Drosophila as a model for unfolded protein response research

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INTRODUCTION

It is estimated that approximately a third of all genes in eukaryotes encode secretory or membrane proteins that are synthesized on the rough endoplasmic reticulum (ER) (1, 2). Peptides that are synthesized into this organelle first undergo chaperone-assisted folding, and a subset is further modified through glycosylation or oxidation to form disulfide bonds (3). Proteins that fail to undergo proper folding and maturation can be toxic to cells, and underlie many metabolic and degenerative diseases that include diabetes and various forms of neurodegeneration (4, 5). Eukaryotic cells have evolved a robust Unfolded Protein Response (UPR), which specifically refers to signaling pathways that regulate gene expression in response to ER stress (6, 7). Naturally, the regulation of the UPR has been a topic that has drawn significant interest in the field.

Cells can suffer from ER stress for a variety of reasons. Perhaps most obvious are mutations that impair the inherent folding properties of an encoded protein (6). These proteins can cause aggregates, and also have the effect of overwhelming the protein folding machinery. ER is also an organelle that stores high concentrations of Ca\(^{2+}\), which in turn is essential for proper ER function. In fact, the Ca\(^{2+}\) pump inhibitor thapsigargin is frequently used among researchers to impose stress in the ER (8). Calnexin and Calreticulin are examples of Ca\(^{2+}\) binding proteins that are specifically involved in the folding of glycosylated proteins in the ER, and inhibition of glycosylation with tunicamycin similarly interferes with protein folding in this organelle (9). In addition, ER has an oxidizing environment that promotes the formation of disulfide bonds between cysteine residues (10). Many proteins in the ER make stable domain structures only when certain disulfide bonds are formed, and inhibition of cysteine oxidation with reducing agents such as DTT also imposes severe stress in the ER. The rapid elucidation of the UPR pathways was possible, in part, due to the facile ER stress assays based on tunicamycin, thapsigargin and DTT treatment on cultured cells.

The term, UPR, was first coined to describe the transcriptional response to mutant viral protein expression in cultured mammalian cells (6). Such transcriptional response is also observed in Drosophila, as documented in detail in more than a hundred inbred Drosophila species that were fed tunicamycin (11). In recent years, there have been significant efforts to go beyond drug treatment experiments, and determine the physiological role of the UPR in animal development, tissue homeostasis, disease models and lifespan regulation. Drosophila has emerged as a popular model organism for those studies, and here I will discuss the recent advances in this area.

IRE1/XBP1 PATHWAY OF THE UPR

The UPR pathway was initially dissected in the baker’s yeast, Saccharomyces cerevisiae (12). It was first found that IRE1 is an essential mediator of ER chaperone induction after ER stress, as such conditions prompt the activation of this transmembrane signaling protein by forcing oligomer formation
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Fig. 1. Regulation and detection of the IRE1/XBP1 pathway. (A) A schematic diagram of the IRE1/XBP1 pathway in Drosophila. IRE1 is an ER stress sensor that directly binds to misfolded peptides in the ER lumen. Upon detecting ER stress, IRE1 activates its RNase domain on the cytoplasmic side. IRE1 works together with a tRNA ligase to catalyze the splicing of XBP1 mRNA. The product of this spliced isoform acts as a transcription factor that induces ER quality control genes. In addition, IRE1 promotes the decay of many mRNAs associated with the ER. (B) The XBP1-GFP reporter used to detect IRE1 activity in vivo. As XBP1 mRNA splicing by IRE1 shifts the reading frame, an XBP1-GFP fusion transgene was designed to have GFP expressed in frame only when IRE1-mediated splicing occurs.

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found that XBP1 mutant cells activate autophagy, indicating that XBP1 is normally active in that tissue to maintain cellular homeostasis (33). The expression pattern and activity of IRE1 and XBP1 suggest possible roles in a number of other tissues. One of those is the gastrointestinal system. In situ hybridization of xbp1 most prominently shows high transcript levels in the salivary gland and the intestines, and this has been further confirmed with a reporter under the control of XBP1’s upstream sequence (24, 32). Aside from the expression pattern, IRE1 activity can be visualized with another reporter, XBP1-GFP (24, 34). This reporter takes advantage of the fact that the splicing of XBP1 results in a reading frame shift of that transcript. By placing GFP after the XBP1 sequence in a specific reading frame, GFP is expressed in frame specifically when IRE1-mediated mRNA splicing occurs (24, 34) (Fig. 1B). In addition to detecting IRE1 activation in pathological conditions, this reporter can detect ER stress in various tissues, including the larval intestine, fat body, glia, certain neurons and developing photoreceptors (28, 34).

A number of adult tissues also show signs of IRE1 activity, including the male reproductive system and the aging adult intestinal stem cells (34, 35). The accessory gland normally secretes many seminal fluid proteins, and correlating with this, XBP1-based reporters are active in this tissue (32, 34). Further aggravation of such inherent ER stress, either by misexpressing mutant proteins, or by knocking down the ER chaperone BiP, leads to excessive activation of XBP1 splicing and infertility (36). In the adult intestine, XBP1 is required for the proper homeostasis of the epithelial cells, and in its absence the cells build up high levels of reactive oxygen species (ROS), which in turn, signal to promote stem cell hyperproliferation and epithelial dysplasia in the aging fly intestine. Conversely, hyper-activation of the IRE1/XBP1 branch by overexpressing the spliced isoform of XBP1 (XBP1-RB, also known as XBP1s) suppresses ER-stress related phenotypes in the intestinal stem cells (35, 37).

**DEVELOPMENTAL DEFECTS ASSOCIATED WITH IRE1/XBP1**

Major signaling pathways involve membrane receptors and ligands that are synthesized in the ER, and therefore, dysfunction of the ER may have a broad effect on those pathways. Intriguingly, the Notch signaling pathway in *Drosophila* appears to be particularly sensitive to the protein-folding environment in the ER. The connection with UPR was first noticed when a genetic screen for a Notch-like phenotype in the fly identified mutations in ero1L, whose normal function is to stimulate disulfide bond formation in the ER. Based on yeast genetic studies, it was assumed at the time that all disulfide bond formation in the ER should be impaired in ero1L mutants, and therefore, loss of this gene would result in a pleiotropic phenotype. However, ero1L mutant cells specifically showed a Notch-like phenotype in *Drosophila*, with Notch protein accumulation in the ER, and activation of the XBP1-GFP reporter (38). Since that study in *Drosophila*, it has been also determined in mammals that disulfide bonds can form without ero1L, indicating that this gene has assumed more specific roles in metazoans (39).

Other mutations that impair Notch maturation in the ER include mutations in the Catsup gene, a *Drosophila* homolog of ZIP7 zinc transporter (40), Rumi that encodes an O-glucosyltransferase (41) and pecanex (42). Overexpression of the spliced isoform of XBP1 suppressed the pecanex phenotype (42).

**THE PERK/ATF4 PATHWAY IN DROSOPHILA**

In parallel to the IRE1/XBP1 branch, the ER transmembrane kinase PERK is activated in response to ER stress and phosphorylates the translational initiation factor eIF2alpha (43) (Fig. 2). The normal role of eIF2alpha is to help change 40S ribosomal subunits with initiator methionyl tRNAs, which is essential for translational initiation. Therefore, the inhibitory phosphorylation by PERK attenuates the overall rate of translational initiation. It is generally understood that such reduction in translation helps to alleviate the protein-folding burden of cells, but excessive activation of PERK by gene overexpression in *Drosophila* tissues can also cause toxicity (44). In addition, such conditions activate downstream signaling pathways. One of the best characterized is that of ATF4, whose synthesis paradoxically increases when eIF2alpha is phosphorylated. The underlying mechanism of this intriguing phenomenon has been described in detail elsewhere (43, 45). In brief, the unique induction of ATF4 synthesis is possible due to a number of small upstream Open Reading Frames (uORFs) in the 5' UTR (Fig. 2B). The last uORF overlaps with ATF4 in a different reading frame, and therefore, inhibits ATF4 translation in unstressed cells. eIF2alpha phosphorylation makes the recognition of this uORF by the 40S ribosomal inefficient, thereby allowing the opportunity for the main ATF4 ORF to be translated. Once synthesized, ATF4 induces many targets that are involved in stress response. The *Drosophila* genome encodes single orthologs of PERK and ATF4 (44, 46). The latter transcript has uORFs in its 5’ UTR, similar to the homologs in other species. While the ATF4 transcript is widely distributed, the translation only occurs in response to stress (46, 47). A *Drosophila* ATF4 reporter was recently made by fusing the ATF4 5' UTR with the coding sequence of dsRed. This reporter is activated in response to mutant membrane protein expression, and also detects stress in a number of normally developing tissues, including the pupal stage photoreceptors (48).

If ATF4 induces many transcripts, only to have their translation blocked by phosphorylated eIF2alpha, a robust gene expression response cannot be mounted. In reality, eIF2alpha phosphorylation occurs only for a few hours, before being de-phosphorylated to allow mRNA translational initiation. In mammals, such negative feedback occurs through ATF4, and

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Fig. 2. Regulation of the PERK/ATF4 pathway in Drosophila. (A) A schematic diagram of the pathway initiated by the elf2alpha kinases, PERK and GCN2. These kinases are activated by distinct types of stress and phosphorylate elf2alpha. This results in the overall translation attenuation, but at least two transcripts in Drosophila enhance their translation during such conditions: ATF4 is a transcription factor that induces stress response genes, and PPP1R15 is a phosphatase subunit that helps to de-phosphorylate elf2alpha as a feedback mechanism. (B) uORFs in the 5' UTR allow enhanced ATF4 synthesis under conditions of elf2alpha phosphorylation. When elf2alpha is active, 40S ribosomes efficiently recognize uORF2 for translation. When elf2alpha is phosphorylated, 40S ribosome's ability to recognize the uORF2 is compromised, bypassing its AUG to allow the recognition of the ATF4 ORF.

its downstream transcription factor CHOP, which transcriptionally induces a phosphatase subunit PPP1R15 (also referred to as GADD34) to dephosphorylate elf2alpha. A recent study shows that Drosophila PPP1R15 is also induced by ER stress, but not through a transcriptional mechanism. Instead, the Drosophila transcript also has a 5' UTR with uORFs that translationally activates this gene upon elf2alpha phosphorylation (Fig. 2A). Once synthesized, PPP1R15 opposes PERK's effect on elf2alpha (49).

Independent studies have reported the presence of ATF4-like 5' UTRs in a number of other transcripts. These include ATF5 and CHOP (C/EBP homologous protein), transcription factors that contribute to the UPR (50), as well as a kinase of unclear function (51, 52). As there have not been any systematic efforts to identify such transcripts in Drosophila, it is likely that additional transcripts specifically translated during UPR remain at large.

ROLE OF THE PERK/ATF4 PATHWAY IN Drosophila DEVELOPMENT

A hypomorphic allele of ATF4, cryptocephal1, was first described in the 1940s as a mutant with heads that fail to emerge from the thorax during the pupal stage of development (53). Null alleles show defects in larval molting and pupariation (46). Consistent with this idea, Drosophila ATF4 transcriptionally induces Ecdysis triggering hormone (ETH) in the endocrine cells, which in turn promote molting. Intriguingly, a recent study reported that ATF4 works as a coactivator of the Ecdysone receptor, which controls numerous target genes involved in metamorphosis (54). As ATF4 protein cannot be synthesized without stress-induced elf2alpha phosphorylation, these observations provide a tantalizing link between metamorphosis and ER stress, which remains to be further characterized.

Consistent with the developmentally essential role of ATF4, we recently reported that the PERK allele e01744, with a Piggybac element inserted in the first intron, causes developmental lethality. Moreover, PERK is active in healthy adult intestinal stem cells, and without PERK, intestinal stem cell proliferation is reduced. Intriguingly, PERK activity in the stem cells has negative consequences on the intestinal epithelium, and knock down of PERK in that tissue prolongs lifespan of Drosophila (37). These observations show that the PERK/ATF4 pathway is also active in healthy tissues. While it plays positive roles in certain tissues as judged by their developmental requirement, the pathway can have negative effects in others.

In mammals, there are a few additional layers of complexity to this pathway. One is the fact that there are four elf2alpha kinases, each mediating distinct stress response. Two of those, PERK and GCN2, are conserved in Drosophila (49) (Fig. 2A). GCN2 is specifically activated by amino acid deprivation, and consistent with this, ATF4's transcriptional targets include amino acid transporters and other metabolic genes (55-57). Adding to the complexity are non-canonical downstream effectors of PERK. Specifically, studies have reported the transcription factors Nrf2 and NF-kappaB to lie downstream of PERK (58-60). Whether these non-canonical axes of PERK signaling also exist in Drosophila, and whether they play physiologically significant roles in vivo remains to be determined.
THE ATF6 PATHWAY OF THE UPR

In vertebrates, ATF6 also plays an important role in the UPR. This protein has a DNA binding domain in addition to a transmembrane domain that tethers the protein at the ER membrane under unstressed conditions. Upon stress, ATF6 is released from the ER and traffics to the Golgi, where it is cleaved by membrane associated proteases to release the cytoplasmic portion (61, 62). Such conditions allow ATF6 to translocate to the nucleus, where it induces many ER quality control genes including XBP1 and ER chaperones (63, 64). There are two ATF6 genes in vertebrates, referred to as ATF6alpha and beta. Single knockout mice are viable, but double knockouts are embryonic lethal, indicative of redundancy of function in the two genes (64).

The *Drosophila* genome encodes a single ATF6 homolog, with conservation not only within the DNA binding domain, but also in the transmembrane and luminal domains. A Piggybac insertion line within the coding sequence of ATF6, PBac LL0743, is available from public stock centers. This insertion line is viable and fertile as homozygotes (unpublished data), indicating that *Drosophila* atf6 does not play a developmentally essential role as in mammals. Whether this gene is required for a proper ER stress response in adult tissues remain to be validated.

THE UPR IN *Drosophila* DISEASE MODELS

The role of IRE1 and XBP1 in *vivo* has been studied most extensively in the context of mutations that impair the folding property of cells in the ER. A well-characterized example is the *Drosophila* *ninaE* mutant alleles that cause age-related photoreceptor degeneration (65, 66). *Drosophila* *ninaE* encodes a light detecting protein, Rhodopsin-1, and certain missense mutant alleles are similar in nature with the human rhodopsin alleles that underlie Autosomal Dominant Retinitis Pigmentosa (ADRP) (67). Although the human alleles have been speculated to impair the encoded protein's folding property, the link between the UPR and retinal degeneration by rhodopsin mutants was first established through the study of the *Drosophila* *ninaE* (;65) mutant. Specifically, the XBP1-GFP reporter was used to show that the IRE1/XBP1 pathway is activated in these mutant photoreceptors, and that the loss of XBP1 accelerated the course of degeneration in this disease model (24). Conversely, enhancing the degradation of misfolded Rhodopsin-1, by overexpressing the HRD1 ubiquitin ligase, suppressed the course of retinal degeneration in *Drosophila* (68). In human ADRP, P23H substitution is the most common mutation, and the equivalent mutant P37H was generated in the fly to establish similar UPR activation (69).

It appears that *Drosophila* Rhodopsin-1 is particularly sensitive to the protein-folding environment in the ER. *Drosophila* genome encodes two Calnexins. Mutation in one of those, *Calnexin 99A*, gives rise to viable adults with significantly reduced Rhodopsin-1 levels in their photoreceptors (70). A number of other genetic conditions impair proper rhodopsin folding in the ER. Most recently, it has been found that a complex of proteins with previously unknown function, termed the ER membrane protein complex (EMC), are required for multpass transmembrane domain protein folding. In photoreceptors, loss of EMC subunits resulted in Rhodopsin-1 misfolding (71). Although excessive ER stress is a cause of cellular dysfunction and cell death, it has been reported that there are milder conditions of rhodopsin misfolding that can protect cells from other kinds of stress. Such effect was observed while examining p53-induced photoreceptor cell death. The authors found that a mutation in *ninaA*, a gene that is normally required for proper Rhodopsin-1 folding, protects against p53-induced cell death (72). The degree of rhodopsin misfolding is probably mild enough to avoid photoreceptor degeneration under these conditions, while activating UPR’s ability to attenuate translation and enhance anti-oxidant response to enhance general stress resistance of cells.

UPR is associated with a number of other neurodegenerative diseases. Research in *Drosophila* established a link between IRE1/XBP1 and a VapB mutation that underlies amyothrophic lateral sclerosis. VapB is a transmembrane protein with an immunoglobulin fold domain, MSB. *Drosophila* encodes a homologous gene, VAP, whose loss results in the disruption of neuromuscular junctions (73). Interestingly, a point mutation in the human gene, P56S, underlies the dominant effects in the motor neuron degeneration of amyothrophic lateral sclerosis (74), but the underlying reason for pathogenesis had remained unclear, before *Drosophila* was employed as a model system. Studies have determined that the P56S equivalent mutation in *Drosophila*, P58S, forms aggregates in the ER and activates the IRE1/XBP1 pathway of the UPR (75, 76). Defects in the ER quality control in this model are partly attributed to a failure of mutant VapB to bind and retain Oxyyster binding protein in the ER (76). In addition, this mutant allele shows a non-autonomous effect, as wild type VAP has an MSB domain that is cleaved off for secretion to bind to Ephrin receptors in neighboring cells, which does not occur in the mutants (75).

Another example of disease associated with UPR is Hereditary spastic paraplegias. These are neurological disorders that show progressive stiffness and spasticity in the lower limbs, due to damages or dysfunction of nerve fibers. A mutation in a reticulon family protein, RTN2, underlies an autosomal dominant form of this disease. *Drosophila* encodes a single reticulon, Rtn1. A recent study has found that the protein product is enriched in axons and is essential for proper organization of smooth ER in the distal parts of axons. Rtn1 mutants activate the UPR, as evidenced by XBP1-GFP expression (77). Mutations in the cytoplasmic aminocacyl-tRNA synthetases are associated with Charcot-Marie-Tooth diseases, a common form of neurological disorder. A recent study has examined the physiological defects associated with phenylalanyl-tRNA synthetase mutations that underlie this disease, and found that,
among others, the mutant protein expression triggers the activation of XBP1-GFP, providing a link between Charcot-Marie-Tooth disease and UPR (78). Very recently, a genetic screen for genes involved in axonal regeneration after injury identified regulators of XBP1 mRNA splicing. The study found that loss of XBP1 reduces axonal regeneration, whereas conditions that enhance XBP1 splicing stimulates it (79).

In a number of genetic conditions, it has been shown that hyper-stimulation of the IRE1/XBP1 pathway helps to suppress phenotypes. An interesting example has been reported in a Drosophila model for Alzheimer’s disease, in which the amyloid-beta 1–42 peptide or mutant tau were overexpressed in the developing eye (80, 81). Such conditions not only activated the XBP1-GFP reporter, but overexpressing the spliced isoform of XBP1 suppressed the phenotype caused by amyloid-beta expression (81). Spliced XBP1 overexpression can also suppress a distinct phenotype associated with the disruption of the ER-mitochondria interface, caused by the knock down of mitofusin (82).

Drosophila has been actively employed to express human gene alleles that underlie diseases. Among those associated that activate the UPR include mutations in Pro-insulin that underlie diabetes (83), alpha-one antitrypsin mutations that underlie lung emphesyma (68, 84), the GBA gene that underlies Gaucher disease (85).

OTHER UPR PATHWAYS

Most studies in the field focus on the three UPR branches, mediated by IRE1, PERK and ATF6, respectively. These pathways are fully activated by ER stress within hours, and quickly become inactivated through feedback mechanisms. Although a number of studies have implicated their role in degenerative diseases, it is difficult to imagine how these pathways can be responsible for age-related degenerative diseases that manifest only after decades of chronic ER stress. Studies from Drosophila point to the role of distinct pathways that contribute to ER-stress inducible cell death, possibly involving Ca2++. Notably, the ER is a major storage site for Ca2++, and excessive leakage into the cytoplasm can trigger cell death.

Interestingly, an RNAi screen for genes that are required for mutant Rhodopsin-1 induced cell death identified stress-activated kinases, CDK5 and MEKK1, which had not been previously implicated in the canonical UPR pathways (47). CDK5 is a kinase that can be activated by ROS or excessive Ca2+ in the cytoplasm, and implicated in other forms of neuronal cell death (86). Interestingly, excessive Ca2+ release into the cytoplasm also occurs in a Drosophila model for Alzheimer’s disease, where amyloid beta peptide overexpression imposes stress in the ER. The amyloid beta overexpression phenotype is suppressed in the mutant background of Ryanodine Receptor, whose normal role is to release Ca2+ from the ER to the cytoplasm (81). Based on this, one can put together a working hypothesis that chronic stress in the ER causes Ca2+ to leak out into the cytoplasm, and initiate a distinct signaling pathway mediated by CDK5 and MEKK1. This particular signaling pathway may better explain gene expression changes that occur after decades of chronic ER stress, as breakdown of ER Ca2+ homeostasis may occur possibly years after chronic exposure to ER stress, whereas PERK, IRE1 and ATF6 mediate acute UPR responses within hours.

CONCLUSION

UPR research in Drosophila has accelerated in recent years, in part due to the availability of new genetic tools. This model organism nicely complements the existing approaches, particularly in the investigation of the normal physiological role of the UPR, and also regarding specific disease mechanisms. Regarding the molecular mechanism of UPR, much progress has been made in the IRE1/XBP1 branch of signaling. Our understanding of the PERK/ATF4 pathway in Drosophila has been gradually improving, but research on the other branches still lag behind. Unbiased genetic approaches in Drosophila may help elucidate other branches of the UPR that may remain at large.

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