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DEPARTAMENTO DE BIOLOGIA VEGETAL

The role of the IRE1/Xbp1 pathway in a *Drosophila* model for Autosomal Dominant Retinitis Pigmentosa (ADRP)

Rita Esperto Costa

Mestrado em Biologia Molecular e Genética

Lisboa 2011

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Mestrado em Biologia Molecular e Genética

Lisboa 2011

Acknowledgments

This research project would not have been possible without the support of many people. Thus, I which to express my gratitude:

To Instituto de Tecnologia Química e Biológica (ITQB) and Instituto Gulbenkian Ciência (IGC) for their facilities.
To Fundação para a Ciência e Tecnologia that financed this project and the Cell Signaling in *Drosophila* laboratory.

To Pedro Domingos, my supervisor, who helped me to overcome every obstacle, always believing in my work and encouraging me to continue.
To all my lab colleagues for their assistance, especially to Fátima for teaching me biomolecular techniques and helping with the cell transfections and to Dina for being very polite and helpful whenever I needed.

To Monica Dias lab for sharing Gateway reagents.

To Rui Gomes, my faculty teacher and supervisor, for his enthusiasm and his teachings.

Finally, to all my beloved families and friends for their understanding and endless love, through the duration of my studies.
A special thank you to Jorge Costa, Paula Esperto, Gil Costa, Rafaela Costa, Diogo Claro, Francisco Freixo, Ana Duarte, Joana Assunção, Ana Nunes, Inês Figueira e Petra Pintado.

Abstract

Neurodegenerative disorders are a major medical problem for society. Growing evidence indicates that endoplasmic reticulum (ER) stress is an important common pathological event in a variety of neurodegenerative diseases. The unfolded protein response (UPR) is triggered by the accumulation of misfolded proteins provoked by ER stress conditions, to restore homeostasis. We are particularly interested in the Ire1/Xbp1 branch of the UPR, in which Ire1 mediates the unconventional splicing of Xbp1 mRNA so that $Xbp1_{spliced}$ can activate the transcription of UPR effectors genes.

Our aim is to investigate the molecular mechanisms regulating the co-localization of Ire1 with Xbp1 mRNA, which are required for the splicing of Xbp1 mRNA. For this purpose we generated two Ire1-tagged transgenes: 1) in which a mcherry tag is between the transmembrane and the kinase domains of Ire1. 2) Ire1 is fused with a GFP tag in its C-terminal. S2 cell transfections show that our Ire1 constructs are being expressed and correctly localize to the ER. Also, cell transfections suggest that both Ire1 constructs are functionally active, since they are able to splice a Xbp1-EGFP reporter.

In parallel, we want to investigate the role of microRNAs (miRNA) in the Xbp1 post-transcription regulation. We started to establish an assay to do miRNA profiling in the context of Ire1/Xbp1 pathway activation. We did preliminary tests where we followed the progression of retinal degeneration induced by light in *wt* flies or in a ADRP fly model. So far, we could not find the right experimental conditions in which ADRP fly eyes degenerate but control flies do not.

Key words: ER stress, UPR, Retinal degeneration, Ire1, Xbp1

Resumo

As doenças neurodegenerativas constituem um problema emergente na sociedade actual, sendo o stress do retículo endoplasmático (RE) um evento patológico comum a uma variedade destas doenças. Quando ocorre stress no RE, um mecanismo celular denominado *unfolded protein response* (*UPR*) é activado, pela acumulação de proteínas *misfolded*, para que a célula possa restaurar a homeostasia. O *UPR* leva a uma diminuição da tradução de proteínas clientes do RE e a um aumento da capacidade de lidar com proteínas *misfolded*, o qual ocorre pela activação da transcrição de uma série de genes do RE, como por exemplo, as chaperones. Paralelamente, o *UPR* tem também como consequência a activação da via de degradação associada ao RE, que degrada proteínas com *folding* incorrecto, através do sistema de ubiquitina-proteossoma. Em conjunto, os mecanismos activados pelo *UPR* permitem restabelecer o funcionamento do RE. Contudo, nos casos em que o stress no RE é severo e prolongado, o *UPR* pode não ser suficiente para contrabalançar os seus efeitos. Nestes casos, são desencadeadas vias de sinalização que culminam na morte celular programada – a apoptose.

O *UPR* é iniciado por transdutores transmembranares do RE que conseguem reconhecer a acumulação de proteínas *misfolded* no lúmen do RE. Existem três transdutores: o *inositol-requiring enzyme 1* (Ire1), o *pancreatic ER kinase* (*PKR*)-*like ER kinase* (PERK) e o *activating transcription factor 6* (ATF6), que activam três ramos do *UPR*, mecanicamente distintos. O Ire1 é o mais conservado destes transdutores, uma vez que está presente em todos os eucariotas. Nos metazoários, depois de activado, Ire1 catalisa a remoção de um intrão do mRNA de *X-box binding protein 1* (Xbp1) – um mecanismo de *splicing* independente do spliceosoma. Quer a forma *spliced*, quer a forma *unspliced* do mRNA de Xbp1 são traduzidas e originam proteínas, mas apenas a forma *spliced* de Xbp1 origina um factor de transcrição eficiente, que activa a expressão de genes-alvo do *UPR* a jusante. A remoção do intrão de Xbp1 causa um *frameshift* durante a tradução, que introduz um novo domínio C-terminal na proteína, o qual é essencial para função desta como factor de transcrição.

A Retinite Pigmentosa (RP) é uma doença caracterizada pela degeneração progressiva dos fotorreceptores, tendo como principais sintomas a cegueira nocturna, seguida pela perda da visão periférica e, numa fase mais avançada, pela perda da visão central. Utilizando um modelo em *Drosophila melanogaster* de RP, conclui-se que a via de sinalização de Ire1/Xbp1 tem um papel relevante na protecção contra a degeneração da retina. Assim, mais estudos

sobre a regulação da via de sinalização de Ire1/Xbp1 poderão permitir uma melhor compreensão da RP.

Neste trabalho, estivemos particularmente interessados na via de sinalização de Ire1/Xbp1 do *UPR*, tendo dois objectivos principais de estudo.

1. O primeiro objectivo foi o de estudar em *Drosophila* o fenómeno de co-localização do Ire1 e do mRNA de Xbp1.

Uma vez que Ire1 é uma proteína transmembranar do RE, para que se possa realizar o *splicing* do mRNA de Xbp1 é necessário que este último, de alguma forma, seja recrutado para a vizinhança do RE. Investigações recentes sugerem diferentes modelos para a co-localização de Ire1 e do mRNA Xbp1. De acordo com o estudo em levedura de Aragon *et al*, sempre que as células estão sob condições de stress, Ire1 "oligomeriza-se" e acumula-se em *foci* na membrana do RE. O mRNA Hac1 (o homólogo funcional de Xbp1 na levedura) é então recrutado por intermédio de um elemento bipartido presente no 3' *untransleted region* (UTR) para estes *foci*. Já Yanagitani *et al* argumentam que a pausa na tradução de Xbp1 *unspliced* é que é necessária para o recrutamento (por intermédio de uma região hidrofóbica denominada HR2) e para o *splicing* do mRNA de Xbp1 em células de mamíferos.

Em *Drosophila*, foi identificada uma região HR2 mas a sua funcionalidade ainda não foi comprovada. O fenómeno de co-localização do Ire1 e do mRNA de Xbp1 continua por explorar.

Neste trabalho, originámos dois transgenes de Ire1 fundidos com uma cauda fluorescente, de modo a ser possível a sua visualização em microscopia confocal. Num dos transgenes, foi inserida uma cauda mcherry entre o domínio transmembranar e o domínio cinase da proteína Ire1. No outro caso, uma cauda GFP foi fundida na extremidade C-terminal de Ire1. Seguidamente, estas construções foram clonadas em vectores pUAST e transfectadas em células S2 para podermos avaliar se elas seriam expressas na célula, se se localizariam correctamente e se estariam funcionais. As transfecções efectuadas mostraram que as nossas construções de Ire1 foram expressas pelas células e localizavam-se, como esperado, no RE. Além disso, as transfecções também sugeriram que as construções de Ire1 são funcionais, já que realizaram o *splicing* do repórter Xbp1-EGFP. No entanto, para que possamos ter a certeza que as nossas construções são viáveis, estes resultados precisam de ser repetidos e ser validados no organismo da *Drosophila*.

2. O segundo objectivo de estudo foi o de investigar a regulação pós-transcricional de Xbp1, mais concretamente, de analisar se os miRNA podem ter uma função neste processo.

A regulação pós-transcricional relativamente à estabilidade, à tradução e à localização do mRNA é muito importante para um rápido controlo da expressão génica. Este tipo de regulação exige que se liguem ao mRNA proteínas de ligação ao RNA (RNA binding proteins - RBPs) e microRNAs (miRNAs). A regulação pós-transcricional da via de sinalização de Ire1/Xbp1 precisa de ser um fenómeno rápido para que o controlo de qualidade do RE não seja comprometido. De facto, os níveis de Xbp1 *spliced* parecem ser rigorosamente controlados para que as células ajustem rapidamente a sua resposta e recuperarem do stress no RE. Apesar disto, nunca foi investigado o envolvimento dos miRNAs e RBPs no contexto da vida de sinalização de Ire1/Xbp1, pelo que nos propusemos fazê-lo.

Com o intuito de investigar a regulação pós-transcricional de Xbp1, nós pretendemos fazer um levantamento da expressão de miRNAs no âmbito da degeneração dos fotorreceptores, utilizando o modelo em mosca de Retinite Pigmentosa Autossómica Dominante (*Autosomal Dominant Retinitis Pigmentosa* - ADRP) que temos no laboratório. Para tal, neste trabalho, decidimos tentar estabelecer um ensaio onde fosse possível estudar o perfil de miRNAs no contexto da via de sinalização de Ire1/Xbp1. Tendo em conta toda a variedade de factores envolvidos na degeneração de fotorreceptores da mosca, foi necessário fazer alguns testes preliminares que acompanharam o desenvolvimento da degeneração da retina induzida pela luz em moscas *wt* ou no nosso modelo de mosca de ADRP. Até à data, não foi possível encontrar as condições experimentais adequadas para o ensaio pretendido: a não degeneração dos olhos das moscas *wt* e a degeneração dos olhos das moscas do nosso modelo de ADRP. Ainda assim, vamos prosseguir com este trabalho, repetindo os testes preliminares com uma população maior e com parâmetros mais controlados. Planeamos ainda tentar encontrar alternativas ao ensaio que estamos a desenvolver, utilizando uma estratégia menos complexa.

Cumpre dizer que relativamente a ambos os objectivos, o trabalho aqui descrito representa o início de um estudo que pretendemos continuar no futuro, no sentido de esclarecer qual o modelo de co-localização de Ire1 e do mRNA de Xbp1 na célula e quais os factores implicados na regulação pós-transcricional de Xbp1.

Palavras chave: Stress do RE, *UPR*, Degeneração da Retina, Xbp1, Ire1

General Index

Acknowledgments iii
Abstract iv
Resumo v
General Index viii
Index of Figures x
Index of Tables xi
Abbreviations xii
1. Introduction 1
1.1. ER Stress and Neurodegenerative Disorders 1
1.2. The Unfolded Protein Response 2
1.2.1. Ire1/Xbp1 signaling pathway of the UPR 3
1.3. Autosomal Dominant Retinitis Pigmentosa 4
1.3.1. Ire1/Xbp1 pathway in the degeneration of photoreceptors 4
1.3.2. Post-transcriptional regulation of IRE1/Xbp1 pathway 5
1.4. Objectives 7
2. Materials and Methods 8
2.1. Drosophila Melanogaster Genetics 8
2.1.1. Generation of transgenic Drosophila melanogaster flies 8
2.1.2. Directed gene expression in Drosophila melanogaster 8
2.1.3. Lines Maintenance 8
2.1.4. Crosses 9
2.2. Microscopy 9
2.2.1. Retinal degeneration assessment across time 9
2.3. Molecular Biology Techniques 9
2.3.1. DNA Purification from Agarose Gel 9

2.3.2. Chemical Transformation of Competent Cells 10
2.3.3. Restriction and Ligation Reactions 10
2.3.4. Purification of Plasmid DNA 10
2.3.5. Molecular Cloning 10
2.3.6. Cell culture and Transfection 11
2.3.7. Immunostaining 12
2.3.8. Total RNA Extraction 12
2.3.9. Reverse Transcriptase Polimerase Chain Reaction (RT-PCR) 12
3. Results and Discussion 13
3.1. Study of Ire1 and Xbp1 mRNA localization and interaction 13
3.1.1. Ire1 fusion with fluorescent tags and cloning 14
3.1.2. Testing for localization and functionality of cloned constructions 18
3.2. Ire1/Xbp1 post-trancriptional regulation in the degerating photoreceptors 23
3.2.1. Premilinary tests for microRNA profiling 24
4. Future Perspectives 29
5. References 30
6. Attachments 33

Index of Figures

Fig. 1. The three major components of the Unfolded Protein response. **3**
Fig. 2. Schematic of Xbp1-EGFP construct which serves as ER stress reporter. **5**
Fig. 3. Xbp1 mRNA 3'UTR structure and conservation . **6**
Fig. 4. Model for the translational pausing of XBP1unspliced mRNA and its recruitment to the ER. **7**
Fig. 5. Comparison of $Xbp1_{unspliced}$ C-terminal sequences from Mouse, Human and *Drosophila*. **14**
Fig. 6. Alignment between Ire1 proteins of Mouse, *Drosophila* and Yeast. **15**
Fig. 7. Schematic of Ire1 constructs plan. **16**
Fig. 8. Strategy for the fusion of mcherry to Ire1 by PCR. **16**
Fig. 9. Cloning of Ire1::mcherry in pUAST. **17**
Fig. 10. Schematic of the Gateway system used. **17**
Fig. 11. Cloning of Ire1 in pTWG. **18**
Fig. 12. Ire1 and calnexin co-localization in S2 cells. **19**
Fig. 13. Ire1 and Xbp1-EGFP expression in S2 cells. **20**
Fig. 14. Xbp1-EGFP splicing occurs only when it is co-transfected with one of our Ire1 constructs. **21**
Fig. 15. RT-PCRs for Ire1 constructs **22**
Fig. 16. Xbp1-EGFP specific band digestion with PstI. **23**
Fig. 17. Retinal Degeneration assessment across time. **26**
Fig. 18. Retinal Degeneration assessment across time using attB lines. **27**
Fig. 19. PWIZ_attB lines hatch already with no DPP image. **27**
Fig. 20. Retinal Degeneration assessment across time using attB lines with constant light. **28**
Fig. 21. Examples of retinal degeneration of attB lines under constant light. **28**

Attachments

Fig.1. Fig.1. Xbp1 Unspliced and Spliced forms. **33**
Fig.2. Biogenesis of miRNA. **33**
Fig.3. Directed gene expression in *Drosophila* melanogaster by the GAL4 system. **34**
Fig.4. Crosses done during the master project. **35**
Fig.5. Vectors used in conventional cloning. **35**
Fig.6. Vectors used in Gateway cloning. **35**

Index of Tables

Attachments

Table 1. Lines used in pseudopupil assays **36**

Table 2. Primers used for conventional cloning of Ire1::mcherry **36**

Table 3. Primers utilized in the Gateway system cloning **36**

Table 4. Primers utilized in RT-PCRs **37**

Table 5. Retinal Degeneration assessment across time **37**

Table 6. Retinal Degeneration assessment across time with attB lines **37**

Table 7. Retinal Degeneration assessment across time with attB lines - constant light **38**

Abbreviations

µg – microgram

µL – microliter

a.a. – amino acid

ADRP – Autosomal Dominant Retinitis Pigmentosa

ATF6 – activating transcription factor 6

BiP – Immunoglobulin Binding protein

BSA – Bovine serum albumin

bZIP – basic leucine zipper

CDS – coding sequence

Cyo – Curly of Oster (dominant genetic marker)

DNA – Deoxyribonucleic acid

eIF2α – initiation factor 2 α

ER – Endoplasmic Reticulum

ERAD – ER-associated degradation

FBS – foetal bovine serum

GFP – green fluorescent protein

GMR – Glass multimer reporter

h – hour

Hac1 – Homologous to Atf/Creb1

HR2 – hydrophobic region 2 (present in Xbp1 unspliced protein)

hsc3 – heat shock cognate protein 3

Ire1 – Inositol-requiring enzyme 1

Ire1-PC – Ire1 isoform C protein

JNK – Jun N-terminal kinase

mg – milligram

min – minutes

miRNA – microRNA

mL – milliliter

mRNA – mensager ribonucleic acid

ninaE – neither inactivation nor after potential E (Rodopsin 1 gene)

ORF – open reading frame

PBS – Phosphate buffered saline

PCR – polymerase chain reaction

PEK – pancreatic eIF-2alpha kinase

pek-1 – human PERK kinase homolog

PERK – Pancreatic ER kinase (PKR)-like ER Kinase

RBPs – RNA binding proteins

Rh1 – Rhodopsin 1

RNA – ribonucleic acid

RP – Retinitis Pigmentosa

R-RNC – mRNA-ribossome-nascent chain

RT-PCR – Reverse Transcriptase Polimerase Chain Reaction

S2 cells – Schneider cells

Sb – Stubble (dominant genetic marker)

Sco – Scutoid (dominant genetic marker)

sec – seconds

TAE – Tris-acetate EDTA

Tm – temperatures of melting

TM6B – *Drosophila* 3rd chromosome balancer

UAS – Upstream activation sequence

UPR – Unfolded Protein Response

UTR – untranslated region

w – gene white

Xbp1 – X-box binding protein 1

1. Introduction

1.1. ER Stress and Neurodegenerative Disorders

Human neurodegenerative diseases constitute a major medical problem that affect millions of people worldwide. These disorders lead to nervous system dysfunction, as a result from the gradual and progressive loss of neural cells[1]. Consequently, they have a great impact on the patient, the family and medical services, with repercussions in social, financial and work life.

The proposed mechanisms of neuronal death have varied widely, puzzling scientists and leading them to ask whether there are effects that are common to diverse diseases. Although different neurodegenerative disorders have different etiologies and key players throughout its progression, they may share common pathological events. Accumulating evidence indicates that endoplasmic reticulum (ER) stress is one of the most important of these common pathological events. ER stress is caused by the accumulation of misfolded proteins in the ER and is observed in the symptomatic and late disease stage of many neurodegenerative disorders[2].

In eukaryotic cells, transmembrane and secreted proteins are synthesized in the ER. The lumen of this organelle provides an oxidative compartment where client-proteins can be co-translationally modified, folded and maturated[3]. It is estimated that about 100mg/mL of polypeptides are present in this compartment, a high concentration that promotes protein aggregation [4]. To deal with this load of proteins, the ER lumen has a very complex and dynamic network of chaperones, foldases and co-factors which prevent abnormal protein aggregation or misfolding and thus ensure the correct folding of ER client proteins[2]. Proteins that fail to reach the native conformation even after the assistance of ER chaperones are retrotranslocated back to the cytosol, where they undergo ubiquitination and degradation by the proteasome, a process named ER-associated degradation (ERAD)[5]. In addition to the function of protein synthesis, modification and folding, the ER also operates as the major calcium intracellular store[2].

Productive folding and ERAD are important aspects of the quality control machinery that guarantees the correct protein native conformations and the absence of protein aggregates in the cell. However, several conditions can contribute to an imbalance between high load of client proteins and insufficient capacity of the ER protein-folding machinery, leading to a ER stress[6] and, in many cases, to the saturation of the proteolytic machinery of the cell[6-7].

Misfolded proteins not only lack their physiologic function but may also become prone to spurious interactions with other proteins or to the formation of insoluble protein aggregates – a pathologic symptom that has been related with many neurodegenerative disorders.

1.2.The Unfolded Protein Response

ER stress is caused by a variety of physiological and other conditions, such as perturbations in calcium homeostasis, energy deprivation, redox changes, ischemia, hyperhomocystinemia, viral infections and mutations that impair client protein folding[3, 7]. The unfolded protein response (UPR) is a cellular mechanism that is triggered by these stress conditions to restore the normal functioning of the ER.

The direct consequences of the UPR are cell cycle arrest, the decrease of the protein load that enters the ER (transient adaptation) and the increase of the capacity of the ER to handle misfolded proteins (long-term adaptation)[3, 8]. These direct consequences are achieved by the attenuation of client-protein synthesis and translocation into the ER, the transcriptional activation of ER chaperones and the enhancement of the degradation of misfolded proteins present in the ER[9]. If homeostasis cannot be reestablished, cells may die by apoptosis as prolonged UPR activation leads to ER calcium release and pro-apoptotic signaling[8, 10].

The UPR is initiated by transmembrane transducer(s) that sense the accumulation of misfolded proteins in the ER. The number of such transducers has increased with evolution, from one in *Saccharomyces cerevisea* (inositol-requiring enzyme 1 (Ire1)) to three in metazoans (Ire1, pancreatic ER kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6)). These ER sensors are maintained in an inactive state by binding of Immunoglobulin Binding protein (BiP), one of the key chaperones of the ER. Upon ER stress, BiP is released from the UPR sensors to help with the folding of accumulating misfolded proteins, allowing the activation of the UPR transducers[11].

In mammalians, after activation, PERK phosphorylates the initiation factor 2 α (eIF2α) to attenuate protein translation and also phosphorylates NRF-2 to trigger the anti-oxidant response[10]; ATF6 is delivered to the Golgi where it is cleaved and then goes to the nucleus to work as a transcription factor that contributes to the increased expression of ER chaperones [8]; and Ire1 performs the splicing of X-box binding protein 1 (Xpb1) mRNA to activate the transcription of UPR effector genes and to activate ERAD (Fig. 1)[10, 12].

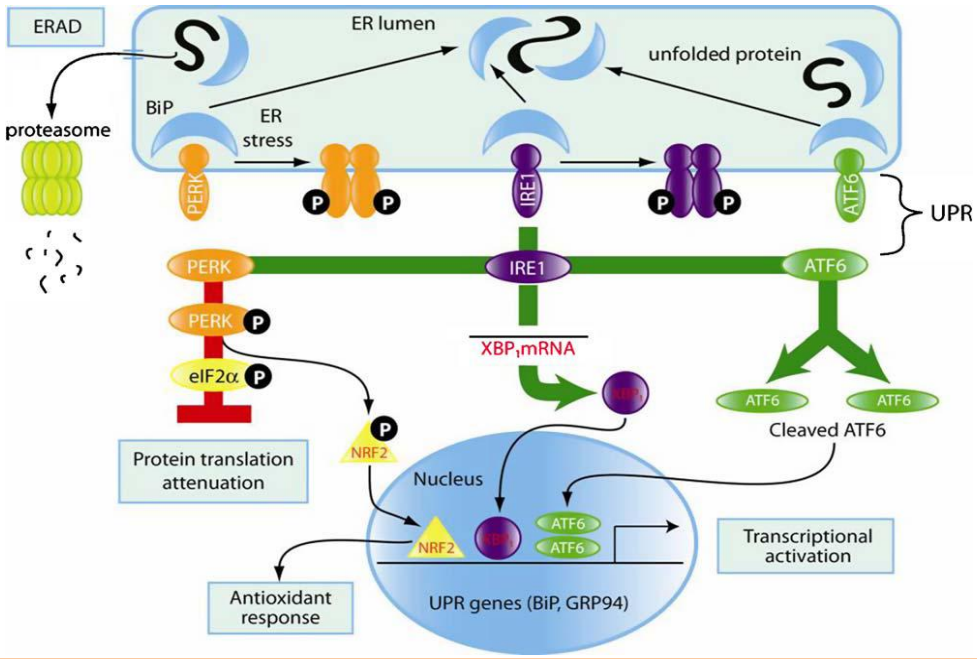

Fig. 1. The three major components of the Unfolded Protein response. PERK, Ire1 an ATF6 are inactive when connected to BiP. Upon ER stress BiP unbinds them and they become active. PERK contributes to attenuation of protein translation and to the antioxidant response. Cleaved ATF6 activates chaperones transcription. Ire1 leads to Xbp1 splicing, to transcription activation of chaperones and to stimulation of ERAD (from [10]).

1.2.1. Ire1/Xbp1 signaling pathway of the UPR

Ire1 is the most conserved among the UPR transducers and it is a major regulator of ER quality control[13]. Ire1 is a type 1 ER-resident transmembrane protein with a lumenal domain that senses ER stress, a transmembrane domain and a cytoplasmic domain which has both endoribonuclease and kinase activities. Under stress conditions, Ire1 becomes activated upon oligomerization and trans-autophosphorylation of the kinase domains [8].

The Ire1 pathway was first described in yeast (*S. cerevisea*)[14-15]. In this organism, once activated, Ire1 cleaves Homologous to Atf/Creb1 (Hac1) mRNA in two sites, removing an intron from it – an unconventional splicing event which is independent of the spliceosome [16-17]. Because this intron has the ability to block translation, only spliced Hac1 mRNA will originate the functional transcription factor which activates downstream gene expression[18]. In metazoans, Ire1 mediates the unconventional splicing of Xbp1 mRNA (the Hac1 functional homologue) (Fig. 1)[13]. In this case, the splicing causes a frame-shift during translation, which introduces a new carboxyl trans-activation domain in Xbp1 protein[19] (Attachment – Fig. 1). Spliced Xbp1 functions as a potent transcription factor for UPR-responsive genes[20]. Contrary to what happens to Hac1, both forms of Xbp1 mRNA (unspliced and spliced) are translated but only the spliced form produces an efficient transcription factor. The $Xbp1_{unspliced}$ is thought to work as negative regulator of the $Xbp1_{spliced}$, promoting its

degradation[21]. In *Drosophila*, the mutant allele *xbp1*^{k13803} is recessive lethal, indicating that *xbp1* is an essential gene during development[9].

Both Xbp1$_{spliced}$ and Hac1 translocate to the nucleus where they bind to DNA by a basic leucine zipper (bZIP) to activate the transcription of UPR target genes that code for ER chaperones, foldases, proteins involved in ER biogenesis, ERAD proteins and other protein folding quality control molecules[8, 22].

1.3.Autosomal Dominant Retinitis Pigmentosa

Retinitis pigmentosa (RP) defines a group of retinal neurodegenerative diseases, that affects about 1.5 million people across the world, in which abnormalities of the photoreceptors lead to progressive visual loss[6, 23]. The common symptoms are the defective dark adaptation or "night blindness", followed by constriction of the peripheral visual field and, eventually, loss of central vision late in the course of the disease. These symptoms result from degeneration of the photoreceptors and consequent impairment of the cone-receptor function[24]. It is estimated that about 30% of RP patients have mutations in rhodopsin (the light sensing protein present in photoreceptors). Actually, over 120 mutations have been identified in rhodopsin that are linked with RP and most of them cause Autosomal Dominant RP (ADRP)[6]. Nathans and Khorana showed that several of these pathogenic mutations fail to fold correctly[25-26].

In a *Drosophila* model for ADRP, flies, as humans, show late-onset retinal degeneration, which is caused by apoptosis of the photoreceptors cells[27].The *Drosophila* Rhodopsin 1 (Rh-1) shows a 22% homology in amino acid (a.a.) sequence with the human rhodopsin. Moreover, several alleles of *Drosophila ninaE* (gene encoding Rh-1) have both molecular and phenotypic characteristics identical to those found in class III ADRP[28-29]. These alleles have mutations in the transmembrane domain or in the “extracellular” portions of Rh1, which interfere with the correct folding of the protein[28, 30].

1.3.1. Ire1/Xbp1 pathway in the degeneration of photoreceptors

In a *Drosophila* model of ADRP, Xbp1 splicing and activation of an ER chaperone, the heat shock cognate protein 3 (hsc3), occur in response to ER stress caused by the accumulation of misfolded Rhodopsin-1 and the Ire1/Xbp1 pathway has a protective role against retinal degeneration[9]. In this study it was designed a fusion protein between Xbp1 and EGFP, in which EGFP is only in frame with Xbp1 when Ire1 mediated splicing occurs (Fig.2). Therefore, the Xbp1-EGFP protein functions as a reporter for ER stress and for the

activation of Ire1. Using this approach, my supervisor and his colleagues saw that NinaE dominant mutations lead to activation of Xbp1-EGFP, implying that Ire1/Xbp1 pathway is activated by rhodopsin mutants that show a degeneration of the retina[9]. Accordingly, they could conclude that the disruption of Xbp1 function enhances retinal degeneration in *ninaE*^{G69D} mutant[9].

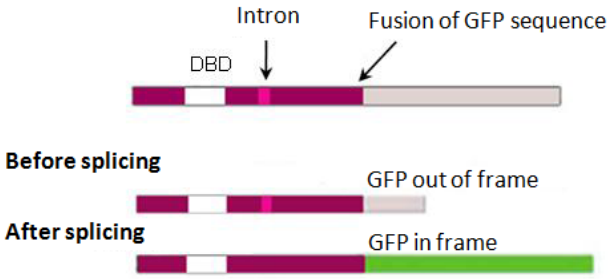

Fig. 2. Schematic of Xbp1-EGFP construct which serves as ER stress reporter. The EGFP gene was cloned downstream of the small intron that is removed by Ire1 in the splicing event. Whenever Ire1 is not activated, Xbp1 is not spliced and the EGFP gene is out of frame because a stop codon is present before it. In situations of ER stress, Ire1 is activated and removes the Xbp1 intron, causing a frameshift that allows the EGFP gene to be in frame with de $Xbp1_{spliced}$. (adapted from [9])

These findings link ER stress to the progression of ADRP and underline the importance of Ire1/Xbp1 pathway to overcome its consequences.

1.3.2. Post-transcriptional regulation of IRE1/Xbp1 pathway

Gene expression is controlled by several mechanisms and is modulated to ensure cellular homeostasis. The post-transcriptional regulation of mRNA stability, translation and localization is very important for rapid control of gene expression. This type of regulation requires factors that bind to mRNAs, such as RNA binding proteins (RBPs) and microRNAs (miRNAs)[31]. miRNAs are approximately 21-nucleotide-long non-coding RNAs which show base complementarities to the 3' unstranslated regions (UTRs) of target mRNAs[32]. miRNAs regulate gene expression through induction of mRNA degradation, repression of translation or by destabilizing mRNA and thereby causing its decay (Attachments, Fig.2)[32]. miRNA and RBPs can counteract or facilitate each other functions by blocking or allowing the accessibility of target transcripts, which highlights the importance of RBPs and miRNAs interaction at the 3'UTR of mRNAs in the regulation of gene expression [33-36]. Hence, identification of miRNAs and RBPs and their target mRNAs may be very advantageous to reveal crucial control steps in many biological contexts.

The post-transcriptional regulation of Ire1/Xbp1 pathway must be a rapid phenomena so that the ER quality control is not compromised. Indeed, Xbp1 spliced levels seem to be tightly controlled so cells can quickly adjust their response and recover from ER-stress. However, the involvement of miRNAs and RBPs has never been investigated in the context of Ire1/Xbp1 pathway.

Another important aspect of the Ire1/Xbp1 pathway is the fact that, once activated, Ire1 needs to be in contact with Xbp1 mRNA, which means that, whenever there is ER stress, Xbp1 mRNAs should concentrate around the ER membrane, where Ire1 is localized. Aragon *et al*, found that the splicing of Hac1 was significantly reduced when the 3'UTR was deleted or replaced by a non-specific one [37]. Further investigation revealed that Hac1 3'UTR forms a stem-loop structure, containing two short motifs (bipartite element) that are highly conserved among all Hac1 orthologues (Fig.3) [37]. The 3'UTR bipartite element was shown to be essential for the recruitment of Hac1 mRNA into Ire1 foci upon ER stress [37]. According to this study, whenever cells are under stress conditions, Ire1 oligomerizes and cluster into foci localized in the ER membrane, to where Hac1 mRNA is recruited, by an unknown mechanism that requires the 3'UTR bipartite element. The targeting of Hac1 mRNA only occurs while it is translational repressed, since the targeting signal is inactivated when Hac1 mRNA translation resumes (although 3'UTR bipartite element is still there)[37]. This suggests the role of translational repression by Hac1 intron not only in facilitating an UPR sensitive synthesis of Hac1 but also in the actual recruitment of its mRNA to vicinity of ER.

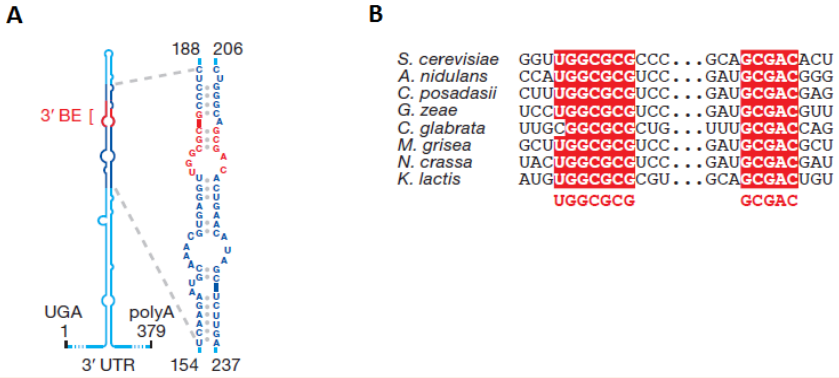

Fig. 3. Xbp1 mRNA 3'UTR structure and conservation **A**. Schematic of 3'UTR stem loop with the Bipartite element shown in expanded view to the right. The two red motifs are in the same region in opposite strands, constituting a Bipartite Element. Positional numbering is from UGA stop codon **B**. Alignment of the 3' BE in HAC1 orthologues (*Saccharomyces cerevisiae, Aspergillus nidulans, Coccidioides posadasii, Gibberella zeae, Candida glabrata, Magnaporthea grisea, Neurospora crassa, Kluyveromyces lactis*). BE- bipartite Element. From[37]

In mammalian cells, Yanagitani *et al*, argue that translational pausing is also required for the targeting and splicing of Xbp1 mRNA, although in a process that is independent of the Xbp1 3'UTR (Fig.4)[38]. Yanagitani and colleagues showed that $Xbp1_{unspliced}$ protein has an hydrophobic region – hydrophobic region 2 (HR2) –, which is a membrane targeting signal, that is responsible for the recruitment of $Xbp1_{unspliced}$ protein and its mRNA to the ER membrane[39]. This hydrophobic region is present in *Homo sapiens*, *Danio rerio*, *Xenopus tropicalis* and *Drosophila melanogaster*. In addition, $Xbp1_{unspliced}$ contains a C-terminal 26-a.a. region, evolutionary conserved, that is required to the translational pausing of

$Xbp1_{unspliced}$, to allow sufficient time for HR2 to target R-RNC complex to the ER[38]. In this model, translation of $Xbp1_{unspliced}$ mRNA is paused just before translation termination, when HR2 is exposed outside of the ribosome tunnel to drag R-RNC complex to the ER membrane (Fig.4)[38]. The amino acids required for this pausing are likely buried inside the ribosome tunnel at this stage.

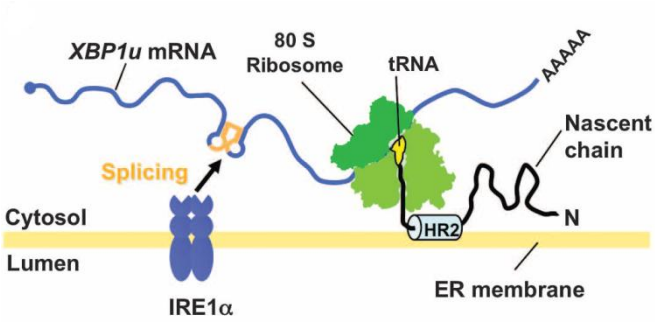

Fig. 4. Model for the translational pausing of XBP1unspliced mRNA and its recruitment to the ER. HR2-hydrophobic region 2. From [38]

Yanagitani *et al* hypothesis leaves aside the role of 3'UTR claimed by Aragon *et al*. Nevertheless, the two studies were done with different strategies since the first was done in *S. cerevisea* organism, using live microscopy, and the second was realized in cell culture and *in vitro*, utilizing mainly a biochemical assay in which the Xbp1 mRNA is detected (or not) in a membrane-rich fraction (ER membrane) or a cytoplasmic fraction.

In *Drosophila*, regulation of Ire1/Xbp1 pathway has not been explored so far and the parties involved in gene expression control and in the gathering of Ire1 and Xbp1 mRNA still need to be revealed. Since *Drosophila* is an organism that is evolutionary between yeast and mammalians, it would be interesting to explore how these processes occur in *Drosophila* and try to understand its relation with other organisms. And even more important, clarification of Ire1/Xbp1 pathway regulation could provide the development of new therapeutic approaches for the prevention of many neurodegenerative disorders associated with ER stress, particularly of ADRP.

1.4.Objectives

The aim of this project is to investigate the regulation of the Ire1/Xbp1 in the UPR, more specifically to try to understand what are the requirements for the targeting of Xbp1 mRNA to the ER membrane and to assess if there is an involvement of miRNAs or RBPs in the regulation of Xbp1 gene expression. To address that purpose several tasks were carried out. The work reported here is the starting point for the investigation we want to pursue in the future and, therefore, has as main objectives:

- The cloning of Ire1 fused with a tag in a *Drosophila* expression vector so that Ire1 can be visualized together with Xbp1 mRNA in live microscopy;
- The evaluation of expression, localization and functionality of cloned constructs by doing transfections of *Drosophila* Schneider cells (S2), imaging and Reverse Transcriptase Polimerase Chain Reactions (RT-PCRs);
- The establishment of an assay to analyze miRNA expression in the context of Ire1/Xbp1 pathway.

2. Materials and Methods

2.1. Drosophila Melanogaster Genetics

2.1.1. Generation of transgenic Drosophila melanogaster flies

All the transgene lines used in this project were inserted into pUAST (to random insertions) or pUAST-AttB vectors (for site-specific insertions). The plasmids were then injected into the fly embryos by BestGeneInc (U.S.A). pUAST-AttB vectors enable the using of øC31 system which allows the insertion of the transgenes in a specific site of the acceptor fly genome[40]. The øC31 system guarantees that all transgenes integrated in the same site will have the same level of transcription and, supposedly, the same level of expression.

2.1.2. Directed gene expression in Drosophila melanogaster

All transgenic flies used in the project are based on a directed gene expression method – the gal4 system. This system was adapted from a yeast transcriptional mechanism and allows the separation of the target gene from its transcriptional activator in two distinct transgenic lines. There are fly lines in which the yeast transcriptional activator GAL4 expression is derived from different genomic enhancers[41]. A GAL4-dependent target gene can be constructed by subcloning any sequence next to the GAL4 binding sites – the UAS sites – in another line. The target gene is silent in the absence of GAL4. To activate the target gene, flies carrying the UAS-Gene are crossed to flies expressing GAL4[41]. In the progeny of this cross, UAS-Gene will be activated in cells where GAL4 is expressed (Attachments, Fig.3).

In this project we utilized GMR-Gal4 (expression in the developing eye), Rh1-Gal4 expression in photoreceptors in the pupal and adult stages) and Actin5C-Gal4 (ubiquitously expression) enhancers.

2.1.3. Lines Maintenance

All flies were kept at 25°C in 70% of humidity in vials with food made from corn flour, sugar, molasses, agar and 10% Nipagin (in ethanol) , exposed to a 12h light/12h dark cycle.

2.1.4. Crosses

Crosses done in this work will be briefly explained here (details -Attachments, Fig. 4).

i) Crosses to the eye degeneration assessment across time

- Rh1Gal4;UASGFP^{ninac}/(cyo);Sb/TM6B was crossed with the lines:

w-;Sco/Cyo;UASninaE^{G69D}/(TM6B) , w-;Sco/Cyo;UASninaE$^{G69D\mathbf{68A4}}$/(TM6B) and w-;Sco/Cyo;UASninaE$^{WT\mathbf{68A4}}$/(TM6B) to obtain:

Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE^{G69D}/(TM6B)

Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{wt\mathbf{68A4}}$/(TM6B)

Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{G69D\mathbf{68A4}}$/(TM6B)

- w;Sco/Cyo;pWIZ /TM6B was crossed with the lines:

Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{G69D\mathbf{68A4}}$/(TM6B)

Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{WT\mathbf{68A4}}$/(TM6B) to obtain flies without eye pigment:

Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{wt\mathbf{68A4}}$/Pwiz

Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{G69D\mathbf{68A4}}$/Pwiz

2.2. Microscopy

2.2.1. Retinal degeneration assessment across time

This method consists in the evaluation of the deep pseudopupil (DPP) image – a marker of eye integrity[42] – in the fly eyes throughout time to stipulate the state of photoreceptors degeneration. 10 flies per vial were kept at 25ºC in a light intensity around 2000 lux. Light cycle of the firsts experiments was 12h light/12h dark and was then changed to a constant light regime. For this assessment fly eyes were analyzed every 3 to 4 days. A fly was considered to have degenerated eyes when at least one of the two eyes had lost the pseudopupil image. The number of flies with degenerated eyes per number of flies alive per line was counted until there were no flies alive (used lines – Attachments, Table 1). The total number of flies of each line was different between the assays but the same within each assay.

2.3.Molecular Biology Techniques

Commercial products and kits were used according to manufacturer's instructions.

2.3.1. DNA Purification from Agarose Gel

DNA purification was done using gels of 0,8% agarose (SeaKem LE, Lonza) run in TAE buffer 1X (Tris-acetate 0,04M, EDTA 0,001M; Lonza). Size of DNA fragments was

estimated by comparison with the molecular marker NZYDNA Ladder III (NZYTech). DNA was extracted from gel using the ZymoClean™ Gel DNA Recovery Kit (Zymo Research).

2.3.2. Chemical Transformation of Competent Cells

The strain E. Clone 10G (Lucigen) was used in every transformations except for propagating vectors containing the *ccd*B gene, in which cases transformations were done with DB3.1™ (Invitrogen) competent cells. Ligation mixture or plasmid DNA was added to 100µl of competent cells. After 1h of incubation on ice cells were subjected a heat shock at 42ºC for 1:30 min. Then cells were again put on ice for 1-2 minutes and, followed the addition of 800 µl of LB medium (Scharlau), they were incubated at 37ºC with agitation for 1h. Finally, cells were plated on LA plates supplemented with the specific antibiotic for posterior selection.

2.3.3. Restriction and Ligation Reactions

Restriction reactions were incubated overnight at 37°C. The enzymes BglI, BglII, Acc65I, EcoRV, PstI, DnaseI (New England Biolabs) were utilized with the appropriate buffers. Ligation reactions were performed using T4 DNA ligase (New England Biolabs) and incubated overnight at 18°C. The ratio between the insert and the vector used was 3:1(in µg).

2.3.4. Purification of Plasmid DNA

In the purification of plasmid DNA on a small scale, it was used the kit NZYMiniprep (NZYTech). In large scale purifications it was used the kit NZYMidiprep (NZYTech).

2.3.5. Molecular Cloning

Conventional cloning

Ire1 was fused with a mcherry tag between the Ire1 residues G394 and S395. Primers were designed to fuse these two proteins by PCR and to flank the final fused DNA fragment with BglII and Acc65I restriction sites (Attachments, Table 2). Ire1I (N-terminal part), Ire1II (C-terminal part) and mcherry were amplified by PCR, from pUAST_IRE1-PC and from pUAST_nlsmcherry, using those primers. For PCRs it was utilized the Phusion™ High-Fidelity DNA polymerase (Finnzymes), which has a proof-reading activity, to create homology zones between the 3 DNA fragments, using the following conditions: initial denaturation step (3min at 98ºC), followed by 32 cycles of denaturation (15sec at 98ºC), annealing (30sec at temperature of melting (Tm)) and elongation (72ºC – for times see Attachments, Table 2) and lastly by a final elongation step (10min at 72ºC). Then they were gel purified and fused by PCR with the same polymerase, using the forward primer for Ire1 I and the reverse primer for Ire1 II and the above conditions (Tm- 60ºC, elongantion time-

2min). After, Ire1::mcherry was gel purified and cloned into an intermediate vector – pJET (Attachments, Fig.5-A) – utilizing Clone JET PCR Cloning kit (Fermentas). Finally, Ire1::mcherry was separated from pJET by restriction digestion with BglII and Acc65I and cloned into pUAST (Attachments, Fig.5-B) that was also digested with these two enzymes. Cloning was confirmed by PCRs, by restriction digestion and by sequencing (STABVIDA).

Gateway® system

Ire1 was fused with a GFP tag in the C-terminal. Primers with attB sites were designed for Ire1 gene (Attachments, Table 3), excluding the stop codon. Ire1 was amplified from pUAST_IRE1-PC by PCR using those primers and Phusion™ High-Fidelity DNA polymerase (Finnzymes), with the following conditions: initial denaturation step (1min at 98ºC), followed by 28 cycles of denaturation (10sec at 98ºC), annealing (30sec at 50ºC) and elongation (2min at 72ºC) and lastly by a final elongation step (10min at 72ºC). Then Ire1 was gel purified and cloned into the pDONR™221 (Invitrogen - Attachments, Fig.6-A) using the Gateway® BP Clonase™ II Enzyme Mix kit (Invitrogen), by recombination between attB sites from Ire1 fragment and attP sites present in the vector. Next Ire1 was transferred from pDONR™22 to pTWG (pUAST_White+_$GFP_{C\text{-}terminal}$ - Attachments, Fig.6-B), using the Gateway® LR Clonase™ II Enzyme Mix kit (Invitrogen), by recombination between attL and attR sites (Results and Discussion for details in Gateway® technology). Cloning was confirmed by PCRs and by sequencing (STABVIDA).

2.3.6. Cell culture and Transfection

Schneider 2 (S2) cells were and cultured in Schneider's *Drosophila* Medium (Gibco®) containing 10% heat-inactivated foetal bovine serum (FBS - Gibco®) and supplemented with 50U/mL penicillin and 50µg/mL streptomycin antibiotics. Cells were maintained at 25ºC with no CO_2 and passed at a cell density between 6 to $20x10^6$ cells/mL, splitting at a 1:2 to 1:5 dilution.

Cell transfections were performed using the Effectene (Qiagen) transfection reagent. The day before transfection, $3x10^5$ cells per well (in a 12 well plate) were seeded on 1mL of growth medium containing serum and antibiotics and were then incubated overnight at normal growth conditions. Cells to be used in immunostaining were seeded on a cover glass inside the well. On the day of transfections, for each of them, 0,3µg of each plasmid was diluted in the DNA-condensation buffer (Qiagen) to a total volume of 75µl, to which was added 2,4µl of Enhancer (Qiagen) per plasmid. Next, an incubation of 5min at room temperature was done, followed by the addition of 3 of transfection reagent Effectene (Qiagen) per plasmid. Then the

mixture was incubated for 10min at room temperature. Meanwhile, cell medium was aspirated, cells were washed with 1mL of PBS 1X and added 1mL of new medium. Then, 125µl of medium was added to the tube containing the transfection complexes. After mixing well, all the volume was added drop-wise onto the cells in each well. To conclude, cells were incubated at normal conditions for 72h for expression of the transfected transgenes.

Following the expression of 72h, cells in a cover glass were fixed by adding 200µl of 4% formaldehyde in 1X PBS (Phosphate buffered saline) and incubating with that for 15min. Next the 200µl were pipeted off and cells were washed with 1X PBS, 3 times for 5min each. For samples not to be immunostained, cells were immediately mounted on a slide.

2.3.7. Immunostaining

Fixed an washed cells had 1h of incubation with blocking buffer (1X PBS, 0,1% Bovine serum albumin (BSA), 0,1% Tween 20, 250mM NaCl). Then 200µl of rabit calnexin primary antibody[43], diluted at 1:400 in blocking buffer, was added to cells and incubated overnight at 4ºC. On the second day, cells were again washed 3 times, as before and were incubated with anti-rabit secondary antibody (Jakson ImmunoResearch), diluted at 1:200 in blocking buffer, for 2h at room temperature. Cells were washed again 3 times and mounted on a slide.

2.3.8. Total RNA Extraction

RNA extraction was done using the RNeasy® Mini Kit (Qiagen) and performing a column DNA digestion step with DnaseI (Promega). There were utilized samples of non transfected S2 cells (control) and of S2 cells transfected with the reagents: ActinGAL4 + pTWG_Ire1 + pUAST_Xbp1-EGFP; ActinGAL4 + pUAST_Ire1::mcherry + pUAST_Xbp1-EGFP and ActinGAL4 + pUAST_Xbp1-EGFP.

2.3.9. Reverse Transcriptase Polimerase Chain Reaction (RT-PCR)

The purified total RNA was used to synthesize the first strand of cDNA with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). It was done a negative control for each sample (with no reverse transcriptase enzyme). PCR amplification of first cDNA strand was done using 2µl of first strand cDNA synthesis reaction mixture as template. It was utilized the NZYTaq DNA polymerase (NZYTech) at standard PCR conditions. To amplify Ire1::GFP, Ire1:.mcherry and Xbp1-EGFP it was realized (for primers, Tms and elongation times- Attachments, Table 4): initial denaturation step (5min at 95ºC), followed by 32 cycles of denaturation (30sec at 95ºC), annealing (30sec at Tm) and elongation (elongation time at 72ºC) and lastly by a final elongation step (5min at 72ºC).

3. Results and Discussion

3.1. Study of Ire1 and Xbp1 mRNA localization and interaction

The Xbp1 mRNA splicing mediated by Ire1 is a crucial step in the regulation of the Ire1/Xbp1 pathway. This splicing reaction differs significantly from conventional mRNA splicing, as it is believed to occur in the cytoplasm, in the absence of the nuclear splicing machinery [44]. Thus, the features that underlie this event are necessarily different from nuclear splicing and yet poorly understood. Given that Ire1 is localized at the ER membrane, it is assumed that Xbp1 mRNA splicing occurs in the cytoplasm, near the ER membrane. For that to happen, Xbp1 mRNA must be targeted to the vicinity of ER membrane by some means. The role of Xbp1 mRNA 3'UTR has not been explored in *Drosophila* and can actually be important in Xbp1 mRNA targeting to the ER/Ire1. The HR2 region exists in *Drosophila* $Xbp1_{unspliced}$, although its functionality has not been proven.

Concerning the importance of 3'UTR in the recruitment of Xbp1 to the ER, we searched in *Drosophila* $Xbp1_{unspliced}$ 3'UTR sequence and identified one UGGCG and two GCGA regions that could represent an evolutionary change of the UGGCGCG and GCGAC regions of the yeast Hac1 bipartite element. However, these regions in *Drosophila* $Xbp1_{unspliced}$ 3'UTR are around 150bp and 190 bp apart from each other and may not be able to form a bipartite element as Hac1 3'UTR motifs do. However, it is also possible that *Drosophila* $Xbp1_{unspliced}$ 3'UTR has another targeting element responsible for its recruitment to the ER. So, further studies on 3'UTR secondary structure and its importance in Xbp1-Ire1 co-localization are needed to analyze this question.

Regarding the Yanagitani *et al* model (Fig.4), we decided to examine the C-terminal 26-a.a. region which was shown to be responsible for the translational pausing of $Xbp1_{unspliced}$ in mammals. The alignment between C-terminal 26-a.a. region of mouse, human and fly $Xbp1_{unspliced}$ (Fig.5) demonstrates that there is not much similarity between the first two and the last. Fig. 5 shows that there was an evolutionary change in the C-terminal of $Xbp1_{unspliced}$ protein between invertebrates and vertebrates since only five of the 15 a.a. that are conserved in human, mouse, chicken, frog and zebrafish ([38] and Fig.5-black arrows) are also conserved in *Drosophila* (Fig.5-green arrows). Interestingly, Yanaginati *et al* showed that the S255 to A255 mutation of human $Xbp1_{unspliced}$ increased translational pausing [38] and, as observed in Fig.5, there is an A297 (homologous position) in the *Drosophila* sequence (Fig.5-red arrow). This finding leads us to ask whether $Xbp1_{unspliced}$ A297 could increase

translation pausing in *Drosophila*, even though fly Xbp1$_{unspliced}$ C-terminal is considerably different from the conserved region identified by Yanaginati *et al*. In addition, the presence of the HR2 region in *Drosophila* Xbp1$_{unspliced}$ lead us to not set aside the hypothesis that translation pausing is also important in *Drosophila*.

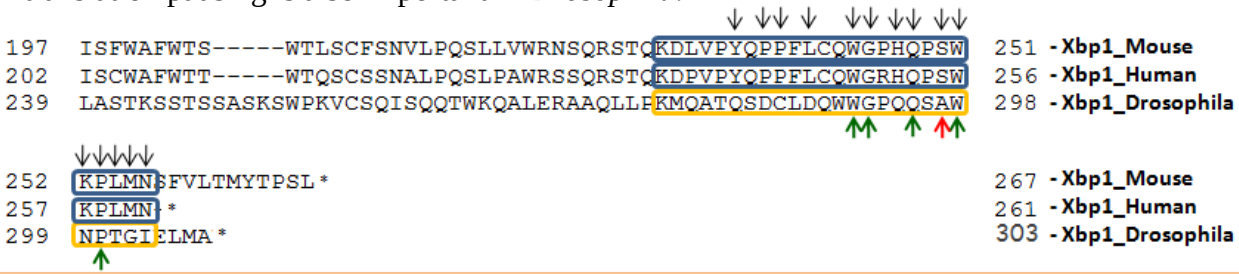

Fig. 5. Comparison of Xbp1$_{unspliced}$ C-terminal sequences from Mouse, Human and *Drosophila*. Boxes (blue for vertebrates and yellow for fly) represent the 26-a.a. conserved sequence suggested to be important for translational pausing by Yanagitani *et al*. Black arrows point a.a. that are conserved in Human, Mouse, Chicken, Frog and Zebrafish according to Yanagitani *et al*. Of these a.a., green arrows point the ones that are also conserved in *Drosophila*. Red arrow represents a S to A difference that may augment the translational pausing according to Yanagitani *et al*.* marks for translation termination.

3.1.1. Ire1 fusion with fluorescent tags and cloning

To investigate how Ire1 and Xbp1 mRNA co-localize in *Drosophila in vivo*, using live microscopy, we decided to create Ire1 protein and Xbp1mRNA linked with fluorescent tags. This project reports the development of the Ire1 constructs.

As explained before, Ire1 protein has three topological domains: the luminal domain, the transmembrane domain and the cytoplasmic domain. The last one, in its turn, is constituted by two functional domains which have, from N-terminal to C-terminal, kinase and endoribonuclease activities, that are conserved from yeast to fly and mouse (Fig.6).

In such a complex protein it is possible that incorporating a tag in it will interfere with its folding or function. Thus, we chose to insert a tag in two different positions within Ire1, using two different strategies. One construct was done inserting a mcherry tag in the middle of Ire1 gene, between the transmembrane and the kinase domains (between G394 and S395 residues, Fig.7-A). The second construct was done by inserting a GFP tag after the Ire1 C-terminus (Fig.7-B). In the first case (Fig.7-A), mcherry was fused with Ire1 by PCR. On the first generation of PCRs the N-terminal part of Ire1 (Ire1 I), the mcherry tag (without the Stop codon) and the C-terminal part of Ire1 (Ire1 II) were amplified using primers that allowed the addition of homology zones between these three DNA fragments (Fig.8). Then, a second generation of PCR was done using the gel-purified first generation PCR products Ire1 I, mcherry and Ire1 II with the forward and reverse primers for Ire1 gene to generate a single DNA fragment (Fig.8).

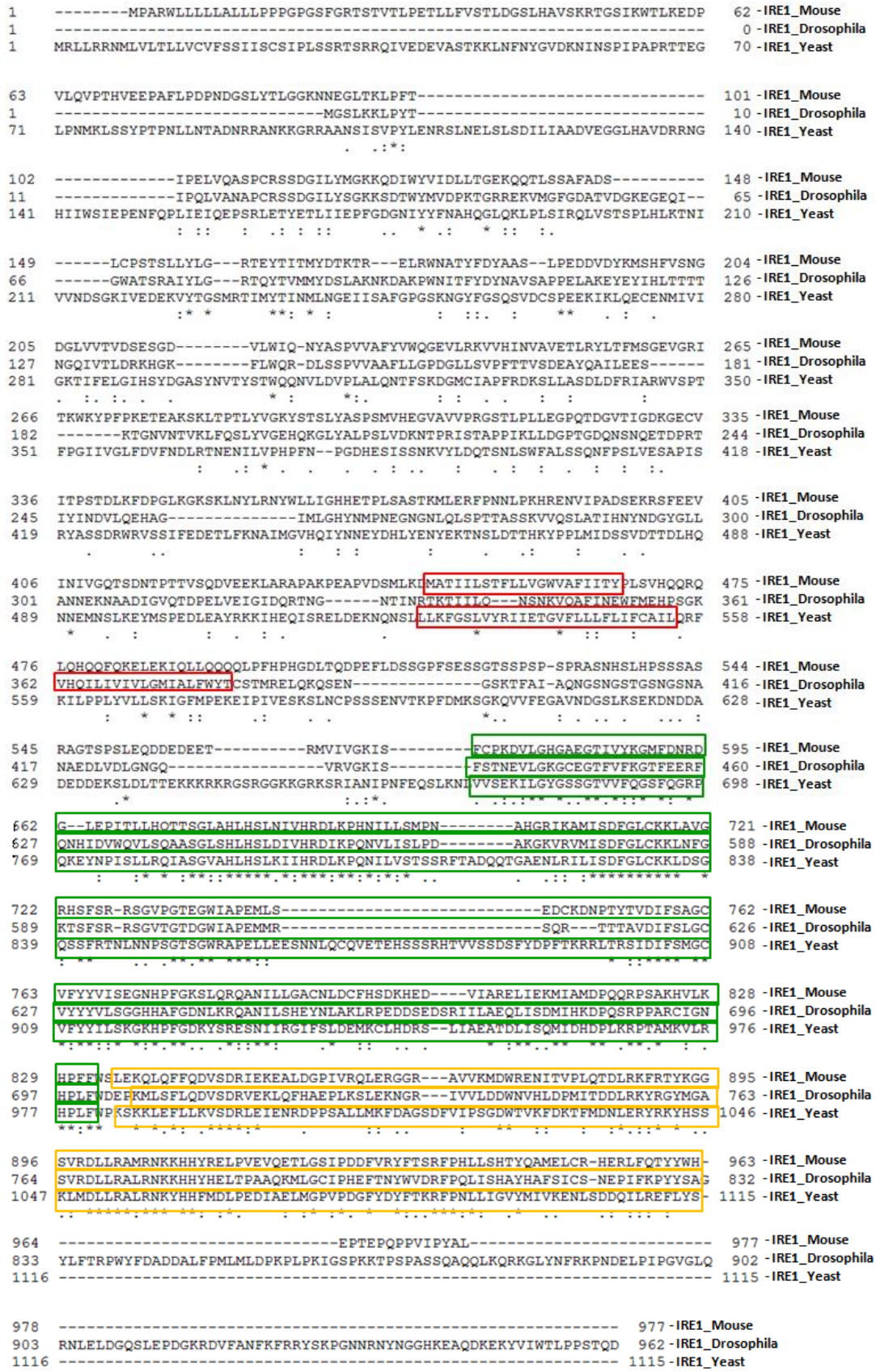

Fig. 6. Alignment between Ire1 proteins of Mouse, *Drosophila* and Yeast. Red boxes- transmembrane domains, green boxes- kinase domains, yellow boxes- endoribonuclease domains.

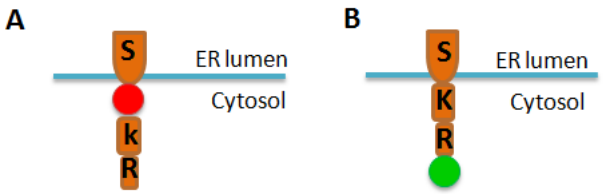

Fig. 7. Schematic of Ire1 constructs plan. **A.** mcherry tag was inserted between the transmembrane and the kinase domain. **B.** GFP tag was inserted in the C-terminal. S- sensing stress luminal domain and transmembrane domain; K- Kinase domain; R- endoRibonuclease domain and C-terminal of Ire1. Red circle-mcherry tag; green circle-GFP tag

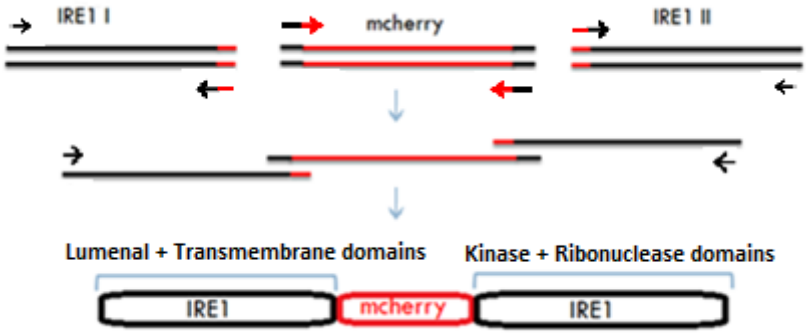

Fig. 8. Strategy for the fusion of mcherry to Ire1 by PCR. On the first generation of PCRs, it was created homology zones between Ire1 I, Ire1 II and mcherry with the different primers. On the second generation of PCR, these three DNA fragments were used as a template to create Ire1::mcherry. Red – mcherry sequences; Black – Ire1 sequences

Ire1 I and Ire1 II were amplified using as template the plasmid pUAST_Ire1-PC. This vector contains the sequence encoding for Ire1 isoform C protein (Ire1-PC), which had been already cloned in the lab from larvae cDNA. In the cloned sequence of Ire1-PC there are two non-synonymous substitutions comparing to the Flybase sequence: CAG instead of CAC (Q137 instead of H137) and AGC instead of ATC (S953 instead of I953). Ire1-PC was proven to induce the splicing of the reporter Xbp1-EGFP and to rescue the $Ire1^{f02170}$ mutant phenotype (data not shown, Dina Coelho unpublished). $Ire1^{f02170}$ mutation is a P element insertion in the Ire1 coding sequence (CDS) so its protein is not functional and cannot perform the splicing of Xbp1-EGFP reporter (data not shown). Thus, we believe that the differences that exist in Ire1-PC correspond to natural polymorphisms and that the cloned Ire1-PC is functional in the cell.

Ire1 I, mcherry and Ire1 II were amplified with primers CH1 and C2, C-3 and C-4 and C-5 and CH6, respectively (Fig.9-A (primers -Attachments, Table 3)). After purification, Ire1 I, mcherry and Ire1 II served as template for the second PCR, in which the primers CH1 and CH6 were used, to create Ire1::mcherry (Fig.9-B (primers -Attachments, Table 3)). Then Ire1::mcherry was cloned in pJET (Fig.9-C) and subcloned into pUAST in the orientation from BglII site to Acc65I site (Fig.9-D). Sequencing proved that Ire1::mcherry was present in pUAST and showed that there were no errors in both sequences of Ire1 (comparing with pUAST_Ire1-PC from which it was amplified) and mcherry.

In the cloning of second construct (Fig.7-B), a GFP tag was fused with Ire1 C-terminal using the Gateway system (Fig.10). Ire1 was amplified again from pUAST_Ire1-PC using primers that were designed not to include the stop codon and also to insert attB sites in each end of the gene (Fig. 11-A (primers-Attachments, Table 4)). Then Ire1 flanked with attB sites

was cloned into pDONR221 (Fig.11-B) and subcloned to pTWG (Fig.11-C). pTWG is a destination vector that has a GFP tag to make C-terminal fusions, upon recombination. The presence of Ire1 in the positive clones was confirmed by PCRs (Fig.11-D). Sequencing of pTWG showed there were no errors in Ire1 sequence comparing with pUAST_Ire1-PC and that it was in frame with GFP tag.

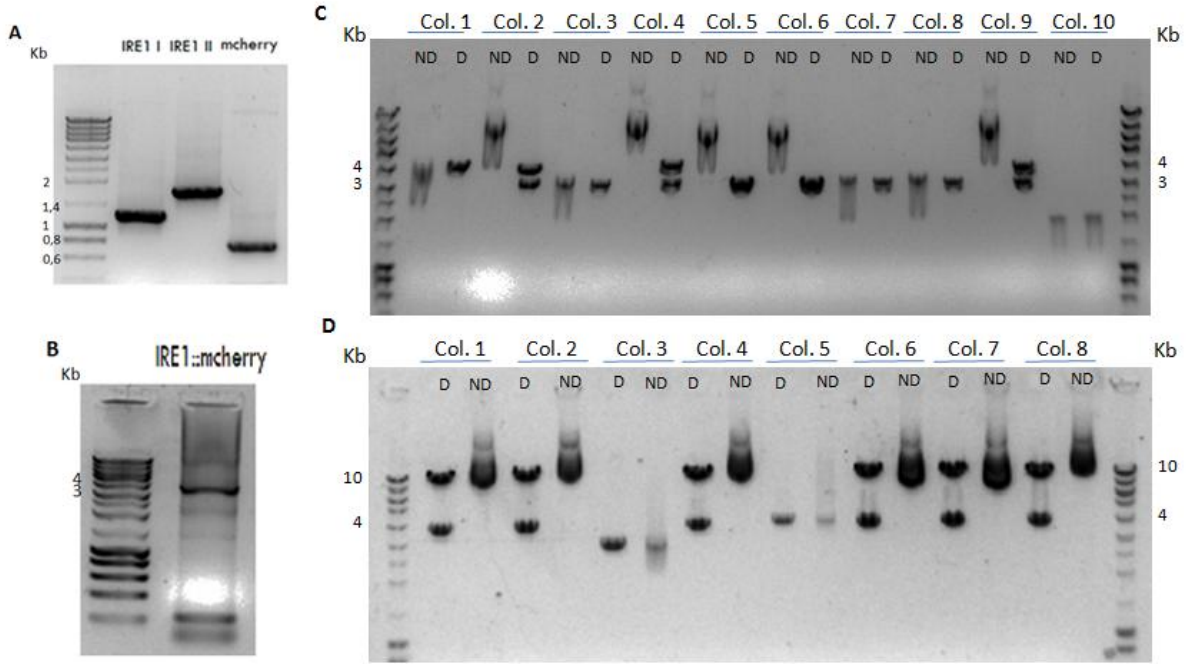

Fig. 9. Cloning of Ire1::mcherry in pUAST. A. Ire1 I, Ire1 II and mcherry fragments after first generation of PCRs **B.** Ire1::mcherry fused by a second generation of PCR **C.** Digestion of plasmid DNA from colonies transformed with ligation mixture of pJET + Ire1::mcherry with Acc65I and BglII. Colonies 2, 4 and 9 are positive for the presence of pJET_Ire1::mcherry. We pursued with the cloning in pUAST using colony 2 **D**. Digestion of plasmid DNA from colonies transformed with ligation mixture of pUAST + Ire1::mcherry with Acc65I and BglII. Colonies 1, 2, 4, 6, 7 and 8 are positive for the presence of pUAST_Ire1::mcherry. We pursued with colony number 2 in our investigation. Col.- colony; D- digested with Acc65I and BglII; ND- non digested.

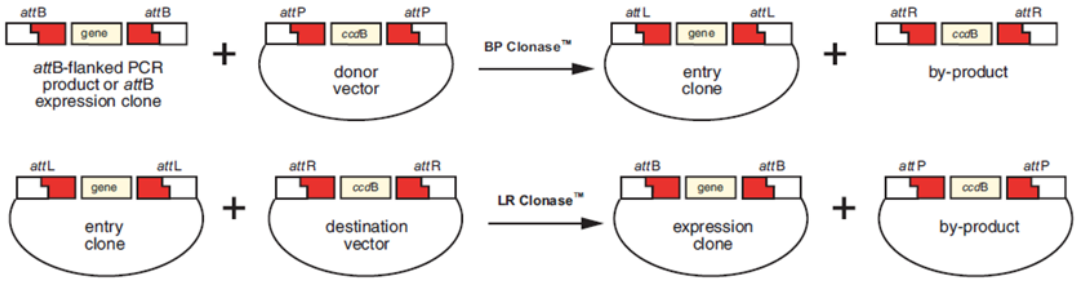

Fig. 10. Schematic of the Gateway system used. The Gateway system allows cloning of any DNA fragment into any expression vector by recombination. First, a gene of interest flanked with attB sites is cloned into a donor vector which has attP sites, by recombination (BP reaction). This recombination is done *in vitro* by the BP Clonase™. The recombination products are an entry clone (donor vector + gene cloned) in which the cloned gene is flanked with attL sites and a by-product (the ccdB gene) flanked with attR sites. Colonies with plasmid in which recombination did not occur dye due to the effect of the ccdB gene present in the donor vector, so there is no need to do a posterior selection of the clones. Then, from an entry clone, the gene of interest can be subcloned to any destination vector (for expression in any organism) by means of LR Clonase™, which performs a recombination between attL sites from an entry clone and attR sites present in a destination vector (LR reaction). The result of this is an expression clone with the gene flanked with attB and a by-product vector with the ccdB gene flanked with attP sites. Again only the expression clone will allow the formation of colonies because of the lethality of the ccdB gene. All recombinations are reversible using the other Clonase, thus a gene can rapidly be moved from an entry clone to destination vector or from an expression clone to a donor vector (from Invitrogen Gateway manual)

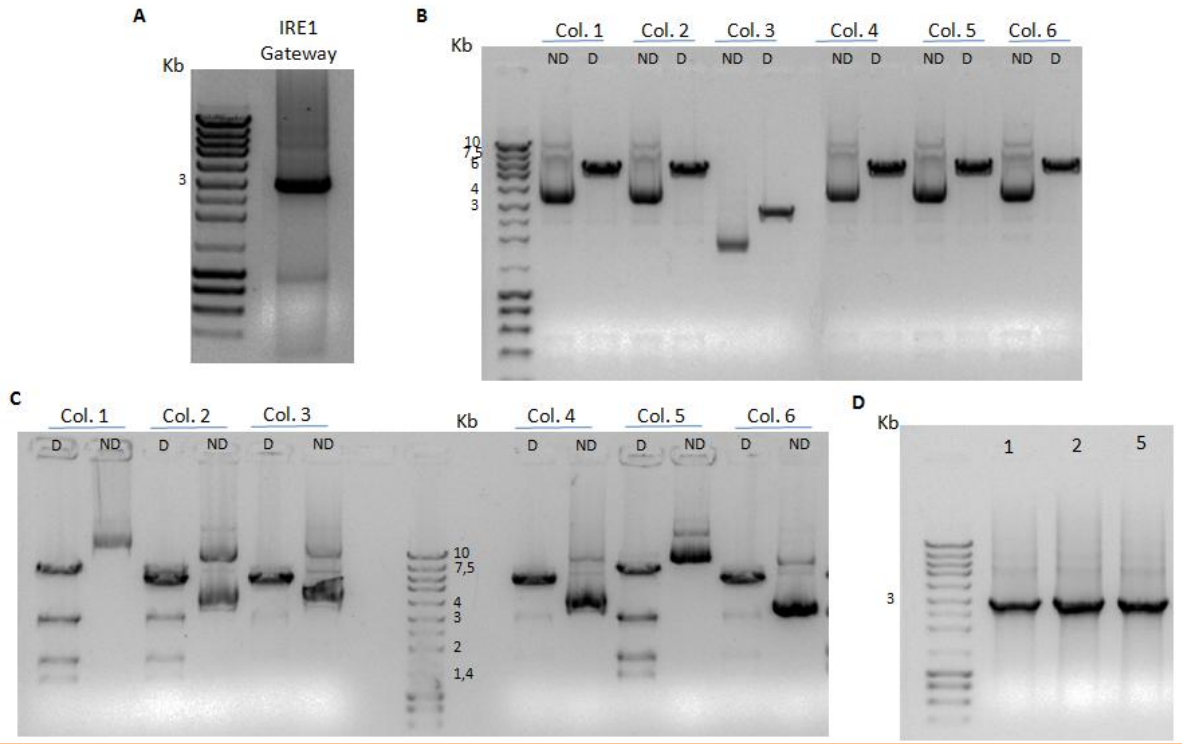

Fig. 11. Cloning of Ire1 in pTWG. A. Ire1 with attB sites after PCR **B.** Digestion of plasmid DNA from colonies transformed with BP reaction with EcoRV to linearize the entry clone. Colonies 1, 2, 4, 5 and 6 are positive for the presence of pDONR221_Ire1. We pursued with the cloning in pTWG using colony 1 **C.** Digestion of plasmid DNA from colonies transformed LR reaction with EcoRV and BglI. Colonies 1, 2, and 5 are positive for the presence of pTWG_Ire1. We pursued with colony number 1 in our investigation. **D.** PCRs for Ire1 gene confirmed its presence in the plasmid pTWG. Col.- colony; D- digested; ND- non digested

3.1.2. Testing for localization and functionality of cloned constructions

After cloning the 2 tagged Ire1 constructs in UAST vectors, we needed to prove whether our constructs were functional. For that purpose we transfected S2 cells with our constructs to see where they localized in the cell and to check if they were able to perform the Xbp1-EGFP reporter splicing. All transfections were done in duplicates with an expression time of 72h. As all transfected transgenes were cloned in UAST plasmids, they were co-transfected with ActinGAL4 to induce expression in S2 cells.

To investigate Ire1::GFP and Ire1::mcherry localization in cells we transfected S2 cells with Ire1::GFP and with Ire1::mcherry and did immunostaining using anti-*Drosophila* Calnexin antibody[43]. Calnexin is an ER transmembrane lectin chaperone which is involved in the folding of glycoproteins[45]. As Ire1 is also an ER transmembrane protein, we used calnexin antibody as an ER marker to verify if our constructs were localizing in the right place in the cell. In Fig.12 we can observe that both Ire1::GFP and Ire1::mcherry have the same pattern of localization as Calnexin, meaning tags are not interfering with the right positioning of Ire1 in cells – the ER.

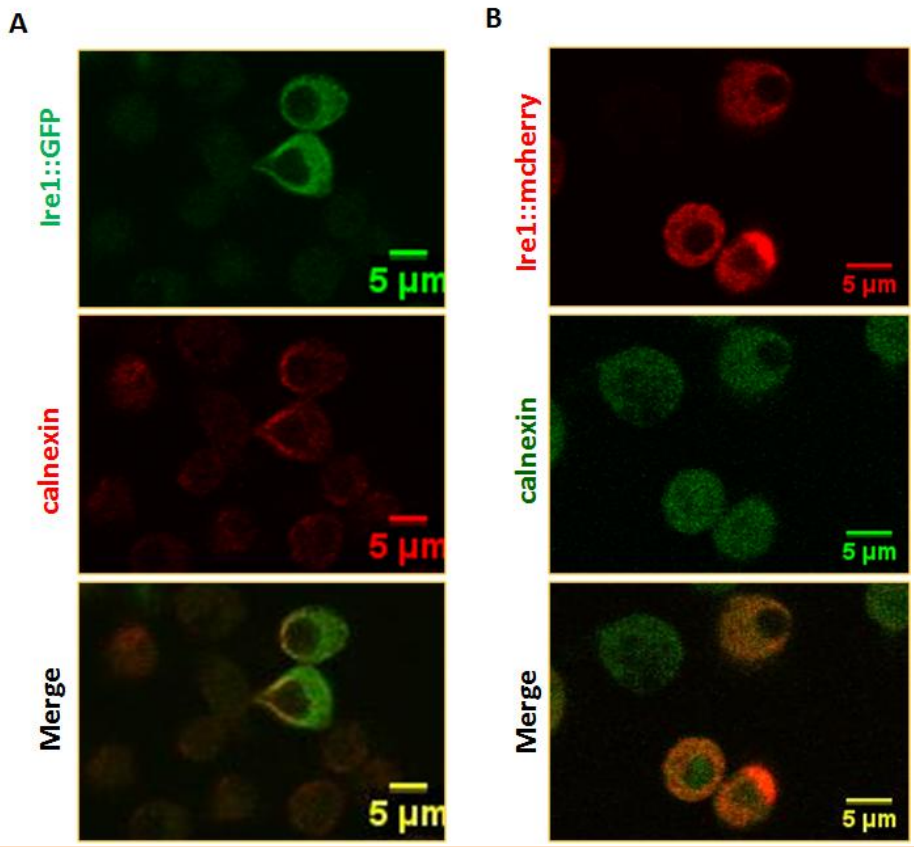

Fig. 12. Ire1 and calnexin co-localization in S2 cells A. Expression of Ire1::GFP and immunostaining for calnexin show the same pattern. **B.** Expression of Ire1::mcherry and immunostaining for calnexin show also the same pattern.

Regarding the functionality of the constructs, we wanted to verify if Ire1::GFP and Ire1::mcherry were able to do the Xbp1-EGFP reporter splicing (Fig.2). To that end, S2 cells were transfected with Ire1::GFP + Xbp1-EGFP, with Ire1::mcherry + Xbp1-EGFP and only with Xbp1-EGFP (negative control). Previous experiments done in our lab led to the conclusion that the over-expression of Ire1, both in S2 cells and in the fly, was sufficient to provoke the Xbp1-EGFP reporter splicing, even in the absence of induced ER stress (data not shown). Accordingly, we expected that only the in the cells transfected with our Ire1 transgenes should be possible to see the splicing of the Xbp1-EGFP reporter.

As observed in Fig.13, both constructs are able to splice the Xbp1-EGFP reporter. In Fig.13-A, Ire1::mcherry shows the same pattern of localization as in Fig.12-B, in the perinuclear and cytosolic space, and the spliced Xbp1-EGFP is localizing in the nucleus of the cell, as it was expected (because Xbp1-EGFP has a nuclear localization signal). Looking at Fig.13-B it is impossible to be sure of the presence or the exact localization of each of the transfected transgenes because both have GFP tags. Nevertheless, comparing Fig.13-B with Fig.12-A and Fig.13-A, we can infer that both Ire1::GFP and Xbp1-EGFP spliced are localizing in the correct place in the cell, ER and nucleus, respectively. Also, the fact that we

have seen cells in which there was only the presence of Ire1::GFP (supposedly due to transfection of Ire1::GFP only) that coincided with the pattern seen in Fig.12-A make us be confident about the presence of Xbp1-EGFP in the nucleus of the cells seen in Fig.13-B.

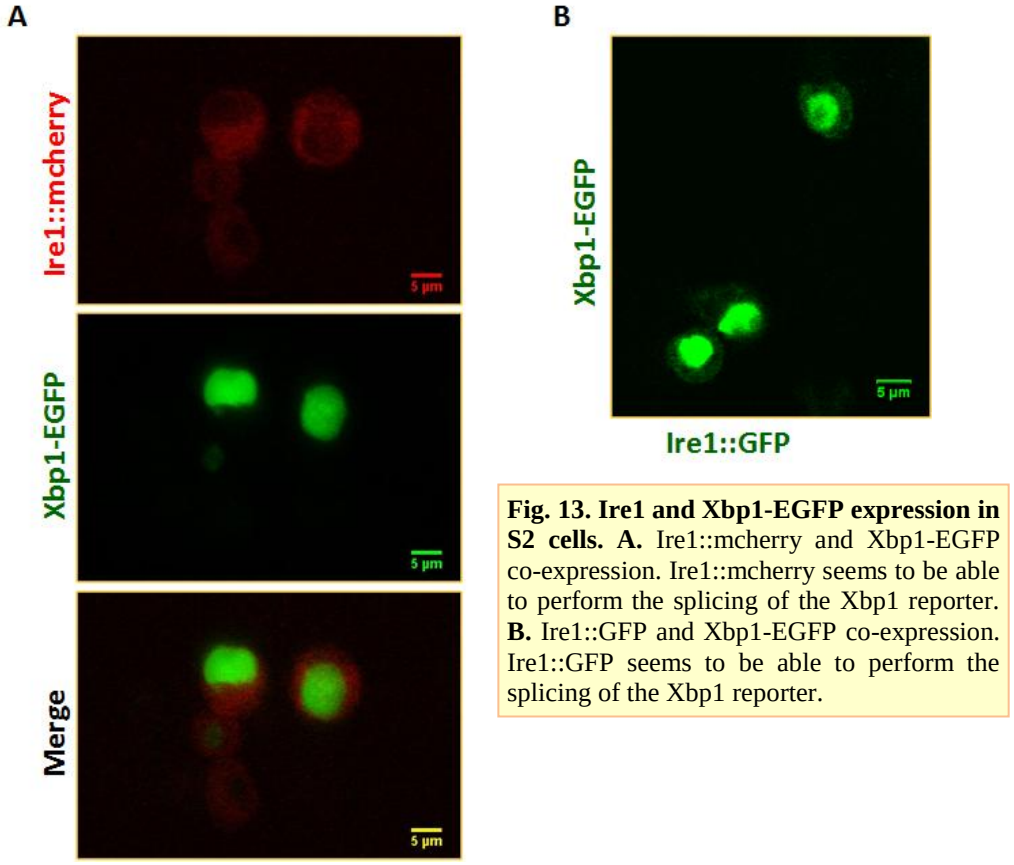

Fig. 13. Ire1 and Xbp1-EGFP expression in S2 cells. A. Ire1::mcherry and Xbp1-EGFP co-expression. Ire1::mcherry seems to be able to perform the splicing of the Xbp1 reporter. **B.** Ire1::GFP and Xbp1-EGFP co-expression. Ire1::GFP seems to be able to perform the splicing of the Xbp1 reporter.

Analyzing Fig.13 we can deduce that Ire1::mcherry and Ire1::GFP are present in the cells and that Xbp1-EGFP reporter is being spliced. Still, we cannot be certain that the splicing is being done by our constructs rather than by endogenous Ire1. One of the experiments to be done to be sure that our constructs, and not endogenous Ire1, are performing the splicing of Xbp1-EGFP reporter is to do a negative control experiment (the transfection of the Xbp1-EGFP alone). In our negative control we cannot observe any GFP (Fig.14-B), indicating that the Xbp1-EGFP splicing only occur in cells transfected with our Ire1 transgenes (Fig.13 and Fig.14-A). However, there is also the possibility that over-expression of our constructs somehow induce ER stress in cells and leads to the activation of the endogenous Ire1 that splices the Xbp1-EGFP reporter. To exclude this possibility an Ire1 mutant rescue experiment in fly is essential (see Future Perspectives). Nevertheless, these results suggest that both our Ire1 constructs are being expressed and that they are functionally active.

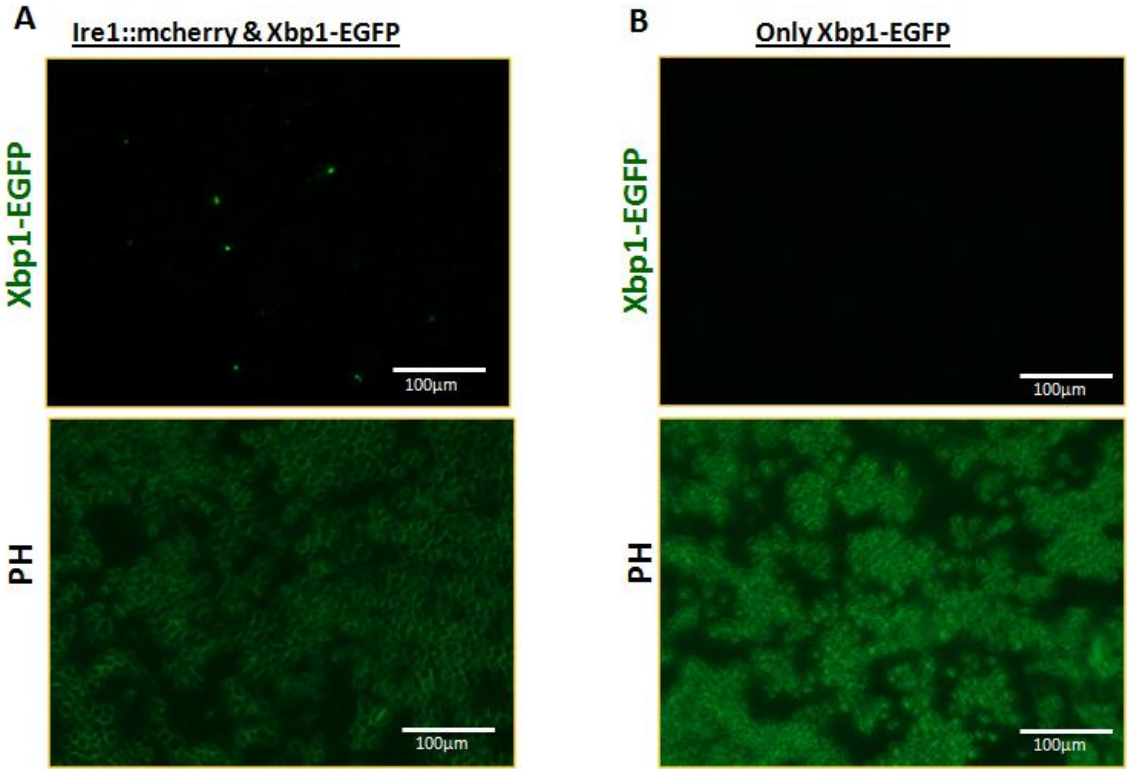

Fig. 14. Xbp1-EGFP splicing occurs only when it is co-transfected with one of our Ire1 constructs. A. Cells transfected with Ire1::mcherry and Xbp1-EGFP. It is possible to see GFP, which means that Xbp1-EGFP splicing occurred. **B.** Cells transfected only with Xbp1-EGFP. It seems that splicing of Xbp1-EGFP did not occur in any cell.

In addition to microscopy imaging, we did RT-PCRs to confirm the above results. We made RT-PCRs for both our Ire1 constructs and for Xbp1-EGFP, using always one primer specific for the tags (Attachments, Table 4), ensuring that we were targeting only the transfected transgenes and not the endogenous genes. All RT-PCRs were done with negative controls (using no reverse transcriptase enzyme) to detect DNA contamination. We could amplify all our targets although we had DNA contaminations (Fig.15). The DNA digestion on column during RNA extraction was not sufficient to eliminate all the DNA from our RNA samples. Subsequent to doing these RT-PCRs (Fig.15), extra DnaseI digestions were done to clean the RNA samples, resulting in partial degradation of RNAs that prevent us to further analyze them (data not shown). Thus, we considered only our initial RT-PCRs results.

In Fig.15-A, we can observe that Ire1::GFP was amplified only in the sample transfected with Ire1::GFP + Xbp1-EGFP (Fig.15-B-1, black arrow) and in the negative control of that same sample (Fig.15-B-1). Fig.15-C shows that RT-PCRs for Ire1::mcherry only amplified it in the sample transfected with Ire1::mcherry + Xbp1-EGFP (Fig.15-C-2, black arrow) and in the negative control of that same sample (Fig.15-C-2-). Consistently, for both Ire1 constructs, there is more intensity in the band of the samples in which it was used reverse transcriptase

enzyme than in the negative controls, suggesting Ire1::GFP and Ire1::mcherry were being expressed by the cells transfected with them.

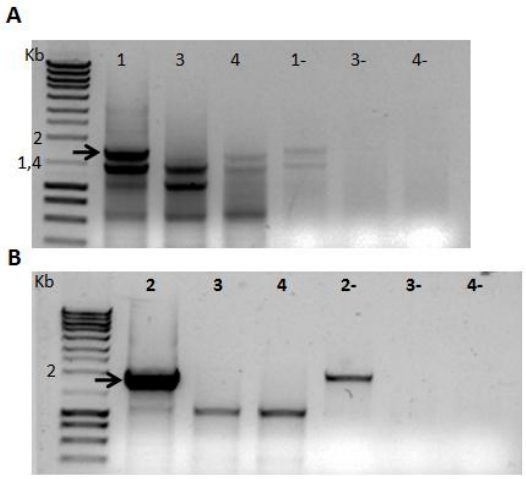

Fig. 15. RT-PCRs for Ire1 constructs A. RT-PCRs for Ire1::GFP **B.** RT-PCRs for Ire1::mcherry. **Templates**: **1** – S2 cells transfected with Ire1::GFP and Xbp1-EGFP; **2** – S2 cells transfected with Ire1::mcherry and Xbp1-EGFP; **3** – S2 cells transfected with Xbp1-EGFP; **4** – S2 cells non transfected (control); **1-** – negative control of S2 cells transfected with Ire1::GFP and Xbp1-EGFP (no RT enzyme); **2-** – negative control S2 cells transfected with Ire1::mcherry and Xbp1-EGFP (no RT enzyme); **3-** – negative control of S2 cells transfected with Xbp1-EGFP (no RT enzyme); **4-** – negative control of S2 cells non transfected (no RT enzyme). Arrows – band corresponding to the Ire1 construct we wanted to amplify

After looking at the expression of the Ire1trangenes we also wanted to confirm if the Xbp1-EGFP reporter was being spliced in the S2 cells transfected with Ire1::GFP and Ire1::mcherry. We performed the RT-PCRs for Xbp1-EGFP and verified that it was present in all the samples except for the non transfected S2 cells samples (positive and negative for RT enzyme), meaning there was DNA contamination in our negative control samples (with no RT enzyme). As Xbp1 unspliced and spliced forms differ only in 23 nucleotides it was not possible to interpret if there was splicing of the reporter just by doing the RT-PCR for Xbp1-EGFP. With the set of primers we used (Attachments, Table 4), the specific Xbp1-EGFP product has two PstI restriction sites: one in the intron (if present) and the other one 26 nucleotides before the "C-terminal" of the amplified PCR product (Fig.16- Restriction Map). Therefore, by doing PstI digestion, the unspliced form is digested in two sites, resulting in two visible bands (because the 26 nucleotide band is not visible on an agarose gel) and the spliced form is only digested in one site and results in one visible band (Fig.16- Restriction Map). So, we gel purified the specific band for Xbp1-EGFP in our RT-PCRs and digested them with PstI. The result observed in Fig.16 suggests that there was splicing in the cells transfected with the reporter and the Ire1 constructs (Fig.16-white arrows) but not in the cells transfected only with the reporter, which is consistent with our imaging results. The samples from RT-PCRs negative controls (with no RT enzyme) here served as a control for the digestion efficiency, as genomic or plasmid Xbp1 has always the intron and, thus, should originate two bands. Once there was total digestion of RT-PCRs negative controls we can assume that the bands we see for samples 1 and 2 (Fig.16-white arrows) do not represent any undigested DNA.

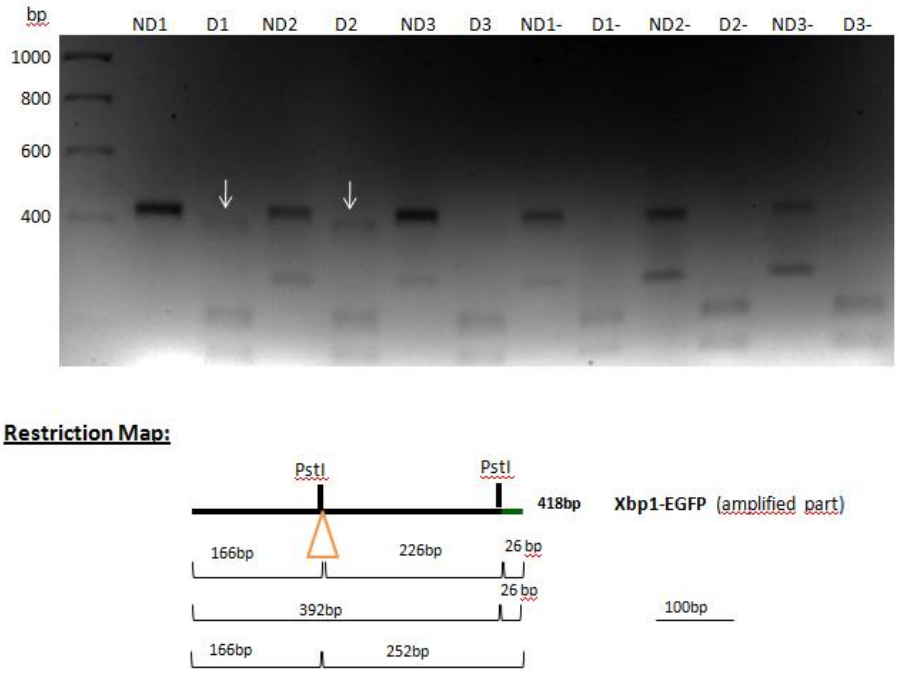

Fig. 16. Xbp1-EGFP specific band digestion with PstI. 1 – S2 cells transfected with Ire1::GFP and Xbp1-EGFP; **2** – S2 cells transfected with Ire1::mcherry and Xbp1-EGFP; **3** – S2 cells transfected with Xbp1-EGFP; **1-** – negative control of S2 cells transfected with Ire1::GFP and Xbp1-EGFP (no RT enzyme); **2-** – negative control S2 cells transfected with Ire1::mcherry and Xbp1-EGFP (no RT enzyme); **3-** – negative control of S2 cells transfected with Xbp1-EGFP (no RT enzyme).

Obviously, these results do not prove by themselves the expression of the transfected constructs because they contain DNA contamination and unspecific PCR products. But, taking the microscopy imaging and the RT-PCRs results together, they corroborate one another, suggesting that S2 cells are expressing the transfected genes and that our Ire1 constructs are localizing correctly and are also functionally active. Nevertheless, repeating these experiments and doing simultaneously a western bolt for the transfected constructs would be necessary to take unambiguous conclusions and be certain about the Ire1::GFP, Ire1::mcherry and Xbp1-EGFP expression, as well as the Xbp1-EGFP splicing by Ire1.

3.2. Ire1/Xbp1 post-trancriptional regulation in the degerating photoreceptors

As already mentioned, miRNAs are important players in the post-transcriptional regulation of gene expression. Despite the identification of hundreds of miRNAs, the biological functions of most of them are poorly understood[46]. Though, it is known that miRNAs are important regulators during neuronal development[47]. In fact, recent findings link miRNAs with neurodegenerative diseases and cancer[46]. It has been shown that mammalian miRNA expression vary in a developmental- or tissue-specific manner, and that, in the case of the mouse visual system, specific miRNAs are expressed in various parts of the eye, suggesting a role in controlling cell differentiation in the retina[48-49]. Additionally,

miRNAs in mouse retina seem to have a high turnover capacity in adaptation to different levels of light[49].

Of all the miRNAs described, we are particularly interested in those related with the UPR and apoptosis, given that previous studies demonstrated that retinal degeneration in ADRP is apoptosis-dependent[27]. Several miRNAs have been associated with cell proliferation and apoptosis in *Drosophila* but none has ever been associated with UPR.

We aim to elucidate the role of miRNAs in the post-transcriptional regulation of the Ire1/Xbp1 signaling pathway of UPR, in the context of ER stress and retinal degeneration. With that intention, we want to evaluate the miRNA expression pattern of flies with degenerating photoreceptors comparing with flies with normal eyes. But, first we need to understand what are the best conditions to perform a comparative assay between *wt* flies (upon no ER stress circumstances) and flies carrying *ninaE* mutations (which lead to UPR activation and photoreceptors degeneration – our ADRP model).

To study miRNA influence in Ire1/Xbp1 pathway we decided to follow the evolution of photoreceptors degeneration that happens in our ADRP model flies, which is provoked by normal exposure to light, and to evaluate miRNA expression in different stages of its progression. However, some features have to be taken into account to assure an adequate comparison between *wt* and ADRP flies miRNA expression. Since flies do not have eyelids, light-induced retinal degeneration occurs even in *wild type* animals, resulting from the acute exposure to very intense illumination or from long-term exposure to continuous moderate levels of light[50]. Also, light-induced blindness is accelerated greatly at higher temperatures, while red-eyed flies seem to slow down the visual impairment process[50]. And curiously, flies carrying *ninaE* mutations, which are known to cause ER stress and activate the UPR, do not show retina degeneration when maintained in the absence of light.

Besides all these features that reflect the variety of factors involved in fly photoreceptors degeneration, it also is important to remind that miRNAs are involved in the regulation of the circadian cycle and that the circadian cycle is mostly regulated by light. Taken everything together, the experiments to study the miRNA expression in the context of ER stress and photoreceptors degeneration needs to be thoroughly designed to assure the right interpretation of the achieved results.

3.2.1. Premilinary tests for microRNA profiling

Considering these previous findings, some preliminary tests needed to be done to define what are the best conditions of light intensity, time of exposure to light and culture

temperature so that *wt* fly eyes do not degenerate early but ninaE^{G69D} fly eyes do, trying not to interfere with circadian cycle. In this way we can diminish artifacts in miRNA patterns comparison interpretation.

In the preliminary assays we examined the deep pseudopupil (DPP)[42] of flies to comprehend the effect of different culture features in the retinal degeneration of *wt* and ADRP flies. The DPP image can be seen by the projection of GFP light inside the eye under the control of the Rh1 Gal4 driver, which shows the trapezoidal shape of the outer R1-R6 photoreceptors that form the ommatidia - the small parts that assemble the compound eye of the fly. When the eye starts to degenerate it gradually looses the DPP image, as the ommatidia losses its shape. So, DPP is a marker for the integrity of the eye. This allows us to outline the optimal experimental conditions, as well as the proper controls required for the comparative assay to be performed in miRNA profiling.

Hence, in the preliminary tests we wanted to access retinal degeneration across time. To do so, we initiated a trial with control and ADRP flies, in which the number of flies with no DPP image in the eyes per number of flies alive per line was counted until there were no flies alive, with a goal of finding a difference in the initiation, in the progression and in the level of retinal degeneration between the different lines.

Initially we tried to use twenty flies per genotype, at 25ºC in a light cycle of 12h light/12h dark with a mean of light intensity of 2200 lux. We started to use lines that already existed in the laboratory, which we named in this preliminary experiment as: Control (Rh1Gal4;UASGFP^{ninac}/(cyo);Sb/TM6B), ninaE^{G69D} (Rh1Gal4;UASGFP^{ninac}/(cyo);ninaE^{G69D}/(TM6B)) and UASninaE^{G69D} (Rh1Gal4; UASGFP^{ninac}/(cyo);UASninaE^{G69D}/(TM6B)). As seen in Fig.17 (see also Attachments, Table 5), in none of the lines was registered a high rate of retinal degeneration and also there was not a considerable difference in that rate between the control and the rest of the lines. To notice, ninaE^{G69D} line was the only that did not registered any retinal degeneration across time. Additionally, the lines did not have the same pattern of survival and, surprisingly, the control line was the one that had the lower survival rate.

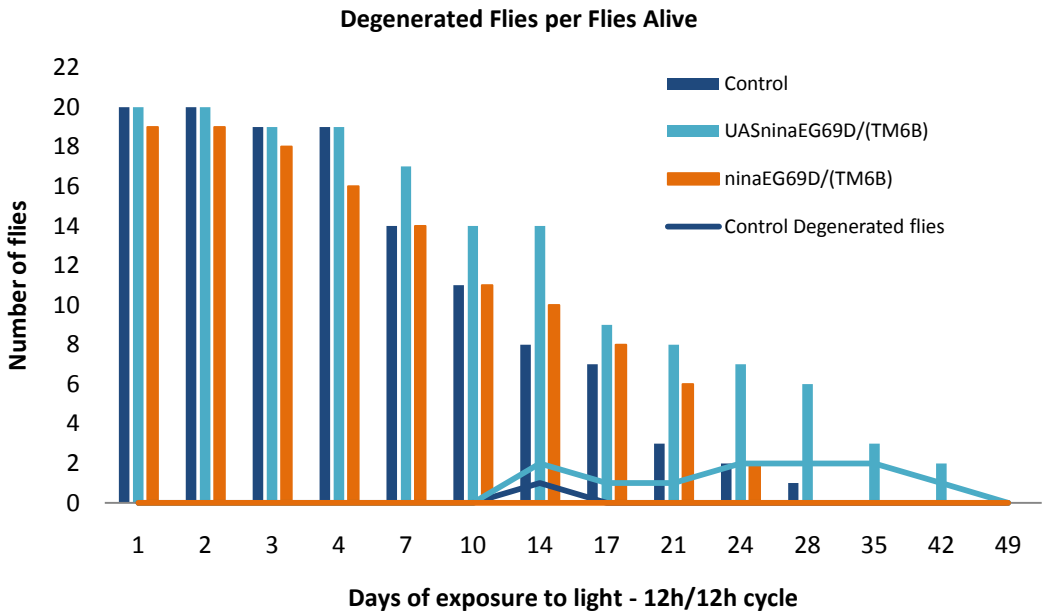

Fig. 17. Retinal Degeneration assessment across time. Number of degenerated flies per number of flies alive per line. Bars- Number of flies alive, lines- number of degenerated flies. Light cycle- 12h of light followed by 12h of darkness. Mean of light intensity- 2200 lux

Since those results were not conclusive, we chose to do the same assay using only two lines which are genetically very similar, differing only in the *ninaE* transgene that they over-express – *nina*E^{wt} or *ninaE*^{G69D}. These lines were created using the øC31 system, integrating the transgenes in the same attP specific site (the 68A4 site). The øC31 system guarantees that the lines resulting from this system will have the same level of transcription and, supposedly, the same level of expression of the transgene. It ensures too that if the insertion of the transgenes by any chance influences the expression of any of the self genes of the flies, it will occur in all the lines, as they will be all in the same conditions. Thus, the results are more reliable to compare within the flies used in the preliminary tests. We named these lines collectively as attB lines, and particularly as: $_{attB}$UASninaE^{wt} (Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{wt\mathbf{68A4}}$/(TM6B)) and $_{attB}$UASninaE^{G69D} (Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{G69D\mathbf{68A4}}$/(TM6B)) (for crosses – Attachments, Fig.4). This time we used a larger population size to assess the retinal degeneration of the attB lines across time. Looking at Fig. 18 (see also Attachments, Table 6), we can observe that $_{attB}$UASninaE^{wt} flies died in a much higher rate than $_{attB}$UASninaE^{G69D}. Concerning retinal degeneration we cannot see much difference between the two lines in study.

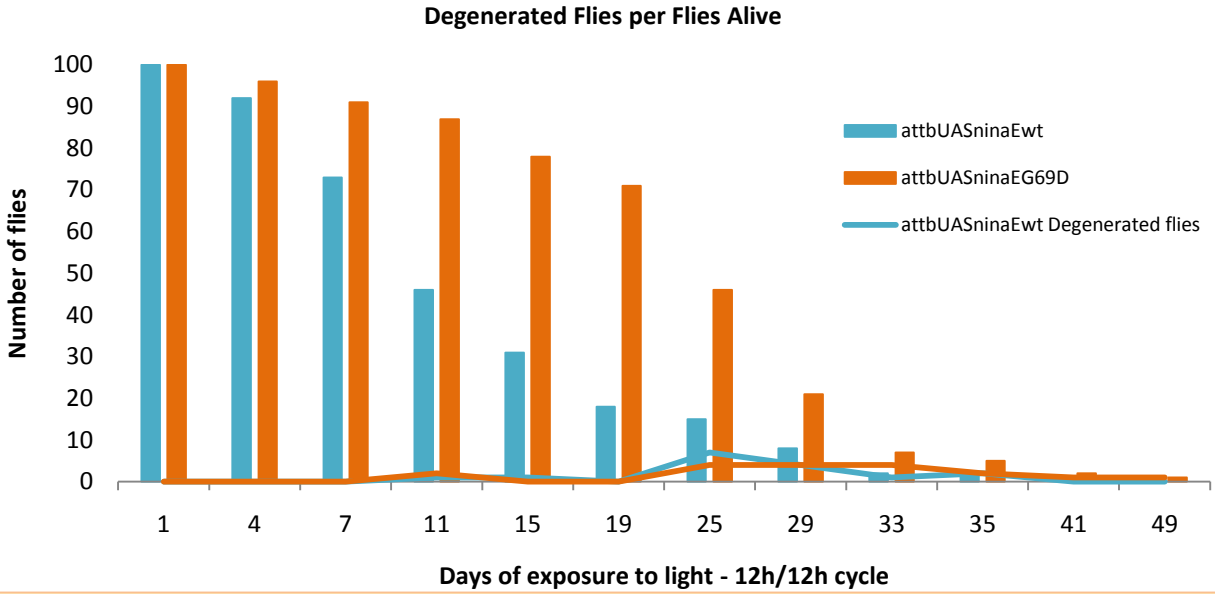

Fig. 18. Retinal Degeneration assessment across time using attB lines. Number of degenerated flies per number of flies alive per line. Bars- Number of flies alive, lines- number of degenerated flies. Light cycle- 12h of light followed by 12h of darkness. Mean of light intensity- 2200 lux

Once we still could not verify a considerable difference between the retinal degeneration of attB lines, we generated fly lines without eye pigment from our attB lines in order to accelerate the retinal degeneration and maybe be able to see a difference in its progression between the two lines (for crosses – Attachments, Fig.4). To do so, we used the pWIZ, a transgenic line expressing RNAi against *white*, the gene encoding the eye pigment in flies, and created pWIZ_attB lines: Pwiz_$_{attB}$UASninaE^{wt} (Rh1Gal4; UASGFP^{ninac}/(Cyo); UASninaE$^{wt\mathbf{68A4}}$/Pwiz) and Pwiz_$_{attB}$UASninaE^{G69D} (Rh1Gal4; UASGFP^{ninac}/(Cyo); UASninaE$^{G69D\mathbf{68A4}}$/Pwiz). Intriguingly, pWIZ_attB lines hatch already with no DPP image (Fig.19), meaning that pWIZ must be interfering with the GFP expression, which prevented us to further use.

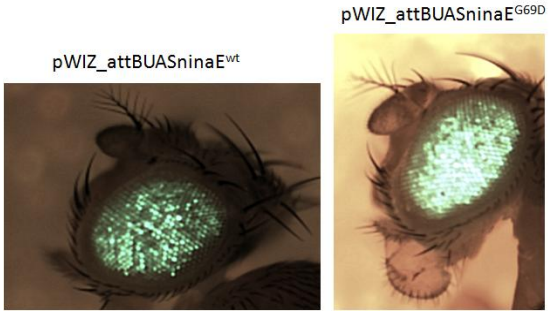

Fig. 19. PWIZ_attB lines hatch already with no DPP image. Flies in figure were 1day old.

Our next step was to use constant light of around 2000 lux to induce a higher retinal degeneration. As observed in Fig.20 (see also Attachments, Table 7), retinal degeneration was considerably higher than in the cases in which was used a 12h light/12h dark cycle. Conversely, $_{attB}$UASninaE^{wt} flies started to degenerate earlier than $_{attB}$UASninaE^{G69D} flies. For example, on day 19th, almost 50% of the $_{attB}$UASninaE^{wt} flies were already degenerated while

only one of the $_{attB}$UASninaE^{G69D} flies was too. Also, the level of degeneration in $_{attB}$UASninaE^{wt} seem to be worse than in the other assays and than in $_{attB}$UASninaE^{G69D} of this assay (Fig.21), since some of $_{attB}$UASninaE^{wt} flies lost almost all the GFP expression in the eye. Again, $_{attB}$UASninaE^{wt} had a lower survival rate comparing to $_{attB}$UASninaE^{G69D}.

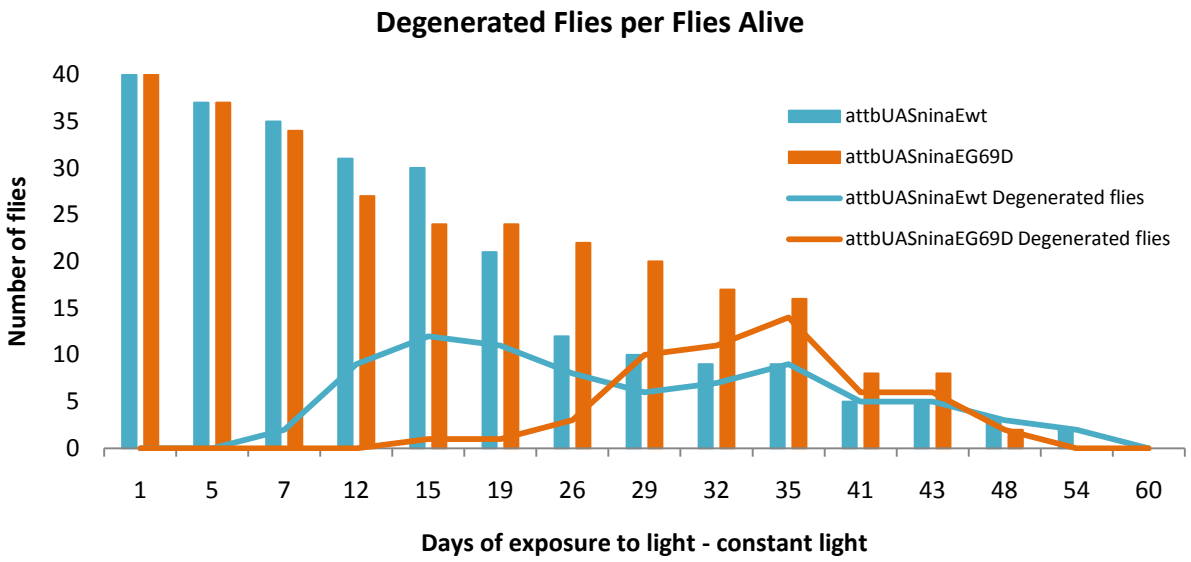

Fig. 20. Retinal Degeneration assessment across time using attB lines with constant light. Number of degenerated flies per number of flies alive per line. Bars- Number of flies alive, lines- number of degenerated flies. Light cycle- constant light. Mean of light intensity- 2000 lux

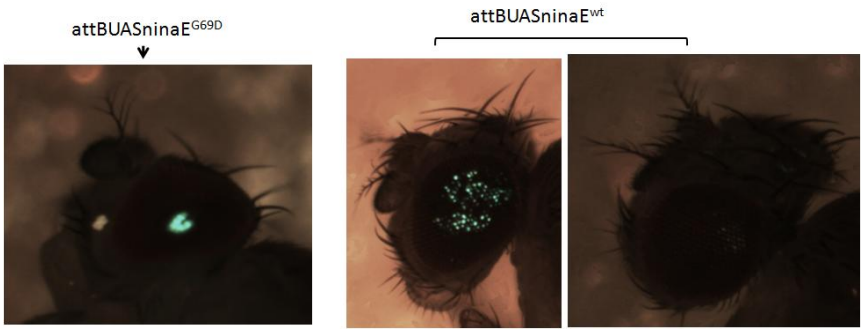

Fig. 21. Examples of retinal degeneration of attB lines under constant light. Most of the attBUASninaE^{G69D} had the DPP image intact. Nearly half of the attBUASninaE^{wt} had already retinal degeneration and some of them showed severe degeneration (photo on right). Photos taken on the 19th day of exposure to constant light. Mean of light intensity- 2000 lux

Given all the preliminary tests, it is clear that we could not yet establish an assay where it would be possible to analyze miRNA expression in the context of Ire1/Xbp1 pathway. To achieve that assay, we are planning to repeat our preliminary tests with three independent crosses for each line and a larger population size to reduce eventual artifacts. Using another strategy, such as the cinnabar and brown mutations, to take out the eye pigment of flies is also a viable option. Still we may need to change our approach into something less complex (see Future Perspectives).

4. Future Perspectives

Concerning section 3.1., we are planning on pursuing our investigation in the *Drosophila* organism. Although cell lines are a helpful tool, they have several limitations since they cannot characterize the complexity of a full being. Therefore, the reported results need to be validated with experiments in the fly. Our plan is to evaluate the Ire1 constructs (which were already sent to by BestGeneInc (U.S.A) for embryo injection and production of transgenic strains) for functionality by doing a rescue experiment of the $Ire1^{f02170}$ mutant phenotype. For that purpose, in the case of Ire1::mcherry, we will generate a fly with the genotype: eyFlpGMRGal4;UASXbp1-EGFP/UASIre1::mcherry;F82B $Ire1^{f02170}$/F82BLacZ and look for Xbp1-EGFP splicing in clones of $Ire1^{f02170}$. If we see Xbp1 splicing in those clones we can be sure that it is being performed by our construct because there is no endogenous functional Ire1 protein in the homozygous mutant clones. In the case of Ire1::GFP, this is not possible to do because Ire1 and Xbp1 reporter have the same tag. To overcome that, we plan to clone a mcherry tag in the C-terminal of Ire1 and then use the same approach to see if the position of the tag in Ire1 protein interferes with its function.

After the validation of the reported results we will analyze by live microscopy the co-localization of Ire1 and Xbp1 mRNA, using Ire1::mcherry (here reported) and Xbp1 mRNA labeled with the MS2-GFP system (ongoing work in the lab - the MS2 binding sites are inserted in the Xbp1 mRNA after the 3'UTR and before the polyA. This reagent will be expressed in cells also expressing MS2-GFP fusion protein, which binds to the respective binding sites allowing the imaging of Xbp1 mRNAs).

In the future, we would like to study the localization of Ire1 and Xbp1 mRNA in an endogenous context, to minimize possible artifacts caused by over-expression of tagged Ire1. To do that we will need to tag Ire1 in the fly genome by modification (recombineering) of a PACMAN reagent (library of bacterial artificial chromosomes with inserts on average of 20 kb covering the *Drosophila* genome and ready for modification and transgenic insertion in the genome). We have already obtained and sent for injection the clone CH322-7H5, which contains the full open reading frame (ORF) of Ire1 and 10 kb of additional 5' genomic sequence, which may or may not contain all the enhancers and promoter of Ire1. To test if CH322-7H5 is a bona-fide genomic rescue construct, we will use this reagent to attempt to rescue $Ire1^{f02170}$ mutant phenotype (for example lethality), after which CH322-7H5 will be modified by recombineering to insert the GFP or mcherry tags in the ORF of Ire1.

Regarding section 3.2, we will repeat our preliminary tests using independent crosses and a larger population size but, at the same time, we will try to set up another strategy for the miRNA profiling experiment. That strategy consists in using a fly line in which there is induced retinal degeneration by over-expression of $Xbp1_{spliced}$ protein – w;sGMRGal4/Cyo;UAS$Xbp1_{spliced}$/Tm6B – to do the miRNA profiling, using w;sGMRGal4/Cyo as a control. We intend to dissect eye imaginal discs of 3rd instar larvae, extract total RNA, evaluate its quality and send for miRNA analysis. From that, we can try to understand if there are any miRNAs candidates, which may have a role in controlling Xbp1 post-transcriptional regulation, and further investigate them.

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6. Attachments

MAPTANTVLITVPRTAITSNNLPKLRPAPLTAALLKVSATPSASPTPSSS
GYASSSNMDDDNMAASQPKAKKRRLDHLTWEEKVQRKKLKNRVAAQTSRD
RKKARMEEMDYEIKELTDRTEILQNKCDSLQAINESLLAKNHKLDSELEL
LRQELAELKQQQQHNTRCISQSNASAGAEGCASTNLGSAASKADPLPQGT
QQVDTQSSARLLAEQLKSSKSLASLWKVVALCLLYKTCLASTKSSTSSAS
KSWPKVCSQISQQTWKQALERAAQLLPKMQATQSDCLDQWWGPQQSAWNP
TGIELMA **DXbp-1u**

MAPTANTVLITVPRTAITSNNLPKLRPAPLTAALLKVSATPSASPTPSSS
GYASSSNMDDDNMAASQPKAKKRRLDHLTWEEKVQRKKLKNRVAAQTSRD
RKKARMEEMDYEIKELTDRTEILQNKCDSLQAINESLLAKNHKLDSELEL
LRQELAELKQQQQHNTRCISQSNASAGAEGCASTNLGSAAGYTTGGHTVV
SASAGGAAEEQQEPGLTLESCGPLPTLQDMLGVDEEFDVKRLEELAESLL
ADITADLETGAGASSPAAAQDAGNAERLPGPMVGPAAERLESDGHRANGL
NVEQEQETEHKVSLNVQMLKINGNPQHTTAAPASRTATITATAAASQLQA
TPDTVYGTYDAKTNSITIVMDGDAVPVNEAVEEIYCDGVSAGDDSTDVIM
KCPPPATSPSQVYLNVMNAVDNSDDEESFDPIDRFLRPRVKAISPLAKSP
ALSLHSATSDHGYESILGSPTSVALTLPADEDDFPWESNFDELFPSLI
DXbp-1s

Fig.1. Xbp1 Unspliced and Spliced forms. DXbp-1u- Xbp1 unslipced, DXbp-1s- Xbp1 spliced. Blue and Red- Different C-terminals comparing the two forms of Xbp1 From [19]

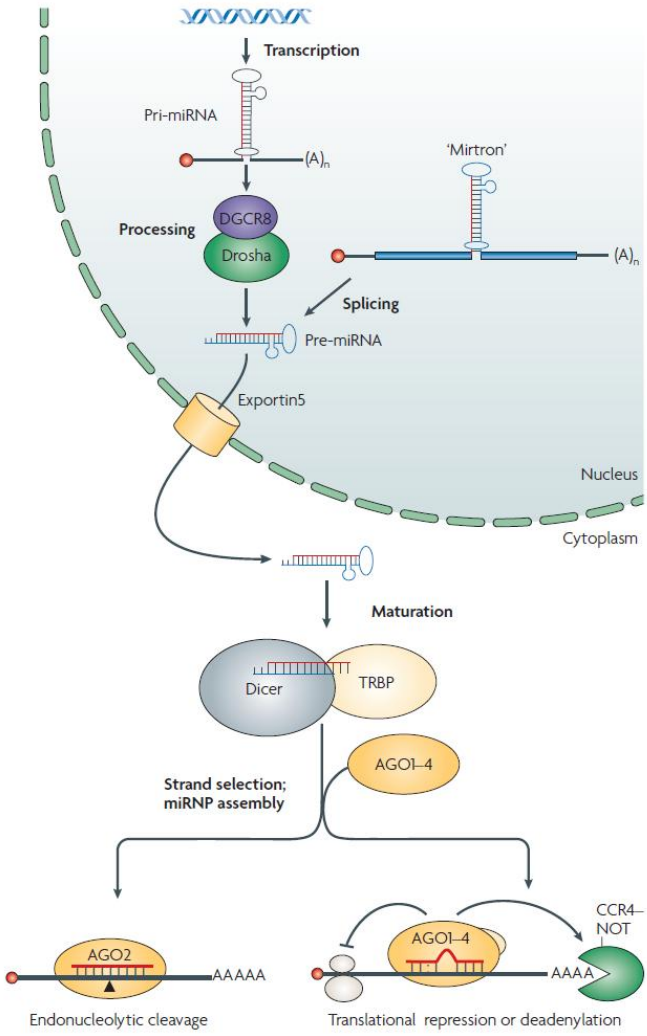


Fig.2. Biogenesis of miRNA. miRNAs are processed from precursor molecules (pri-miRNAs), which are either transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase II transcripts. In animals, pri-miRNAs fold into hairpin structures and are processed by Drosha and Dicer, RNase III type endonucleases. Both Drosha and Dicer function in complexes with proteins containing dsRNA-binding domains (dsRBDs). In mammals, the Drosha–DGCR8 complex processes pri-miRNAs to ~70-nucleotide hairpins known as pre-miRNAs. Then pre-miRNAs are transported to the cytoplasm by *exportin5*, where they are cleaved by Dicer to yield ~20-bp miRNA duplexes. One strand is then selected to function as a mature miRNA, while the other strand is degraded. Following their processing, miRNAs are assembled into ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs). The key components of miRNPs are proteins of the Argonaute (AGO) family. The mature miRNA then binds to complementary sites in the 3'UTR of them RNA target to negatively regulate gene expression. If perfect complementarity is achieved the mRNA is cleaved endonucleolytically in the middle of the miRNA–mRNA duplex. If there is imperfect complementarity, gene expression is blocked at the level of protein translation ou mRNA destabilization. From [32]

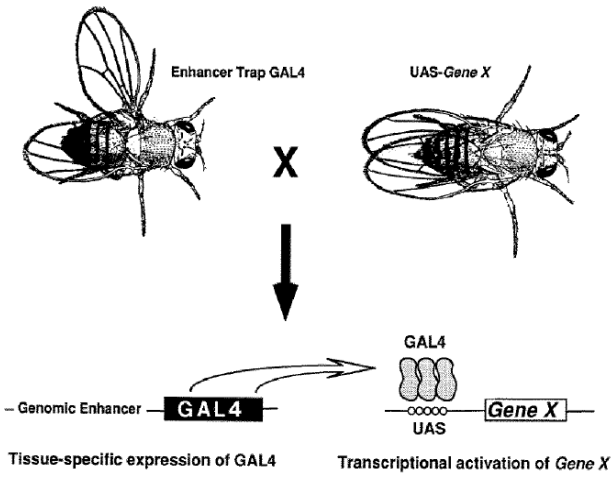
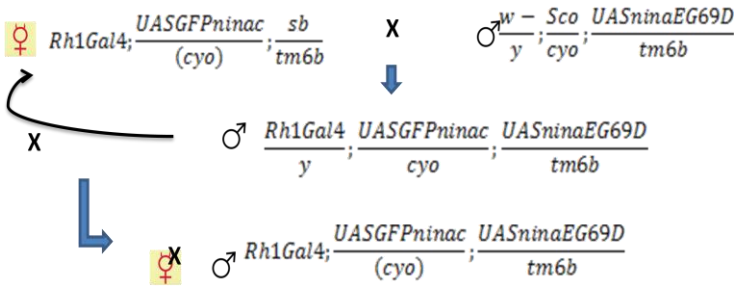
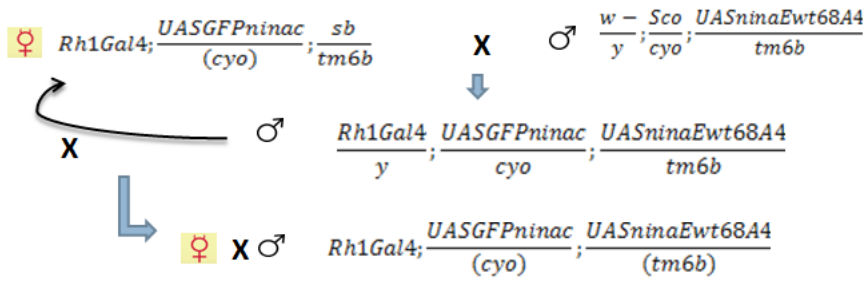


Fig.3. Directed gene expression in *Drosophila* melanogaster by the GAL4 system. The gene encoding the yeast transcriptional activator GAL4 was inserted randomly into the *Drosophila* genome to create various lines in which one GAL4 expression is drived from a different genomic enhancer. Then, a GAL4-dependent target gene can then be constructed by subcloning any sequence next to the GAL4 binding sites – the UAS sites. To activate the target gene in a cell- or tissue-specific pattern, flies carrying the UAS-Gene X are crossed to flies expressing GAL4. In the progeny of this cross, UAS-Gene X will be activated in cells where GAL4 is expressed (from [41])

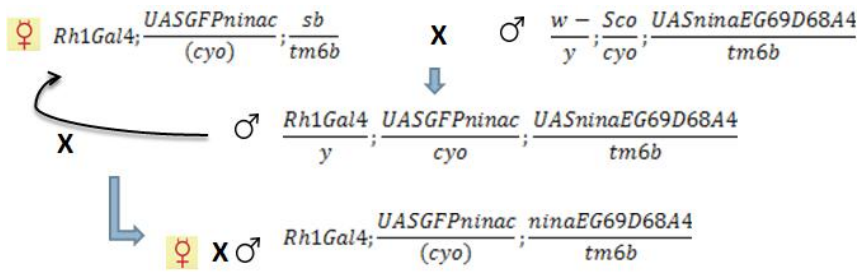
Cross 1



Cross 2



Cross 3



Cross 4

$$\male\ w;\ \frac{Sco}{Cyo};\ \frac{pWIZ}{(tm6b)}\quad \text{X}\quad \female\ Rh1Gal4;\frac{UASGFPninac}{(cyo)};\frac{UASninaEG69D/wt}{(tm6b)}\qquad \text{Attb lines}$$

$$\Downarrow$$

$$Rh1Gal4;\frac{UASGFPninac}{cyo};\frac{UASninaEG69D/wt}{pWIZ}$$

Fig.4. Crosses done during the master project

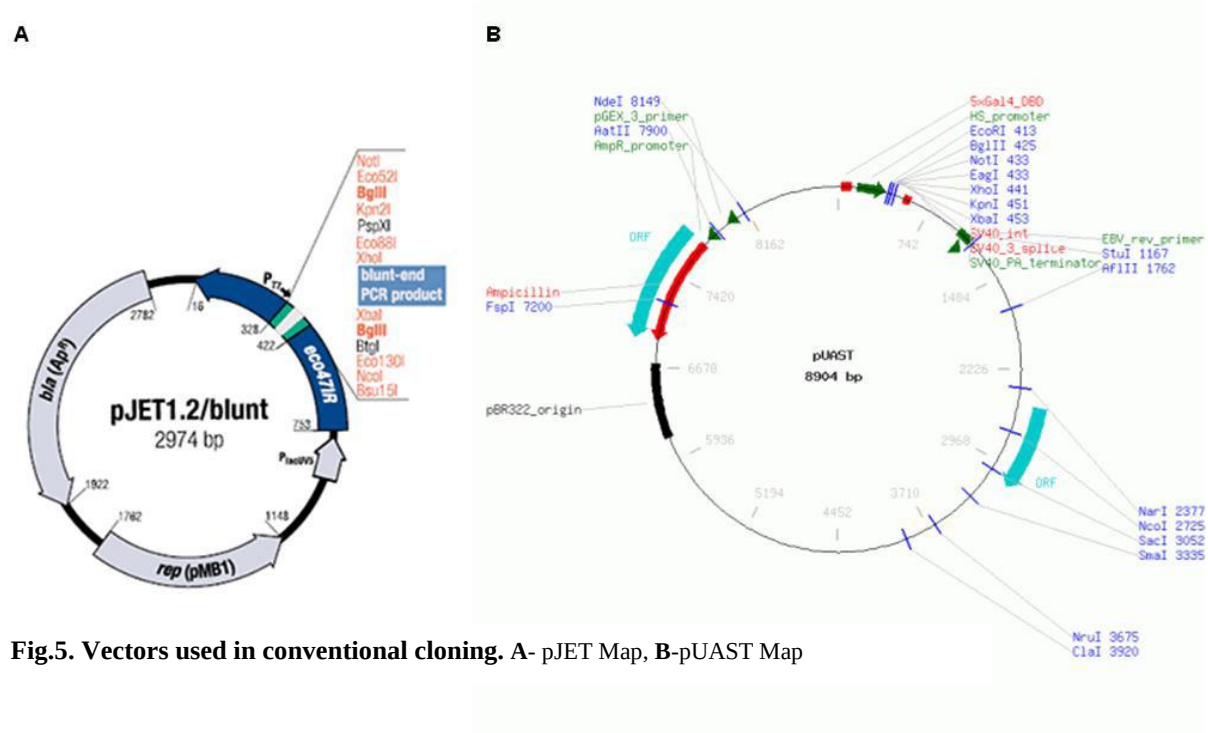


Fig.5. Vectors used in conventional cloning. A- pJET Map, **B**-pUAST Map

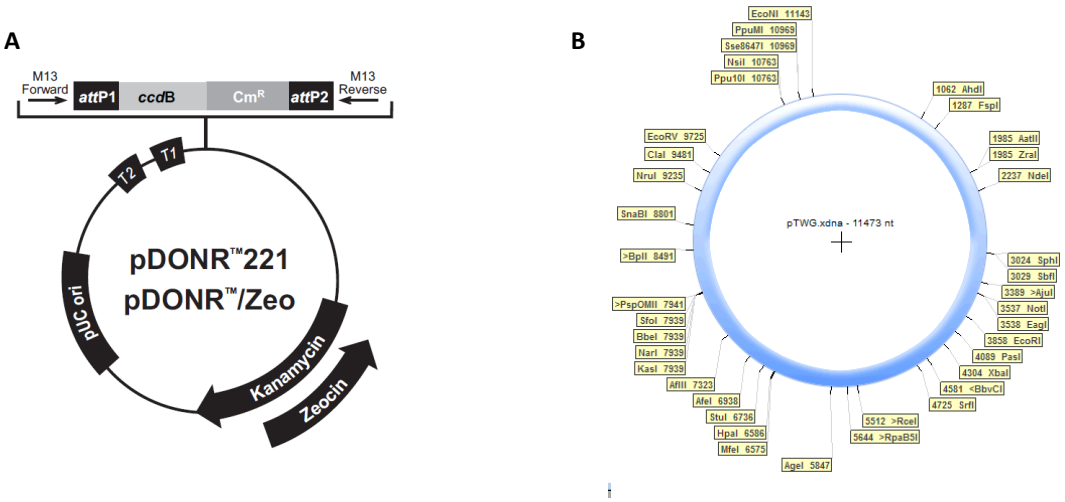


Fig.6. Vectors used in Gateway cloning. A-pDONR^{TM}221 Map, **B**- pTWG Map with its unique restriction sites

Table 1. Lines used in pseudopupil assays

Name given in the experiences	Genotype
Control	Rh1Gal4;UASGFP^{ninac}/(cyo);Sb/TM6B
UASninaE^{G69D}	Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE^{G69D}/(TM6B)
ninaE^{G69D}	Rh1Gal4;UASGFP^{ninac}/(cyo);ninaE^{G69D}/(TM6B)
$_{attB}$UASninaE^{wt}	Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{wt\mathbf{68A4}}$/(TM6B)
$_{attB}$UASninaE^{G69D}	Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{G69D\mathbf{68A4}}$/(TM6B)
Pwiz_$_{attB}$UASninaE^{wt}	Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{wt\mathbf{68A4}}$/Pwiz
Pwiz_$_{attB}$UASninaE^{G69D}	Rh1Gal4;UASGFP^{ninac}/(cyo);UASninaE$^{G69D\mathbf{68A4}}$/Pwiz

Table 2. Primers used for conventional cloning of Ire1::mcherry

Primers	Features
PCR Primers IRE1 I	**Time of elongation**
IRE1 CH1	1min10sec
5'- C GTT AAC AGA TCT ATG GGC AGT CTT AAG AAG TTA CC – **BglII site**	
C-2 -- **Tm: 65,2**	**Tm**
5'- CTC GCC CTT GCT CAC CAT ACC ATT CTC GCT CTG CTT TTG CAG	55ºC
PCR Primers mCherry	**Time of elongation**
C-3	50sec
5´- CAA AAG CAG AGC GAG AAT GGT ATG GTG AGC AAG GGC GAG GAG GAC	
C-4	**Tm**
5'- GGC TAT GGC AAA GGT TTT TGA GTA CAG CTC GTC CAT GCC GCC GGT	60ºC
PCR Primers IRE1 II	**Time of elongation**
C-5	1min10sec
5'- GGC GGC ATG GAC GAG CTG TAC TCA AAA ACC TTT GCC ATA GCC CAA	
IRE1 CH6	**Tm**
5'- TCT AGA GGT ACC TCA ATC CTG CGT TGA AGG – **Acc65I site**	55ºC

Red - mcherry sequences, Blue - Ire1 sequences, Grey - homology zones created by the PCRs

Table 3. Primers utilized in the Gateway system cloning

Primer name	Primer sequence
Ire1_forward_gateway	5' GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ACC ATG GGC AGT CTT AAG AAG TTA
Ire1_reverse_gateway	5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ATC CTG CGT TGA AGG TGG CAG CGT

Green - attb sequences, Red - the initiation codon of Ire1

Table 4. Primers utilized in RT-PCRs

Gene	Primer name	Primer sequence	
Ire1::mcherry	IRE1 CH1	5' C GTT AAC AGA TCT ATG GGC AGT CTT AAG AAG TTA CC	Tm- 58ºC
	C-4	5' GGC TAT GGC AAA GGT TTT TGA GTA CAG CTC GTC CAT GCC GCC GGT	Elongation time – 2min10sec
Ire1::GFP	IRE1-5	5'	Tm- 46ºC
	IRE1 CH6	5' TCT AGA GGT ACC TCA ATC CTG CGT TGA AGG	Elongation time – 2min10sec
Xbp1-EGFP	Xbp1-n	5'	Tm- 60ºC
	GFP-Rw2	5'	Elongation time – 1min10sec

Table 5. Retinal Degeneration assessment across time. Light cycle- 12h light/12h dark

Fly line	Sb/TM6B	UASninaEG69D/(TM6B)	ninaEG69D/(TM6B)
Day	**Degenerated flies /Flies alive**	**Degenerated flies /Flies alive**	**Degenerated flies /Flies Alive**
1	0/20	0/20	0/19
2	0/20	0/20	0/19
3	0/19	0/19	0/18
4	0/19	0/19	0/16
7	0/14	0/17	0/14
10	0/11	0/14	0/11
14	1/8	2/14	0/10
17	0/7	1/9	0/8
21	0/3	1/8	0/6
24	0/2	2/7	0/2
28	0/1	2/6	0/0
35	0/0	2/3	0/0
42	0/0	1/2	0/0
49	0/0	0/0	0/0

Table 6. Retinal Degeneration assessment across time with attB lines. Light cycle- 12h light/12h dark

Lines	attbUASninaE^{wt}	attbUASninaE^{G69D}
Day	**Degenerated flies /Flies alive**	**Degenerated flies /Flies alive**
1	0/100	0/100
4	0/92	0/96
7	0/73	0/91
11	1/46	2/87
15	1/31	0/78
19	0/18	0/71
25	7/15	4/46
29	4/8	4/21
33	1/2	4/7
35	2/2	2/5
41	0/0	1/2
49	0/0	1/1

Table 7. Retinal Degeneration assessment across time with attB lines. Light cycle- constant light

Lines	attbUASninaE^{wt}	attbUASninaE^{G69D}
Day	**Degenerated flies /Flies alive**	**Degenerated flies /Flies alive**
1	0/40	0/40
5	0/37	0/37
7	2/35	0/34
12	9/31	0/27
15	12/30	1/24
19	11/21	1/24
26	8/12	3/22
29	6/10	10/20
32	7/9	11/17
35	9/9	14/16
41	5/5	6/8
43	5/5	6/8
48	3/3	2/2
54	2/2	0/0
60	0/0	0/0