RESEARCH ARTICLE

Exploring the Conserved Role of MANF in the Unfolded Protein Response in Drosophila melanogaster

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Abstract

Disturbances in the homeostasis of endoplasmic reticulum (ER) referred to as ER stress is involved in a variety of human diseases. ER stress activates unfolded protein response (UPR), a cellular mechanism the purpose of which is to restore ER homeostasis. Previous studies show that Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) is an important novel component in the regulation of UPR. In vertebrates, MANF is upregulated by ER stress and protects cells against ER stress-induced cell death. Biochemical studies have revealed an interaction between mammalian MANF and GRP78, the major ER chaperone promoting protein folding. In this study we discovered that the upregulation of MANF expression in response to drug-induced ER stress is conserved between Drosophila and mammals. Additionally, by using a genetic in vivo approach we found genetic interactions between Drosophila Manf and genes encoding for Drosophila homologues of GRP78, PERK and XBP1, the key components of UPR. Our data suggest a role for Manf in the regulation of Drosophila UPR.

Introduction

The accumulation of unfolded or misfolded proteins causes disturbances in endoplasmic reticulum (ER) homeostasis, a phenomenon referred to as ER stress. ER stress in turn activates the unfolded protein response (UPR) (reviewed e.g. in [1–3]). In order to overcome ER stress, UPR leads to attenuation of protein synthesis, enhancement of degradation of unfolded proteins, and activation of specific signalling cascades. These events aim to reduce the overall protein load in the ER and to enhance the protein folding capacity by selective transcription of chaperones. UPR is activated through three signalling cascades by ER transmembrane sensor proteins PERK (PKR-like endoplasmic reticulum kinase), IRE1 (inositol requiring enzyme 1), and ATF6 (activating transcription factor 6). All of these three proteins are maintained inactive in
normal cellular status by binding to the major ER chaperone GRP78/BiP (Glucose-regulated protein 78/Binding immunoglobulin protein). Upon ER stress, GRP78 is dissociated from the sensor proteins which are subsequently activated. The most ancient of these signalling cascades is mediated by IRE1, the sole branch of UPR identified in Saccharomyces cerevisiae. IRE1 has kinase activity and endoribonuclease activity needed for degradation of mRNAs in order to relieve the protein synthesis load. IRE1 is also responsible for the unconventional splicing and thus activation of transcription factor XBP1 (X-box Binding Protein-1), a positive regulator of ER chaperone and other UPR related gene expression. Activated PERK attenuates overall protein synthesis through phosphorylating and thus inhibiting EIF2α (eukaryotic translation initiation factor 2, subunit 1 alpha). However, the decreased activation of EIF2α results in an upregulated translation of specific target mRNAs including ATF4 (activating transcription factor 4) [4,5]. The third signalling pathway is mediated through ATF6, a transcription factor activated by its cleavage and translocation to the nucleus.

In Drosophila, both IRE1- and PERK-mediated UPR signalling cascades are conserved. The amino acid sequence of the Drosophila homologue of ATF6 is highly similar to its mammalian counterpart, but experimental evidence for its involvement in Drosophila UPR is lacking [1,2]. Similar to mammals, the expression of Drosophila homologue of GRP78, Hsc3 (Heat shock protein cognate 3), is upregulated upon induced ER stress in Xbp1-dependent manner [6–9] but no biochemical data are available to show its association with ER stress sensor proteins.

The MANF/CDNF family of neurotrophic factors was first characterized based on its trophic function on dopaminergic neurons in vitro and in vivo [10,11]. When injected into the brain, recombinant mammalian MANF (Mesencephalic Astrocyte-derived Neurotrophic Factor) and CDNF (Cerebral Dopamine Neurotrophic Factor) protect and repair dopaminergic neurons in toxin-induced rodent models of Parkinson’s disease (PD) in vivo [11–13]. The sole Drosophila homologue, DmManf, is expressed in and secreted from glial cells and supports the dopaminergic system in non-cell-autonomous manner [14]. The role of MANF as an extracellular trophic factor is further supported by the evidence that mammalian MANF is protective against ischemic injury in both neurons and cardiomyocytes [15,16]. However, the biology of MANF is not thoroughly understood. Intriguingly, MANF localizes to the ER and has a protective role against ER stress in vitro and in vivo [17–21]. Additionally, mammalian MANF binds GRP78 in Ca2+-dependent manner in vitro and this binding may regulate MANF secretion [16]. MANF can be retained in the ER by its C-terminal signal sequence, RTDL in human and RSEL in Drosophila [16,22]. Experimental evidence suggests that mammalian MANF interacts with KDEL-R [KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor] and that the C-terminal RTDL sequence of MANF is responsible for this interaction [23]. The relevance of KDEL-R as a mediator of the functions of MANF has not been explored in vivo, yet. Recently, MANF was also shown to regulate the expression of ER-resident protein CRELD2 [24].

Both in vivo and in vitro studies have shown that MANF is upregulated after chemically induced ER stress [17,18,25] and by misfolded mutant proteins accumulating in the ER [17,26]. Mammalian MANF expression is activated upon ER stress by ATF6 and XBP1 through an ER stress response element II found in the promoter region of MANF [17,27]. Based on a knockout mouse model, MANF was found to be essential for the survival of pancreatic β-cells and its loss resulted in severe diabetes due to reduction of beta cell mass and activation of UPR in the pancreatic islets [21]. The protective role against 6-OHDA induced and ischemic neuronal damage has been suggested to rise from the ER-related functions of MANF as these processes have been shown to induce ER stress (reviewed in [28,29]) [17,30].

In Drosophila, the loss of DmManf is associated with upregulated expression of genes involved in UPR [20]. Additionally, the overexpression of DmManf resulted in downregulation of several UPR-related genes [20]. Here we show that, similar to mammalian MANF, the...
expression of \( \text{DmManf} \) is induced in response to ER stress in vitro. Further, we applied the transgenic approaches for gene silencing in vivo to reveal genetic interactions between \( \text{DmManf} \) and genes with known functions in the maintenance of ER homeostasis and in UPR.

**Materials and Methods**

**Fly Strains**

Fly stocks and crosses were maintained at 25°C. The following fly lines were used in the study: \( w^− \), UAS-\( \text{DmManf} \)\(^{133} \) (line L3), UAS-\( \text{DmManf} \)\(^{135} \) (line L5) and \( \text{DmManf} \)\(^{496} / \text{TM6 Sb EYFP} \) [14], 69B-GAL4 (Bloomington Drosophila Stock Center (BDSC) #1774) [31], da-GAL4 (BDSC #5460) [32], MS1096-GAL4 (BDSC #8860) [33], tub-GAL4/TM6 Sb EYFP (BDSC #5138) [34], UAS-mCD8-GFP (BDSC #5130) [34], UAS-Hsc3 (BDSC #5843) [35], T(2;3)SM6a-TM6B Tb translocation balancer (originating from \( \text{pnut}^{XP}/T(2;3)\text{SM6a-TM6B Tb, BDSC #5687} \) was used in viability studies (referred as SM6-TM6). UAS-RNAi lines (listed in S1 Table) were obtained from BDSC [36] and Vienna Drosophila RNAi Center [37]. Adult flies were imaged with ProgRes SpeedXT camera (Jenoptik). Genes were annotated according to Flybase [38].

**Cell Culture**

Schneider 2 (S2) cells were cultured in M3-BPYE medium (Shields and Sang M3, 0.5 g/l KHCO\(_3\), 1.0 g/l yeast extract, 2.5 g/l bactopeptone and 10% fetal bovine serum, pH 6.6) at 25°C. Cells were treated with DMSO, 1 \( \mu \)M thapsigargin (Molecular Probes), 1 mM DTT (Promega) or 10 \( \mu \)g/ml tunicamycin for 20 hours, collected and total RNA was extracted with NucleoSpin \(^1\) RNA II (Macherey-Nagel). In-column DNase treatment was performed according to manufacturer’s instructions. Samples were collected from three biological replicates. For agarose gel electrophoresis analysis total RNA was extracted with the TRIZOL reagent (Gibco BRL, Life Technologies).

**Quantitative RT-PCR**

Larvae were grown at 25°C on apple juice plates with yeast paste and collected 50–54 h after egg laying (AEL) for 2\(^{nd}\) instar larval samples or 20–26 h AEL for early 1\(^{st}\) instar larval samples. Five wandering larvae were collected for 3\(^{rd}\) instar larval samples and each genotype was collected as three biological replicates. Larvae were snap frozen and stored -80°C until RNA extraction. NucleoSpin \(^1\) RNA II (Macherey-Nagel) was used in extraction and purification of total RNA. In-column DNase treatment was performed according to manufacturer’s instructions. First strand cDNA was synthesized from total RNA (1 \( \mu \)g) using RevertAid Premium Reverse Transcriptase (Thermo Scientific) and Oligo(dT\(_{18}\)) primer at 53°C according to manufacturer’s instructions. Expression of \( \text{DmManf} \) mRNA was quantified by LightCycler \(^{\text{\textregistered}}\) 480 Real-Time PCR System with Lightcycler 480 SYBR Green I master mix (Roche). Primer pairs and their PCR efficiencies are presented in S2 Table. PCR efficiency (E) of each primer pair was determined from a relative standard curve. Equation E \( \text{Cp} \) in which \( \text{Cp} \) indicates a crossing point was used to calculate relative concentration of mRNA in each sample. \( \text{RpL32} \) was used for normalization. Each sample was analysed as a duplicate.

**Statistical Analysis**

Microsoft \(^{\text{\textregistered}}\) Excel Analysis ToolPak (Microsoft \(^{\text{\textregistered}}\) Office Professional Plus 2010) was used for all statistical analyses. For qPCR analyses, two-tailed Student’s t-test was used. For pupal viability studies, \( \text{Tb}^+ \) and \( \text{Tb}^- \) pupae were counted, the number of \( \text{Tb}^+ \) pupae was divided by the number of all pupae and normalized to experimentally determined ratio from tub-GAL4/TM6.
Tb Sb EYFP crossed to wild type and to wild type balanced with SM6-TM6 translocation balancer (S3 Table). For preliminary analyses two vials were counted and statistical analysis was done based on six vials with minimum of 40 pupae.

### Results

**DmManf Expression is Upregulated in Response to Drug-Induced ER Stress in vitro**

In mammals, MANF is upregulated after chemically induced ER stress [17,18]. To study whether DmManf is involved in Drosophila ER stress, we used Schneider 2 cells and induced ER stress by thapsigargin (TG), dithiothreitol (DTT) and tunicamycin (TM). TG depletes Ca\(^{2+}\) from the ER by inhibiting Ca\(^{2+}\) ATPase [39], DTT reduces the disulphide bridges leading to accumulation of unfolded proteins [40], and TM inhibits N-linked glycosylation [41]. The induction of ER stress was monitored by measuring the mRNA levels of Drosophila GRP78 homologue Hsc3 and Xbp1 [total (Xbp1t) and spliced (Xbp1s) forms separately] by qPCR analysis and by evaluating the proportions of unspliced and spliced transcripts of Xbp1 (Xbp1\(^u\) and Xbp1\(^s\), respectively) by agarose gel electrophoresis. In agreement with previous studies [6,8], we detected the upregulation of Hsc3 in response to TG, TM and DTT indicating that ER stress was indeed induced (Fig 1A). Under the control conditions, the Xbp1\(^u\) transcript was prevalent (Fig 1B). TG and DTT treatment induced the splicing of the Xbp1 transcripts (Fig 1B). The splicing of Xbp1 was also detected by qPCR analysis (S1A Fig). While total amount of Xbp1 (Xbp1t) was unaltered, the level of Xbp1\(^s\) was increased. Additionally, the ratio of Xbp1\(^s\) to Xbp1\(^t\) (Xbp1\(^s\):t), a commonly used readout of UPR induction, was increased. To study whether the expression of DmManf was altered in response to drug-induced ER stress in vitro we measured DmManf mRNA levels by qPCR analysis. We found that DmManf mRNA levels were increased in response to drug-induced ER stress (Fig 1A). These data demonstrated that upregulation of MANF mRNA in response to drug-induced ER stress is conserved between mammals and Drosophila.

**Overexpression of DmManf Induces Unconventional Splicing of Xbp1 but does not Alter Hsc3 Expression**

Next, we examined whether altering DmManf expression level induces ER stress in vivo. The abolishment of both maternal and zygotic DmManf results in lethality at the end of embryogenesis before hatching [14]. Zygotic DmManf\(^{\Delta 96}\) mutants die during first larval molt due to the persisting maternal contribution of DmManf which promotes the survival of the larvae [14]. In this work, we extracted RNA from larvae at their first larval molt and measured Hsc3 and Xbp1 mRNA levels by qPCR. In the zygotic DmManf\(^{\Delta 96}\) mutant larvae, both Hsc3 and Xbp1\(^s\) expression was slightly decreased suggesting that UPR was not induced (Fig 1C). The level of Xbp1\(^t\) was not changed.

We also tested if the overexpression of DmManf would affect the mRNA expression of Hsc3 and Xbp1. UAS-DmManf construct was driven with ubiquitous tub-GAL4 and wandering 3\(^{rd}\) instar larvae were collected to gain more long-term DmManf overexpression. Overexpression of DmManf did not alter Hsc3 or Xbp1\(^t\) expression levels, but did increase the amount of Xbp1\(^s\) (Fig 1D). Thus, the Xbp1\(^s\):t ratio was increased as well. This suggests that overexpression of DmManf induces UPR similar to drug-induced ER stress in vitro (S1A Fig). However, the mRNA level of Hsc3 was not altered suggesting that the transcriptional regulation via Xbp1\(^s\) was not activated.
Fig 1. Drug-induced ER stress upregulates DmManf expression. A–B) In Schneider 2 (S2) cells, ER stress was induced by thapsigargin (TG), tunicamycin (TM) and dithiothreitol (DTT). DMSO was used as a control treatment. A) The mRNA levels of DmManf and Hsc3 were analysed by qPCR, values were normalised to control treatment (DMSO). B) RT-PCR and agarose gel electrophoresis analysis revealed two transcripts of Xbp1, unspliced (Xbp1<sup>u</sup>) and spliced (Xbp1<sup>s</sup>). RpL32 was used as a loading control. C–D) qPCR analysis of Hsc3 and Xbp1 expression in DmManf mutant (C) and DmManf overexpressing (D) larvae. Expression of Hsc3 was not altered but Xbp1<sup>s</sup> mRNA level was increased in response to overexpression of DmManf. The overexpression of DmManf resulted in 165-fold increase in DmManf mRNA level (±23, <i>P</i> < 0.001, not shown). Xbp1<sub>t</sub>, total amount of Xbp1; Xbp1<sub>s</sub>, spliced-specific transcript of Xbp1; Xbp1<sub>s,t</sub>, proportion of Xbp1<sub>s</sub> out of Xbp1<sub>t</sub>. OE, overexpression. Average ± standard deviation. *, <i>P</i> < 0.05; **, <i>P</i> < 0.01; ***, <i>P</i> < 0.001 versus control, Student’s t-test.

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To monitor the extent of overexpression by UAS/GAL4 system, we measured the level of
DmManf mRNA by qPCR and found massive upregulation of DmManf transcript (Fig 1D).
Since DmManf enters the secretory pathway, it is possible that any observed effects of DmManf
overexpression might be due to increased overall protein synthesis load in the ER and not spe-
cifically induced by DmManf. Thus, we used GFP protein with a membrane-directing tag
(UAS-mCD8-GFP) as a control for a protein synthesized in the ER. The expression of UAS-
mCD8-GFP with tub-GAL4 did not increase the expression levels of Hsc3, Xbp1t or Xbp1s (Fig
1D). This suggests that the alterations caused by overexpression of DmManf were not because
of the increased overall protein load in the ER but related to DmManf activity.

DmManf Genetically Interacts with Genes Involved in the ER Stress and
UPR

To study further whether DmManf interacts with genes involved in the Drosophila UPR, we used
targeted UAS-RNAi transgenes to inactivate a selected set of genes with known function in Dros-
ophila ER and ER stress (Fig 2A). Based on our previous studies, we selected semi-ubiquitous
69B-GAL4 driver [20,31] to knock down target genes for two reasons. First, ectopic DmManf in
the 69B-GAL4 expression pattern is sufficient to substitute for the loss of endogenous DmManf
protein [14]. Second, by comparing the transgene expression in ubiquitous da-GAL4 and semi-
ubiquitous 69B-GAL4 pattern we were able to reveal the significance of mutations in the DmManf
gene for rescuing DmManfΔ96 mutant lethality [22]. In addition to semi-ubiquitous 69B-GAL4
driver, we also wanted to have a more specific expression pattern for the knockdown experiments
and used wing driver MS1096-GAL4 [33,42]. We compared the observed phenotypes in wild type
and DmManf-overexpressing backgrounds to detect whether abundant DmManf expression
would affect the knockdown of target genes. The UAS-RNAi lines with distinct phenotypes in
these backgrounds were further analysed with ubiquitous tub-GAL4 driver to knock down the
selected genes with and without DmManf overexpression (S3 Table). To verify the specificity of
DmManf overexpression on the observed genetic interactions, we used UAS-mCD8-GFP con-
struct as a control for overexpression of a protein processed in the ER. In our previous and current
work, we have never detected any obvious phenotypes in flies overexpressing DmManf with a
variety of GAL4 drivers in wild type background (Fig 2B and S2A Fig,[20,43]). Surprisingly,
simultaneous overexpression of DmManf strongly enhanced the phenotypes caused by knock-
down of Hsc3, PEK (pancreatic elF-2alpha kinase, homologue to human PERK), Xbp1 and sip3
(septin interacting protein 3) (Fig 2A, these results are described in detail below).

DmManf Interacts with Hsc3, Drosophila Homologue of GRP78

Knockdown of Hsc3 in the wing with MS1096-GAL4 driver in wild type background resulted
in severely malformed wings (Fig 3A). This wing phenotype was further worsened when
DmManf was simultaneously overexpressed whereas it was not affected by simultaneous
expression of UAS-mCD8-GFP (Fig 3A). The knockdown of Hsc3 with semi-ubiquitous
69B-GAL4 and ubiquitous tub-GAL4 drivers was lethal in both wild type and DmManf-over-
expressing backgrounds (Fig 2A and S3 Table).

To investigate whether silencing of Hsc3 affected endogenous DmManf expression, we
knocked down Hsc3 and analysed DmManf mRNA levels by qPCR analysis. The knockdown
of Hsc3 with tub-GAL4 resulted in lethality prior to 2nd instar larval stage (S3 Table). Thus, we
analysed the DmManf mRNA levels at the early 1st larval stage and selected the semi-ubiqui-
tous 69B-GAL4 driver instead of ubiquitous tub-GAL4. Although the UAS/GAL4 system is
activated [31] and gene expression silenced by UAS-RNAi only from the embryonic stage 9
onwards, we detected a notable decrease in Hsc3 mRNA levels. The mRNA levels of DmManf
**A**

| Gene name                              | Symbol | Human homologue | RNAI library | Role in ER stress/ER |
|----------------------------------------|--------|----------------|--------------|----------------------|
| Heat shock protein cognate 3          | Hsc3   | GRP78/BiP/HSPA5| Bl (OE vs. wt) | Protein folding, binding of ERSSs |
| pancreatic elf-2alpha kinase           | PEK    | PERK           | GD (OE vs. wt) | ERSS, phosphorylation of eIF2α |
| X box binding protein-1               | Xbp1   | Xbp1           | GD           | Transcriptional regulation |
| septin interacting protein 3           | sip3   | HRD1           | KK           | ERAD |
| Calcium ATPase at 60A                  | Ca-P60A| SERCA          | GD           | Cellular Ca²⁺ homeostasis |
| eukaryotic translation initiation factor 2alpha | eIF2α  | EIF2S1        | KK           | Protein synthesis |
| von Hippel-Lindau                      | Vhl    | VHL            | KK           | Proteasomal degradation |
| Inositol-requiring enzyme-1            | Ire1   | IRE1           | GD           | ERSS, splicing of Xbp1 |
| Att6                                   | Att6   | ATF6           | GD           | ERSS, transcriptional regulation |
| Activating transcription factor 3      | Atf3   | ATF3           | GD           | Transcriptional regulation |
| TNF-receptor-associated factor 6       | Traf6  | TRAF6          | KK           | Apoptosis |
| cryptocephal                           | crc    | ATF4           | GD           | Transcriptional regulation |
| Hrd3                                   | Hrd3   | HRD3           | GD           | Protein transport to ERAD |
| Protein disulfide isomerase            | Pdi    | PDI            | GD           | Protein folding |

**B**

**Fig 2. Overview of genetic interactions between DmManf and selected ER- and UPR-related genes.** A) UAS-RNAi lines were crossed to MS1096-GAL4 and 69B-GAL4 driver lines in wild type and DmManf-overexpressing backgrounds. The observed phenotypes of knockdown flies in DmManf-overexpressing background (OE vs. wt) were compared to the phenotype of knockdown flies in wild type background. Yellow (stronger phenotype) represents affected phenotypes. Light gray (no phenotype in either background), gray (phenotype similar in both backgrounds) and dark gray (lethal phenotype in both backgrounds) represent cases where the overexpression of DmManf did not affect the phenotype caused by knockdown of target gene. As a comparison, results from our previously published microarray analysis (MAA) [20] are presented; red and blue indicate up- and down-regulation of the target gene, respectively. Mutant larvae stands for zygotic DmManf<sup>Δ96</sup> mutant larvae, OE larvae for 69B-GAL4>UAS-DmManf<sup>Δ3</sup> larvae, and mutant embryos.
showed a 1.5-fold increase (Fig 3B) indicating that the knockdown of Hsc3 resulted in upregulation of DmManf expression. These data demonstrated that the genetic interaction between MANF and Hsc3/GRP78 is conserved between Drosophila and mammals.

DmManf and Hsc3 do not Complement each other

Ubiquitous knockdown of Hsc3 in wild type background was lethal before 50 hours AEL (S3 Table) and this lethality was not rescued by simultaneous overexpression of DmManf (S3 Table). To further study the genetic interaction between DmManf and Hsc3, we used a UAS-Hsc3 construct to overexpress Hsc3 [35]. Ubiquitous overexpression of Hsc3 with tub-GAL4 driver in wild type background did not affect overall viability and no obvious phenotypes were detected in the adult flies (S2A–S2C Fig). Furthermore, simultaneous overexpression of Hsc3 and DmManf did not affect viability and showed no obvious phenotype in the emerged adults (S2A–S2C Fig).

The loss of zygotic DmManf results in lethality at early larval stage [14]. To examine whether overexpression of Hsc3 could complement for the lack of DmManf, we crossed UAS-Hsc3; DmManfΔ96/TM6 males to da-GAL4 DmManfΔ96/TM6 females. We could not detect homozygous DmManfΔ96 mutant pupae or adults (number of pupae analysed = 141). Thus, the ubiquitous overexpression of Hsc3 failed to rescue DmManfΔ96 mutant lethality. Taken together, these data indicate that both Hsc3 and DmManf are necessary for fly viability but unable to complement each other.

Genetic Interaction between DmManf and ER Stress Sensor PEK

Knockdown of PEK with MS1096-GAL4 showed more severe wing phenotype together with DmManf overexpression in comparison to wild type background (Fig 4A). With semi-ubiquitous

![Fig 3. DmManf genetically interacts with Hsc3, the Drosophila homologue of the mammalian ER chaperone GRP78.](http://example.com/f3.png)
69B-GAL4 driver, the knockdown of PEK was viable in wild type background (Fig 4B). However, together with DmManf overexpression the knockdown of PEK with 69B-GAL4 was lethal at pupal stage (Fig 4B). In wild type background the ubiquitous knockdown of PEK was viable (S2D and S2E Fig, S3 Table). Interestingly, simultaneous overexpression of DmManf worsened the ubiquitous knockdown of PEK to lethality at larval stage (S2D Fig, S3 Table). Simultaneous expression of UAS-mCD8-GFP did not affect the PEK knockdown either by MS1096-GAL4, 69B-GAL4 or tub-GAL4 drivers (Fig 4A and 4B, S3 Table) indicating that the observed changes caused by overexpression of DmManf were due to increased DmManf activity.

To verify the knockdown of PEK expression by UAS-PEK-RNAi construct, we measured the PEK transcript levels by qPCR analysis in ubiquitous PEK knockdown larvae 50–54 h AEL. Indeed, the expression level was significantly decreased (S1B Fig). To further elaborate
the genetic interaction of \textit{DmManf} with \textit{PEK}, we investigated the effect of ubiquitous knockdown of \textit{PEK} on \textit{DmManf} expression by analysing \textit{DmManf} mRNA levels in \textit{PEK} knockdown larvae. Interestingly, ubiquitous knockdown of \textit{PEK} increased the mRNA levels of \textit{DmManf} (Fig 4C). This qPCR analysis together with the \textit{in vivo} phenotypic data indicates genetic interaction between \textit{DmManf} and \textit{PEK}, the functionally conserved \textit{Drosophila} homologue of mammalian UPR transducer \textit{PERK}.

Overexpression of \textit{DmManf} Alters \textit{Xbp1} Knockdown Phenotype

In wild type background, the knockdown of \textit{Xbp1} with either wing driver MS1096-GAL4 or semi-ubiquitous 69B-GAL4 showed barely notable phenotype in adult wings (Fig 5A and 5B). When \textit{Xbp1} was knocked down with these drivers together with \textit{DmManf} overexpression, the adults showed clearly stronger wing phenotypes (Fig 5A and 5B). In wild type background the ubiquitous knockdown of \textit{Xbp1} with \textit{tub-GAL4} was partially lethal at larval stage (S2D and S2E Fig, S3 Table). In \textit{DmManf}-overexpressing background, ubiquitous knockdown of \textit{Xbp1} resulted in complete larval lethality (S2D Fig, S3 Table). Simultaneous expression of UAS-mCD8-GFP did not alter \textit{Xbp1} knockdown with any of the GAL4 drivers used (Fig 5A and 5B, S3 Table).

Again, we quantified by qPCR the expression level of \textit{DmManf}, \textit{Hsc3} and \textit{Xbp1} mRNAs in ubiquitous \textit{Xbp1}-knockdown larvae. Both total amount and spliced form of \textit{Xbp1} mRNA showed severely reduced expression level indicating a successful knockdown by UAS-\textit{Xbp1}-RNAi transgene (Fig 5C). The ubiquitous knockdown of \textit{Xbp1} with \textit{tub-GAL4} only slightly reduced the \textit{DmManf} and \textit{Hsc3} mRNA levels (Fig 5C). However, overexpression of \textit{DmManf} in wild type background resulted in increased level of \textit{Xbp1s} transcript (Fig 1D). Thus, the data presented in this study strongly indicated that \textit{DmManf} and \textit{Xbp1} interact with each other.

Upon UPR, the splicing of \textit{Xbp1} is carried out by \textit{Ire1}, one of the ER stress sensor proteins. In our study, we also examined the genetic interaction between \textit{DmManf} and \textit{Ire1}. The knockdown of \textit{Ire1} with MS1096-GAL4, 69B-GAL4 and \textit{tub-GAL4} did not show any obvious phenotype in the adult flies either in wild type and \textit{DmManf}-overexpressing backgrounds (Fig 2A). The \textit{Ire1} mutant larvae die prior 72 h AEL [44]. Most likely, the knockdown by UAS-\textit{Ire1}-RNAi construct was insufficient to reduce the \textit{Ire1} expression level enough to detect any genetic interactions in this study.

\textit{DmManf} Interacts with \textit{sip3}, a Gene Encoding for a Component of ER-Associated Degradation (ERAD)

The simultaneous overexpression of \textit{DmManf} also affected the knockdown of \textit{sip3} (septin interacting protein 3) (Fig 2A). \textit{sip3} encodes for the \textit{Drosophila} homologue of human synoviolin/HRD1, an ER resident E3 ubiquitin ligase with specific function in ERAD. In wild type background, the knockdown of \textit{sip3} with wing driver MS1096-GAL4 showed subtly uneven wing phenotype (S2F Fig). The simultaneous overexpression of \textit{DmManf} enhanced this phenotype to mildly wrinkled wings (S2F Fig). Co-expression of UAS-mCD8-GFP did not affect the \textit{sip3} knockdown phenotype (S2F Fig). The ubiquitous knockdown of \textit{sip3} with \textit{tub-GAL4} resulted in lethality prior to pupal stage in both wild type and \textit{DmManf}-overexpressing backgrounds (S3 Table). In our previous study we found that \textit{sip3} was downregulated in \textit{DmManf}\textsuperscript{Δ96} mutant larvae. Taken together, these data demonstrated a genetic interaction between \textit{DmManf} and \textit{sip3}.

\textit{DmManf} does not Genetically Interact with the ER Stress Sensor \textit{Atf6}

Previous studies on mammalian systems have suggested an interaction between MANF and \textit{ATF6}, one of the three ER stress sensor proteins [17,27]. In the current study, we found a
genetic interaction between \textit{DmManf} and \textit{PEK}, a gene encoding for another ER stress sensor protein. We did not detect any phenotype in the \textit{Atf6} knockdown flies by either MS1096-GAL4, 69B-GAL4 or \textit{tub}-GAL4 drivers (Fig 2A). Simultaneous overexpression of \textit{DmManf} did not affect \textit{Atf6} knockdown by MS1096-GAL4 or 69B-GAL4 drivers (Fig 2A). We also measured the \textit{Atf6} mRNA levels by qPCR in \textit{DmManf} mutant (S1C Fig) and overexpressing (S1D Fig) larvae and could not detect alterations in the \textit{Atf6} expression. Further, we analysed the \textit{DmManf} mRNA level by qPCR analysis in ubiquitous \textit{Atf6} knockdown larvae. The
$DmManf$ expression level was not altered while $Atf6$ expression level was decreased (S1C Fig). Taken together, our data suggest that there is no genetic interaction between $DmManf$ and $Atf6$ in this experimental setup.

**Discussion**

Increasing evidence indicates that ER stress and UPR play a major role in a variety of human diseases including diabetes mellitus and neurodegenerative disorders (reviewed e.g. in [3,45]). MANF is a secreted protein [14,30], but also localizes to the ER and has a role in mammalian UPR [17–20]. In this study, we examined the role of $DmManf$ in UPR in the *Drosophila* model. We show that the upregulation of MANF mRNA expression by ER stress-inducing agents is conserved in *Drosophila* S2 cells. Additionally, we found genetic interaction between $DmManf$ and genes known to function in the ER and UPR. A schematic presentation of the interactions discovered is presented in Fig 6.

One of the interacting partners was $Hsc3$, the *Drosophila* homologue of mammalian chaperone GRP78. The silencing of $Hsc3$ in the wing resulted in an abnormal wing phenotype in wild type background. This wing phenotype was stronger in $DmManf$-overexpressing background. In cultured mammalian cells MANF has been shown to bind GRP78 in Ca$^{2+}$-dependent manner and the loss of interaction between mammalian MANF and GRP78 was associated with increased secretion of MANF [16]. In line, the knockdown of $Hsc3$ could lead to increased secretion of DmManf and lead to deprivation of intracellular DmManf. In our previous study, we noticed that the deletion of ER retention signal RSEL increased the secretion of DmManf in S2 cells and decreased its functionality in rescue experiments in vivo [22]. Based on the physical interaction found between mammalian MANF and GRP78, the simultaneous overexpression of $DmManf$ and knockdown of $Hsc3$ could also result in the abundant DmManf binding the residual Hsc3 and preventing other important cellular functions of Hsc3. Alternatively, the loss of $Hsc3$ could lead to decreased protein folding capacity in the ER and activation of UPR. The vast amount of DmManf protein could exhaust this already disturbed cellular state.

![Fig 6. A simplified presentation of UPR and genetic interactions (coloured in orange) discovered for *Drosophila Manf*. ER stress sensor proteins IRE1, PERK and ATF6 reside on ER membrane. The role of ATF6 in *Drosophila* UPR is uncharacterised (in gray). Upon ER stress, transcription factor XBP1 is spliced by IRE1 and activated. XBP1 directs expression of chaperones, including GRP78. In cultured mammalian cells, XBP1 is also suggested to regulate MANF expression. HRD1, an ER resident E3 ubiquitin ligase, functions in ERAD, a process in which terminally misfolded proteins are degraded in the cytosol. In this study, we found that DmManf genetically interacts with Hsc3/GRP78, Xbp1, Pek/PERK and sip3/HRD1 but the functional role of DmManf in Drosophila UPR remains to be solved in future. In blue lines are presented the regulatory genetic interactions our data suggest. Dashed lines indicate speculative interactions.](https://doi.org/10.1371/journal.pone.0151550.g006)
In previous studies, mammalian MANF has been suggested to have chaperone-like functions, e.g. by binding unfolded proteins in vitro [46] but the putative chaperone activity remains unconfirmed [17,46]. The major ER chaperone Hsc3 and DmManf clearly have distinct roles as either the overexpression or the loss of one could not complement for the loss of the other. However, our study indicates that the interaction between MANF and GRP78 [16] is conserved. In future, the functional significance of this intriguing interaction deserves to be addressed in detail.

We also found that DmManf genetically interacted with PEK/PERK, an ER stress sensor protein. Similar to the silencing of Hsc3, we found that simultaneous overexpression of DmManf worsened the phenotypes observed in PEK knockdown flies. Previous studies have indicated functional conservation of PERK in Drosophila and mammals (reviewed in [1,2]). The Drosophila homologue to ATF4, the downstream target of activated PERK and selectively upregulated by UPR, showed no genetic interaction with DmManf in our study (Fig 2A). We have previously shown that the abolishment of both zygotic and maternal DmManf resulted in increased phosphorylation of eIF2α, another molecular marker used for detecting ER stress [20]. In this study, we abolished only the zygotic DmManf while maternal DmManf was still present. The loss of zygotic DmManf alone did not induce UPR when evaluated by other readouts, i.e. increased Hsc3 mRNA level and splicing of Xbp1. Although the zygotic DmManfΔ96 mutant larvae show only low amount of persisting maternal DmManf mRNA and protein (Fig 1C and [14]), it could be sufficient to prevent the induction of UPR.

Additionally, we discovered a genetic interaction between DmManf and Xbp1, a transcription factor mainly responsible for the regulation of UPR-induced genes. Upon UPR, the mRNA of Xbp1 is spliced by IRE1 and translated into a transcriptional activator of chaperone expression in response to the increased protein folding demand [47,48] (reviewed e.g. in [49]). According to previous studies, the spliced form of Xbp1 could mediate the UPR-upregulated MANF in mammals [27,50,51]. MANF has been suggested to have protective role against ER stress [15,16,18,21,30,46]. During normal development, ER stress is detected in the secretory cells and the silencing of Xbp1 disturbs this developmental ER stress [44,52–54]. Both mammalian and Drosophila MANF has been shown to have especially high expression levels in secretory tissues [14,17,20,30]. We found that overexpression of DmManf increased Xbp1's mRNA level but the knockdown of Xbp1 did not affect DmManf expression levels. Also, the mRNA levels of Hsc3 were not upregulated in Xbp1 knockdown larvae. This could indicate the lack of transcriptional activation of DmManf and Hsc3 expression by Xbp1 in Xbp1 knockdown larvae. Therefore, knockdown of Xbp1 could compromise the regulation of DmManf expression in the developmental ER stress and deteriorate its function in the secretory cells.

ERAD is a cellular process aiming to clear out the unfolded and misfolded proteins from the ER (reviewed e.g. in [55]). According to our previous transcriptome analysis, sip3 was downregulated in DmManfΔ96 mutant larvae [20]. In this study, we also found a genetic interaction between DmManf and sip3. Sip3 encodes for a homologue to mammalian ER resident E3 ubiquitin ligase synoviolin/HRD1 with specific function in ERAD. Mammalian MANF is upregulated by ERSE-II (ER stress response element II) found in its promoter region [17]. Interestingly, ERSE-II is also found in ERAD-related components HERPUD1 (homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1, also known as HERP) [56] and VIMP (VCP-interacting membrane protein, also known as selenoprotein S) [57]. ERSE-II has been hypothesized to regulate the protein quality control and degradation of misfolded proteins during ER stress suggesting that MANF could also have a role in these functions [17].

Surprisingly, we discovered that the overexpression of DmManf led to enhanced phenotypes in flies of which a UPR-related gene was knocked down. Thus far, overexpression of DmManf with any GAL4 driver we have tested has never resulted in a detectable phenotype or altered
viability (Fig 2B, S2A Fig, [20,43]). According to our previous microarray analysis, DmManf overexpression led to downregulation of UPR-related genes [20]. This suggests that the overexpression of DmManf would disturb UPR signalling. Hypothetically, in wild type background cells would be able to deal with the increased DmManf expression and the subsequent downregulation of UPR-related genes whereas the additional knockdown of an important component of UPR, e.g. Hsc3, PEK or Xbp1, could compromise the cell homeostasis.

An alternative explanation for our observations in interaction studies between UPR genes and DmManf would be that DmManf is actually a substrate for UPR. Then, the abundant expression of DmManf by UAS/GAL4 would rather model the effects of increased overall protein synthesis in ER than indicate specific ER-related functions for DmManf. DmManf enters the secretory pathway [14] and its ectopic expression may cause stress to the protein folding machinery in the ER. Although the Xbp1s mRNA level was increased, the expression of Hsc3 was not altered indicating that overexpression of DmManf induces mild UPR. However, we did not see similar effects with overexpression of membrane-directed GFP suggesting that the observed phenomena were specific for DmManf.

In our previous microarray study, we found that the total loss of DmManf is associated with upregulated expression of genes involved in UPR [20]. However, in the current study we found that the mRNA levels of Hsc3 and Xbp1 were mildly decreased in DmManf mutant larvae. In the previous study, transcriptome analysis was done from the embryonic DmManf mutants lacking both maternal and zygotic DmManf. In the current study, we collected RNA from zygotic DmManf mutants with the persisting maternal DmManf mRNA and protein [14]. The maternal DmManf is apparently sufficient to prevent induction of UPR and upregulation of UPR related genes.

This work provides evidence for the contribution of DmManf in Drosophila UPR. Further biochemical studies on the interaction between DmManf and UPR genes in Drosophila are needed to elucidate the details of this process.

Supporting Information

S1 Fig. Additional qPCR analyses. A) In Schneider 2 (S2) cells treated with ER stress-inducing drugs thapsigargin (TG), tunicamycin (TM) and dithiothreitol (DTT) the mRNA level of Xbp1s is increased while Xbp1t remains unaltered resulting in increase of Xbp1 s:t ratio. Xbp1t, total amount of Xbp1; Xbp1s, spliced-specific transcript of Xbp1; Xbp1 s:t, proportion of Xbp1s out of Xbp1t. B) In ubiquitous PEK knockdown larvae, mRNA level of PEK was decreased. C) The Atf6 mRNA level was not altered in zygotic DmManf mutants. Ubiquitous knockdown of Atf6 showed decreased expression level of Atf6 but did not alter DmManf mRNA expression. D) In 3rd instar wandering larvae, ubiquitous DmManf overexpression did not affect Atf6 expression. KD, knockdown. Average ± standard deviation. *, P<0.05; **, P<0.01; ***, P<0.001 versus control, Student’s t-test.

(TIF)

S2 Fig. Phenotypic analyses of DmManf genetic interactors. A–C) Ubiquitous overexpression of Hsc3 does not affect fly viability. A) Overexpression of DmManf or Hsc3 with tub-GAL4 showed no phenotype in adult flies. B–C) Viability of Hsc3 overexpression pupae (B) or adults (C) was not affected by overexpression of DmManf. D–E) Ubiquitous knockdown of PEK and Xbp1 with tub-GAL4 was viable (PEK) and only partially lethal (Xbp1) at pupal stage (D). Knockdown of PEK was also partially viable at adult stage (E). With DmManf overexpression, the ubiquitous knockdown of both PEK and Xbp1 was completely lethal at larval stage. F) Knockdown of sip3 with wing driver MS1096-GAL4 results in wrinkled wing phenotype in DmManf-overexpressing background. Scale bar 1 mm (in A and F). Amount of pupae analysed...
in B–C and D–E are presented in S3 Table. Proportion of Tb+ pupae was normalized to experimentally determined proportion of Tb+ pupae (see S3 Table, wild type and wild type/SM6-TM6). OE, overexpression.

(TIF)

S1 Table. List of UAS-RNAi lines used in the study. Symbols used: Tf ID, transformant line identification; Collection, RNAi library where GD = Vienna Drosophila RNAi Center (VDRC) GD library, KK = VDRC KK library, BL = TRiP-3 collection available in Bloomington Drosophila Stock Center. According to the VDRC datasheet, the UAS-Xbp1-RNAi construct in transformant line 109312 targets both unspliced and spliced Xbp1 transcripts. (DOCX)

S2 Table. List of primer pairs used in the qPCR analysis and their PCR efficiencies (E). Hsc3 and Pek primers were designed with FlyPrimerBank (http://www.flyrnai.org/FlyPrimerBank) [58]. DmManf and RpL32 were adopted from [20], Xbp1t from [59], Xbp1s from [60] and Atf6 from [61]. (DOCX)

S3 Table. Results from ubiquitous knockdown studies of UAS-RNAi lines. tub-GAL4/TM6 Tb Sb EYFP females were crossed to UAS-x-RNAi (wild type background), UAS-x-RNAi; UAS-DmManf-OE/SM6-TM6 (+ UAS-DmManf-OE) or UAS-x-RNAi; UAS-mCD8-GFP/SM6-TM6 (+ UAS-mCD8-GFP) males. Since UAS-Hsc3-RNAiBL construct was inserted in 3rd chromosome and the insertion was lethal, UAS-Hsc3-RNAiBL/SM6-TM6 and UAS-DmManf-OE; UAS-Hsc3-RNAiBL/SM6-TM6 males were used. Columns: Tb+ and Tb-, amounts of Tb+ and Tb- pupae in crosses; Pupae, normalized proportion of Tb+ of all pupae, wild type or wild type/SM6-TM6 were used to normalize proportions; Adults, proportion of emerged adults out of Tb+ pupae. OE, overexpression; ND, not determined. n of analysed vials = 6 (except 1, n = 2 vials). (DOCX)

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Author Contributions

Conceived and designed the experiments: RL PL MP MS TIH. Performed the experiments: RL PL JK MP TIH. Analyzed the data: RL PL. Wrote the paper: RL PL JK MP MS TIH.

References

1. Ryoo HD, Steller H. Unfolded protein response in Drosophila: Why another model can make it fly. Cell Cycle. 2007; 6: 830–835. PMID: 17387279
2. Rasheva VI, Domingos PM. Cellular responses to endoplasmic reticulum stress and apoptosis. Apoptosis. 2009; 14: 996–1007. doi: 10.1007/s10495-009-0341-y PMID: 19360473
3. Dufey E, Sepúlveda D, Rojas-Rivera D, Hetz C. Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 1. An overview. Am J Physiol Cell Physiol. 2014; 307: C582–C594. doi: 10.1152/ajpcell.00258.2014 PMID: 25143348
4. Hinnebusch AG. Gene-specific translational control of the yeast GCN4 gene by phosphorylation of eukaryotic initiation factor 2. Mol Microbiol. 1993; 10: 215–223. doi: 10.1111/j.1365-2958.1993.tb01947.x PMID: 7934812
5. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell. 2000; 6: 1099–1108. PMID: 11106749

6. Plongthongkum N, Kullawong N, Panyim S, Tirasophon W. Ire1 regulated XBP1 mRNA splicing is essential for the unfolded protein response (UPR) in Drosophila melanogaster. Biochem Biophys Res Commun. 2007; 354: 789–794. doi: 10.1016/j.bbrc.2007.01.056 PMID: 17266933

7. Loewen CA, Feany MB. The unfolded protein response protects from tau neurotoxicity in vivo. PLoS ONE. 2010; 5. doi: 10.1371/journal.pone.0013084

8. Malzer E, Szajewska-Skuta M, Dalton LE, Thomas SE, Hu N, Skaer H, et al. Coordinate regulation of eif2a phosphorylation by PPP1R15 and GCN2 is required during Drosophila development. J Cell Sci. 2013; 126: 1406–1415. doi:10.1242/jcs.117614 PMID: 23418347

9. Sekine SU, Haraguchi S, Chao K, Kato T, Luo L, Miura M, et al. Meigo governs dendrite targeting specificity by modulating Ephrin level and N-glycosylation. Nat Neurosci. 2013; 16: 683–691. doi:10.1038/nn.3389 PMID: 23624514

10. Petrova PS, Raibekas A, Pevsner J, Vigo N, Anafi M, Moore MK, et al. MANF: A new mesencephalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. J Mol Neurosci. 2003; 20: 173–187. doi: 10.1385/JMN:20:2:173 PMID: 12794311

11. Lindholm P, Voutilainen MH, Laurén J, Peränen J, Leppäläinen V-M, Andressoo J-O, et al. Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. Nature. 2007; 448: 73–77. doi: 10.1038/nature05957 PMID: 17611540

12. Voutilainen MH, Bäck S, Pörsti E, Toppinen L, Lindgren L, Lindholm P, et al. Mesencephalic astrocyte-derived neurotrophic factor is neurorestorative in rat model of Parkinson’s disease. J Neurosci. 2009; 29: 9651–9659. doi: 10.1523/JNEUROSCI.0833-09.2009 PMID: 19641128

13. Airavaara M, Harvey BK, Voutilainen MH, Shen H, Chou J, Lindholm P, et al. CDNF Protects the Nigrostriatal Dopamine System and Promotes Recovery After MPTP Treatment in Mice. Cell Transplant. 2012; 21: 1213–1223. doi: 10.3727/096368911X600948 PMID: 21943517

14. Palgi M, Lindström R, Peränen J, Piepponen TP, Saarma M, Heino TI. Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. Proc Natl Acad Sci U S A. 2009; 106: 2429–2434. doi: 10.1073/pnas.0810996106 PMID: 19164766

15. Airavaara M, Chiocco MJ, Howard DB, Zuchowski KL, Peränen J, Liu C, et al. Widespread cortical expression of MANF by AAV serotype 7: Localization and protection against ischemic brain injury. Exp Neurol. 2010; 225: 104–113. doi: 10.1016/j.expneurol.2010.05.020 PMID: 20685313

16. Glembotski CC, Thuerauf DJ, Huang C, Vekich JA, Gottlieb RA, Doroudgar S. Mesencephalic astrocyte-derived neurotrophic factor protects the heart from ischemic damage and is selectively secreted upon sarco/endoplasmic reticulum calcium depletion. J Biol Chem. 2012; 287: 25893–25904. doi: 10.1074/jbc.M112.356345 PMID: 22637475

17. Mizobuchi N, Hoseki J, Kubota H, Toyokuni S, Nozaki J-I, Naitoh M, et al. ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element. Cell Struct Funct. 2007; 32: 41–50. doi: 10.1247/csf.07001 PMID: 17507765

18. Apostolou A, Shen Y, Liang Y, Luo J, Fang S. Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. Exp Cell Res. 2008; 314: 2454–2467. doi: 10.1016/j.yexcr.2008.05.001 PMID: 18561914

19. Tadimalla A, Belmont PJ, Thuerauf DJ, Glassy MS, Martindale JJ, Gude N, et al. MANF is Indispensable for the Proliferation and Survival of Pancreatic β Cells. Cell Rep. 2014; 7: 366–375. doi: 10.1016/j.celrep.2014.03.023 PMID: 24726366

20. Lindholm P, Voutilainen MH, Laurén J, Peränen J, Leppäläinen V-M, Andressoo J-O, et al. Characterization of the Structural and Functional Determinants of MANF/CDNF in Drosophila In Vivo Model. PLoS ONE. 2013; 8. doi: 10.1371/journal.pone.0073928

21. Henderson MJ, Richie CT, Airavaara M, Wang Y, Harvey BK. Mesencephalic astrocyte-derived neurotrophic factor (MANF) secretion and cell surface binding are modulated by KDEL receptors. J Biol Chem. 2013; 288: 4209–4225. PMID: 23255601
24. Oh-hashi K, Norisada J, Hirata Y, Kiuchi K. Characterization of the role of MANF in regulating the secretion of CRELD2. Biol Pharm Bull. 2015; 38: 722–731. doi: 10.1248/bpb.b14-00825 PMID: 25947918
25. Girardot F, Monnier V, Tricoire H. Genome-wide analysis of common and specific stress responses in adult Drosophila melanogaster. BMC Genomics. 2004; 5. doi: 10.1186/1471-2164-5-74
26. Nundlall S, Rajpar MH, Bell PA, Clowes C, Zeeff LAH, Gardner B, et al. An unfolded protein response is the initial cellular response to the expression of mutant matrilin-3 in a mouse model of multiple epiphyseal dysplasia. Cell Stress Chaperones. 2010; 15: 835–849. doi: 10.1007/s12192-010-0193-y PMID: 20428984
27. Oh-Hashi K, Hirata Y, Kiuchi K. Transcriptional regulation of mouse mesencephalic astrocyte-derived neurotrophic factor in Neuro2a cells. Cell Mol Biol Lett. 2013; 18: 398–415. doi: 10.2478/s11658-013-0096-x PMID: 23864333
28. DeGraciac DJ, Montie HL. Cerebral ischemia and the unfolded protein response. J Neurochem. 2004; 91: 1–8. doi: 10.1111/j.1471-4159.2004.02703.x PMID: 15379881
29. Holtz WA, O’Malley KL. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. J Biol Chem. 2003; 278: 19367–19377. doi: 10.1074/jbc.M211821200 PMID: 12598533
30. Lindholm P, Peränen J, Andressoo J-O, Kalkkinen N, Kokaia Z, Lindvall O, et al. MANF is widely expressed in mammalian tissues and differentially regulated after ischemic and epileptic insults in rodent brain. Mol Cell Neurosci. 2008; 39: 356–371. doi: 10.1016/j.mcn.2008.07.016 PMID: 18718866
31. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 1993; 118: 401–415. PMID: 8223268
32. Wodarz A, Hinz U, Engelbert M, Knust E. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of drosophila. Cell. 1995; 82: 67–76. PMID: 7606787
33. Capdevila J, Guerrero I. Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. EMBO J. 1994; 13: 4459–4468. PMID: 7925288
34. Lee T, Luo L. Mosaic analysis with a repressible neurotechnique cell marker for studies of gene function in neuronal morphogenesis. Neuron. 1999; 22: 451–461. PMID: 10197526
35. Elefant F, Palter KB. Tissue-specific expression of dominant negative mutant Drosophila HSC70 causes developmental defects and lethality. Mol Biol Cell. 1999; 10: 2101–2117. PMID: 10397752
36. Ni J-Q, Liu L-P, Binari R, Hardy R, Shim H-S, Cavallaro A, et al. A Drosophila resource of transgenic RNAi lines for neurogenetics. Genetics. 2009; 182: 1089–1100. doi: 10.1534/genetics.109.103630 PMID: 19487563
37. Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, et al. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature. 2007; 448: 151–156. doi: 10.1038/ nature05954 PMID: 17625586
38. McQuilton P, St Pierre SE, Thurmond J, Gelbart W, Brown N, Kaufman T, et al. FlyBase 101—the basics of navigating FlyBase. Nucleic Acids Res. 2012; 40: D706–D714. doi: 10.1093/nar/gkr1030 PMID: 22127867
39. Jämsä E, Simonen M, Makarow M. Selective retention of secretory proteins in the yeast endoplasmic reticulum by treatment of cells with a reducing agent. Yeast. 1994; 10: 355–370. PMID: 8017105
40. Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2+-ATPase. Proc Natl Acad Sci U S A. 1990; 87: 2466–2470. PMID: 2138778
41. Olden K, Pratt RM, Jaworski C, Yamada KM. Evidence for role of glycoprotein carbohydrates in membrane transport: Specific inhibition by tunicamycin. Proc Natl Acad Sci U S A. 1979; 76: 791–795. PMID: 218220
42. Neumann CJ, Cohen SM. Distinct mitogenic and cell fate specification functions of wingless in different regions of the wing. Development. 1996; 122: 1761–1789. PMID: 8674417
43. Stratoulias V, Heino T. Analysis of the conserved neurotrophic factor MANF in the Drosophila adult brain. Gene Expr Patterns. 2015; 18: 8–15. doi: 10.1016/j.gep.2015.04.002 PMID: 25917377
44. Ryoo HD, Li J, Kang M-J. Drosophila XBP1 Expression Reporter Marks Cells under Endoplasmic Reticulum Stress and with High Protein Secretory Load. PLoS ONE. 2013; 8. doi: 10.1371/journal.pone.0075774
45. Wang S, Kaufman RJ. The impact of the unfolded protein response on human disease. J Cell Biol. 2012; 197: 857–867. doi: 10.1083/jcb.201110131 PMID: 22733998
46. Hartley CL, Edwards S, Mullan L, Bell PA, Fresquet M, Boot-Handford RP, et al. Armet/Manf and Creld2 are components of a specialized ER stress response provoked by inappropriate formation of
47. Rueggsegger U, Leber JH, Walter P. Block of HAC1 mRNA translation by long-range base pairing is released by cytoplasmic splicing upon induction of the unfolded protein response. Cell. 2001; 107: 103–114. doi: 10.1016/S0092-8674(01)00505-0 PMID: 11595189

48. Ryoo HD, Domingos PM, Kang M-J, Steller H. Unfolded protein response in a Drosophila model for retinal degeneration. EMBO J. 2007; 26: 242–252. doi: 10.1038/sj.emboj.7601477 PMID: 17170705

49. Patil C, Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: The unfolded protein response in yeast and mammals. Curr Opin Cell Biol. 2001; 13: 349–356. doi: 10.1016/S0955-0674(00)00219-2 PMID: 11343907

50. Lee A-H, Iwakoshi NN, Glimcher LH. XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response. Mol Cell Biol. 2003; 23: 7448–7459. doi: 10.1128/MCB.23.21.7448-7459.2003 PMID: 14559994

51. Yang S, Huang S, Gaertig MA, Li X-J, Li S. Age-dependent decrease in chaperone activity impairs MANF expression, leading to Purkinje Cell degeneration in inducible SCA17 Mice. Neuron. 2014; 81: 349–365. doi: 10.1016/j.neuron.2013.12.002 PMID: 24462098

52. Iwawaki T, Akai R, Kohno K, Miura M. A transgenic mouse model for monitoring endoplasmic reticulum stress. Nat Med. 2004; 10: 98–102. doi: 10.1038/nm970 PMID: 14702639

53. Souid S, Lepesant J-A, Yanicostas C. The xbp-1 gene is essential for development in Drosophila. Dev Genes Evol. 2007; 217: 159–167. doi: 10.1007/s00427-006-0124-1 PMID: 17206451

54. Sone M, Zeng X, Larese J, Ryoo HD. A modified UPR stress sensing system reveals a novel tissue distribution of IRE1/XBP1 activity during normal Drosophila development. Cell Stress Chaperones. 2013; 18: 307–319. doi: 10.1007/s12192-012-0383-x PMID: 23160805

55. Stolz A, Wolf DH. Endoplasmic reticulum associated protein degradation: A chaperone assisted journey to hell. Biochim Biophys Acta Mol Cell Res. 2010; 1803: 694–705. doi: 10.1016/j.bbamcr.2010.02.005

56. Kokame K, Kato H, Miyata T. Identification of ERSE-II, a New cis-Acting Element Responsible for the ATF6-dependent Mammalian Unfolded Protein Response. J Biol Chem. 2001; 276: 9199–9205. doi: 10.1074/jbc.M010486200 PMID: 11112790

57. Schulze A, Standera S, Buenger E, Kikkert M, Van Voorden S, Wiertz E, et al. The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. J Mol Biol. 2005; 354: 1021–1027. doi: 10.1016/j.jmb.2005.06.020 PMID: 16289116

58. Hu Y, Sopko R, Foos M, Kelley C, Flockhart I, Ammeux N, et al. Fly Primer Bank: an online database for Drosophila melanogaster gene expression analysis and knockdown evaluation of RNAi reagents. G3 (Bethesda). 2013; 3: 1607–1616.

59. Benosman S, Ravanan P, Correa RG, Hou Y-C, Yu M, Gulen MF, et al. Interleukin-1 Receptor-Associated Kinase-2 (IRAK2) Is a Critical Mediator of Endoplasmic Reticulum (ER) Stress Signaling. PLoS ONE. 2013; 8. doi: 10.1371/journal.pone.0064256

60. Maor G, Rencus-Lazar S, Filocamo M, Steller H, Segal D, Horowitz M. Unfolded protein response in Gaucher disease: From human to Drosophila. Orphanet J Rare Dis. 2013; 8. doi: 10.1186/1750-1172-8-140

61. Kim A-Y, Seo JB, Kim W, Choi HJ, Kim S-Y, Morrow G, et al. The pathogenic human Torsin A in Drosophila activates the unfolded protein response and increases susceptibility to oxidative stress. BMC Genomics. 2015; 338. doi: 10.1186/s12864-015-1518-0