



# Mild Endoplasmic Reticulum Stress Protects From Cell Death: The Role Of Autophagy

Clémence Levet

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**Clémence LEVET**

# Mild Endoplasmic Reticulum Stress Protects From Cell Death : The Role Of Autophagy

Directeur de thèse : Pr Bertrand MOLLEREAU

JURY : Dr Magali SUZANNE, Rapporteur  
Dr Hervé TRICOIRE, Rapporteur  
Dr Serge BIRMAN, Examineur  
Dr Matthias FAURE, Examineur  
Dr Sébastien GAUMER, Examineur

This thesis has been performed in the “apoptosis and neurogenetic” group headed by the Pr Bertrand Mollereau in the Laboratory of Molecular Biology of the Cell at the Ecole Normale Supérieure de Lyon.

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# Abstract

The last years have been very successful in identifying mechanisms, which control apoptosis in metazoan. However, the regulation of cell death in specific cell type remains to be determined. An excess of neuron apoptosis can lead to neurodegenerative diseases such as Huntington, Parkinson or Alzheimer diseases. Neurodegeneration is usually associated to Endoplasmic Reticulum stress (ER stress), autophagy or oxidative stress. However, the role of these mechanisms in the regulation of neurodegeneration is not clearly established. To test the role of ER stress in the regulation of neuronal death, we used several models of neurodegeneration in *Drosophila* and mammals. First, we have shown that the genetic induction of ER stress protected photoreceptors of the *Drosophila* eye from apoptosis. Then, we have shown that the protective effect of ER stress is conserved in both *Drosophila* and mouse models of Parkinson disease. In order to characterize the protective effect of ER stress, we have studied the activation of protective mechanisms upon ER stress. We have shown that in the *Drosophila* retina, ER stress can induce an anti-oxidative response and autophagy. Interestingly, autophagy is only activated in presence of both ER stress and cell death signal. We have focused on the role of autophagy in the protective effect of ER stress. We have shown that the activation of autophagy was required for the protective effect of ER stress. Thus, we have shown that ER stress response is not only involved in the reduction of misfolded protein accumulation, but can also protect neurons from cell death by activating autophagy.

Keyword: Apoptosis, endoplasmic reticulum stress, autophagy, neurodegeneration, Parkinson disease, *Drosophila*, mouse



# Titre et résumé

## Un stress modéré du réticulum endoplasmique protège de la mort cellulaire : rôle de l'autophagie

Ces dernières années ont été très fructueuses pour l'identification des mécanismes fondamentaux de l'apoptose chez les métazoaires. Cependant, il reste beaucoup à apprendre sur la manière dont sont régulés les programmes de mort en fonction du type cellulaire. Un excès d'apoptose des neurones peut conduire à différentes pathologies neurodégénératives comme les maladies de Huntington, Parkinson ou Alzheimer. La neurodégénérescence est souvent associée à un stress du réticulum endoplasmique (RE), à l'autophagie et à un stress oxydatif. Cependant le rôle de ces mécanismes dans la régulation de la neurodégénérescence n'a pas été clairement établi. Afin de tester l'implication du stress du RE dans la régulation de la mort neuronale, nous avons utilisé différents modèles de neurodégénérescence chez la *Drosophila melanogaster* et les mammifères. Nous avons d'abord montré que l'induction d'un stress modéré du réticulum protégeait les photorécepteurs de l'œil de drosophile contre l'apoptose. Nous avons ensuite montré que l'effet protecteur du stress du RE était conservé dans les modèles de maladie de Parkinson chez la drosophile et la souris. Afin de caractériser l'effet protecteur du stress du réticulum, nous avons étudié l'activation de mécanismes protecteurs lors d'un stress du réticulum. Nous avons montré que dans la rétine de drosophile l'activation du stress du RE dans un contexte de mort neuronale induisait une réponse anti-oxydante et l'autophagie. Nous nous sommes plus particulièrement intéressés au rôle de l'autophagie dans le rôle protecteur du stress du RE. Nous avons montré que l'activation de l'autophagie était nécessaire à l'effet protecteur du stress du RE contre l'apoptose. Nous avons donc pu mettre en évidence que la réponse au stress du RE ne participait pas uniquement à réduire l'accumulation de protéines mal-conformées mais permettait aussi de protéger les neurones contre la mort cellulaire en activant l'autophagie.

Mots clés : Apoptose, stress du réticulum endoplasmique, autophagie, neurodégénérescence, maladie de Parkinson, drosophile, souris



# Résumé substantiel

Ces dernières années ont été très fructueuses pour l'identification des mécanismes fondamentaux de l'apoptose chez les métazoaires [19, 20]. Cependant, il reste beaucoup à apprendre sur la manière dont sont régulés les programmes de mort en fonction du type cellulaire. Un excès d'apoptose peut conduire à différentes pathologies neurodégénératives comme les maladies de Huntington, Parkinson ou Alzheimer. Le stress du réticulum endoplasmique (RE), le stress oxydatif et l'autophagie sont associés à la neurodégénérescence [15, 21-24]. Cependant, leur importance dans la progression de ces maladies n'a pas encore été déterminée.

Le RE est un organelle par lequel transitent les protéines sécrétées et les protéines transmembranaires. Ces protéines acquièrent leur bonne conformation grâce aux protéines chaperonnes résidentes et vont s'associer en complexes. Seules les protéines correctement repliées et associées pourront sortir du RE et transiter par les voies de sécrétion. Ce mécanisme de contrôle permet de s'assurer de la qualité des protéines sécrétées ou associées à la membrane. La présence de protéines anormalement conformées induit une voie de signalisation appelée la réponse UPR (pour « Unfolded Protein Response ») [28]. La réponse UPR est une réponse adaptative au stress du RE qui est conservée au cours de l'évolution. En effet, la plupart des effecteurs de la réponse UPR identifiés chez les vertébrés ont un homologue chez la drosophile [35]. La réponse UPR chez les vertébrés est activée par un senseur du stress du réticulum : BiP. En absence de stress du RE, BiP est associé à des récepteurs transmembranaires du RE: PERK (pour « PKR-like ER kinase »), ATF6 (pour « activating transcription factor 6 ») et IRE1 (pour « inositol-requiring enzyme 1 »). Lors d'un stress du RE, BiP se dissocie de ces partenaires, il se fixe sur les protéines mal-conformées afin de jouer son rôle de chaperonne. Les voies PERK, ATF6 et IRE1 sont alors activées. Elles vont induire l'expression de chaperonnes, activer les voies de dégradation des protéines et réduire la synthèse protéique. Des études suggèrent qu'une réponse UPR intense et prolongée induit l'activation du programme d'apoptose [37]. Cependant, il n'est pas établi si la réponse UPR est la cause de la mort neuronale dans les maladies neurodégénératives. La conservation des mécanismes de mort cellulaire au cours de l'évolution nous permet d'utiliser des modèles génétiques simples, comme la drosophile, pour mieux comprendre la régulation de la mort cellulaire chez l'homme.

L'œil de *Drosophila melanogaster* est un excellent modèle pour l'étude *in vivo* des maladies neurodégénératives. En effet, l'expression ectopique de protéines pathologiques dans la rétine induit la dégénérescence progressive des cellules photoréceptrices (PR) avec des caractéristiques similaires à celles qui sont observées dans les maladies humaines [41]. Nous avons établi un modèle de neurodégénérescence chez la drosophile dépendant de la surexpression des gènes pro-apoptotiques *reaper* et *Dp53* (homologue drosophile du gène *p53* de mammifère). La surexpression de *reaper* induit l'apoptose des cellules photoréceptrices de l'œil. Un crible génétique nous a permis d'identifier les mutations du gène *ninaA* (homologue de la cyclophiline A humaine) comme suppresseurs de la mort cellulaire. *NinaA* code une chaperonne nécessaire à la maturation de la Rhodopsine-1 dans le réticulum. L'absence de *NinaA* provoque l'accumulation de protéines Rh1 mal conformées induisant un stress du RE et une réponse UPR dans les cellules photoréceptrices de l'œil de drosophile. Nous avons observé que la réponse UPR, qui est normalement dédiée à éviter l'accumulation des protéines mal conformées, génère des signaux anti-apoptotiques.

Nous avons montré que le stress du RE protège les neurones contre la mort cellulaire dans des modèles de maladie de Parkinson. Nous avons utilisé la surexpression de  $\alpha$ -synucléine dans les neurones dopaminergiques ainsi qu'un traitement au paraquat comme modèles de la maladie de Parkinson *in vivo* chez la drosophile. Nous avons montré que l'induction chimique du stress du RE avec de la tunicamycine protégeait les neurones contre la mort cellulaire et améliorait la motricité des drosophiles. De plus, l'induction d'un stress du RE par la tunicamycine protège les neurones dopaminergiques de souris *in vivo* contre la mort cellulaire induite par la 6-hydroxydopamine (6-OHDA). L'injection de 6-OHDA dans le cerveau des souris induit un stress oxydatif, la mort des neurones dopaminergiques ainsi que des défauts locomoteurs. Le stress du RE, induit par un prétraitement à la tunicamycine, active donc des mécanismes qui protègent les neurones contre la mort cellulaire. En particulier la réponse UPR active des mécanismes protecteurs tels que la réponse anti-oxydante et l'autophagie qui pourraient participer à l'inhibition de l'apoptose des neurones.

Les cellules exposées à un stress du RE induisent une réponse anti-oxydante qui pourrait les protéger contre la mort cellulaire. La réponse anti-oxydante est activée pour protéger les cellules contre l'apoptose induite par la présence de formes réactives de l'oxygène. La réponse anti-oxydante fait appel à différentes protéines anti-oxydantes comme la Superoxyde dismutase, la Catalase, la Glutathion S transférase ou la Ferritine [45, 46]. Nous avons montré que la réponse anti-oxydante et en particulier la Ferritine étaient activées lors d'un stress du

réticulum. Ces protéines permettent de réduire le stress oxydatif mais leur contribution dans la protection contre la mort cellulaire lors d'un stress du RE n'est pas démontrée.

Les cellules exposées à un stress du RE induisent l'autophagie qui pourrait contribuer à les protéger de la mort cellulaire. L'autophagie permet aux cellules de survivre dans des conditions de carence alimentaire, de stress ou en présence d'organelles défectueuses. Le cytoplasme et les organelles sont emprisonnés dans des vacuoles où ils seront dégradés. Il a été montré que l'autophagie pouvait être activée lors d'un stress du RE pour limiter l'expansion du RE et ainsi permettre la survie des cellules exposées à un fort stress du RE [47, 48]. Nous avons montré que l'autophagie était activée lors d'un stress du réticulum associé à un signal de mort cellulaire dans les photorécepteurs de l'œil de drosophile ainsi que dans les neurones dopaminergiques de souris. De plus, nous avons montré que l'activation de l'autophagie est requise pour l'effet protecteur du stress du RE contre l'apoptose dans la rétine de drosophile. Nous avons donc montré que l'activation de l'autophagie par un stress du RE combiné à un signal de mort cellulaire protégeait les cellules contre l'apoptose. L'implication de l'autophagie dans l'effet protecteur du stress du réticulum endoplasmique n'exclue pas un rôle potentiel de la réponse anti-oxydante qui reste cependant à être démontrée. Le stress modéré du RE ainsi que l'autophagie offrent donc des nouvelles perspectives thérapeutiques contre les maladies neurodégénératives.





# List of Publications

- Mendes C, **Levet C**, Chatelain G, Dourlen P, Fouillet A, Dichtel-Danjoy ML, Gambis A, Ryoo HD, Steller H and Mollereau B. ER stress protects from neurodegeneration in *Drosophila* EMBO Journal (2009), 28(9):1296-307.
- **Levet C\***, Fouillet A\*, Virgone A, Robin M, Dourlen P, Touret M, Rieusset J, Belaidi E, Ovize M, Nataf S, Mollereau B. ER stress inhibits neuronal death by promoting autophagy. Autophagy (2012), 1;8(6):915-26
- Dichtel-Danjoy ML, Ma D, Dourlen P, Chatelain G, Napoletano F, Robin M, Corbet M, **Levet C**, Hafsi H, Hainaut P, Ryoo HD, Bourdon JC, Mollereau B. *Drosophila* p53 isoforms differentially regulate apoptosis and apoptosis-induced proliferation. Cell Death and Differentiation (2012)



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# Abbreviations

4E-BP	eIF4E binding protein
6-OHDA	6-hydroxydopamine
AIF	Apoptosis-inducing factor
ALS	Amyotrophic Lateral Sclerosis
AMD	Age-related macular degeneration
Apaf1	Apoptotic protease activating factor 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
Atg gene	AuTophagy gene
Bcl2	B-cell lymphoma 2
BH	Bcl-2 homology domains
BI-1	Bax inhibitor 1
BIR domain	Baculovirus IAP repeats domain
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CARD	Caspase-recruitment domain
ced gene	<i>Caenorhabditis elegans</i> Death Genes
Chk2	Checkpoint kinase 2
CHOP	C/EBP homologous transcription factor
PI3K	Phosphoinositide 3-kinase
CNS	Central Nervous System
CNX	Calnexin
CRT	Calreticulin
Cvt	Cytoplasm-to-vacuole targeting
d4E-BP	<i>Drosophila</i> 4E-BP
DAMM	Death associated molecule related to Mch2
DCP-1	Death caspase-1
DD	Death domain
Debcl	Death executioner Bcl-2 homologue
DECAY	Death executioner caspase related to Apopain/Yama
DED	Death effector domain
dFADD	<i>Drosophila</i> Fas-associated death domain
DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DIAP	<i>Drosophila</i> inhibitor of apoptosis
DiI	1,1'-dihexadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DR	Death receptor
DREDD	Death related ced-3/Nedd2-like protein
DRICE	<i>Drosophila</i> Interleukin-1 $\beta$ -converting enzyme
<i>Drosophila</i>	<i>Drosophila melanogaster</i>
dTOR	<i>Drosophila</i> Target of rapamycin
dTRAF	<i>Drosophila</i> TNF-receptor-associated factor
DTT	Dithiothreitol
EM	Electron microscopy
ER	Endoplasmic reticulum
ERAD	ER associated degradation
Ero1	Oxidoreductin 1



ERQC	ER quality control
FADD	Fas-associated death domain
FIP200	FAK family interacting protein of 200 kD
GFP	Green Fluorescent protein
GH3 domain	Grim Homology 3 domain
GRP	Glucose-regulated protein
hid	Head involution defective
Hsc3	Heat shock protein cognate 3
HSPs	Heat shock proteins
HTRA2	High-temperature-requirement protein A2
Htt	Huntingtin
IAPs	Inhibitor of Apoptosis Proteins
IBM	IAP binding motif
ICE	Interleukin-1 $\beta$ -converting enzyme
IRE1	Inositol-requiring enzyme 1
ITA	Inhibitor of T cell apoptosis
JNK	c-Jun N-terminal kinases
Lamp	Lysosome associated membrane protein
LC3	Microtubule-associated protein 1 light chain 3
MAMs	Mitochondria-associated membranes
Mdm2	Mouse double minute 2
MOM	Mitochondria outer membrane
MOMP	Mitochondria outer membrane permeabilization
NF-kB	Nuclear factor-kappa B
NinaA	Neither inactivation nor afterpotential A
Nrf2	Nuclear Factor Erythroid 2p45-Related Factor-2
PCD	Programmed cell death
PDI	Protein disulfide isomerase
PE	Phosphatidylethanolamine
PERK	PKR-like ER kinase
PRCs	Photoreceptor cells
ref(2)P	Refractory to sigma P
RFP	Red fluorescent protein
Rh1	Rhodopsin 1
RHG	Reaper, Hid, Grim
RIDD	Regulated IRE1-dependant decay
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RP	Retinitis pigmentosa
Rpr	Reaper
SERCA	Sarco/Endoplasmic Reticulum Calcium ATPase
SMAC	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
TOR	Target of rapamycin
TORC	TOR complex
TRAF	Tumor necrosis factor-receptor-associated factor
UPR	Unfolded protein response
XBP1	Factor X box-binding protein 1
$\alpha$ -Syn	$\alpha$ -Synuclein

# **1. Introduction**



# 1.1. Programmed cell death

## 1.1.1. Cell death : morphological and biochemical characterization

Development and homeostasis of multicellular organisms depends on the proper balance between proliferation, differentiation and cell death. The metazoan development is characterized by a fast increase of cell number. Some cells differentiate and others are eliminated via cell death to lead to functional adult tissues [4, 49]. Dying cells have been observed during embryogenesis and metamorphosis and caught the attention of the biologists during the 19<sup>th</sup> and 20<sup>th</sup> century. By the mid-20th century, it was clear that cell death was a naturally occurring process necessary for development [50]. Developmental cell death is essential for the development of most of the pluricellular organism such as insects and vertebrates [51]. Cell death has also been observed during *C. elegans* development, however this cell death is not essential for development. This observation considerably helped the identification of cell death defective mutant and genes required for death during development [52, 53]. The specific cell death during *C. elegans* development suggests that the cell death process is tightly controlled at the level of the organism [54]. The term “programmed cell death” (PCD) defined cell death not as random or accidental in nature, but as a sequence of controlled steps leading to locally and temporally defined self destruction [55]. The term PCD was introduced in 1964 during the characterization of inter-segmental muscle degeneration of the silkworm [56]. This term is now used to define temporally and locally regulated cell elimination during development or tissue homeostasis.

Different programmed cell death types occur to kill cell during development and tissue homeostasis. Three types of programmed cell death have been identified: apoptosis, autophagic cell death and necrotic cell death [57]. These three PCD are not equivalent regarding the control of cell death during development and tissue homeostasis. Apoptosis is the main PCD. It is involved both during development and the life of an organism. During development, cell death allows the elimination of transitory organs and tissues. For example the tail of the amphibian and the larval tissue of *Drosophila* are removed by apoptosis [19, 58]. Cell death is also involved in tissue remodeling and organ shaping such as the digit formation during mammals development or the rotation of genitalia in *Drosophila*

development [59, 60]. Almost all developmental programmed cell death requires apoptosis. Apoptosis is characterized by apoptotic corpses formation and the activation of specific effectors, the caspases. This mechanism was observed in almost all dying tissues during development and thus, it has been thus proposed that apoptosis was the only developmental PCD. However, rare exceptions have shown that developmental cell death PCD could be caspase independent [4, 61]. Two *Drosophila* tissues, the salivary gland and the mid-gut, are eliminated via autophagy, a caspase independent PCD [62, 63]. Autophagic cell death has also been observed in *Dictyostelium* [64]. However, the role of autophagic cell death during development was only observed so far in these two tissues in *Drosophila* and in *Dictyostelium*. Apoptosis is thus the major developmental cell death. In addition to development, apoptosis is also involved in tissue homeostasis. The PCD which occurs during tissue homeostasis is not only dependent of apoptosis it can also involve autophagic cell death and necrotic cell death [65]. Indeed, the inhibition of caspase in Apaf1 mutant mice does not suppress interdigital cell death. The interdigital death in absence of apoptosis involves necrosis [66]. In addition, necrosis also participates to interdigital cell death in mouse, T-cell elimination especially upon *Toxoplasma gondii* infection or in mouse neurons upon ischemia [67, 68]. To conclude, apoptosis can be considered as a developmental programmed cell death whereas necrosis and autophagy, few exceptions excluded, are restricted to tissue homeostasis control.

### **1.1.2. Apoptosis**

#### **1.1.2.1. Morphological and biochemical characterization**

Apoptosis is a cell death mechanism. The term “Apoptosis” has been first used by Hippocrate and Galen in the first century BC as a medical term to describe “falling off of the bones” upon bone fracture and also “dropping of the scabs” [69, 70]. Then, Kerr, Wylie and Currie used the term apoptosis, meaning “falling off” as leaves from trees, to name programmed cell death [71]. Apoptosis describes the stereotypical and morphological features culminating in the controlled self-destruction of a cell [71-73]. Apoptosis was defined as a basic biological process, which allow the turnover of cells in a normal tissue and also the removal of cell in diseased tissues [74]. Upon apoptosis, dying cells present several features, which have been characterized using electron microscopy. Apoptotic cells arbor chromatin condensation and

DNA fragmentation [75, 76]. The nuclear changes of apoptotic cell are associated to cytoplasmic changes [58]. As the global cell size diminishes, the organelles concentrate in the cytoplasm, however, they are still intact. Moreover, the cytoskeleton of the cell is disorganized leading to plasma membrane blebbing [77]. During all this process, the apoptotic cells lose adhesion with neighboring cells [73]. Finally, cells break up into apoptotic bodies, which are eliminated by surrounding cells via phagocytosis [59, 78]. This phagocytosis is induced by changes in the plasma membrane of apoptotic cells, which are identified as a “eat me” signal by surrounding healthy cells. Upon all the steps of the apoptotic process, the plasma membrane is conserved and there is no leakage of intracellular content [75]. Because apoptotic cells are eliminated by phagocytosis before membrane rupture, apoptosis does not activate the immune response.

Apoptosis is the main programmed cell death which occurs during development and homeostasis of multicellular organism. During development, apoptosis allow the elimination of transitory organs and tissues. For example the tail of the amphibian is removed by apoptosis [19, 58]. In addition, apoptosis is required during development and all life of an organism for cellular homeostasis and the elimination of unwanted or damaged cells [79, 80]. Indeed,  $10^7$  cells are eliminated daily among the  $10^{14}$  cells, which composed the human body. For example, apoptosis allows the elimination of the unwanted B and T lymphocytes. Indeed, newly synthesized lymphocytes carry T-cell receptors which can virtually recognize all kind of proteins. In the thymus, T lymphocytes that recognize auto-antigens are eliminated via apoptosis [81-83]. This clonal selection occurs in the thymus for the T lymphocytes while it takes place in germinal centers of lymphoid organs for B lymphocytes [83, 84]. Therefore apoptosis is involved in the maturation of the immune system [85]. Apoptosis is also required in other mammalian tissues such as the turnover of epithelial cell in mouse [86]. Moreover, specific cell elimination is involved during development in both vertebrates and invertebrates. For example, *Drosophila* leg morphogenesis requires apoptosis of cell localized at the presumptive joint. Indeed, in this model, caspases inhibition prevent apoptosis in the leg imaginal disc leading to altered leg joints in the fly [80]. Another example of specific cell apoptosis is the elimination of neurons during development to form the mature nervous system [87-89]. Thus, apoptosis is essential for organism development and also the maintenance of tissue function. Any impairment in the control of apoptosis can be deleterious. Thus apoptosis needs to be tightly controlled.

### 1.1.2.2. Apoptosis: a programmed cell death

Apoptosis is a genetically controlled cell death process. The discovery of the *ced* genes in *C. elegans* has been an important step in the identification and characterization of genes regulating apoptosis. As the cell lineage of *C. elegans* has been identified, it is an ideal model to follow cells during development and to study developmental apoptosis in a living organism [88, 90]. Genetic screens have led to the identification of apoptosis regulators in *C. elegans* named *cell death-defective* (*ced*) genes. Ced-3 and Ced-4 have been identified as the main executioners of cell death. Indeed *ced-3* mutant has up to 20% more neural cells than wild-type animals [91]. Moreover, *ced-4* mutant also presents an excess of cell survival. As the combination of *ced-3* and *ced-4* mutant did not have enhanced effect compared to the single mutant, this suggests that these two proteins act sequentially to induce apoptosis [91]. This hypothesis has been supported by the overexpression of *ced-4* which induced *ced-3* activation [92]. The apoptotic pathway has been completed in *C. elegans* with the identification of other cell death inducer such as Egl-1 (Egg laying defective-1). *Egl-1* mutant arbors excessive cell number due to remaining cells, which were programmed to die [93]. Egl-1 is thus a pro-apoptotic factor. The pro-apoptotic effect of Egl-1 is linked to Ced-3 and Ced-4 as the overexpression of *egl-1* induced a Ced-3/Ced-4 dependent cell death [93]. These results show for the first time the existence of a molecular cascade, which induces apoptosis. In addition to pro-apoptotic genes, the *C. elegans* screens also led to the identification of anti-apoptotic gene. Indeed, the loss of function of *ced-9* increased cell death during *C. elegans* development. Respectively, *ced-9* overexpression led to supernumerary cells in the adult *C. elegans* [94]. Thus, *ced-9* has been identified as pro-survival factor. In addition to *ced-3*, *ced-4* and *ced-9*, other *ced* genes regulate apoptotic process. *Ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-8*, *ced-10* and *ced-12* are involved in phagocytosis of apoptotic cell corpses [95]. These proteins are required for the recognition and the degradation of apoptotic corpses by engulfing cell. Thus, the genes involved in the regulation of apoptosis in *C. elegans* share homology with genes in *Drosophila* and mammals. *Ced-3* and *ced-4* are respectively the homologs of the mammalian *caspase-9* and *apaf-1*. *Egl-1* is the homolog of BH3-only protein, a pro-apoptotic member of the Bcl2 family. In addition Ced-9 is the homolog of anti-apoptotic members of the Bcl-2 family such as Bcl-2 or Bcl-XL. The *ced* genes involved in apoptotic cell corpse engulfment are conserved in *Drosophila* and mammals and these *ced* homologs are also involved in phagocytosis [96-98]. These homologies suggest that mechanism, which regulate cell death in metazoans, are conserved in evolution (**Table 1**).

Gene Family	Worm	Fly	Mouse
Caspases	ced-3, csp-1, csp-2	dredd, dronc, strica, dcp-1, drice, decay, damm	caspases 1-14
Bcl-2	ced-9, egl-1	debcl-1, buffy	bcl-2, bcl-xl, bcl-w, mcl-1, bax, bak, bok, bik, bid, bad, bim
APAF-1	ced-4	dark/dapaf-1/hac-1	apaf1
IAP	bir-1, bir-2	diap-1, diap-2, dbruce, deterin	xiap, c-iap1, c-ap2, hILP-2, mI-iap, niap, survivin, bruce
RHG domain	?	rpr, hid, grim, sickle	smac/diablo, omi/htrA2
Genes involved in the engulfment process	ced-1, -2, -5, -6, -7, -10, -12	draper, MBC	srec, crk-II, dock180, gulp, ABC transporter, rac1, elmo1

**Table 1:** Apoptotic genes conservation upon evolution (adapted from [4])

### 1.1.3. Caspases : the actors of apoptosis

#### 1.1.3.1. The identification of caspases

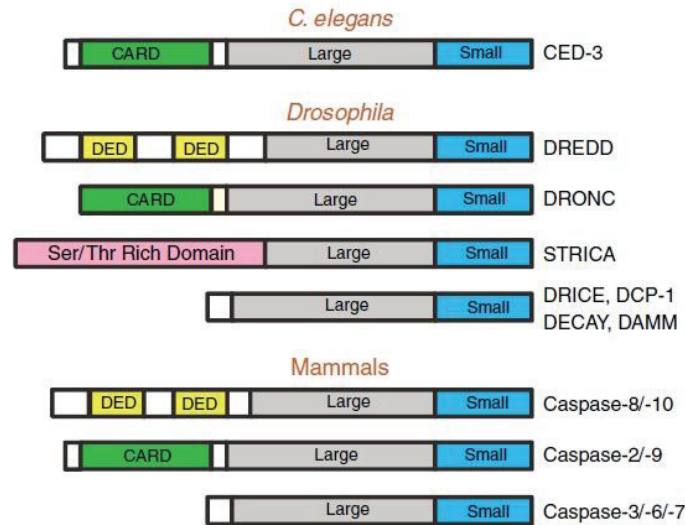
Caspases are proteases conserved throughout evolution, which are involved in apoptosis induction and execution. The first caspase, interleukin-1 $\beta$ -converting enzyme (ICE also known as Caspase-1), has been identified in humans [99, 100]. However, ICE has not been identified for its role in apoptosis but in the immune response, as it is required for the processing of the inactive interleukin-1- $\beta$  into the pro-inflammatory cytokine [101, 102]. The role of ICE in apoptosis has been first suggested with the identification of its homolog in *C. elegans*, the pro-apoptotic gene *ced-3* [103]. A domain essential for ICE activity presents 43% of homology with *ced-3* [104]. The high homology between ICE and *ced-3* suggests that ICE could be a pro-apoptotic factor. This hypothesis has been confirmed by the overexpression of the rat ICE in cells, which activates cell death [104]. The discovery of Ced-3/ICE established the role of caspases as cell death effectors in the apoptotic pathway [104, 105]. Several members of the caspase family have been identified in metazoan. *C. elegans*



genome contains four caspases: *ced-3*, *csp-1* (*caspase homolog-1*), *csp-2* and *csp-3* [106]. In *Drosophila*, seven caspases have been identified : Dredd, Dronc, Strica, Drice, Dcp-1, Decay, Damm [6]. Fourteen caspases have been characterized in mammals : Caspase-1 to Caspase-10 and Caspase-14 found in human and Caspase-11 to Caspase-13 found in other mammals [107]. Thus the caspases appear as a protein family conserved in evolution. The majority of the caspases are involved in apoptosis execution. However, some Caspases have non-apoptotic function [108]. Indeed, caspases can be implicated in immune response and neuron differentiation and development in mammals [108]. Caspases are also implicated in sperm differentiation and neuroblast formation in *Drosophila* [109, 110]. Moreover, DRONC, the *Drosophila* homolog of Caspase-8 is involved in apoptosis induced proliferation [111].

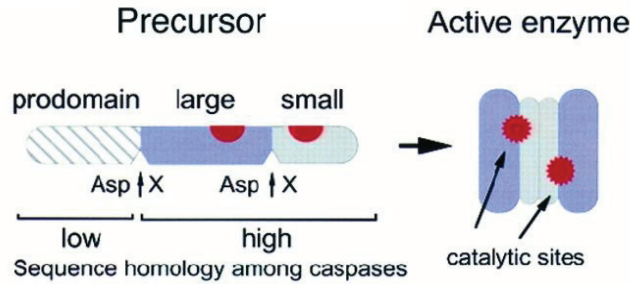
#### 1.1.3.2. Structure and function of caspases

Caspases are present in the cell as a catalytic inactive zymogen, which is activated by cleavage. Caspase zymogens contain three domains: a large subunit (~20 kDa), and a small subunit (~10 kDa), which are highly conserved throughout evolution and also a variable N-terminal domain named the prodomain (**Figure 1**). The variable N-terminal domain defines two types of caspases. The initiator caspases contain a long N-terminal domain, whereas the effector caspases contains a short N-terminal domain. The N-terminal domain of initiator caspases can be a 'caspase-recruitment domain' (CARD), 'death effector domain' (DED) or 'pyrin domain' (PYD). Even if caspases have different N-terminal domain, they all carry a protease activity. Most, caspases are cysteine proteases that cleave their substrates after an aspartate residue. For example, ICE cleaves inactive interleukin-1- $\beta$  and requires an aspartate residue at the cleavage site [99, 101]. This specific cleavage lead to the designation of these proteins as caspases : CysteinyI ASpartate-specific proteASE [112]. However, this protease activity is not present in the zymogen. To be activated, effector caspases have to be cleaved. The caspase activation requires specific cleavage at select aspartate residues. Activation involves proteolytic processing of the pro-enzyme between the large and the small subunit. After their cleavage, large and small subunits associate to form a heterodimer [102]. The heterodimers associate to form a heterotetramer. The heterotetramer has two protease catalytic sites, which can cleave protease targets (**Figure 2**).



**Figure 1:** The initiator and effector caspases in *C. elegans*, *Drosophila*, and mammals. Only caspases with potential or demonstrated role in apoptosis are shown. The small and large subunits, and the protein and protein interaction motifs (such as CARD and DED) are shown. Note that the long pro-domain in STRICA does not contain any protein-protein interaction motif(s). The relative lengths of various domains and subunits are not drawn to size and only serve as a guide here to demonstrate the various types of apoptotic caspases found in *C. elegans*, *Drosophila* and mammals. (adapted from [6])

Caspase family has been divided into 3 sub-groups. The 3 groups are characterized by both the function and the structure of the caspases. The first group contains the caspases, which do not have an apoptotic function. These caspases are the mammalian Caspases-1, -4, -5, -11, -12 and -13, which are involved in inflammation [107]. Indeed, as discussed previously Caspase-1 is an interleukin-1 $\beta$ -converting enzyme and is therefore implicated in the immune response. Caspases-4, -5, -11 and -13 have been also identified as activators of interleukin. These caspases participate to the inflammatory response and could trigger apoptosis specifically in response to pathogens. The second group of caspases contains apoptotic caspases. This group is sub-divided in two: the initiator caspases and the effector caspases. The initiator caspases are the human Caspases-2, -8, -9 and -10, the *Drosophila* DREDD and DRONC the respective homologs of Caspases-8 and -9 and the *C. elegans* Ced-3. The effector caspases are Caspase-3, -6 and -7 in human and DRICE, Dcp-1 in *Drosophila*. The two groups of apoptotic caspases are defined by their divergence in the N-terminal domain and in their specific function in the apoptotic pathway.



**Figure 2:** Processing of caspase precursor to form active enzyme. The NH<sub>2</sub>-terminal domain, which is highly variable in length (23 to 216 amino acids) and sequence, is involved in regulation of the caspases. Caspase zymogens are activated by proteolytic cleavage on two Aspartate. Two processed precursors assemble into a heterotetrameric active enzyme with active catalytic site. (adapted from [1])

#### 1.1.3.2.1. The initiator caspases

The initiator caspases are upstream caspases. They are characterized by a long N-terminal prodomain. This domain is variable between caspases and contains homotypic protein-protein motifs. Several N-terminal domains have been identified: “caspase-recruitment domain” (CARD), “death effector domain” (DED) or “pyrin domain” (PYD) (**Figure 1**). It has been proposed that these domains control caspase regulation upon apoptosis induction. These domains could therefore control the specific activation of certain caspases in presence cell death induction signals via either the intrinsic or the extrinsic pathways (these pathways will be further described in 1.1.4 Part). However, despite their specific activation, they have common downstream targets. The common feature of the initiator caspases is their ability to autoactivate and also to activate downstream caspases: the effector caspases. The autoactivation of caspases requires the formation oligomers or multimers of pro-initiator caspase and not cleavage. The best characterized multimeric complex for initiator caspase activation is the apoptosome. In this structure, the mammalian pro-caspase-9 forms aggregates in association with Apaf-1 and Cytochrome C [113]. The pro-caspase 9 is activated by the proximity with other pro-caspases within the complex. Then, the activated initiator caspases cleaves itself and effector caspases. The activation of effector caspases by initiator caspases is required for apoptosis induction.

In mammals, Caspases-8, -9 and -10 are the main initiator caspases. The effector caspases are activated by both the intrinsic and the extrinsic pathways, which respectively are mitochondria dependant and independent. Caspase-9, the Ced-3 homolog, is the only caspase

involved in the mitochondria pathway. The activation of Caspase-9 involves the apoptosome. The apoptosome is composed of Caspase-9, Apaf-1 and Cytochrome C. In presence of apoptotic signal, Cytochrome C is released from the mitochondria and binds to Apaf-1. Caspase-9 is recruited in the apoptosome via its CARD domain, which mediates its interaction with Apaf-1 [113]. In this complex, Caspase-9 forms homodimers and auto-activates [114]. Caspase-9 activation is required for apoptosis of several cell types. Caspase-9 has been first identified as a member of the caspase family involved in cytotoxic T cell-mediated apoptosis [115]. Then, the analysis of *caspase-9* mutant revealed a more general role of Caspase-9 in apoptosis regulation during mammal development. Indeed, mouse mutant for *caspase-9* exhibits developmental defects, which leads to embryo lethality [116]. This lethality is associated to cell death deficiency. This blockage of apoptosis in *caspase-9* KO mice is characterized by the absence of Caspase-3 cleavage and of lack of DNA fragmentation in cells intended to die [117]. Thus Caspase-9 appears as an upstream actor of apoptosis, which leads to the activation of other caspases. In addition to Caspase-9, effector caspases can also be activated by Caspases-8 and -10. Unlike Caspase-9, Caspases-8 and -10 are not activated in the apoptosome and their activation does not require mitochondria. They are activated in another activation platform, the death-inducing signaling complex (DISC) and are involved in the extrinsic pathway. The integration of Caspases-8 and -10 in the DISC complex requires their N-terminal DED domain. In this complex Caspases-8 and -10 form homodimers and autoactivate [118, 119]. The activated Caspase-8 and -10 leads to effector caspase activation such as Caspase-3.

The *Drosophila* genome contains three initiator caspases: Dronc, Dredd and Strica (**Figure 1**). Dronc, the homolog of Ced-3/Caspase-9, has been identified as an initiator caspase. As its mammalian homolog, Dronc activation could involve the formation of the apoptosome as suggested by the aggregation of Dronc next to the mitochondria [120]. However, the role of apoptosome in the activation of caspases is controversial and will be discuss in the 1.1.4.2.1 Part. In *Drosophila*, the regulation of Dronc involves mainly the inhibitor of apoptosis proteins (IAPs), which inhibit caspase activity [121] (see 1.1.5 Part). Dronc activity is required during *Drosophila* development in several tissues such as the larval brain and the eye imaginal disc [6, 122-124]. Moreover, as *dronc* mutations are lethal at the pupal stage, it suggests that Dronc has also a role in apoptosis induction upon metamorphosis [122, 124]. In addition to its role in *Drosophila* development and metamorphosis, Dronc also initiates cell death induced by ectopic factors such as X-ray irradiation and pro-apoptotic genes over-

expression such as reaper [125]. Thus, Dronc appears as a central actor of apoptosis in *Drosophila*, which is activated by several signals and in different tissues. Cell death regulation during *Drosophila* development appears to be mediated by a temporal and special regulation of Dronc [126]. Indeed ectopic expression of *dronc* leads to excess of cell death any modification of *dronc* expression leads to development defect. Dronc mediated apoptosis requires the activation effector caspase such as Drice or Dcp1. These effector caspases can also be activated by other initiator caspases such as Dredd and Strica. However, these caspases are less efficient for Drice activation compare to Dronc. Indeed, even if Dredd is able to activate downstream caspases, Dredd is more involved in immunity than apoptosis regulation. Strica is another *Drosophila* initiator caspase which can trigger apoptosis [127]. However, the downstream effectors of Strica have not been identified. In conclusion, Dronc, Dredd and Strica are all initiator caspases but Dronc is the main player of apoptosis in *Drosophila*.

#### **1.1.3.2.2. The effector caspases**

The effector caspases are the downstream actors of apoptosis. The effector caspases are characterized by a small N-terminal domain and as initiator caspases contains a large and a small subunit (**Figure 1**). Their activation requires the cleavage of the large and the small subunit. However, unlike initiator caspases, the prodomain of the effector caspases prevents their autoactivation. Thus, the effector caspases require other proteases for their activation. The main proteases involved in effector caspase activation are the initiator caspases [128]. Thus, the two classes of caspases act in the same pathway to trigger apoptosis. Once activated the effector caspase cleave cellular substrates for the completion of apoptosis.

In mammals there are three effector caspases: Caspases-3, -6, and -7. Caspase-3 is the mammalian homolog of Ced-3. It has therefore been proposed that this protein could participate in apoptosis [129]. To test this hypothesis, *caspase-3* knockout mice have been studied. These mutants exhibit developmental defects and lethality similar to *caspase-9* mutants. *Caspase-3* knockout mice harbor tissue hyperplasia and supernumerary cells. Thus Caspase-3 is required for developmental apoptosis [130]. The classification of Caspase-3 as an effector caspase result from the structure of its prodomain and its incapacity to autoactivate [131]. In addition to Caspase-3, Caspases-7 and -6 have also been identified as effector caspases as they carry a short pro-domain (**Figure 1**). It has been shown that in dying cell

Caspases-3, -7 and -6 can be activated and lead to apoptosis [132-134]. The fact that several effector caspases are activated upon apoptosis suggests that they could have specific function. Particularly, it has been shown that Caspases-3 and -7 had different subcellular localization [132, 134]. Indeed, Caspase-3 appeared as a cytoplasmic protein, however Caspase-7 was translocated to the endoplasmic reticulum [132]. Therefore, due to their specific localization, Caspases-3 and -7 can have different targets which lead to apoptosis. Several cytoplasmic targets of the effector caspases have been identified as discuss in 1.1.3.3 Part. However the specific targets of Caspases-7 and -6 remain to be further studied.

Drice and Dcp1 are two effector caspases in *Drosophila*. Drice and Dcp1 are caspases with a short pro-domain which are respectively the homologs of Caspase-7 and Caspase-3. This homology suggests that Drice and Dcp1 are effector caspases. It has been shown that Dronc physically interacts with and cleaves Drice both *in vivo* and *in vitro* [135, 136]. However, in addition to Drice, Dronc can activate other effector caspases. Indeed, Dronc mutation is lethal at the larval stage whereas Drice mutation induces pupal lethality [122, 137]. This result suggests that Dronc activity not only relies on Drice but could involve other effector caspases such as Dcp1. Indeed, *Dcp1* RNAi suppresses *dronc* over-expression induced apoptosis whereas *dronc* RNAi does not inhibit *dcp1* over-expression induced cell death in the eye [138]. This experiment suggests that Dcp1 is a downstream target of Dronc. Thus, Drice and Dcp1 are effector caspases which trigger apoptosis. These caspase are involved for *Drosophila* development [139, 140]. *Drice* or *dcp1* mutant can lead to developmental lethality [137, 141]. *Drice1* mutant is lethal for 80% of pupae whereas *dcp1* mutant is lethal for only 20% of the pupae [137]. In addition, *drice* mutant adults have defects in formation of eyes, arista and wings, which suggests that Drice inhibition alters cell death upon the development of these tissues [137]. As the penetrance of *drice* mutant phenotype is higher in *dcp1* mutant, Drice appears as the major initiator caspase involves in development. However *dcp1-drice* double mutant enhances *drice* single mutant lethality, which shows that the two effector caspase acts in parallel in apoptosis upon development [137]. Moreover, it has been shown that, even if both Drice and Dcp1 are both activated by irradiation, Drice is a more effective inducer of irradiation induced cell death than Dcp1 [142]. These results suggest that Dcp1 is less efficient for apoptosis. Indeed, Dcp1 is not able to compensate Drice impairment. Interestingly, if *dcp1* is expressed with *drice* promoter, Dcp1 activates apoptosis after irradiation. Thus the difference between Dcp1 and Drice regarding apoptosis induction is not due to their intrinsic activity but to their pattern and level of expression [142]. Thus it seems



that in some cases, apoptosis can specifically require Drice and not Dcp1 [141]. For example, it has been shown that S2 cell death is only dependent of Drice. But, upon development, both Dcp1 and Drice are required. Those results suggest that in *Drosophila*, apoptosis requires either Dcp1 or Drice or both effector caspases.

### **1.1.3.3. Effector caspases targets**

Activated caspases cleave cellular substrates causing morphological changes, and ultimately leading to apoptosis. Over 1000 caspase substrates have been identified [143, 144]. There are still uncertainties concerning which substrates are functionally required in the apoptotic process. Nevertheless, some effector caspase targets can be linked to apoptosis features such as DNA fragmentation. The DNA fragmentation can be mediated by DNase activation during apoptosis. A DNase, DNA Fragmentation Factor (DFF), has been identified as a target of Caspase-3. Caspase-3 mediated cleavage of DFF produces an active DNase and thus leads to DNA fragmentation [145]. DNase activation could also be triggered by the inhibition of DNase inhibitors. Indeed, caspase-3 cleaves the inhibitor of the caspase-activated DNase (ICAD), which leads to the activation of CAD and DNA fragmentation [146, 147]. In addition to DNA fragmentation, apoptotic cell shape changes and apoptotic cells finally detach from the neighboring cell. Cell shape alteration upon apoptosis involves cytoskeleton disorganization [77]. For example, one structural component of the cell, the nuclear Lamin, is cleaved by effector caspases, which facilitates the orderly disassembly of the cell [148, 149]. Moreover, effector caspases cleave  $\beta$ -catenin both in *Drosophila* and in mammals, promoting the loss of contact of the apoptotic cell with neighboring cells and thought to facilitate cellular clearance and engulfment [150, 151].

In addition to caspases targets, which are involved in apoptosis, other targets have been identified. Indeed, even if caspases are predominantly involved in cell death, they also function in non-apoptotic processes. A variety of mammalian caspases have been demonstrated to have functions in a range of differentiation processes, including muscle specification, terminal nucleation of red blood cells, and lens epithelial cell development in the eye [152, 153]. For example, myoblast differentiation to form muscle requires the activation of caspases. Indeed, caspase inhibition prevents myofiber formation and expression of muscle-specific proteins. Interestingly, it has been shown that in the differentiating myoblasts as in apoptotic cells, there is a reorganization of the cytoskeleton. Thus both in

differentiating and dying cell, caspases could induce actin remodeling [152]. As mammalian caspases, the *Drosophila* caspases are involved in non apoptotic functions. *Drosophila* caspases have been involved in a myriad of cellular processes during development. That includes cell border migration, sperm differentiation or Sensory Organ Precursor (SOP) specification [137, 154, 155]. For example, caspases are required for spermatid individualization upon sperm differentiation. Caspase activation is detected in differentiating sperm, however, apoptosis is not activated. The inhibition of caspases prevents spermatid individualization defects which leads to male sterility [109]. The targets of caspases specifically involved in these mechanisms remain to be characterized.

#### **1.1.4. Extrinsic and intrinsic pathways of apoptosis**

Caspase induction is regulated by two different pathways in mammal cells depending of the origin of the death signal: the extrinsic and the intrinsic pathways. The extrinsic pathway involved cell death receptors of the tumor necrosis factor (TNF) family, which respond to extracellular apoptotic signals. The intrinsic pathway involves mitochondria and is activated in response to internal signals such as genotoxic damage, oxidative stress, heat shock and developmental signals.

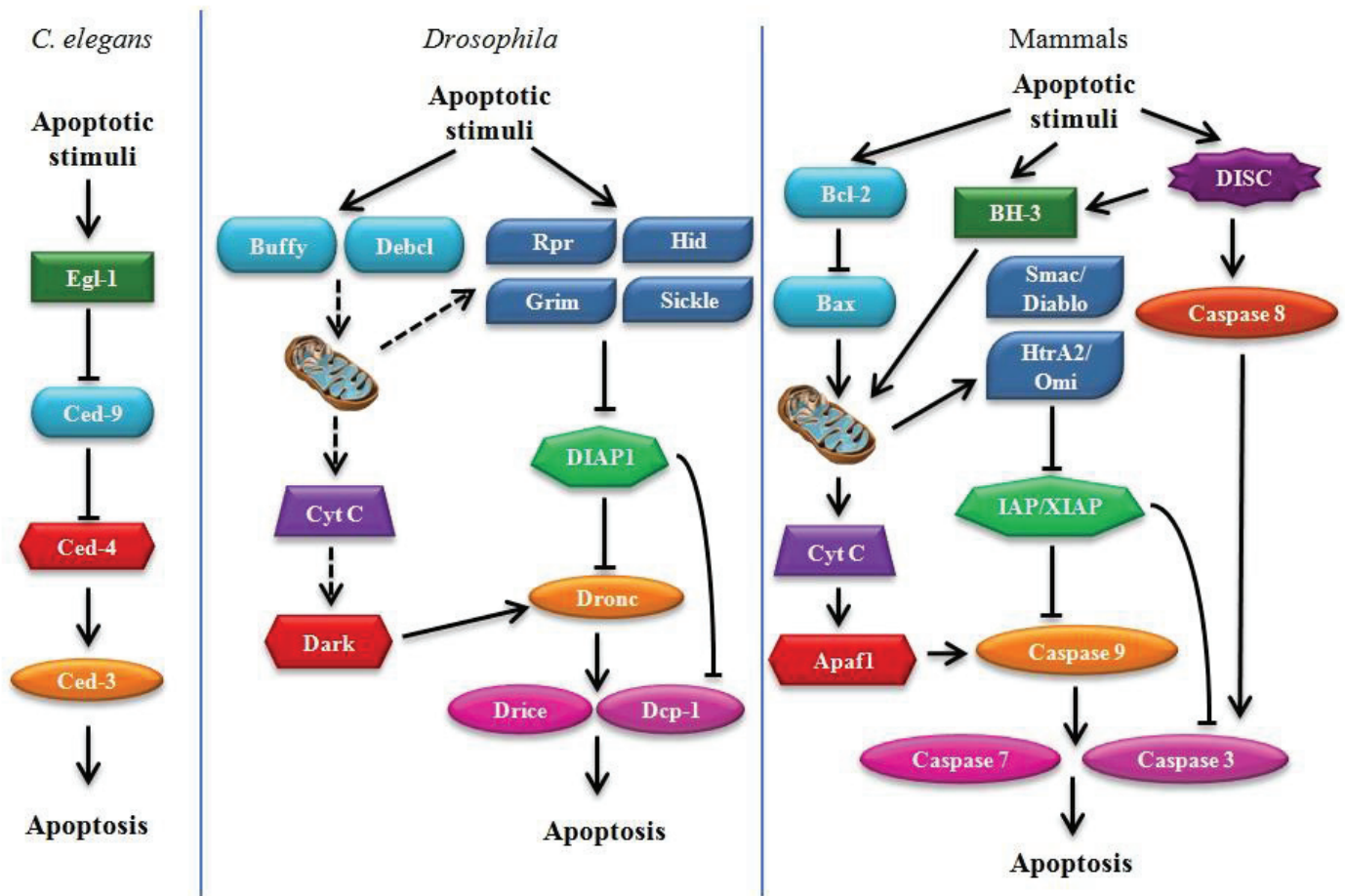
##### **1.1.4.1. The extrinsic pathway**

The extrinsic pathway is a receptor-mediated pathway, which leads to caspase activation. Apoptosis can be activated by extracellular signals. These signals are sensed by receptors localized on the plasma membrane called the death receptors, which transduce signals in the cytoplasm to induce apoptosis. These receptors are members of the tumor necrosis factor (TNF) receptor family. Twenty-nine TNF receptors (TNFRs) have been identified in mammals [156]. TNFR family members are involved in different cellular processes such as inflammation and apoptosis [157]. Six members of the TNF receptor family are known to be involved in apoptosis: Fas, TNFR1, DR3, TNF-related apoptosis-inducing ligand (TRAIL)-R1 and -R2 and DR6 [158-160]. These receptors are characterized by cysteine-rich domains (CRDs). The extracellular domains of the TNFRs allow the specific binding of the ligand. In presence of the ligands, the receptors adopt a tertiary structure. When the TNFRs form trimers, they activate downstream signals in the cytoplasm [161]. The intracellular part of



TNFRs contains a death domain (DD), which functions as a protein-protein binding site. Indeed on their DD domain, TNFRs bind to adaptor proteins, which also have a DD. For example, Fas-associated death domain (FADD) and the TNF-receptor-associated factor (TRAF) are two adaptor proteins, which respectively bind to Fas and TNFR1. The adaptor proteins in addition to the DD also have a death effector domain (DED). This DED allows the recruitment of caspases in the complex. Indeed, caspase-8 carries a DED, which interacts with the DED of the adaptor protein. The protein interactions lead to the formation of a complex, which contains TNFR-adaptor proteins-caspases, called death-inducing signaling complex (DISC). The DISC is a protein platform where initiator caspases dimerize and autoactivate leading to activation of effector caspases such as caspase-3 and to apoptosis (**Figure 3**). Thus, the DISC is an effective caspase activation platform. However, the DISC also contains proteins, which prevent caspase activation. For example, cellular-FLICE inhibitory protein (c-FLIP) can be associated to FADD [162]. c-FLIP contains a DED, which competes with the binding site of caspases inhibiting their activation in the DISC complex [163]. In addition to c-FLIP, DISC mediated caspase activation can be regulated by cyclic AMP (cAMP). cAMP prevent the formation of DISC inhibiting the recruitment of adaptor proteins to TNFR1 [164]. DISC is a platform where the activation of the initiator Caspase-8 is tightly controlled.

Homologs of the extrinsic pathway have been characterized in *Drosophila*. Genetic screens have identified the first invertebrate TNF superfamily ligand, Eiger, and the TNFR-1 homolog, Wengen [165-167]. Although both genes are potent inducers of apoptosis, the mechanism involved seems to be distinct from the mechanism used by their mammalian counterparts. Wengen does not require the caspase-8 homolog Dredd to promote apoptosis [168]. Instead, Eiger and Wengen-induced cell death is entirely dependent on the JNK pathway [165, 167, 168]. Interestingly, the activation of JNK pathway can lead to rpr activation and thus caspase activation [168]. However, it has been shown that caspase inhibition by P35 or Dronc-dominant negative only partially suppresses Eiger induced cell death [165]. This suggests that caspase activation triggered by Eiger is not essential for cell death induction. Thus, Eiger induces mainly cell death via a caspase independent cell death. Thus as in mammals, TNF in *Drosophila* participates in apoptosis regulation. However, the *Drosophila* TNF only has a limited function in apoptosis control via caspase regulation, rather it regulates cell death via JNK pathway which induces cell death by caspase independent mechanisms which could be necrosis.



**Figure 3:** The apoptotic pathways in *C. elegans*, *Drosophila* and mammals. Functional homologs across species are represented by the same color and shape. Arrows represent relation between apoptotic pathway components. The dashed arrows are hypotheses that require further studies.

#### 1.1.4.2. The intrinsic pathway

##### 1.1.4.2.1. Mitochondria and apoptosis

Mammalian mitochondria are pivotal regulators of the intrinsic pathway of apoptosis. Mitochondria contain in the intermembrane space apoptogenic factors such as Cytochrome C, second mitochondria-derived activator of caspases (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low pI (Diablo), high-temperature-requirement protein A2 (HtrA2)/Omi [169-176]. These factors are released to the cytoplasm upon apoptosis induction, they are. The release of these factors in the cytoplasm induces caspases activation and DNA fragmentation. The main apoptotic regulation from the mitochondria is mediated by Cytochrome C release and apoptosome formation. Apoptotic signals induces the release of Cytochrome C in the

cytoplasm, which trigger the multimerisation of Apaf1 [177]. This complex recruits pro-caspase-9 forming the apoptosome. In the apoptosome, Caspase-9 is autoactivated, which leads to the activation of effector caspases. The caspase activation mediated by mitochondria and the apoptosome constitutes the intrinsic pathway of apoptosis (**Figure 3**). In addition to apoptosome formation, mitochondria can trigger apoptosis via other mechanisms. Caspases can be activated by Smac/Diablo and HtrA2/Omi. Indeed, they activate caspases via the inactivation of the inhibitor apoptosis proteins (IAPs). Their role in caspase regulation will be discussed later in the 1.1.5 Part.

Mitochondria outer membrane permeabilization (MOMP) is essential for the release of pro-apoptotic factors: Cytochrome C, Smac/Diablo, HtrA2/Omi. MOMP can be triggered by specific pores in the mitochondrial membrane [178]. These pores are formed by proteins of the Bcl2 family such as Bax and Bak and are described as the most effective Cytochrome C transporter. Bax and Bak are pro-apoptotic members of the Bcl2 family which can form dimers. The pore can be either homodimer of Bak and Bak or heterodimer Bax/Bak. These different types of dimer are all efficient for the transport of Cytochrome C. It has been shown that Bax and Bak have redundant function. Indeed, it has been shown that the mutant mice for either Bax or Bak are healthy viable whereas the double mutant mice *bax*<sup>-/-</sup> *bak*<sup>-/-</sup> have developmental defects that can lead to perinatal death [179]. Moreover, it has been shown that in *bax*<sup>-/-</sup> *bak*<sup>-/-</sup> mouse cells, the release of Cytochrome C is inhibited upon apoptosis induction. These results indicate that Bax and Bak have redundant function in the released of Cytochrome C from mitochondria. The role of Bcl2 family in mitochondria permeabilization will be further described in the 1.1.4.2.2 Part. The addition to Bax and Bak pores, other transporter localized in the mitochondria membrane have been identified such as Voltage Dependent Anion Channel (VDAC) or Permeability transition pore (PTP). These transporters have been mostly implicated in the necrosis process via the induction of mitochondria swelling and MOM rupture [178, 180, 181]. However, several experiments also shown that these transporter can be involved in Cytochrome C, AIF or Smac/Diablo released from the mitochondria to the cytoplasm [182-185]. These transporters have therefore been related to apoptosis regulation. Via the released of pro-apoptotic compound, mitochondria is essential for apoptosis regulation in mammals [186, 187].

Mitochondria fragmentation could induce apoptosis independently of Cytochrome C. During apoptosis, mitochondria undergo various structural changes. These morphological changes are

due to fission and fusion processes, which could therefore play a role in apoptosis. Indeed, Dynamin-related protein 1 (Drp1) a protein involved in fission of mitochondria is linked to apoptosis. Inhibition of Drp1 blocks mitochondria fission and inhibits apoptosis [188]. Moreover, Drp1 co-localizes with Bax, a Bcl-2 family member, which suggests that Bax could mediate mitochondria fragmentation upon apoptosis. It has been shown in mammals and *C. elegans* that Bax overexpression promoted both mitochondrial fragmentation and Cytochrome C released [189, 190]. Interestingly these two mechanisms are independent, as Bcl-XL overexpression block Cytochrome C release but not mitochondria fragmentation [190]. Mitochondria fragmentation could be involved in the released of mitochondrial Smac/Diablo and thus leads to apoptosis.

The role of mitochondria in the regulation of apoptosis in *Drosophila* is controversial. Orthologs of the mitochondrial pro-apoptotic factors have been identified in *Drosophila* but their role in caspase activation remains unclear. The *Drosophila* genome encodes orthologs of Cytochrome C and members of the Bcl-2 family [109, 191-194]. The existence of all these components of the intrinsic pathway suggests that it could be involved in caspase regulation in *Drosophila*. The role of mitochondria in cell death induction has been studied. Mitochondria from apoptotic cells are capable to induce caspase activation and cleavage of Poly-(ADP-ribose) polymerase (PARP), a substrate for active caspases in *Drosophila* S2 cell [195, 196]. This result suggests that mitochondria of dying cells are associated with caspase activation. Interestingly, Dronc, a *Drosophila* effector caspase, has been shown to localize near mitochondria, suggesting that an apoptosome may form in contact to mitochondria [136]. However, opposite results have been shown *in vitro*. In this experiment, mitochondria from apoptotic S2 cells were incapable to induce caspase activation, suggesting that *Drosophila* mitochondria do not play a role in caspase activation [197]. These contradictory results failed to determine the precise role of mitochondria in caspases regulation. To determine a potential role of mitochondria in apoptosis induction, the role of Cytochrome C has been studied. First, the activity of *Drosophila* Cytochrome C has been tested. Purified *Drosophila* Cytochrome C is able to replace human Cytochrome C in activating caspases through Apaf-1, which suggest that Cytochrome C function is conserved in evolution [198]. In addition, Cytochrome C can activate *Drosophila* caspases in embryonic lysates [196]. Even if, Cytochrome C is able to activate caspases, it has to be released from the mitochondria upon cell death induction to activate caspases in the cytoplasm. It has been observed that upon apoptosis induced by reaper over-expression, Cytochrome C is released from mitochondria [199]. However, it has also

been shown by another group that Cytochrome c is not released from the mitochondria but rather its form is altered to expose a new epitope during apoptosis [195]. The exposition of Cytochrome C at the surface of the mitochondria could explain the concentration of caspases forming an apoptosome like structure next to mitochondria [136]. Moreover, *Drosophila* Apaf-1 related killer (Dark), a component of the apoptosome, carries a WD repeat domain, which is a potent interaction domain of Cytochrome C [200]. However, structural data suggests that the *Drosophila* Cytochrome C is not required for apoptosome assembly [201]. In addition, no link has been identified between *Drosophila* Apaf-1 related killer (Dark), and Cytochrome C suggesting that the apoptosome formation could be independent of Cytochrome C [202]. The role of Cytochrome C is thus a controversial issue for the execution of apoptosis in *Drosophila* [203]. In vitro studies addressing its functional role in the execution of apoptosis revealed that Cytochrome C is not involved in caspases activation. Initial studies knocking-down Cytochrome C by RNAi technology failed to suppress grim, rpr and stress-induced cell death in *Drosophila* S2 and SL2 cells [204]. Moreover, Cytochrome C knocked-down resulted in no effect on Dronc activation or on the execution of apoptosis in SL2 and BG2 cells [205]. Moreover, in condition where Cytochrome C released from the mitochondria have been observed Cytochrome C is not involved in caspase activation [199]. However, in vivo experiments shown that Cytochrome C can be involved in caspase activation and apoptosis during development. Genetic evidences in fly testis support a role for Cytochrome C in caspase activation, contrary to what was observed in *Drosophila* cell lines. Arama and colleagues have shown that sperm terminal differentiation requires caspase activation and that this activation is dependent on the presence of Cytochrome C. Loss of Cytochrome C transcript results in male sterility due to defective differentiation, as a consequence of loss of caspase activity [109, 206]. Thus Cytochrome C is required for caspase activation in an apoptotic-like process necessary for sperm maturation. Moreover, it has also been shown that Cytochrome C is required during *Drosophila* eye development. Indeed, supernumerary inter-ommatidial cells are eliminated by apoptosis during development [194]. In addition, it has been shown that adult *Drosophila* mutant for Cytochrome C have extra bristle in the scutellum [194]. These examples show that Cytochrome C is involved in apoptosis. The apparent conflicting results found on the role of mitochondria and Cytochrome C in apoptosis regulation could be due to the different cell types and different contexts used in these studies. The involvement of mitochondria and Cytochrome C in caspases regulation in *Drosophila* could be dependent of the cell type and of the cell death signals. Thus the mitochondria pathway is essential for apoptosis in mammals, and is likely involved in

*Drosophila* to regulate apoptosis in a limited cell type number. Cytochrome C is not required in the majority cell apoptosis, however, the *Drosophila* ortholog of the apoptosome component Apaf-1 named Dark is involved in apoptosis and in particular in Dronc activation [207]. Indeed, *dark* mutant larvae exhibit reduction of cell death in the brain, the wing disc, the eye disc and in the salivary gland [207]. Interestingly, *dronc* and *dark* mutants have similar phenotype, which support the role of Dark in Dronc activation. However, it has been shown that Dark expression is not sufficient to induce Dronc activation [208]. Therefore, it has been proposed that Dark requires an addition factor to activate Dronc. Thus as in mammals, *Drosophila* initiator caspase can be activated in an apoptosome like structure. However, in *Drosophila*, the apoptosome like structure is not the main regulator of caspase activity. A model involving the inhibitor apoptosis proteins (IAPs) have been proposed for caspase activity regulation [209]. In this model, it has been proposed that caspases are constitutively activated but their activity is block by IAP. The mechanism of IAPs mediated caspase inhibition will be discussed in 1.1.5 part. In presence of apoptotic signal IAPs could be inhibited leading to caspase activation [209]. To conclude, *Drosophila* homologs of the intrinsic pathway can induce caspase activation however, it seems that the fine control of caspase activity in response to apoptotic signal is mediated by the IAPs.

#### **1.1.4.2.2. BCL-2 family**

The Bcl-2 family members are involved in apoptosis regulation. They are localized in the outer membrane of the mitochondria and control the integrity of the mitochondrial membrane and the released of the Cytochrome C. The Bcl-2 family members have been identified in *C. elegans* as modifier of developmental apoptosis pattern. The Bcl-2 family members have been shown to be either pro- or anti-apoptotic. The anti-apoptotic member of the Bcl-2 family in *C. elegans* is Ced-9 [94]. Ced-9 can prevent the cell death induced by Ced-3, and this anti-apoptotic activity of Ced-9 requires Ced-4, suggesting that Ced-9 acts at least in part through Ced-4 to prevent Ced-3-induced cell death. In contrast, Egl-1 is a pro-apoptotic factor of the Bcl-2 family, which is observed only in dying cells [93]. In mammals, several pro and anti-apoptotic factors Bcl-2 family members have been identified. The anti-apoptotic members are Bcl-2, Bcl-XL, Bcl-W, mcl-1 and Bfl-1/A1. Bcl-2 was demonstrated to be a potent suppressor of the process of programmed cell death in mammals. Indeed, the expression of exogenous Bcl-2 could protect cells from apoptosis induced by several mechanisms such as infection or sepsis [210, 211]. In addition to its protective effect against cell death in



mammals, Bcl-2 can also block cell death in *C. elegans*. Indeed, mammalian *bcl-2*, the *ced-9* homolog, over-expression reduces cell death in *C. elegans*, which confirms the conservation of cell death pathway from mammals to *C. elegans* [212-214]. Like the anti-apoptotic members of the Bcl-2 family, the pro-apoptotic members are conserved throughout evolution. The homologs of the pro-apoptotic Egl-1 have been identified in mammals among them Bax, Bad and Bid [215]. Indeed, Bax overexpression leads to Cytochrome C release and apoptosis [216, 217]. Thus, in mammals as in *C. elegans*, two classes of Bcl-2 family proteins can be distinguished: the pro and anti-apoptotic classes. These classes of Bcl-2 family members are classified depending on their function and their structure. The Bcl-2 family members carry Bcl-2 homology domains (BH): BH1, BH2, BH3 and BH4. The anti-apoptotic members harbor at least three BH domains. Whereas the pro-apoptotic members contain at least the BH3 domain [215]. The Bcl-2 family members, which only contain the BH3 domain form a sub-family of Bcl-2 proteins: the BH3-only proteins, which play a key role in apoptosis induction [218].

The balance between pro- and anti-apoptotic Bcl-2 family members at the mitochondrial membrane determines cell fate. The Bcl-2 family members carry BH domains, which are protein-protein interaction domains. The BH domains allow dimer formation. Bcl-2 family members such as Bcl-2, Bax and Bak can interact and form heterodimers or homodimers. The dimers of Bcl-2 family members are involved in the control of apoptosis. Apoptotic signals induce Bax and Bak translocation to the mitochondrial membrane and formation of Bax and Bak homodimers, which leads to mitochondrial membrane permeabilization and apoptosis [216, 219, 220]. Bax homodimers form pore in the mitochondria membrane and allow the released of Cytochrome C in the cytoplasm [221]. The formation of Bax homodimer is controlled by anti-apoptotic Bcl-2 family members and BH3-domain-only proteins. Bcl-2 and Bcl-XL can form heterodimers with Bax. These heterodimers are unable to induced apoptosis. BH3-domain-only proteins such as Bid or Bad bind to Bcl-2 preventing its interaction with Bax [222] [223]. This titration of Bcl-2 increases the Bax homodimer formation and leads to apoptosis [223]. Thus the ratio between Bcl-2 and Bax is important to control the formation of hetero and homodimer and thus apoptosis [216]. Indeed, *bcl-2* overexpression protects form apoptosis and this protective effect is reduced if *bax* is co-expressed with *bcl-2*. Apoptosis regulation by the Bcl-2 family members is mediated by both the balance between pro and anti apoptotic factor and the specific inhibition of anti-apoptotic members by the pro-apoptotic factors [224].

Bcl-2 family members are regulated by several intracellular mechanisms. The intrinsic pathway is activated by intra cellular defects such as DNA damage, oxidative stress, and endoplasmic reticulum stress or mitochondria dysfunction. These stresses induce the translocation of Bax to the mitochondrial membrane inducing MOMP. In addition, mitochondria-mediated cell death can also be activated by the extrinsic pathway. The Bcl-2 family members are the link between intrinsic and extrinsic pathway. Bid, a BH3-domain only protein, is cleaved by Caspase-8 activated in the DISC [225]. The activated Bid goes to the mitochondria where it binds to Bcl-2. The binding of Bid to Bcl-2 favors the dimerization of Bax and leads to the activation of the intrinsic pathway. Thus upon extracellular apoptotic signal, BH3-domain only proteins enhance cell death by activating the mitochondrial pathway.

The role of Bcl-2 family members and in the control of apoptosis is not defined in *Drosophila*. The anti-apoptotic function of the Bcl-2 family is conserved throughout evolution. Indeed, mammalian Bcl-2 overexpression in *Drosophila* prevented cell death during embryogenesis, and wing and eye development [226]. These results shown that the Bcl-2 pathway is functional in *Drosophila*. In *Drosophila*, two members of the Bcl-2 family have been identified, named Debcl/Drob-1/ dBorg-1/dBok and Buffy/dBorg-2 [191, 192, 227]. Debcl and Buffy carry the BH1, BH2, BH3, and C-terminal transmembrane domains of the Bcl-2 family of proteins [228, 229]. Debcl shares an high homology with the pro-apoptotic member of the Bcl-2 family in mammals, Bok, which is a protein involved in pore formation in the mitochondria membrane [230]. Thus, this homology suggests that Debcl can be involved in apoptosis induction in *Drosophila* [226]. Debcl has been shown to be a killer protein *in vitro* and *in vivo*. Overexpression of *debcl* induces cell death both in *Drosophila* and mammalian cells. Ectopically expressed *debcl* is localized to the mitochondria and induces cell death. A mutant Debcl protein that lacks the C-terminal transmembrane domain loses both the mitochondrial localization and cell killing ability, indicating that Debcl triggers the cell death pathway at the mitochondrial level [227]. On the contrary, the other member of the Bcl-2 family identified in *Drosophila*, Buffy, is an anti-apoptotic factor [193]. It seems that as in mammals, cell death regulation by the Bcl-2 family in *Drosophila* involves the ratio between pro and anti-apoptotic factors. Indeed, it has been shown that Debcl can inhibit the anti-apoptotic function of Buffy [231]. Thus, as in mammals, the Bcl-2 family members seem to be involved in cell death regulation (**Figure 3**). However, Debcl induces cell death is not



blocked by caspases inhibitors showing that this cell death is caspase independent [191, 192, 227, 230]. In addition, it has been shown that *Debcl* is not involved in PCD during *Drosophila* development. Indeed, *debcl* gene deletion did not alter fly development [231]. However, *Debcl* seems to be required for PCD in the developing central nervous system. Indeed, *debcl* knock out *Drosophila* specifically present extra cells in Central Nervous System of the embryo [232]. These two articles present contradictory results. It has been proposed the *debcl* deletion published by Sevrioukov was only a hypomorphic allele and thus was not possible to detect supernumary cells. Although, it appears that contrary to mammals where *bak*<sup>-/-</sup> *bak*<sup>-/-</sup> double mutation is lethal, *debcl* mutation is not lethal in flies. Thus pathway which involves Bcl-2 family is essential for mammal development but not in *Drosophila*. Thus, it seems that *Drosophila* pro-apoptotic Bcl-2 family members have diverged from their mammalian homologs and cannot activates caspase.

### 1.1.5. Inhibitor of Apoptosis Proteins (IAPs)

In healthy cells, caspase are expressed but their activation is prevented by the Inhibitor of Apoptosis Proteins (IAPs). In apoptotic cell, several mechanisms have been identified that lead to caspase activation while these mechanisms are kept in check by IAPs in living cells. The IAPs have been identified in baculovirus. The baculovirus express proteins such and the IAPs and P35, which inhibit apoptosis to maintain infected cells alive [233-239]. Viral IAPs and P35 protect insect and mammalian cells from virus-induced cell death. Moreover, IAPs and P35 overexpression protect cells from apoptosis induced by caspases over-expression [240]. These results indicate that caspases inhibitory mechanisms are conserved in evolution and IAP or P35 homologs could exist in metazoans. No metazoan homologs of P35 have been identified. However, it has been shown that IAPs are conserved throughout evolution. Indeed genome analyses, lead to the identification of genes with high homology with the viral IAPs [241]. Cellular IAP homologs have been identified in several species. In *Drosophila*, there are two IAPs: DIAP1 and DIAP2 [242]. ITA (inhibitor of T cell apoptosis) is an IAP localized in the T lymphocytes in chicken [243, 244]. Several mammalian IAPs have been identified such as NAIP, cIAP1, cIAP-2 and XIAP [242]. The mammalian and invertebrates IAPs are as the viral IAPs involved in the inhibition of apoptosis. Indeed, mutations in *Drosophila* IAP1 (DIAP1) enhanced developmental apoptosis that depends on *rpr* and *hid* [245]. Moreover,

DIAP2 has been found to bind and inhibit the caspase Drice [246]. In addition, various human IAP homologs have been reported to block death induced by serum withdrawal, staurosporine, menadione, caspase overexpression and Sindbis virus infection [241]. IAPs are thus able to block cellular stresses. In particular, it has been shown that IAP expression is enhanced by endoplasmic reticulum (ER) stress. Indeed, chemical induction of ER stress with tunicamycin or thapsigargin activates IAP expression, which prevents ER stress induced apoptosis [247]. In addition to stress resistance, endogenous human IAPs are linked with pathologies such as degenerative diseases or cancer. Indeed the human Neuronal Apoptosis Inhibitory Protein (NAIP) mutation is associated to Spinal Muscular Atrophy [248]. This suggests that endogenous IAPs prevent degeneration. Reciprocally, the overexpression of IAPs protects neurons against ischemia induced cell death [249]. In addition to degenerative diseases, IAPs are also involved in cancer. The expression of IAPs is altered in several cancers such as lung, prostate or breast cancers [108, 250, 251]. Furthermore, it has been shown that IAPs inhibition either genetically or chemically enhance cancer cell death induced by irradiation [250, 252].

The members of the IAP family inhibit caspase activity via steric and ubiquitin-mediated degradation. IAP activity is mediated by 2 characteristic motifs: RING finger and Baculovirus IAP repeats (BIR) domain. In *Drosophila* and in mammals, only the BIR domains of the IAPs are required for their anti-apoptotic activity. The BIR domains mediate the interaction IAP interaction with both initiator and effector caspases. Indeed, it has been shown in mammals that IAPs interact with Caspases-3, -7 or -9. The BIR domain of IAPs binds to the IAP-binding motifs (IBMs) present on the caspases [253]. The binding of IAPs to caspases can either prevent caspase activation or inhibits caspase activity. On one hand, IAPs can prevent zymogen caspase activation by both intrinsic and extrinsic pathway. For example, the human IAPs can bind to the zymogen Caspase-9. This binding prevents the activation of Caspase-9 in the apoptosome [254]. Thus, IAPs inhibit apoptosome and prevent initiator or effector caspase activation [255, 256]. Moreover IAPs can inhibit caspases via the regulation of the extrinsic pathway. Via binding to TNFR-associated factor 2 (TRAF2), c-IAP1 and c-IAP2 are recruited to TNFR signaling complex. This complex can either activates caspases or NF- $\kappa$ B. When TNFR complex contains IAP, it induces NF- $\kappa$ B preventing caspases activation [257, 258]. The IAPs apparently provide a safeguard mechanism against minimal activation of the apoptosis program. On the other hand, human IAPs can also bind to activated effector caspases such as Caspase-3 or Caspase-7. The binding of IAPs to activated caspases prevents

the cleavage of their downstream targets. As the human IAPs, the *Drosophila* IAPs interact with caspases such as Dronc, Drice and Dcp-1 to block their proapoptotic function [246, 259, 260]. It has been proposed that DIAP1 functions upstream to block Drice and Dronc activation [261]. The binding of IAPs block the interaction of caspases to other proteins and thus prevents caspases activation. In addition to this steric inhibition, the IAP binding to caspases blocks their activity by ubiquitination and proteasome mediated degradation. Indeed, IAPs have a E3 ubiquitin ligase activity [262]. It has been shown that in *Drosophila*, Diap1 can promote ubiquitination of the caspase Dronc in a RING-dependent manner [263]. Moreover, Dronc accumulates in DIAP1 mutant [264]. These results suggest that ubiquitination of caspase by DIAP1 leads to proteasome-mediated degradation of caspases. Mammalian IAPs also inhibits the pro-apoptotic function of caspases by ubiquitination. In humans, XIAP and cIAP2 induce effector caspase ubiquitination *in vitro* [265]. Moreover, it has been shown that cIAP1 binding to Caspases-3 and -7 induces their ubiquitination [266]. The ubiquitination of caspases leads to their degradation [267]. However it has also been shown that IAP mediated ubiquitination of caspase could inhibits caspases independently of ubiquitination. Indeed, Bergmann and col. shown that the accumulation of *Dronc* in *DIAP1* mutant was not due to inhibition of degradation [268]. Indeed, proteasome inhibition did not induce ubiquitinated caspase accumulation [269]. Furthermore, they have shown that DIAP1 controls processing and activation of Dronc [268] [269]. In addition, it has been shown that DIAP1 mediated ubiquitination blocks specifically caspase proapoptotic activity but not their non-apoptotic function. These results suggest that DIAP1 mediated ubiquitination does not induce caspase degradation but rather results in a selective inhibition of caspase activities [270]. It has been proposed that caspase ubiquitination could sterically interferes with substrate entry and reduces the caspases proteolytic activity [269]. The cellular levels of IAPs may determine the difference in sensitivities to apoptosis-inducing stimuli in various cell types. For this reason, the regulation of IAPs levels becomes an important issue in apoptosis.

### **1.1.6. IAP antagonists**

#### **1.1.6.1. IAP antagonist characteristics**

In *Drosophila*, the major inducers of apoptosis are inhibitors of IAPs. The inhibitors of IAPs are called RHG for Reaper, Hid, Grim, which were the first three members identified in

*Drosophila*. These genes have been identified in a screen looking for a lack of developmental apoptosis using genome deficiencies. Embryos homozygous for the deletion H99 showed a lack of developmental apoptosis visible in stage 11 embryos [271]. This deletion covers a small region, which contains the three genes *reaper (rpr)*, *head involution defective (hid)* and *grim*. In addition to these three genes, two other members of the RHG family have been identified : *Sickle* and *Jafrac2* [272]. All the members of the RHG family are characterized by their ability to induce caspase dependent cell death. The over expression of the RHG is sufficient to induce apoptosis. Indeed the ectopic expression of *rpr* or *grim* induces apoptosis either in cell culture or in *Drosophila* tissue [273, 274]. For example, the ectopic expression of *rpr* or *hid* early during *Drosophila* eye development and also in the adult eye leads to increased apoptosis [275]. This cell death induction can be totally inhibited by P35, which indicate RHG-induced cell death is only caspases dependent. The proapoptotic function of the RHG proteins is carried by a common domain of the RHG family members. Rpr, Hid and Grim have an N-term conserved sequence of 4 amino-acids named the IAP Binding Motif (IBM). The IBM domain is involved in the binding to IAPs. The binding of RHG with IAPs could titrate IAP, which cannot bind to caspases leading to caspase activation. The interaction of RHG with IAPs is required but not sufficient for IAP inhibition [276]. In addition to IAP sequestration, the binding to RHG promotes IAPs auto-ubiquitination via the RING domain. The ubiquitination of IAPs targets them to degradation. Indeed, Rpr induces DIAP1 ubiquitination which finally induces its degradation in the eye disc and adult eye [277]. Rpr can trigger DIAP1 ubiquitination via two mechanisms. On one hand, Rpr induces the auto-ubiquitination of DIAP1. On the other hand, Rpr induces DIAP1 ubiquitination by UBCD1, an ubiquitin-conjugating enzyme [277]. In addition, Hid can also stimulate DIAP1 ubiquitination and degradation in the embryo [278]. In addition to ubiquitin mediated degradation, it has been proposed the RHG inhibits DIAP via translation regulation. Indeed, it has been shown that Grim and Rpr can promote a general suppression of protein translation in the embryo [278-280]. Inhibiting IAPs, RHG proteins suppress IAPs mediated caspase inhibition, which induces apoptosis [263]. For example, Hid overexpression induces DCP-1 activation via DIAP1 inhibition [260]. Thus, *Drosophila* RHG proteins are major inducers of apoptosis by inhibiting IAPs. Moreover, RHG genes can regulate cell death via the mitochondria. Indeed the RHG genes possess a Grim Homology 3 domain (GH3 domain), which induces their mitochondrial localization. The role of mitochondrial localization in apoptosis regulation will be further described in 1.1.6.2 Part. As RHG mitochondrial localization could be involved in their pro-apoptotic function, the link between RHG and the

Bcl-2 family has been studied. It has been shown that human *bcl-2* overexpression suppressed *rpr*-induced cell death [281]. Thus Reaper as the pro-apoptotic members of the Bcl-2 family can be inhibited by Bcl-2. Moreover, it has been shown in *Drosophila* that Reaper induced apoptosis by mitochondria fragmentation but not by Cytochrome C release. Thus, as the proapoptotic members of the Bcl-2 family, Reaper pro-apoptotic function can involve mitochondria modification and be inhibited by Bcl-2. Thus in addition to IAP inhibition which is the major effector of reaper induced apoptosis, reaper activity can be dependent of the mitochondria.

The RHG genes are involved in developmental apoptosis. The loss of one of these genes partially suppresses cell death [282-284]. This indicates that these genes participate in developmental cell death but as single mutant does not suppress all cell death, they should have at least partially redundant function. One of the RHG can be specifically implicated in specific developmental cell death. For example, it has been shown that *hid* mutation suppressed developmental cell death in the drosophila eye. Indeed, *hid* mutation totally suppresses Caspase activation in pupal retina which leads to extra interommatidial cells in the adult eye [285]. This result suggests that Hid is the main RHG gene required to control apoptosis during eye development [285]. On the other hand, *rpr* and *grim* are required for neuroblast cell death upon central nervous system (CNS) development [283, 286]. Indeed, Rpr and Hid inhibition induces inappropriate survival of both neurons and neuroblasts in the larval CNS. Interestingly, it has been shown that Rpr or Hid expression alone is not sufficient to induce ectopic cell death in the CNS. Ectopic cell death in the CNS is only observed when both Rpr and Hid are expressed [284]. This result shows that in the CNS, Rpr and Hid cooperate to induce apoptosis. These coordinated effects of RHG can be due to their different binding affinity to IAP BIR domains. Rpr, Hid and Grim are able to bind both BIR-1 and BIR-2 of DIAP1 and thus suppress the inhibition of both effector and initiator caspases whereas Sickie and Jafrac2 bind preferentially to BIR-2, which leads only to initiator caspases activation [287]. Thus all the members of the RHG family are not equivalent but they are all able to induce caspase activation. It has been shown that the activation of apoptosis is enhanced by the cooperation between RHG proteins. The RHG proteins contain helicase domain, which allows the formation of homodimer and heterodimer. The interaction between *rpr* and *hid* targets *rpr* to the mitochondria and potentiates *rpr*-induced apoptosis [288]. Thus, the RHG are efficient inducer of apoptosis which can function independently or in cooperation.

In mammals, proteins that contain IBM-domain have been identified. Four mammalian proteins with an IAP binding activity have been found: Smac/Diablo, Omi/HtrA2, Arts and Survivin [172, 175, 289-291]. These four proteins contain an N-terminal IBM domain, which allows their interaction with IAPs [276, 292, 293]. Via this interaction, Smac/Diablo, Omi/HtrA2 and Arts suppress IAP binding to caspases leading to apoptosis. The pro-apoptotic effect of these proteins has been confirmed by overexpression experiment. The overexpression of Smac/Diablo in cell culture leads to cell apoptosis. Even if ectopic expression of mammalian IBM-domain proteins is able to induce apoptosis, the role of these proteins *in vivo* for apoptosis induction remains unclear. Indeed, mice deficient for either Smac/Diablo or Omi/HtrA2 are viable and do not have apoptosis defects. This suggests that those proteins are not required *in vivo* in mice or that they have redundant function [294-296]. Arts is another IBM carrying protein that is required both *in vivo* and *in vitro* for the regulation of apoptosis. Arts can interact with and inhibit IAPs and thus leads to caspase activation [297]. *Arts* mutant mice present an increased number of hematopoietic stem cells. In addition, Arts inhibition also enhances tumor development in mice [298]. Reciprocally, a peptide derived from Arts binds to and antagonizes XIAP promoting apoptosis in cancer cell lines [299]. This result suggests that IAP inhibitors can be functional *in vivo* and *in vitro*.

#### **1.1.6.2. IAP antagonist regulation**

RHG family activity is regulated by their intracellular localization, their degradation or at the transcriptional level. In mammals, the RHG family members are localized in the mitochondria. In presence of apoptotic signals, the MOMP leads to the release of Smac/Diablo and Omi/HtrA2 in the cytoplasm of the cell. In the cytoplasm, the RHG family members can bind to and inhibit IAPs leading to caspase activation [293]. The mitochondria localization is required for the activation of several members of the RHG family. Indeed, the IBM motif of Smac/Diablo, Omi/HtrA2 is not located at the N terminus of the precursor forms of these proteins, and requires proteolytic processing for N-terminal exposure. At their N terminus, Smac/Diablo, Omi/HtrA2 and Jafrac contain a signal sequence, which is cleaved after their mitochondrial import. The cleavage of N-terminus signal in the mitochondria forms the active protein with an IBM domain at the N-terminus [172, 174, 272, 289]. Thus, the mitochondrial localization is essential for Smac/Diablo, Omi/HtrA2 and Jafrac activation. Similarly to Smac/Diablo, Omi/HtrA2, the *Drosophila* Jafrac2 activation requires its localization in an organelle. Jafrac2 is a protein localized in the endoplasmic reticulum (ER).



Upon apoptotic signal, its N-terminus ER localization signal is cleaved and Jafrac2 is released in the cytoplasm leading to IAP inhibition, and thus Dronc activation [272]. As their mammalian homologs, Reaper, Hid, Grim and Sickie can localize to the mitochondria upon apoptotic stimuli [199, 300, 301]. The translocation of the RHG seems to be dependent of Grim Homology 3 (GH3) domain [302, 303]. However, the role of mitochondrial localization of the *Drosophila* RHG members in their pro-apoptotic function remains controversial. On one hand, mitochondria localization seems to be required for Reaper and Grim pro-apoptotic function. Indeed, it has been shown that the GH3 domain of Rpr is required for Rpr mediated IAP inhibition [301, 303]. It has been shown, *in vitro*, that rpr over-expression induced mitochondria fragmentation which required Rpr GH3 domain. In addition, Grim overexpression also induces mitochondria dysfunction and fragmentation leading to apoptosis [304]. Those results suggest that RHG mediated induction of apoptosis could require mitochondrial localization. On the other hand, several experiments showed that RHG mitochondrial localization is not required for its pro-apoptotic activity. Indeed, the inhibition of Rpr mitochondrial localization does not suppress rpr activity, it only reduces its efficiency. Moreover, Rpr can induce apoptosis independently of the mitochondria via translation regulation [280]. Thus, the requirement of Rpr mitochondrial localization in apoptosis induction is debated. In *Drosophila*, RHG activity is dependent of their transcriptional activity. Rpr and Hid levels are upregulated by apoptotic signals such as irradiation in *Drosophila* embryos. It has been shown that upon irradiation, Rpr and Hid expression is dependent of P53 [305-307]. Indeed, it has been shown that rpr and hid promoters contain a p53 responsive element. Thus rpr and Hid expression seems to be dependent of P53. P53 is a protein involved in several mechanisms and in particular cell death regulation. P53 is sensible to cellular stresses such as DNA damage and in these conditions leads to cell death via Rpr and Hid induction. In *Drosophila*, RHG family members are transcriptionally regulated. However, unlike in *Drosophila*, all known mammalian of Smac/Diablo and Omi/HtrA2 proteins are ubiquitously expressed and therefore does not appear to be transcriptionally regulated [172, 289]. The RHG family member regulatory mechanisms are not conserved in evolution. In *Drosophila*, they are regulated at the transcriptional level. However, in mammals, their activation is dependent of their mitochondrial localization.

### 1.1.7. P53

P53 is a tumor suppressor gene which regulates multiple functions such as proliferation, DNA repair and apoptosis among others [308]. P53 is a transcription factor, which contains a DNA binding domain, a tetramerization domain and a transactivation domain [309, 310]. P53, in particular its transcription activation domain, is found to be mutated in 50% of cancers [311, 312]. Thus P53 has been defined as a tumor suppressor [313-315]. This tumor suppressor activity of P53 is associated to the control of DNA integrity, of cell proliferation and of cell death. Indeed, it has been shown that P53 can induce cell cycle arrest and cell death in response to several stresses such as DNA damage, hypoxia, oxidative stress, or viral infections [316]. For example, when DNA damage are detected, P53 induces cell cycle arrest to favor DNA repair and thus prevent the accumulation of mutation in the cell [317]. However, if the damages are not repaired, P53 activates apoptosis. P53 induces cell death via caspase dependant and caspase independent mechanisms. On one hand, P53 activates apoptosis. Indeed, P53 induces the expression of pro-apoptotic factors such as Reaper or Hid in *Drosophila*. In addition, P53 can activate apoptosis via its transcriptional activity. P53 can also induce apoptosis independently of transcription regulation via the mitochondrial pathway. Upon cellular stress, P53 goes to the mitochondria where it interacts with Bcl-2 and Bcl-XL, activating the mitochondrial pathway of apoptosis [318-321]. On the other hand, P53 induces caspase independent cell death. In mammals, it has been shown that P53 could induce a necrotic process via mitochondria. Upon oxidative stress, P53 induces the opening to the permeability transition pore (PTP) of the mitochondria. PTP opening induces cell death but, caspases are not activated and necrosis markers are detected [322]. In addition, to necrosis, P53 can induce autophagic cell death [323]. Altogether, the experiments performed on P53 shown that P53 is a powerful cell death inducer. It can activate different forms cell death via apoptosis, necrosis and autophagy. In addition to cell death, P53 is also involved in apoptosis-induced proliferation. Apoptosis-induced proliferation is a process conserved in metazoan by which damaged cells entering apoptosis signal the surrounding unaffected cells to divide so as to recoup the tissue loss [324, 325]. For example, *Drosophila* wing imaginal discs submitted to  $\gamma$ -irradiation or genetically induced cell death undergo apoptosis-induced proliferation [326]. In this context, P53 inhibition prevents proliferation [327]. Thus in *Drosophila*, apoptosis-induced proliferation required P53. P53 is a complex transcription which control several mechanisms from cell proliferation to cell death.



P53 function can be regulated by several mechanism involving P53 isoforms and phosphorylation. It has been shown the human *p53* locus can produce twelve isoforms of P53 proteins. The twelve isoforms present variability either at the N terminal domain (transactivation domain) or at their C terminal domain (oligomerization domain). The P53 isoforms do not have the same activity and they can regulates each other [308]. Indeed, the P53 tetramer, which is required for its transcriptional activity, can contain full length or truncated isoforms. Depending of the tetramer composition the affinity of P53 to it binding site is modified [328]. Thus P53 isoforms can alter P53 targets expression. In addition to P53 isoforms, P53 can be regulated by post-translational modification such as phosphorylation or ubiquitination [329]. P53 can be phosphorylated by ATM (ataxia-telangiectasia mutated) and Chk2 (Checkpoint kinase 2) at distinct sites leading to P53-dependent cell cycle arrest or apoptosis [330, 331]. Moreover, P53 activity can be regulated by the ubiquitin ligase mouse double minute 2 (Mdm2) [332, 333]. In mouse, Mdm-2 binds to P53 and targets it to degradation in the proteasome preventing its activation. In response to cellular stresses such as DNA damage, mdm2 is inhibited and dissociates from P53 [334]. In absence of Mdm2 P53 can tetramerize and bind to the promoter of its targets [335, 336].

P53 is a cell death inducer in *Drosophila*. The *Drosophila* homolog of P53, DmP53, has been first identified for its pro-cell death function [306, 337]. In addition to its pro-apoptotic function, DmP53 is also involved in cell cycle, cellular differentiation, for apoptosis induced proliferation [327, 338]. Apoptosis induced proliferation defines the proliferation of cells which are next to dying cell. This process requires DmP53 and the activation of the caspase Dronc [327]. In addition to cell proliferation, DmP53 is also implicated in the regulation of cell differentiation. For example, DmP53 overexpression during the eye development altered cell differentiation [338]. DmP53 regulation is thus essential for the control of all these processes. In *Drosophila*, DmP53 is regulation by the existence of several isoforms and by phosphorylation. However, contrary to mammals, DmP53 is independent of Chk2 and Mdm2. Indeed, the *Drosophila* homolog of Chk2 induces cell death independently of DmP53 [339]. DmP53 activity in *Drosophila* can be regulated by P53 isoforms. In *Drosophila* as in mammals, several isoforms have been identified [340]. The first DmP53 protein identified corresponds to a truncated P53 and has been named DΔp53. The full length P53 has then been identified and named Dp53. It has been shown that these isoforms have different function in the control of apoptosis and apoptosis induced proliferation [307]. In particular, P53 isoforms

can activate different RHG gens to trigger apoptosis. It has been shown that *rpr* promoter contain a P53 response element which trigger their expression in presence of P53 [341]. Moreover, *hid* expression is also induced by P53. However, it has been shown that *rpr* and *hid* expression is dependant of P53 isoforms. The overexpression of DP53 preferentially induced *rpr* whereas DAP53 induced *hid* [307]. Thus in *Drosophila* both DP53 and DAP53 could induce apoptosis but via different pathways.

### **1.1.8. Neurodegenerative diseases**

#### **1.1.8.1. Neuronal cell death in neurodegenerative diseases**

The deregulation of cell death causes the apparition of several pathologies. An excess of cell survival can lead to tumor formation and cancer. On the contrary, an excess of cell death leads to degenerative diseases. The cell death deregulation can specifically concern one cell type. For example, an excess of neuron death can leads to neurodegenerative diseases such as Alzheimer, Parkinson, Huntington diseases, Amyotrophic Lateral Sclerosis (ALS), age-related macular degeneration (AMD) or retinis pigmentosa. These neurodegenerative pathologies are characterized by the type and the localization of the neurons affected by cell death. Alzheimer disease is characterized by the loss of neurons more specifically in hippocampal and neocortical areas [342, 343]. AMD or retinis pigmentosa are due to the loss of photoreceptor in the eye [344]. Parkinson disease is a neurodegenerative disease characterized by the loss of dopaminergic neurons. Parkinson disease is the second most common neurodegenerative disease. This disease is characterized by the protein aggregates named the Lewis bodies and to oxidative stress in the dopaminergic neurons [345]. These protein aggregates are mostly composed of  $\alpha$ -Synuclein. The cause of Lewis bodies formation in the neuron is not totally elucidated. Sporadic and familial forms of the Parkinson disease have been identified. The causes of sporadic form of Parkinson disease, which represent 95% of the cases, are not identified. Dominantly inherited Parkinson disease is linked to mutations in the gene encoding Parkin, DJ-1, Pink,  $\alpha$ -Synuclein or HTRA2 [346, 347]. Neurons loss in neurodegenerative disease can be induced by different signal which all lead to neuron programmed cell death.

The elimination of neurons in neurodegenerative disorders is mediated by a mixed cell death that involves several death mechanisms, including apoptosis, autophagic cell death and

necrosis. In particular, apoptosis has been detected upon neurodegeneration. In patient brain with neurodegenerative disease, activated caspases have been detected. The activation of apoptosis in dying neurons has been also observed in animal neurodegenerative disease models. It has been suggested that the induction of apoptosis could be mediated by mitochondria dysfunction. Indeed, mitochondria dysfunction has been detected in neurodegenerative diseases such as Alzheimer, Parkinson, Huntington diseases or ALS. Mitochondria dysfunction can lead to reactive oxygen species (ROS) production or Cytochrome C released and caspases activation [348]. Interestingly, several proteins implicated in familial Parkinson disease such as Parkin, DJ-1, Pink,  $\alpha$ -Synuclein or HTRA2 are localized to the mitochondria, which suggest that mitochondria dysfunction are related to Parkinson disease. Moreover, it has been shown that the alteration of mitochondria function by knock down of the *Drosophila* mtDNA polymerase  $\gamma$  induces progressive degeneration of dopaminergic neurons and locomotor defects [349]. In addition to mitochondrial dysfunction and apoptosis, it has been proposed that neuron death could be triggered by autophagy. Indeed, autophagy is enhanced upon neurodegeneration [350]. The role of autophagy induction in neurodegenerative diseases will be further described in 1.2.3 Part. Several markers of programmed cell death such as caspases or autophagy are detected upon neurodegeneration however, the mechanisms which control neuron death remains to be characterized in neuron degenerative diseases. To identify regulator of neuron death upon neurodegenerative disease, *in vivo* and *in vitro* models are used.

#### **1.1.8.2. Models of neurodegeneration**

Neurodegenerative models are used to characterize neuronal death process. The conservation of the cell death machinery allows the utilization of animal models to study neurodegenerative diseases. Neurodegenerative models are described in primates, rodent *Drosophila* and *C. elegans*. Mouse model of Parkinson disease have been developed. These models used both genetic and chemical induction of neuron death. For example, a modified virus carrying  $\alpha$ -Synuclein can be specifically injected in rat substantia nigra to induce the expression of this protein in the dopaminergic neurons. This ectopic expression of  $\alpha$ -Synuclein induces dopaminergic neuron death [17]. Moreover, chemical oxidative stress inducer such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or Rotenone can be injected intra-peritoneally or specifically in the striatum of the mouse brain to induce dopaminergic neuron loss [351, 352]. After peritoneal injection of oxidative stress

inducers, the drug can enter in the brain where it will more specifically induce dopaminergic neuron loss as they are more sensitive to oxidative stress than other cells [353]. The stereotaxic injection of drugs in the brain offers a more specific model for Parkinson disease. Indeed, 6-OHDA can be injected in the striatum of the mouse where are localized the projection of dopaminergic neurons. The dopaminergic neurons will specifically uptake the 6-OHDA, which lead to the specific death of dopaminergic neurons [352]. In addition to mouse models, *Drosophila* models of Parkinson diseases have been developed. *Drosophila* is as a powerful model of neurodegenerative disease. Indeed, in *Drosophila*, overexpression of mutated proteins implicated in the human pathology induces neuron cell death and behavior dysfunctions. For example in PD, several proteins such as Parkin, DJ-1, Pink,  $\alpha$ -Synuclein or HTRA2 are involved in the pathology and have a conserved function in *Drosophila*. The specific overexpression of human  $\alpha$ -Synuclein in the dopaminergic neurons of *Drosophila* induces a progressive loss of the dopaminergic neurons and locomotor impairment [354]. Moreover, *pink* and *parkin* mutations also induce neuron death. In addition to genetic models, chemical models of Parkinson disease are used in *Drosophila*. In Parkinson disease, neurons harbor an oxidative stress, which have been linked to cell death. Chemical induction of oxidative stress has thus been used as models of Parkinson disease. For example, paraquat treatment induces oxidative stress, which leads to lethality. This model has been used to study transcriptional response to cellular stress and to identify the identified modulators of oxidative stress sensitivity [355, 356]. Interestingly, the dopaminergic neurons are highly sensitive to oxidative stress, thus a systemic oxidative stress will more particularly induce death of dopaminergic neurons. For example, *Drosophila* feed with either with low dose of paraquat or rotenone present progressive loss of neurons and in particular dopaminergic neuron [357]. This loss of neuron is associated to locomotor defects. Neurons death regulation in these models can be studied using the powerful genetic tools available in *Drosophila*. These models recapitulate Parkinson disease symptoms such as oxidative stress, dopaminergic neuron loss and locomotor defect. These models are used to characterize the neuron death process in Parkinson disease, but also to identify modulators of neuron death and therefore potential therapies for Parkinson disease [351, 352, 355, 356, 358].



## **1.2. Autophagy** Autophagy, a degradation process

### **1.2.1.1. Autophagy characteristics**

#### **1.2.1.1.1. Autophagy, definition and identification**

The autophagic process is an evolutionarily conserved cellular process marked by the degradation of cellular components in the lysosomes. Autophagy has been first observed in mammals. Vacuolar structures containing mitochondria and other cytoplasmic components have been observed in rat liver cells and in rat hepatoma [359, 360]. However, the mechanisms and the role of autophagic degradation have been first characterized in yeast [361, 362]. Autophagy is activated in yeast upon starvation [363]. It has been shown that this process was required for the recycling of cellular components to produce energy for the cell to survive despite nutrient deprivation. The degradation process of autophagy has also been observed in different organisms such as *Dictyostelium*, *C. elegans*, *Drosophila* and mammals [359, 360, 363-367]. In these organisms autophagy is an essential cellular process for development, cell death, stress response and immunity. The roles of autophagy will be further described in 1.2.1.1.3 part.

#### **1.2.1.1.2. Micro-autophagy, macro-autophagy and chaperone mediated autophagy**

Three types of autophagy have been described: micro-autophagy, macro-autophagy and chaperone mediated autophagy [368, 369]. These three types of autophagy are characterized by the entry of cellular components targeted for degradation in the lysosome. Micro-autophagy is the direct uptake of the cytoplasm in the lysosome. It is characterized by the invagination of the membrane of the lysosome, which forms a vacuole containing cytoplasm inside the lysosome [370]. As micro-autophagy, macro-autophagy is also responsible for the degradation of part of the cytoplasm. However, in macro-autophagy, the cytoplasm is not directly engulfed into the lysosomes. Rather, double membrane vesicles called autophagosomes form and engulf part of the cytoplasm [362]. The vesicles that contain organelles and cytoplasmic proteins fuse with the lysosome and are degraded [371]. In addition to micro- and macro-autophagy, which allow the degradation of part of the cytoplasm, chaperone-mediated autophagy targets directly and specifically certain proteins to

degradation [372]. These proteins contain targeting sequences that mediate their interaction with chaperone proteins in the cytoplasm and facilitate the binding to lysosome associated membrane protein (Lamp) type 2a [373]. Such interaction allows the direct transport of specific proteins across the lysosomal membrane [372].

#### **1.2.1.1.3. Roles of autophagy**

Autophagy has been detected during the developmental stages of several organisms such as *Dictyostelium*, *C. elegans*, *Drosophila*, and mouse. *Dictyostelium* is a soil amoeba that feeds on bacteria. Upon starvation, this organism enters a complex developmental cycle to produce a multicellular organism. This specific developmental step requires the induction of autophagy. Indeed autophagy inhibition induces developmental defects such as the absence of fruiting bodies. Therefore, *Dictyostelium* mutants for autophagy are more sensitive to starvation conditions [367]. Autophagy is also required for the development of multicellular organisms [374]. For example, upon starvation or temperature fluctuation, *C. elegans*, enters a unique developmental stage named the Dauer stage [375, 376]. Mutant worms with impaired autophagy cannot enter in the Dauer stage and have reduced life span. Thus autophagy is essential for stress adaptation in multicellular organisms. Autophagy is also required for normal *Drosophila* and mouse development. For example, autophagy is activated in the larval salivary gland, in the fat body of *Drosophila* during pupariation. Mutations blocking autophagic process are lethal during *Drosophila* metamorphosis [377]. Moreover during mouse embryonic development impairment of autophagy gene function results in early lethality embryo [378, 379].

Autophagy is also implicated in cellular response to pathogens. Autophagy is activated after cellular infection with viruses or bacteria. For example, Herpes Simplex virus infections increase autophagy and viral particles are found inside the autophagic vacuoles [380]. Bacterial infections by *Streptococcus pyogenes* or *Coxiella burnetii* also induce autophagy in infected cells [381, 382]. Thus both virus and bacteria activate autophagy. Interestingly, it has been shown that a pathogen receptor localized at the surface of the cell can trigger autophagy. Indeed, pathogen receptor CD46, which can recognize both virus and bacteria, can induce autophagy in presence of pathogen [5, 383]. Via the activation of cellular receptor, pathogen could thus activate autophagy. The role of autophagy in the infection process is complex [8]. On one hand, autophagy limits pathogen infection via direct degradation or by activating the

adaptative immune response. During the direct degradation process, viruses and bacteria can be engulfed in autophagic vacuoles which are degraded after fusion with the lysosomes [384]. Hence infection is limited. Indeed, it has been shown that upon pathogen infection such as Sindbis virus or *Streptococcus pyogenes*, autophagy activation reduces pathogen amount [381]. Autophagy is also linked to the activation of the adaptive immune response. For example, autophagy promotes the localization of the pathogen proteins on the MCH-II complex at the surface of infected cells, allowing their recognition by T lymphocyte [8, 385, 386]. However, the activation of autophagy can also favor pathogen proliferation because pathogens have developed strategies to adapt to autophagy induction. It has been shown that several pathogens can inhibit autophagic vacuole degradation. For example, the bacteria *Coxiella burnetii* multiplies in acidic vacuoles with autophagic vacuole characteristics [387]. This adaptation to autophagy induction enhances pathogen infection. Moreover, it has been shown that the autophagy machinery can be specifically used to enhance viral replication. For example, autophagy inhibition reduced replication of viruses such as influenza A virus, encephalomyocarditis virus, Hepatitis C virus and Human immunodeficiency virus-1 [388-390]. Thus, autophagy has a dual role in viral infection control. The regulation of the autophagic process is thus essential for the response to pathogen infections.

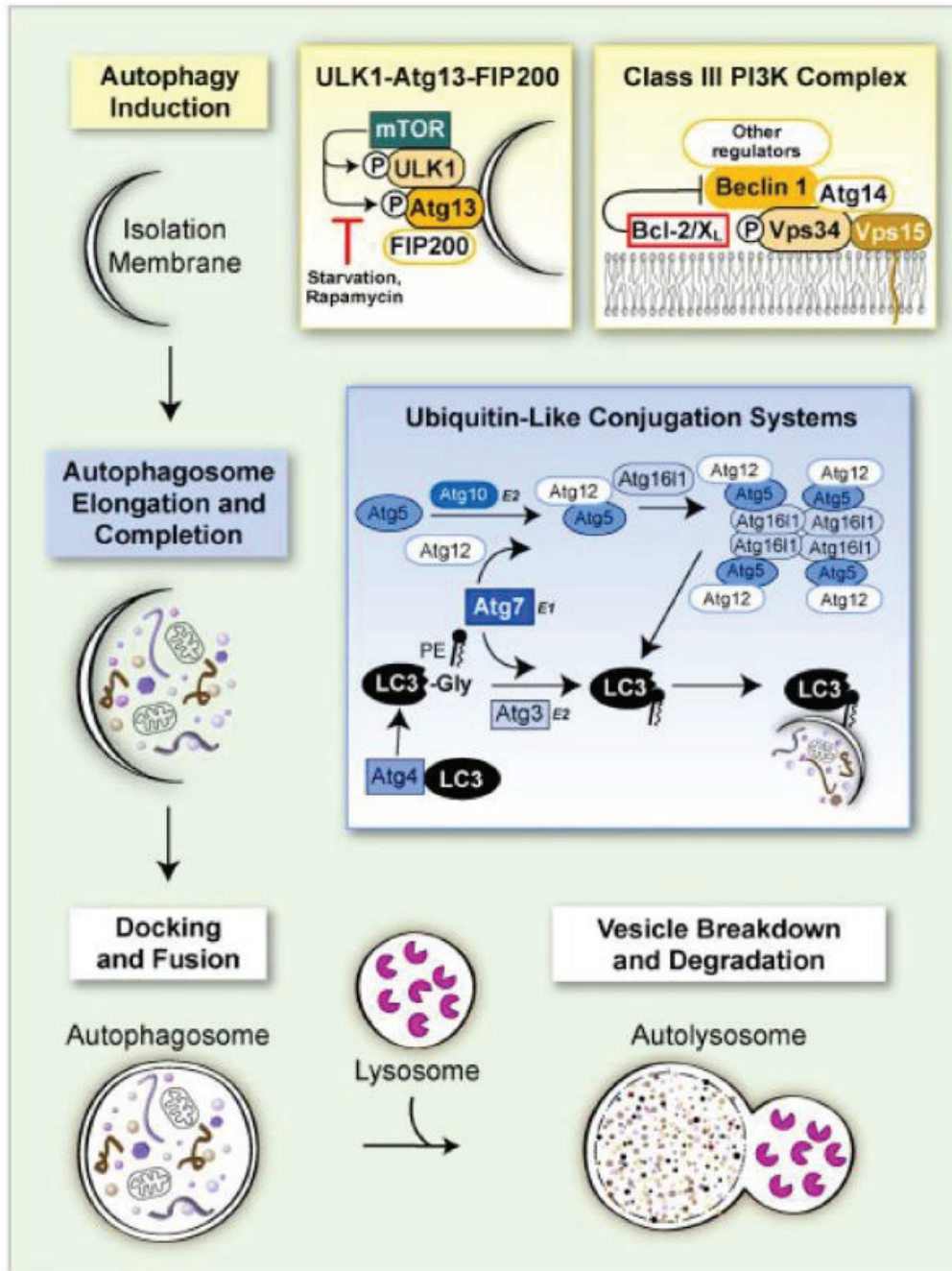
#### **1.2.1.1.4. The autophagy machinery : Atg genes**

Autophagy is a genetically controlled process. The genes involved in the autophagic pathway have been first identified in yeast via genetic screens. The first screens identified several types of autophagy-defective mutants, some of which block autophagy during starvation, some are defective in the degradation of the cytosol with or without starvation [361] [391]. Further screens have been performed to identify other genes involved in autophagy regulation [392]. Finally, it has been found that 65 yeast genes are involved in autophagy [393]. The genes involved in autophagy in yeast are conserved from yeast to mammals [394]. As genes involved in autophagy have been characterized independently, they had several names. In 2003, Klionsky published a review to unify the nomenclature of autophagic genes which are now called *atg* (for AuTophagy) [393].

Autophagic genes homologs have been identified in *Drosophila*. Several paradigms of autophagy, such as in the salivary gland and the gut, have been described in *Drosophila* development [62, 63, 395]. In 2004, Scott et al. identified 11 genes which share significant sequence identity with the yeast *atg* genes [44]. To confirm the involvement of these genes in



the induction of autophagy, the effect of their mutation has been studied upon autophagy. Mutants for the *atg1*, *atg12* and *atg18* and expression of the RNAi for *atg5*, *atg7* and *atg12* homologs reduce the induction of autophagy by starvation in the *Drosophila* larval fat bodies [44]. Moreover, the role of *Drosophila atg8a* and *atg8b* (the homologues of the yeast *atg8*) in autophagic vacuole formation has been studied [44]. It has been shown that Atg8a and Atg8b are evenly dispersed in fed animals; upon starvation, they adopt a punctuate pattern of dots and ring-shaped structures. This experiment confirms that the *Drosophila* Atg8 is involved in autophagy. Altogether, these results have shown that seven *Drosophila* homologues of *atg* genes are involved in starvation-induced autophagy [44]. In addition to the *atg* genes, other homologs in the autophagic pathway have been identified. For example, *blue cheese (bchs)*, the homolog of autophagy-linked FYVE protein (ALFY), is a protein involved in the specific degradation of ubiquitinated proteins [396]. Mutation of *bchs* induces the accumulation of ubiquitin-conjugated protein aggregates throughout the adult central nervous system. This observation suggests that Bchs, as its mammalian ortholog ALFY, is essential for the autophagic clearance of protein aggregates. The over-expression of Bchs in the *Drosophila* retina induces a rough eye phenotype, which has been used as a read-out for a screen to identify regulators of degradation pathway [397]. This screen identified Atg1, Atg6 and Atg18 as enhancer of the eye phenotype. In addition to eye and fat body, the salivary gland has been used as a developmental model to identify the role of the autophagic genes homolog of the in *Drosophila*. Autophagy has been detected in the dying cells of the salivary gland during *Drosophila* development. Indeed, during *Drosophila* metamorphosis, an ecdysone peak induces the destruction of the salivary gland by autophagy activation [395]. Gorski et al. performed serial analysis of gene expression (SAGE) on salivary glands undergoing autophagic cell death [398]. They showed that ten homologues of the autophagic genes are up-regulated. The role of *atg* genes in autophagy induction in salivary gland has been strengthened by the study of *atg* mutants. It has been shown that salivary gland destruction is suppressed in *atg8* or *atg18* mutants, or in *Drosophila* expressing an *atg1* kinase dead mutant. Thus, Atg8, Atg18 and Atg1 are required for autophagy activation. Finally, the autophagic gene induction has been further characterized using several autophagy models in *Drosophila* such as irradiation and cellular stresses. Lee et al. found that several *Drosophila atg* genes such as *atg2*, *atg4*, *atg5*, and *atg7* that are transcriptionally up-regulated in autophagic cells [399]. Moreover, it has been shown that Atg1, Atg3, Atg4 and Atg7 were required for the autophagic process in *Drosophila* [44, 400, 401].



**Figure 4:** Schematic of the autophagy pathway. The process of autophagy begins with the nucleation of an autophagic isolation membrane that eventually encloses the cytoplasmic and/or microbial cargo destined for lysosomal degradation. The mammalian target of rapamycin (mTOR) kinase plays a major role in suppressing autophagy induction by binding to ULK1-Atg13-FIP200 complexes and hyperphosphorylating ULK1 and Atg13. Vesicle nucleation is also promoted through the activation of the class III phosphatidylinositol 3-phosphate (PIP3) kinase Vps34, which contains the essential mammalian autophagy proteins Beclin 1 and Atg14. The second major step of autophagy involves the elongation of the isolation membrane to form an autophagosome which has two phospholipid bilayer membranes with the selected cargo sequestered within the inner membrane. Two ubiquitin-like conjugation systems mediate this step by modifying two ubiquitin-like molecules, Atg5 and LC3, so that they can associate with the isolation membrane and promote its curvature and expansion. The last step of autophagy is the maturation of the autophagosome. This step involves the docking of completed autophagosomes with lysosomes and the lysosomal enzyme-mediated breakdown and degradation of the inner membrane of the autophagosome and its constituents (adapted from [8]).

### **1.2.1.2. The control of autophagy by the autophagic genes**

Autophagy is characterized by the formation of autophagic vacuoles. The vacuole formation requires different steps: the initiation phase, the elongation phase and finally the maturation phase (**Figure 4**). These three steps are controlled by the *atg* genes.

#### **1.2.1.2.1. Initiation phase**

The initiation phase consists in the recruitment of sequestering membrane, which will form the autophagic membrane (**Figure 4**). This sequestering membrane, also named phagophore, is the first step of vacuole formation. Its origin has not been clearly identified. Some evidence suggests that it is formed from the Golgi apparatus as the membrane contains Golgi-specific proteins [402-404]. In contrast, ER proteins but not Golgi markers have been detected at the surface of autophagic vacuoles in starved rat liver cell [405]. Moreover, in NIH 3T3 and rat kidney cells, electron microscopy images show that membranes of the ER and of the autophagic vacuole are interconnected [29, 406]. Therefore, the sequestering membrane could originate from the endoplasmic reticulum (ER) [407]. However, other experiments have shown that the membrane forming autophagic vacuoles do not carry specific proteins of the ER [408]. Finally, it has also been proposed that newly formed autophagosomes emerged from mitochondria or plasma membrane [409, 410]. Thus, the origin of the sequestering membrane could be heterogeneous and is still controversial [411, 412]. One hypothesis is that depending of the cell type, autophagic vacuoles are formed from different structure. Another hypothesis is that autophagic vacuoles can be formed from several structures. The recruitment of sequestering membrane is dependent on the upstream autophagy activation signals. These signals will be described below.

#### **1.2.1.2.2. Elongation phase**

The elongation phase is characterized by the elongation of the sequestering membrane to form the autophagic vacuole. The elongation process is dependent on the Atg proteins, which are involved in two ubiquitin-like conjugation systems (**Figure 4**) [413]. The first ubiquitin-like conjugation system involves Atg8-phosphatidylethanolamine (PE). The second ubiquitin-like conjugation system involves the complex Atg5-Atg12 - Atg16 [414]. These two complexes allow the elongation of the sequestering membrane, which could be mediated by

the tethering and hemifusion of liposomes [415]. The extremities of the sequestering membrane fuse to form the autophagic vacuole named the autophagosome.

#### 1.2.1.2.2.1. Atg8-phosphatidylethanolamine (PE) is required for the elongation phase

Atg8 (Apg8/aut7/LC3) is the most conserved Atg protein. In yeast, there is only one *atg8* gene; in higher eukaryotes, *atg8* is a multigene family. The first member of the Atg8 family has been identified in rat as a light chain of the microtubule-associated protein 1 (MAP1-LC3) [416]. However, its function was first described in microtubule dynamics. Atg8, the yeast homolog of LC3, has been identified as a protein associated to the autophagic vacuole and is required for vacuole formation [417-419]. The mammalian LC3 is a cytoplasmic protein, which becomes associated with the autophagic vacuole membrane upon autophagy induction [420]. Moreover, other orthologs of yeast Atg8 have been identified in mammals such as GATE16, GABA(A) receptor-associated protein, and Atg8L, all are associated to autophagy vacuoles [420-422]. Thus, the autophagic function is conserved upon the Atg8 family. In *Drosophila*, two *atg8* genes have been found: *atg8a* and *atg8b*; both are involved in autophagy. They localize to autophagosomes [423, 424]. However, even if Atg8 is a major protein in the autophagic pathway, adult flies mutant for *atg8a* are viable whereas other mutant of *atg* genes as lethal [397]. These data suggest that Atg8a and Atg8b have redundant functions in *Drosophila*. To confirmed this hypothesis, the phenotype of the double mutant *atg8a;atg8b* should be tested.

The recruitment of Atg8/LC3 to the surface of autophagic vacuole requires Atg8 maturation. Atg8 maturation is mediated by protein cleavage, and the lipid phosphatidylethanolamine (PE) [425]. After its synthesis, Atg8/LC3 is processed at its carboxy-terminal region and is cleaved by the cysteine protease Atg4 (**Figure 4**). The cleavage of Atg8/LC3 C-terminal by Atg4 is the first step of Atg8/LC3 maturation, which is required for Atg8/LC3 lipidation [426]. Interestingly, the cleavage of Atg8/LC3 by Atg4 is conserved in evolution. The yeast Atg4 has homologs in mammals (HsAtg4A/HsApg4A/autophagin-2, HsAtg4B/HsApg4B/autophagin-1, HsAutl1/autophagin-3, and autophagin-4) and in *Drosophila* (Atg4) [427, 428]. All Atg4 homologs are cysteine proteases which are required for the cleavage of the C-terminal of Atg8 family members. The conservation of Atg4 cleavage activity confirmed the key role of this protein in Atg8 regulation. The cleavage of

Atg8 leads to the pre-activation of Atg8/LC3. For the mammalian LC3, this pre-active form is called LC3I [420]. LC3I can be activated by autophagic signal. The activation of Atg8/LC3 is a ubiquitin like system which involves Atg8 as a ubiquitin like protein [425] [413] (**Figure 4**). LC3 binds to Atg7, an E1-like enzyme, via thioester bond [426]. Then, Atg3, an E2-like enzyme, binds to the Atg7-LC3 complex. In this complex, LC3 is transferred from Atg7 to Atg3. The binding of LC3 to Atg3 catalyzes the conjugation of LC3 with the lipid phosphatidylethanolamine (PE). The lipidated form of Atg8/LC3 is named LC3II or Atg8-PE. The detection of Atg8-PE is classically used as a marker of autophagy induction, as it will be discussed latter in 1.2.1.4 Part. Atg8-PE can bind to the sequestering membrane both on the internal and external side [420]. The binding of Atg8-PE on the sequestering membrane is essential for the elongation phase and thus the formation of the autophagosome. Autophagic membranes without Atg8 can elongate but their extremities cannot fuse and thus autophagic vacuoles cannot be formed. Atg8-PE can be found on the inner and outer membrane of the autophagic vacuole. The internal Atg8-PE is sequestered in the autophagic vacuole and is degraded after the fusion with the lysosome. The external Atg8-PE is removed from the membrane and recycled to regenerate Atg8. The removal of Atg8-PE from the outer membrane of the autophagic vacuole is essential to for the maturation phase [429].

#### 1.2.1.2.2.2. Atg5-Atg12-Atg16 complex is required for the elongation phase

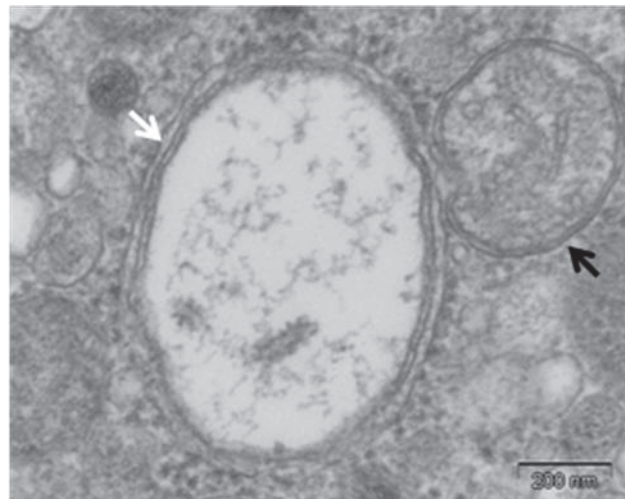
Atg12-Atg5-Atg16 is the other ubiquitin-like conjugation system involved in the elongation of the autophagic vacuole (**Figure 4**). This ubiquitin-like conjugation system utilizes Atg7 as an E1-like enzyme [430]. Atg12 binds Atg7 to via its c-terminal glycine residue. Atg12 is then transferred to another E1-like enzyme, Atg10 [431]. The binding of Atg12 to Atg10 facilitates the formation of the Atg5-Atg12 complex. Atg12 is covalently attached to Atg5 through its COOH-terminal glycine and an internal lysine residue of Atg5. This interaction between Atg12 and Atg5 is conserved in yeast and mammals [414]. Besides Atg5 and Atg12, a third protein, Atg 16, is recruited in the complex [432, 433]. Atg16 can form homo-oligomers linking several atg5-Atg12 complexes.

The Atg12-Atg5-Atg16 complex is bound to the sequestering membrane; the complex is required for membrane biogenesis and in particular for the autophagic membrane elongation [432, 434]. In addition, it has been proposed that this complex acts as a membrane coat, which assists the membrane curve during autophagosome formation. In addition to membrane



remodeling, Atg12-Atg5-Atg16 complex is required for to activation of Atg8/LC3. As discussed previously, Atg8/LC3 binding to the forming vacuole requires Atg8/LC3 lipidation. However, the Atg8/LC3 lipidation is not sufficient to target Atg8/LC3 to the autophagic membrane. Without the Atg12-Atg5-Atg16 protein complex, Atg8/LC3 does not localize to the autophagic membrane. Indeed, in *atg5* mutants, Atg8/LC3 is lipidated but is not localized on autophagic membrane [435]. It has therefore been proposed that the Atg12-Atg5-Atg16 is required for Atg8/LC3recruitment to the autophagic membrane.

The elongation of the sequestering membrane leads to the fusion of the two extremity of autophagosome membrane. This process results in the formation of the autophagic vacuole that contains part of the cytoplasm including mitochondria, endoplasmic reticulum or proteins (**Figure 5**).



**Figure 5** : Autophagic vacuoles in *Drosophila* photoreceptor observed by electron microscopy. The autophagic vacuoles are surrounded by a double membrane. The white arrow indicates an empty autophagic vacuole. The black arrow indicates a vacuole whose content has not been degraded.

#### 1.2.1.2.3. Maturation phase

The maturation of autophagosome is characterized by the fusion of the autophagic vacuole with the lysosome. To complete the autophagic process, the autophagic vacuoles have to be degraded. The degradation process is a rapid event as the autophagosomes is short-lived ( $t_{1/2}$  about 8 min) [436, 437]. The degradation process is mediated by the fusion of the autophagic vacuoles with lysosomes. The lysosome is a degradative organelle which is characterized by an acidic environment containing hydrolytic enzymes. Autophagic vacuoles are shown to bear

lysosomal markers, indicating that the autophagic vacuoles fuse with the lysosome [40, 394, 438]. The fusion of the autophagic vacuole with lysosome leads the acidification of the autophagic vacuole and enables the entrance of hydrolases in the vacuole to degrade its contents. Thus, the mature autophagic vacuole is the autophagolysosome. The environment of the autophagolysosome leads to the degradation of the autophagic vacuole content.

The fusion of the autophagic vacuoles to the lysosome involves Atg proteins and the cytoskeleton. Autophagic vacuoles are associated to the microtubules. As described previously, Atg8/LC3, a protein localized on the surface of autophagic vacuole, can bind to microtubules [416, 439]. This binding to microtubule is essential for vacuole maturation. Indeed, microtubule disruption with vinblastin or nocodazole delayed the fusion of autophagic vacuoles with the lysosomes [440-442]. Conversely, microtubule stabilization by taxol enhances the fusion between autophagic vacuoles and lysosomes [443]. Thus the association of autophagic vacuoles to the microtubule is essential for the maturation process of autophagosome. Moreover, it has been shown that the movement of autophagic vacuoles along the microtubules is also essential for the fusion with lysosomes. The movement of the autophagic along the microtubule is mediated by dynein. Dynein inhibition in mouse cell prevented the degradation process due to the absence of fusion between the autophagic vacuole and the lysosome [444]. It has been proposed and the newly formed autophagic vacuole migrates along the microtubule intracellularly in a dynein- dependant, manner to get closer to the lysosomes [444]. When the autophagic vacuole and the lysosome are in contact, their membranes fuse. The membrane fusion is controlled by proteins localized either on the autophagic vacuoles or on the lysosome. LC3/Atg8, which is localized on the surface of the autophagic vacuoles, inhibits the fusion process. Therefore, Atg8 undergoes the second cleavage by Atg4 to be removed from the outer membrane of autophagosome prior to membrane fusion [415, 427]. The inhibition of Atg4 mediated Atg8 cleavage prevents Atg8 release from autophagic vacuoles, which results in a partial defect in autophagic degradation [368]. Thus Atg8 removal from the autophagic vacuole is essential for fusion with the lysosome. In addition to Atg8 removal, the localization of Rab family members to the autophagic membrane is also required for the fusion process. The Rab proteins are small GTPases involved in the transport of the endocytic and exocytic vesicles. Interestingly, Rab7 is found on the membrane autophagic vacuole. Rab7 inhibition prevents the fusion of the autophagic vacuoles to the lysosome. Thus, Rab7 is required for the lysosomal fusion. In addition to Rab7, other proteins, such as SKD1 and Cathepsin, are also localized in the

autophagic vacuole and are also required of the fusion with the lysosome [445]. Lysosomal proteins are also involved for the fusion process. For example, Lamp-2 is a lysosomal protein required for the fusion of lysosomes with autophagic vacuoles [446]. In lamp-2 deficient mice, autophagic vacuoles abnormally accumulated in several tissues such as muscle, liver, pancreas or spleen due to the absence of fusion between the lysosome and autophagic vacuole [447, 448]. Thus the lysosomal Lamp-2 is essential for the maturation process.

#### **1.2.1.3. Autophagy : a random and a specific degradation process**

Autophagy is both a random and a specific degradation process. Autophagy was first been described as a random form of cell degradation. It was proposed that autophagic vacuoles engulfed randomly the cytoplasm localized next to the sequestering membrane. However, it has been shown that in addition to random degradation, cellular components can be specifically targeted to autophagic vacuoles [449, 450]. For example, damaged organelles such as mitochondria or ER are specifically degraded via autophagy [451, 452]. In addition, autophagy specifically induces the degradation of long-lived proteins. This specific degradation is mediated by autophagic receptors and autophagic adaptors. The receptor protein interacts with proteins of the autophagic vacuole membrane such as LC3/Atg8 and with the autophagic targets such as ubiquitinated proteins. These receptor proteins are conserved in evolution. One of the best characterized autophagic receptors is P62 [453]. P62 has an interaction domain for ubiquitin and an Atg8 binding domain. It targets ubiquitinated proteins to the autophagic vacuoles for degradation; it also targets some organelles such as mitochondria for degradation [454]. Indeed, P62 can target mitochondria to autophagic vacuoles upon ischemia preconditioning in mouse cardiomyocytes *in vitro*. Ischemia preconditioning activates mitophagy. However, P62 depletion (siRNA) attenuates mitochondria loss. This suggests that P62 is involved in mitophagy [455]. In addition to receptor proteins, the adaptor proteins are also involved in the targeting to autophagic vacuoles. The only adaptor protein in mammals is the ortholog of autophagy-linked FYVE protein, ALFY. ALFY is involved in the degradation of ubiquitinated proteins and potentiates P62 function [456]. These adaptor proteins can interact with proteins of the autophagic vacuole membrane, the targets of autophagic vacuoles and the autophagic receptors. The adaptor proteins are not well characterized but they seem to enhance the targeting to autophagic vacuoles. Thus via the receptor and the adaptor proteins, autophagy can specifically degrade some cellular components such as organelles or protein aggregates.



Through specific degradation, autophagy controls several cellular processes and is implicated in several diseases. For example, the autophagic receptor P62 regulates the activity of several pathways such as NF- $\kappa$ B, anti-oxidative response or apoptotic pathways [453, 457]. P62 can enhance the NF- $\kappa$ B pathway activation via TRAF6 degradation. Indeed, P62 physically interacts with TRAF6 (tumor necrosis factor receptor-associated factor 6) and induces its degradation in autophagic vacuoles. The inhibition of TRAF6 mediated by P62 induces the activation of the NF- $\kappa$ B pathway and enhances cell survival [458, 459]. On the other hand, P62 can control cell death via Caspase-8 regulation. P62 have a Caspase-8 interacting domain. Interestingly, the interaction between P62 and caspase 8 leads to the formation of Caspase-8 dimers and thus Caspase-8 activation [460-462]. On the contrary, P62 interaction with Caspase-8 could target this protease to degradation in the autophagy activation and thus inhibits apoptosis. In addition to regulation of apoptosis, P62 can control the anti-oxidative response by interacting with Keap1, degrading Keap1 and thus preventing Keap1 binding to Nrf2 [463-465]. The inhibition of Keap1 leads to the activation of Nrf2 and downstream targets which are involved in the antioxidant response [466]. As autophagy specifically regulates several cellular pathways, it is an important process in several pathologies such as cancers or neurodegenerative diseases. By controlling of NF- $\kappa$ B and apoptotic pathway, P62 is essential for the control of cell proliferation of cancer cells [467]. In addition to cancer, the specific degradation mediated by autophagy is involved in neurodegenerative diseases. For example, it has been shown that P62 participates in the degradation of ubiquitinated mitochondria mediated by PINK1–Parkin, whose mutations are responsible for autosomal recessive Parkinsonism [468, 469]. Autophagy could limit oxidative stress in Parkinson disease through the degradation of damaged mitochondria. In addition, autophagy specific degradation is involved in Huntington disease via the degradation of protein aggregates. It has been shown that P62 facilitates the degradation of protein aggregates in cells which over-express mutated Huntingtin (Htt). The inhibition of P62 induces the accumulation of Htt aggregates which enhance cellular toxicity [470]. Moreover, the activation of P62 enhances Htt degradation [454]. Thus autophagy mediated specific degradation is involved in several cellular processes and is essential to maintain cellular homeostasis.

#### 1.2.1.4. Autophagy detection

Autophagic vacuoles are detected via different methods such as electron microscopy, immunostaining or luminescent markers (**Table 2**, [471-473]). Autophagic vacuoles have been first observed by Electron microscopy (EM), which revealed their double membrane structure (**Figure 5**, [26, 29]). Because autophagy is a degradative process, the content of the vacuoles often appears partially or totally degraded on electron microscopy (EM) photographs. In addition to EM images, other tools have been developed to visualize autophagic vacuoles. For example, autophagic vacuoles can be visualized by the detection of proteins associated to the membrane of autophagic vacuoles, such as Atg8 or Atg5. The most widely-used molecular marker for detecting autophagic vacuoles is the autophagy membrane protein LC3/Atg8 Antibodies against LC3/Atg8 or fluorescently tagged LC3/Atg8 have been

Autophagic Markers	Models	References
Electron Microscopy	In vitro in cell culture, tobacco hawkmoth, <i>Manduca sexta</i> , rat cervical ganglion cells , rat sympathetic neurons, Animal Cell Cultures, in situ mammals	Joubert 2009, Schwartz 1992, Xue 1999, Eskelinen 2008, Ylä-Anttila 2009, Liang 1999
Measurement of degradation of long-lived proteins	human MCF7 breast carcinoma cells	Liang 1999
LC3 western Blot	Mammalian cells: MEF, HEK293, HeLa, SH-SY5Y	Mizushima 2007, Fouillet 2012
GFP-LC3 cell line	Mammalian cells: Hela, MEF Drosophila Cells : S2, l(2)mbn	Joubert 2009, Bampton 2005
mRFP-GFP-LC3 cell line	Hela	Joubert 2009, Kimura 2007
GFP-LC3 in vivo in model organisms	Mouse, <i>Drosophila</i> , <i>C. elegans</i>	Joubert 2009, Kimura 2007, Fouillet 2012
LysoTracker	CHO cells, <i>Drosophila</i>	Munafó 2001, Scott 2004

**Table 2:** Autophagic markers

References : Joubert 2009 [5], Schwartz 1992 [12], Xue 1999 [16], Eskelinen 2008 [26], Ylä-Anttila 2009 [29], Liang 1999 [30], Mizushima 2007 [34], Fouillet 2012 [39], Bampton 2005 [40], Kimura 2007 [42], Munafó 2001 [43], Scott 2004 [44]

used to detect LC3/Atg8 in cell culture yeast, *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Drosophila melanogaster* and mice [366, 375, 381, 420, 423, 424, 474]. LC3/ATG8 is a cytoplasmic protein that normally appears diffused in healthy wild type cells. Upon autophagy activation, LC3/Atg8 is translocated to the autophagic vacuole membrane and assumes punctuate structures. As the translocation of LC3/atg8 is primed by the lipidation of this protein, the lipidation status of LC3/Atg8 can be detected by western blot at 18KDa. The lipidated LC3/Atg8 (ATG8-PE or LC3II) migrates farther than the native LC3/Atg8 due to the hydrophobic nature of PE. LC3II migrates as a 16KDa protein [34, 475]. In addition to LC3/Atg8, Atg5 is another protein associated to autophagic vacuole membrane [424]. In addition to autophagic proteins, specific dyes such as monodansylcadaverine, LysoSensor and LysoTracker label autophagic vacuoles [423, 476]. For example, LysoTracker is a dye that is retained in the acidic compartment of the cell, such as the autophagolysosomes [423]. Together, these markers are used to monitor the amount of autophagic vacuoles in cell. However, it is worth emphasizing that an accumulation of autophagic vacuoles could imply either an increase of autophagy or a blockade of degradation. For example, in *Drosophila*, atrophin mutants present an accumulation of autophagic vacuoles, which is not due to autophagy induction but to an inhibition of the fusion between autophagic vacuoles and lysosomes [477]. Thus, it appears that autophagic vacuole accumulation can be either due to autophagy induction or to degradation inhibition. To test if the autophagic flux is functional, several techniques are used to detect the fusion between autophagic vacuole and lysosomes. The fusion between the autophagic vacuoles and the lysosomes can be detected by co-localization with markers of both autophagic vacuoles such as LC3/Atg8 and lysosomes such as Lamp1 [40]. Another tool to evaluate autophagy flux is LC3/Atg8 fused with both RFP and GFP. In newly formed autophagic vacuoles, both GFP and RFP are fluorescent; whereas in an acidic environment, GFP fluorescence is inhibited. After fusion with the lysosomes, LC3/Atg8-GFP-RFP is trapped in an acidic compartment and hence only RFP labeling remains [42]. In addition to the detection of autophagolysosome, the autophagic flux is evaluated looking at protein degradation in the autophagic vacuoles. The degradation efficiency can be evaluated by monitoring LC3/Atg8 fused with GFP with Western blot. As GFP is more resistant to lysosomal degradation than LC3/Atg8, if LC3/Atg8-GFP is degraded in the autophagolysosome, a band corresponding to GFP alone is detected [424]. Moreover, the autophagic flux can be studied by looking at the amount of proteins targeted to degradation in the autophagolysosomes such as long-lived proteins or P62. The degradation of long life proteins by autophagy can be monitored *in vitro* by radioactivity [478]. The amount

of P62 and its *Drosophila* homolog Ref(2)P can be detected both *in vitro* and *in vivo* with specific antibodies or via the detection of GFP-P62 protein fusion [470, 479]. The presence of significant amount of P62 indicates a blockage of autophagic flux.

## **1.2.1. Autophagy regulation pathways**

### **1.2.1.1. Atg1 controls the autophagic process**

Atg1 was first identified in yeast as an autophagy inducer. The *atg1* null mutant yeast has a defect in the autophagic process [480]. Atg1 is conserved in evolution from yeast to mammals. In mammals, Atg1 has been identified as Unc-51-like kinases 1 (ULK1) and Unc-51-like kinases 2 (ULK2). The Atg1 homologs have a conserved function in autophagy activation. Moreover, in *Drosophila*, *atg1* overexpression induces autophagy [401]. In particular, Atg1 controls the elongation and the maturation steps of autophagy. The activation of Atg1 plays a structural role for efficient recruitment of Atg proteins to the sequestering membrane. For example, in absence of Atg1, Atg8 is not recruited to the sequestering membrane [481]. As Atg1 is required for the binding of Atg8 on sequestering membrane, Atg1 is required for the elongation steps [482, 483]. In addition to the elongation phase, Atg1 is also involved in the maturation step. It has been shown that the recruitment of Atg1 on the autophagic vacuole, via its interaction with Atg8, is required for vacuole fusion with the lysosome. Indeed, mutations in the Atg1 cause a significant defect in autophagy mediated degradation [483]. Thus, Atg1 is involved at different steps of the autophagic process. Indeed, Atg1 controls both autophagic vacuole formation and maturation.

Atg1 activity is regulated by a multi-protein complex called the Atg1 complex. In yeast, the Atg1 complex contains Atg1, Atg17, Vac8, and Cvt9 [484]. The interaction between Atg1, Atg13 and Atg17 controls Atg1-mediated autophagic regulation. In nutrient-rich condition, Atg13 is hyperphosphorylated and cannot interact with Atg1 for its activation. In contrast, in autophagic condition such as starvation or rapamycin treatment, Atg13 is dephosphorylated, which leads to Atg1-Atg13 interaction. Atg13 thus mediates Atg1 homodimerization, induces its activation and the subsequent generation of autophagosomes [485]. Atg1-Atg13 complex formation is controlled by Atg17. Atg17 interacts with Atg13 and is required for Atg13 mediated activation of Atg1 [486]. In mammal, a complex similar to Atg1-Atg13-Atg17 has

been identified. ULK1, the homolog of Atg1, is a component of a complex consisting of Atg13, FIP200 (focal adhesion kinase (FAK) family interacting protein of 200 kDa), and Atg101 [484]. It has been shown that in mammals Atg13 also induces atg1 self-interaction for activation. It has been proposed that FIP200 is the functional homolog of Atg17 [484, 487, 488]. Activation of ULK1 in this complex induces autophagic vacuole formation. Thus, both in yeast in mammals, atg1 activation requires the formation of a multi-protein complex.

#### **1.2.1.2. TOR is a key regulator of autophagy**

Target of rapamycin (TOR) is a highly conserved phosphatidylinositol (PtdIns) kinase related serine-threonine protein kinase. It regulates several cellular functions [489]. In yeast, two genes encode the proteins TOR1 and TOR 2 [490]. TOR1 and TOR2 are members of distinct complexes which are involved in cell growth, translation and transcription control, stress response and autophagy. In *Drosophila*, only one TOR protein has been identified: dTOR. Like its yeast homologue, dTOR has conserved function in regulating mRNA translation, autophagy, transcription, metabolism, cell survival, proliferation and growth [491]. Like in *Drosophila*, only one protein, mTOR, has been identified in mammals. However, it has been shown that mTOR can be recruited in two distinct cellular complexes: TORC1 and TORC2. TORC2 regulates the oncogene Akt and also actin polymerization. TORC1 is involved in the control of translation via the regulation of eIF4F (elongation initiation factor 4F) and p70 ribosomal S6 kinase [492, 493]. In addition, TORC1 is an inhibitor of autophagy [494].

The TOR kinase pathway is a key nutrient-sensing signal pathway that controls autophagy. The TOR kinase pathway provides the major inhibitory signal to dampen autophagy when nutrients are abundant. The key component of this pathway, Tor, inhibits autophagy. In normal condition, active Tor prevents autophagy activation. Upon starvation or after treatment with rapamycin, Tor is inhibited, leading to autophagy induction [423, 495]. Thus Tor is able to sense nutrient levels and controls autophagy activation. Tor has several downstream targets such as Atg13, which controls vacuole formation. Tor induces the phosphorylation of Atg13, thus preventing Atg1 interaction and activation, which in turn suppresses autophagy. In addition to its role in regulating Atg13 and Atg1 activity, Tor controls the expression of other proteins involved in the autophagic process. For example, Tor can inhibit transcription factors such as TFEB and thus prevent the expression of autophagic and lysosomal genes such as Atg9 and Lamp1 [496].

Tor activity is controlled by several signals. First, TOR can be regulated by amino acid amount and by the insulin pathway. Amino acids mediate TOR-dependent signaling by activating the Class III PI3K: Vps34 [497-499]. In addition to amino acid, TOR is regulated by the insulin pathway. Insulin activates TOR via the insulin receptor, the insulin receptor substrates (IRS 1 and 2), class I PI3K and Akt/protein kinase B (PKB). In the presence of insulin, Class I PI3K is activated by IRS thereby providing a link between PI3K-I activation and nutrient availability [500]. Activation of class I PI3K leads to the phosphorylation of plasma membrane lipids that recruit and activate Akt/ PKB, a downstream negative regulator of autophagy. Activation of Akt/PKB is also regulated by the tumor suppressor phosphatase and tensin homologue PTEN, which prevents the accumulation of phosphates on lipids and positively regulates autophagy. Activated Akt/PKB represses the positive regulators of autophagy, the tuberous sclerosis complex 1 (TSC1) and TSC2 [501]. TSC1 and TSC2 repress the small G protein Rheb and induce TOR [502, 503].

#### **1.2.1.3. PI3K/beclin complex induces autophagy**

Autophagy is regulated by the PI3K/beclin complex. In yeast, *Drosophila*, and mammalian cells, following autophagy induction by the Atg1 complex, a PI3P-enriched structure appears at the site of autophagosome formation [504, 505]. It has thus been proposed that PI3P is important for the formation of autophagic vacuole. One hypothesis is that (PIP3) recruits additional factors. This hypothesis has been confirmed by the observation that several yeast Atg proteins bind to PIP3 and localize to the autophagosome in a phosphatidylinositol 3-kinase (PI3K)-dependent manner [506]. As a result, it was proposed that PI3P kinase is involved in the activation of autophagy. The single yeast PI3K, Vps34, is required for a variety of membrane trafficking events, including autophagy. It has been shown that Vps34 can interact with autophagic proteins Atg14 and Atg6, and this interaction leads to the formation of a protein complex that contains Vps34, Vps15, Atg6, and Atg14. Interestingly, the members of this complex are conserved in both *Drosophila* and mammals [507]. For example, Beclin-1, the mammalian homolog of Atg6, was originally isolated as a Bcl-2-interacting protein that inhibits Bcl-2. It was the first reported autophagy-related tumor suppressor gene reported [30, 508]. Like the yeast Atg6, Beclin 1 forms a complex with the class III PI3-kinase Vps34 [509, 510]. The PI3-K/Atg6 is thus an evolutionarily conserved complex and is essential for autophagy regulation. In particular Vps34 function is needed for



autophagosome formation. Indeed, a PI3-K inhibitor, 3-methyladenine (3-MA), is a classical inhibitor of autophagic vacuole formation [511]. Moreover, PI3-K mutation in *Drosophila* prevents autophagy induction [512]. Reciprocally, increasing the level of the class III PI3-K stimulates macroautophagy in human colon cancer cells [513]. Thus PI3-K is an important inducer of autophagy. However, the mechanisms which mediate autophagy induction by PI3-K/Atg6 complex are still unknown. It has only been shown in *Drosophila* that PI3-K alteration fails to modify TOR signaling, suggesting that PI3-K/Atg6 and Tor are two independent regulators of autophagy.

### **1.2.2. Autophagy, dual roles in cell survival and cell death**

The role of autophagy in controlling cell death is controversial [514-517]. The study of autophagy in yeast upon starvation leads to the hypothesis that autophagy is a protective mechanism. In contrast, the observation of autophagy in the pluricellular organism dying cells leads to the hypothesis that autophagy is an alternative cell death mechanism both *in vivo* and *in vitro*. More recent results questioned the role of autophagy in cell death in particular in neurodegenerative diseases in which autophagy function appears as a critical survival mechanism.

#### **1.2.2.1. Autophagic cell death**

Autophagic cell death is one of the three types of programmed cell death. The major type of programmed cell death is apoptosis. Necrosis and autophagy are the other two known types of cell death. The autophagic cell death has been defined in 1990 as a cell death process characterized by the presence of autophagic vacuoles in the dying cells [518, 519]. Autophagic features have been observed in dying cells during the development of several organisms such as midge, *Drosophila*, *Dictyostelium*, silkworm, frog, mouse and chicken [518]. During *Drosophila* development, autophagy has been detected in dying cells in salivary gland and also in midgut [62, 395, 520]. Autophagy is also activated in the dying cells in *Dictyostelium*. Moreover, during vertebrate development, autophagy has been detected in dying cells such as in frog tails, in the palatal epithelium during closure in mouse embryos or in feather melanocytes in chickens. In addition to its role in development, autophagy is also implicated in pathological cell death such as in neurodegenerative diseases. Autophagy

features have also been detected in neurons in Parkinson, Alzheimer or Huntington disease patient brain [521-523]. The observation of autophagic features in the dying cells leads to the hypothesis that autophagy is a cell death mechanism. To test if autophagy is a cell death process, experiments have been performed both *in vitro* and *in vivo*. It has been shown that autophagy is required for cell death in several *in vitro* models. Indeed, the pharmacologic inhibitor of autophagy 3-methyladenine (3-MA), a nucleotide derivative that blocks autophagy can reduce cell death *in vitro* [511, 513, 524]. For examples, it has been shown that 3-MA treatment delays or partially inhibits death in starved hepatocytes from carcinogen-treated rats [525], in anti-estrogen-treated human mammary carcinoma cells [526], in chloroquine-treated cortical neurons [527], in nerve growth factor-deprived sympathetic neurons [16], in serum- and potassium-deprived cerebellar granule cells [528], in serum-deprived PC12 cells [529], in TNF-treated human T lymphoblastic leukemia cells [530] and in carcinoma cell death [531]. In addition, autophagy acting as a cell death mechanism has also been shown *in vitro* in several diseases model such as cancer and neurodegenerative diseases. It has been shown that ectopic autophagy activation induces cancer cell death [532, 533]. In addition, anti-cancer therapies such as irradiation, Atg4c, Baxinteracting factor-1 (Bif-1), BH3-only proteins or DAPkinase overexpression induce autophagy [534-536]. Autophagy is thus a cell death inducer in cancer cells and can act potentially as a target for cancer therapy [537, 538]. Autophagy has also been implicated in neurodegenerative disease models. *In vitro*, overexpression of LRRK2 or MPP (1-methyl-4-phenylpyridine) treatment, which are cellular models for Parkinson disease, induce autophagy [539]. The RNAi mediated inhibition of autophagy reduces LRRK2 induced cell death [539]. The role of autophagy in cell death has also been studied in *in vivo* autophagic models in *Drosophila* and *Dyctyostelium*. During *Drosophila* development, autophagic vacuoles have been observed in salivary gland and midgut dying cells [62, 63, 395]. Mutations or RNAi for *atg* genes inhibit autophagy and prevent salivary gland and midgut cell death [63, 540-542]. These results show that autophagic cell death is required *in vivo* for cell elimination in *Drosophila* salivary gland and midgut during development. Conversely, induction of autophagy induction enhances cell death in the salivary gland and fat bodies [401, 541]. In *Dyctyostelium*, it has been shown that cell death is independent of caspases and instead requires the autophagic machinery [543]. However, the existence of autophagic cell death has not been characterized *in vivo* during vertebrate development.



#### 1.2.2.2. Autophagy, a survival mechanism

Autophagy is activated upon cellular stresses and confers cellular protection. It has first been shown in yeast that autophagy is activated upon nutrient deprivation. The inhibition of autophagy by atg gene mutations or chemical agent increased starvation toxicity [363]. This result shows that autophagy is essential for survival upon starvation [544]. In this context, autophagy facilitates the recycling of cellular components to produce energy. Moreover it has been shown that *in vitro*, nutrient deprivation induces the formation of autophagic vacuoles in the insect and mammalian cells [43, 545]. *In vivo*, autophagy has been detected after starvation in several *Drosophila* tissues such as in the ovary and fat bodies [44, 546]. *In situ* stainings also show that the liver tissue of a starved mouse presented autophagic activation [545]. Based on these observations, autophagy seems to be activated to recycle cell energy and thus favors survival upon starvation. Activation of autophagy not only maintains energy level during starvation, but also removes the toxic components of the cell such as damaged mitochondria and other organelles, intracellular pathogens, and protein aggregates. The specific degradation of mitochondria by autophagy, named mitophagy, is important for cell survival [547]. Mitochondria are an important cell death inducer through the release of Cytochrome C. It has been shown that the amount of cytoplasmic Cytochrome C released via rotenone-induced apoptosis is reduced by rapamycin treatment [548]. Therefore, the degradation of damage mitochondria via autophagy could reduce apoptosis induction. Moreover, mitochondria can also produce ROS to induce an oxidative stress which is toxic for cell. It has been shown that the release of ROS induces autophagy, particularly mitophagy [549]. Thus the specific degradation of mitochondria which releases ROS via the autophagy prevents oxidative stress and protects against cell death [550, 551]. In addition to mitochondria, autophagy can degrade other organelles with impaired function to maintain cell homeostasis. For example, several cellular homeostasis alterations such as aging or infection can cause endoplasmic reticulum malfunction, which leads to the accumulation of misfolded proteins in the ER that induces ER stress and ultimately cell death. In these contexts, autophagy can specifically degrade the malfunctioning ER to reduce the ER stress and prevent the induction of cell death [452, 552]. Thus, via its role in the control of cellular homeostasis and in the regulation of stress, autophagy is an essential mechanism for cell survival upon aging.

Autophagy can reduce neurodegeneration that is usually marked by protein aggregates and organelles degradation. Protein aggregates are found in neurodegenerative diseases such as Huntington or Alzheimer diseases. Defects in protein ubiquitination and in protein folding lead to the formation of protein aggregates. Nascent proteins are folded in the cytoplasm. However, if these proteins are mutated or if the cells are submitted to stresses such as oxidative stress, the proper folding is disrupted. The misfolded proteins in the cytoplasm can form aggregates which are toxic for the cells. It has been shown that autophagic vacuoles accumulate in neurons of neurodegenerative diseases [350]. Autophagy has been detected in the brain of patient with Huntington, Alzheimer and Parkinson diseases [521, 553, 554]. Moreover, the model animals of these diseases also present induction of autophagy. For example, Human Huntingtin (Htt) overexpression induces autophagy in mouse striatal cells [555, 556]. As discussed previously, the activation of autophagy in neurodegenerative diseases was first proposed to be deleterious. However, several experiments showed that autophagy could in fact protect from neurodegeneration. Indeed, it has been shown that *atg5*<sup>-/-</sup> or *atg7*<sup>-/-</sup> knockout mice present an excess of neuron death [557, 558]. These results show that basal autophagy is required for neuron survival. It has been proposed that autophagy could prevent neurodegenerative disease. Indeed, it has been shown that autophagic flux was blocked in Alzheimer disease models, which has been associated to neuron death [559]. Indeed, the inhibition of autophagic flux leads to the accumulation of autophagic vacuoles and protein aggregates which are toxic for the cell. It has thus been proposed that the reactivation of the autophagic flux could reduce neuron death [559]. Moreover, the protective role of autophagy has further been shown in several neurodegenerative disease models [560, 561]. For example, autophagy activation mediated by Tor inhibition enhances cell survival in Huntington disease model [562]. Moreover, autophagy is also protective in Parkinson disease model both *in vitro* and *in vivo*. *In vitro*, rotenone treatment induced neuron death, which was inhibited by rapamycin treatment [548]. In mouse Parkinson model, knocking out *Atg5* or *Beclin 1* genes aggravates motor deficits and the animals display abnormal reflexes. Ubiquitin-positive inclusion bodies accumulate in the neurons in the CNS of these mice. Autophagy induction is also protective in the Alzheimer disease models. For example, in cultured rat cells expressing human APP (amyloid beta (A4) precursor protein), genetic reduction of expression of *Beclin 1*, an inducer of autophagy, promotes intraneuronal A $\beta$  accumulation, extracellular A $\beta$  deposition and cell death. The protective effect of autophagy in neurodegenerative disease has been associated to protein aggregate degradation. In neurodegeneration related to protein aggregates such as Huntington, Alzheimer and Parkinson

diseases, the autophagic vacuoles co-localize with protein aggregates. Furthermore, overexpressed Huntingtin localized to autophagic vacuoles [555]. This observation supports the hypothesis that autophagy is activated to degrade protein aggregates [560, 563-565]. It has been shown that polyQ proteins aggregates are degraded in autophagic vacuoles [566]. In addition,  $\alpha$ -Synuclein aggregates which induce Parkinson disease is also degraded in autophagic vacuoles [567]. By eliminating protein aggregates, autophagy is proposed to be protective [560, 561]. Indeed autophagy activation mediated by Tor inhibition enhances cell survival in Huntington disease model [562]. In summary, in neurodegenerative disease, the elimination of protein aggregates and damaged organelles via autophagy could promote cellular survival during neurodegenerative processes. Thus autophagy appears as a protective mechanism upon cellular stress. Moreover, it has been shown that basal autophagy is also protective in neurons. The implication of autophagy in neuron survival suggests that autophagy activation could be an interesting target for neurodegenerative diseases therapies [561, 568].

Autophagy has a dual role in cell death regulation. It has been shown that autophagy activation could act in either a pro-survival or a pro-apoptotic pathway. Basal autophagy could enable the degradation of potentially toxic cellular components and thus maintain cellular homeostasis promotes cell survival [557, 569]. Autophagy induction reduces cell death *in vitro*. For example, autophagy induction via rapamycin treatment enhances CHO cell survival [570]. On the other hand, autophagy can also participate to cell death both *in vivo* and *in vitro* [519]. It has been proposed that this opposite effects of autophagy could depend of the level of autophagy activation. When autophagy exceeds a threshold, autophagy could switch to cell death. However the existence of such threshold remains to be elucidated.

## 1.3. Endoplasmic Reticulum

### 1.3.1. Identification of the ER

The endoplasmic reticulum (ER) has been identified as a basophilic multivesicular compartment in the cytoplasm. A basophilic compartment has been first observed in the cytoplasm of cell from mammalian salivary gland and pancreas by Garnier et al. in 1897 [571]. However, the existence of this basophilic compartment was controversial at the time and thought to be an artifact. In 1950s, the presence of the basophilic filamentous structure was supported by electron microscopy techniques [571-573]. Particularly in 1945, Porter et al. described *in vitro* a "lace-like reticulum" in the cytoplasm of cultured avian cells [572]. The electron microscopy observations led to the identification of two characteristic features of this structure: its reticular disposition and its vesicular character [574]. These vesicular elements of the reticulum are localized in the endoplasm, which is the inner part of the cytoplasm. The shape and the position of this organelle gave its name to this structure: the endoplasmic reticulum (ER) [575].

The ER is a structure conserved in evolution in eukaryotic cell. The ER has been observed by electron microscopy *in vitro* and *in situ* in more than 40 different mammalian and avian cell types [574-576]. Moreover, the development of fluorescent markers allowed the identification of ER in different species like yeast, *Drosophila* or sea urchin [577-579]. Interestingly, the ER is absent of the red blood cell [580], which suggests that the ER is present only in nucleated cells. Therefore, the ER is an ubiquitous organelle of the nucleated cells [581]. This suggests that the ER is part of the fundamental organization of the cell and involved in essential cellular functions.

### 1.3.2. The structure of the ER

#### 1.3.2.1. The vesicular / tubular organization

Over the years, the structure of the ER has been studied using light microscopy, fluorescent and also electron microscopy. The electron microscopy images showed that the ER was composed of vesicles called cisternae. The vesicles are delimited by a simple membrane and contain the ER lumen [582]. Structure of the ER in three dimensions has been characterized

by the several visualization techniques. Dyes which label membranes have been used to stain the ER *in vitro* and *in vivo*. The fluorescent dicarbocyanine DiIC<sub>12</sub>(3) (DiI) labels the membrane of the ER in cell culture. This dye has allowed the visualization of the three dimensional organization of the ER in living cells for example in sea urchin eggs [583]. The ER appeared as interconnected sheets of membrane arranged in apparently random orientations. In addition, the dye was able to diffuse in all the ER, which suggests that all the vesicles of this organelle are interconnected [579]. The ER can also be visualized looking at proteins localized in the ER membrane or in the lumen. These proteins can be directly detected using antibodies. For example, in *Drosophila*, antibodies can be used to detect endogenous ER resident proteins like Bop, Septin, Calreticulin or Protein disulfide isomerase (PDI) [584, 585]. Moreover, by using the ER resident proteins, other markers of the ER have been created to detect the ER *in vivo*. The ER resident proteins have been used as tagged proteins to visualize the ER. In yeast, Sec63p is a protein of the ER membrane. The Sec63p tagged with GFP labeled the ER and confirmed that the ER in yeast, as in mammals cells, has a tubular structure [577]. Moreover, GFP-tagged proteins have also been used in *Drosophila* to study ER structure. PDI which is a protein localized on the ER membrane has been fused to GFP. The PDI-GFP allows to visualize ER shape upon spermatogenesis in *Drosophila*. It shows that in non dividing cells, the ER is localized in all the cytoplasm [586]. However, the GFP signal is stronger at the periphery of the nucleus, which suggests that the ER is more concentrated around the nucleus [586]. In addition to ER resident proteins tagged with GFP, protein fusions between an ER localization signal (KDEL) and fluorescent markers can also be used to visualize the ER. KDEL is a peptide sequence required and sufficient to address proteins to the ER. Thus, KDEL can be used as a tag to target proteins to the ER. The GFP-KDEL and RFP-KDEL fusions target GFP and RFP to the ER and label this organelle [587, 588]. All these tools allow the visualization of the ER and are used to study ER reorganization upon cellular processes such as cell division or cell differentiation [589-591]. Moreover, they are used to test the localization of a protein in the ER, but also evaluated their accumulation in this organelle upon cellular stresses [592, 593].

### **1.3.2.2. ER and other organelles**

The ER is interconnected with other cellular organelles such as the nucleus, the Golgi and the mitochondria. In particular the structural relationship between the ER and the nucleus has been widely studied. On electron microscopy images, it has been observed that the ER

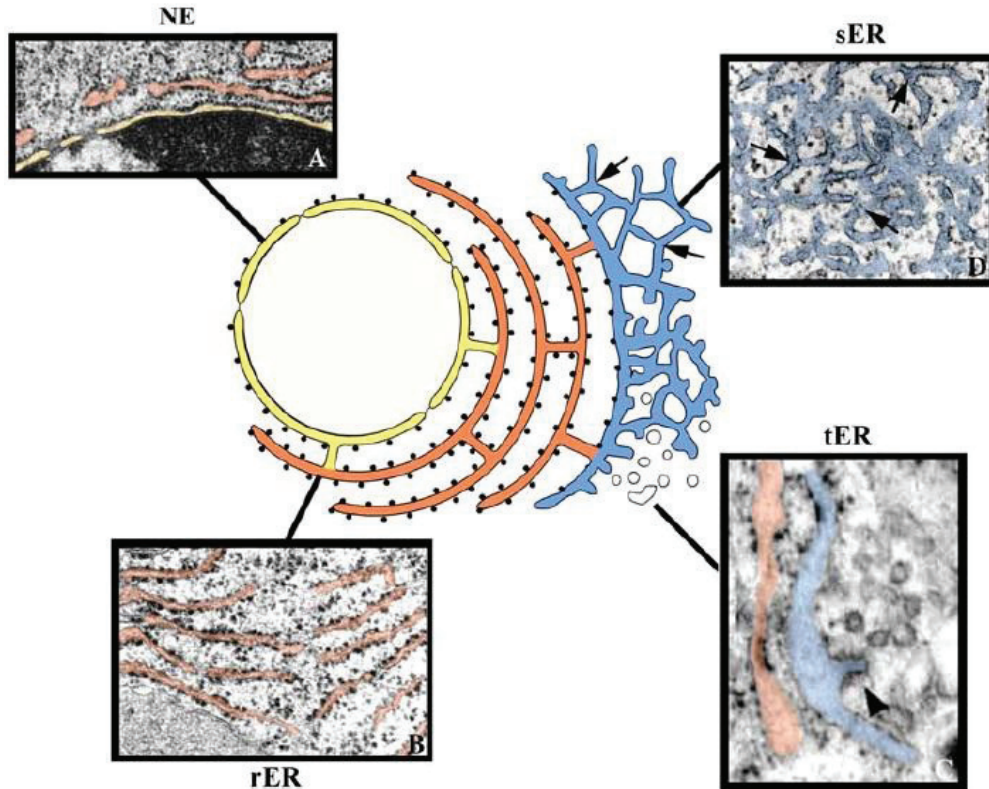
membrane was continuous with the nuclear envelope [594]. In addition, the dyes which stain the ER like DiI also stain the nuclear envelope, which suggests that the ER and the nuclear envelope are formed by the same membrane [579]. It has been shown that the proteins of the nuclear envelope are produced by the ER. It has therefore been proposed that the nuclear envelope is a subdivision of the ER. Moreover, experiments have shown that the nucleus and the ER undergo coordinated movement in yeast [581]. This confirmed that the ER and the nucleus are structurally coupled. However, it has been shown that the composition of the ER membrane and the nuclear envelope are different which shows that these two membranes are functionally different. In particular only the nuclear envelope can interact with the chromatin [595, 596]. Therefore, even if the ER and the nucleus are delimited by a continuous membrane, the ER membrane and the nuclear envelope composition are not identical. Thus, the ER and the nucleus are structurally linked but they are functionally independent.

The ER is also linked with other organelles such as mitochondria and Golgi [597]. The link between mitochondria and ER has caught the attention of the researchers since the observation of mitochondria in the ER cellular fraction [598, 599]. Indeed, after cellular fragmentation, the ER fraction can contain mitochondria which suggests that these organelles have a close relationship [600]. The relationship between ER and mitochondria has been further studied by electron microscopy. On rat liver section, cluster of ER and mitochondria have been observed [601]. Moreover, it has been shown that the ER and mitochondria outer membranes were in close association [602]. This interaction between ER and mitochondria has been observed both in yeast and mammals. In yeast, the interaction domain between ER and mitochondria has been named ER-mitochondria encounter structure (ERES) [603]. In mammals, this domain is the mitochondria-associated membranes (MAMs) [604]. The MAMs define the interface between the ER and the mitochondria and therefore are involved in crosstalk between the ER and the mitochondria functions. MAMs contain multiple phospholipid- and glycosphingolipidsynthesizing enzymes, including long-chain fatty acid-CoA ligase type 4 (FACL4) and phosphatidylserine synthase-1 (PSS-1), but also chaperones such as PACS-2 (phosphofurin acidic cluster sorting protein 2), Grp75 (glucose-regulated protein 75) and Sig-1R (Sigma-1 receptor) [605]. MAMs are involved in  $\text{Ca}^{2+}$  flux between the ER and the mitochondria [606-609]. Indeed, a higher concentration of  $\text{Ca}^{2+}$  has been found in the mitochondria next to ER association domain [610]. Moreover, proteome analyses have shown that  $\text{Ca}^{2+}$  transporters such as inositol-1,4,5-trisphosphate receptors (IP3R) and voltage dependent anion channel (VDAC) were localized in MAMs [598, 605, 607]. This

confirms that MAMs are an active  $\text{Ca}^{2+}$  transport platform. In addition to  $\text{Ca}^{2+}$  flux regulation, MAMs are also involved in lipid metabolism [598, 611]. Therefore, MAMs dysfunction has been associated to diabetes [611]. Thus, ER-mitochondria interaction is essential for cell metabolism. In addition to mitochondria, the ER is also associated to the Golgi apparatus. Vesicular structures have been detected between the ER and the Golgi [612]. These vesicles are formed by the ER membrane budding and then fuse to the Golgi [613, 614]. These vesicles represent the ER-Golgi intermediate compartment (ERGIC) [615-617]. The ERGIC is composed of non-clathrin coats named COPII [617-619]. COPII vesicles are in charge of protein transfer between the ER and the Golgi. They contain proteins from the ER which are addressed to the Golgi and then secreted. Indeed, radioactive pulse to label newly synthesized proteins have shown that proteins are first produced in the ER and then transit in the Golgi apparatus before their secretion [620]. Thus the ER and the Golgi interaction via ERGIC is essential for the secretory pathway [619, 620].

The subcompartments of the ER can be identified depending of their shape and their link with other organelles. The ER can be divided in four regions: the nuclear envelope, the rough ER, the smooth ER and the transitional ER [621, 622] (**Figure 6**). The four compartments of the ER have a radial organization from the nucleus to the cellular periphery. As discussed previously, the ER and the nucleus are structurally linked therefore the nuclear envelope has been defined as one subcompartment of the ER [581, 594, 623]. Associated to the nuclear envelope, the ER forms interconnected tubular structures. The membranes of the tubular structure are associated with ribosomes [624]. As the ribosomes have been visualized as dark granules on electron microscopy images the tubular ER appeared rough. Therefore, the tubular ER associated with ribosome was named the rough ER. Another part of the ER is composed of interconnected vesicles, which are not linked with ribosomes [597]. In opposition to the rough ER, this compartment is named the smooth ER. The smooth and rough ER have some lumen contents specificity but the main difference between these two compartments is the binding of ribosome. The last compartment of the ER is the transitional ER. It is formed of independent vesicles, which are localized next to the Golgi [597]. The transitional ER makes the link between the ER and the Golgi [625].





**Figure 6:** Different subcompartments of the endoplasmic reticulum. The ER is composed of continuous but distinct subdomains. **a** The nuclear envelope (NE) is shown with nuclear pores and ribosomal particles attached to the outer membrane. **b** The rough ER (rER) is continuous with the NE and consists of stacked flattened saccules, whose limiting membranes have numerous attached ribosomal particles. **c** Transitional ER (tER) is composed of a rER subdomain continuous with the rER and a smooth ER (sER) subdomain consisting of buds and tubules devoid of associated ribosomes (*arrowhead points to a coated bud*). **d** In some cells (e.g., steroid secreting cells and hepatocytes) the sER is composed of a large network of interconnecting tubules showing tripartite junctions (*arrows*). (from [9])

#### 1.3.2.3. Rough and Smooth Endoplasmic reticulum : ribosome binding

Ribosomes are specifically localized at the surface of the rough endoplasmic reticulum. Ribosomes were first identified as macromolecules that localized in the microsomal fraction of cell lysate which contained the ER fragment [582, 626, 627]. This result suggests that ribosomes could be in association with the ER. In Palade's work (1954), electron microscopy shown that the ribosomes are localized either free in the cytoplasm or in contact with the ER [627, 628]. Interestingly, electron microscopy experiments showed that the distribution of the granules is not homogenous through the ER. Indeed, the granules are in contact with the ER with tubular feature, however the vesicular structures of the ER do not have ribosomes at their surface [573, 580, 624]. Judging from the ribosome localization observed on electron microscopy images, the ER has been subdivided in two parts: the rough ER which shows



ribosomes at its surface and the smooth ER which has no ribosome (**Figure 6**). The fact that ribosomes are bound specifically on the rough ER and not on the smooth ER suggests that these two compartments could have different functions. Due to its association with the ribosomes, it has been proposed that the rough ER could be involved in protein translation. On the contrary smooth ER could have a function independent of ribosomes activity such as synthesis of lipids and steroids, metabolism of carbohydrates, regulation of calcium concentration and export of proteins to the Golgi.

### **1.3.3. Function of the ER**

#### **1.3.3.1. Protein maturation, folding and export**

##### **1.3.3.1.1. Secreted and membrane proteins are matured in the ER**

Secreted and transmembrane proteins are folded in the ER. The proteins synthesized by the ribosomes at the surface of the rough ER enter in the ER and are addressed to the Golgi apparatus to be secreted or targeted to membranes [629-632]. However, the newly synthesized proteins of the ER are not addressed directly to the Golgi. Before their entrance in the Golgi, proteins have to be matured to become functional in the ER. The proteins stay in the ER until they are matured and folded. The maturation process of the protein takes place during and after translation in the ER [633]. The maturation process in the ER involved several protein modifications such as signal sequence cleavage, N-linked glycosylation, disulphide-bond formation and reshuffling, addition of glycosylphosphatidylinositol anchors and insertion of membrane proteins in the lipid bilayer [634]. The formation of disulfide bond is the main protein maturation process, which occurs in the ER. The disulfide bond formation is dependent on the oxidative environment of the ER. Indeed, disulfide bond formation can be inhibited by dithiothreitol (DTT) a reducing agent, which blocks protein maturation and thus leads to the accumulation of misfolded protein in the ER [635]. These results show that the oxidative environment of the ER is essential for protein maturation. However, the oxidative environment is not the only parameter which favors disulfide bond formation in the ER. The disulfide bond formation also requires the action of enzymes of endoplasmic reticulum such as oxidoreductin 1 (Ero1) in yeast or Ero1a and Ero1b in mammalian cells [636, 637]. In addition to disulfide bond formation, resident proteins of the ER can be

involved in other maturation processes such as glycosylation [638]. Glycosylation process involves several proteins which have been identified via mutant studies [639]. The mutant studies identified enzymes such as Oligosaccharyl transferase and chaperone proteins such as lectin as component of the glycosylation process [638, 640]. Thus the maturation steps of the protein require resident proteins of the ER such as chaperone proteins and enzymes. The chaperone proteins bind to the newly synthesized protein and favor their maturation and proper folding and are considered as the ER quality control (ERQC).

#### **1.3.3.1.2. Quality control system**

The maturation and folding of the newly synthesized proteins are controlled in the ER. The ER quality control (ERQC) is responsible of the folding and the maturation of newly synthesized protein in the ER. The newly synthesized proteins do not have their 3 dimensional organization and therefore harbor hydrophobic domains. These domains can interact with each other and form protein aggregates in the ER that cannot be exported and can be toxic for the cell. Accordingly, the newly synthesized proteins have to be rapidly recognized and folded after their synthesis in the ER. Newly synthesized proteins are recognized and folded by the ERQC actors. Nevertheless, unfolded protein can accumulate in the ER. In the presence of unfolded protein, the ERQC will activate the degradation of the proteins. Therefore, two distinct roles of the ERQC have been proposed: (1) the maturation of the proteins to allow their exportation, (2) the degradation of unfolded proteins.

##### **1.3.3.1.2.1. Folding machinery: chaperone proteins**

The chaperone proteins are required of the proper folding and assembly of proteins. They are essential for the control of protein maturation. The chaperone proteins bind to unfolded proteins and control their folding [641]. Thus, these proteins are required in cell compartment in which proteins are synthesized like the cytoplasm, mitochondria and ER [642]. The ER is a key organelle for protein folding. Indeed the secreted proteins which represent 30% of the proteins produced in the cell are folded in the ER [643]. Thus, the ER, as the cytoplasm, contains resident chaperones, which are required for the folding of newly synthesized proteins. The chaperone proteins bind to the nascent peptides. The chaperones interaction with the peptides controls their folding and prevents accumulation of misfolded proteins [644, 645]. As chaperone proteins are in charge of protein folding control, they are more

specifically required upon cellular stresses, which alter protein folding. Thus, upon these stresses, more chaperone proteins are required to improve the folding capacity and quality control. Interestingly, it has been shown that upon heat shock stress, some proteins are upregulated: the heat shock proteins (Hsps). Moreover, *hsp* mutant yeasts are more sensitive to high temperature than the wild-type and cannot grow at 37°C. Thus Hsps are chaperone proteins, which appear as essential for cell resistance to stresses [646, 647]. The chaperones from the Hsps family have been found in the cytoplasm and in the ER where they control protein folding. The main family of cytoplasmic chaperones, which is conserved in evolution from bacteria to mammals, is the Hsp70 family [648]. Hsp70s are composed of an N-terminal ATPase domain and a C-terminal substrate-binding domain. Via their substrate-binding domain, Hsp70s bind to hydrophobic residues such as leucine flanked by positively charged residues of their substrate [649]. This interaction between Hsp70s and their substrates prevent protein aggregation. Moreover, it has been shown that Hsp70s can also disrupt already formed aggregates. In addition to protein aggregates elimination, Hsp70 can prevent cell death directly inhibiting the apoptotic pathway. Indeed, it has been shown that upon cellular stress, Hsp70 interacts with Apaf-1 prevent apoptosome formation and thus caspase activation [650]. Hsp70 is thus essential to maintain cell survival upon cellular stresses such as heat shock. Moreover, Hsp70 is implicated in diseases associated to protein aggregates such as Parkinson's disease or Tauopathies. Indeed, it has been shown in a *Drosophila* Parkinson model that human *hsp70* overexpression reduced  $\alpha$ -Synuclein toxicity. Reciprocally, *Drosophila* Hsp70 protein, Hsc4, inhibition enhanced  $\alpha$ -Synuclein toxicity [651]. Thus, cytoplasmic chaperone machinery in *Drosophila* helps to protect neurons against degeneration [652]. Moreover, it has been shown that Hsp70 prevented Tau aggregation *in vitro* [653]. Thus via its chaperone activity, Hsp70 could prevent neurodegenerative diseases. Hsp70s appear as essential cytoplasmic chaperones which allow cell survival in divers condition. In addition to Hsp70s, other Hsps have been identified for their chaperone activity such as Hsp90s. As Hsp70, Hsp90 prevents protein aggregates accumulation [654]. The function of Hsps chaperone can require complex formation with co-chaperones such as Hsp100 and Hsp40. In these complexes, Hsps cooperate to bind to protein aggregates. Indeed, Hsp100 recruitment in protein aggregates requires Hsp70 in yeast and in bacteria [655]. Thus cytosolic chaperones are proteins conserved in evolution, which cooperate to fold cytoplasmic proteins.

The ER contains resident proteins, like chaperones which are essential for ERQC. The ER chaperones have an ER localization signal to maintain these proteins in the ER. An ER localization domain have been detected on ER chaperones and enzymes like BiP, Calnexin (CNX), Calreticulin (CRT), GRP94, protein disulfide isomerase (PDI), ERp57, and ERp72. The two major ER localization signals are HDEL and KDEL. In Yeast, the ER localization signal has been identified as a His-Asp-Glu-Leu sequence named HDEL [656]. In mammals, the ER localization signal is KDEL (Lys-Asp-Glu-Leu). The HDEL and KDEL signals induce the targeting of protein to the ER. However, it has also been shown that the KDEL signal does not prevent proteins from importation in the Golgi but, it retrieves the proteins from the post-ER compartment to the ER [657]. Therefore, it suggests that HDEL can be specifically recognized to re-addressed proteins in the ER. The HDEL signal can be recognized by specific proteins such as ERD2, which actively maintains the ER proteins in the ER [658-660]. The ER retrieval sequences are essential for protein localization in the ER and in particular for chaperone localization.

The 4 major chaperones of the ER are BiP, calreticulin, protein disulfide isomerase (PDI), and endoplasmic reticulum chaperonin [644]. All these chaperones have specific features and roles in protein folding.

BiP is a conserved heat shock protein 70 (Hsp70) family member localized in the ER [661]. The Hsp70 family member localized in the ER is a 78 kDa glucose regulated protein named BiP/GRP78 [662]. BiP/GRP78 is conserved throughout evolution from yeast to mammals [663, 664]. Depending of the species, BiP/GRP78 have different names, for example, in *Drosophila*, BiP/GRP78 is also named Heat shock protein cognate 3 (Hsc3). In *C. elegans*, there are 2 homologues of BiP: Hsp-3 and Hsp-4. The BiP/GRP78 homologues have conserved domains, characteristics and functions. BiP/GRP78 has an ER localization signal, HDEL in mammals or KDEL in yeast, which retrieves BiP from the Golgi to the ER [656, 663]. BiP/GRP78 has a peptide-binding site, which is dependant of ATP [665]. BiP/GRP78 also has a N-terminal ATPase domain and a C-terminal substrate-binding domain.

BiP binds to unfolded peptides. BiP binds to unfolded protein via its peptide binding site. As for other members of the Hsp70 family, BiP binding to its targets depends of ATP [643]. BiP binds tightly to ATP and its target proteins. However, when ATP is hydrolysed, BiP releases its target proteins [666]. BiP has several target proteins like influenza virus hemagglutinin precursor [667], E1 protein of the Sindbis virus [668], H chain and the L chain of immunoglobulin complex [669]. BiP targets are various but their common characteristic is the

presence of hydrophobic amino acids which allow BiP binding [670]. The best described motif of BiP binding site is Hy(W/X)HyXHyXMy, where Hy is a bulky aromatic or hydrophobic residue (most frequently tryptophan, phenylalanine or leucine, but also methionine and isoleucine), W is tryptophan, and X is any amino acid [671]. This sequence is characterized by the presence of hydrophobic amino acids. The interaction between hydrophobic amino acid of different proteins can lead to aggregates formation. Therefore, BiP binding blocks hydrophobic sites, preventing protein aggregation [670]. Moreover, it has been shown that BiP could also dissociate already formed protein aggregates and favors their proper folding. By preventing the inhibition of protein aggregation, BiP favors the quality of protein folding [672]. Indeed, it has been shown that in cell infected by influenza, that BiP interacts more with misfolded influenza virus hemagglutinin precursor HA0 than with the correctly folded HA0 [635]. This result shows that BiP binds preferentially unfolded proteins and detaches from correctly folded proteins, which suggests that BiP binds to the unfolded proteins until they are matured. Moreover, BiP binding on unfolded protein acts as an ER retention signal. Indeed, the overexpression of BiP in CHO cell reduces the secretion of specific BiP-binding proteins such as factor VIII [673]. It has also been shown that BiP can sequester a component of a heterodimer if the other component is not folded properly. For example, two membrane glycoproteins, E1 and PE2, of Sindbis virus assemble into heterodimers within the endoplasmic reticulum. If E1 is mutated and cannot be folded, PE2 is maintained in the ER via an increase of BiP binding [668]. These results show that BiP influences protein retention or secretion from the ER. In conclusion, BiP is an ER resident protein involved in proteins folding and proteins export [674]. Via its role in the control of misfolded protein secretion, BiP has a role in the unfolded protein response. The role of BiP in the unfolded protein response will be further described in 1.3.4.2 Part.

Endoplasmin is a chaperone localized in the ER. Interestingly, it has been shown that endoplasmin is one of the most abundant proteins in mammalian cells [675]. It is a glucose regulated protein of 94kDa and has therefore been also named Grp94. Endoplasmin and BiP are glucose-regulated protein (GRP) that share common features. Indeed, as for BiP, Endoplasmin binding to unfolded proteins and chaperone activity requires ATP [676]. The chaperone activity of endoplasmin is required in the immune response as it can fold Toll Like Receptor and integrins in  $\beta$  cells [677, 678]. In addition to its own chaperone activity, endoplasmin acts also in association with BiP. Indeed, endoplasmin can associate with BiP to fold the H chain and the L chain of immunoglobulin [669]. Moreover, BiP and endoplasmin

binds to rotavirus proteins which transit in the ER for their maturation [679]. Thus, via this folding activity endoplasmic reticulum is involved cellular functions such as the immune response.

Calnexin (CNX) and Calreticulin (CRT) are two lectin chaperones localized in the ER. Calnexin is a type-I integral membrane protein and calreticulin is the soluble homolog of Calnexin in the ER lumen. CNX and CRT share sequence homology and have common domains. Calnexin and Calreticulin have an unusual three-dimensional structure with a globular domain and a proline-rich arm called the P domain, and a short cytosolic tail [643, 680]. CNX and CRT have a N terminal domain which has structure plasticity and allows the interaction with several targeted proteins like the Class 1 Major Histocompatibility Complex protein [681]. The globular domain is thought to bind a single  $\text{Ca}^{2+}$  ion and is also involved in glucose binding (lectin domain) [680]. The C-terminal domain of calreticulin displays low-affinity and high-capacity  $\text{Ca}^{2+}$  binding and is thought to buffer luminal  $\text{Ca}^{2+}$  [682, 683]. CNX and CRT are chaperones with specificity for glycoproteins maturation [684]. Glycoproteins are formed via glycosylation which consists in the co-translationally transfer of a preassembled oligosaccharide to nascent polypeptides. Glycoproteins represent 80% of proteins in the secretory pathway. Thus, the glycosylation is an essential protein maturation step which occurs in the ER [643]. This glycosylation process is mediated by chaperone in the ER. CNX is a chaperone involved in glycoprotein maturation. For example, CNX binds to G glycoproteins of the vesicular stomatitis virus. This binding of CNX is required for the proper maturation of the G glycoprotein. Indeed, if G glycoproteins do not bind to calnexin, G glycoproteins are not functional [685, 686]. Moreover, it has been shown that the inhibition of glycosylation by tunicamycin or castanospermine suppresses CNX binding to its targets such as G glycoproteins and influenza virus hemagglutinin (HA) [687]. Thus, mammalian CNX appears as an essential chaperone for glycoproteins maturation. The CNX *Drosophila* homologue is also involved in protein maturation [683]. Indeed the *Drosophila* CNX is required for the folding of Rhodopsin 1 (Rh1) a light sensitive protein. CNX mutations lead to a reduced level of Rh1 in the photoreceptor cells of the *Drosophila* eye. In addition to the protein folding, CNX is also involved in protein export control. In absence of CNX binding, non-functional G glycoproteins are exported to cell membrane, suggesting that calnexin has a role in protein retention in the ER [685]. However, even if CNX has been identified as a chaperone specific for glycoprotein, it seems that CNX could be involved in the maturation of unglycosylated protein. Indeed, CNX/CRT are involved in the immune response as they fold



Class 1 Major Histocompatibility Complex protein *in vivo* [643, 681]. Therefore, CNX and CRT appear as major chaperones of the ER.

Protein disulfide isomerase (PDI) is a chaperone protein of the ER. PDI is a member of the PDI family which contains 20 members in human among which ERp57 and ERp72. The PDI family members are characterized by thiol–disulfide oxidoreductases activity [688, 689]. PDI can catalyze the disulphide bond formation, which occurs upon protein maturation in the ER [690]. As a lot of protein maturation required disulphide bond formation, PDI is a key chaperone of the ER. PDI is required for the folding of proteins involved in different cellular functions such as ER-associated degradation (ERAD), trafficking, calcium homeostasis, antigen presentation and virus entry. Moreover, it has been shown that PDI is also involved in secreted protein maturation. PDI enhances by 3,000 to 6,000 the rate of folding of bovine pancreatic trypsin inhibitor [691]. Thus, PDI, probably due to its catalyzing activity, permits the massive secretion of digestive enzymes by the pancreatic cells. PDI is an ER chaperone protein which enhances protein folding and also prevents protein aggregation [692].

In addition to chaperones that can fold a large number of proteins, other chaperones are involved in the folding of specific proteins or in specific tissue. On one hand, specific chaperones can be only expressed in one tissue to fold one protein. For example, the NinaA chaperone in *Drosophila* is specifically expressed in the photoreceptor cells (PRCs) and folds only the Rhodopsin1 protein [7, 693]. On the other hand, other specific chaperones can be expressed ubiquitously but only regulate the folding of one specific protein. For example, Hsp47, which belongs to the serine protease inhibitor superfamily, specifically binds to collagen and is involved in its maturation [694, 695]. Moreover, receptor associated protein (RAP) is a chaperone dedicated to lipoprotein receptor-related protein (LRP) maturation in the ER [696]. LRP influences the export of LRP to the cytoplasmic membrane and regulates the binding of LRP ligand. Thus, the ER contains many chaperones which could either fold a large proportion of the ER proteins or have more specific targets.

Chaperones collaborate to fold proteins. It has been shown that the same peptide can bind several chaperones. However, two chaperones cannot bind simultaneously to the same peptide. Thus, depending of the peptide sequence one chaperone will preferentially binds to the peptide. For example, the position of the putative glycosylation sites induces the binding of either BiP or calnexin/calreticulin. The glycosylation sites localized at the N-terminus such

as on P62, a structural protein of the semliki forest virus, induces the binding of calnexin/calreticulin rather than BiP. On the contrary, the glycosylation localized on the C-terminus of the protein, such as the E1 protein of the Semliki forest virus, leads to the binding of BiP [697]. Interestingly, there is a competition between the chaperone proteins and the first proteins which bind to the peptide inhibits the binding of other chaperones [697]. However, the maturation of newly synthesized peptide can required several chaperone proteins. Thus, even if two chaperones cannot bind to the same peptide simultaneously, chaperones can bind sequentially to the same peptide. For example, BiP and CNX cannot interact at the same time with proteins such as glycoprotein G of vesicular stomatitis virus (VSV) or thyroglobulin1, there is a sequential binding of BiP and CNX. The order of chaperone binding depends of the proteins. For example, the maturation of glycoprotein G of vesicular stomatitis virus (VSV), and the E1 of the semliki forest virus (SFV) requires first the bind of BiP/GRP78 and then CNX [685, 697]. In contrast, thyroglobulin1 maturation requires first the interaction of CNX followed by BiP [698]. Protein maturation in the ER is a complex mechanism which can require the action of several chaperones to produce functional proteins.

#### 1.3.3.1.2.2. ERAD

The ER associated degradation (ERAD) is in charge of the specific degradation of unfolded proteins of the ER in the proteasome [699]. If the misfolded proteins are not exported to the Golgi, they accumulate in the ER, which can be toxic. Thus, to prevent this toxicity, proteins that cannot be refolded by the chaperones are targeted to degradation in the proteasome. This specific degradation of misfolded protein in the ER is named ERAD. The ERAD process requires the ER localization of the misfolded proteins. Indeed, the misfolded proteins such as misfolded G glycoproteins of vesicular stomatitis virus that can enter in the Golgi have to be re-addressed to the ER to be degraded [686]. It has therefore been proposed that the ER localization of the misfolded protein is important of their degradation. ERAD requires ER resident proteins such as the ER chaperones, and lectin-like ERAD factor [700, 701]. BiP and CNX chaperones recognize misfolded proteins and bind to them. In addition to chaperone, misfolded proteins can be recognized by other ER resident protein. In particular, misfolded glycoprotein are recognized by lectin-like ERAD factor such as Yos9p in yeast [701, 702]. The recognition of the misfolded proteins by BiP, Calnexin or Yos9p targets misfolded proteins to the retrotranslocon. The retrotranslocon is an ER transporter which is dedicated to the misfolded protein exit from the ER. The exit of protein for degradation is required for



protein degradation. It has been shown in yeast that the degradation of misfolded protein required cytosolic component [700]. The cytosolic degradative component involved in ERAD is the proteasome. The targeting of misfolded proteins to the proteasome requires their ubiquitination. The cytoplasmic part of the misfolded proteins is ubiquitinated during their export via the retrotranslocon. The ubiquitination involves enzymes from the cytosol: an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. This ubiquitination targets the ER misfolded proteins to the proteasome where they are degraded.

### **1.3.3.2. Calcium**

Calcium homeostasis is essential for cell and ER functions. Intracellular calcium levels control several cellular functions such as adhesion, cell motility, gene expression and proliferation. Variation of  $\text{Ca}^{2+}$  levels modifies transcription factors activity and thus controls cellular activity [703, 704]. Moreover, the calcium level is important for cellular organelle functions. Calcium level controls mitochondrial functions and alteration of  $\text{Ca}^{2+}$  level can lead to mitochondria-induced apoptosis [705].  $\text{Ca}^{2+}$  has also a specific role on ER function. Indeed, alteration of calcium flux can either induce accumulation of misfolded proteins in the ER or enhance protein export to the Golgi. For example, the reduction of calcium level in the ER by thapsigargin treatment, which inhibits the calcium transporter Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA), induces the accumulation of misfolded proteins in the ER [706]. On the contrary, the increase of  $\text{Ca}^{2+}$  level in the ER, by treatment with a  $\text{Ca}^{2+}$  ionophore which allows transport ions across the lipid bilayer, reduces the export of protein from the ER to the Golgi in human hepatoma HepG2 [707]. In this situation, the retention of protein in the ER could be due to defect in protein folding [708]. These results suggest that  $\text{Ca}^{2+}$  homeostasis in the ER controls proteins folding and chaperone activity of the ER. It has been shown that  $\text{Ca}^{2+}$  level can modify chaperone function. Indeed, several chaperone proteins of the ER have calcium binding site as calreticulin, PDI and BiP [709, 710].  $\text{Ca}^{2+}$  level can alter calreticulin function by altering its conformation [711]. Thus  $\text{Ca}^{2+}$  levels control ER protein folding by regulating chaperone activity.  $\text{Ca}^{2+}$  regulation is thus essential for both ER protein folding and export.

Intracellular  $\text{Ca}^{2+}$  levels are controlled by active transport and storage in cell compartment. The cytoplasmic  $\text{Ca}^{2+}$  level is controlled by both the import of extracellular  $\text{Ca}^{2+}$  and the  $\text{Ca}^{2+}$  flux between the cytoplasm and the  $\text{Ca}^{2+}$  storage organelles such as the ER. On one hand the

Ca<sup>2+</sup> levels are regulated via plasma membrane Ca<sup>2+</sup> channels that regulate Ca<sup>2+</sup> exchanges between the cytoplasm and extracellular compartment: store-operated calcium (SOC). The entry of extracellular Ca<sup>2+</sup> is induced by the reduction of intracellular Ca<sup>2+</sup> level. In human cells, when the Ca<sup>2+</sup> diminishes in the ER, the Ca<sup>2+</sup> sensor, stromal interacting molecule 1 (Stim1) forms oligomers [712]. These oligomers are translocated to the plasma membrane and interact with Orai1 a component of calcium-release activated calcium (CARC), a plasma membrane Ca<sup>2+</sup> channel [710, 713-715]. The association of Stim1 and Orai1 leads to the opening of the channel and therefore the entrance of extracellular Ca<sup>2+</sup> in the cytoplasm. On the other hand, there is an intracellular regulation of Ca<sup>2+</sup> levels involving exchange between organelles and cytosol. The main organelle which controls Ca<sup>2+</sup> homeostasis is the ER. The ER is the main calcium storage organelle. This organelle can capture or release Ca<sup>2+</sup> in the cytoplasm. The Ca<sup>2+</sup> gradient between the ER and the cytoplasm is controlled by Ca<sup>2+</sup> binding proteins, Ca<sup>2+</sup> transporters and channels [716]. Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA) is a calcium pump localized in the ER. SERCA is present in almost all the eukaryote suggesting an important role of these pump in cell function [717]. When high levels of Ca<sup>2+</sup> are reached in the cytoplasm, SERCA is activated; Ca<sup>2+</sup> enters in the ER and the cytoplasmic Ca<sup>2+</sup> level decreases. The Ca<sup>2+</sup> entering in the ER is sequestered by Ca<sup>2+</sup> binding proteins such as Calsequestrin. Calsequestrin has been first identified in the muscular cells where it localizes in the interior membrane of the sarcoplasmic reticulum, which is the smooth ER found in smooth and striated muscle. Calsequestrin has an high affinity for Ca<sup>2+</sup> and is able to sequester calcium in the sarcoplasmic reticulum [718]. Other Ca<sup>2+</sup> binding proteins have been identified in the ER. The main Ca<sup>2+</sup> buffering protein in the ER is Calreticulin. These Ca<sup>2+</sup> binding proteins participate in the maintenance of the Ca<sup>2+</sup> pool by sequestering Ca<sup>2+</sup> in the ER. Indeed, the overexpression of Calreticulin in the ER leads to the accumulation of Ca<sup>2+</sup> in the ER [719]. Ca<sup>2+</sup> binding proteins are thus required to sequester Ca<sup>2+</sup> in the ER if the cytoplasmic Ca<sup>2+</sup> concentration is too high. In addition to Ca<sup>2+</sup> uptake and storage, ER can also release Ca<sup>2+</sup>. If Ca<sup>2+</sup> levels are too low in the cytoplasm, Ca<sup>2+</sup> is released from the ER. The export of Ca<sup>2+</sup> from the ER involves the cytoplasmic inositol-1,4,5-trisphosphate (IP3) [720]. IP3-receptor (IP3-R) is localized on the ER membrane. When calcium level is low in the cytoplasm, IP3 binds to IP3-R. IP3-R is calcium channel and the binding of IP3 in its receptor leads to the opening of the channel. Ca<sup>2+</sup> channel opening induces Ca<sup>2+</sup> exit from the ER and leads to cytoplasmic Ca<sup>2+</sup> level increased. Thus the ER Ca<sup>2+</sup> homeostasis is controlled by two Ca<sup>2+</sup> transporters SERCA and IP3R which have opposite effect on Ca<sup>2+</sup> flux. The functional homologues of the Ca<sup>2+</sup> trafficking regulators have been identified in *C. elegans*,

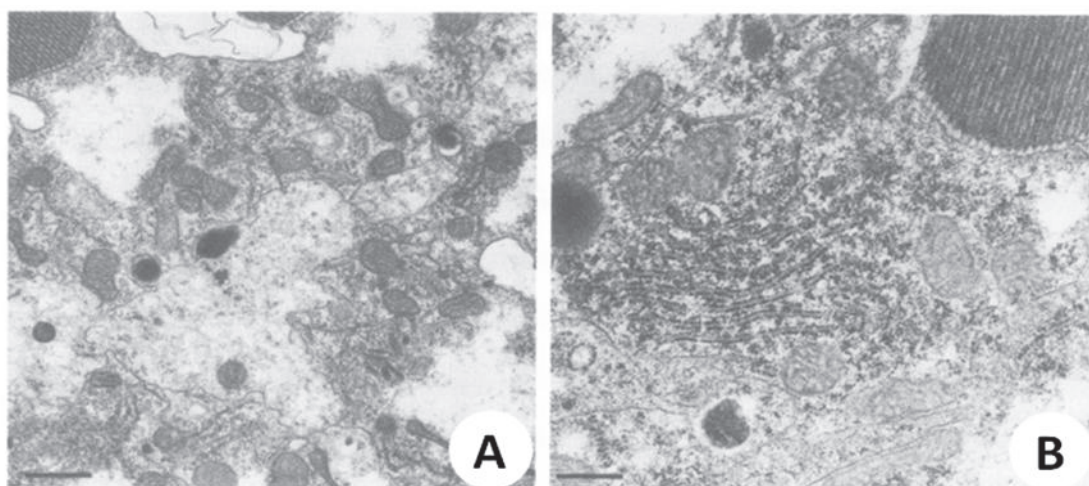
*Drosophila* and humans [714, 715, 717, 721, 722]. These  $\text{Ca}^{2+}$  trafficking regulators are all involved in the control of intracellular  $\text{Ca}^{2+}$  homeostasis. Control of intracellular  $\text{Ca}^{2+}$  homeostasis is thus a highly conserved mechanism in evolution which confirms the essential role of  $\text{Ca}^{2+}$  for cellular functions.

### **1.3.4. ER stress and the unfolded protein response**

#### **1.3.4.1. ER stress**

The ER stress is caused by the accumulation of unfolded proteins in the ER. The accumulation of unfolded proteins can be due to the expression of high amount of newly synthesized proteins or to defects in the folding machinery. For example, ER stress has been detected *in vivo* in specialized cells, which produce high level of secreted or transmembrane proteins such as pancreatic, salivary gland or photoreceptor cells [723]. For example, pancreatic cells in mammals produce large amount of insulin, B-lymphocytes secrete antibodies and photoreceptor cells produce opsin [38, 724-727]. These cells produce large amount of proteins, which transit in the ER. Thus proteins accumulating in the ER generate an ER stress. In addition, the accumulation of unfolded protein can be caused by a reduction of the folding efficiency. Folding efficiency can be altered by ER environment changes or modifications of the amino acid sequence of folded proteins. The ER environment is very important for protein folding. Modification of the temperature,  $\text{Ca}^{2+}$  levels or reducing condition can induce the accumulation of misfolded proteins. For example, ER stress can be induced by oxidative stress. Indeed,  $\text{H}_2\text{O}_2$  treatment on PC12 cell induced ER stress [728]. Another oxidative stress inducer, the paraquat, activates the UPR *in vitro* in rat dopaminergic neurons [729]. Moreover, ER stress can be induced by drugs, which directly modify the ER environment or functions. For example, the dithiothreitol (DTT) is a reducing agent which alter the oxidative environment of the ER, thapsigargin alters  $\text{Ca}^{2+}$  homeostasis in the ER, and Tunicamycin inhibits glycosylations [635, 706, 730]. All these drugs alter protein folding in the ER and therefore lead to ER stress [35, 731]. In addition to ER environment condition, ER stress can be induced by mutations in the amino acid sequence of both the chaperones and the folded protein. For example, ER stress has been detected in the pancreas of Akita mouse, which carries a conformation-altering missense mutation in Insulin 2 [732]. ER stress is also detected *in vitro* in cell expressing mutated P23H Rhodopsin, which accumulates in the ER

[733]. Rhodopsin mutations also induce ER stress in vivo in rat model for autosomal dominant retinitis pigmentosa (ADRP) [38, 727]. In all these models, protein mutations prevent their folding, therefore proteins accumulate in the ER inducing ER stresses. The accumulation of misfolded proteins can be induced by the mutation of the chaperone. For example, mutation in the chaperone Neither inactivation nor afterpotential A (NinaA) prevent Rhodopsin1 (Rh1) folding in the *Drosophila* photoreceptors [693]. Thus *ninaA* mutation leads to Rh1 accumulation, ER expansion and ER stress (**Figure 7**). Misfolded protein accumulation in the ER has also been associated to several human pathologies. Indeed, ER stress has been detected in patients with neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), retinitis pigmentosa (RP), Parkinson or Huntington diseases [27, 32, 734, 735]. The cause of ER stress induction in these diseases is not clearly identified. As oxidative stress has also been detected in these diseases, it has been suggested that this oxidative stress could be the cause of misfolded protein accumulation and ER stress. The role of ER stress in cell death observed in these diseases is not clearly identified. The role of ER stress in cell death regulation and in particular in neurodegenerative disease will be discussed in 1.3.5 and 1.3.6 Parts. It has been shown that the accumulation of the misfolded protein in the ER can be deleterious for cell [686, 734-736]. However, even if some cells contain a lot of unfolded protein in the ER, like secreting cells, they survive to this stress. To cope with the accumulation of misfolded protein in the ER, the cells have an adaptive response named the unfolded protein response (UPR).



**Figure 7:** Mutation in NinaA leads to an accumulation of ER. (A) Photoreceptor cells from flies with functional *ninaA* display normal ER morphology (bar = 0.5  $\mu$ m). (B) Photoreceptor cells from flies that carry mutant *ninaA* (*ninaA*<sup>P269</sup>) display accumulations of ER cisternae (bar = 0.25  $\mu$ m). (adapted from [7])

#### 1.3.4.2. The UPR

The unfolded protein response (UPR) is activated upon ER stress. The UPR involves resident proteins of the ER, which directly or indirectly sense the presence of unfolded proteins. In mammals, the UPR is mediated by three transmembrane proteins: inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6). Upon ER stress IRE1, PERK and ATF6 are activated. In normal conditions, the chaperone BiP/GRP78 binds to and inhibits IRE1, PERK and ATF6. When misfolded proteins accumulate in the ER, the chaperone BiP binds to them [635], and therefore released its inhibition on IRE1, PERK and ATF6. In this situation, IRE1, PERK and ATF6 activate their downstream targets (**Figure 8**). Upon ER stress, these three proteins induce three pathways, which are called the UPR. The aim of the UPR is to reduce the unfolded protein load in the ER [737-739]. To achieve this goal, the UPR acts on different levels: (1) the increase of protein degradation via ERAD, (2) the increase of expression of chaperones such as BiP/GRP78 for protein folding [740] and (3) the attenuation of translation to reduce the amount of proteins which enter in the ER. The three branches of the UPR act differently on these three mechanisms.

The UPR is a conserved mechanism in evolution, however, depending of the organism, the UPR effectors are different. In mammals, the UPR is complex and involves three different pathways mediated by IRE1, PERK and ATF6. However, all these branches are not conserved in evolution (**Figure 8**). IRE1 is the only branch of the UPR conserved from yeast to mammals [737]. In yeast, IRE1 pathway is the only branch of the UPR as there is not homologue of PERK or ATF6. As in yeast, *Drosophila* UPR involves the IRE1 pathway. In addition, as in mammal, it also involves PERK pathway. The role of the third branch of the UPR, ATF6, has not been characterized in *Drosophila*. Indeed, even if a *Drosophila* homologue of ATF6 has been identified, its role in UPR is not elucidated. IRE1 is thus the more conserved branch of the UPR and the other branches, PERK and ATF6, appeared later in evolution.

##### 1.3.4.2.1. IRE1/Xbp1

The inositol-requiring enzyme 1 (IRE1) is a transmembrane protein of the ER which has a kinase and endoribonuclease activity conserved in evolution. IRE1 is localized on the ER

membrane of all eukaryotic cells [741] (**Figure 8**). IRE1 has been first identified in yeast then in *C. elegans*, *Drosophila* and mammals [742, 743]. Mice and human homologues of IRE1 have been identified by sequence homology compare to the yeast sequences [744, 745]. Mammals have two IRE1 genes: IRE1 $\alpha$  is essential for viability and is broadly expressed, and IRE1 $\beta$  is expressed only in the gastrointestinal mucosa which is a tissue often exposed to ER stress. IRE1 is characterized as a type 1 transmembrane protein containing a cytoplasmic domain, which contains a Ser/Thr protein kinase domain and a domain homologous to RNase L [745].

IRE1 activation is a major branch of the UPR. IRE1 is essential to protect cell from ER stress. Indeed, the studies of IRE1 mutants showed that IRE1 was required for cell survival in particular upon ER stress. Mutant mice for IRE1 $\beta$  are more sensitive to dextran, an ER stress inducer, than control mice. This shows that IRE1 is essential for ER stress protection [746]. IRE1 protective effect is mediated by the activation of major player of the UPR. For example, IRE1 inhibition in *C. elegans* blocks Hsp4 (BiP homologue). This result shows that IRE1 pathway induces the expression of the Hsp4. Thus, via the activation of the chaperone proteins, IRE1 pathway can enhance folding capacity of the ER, which participates in the UPR. Upon ER stress, IRE1 activity is regulated by the formation of IRE1 dimers and oligomers on the membrane of the ER [747, 748]. Indeed, it has been shown that quercetin, a drug which induces IRE dimerization, activates the IRE1 branch of the UPR even in absence of ER stress [749]. Thus the regulation of IRE1 dimerization appears essential for the regulation of IRE1. Dimerization of IRE1 involves the N-terminal luminal domain (NLD). NLD is conserved in evolution as the yeast sequence can be replaced by the human sequence without any change in IRE1 activity [750]. This domain is characterized by its capacities to bind with other NLD to form dimers [751]. The NLD mediated formation of IRE1 oligomers can be controlled by BiP or misfolded proteins binding on IRE1 [752]. Indeed, it has been shown that IRE1 NLD can interact with BiP/GRP78 [751]. The interaction of BiP on NLD prevents IRE1 dimerization leading to IRE1 inhibition. Upon ER stress, BiP/GRP78 preferentially binds to misfolded protein and detaches from IRE1. This allows the dimerization of IRE1 and the activation of IRE1 [753, 754]. However, in yeast, it has been shown that BiP is not required for IRE1 regulation. Indeed, in BiP mutant, IRE1 was almost normally regulated in an ER-stress dependent manner [755]. This IRE1 regulation independent of BiP is controlled by the direct binding of misfolded proteins to IRE1 in yeast [755]. It has been proposed that upon ER stress unfolded protein interacts with IRE1



enhancing the formation of oligomers. As IRE1 has a Ser/Thr protein kinase activity, the oligomerization of IRE1 by either BiP or misfolded proteins induces its trans-autophosphorylation. Autophosphorylation of IRE1 controls its activity. Indeed, the inhibition of IRE1 phosphorylation leads to the over-activation of IRE1 which could be deleterious for the cell [756].

IRE1 has also an endoribonuclease activity which promotes unconventional splicing of XBP1/Hac1, a transcription factor (**Figure 8**). The splicing of factor X box-binding protein 1 (XBP1) mRNA consists in the elimination of an intron of 26 nucleotides in mouse and 21 nucleotides in *Drosophila* [35, 757]. The elimination of the intron by IRE1 induces a shift in the coding sequence of XBP1, which leads to the production of a protein named XBP1 spliced (XBP1s) which is shorter than the one translated from the unspliced mRNA [757]. XBP1s is a transcriptionally active XBP1 which stimulates the transcription of its target genes. In *Saccharomyces cerevisiae*, IRE1 splices the mRNA encoding the transcription factor Hac1, the homolog of XBP1. The splicing of Hac1 mRNA by IRE1 allows the translation of a functional transcription factor. The functional Hac1 induces the expression of all the proteins involved in the UPR like chaperones, ERAD associated proteins or Hac1 itself [758, 759]. Thus, in yeast and in metazoans, IRE1 controls the expression of all the UPR effectors via the splicing of *hac1* mRNA and *xbp1* mRNA respectively [731, 757, 760]. The splicing of *xbp1* mRNA by IRE1 is a conserved mechanism activated upon ER stress. For example, in the *Drosophila* eye, the splicing of *xbp1* mRNA can be detected upon ER stress induced by accumulation of mutated of Rhodopsin1 in the ER [761]. Importantly, misfolded protein need to accumulate in the ER to induce IRE. For example, in some neurodegenerative disease model, cytoplasmic protein aggregation did not activates the UPR. Huntingtin-Q128 (Htt-Q128) or Tau R406W, which are classical models of cytoplasmic aggregates, have been overexpressed in the *Drosophila* eye disc. In these models, misfolded protein accumulation in the cytoplasm do no induces the splicing of *xbp1* mRNA [761]. These results show that in *Drosophila* as in mammals, the alternative splicing of *xbp1* mRNA by IRE1 is specifically induced by misfolded protein accumulation on the ER. *Xbp1* mRNA splicing leads to the production of a functional transcription factor XBP1s which induces the expression of different targets. The XBP1 targets are characterized by the presence of XBP1s binding sites in their promoter. XBP1s can bind to different binding sites such as the unfolded protein response element (ERSE I and II) and also UPRE A or UPRE B [762, 763]. Among the genes which contain XBP1 binding sites, there are luminal ER chaperones and proteins involved in

ERAD. For example, in *C. elegans*, XBP1s induces the expression of major proteins of the UPR such as BiP/GRP78 [757]. In mammals, targets of XBP1s are also involved in the ER quality control. Thus, via the activation of XBP1, IRE1 improves ER folding capacities and activates the ERAD. Moreover, XBP1s induces the expression of itself and also of PERK and ATF4 component of another branch of the UPR. This result suggests that XBP1s induces an amplification loop of the UPR [762].

XBP1 function has evolved in evolution. In yeast, IRE1-HAC1 pathway is the only component of the UPR and therefore is in charge of the transcription regulation of all UPR associated genes such as ER chaperones or ERAD associated factors (**Figure 8**). However, in mammals, only some of the UPR associated factors are controlled by XBP1. For example, it has been shown that XBP1 did not induce CHOP expression which is a key downstream target of the UPR [757, 763]. Moreover, it has been shown that XBP1 mutation in MEF only partially inhibited BiP expression [763]. These results indicate that, in mammals contrary to yeast, UPR mediated transcription activation is not only controlled by IRE1-XBP1 pathway. The other branches of the UPR, ATF6 and PERK, are also involved in the transcriptional regulation of UPR associated genes (**Figure 8**). The role of ATF6 and PERK pathway in transcription activation will be respectively described below.

IRE1 reduces general protein expression via mRNA decay. IRE1 has an endoribonuclease activity which can cleave *xbp1* mRNA but also other mRNA. IRE1 activation leads to the degradation of specific mRNA [764, 765]. The mRNA degraded by IRE1 contains specific sequence which contain hydrophobic residue. The degradation of these mRNA by IRE1 is called the regulated IRE1-dependant decay (RIDD), which is conserved from *Drosophila* to mammals [766-768]. In *Drosophila*, 120 mRNAs degraded by IRE1 have been identified such as Laminin B1 subunit 1, Neural cell adhesion molecule 1, Scavenger receptor class A, member 3 (Scara3) or Mannose receptor C type 2 [767]. Moreover, ER chaperone such as BiP has been identified as a target of RIDD [768]. Thus via RIDD, IRE1 can regulate different cellular function but also regulates ER chaperone expression. Interestingly, the 28S ribosomal mRNA is another target of RIDD. Via the degradation of 28S ribosomal mRNA, RIDD reduces ribosome activity and thus leads to a global decrease of translation [769]. Therefore, via RIDD, IRE1 could reduce amount of newly synthesized proteins in the ER and thus limits ER stress. On the contrary, as *bip* mRNA is a target of RIDD, this mRNA decay can reduce the folding capacity of the ER enhancing ER stress. Interestingly, RIDD activation correlates



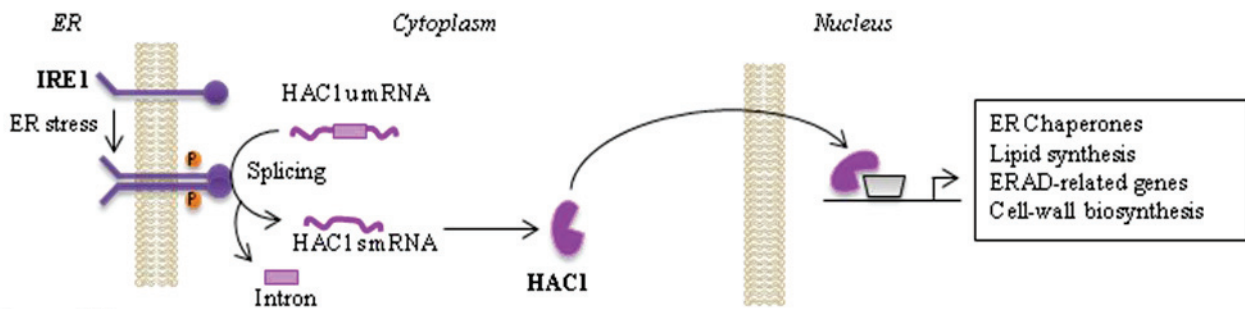
with apoptotic cell which confirm the deleterious effect of RIDD. Moreover, the endoribonuclease activity of IRE1 cleaves *ire1* mRNA itself [770]. This suggests the existence of a negative feedback loop in IRE1 activation that requires RIDD.

IRE1 activates the c-Jun NH2-terminal kinase (JNK) pathway via the interaction with JNK regulators. JNK pathway is activated upon ER stress. Indeed, P54, a c-Jun NH2-terminal kinase (JNK), is activated in human cell treated with the ER stress inducers tunicamycin and thapsigargin [771-773]. The induction of JNK upon ER stress is dependent of the UPR and in particular of the IRE pathway. Indeed, in rat cells, IRE1 over-expression induces JNK activation [774]. The cytoplasmic part of IRE1 can interact with TRAF2 and JIK (human STE20-related serine/threonine Kinase), which mediates JNK pathway regulation. In normal cell, IRE1 interacts with JIK. Upon ER stress, TRAF2 binds to IRE1/JIK, which induced the recruitment apoptosis-signaling regulating kinase 1 (ASK1) in the complex [775, 776]. ASK1 recruitment in the complex leads to JNK pathway [774]. Interestingly, the inhibition of either JIK or TRAF2 suppresses the induction of JNK pathway upon ER stress [775]. Thus, UPR via IRE1/JIK/TRAF2/ASK1 complex activates JNK pathway [773, 774]. It has been proposed that in mammalian cells JNK activation in response to ER stress may be an important to determine cell to live or die [772]. The role of JNK in cell fate determination upon ER stress will be described in 1.3.5.2 Part.

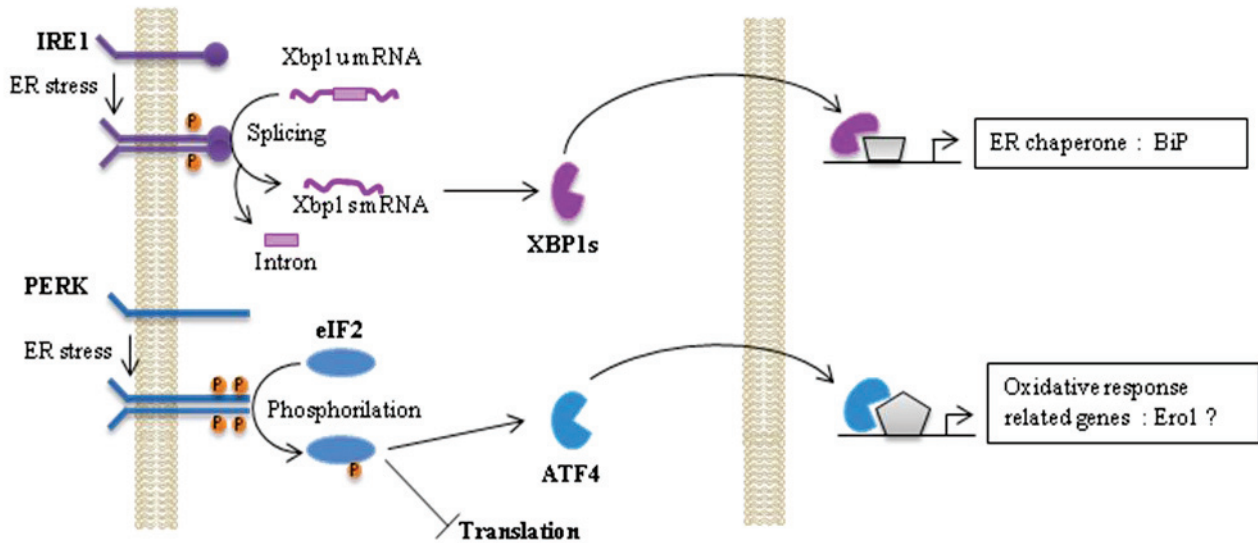
#### 1.3.4.2.2. ATF6

Activating transcription factor 6 (ATF6) is a transcription factor conserved in evolution in all metazoan. There is one ATF6 gene in *C. elegans* and *Drosophila* and two ATF6 genes in mammals: ATF6 $\alpha$  and ATF6 $\beta$  [777, 778]. ATF6 is important for UPR in mammals. Indeed, MEF mutant for ATF6 $\alpha$  have an increased sensitivity to ER stress [779]. The role of Atf6 is conserved in *C. elegans*. Even if *C. elegans* treated with Atf6 or Ire1 RNAi are viable, the combination of ATF6 and IRE1 inhibition induced larval lethality [777]. This result suggests that ATF6 and IRE1 have redundant effect. Moreover, it has been shown that XBP1 and ATF6 have common targets [777]. Altogether, these results show that, ATF6 function in the UPR is partially redundant with IRE1 in *C. elegans*. ATF6 function is thus conserved in *C. elegans* and in mammals. However, the involvement of ATF6 in *Drosophila* UPR has not been characterized yet.

## Yeast



## Drosophila



## Mammals

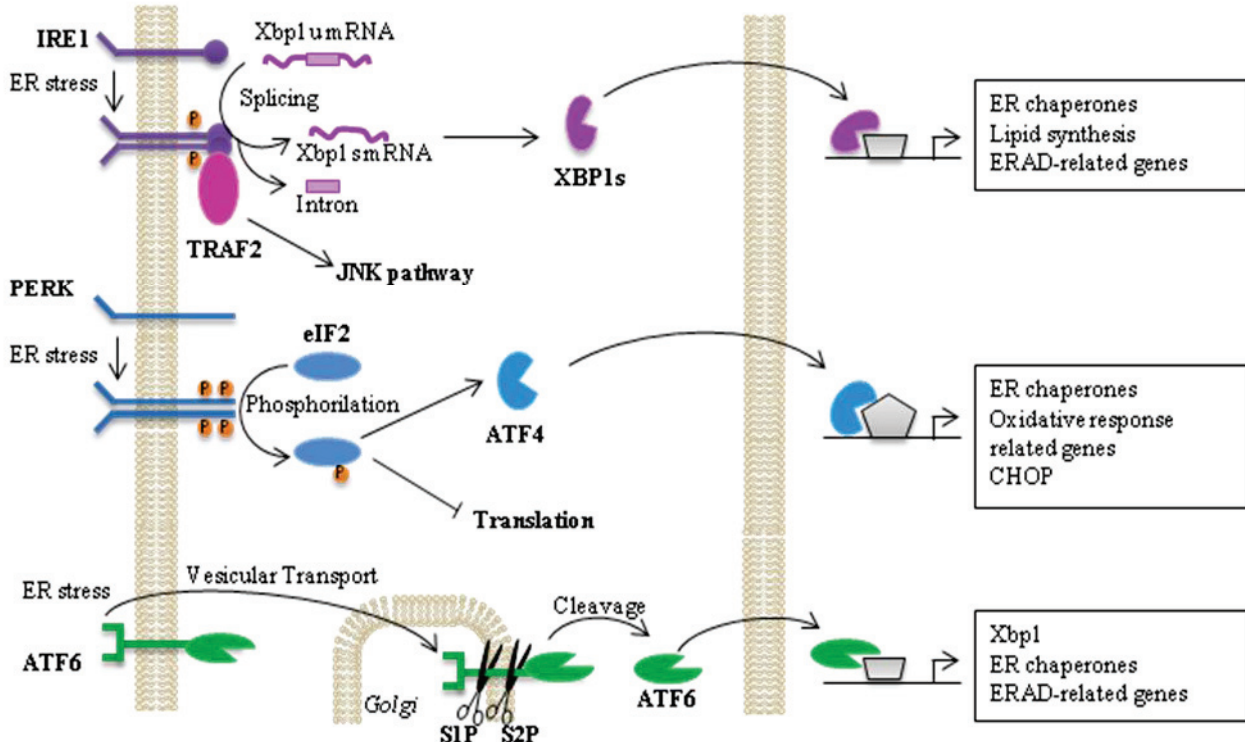


Figure 8: UPR pathways in yeast, *Drosophila* and mammals

ATF6 is a transcription factor localized on the ER membrane. ATF6 contains a transmembrane domain, which anchors ATF6 in the ER membrane. The cytoplasmic domain of ATF6 contains a bZIP domain and the luminal domain contains CD1 and CD2 domains which sense ER stress and are required for ATF6 activation [780]. Upon ER stress condition, ATF6 is activated and is translocated in the Golgi [780] (**Figure 8**). In this organelle, ATF6 is then cleaved by proteases of the Golgi: the site-1 proteases in its ER luminal domain and by site-2 proteases in its transmembrane domain [781]. The cleavage of ATF6 produces a 56kDa protein which, contrary to full length ATF6, is not associated to the membrane but exported to the nucleus [782]. The cytoplasmic domain of ATF6 enters in the nucleus and acts as a transcription factor [780].

ATF6 is a bZIP transcription factor involved in the UPR. ATF6 $\alpha$ , as XBP1, binds on ERSE sequence and leads to the expression of chaperone proteins and ERAD associated protein [763]. Indeed, ATF6 $\alpha$  or ATF6 $\beta$  inhibition in MEFs dramatically reduces the expression of ERAD components, ER chaperones [779]. In addition to genes with ERSE sequence, ATF6 has other targets involved in the UPR. In contrast to XBP1, ATF6 induces the expression of CHOP [783]. Moreover, ATF6 induces the expression of XBP1 [763, 784, 785]. Thus the ATF6 pathway enhances XBP1 function by increasing its expression. Furthermore, ATF6 also enhances XBP1 activity by forming heterodimers with XBP1. These heterodimers have a higher transcriptional activity than the homodimers. These results indicate that ATF6 enhances XBP1 activity and thus the IRE1 pathway. In mammals, ATF6 is a key component of UPR via the transcription regulation and the enhancement of other branch of the UPR.

#### 1.3.4.2.3. PERK

PKR-like ER kinase (PERK) is an ER transmembrane kinase involved in the UPR (**Figure 8**). PERK has an ER luminal domain similar to IRE1 [786]. Indeed the IRE luminal domain can be replaced by PERK luminal domain without affecting IRE1 function [750]. This experiment shows that PERK luminal domain, as IRE1 luminal domain, is able to sense ER stress. PERK can sense ER stress via its interaction with BiP. Upon ER stress, BiP is detached from PERK, which forms dimers via the interaction of luminal domain [787]. PERK dimers formation leads to its autophosphorylation on both serine and threonine residues via kinase activity [788]. Thus, the activation of PERK and IRE1 upon ER stress is very similar, with dimerization and autophosphorylation. However, these pathways diverge on the kinetic of their activation and

their downstream effects in the UPR. PERK is the first pathway activated upon ER stress. PERK downstream effectors are thus the first effectors of the UPR. Upon ER stress, PERK induces eIF-2 phosphorylation which leads to translation attenuation [789].

PERK induces a general attenuation of protein translation via the phosphorylation of eIF-2 $\alpha$  upon ER stress that will reduce the load of newly synthesized proteins in the ER. Activated PERK specifically phosphorylates eIF-2 $\alpha$  on serine-51 [788] (**Figure 8**). Indeed, the cytoplasmic domain of PERK contains a protein-kinase domain similar to interferon-inducible RNA-dependent protein kinase (PKR) and haem-regulated eIF-2 $\alpha$  kinase (HRI), which are known kinase for eIF-2 $\alpha$ . In response to ER stress, PERK could therefore phosphorylate and activate eIF-2 $\alpha$ . eIF-2 $\alpha$  interferes with the translation of mRNA into proteins. The activation of eIF-2 $\alpha$  induces a general reduction of protein synthesis upon ER stress. Indeed, eIF2 mutant MEFs cannot reduce protein translation [790]. This result confirmed that translation inhibition is mediated by eIF-2 $\alpha$  upon ER stress. The inhibition of protein synthesis is required for ER stress protection. Indeed, PERK mutant cells are more sensitive to ER stress induced by tunicamycin. However, a treatment with cycloheximide, an inhibitor of protein synthesis, protects PERK mutants from ER stress [789]. So, the reduction of protein synthesis via the PERK branch of the UPR improves cell survival upon ER stress. However, cycloheximide does not rescue totally PERK mutant hypersensitivity to ER stress suggesting that PERK has other downstream effectors.

Despite its role in translation attenuation, PERK can induce a specific transcription increase via eIF-2 $\alpha$  phosphorylation. Upon PERK activation, phosphorylated eIF-2 $\alpha$  specifically induces the specific stress-related mRNA such as the activating transcription factor 4 (ATF4) and GADD34 [791]. ATF4 targets can control pro-survival genes that are related to redox balance, amino acid metabolism, protein folding and autophagy [792, 793]. Thus, PERK can control cell death or survival via eIF2 mediated translation activation [794]. For example, the transcription factor Nrf2, which control the anti-oxidative response, is a target of the PERK branch [795]. When PERK is activated, the Nrf2-Keap1 complex dissociates and Nrf2 induces the expression of anti-oxidant proteins which enhance cell survival [796]. However, PERK can also activates toxic protein expression like C/EBP homologous transcription factor (CHOP) also referred as growth arrest and DNA damage gene 153 (Gadd153), which is an apoptosis inducer activated by ER stress [797]. Gadd34, another target of PERK, induces a negative feedback on eIF-2 $\alpha$  inducing its dephosphorylation and thus regulates the PERK

branch of the UPR [798]. The inhibition of eIF2 $\alpha$  by Gadd34 reactivates protein translation and could therefore enhance the expression of UPR proteins. Thus, PERK branch of the UPR induces both a general reduction of protein synthesis and a specific induction of transcription which can control cell death or survival

### **1.3.5. UPR and cell death**

Upon ER stress, two types of UPR can be distinguished depending on the stress level and on the downstream effectors of the UPR. After ER stress, mammalian cells first activate IRE1, PERK and ATF6 to reduce ER stress. However, if the ER stress is severe or prolonged, the UPR cannot control anymore the stress and the three branches of the UPR switch to cell death induction [737, 799].

#### **1.3.5.1. UPR : an adaptive response**

UPR activation allows the cell to cope with ER stress. The 3 branches of the UPR: IRE1, ATF6 and PERK reduce the ER stress via transcription and translation regulation. IRE1 and ATF6 are dedicated for transcription induction of chaperones and ERAD associated proteins. PERK inhibits translation and also induces specific transcription. These pathways are essential for cell survival upon ER stress. Indeed, mutants for these pathways present enhanced cell sensitivity to ER stress. For example, the inhibition of eIF-2 $\alpha$  phosphorylation leads to pancreatic  $\beta$  cell death [790]. Moreover, XBP1 over-expression protects neurons from axon injury induced cell death [15]. On the contrary, the chemical induction of XBP1 with quercetin protects against ER stress caused by calcium dynamics deregulation in intestinal epithelial cells [800]. In addition, extinction of a single UPR branch in Multiple Myeloma cells promotes a caspase-independent programmed cell death [801]. The activation of UPR upon ER stress is thus essential for cell survival. However, even if all the UPR branches seem to be required for cell survival upon ER stress, they are not identical and do not respond the same way to ER stress.

The three branches of the UPR are differentially activated depending of the ER stress inducer. IRE1, PERK and ATF6 do not have the same sensitivity to ER stresses. This different sensitivity allows the adaptation of UPR to stress exposition. Depending of the ER stress inducers, the UPR pathways are differentially activated [802]. IRE1 phosphorylation occurs

rapidly after different ER stresses: glycosylation inhibition with tunicamycin,  $\text{Ca}^{2+}$  alteration with thapsigargin or disulfide bond disruption with DTT. On the contrary, ATF6 and PERK respond more specifically to certain stresses. ATF6 is more efficiently activated by disulfide bond disruption and PERK responds better to changes in  $\text{Ca}^{2+}$  release [802]. Therefore, IRE1 branch of the UPR seems to be a general response to ER stress whereas ATF6 and PERK seem to be more specific of some ER stress inducers.

In addition to the specific sensitivity to ER stress inducers, there is a time dependent activation of the UPR. Indeed, each branch of the UPR has different effects. These effects have to be coordinated to allow the activation of an effective UPR. For example, it has been shown that upon ER stress, eIF-2 $\alpha$  phosphorylation inhibits protein translation to reduce protein accumulation in the ER whereas, the other branches of the UPR, IRE1 and ATF6, requires the activation of translation to produce chaperone and ERAD associated proteins. Thus, eIF-2 $\alpha$  mediated translation inhibition has to be downregulated to allow the activation of an effective UPR. Activation of eIF-2 $\alpha$  induces its own inhibitor GADD34. GADD34 acts as negative feedback loop on eIF-2 $\alpha$ , which reactivates protein translation. Another feedback loop, which involves IRE1, ATF6 and BiP/Grp78, controls the UPR. Indeed IRE1 and ATF6 pathways induce the expression of BiP/Grp78. As discussed previously, BiP/GRP78 can bind to and inhibits IRE1 and ATF6. Thus IRE1 and ATF6 induce their own inhibitor which prevents over-activation of the UPR. UPR is thus a reversible mechanism and when the ER stress diminishes, the UPR is blocked. The inhibition of UPR branches after the coming back to normal situation is key for cell survival.

ER stress could activate a hormesis process which enhances cell tolerance to cellular stresses. Hormesis is a cellular protective signal induced by exposure to a low dose of stress-inducing agent that allows the cell to better respond to a second insult [803, 804]. The hormesis process is also termed pre-conditioning. The hormesis consists in the pre-activation of protective cellular response. The hormesis process can be induced by several mild stresses such as irradiation, antibiotics, alcohols or metals treatment [805-810]. Hormesis has medical perspective. Indeed, it has been shown that ischemia pre-conditioning can reduce infarct size [811]. The protective effect of hormesis has been associated to the activation of protective mechanisms by intracellular organelles such as ER or mitochondria. The first organelle linked hormesis is mitochondria. Stimulation of ROS production by the mitochondria at a sub-lethal level enhances organism survival. This mitochondria mediated hormesis is named mitohormesis [812]. In addition to mitochondria, it has been shown that the ER stimulation



can also activate a hormesis process. Indeed, ER pre-conditioning effect has been observed in different cell injury contexts. For example, ER stress can protect from retinal endothelial inflammation [813]. ER stress also enhances oxidative stress tolerance [814, 815]. The induction of mild ER stress is thus a preconditioning mechanism called ER-hormesis, which enhances resistance to cellular insults.

#### **1.3.5.2. UPR activation can lead to cell death**

ER stress and UPR can lead to cell death. The UPR helps the cell to cope with the ER stress, but if ER stress is prolonged and cannot be reduced by the UPR, the UPR induces cell death mechanisms. Indeed high doses of chemical ER stress inducers lead to cell death. For example, thapsigargin induced apoptosis of Jurkat T cell [772]. Tunicamycin treatment on human neuroblastoma SK-N-SH cells induced classical features of apoptotic cell death. Moreover, the reduction of ER stress can increase cell survival upon stress condition. The inhibition of ER stress with VPA, a histone deacetylase (HDAC) inhibitor, protects neurons from apoptosis following ischemia reperfusion [816]. In addition, ER stress contributes to CRH-induced hippocampal neuron apoptosis [817]. ER stress can lead to cell death and several components of apoptosis induction have been detected upon ER stress [737, 818].

Caspases are involved in ER stress-induced cell death. The Caspases-12, -4 and -2 are localized in the ER [37, 819, 820]. They could be involved in ER stress-induced apoptosis [739]. The first caspase identified associated to ER stress was Caspase-12. It has been shown that upon ER stress, the pro-caspase-12 is cleaved and forms dimers. The activation of the initiator Caspase-12 involves either IRE1 or  $\text{Ca}^{2+}$  levels. Caspase-12 is activated by IRE1 pathway and in particular by the IRE1 interacting protein TRAF2 [775]. In basal condition, the Caspase-12 interacts with TRAF2. Upon ER stress, TRAF2 favors Caspase-12 phosphorylation and dimerization [775]. Caspase-12 activation is also  $\text{Ca}^{2+}$  dependent. Upon ER stress, the increase level of  $\text{Ca}^{2+}$  in the cytoplasm triggers Calpain activation, a calcium-activated neutral cysteine endopeptidase, which activates Caspase-12 [37]. Upon ER stress Caspase-12 interacts with Caspase-9 and triggers Caspase-3 activation and apoptosis [821]. Caspase-12 activates caspases like Caspases-9 and -3 independently of APAF-1 and Cytochrome C [822, 823]. Thus ER stress induces cell death independently of the mitochondria via Caspase-12. Caspase-12 is a functional gene in mouse, however, the *caspase-12* gene in human produces a non functional protein [824]. This observation suggests

that other caspases could be involved in ER stress induced apoptosis. In human, Caspase-4 is an ER localized caspase. This caspase is activated upon ER stress and like Caspase-12 forms dimers. The reduction of *caspase-4* expression by small interfering RNA decreases ER stress-induced apoptosis [819]. It has thus been proposed that Caspase-4 is the human counterpart of Caspase-12 and induce apoptosis upon ER stress. Interestingly, Caspase-4 is upregulated in the brain of patient with infantile neuronal ceroid lipofuscinosis (INCL), which is a neurodegenerative disease associated to ER stress. *In vitro* experiment shown that Caspase-4 inhibition is cytoprotective in INCL cells [825]. These results show that Caspase-4 is required for ER stress mediated apoptosis. Even if Caspases-4 and -12 seem to be involved in ER stress induced apoptosis, cells which do not express these caspases are still sensitive to ER stress which suggests the activation of other cell death inducers upon ER stress [826]. In mammals, ER stress-induced cell death also involves Caspase-2 [827]. Indeed Caspase-2 inhibition rescues from ER stress induced cell death [828]. Cell death induced by Caspase-2 has been further characterized. It has been shown that Caspase-2 induced cell death via the mitochondria and is dependant of the BH3-only protein BID [828]. Thus in mouse, ER stress mediated apoptosis involves, Caspases-2, -4 and -12 whereas in human, ER stress mediated apoptosis caspases 2 and 4 but not caspase 12.

The UPR pathways induce apoptosis by regulating Bcl-2 family. CHOP, a target of PERK and ATF6, can regulate Bcl-2 family gene expression such as Bcl-2, BIM or MCL-1 [829]. CHOP inhibits the expression of the Bcl-2, an anti apoptotic factor and up regulates pro-apoptotic factors like BH3 only protein BIM or MCL-1 [739]. Indeed, it has been shown that upon ER stress induced by several drug such as thapsigargin, the pro-apoptotic member of bcl2 family BIM is overexpressed. BIM overexpression is mediated by CHOP [830]. In addition to proapoptotic Bcl-2 family members activation, CHOP inhibits prosurvival members of the Bcl-2 family. CHOP overexpression enhances MEF sensitivity to ER stress inducer such as thapsigargin. This enhanced sensitivity is associated to Bcl2 downregulation [831]. Thus, CHOP is a key inducer of the ER stress mediated apoptosis via the mitochondrial pathway and the regulation of Bcl-2 family member expression [739, 830]. The Bcl-2 family members can also be regulated by the IRE1 pathway. IRE1 physically bind to Bax and Bak, which are pro-apoptotic members of the Bcl-2 family [832]. The interaction between Bax, Bak and IRE1 regulates IRE1 activity. Indeed, *bak*<sup>-/-</sup> *bax*<sup>-/-</sup> double mutant prevent IRE1 mediated activation of JNK [833]. Moreover, Bax and Bak are required for ER stress induced cell death as the double mutant cells for Bak and Bax are resistant to ER stress cell death



[834]. It has thus been proposed that Bak and Bax induced ER stress mediated cell death via the JNK pathway. The regulation of the Bcl-2 family mediates the ER stress induced cell death.

In addition to Bcl-2 family regulation, CHOP induces cell death enhancing misfolded protein accumulation in the ER. Indeed, CHOP enhances ER stress blocking the UPR and favoring protein aggregation formation. CHOP activates Gadd34, an inhibitor of, eIF-2 $\alpha$ . Gadd34 mediates dephosphorylation of eIF-2 $\alpha$ , which reactivates protein translation leading to the increase of unfolded protein accumulation in the ER. Therefore CHOP, via Gadd34 and eIF2 inhibition, enhances ER stress [835]. Moreover, CHOP enhances ER stress via the induction of protein aggregation. CHOP induces the overexpression of Ero1, mammalian homolog of a yeast ER oxidase. Ero1 enhances disulfide bond formation and increase protein aggregate formation which is toxic for cells. On the contrary Ero1 mutant cells are protected against ER stress [835]. Thus by overexpressing Ero1, CHOP increases protein aggregation in the ER and thus enhances ER stress.

JNK pathway is involved in ER stress induced cell death. IRE1 is associated with regulators of the JNK pathway such as TRAF2 and c-Jun NH2-terminal inhibitory kinase (JIK) and also Cdk5 and Mekk1. Upon ER stress, IRE1 binds to TRAF2 and JIK inducing JNK pathway. In addition, *cdk5* or *mekk1* mutations prevent ER stress mediated JNK activation in the *Drosophila* eye. JNK pathway has been involved in cell death regulation and could lead to cell death. Upon ER stress, the inhibition of JNK by dominant negative MKK4/7 and JNK1/2 suppresses cell death [773]. Moreover the thapsigargin induced cell death requires the activation of JNK in Jurkat cells [772]. These results show that JNK activation via the IRE1 pathway leads to cell death. Moreover, the connection between IRE1 and JNK pathway in the induction of cell death has been highlighted *in vivo* in rat ischemia [836]. Indeed, upon ischemia reperfusion, UPR and JNK activation markers are detected in rat brains. Interestingly IRE1 and JNK activation correlates with the activation of caspase-12. The inhibition of JNK prevent the Caspase-12 inhibition [836]. Thus, it has thus been proposed that upon ischemia reperfusion, IRE1 mediated JNK activation induces apoptosis.

### **1.3.5.3. Switch from adaptive response to apoptosis**

Upon ER stress the UPR activates different mechanisms, which could be either protective or deleterious for cells. An important question is how cells switch from an adaptive response to cell death. Several studies showed that there was a time regulation of the UPR branches, which can control cell fate. After ER stress induction, IRE1, ATF6 and PERK are activated. But, when the UPR is prolonged and toxic, IRE1 and ATF6 branches of the UPR are switch off and only the PERK pathway remains activated [733, 837]. Thus IRE1 (and ATF6) may be the protective pathways of the UPR. The protective role of IRE1 has been supported by the artificial induction of IRE1 upon sustained ER stress, which improved cell survival [733]. On the contrary, PERK specific activation leads to cell death via CHOP activation [837]. It has therefore been proposed that IRE1 pathway controls survival while PERK is associated with cell death.

IRE1 activity is timely controlled upon ER stress. Upon prolonged ER stress, the IRE1 branch of the UPR is downregulated. Therefore, modulators of IRE1 activation should exist to control IRE1 activity. Heat shock protein 90 (Hsp90) is a modulator of IRE1. Hsp90 is a cytoplasmic protein which binds to kinases and promotes their stabilization. Hsp90 interacts with the cytoplasmic domain of IRE1. Hsp90 stabilizes IRE1 and thus could enhance IRE1 activity. Indeed, inhibition of Hsp90 leads to the enhanced degradation of IRE1 [838]. Another regulator of IRE1 function is Bax inhibitor 1 (BI-1). BI-1 has been identified as a suppressor of Bax induced cell death [839]. In addition to its role in the regulation of apoptosis, BI-1 has been associated to UPR. Indeed, BI-1 is a transmembrane protein of the ER, which is involved in the regulation of the IRE1. Indeed, it has been shown that BI-1 interacts with and inhibits IRE1 [840]. The inhibition of BI-1 in MEF cell leads to the activation of IRE1 [840, 841]. On the contrary, over-expression of BI-1 suppresses IRE1 activity [840]. Moreover, BI-1 inhibits IRE1 mediated activation of autophagy [842]. Thus BI-1 regulation could therefore be involved in the control of IRE1 activity. BI-1 is regulated by bifunctional apoptosis regulator (Bar). Bar is an ER-associated RING-type E3 ligase. Bar interacts with BI-1, promotes BI-1 ubiquitination and leads to BI-1 degradation in the proteasome [843]. Interestingly, Bar level is reduced upon a prolonged ER stress. These results suggests that upon moderates ER stress, BI-1 could be degraded via its interaction with Bar which could allow the activation of IRE1. However, upon prolonged UPR, Bar levels is

reduced leading to the increase of BI-1 level and the inhibition of IRE1. IRE1 switch off upon prolonged ER stress could therefore depend of the degradation of BI-1 via Bar.

The regulation of IRE1 downstream effectors is a key factor for cell fate upon ER stress. The IRE1 pathway has been defined more as a protective pathway as its activation is reduced upon toxic ER stress. However, IRE1 can induce both a protective pathway via XBP and a deleterious pathway via JNK and RIDD. It has been shown that IRE1 can either induce XBP1 or JNK depending of experimental conditions. IRE1 can specifically induce XBP1 splicing. Indeed, ectopic IRE1 expression upon ER stress induces only XBP1s and not JNK activation [733]. Moreover, a fragment of the kinase domain of IRE1 (F6) has been identified as a specific regulator of XBP1 splicing by IRE1 [844]. F6 enhances IRE1 oligomerization and XBP1 splicing. However, neither JNK nor RIDD are activated by F6. Interestingly, the specific activation of XBP1 without JNK by F6 enhances cell resistance to ER stress which confirms that XBP1 is protective whereas JNK is deleterious [844]. IRE1 and ATF6 overexpression showed that IRE1 is able to induce specifically XBP1 without JNK. Thus, IRE1 cofactors could exist *in vivo* to regulate IRE1 specific downstream activity. Hsp72 has been identified as a regulator of IRE1. Indeed, the specific induction of XBP1s by IRE1 can be induced by Hsp72 overexpression. Hsp72 also blocks JNK activation and protects from cellular stresses [845]. On the contrary, JNK can be specifically induced by IRE1. Indeed, the overexpression of Bax in the ER which interacts with IRE1, leads to the induction of JNK. In this context of Bax overexpression, JNK is activated but not XBP1 and this leads to cell death [846]. Thus, Hsp72 seems to favor XBP1 inductor whereas BAX favors JNK activation. In addition to IRE1 cofactors, the specific induction of XBP1 or JNK could also be controlled by the phosphorylation or the oligomerization of IRE1. The overexpression of a fragment of IRE1 kinase domain which does not enhance IRE kinase activity but favors oligomerization of IRE1, enhances XBP1 splicing and inhibits JNK [844]. Moreover, mutations in IRE1 kinase domain abolish its kinase activity but do not alter its RNase activity [756]. It suggests that IRE1 phosphorylation is not required for XBP1 splicing but could be required for apoptotic signal induction [768]. These experiments suggest that oligomerization of IRE1 favors XBP1 splicing whereas, IRE1 phosphorylation favors JNK pathway induction. IRE1 can more specifically induce either XBP1 or JNK and thus switches from survival to apoptotic signal.

### 1.3.6. ER stress is detected in neurodegenerative diseases

Diseases	URP detection	Articles
ALS	Induction of <b>elf2a</b> , <b>ATF4</b> , <b>Gadd45</b> in motoneurons in FALS <b>mice</b> model	Saxena 2009
	Induction of <b>XBP1</b> and <b>CHOP</b> in <b>cell</b> over-expressing mutant FUS	Farg 2012
Parkinson	Increased levels of <b>GRP78</b> , <b>GRP94</b> , and <b>PDI</b> , activation of <b>xbp1</b> in $\alpha$ -synucleinopathy <b>mouse</b> model	Colla 2012
	Induction of <b>PERK</b> and <b>ATF6</b> in <b>rat</b> model of Parkinson	Gorbatyuk 2012
	<b>CHOP</b> , <b>IRE1</b> , <b>PERK</b> , <b>BiP</b> increased after 6-OHDA treatment on <b>cell</b>	Ryu 2002
Alzheimer	<b>XBP1</b> activation in <i>Drosophila</i> tauopathy model	Loewen 2010
Huntington	Enhancement of <b>XBP1</b> splicing in mHtt <b>mouse</b> model	Vidal 2012
optic nerve crush injury	increased <b>CHOP</b> , <b>Gadd45</b> , <b>BiP</b> levels and splicing of <b>xbp1</b> in <b>mouse</b>	Hu 2012
Ischemia reperfusion	Variation of <b>XBP1</b> after ischemia reperfusion in <b>rat</b>	Lehotsky 2009
Retinis pigmentosa	<b>eIF2a</b> , <b>ATF4</b> , <b>ATF6</b> and <b>XBP1</b> genes were upregulated in <b>Rat</b> RP model	Shinde 2012
spinal cord injury	induction of <b>XBP1s</b> , <b>BiP</b> , and <b>CHOP</b> after spinal cord injury in <b>mouse</b>	Valenzuela 2012

**Table 3:** Unfolded protein response detection in animal models of neurodegenerative diseases

Reference : Saxena 2009 [2], Farg 2012 [10], Colla 2012 [13], Gorbatyuk 2012 [17], Ryu 2002 [18], Loewen 2010[11], Vidal 2012[27], Hu 2012 [15], Lehotsky 2009 [36], Shinde 2012 [38], Valenzuela 2012 [25]

ER stress is associated to neurodegenerative diseases. ER stress has been detected upon neurodegeneration *in situ*, *in vivo*, and *in vitro* (**Table 3**, [847, 848]). UPR markers have been detected in tissues of neurodegenerative diseases patients. For example, ATF4 induction and XBP1 splicing have been detected in spinal cord tissue of ALS patients [32]. UPR activation has also been detected in post-mortem brain of patient with Parkinson, Alzheimer or Huntington diseases. Indeed, PERK and eIF2 phosphorylation are detected in dopaminergic neurons in the substantia nigra of Parkinson disease cases [849, 850]. Moreover, phosphorylated PERK, IRE1, and eIF2 and detected in hippocampus of the Alzheimer disease

patients [851]. *Xbp1* mRNA splicing is detected in brain of Huntington disease patient [27]. The induction of ER stress and the activation of UPR upon neurodegeneration have been further studied in animal models of neurodegenerative diseases. It has been shown that these models recapitulate the UPR activation observed in patients (**Table 3**). Indeed, the activation of the Perk pathway has been observed in mice model of ALS [2]. Moreover, in vitro model of ALS also shown that the Xbp1 splicing was activated [10]. It has also been shown both *in vitro* and *in vivo* in Parkinson disease model that the three branches of the UPR are activated [13, 17, 18]. The IRE1 pathway is also activated in *Drosophila* model of Alzheimer disease and in mice model of Huntington disease. Altogether, these results suggest that the activation of UPR pathways and thus ER stress is associated to neurodegeneration. However, the role of UPR activation in neurodegenerative disease progression remains to be characterized.

## **2. Results**





## 2.1. Article 1 : ER stress protects from retinal degeneration

The study of the cell death regulator pathway is essential to understand pathologies such as neurodegenerative disease. Cell death allows the proper function of an organism. Any deregulation of cell death process can induce pathologies such as cancer or degenerative diseases. To characterize and find therapies for these diseases, it is essential to characterize the regulation of cell death process. The last years have been very successful in identifying mechanisms, which control apoptosis in metazoan. However, the regulation of cell death in specific cell type remains to be determined. An excess of neuron apoptosis can lead to neurodegenerative diseases such as Huntington, Parkinson or Alzheimer diseases. Neurodegeneration is usually associated to Endoplasmic Reticulum stress (ER stress), autophagy or oxidative stress [21-24]. However, the role of these mechanisms in the regulation of neurodegeneration is not clearly established. The objective of this article was to characterize the role of ER stress in the control of neuron apoptosis.

The ER is an organelle where secreted and transmembrane proteins are folded. These proteins acquire their proper conformation via their interaction with resident ER chaperones. Only properly folded proteins can be exported from the ER to be secreted or addressed to membrane. The accumulation of misfolded or unfolded proteins induces an ER stress can be deleterious for the cell. Thus, the folding process has to be tightly control by the ER quality control system. In presence of misfolded protein, the ER quality control system induces the unfolded protein response (UPR). The UPR is an adaptive response to the ER stress, which is conserved in evolution. Indeed, most the effectors of the UPR identified in mammals are conserved in *Drosophila* [35]. In mammals, the UPR is activated by BiP, a sensor of the ER stress. In basal condition, BiP is associated the transmembrane proteins of the ER: PERK, ATF6 and IRE1. Upon ER stress, BiP binds to the misfolded protein and thus released its inhibition on IRE1, PERK and ATF6. These proteins activate three pathways which induce chaperone expression, the activation of the ER associated degradation and the reduction of protein synthesis. These processes allow the cell to cope with ER stress. However, several studies have shown that an intense and prolonged ER stress can lead to the activation of cell death [37]. The UPR can thus be toxic for the cell. However, this toxic effect of ER stress and

UPR activation in neurodegenerative diseases has not been established. The conservation of the cell death regulatory pathways and of the UPR leads us to use simple genetic model such as *Drosophila*, to study the role of the ER stress and UPR in the regulation of neuronal death.

The *Drosophila* eye is an excellent model to study *in vivo* neurodegenerative diseases. Indeed, the ectopic expression of proteins, associated to neurodegenerative diseases, in the *Drosophila* retina induces the progressive loss of photoreceptor cells (PRCs) [41]. For example, the overexpression of protein with polyglutamine repeats induced a small eye phenotype due to an excess of cell death [852]. We have established a new model of neurodegeneration in the *Drosophila* eye via the overexpression of pro-apoptotic genes such as *reaper* or *P53* in the differentiated PRCs. The over-expression of *reaper* induces a progressive apoptosis of the PRCs in the *Drosophila* eye.

We identified ER stress as a protective process from neurodegeneration. We used the over-expression of *Reaper* or *P53* in the *Drosophila* retina as a model of neurodegeneration to identify new regulators of neuronal cell death. We identified mutations in *ninaA* as suppressor of neuron apoptosis. *NinaA* is a chaperone protein required for the folding of Rhodopsin-1 (Rh1) in the reticulum. The inhibition of *NinaA* induces the accumulation of misfolded Rh1 leading to ER stress and UPR activation. We have shown that the protective effect of *ninaA* mutation was due to the activation of ER stress. We have thus shown that ER stress activation in the *Drosophila* eye can lead to protect neurons from apoptosis. Moreover, we have shown *in vitro* that UPR mediates the protective effect of ER stress. The chemical induction of ER stress with tunicamycin or thapsigargin protected S2 cells from cell death induced by cycloheximide, H<sub>2</sub>O<sub>2</sub> or ultra-violet exposition. However, the RNAi mediated inhibition of *xbp1* suppressed the protective effect of tunicamycin.

The cell exposed to ER stress induced an anti-oxidative response, which could protect from cell death. The anti-oxidative is activated to protect cells from apoptosis induced by reactive oxygen species (ROS). ROS can be released from the mitochondria or the ER. The oxidative stress can also be induced by iron. Indeed, free iron can produce free radical via the Fenton reaction. Thus to limit ROS production, the anti-oxidant response involves ferritin, a multi-protein complex which sequester iron. In addition, the anti-oxidant response mainly activates enzymes that directly act on the ROS synthesis pathway such as superoxide dismutase (SOD), catalase and Glutathion S transferase [45]. We have shown that anti-oxidant response and in

particular ferritin are activated upon ER stress. A ferritin transcriptional activity reporter is activated in *ninaA* mutant *Drosophila* PRCs. Moreover, a target of the anti-oxidative stress d4-EBP is induced in *ninaA* mutant retina. Thus, we have shown that the anti-oxidant response is activated upon ER stress. However, the contribution of anti-oxidant response in the protective effect of ER stress remains to be elucidated.



# ER stress protects from retinal degeneration

César S Mendes<sup>1,4</sup>, Clémence Levet<sup>2</sup>,  
Gilles Chatelain<sup>2</sup>, Pierre Dourlen<sup>2</sup>,  
Antoine Fouillet<sup>2</sup>, Marie-Laure  
Dichtel-Danjoy<sup>2</sup>, Alexis Gambis<sup>1</sup>,  
Hyung Don Ryoo<sup>3</sup>, Hermann Steller<sup>1</sup>  
and Bertrand Mollereau<sup>2,\*</sup>

<sup>1</sup>Howard Hughes Medical Institute, The Rockefeller University, New York, NY, USA, <sup>2</sup>Laboratory of Molecular Biology of the Cell, Ecole Normale Supérieure de Lyon, Lyon, France and <sup>3</sup>Department of Cell Biology, New York University School of Medicine, New York, NY, USA

The unfolded protein response (UPR) is a specific cellular process that allows the cell to cope with the overload of unfolded/misfolded proteins in the endoplasmic reticulum (ER). ER stress is commonly associated with degenerative pathologies, but its role in disease progression is still a matter for debate. Here, we found that mutations in the ER-resident chaperone, Neither inactivation nor afterpotential A (*NinaA*), lead to mild ER stress, protecting photoreceptor neurons from various death stimuli in adult *Drosophila*. In addition, *Drosophila* S2 cultured cells, when pre-exposed to mild ER stress, are protected from H<sub>2</sub>O<sub>2</sub>, cycloheximide- or ultraviolet-induced cell death. We show that a specific ER-mediated signal promotes antioxidant defences and inhibits caspase-dependent cell death. We propose that an immediate consequence of the UPR not only limits the accumulation of misfolded proteins but also protects tissues from harmful exogenous stresses.

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## Introduction

The unfolded protein response (UPR) is an evolutionary conserved adaptive response to perturbations of normal endoplasmic reticulum (ER) physiology. Strikingly, the UPR is implicated in several human pathologies, such as neurodegeneration, diabetes and cancer (Marciniak and Ron, 2006; Lin *et al.*, 2008). The UPR engages several responses, including transcriptional upregulation of ER-resident chaperones, selective inhibition of translation and activation of ER-associated degradation (ERAD) (reviewed in Ron and Walter, 2007). Despite a large body of work in this field, little is

known about the cellular consequences of the UPR upon physiological levels of ER stress *in vivo*.

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

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

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

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

\*Corresponding author. Laboratory of Molecular Biology of the Cell, Ecole Normale Supérieure de Lyon, 9 rue du Vercors, Lyon 69007, France. Tel.: +33 472 728 163; Fax: +33 472 728 674; E-mail: bertrand.mollereau@ens-lyon.fr

<sup>4</sup>Present address: Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY, USA

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

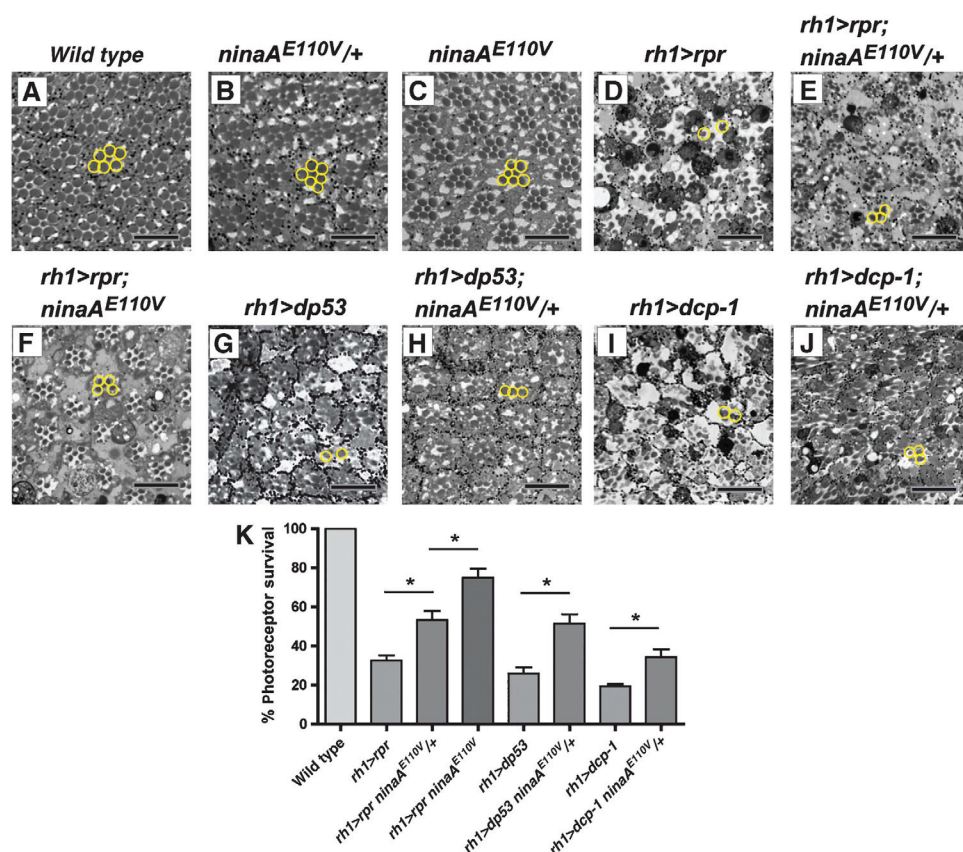
## Results

### PRCs exposed to ER stress exhibit death resistance

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

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

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



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



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

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

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

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

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

We next tested whether the resistance to PRC death, induced by loss of *ninaA*, is due to the accumulation of misfolded Rh1 in the ER. If true, reducing the amount of Rh1 in *ninaA* mutant retinas should reduce resistance to PRC death. Using a *rh1* null allele (*ninaE<sup>117</sup>*, here termed *rh1<sup>117</sup>*), we found that double heterozygous mutants for *ninaA* and *rh1* exhibited similar death sensitivity as wild-type PRCs (Figure 2B–E and G). In addition, PRCs carrying a heterozygous *rh1* null mutation in an otherwise wild-type background were not protected from cell death (Figure 2D). This indicates that it is the accumulation of unfolded Rh1 in the *ninaA* mutant retina responsible for the death resistance of PRCs and not the loss of Rh1 function. We then tested whether cells exposed to unfolded Rh1 in the ER could phenocopy the protective effect seen in *ninaA* mutant retinas. There are several dominant *rh1* mutations leading to Rh1 misfolding and accumulation in the ER (Colley *et al*, 1995). PRCs maintain their integrity for up to 45 days under normal light and temperature conditions, but dominant *rh1* mutations cause a slow age-dependent retinal degeneration when exposed to constant light. (Kurada and O'Tousa, 1995, and data not shown). We examined PRC death sensitivity in *rh1<sup>G69D</sup>* retina in 2-day-old adults submitted to *dp53* or *rpr* expression. As in *ninaA* mutant retina, PRCs exhibited strong resistance to *dp53* (Figure 2F and G) and *rpr* expression (data not shown).

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

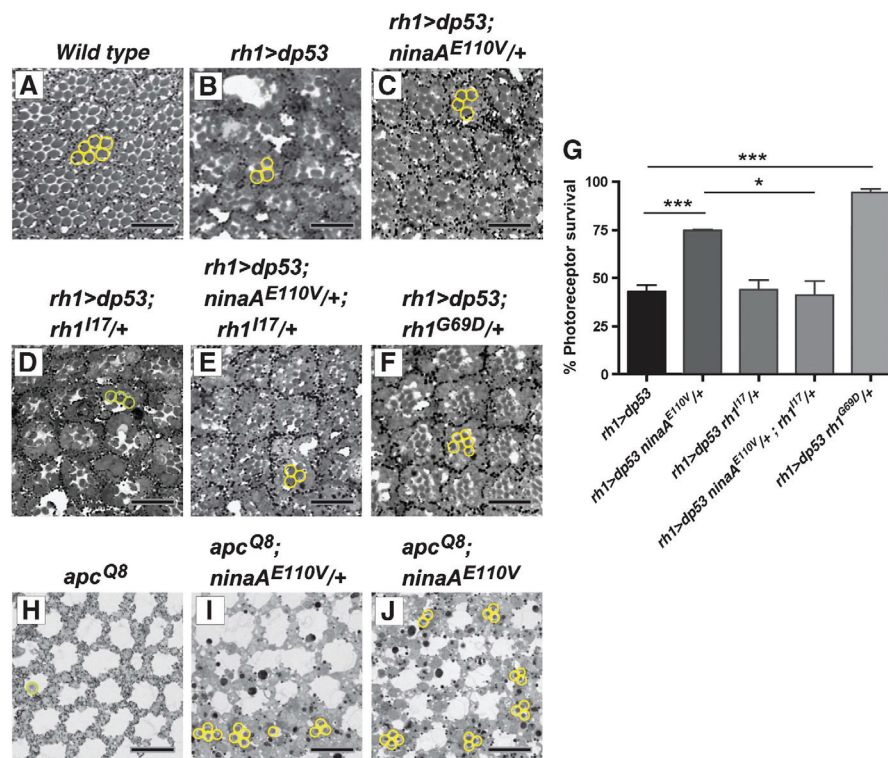
accumulation of unfolded Rh1 in the ER is responsible for the increased resistance to death in PRCs.

### Misfolded Rh1 induces an UPR

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

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





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

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

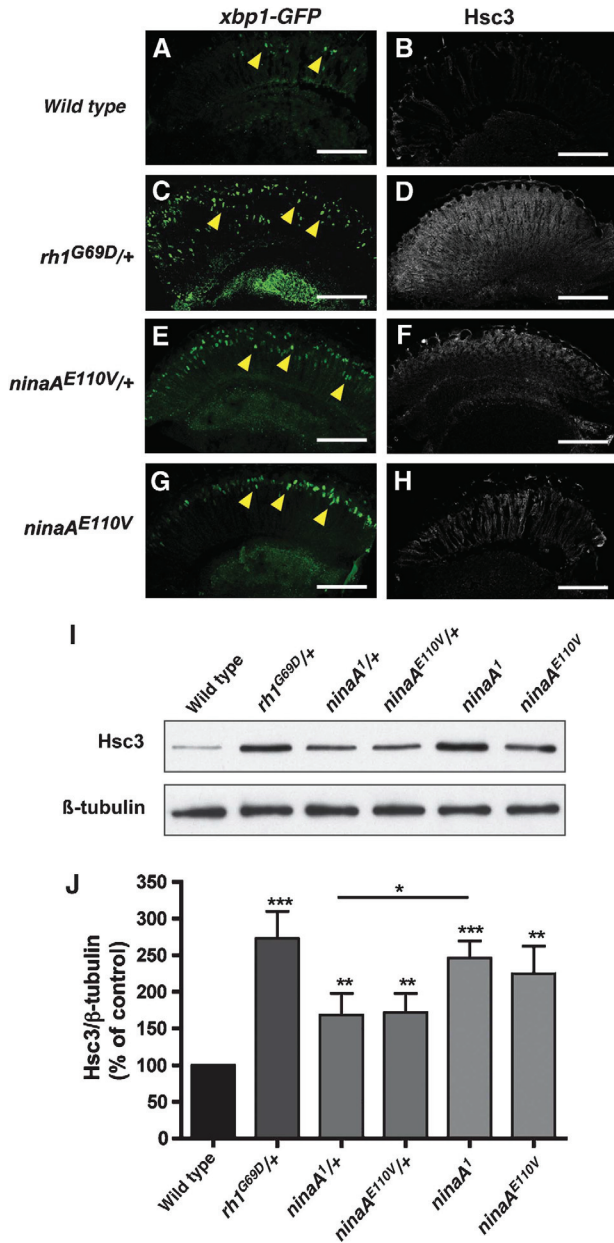
#### Prolonged and moderate ER stress does not lead to neurodegeneration

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

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

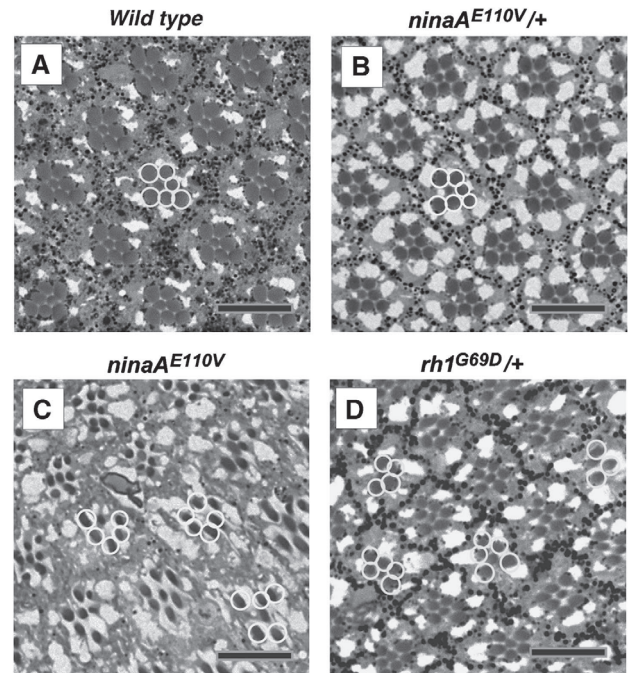
#### An ER-mediated signal inhibits caspase activation

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

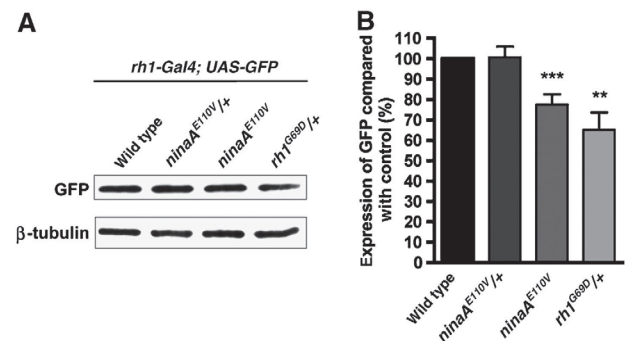


**Figure 3** *xbp1* splicing and Hsc3 expression are increased in *ninaA* mutant retinas. Horizontal retina cryosections are stained with an antibody raised against GFP (A, C, E, G) or against Hsc3 (B, D, F, H) in 7-day-old flies. *xbp1:GFP* is expressed in PRCs under the control of *rh1* promoter (*rh1* > *xbp1:GFP*). Scale bar, 100 μm. (A) Wild-type PRCs expressing *xbp1:GFP* show weak GFP staining. (C) *rh1<sup>G69D</sup>/+*, (E) *ninaA<sup>E110V</sup>/+* or (G) *ninaA<sup>E110V</sup>* retina expressing *xbp1:GFP* display an increased GFP staining in PRC nuclei (yellow arrowheads). (B) Wild-type retinas do not exhibit Hsc3 staining. (D) *rh1<sup>G69D</sup>/+* retinas show a marked Hsc3 staining. (F) *ninaA<sup>E110V</sup>/+* and (H) *ninaA<sup>E110V</sup>* retinas show weak Hsc3 staining. (I) Western blot analysis performed on fly eye tissue. *ninaA<sup>E110V</sup>/+* or *ninaA<sup>1</sup>/+* exhibit a clear Hsc3 increase compared with wild-type retina. Hsc3 protein level is further increased in *ninaA<sup>1</sup>* compared with *ninaA<sup>1</sup>/+*. The Hsc3 level in *rh1<sup>G69D</sup>/+* is increased to a level similar to that in *ninaA* amorphs. Representative blot of four independent experiments. (J) Quantification of Hsc3 immunoreactivity. The results are expressed as mean percentage of wild-type control ± s.e.m. of four independent experiments (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 in Student's *t*-test).

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



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



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

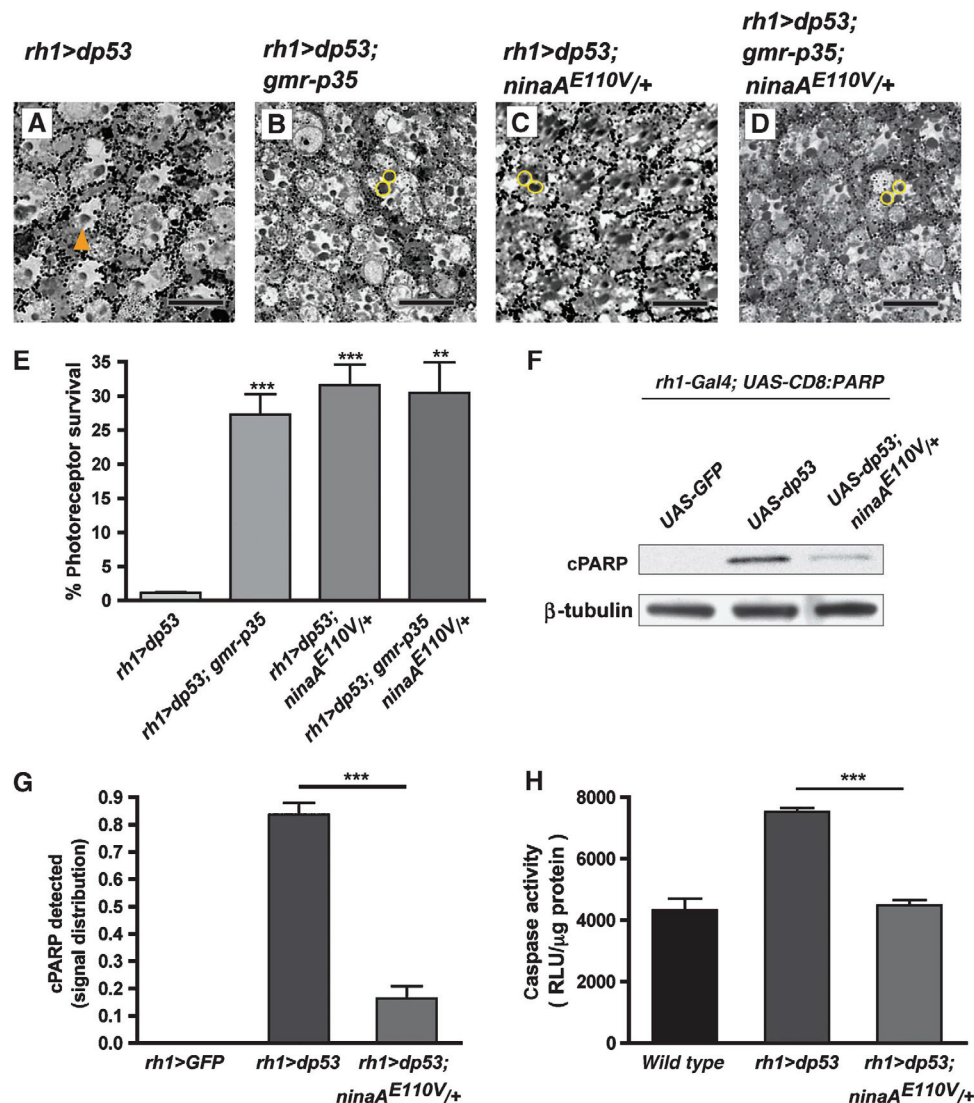
β-galactosidase activity with an ONPG assay in *ninaA<sup>E110V</sup>* and *rh1<sup>G69D</sup>* mutants (Supplementary Figure 3). No difference was observed between the different genotypes, indicating



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

Next, we examined whether ER stress inhibits caspase activation. If *ninaA* mutants block primarily caspases sensitive to the baculovirus caspase inhibitor p35,

then *ninaA* mutations should not show an additive effect on PRC protection mediated by p35 expression. We took advantage that p35 only partially suppresses *dp53*-induced death (Hsu *et al*, 2002; Figure 6 and data not shown). We found that *dp53*-induced PRC death was equally inhibited by the expression of p35 or by *ninaA* mutation. Moreover, p35-mediated caspase inhibition was not enhanced by *ninaA* mutations (Figure 6A–E). These results suggest that *ninaA* mutation blocks primarily p35-inhibitable caspases in PRCs submitted to *dp53*-mediated PRC death.



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

We then examined the effector caspase activation levels in PRCs submitted to ER stress by measuring PARP cleavage and using a DEVD-based caspase activity assay. We first assayed cleavage of the genetically encoded caspase probe CD8:PARP:Venus as a read-out for effector caspase activation (Williams *et al*, 2006). We found that loss of *ninaA* strongly suppressed the *dp53*-induced cleavage of CD8:PARP:Venus in PRCs as visualized by western blot analysis (Figure 6F and G). Next, we measured effector caspase activity in retina extracts using a caspase activity assay (Caspase Glo<sup>®</sup>). DEVD cleavage is strongly impaired in heterozygous *ninaA*<sup>E110V</sup> mutants compared with wild-type retinas (Figure 6H), suggesting an inhibition of *Drosophila* interleukin-1 converting enzyme (Drice) activity (Fraser *et al*, 1997). Together with the observation that *ninaA* mutants inhibit *dcp-1*-induced apoptosis (Figure 1I and J), our results indicate that an ER-mediated protective signal inhibits the activation of effector caspases.

### An antioxidant response is induced upon ER stress

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

The *Drosophila* eIF4E binding protein (d4E-BP) is induced in response to oxidative stress (Tettweiler *et al*, 2005). Although d4E-BP is not an antioxidant protein *per se*, it downregulates translation of cap-dependent mRNA and mediates survival in animals exposed to oxidative stress. Whether ER stress is capable of inducing *d4E-BP* expression is not known. We examined *d4E-BP* levels in retinas submitted to ER stress. We found that PRCs under ER stress exhibit an increase of *d4E-BP* transcriptional activity and protein expression compared with wild-type conditions (Figure 7E–I; Supplementary Figure 4B). We used the enhancer trap line Pz [*d4E-BP*<sup>P06270</sup>] to evaluate *d4E-BP* transcriptional activity in

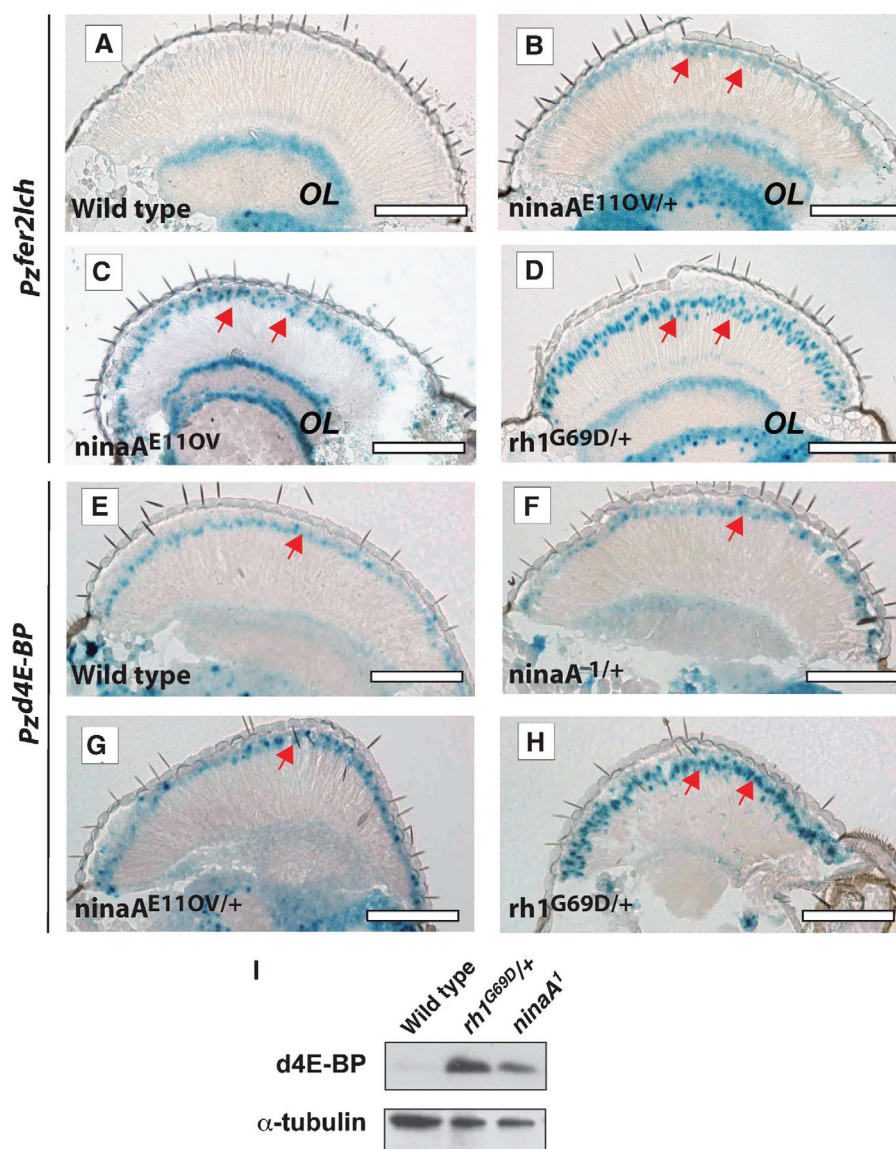
*ninaA* and *rh1* mutant retinas. We observed that the *rh1*<sup>G69D</sup> mutant exhibited a marked increase in  $\beta$ -galactosidase activity staining compared with the wild-type retina (Figure 7H). Increased staining in *ninaA*<sup>E110V</sup> and *ninaA*<sup>1</sup> heterozygotes was modest, which is consistent with the fact that *ninaA* heterozygous mutants exhibit a weaker UPR than in *rh1*<sup>G69D</sup> retinas (Figure 3J). We next tested whether the upregulation of *d4E-BP* transcriptional activity is associated with an increase of d4E-BP protein expression in western blots of *ninaA* or *rh1* mutant retinas (Figure 7I). Retinas submitted to ER stress (*ninaA*<sup>1</sup>, *ninaA*<sup>E110V</sup> and *rh1*<sup>G69D</sup> alleles) exhibited an increase of d4E-BP protein compared with wild type (Figure 7I, and data not shown). This result shows that PRCs under ER stress trigger a stress response marked by the induction of *ferritin* and *d4E-BP* genes.

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

We then explored whether an ER-mediated stress response is capable of protecting from reactive oxygen species (ROS)-induced cell death. We used an *in vitro* approach in which pharmacologically induced ER stress protects cultured cells from apoptosis. *Drosophila* S2 cells can activate an UPR when submitted to tunicamycin (Tm, a glycosylation inhibitor) or thapsigargin treatment (Tg, an ER calcium storage inhibitor) (Plongthongkum *et al*, 2007; Ryoo *et al*, 2007). When pre-treated with a sub-lethal dose of Tm or Tg, S2 cells exhibit oxidative stress resistance to H<sub>2</sub>O<sub>2</sub> (Figure 8A, and data not shown). This result suggests that moderate ER stress can protect cells from oxidative stress. We did not observe an increase in *ferritin* expression in S2 cells, suggesting that these cells may induce the expression of other antioxidant genes to promote an antioxidant response. In contrast, we found that Tm or Tg treatments induced a dose dependent increase of d4E-BP proteins (Figure 8B). This result is consistent with the fact that PRCs submitted to ER stress exhibit an increase of d4E-BP protein expression.

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

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



**Figure 7** ER stress triggers upregulation of the *fer2lch* and *d4E-BP* genes. (A–H) Horizontal retinal cryosections stained for nuclear β-galactosidase activity from flies carrying the transposable P-element line inserted in *fer2lch* (Pz[ry, lacZ]<sup>fer2lch035</sup>) (A–D) or *d4E-BP* (Pz[ry, lacZ]<sup>d4E-BP</sup>) (E–H). Arrows point to nuclear β-galactosidase activity staining in PRC nuclei (B–H). (A) Wild-type retinas exhibit a *fer2lch* basal background staining in the optic lobe but no apparent staining in the retina. (B) *ninaA<sup>E110V/+</sup>*, (C) *ninaA<sup>E110V</sup>* and (D) *rh1<sup>G69D/+</sup>* retinas exhibit increased β-galactosidase activity staining at the level of outer PRC nuclei. (E) Wild-type retina exhibit some *d4E-BP* staining in nuclei. *d4E-BP* expression is merely increased in *ninaA<sup>1/+</sup>* (F) and *ninaA<sup>E110V/+</sup>* (G) mutant retinas, but a marked increase is observed in *rh1<sup>G69D/+</sup>* (H). Scale bar, 100 μm. (I) Western blot analysis of d4E-BP protein levels in *rh1<sup>G69D/+</sup>* and *ninaA<sup>1</sup>* compared with wild-type fly eye tissues. Representative blot of three independent experiments.

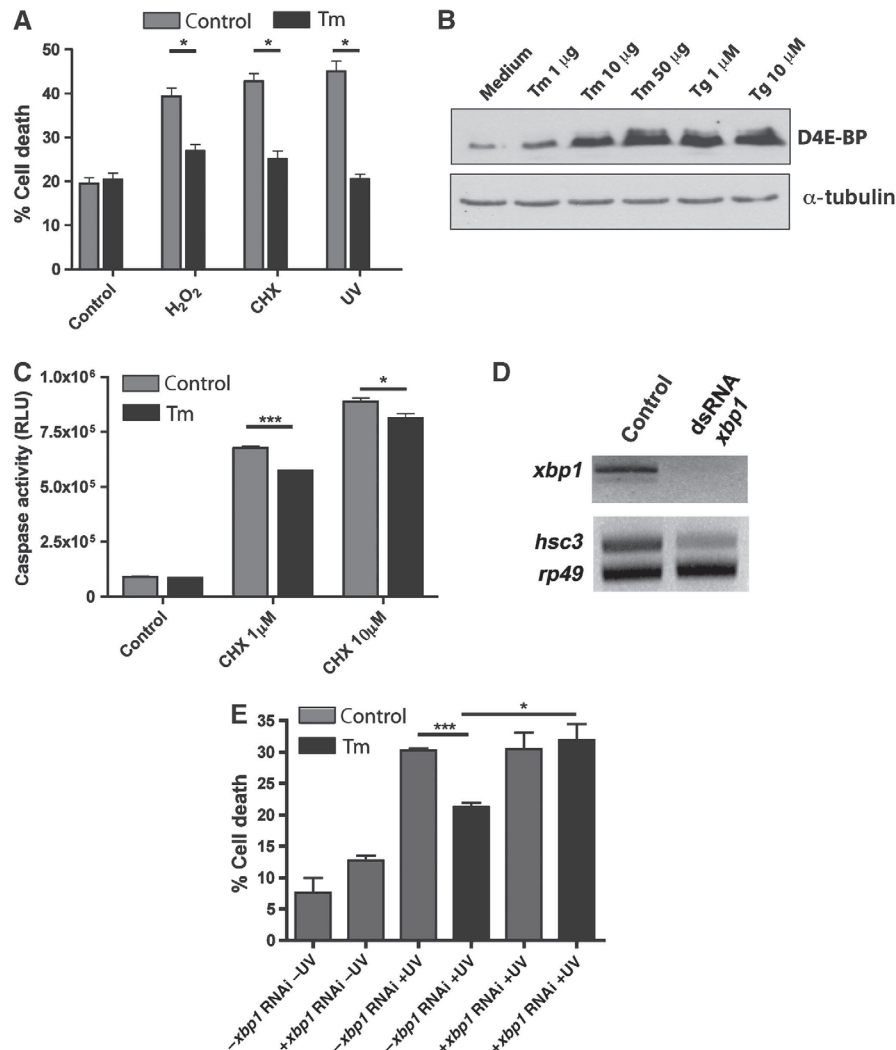
## Discussion

In pathological conditions such as diabetes and neurodegenerative diseases, a massive accumulation of misfolded proteins in the ER induces a UPR, which in turn triggers apoptosis (Marciniak and Ron, 2006). Here we show that a more moderate activation of the UPR (*ninaA* and *rh1* mutants) can mediate a survival response. The UPR protects PRCs from apoptosis induced by the expression of pro-apoptotic genes, *rpr*, *dp53* and *dcp-1*, as well as the degeneration observed in *apc* mutants (Figure 1). We found that the ER inhibits caspase activation and triggers an antioxidant response (Figures 6 and 7). In addition, we showed that a pharmacological pre-treatment with sub-lethal dose of the ER inducers (Tm or Tg) protects S2 cells from H<sub>2</sub>O<sub>2</sub>,

CHX or UV exposure (Figure 8, and data not shown). Our results indicate that moderate ER stress mediates a cellular response that inhibits additional external insults and allows the cells to have a survival response to the initial ER stress.

The ER stress-mediated protective effect can be assimilated as pre-conditioning or an adaptative stress response, also termed hormesis. Hormesis is a cellular protective signal induced by exposure to a low dose (or mild) stress-inducing agent that allows the cell to better respond to a second insult (for review Mattson, 2007). Our work is an example of ER-mediated hormesis (or ER-hormesis), because low levels of ER stress cause the UPR to protect the cells against cell death, whereas high levels of ER stress cause the UPR to trigger the apoptosis pathway.





**Figure 8** Mild ER stress protects S2 cells from oxidative and apoptotic insults. (A) Percentage of cell death was evaluated by trypan blue uptake in untreated (control, gray columns) and pre-treated S2 cells with 1 μg/ml of Tm (black columns), which were then submitted to H<sub>2</sub>O<sub>2</sub> (500 nM), CHX (1 μM) or UVC exposure (300 mJ/cm<sup>2</sup>). The results are expressed as mean percentage of cell death ± s.e.m. of three independent experiments. (B) Tm or Tg treatment increased d4E-BP expression in a dose-dependent manner. d4E-BP expression is visualized by western blot analysis on S2 cells treated with Tm or Tg for 6 h. (C) Tm pre-treatment reduces caspase activity induced by CHX treatment. Cells were either untreated (control, gray columns) or pre-treated with 1 μg/ml of Tm (black columns) then submitted to CHX (1 and 10 μM). The results are expressed as RLU (relative luminescent unit) ± s.e.m. of one experiment (triplicates) representative of three independent experiments. (D) *xbp1* knock down by dsRNA strongly reduced the amount of *xbp1* and *hsc3* transcripts. The expression of the ribosomal *rp49* gene was used as internal control for RNA extraction and RT-PCR efficiency. Its expression is constant in control and treated cells. (E) Knock down of *xbp1* reverts the protective effect of Tm. The results are expressed as mean percentage of cell death ± s.e.m. of three independent experiments. (in A, B, C and E, \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 in Student's *t*-test).

### What makes ER stress lethal?

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

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

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

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

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

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

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

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

In support of an ER-mediated antioxidant response, we show S2 cells, pre-treated with Tm or Tg, are resistant to ROS exposure (H<sub>2</sub>O<sub>2</sub>) (Figure 8A, and data not shown). Similarly, it has been shown that UPR activation counteracts ROS accumulation and cell death induced by tumour necrosis factor alpha in mouse embryonic fibroblasts (Xue *et al*, 2005). Together, these data argue that the UPR can limit ROS accumulation, inhibiting apoptosis in a conserved process.

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

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

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

#### Relevance to pathology

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

## Materials and methods

### Fly stocks

The following genotypes were used: Canton S (CS) and *cnbr* as wild-type strains. The *ninaA*<sup>1</sup> (*ninaA*<sup>W208@</sup>), *pzfer1lch*<sup>0451</sup>, *pzfer2lch*<sup>035</sup>, *pzthor*<sup>1(2)06270</sup> alleles were obtained from the Bloomington Stock Center. The *ninaA* alleles *ninaA*<sup>E110V</sup>, *ninaA*<sup>Q137L</sup> and *ninaA*<sup>G98D</sup> were a gift from Charles Zuker (Ondek *et al*, 1992). The *apc*<sup>Q8</sup> allele was a gift of Yashi Ahmed. The *rh1* alleles, *ninaE*<sup>117</sup> and *ninaE*<sup>G69D</sup> (termed *rh1*<sup>117</sup> and *rh1*<sup>G69D</sup>, respectively in the text) were a gift from Joseph O'Tousa (Kurada and O'Tousa, 1995). The *rh1-Gal4* driver, a gift from Jessica Treisman, was used for ectopic expression in the outer PRCs (Mollereau *et al*, 2000). The *uas-CD8:PARP:venus* stock was a gift from Darren Williams (Williams *et al*, 2006). The following genetic combinations were used to express transgenes in adult outer PRCs: (1) *rh1-Gal4; uas-rpr*, (2) *rh1-Gal4; uas-dp53*, (3) *rh1-Gal4; uas-dcp-1*, (4) *rh1-Gal4; uas-xbp1:GFP*, (5) *rh1-Gal4; GMR-p35*, (6) *rh1-Gal4; uas-GFP* and (7) *rh1-Gal4; uas-CD8:PARP:venus*. Flies were maintained at 25°C and a 12:12 h light cycle.

### Caspase assay

S2 cells were seeded in 96-well culture plates at 15 × 10<sup>3</sup> cells per wells. After a 4-h Tm treatment, cells were submitted to CHX for 5 h and 30 min. Caspase-Glo<sup>®</sup> 3/7 reagent (Promega, France) was added (V/V) to wells and incubated for 1 h 30 min. Luminescence was measured by a luminometer (Veritas<sup>™</sup> microplate luminometer).

### RT-PCR

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



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

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

#### Supplementary data

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

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## 2.2. Article 2 : ER stress inhibits neuronal death by promoting autophagy

### 2.2.1. ER stress is protective in neurodegenerative disease

The ER stress and the UPR are associated to neurodegenerative disease. UPR activation has been detected in brain patient with neurodegenerative diseases such as Alzheimer, Parkinson or Huntington diseases. However, the role of ER stress upon neurodegeneration is still under debate. Indeed inhibition of the IRE1 or PERK pathways in neurodegenerative disease models can either prevent or enhance neuron death. We have previously shown that the ER preconditioning protects neurons in *Drosophila* from apoptosis. We used models of Parkinson disease in *Drosophila* and mouse to test the role of ER preconditioning on neuron cell death.

*Drosophila* and mouse are used as model for Parkinson disease. Several models of Parkinson disease mimic this pathology in *Drosophila* and in mouse. We used the over-expression of human  $\alpha$ -Synuclein specifically in the dopaminergic neurons as *Drosophila* Parkinson model. The overexpression of human  $\alpha$ -syn induces the progressive loss of dopaminergic neurons in the *Drosophila* brain. In mouse, the loss of dopaminergic neuron can be induced by stereotaxic injection of 6-hydroxydopamine in the striatum. We used these models to study the role of ER stress in Parkinson disease progression.

Pre-treatment with tunicamycin induces a protective ER stress in Parkinson disease models. ER stress can be induced in *Drosophila* by tunicamycin feeding. In addition, intraperitoneal injection of tunicamycin activates the unfolded protein response in the mouse brain. We have shown that these treatments are toxic neither in *Drosophila* nor in mouse and considered these treatments as induction of mild ER stress. We have shown that tunicamycin pre-treatment protected dopaminergic neuron from cell death in Parkinson disease models. The neurons rescued by tunicamycin treatment are functional. Indeed, in *Drosophila*, tunicamycin treatment maintains dopaminergic neuron function as suggested by the expression of pale and vmat, which are involved in the synthesis and transport of dopamine. In addition, in mouse, tunicamycin maintains locomotor activity despite the injection of 6-OHDA. We thus have

shown that mild ER stress protects neurons in neurodegenerative disease model. We next studied the protective mechanism activated by ER stress.

### **2.2.2. Autophagy mediates the protective effect of ER stress**

Autophagy is a degradative process conserved in evolution which is implicated in the regulation of cell death. Autophagy is activated upon starvation or cellular stress. The autophagic vacuoles are formed to sequester part of the cytoplasm. These vacuoles fuse with lysosomes leading to the degradation of the vacuole content. This degradative process is involved in cell survival. Indeed, autophagy degrades cellular component to produce energy required for cell survival. Moreover, autophagy allows the elimination of damaged mitochondria that released Cytochrome C or reactive oxygen species, which are toxic for cells [853]. Autophagy could thus inhibit apoptosis. On the contrary, several studies shown that autophagy can induce cell death. For example, it has been shown that autophagy induced the elimination of cells during *Drosophila* development [541]. Autophagy is thus a mechanism implicated in the regulation of cell death.

Autophagy is associated to neurodegenerative diseases. Autophagy has been detected in brain patient with neurodegenerative diseases such as Alzheimer or Parkinson diseases [847, 854]. These diseases are characterized by presence of protein aggregates, which are toxic for cells [855]. The elimination of protein aggregates could allow cell survival. However, the role of autophagy in the progression of neurodegenerative disease is not yet elucidated.

Autophagy is activated upon ER stress associated to cell death signal. We have studied autophagy induction in our models of ER stress and cell death. Interestingly, we have shown that mild ER stress alone is not sufficient to induce autophagy neither in *Drosophila* nor mouse. Interestingly, we have shown that autophagy is activated when mild ER stress is associated in cell death induction. Indeed, we have shown that autophagy is activated in the *Drosophila* PRC mutated for *ninaA* which expressed rpr. Autophagy is also activated in dopaminergic neurons of mice treated with both tunicamycin and 6-OHDA. Mild ER stress and cell death signals combination is thus required for the autophagy induction both in *Drosophila* retina apoptosis and Parkinson mice brain.

Finally, we have shown that autophagy is required for mild ER stress protective effect in the *Drosophila* retina. The inhibition of autophagy with *atg* gene mutants or RNAi prevents the protective effect of *ninaA* mutation upon *reaper* overexpression in the *Drosophila* retina. Moreover, the direct induction of autophagy via Atg1 over-expression protected *Drosophila* photoreceptors from apoptosis. Our experiments show that the mild ER stress protects from neurons apoptosis *in vivo* in the *Drosophila* eye via the induction of autophagy. The protective effect of autophagy in ER stressed dopaminergic neuron in mice remains to be identified.



# ER stress inhibits neuronal death by promoting autophagy

Antoine Fouillet,<sup>1†</sup> Clémence Levet,<sup>1†</sup> Angélique Virgone,<sup>2</sup> Marion Robin,<sup>1</sup> Pierre Dourlen,<sup>1</sup> Jennifer Rieusset,<sup>3</sup> Elise Belaidi,<sup>4</sup> Michel Ovize,<sup>4</sup> Monique Touret,<sup>2</sup> Serge Nataf<sup>2</sup> and Bertrand Mollereau<sup>1,\*</sup>

<sup>1</sup>Ecole Normale Supérieure de Lyon; Laboratoire de Biologie Moléculaire de la Cellule; Lyon, France; <sup>2</sup>Université de Lyon; Lyon, France; <sup>3</sup>UMR INSERM Faculté de Médecine Lyon-Sud; Lyon, France; <sup>4</sup>INSERM and Service d'Explorations Fonctionnelles Cardiovasculaires Hospices Civils de Lyon; Université Claude Bernard Lyon 1; Lyon, France

<sup>†</sup>These authors contributed equally to this work.

**Keywords:** ER stress, autophagy, Parkinson, apoptosis, *Drosophila*, mouse

Endoplasmic reticulum (ER) stress has been implicated in neurodegenerative diseases but its relationship and role in disease progression remain unclear. Using genetic and pharmacological approaches, we showed that mild ER stress (“preconditioning”) is neuroprotective in *Drosophila* and mouse models of Parkinson disease. In addition, we found that the combination of mild ER stress and apoptotic signals triggers an autophagic response both in vivo and in vitro. We showed that when autophagy is impaired, ER-mediated protection is lost. We further demonstrated that autophagy inhibits caspase activation and apoptosis. Based on our findings, we conclude that autophagy is required for the neuroprotection mediated by mild ER stress, and therefore ER preconditioning has potential therapeutic value for the treatment of neurodegenerative diseases.

## Introduction

The unfolded protein response (UPR) is an evolutionarily conserved adaptive response to perturbations of normal endoplasmic reticulum (ER) physiology,<sup>1–3</sup> and is characteristic of several neurodegenerative diseases. Whether ER stress plays a causative role in certain disease conditions is still being debated.<sup>4,5</sup> To cope with the aberrant accumulation of unfolded proteins, cells trigger the UPR causing the activation of the ERN1/IRE1, ATF6 and EIF2AK3/PERK pathways.<sup>6</sup> Depending on the level of UPR activation and which components of the pathway are activated, ER stress can lead to either cellular death or survival. Specifically, sustained and full-fledged UPR that involves GADD153/CHOP and CASP12/caspase 12 activations is detrimental to the cell.<sup>7,8</sup> By contrast, mild ER stress (ER preconditioning) induces selective activation of X box binding protein (XBP1) accompanied by cellular protection.<sup>9,10</sup> ER preconditioning induces a cytoprotective response, named ER-hormesis, that protects the cell against a stronger insult.<sup>10,11</sup> ER preconditioning has been shown to induce cytoprotection in ischemia/hypoxia models.<sup>12</sup> However, in other models of neurodegenerative diseases, the cellular mechanism that elicits ER-mediated cytoprotection remains to be explored. A candidate mechanism for the ER-mediated protection is autophagy. It has been proposed in yeast that UPR activation stimulates autophagy, which in turn acts as a protective mechanism limiting ER expansion.<sup>13</sup> Mutations in *Drosophila* or mouse *atg* genes lead to

spontaneous neurodegeneration, suggesting that basal autophagy is neuroprotective.<sup>14–16</sup> In addition, defective autophagic responses are observed in several neurodegenerative diseases including both Alzheimer and Parkinson diseases.<sup>17,18</sup>

Because the UPR and autophagic responses are evolutionarily conserved,<sup>2,3,19,20</sup> we studied the protective mechanisms mediated by mild ER stress in *Drosophila* and mouse model of Parkinson disease (PD). First, we found that mild ER stress is protective in *Drosophila* and mouse models of Parkinson disease. In addition, we show that combination of mild ER stress and apoptotic signal induces autophagy, which in turn mediates neuroprotection. We discuss the implications of our findings in the light of the antagonistic relationship between autophagy and apoptosis, as well as the physiological relevance of ER stress and autophagy in neurodegenerative diseases.

## Results

**Mild ER stress is neuroprotective in *Drosophila* and mouse models of PD.** We have previously shown that mild ER stress inhibits cell death both in vitro and in vivo in *Drosophila*. *Drosophila* S2 cells pretreated with a mild dose of tunicamycin (Tm), a chemical inducer of the UPR, exhibited increased resistance to cell death. Similar resistance to cell death was observed in adult *Drosophila* photoreceptor neurons (PRN) where the UPR was genetically induced by mutations in *neither inactivation nor afterpotential A* (*ninaA*), a gene encoding a

\*Correspondence to: Bertrand Mollereau; Email: bertrand.mollereau@ens-lyon.fr  
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chaperone specific for the folding of Rhodopsin-1 (Rh1).<sup>10</sup> In these experimental conditions, the activation of the Ire1-Xbp1 pathway is well tolerated, does not induce cell lethality but instead increases resistance to exogenous apoptotic insults.

To determine if mild ER stress-mediated protection is effective in *Drosophila* PD model, we first confirmed that Tm feeding could activate UPR in the *Drosophila* brain tissues. *hsc3/bip* expression, a hallmark of UPR activation, was detected by RT-PCR in the heads of flies fed on two doses of Tm (1 µg/ml and 10 µg/ml) (Fig. S1A and S1B). Furthermore, we observed an increase of spliced and unspliced forms of *xbp1* (Fig. S1C). In our assays, we chose the weakest dose of Tm (1 µg/ml) to induce a mild ER stress in *Drosophila*.

To establish a previously reported *Drosophila* PD model, we expressed the gene encoding SNCA/human  $\alpha$ -synuclein (*hu- $\alpha$ -syn*) in all neurons with *elav-gal4* or selectively in dopaminergic (DA) neurons with *th-gal4*.<sup>21,22</sup> Next, we examined if Tm feeding could improve locomotor functions and dopaminergic neurons viability in *hu- $\alpha$ -syn* expressing flies. Flies expressing *hu- $\alpha$ -syn* using the pan-neuronal driver *elav* exhibited progressive decrease of motility compared with control (Fig. 1A). However, Tm feeding clearly improved climbing ability in 21, 28 and 35 d old flies (Fig. 1B).

To assess the toxicity of *hu- $\alpha$ -syn* expression on DA neurons, we measured DA neurons viability and the transcriptional activity of *pale*, a gene responsible for dopamine metabolism and vesicular monoamine transporter (*vmat*), a gene specific for dopamine transport.<sup>23–25</sup> After 42 d of *hu- $\alpha$ -syn* expression in DA neurons, we observed 30% loss of DA neurons in the protocerebral posterior medial clusters (PPM1/2) compared with control flies (Fig. 1C). We then detected by RT-qPCR a decrease of *pale* and *vmat* expression in *th > hu- $\alpha$ -syn* flies starting from 20 d onward (Fig. 1D and E). In *hu- $\alpha$ -syn* expressing flies that were regularly fed on Tm diet, we observed a rescue of DA neuron number in the PPM1/2 cluster (Fig. 1C). Similarly, the expression of *pale* and *vmat* was restored following Tm treatment (Fig. 1D and E). Together these results show that Tm feeding induces the UPR in *Drosophila* brain and is neuroprotective in the *hu- $\alpha$ -syn* model of Parkinson disease.

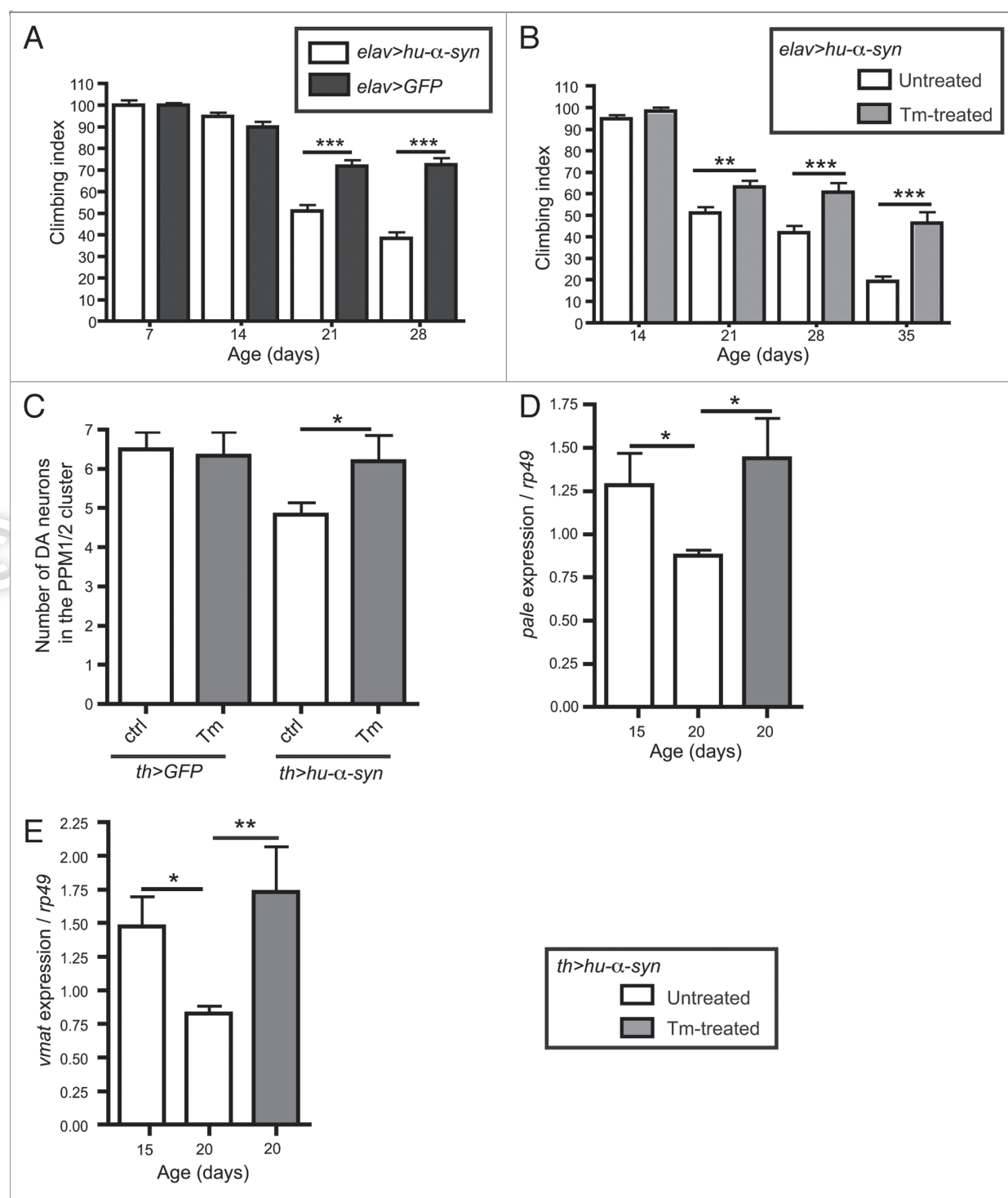
To validate these findings in a mammalian PD model, we tested whether mild ER-stress can induce neuroprotection in the 6-OHDA mouse model and in the human SH-SY5Y neuroblastoma cells.<sup>26–28</sup> The 6-OHDA mouse model recapitulates the common features of PD, including the loss of dopaminergic (DA) neurons and gradual onset of locomotor dysfunction.<sup>29,30</sup> We first assessed if Tm activates the UPR in the mouse brain. We monitored UPR activation by visualizing spliced *xbp1* (*xbp1s*) and *bip* mRNA (Fig. S1D and S1E) after intraperitoneal (I/P) injection of Tm. Increases in *xbp1s* and *bip* mRNA were detected at a low dose of Tm (0.1 mg/kg) in the *substantia nigra* (SN), where DA neurons of the nigra-striatal pathway are located (Fig. S1D and S1E). No toxic effect or locomotor deficit was observed following chronic injection of Tm (0.1 mg/kg) into mice (Fig. S1H–J). Moreover, following Tm injection at 0.1 mg/kg, the expression of *chop*, a transcription factor inducing cell death,<sup>31</sup> was not increased in the SN of mice (Fig. S1F and S1G). The

*chop* level was only elevated at high doses of Tm (4.5 mg/kg, ED<sub>50</sub>) whereas *xbp1s* remained at the basal level at this dose (Fig. S1G and data not shown). These findings show that low doses of Tm activate a nontoxic, mild UPR in the SN.

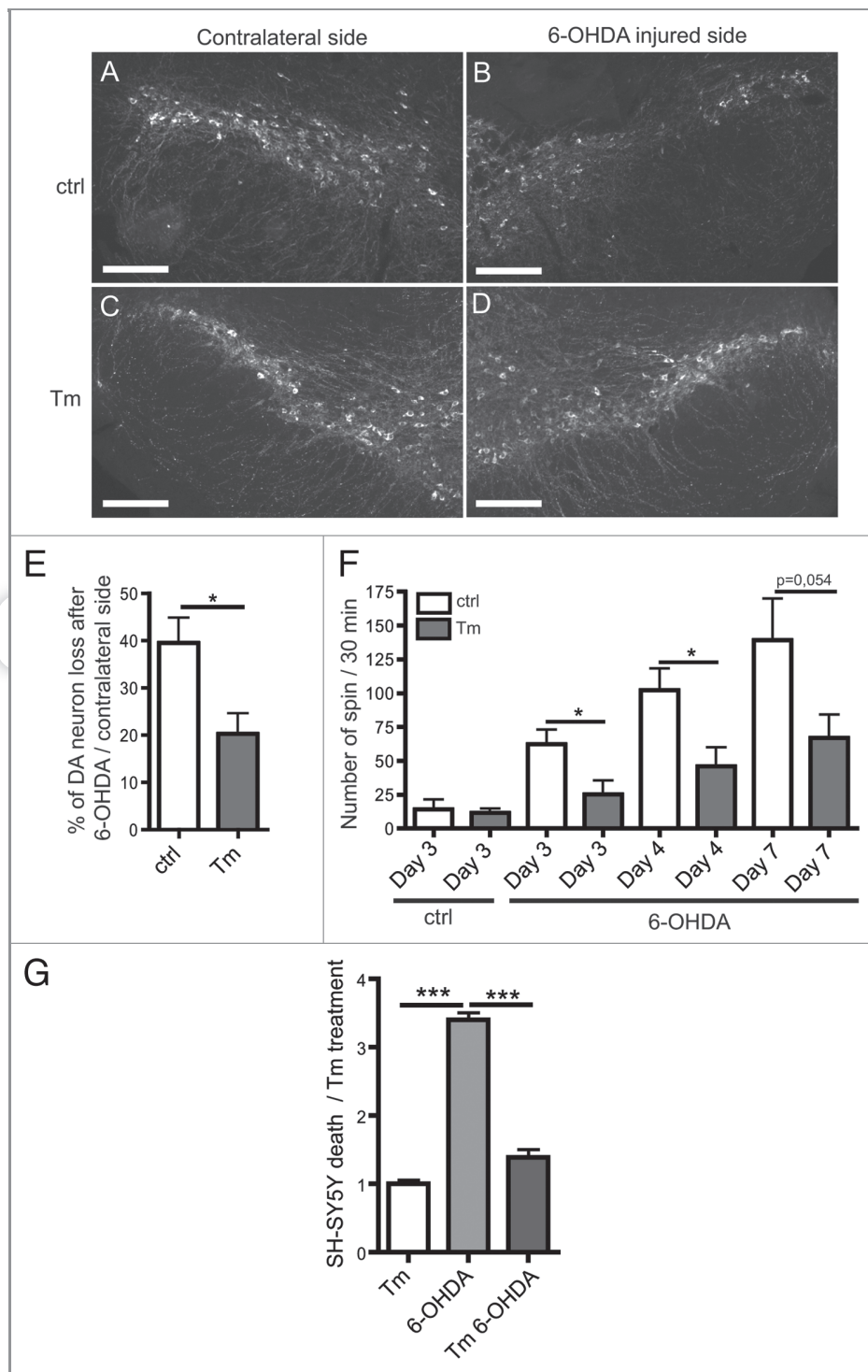
Stereotaxic injection of 6-OHDA into the mouse left striatum induces an asymmetrical loss of DA in the nigra-striatal circuit (Fig. 2A, B, E and ref. 32). To determine if mild ER stress is protective against the 6-OHDA-induced DA loss, we counted TH-positive DA neurons in the bilateral SN (Fig. 2A–E). After 6-OHDA injection, 20% more DA neurons remained in Tm-treated (0.1 mg/kg) than nontreated mice (Fig. 2E). Similarly in the human SH-SY5Y neuroblastoma cell line, we observed that Tm treatment reduced cell death induced by 6-OHDA (Fig. 2G). In addition, in both SN extracts and in SH-SY5Y cells, caspase activation induced by 6-OHDA was significantly inhibited by Tm treatment (Fig. S2A and S2B). These results indicate that Tm treatment-induced ER stress mediates neuron survival by blocking apoptosis both in vitro and in vivo.

Next, we studied the effects of Tm treatment on the rotational behavior in the 6-OHDA mouse model (Fig. 2F). The 6-OHDA lesion triggered a progressive increase of unilateral rotational behavior induced by apomorphine treatment. Tm pretreatment markedly reduced the number of turns, indicating that Tm antagonizes 6-OHDA-induced rotational behavior. In summary, our results indicate that Tm treatment protects against the toxic effects of 6-OHDA both in the mouse model and in human neuroblastoma cell line (Fig. 2). These findings are pertinent to Parkinson disease progression and treatment.

**Autophagy activation is required for the ER-mediated protection.** Autophagy and cell death are highly conserved cellular processes during evolution.<sup>19,20,33–35</sup> We therefore chose to study the contribution of autophagy in the ER-mediated protection against neuronal cell death in *Drosophila*. To achieve this goal, we examined autophagy activation in *Drosophila* retina submitted to genetically-induced ER stress (*ninaA* mutant) and apoptotic signal (*reaper* overexpression). In *ninaA* mutant PRN where apoptosis was induced by the expression of *reaper* (*rpr*) under the control of *rh1* promoter,<sup>10</sup> we coexpressed the GFP-LC3/Atg8 reporter fusion protein construct (referred to as GFP-LC3) and sought GFP-LC3 puncta in dissected whole-mount retina<sup>36</sup> (Fig. 3A–D and A'–D'). We found that PRNs subjected to mild ER stress and apoptosis exhibited a marked increase of GFP-LC3 puncta (Fig. 3D, D' and E). In contrast, diffuse GFP-LC3 staining similar to that in wild-type controls was observed in *ninaA* mutant PRN and in PRN expressing *rpr* alone (Fig. 3A–C and 3A'–C'). LC3-I was converted into the active form of LC3 (LC3-II) only in *ninaA* mutant PRN subjected to *rpr* expression (Fig. 3F). On the western blot, we could also detect in this latter condition the appearance of free GFP, which is more resistant to lysosomal degradation than LC3 (Fig. S3A). This result suggests that autophagosomes have fused with lysosomes and that autophagic flux is functional. Next, we examined the expression of Ref(2)P/p62, as an indirect mean to evaluate the flux of autophagy in vivo. Ref(2)P/p62 is a multifunctional scaffold protein that is retained in autophagic vacuoles when the



**Figure 1.** Tm is protective in the  $\alpha$ -syn Drosophila Parkinson disease model. Flies expressing *hu-α-syn* in all neurons (A and B) with *elav-gal4* driver, or in the dopaminergic neurons (C–E) with the tyrosine hydroxylase driver (*th-gal4*). (A) Climbing ability of aged matched flies expressing *hu-α-syn* and control flies expressing GFP in all neurons (n = 100–120 flies). (B) Climbing ability of flies expressing *hu-α-syn* with or without Tm treatment (n = 100–120 flies). (C) Number of DA neurons in the PPM1/2 brain cluster in *hu-α-syn* or GFP expressing flies with or without Tm treatment. (D and E) Expression of *pale* or *vmat* mRNA normalized to *rp49* in flies expressing *hu-α-syn* with or without Tm (n = 15 flies). \*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001 in Student's t-test.



**Figure 2.** Tm is protective in the 6-OHDA mouse Parkinson disease model. (A–D) Sections of the *substantia nigra* (SN) obtained 4 d after 6-OHDA treatment with or without Tm pre-treatment (0.1 mg/kg). DA neurons are visualized by immunostaining for tyrosine hydroxylase (TH). (E) Quantification of DA neuron loss after 6-OHDA injection normalized to the contralateral side. (F) Rotational behavior of mice after 6-OHDA injection. The graph shows the number of unilateral turns made by mice on days 3, 4 and 7 after the 6-OHDA injection with or without Tm ( $n = 6-7$ ). (G) In vitro experiments on SH-SY5Y to assess the cell viability after Tm and 6-OHDA treatments. Cell viability was evaluated using trypan blue after Tm and 6-OHDA treatments. Quantification of cell death is normalized to Tm treatment. \* $p \leq 0.05$ , \*\*\* $p < 0.001$  in Student's t-test. Scale bar: 200  $\mu\text{m}$ .

process of autophagy is compromised. Ref(2)P accumulation was observed in *atg8a* mutant as previously described,<sup>37</sup> but not in *ninaA* mutant retina expressing *rpr* (Fig. S3B). These results show that the autophagy flux is functional in *ninaA* mutant PRN expressing *rpr* and indicate that mild ER stress activates autophagy, when combined with an apoptotic signal.

In the light of our findings in the *Drosophila* PRN, we examined autophagy activation of DA neurons in the SN of mice subjected to Tm and 6-OHDA treatments (Fig. 3G–K). Basal LC3 levels were observed in DA neurons in both control mice and mice treated with Tm or 6-OHDA alone (Fig. 3G'–I'). By contrast, punctate LC3 staining was higher in DA neurons in animals subjected to both Tm and 6OHDA treatments (Fig. 3J' and K). We also examined autophagy activation in SH-SY5Y cells submitted to Tm and 6-OHDA treatments. 6-OHDA induced an increased of LC3II form compared with untreated cells. Moreover, we detected a 2-fold increase in LC3II form by combined treatment with Tm and 6-OHDA compared with 6-OHDA alone (Fig. 3L). The increase of LC3II form was only observed in the presence of bafilomycin A<sub>1</sub> that alters the lysosomal pH and prevents lysosomal degradation (Figs. 3L and S3C). This result indicates that the autophagic flux is functional in SH-SY5Y cells submitted to Tm and 6-OHDA treatments. Thus, autophagy is specifically increased in DA and SH-SY5Y neurons in response to a combination of Tm and 6-OHDA and may therefore be responsible for the neuroprotection.

We then investigated whether autophagy activation is required for the ER-mediated protection in *Drosophila* PRN and in human SH-SY5Y cell line. We first inactivated autophagy in *Drosophila* PRN with mutations and RNAi knockdown of components of the autophagy pathway. We used the cornea neutralization technique to evaluate PRN viability in living flies.<sup>38,39</sup> We found that in the presence of ectopic *rpr* expression, PRN loss was greater in double-mutant *ninaA*<sup>E110V</sup>/*atg1Δ3D* flies than in flies carrying only *ninaA*<sup>E110V</sup> mutation (Fig. 4A–F). Similarly, expression of a transgenic *atg6-IR* abolished *ninaA* mutant-mediated protection in *Drosophila* PRN (Fig. S3D). We then examined the role of *Drosophila* Ref(2)P/p62, a protein required for the formation of protein aggregates that are eliminated by autophagy in *Drosophila* brain.<sup>37</sup> We found that the expression of transgenic *ref(2)P-IR* suppressed PRN protection in *ninaA* mutant (Fig. 4G–L). These results indicate that autophagic clearance contributes to the ER-mediated protection. Next, we examined if Tm-mediated protection required autophagy in SH-SY5Y cell line. To achieve this goal, we performed a siRNA treatment against *atg8/LC3*, which knocked down LC3 expression on a western blot (Fig. 4M). We found that LC3 knockdown abolished Tm-mediated protection in SH-SY5Y cell submitted to 6-OHDA (Fig. 4N). In addition, we used 3MA which inhibited autophagy induced by starvation or 6-OHDA/Tm treatments (Fig. S3E and S3F). We observed that the 3MA treatment also suppressed Tm-mediated protection in SH-SY5Y cell submitted to 6-OHDA (Fig. 4O). Altogether these results demonstrate that autophagy is required for ER-mediated protection.

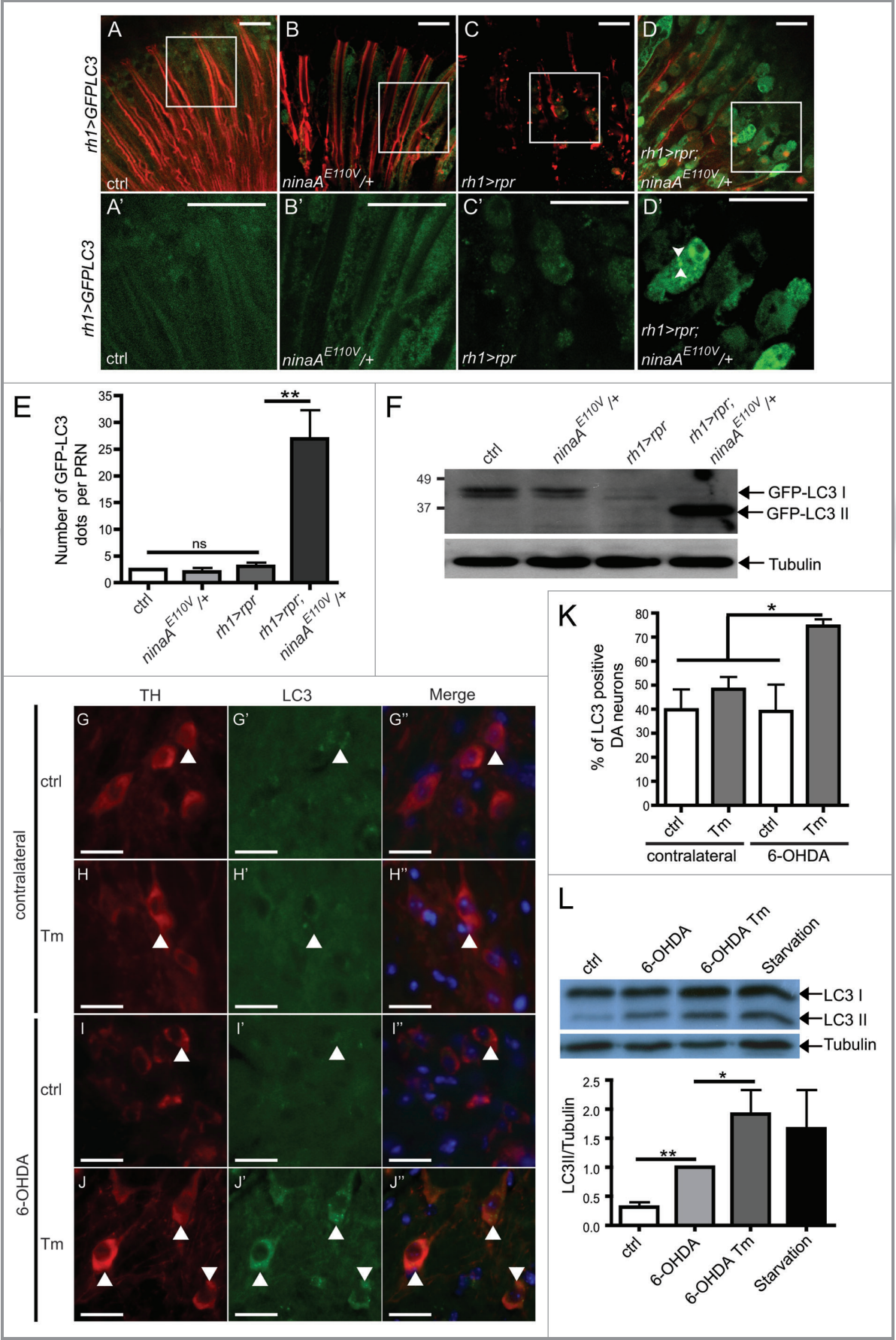
Last, we investigated the controversial issue of whether autophagy inhibits apoptosis.<sup>16,40</sup> We found that ectopic *atg1* expression rescued the viability of *Drosophila* PRN from *rpr*-induced apoptosis (Fig. 5A–D). In addition, rapamycin, an activator of autophagy,<sup>41</sup> inhibited *Drosophila* S2 cell death induced by cycloheximide (CHX) or UVC and suppressed caspase activation (Fig. 5E and F). These results demonstrate that autophagy inhibits apoptosis.

## Discussion

Our study provides new insight that mild ER stress promotes neuroprotection via the activation of autophagy. We have defined in vitro and in vivo experimental conditions in which the activation of UPR does not induce cell or organism lethality but rather promotes an adaptive response that protects from apoptotic stimuli. We show that a mild dose of tunicamycin (Tm) activates Ire1-Xbp1 and promotes protective autophagy in response to apoptotic stimuli. We have previously proposed that a preferential activation of Ire1-Xbp1 is responsible for protection in *Drosophila* S2 cells.<sup>10</sup> This hypothesis is supported by our new results in which we show that mild dose of Tm induces the Ire1-Xbp1 pathway but not *chop* expression in mouse brains (Fig. S1). It is also possible that upon mild ER stress, *chop* is induced with a different kinetic than Ire1-Xbp1 and leads to a partial activation of its transcriptional targets as previously proposed.<sup>42</sup> Thus, an adaptive response to mild ER stress may alter *chop* expression or Ddit3/Chop activity and promotes survival. In a recent study, it was shown that adaptive suppression of the Atf4/Chop branch by toll-like receptor engagement, promotes survival in response to prolonged ER stress.<sup>43,44</sup> It remains to be demonstrated whether selective activation of Ire1-Xbp1 or suppression of Atf4/Chop promotes neuroprotection in *Drosophila* and mouse Parkinson disease models.

Several previous studies investigated the link between the UPR and autophagy but a lot remains to be understood on how the UPR activates autophagy and promotes neuroprotection. Yeast cells subjected to severe ER stress manifest an autophagic response, which counterbalances ER expansion.<sup>13</sup> In this model, severe ER stress alone induces autophagy, which in turn limits ER expansion. In a recent study, it was shown that mild ER stress promotes cardioprotection against an ischemic/reperfusion injury.<sup>45</sup> In this model, autophagy activation could reduce subsequent lethal ischemic reperfusion injury. Another study reports that *xbp1* deficiency induces autophagy in a mouse model of the amyotrophic lateral sclerosis.<sup>46</sup> The *xbp1* deficiency leads to an unexpected rescue of *sod1* mutant motor neurons. Although several interpretations have been suggested to explain this result, it is possible that *xbp1* deficiency induces an increase in basal ER stress leading to autophagy. From our results, we propose a model in which mild ER stress primes the cells to trigger neuroprotective autophagy upon an apoptotic stimulus. Our results also indicate that autophagy is neuroprotective, and we further delineate a distinct mechanism by which UPR regulates autophagy. The understanding of the complex relationship between UPR, autophagy and apoptosis probably resides in the identification





and characterization of key factors that integrate these stress responses. In a recent study, it was shown that these responses are controlled by Bax inhibitor-1 (BI-1). BI-1 is a factor that inhibits IRE1- $\alpha$ , controls autophagy and apoptosis.<sup>47</sup> In cells lacking BI-1, IRE1- $\alpha$  is activated and induces autophagy, promoting cell survival. A role of BI-1 remains to be investigated in the control of autophagy in cells submitted to mild ER stress and apoptotic signal.

Previous work has identified a link between apoptosis and autophagy.<sup>48</sup> The authors have shown that stimulating cell death with TNF $\alpha$  in the presence of caspase inhibitors induced autophagy in L929 fibroblastic mouse cells.<sup>48</sup> Based on their hypothesis, TNF $\alpha$  stimulates an alternative autophagic death program when caspases are inhibited. In our hands, we found that inhibition of caspases with p35 did not induce autophagy in PRN submitted to apoptosis by *rpr* expression (data not shown). We favor a model in which combined signals of the UPR and apoptosis induce autophagy. In addition, our results differ from the one presented by Lenardo and col.,<sup>48</sup> as we demonstrated in several in vivo and in vitro models that autophagy is protective in cells submitted to mild ER stress. As discussed elsewhere, the opposite functions of autophagy on survival and death may depend on cell type and the level of autophagy activation.<sup>49</sup>

Autophagy has been proposed to inhibit cell death, however its role in the inhibition of apoptosis is a controversial subject.<sup>16,40</sup> We have shown that autophagy inhibits caspase activation and apoptosis in several in vitro and in vivo paradigms (Figs. 4 and 5). We have observed a concomitant increase of autophagic markers and decrease of caspase activation in cell submitted to both mild ER stress and apoptosis (Figs. 2, 3 and S2). Our findings suggest that cells switch from an apoptotic to an autophagic response when submitted to both mild ER stress and apoptotic signal. However, how autophagy inhibits apoptosis remains to be uncovered. Autophagosomes could engulf and degrade impaired mitochondria (mitophagy) to prevent the subsequent activation of apoptotic pathway.<sup>50</sup> Another hypothesis is that autophagy could directly sequester pro-apoptotic factors, such as caspases, and promote their degradation as previously proposed in a mouse model of Alzheimer disease.<sup>51</sup> Further work is required to elucidate this mechanism.

**Relevance to pathology.** We found that mild ER stress is protective in the Parkinson 6-OHDA mouse model, showing that maintaining UPR at a moderate level could protect against Parkinson disease. After injection in the striatum, 6-OHDA is selectively taken in DA by retrograde transport.<sup>52</sup> 6-OHDA induces an oxidative burst and caspase activation, which leads to DA death.<sup>28</sup> We show that Tm treatment activates mild UPR

responses, correlates to reduced DA death and improved locomotor function in mice bearing 6-OHDA lesions. Moreover, mild ER stress protects DA neurons of the SN from 6-OHDA-induced death by limiting caspase activation (Fig. S2) as previously observed in human neuroblastoma cell lines.<sup>28</sup> As in the fly paradigm, the increased autophagy in DA submitted to Tm and 6-OHDA suggests that autophagy is an active player of neuroprotection in mice. Our results incite new investigations into therapeutic possibilities to trigger and maintain ER stress at a moderate level, so that the stress response protects against or delay the onset of neurodegeneration, or retard the disease progress.

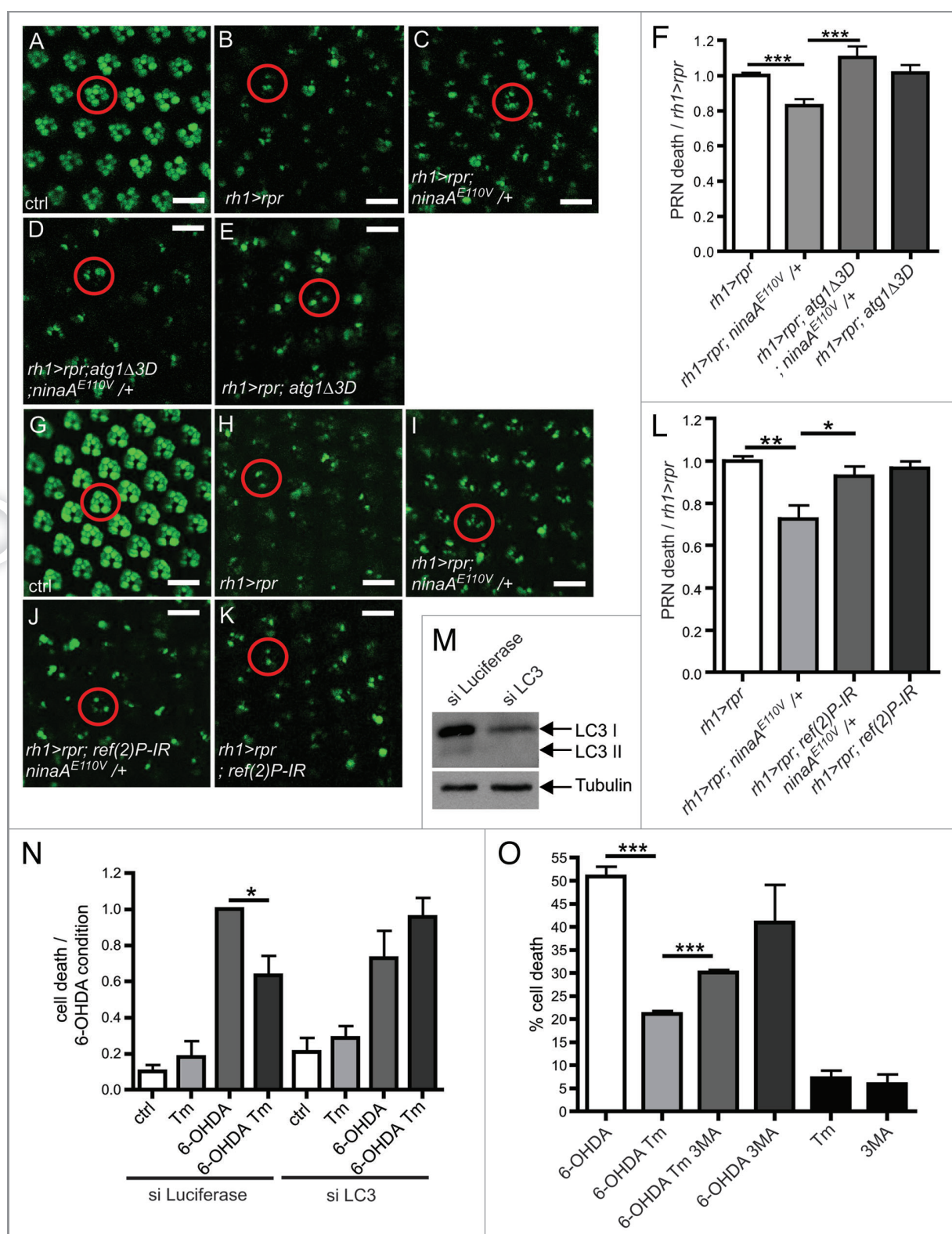
## Materials and Methods

**Drosophila genetics.** Flies were maintained at 25°C in a 12:12 h light cycle. The wild-type flies used for this study were Canton<sup>S</sup> strain. The *ninaA<sup>E110V</sup>* fly stock is a kind gift from Charles Zuker.<sup>53</sup> The *rh1-gal4* fly stock is a generous gift from Jessica Treisman.<sup>38</sup> *UAS-reaper (rpr)*, *UAS-lacZ* and *atg8<sup>KQ70569</sup>*, *elav-gal4* and *th-gal4* were obtained from Bloomington stock. *UAS-atg1* and *atg1 $\Delta$ 3D* stocks were a kind gift from Thomas Neufeld,<sup>54</sup> *UAS-GFP-LC3* was a kindly provided by Harald Stenmark,<sup>36</sup> *UAS-atg6-IR* were kindly obtained from Udai Bhan Pandey and *UAS-hu- $\alpha$ -syn* was kindly given by Mel Feany. The following genetic combinations were used to express transgenes in adult outer PRN: (1) *rh1-gal4; UAS-GFP; UAS-rpr*, (2) *rh1-gal4; UAS-GFP*, (3) *rh1-gal4; UAS-GFP-LC3*, (4) *rh1-gal4; UAS-GFP-LC3; UAS-rpr*, (5) *rh1-gal4; UAS-atg1*, (6) *rh1-gal4; ninaA<sup>E110V</sup>/UAS-GFP-LC3*, (7) *rh1-gal4; ninaA<sup>E110V</sup>/UAS-GFP-LC3; UAS-rpr*, (8) *rh1-gal4; UAS-rpr*, (9) *rh1-gal4; ninaA<sup>E110V/+</sup>; UAS-rpr*, (10) *rh1-gal4;ninaA<sup>E110V</sup>; UAS-rpr*, (11) *rh1-gal4; ninaA<sup>E110V</sup>/UAS-GFP; UAS-rpr*, (12) *rh1-gal4; ninaA<sup>E110V</sup>/UAS-GFP; UAS-rpr/atg1 $\Delta$ 3D*, (13) *rh1-gal4; UAS-GFP/ UAS-lacZ; UAS-atg1*, (14) *rh1-gal4; UAS-GFP; UAS-atg1/ UAS-rpr*. (15) *elav-gal4; UAS-hu- $\alpha$ -syn*; (16) *elav-gal4; UAS-GFP*; (17) *UAS-hu- $\alpha$ -syn; th-gal4* (18) *UAS-lacZ; th-gal4*.

**Drosophila pharmacological treatments.** ER stress was pharmacologically induced using tunicamycin (Tm; Covalab, 11089-65-9), an inhibitor of protein glycosylation. Twenty males and 20 females aged for 24 h were collected and starved for 5 h on 0.8% agarose, 1X PBS medium. Flies were then transferred in vial containing food (0.8% agarose, 10% sucrose, 1X PBS medium) supplemented with Tm (1 $\mu$ g/ml; 10 $\mu$ g/ml) or vehicle solution (Dimethyl sulfoxide, 2% Sigma Aldrich, D8418) for 4 h.

**Immunostaining on eye whole mount.** GFP-LC3 labeling was performed on *Drosophila* whole-mount retina as previously

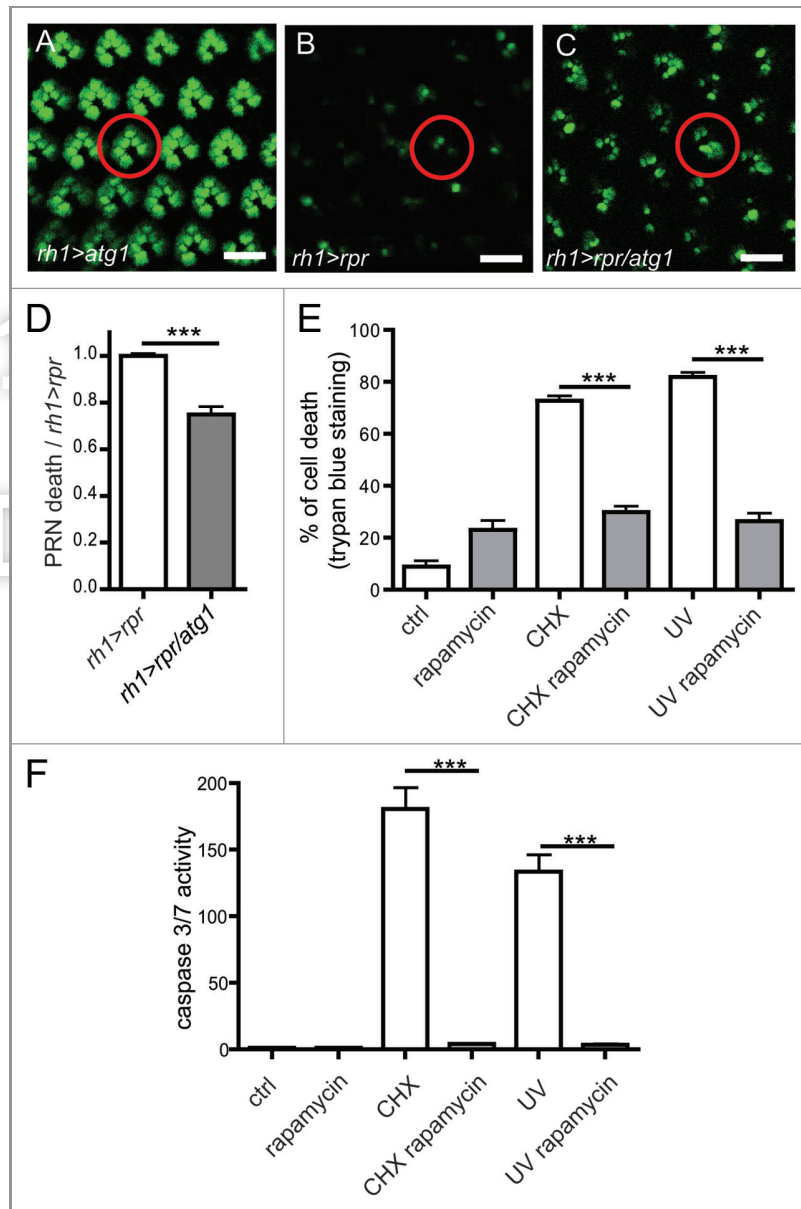
**Figure 3 (See opposite page).** Activation of autophagy by combined ER stress and cell death signals. (A–D') Whole-mount adult retina from flies expressing GFP-LC3 in PRN. GFP-LC3 is in green and phalloidin labels PRN rhabdomeres in red. (A'–D') Higher magnification view of GFP staining in the area surrounded by a white rectangle in (A–D). Scale bar: 10  $\mu$ m. (E) Quantification of the number of GFP-LC3 dots per PRN. (F) western blot with anti-GFP antibody showing the conversion of GFP-LC3-I to GFP-LC3-II in *Drosophila* retina. The western blots shown are representative of three independent experiments. (G–J') *Substantia nigra* sections after Tm and 6-OHDA injections in mice. (G–J) Anti-TH in red. (G'–J') Anti-LC3 in green. (G''–J'') The merge shows TH, LC3 and DAPI (blue) for nuclei. (K) The graph shows the percentage of TH positive cells with LC3 punctates. Between 220 and 250 neurons from each of three different mice were assessed. Scale bar: 20  $\mu$ m. (L) western blot and quantification showing the conversion of LC3-I to LC3-II in SH-SY5Y cells treated or not treated with Tm and 6-OHDA in the presence of bafilomycin A<sub>1</sub>. Cells under starvation are used as a positive control. The western blots shown are representative of three independent experiments. \*p  $\leq$  0.05, \*\*p  $\leq$  0.01 in Student's t-test.



**Figure 4.** For figure legend, see page 923.



**Figure 4 (See opposite page).** Autophagy is required for ER-mediated neuroprotection. (A–E and G–K) Visualization of PRN viability in 16 h-old living flies expressing *rh1 > GFP*. (A–E) Visualization of PRN in retina overexpressing *rpr* (*rh1 > rpr*) and mutant for *ninaA<sup>E110V/+</sup>* and *atg1Δ3D*. (F) Quantification of PRN loss in the various mutants (B–E) relative to *rh1 > rpr* (*n* = 10). (G–K) Visualization of PRN in retina overexpressing *rpr* (*rh1 > rpr*), *ref(2)P-IR* and mutant for *ninaA<sup>E110V/+</sup>*. (L) Quantification of PRN loss in the various mutants (H–K) relative to *rh1 > rpr* (*n* = 10). (M) western blotting showing LC3/II levels after siRNA against LC3 in SH-SY5Y cell compared with control (siRNA Luciferase). (N) SH-SY5Y cell viability was assessed by trypan blue exclusion after treatments with Tm, 6-OHDA and siRNA against LC3 or luciferase as control (*n* = 3). (O) SH-SY5Y cell viability was assessed by trypan blue exclusion after Tm and 6-OHDA treatments. 3-MA treatment is used to block autophagy. \**p* ≤ 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 in Student's t-test. Scale bar: 10 μm. The abbreviations used: *rh1-gal4; UAS-GFP* (*rh1 > GFP*), *rh1-gal4;UAS-rpr* (*rh1 > rpr*).



**Figure 5.** Autophagy inhibits cell death. (A–C) Visualization of PRN viability in 16 h-old living flies expressing GFP. PRN express *rpr* (*rh1 > rpr*) and *atg1* (*rh1 > atg1*). (D) Quantification of PRN loss in (B and C) relative to *rpr* (*n* = 6–7). (E) In vitro experiments on S2 cells to assess the cell viability after rapamycin and cycloheximide (CHX) treatments and UVC irradiation. Cell death was monitored by FACS analysis after incorporation of propidium iodide. (F) Caspase activity in S2 cells subjected rapamycin and cycloheximide (CHX) treatment and UVC irradiation. Results are expressed as ratio of caspase activity relative to control values (*n* = 3). Scale bar: 10 μm. \*\*\**p* < 0.001 in Student's t-test. The abbreviations used: *rh1-gal4;UAS-rpr* (*rh1 > rpr*), *rh1-gal4; UAS-atg1* (*rh1 > atg1*).

described.<sup>55</sup> Briefly, *Drosophila* heads were bisected in the middle with a scalpel. Brain tissue was removed to expose retina underneath. The retinæ were fixed in 4% PFA for 15 min. GFP-LC3 was revealed by immunostaining using a rabbit anti-GFP antibody (1/200, Invitrogen, A-6455) followed by an anti-rabbit secondary antibody (Alexa 488 1/400, Invitrogen, A-21206). Photoreceptor rhabdomeres were visualized using Actin coupled with phalloidin staining (1/400, Sigma Aldrich, 77418-1EA). Retinæ were mounted in DAPI mounting media (Vectashield, AbCys, H1500). Fluorescent images were obtained using a Leica SP5 confocal microscope.

**Mouse protocol.** All animal protocols were approved by the regional ethics committee for animal experiments, Rhône Alpes (authorization n°153). C57Bl6/J female mice (10 weeks old) were used for this study. Tm (Covalab, 11089-65-9) was administered by intraperitoneal (I/P) injection (0.01 mg/kg, 0.1 mg/kg or 4.5 mg/kg). Eighteen hours after Tm treatment, 8 µg of 6-hydroxydopamine (6-OHDA, 4 µg/µl, Tocris, 2547) in 0.02% ascorbic acid was injected stereotactically into the left striatum of the mouse brain to induce Parkinson disease-like injury.<sup>56</sup> Rotational behavior tests were performed on 6-OHDA-treated mice to evaluate alterations of the nigra-striatal pathway. Rotational asymmetry was induced by I/P injection of apomorphine at 0.6 mg/kg (Sigma Aldrich, A4393) on days 3, 4 and 7 as described.<sup>30</sup> Motor behavior was tested to assess motricity following Tm injection. Walking distance (cm) was measured over a period of 2 min three times for Tm-treated (n = 7) and control mice (n = 8).

**Immunostaining on mice brain sections.** Visualization of dopaminergic neurons was performed in mice substantia nigra sections. Mice were sacrificed by lethal I/P injection of pentobarbital, then perfused intracardially with saline solution and 4% PFA for fixation. Brains were extracted, further post-fixed in 4% PFA for 2 h, transferred to 30% sucrose solution at 4°C, and serially freeze-sectioned. Fourteen µm-thick floating brain sections were transferred into blocking solution (PBS-triton 0.1%, 4% BSA, 10% normal goat serum) for 1 h at RT. DA neurons were visualized using an anti-Tyrosine Hydroxylase antibody (α-TH, 1:2000, Millipore, Ab152) and anti-LC3 antibody (1:800, Cell Signaling, 2775S). Specifically, brain sections were incubated with the primary antibodies at 4°C overnight and with secondary antibodies Alexa 555 (1:500, Invitrogen, A21424) and Alexa 488 (1:500, Invitrogen, A-21206) at RT for 2 h in the dark. Fluorescent images were taken using an ApoTome Imager M2 with an AxioCam MRm (Zeiss). The loss of DA neurons after 6-OHDA treatment was defined as the percent of TH positive cells in the 6-OHDA injured side compared with the contralateral side. Autophagy level in DA neuron was defined as the percent of TH positive cells with LC3 punctates.

**Cell culture.** *Drosophila* S2 cells were cultured in *Drosophila* Schneider medium (Invitrogen, 21720024) supplemented with 10% fetal bovine serum. Cells were pre-incubated with 0.4 µg/ml of rapamycin for 40 h, then subjected to treatment with 10 µM

cycloheximide (Sigma-Aldrich, C1988) or 300 mJ/cm<sup>2</sup> UV C (UVC) with a UV irradiator (Vilber Lourmat 254 nm, LBX). After 8 h, the cells were stained with 50 µg/ml propidium iodide and analyzed by flow cytometry (FACSCalibur4C) to measure cell death.

SH-SY5Y neuroblastoma cell line were cultured in DMEM: HamF12 (1:1) supplemented with L-Glu plus nonessential amino acid (1%) and 10% FCS (Invitrogen, 10270106). Cells were pre-incubated with 0.5 µg/ml of Tm for 4 h, then subjected to treatment with 50 µM 6-OHDA (Tocris, 2547). After 16 h the cells were stained with 50 µg/ml propidium iodide and analyzed by flow cytometry (FACSCalibur4C) to measure cell death. For starvation, SH-SY5Y were maintained for 24 h in DMEM: HamF12 (1:1) supplemented with L-Glu plus nonessential amino acid (1%) without FCS.

**Inhibition of autophagy in SH-SY5Y.** Inhibition of autophagy was performed via RNAi as previously described.<sup>57</sup> Briefly, 40 nM of small interfering RNA (siRNA) sequences targeting LC3 (5'-GAAGGCGCUUACAGCUCAA-3') or siRNA targeting luciferase, used as negative control (5'-CGUACGCGGAAUACUUCGA-3'), were introduced in 0.1% lipofectamine 2000 (Invitrogen, 11668019) at day 1. siRNA experiment was repeated 48 h following the first siRNA. Inhibition of autophagy was assessed by western blotting experiment using LC3 antibody (Cell Signaling, 2775S). Autophagy was also inhibited by 3-methyladenine (3-MA; Sigma Aldrich, M9281). Cells were incubated with Tm (0.5 µg/ml) for 4 h, then subjected to 3-MA treatment at 10 µM and 6-OHDA at 50 µM (Tocris, 2547) for 16 h. Cell viability was assessed by trypan blue staining (Sigma Aldrich, T8154). In **Figure 3L**, autophagy flux was inhibited by adding bafilomycin A<sub>1</sub> to the cells at 10 nM for 12 h.

**Statistical analysis.** Data from mRNA expression, photoreceptor cell survival, autophagy activation, 6-OHDA cytotoxicity, rotatory and motricity behavior assays were analyzed using Student's t-test (2-group comparison). Level of significance was set at  $p \leq 0.05$ .

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#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Supplemental Materials

Additional Material and Methods may be found here: [www.landesbioscience.com/journals/autophagy/article/19716](http://www.landesbioscience.com/journals/autophagy/article/19716)

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### **3. Discussion**





## 3.1. ER stress in diseases

### 3.1.1. ER stress and UPR activation in neurodegenerative diseases

The ER stress is detected in several models of neurodegeneration such as Alzheimer, Parkinson and Huntington diseases and Amyotrophic Lateral sclerosis (ALS) [847, 848]. ER stress and the UPR have been detected both in patient tissues and in animal models of these diseases. Indeed, XBP1 splicing, IRE1 and PERK phosphorylation has been detected in brain of Alzheimer or Parkinson disease patient [850, 851]. Moreover, both IRE1 and PERK pathways are activated in *Drosophila* and mouse models of neurodegenerative diseases [2, 10, 13, 17, 18]. However, the mechanisms of ER stress induction in neurodegenerative disease are not yet fully elucidated. For example it is not clear whether cytoplasmic protein aggregates that are often present in neurodegenerative diseases induce ER stress [761]. Two hypotheses have been proposed to explain the activation of ER stress in neurodegenerative disease model. The first hypothesis is that in neurodegenerative disease models, some protein aggregates also accumulate in the ER. For example, it has been shown that  $\alpha$ -Syn, a cytoplasmic protein, accumulated in the ER, binds to ER chaperones, and  $\alpha$ -Syn overexpression sensitizes neuronal cells to ER Stress-induced toxicity [13]. The second hypothesis is that cytoplasmic protein aggregates could indirectly trigger ER stress. Indeed, both protein aggregates and misfolded proteins are targeted to proteasome degradation. Thus, ER stress could therefore be triggered by a saturation of the proteasome induced by the cytoplasmic aggregates [856]. ER stress and the UPR are thus activated upon neurodegeneration. However the role of the ER stress and UPR in the neurodegenerative diseases progression remains to be elucidated.

#### 3.1.1.1. ER stress and the regulation of neurodegeneration

The activation of mild ER stress is protective against neurodegeneration whereas a strong ER stress is deleterious. The effect of ectopic modulation of the UPR via and ER stress induction has been studied in several models of neurodegeneration (Table 4). ER stress can be induced in several manners by several mechanisms either chemical or genetic. We will distinguish models in which a potent ER signals induce cell death and models in which a sub-lethal activation promotes protection. Several examples have shown that ER stress and activation of the UPR can lead to cell death. The induction ER stress by treatment with tunicamycin or

thapsigargin induced cortical neurons apoptosis in mice [857]. In addition, the deleterious effect of ER stress has also been observed in neurodegenerative disease models. For example, IRE1 overexpression enhanced Huntingtin aggregation and cell death [33]. Interestingly, in these contexts, ER stress led to CHOP and the proapoptotic Bcl-2 family members activation [857]. These two factors are known actors of ER induced cell death. For example, *chop* knock-out increased neuron survival after optic nerve injury [15]. As in these models, UPR activation induces pro-apoptotic factors and leads to cell death, we considered this stress as a strong ER stress. It has thus been proposed that strong ER stress could favor neurodegenerative disease progression. In contrast to the pro-apoptotic role of a strong ER stress, the induction of a sub-lethal ER stress leads to neuroprotection. Indeed, UPR can be activated without leading to cell death. Upon sub-lethal ER stress, CHOP is not activated and the cells can handle the ER stress [733]. This sub-lethal ER stress is considered as a mild ER stress. We have shown that a genetic induction of mild ER stress by the *ninaA* mutation protected neurons from apoptosis. NinaA is a chaperone localized in the ER, which is dedicated to the folding of Rh1. *NinaA* mutation blocks its folding capacity and leads to Rh1 accumulation and ER stress. This accumulation of misfolded rh1 induced a mild ER stress, which protects from ectopic apoptosis *in vivo* in the *Drosophila* eye. Moreover, we have shown that mild ER stress is also protective in *Drosophila* and mouse Parkinson models *in vivo*. Our results suggest the activation and the maintaining of ER stress at a sub-lethal level could offer therapeutic perspectives to prevent or delayed neurons death in neurodegenerative diseases. In our models, neuron death is inhibited by a mild ER stress that precedes a second stress or injury. Thus this protective effect of mild ER stress can be considered as a preconditioning also termed ER-hormesis. The ER preconditioning effect has been observed in different cell injury contexts. For example, ER preconditioning can protect from retinal endothelial [858]. ER preconditioning also enhance oxidative stress tolerance [859]. Our results showed for the first time ER-hormesis in a neurodegenerative context. The induction of mild ER stress is thus an ER-hormesis process, which enhances resistance to cellular insults.

Hormesis is a protective mechanism, which allows cellular tolerance to several stresses. ER hormesis and mito-hormesis are two type of preconditioning. Interestingly, the ER and the mitochondria are two linked organelles. In addition, it has been shown that mild ER stress could protect cells against oxidative stress [859]. Thus the induction of mild ER stress could lead to mitochondria dysfunction and activation of mito-hormesis. The role of mito-hormesis upon mild ER stress remains to be tested.

ER stress pathway	Protective effect upon neurodegeneration	Deleterious effect upon neurodegeneration
IRE1/XBP1	Xbp1 inhibition suppresses the protective effect of chemical induced ER stress in Drosophila cell culture (Mendes 2009)	Xbp1 inhibition is protective in ALS (Hetz 2009 )
	XBP1 inhibition enhances cell death induced by tau over-expression (Loewen 2010 )	IRE increases aggregation of mtHTT in cell culture (Lee 2012 )
	XBP1s induction locally into the striatum reduces mutant Huntingtin aggregation in a mouse model (Zuleta 2012 )	Xbp1 deficiency protects neurons in mouse Htt model (Vidal 2012 )
	XBP1 overexpression enhances survival after nerve injury in mice (Hu 2012)	
	XBP1 deficiency attenuates locomotor recovery after spinal cord injury in mice (Valenzuela 2012)	
PERK/EIF2 and ATF4	Perk inhibition increased sensitivity to 6-OHDA (Ryu 2002 )	Salubrinal augments disease manifestation of FALS mice (Saxena 2009 )
	ATF4 deficiency attenuates locomotor recovery after SCI (Valenzuela 2012 )	Salubrinal, extends lifespan of $\alpha$ -Syn in A53TaS Tg mouse (Colla 2012 )
BIP	BiP reduces apoptosis caused by overexpression of human wt $\alpha$ -syn (Gorbatyuk 2012 )	
sigma-1 receptor	Sig inhibition increases Dopamine induced death (Mori 2012 )	

**Table 4:** UPR activation has both protective and deleterious effect upon neurodegeneration

References : Mendes 2009 [3], Loewen 2010 [11], Zuleta 2012 [14], Hu 2012 [15], Valenzuela 2012 [25], Ryu 2002 [18], Gorbatyuk 2012 [17], Mori 2012 [31] , Hetz 2009 [32], Lee 2012 [33], Vidal 2012[27], Saxena 2009 [2], Colla 2012 [13].

### 3.1.1.2. UPR branches control ER stress effect in neurodegeneration

The specific activation of UPR branches can control cell fate upon ER stress. Upon ER stress, the three branches of the UPR, IRE1, PERK and ATF6 are activated. All these branches are not equivalent regarding cell survival regulation. Each branch of the UPR can induce both pro-survival and pro-apoptotic pathways. However, it has been proposed that upon ER stress, IRE1/XBP1 was more a prosurvival branch, whereas PERK/ATF4 was deleterious. The respective role of UPR branches has been tested in the protection mediated by a mild ER stress upon neurodegeneration.

The specific activation of UPR branches controls neuron fate upon ER stress in neurodegenerative disease. IRE1 and PERK pathways can protect from neurodegeneration. We have shown that *xbp1* is required for the protective effect of tunicamycin against apoptotic insult in S2 cells. Moreover, the protective role of IRE/XBP1 pathway has been highlighted in several neurodegenerative models. It has been shown that XBP1 inhibition enhances cell death induced by tau over-expression [11]. Moreover, it has been shown Xbp1 activation locally into the striatum reduced mutant Huntingtin aggregation in a mouse model [14]. A protective effect of XBP1 have been also detected upon nerve injury in mice [15]. In this model, *xbp1* overexpression enhances survival of in axotomized retinal ganglion cells. In addition to IRE1 pathway, PERK pathway can protect from neuron death. Indeed, perk inhibition enhances sensitivity to 6-OHDA [18]. Similarly, eIF2 phosphorylation induced by salubrinal extended life span of  $\alpha$ -Syn expressing mouse [13]. On the contrary, it has been shown that UPR branches can be deleterious upon neurodegeneration. IRE1 pathway can be deleterious as suggested by the increase aggregation of Huntingtin in neuronal cells overexpressing IRE1 [33]. XBP1, a downstream effector of IRE1, can also be deleterious in vivo in ALS and Huntington disease models [27, 32]. Indeed, *xbp1* deficiency decreases Huntingtin level and reduces apoptosis in mouse model of Huntington disease [27]. In addition, *xbp1* deficient also prevent SOD1 aggregation in ALS model, which enhances cell survival [32]. The PERK pathway can also be deleterious upon neurodegeneration. Indeed, eIF2 phosphorylation induced by salubrinal enhanced symptoms of the disease, such as locomotor defect, of FALS mice [2]. Moreover, *atf4* deficiency attenuates locomotor recovery after Spinal Cord Injury [25]. Furthermore, the inhibition of the pro-apoptotic factor CHOP, which is a downstream target of the PERK and ATF6 pathways, rescues degeneration. Indeed,

*chop* deletion attenuated neurotoxin-induced Parkinson's disease [860]. Thus each branches of the UPR can be either deleterious or protective upon ER stress. The dual role of IRE1 pathway in neurodegeneration control, it could be mediated by the two major downstream targets XBP1 and JNK. The relative levels of XBP1 and JNK activation could control the pro-survival or pro-death effect of IRE1 upon neurodegeneration. One hypothesis is that when XBP1 is predominantly activated, the IRE1 pathway is protective while if XBP1 is inhibited and JNK activation persists, IRE1 pathway enhances neurodegeneration. The dual effect of PERK pathway could also depend of the activation of its downstream targets. It seems that PERK mediated eIF2 activation is protective via protein synthesis inhibition whereas ATF4, via CHOP activation is more deleterious. For example, in the immune response, the activation of Toll-like receptors (TLRs) prevent CHOP activation by the PERK pathway to maintain cell survival despite the activation of the UPR [861, 862]. Thus, modulation of the UPR pathways, could favors cell survival of cell death. Altogether, these results could be summarized in a model where the specific activation of the UPR branches control the neurodegeneration process (**Figure 9**). IRE1 and PERK can enhance degeneration via the induction of JNK and ATF4-CHOP. However, the activation of XBP1 and eIF2 via IRE1 and PERK respectively is neuroprotective.

Our model (Figure 9) implies that mild ER stress is neuroprotective via the activation of autophagy and that XBP1 contributes its activation. However, two studies show that XBP1 depletion promotes protective autophagy in ALS and Huntington disease models of degeneration [27, 32]. This suggests that XBP1 directly antagonizes protective autophagy which does not support our model. We rather favor alternative hypotheses in which XBP1 depletion induces autophagy via indirect compensatory mechanisms. The lack of XBP1 could lead to the accumulation of misfolded proteins in the ER and activate alternative UPR pathways. For example, XBP1 deficiency could trigger PERK activation, and in turn activate autophagy. This is supported by results showing that PERK/eIF2 induces the expression of atg12 and autophagy [863]. Alternatively, XBP1 deficiency could favors IRE1 mediated JNK activation which lead to autophagy induction. Thus in these models XBP1 would not antagonize autophagy but its absence would stimulate ER stress associated protective pathways.

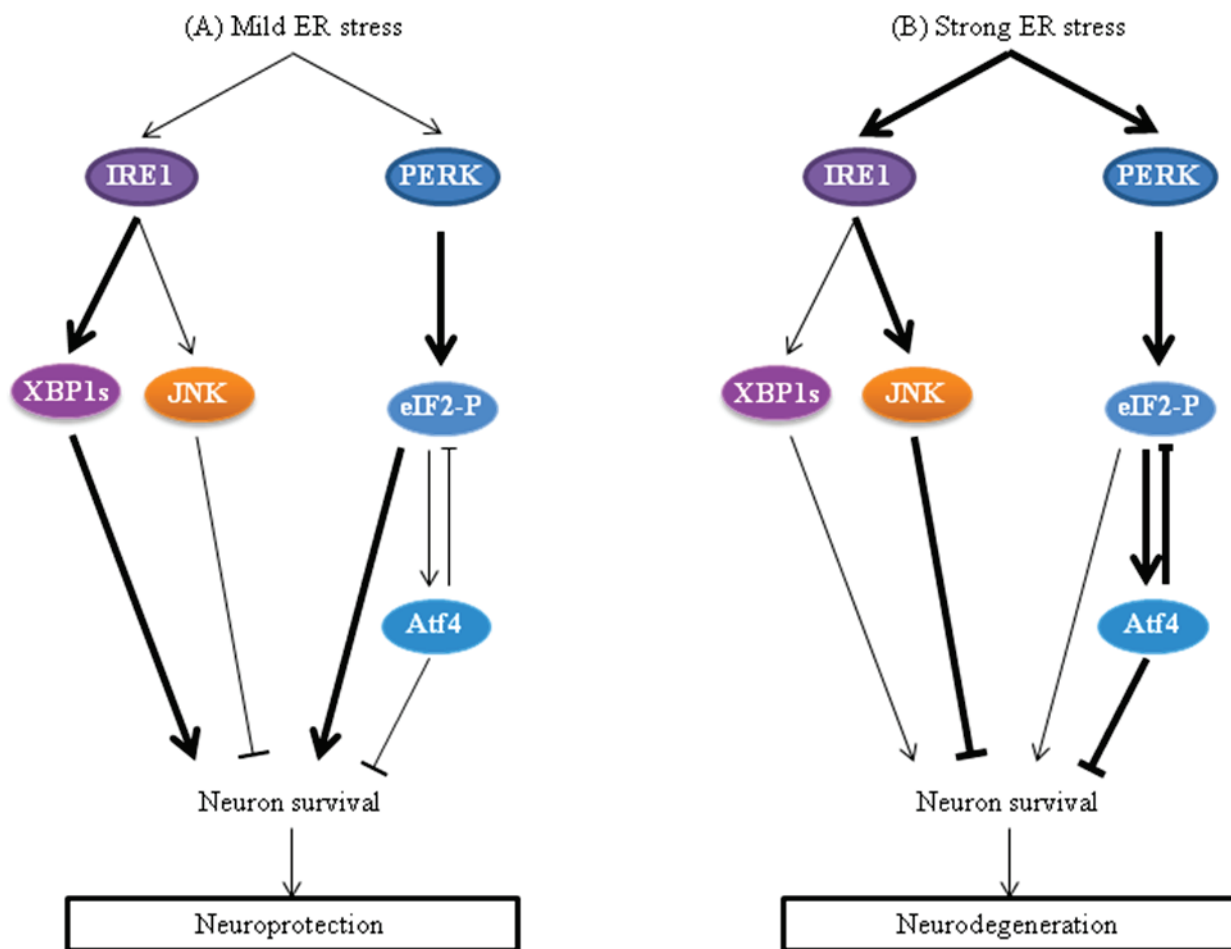


Figure 9: Model describing the role of UPR pathways in neurodegeneration. (A) Upon mild ER stress, the three UPR branches IRE1, ATF6 and PERK are activated and could predominantly activates protective effectors. Upon mild ER stress, XBP1 splicing and eIF2 phosphorylation could be respectively induced by IRE1 and PERK to enhance neurons survival. (B) Upon strong ER stress, the UPR could switch from a protective to a toxic response which leads to neuron death. Upon strong ER stress, IRE-1 and PERK could predominantly activate JNK and ATF4 which enhance neurons death.

### 3.1.1.3. Relevance to pathology

ER stress could be involved in the control of neurodegenerative disease progression. ER stress is detected in several models of neurodegenerative disease. However, the role of this ER stress as protective or deleterious is debated. We have shown that moderate ER stress is protective in animal models of Parkinson disease. Therefore, our results suggest that ER stress which occurs in neurodegenerative diseases could activate protective mechanism which protects the cell from neuron death. However, if ER stress is prolonged, it could switch the UPR functions from pro-survival to pro-apoptotic. Thus, two phases could be distinguished in neurodegenerative diseases: in the first phase UPR would allow the cell to cope with the



accumulation of pathological proteins, in the second phase, the overloaded ER would ultimately lead to cell death. The switch from a protective UPR to a pro-apoptotic signal has been observed in vitro in HEK293 cells over-expressing mutant Rhodopsin or treated with tunicamycin to induce ER stress. In these models, it has been shown that ER stress first induces the three branches of the UPR, which protects the cells from ER stress [733]. However, if the ER stress is prolonged the IRE1 and ATF6 pathways are turn-off and only PERK pathway remains activated. Thus partial activation of the UPR is deleterious of the cells [733]. These results shown that in neurodegenerative disease model, UPR activation can be first protective and become deleterious if its activation is prolonged. This hypothesis on the role of ER stress in neurodegenerative disease could explain the late apparition of the symptoms. In this scenario, the moderate ER stress would allow the organism to delay the onset of the pathology to old ages. Our results incite new investigations into therapeutic possibilities to trigger and maintain ER stress at a moderate level, so that the stress response protects against or delay the onset of neurodegeneration, or retard the progression of the disease. Hence neurodegeneration could be delayed by the specific stimulation of the protective branches of the UPR such as XBP1.

## **3.2. Protective mechanisms activated by ER stress**

### **3.2.1. Anti-oxidant response**

#### **3.2.1.1. The relationship between the anti-oxidant response and ER stress**

ER stress induces oxidative stress and an anti-oxidant response. In ER stress conditions, oxidative stress has been detected. In several models, the induction of ER stress is associated with ROS accumulation in the cells. For example, both ROS and ER stress are detected after viral infection such as Hepatitis C virus [864]. Moreover, ROS and UPR activation have also been associated in neurodegenerative diseases [865]. In addition to oxidative stress induction, ER stress can also trigger an anti-oxidant response. We have shown the presence of an anti-oxidant response upon ER stress in the *Drosophila* retina. Indeed the *ninaA* mutant retina, which presents a mild ER stress, has less carbonylated proteins suggesting that upon ER stress, an anti-oxidative response is activated. Moreover, the anti-oxidative protein Ferritin is

up-regulated in *ninaA* mutant retina [3]. Furthermore, we have shown that ER preconditioning is able to suppress both H<sub>2</sub>O<sub>2</sub> and 6-OHDA-induced cell death [3]. The role of ER preconditioning in the inhibition of oxidative stress has been observed by other groups. Hung et al showed that Tunicamycin treatment inhibited oxidative stress induced cell death *in vitro* [859]. Moreover, it has been shown that the induction of ER stress proteins (Chaperones Grp78 and Calreticulin) prevents disturbances of intracellular Ca<sup>2+</sup> homeostasis, and thus oxidative stress reduction [866]. Altogether, these results indicate that ER stress can trigger an anti-oxidant response, which protects from cells death. The anti-oxidant response induced by ER stress could be induced by a direct transcriptional activation of anti-oxidant genes via the UPR. Indeed, it has been shown that PERK induces the expression of Nrf2 [867]. Nrf2 is a transcription factor, which binds on ARE element located on several promoter such as Nrf2 binds on ARE element which are located on several promoter such as glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and heme oxygenase-1 (HMOX-1) [466, 795, 868]. Therefore, the UPR pathways can lead to the activation of anti-oxidative genes.

In addition to the direct induction of the anti-oxidant genes, the anti-oxidant response could be indirectly mediated by the induction of oxidative stress by ER stress. It has been proposed that ER stress induces an oxidative stress, which in turn promotes an anti-oxidant response [869, 870]. Indeed, the ER can produce reactive oxygen species (ROS), which will trigger the oxidative stress. Indeed, the folding of oxidative protein in the ER can produce ROS. For example, disulfide bond formation by PDI and ERO1 during oxidative protein folding in the ER produces hydrogen peroxide and generates oxidative stress [871]. Thus ER activation can produce oxidative stress. In addition to the direct production of ROS by the ER, ROS can be produced via the mitochondria. The ER and mitochondria communicate via MAMs (see Part 1.3.2.2). Thus, alteration of the ER leads to the modification of mitochondrial function. For example, it has been shown that ER stress in diabetes is associated to mitochondria mediated oxidative stress [611]. Mitochondria dysfunction could be due to a defect in the Ca<sup>2+</sup> flux between the ER and the mitochondria [598]. Upon ER stress, the ER calcium level is increased. The high level of Ca<sup>2+</sup> in the ER leads to the transfer of Ca<sup>2+</sup> to the mitochondria. Thus, Ca<sup>2+</sup> level increases in the mitochondria altering organelle homeostasis. In this stress condition, mitochondria produce ROS leading to oxidative stress and the activation of the anti-oxidant response.

### 3.2.1.2. Anti-oxidant response and neurodegeneration

Neurodegenerative diseases are associated to oxidative stress. ROS have been detected in the brain patient with ALS, Alzheimer, Huntington or Parkinson diseases. This observation has been made in animal models of Alzheimer, ALS and Parkinson diseases [872, 873]. Interestingly, the induction of oxidative stress by the administration of 6-OHDA or paraquat is used as Parkinson disease model. It has thus been proposed that oxidative stress plays a crucial role in neuronal death and in the pathogenesis of the neurodegenerative diseases. For example, it has been shown that in a *Drosophila* Alzheimer disease model, the oxidative stress contributes to neuron death [874]. Moreover, the over-expression of anti-oxidant enzymes, such as heme oxygenase-1 (HO-1) and superoxide dismutase (SOD)-1/-2 can prevent neuronal death in neurodegenerative diseases models. For example, overexpression of SOD2 reduces hippocampal superoxide and prevents memory deficits in mouse model of Alzheimer disease [875]. Reciprocally, blocking the anti-oxidant response enhances neurons death. In SOD2+/- mice the onset of hAPP/A $\beta$ -dependent behavioral abnormalities is accelerated and it worsened a range of AD-related molecular and pathological alterations [876]. Thus anti-oxidant response can be a protective mechanism upon neurodegeneration. Interestingly, we have shown that protective ER stress can trigger anti-oxidant response. Indeed, we have shown that transcriptional level of the anti-oxidant gene, *ferritin*, is induced upon ER stress induction on *ninaA*<sup>E110V</sup> mutant retina [3]. Furthermore, we have shown that antioxidant response element was activated in *ninaA*<sup>E110V</sup> mutant retina (unpublished results). These results show that mild ER stress induces the anti-oxidant response in *Drosophila* retina. However, the inhibition of ferritin did not suppress the protective effect in *ninaA*<sup>E110V</sup> mutant retina (unpublished results). Thus, Ferritin does not seem to be required for the protective effect of mild ER stress. However, other anti-oxidant proteins remain to be tested for their role in the protective effect of mild ER stress. Thus, the protective effect of mild ER stress could be mediated in part by the activation of an anti-oxidant response.

### 3.2.2. 4EBP

4E-BP is an important regulator of overall translation levels in cells upon cellular stresses. 4E-BP binds to translation regulator eIF4E (Eukaryotic translation initiation factor 4E) that is

involved in the regulation of cap dependent translation. The binding of 4E-BP to eIF4E prevents the recruitment of the ribosomal subunit on mRNA. Thus, 4E-BP inhibits translation [877]. Inhibition of cap-dependent translation by 4E-BP is essential for survival under stress conditions. 4E-BP has been shown to be important for survival under a wide variety of stresses, including starvation, oxidative stress, unfolded protein stress and immune challenge. Many cellular stressors result in the rapid cessation of cap-dependent translation, which is accompanied by the concomitant promotion of cap-independent translation of essential pro-survival factors [878, 879]. The cap-independent translation induced by cellular stress involves internal ribosome entry site (IRES) located on mRNA such as XIAP, BiP or Apaf1 [880]. It has been shown that 4E-BP is also involved in oxidative stress regulation. Indeed, the *Drosophila* 4E-BP (d4E-BP) is induced in response to oxidative stress [881]. Although d4E-BP is not an anti-oxidant protein per se, it downregulated translation of cap-dependent mRNA and mediates survival in animals exposed to oxidative stress. In addition to oxidative stress, 4E-BP is also activated upon ER stress. We have shown that d4E-BP is activated both in *Drosophila* retina and in S2 cells exposed to ER stress [3]. As 4E-BP is activated upon ER stress, this suggests that 4E-BP could be regulated by the UPR. The link between 4E-BP and UPR pathway has been characterized in mammals. It has been shown that 4E-BP is activated via ATF4 upon ER stress, which enhances cell survival [882]. Indeed, it has been shown that 4E-BP favors internal ribosome-entry site (IRES) dependant translation [883]. In particular, BiP contains an IRES in its mRNAs. Thus 4E-BP could favor BiP activation limiting ER stress. In addition, IAP also contains an IRES. Thus via the activation of IRES, 4E-BP could inhibit apoptosis. Thus 4E-BP targets participate to UPR and also can directly control apoptosis. 4E-BP could thus mediate the protective effects of mild ER stress. We have to test the effect of 4E-BP inhibition on ER stress protective effect.

4E-BP activation can protect neurons in Parkinson models. The study of *Drosophila* Parkinson disease models highlights the role of 4E-BP in neuron survival. The over-expression of the human leucine-rich repeat kinase 2 (LRRK2), a protein frequently mutated in Parkinson disease, induces dopaminergic neuron loss. It has been shown that human LRRK2 and the *Drosophila* ortholog of LRRK2 phosphorylate 4E-BP. The phosphorylation of 4E-BP induces its binding to eIF4 leading to induction of protein synthesis [884]. Thus it suggests that chronic inactivation of 4E-BP by LRRK2 with pathogenic mutations deregulates protein translation, eventually resulting in age-dependent loss of DA neurons. Thus 4E-BP appears as a pro-survival factor in neurons. The pro-survival role of 4E-BP has been

confirmed in the *Drosophila* Parkinson model, Pink and Park [885]. In these models, 4E-BP overexpression suppresses Park and Pink1 mutant phenotypes. Thus 4E-BP protects neurons from cell death. This protective effect can be mediated by the translation regulation by 4E-BP. Interestingly, we have found that 4E-BP is activated by protective ER stress in *Drosophila* neurons. Thus mild ER stress could protect from cell death in LRRK2 *Drosophila* model via the activation of 4E-BP. This hypothesis remains to be tested in the LRRK2 *Drosophila* model [886].

### 3.2.3. Autophagy

#### 3.2.3.1. Autophagy and ER stress

Autophagy activation upon ER stress can control cell fate. The ER stress is characterized by an overload of the ER by unfolded or misfolded proteins. To reduce the ER stress, misfolded proteins have to be degraded. Several degradative processes can be activated upon ER stress: the ER associated degradation (ERAD) and autophagy [887, 888]. ERAD consists in the degradation of ER ubiquitinated proteins in the proteasome. In addition to ERAD, autophagy is also activated to reduce ER stress via the degradation of entire part of the ER [47]. This specific degradation of the ER in autophagic vacuoles is named the ER-phagy [452]. We can distinguish models in which the activation of autophagy upon ER stress is protective and other models in which autophagy is deleterious. The protective effect of autophagy has been detected in several neurodegenerative disease models. We have shown that the ER stress mediated activation of autophagy protected photoreceptor cell from apoptosis in *Drosophila* [3]. In addition, ER stress induced autophagy is also protective in neurodegenerative disease model in mice cells *in vitro*. Indeed, expanded polyglutamine 72 repeat (polyQ72) aggregates induces ER stress mediated cell death *in vitro*. In this model, autophagy is activated to reduced ER stress mediated cell death [863]. Thus ER stress mediated autophagy has a beneficial role in neurodegenerative disease but detrimental in cancer cells. However, the activation of a protective induction of autophagy is deleterious for cancer cells. The protective effect of autophagy upon ER stress has been shown in cancer cells [48, 889-892]. Indeed, proteasome inhibition induces ER stress and autophagy, which blocks cell death in colon cancer cell line HCT116 [893]. In addition, ceramides induce a protective autophagy via PERK activation in MEF cells [891, 892]. Altogether, these experiments showed that ER

stress and UPR can activate autophagy to protect from cell death, which could prevent neurodegeneration or enhance cancer cell survival. In contrast to the prosurvival effect of ER stress induced autophagy, the activation of autophagy can enhance cell death upon ER stress in several models such as glioma cells exposed to Cannabinoid, CCD-18Co, MEFS, HCT116 or HEK293T cells [889, 894, 895]. For example, Cannabinoid treatment induces glioma cell death via ER stress mediated autophagy activation [894]. In addition, tunicamycin treatment induces ER stress mediated cell death via the activation of autophagy [895]. Therefore, ER stress induced autophagy can be either cytotoxic or cytoprotective [896]. The mechanism, which controls the decision between an adaptive role and a cell death induction via autophagy, is not understood. It has been suggested that autophagy effect can be dependant of the cellular context or of the autophagy induction pathway.

ER stress regulates autophagy via distinct pathways. Upon ER stress, the UPR is activated, and in some situation it can lead to autophagy induction. The IRE1 branch of the UPR can induce autophagy [48]. The activation of autophagy by IRE1 can be mediated by JNK activation. It has been proposed that via the phosphorylation of Bcl-2, JNK activates beclin leading to autophagy induction [897-899]. IRE1 mediated JNK activation could therefore lead to autophagy activation upon ER stress. IRE1 activation is induced by ER stress but can also be regulated by Bax inhibitor 1 (BI-1). BI-1 is a factor that inhibits IRE1- $\alpha$ , controls autophagy and apoptosis. In cells lacking BI-1, IRE1- $\alpha$  is activated and induces autophagy. This result confirms that IRE pathway can induce autophagy. In addition to IRE1, PERK, another branch of the UPR control autophagy. It has been shown that eIF2, a downstream effector of PERK is required for autophagy induction by PolyQ proteins and viral infection [863, 900]. This result suggests that PERK can induce autophagy. In addition, ER stress can induce autophagy independently of the UPR. ER stress can induce cellular homeostasis alteration. Indeed, it has been shown that ER stress induces calcium export from the ER to the cytoplasm. The increase of  $\text{Ca}^{2+}$  level in the cytoplasm can trigger apoptosis. Several proteins such as death associated protein kinase 1 (DAPK) and AMPK have been involved in the induction of autophagy by  $\text{Ca}^{2+}$  [552, 901]. In addition to  $\text{Ca}^{2+}$ , autophagy can be activated by the alteration of proteasome function. The proteasome degrades misfolded proteins of the ER. Indeed, upon ER stress, the ER associated degradation (ERAD) is activated, which induces the export of misfolded proteins from the ER to the cytosol where they are degraded via the proteasome. However, the proteasome overload by misfolded protein upon ER stress induces autophagy activation as an alternative degradative process [893]. Moreover, it has been shown



that proteasome inhibition triggers autophagy activation [902]. Thus ER stress can induce autophagy by several pathways: UPR,  $\text{Ca}^{2+}$  or proteasome overload. Interestingly, several pathways involved in ER stress mediated autophagy activation, such as JNK or Bcl2, are also involved in cell death regulation. The implication of those pathways in autophagy regulation upon ER stress could therefore be related to the decision between cell survival and cell death in autophagic contexts.

#### **3.2.3.2. Selective autophagy degradation**

The selective degradation by autophagy may be required for protective ER stress. We have shown that the activation of autophagy protects photoreceptor cell from apoptosis. However, how autophagy inhibits apoptosis remains to be determined. On one hand, autophagy could be required to limit ER stress and maintain the protective effect of ER stress. Indeed, it has previously been shown that autophagy can specifically trigger ER degradation [452]. This specific degradation of the ER allows the reduction of ER stress. Thus autophagy activated upon protective ER stress could be required to maintain the ER stress at a low level preventing ER stress-induced cell death. On the other hand, autophagy could directly control apoptosis via specific degradation of toxic components such as damage organelles or pro-apoptotic proteins. For example, a selective autophagy termed mitophagy can degrade specifically mitochondria [547, 903]. Mitochondria are a key inducer of apoptosis and oxidative stress. Thus, the degradation of mitochondria in the autophagic vacuoles can prevent cell death induction. In addition to mitochondria degradation, autophagy can specifically degrade ubiquitinated proteins. The specific addressing of ubiquitinated proteins to the autophagic vacuoles is mediated by P62. Interestingly, in our model of autophagy-mediated neuroprotection, we have found that P62 is required for the apoptosis inhibition [39]. Thus, p62 could mediate the degradation of pro-apoptotic proteins in autophagic vacuoles. Several actors of the apoptotic pathway such as caspases are ubiquitinated. Thus, these proteins could be targeted to autophagic vacuole via P62. In particular, caspases have been detected in autophagic vacuole in brain tissues of Alzheimer disease deceased patients [904]. Thus, caspases degradation in autophagic vacuole could be responsible of apoptosis inhibition by autophagy. To test this hypothesis, we want to test if caspases are localized in the autophagic vacuoles in ER stress and apoptotic context.

### 3.2.3.3. How ER stress combined with an apoptotic signal can induces autophagy?

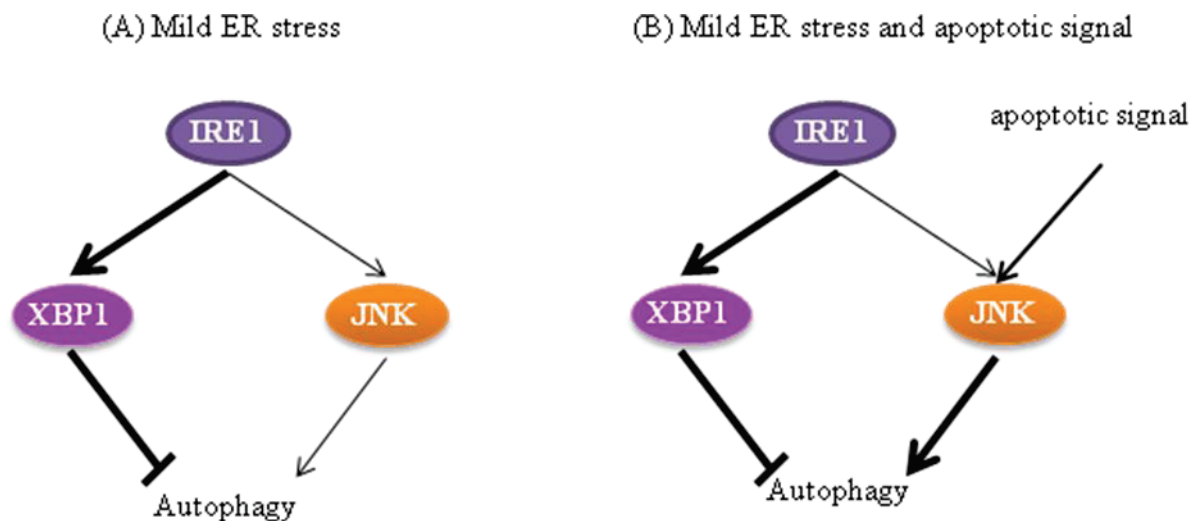


Figure 10: Model describing the induction of autophagy by apoptotic signal and ER stress via JNK regulation. (A) Upon mild ER stress IRE1 activates mainly XBP1. The weak activation of JNK by IRE1 could not be sufficient to activate autophagy. (B) Mild ER stress and apoptotic signal activate JNK independently. Upon mild ER stress combined with apoptotic signal, JNK could be highly activated upon a threshold, which could lead to autophagy activation.

Autophagy can be specifically activated when both ER stress and cell death signals are activated. We have shown both in *Drosophila* retina and in mouse model of Parkinson disease that a combination of ER stress and cell death signal leads to the activation of autophagy [39]. In these models, ER stress or apoptosis induction alone are not sufficient to induce autophagy. One hypothesis is that in our model ER stress and apoptosis are not able to activate sufficient level of a downstream target to induce autophagy. However, when combined, ER stress and apoptosis downstream signals have additive effects, which lead to autophagy induction. This theoretical model explains how autophagy could be activated; however, the understanding of the complex relationship between UPR, autophagy and apoptosis resides in the identification and characterization of key factors that integrate these stress responses. Several proteins involved in the activation of ER stress in the control of autophagy are also involved in the regulation of apoptosis such as JNK and Beclin. Therefore, these proteins could be involved in autophagy activation by both ER stress and apoptotic signal. In particular, the JNK pathway is an interesting candidate for autophagy regulation by ER stress and apoptosis. Indeed, JNK is a pathway known to induce the activation of autophagy [905]. Moreover, it has been shown that JNK activation is triggered by both ER stress and apoptotic signals. On one hand, JNK pathway can be activated by apoptotic signals. Indeed, the overexpression of the pro-apoptotic

gene reaper induced the activation of JNK pathway [906]. On the other hand, UPR and in particular IRE1 pathway can activate JNK [773, 774]. Indeed, it has been shown that IRE1 overexpression induces JNK activation and reciprocally, IRE1 inhibition prevents ER stress mediated JNK activation [774]. Thus, via the induction of JNK, IRE1 could trigger autophagy. Contrary to the activation of JNK, IRE1 can prevent autophagy via XBP1. Indeed, Xbp1 deficiency induces autophagy *in vitro* in ALS model [32, 907]. Thus a model involving IRE1, XBP1 and JNK in the regulation of apoptosis can be proposed. Upon mild ER stress, UPR activation is not sufficient to induce autophagy because IRE1 only induces a mild JNK activation. In the presence of apoptotic signal, JNK is further activated and combined with XBP1 activation leads to autophagy [906]. Thus, mild ER stress and apoptotic signal could synergize to induce JNK activation and autophagy (Figure 10). In addition to JNK pathway, autophagy activation in presence of ER stress and cell death signals could involve Bax-inhibitor 1 (BI-1). BI-1 is a factor that inhibits IRE1- $\alpha$ , controls autophagy and apoptosis. In cells lacking BI-1, IRE1- $\alpha$  is activated and induces autophagy, promoting cell survival. Thus the inhibition BI-1 could be involved in our model of ER stress mediated neuroprotection. However, the regulation of BI-1 by ER stress and apoptosis remains to be investigated.



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## Appendix : Dichtel-Danjoy et *al.*2012

Dichtel-Danjoy ML, Ma D, Dourlen P, Chatelain G, Napoletano F, Robin M, Corbet M, **Levet C**, Hafsi H, Hainaut P, Ryoo HD, Bourdon JC, Mollereau B. Drosophila p53 isoforms differentially regulate apoptosis and apoptosis-induced proliferation. Cell Death and Differentiation (2012)

# *Drosophila* p53 isoforms differentially regulate apoptosis and apoptosis-induced proliferation

M-L Dichtel-Danjoy<sup>1,5</sup>, D Ma<sup>1,5</sup>, P Dourlen<sup>1</sup>, G Chatelain<sup>1</sup>, F Napoletano<sup>1</sup>, M Robin<sup>1</sup>, M Corbet<sup>1</sup>, C Levet<sup>1</sup>, H Hafsi<sup>2</sup>, P Hainaut<sup>2</sup>, HD Ryoo<sup>3</sup>, J-C Bourdon<sup>4</sup> and B Mollereau<sup>\*,1</sup>

Irradiated or injured cells enter apoptosis, and in turn, promote proliferation of surrounding unaffected cells. In *Drosophila*, apoptotic cells have an active role in proliferation, where the caspase Dronc and p53 induce mitogen expression and growth in the surrounding tissues. The *Drosophila* p53 gene structure is conserved and encodes at least two protein isoforms: a full-length isoform (Dp53) and an N-terminally truncated isoform (DΔNp53). Historically, DΔNp53 was the first p53 isoform identified and was thought to be responsible for all p53 biological activities. It was shown that DΔNp53 induces apoptosis by inducing the expression of IAP antagonists, such as Reaper. Here we investigated the roles of Dp53 and DΔNp53 in apoptosis and apoptosis-induced proliferation. We found that both isoforms were capable of activating apoptosis, but that they each induced distinct IAP antagonists. Expression of DΔNp53 induced Wingless (Wg) expression and enhanced proliferation in both 'undead cells' and in 'genuine' apoptotic cells. In contrast to DΔNp53, Dp53 did not induce Wg expression in the absence of the endogenous p53 gene. Thus, we propose that DΔNp53 is the main isoform that regulates apoptosis-induced proliferation. Understanding the roles of *Drosophila* p53 isoforms in apoptosis and in apoptosis-induced proliferation may shed new light on the roles of p53 isoforms in humans, with important implications in cancer biology.

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Epithelial tissues have the intrinsic capability to repair and regenerate, following irradiation or genetically induced cell death. However, how epithelial cells respond to injury and recover is not well understood. In the past few years, studies from metazoan models, such as *Drosophila*, forged the concept of apoptosis-induced proliferation, a process by which damaged cells entering apoptosis signal the surrounding unaffected cells to divide so as to recoup the tissue loss.<sup>1,2,3</sup> Using *Drosophila* developing imaginal discs as a model, several groups have demonstrated that fly wing imaginal discs submitted to  $\gamma$ -irradiation or genetically induced cell death undergo apoptosis-induced proliferation.<sup>4,5</sup> Apoptosis-induced proliferation requires *Drosophila* p53 and the caspase Dronc, and involves the release of mitogens such as Wingless (Wg) and Decapentaplegic (Dpp) that induce the growth of the surrounding tissues.<sup>6–8</sup> Apoptosis-induced proliferation has also been observed in hydra, where dying cells express Wnt that is required for cell division.<sup>9</sup> A recent study showed that when injected into mice, irradiated mouse embryonic fibroblasts can induce sustained growth of feeder tumour cells.<sup>10</sup> Specifically, this study shows that caspase 3, the executioner of apoptosis, stimulates prostaglandin E2 expression and growth of surviving tumour cells. Other studies also demonstrate that mice deficient for the p53 inhibitor, MDM2, develop intestinal hyperplasia due to the

activation of the canonical Wnt and EGFR pathways.<sup>11</sup> Together, these results suggest that apoptosis-induced proliferation is a fundamental and conserved process by which epithelial tissues recover and regenerate after injury, and that p53 has an active role in both apoptosis and compensatory growth in mice and in *Drosophila*.

The p53 protein is the product of a well-known tumour suppressor gene, *TP53*. It is mutated in more than 50% of human cancers. Initial studies of p53 functions have highlighted its key role as a stress-induced factor, particularly in response to DNA damage. The results from decades of studies coined p53 as the 'guardian of the genome', as it induces DNA repair, cell cycle arrest or apoptosis after exposure to genotoxic stress,<sup>12,13</sup> thus preventing the sequential accumulation of genetic alterations that underpins progression towards neoplasia. However, p53 is present in many lower eukaryotes, including *Drosophila*, where cancer is not a prevalent biological phenomenon. This paradox leads many to postulate that the tumour suppression function of p53 in vertebrates has probably evolved for some hitherto unappreciated primordial regulatory functions.<sup>14–16</sup> However, the exact nature of such primordial functions has remained elusive.

Until recently, TP53 was thought to be expressed as a single major transcript. This view was radically transformed in

<sup>1</sup>Group Apoptosis and Neurogenetics, Laboratory of Molecular Biology of the Cell, CNRS UMR5239, Ecole Normale Supérieure de Lyon, UMS3444 Biosciences Lyon Gerland, Université de Lyon, Lyon, France; <sup>2</sup>International Agency for Research on Cancer, Lyon, France; <sup>3</sup>Department of Cell Biology, New York University School of Medicine, New York, NY, USA and <sup>4</sup>Department of Surgery and Molecular Oncology, European Associated Laboratory University of Dundee/Inserm U858, Dundee, UK  
\*Corresponding author: B Mollereau, Group Apoptosis and Neurogenetics, Laboratory of Molecular Biology of the Cell, CNRS UMR5239, Ecole Normale Supérieure de Lyon, UMS3444 Biosciences Lyon Gerland, Université de Lyon, 69007 Lyon, France. Tel: +33 4 72 72 81 63; Fax: +33 4 72 72 86 74; E-mail: bertrand.mollereau@ens-lyon.fr

<sup>5</sup>These authors contributed equally to this work.

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the last 15 years by the discovery of two vertebrate p53 paralogs, *TP63* and *TP73*. These two genes encode several protein isoforms with diverse functions in neuronal development, morphogenesis, immune response and responses to specific stress.<sup>17</sup> Subsequent to the discovery of the  $\Delta N$  isoforms of p63 and p73, studies have revealed that p53 express up to 12 protein isoforms generated by alternative splicing sites, codon initiation sites and internal promoter.<sup>18,19</sup>

In invertebrate animals, only one gene represents the *TP53* gene family. It resembles *TP53* more than it resembles *TP63* and *TP73*. In *Drosophila*, the *p53* gene structure is highly conserved compared to its mammalian homologue. *Drosophila p53* (*Dp53*) gene structure was recently reviewed; it contains two alternative promoters and encodes three possible protein isoforms, *Dp53*, *D $\Delta$ Np53* and *Dp53 $\Delta$ C*.<sup>19</sup> Experimental evidence only confirms the presence of the full-length *Dp53* and *D $\Delta$ Np53*. Therefore, in this study, we focused on *Dp53* full-length isoform corresponding to the human full-length (TA) p53 that includes a full transactivation domain and *D $\Delta$ Np53*, a general counterpart of the N-terminal truncated human p53 forms. *D $\Delta$ Np53* is encoded by an mRNA transcribed from an internal promoter like the N-terminally truncated human  $\Delta 133p53$ , but unlike  $\Delta 133p53$ , it contains a truncated trans-activation domain followed by a complete DNA-binding domain and an oligomerization domain such as that found in the human  $\Delta 40Np53$ .<sup>18</sup> These findings raise the possibility that *Dp53* and *D $\Delta$ Np53* are the respective functional homologues of the human *TAp53* and  $\Delta 40/\Delta 133p53$ .

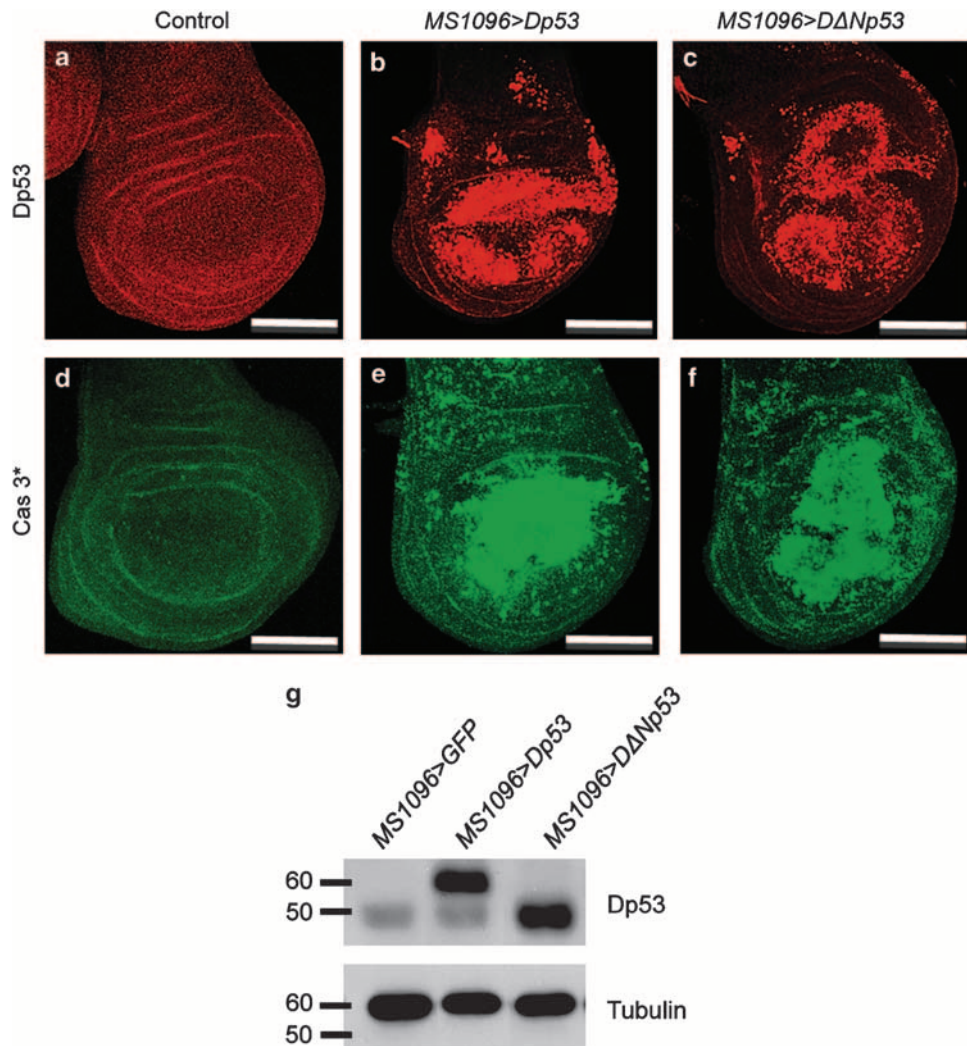
*D $\Delta$ Np53* was the first p53 isoform identified in *Drosophila* and was thought to be the only p53 isoform for several years; it was therefore initially named *Dp53* or *Dmp53* in earlier publications.<sup>20–22</sup> The subsequent identification of a form of *Drosophila p53* matching the mammalian full-length (TA) p53 protein has led to a reassessment of this nomenclature, with the name *Dp53* to designate the full-length protein isoform and *D $\Delta$ Np53* for the N-terminal truncated form. Studies on fly primordial germ cells, imaginal discs and adult photoreceptor cells have highlighted the role of *D $\Delta$ Np53* in regulating apoptosis.<sup>21,23–26</sup> *D $\Delta$ Np53* induces apoptosis through the Reaper-Hid-Grim (RHG) cascade. It was proposed that *D $\Delta$ Np53* directly activates the expression of *reaper* (*rpr*), whose protein product activates caspases by inhibiting *DIAP1* (*Drosophila* inhibitor of apoptosis protein).<sup>21,22</sup> In addition to its apoptotic function, the *Dp53* locus (*Dp53* and/or *D $\Delta$ Np53*) regulates several biological functions, such as cell cycle, DNA repair, aging and apoptosis-induced proliferation.<sup>3,27–30</sup>

Here we have investigated the role of *Dp53* and *D $\Delta$ Np53* in apoptosis and apoptosis-induced proliferation. We found that both isoforms were capable of activating apoptosis, but that each induced distinct RHG family members to inhibit *DIAP1*. Strikingly, we observed that *D $\Delta$ Np53* induced *wg* expression and enhanced proliferation in the wing imaginal disc, suggesting that *D $\Delta$ Np53* promotes apoptosis-induced proliferation. In contrast to *D $\Delta$ Np53*, *Dp53* did not induce *wg* expression in the absence of the endogenous *p53* gene. Thus, we propose that *D $\Delta$ Np53* is the main p53 isoform that regulates apoptosis-induced proliferation. The physiological consequences of these dual functions of p53 isoforms on apoptosis and apoptosis-induced proliferation are discussed.

## Results

**Dp53 and D $\Delta$ Np53 activate distinct RHG genes to induce apoptosis.** To study the respective functions of *Dp53* and *D $\Delta$ Np53*, we undertook a gain-of-function approach. We generated *UAS-Dp53* and *UAS-D $\Delta$ Np53* *Drosophila* transgenic lines for tissue-specific expression using the UAS/GAL4 system. To eliminate expression level variations due to position effects,<sup>31</sup> we targeted individual *UAS-Dp53* and *UAS-D $\Delta$ Np53* insertions to the same chosen genomic region using the site-specific  $\phi C31$  integrase system. *Dp53* and *D $\Delta$ Np53* cDNAs were expressed in the wing imaginal disc using the MS1096 driver that is specific to the wing pouch and hinge areas (Figure 1 and Supplementary Figure 1).<sup>32</sup> We observed robust production of *Dp53* in wing imaginal discs using an anti-*Dp53* antibody that recognizes the DNA-binding domain common to the *Dp53* and *D $\Delta$ Np53* isoforms (Figures 1a–c). Similar levels of *Dp53* and *D $\Delta$ Np53* isoforms were detected by western blot analysis (Figure 1g). We found that both isoforms induced caspase activation in wing imaginal discs, indicating that their expression leads to apoptosis (Figures 1d–f and Supplementary Figure S2).

To investigate the mechanisms by which *Dp53* and *D $\Delta$ Np53* overexpression lead to apoptosis, we examined *rpr* and *hid* expression (Figure 2). We used a *rpr*<sup>XRE</sup>-lacZ (*rprZ*) reporter, which carries a 2.2-kb genomic region necessary for *rpr* induction in response to irradiation.<sup>25</sup> Although *Dp53* induced robust *rprZ* activation, *D $\Delta$ Np53* led to only a weak *rprZ* response in the wing imaginal discs (Figures 2b, c and g). To confirm this result, we tested how both *Dp53* isoforms can activate p53RE-GFPnls, another *rpr* activity reporter, which contains a 150-bp *rpr* enhancer sequence embedding a consensus p53-binding site.<sup>15</sup> We found *Dp53* induced greater levels of GFP than *D $\Delta$ Np53* (Figures 2e and f). Furthermore, we found that *Dp53* production (*en*>*Dp53*) led to the formation of blisters in the adult wings (Supplementary Figure S3). Although we currently do not know what p53-related biological process is responsible for the blister formation, we found that the incidence of wing blisters was significantly reduced in an *rpr* mutant (Supplementary Figure S3 and Table S1a). Because of the pupal lethality induced by the expression of *D $\Delta$ Np53* (*en*>*D $\Delta$ Np53*), we could not test whether *rpr* mutant reduces wing blisters in this condition. Instead, we showed that *rpr* mutant partially suppressed pupal lethality induced by *D $\Delta$ Np53* (*en*>*D $\Delta$ Np53*), suggesting that the *D $\Delta$ Np53*-mediated phenotype involves *rpr* (Supplementary Table S1b). Next, we examined *hid* induction by *Dp53* and *D $\Delta$ Np53* using an anti-Hid antibody.<sup>4</sup> However, as both *Dp53* isoforms lead to rapid elimination of apoptotic cells (data not shown), *Hid* expression was hard to detect. To overcome this difficulty, we examined the induction of *Hid* expression by *Dp53* isoforms with the *engrailed* driver in *dronc*-null wing discs, where the cells were kept 'undead' (Figures 2h–j). The *engrailed* driver is expressed in the posterior part of the wing imaginal disc in a clearly delineated domain (Supplementary Figure S1a). We observed much stronger *Hid* staining in the *engrailed* domain where *D $\Delta$ Np53* was overexpressed compared with *Dp53*. Together, these experiments support that *rpr* is a primary target during *Dp53*-mediated apoptosis and suggest that *Dp53* is responsible for



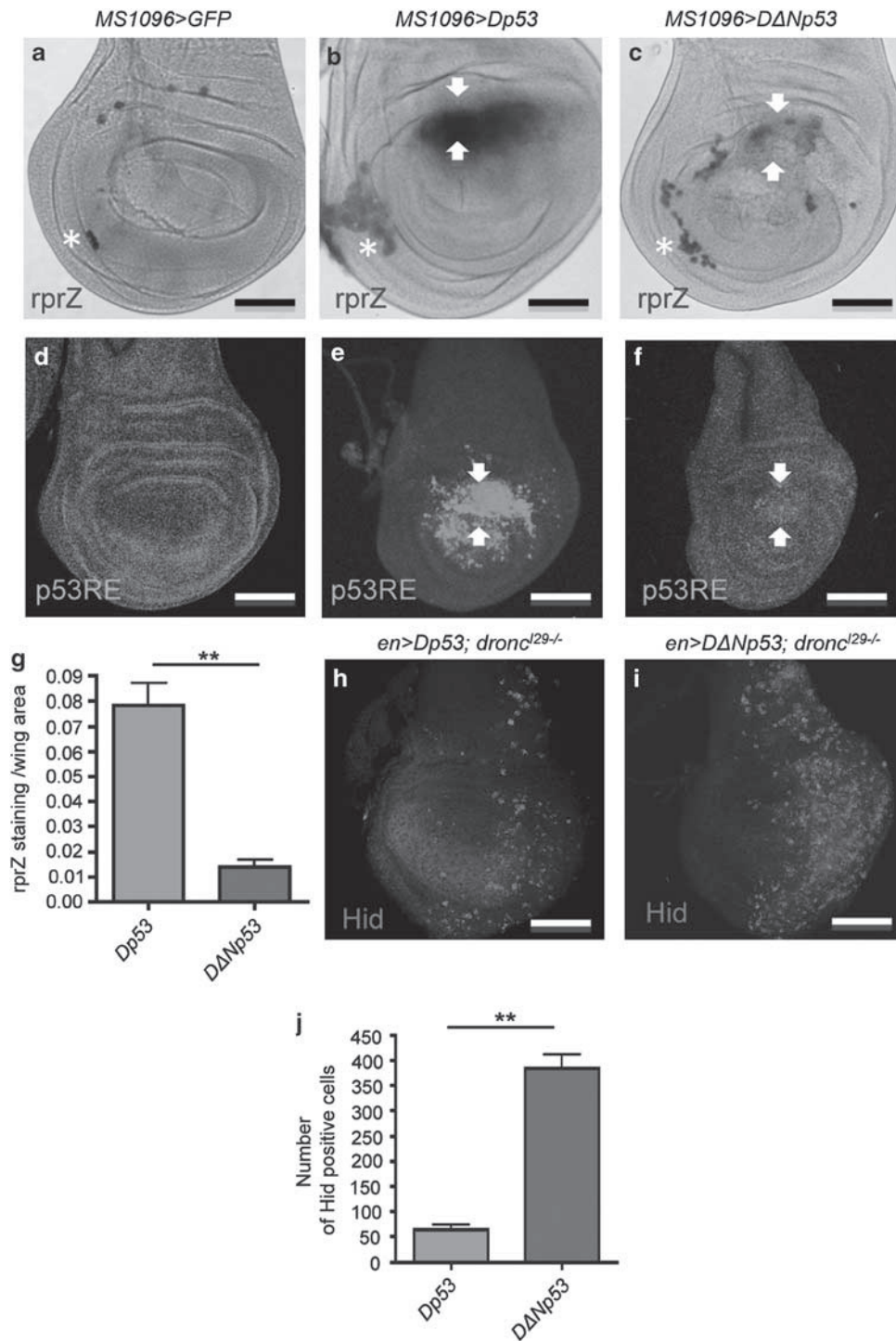
**Figure 1** *Dp53* or *DΔNp53* expression induces caspase activation in wing imaginal discs. (a–f) Ectopic production of *Dp53* or *DΔNp53* using the *MS1096* driver. (a–c) *Dp53* and *DΔNp53* protein isoforms are detected by immunostaining with an anti-p53 antibody (25F4) directed against the common C terminus domain. (a) *MS1096 > GFP* is used as a negative control. *Dp53* (b) and *DΔNp53* (c) are detected in the *MS1096* domain of expression. (d–f) Wing imaginal discs were stained using an anti-cleaved caspase 3 antibody (Cas 3\*). Elevated levels of *Dp53* (*MS1096 > Dp53* in e) or *DΔNp53* (*MS1096 > DΔNp53* in f) induce strong caspase 3 staining in the *MS1096* domain. Caspase activation is not detected in control wing discs (*MS1096 > LacZ* in d). (g) Western blot analysis of *Dp53* and *DΔNp53* in the wing imaginal discs using an anti-p53 antibody (C11) against the common C terminus domain. *MS1096 > Dp53* and *MS1096 > DΔNp53* show a band around 60 kDa and 50 kDa, respectively. The endogenous level of *DΔNp53* is detected in the wild-type control (*MS1096 > GFP*). Tubulin is used as loading control. Scale bars are 100 μm

damage-induced transcription of *rpr*. In contrast, *DΔNp53* is a poor activator of *rpr* and favors *hid*-mediated apoptosis.

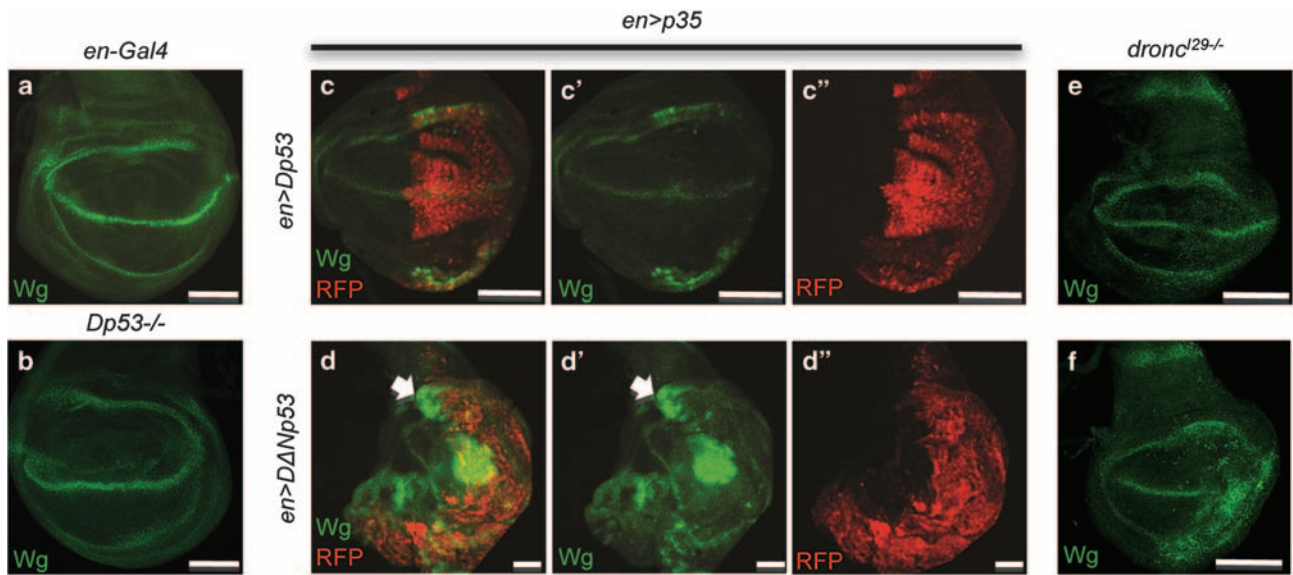
***Dp53* and *DΔNp53* differentially regulate apoptosis-induced proliferation.** Johnston and colleagues<sup>7</sup> have proposed that *Dp53* gene promotes the expression of mitogens, such as *Wg*, which is required for apoptosis-induced proliferation. However, the specific roles of the *Dp53* and *DΔNp53* isoforms in activating *Wg* have not been defined. To study the roles of *Dp53* and *DΔNp53* in apoptosis-induced proliferation, we examined *wg* expression and cell proliferation after *Dp53* or *DΔNp53* proteins were produced in the developing wing tissues (Figures 3 and 4). We first used the ‘undead cell’ model in which apoptosis is initiated by the expression of *Dp53* isoforms but its execution

is inhibited by expressing the inhibitor of caspase p35 (Figure 3). We found that *DΔNp53* induced strong and widespread *wg* expression associated with hyperproliferative tissue in a deformed wing disc (Figure 3d). In this context, we determined whether *wg* expression was induced in neighbouring unaffected cells, namely, in a cell-non-autonomous manner. We observed that *DΔNp53* induced *wg* expression both inside and outside of the *engrailed* domain of expression labelled with RFP (Figures 3d, d’ and d’'). This result indicates that *DΔNp53* induces *wg* expression both in a cell-autonomous and non-autonomous manner. In contrast to *DΔNp53*, when ectopically expressed, *Dp53* was only able to induce a moderate increase of *wg* that mainly resulted in a thickening of the *Wg* endogenous pattern of expression within the *engrailed* domain (Figure 3c). Moreover, *Wg*





**Figure 2** *Dp53* and *DΔNp53* use distinct RHG for apoptosis. (a–c) Wing imaginal discs carrying a *rpr<sup>XRE</sup>-lacZ* (*rprZ*) was stained for  $\beta$ -galactosidase activity. A strong induction of *rprZ* is observed upon overproduction of *Dp53* (*MS1096>Dp53* in b; arrows). In contrast, weak *rprZ* induction is observed upon overproduction of *DΔNp53* (*MS1096>DΔNp53* in c; arrows). No *rprZ* induction is observed in the control disc (*MS1096>GFP*; a). White stars mark LacZ-positive phagocytes. (d–f) GFP fluorescence is observed in wing imaginal discs carrying *p53R-GFPnls* (*p53 RE*). Stronger GFP labelling is observed in *Dp53* expression discs (e) compared with *DΔNp53* expression discs (f), or in control wing discs (d). (g) Quantification of the *rprZ* staining area relative to the total wing area. (h and i) Hid protein was visualized by immunostaining in ‘undead cells’ with an anti-Hid antibody in wing imaginal discs. *Dp53* overproduction induces a mild *hid* expression (*en>Dp53; dronc<sup>129-/-</sup>* in h). *DΔNp53* induces a strong *hid* expression in the *engrailed* domain of expression (*en>DΔNp53; dronc<sup>129-/-</sup>* in i). Scale bars are 100  $\mu$ m. (j) Quantification of Hid-positive cells per wing in h and i. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , in Student’s *t*-test



**Figure 3** *DΔNp53* induced *wg* expression in undead cells. (a–f) Wg protein was stained with an anti-Wg antibody (green). (a, b) The *wg* expression in wild-type (a) and in *p53*-null wing discs (b). (c and d) Double staining Wg (green) and RFP (red). Overproduction of *Dp53* or *DΔNp53* in wing imaginal discs expressing *p35* and RFP (*en* > *p35* > RFP). The *engrailed* domain expression is visualized by RFP. Wg (c' and d') and RFP (c'' and d'') single fluorescent channels are shown. (c, c' and c'') A mild induction of Wg is induced by *Dp53* overproduction (*en* > *Dp53*) resulting in broadening of the endogenous Wg expression pattern in the *engrailed* domain. (d, d' and d'') The overproduction of *DΔNp53* (*en* > *DΔNp53*) induces a strong and widespread induction of Wg inside and outside the *engrailed* domain of expression (arrow). (e and f) Overproduction of *Dp53* or *DΔNp53* in wing imaginal discs mutant for *dronc*<sup>129</sup>. (e) A mild induction of Wg is induced by *Dp53* overproduction (*en* > *Dp53*; *dronc*<sup>129-/-</sup>) resulting in broadening of the endogenous Wg expression pattern in the *engrailed* domain. (f) The overproduction of *DΔNp53* (*en* > *DΔNp53*; *dronc*<sup>129-/-</sup>) induces a strong and widespread induction of Wg. Scale bars are 100 μm

expression pattern was completely normal in *p53*-null wing disc, indicating that endogenous *p53* gene does not regulate *wg* expression (Figures 3a and b). Next, we examined whether the induction of *wg* by *Dp53* isoforms required *dronc*. We found that in *dronc* mutant wing discs, *DΔNp53* also induced stronger *wg* expression than *Dp53* (Figures 3e and f). From these results, we conclude that the regulation of *wg* expression by *Dp53* isoforms is *dronc* independent.

Next, we asked if the regulation of *wg* by *Dp53* isoforms can be detected in 'genuine' apoptotic cells. To achieve this goal, we used the strong MS1096 wing imaginal disc driver (Figure 4). As in the 'undead' cell model, we observed that *DΔNp53* induced strong *wg* expression in 'genuine' apoptotic cells (Figure 4c). The increased level of *wg* expression was clearly detected in the dorsal part of the wing pouch region where the MS1096 is the strongest (Supplementary Figure S1b). The increased *wg* expression was associated with tissue accumulation and folding, suggesting hyperproliferation (Figures 4c and f). In contrast to *DΔNp53*, *Dp53* expression did not alter the overall pattern of *wg* expression, but resulted in a thickening of the endogenous *wg* expression pattern (Figure 4b).

Next, we used a PCNA-EmGFP reporter that monitors E2f1 activity and EdU staining for cell proliferation.<sup>33</sup> We observed enhanced PCNA-EmGFP labelling in the presence of *DΔNp53*, indicating increase cell proliferation (Figure 4i). In contrast, PCNA-EmGFP was only weakly induced by *Dp53*, suggesting that *Dp53* induces little proliferation compared with *DΔNp53* (Figures 4h and i). We also evaluated proliferation by EdU, a thymidine analogue that stains cells that have

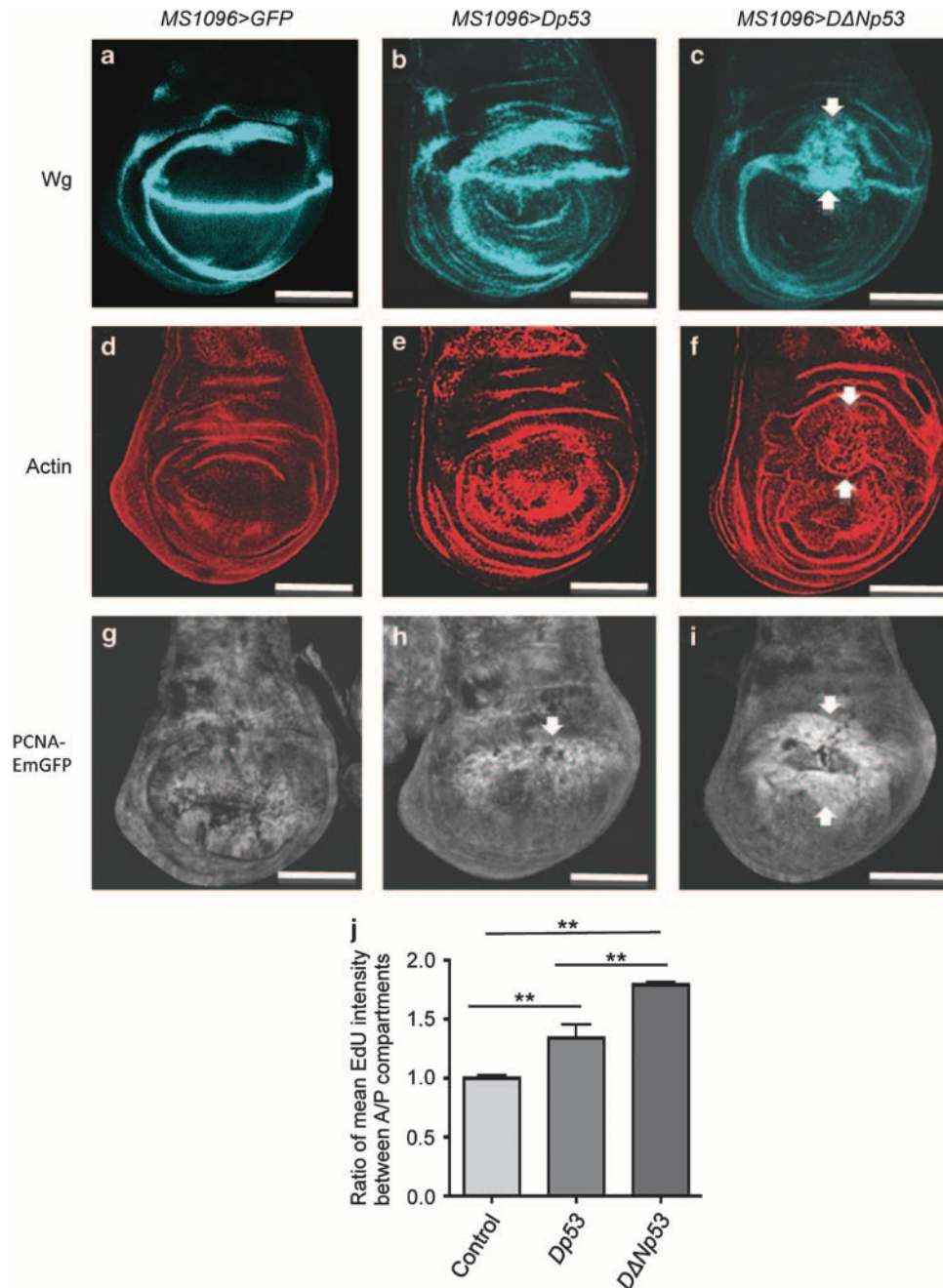
transited to S phase (Figure 4j). Consistent with the PCNA-EmGFP assay result, the EdU staining revealed that *DΔNp53* induces more proliferation than *Dp53*.

*Drosophila p53* gene is proposed to act in a feedback loop to self-amplify and promote apoptosis-induced proliferation.<sup>7,34</sup> Therefore, we wanted to examine how the endogenous *p53* gene contributes to the observed overexpression phenotype. We produced *DΔNp53* or *Dp53* in *p53*-null flies.<sup>35</sup> First, we found that elevated levels of *Dp53* or *DΔNp53* led to robust caspase activation, indicating that each isoform can induce apoptosis in the absence of the endogenous *p53* gene (Figures 5a and b). Next, we observed that *DΔNp53* retained the ability to increase *wg* expression in the *p53*-null flies (Figures 5d and d'). This suggests that *DΔNp53* overexpression alone is sufficient to induce *wg* expression. In contrast, in *p53*-null wing discs, we observed that *Dp53* expression no longer induced any thickening of *wg* endogenous expression pattern. Rather, we observed a reduction of *wg* expression, which could be attributed to apoptosis of Wg-positive cells in this area (Figure 5c'). Together, these results show that *DΔNp53*, but not *Dp53*, is the positive regulator of *wg* expression.

## Discussion

The discovery of multiple p53 isoforms raises the question of their functional specificity in the spectrum of p53-mediated biological responses. In *Drosophila*, as the first and only p53 isoform identified in almost a decade, the truncated *DΔNp53* isoform was initially presumed responsible for all p53 activities.

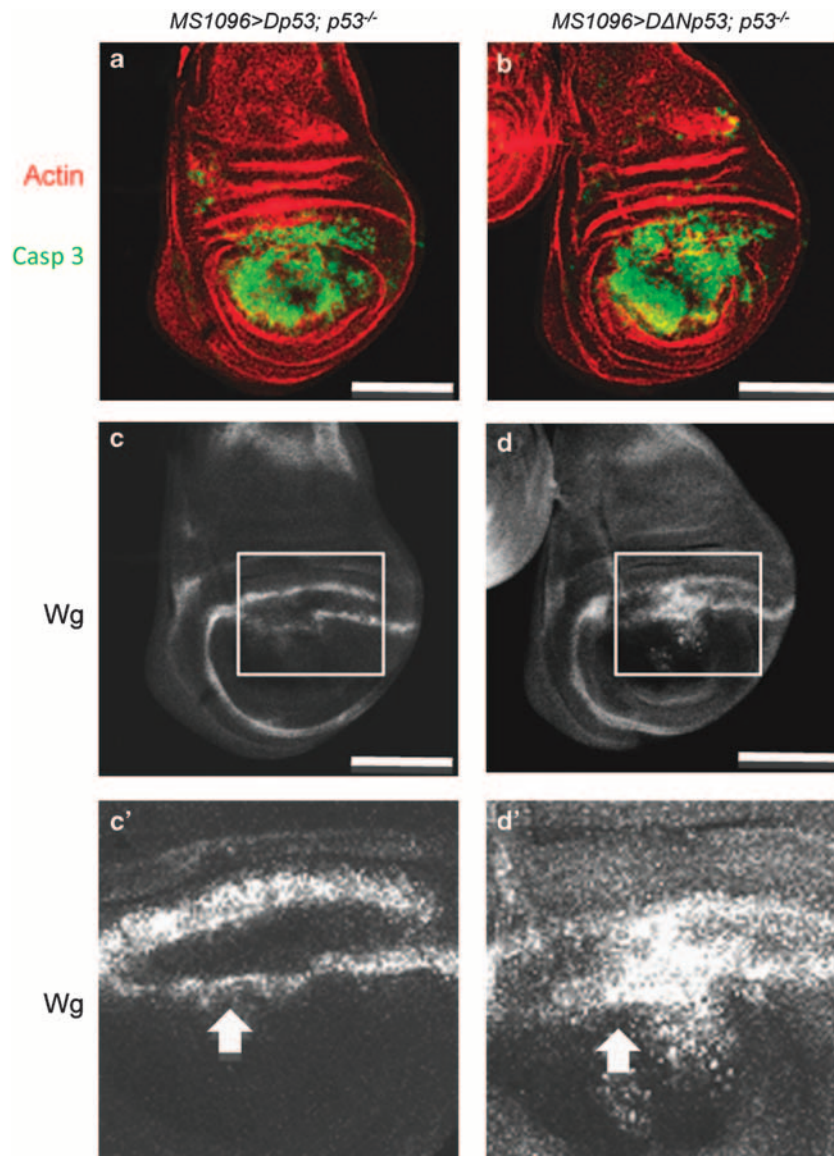




**Figure 4** DΔNp53 induced *wg* expression and enhanced proliferation in wing imaginal discs. (a, d and g) Control wing imaginal discs (*MS1096> LacZ*). (b, e and h) Wing imaginal discs overproducing Dp53 (*MS1096> Dp53*). (c, f and i) Wing imaginal discs overproducing DΔNp53 (*MS1096> DΔNp53*). (a–c) Wg protein was stained with an anti-Wg antibody. (d–f) Actin was stained with phalloidin coupled with TRITC. (g–i) GFP fluorescence in wing imaginal discs carrying PCNA-EmGFP. (c) The overproduction of DΔNp53 leads to a strong increase of *wg* expression in the *MS1096* domain. Increased *wg* expression by DΔNp53 is associated with tissue folding as visualized with actin staining (f) and with enhanced proliferation visualized with the PCNA-EmGFP reporter (i). Dp53 overproduction does not alter the overall Wg pattern but leads to an apparent thickening of the endogenous Wg domain (b). Dp53 overproduction does not induce tissue folding (e) and only induces some PCNA-EmGFP expression (h). Scale bars are 100  $\mu$ m. (j) Quantification of EdU staining between the anterior and posterior compartments in control (*en> p35*) and wing discs overproducing Dp53 (*en> Dp53*) or DΔNp53 (*en> DΔNp53*). \*\* $P \leq 0.01$ , in Student's *t*-test

The identification of the full-length Dp53 isoform that contains a full N-terminal transactivation domain challenged this presumption. Here, using gain-of-function studies, we examined the role of these two isoforms in apoptosis and apoptosis-induced proliferation. We found that both Dp53

isoforms activate apoptosis but preferentially activate different DIAP antagonists (Rpr or Hid) for caspase activation (Figures 1, 2 and Supplementary Figure S2). We showed that DΔNp53 promotes *wg* expression and cell proliferation, independently of endogenous p53, whereas Dp53 is unable



**Figure 5** Dp53 and DΔNp53 differentially regulated *wg* expression. (a–d) Overexpression of Dp53 or DΔNp53 in *p53* mutant flies. Dp53 (a and c) (*MS1096>Dp53; p53*<sup>-/-</sup>) and DΔNp53 (b and d) (*MS1096>DΔNp53; p53*<sup>-/-</sup>) are expressed in *p53* mutant wing imaginal discs. (a and b) Overproduction of Dp53 or DΔNp53 leads to strong active caspase 3 staining (green). (c and c') The overproduction of Dp53 inhibits *wg* expression at the dorso-ventral boundary (arrow). (d and d') The overproduction of DΔNp53 induces *wg* expression (arrow). c' and d' are magnified views of the rectangles shown in c and d, respectively. Scale bars are 100 μm

to do so (Figures 3–5). We also found Dp53 to be primarily responsible for damage-induced transcriptional activation of *rpr*, whereas DΔNp53 is the p53 isoform dedicated to promoting apoptosis-induced proliferation.

The landmark study of Abrams and colleagues<sup>21</sup> showed that DΔNp53 binds a DNA damage response element in the *rpr* regulatory region, which is responsible for the induction of apoptosis in response to irradiation. Here we showed that in wing imaginal discs, Dp53 is a stronger inducer of *rpr* expression than DΔNp53 (Figure 2). Moreover, we showed that DΔNp53 strongly induced *hid* expression, whereas Dp53 was only a weak inducer. Together, these observations suggest that the transcriptional competence of DΔNp53 differs from that of Dp53, and is consistent with a previous

study showing that *hid* is transcriptionally induced by DΔNp53 in eye and wing imaginal discs.<sup>25,28,34</sup> These results also suggest that some intrinsic ability to distinguish its activity for *rpr* and *hid* expressions is embedded in the N-terminus of the full length Dp53. Therefore, we propose that Dp53 is responsible for the damage-mediated activation of *rpr* for apoptosis, whereas DΔNp53 promotes apoptosis by inducing expression of *hid*. The physiological consequences of this functional segregation in apoptosis regulation by p53 isoforms remain to be determined.

Previous works have shown that apoptotic cells secrete morphogens that induce proliferation of surrounding cells.<sup>4,36,37</sup> Although more clearly detected in 'undead cells', mitogen gene expression and extra proliferation have also

been detected in genuine apoptotic cells.<sup>4,36,38</sup> It was proposed that the initiator caspase Dronc leads to *Dp53* expression, which in turn activates mitogen gene expression,<sup>7,34</sup> but the specific roles of *Dp53* and *DΔNp53* remain to be established. Here we showed that *DΔNp53* is a potent inducer of *wg* expression both in the 'undead cell' and genuine apoptotic cell models (Figures 3–5). Specifically, we showed that *DΔNp53* induced *wg* expression independently of *dronc* (Figure 3f). This indicates that *DΔNp53* acts downstream of the apoptotic pathway to induce proliferation via the expression of *wg*. Thus, like JNK,<sup>39</sup> *DΔNp53* promotes proliferation independently of the apoptotic cascade. Further analysis will be required to determine the relationship between JNK and p53 isoforms in the induction of proliferation.

Wells *et al.*<sup>7</sup> proposed that in the apoptosis-induced proliferation process, there is a feedback loop that activates *wg* expression in 'undead cells' via Dronc and *Dp53*. Our results are consistent with such a feedback mechanism in which *Dp53* and *DΔNp53* induce apoptosis via *rpr* and *hid*, which in turn amplifies *DΔNp53* via Dronc to promote *wg* expression. Our results also suggest that the feedback loop not only functions in 'undead cells' but also in genuine apoptotic cells. Together, we propose that p53 isoforms act both upstream and downstream of the apoptotic pathway to promote *wg* expression and proliferation.

Our results show that *DΔNp53* is a potent inducer of *wg* expression in both wild-type and *p53*-null wing discs. In contrast, *Dp53* only weakly increased *wg* expression in wild-type but not in *p53*-null flies (Figures 3–5). Therefore, the weak induction of *wg* expression by *Dp53* in wild-type disc is likely dependent on the endogenous *p53* gene. Further investigations will be required to determine if *DΔNp53* is the only p53 isoform regulating *wg* expression or if another isoform such as *Dp53ΔC* or the one encoded by the recently annotated p53-RD transcript (Flybase) contribute as well to the regulation of *wg* expression.

One of the most intensely debated questions regarding *Drosophila* ΔNp53 isoforms is whether they have their own biological activity or exert a dominant negative activity on p53.<sup>40–42</sup> The fact that *DΔNp53* induced *Wg* expression independently of endogenous *p53* gene indicates that *DΔNp53* does not require *p53* for this function. In vertebrate studies, zebrafish Δ113p53 and human Δ133p53 do not act exclusively in a dominant-negative manner toward *p53* but differentially regulate p53 target gene expression to modulate p53 function.<sup>41,42</sup> Similarly, our results show that *Drosophila* p53 isoforms have the capacity to use distinct targets to orchestrate their biological functions; we have shown that *Dp53* promotes *rpr* expression, whereas *DΔNp53* activates *Hid* and *Wg* expression in wing epithelium (Figures 2–5). Overall, we propose that balancing apoptosis and apoptosis-induced proliferation may represent one primordial function of the *TP53* gene family, and that this function requires the expression of *Dp53* and *DΔNp53* isoforms in a tightly controlled manner. In vertebrate, this primordial functional capacity may be differently exploited by TP53, TP63 and TP73 to regulate specific aspects of death/proliferation in the equilibrium, depending upon tissues and physiological contexts.

## Material and Methods

**UAS-Dp53 and UAS-DΔNp53 transgenic lines.** *Dp53* and *DΔNp53* cDNAs were cloned (Kpn1/Xba1) into a pUAST-w + -attB transgenic fly vector. Best Gene, Inc. (Chino Hills, CA, USA) generated transgenic lines using φC31 integrase-mediated transgenesis. Vector DNA was injected in embryos carrying attP docking sites (strain 9736 at 53B2 and strain 9750 at 65B2). *W*<sup>+</sup> embryos were selected and for establishing stable transgenic fly stocks.

**Fly stocks.** The following transgenic and mutant fly stocks were used: *MS1096-Gal4*, *en-Gal4*, *uas-lacZ* and *uas-RFP* (Bloomington stock); *uas-GFP*,<sup>43</sup> *rpr*<sup>XRE</sup>-*lacZ* (*rprZ*) and *dronc*<sup>J29</sup> (kind gifts from A Bergmann<sup>25</sup>); *p53R-GFPnls* (*p53* RE; a generous gift from J Abrams<sup>15</sup>), *rpr87*,<sup>44</sup> deficiency (3L)H99 (*Df*(3L)H99, referred to as H99<sup>45</sup>), *PCNA-EmGFP*<sup>33</sup> and *p53*-null (*p53* [5A-1-4]).<sup>35</sup> The following genetic combinations were used to express transgenes in wing imaginal discs: (1) *MS1096-Gal4;uas-GFP* (*MS1096 > GFP*), (2) *MS1096-Gal4;uas-Dp53* (*MS1096 > Dp53*), (3) *MS1096-Gal4;uas-DΔNp53* (*MS1096 > DΔNp53*), (4) *en-Gal4;uas-Dp53* (*en > Dp53*), and (5) *en-Gal4;uas-DΔNp53* (*en > DΔNp53*). Flies were raised under standard conditions at 25 °C.

Additional information can be found in the supplemental information.

## Conflict of Interest

The authors declare no conflict of interest.

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