6B/ + and tested if the presence of CH322-12H15 can revert the suppression effect of CG6758 mutant clones in the “glossy” eye.

In fact, the eyes of the eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, Su357/ FRT42D, UbiGFP; CH322-12H15/ + flies were totally “glossy” and no suppressor clones were visible (**fig.25**).

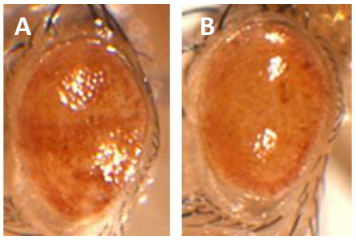


Fig.25: The [P]acman reagent CH322-12H15 is able to revert the suppression effect of CG6758 mutations. (A) GMR-GAL4>UAS-Xbp1s “glossy” eye with suppressor mutation Su357. **(B)** With the [P]acman reagent CH322-12H15 in the background. Note: The suppression effect of Su357 is completely reverted. Mutations in CG6758 seem to be the cause for the suppression effect.

Furthermore, the [P]acman reagent CH322-12H15 was able to rescue lethality of the CG6758 mutations.

2.7. Rescue of the suppression effect with CG6758 cDNA

We also rescued the suppression effect of CG6758 mutations with the co-expression of CG6758 cDNA. We cloned CG6758 cDNA into the Gateway vectors pTFHW and pTWG to have tagged versions of CG6758. The pTFHW vector generates an N-terminally tagged fusion protein (3xFLAG-3xHA) and the pTWG vector a C-terminally tagged fusion protein (GFP). With both UAS-constructs we were able to revert the suppression effect in the "glossy" eye when over-expressed with GMR-GAL4 (**fig.26**). These results confirm that CG6758 mutations, and not any other mutation that may be present in the chromosomes, lead to the suppression effect in the Xbp1s induced "glossy" eye.

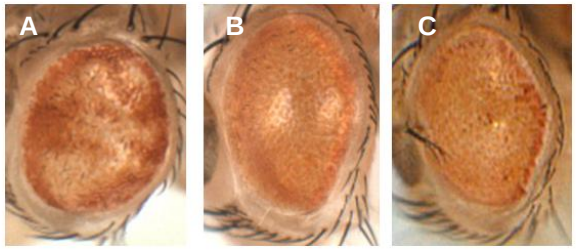


Fig.26: Rescue of the suppression effect with CG6758 cDNA. (A) Mutations of CG6758 (here: Su209) suppress the GMR-Gal4>UAS-Xbp1s "glossy" eye phenotype. With CG6758 cDNA CG6758-GFP **(B)**, 3xHA-3xFlag-CG6758 **(C)**) the suppression effect of the CG6758 mutations (here: Su209/ Su218) is completely reverted when the constructs are co-expressed by GMR-GAL4. Mutations in CG6758 are the cause for the suppression effect.

2.8. RNA interference of SCF complex components can partially rescue the “glossy” eye phenotype

Since the function of CG6758 is unknown, we tested if CG6758 is acting as part of an SCF complex, being responsible for ubiquitination of substrates. Therefore, we tested RNA-interference (RNAi) lines for the different components of an SCF complex for their ability to suppress the “glossy” eye phenotype (**fig.27**).

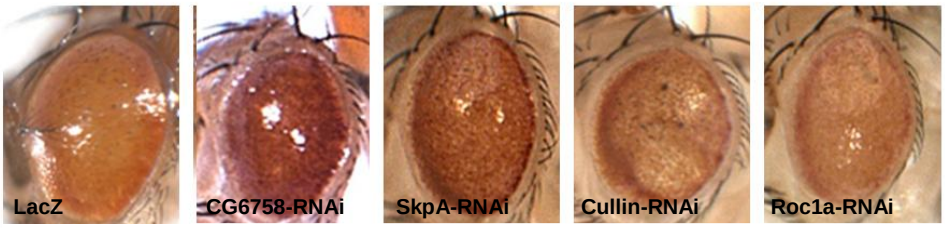


Fig.27: RNAi of SCF complex components can partially rescue the “glossy” eye phenotype. GMR-GAL4 driven expression CG6758-RNAi is the strongest suppressor among the tested components, SkpA-RNAi and Cullin-1-RNAi construct expression is weaker and Roc1a-RNAi does not suppress the “glossy eye” phenotype. Suppression with the RNAi constructs is mainly a rescue of the pigmentation. GMR-GAL4>UAS-LacZ serves as a control.

RNAi constructs were over-expressed in the “glossy” eye by the GMR-GAL4 driver. RNAi constructs for CG6758, SkpA, Cullin-1 and Roc1a were tested. In terms of eye pigmentation, CG6758-RNAi was the most potent suppressor, SkpA- and Cullin-RNAi showed less suppression and with the Roc1a-RNAi construct, no suppression was observed.

The GMR-GAL4 driven expression of UAS-LacZ served as a control construct, with that control, no rescue of pigmentation could be observed, although the UAS-LacZ construct expresses the *mini white* gene strongly in the used line.

Since there was partial suppression with SkpA-RNAi and Cullin-RNAi, we proposed that CG6758 may act in an SCF ubiquitin ligase complex.

Discussion:

The UPR is activated when unfolded or misfolded proteins accumulate in the ER and serves to restore ER homeostasis by translational attenuation, the induction of chaperones and ERAD. However, if ER stress is too strong or prolonged, cells die. In mammals, IRE1 signaling is known to be involved in apoptosis and autophagy, one link is JNK activation via IRE1-Traf2 interaction (Nishito et al., 2002, Urano et al., 2000). Depending on what signaling JNK triggers, this can lead to apoptosis or autophagy. There are also JNK-independent links between IRE1 activation and cell death, for example it could be shown that RIDD leads to degradation of microRNAs that normally repress translation of Caspase-2 mRNA, and thus sharply elevates protein levels of this initiator protease of the mitochondrial apoptotic pathway (Upton et al., 2012).

The pathways linked to ER stress induced apoptosis and autophagy are independently of Xbp1s signaling, however over-expression of Xbp1s in the *Drosophila* eye driven by GMR-GAL4 leads to a “glossy” eye phenotype, which is due to cell death of the cells posterior to the morphogenetic furrow. We made use of this atrophic eye phenotype to identify genes which are involved downstream of Xbp1s induced cell death. We performed a mosaic genetic screen to identify EMS induced mutations that suppress the “glossy” eye phenotype in terms of pigementation (and ommatidial structure). We showed that our screen is amenable to the identification of Xbp1s downstream targets as we could recover 32 lethal suppressor

mutations. Since we found four complementation groups, lethality is due to a hit in the same gene that also causes the suppression effect and we were able to identify three of the four complementation groups by mapping with the Bloomington deficiency kit and sequencing of candidate genes. Complementation group one consists of four alleles of Xeroderma pigmentosum group D (Xpd), complementation group two consists of two alleles of Ell-associated factor (Eaf) and for complementation group three we could recover 14 alleles of the Fbox protein CG6758.

Although those complementation groups could be potential downstream mediators of Xbp1s induced cell death, it was also possible that those mutations solely reduced the GMR-GAL4>UAS-Xbp1s transgene expression, e.g. due to modification of GMR-GAL4 or an overall repression of transcription. We tested this possibility by investigating if the mutations lead to reduced GMR-GAL4>UAS-DsRed expression and found for the first, the second and the fourth complementation group this to be true. Since Xpd and Eaf (complementation group one and two) are involved in the process of transcription, one can assume that overall transcription is repressed in the mutant clones. Since complementation group four was not mapped and we also observed a reduced DsRed expression, we decided to stop the mapping process for this complementation group. For CG6758 (complementation group three) we did not observe any reduced DsRed expression, neither in eye imaginal discs nor at later stages in the pupa, making CG6758 a potential downstream mediator of Xbp1s. We excluded the possibility that mutations of CG6758 modulate the Xbp1s protein levels, by staining eye imaginal discs and pupal eyes with an HA antibody recognizing Xbp1s-HA. The protein levels of Xbp1s were not altered in CG6758 mutant clones. We concluded that CG6758 acts downstream of Xbp1s. It is also possible that CG6759 somehow regulates Xbp1s activity independently of Xbp1s protein levels. For example, CG6758 could promote the degratation of a negative regulator/ co-factor of Xbp1s.

The best method to test whether a distinct mutation is responsible for a given phenotype, is to test a rescue construct for its ability to revert the phenotype. In our case, we tested if mutations in CG6758 are responsible for the suppression effect in the Xbp1s induced “glossy” eye. We tested the [P]acman reagent CH322-12H15 for its rescue ability. With this construct in the background the suppression effect of the CG6758 mutations could be rescued. Furthermore, we could also rescue lethality of the CG6758 alleles. The construct spans the CG6758 ORF and 20kb upstream as well as 2,7kb downstream of CG6758. In this type of rescue experiments with genomic regions, failure in rescuing could be due to missing regulatory sequences of the gene of interest. Alternatively, it is also possible that the ability to rescue is due to a gene neighbouring the gene of interest (CG6758, in this case), which is also covered by the rescuing construct. So, we also performed the rescue experiment with cDNA of CG6758. We confirmed that GMR-GAL4 driven expression of CG6758-GFP and 3xHA-3xFlag-CG6758 cDNA was able to revert the suppression effect of the CG6758 mutations in the Xbp1s induced “glossy” eye.

To analyse whether there is a hint that CG6758 possibly acts in an SCF ubiquitin ligase complex, we conducted RNAi for the different complex components in the background of Xbp1s and found for CG6758-RNAi and RNAi of Skp1 and Cullin a partial rescue of eye pigmentation. If CG6758 acts in an SCF complex, knockdown of the complex components should lead to a suppression of the “glossy” eye phenotype. A proof that CG6758 acts in an SCF complex could be provided by Co-IP between CG6758 and components of the SCF complex, in transient transfections in S2 and human HEK cells such an interaction was found between CG6758 and Skp1 as well as Cullin (Catarina Gaspar, personal communication).

Acknowledgements and autor contribution

We thank Vanya Rasheva for setting up the screen by generating the appropriate fly stocks, additionally we thank her for the contribution of the suppressor mutants up to Su233.

Vanya Rasheva performed the mapping of the the first complementation group.

We also thank the Bloomington *Drosophila* stock center for providing deficiency stocks to do our mapping analysis and for the CG6758 cDNA clone RH08189 and the RNAi lines. We thank the [P]acman resource center for providing the two [P]acman reagents, CH322-12H15 and CH322-114L3. Furthermore, we thank Sören Prag for the *Drosophila* Gateway vectors pTFHW and pTWG and Florence Janody for the T155-GAL4, UAS-Flp line. Finally we thank H. Bellen for the senseless antibody.

References

Brogna, S., Wen, J. (2006). Nonsense-mediated mRNA decay (NMD) mechanisms. Nature Structural & Molecular Biology 16, 107-113

Cameroni, E., Stettler, K., Suter, B. (2010). On the traces of XPD: cell cycle matters -untangling the genotype-phenotype relationship of XPD mutations. Cell Division 5:24.

Campos, I., Geiger, J.A., Santos, A.C., Carlos, V., Jacinto, A. (2010). Genetic screen in Drosophila melanogaster uncovers a novel set of genes required for embryonic epithelial repair. Genetics 184 (1): 129-140.

Chen, J., Larochelle, S., Li, X., Suter, B. (2003). Xpd/Ercc2 regulates CAK activity and mitotic progression. Nature 424, 228-230

Dui, W., Lu, W., Ma, J., Jiao, R. (2012). A systematic phenotypic screen of F-box genes through a tissue-specific RNAi-based approach in Drosophila. J Genet Genomics. 39 (8): 397-413.

Franch-Marro, X., Wendler, F., Guidato, S., Griffith, J., Baena-Lopez, A., Itasaki, N., Maurice, M. M., Vincent, J.-P. (2008). Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. Nature Cell Biology 10, 170-177.

Golic K.G. (1991). Site-specific recombination between homologous chromosomes in Drosophila. Science. 252(5008), 958-61.

Hermand, D. (2006). F-box proteins: more than baits for the SCF? Cell Div. 1: 30

Kong, S. E., Banks, C. A., Shilatifard, A., Conaway, J. W., Conaway, R. C. (2005). ELL-associated factors 1 and 2 are positive regulators of RNA polymerase II elongation factor ELL. Proc Natl Acad Sci USA 102(29):10094-8.

Liu, X., Lengyel, J. A. (2000). Drosophila arc Encodes a Novel Adherens Junction-Associated PDZ Domain Protein Required for Wing and Eye Development. Developmental Biology 221, 419-434.

Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., Ichijo, H. *(2002*). ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev. 16, *1345*–1355.

Ryoo, H.D., Domingos, P.M., Kang, M.-J., Steller, H. (2007). Unfolded protein response in a Drosophila model for retinal degeneration. EMBO J. *26*, 242–252.

Skaar, J. R., Pagan, J. K., Pagano, M. (2009). SnapShot: F Box Proteins I. Cell Volume 137, Issue 6, Pages 1160-1160.e1.

Smith, E. R., Winter, S., Eissenberg, J. C, Shilatifard, A. (2008). Regulation of the transcriptional activity of poised RNA polymerase II by the elongation factor ELL. PNAS vol. 105; no. 25; 8575-8579.

Tabata, T. (2001). Genetics of morphogen gradients. Nature Reviews Genetics 2, 620-630.

Upton, J. P., Wang, L., Han, D., Wang, E. S., Huskey, N. E., Lim, L., Truitt, M., McManus, M. T., Ruggero, D., Goga, A., Papa, F. R., Oakes, S. A. (2012). IRE1α cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. Science 338 (6108): 818-22.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., Ron, D.(2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 287 (5453): 664-6.

Chapter III

The role of CG6758 in the regulation of cell death induced by Xbp1s

Summary

In a *Drosophila* model for Autosomal Dominant Retinitis Pigmentosum (ADRP), Xbp1 was shown to have a protective function. However, over-expression of the active transcription factor Xbp1s with the GMR-GAL4 driver leads to an atrophic eye phenotype ("glossy" eye) with cell death of lattice and pigment cells.

To analyse the cell death induced by Xbp1s, we generated eyes mosaic for Xbp1s over-expressing cells and found by TUNEL staining and by use of the CPV reporter, that apoptosis is involved in the cell death process. Mosaic adult eyes have only few Xbp1s over-expressing cells, presumably due to apoptosis of most of these cells during development. The number of Xbp1s over-expressing cells could be rescued by the co-expression of P35, an inhibitor of caspases. Furthermore we observed autophagy activity. Eyes completely "glossy" due to heterozygous GMR-GAL4 driven over-expression of Xbp1s can only be partially rescued by the co-expression of P35. Diap-1 (*Drosophila* Inhibitor of Apoptosis Protein-1) co-expression also leads only to a partial rescue of the eye phenotype, suggesting that there are additional mechanisms besides apoptosis that are involved in the induction of the "glossy" eye phenotype by Xbp1s. The co-expression of Xbp1unspliced (Xbp1u) was the most potent suppressor of the heterozygous Xbp1s over-expressing eye phenotype. We analysed cell death in "glossy" eye imaginal discs with clones of the CG6758 mutations to test if apoptosis is reduced in homozygous mutant clones. We found with TUNEL staining only little apoptosis in the CG6758 mutant cells and with the CPV apoptosis marker we could not detect any cleaved parp which is the substrate for caspase 3. Since the whole "glossy" eye can only be partially rescued by apoptosis inhibitors and we could

only detect little apoptosis with TUNEL staining, we analysed autophagy levels in those discs and found no ATG8-GFP foci in the Xbp1s over-expressing GMR domain.

Introduction

Cone cells are the ommatidial cells that secrete the lens-forming material. "Glossy" phenotypes are characteristic of retinas with faulty lens secretion due to defective cone cells (Cagan and Ready, 1989; Fu and Noll, 1997).

A loss of cone cells either due to cell death or due to impaired specification can be responsible for the Xbp1s induced "glossy" eye phenotype.

Cell death can be apoptotic, autophagic or necrotic. Apoptotic cell death is conferred by caspases and can be inhibited by IAPs (Diap in *Drosophila*) (Tenev et al., 2004) or the baculoviral protein P35 (Clem at al., 1991). The core components of autophagic cell death are the ATG proteins (Mizushima, 2005). Necrosis is a form of cell injury, leading to loss of cell membrane integrity and an uncontrolled release of cellular products into the intracellular space.

The UPR is associated with apoptosis as well as autophagy (see 1.4.1. and 1.4.2.), however Xbp1s signaling was known so far to be "protective" for cells in most paradigms (e.g. Sado et al., 2009) To test if prolonged and strong Xbp1s signaling can induce cell death, one needs to analyse if there are underlying cell death mechanisms, this can be done by special markers for the cell death types or by inhibiting the different forms of cell death.

In a *Drosophila* model for Autosomal Dominant Retinitis Pigmentosa (ADRP), where flies are heterozygous for the $Rh1^{G69D}$ allele ($ninaE^{G69D/+}$), it was shown that Xbp1 has a protective role against retinal degeneration (Ryoo et al., 2007). Reduction of Xbp1 gene dosage accelerated retinal degeneration of these animals. In the ADRP model the Ire1/Xbp1 pathway is activated, which was analysed with a specific UPR marker, xbp1-EGFP, in which EGFP is only expressed in frame in the presence of ER stress and Ire1 activation (Ryoo et al., 2007).

However, expressing the spliced form of Xbp1 (Xbp1s) under the control of GMR-GAL4 in the developing *Drosophila* eye, leads either to cell death or impaired specification of ommatidial cells (**fig.11**).

Material and Methods

Apoptosis markers:

TUNEL staining

TUNEL staining was done with the ApopTag Fluorescein In Situ Apoptosis Detection Kit from Millipore.

Eye imaginal discs from flies with the following genotypes were TUNEL stained: eyFlp, GMR-GAL4;; FRT82B/ FRT82B, UAS-DsRed, UAS-Xbp1s and eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, UbiRFP/ FRT42D, Su218.
Eye discs were dissected and immuno-stained with rat-anti-Elav (for protocol see Chapter II, p.56), followed by analysis of the discs under the confocal microscope (Zeiss LSM710).

CPV reporter

Eye imaginal discs of flies with the following genotypes were dissected and immuno-stained with rabbit-anti-cleaved PARP (Abcam ab2317) following the protocol in Chapter II, p.55: eyFlp, GMR-GAL4; UAS-CPV; FRT82B/ FRT82B, UAS-DsRed, UAS-Xbp1s and eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, UbiRFP/ FRT42D, Su218; UAS-CPV. The UAS-CPV flies were obtained from Florentin and Arama, 2012.

Apoptosis inhibitors:

Co-expression of GMR-P35, GMR-GAL4>UAS-P35 and GMR-GAL4>UAS-Diap was performed in adult whole “glossy” eyes (GMR-GAL4>UAS-Xbp1s).

Autophagy markers:

The autophagy markers UAS-LC3-GFP and UAS-ATG8-GFP were used (Rusten et al., 2004).

Results

3.1. Cell death induced by Xbp1s

Since the Xbp1s construct is C-terminally tagged with HA, we also tested whether untagged Xbp1s (Yanicostas) causes a “glossy” eye phenotype, when over-expressed by the GMR-GAL4 driver, to exclude that the HA tag is responsible for the cell death. GMR-GAL4 driven over-expression of UAS-Xbp1s without tag causes the same eye phenotype as our tagged construct **(fig.11).**

We tested whether the cell death observed in the eye is due to apoptosis by analyzing mosaic eye discs with clones over-expressing Xbp1s with two different apoptosis markers, TUNEL staining and the CPV reporter (CD8-parp-Venus) (Florentin and Arama, 2012). The CPV reporter is cleaved specifically in its parp portion (DEVD'G) by caspase-3, leading to a membrane-bound CD8-parp portion and a cytoplasmic parp-Venus portion,

which can specifically be recognized by an antibody against cleaved parp (**fig.28**).

With both TUNEL staining and the CPV reporter, we could observe apoptosis in Xbp1s over-expressing cell clones **(fig.29, 30)**.

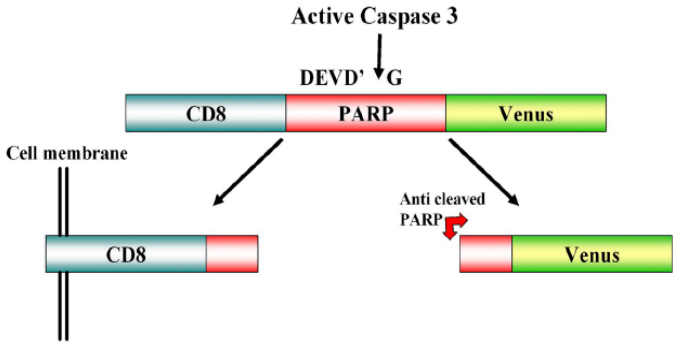


Fig.28: CPV reporter to detect active caspase 3. The CPV reporter consists of the membrane-bound CD8, the parp protein and a venus portion. Active caspase-3 cleaves its substrate parp at the motif DEVD*G which leads to relief of the venus and parp portion from the membrane. Furthermore, cleaved parp can be recognized by a specific antibody.

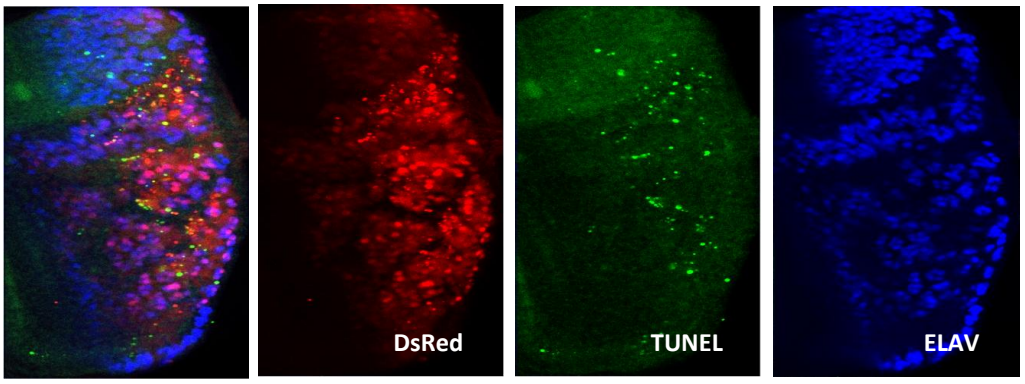


Fig.29: There is apoptosis detection in Xbp1s over-expressing clones by TUNEL staining. TUNEL staining (in green) reveals apoptotic cells in Xbp1s over-expressing cell clones marked by DsRed (eyFlp, GMR-GAL4;; FRT82B/ FRT82B, UAS-DsRed, UAS-Xbp1s). Elav (blue) is a marker of the photoreceptors.

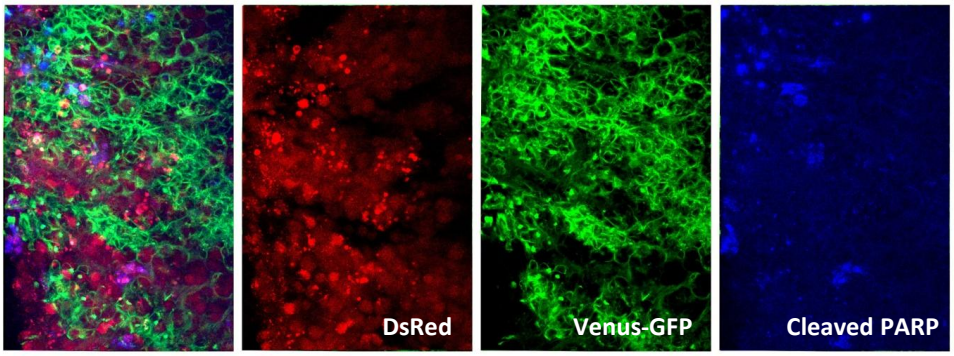


Fig.30: There is apoptosis detection by the CPV reporter in Xbp1s over-expressing clones. Xbp1s over-expressing cells (DsRed) have less membrane bound CPV reporter (GFP). The cleaved parp antibody (in blue) recognizes cleaved parp in Xbp1s over-expressing cells. There is active caspase-3 in Xbp1s over-expressing cell clones. Genotype: eyFlp, GMR-GAL4; UAS-CPV; FRT82B/ FRT82B, UAS-DsRed, UAS-Xbp1s

Adult eyes which are mosaic for cells over-expressing UAS-Xbp1s have only few “glossy” areas **(fig.31a,b)**, presumably because most of those cells die during development and are replaced by “wildtype” cells. The co-expression of the baculoviral caspase inhibitor P35 could rescue the clone number and size, as well as it rescued partially the “glossyness” in heterozygous tissue (which can be identified by less DsRed expression than in tissue homozygous for the UAS-Xbp1s construct) **(fig.31d,e)**.

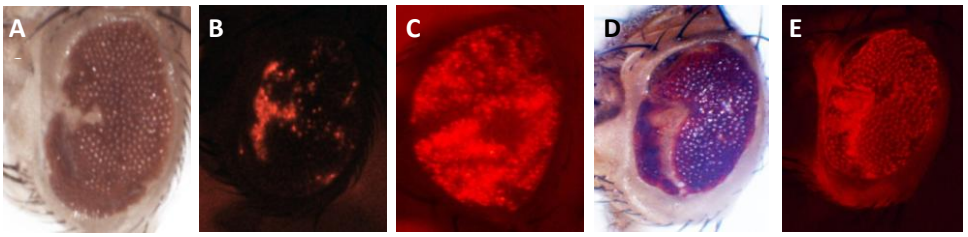


Fig.31: Xbp1s over-expressing cell clones in the adult eye are very small and can be partially be rescued by co-expression of P35. (A) Flies with the genotype eyFlp, GMR-GAL4; CyO/ Sco; FRT82B/ FRT82B, UAS-DsRed, UAS-Xbp1s have only very small "glossy" clones in the eye, which can be visualized by DsRed **(B)**. **(C)** As control, in flies with the genotype eyFlp, GMR-GAL4; CyO/ Sco; FRT82B/ FRT82B, UAS-DsRed around two thirds of the eye consists of DsRed expressing cells, indicating that Xbp1s over-expressing cell clones are removed from the eye during development. **(C)** When P35 is co-expressed, the eyes consists more than 2/3rd of Xbp1s over-expressing cell clones and the ommatidial pattern can be partially restored. The clones are visualized by DsRed **(E).** Flies in D and E have the genotype: eyFlp, GMR-GAL4; CyO/ P35; FRT82B/ FRT82B,

We tried to rescue the "glossy" eye phenotype when the whole eye is "glossy" by co-expression of the baculoviral caspase inhibitor P35, but could only observe little restoration of the ommatidial patterning **(fig.32c,d)**, the same is true if we co-expressed Diap **(fig.32e)**. Co-expression of UAS-LacZ served as a control.

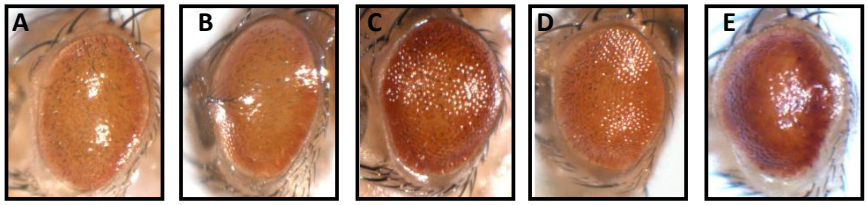


Fig.32: Rescue of the glossy eye phenotype with apoptosis inhibitors. (A) GMR-GAL4>UAS-Xbp1s. **(B)** GMR-GAL4>UAS-Xbp1s, UAS-LacZ. **(C)** GMR-GAL4>UAS-Xbp1s, GMR-P35. **(D)** GMR-GAL4>UAS-Xbp1s, UAS-P35. **(E)** GMR-GAL4>UAS-Xbp1s, UAS-Diap

On the other hand, co-expression of Xbp1unspliced (Xbp1u) led to a strong suppression of the "glossy" eye and restoration of the ommatidial patterning (**fig.33**).

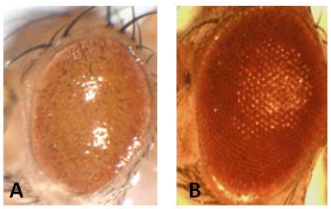


Fig.33: Xbp1u rescues the glossy eye phenotype. (A) "Glossy" eye due to GMR-GAL4>UAS-Xbp1s with LacZ in the backround as a control. **(B)** Co-expression of Xbp1u by GMR-GAL4 rescues the "glossy" eye phenotype.

Since it is also possible that other forms of cell death contribute to the "glossy eye" phenotype, we analysed autophagy in Xbp1s over-expressing cell clones (**fig.34**). Autophagy can be pro-survival and pro-death, depending on the context (Depnath et al., 2005). In the Xbp1s over-expressing cell clones we found foci with elevated levels of the LC3-GFP autophagy marker (**fig.34**). This result is consistent with what was observed in the *Drosophila* fat body, where Xbp1s over-expression also induces Atg8-GFP (Arsham and Neufeld, 2009).

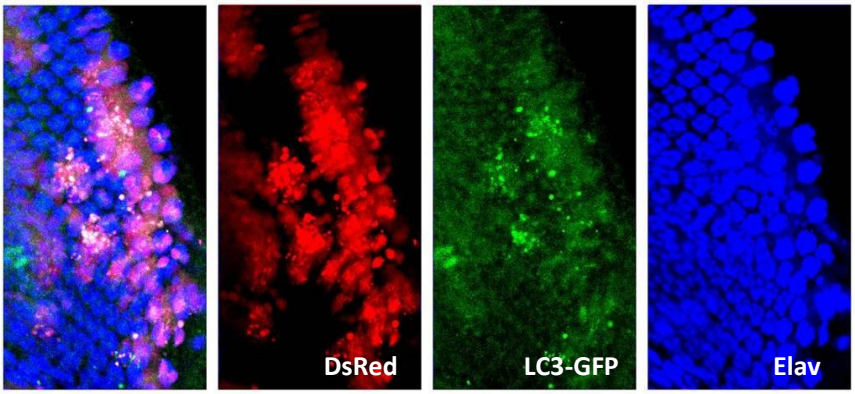


Fig.34: There is active autophagy in Xbp1s overexpressing cells. Flies have the genotype eyFlp, GMR-GAL4; CyO/ LC3-GFP; FRT82B/ FRT82B, UAS-DsRed, UAS-Xbp1s. Xbp1s over-expressing cell clones (DsRed) show elevated levels of LC3-GFP staining, indicating active autophagy. As a control photoreceptors are stained with Elav (blue) to make sure that clones lacking DsRed are no holes in the disc.

3.2. Cell death in CG6758 mutant clones

Since there is reduced “glossyness” in CG6758 mutant clones in the GMR-GAL4>UAS-Xbp1s eye and we could also detect apoptosis in Xbp1s over-expressing cell clones, we asked whether there is reduced apoptosis in CG6758 mutant clones. Therefore, we used the same two apoptosis markers as we used to detect cell death induced by Xbp1s in clones, the CPV reporter and TUNEL staining. With TUNEL staining we just saw very little apoptosis in the eye discs (**fig.35**) and with the CPV reporter we could not detect active caspase-3 at all, visualized by no cleaved parp staining and membrane bound Venus-GFP (**fig.36**).

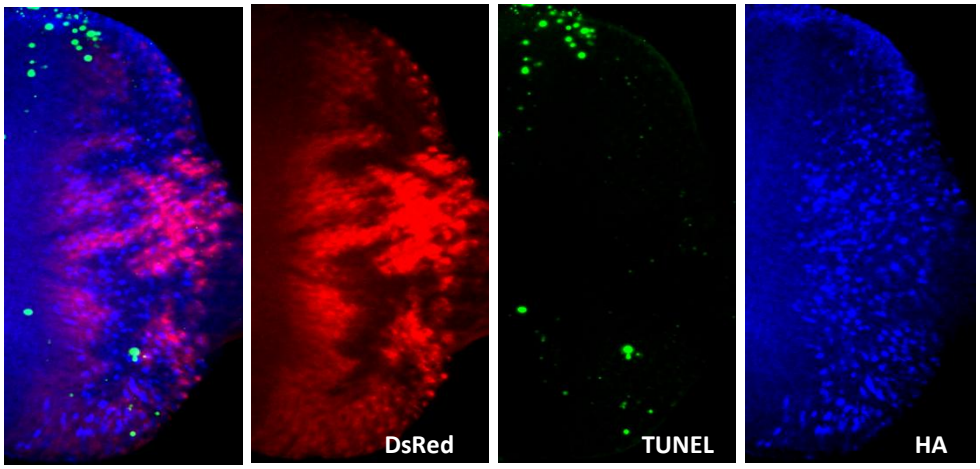


Fig.35: TUNEL staining in CG6758 mutant “glossy” eye discs reveals on very little TUNEL positive cells. Flies have the genotype eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, UbiRFP/ FRT42D, Su218. Absence of RFP marks homozygous mutant clones. There is only very little TUNEL staining (green) in those discs. Blue marks the HA-antibody indicating that the fly is from the right genotype and that there are no holes in the disc.

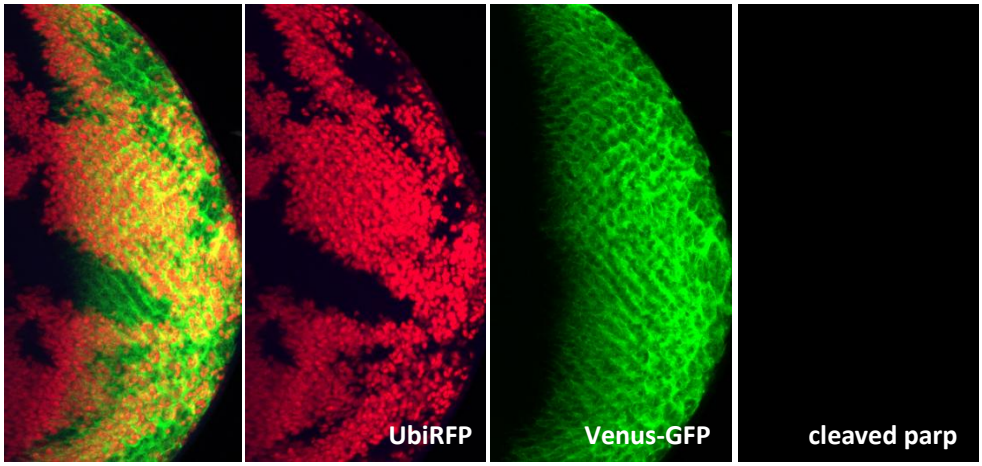


Fig.36: The CPV reporter is not cleaved in CG6758 mutant "glossy" eye discs. Flies have the genotype eyFlp, GMR-GAL4; FRT42D, UbiRFP/ FRT42D, Su218; CPV/ UAS-Xbp1s. Absence of RFP marks homozygous mutant clones. In the whole GMR-GAL4 domain, Venus-GFP is membrane-bound and no cleaved parp staining is visible, indicating there is no apoptosis.

The over-expression of Xbp1s in clones leads to an apoptosis pathway, and we wanted to test if there is any involvement of CG6758 in the apoptotic canonical pathway by testing if CG6758 mutations show any suppression of the GMR-Hid eye (Hid induces cell death by directly binding and inactivating Diap-1, see 1.4.1.). We did not observe any suppression of the atrophic eye phenotype in mosaic animals (**fig.37**). This result indicates that mutations in CG6758 are not general suppressors of apoptotic cell death but are instead specific suppressors of the Xbp1s phenotype.

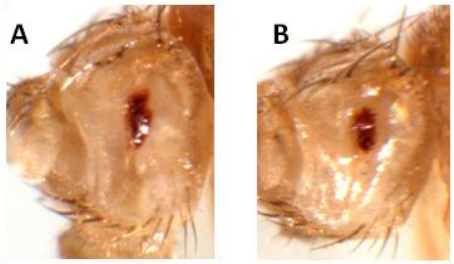


Fig.37: CG6758 mutant clones in the GMR-Hid eye can not rescue the phenotype. (A) GMR-Hid eye. **(B)** GMR-Hid eye mosaic for a CG6758 mutation (Su218) presents no suppression. Genotype: eyFlp, GMR-hid; FRT42D, Su218/ FRT42D

Since we detected autophagy in Xbp1s over-expressing cell clones we also tested autophagy in whole "glossy" eye discs (**fig.38**). We wanted to test if there is a contribution of autophagy to the cell death leading to the atrophic eye, since there is only partial rescue with caspase inhibitors and only little apoptosis detection (TUNEL).

We expressed in "glossy" eye discs the autophagy marker UAS-ATG8-GFP by the GMR-GAL4 driver to analyse if there is autophagy at all (**fig.38**). In the GMR-GAL4 domain, we could not detect any ATG8-GFP punctae as marker for active autophagy.

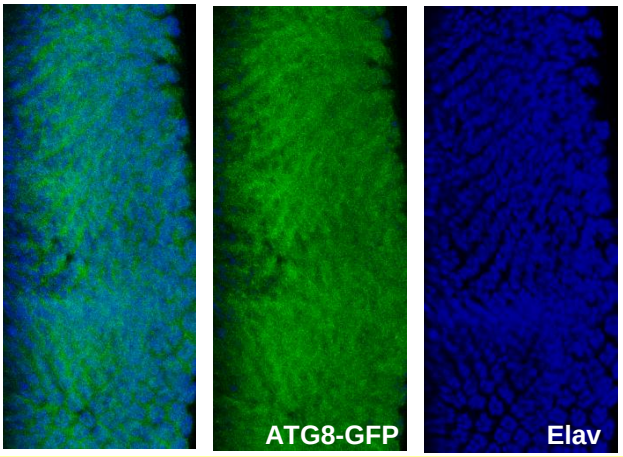


Fig.38: There is no ATG8 induction in whole „glossy" eye discs. UAS-ATG8-GFP expression is driven by the GMR-GAL4 driver and there are no ATG8-GFP foci (ATG8 activation) in the GMR-domain which is marked by Elav staining (blue). Genotype: GMR-GAL4>UAS-Xbp1s, UAS-ATG8

Discussion

Although Xbp1 has a protective role during the disease process of ADRP, the over-expression of the spliced form with the GMR-GAL4 driver leads to cell death and an atrophic “glossy” eye phenotype. We analysed the cell death process which leads to the Xbp1s induced phenotype with markers for apoptosis and autophagy, which revealed apoptotic and autophagy activity in cell clones over-expressing Xbp1s. We could detect apoptosis with TUNEL staining as well as with the CPV reporter, autophagy was detected by use of the LC3-GFP marker.

It was possible to partially rescue heterozygous cell clones over-expressing Xbp1s when P35 was co-expressed, although homozygous clones were still “glossy”. Furthermore, it was possible to rescue the clone number and size of heterozygous Xbp1s over-expressing clones. Adult eyes with cell clones over-expressing Xbp1s consists only very little of Xbp1s over-expressing cell clones, suggesting that those cells are removed during pupal stages by cell death and that “wildtype” cells outcompete their neighbour cells by competitive proliferation, leading to apoptosis of the “glossy” clones. This apoptosis of looser cells which is due to competitive proliferation can be blocked by P35, the “wildtype” cells undergo compensatory proliferation. In the whole “glossy eye” there is also only partial rescue of the “glossyness” by P35 as well as by Diap, suggesting that cell death is triggered only partially by apoptosis. Furthermore there is only little TUNEL staining and the CPV reporter is not cleaved by caspase-3. Maybe the underlying cell death pathway leading to Xbp1s induced “glossyness” is not solely apoptosis. In whole “glossy” eye discs there is no active autophagy indicated by the ATG8-GFP reporter. Since we could detect autophagy in Xbp1s over-expressing cell clones, it can be that autophagy contributes to the cell death conferred by Xbp1s. Autophagy can be pro-death or pro-survival, depending on the context (Depnath et al., 2005).

The most potent suppressor of Xbp1s induced cell death was Xbp1u. In mammals, Xbp1u shuttles between the cytoplasm and the nucleus where it interacts with Xbp1s, leading to the degradation of both proteins (Calfon et al., 2002; Yoshida et al., 2006). Maybe in *Drosophila* such a mechanism also exists.

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We thank E. Arama for the flies with the CPV reporter and E. Rusten for the flies with the ATG8-GFP and the flies with the LC3-GFP. Furthermore, we thank the Bloomington stock center for fly stocks. We also thank C. Yanicostas for flies with the Xbp1s construct without tag.

References

Arsham, A. M., Neufeld, T. P. (2009). A genetic screen in Drosophila reveals novel cytoprotective functions of the autophagy-lysosome pathway PLoS ONE, 4 (6) pp. e6068.

Clem RJ, Fechheimer M, Miller LK. (1991). Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science 254 (5036): 1388-90.

Debnath, J., Baehrecke, E. H., Kroemer, G. (2005). Does autophagy contribute to cell death? Autophagy 1 (2): 66–74.

Florentin, A. and Arama, E. (2012). Caspase levels and execution efficiencies determine the apoptotic potential of the cell. JCB, vol. 196, number 4.

Cagan, R. L., Ready, D. F. (1989). Notch is required for successive cell decisions in the developing Drosophila retina. Genes Dev 3: 1099–1112.

Fu, W., Noll, M. (1997). The Pax2 homolog sparkling is required for development of cone and pigment cells in the Drosophila eye. Genes Dev 11: 2066–2078.

Mizushima, N. (2014). Atophagy: Process and Function. Genes Dev. 21: 2861-2873

Rusten, E., Lindmo, K., Juhász, G., Sass, M., Seglen, P. O., Brech, A., Stenmark, H. (2004). Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. Developmental Cell, vol. 7 (2) pp. 179-92

Ryoo, H.D., Domingos, P.M., Kang, M.-J., and Steller, H. (2007). Unfolded protein response in a Drosophila model for retinal degeneration. EMBO J. *26*, 242–252.

Sado M, Yamasaki Y, Iwanaga T, Onaka Y, Ibuki T, Nishihara S, Mizuguchi H, Momota H, Kishibuchi R, Hashimoto T, Wada D, Kitagawa H, Watanabe TK. (2009). Protective effect against Parkinson's disease-related insults through the activation of XBP1. Brain Res. 1257: 16-24.

Tenev, T., Zachariou, A., Wilson, R., Ditzel, M., Meier, P. (2004). IAPs are functionally non-equivalent and regulate effector caspases through distinct mechanisms. Nat. Cell Biol. 7, 70-77.

Chapter IV

Testing potential candidate substrates

Summary

Two human proteins are described to have some homology to *Drosophila* CG6758, namely FBXO42/JFK and the more distantly related FBXO6. Since some of the substrates for these two proteins are known, we tested the fly homologues as potential substrates for CG6758. CHK1 is a substrate of FBXO6 and P53 is a known substrate of FBXO42/JFK. We tested the fly homologues Grapes (CHK1) and Dp53 as candidate substrates of CG6758. We analysed if in homozygous CG6758 mutant clones in GMR-GAL4>UAS-Xbp1s "glossy" eye discs, there are elevated levels of these potential substrates. This would be the expected result if CG6758 is mediating the SCF complex-dependent ubiquitination and degradation of these candidate substrates. We found reduced levels of Grapes in CG6758 homozygous mutant clones and for Dp53 there was no difference in protein levels between CG6758 homozygous mutant clones and control tissue. These results exclude that Dp53 or Chk1 are substrates for the CG6758. In addition, we tried to rescue the GMR-P53 atrophic eye phenotype with over-expression of CG6758 cDNA and found no suppression.

To test if CG6758 mutations can also suppress the cell death conferred by GMR-P53, we tested our CG6758 mutations in the GMR-P53 atrophic eye and we found no suppression, arguing that GMR-P53 mediated cell death is independent of CG6758.

We also tested other proteins as potential candidate substrates for CG6758, namely Diap-1, the AAA-type ATPase Vcp, CycE and CycB, the phospho-histone-3 marker, the AAA-type ATPase CG16789, which is approximately 3-fold upregulated in adult Xbp1s induced "glossy" eyes (semiquantitative RT-PCR) and 7-fold upregulated in "glossy" eye imaginal discs (microarray, Domingos, unpublished) and seems to interact with CG6758 (Drosophila Protein Interaction Map, Mintseris

et al., 2007) and finally Xbp1u. For all the tested proteins, the results were negative, but with the phospho-histone-3 marker we found a disturbed mitotic pattern in the eye discs. To analyse if cone cells are specified in the "glossy" eye discs, we stained discs with clones over-expressing Xbp1s for Cut, a cone cell marker and could identify cone cells in the "glossy" clones.

Introduction

Our goal is to identify the downstream mechanisms that are required for Xbp1s induced cell death. We found that mutations in CG6758 are able to suppress the Xbp1s induced “glossy” eye. CG6758 encodes an Fbox protein acting in an SCF ubiquitin ligase complex. However, to elaborate Xbp1s cell death signaling, it is necessary to identify the substrates of CG6758 and to understand how accumulation of these substrates in CG6758 mutants impair Xbp1s signaling. One possible approach is to test if known substrates of the mammalian Fbox protein with higher homology to CG6758 are also regulated in the fly by CG6758.

The two human proteins with higher homology to CG6758 are FBXO42 (considered to be the orthologue of CG6758) and FBXO6. P53 was shown to be a substrate of FBXO42 (Sun et al., 2009) and Chk1 was shown to be a substrate of FBXO6 (Zhang, Y. W. et al., 2009).

Chk1 is necessary for the G2-M DNA-damage checkpoint (Fogarty et al., 1997; Liu, G. et al., 2000; Zachos et al., 2003). For the activation of Chk1, ATR-dependent phosphorylation is required, activated Chk1 phosphorylates downstream targets and thereby triggers different cellular responses such as transcription regulation, energy consumption alteration, cell cycle arrest or delay, DNA repair or cell death if the damage is too severe to repair. Furthermore, it was shown that Chk1 is phosphorylated during ER stress as a consequence of eIF2α phosphorylation and subsequent translational repression. CDC25A is destabilized during ER stress in a Chk1-dependent manner to cause G2 cell cycle delay (Malzer et al., 2010).

Drosophila Grapes regulates syncytial cell division fidelity, mitotic entry and CycA degradation and thereby plays a crucial role during embryogenesis. Grapes was found in a modifier screen of over-expression of the integrated stress response kinase PERK, which results in a atrophic “glossy” eye phenotype similar to the one obtained by Xbp1s over-expression.

Knockdown of Grapes by RNAi rescued the eye phenotype (Malzer et al. 2010).

P53 is a tumour suppressor protein that integrates endogenous and exogenous signals to modulate cell fate to stress and cellular environments. The human P53 regulates as a transcription factor the expression of genes involved in cell cycle regulation, induction of apoptosis and DNA repair after DNA damage (reviewed in Chumakov, 2007). The *Drosophila* genome contains one single P53 family member, Dp53. In contrast to human P53, it does not excert DNA damage induced cell cycle arrest, but the pro-apoptotic function is well conserved. Upon apoptotic stimulation, Reaper, Hid and Grim inhibit Diap which in turn leads to the activation of Dronc, DriCE and Dcp-1. It was shown in the developing *Drosophila* eye that Dp53 induced apoptosis is primarily dependent on Hid but not on Reaper and occurs through the canonical apoptosis pathway (Fan et al., 2010). Furthermore, Dp53 also inhibited cellular differentiation of photoreceptors and cone cells in the eye, independently of its apoptotic function (Fan et al., 2010).

Besides testing Chk1 and Dp53 as potential candidate substrates for CG6758 dependent ubiquitination, we also analysed levels of different proteins. We tested levels of Diap, an anti-apoptotic protein which suppresses apoptosis by inhibiting Reaper, Hid and Grim activity as well as it inhibits the functions of caspases (Tenev et al., 2004) and it was shown to partially rescue the “glossy” eye phenotype (Chapter III, p.89). Furthermore we tested the two AAA-type ATPases Vcp and CG16789, which seems to be an interactor of CG6758 (DPIM) and which is upregulated in “glossy” eye discs (microarray; Domingos, P., unpublished) and adult “glossy” eyes (semiquantitative RT-PCR). We also tested CycB and CycE levels as well as the mitotic pattern in “glossy” eye imaginal discs, to analyse if the specification of cone cells is the underlaying process of “glossyness”. Finally we tested Xbp1u as the substrate for CG6758. Xbp1u in mammals

is known to interact with Xbp1s, leading to the degradation of Xbp1s/Xbp1u (Yoshida et al., 2006). Xbp1u is a target gene of Xbp1s as seen in its upregulation in “glossy” eye imaginal discs by microarray analysis (Domingos, P; unpublished). Furthermore, UAS-Xbp1u transgene expression can suppress the Xbp1s induced “glossy” eye phenotype (see Chapter III, p.90).

Material and Methods

Immunofluorescentstaining:

Flies with the genotype eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, UbiRFP or UbiGFP/ FRT42D, Su218 were generated and after dissection of eye imaginal discs immuno-stained with rat anti-CHK1 (Abcam ab47574)/ mouse anti-Dp53 (anti-P53 H3-s from Developmental Studies Hybridoma Bank)/ guinea pig anti-Diap (kindly provided by H. D. Ryoo)/ rat anti-Vcp (kindly provided by D. McKearin)/ mouse anti-CycB (F2F4 from Developmental Studies Hybridoma Bank)/ mouse anti-CycE / rabbit anti-PH3 (Abcam ab5176) and rat anti-Elav (DSHB, 7E8A10) (for protocol see Chapter II, p.56), followed by analysis of the discs under the confocal microscope (Zeiss LSM710).

Analysis of eye discs with Xbp1s over-expressing cell clones:

Flies with the genotype eyFlp, GMR-GAL4; (); FRT82B/ FRT82B, UAS-DsRed, UAS-Xbp1s were generated and the eye discs were dissected. The dissected eye discs were immuno-stained with rat anti-PH3 (Abcam ab5176) / mouse anti-Cut (2B10 from DSHB) and rat anti-Elav (DSHB,

7E8A10) (for protocol see Chapter II, p.56), followed by analysis of the discs under the confocal microscope (Zeiss LSM710).

Rescue of the GMR-p53 eye phenotype with 3xHA-3xFlag-CG6758 cDNA:

Flies of the genotype GMR-p53 were crossed to flies with UAS-3xHA-3xFlag-CG6758.

CG6758 mutant clones in the GMR-p53 eye:

Flies with the genotype eyFlp, GMR-GAL4, FRT42D, UbiRFP/ FRT42D, Su218; GMR-P53/ + were established and analysed under the dissecting microscope.

Semiquantitative RT-PCR:

Fly heads of GMR-GAL4>UAS-Xbp1s flies and control flies (GMR-GAL4) were collected (150 flies each) by freezing the flies in a tube in liquid nitrogen and vortexing the flies. The heads were pooled in 30µl H2O and smashed with a pestle. For the isolation of total RNA, Zymo Quick-RNA mini-prep kit was used following the manufacturer protocol. For cDNA synthesis the Thermo/Fermentas Revert Aid First Strand kit was used. The semi-quantitative RT-PCR for CG16789 and the housekeeping gene RP49 was performed with the following primers:

FP-CG16789RT: ccgtgctctttgatctgactcc

RP-CG16789RT: gacgggatctcgtgagcacag

FP-Rp49: agatcgtgaagaagcgcaccaagc

RP-Rp49: gcaccaggaacttcttgaatccgg

CG16789 levels in CG6789 mutant eye clones:

The full length cDNA clone RH08189 of CG16789 was used as a template for PCR of CG16789 cDNA. The CG16789 PCR product was cloned into the Gateway vector PTWG following the Gateway protocol. Embryos were injected with the CG16789-GFP plasmid and random P-element transformation was performed by Stabvida.

Flies with the genotype eyFlp, GMR-GAL4; FRT42D, UbiRFP/ FRT42D, Su218, UAS-CG16789-GFP were generated and the eye discs were dissected. The dissected eye discs were immuno-stained with rat anti-Elav (DSHB, 7E8A10) (for protocol see Chapter II, p.56), followed by analysis of the discs under the confocal microscope (Zeiss LSM710).

Xbp1u levels in CG6758 mutant eye clones:

Flies with the genotype eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, UbiRFP/ FRT42D, Su218; UAS-Xbp1_HA_GFP were generated and the eye discs were dissected. The dissected eye discs were incubated over night with DTT (2mM) to induce ER stress, leading to splicing of the UAS-Xbp1_HA_GFP transgene (Cairrao, F.; unpublished) which results in a frameshift and expression of Xbp1u-GFP. The discs were immuno-stained with rabbit anti-GFP (Abcam ab32146) and rat anti-Elav (DSHB, 7E8A10) (for protocol see Chapter II, p.56), followed by analysis of the discs under the confocal microscope (Zeiss LSM710).

Results

4.1. Grapes as a potential substrate for CG6758

To test whether Grapes is the substrate for CG6758 dependent ubiquitination, we generated “glossy” eye imaginal discs mosaic for Su218. Homozygous mutant clones were marked by the absence of RFP and the discs were stained with an antibody against CHK1 (**fig.39**). For a substrate of CG6758 one would expect an accumulation of the substrate in the CG6758 homozygous mutant clones. However, we found a reduction of Grapes staining in the clones lacking RFP, excluding Grapes as a substrate for CG6758.

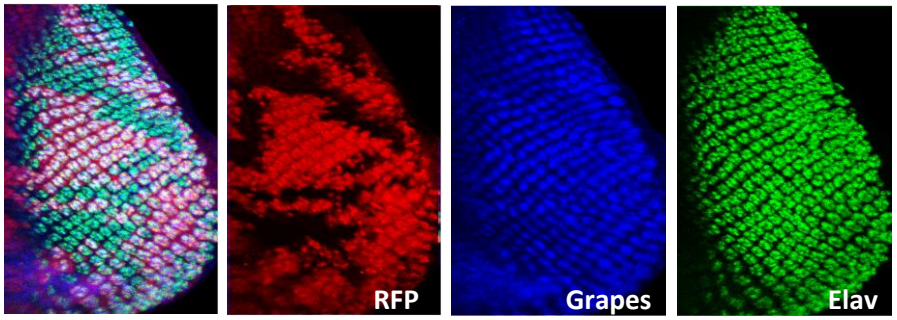


Fig.39: Reduced levels of Grapes in CG6758 mutant clones in Xbp1s over-expressing discs indicate that grapes is not the substrate of CG6758. Flies have the genotype eyFlp, GMR-GAL4; FRT42D, UbiRFP/ FRT42D, Su218; UAS-Xbp1s/ +. In cell clones homozygous mutant for CG6758, marked by the absence of RFP, there are reduced levels of Grapes staining (in blue). Elav (green) serves as a control to be sure that cells lacking RFP are no holes in the discs. Note: Grapes is not the substrate of CG6758.

4.2. Dp53 as a potential substrate for CG6758

To analyse if Dp53 is the substrate of CG6758 we checked Dp53 levels in CG6758 “glossy” mosaic eye discs (**fig.40**). We did not observe any

difference in Dp53 levels in homozygous CG6758 mutant clones and surrounding control tissue, excluding Dp53 as a substrate for CG6758.

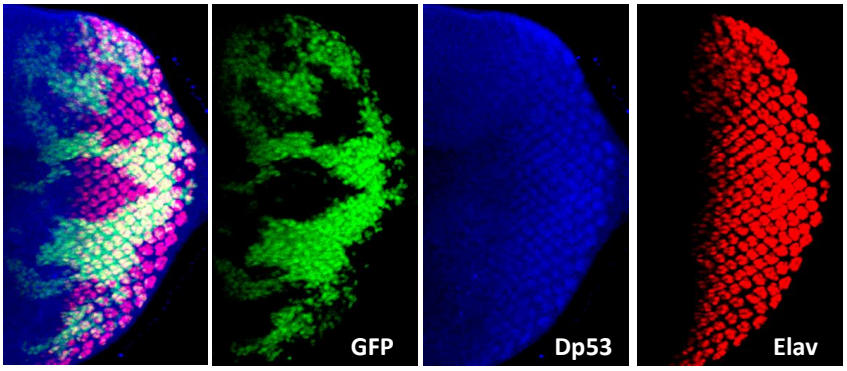


Fig.40: There are no elevated Dp53 levels in CG6758 mutant clones in Xbp1s over-expressing discs excluding p53 as the substrate for CG6758. Flies have the genotype eyFlp, GMR-GAL4; FRT42D, UbiGFP/ FRT42D, Su218; UAS-Xbp1s/ +. In cell clones homozygous mutant for CG6758, marked by the absence of UbiGFP, there are no elevated levels of Dp53 staining. Elav serves as a control to be sure that cells lacking RFP are no holes in the disc. Note: Dp53 is not the substrate of CG6758.

Another way to test if Dp53 is the substrate of CG6758 is the over-expression of CG6758 in the atrophic GMR-Dp53 eye, since over-expression should lead to degradation of Dp53. We tested 3xHA-3xFlag-CG6758 over-expression and found no rescue of the eye phenotype (**fig.41**).

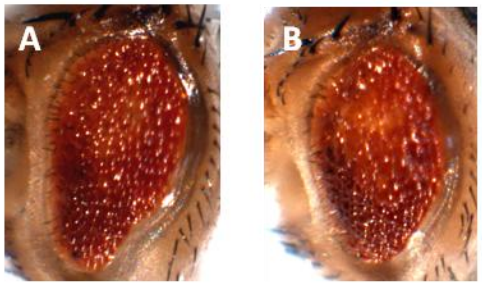


Fig.41: There is no rescue of the GMR-Dp53 eye with CG6758 over-expression. (A) GMR-Dp53 over-expressing eye. **(B)** Co-expression of 3xHA-3xFlag-CG6758. Note: Dp53 is not the substrate of CG6758.

Anyway we wanted to know if CG6758 mutations maybe suppress the cell death induced by GMR-Dp53 and generated clones of Su218 in the GMR-Dp53 eye (**fig.42**). CG6758 mutations do not suppress the atrophic eye phenotype.

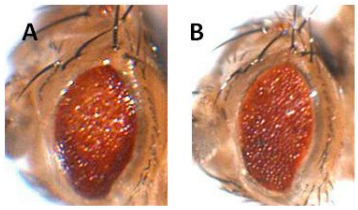


Fig.42: Mutations of CG6758 do not suppress the GMR-Dp53 eye. (A) GMR-Dp53 eye. **(B)** Clones of Su218 are generated in the GMR-Dp53 eye. Note: CG6758 does not play a role in P53 mediated cell death.

4.3. Diap as a potential candidate substrate for CG6758

We wanted to know if increased Diap levels in the CG6758 mutant eye clones are responsible for the suppression effect conferred by the CG6758 alleles.

So we tested the levels of Diap in CG6758 mutant clones in comparison to “wildtype” and heterozygous tissue in eye imaginal discs which had Xbp1s in the background (**fig.43**).

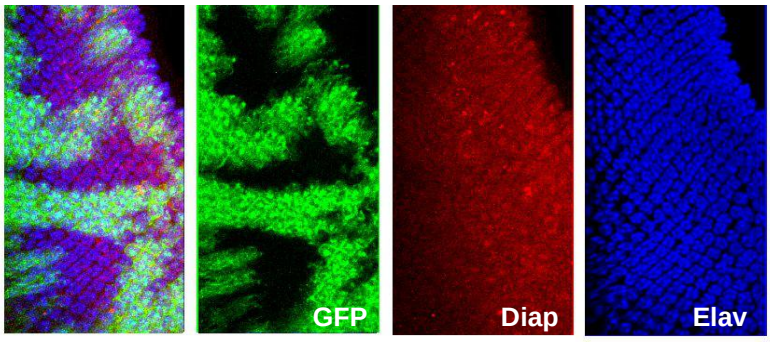


Fig.43: There are no elevated Diap levels in homozygous CG6758 mutant clones in Xbp1s over-expressing discs which indicates that Diap is not the substrate of CG6758. Absence of GFP indicates the homozygous mutant CG6758 clones, red are the Diap levels which are equal in the GMR domain, marked by stained photoreceptors with Elav (blue). Note: Diap is not the substrate of CG6758.

We could not see any difference in Diap levels in the homozygous mutant clones compared with the surrounding heterozygous and “wildtype” tissue, excluding Diap1 as a substrate of CG6758 conferred ubiqutination.

4.4. Vcp as a potential candidate substrate for CG6758

We tested Vcp as a potential candidate substrate of CG6758.

Also for Vcp, the protein levels were equal in homozygous mutant clones and surrounding heterozygous and “wildtype” tissue, excluding Vcp as a substrate of CG6758 (**fig.44**).

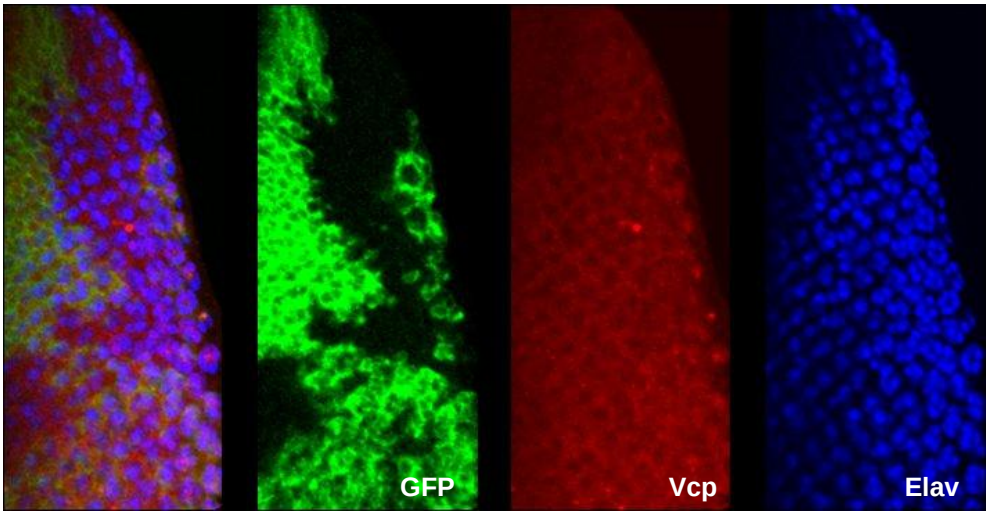


Fig.44: There are no elevated Vcp levels in homozygous CG6758 mutant clones in Xbp1s over-expressing discs, excluding Vcp as the substrate for CG6758. Absence of GFP indicates the homozygous mutant CG6758 clones, red are the Vcp levels which are equal in the GMR domain, marked by stained photoreceptors with Elav (blue). Note: Vcp is not the substrate of CG6758.

4.5. Disturbed cell cycle as the reason for CG6758 induced cell death

It could have been possible that a disturbed cell cycle is the cause for the "glossy" eye phenotype, for instance the SMW could be blocked, which inhibits differentiation of cone cells, responsible for secreting the overlying lens material. Degradation of the cone cells or missing cone cells lead to a "glossy" eye phenotype. So, first we checked the two cyclins, CycE and CycB for being decreased in heterozygous or "wildtype" tissue compared to CG6758 mutant clones in eye imaginal discs. CycE drives cells from G1 to S-phase in the SMW. In *Drosophila*, the CycE-CDK2 complex is both sufficient and rate-limiting for G1-S-phase transition (Knoblich et al., 1994; Richardson et al., 1995; Sauer and Lehner, 1995). The CycE-CDK2 complex activates the Retinoblastoma protein (Rb), which in turn leads to the activation of the E2F/Dp transcription factors and S-phase promoting genes are expressed. The cycB-CDK1 complex is the main regulator of the G2-M-transition for the sychronized cells behind the MF and multiple factors control its activity (Harper and Elledge, 1996; Lew and Kornbluth, 1996).

For both cyclins we found no accumulation in the CG6758 mutant area (fig.**45, 46**), arguing that these two cyclins are not responsible for the cell death induced by Xbp1s.

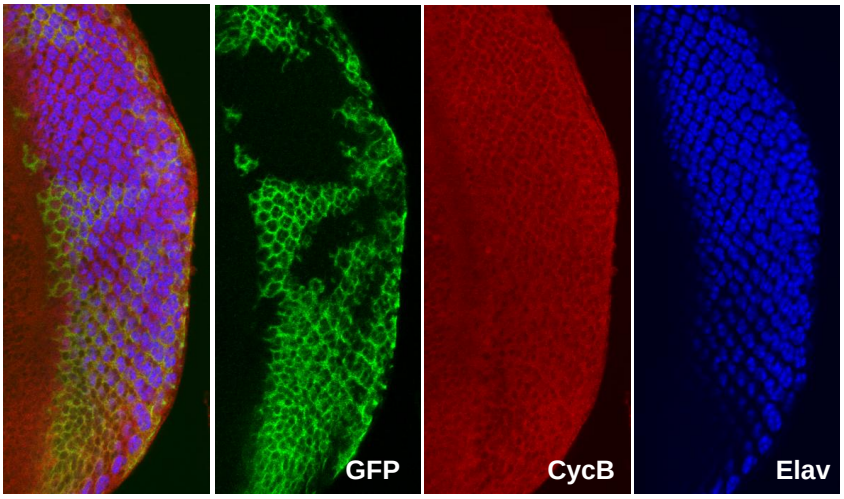


Fig.45: There are no elevated CycB levels in homozygous CG6758 mutant clones in Xbp1s over-expressing discs, excluding CcyB as that substrate for CG6758. The absence of GFP marks the homozygous CG6758 mutant clones. CycB levels (red) are equal in the GMR domain, marked by Elav staining (blue). Note: CycB is not the substrate of CG6758.

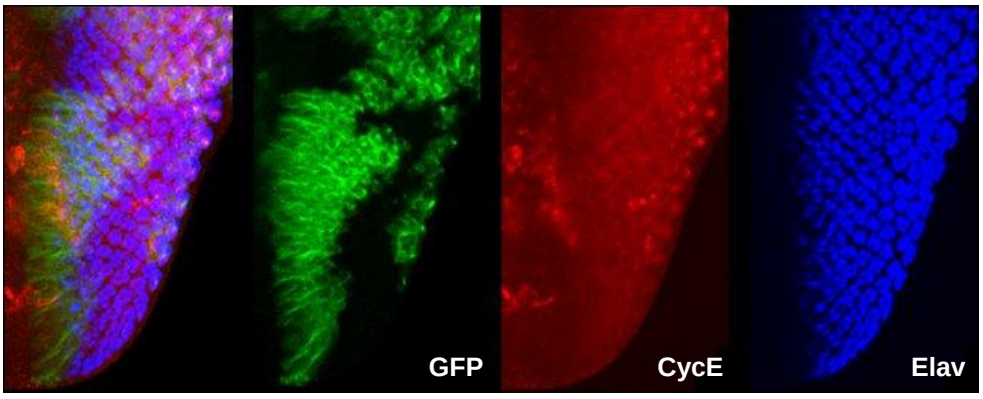
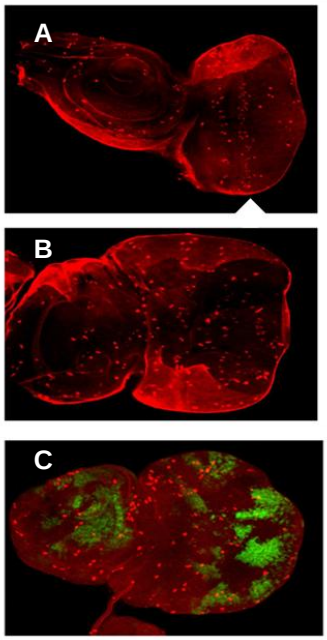


Fig.46: There are no elevated levels of CycE in homozygous CG6758 mutant clones in Xbp1s over-expressing discs, excluding CycE as the substrate for CG6758. The absence of GFP marks the homozygous CG6758 mutant clones. CycE levels (red) are equal in the GMR domain, marked by Elav staining (blue). Note: CycE is not the substrate of CG6758.

Next we analysed phosphohistone-3 (PH3) levels in control eye imaginal discs, discs over-expressing GMR-GAL4 driven UAS-Xbp1s (with and without clones of a CG6758 mutation) and discs with clones over-expressing Xbp1s (GMR-GAL4 driven). Phosphohistone-3 is an immune-marker specific for cells undergoing mitosis. With this immune-marker we analysed the mitotic pattern in the different discs and found that there is no clear SMW in eye discs, which over-express Xbp1s (**fig.47a**), this disturbed pattern of PH3 staining could also not be rescued in the clones of Su218 (marked by the absence of GFP) (**fig.47b**). In clones of Xbp1s over-expression (in red), it seems that there is no SMW at the border between the clones and the SMW (**fig.47d**).

Since we observed a disturbed pattern of PH3 staining, we decided to analyse if cone cells are specified in Xbp1s over-expressing cell clones, since the cone cells give rise to the lens material.
We could clearly detect cone cells in the Xbp1s over-expressing cell clones indicating that a second round of mitosis posterior to the MF has occurred (**fig.48**).



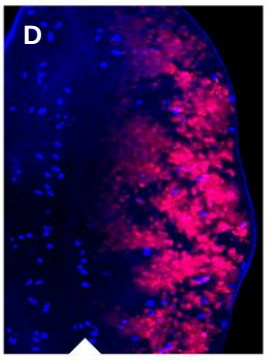


Fig.47: Disturbed mitotic patterning in Xbp1s over-expressing eye imaginal discs. (A) Control discs. Flies have the genotype w-; FRT42D/FRT42D. **(B)** Whole glossy eye imaginal discs. Flies have the genotype eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, UbiGFP/ (*CyO*). **(C)** Whole glossy eye imaginal discs with mutant clones for Su218. Flies have the genotype eyFlp, GMR-GAL4, UAS-Xbp1s; FRT42D, UbiGFP/ FRT42D, Su218. **(D)** Eye imaginal discs with clones over-expressing Xbp1s posterior to the MF. Flies have the genotype eyFlp, GMR4; (); FRT82B, UAS-Dsred, UAS-Xbp1s/ FRT82B. The discs were stained with PH3 (red in A, B and C; blue in D); the SMW is indicated by an arrowhead. Note: The mitotic patterning is disturbed in Xbp1s over-expressing discs, there is no clear SMW visible (B, C). At the border of Xbp1s over-expressing cell clones there is no normal mitotic pattern (D).

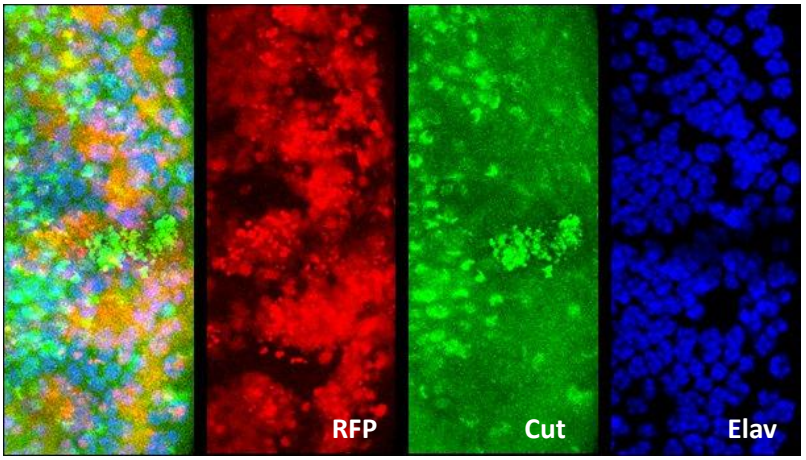


Fig.48: Cone cells are specified in Cut in Xbp1s over-expressing clones indicated by positive cut staining. The fly has the genotype eyFlp, GMR-GAL4; (); FRT82B, UAS-DsRed, UAS-Xbp1s/ FRT82B. Cell clones over-expressing GMR-driven UAS-Xbp1s are marked in red, cut staining (green) indicates the cone cells and Elav staining (blue) is a control that there are no holes in the disc due to the preparation.

4.6. CG16789 as a potential candidate substrate for CG6758

CG16789 seems to be an interactor of the Fbox protein CG6758 (DPIM), and since it is around 7-fold upregulated in “glossy” eye discs, observed in a microarray (Domingos, P.; unpublished) and approximately 3-fold upregulated in adult “glossy” eyes in a semiquantitative RT-PCR (**fig.49**), we asked whether CG16789 could be substrate of CG6758. CG16789 encodes an AAA-type ATPase with unknown cellular function.

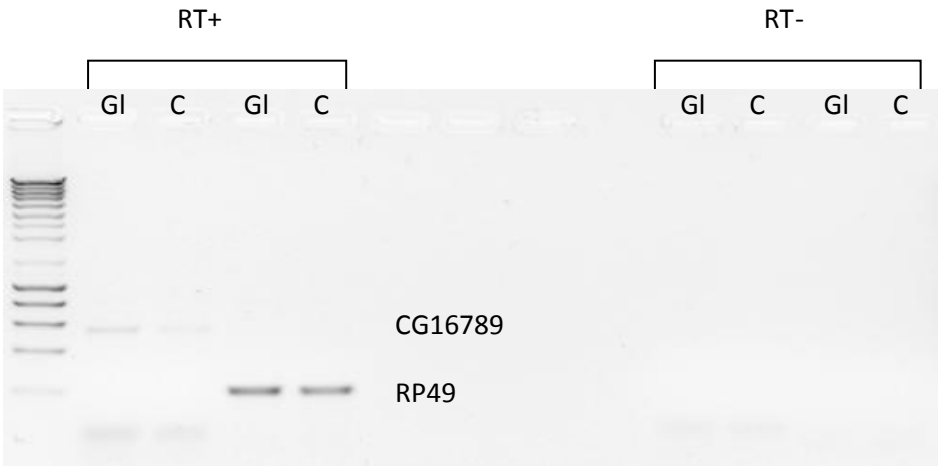


Fig.49: In adult „glossy" eyes, the ATPase CG16789 is upregulated. Semiquantitative RT-PCR reveals an upregulation of the factor ~3. Gl: "glossy" eyes; C: Control eyes.

We tested two different CG16789-RNAi lines (v32730, v103617) for enhancement of the "glossy" eye phenotype, but did not observe any change in the atrophic eye appearance (**fig.50**). If CG16789 is the substrate of CG6758 one would expect an enhancement of the "glossyness", due more reduction in the levels of CG16789.

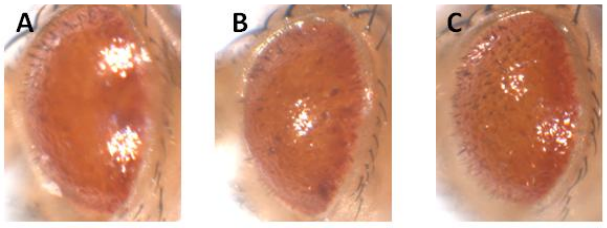


Fig.50: CG16789-RNAi has no effect on the GMR-GAL4>UAS-Xbp1s induced "glossy" eye. (A) GMR-GAL4>UAS-Xbp1s eye. **(B, C)** Co-expression of CG16789 RNAi (v103617).

Next we tested if RNAi for CG16789 in the background (v103617 recombined to the 3rd chromosome) of CG6758 mutant mosaic eyes suppresses the rescue effect of CG6758 mutant clones. We did not observe any rescue (**fig.51**), which one would expect if CG16789 is the substrate of CG6758, since loss of CG6758 functioning in the mutant clones would have been compensated.

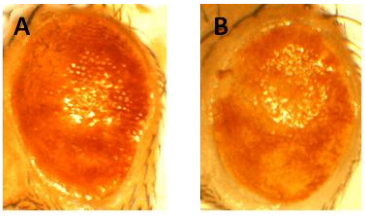


Fig.51: CG16789-RNAi has no effect on the suppression effect of CG6758 mutants. (A) Su209 suppresses the "glossy" eye phenotype. (B) Co-expression of CG16789-RNAi (v103617). Note: Also with co-expression of CG16789-RNAi there are suppressor clones visible. CG16789 is not the substrate of CG6758.

Finally, we cloned CG16789 cDNA into the fly gateway vector pTWG, which results in a GFP fusion protein and transgenic flies were generated. We created flies mosaic for CG6758 mutant clones with the CG16789-GFP construct in the background and tested whether in clones of CG6758 mutants there are elevated levels of the GFP-signal (**fig.52**). GFP levels were equal in the GMR-domain where the CG16789-GFP construct was expressed. Anyway we did not have Xbp1s expression in the background, which maybe is needed to provide factors involved in CG6758 functioning.

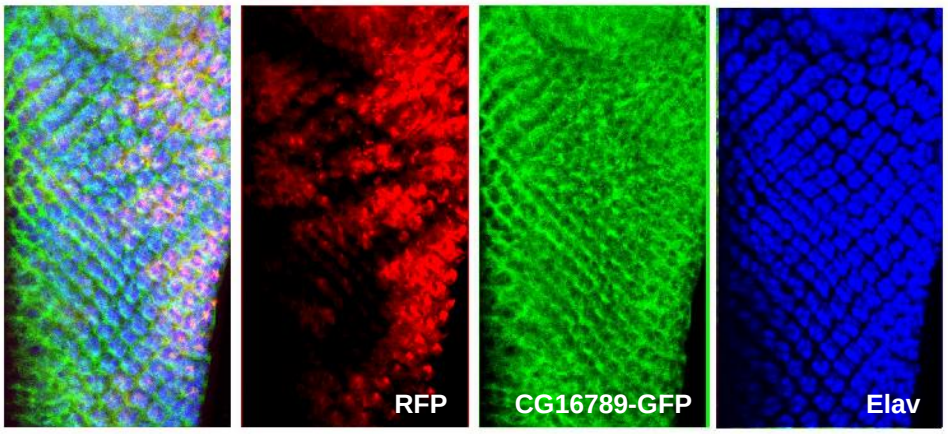


Fig.52: There are no elevated CG16789-GFP levels in CG6758 mutant clones, indicating that CG16789 is not the substrate of CG6758. Fly with the genotype eyFlp, GMR-GAL4; FRT42D, myrRFP/ FRT42D, Su218; UAS-CG16789-GFP/ +. The absence of Red marks the clones homozygous for the CG6758 mutation, green is the expression of CG16789, which is equal in mutant clones and surrounding heterozygous and "wildtype" tissue (red). Elav (blue) stains photoreceptors and is a control if absence of RFP is due to holes in the disc. Note: The over-expressed CG16789-GFP is no substrate of CG6758.

4.7. Xbp1u as a potential candidate substrate for CG6758

Finally we tested Xbp1u as a potential candidate substrate for CG6758-mediated ubiquitination. Xbp1u in mammals is known as a negative regulator of Xbp1s, it shuttles between the cytoplasm and the nucleus, where it binds Xbp1s (Calfon et al., 2002; Yoshida et al., 2006). The interaction of Xbp1s and Xbp1u leads to their degradation. In mammals as well as in *Drosophila,* Xbp1 is transcribed as a target gene of Xbp1s (Acosta-Alvear et al., 2007, Domingos, P.; unpublished). Furthermore we showed that Xbp1u can rescue the "glossy" eye phenotype (Chapter III, p.90), so maybe also in flies there exists this negative feedback-loop, where Xbp1s leads to transcription of Xbp1, which results in the Xbp1u form and shuts down Xbp1s signaling.

We analysed if in clones homozygous for the CG6758 mutations there are elevated levels of Xbp1u (**fig.53**), but could not find any difference compared to surrounding heterozygous and “wildtype” tissue.

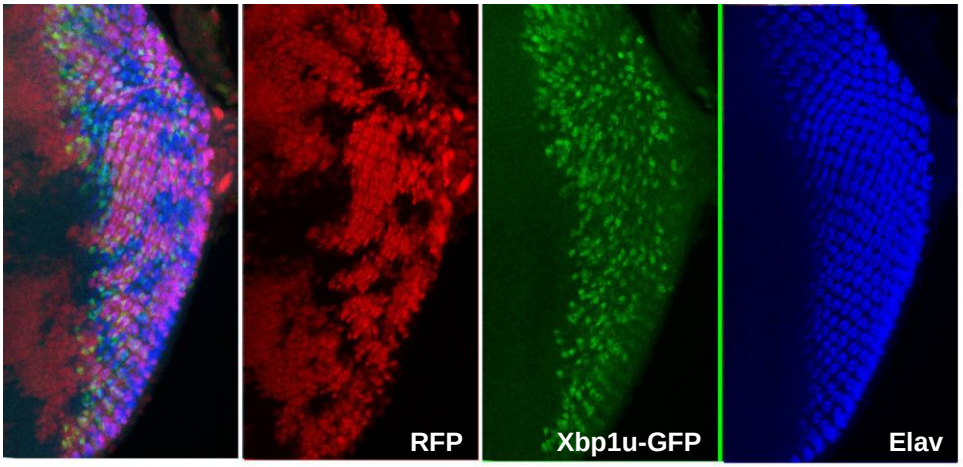


Fig.53: There are no elevated Xbp1u levels in homozygous CG6758 mutant clones, excluding Xbp1u as the substrate for CG6758. The absence of RFP marks the homozygous CG6758 mutant clones, there are no elevated levels of Xbp1u, excluding Xbp1u as a substrate for CG6758. Staining of the photoreceptors with Elav serves as an internal control to be sure that the absence of RFP is not due to holes in the disc.

Discussion

To find the substrate of CG6758 destined for ubiquitination, followed by proteasomal degradation, we tested the known substrates of the Fbox proteins FBXO6 and FBXO42, the annotated human homologues of CG6758. The known substrates are CHK1 and P53, respectively, so we analysed the protein levels of Grapes (dCHK1) and Dp53 in CG6758

mutant mosaic eye discs to analyse whether those potential substrates are elevated in the homozygous mutant clones.

For Grapes we found reduced protein levels in the homozygous mutant clones. The reduced Grapes levels could be a secondary effect, which could be explained by less pro-death ER stress signaling conferred by Xbp1s. The different UPR branches cross-talk and Grapes is known to undergo transient activation during ER stress mediated by PERK (Malzer et al., 2010), meaning that in the homozygous mutant clones there is less Grapes activation, maybe reflected in lower protein levels.

By testing Dp53 as a potential substrate of CG6758, we did not observe any differences in protein levels between the homozygous mutant clones and the surrounding heterozygous and “wildtype” tissue. We also tested the over-expression of UAS-3xHA-3xFlag-full length-CG6758 in the atrophic eye phenotype caused by GMR-Dp53, if Dp53 is the substrate of CG6758. If Dp53 would be the substrate of G6758, the over-expression of CG6758 should rescue the GMR-Dp53 eye phenptype due to Dp53 degradation, but we did not observe any rescue and excluded Dp53 as the substrate for CG6758.

Anyway, we wanted to know, whether clones homozygous mutant for CG6758 suppress GMR-Dp53 induced cell death or if cell death conferred by CG6758 is ER stress specific. Clones homozygous for CG6758 can not suppress the atrophic eye phenotype induced by GMR-Dp53.

We tested more potential candidate substrates for CG6758 mediated ubiquitination, but did not find any positive result. We tested the anti-apoptotic protein Diap, the AAA-type ATPase Vcp, which is involved in ERAD, the cell cycle regulators CycB and CycE as well as the mitotic pattern in “glossy” eye imaginal discs and cut expression for the presence of cone cells in the “glossy” eye. Furthermore we also tested the potential CG6758 interactor CG16789 which is also an AAA-type ATPase and is upregulated in larval and adult “glossy” eyes upon Xbp1s over-expression.

Finally, we tested Xbp1u.

In our analysis for enrichment of potential candidate substrates of CG6758, we put Xbp1s over-expression in the background (except for CG16789-GFP levels), since it is possible that this over-expression provides factors for CG6758 functioning.

The first potential candidate substrate we tested was Diap. Increased levels of Diap in the CG6758 mutant clones would lead to more suppression of apoptosis induced by Xbp1s and to suppressor clones in the adult “glossy” eye. Since there were no increased Diap levels in homozygous mutant clones in the “glossy” eye discs, we excluded Diap as a substrate for CG6758.

Next we tested the AAA-type ATPase Vcp which is involved in ERAD, where it plays a crucial role in shuttling the misfolded or unfolded proteins to the proteasome (see 1.2.) for degradation. Enrichment of Vcp could lead to a better clearance of misfolded or unfolded proteins which accumulate in the ER upon prolonged or strong Xbp1s driven transcription. But also for Vcp, we did not find any difference in protein levels in homozygous mutant, heterozygous and “wildtype” clones, excluding Vcp as a substrate for CG6758.

We examined, if a disturbed SMW is responsible for the suppression effect of CG6758 mutant clones in the GMR-GAL4>UAS-Xbp1s induced “glossy” eye. We tested levels of CycB, which in *Drosophila* is responsible for G2-M transition of synchronized cells behind the MF when in complex with Cdk1 and CycE levels, the CycE/Cdk2 complex drives the cells into S-phase of the SMW. The degradation of one of the two proteins in the CG6758 “wildtype” or heterozygous clones in CG6758 mutant mosaic “glossy” eye discs would result in a block in the SMW and no cone cells, which secrete the lens-forming material, would differentiate (see 1.5.). The loss or degradation of the lens is the cause for “glossy” eyes. We did not observe any differences of those protein levels in the mosaic eyes, however staining “glossy” eye discs with the mitosis marker PH3 revealed a disturbed SMW

pattern. Staining mosaic mutant “glossy” eye discs with PH3 did not show a restoration of the SMW pattern in the homozygous mutant clones. We also tested the SMW pattern in cell clones over-expressing Xbp1s, here it seems that the border of the cell clones is the area where the SMW is disturbed.

To test if a disturbed SMW which would result in no differentiation of cone cells is responsible for the “glossy” eye phenotype, we stained discs with Xbp1s over-expressing cell clones with an antibody against Cut, positive cut staining indicates the presence of cone cells. We found positive cut signal in the Xbp1s over-expressing cell clones, arguing that the Xbp1s induced “glossy” eye phenotype does not result from differentiation defects but from cell death of the cone (and additional) cells.

Afterwards, we wanted to know if CG16789, is the substrate of CG6758. Therefore, we tested two different available CG16789 RNAi lines for an enhancement of the Xbp1s induced “glossy” eye phenotype and did not observe any effect in the eye. However, RNAi lines not always provide sufficient knock down of the endogenous protein. Next we generated flies with mosaic eyes for CG6758 mutations and CG16789-RNAi in the background to test whether CG16789 is the substrate of CG6758, If this would be the case, one could expect that there are no suppressor clones visible in the “glossy” eye, because the loss of CG6758 funtioning would be compensated. No reversion of suppression in the mosaic eyes was visible, although maybe the used RNAi-line was not effective. We then tested whether UAS-CG16789 transgene expression is enhanced in the homozygous mutant clones due to impaired proteasomal degradation mediated by CG6758 substrate binding and ubiquitination of CG16789. We did not see any difference in the homozygous mutant clones compared to heterozygous and “wildtype” clones. However in this case, we did not provide Xbp1s over-expression in the background which is maybe required for the degradation of CG16789 due to delivery of essential factors. Since

in those flies, there was no Xbp1s in the backround, we also analysed if the over-expression of CG16789 could rescue the “glossy” eye phenotype conferred by GMR-GAL4>UAS-Xbp1s. We did not see any difference in the “glossy” eye phenotype and could exclude CG16789 as substrate for CG6758.

As last potential candidate substrate, we tested Xbp1u. Xbp1u in mammals is known to interact with Xbp1s, leading to the degradation of Xbp1s/Xbp1u (Yoshida et al., 2006). Xbp1u is a target gene of Xbp1s as seen in its upregulation in “glossy” eye imaginal discs by microarray analysis (Domingos, P; unpublished). Furthermore, UAS-Xbp1u transgene expression can suppress the Xbp1s induced “glossy” eye phenotype (see Chapter III p.90), suggesting that also in *Drosophila* the Xbp1s/Xbp1u complex is degraded. If Xbp1u is the substrate for CG6758, then in homozygous mutant clones there should be more Xbp1u accumulation and more degradation of Xbp1s, to due Xbp1s/Xbp1u complex formation and reduced Xbp1s levels would lead to a suppression of the “glossy” eye phenotype. However by checking UAS-Xbp1u transgene levels in mutant mosaic “glossy” eye discs, we did not observe any difference of Xbp1u expression in homozygous CG6758 mutant clones, compared to heterozygous and “wildtype” tissue, excluding Xbp1u as a substrate of CG6758.

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References

Acosta-Alvear, D., Zhou, Y., Blais, A., Tsikitis, M., Lents, N.H., Arias, C., Lennon, C.J., Kluger, Y., and Dynlacht, B.D. (2007). XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. Mol. Cell *27*, 53–66.

Arsham, A. M., Neufeld, T. P. (2009). A genetic screen in Drosophila reveals novel cytoprotective functions of the autophagy-lysosome pathway PLoS ONE, 4 (6) pp. e6068.

Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G., and Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature *415*, 92–96.
Chumakov, P. M. (2007). Versatile Functions of p53 Protein in Multicellular Organisms. Biochemistry (Mosc) 72 (13): 1399–1421.

Fan, Y., Lee, T. V., Xu, D., Chen, Z., Lamblin, A.-F., Steller, H., Bergmann, A. (2010). Dual roles of Drosophila p53 in cell death and cell differentiation. Cell Death Differ 17 (6): 912-21.

Fogarty, P., Kalpin, R. F., Sullivan, W. (1994). The Drosophila maternal-effectmutation grapes causes a metaphase arrest at nuclear cycle 13. *Development* 120, 2131-2142.

Harper, J. W., Elledge, S. J. (1996). Cdk inhibitors in development and cancer. Curr. Opin. Genet. Dev. 6, 56-64.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R., Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell, 77, pp. 107-120.

Lew, D. J., Kornbluth, S,. (1996). Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr. Opin. Cell Biol. 8: 795-804.

Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A. et al. (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. 14, 1448-1459.

Malzer, E., Daly, M.-L., Moloney, A., Sendall, T. J., Thomas, S. E., Ryder, E., Ryoo, H., Crowther, D. C., Lomas, D. A., Marciniak, S. J. (2010).

Impaired tissue growth is mediated by checkpoint kinase 1 (CHK1) in the integrated stress response. Journal of Cell Science 123, 2892-2900.

Mintseris, J., Obar, R. A., Celniker, S., Gygi, S. P., Vijay-Raghavan, K., Artavanis-Tsakonas, S. (2009). DPiM: the Drosophila Protein Interaction Mapping project.

Richardson, H., O'Keefe, L.V., Marty, T., Saint, R. (1995). Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the Drosophila eye imaginal disc. Development 121(10): 3371--3379.

Sauer , K., Lehner, C. F. (1995). The role of cyclin E in the regulation of entry into S phase. Prog Cell Cycle Res. 1: 125-39.

Sun. L., Shi, L., Li, W., Yu, W., Liang, J., Zhang, H., Yang, X., Wang, Y., Li, R., Yao, X., Yi, X., Shang, Y. (2009). JFK, a Kelch domain-containing F-box protein, links the SCF complex to p53 regulation. Proc Natl Acad Sci U S A. 106(25):10195-200.

Tenev, T., Zachariou, A., Wilson, R., Ditzel, M., Meier, P. (2004). IAPs are functionally non-equivalent and regulate effector caspases through distinct mechanisms. Nat. Cell Biol. 7, 70-77.

Yoshida, H., Oku, M., Suzuki, M., and Mori, K. (2006). pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. J. Cell Biol. *172*, 565–575.

Zachos, G., Rainey, M. D., Gillespie, D. A. (2003). Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects. EMBO J. 22, 713-723.

Zhang, Y. W., Brognard, J., Coughlin, C., You, Z., Dolled-Filhart, M., Aslanian, A., Manning, G., Abraham, R. T., Hunter, T. (2009). The F box protein Fbx6 regulates Chk1 stability and cellular sensitivity to replication stress. Mol. Cell 35 (4): 442-53.

Chapter V

CG6758 is involved in the GMR-GAL4>UAS-Rh-1 ER stress model

Summary

Over-expression of Rhodopsin-1 wildtype (Rh-1^{WT}) or mutated Rhodopsin-1 (Rh-1^{G69D}) with the GMR-GAL4 driver leads to ER stress, cell death and an atrophic "glossy" eye phenotype, similar to that seen by GMR-GAL4>UAS-Xbp1s. ER stress occurs, because Rhodopsin-1 is normally expressed in the pupa and the folding capacity of the ER in eye imaginal discs (GMR-GAL4 domain) is not sufficient for the processing of the over-expressed proteins. We tested our CG6758 mutations in this "glossy" eye phenotype to analyse an involvement in this cell death pathway. We observed a suppression of "glossyness" in the adult eye as well as suppression of apoptosis in cell clones homozygous mutant for Su218, arguing that CG6758 also plays a role in this ER stress model.

Furthermore, we found reduced levels of Rh-1 in homozygous mutant clones, suggesting that CG6758 is somehow regulating the degradation of Rh1 by ERAD. For example, CG6758 could have as a substrate an ERAD factor, which would accumulate in CG6758 mutant cells and lead to the increased clearance of Rh-1. One ERAD factor that is responsible for the degradation of Rh-1 is Hrd1 and we tested whether Hrd1 accumulates in the homozygous CG6758 mutant clones. We did not find an accumulation of Hrd1 in the homozygous CG6758 mutant clones.

To be sure that the proteasomal degradation by an ERAD factor is responsible for the decreased levels of Rh-1^{WT} in the homozygous mutant clones, we inhibited the proteasome with MG132. Compared to control discs, the inhibition of the proteasome leads to a restoration of Rh-1^{WT} levels in the homozygous mutant cells, demonstrating that Rh-1^{WT} is normally degraded in those clones.

Introduction

The GMR-GAL4 driven over-expression of Rh-1 (wildtype or mutated form) leads to an atrophic “glossy” eye phenotype, similar to the phenotype seen by our GMR-GAL4>UAS-Xbp1s over-expression This phenotype results from over-expressing Rh-1 in the larval eye and thereby overloading the ER capacity in the larvae, since Rh-1 is normally expressed first in the pupa. Exceeding the folding capacity of the larval ER leads to ER stress (Kang et al., 2009). The GMR-GAL4>UAS-Rh-1 eye phenotype can be rescued by co-expression of ERAD components like the *Drosophila* Hrd1, resulting in partial restoration of the “glossy” eye (Kang et al., 2009). Furthermore, it was shown that the atrophic eye phenotype observed by Rh-1 mis-expression is due to elevated levels of apoptosis in the GMR domain (Kang et al., 2009).

Materials and Methods

The pUAST-Rh-1^{WT} and pUAST-Rh-1^{G69D} vectors were amplified in DB3 cells and a Midiprep with the NZY tech Midi Prep kit was performed. Sequencing of the vectors was done by Stabvida. The vectors were injected into embryos (BestGene) and stocks were generated by site-specific recombination into the 68A4 site on the third chromosome. With the Flp/FRT system we were able to generate clones of our CG6758 alleles on the second chromosome in the background of Rh-1 over-expression on the third (eyFlp, GMR-Gal4; FRT42D, UbiRFP/ FRT42D, Su218; UAS-Rh-1^{WT} or Rh-1^{G69D}/ +. In the stock with wildtype Rh-1, apoptosis was analysed with the CPV reporter. Therefore, flies with the genotype eyFlp, GMR-GAL4; FRT42D, myrRFP/ FRT42D, Su218; CPV reporter/Rh-1^{WT} were generated.

Staining with Elav to visualize cleaved parp was performed (for protocol see Chapter II, p.56).

To analyse Rh-1^{WT} levels in the CG6758 mosaic mutant disc, we stained the discs with an Rh-1 antibody (4C5-s from DSHB) and Elav antibody (7E8A10 from DSHB) (for protocol see Chapter III, p.56).

For Hrd1 staining in “glossy” eye discs we generated the following flies: eyFlp, GMR-Gal4; FRT42D, UbiRFP/ FRT42D, Su218; UAS-Xbp1s/ +. Immuno-staining was performed with a Hrd1 antibody (a gift from Toshihiro Nakajima) and an Elav antibody (for protocol see Chapter III, p.56).
We also stained for Hrd1 in “glossy” eye discs induced by Rh1^{WT} expression. Therefore the flies with the genotype eyFlp, GMR-Gal4; FRT42D, UbiRFP/ FRT42D, Su218; UAS-Rh-1^{WT}/ + were generated.
For over-expression of Hrd1-myc and Rh-1^{WT}, we used flies with the genotype eyFlp, GMR-Gal4; FRT42D, UbiGFP/ FRT42D, Su218; UAS-Hrd1-myc/ UAS-Rh1^{WT} .The staining with a Hrd1 and an Elav antibody was performed using the protocol in Chapter II, p.55.

To test if Rh-1^{WT} levels are reduced in CG6758 homozygous mutant clones due to proteasomal function, we used the proteasome inhibitor MG132, eye imaginal discs were incubated at RT over night with 200µM MG132 (in S2 media), control discs were incubated in S2 media.

All the discs were analysed under the confocal microscope (Zeiss LSM710).

Results

5.1. CG6758 mutations in the GMR-GAL4>UAS-Rh-1 “glossy” eye

Over-expression of wildtype and mutated Rhodopsin-1 (Rh-1^{WT}, Rh-1^{G69D}) in the eye imaginal discs also leads to a “glossy” eye phenotype in the adult, similar to the phenotype observed when over-expressing Xbp1s by the GMR-GAL4 driver. The eyes are much smaller than wild type eyes due to apoptosis. This phenotype is due to overloading the capacity of the ER in the eye imaginal discs since Rhodopsin-1, which is translated into the ER, is normally only expressed by late pupal stages (Kang et al., 2009). We chose the GMR-GAL4>UAS-Rh-1 eye as another model for ER stress and analysed if our alleles of CG6758 also suppress this “glossy” eye phenotype. We could observe a suppression of the GMR-GAL4>UAS-Rh-1^{WT} and GMR-GAL4>UAS-Rh-1^{G69D} “glossy” eye phenotype (**fig.54,55**), however flies with the right genetic constitution and the UAS-Rh-1^{G69D} transgene were only viable at 18°C, meaning that the “glossyness” of the eye was not so dominant due to a weaker transgene expression. For the GMR-GAL4>UAS-Rh-1^{WT} eye, we also generated eyes composed exclusively of homozygous mutant clones by the so called EGUF, Hid technique (Stowers and Schwarz, 1999). In those eyes one can easily see a restoration of the eye size and the ommaditial patterning (**fig.54**).

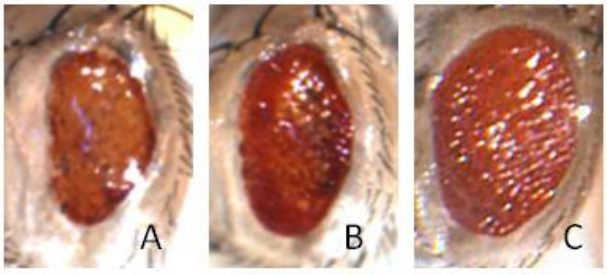

Fig.54: CG6758 mutations suppress the GMR-GAL4>UAS-Rh-1^{WT} “glossy” eye. (A) GMR-GAL4>UAS-Rh-1^{WT} “glossy eye”. **(B)** Su218 clones suppress the “glossy” eye phenotype. **(C)** “Glossy” eyes composed solely of Su218 clones have nearly normal size.

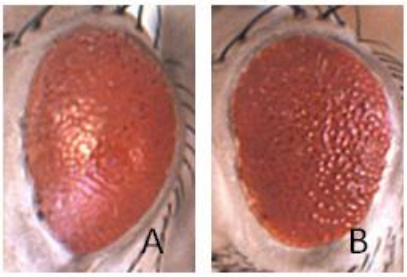

Fig.55: CG6758 mutations suppress the GMR-GAL4>UAS-Rh-1^{G69D} "glossy" eye. (A) GMR-GAL4>UAS-Rh-1^{G69D} "glossy" eye. The fly was raised at 18°C, at RT or higher temperatures the eye looks as "glossy" as the GMR-GAL4>UAS-Rh-1^{WT} eye in fig.49 **(B)** Su218 clones suppress the "glossy" eye phenotype.

Since it was shown, that apoptosis leads to the GMR-GAL4>UAS-Rh-1 "glossy eye" phenotype, we checked if we can detect reduced levels of apoptosis in our homozygous mutant clones in eye imaginal discs.

We chose the GMR-GAL4>UAS-Rh-1^{WT} eye for this purpose and used the CPV apoptosis reporter to detect apoptosis. We could clearly detect less apoptosis in clones homozygous for Su218 (**fig.56**).

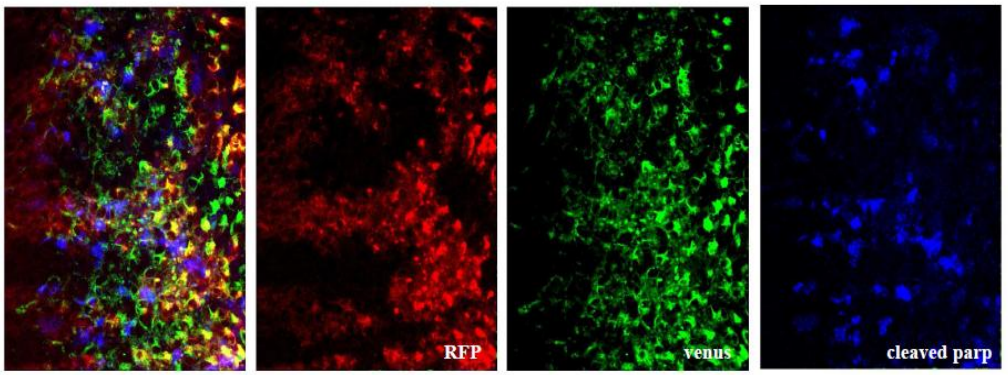

Fig.56: Apoptosis is suppressed in CG6758 mutant clones in GMR-GAL4>UAS-Rh-1^{WT} "glossy" eye discs. Visualization of apoptosis with the CPV reporter. The genotype of the fly is eyFlp ,GMR-Gal4; FRT42D, UbiRFP/ FRT42D, Su218; CPV/ UAS-Rh1^{WT}. In homozygous mutant clones marked by the absence of RFP there is less membrane-bound Venus-GFP and no cleaved parp staining, apoptosis is suppressed in the homozygous mutant clones.

5.2. Reduced apoptosis in CG6758 mutants due to proteasomal degradation of Rh-1

To test if reduced levels of Rh-1^{WT} in the homozygous mutant clones are responsible for the suppression effect and less cell death, we analysed Rh-1^{WT} levels with a Rh-1 antibody in eye discs mosaic for Su218 and could detect reduced Rh-1^{WT} levels (**fig.57**)

Since Rh-1^{WT} levels are reduced in clones homozygous for Su218, we assumed that the substrate of CG6758 is an ERAD factor, since ERAD is responsible of clearing the cells of over-expressed Rh-1^{WT}. Hrd1 is one of the ERAD factors known to promote the degradation of misfolded Rh-1 (Kang et al., 2009). To test the possibility that Hrd1 is a substrate for CG6758, we stained CG6758 mutant eye discs (Xbp1s construct in the background) with a Hrd1 antibody to analyse whether in homozygous mutant clones there are elevated levels of endogenous Hrd1. We could not see any difference in the mutant and the “wildtype” clones (**fig.58**).

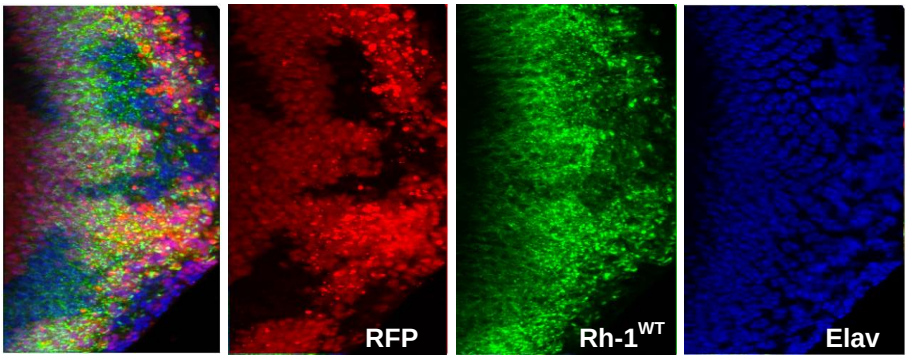

Fig.57: Reduced Rh-1^{WT} levels in CG6758 mutant clones lead to the suppression effect. The fly has the genotype eyFlp, GMR-Gal4; FRT42D, UbiRFP/ FRT42D, Su218; +/ UAS-Rh1^{WT}. In clones homozygous for Su218, marked by the absence of RFP, there are reduced levels of Rh-1^{WT} as seen with the Rh-1 antibody (GFP). Elav staining marks the photoreceptors and serves as a control to ensure that the area which lacks RFP are no holes in the eye disc.

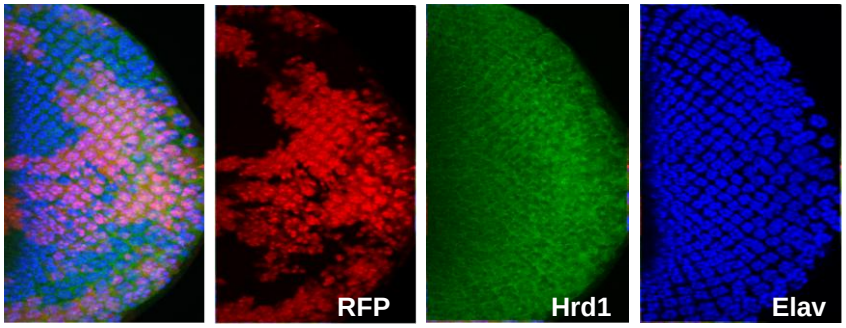

Fig.58: There are no elevated Hrd1 levels in CG6758 mutant clones in GMR-GAL4>UAS-Xbp1s "glossy" eye discs, excluding Hrd1 as the substrate for CG6758. The fly has the genotype eyFlp, GMR-Gal4; FRT42D, UbiRFP/ FRT42D, Su218; UAS-Xbp1s/ +. In clones homozygous for Su218, marked by the absence of RFP, Hrd1 levels (GFP) are equal to the surrounding "wildtype" and heterozygous tissue. Elav staining marks the photoreceptors and serves as a control that the area which lacks RFP are no holes in the eye disc due to the preparation. Note: In Xbp1s over-expressing discs, Hrd1 can be excluded as the substrate for CG6758.

We also tested Hrd1 levels in homozygous mutant clones for Su218 with Rh-1^{WT} in the background and did not observe any difference in endogenous Hrd1 levels when compared to "wildtype" tissue (**fig.59**).

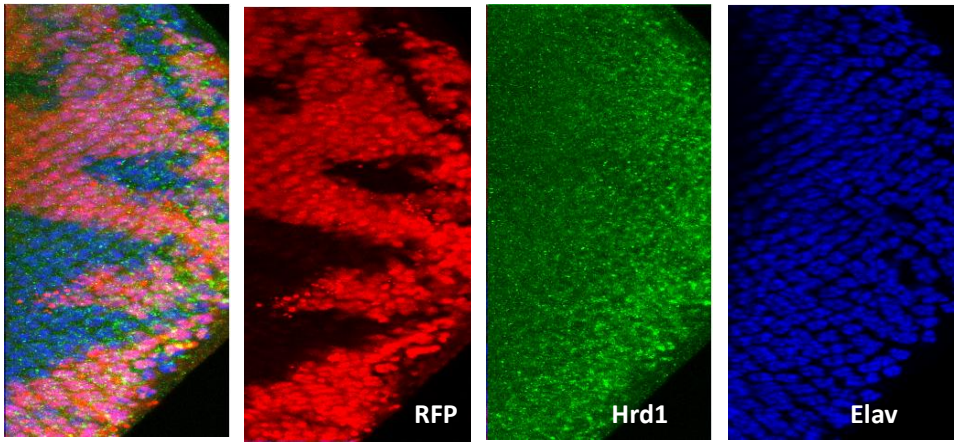

Fig.59: There are no elevated Hrd1 levels in CG6758 mutant clones in GMR-GAL4>UAS-Rh-1^{WT} "glossy" eye discs, excluding Hrd1 as the substrate for CG6758. The fly has the genotype eyFlp, GMR-Gal4; FRT42D, UbiRFP/ FRT42D, Su218; UAS-Rh-1^{WT}/ +. In clones homozygous for Su218, marked by the absence of RFP, Hrd1 levels (GFP) are equal to the surrounding "wildtype" and heterozygous tissue. Elav staining marks the photoreceptors and serves as a control that the area which lacks RFP are no holes in the eye disc. Note: Hrd1 can be excluded as substrate for CG6758.

Next we tested, wether we could observe any difference in Hrd1 levels in the mutant clones when compared to "wildtype" tissue when UAS-Hrd1-myc is over-expressed, furthermore we wanted to see if over-expressed Rh-1^{WT} colocalizes with Hrd1. We generated flies with the genotype eyFlp, GMR-Gal4; FRT42D, UbiGFP/ FRT42D, Su218; UAS-Hrd1-myc/ UAS-Rh1^{WT}. As expected, there are reduced levels of over-expressed Rh1^{WT} in the Su218 mutant clones, but we could not find different Hrd1-myc levels in the homozygous mutant clones when compared to the control tissue (**fig.60**).

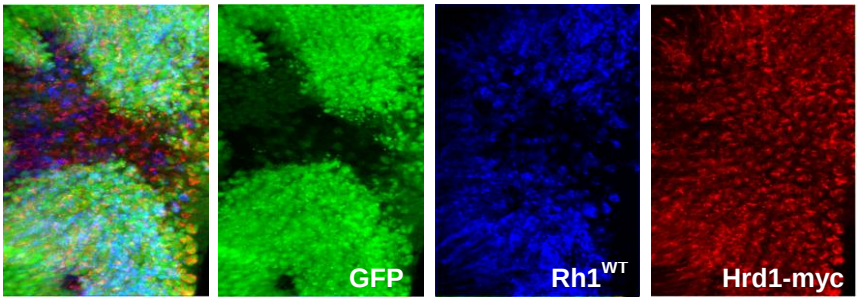

Fig.60: There are no elevated over-expressed Hrd1 levels but reduced Rh-1^{WT} in homozygous mutant CG6758 clones. The fly has the genoype eyFlp, GMR-Gal4; FRT42D, UbiGFP/ FRT42D, Su218; UAS-Hrd1-myc/ UAS-Rh1^{WT}. Clones homozygous for the CG6758 mutation are marked by the absence of GFP. Rh-1 levels are reduced in those clones, Hrd1 levels are the same in mutant and control tissue.

To confirm that reduced levels of over-expressed Rh-1^{WT} in Su218 mutant clones are due to proteasomal degradation, we tested the influence of the proteasome inhibitor MG132 on the Rh-1^{WT} levels in those discs. Eye discs were incubated in 200µM MG132 in Schneider S2 media over night before fixation, as control, we also incubated discs solely in Schneider S2 media over night. The incubation took place at room temperature. At 25°C every row of photoreceptors develops approximately every two hours, meaning that for an incubation of 16h in MG132, there should be eight rows of photoreceptors in the anterior part of the GMR domain in which the proteasome should have been inhibited since the onset of photoreceptor differentiation. We compared the over-expressed Rh-1^{WT} levels in Su218 mutant clones in the anterior GMR domain of MG132 incubated discs with those of control discs and found that incubation with MG132 leads to elevated levels of Rh-1^{WT} (**fig.62**), meaning Rh-1^{WT} is cleared from the homozygous mutant clones by proteasomal function.

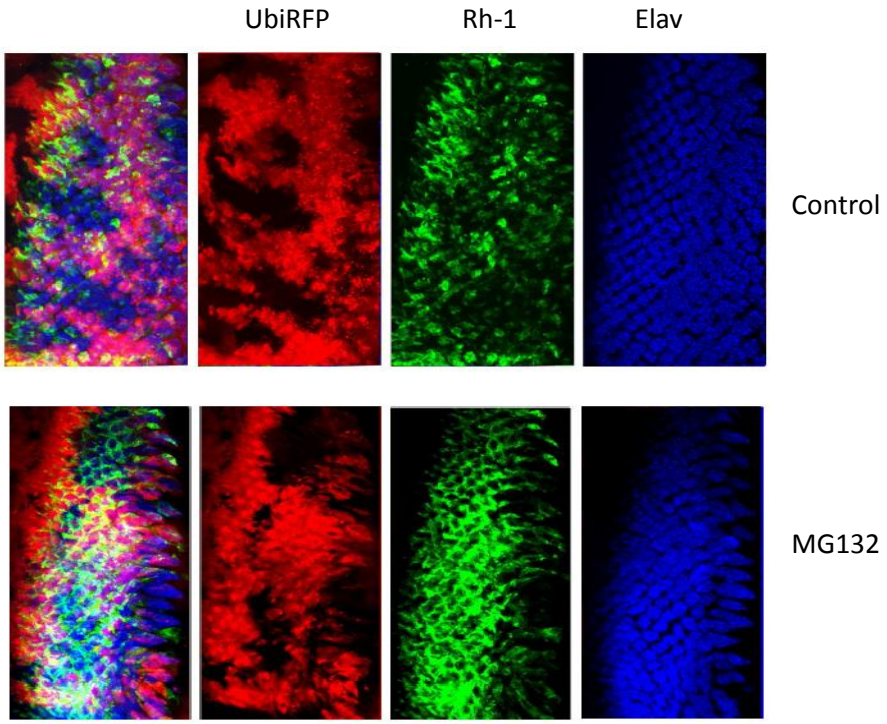

Fig.61: Rh-1^{WT} levels are reduced in CG6758 homozygous mutant clones due to proteasomal function. CG6758 homozygous mutant clones are marked by the absence of UbiRFP, Elav serves as a control to be sure that lack of RFP is not due to holes in the discs. In the control experiment where discs were incubated with S2 media, Rh-1 levels are reduced in homozygous mutant clones, whereas when discs are incubated with MG132, Rh-1 levels are restored in the anterior part of the GMR-GAL4

Discussion

Over-expression of Rh-1 with the GMR-GAL4 driver leads to ER stress, apoptosis and a “glossy” eye phenotype which is an amenable model to screen for suppressors of this phenotype. In our ER stress model where we mimic strong and prolonged ER stress by the over-expression of Xbp1s we

identified CG6758 as a downstream mediator of the Xbp1s induced cell death. We showed that CG6758 mutations also suppress the “glossy” eye phenotype caused by the GMR-GAL4 driven over-expression of Rh-1, which is a valid model for ER stress induced cell death, since it was shown that the co-expression of ERAD factors can suppress this atrophic eye phenotype (Kang et al., 2009). The suppression of the GMR-GAL4>UAS-Rh-1 eye phenotype by CG6758 suppressor mutations obtained from the Xbp1s over-expression screen makes our model valid as another ER stress induced cell death model and shows that the GMR-GAL4>UAS-Xbp1s induced cell death is not an artefact.

In the GMR-GAL4>UAS-Rh-1 atrophic eye, apoptosis is a mechanism responsible for cell death and mutations of CG6758 homozygous mutant clones suppress the apoptosis in this model. In GMR-GAL4>UAS-Xbp1s discs, we could only detect little apoptosis and we could not show that there is a suppression of apoptosis by CG6758 mutations.

We also showed that reduced levels of Rh-1^{WT} in CG6758 homozygous mutant clones are responsible for the suppression of cell death which leads to the assumption that an ERAD factor, responsible for the degradation of Rh-1^{WT}, is the substrate for CG6758 dependent ubiquitination. In CG6758 homozygous mutant clones, the substrate of CG6758 should accumulate and an accumulation of an ERAD factor responsible for Rh-1^{WT} clearance would lead to the increased degradation of Rh-1^{WT}. Hrd1 is one ERAD factor known to be involved in the degradation of Rh-1^{WT}, so we tested Hrd1 levels in homozygous mutant clones of GMR-GAL4>UAS-Xbp1s and GMR-GAL4>UAS-Rh-1^{WT} discs, we found no accumulation of Hrd1 in the clones. We also checked over-expressed Hrd1-myc levels in GMR-GAL4>UAS-Rh-1^{WT} discs and found no accumulation of Hrd1-myc in the homozygous mutant clones. We excluded Hrd1 as the substrate of CG6758.

To show that proteasomal degradation is responsible for the decreased levels of Rh-1^{WT} in CG6758 homozygous mutant clones, we made use of the proteasome inhibitor MG132. Inhibition of the proteasome restored Rh-1^{WT} levels in the mutant clones. From these experiments we can conclude that the substrate of CG6758 is an ERAD component responsible for the degradation of mifolded Rh-1 in the ER.

Acknowledgements

We thank H. D. Ryoo for providing the attB-pUAST-Rh-1^{WT} and attB-pUAST-Rh-1^{G69D} vectors. We thank E. Arama for the CPV reporter and Toshihiro Nakajima for the Hrd1 antibody.

References

Kang, M.-J., Ryoo, H. D. (2009). Suppression of retinal degeneration in Drosophila
by stimulation of ER-associated degradation. PNAS vol. 106, no. 40, 17043–17048

Stovers, R. S. and Schwarz, T. L. (1999). A Genetic Method for Generating Drosophila Eyes Composed Exclusively of Mitotic Clones of a Single Genotype. Genetics 152: 1631–1639.

Chapter VI

Discussion

The UPR is activated under conditions of ER stress, due to unfolded or misfolded proteins in the ER and serves to restore ER homeostasis by translational attenuation to prevent that newly synthesized proteins enter the overloaded ER and by upregulation of ERAD components, chaperones and folding enzymes. However, if ER stress is too strong or prolonged, the different UPR branches do not only elicit protective mechanisms, but also induce cell death.

In humans, IRE1 activity was shown to induce apoptosis via association with TRAF2 and activation of JNK signaling (Nishitoh et al., 2002, Urano et al., 2000; Verfaillie et al., review, 2010). Furthermore, RIDD conferred by the endonuclease activity of IRE1α is responsible for the induction of apoptosis by degradation of microRNAs that normally repress translation of caspase-2 mRNA (Upton et al., 2012).

The pathways which link IRE1 activity to cell death are independent of Xbp1s signaling and so far it was not shown that Xbp1s activity can lead to cell death.

With this work we show for the first time that prolonged and strong Xbp1s signaling leads to cell death by using the *Drosophila* eye as our model system. In our ER stress model we over-express Xbp1s with the GMR-GAL4 driver which leads to cell death in all cells posterior to the morphogenetic furrow. In the external eye structure this cell death is visible as the so called “glossy” eye phenotype which is due to loss of cone, lattice and pigment cells. The “glossy” eye phenotype is amenable for suppressor screens and we decided to perform a mosaic genetic screen by inducing point mutations in the fly genome and searching for mutations that can suppress the “glossyness” and potentially act downstream of Xbp1s in the cell death pathway. Therefore, we made use of the Flp/FRT technique. (Golic, 1991).

By complementation analysis, mapping with deficiencies and sequencing of candidate genes, we found four complementation groups among our

suppressors, with mutations in Xpd, in Eaf and CG6758. The fourth complementation group was not mapped.

However, to be sure that our generated alleles are real downstream targets of Xbp1s cell death signaling, we had to exclude that the suppression effect is due to reduced UAS-Xbp1s transgene expression or due to modulation of Xbp1s protein levels. Both possibilities could only be excluded for the CG6758 alleles, so we proceeded our studies with CG6758, a real downstream mediator of Xbp1s.

To be sure that the mutations found in CG6758 are responsible for rescuing the GAL4>UAS-Xbp1s “glossy” eye phenotype, we reverted the suppression effect of the alleles by co-expression of CG6758 cDNA and a genomic rescue construct.

CG6758 encodes an Fbox protein with unknown biological function (Flybase). Fbox proteins are known to be part of SCF E3 ubiquitin ligase complexes, whereby the Fbox protein binds the substrate destined for proteasomal degradation (Kipreos and Pagano, 2000). Fbox proteins can also act independently of SCF complexes in various contexts (reviewed in Hermand 2006). To analyse if CG6758 acts in an SCF complex, we performed RNAi of different SCF complex components in the GMR-GAL4>UAS-Xbp1s “glossy” eye. RNAi for CG6758, for SkpA and for Cullin-1 could partially rescue the eye phenotype, giving a hint that CG6758 acts in an SCF E3 ubiqutin ligase complex. Furhtermore CG6758 seems to co-IP with Skp1 and Cullin1 in transiently transfected S2 and HEK cells (personal communication, Catarina Gaspar).

By analysing cell death mechanisms leading to the Xbp1s induced atrophic eye phenotype, we found in cell clones over-expressing Xbp1s apoptosis and autophagy. The adult eye with Xbp1s over-expressing cell clones is only very little composed of Xbp1s expressing cells. This is not true when co-expressing P35, however P35 can only partially rescue the “glossyness”. We assume that Xbp1s over-expressing cells are removed during eye development by apoptosis and replaced by “wildtype” tissue.

Co-expression of P35 inhibits apoptosis of Xbp1 over-expressing cells, but is not fully able to rescue the “glossyness”, as it seems that only heterozygous tissue for Xbp1 over-expression can be partially rescued.
We also analysed apoptosis in the GMR-GAL4>UAS-Xbp1s eye and did not observe any induction of apoptosis in the larvae. However, we could see a partial rescue of the GMR-GAL4>UAS-Xbp1s “glossy” eye when P35 and Diap were co-expressed. It is likely that apoptosis contributes to the atrophic eye phenotype later in development, but apoptosis does not seem to be the sole mechanism of cell death in our model. Besides analysing apoptosis in Xbp1s over-expressing cell clones and GMR-GAL4>UAS-Xbp1s discs, we also tested markers for autophagy in these both contexts. We found autophagy activity in Xbp1s over-expressing cell clones but not in discs of GMR-GAL4>UAS-Xbp1s over-expression.
Since there is only partial rescue of “glossyness” with apoptosis inhibitors, but autophagy activity in Xbp1s over-expressing cell clones, one other mechanism that could contribute to the death of cells may be autosis, a form of non-apoptotic cell death recently described (reviewed in Liu et al., 2015). In our setting, the autophagy machinery may be essential for cell death. One unique morphological feature of autosis is ER dilatation. It was shown that XBP1s activity increases synthesis of phosphatidylcholine, a key ER lipid and induced the expansion of the ER. Cells over-expressing XBP1s had enhanced activity of the cytidine diphosphocholine pathway of phosphatidylcholine biosynthesis and exhibited elevated levels of membrane phospholipids, increased surface area and volume of rough ER (Sriburi et al., 2004).

We tested if Xbp1u can revert the “glossy” eye phenotype and found that Xbp1u can strongly suppress the Xbp1s induced cell death, thus it is likely that also in *Drosophila* Xbp1u interaction with Xbp1s leads to degradation of the Xbp1u/ Xbp1s complex as seen in mammals (Yoshida et al., 2006).

Next we wanted to find the substrate for CG6758 mediated ubiquitination and proteasomal degradation. Therefore we tested the substrates of the CG6758 human homologues (FBXO6 and FBXO42), namely CHK1 (Grapes in *Drosophila*) and P53. For a putative substrate of CG6758, one can expect an accumulation of protein levels in CG6758 homozygous mutant clones, which we did not find, neither for Grapes nor for Dp53. We further tested other potential substrates for being degraded by the SCF^{CG6758} complex, such as Diap, Vcp, several cell cycle components, CG16789 and Xbp1u, but we did not find the substrate of CG6758 among these proteins.

To be sure that our Xbp1s ER stress model is valid and not an artifact, we tested our CG6758 alleles in another ER stress model, where the over-expression of Rh-1^{WT}/ Rh-1^{G69D} by the GMR-GAL4 driver leads to ER stress and apoptosis and a “glossy” eye phenotype (Kang et al., 2009), similar to what is seen for Xbp1s over-expression. Our CG6758 alleles also suppress this “glossy” eye phenotype induced by GMR-GAL4 driven over-expression of Rh-1. Furthermore we could show that CG6758 mutants suppress apoptosis, by reducing the Rh-1^{WT} levels in the homozygous mutant clones. This is likely due to proteasomal degradation of Rh-1^{WT}, since inhibition of the proteasome with MG132 led to the restoration of Rh-1^{WT} protein levels. These results lead us to the conclusion that the substrate of CG6758 should be an ERAD factor which accumulates in the CG6758 mutant clones and causes the increased clearance of misfolded Rh-1 from the ER.

We analysed if Hrd1 is the substrate of CG6758, since Hrd1 was shown to rescue the Rh-1^{WT} eye (Kang et al., 2009). We did not observe any accumulation of Hrd1 in homozygous CG6758 mutant clones, neither endogenous Hrd1 accumulation nor accumulation of over-expressed Hrd1-myc.

We conclude that prolonged and strong Xbp1s signaling leads to cell death and that CG6758 is acting downstream of Xbp1s in this cell death pathway. Since CG6758 is an Fbox protein acting in an SCF complex mediating

protein ubiquitination, we assume that substrate accumulation in CG6758 mutants somehow leads to suppression of the Xbp1s induced cell death. From our experiments in the GMR-GAL4>UAS-Rh-1^{WT} eye, we believe that the substrate of CG6758 could be an ERAD component that is involved in degradation of misfolded Rh-1, because in CG6758 mutants Rh-1 degradation is increased. Xbp1s activates the transcription of many ERAD components (Shoulders et al 2013; Acosta-Alvear et al 2007), including Hrd1, Edem, DERL1 and Vcp. The transcriptional activation of ERAD components should be protective to the cell, since it causes the increased degradation of misfolded proteins from the ER. However, under certain conditions ERAD over-activation may lead to cell death. This was demonstrated by suppression of retinal pathology caused by misfolded Rhodopsin when Vcp was inactivated (Griciuc et al., 2010).

It could be possible that the sustained and strong transcriptional activity of the over-expressed Xbp1s by the GMR-GAL4 driver leads by itself to ER stress, since the transcriptional targets include many ER resident proteins (ERAD factors, chaperones) which also need to be properly folded. In this context one could explain that the impaired function of CG6758 leading to the accumulation of an ERAD factor is responsible for suppression of the "glossy" eye phenotype, since this ERAD factor could help to alleviate the induced ER stress.

This ERAD factor which could be the substrate of CG6758 mediated ubiquitinationcould also be responsible for the clearance of misfolded Rh-1 from mis-expressing eye imaginal discs in homozygous mutant clones in the GMR-GAL4>UAS-Rh-1^{WT} eye, thereby suppressing apoptosis and the atrophic eye phenotype.

The identification of cell death pathways conferred by the UPR transducer Xbp1s and the involvement of mediators in this process will provide new insights into molecular and cellular mechanisms underlying neurodegenerative diseases caused by ER stress.

Acosta-Alvear, D., Zhou, Y., Blais, A., Tsikitis, M., Lents, N.H., Arias, C., Lennon, C.J., Kluger, Y., and Dynlacht, B.D. (2007). XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. Mol. Cell *27*, 53–66.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter P. (2002). The Endoplasmic Reticulum. Molecular Biology of the Cell.

Aragón, T., van Anken, E., Pincus, D., Serafimova, I.M., Korennykh, A.V., Rubio, C.A., and Walter, P. (2009). Messenger RNA targeting to endoplasmic reticulum stress signalling sites. Nature *457*, 736–740.

Baker, N. E. (2001). Cell proliferation, survival, and death in the Drosophila eye. Semin. Cell Dev. Biol. 12**:** 499-507.

Baker, N. E., Yu, S. Y. (2001). The Egf receptor defines domains of cell cycle progression and survival to regulate cell number in the developing Drosophila eye. Cell 104: 699–708.

Baker, E. K., Colley, N. J., Zuker, C. S. (1994). The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. EMBO J. 13 (20): 4886-4895.

B'chir, W., Vale, A.C. M., Carraro, Averous, J., Jousse, C., Muranishi, Y., Parry, L., Stepien, G., Fafournoux, P., Bruha, A. (2013). The eIF2a/ATF4 pathway is essential for stress-induced autophagy gene expression. Nucleic Acids Research, Vol. 41, No. 16, 7683–7699.

Bergmann, A., Agapite, J., McCall, K., Steller, H. (1998). The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. Cell 95 (3): 331-341.Bergmann A, Tugentman M, Shilo BZ, Steller H. (2002). Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling. Dev Cell 2 (2): 159-170.

Bernales, S., McDonald, K. L., Walter, P. (2006). Autophagy Counterbalances Endoplasmic Reticulum Expansion during the Unfolded Protein Response. PLoS Biol. 4: e423.

Bernasconi, R., Pertel, T., Luban, J., and Molinari, M. (2008). A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. J. Biol. Chem. *283*, 16446–16454.

Bernasconi, R., Soldà, T., Galli, C., Pertel, T., Luban, J., and Molinari, M. (2010). Cyclosporine A-sensitive, cyclophilin B-dependent endoplasmic reticulum-associated degradation. PloS One 5.

Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P., and Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat. Cell Biol. *2*, 326–332.

Blumenstiel JP, Noll AC, Griffiths JA, Perera AG, Walton KN, Gilliland WD, Hawley RS, Staehling-Hampton K. 2009. Identification of EMS-induced mutations in Drosophila melanogaster by whole-genome sequencing. Genetics 182: 25–32.

Bordallo, J., Plemper, R. K., Finger, A., Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol. Biol. Cell (1): 209-22.

Braitenberg, V. (1967). Patterns of projection in the visual system of the fly. I. Retina-lamina projections. Exp. Brain Res 1967;3:271–298.
Brennan, C. A., Moses, K. (2000). Determination of *Drosophila* photoreceptors: timing is everything. CMLS, Cell. Mol. Life Sci. 57, 195–214

Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M., Abrams, J. M. (2000). Drosophila p53 binds a damage response element at the reaper locus. Cell 101:103-113.

Brogna, S., Wen, J. (2006). Nonsense-mediated mRNA decay (NMD) mechanisms. Nature Structural & Molecular Biology 16, 107-113.

Cagan, R. L., Ready, D. F. (1989). Notch is required for successive cell decisions in the developing Drosophila retina. Genes Dev 3: 1099–1112.

Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G., and Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 415, 92–96.

Cameroni, E., Stettler, K., Suter, B. (2010). On the traces of XPD: cell cycle matters -untangling the genotype-phenotype relationship of XPD mutations. Cell Division 5:24.

Campos, I., Geiger, J.A., Santos, A.C., Carlos, V., Jacinto, A. (2010). Genetic screen in Drosophila melanogaster uncovers a novel set of genes required for embryonic epithelial repair. Genetics 184 (1): 129-140.

Campos-Ortega, J., Hofbauer, A. (1977). Cell clones and pattern formation: On the lineage of photoreceptor cells in the compound eye of Drosophila. Rouxs Arch. Dev. Biol.181, 227–245.

Carvalho, P., Goder, V., Rapoport, T. A. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell. 126*:* 361–373*.*

Carvalho, P., Stanley, A. M., Rapoport, T. A. (2010). Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. Cell 143 (4): 579-91.

Chai, J., Yan, N., Huh, J. R., Wu, J. W., Li, W., Hay, B. A. and Shi, Y. (2003). Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent Dronc ubiquitination. Nat. Struct. Biol. 10, 892-898.

Chapman, R.E., and Walter, P. (1997). Translational attenuation mediated by an mRNA intron. Curr. Biol. CB *7*, 850–859.

Chen, G., Goeddel, D. V. (2002). TNF-R1 Signaling: A Beautiful Pathway. Science Vol. 296 no. 5573 pp. 1634-1635

Chen, J., Larochelle, S., Li, X., Suter, B. (2003). Xpd/Ercc2 regulates CAK activity and mitotic progression. Nature 424, 228-230.

Cheng, E.-Y. A., Wei, M. C., Weiler, S., Flavell, R. A.,,2 Mak, T. W., Lindsten, T., Korsmeyer, S. J. (2001). BCL-2, BCL-XL Sequester BH3 Domain-Only Molecules Preventing BAX- and BAK-Mediated Mitochondrial Apoptosis. Mol. Cell 8 (3): 705-11.

Chowdhury, I, Tharakan, B., Bhat, K. B. (2008). Caspases: an update. Comparative Biochemistry and Physiology B, vol. 151, no. 1, pp. 10–27.

Christianson, J.C., Shaler, T.A., Tyler, R.E., Kopito, R.R. (2008). OS9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD. Nat. Cell Biol. 10, 272–282.

Chumakov, P. M. (2007). Versatile Functions of p53 Protein in Multicellular Organisms. Biochemistry (Mosc) 72 (13): 1399–1421.

Clem RJ, Fechheimer M, Miller LK. (1991). Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science 254 (5036): 1388-90.

Cohen, S. M. (1993). Imaginal disc development. In Drosophila Development, A. Martinez-Arias and M. Bate, eds. (Cold Spring Harbor: Cold Spring Harbor Press), pp. 747-841.

Colley, N. J., Baker, E. K., Stamnes, M. A., Zuker, C. S. (1991). The cyclophilin homolog ninaA is required in the secretory pathway. Cell 67 (2): 255-63.

Colley, N. J., Cassill, J. A., Baker, E. K., Zuker, C. S. (1995). Defective intracellular transport is the molecular basis of rhodopsin-dependent dominant retinal degeneration. Proc. Natl. Acad. Sci. USA 92, 3070–3074.

Connor, J. H., Weiser, D. C., Li, S., Hallenbeck, J. M., Shenolikar, S. (2001). Growth arrest and DNA damage inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. Mol. Cell. Biol. 21, 6841–6850.

Cook, T., and Desplan, C. (2001). Photoreceptor subtype specification: from flies to humans. Semin. Cell Dev. Biol. *12*, 509–518.

Cooper JL, Greene EA, Till BJ, Codomo CA, Wakimoto BT, Henikoff S. 2008. Retentionofinduced mutations in a Drosophila reverse-genetic resource. Genetics 180: 661–667.

Cox, J.S., Shamu, C.E., and Walter, P. (1993). Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell *73*, 1197–1206.

Cox, J.S., and Walter, P. (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell *87*, 391–404.

Degterev, A., Boyce, I., Yuan, J. (2003). A decade of caspases.Oncogene, vol. 22, no. 53, pp. 8543–8567, 2003.

Denic V., Quan E. M., Weissman, J. S. (2006). A Luminal Surveillance Complex that Selects Misfolded Glycoproteins for Endoplasmic Reticulum-Associated Degradation. Cell; 126, 349-359.

Debnath, J., Baehrecke, E. H., Kroemer, G. (2005). Does autophagy contribute to cell death? Autophagy 1 (2): 66–74.

Dorstyn, L., Colussi, P. A., Quinn, L. M., Richardson, H. and Kumar, S. (1999). DRONC, an ecdysone-inducible Drosophila caspase. Proc. Natl. Acad. Sci. USA 96, 4307-4312.

Dui, W., Lu, W., Ma, J., Jiao, R. (2012). A systematic phenotypic screen of F-box genes through a tissue-specific RNAi-based approach in Drosophila. J Genet Genomics. 39 (8): 397-413

Ellgaard, L., and Helenius, A. (2003). Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. *4*, 181–191.

Ernst, R., Mueller, B., Ploegh, H. L., Schlieker, C. (2009). The otubain YOD1 is a deubiquitinating enzyme that associates with p97 to facilitate protein dislocation from the ER. Mol. Cell. 36 (1): 28-38.

Fan, Y., Lee, T. V., Xu, D., Chen, Z., Lamblin, A.-F., Steller, H., Bergmann, A. (2010). Dual roles of Drosophila p53 in cell death and cell differentiation. Cell Death Differ 17 (6): 912-21.

Field, M.C., Moran, P., Li, W., Keller, G. A., Caras, I. W. (1994). Retention and degradation of proteins containing an uncleaved glycosylphosphatidylinositol signal. J. Biol. Chem. 269 (14): 10830-7.

Florentin, A. and Arama, E. (2012). Caspase levels and execution efficiencies determine the apoptotic potential of the cell. JCB, vol. 196, number 4.

Fogarty, P., Kalpin, R. F., Sullivan, W. (1994). The Drosophila maternal-effect mutation grapes causes a metaphase arrest at nuclear cycle 13. Development 120, 2131-2142.

Foresti, O., Rodriguez-Vaello, V., Funaya, C., Carvalho, P. (2014). Quality control of inner nuclear membrane proteins by the Asi complex. Science Vol. 346 no. 6210 pp. 751-755.

Franch-Marro,X., Wendler, F., Guidato, S., Griffith, J., Baena-Lopez, A., Itasaki, N., Maurice, M. M., Vincent, J.-P. (2008). Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. Nature Cell Biology 10, 170 – 177

Freel, C. D., Richardson, D. A., Thomenius, M. J., Gan, E. C., Horn, S. R., Olson, M. R., Kornbluth, S. (2008). Mitochondrial localization of Reaper to promote inhibitors of apoptosis protein degradation conferred by GH3 domain-lipid interactions. J. Biol. Chem. 4;283(1):367-79.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell 87: 651-660.

Freeman, M. (1997). Cell determination strategies in the Drosophila eye. Development 124, 261-270.

Frenkel, Z., Shenkman, M., Kondratyev, M., Lederkremer, G. Z. (2004). Separate roles and different routing of calnexin and ERp57 in endoplasmic reticulum quality control revealed by interactions with asialoglycoprotein receptor chains. Mol. Biol. Cell 15 (5): 2133-42.

Fu, W., Noll, M. (1997). The Pax2 homolog sparkling is required for development of cone and pigment cells in the Drosophila eye. Genes Dev 11: 2066–2078.

Gardner, R.G., Swarbrick, G.M., Bays, N.W., Cronin, S.R., Wilhovsky, S., Seelig, L., Kim, C., Hampton, R.Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J. Cell Biol. 151(1), 69-82.

Gardner, B.M., and Walter, P. (2011). Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. Science *333*, 1891–1894.

Gauss, R., Sommer, T., Jarosch, E. (2006a). The Hrd1p ligase complex forms a linchpin between ER-lumenal substrate selection and Cdc48p recruitment. EMBO J. 25:1827–1835

Gauss R, Jarosch E, Sommer T, Hirsch C. (2006b). A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. Nat Cell Biol 8, 849–854.

Gauss, R., Kanehara, K., Carvalho, P., Ng, D. T., Aebi, M. (2011). A complex of Pdi1p and the mannosidase Htm1p initiates clearance of unfolded glycoproteins from the endoplasmic reticulum. Mol. Cell 42 (6): 782-93.

Golic, K.G. (1991). Site-specific recombination between homologous chromosomes in Drosophila. Science. 252(5008), 958-61

Ghosh , R, Wang, L, Wang, ES, Perera, BG, Igbaria, A, Morita, S, Prado, K, Thamsen, M, Caswell, D, Macias, H, Weiberth, KF, Gliedt ,MJ, Alavi, MV, Hari, SB, Mitra, AK, Bhhatarai, B, Schürer, SC, Snapp, EL, Gould, DB, German, MS, Backes, BJ, Maly, DJ, Oakes, SA, Papa, FR. (2014). Allosteric inhibition of the IRE1α RNase preserves cell viability and function during endoplasmic reticulum stress. Cell 158 (3): 534-48.

Griciuc, A., Aron, L., Roux, M. J., Klein, R., Giagrande, G., Ueffing, M. (2010). Inactivation of VCP/ter94 suppresses retinal pathology caused by misfolded rhodopsin in Drosophila. PLoS Genet (2010) vol. 6 (8).

Hardie, R. C. (1985). Functional organization of the fly retina. In Sensory Physiology 5 (ed. D. Ottoson), Vol. 5, pp. 1-79. Tokyo: Springer-Verlag, Berlin, Heidelberg, New York.

Harding, H., Zhang, Y., Ron, D. (1999) Translation andprotein folding are coupled by an endoplasmic reticulum resident kinase. Nature 397, 271–274.

Harmon, B., Sedat, J. (2005). Cell-by-Cell Dissection of Gene Expression and Chromosomal Interactions Reveals Consequences of Nuclear Reorganization. PLoS Biol. 3 (3): e67

Harper, J. W., Elledge, S. J. (1996). Cdk inhibitors in development and cancer. Curr. Opin. Genet. Dev. 6, 56-64.

Hawkins, C. J., Yoo, S. J., Peterson, E. P., Wang, S. L., Vernooy, S. Y., Hay, B. A. (2000). The Drosophila caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. J. Biol. Chem. 275, 27084-27093.

Hay, B.A., Wassarman, D. A., Rubin, G.M. (1995). Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell 1995, 83:1253-1262.

Haynie, J. L., Bryant, P. J. (1986). Development of the eye-antenna imaginal disc and morphogenesis of the adult head in Drosophila melanogaster. J. Exp. Zool. 237: 293-308

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol. Biol. Cell *10*, 3787–3799.

Heberlein, U., Moses, K. (1995). Mechanisms of Drosophila retinal morphogenesis: the virtues of being progressive. Cell 81(7): 987-90.

Helenius, A., Trombetta, E. S., Hebert, D. N., Simons, J. F. (1997). Calnexin, calreticulin and the folding of glycoproteins. Trends Cell Biol. 7, 193-200

Hermand, D. (2006). F-box proteins: more than baits for the SCF? Cell Div. 1: 30

Hetz,, C., Bernasconi, P., Fisher, J., Lee, A.H., Bassik, M.C., Antonsson, B., Brandt, G. S., Iwakoshi, N. N., Schinzel, A., Glimcher, L. H., Korsmeyer,

S. J. (2006). Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. Science 312, 572–576.

Hetz, C., and Glimcher, L.H. (2009). Fine-tuning of the unfolded protein response: Assembling the IRE1alpha interactome. Mol. Cell *35*, 551–561.

Hinnebusch, A. G., Natarajan, K. (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. Eukaryot. Cell 1, 22–32

Holley, C. L., Olson, M. R., Colon-Ramos, D. A., Kornbluth, S. (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. Nat. Cell Biol. 4, 439-444

Hollien, J., Lin, J.H., Li, H., Stevens, N., Walter, P., and Weissman, J.S. (2009). Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J. Cell Biol. *186*, 323–331.

Hollien, J., and Weissman, J.S. (2006). Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science 313, 104–107.

Horn SC, Hanna J, Hirsch C, Volkwein C, Schutz A, Heinemann U, Sommer T, Jarosch E. Usa1 functions as a scaffold of the HRD-ubiquitin ligase. Mol Cell 2009;36:782–793.

Hosokawa, N., Wada, I., Nagasawa, K., Moriyama, T., Okawa, K., Nagata, K. (2008). Human XTP3-B forms an endoplasmic reticulum quality control scaffold with the HRD1 SEL1L ubiquitin ligase complex and BiP. J. Biol. Chem. 283: 20914–20924.

Houck, S. A., Ren, H. Y., Madden, V. J., Bonner, J. N., Conlin, M. P., Janovick, J. A., Conn, P. N., Cyr, D. M. (2014). Quality control autophagy degrades soluble ERAD-resistant conformers of the misfolded membrane protein GnRHR. Mol. Cell 54 (1): 166-179.

Igaki, T, Yamomoto-G, Y., Tokushige, N., Kanda, H., Miura. M. (2003). Down-regulation of DIAP1 triggers a novel Drosophila cell death pathway mediated by Dark and DRONC. J. Biol. Chem. 277 (26): 23103-6

Iida, Y., Fujimori, T., Okawa, K., Nagata, K., Wada, I., Hosokawa, N. (2011). SEL1L protein critically determines the stability of the HRD1-SEL1L endoplasmic reticulum-associated degradation (ERAD) complex to optimize the degradation kinetics of ERAD substrates. J. Biol. Chem. 13; 286 (19): 16929-39.

Jousse, C., Oyadomari, S., Novoa, I., Lu, P., Zhang, Y., Harding, H.P., and Ron, D. (2003). Inhibition of a constitutive translation initiation factor 2alpha

phosphatase, CReP, promotes survival of stressed cells. J. Cell Biol. *163*, 767–775.

Kang, M.-J., Chung, J., Ryoo, H. D. (2012). Cdk5 and Mekk1 mediate a Pro-Apoptotic Signaling Response to Endoplasmic Reticulum Stress in a Drosophila Model of Autosomal Dominant Retinitis Pigmentosa. Nat. Cell Biol.; 14 (4): 409-415

Kang, M.-J., Ryoo, H. D. (2009). Suppression of retinal degeneration in Drosophila by stimulation of ER-associated degradation. PNAS vol. 106, no. 40, 17043–17048

Kanuka, H., Hisahara, S., Sawamoto, K., Shoji, S., Okano, H. and Miura, M. (1999a). Proapoptotic activity of Caenorhabditis elegans CED-4 protein in Drosophila: implicated mechanisms for caspase activation. Proc. Natl. Acad. Sci. USA 96, 145-150.

Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H., Miura, M. (1999b) Control of the cell death pathway by Dapaf-1 a Drosophila Apaf-1/CED-4 related caspase activator. Mol. Cell 4:757-769.

Kimata, Y., Kimata, Y.I., Shimizu, Y., Abe, H., Farcasanu, I.C., Takeuchi, M., Rose, M.D., and Kohno, K. (2003). Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. Mol. Biol. Cell *14*, 2559–2569.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R., Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell, 77, pp. 107–120.

Kokame, K. (2000). Identification of ERSE-II, a New cis-Acting Element Responsible for the ATF6-dependent Mammalian Unfolded Protein Response. J. Biol. Chem. *276*, 9199–9205.

Kong, S. E., Banks, C. A., Shilatifard, A., Conaway, J. W., Conaway, R. C. (2005). ELL-associated factors 1 and 2 are positive regulators of RNA polymerase II elongation factor ELL. Proc Natl Acad Sci USA 102(29):10094-8.

Kostova, Z., Tsai, Y. C., and Weissman, A. M. (2007). Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. Semin. Cell Dev. Biol. 18, 770 –779

Kumar, J. P., Tio, M., Hsiung, F., Akopyan, S., Gabay, L., Seger, R., Shilo, B.-Z., Moses, K. (1998). Development (Cambridge, UK) 125:3875–3885.

Kumar, J. P., Ready, D. F. (1995). Rhodopsin plays an essential structural role in Drosophila photoreceptor development. Dev. Camb. Engl. *121*, 4359–4370.

Kunes, S., Steller, H. (1993). Topography in the Drosophila visual system. Curr. Opin. Neurobiol. 3 (1): 53-9. Review.

Lee, A. H., Iwakoshi, N.N., and Glimcher, L.H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol. Cell. Biol. *23*, 7448–7459.

Lee, A. H., Chu, G. C., Iwakoshi, N. N, Glimcher, L. H. (2005). XBP-1 is required for biogenesis of cellular secretory machinery of exocrine glands. EMBO J. 24: 4368–80.

Lépine,S, Allegood, J. C., Park, M., Dent, P.,Milstien, S., Spiegel, S. (2010). Sphingosine-1-phosphate phosphohydrolase-1 regulates ER stress-induced autophagy. Cell Death and Differentiation 18, 350–361.

Lew, D. J., Kornbluth, S,. (1996). Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr. Opin. Cell Biol. 8: 795-804.

Li, J., Lee, B., Lee, A. S. (2006). Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. J. Biol. Chem. 281, 7260-7270.

Li, J., Ni, M., Lee, B., Barron, E., Hinton, D. R., Lee, A. S. (2008). The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ. 15 (9): 1460-71.

Li, G., Scull, C., Ozcan, L., Tabas, I. (2010). NADPH oxidase links endoplasmic reticulum stress, oxidative stress, and PKR activation to induce apoptosis. J Cell Biol. 191:1113–1125.

Liu, C. Y., Schröder, M., Kaufman, R. J. (2000). Ligand-independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. J. Biol. Chem. 275 (32): 24881-5.

Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G.,Carattini-Rivera, S., DeMayo, F., Bradley, A. et al. (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. 14, 1448-1459.

Liu, C. Y., Hetty, N., Wong, N., Schauerte, J. A., Kaufman, R. J. (2002). The Protein Kinase/Endoribonuclease IRE1α That Signals the Unfolded

Protein Response Has a Luminal N-terminal Ligand-independent Dimerization Domain. J. Biol. Chem. 277:18346-18356.

Liu, Y., Levine, B. (2015). Autosis and autophagic cell death: the dark side of autophagy. Cell Death Differ. 22(3):367-376.

Liu, X., Lengyel, J. A. (2000). Drosophila arc Encodes a Novel Adherens Junction-Associated PDZ Domain Protein Required for Wing and Eye Development. Developmental Biology 221, 419 – 434.

Lohmann, I., McGinnis, N., Bodmer, M., McGinnis, W. (2002) The Drosophila Hox gene Deformed sculpts head morphology via direct regulation of the apoptosis activator reaper. Cell 110: 457-466.

Lu, P.D., Harding, H.P., Ron, D. (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J. Cell Biol. 167, 27–33.

Lu, M., Lawrence, D.A., Marsters, S., Acosta-Alvear,D., Kimmig, P., Mendez, A.S., Paton, A.W., Paton, J.C., Walter, P., Ashkenazi, A. (2014). Cell death. Opposing unfolded-protein-response signals converge on death receptor 5 to control apoptosis. Science 345 (6192): 98-101.

Lütcke, H. (1995). Signal recognition particle (SRP), a ubiquitous initiator of protein translocation. Eur. J. Biochem. FEBS *228*, 531–550.

Ma, C., Zhou, Y., Beachy, P. A., Moses, K. (1993). The segment polarity gene hedgehog is required for progression of the morphogenetic furrow in the developing Drosophila eye. Cell 75 (5): 927-38.

Ma, Y., Brewer, J.W., Alan Diehl, J., and Hendershot, L.M. (2002). Two Distinct Stress Signaling Pathways Converge Upon the CHOP Promoter During the Mammalian Unfolded Protein Response. J. Mol. Biol. *318*, 1351–1365.

Malzer, E., Daly, M.-L., Moloney, A., Sendall, T. J., Thomas, S. E., Ryder, E., Ryoo, H., Crowther, D. C., Lomas, D. A., Marciniak, S. J. (2010). Impaired tissue growth is mediated by checkpoint kinase 1 (CHK1) in the integrated stress response

Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H. P., Ron, D. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev. 18, 3066–3077

McNaught, K. S., Olanow, C. W., Halliwell, B., Isacson, O., Jenner, P. (2001). Failure of the ubiquitin-proteasome system in Parkinson's disease. Nat. Rev. Neurosci. (8):589-94.

Miller, D. T., Cagan, R. L. (1998). Local induction of patterning and programmed cell death in the developing *Drosophila* retina. Development 125 , pp. 2327–2335

Mills, K., Daish, T., Harvey, K. F., Pfleger, C. M., Hariharan, I. K., Kumar, S. (2006). The Drosophila melanogaster Apaf-1 homologue ARK is required for most, but not all, programmed cell death. J. Cell Biol. 172:809–815.

Mintseris, J., Obar, R. A., Celniker, S., Gygi, S. P., Vijay-Raghavan, K., Artavanis-Tsakonas, S. (2009). DPiM: the Drosophila Protein Interaction Mapping project.

Mizushima, N. (2005). The pleiotropic role of autophagy: from protein metabolism to bactericide. Cell Death Differ. 12 Suppl 2: 1535-41. Review.

Mizushima, N. (2014). Atophagy: Process and Function. Genes Dev. 21: 2861-2873.

Molinari, M., Calanca, V., Galli, C., Lucca, P., and Paganetti, P. (2003). Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science *299*, 1397–1400.

Mollereau, B., and Domingos, P.M. (2005). Photoreceptor differentiation in Drosophila: from immature neurons to functional photoreceptors. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *232*, 585–592.

Mori, K., Kawahara, T., Yoshida, H., Yanagi, H., and Yura, T. (1996). Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. Genes Cells Devoted Mol. Cell. Mech. *1*, 803–817.

Nedelsky, M. B., Todd, P. K., taylor, J. P. (2008). Autophagy and the ubiquitin-proteasome system: collaborators in neuroprotection. Biochem. Biophys. Acta 1782 (12): 691-699.

Ng, D. T., Brown, J. D., and Walter, P. (1996). Signal sequences specify the targeting route to the endoplasmic reticulum membrane. J. Cell Biol. 134, 269 –278.

Nikawa, J., Akiyoshi, M., Hirata, S., and Fukuda, T. (1996). Saccharomyces cerevisiae IRE2/HAC1 is involved in IRE1-mediated KAR2 expression. Nucleic Acids Res. *24*, 4222–4226.

Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., Ichijo, H. (2002). ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev. 16, *1345*–1355.

Novoa, I., Zeng, H., Harding, H., Ron, D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol. 153, 1011–1022.

Oda, Y., Hosokawa, N., Wada, I., and Nagata, K. (2003). EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. Science *299*, 1394–1397.

Ogata, M., Hino, S., Saito, A., Morikawa, K., Kondo, S., Kanemoto, S., Murakami, T., Taniguchi, M., Tanii, I., Yoshinaga, K., Shiosaka, S., Hammarback, J. A., Urano, F., Imaizumi, K. (2006). Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol. Cell Biol. 26(24):9220-31.

Ohoka, N., Yoshii, S., Hattori, T., Onozaki, K., Hayashi, H. (2005). TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. EMBO J. 23; 24 (6): 1243-55.

Oikawa, D., Kimata, Y., Kohno, K., and Iwawaki, T. (2009). Activation of mammalian IRE1alpha upon ER stress depends on dissociation of BiP rather than on direct interaction with unfolded proteins. Exp. Cell Res. *315*, 2496–2504.

Okamura, K., Kimata, Y., Higashio, H., Tsuru, A., and Kohno, K. (2000). Dissociation of Kar2p/BiP from an ER sensory molecule, Ire1p, triggers the unfolded protein response in yeast. Biochem. Biophys. Res. Commun. *279*, 445–450.

Okuda-Shimizu Y., Hendershot, L. M. (2007). Characterization of an ERAD pathway for nonglycosylated BiP substrates, which requires Herp. Mol Cell. 2007; 28:544–554. [

Oliver, J. D., van der Wal, F., J., Bulleid, N. J., High, S. (1997). Interaction of the Thiol-Dependent Reductase ERp57 with Nascent Glycoproteins. Science Vol. 275 no. 5296 pp. 86-88.

Ondek, B., Hardy, R. W., Baker, E.K., Stamnes, M. A., Shieh, B. H., Zuker, C. S. (1992). Genetic dissection of cyclophilin function. Saturation mutagenesis of the Drosophila cyclophilin homolog ninaA. J. Biol. Chem. (23): 16460-6.

O'Tousa, J. E., Baehr, W., Martin, R. L., Hrish, J., Pak, W. L., and Applebury, M. L. (1985). The Drosophila ninaE gene encodes an opsin. Cell 40 (4), 839-850.

Papa, F. R., Zhang, R., Shokat, K., Walter, P. (2003). Bypassing a kinase activity with an ATP-competitive drug. Science. 302: 1533–1537.

Pines, J., Rieder, C. L. (2001). Re-staging mitosis: a contemporary view of mitotic progression. Nat Cell Biol. 3 (1): E3-6.

Puthalakath, H., O'Reilly, L. A., Gunn, P., Lee, L., Kelly, P. N., Huntington, N. D., Hughes, P. D., Michalak, E. M., McKimm-Breschkin, J., Motoyama, N., et al. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 129, 1337-1349

Rabinovich, E., Kerem, A., Frohlich, K. U., Diamant, N., Bar-Nun, S. (2002). AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol. Cell. Biol. 22: 626–34

Ready, D. F., Hanson, T. E., Benzer, S. (1976). Development of the Drosophila retina, a neurocrystalline lattice. Dev. Biol. 53, 217-240.

Richardson, H., O'Keefe, L.V., Marty, T., Saint, R. (1995). Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the Drosophila eye imaginal disc. Development 121(10): 3371—3379.

Riedl, S. J., Salvesen, G. S. (2007). The Apoptosome: signaling platform of cell death. Nature: Molecular Cell Biology, 8, 405-412.

Rivett, A.J., Palmer, A., Knecht, E. (1992). Electron microscopic localization of the multicatalytic proteinase complex in rat liver and in cultured cells. J. Histochem. Cytochem. 40: 1165-72.

Robson, A., and Collinson, I. (2006). The structure of the Sec complex and the problem of protein translocation. EMBO Rep. *7*, 1099–1103.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, Dick, L., Hwang, D., Goldberg, A. L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules.Cell 78:761-71

Rodriguez A., Oliver, H., Zou, H., Chen, P., Wang, X., Abrams, J. M.(1999). Dark is a Drosophila homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. Nature Cell Biol. 1:272–279.

Ron, D., Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nature Reviews Molecular Cell Biology 8, 519-529.

Rusten, E., Lindmo, K., Juhász, G., Sass, M., Seglen, P. O., Brech, A., Stenmark, H. (2004). Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. Developmental Cell, vol. 7 (2) pp. 179-92

Ryoo, H. D., Bergmann, A., Gonen, H., Ciechanover, A. Steller, H. (2002). Regulation of Drosophila IAP1 degradation and apoptosis by reaper and ubcD1. Nat. Cell Biol. 4, 432-438.

Ryoo, H.D., Domingos, P.M., Kang, M.-J., and Steller, H. (2007). Unfolded protein response in a Drosophila model for retinal degeneration. EMBO J. *26*, 242–252.

Sado, M., Yamasaki, Y., Iwanaga, T., Onaka, Y., Ibuki, T., Nishihara, S., Mizuguchi, H., Momota, H., Kishibuchi, R., Hashimoto, T., Wada, D., Kitagawa, H., Watanabe, T.K. (2009). Protective effect against Parkinson's disease-related insults through the activation of XBP1. Brain Res. 1257: 16-24.

Sauer , K., Lehner, C. F. (1995). The role of cyclin E in the regulation of entry into S phase. Prog Cell Cycle Res. 1: 125-39.

Schemer, K., Bey, F. (1994). The prosomes (multicatalytic proteinases; proteasomes) and their relationship to the untranslated messenger ribonucleoproteins, the cytoskeleton, and cell differentiation. Prog. Nucleic Acid Res. Mol. Biol. 49:1-64

Schuck, S., Callhager, C. M., Walter, P. (2014). ER-phagy mediates selective degradation of endoplasmic reticulum independently of the core autophagy machinery. J. Cell Sci. 127 (Pt 18): 4078-88.

Seimon, T. A., Obstfeld, A., Moore, K.J., Golenbock, D. T., Tabas, I. (2006). Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. Proc Natl Acad Sci USA. 103:19794–19799.

Shaffer, A.L., Shapiro-Shelef, M., Iwakoshi, N.N., Lee, A.-H., Qian, S.-B., Zhao, H., Yu, X., Yang, L., Tan, B.K., Rosenwald, A., et al. (2004). XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 21, 81–93.

Shamu, C.E., and Walter, P. (1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. EMBO J. *15*, 3028–3039.

Shoulders, M. D., Ryno, L. M., Genereux, J. C., Moresco, J. J., Tu, P. G., Wu, C., Yates III, J. R., Su, A. I., Kelly, J. W., Wiseman, R. L. (2013). Stress-Independent Activation of XBP1s and/or ATF6 Reveals Three Functionally Diverse ER Proteostasis Environments. Cell Rep (2013) pp. 1279-1292.

Sitia, R., Braakman, I, (2003).Quality control in the endoplasmic reticulum protein factory. Nature 426, 891-894.

Sidrauski, C., Cox, J.S., and Walter, P. (1996). tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. Cell 87, 405–413.

Sidausky, C. and Walter, P. (1997). The Transmembrane Kinase Ire1p Is a Site-Specific Endonuclease That Initiates mRNA Splicing in the Unfolded Protein Response. Cell, Vol. 90, 1031–1039

Skaar, J. R., Pagan, J. K., Pagano, M. (2009). SnapShot: F Box Proteins I. Cell Volume 137, Issue 6, Pages 1160-1160.e1

Smith, E. R., Winter, S., Eissenberg, J. C, Shilatifard, A. (2008). Regulation of the transcriptional activity of poised RNA polymerase II by the elongation factor ELL. PNAS vol. 105; no. 25; 8575-8579.

Smith, M. H., Ploegh, H. M., Weissman, J. M. (2011). Road to Ruin: Targeting Proteins for Degradation in the Endoplasmic Reticulum. *Science*: Vol. 334 no. 6059 pp. 1086-1090

Song, Z. and Steller, H. (1999). Death by design: mechanism and control of apoptosis. Trends in Cell Biology 9: M49–M52.

Souid, S., Lepesant, J.-A., and Yanicostas, C. (2007). The xbp-1 gene is essential for development in Drosophila. Dev. Genes Evol. *217*, 159–167.

Sowa, M. E., Bennett, E. J., Gygi, S. P., Harper, J. W. (2009). Defining the human deubiquitinating enzyme interaction landscape. Cell 138, 389-403.

Sriburi, R., Jachowski, S., Mori, K., and Brewer, J.W. (2004). XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. J. Cell Biol. *167*, 35–41.

Srivastava, M., Scherr, H., Lackey, M., Xu, D., Chen, Z., Lu, J., Bergmann, A. (2007). ARK, the Apaf-1 related killer in Drosophila, requires diverse domains for its apoptotic activity. Cell Death Differ. 14: 92-102.

St. Johnston, D. (2002). The art and design of genetic screens: Drosophila melanogaster. Nature Reviews Genetics 3, 176-188.

Stovers, R. S. and Schwarz, T. L. (1999). A Genetic Method for Generating Drosophila Eyes Composed Exclusively of Mitotic Clones of a Single Genotype. Genetics 152: 1631–1639.

Sun L, Shi L, Li W, Yu W, Liang J, Zhang H, Yang X, Wang Y, Li R, Yao X, Yi X, Shang Y. (2009). JFK, a Kelch domain-containing F-box protein, links

the SCF complex to p53 regulation. Proc Natl Acad Sci U S A. 106 (25): 10195-200.

Swanson, R., Locher, M., Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev. 15 (20): 2660-74.

Tabas, I., Ron, D. (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nature Cell Biology 13, 184–190.

Tabata, T. (2001). Genetics of morphogen gradients. Nature Reviews Genetics 2, 620-630.

Tam, A.B., Koong, A. C., Niwa, M. (2014). Ire1 Has Distinct Catalytic Mechanisms for XBP1/ HAC1 Splicing and RIDD. Cell Reports Vol.9, Issue 3 p850-858.

Tenev, T., Zachariou, A., Wilson, R., Ditzel, M., Meier, P. (2004). IAPs are functionally non-equivalent and regulate effector caspases through distinct mechanisms. Nat. Cell Biol. 7, 70-77.

Thomas, B. J, Gunning, D. A., Cho, J., Zipursky, S. L. (1994). Cell cycle progression in the developing Drosophila eye: Roughex encodes a novel protein required for the establishment of G1. Cell, 77**:** 1003-1014.

Timmins, J. M., Ozcan, L., Seimon, T. A., Li, G., Malagelada, C., Backs, J., Backs, T., Bassel-Duby, R., Olson, E. N., Anderson, M. E., Tabas, I. (2009). Calcium/calmodulin-dependent protein kinase II links endoplasmic reticulum stress with Fas and mitochondrial apoptosis pathways. J Clin Invest. 2009; 119: 2925–2941.

Tirasophon, W., Welihinda, A.A., and Kaufman, R.J. (1998). A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. Genes Dev. 12, 1812–1824.

Tomlinson, A., and Ready, D. F. (1987). Cell fate in the Drosophila Ommatidium. Dev. Biol.123, 264–275**.**

Upton J. P., Austgen, K., Nishino, M., Coakley, K. M., Hagen, A., Han, D., Papa, F. R., Oakes, S. A. (2008). Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. Mol. Cell. Biol. 28, 3943-3951

Upton, J. P., Wang, L., Han, D., Wang, E. S., Huskey, N. E., Lim, L., Truitt, M., McManus, M. T., Ruggero, D., Goga, A., Papa, F. R., Oakes, S. A.

(2012). IRE1α cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. Science 338 (6108): 818-22.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., Ron, D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 287 (5453): 664-6.

Ushioda, R., Hoseki, J., Araki, K., Jansen, G., Thomas, D. Y., Nagata, K. (2008). ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. Science 321, 569–572.

Vashist, S., Ng, DT. (2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. J Cell Biol 165:41–52.

Vattem, K.M., and Wek, R.C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc. Natl. Acad. Sci. U. S. A. 101, 11269–11274

Vermeulen, K., Van Bockstaele, D. R., Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 36 (3): 131-49.

Wajant, H. (2003). The Fas Signaling Pathway: More Than a Paradigm. Science Vol. 296 no. 5573 pp. 1635-1636

Wang, Y., Shen, J., Arenzana, N., Tirasophon, W., Kaufman, R.J., and Prywes, R. (2000). Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. J. Biol. Chem. *275*, 27013–27020.

Webel, R., Menon, I., O'Tousa, J. E., Colley, N. J. (2000). Role of asparagine-linked oligosaccharides in rhodopsin maturation and association with its molecular chaperone, NinaA. J. Biol. Chem. 275, 24752–24759.

Welihinda, A.A., and Kaufman, R.J. (1996). The unfolded protein response pathway in Saccharomyces cerevisiae. Oligomerization and trans-phosphorylation of Ire1p (Ern1p) are required for kinase activation. J. Biol. Chem. *271*, 18181–18187.

Weston, C. R., Davis, R. J. (2007). The JNK signal transduction pathway. Curr. Opin. Cell Biol. 19 (2): 142-9.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K., Steller, H. (1994). Genetic control of programmed cell death in Drosophila. Science 264: 677–683.

Wolff, T., Ready, D. F. (1991a). The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. Development 113 (3): 841-850.

Wolff, T. and Ready, D.F. (1991b) Cell death in normal and rough eye mutants of Drosophila. Development, 113, 825-839.

Wolff, T., and Ready, D.F. (1993). Pattern formation in the Drosophila retina. In The Development of Drosophila Melanogaster, M. Bate, and A.M. Arias, eds. (New York: Cold Spring Harbor Laboratory Press), pp. 1277–1325.

Yamaguchi, H, Wang, H. G. (2004). CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J. Biol. Chem. 279 (44): 45495-502.

Yanagitani, K., Kimata, Y., Kadokura, H., and Kohno, K. (2011). Translational pausing ensures membrane targeting and cytoplasmic splicing of XBP1u mRNA. Science *331*, 586–589.

Ye, Y., Meyer, H.H., Rapoport, T. A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 414, 652-656.

Ye, J., Rawson, R.B., Komuro, R., Chen, X., Davé, U.P., Prywes, R., Brown, M.S., and Goldstein, J.L. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol. Cell *6*, 1355–1364.

Yokokura, T., Dresnek, D., Huseinovic, N., Lisi, S., Abdelwahid, E., Bangs, P. and White, K. (2004). Dissection of DIAP1 functional domains via a mutant replacement strategy. J. Biol. Chem. 279, 52603-52612

Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M., Clem, R. J., Muller, H. A., Hay, B. A. (2002). Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. Nat. Cell Biol. 4, 416-424

Yorimitsu T, Nair U, Yang Z, Klionsky DJ. (2006). Endoplasmic reticulum stress triggers autophagy. J. Biol. Chem. 281 (40): 30299-304.

Yoshida, H., Haze, K., Yanagi, H., Yura, T., and Mori, K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. J. Biol. Chem. *273*, 33741–33749.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2000). ATF6 Activated by Proteolysis Binds in the Presence of NF-Y (CBF) Directly to the cis-Acting Element Responsible for the Mammalian Unfolded Protein Response. Mol. Cell. Biol. *20*, 6755–6767.

Yoshida, H., Matsui, T., Hosokawa, N., Kaufman, R.J., Nagata, K., and Mori, K. (2003). A Time-Dependent Phase Shift in the Mammalian Unfolded Protein Response. Dev. Cell *4*, 265–271.

Yoshida, H., Oku, M., Suzuki, M., and Mori, K. (2006). pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. J. Cell Biol. *172*, 565–575.

Zachos, G., Rainey, M. D., Gillespie, D. A. (2003). Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects. EMBO J. 22, 713-723.

Zhang, Y., Lin, N., Carroll, P. M., Chan, G., Guan, B., Xiao, H., Yao, B., Wu, S. S., Zhou, L. (2006). Epigenetic blocking of an enhancer region controls irradiation-induced proapoptotic gene expression. Dev. Cell 2008;14:481–493.

Zhang, Y. W., Brognard, J., Coughlin, C., You, Z., Dolled-Filhart, M., Aslanian, A., Manning, G., Abraham, R. T., Hunter, T. (2009). The F box protein Fbx6 regulates Chk1 stability and cellular sensitivity to replication stress. Mol. Cell 35 (4): 442-53.

Zhou L, Song Z, Tittel J, Steller H. (1999). HAC-1, a Drosophila homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. Mol. Cell 4 (5): 745-755.

Zuker, C., Cowman, A., Rubin, G. M. (1985). Isolation and structure of a rhodopsin gene from D. melanogaster. Cell 40, 851-858.

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I X - **List of figures**

X – **Abbreviations**

aa: amino acid

AAA-ATPase: ATPase associated with diverse cellular activities

ADRP: Autosomal Dominant Retinitis Pigmentosa

AMPK: adenosine monophosphate-activated protein kinase

Apaf-1: apoptotic protease activating factor 1

Ark: Apaf-1-related killer

ASK1: apoptosis signaling-regulating kinase 1

ASK1: apoptosis signal-regulating kinase 1

ATF: Activating Transcription Factor

ATF6f: ATF6fragment

ATG: autophagy-related

Ato: Atonal

ATP: Adenosintriphosphate

ATR: ataxia telangiectasia and Rad3-related *protein*

BAC: bacterial artificial chromosome

BAK: Bcl-2 homologous antagonist killer

BAX: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

Bip: Binding immunoglobulin protein

Bp: base pairs

bZIP: basic Leucine zipper

CAK: CDK activating kinase

CARD: caspase activation and recruitment domain

CaMK: Calcium monitoring kinase

Cdc48: cell division protein 48

Cdk: cyclin dependent kinase

CDKI: Cdk inhibitor

cDNA: complementary DNA

CHK1: Checkpoint kinase 1

Chop1: CCAAT enhancer-binding homologous protein 1

CHOP: C/EBP homologous protein

CNX: Calnexin

COP: Coat Protein

CPV: CD8-parp-Venus

CreP: constitutive repressor of eIF2α phosphorylation

CRT: Calreticulin

CTD: carboxil terminal domain

C-terminal: Carboxy-terminal

Cy: Curly

Cyc: cyclin

CyO: Curly of Oister

Dcp-1: mRNA-decapping enzyme 1

Der: Derlin

DIAP: *Drosophila* IAP

DNA: Desoxyribonucleine acid

DR5: death receptor-5

Dredd: Death related ced-3/Nedd2-like protein

DrICE: *Drosophila* ICE

Dronc: Drosophila melanogaster NEDD2-like caspase

DSHB: Developmental Studies Hybridoma Bank

DTT: Dithiothreitol

E: enhancer

Eaf: Ell-associated factor

EDEM: ER degradation-enhancing α-mannosidase– like *protein*

EGF: Epidermal Growth Factor

EGUF: eyGAL4/UAS-flp

Elav: embryonic lethal abnormal vision

eIF2α : eukaryotic translation initiation factor 2 α

Ell: elongation factor eleven-nineteen lysine-rich in leukemia transcription factor

EMS: Ethyl methanesulfonate

ER: Endoplasmic Reticulum

ERAD: ER Associated Degradation

ERGIC: ER–Golgi intermediate compartment

ERO1: ER oxireductin 1

ERSE: ER stress response element

Flp: Flipase

FP: forward primer

FRT: flipase recognition target

G: gap

g: gram

GADD34: growth arrest and DNA-damage-inducible protein-34)

Gcn: general control non-derepressible

GFP: green fluorescent protein

Glc: Glucose

GMR: Glass Multimer Reporter

GPI: Glycosylphosphatidylinositol

GTP: Guanosinetriphosphate

GWS: Gateway System

HA: hemagglutinin

Hac1: homologous to ATF/CREB1

Hh: hedgehog

Hid: head involution defective

HR: hydrophobic region

HRD: HMG-CoA reductase degradation

HRI: haem-regulated inhibitor kinase

IAP: inhibitor of apoptosis protein

IP3R1: inositol 1,4,5-triphosphate receptor, type 1

IRE1: Inositol-requiring Enzyme 1

ISR: Integrated Stress Response

JNK: c-Jun N-terminale Kinase

L: liter

LC3: Microtubule-associated protein light chain 3

M: mitosis

M: Mol

MAP kinase: mitogen activating protein kinase

Mekk1: MAP/ERK kinase kinase 1

ml: milli liter

MF: morphogenetic furrow

mRNA: messenger RNA

NER: nucleotide excision repair

NFkB: nuclear factor κB

Nina: neither inactivation nor afterpotential

OS-9: osteosarcoma amplified 9

ORF: open reading frame

PBS: phosphate buffered saline

PBT: phosphate buffered saline with triton

PCR: Polymerase chain reaction

PERK: Protein Kinase RNA-like Endoplasmic Reticulum Kinase

Pdi1p: Protein disulfide isomerase 1p

PH3: phosphohistone-3

PKR: Protein kinase R

Pol: Polymerase

PP1: protein phosphatase 1

R: photoreceptor

Rb: Retinoblastoma protein

RFP: red fluorescent protein

Rh: rhodopsin

RIDD: Regulated Ire1 Dependent Decay

RING: Really interesting gene

RNAi: RNA-Interference

RNC: mRNA- ribosome-nascent chain

RP: Retinitis pigmentosa

RP: reverse primer

RT: room temperature

RT-PCR: Reverse Transcriptase PCR

S: synthesis

S1P: site 1 protease

S2P: site 2 protease

SCF: Skip-Cullin-Fbox

Sco: Scutoid

Ser: Serine

SMW: second mitotic wave

Spi: Spiz

SRP: Signal recognition Particle

Su: suppressor

TF: transcription factor

Thr: Threonine

TM6b: Third Multiple 6b

TNF: tumour necrosis factor

TPR: tetratricopeptide repeats

TRAF2: tumor necrosis associated factor 2

TRB3: Tribbles related protein 3

TRC8: Tricornered 8

Trl1: tRNA ligase 1

tRNA: transfer RNA

TUNEL: TdT-mediated dUTP-biotin nick end labeling

UAS: upstream activating sequence

UDP: Uracildiphosphate

uORF: upstream open reading frame

UPR: Unfolded Protein Response

USP13: ubiquitin specific peptidase 13

Vcp: Valosin containing protein

Vps35: Vacuolar protein sorting 35

Xbp1s: Xbox binding protein 1

Xbp1s: Xbp1spliced

Xbp1u: Xbp1unspliced

Xpd: Xeroderma pigmentosum group D