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The *xbp-1* gene is essential for development in *Drosophila*

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Abstract

We report here the characterisation of *Dxbp-1,* the *Drosophila* homologue of the *xpb-1* gene, which encodes a "bZIP"-containing transcription factor that plays a key role in the Unfolded Protein Response (UPR), an evolutionarily conserved signalling pathway activated by an overload of misfolded proteins in the endoplasmic reticulum (ER). *Dxbp-1* is ubiquitously transcribed, and high levels are found in embryonic salivary glands and in the ovarian follicle cells committed to the synthesis of the respiratory appendages. Loss-of-function of *Dxbp-1* induced a recessive larval lethality, thus revealing an essential requirement for this gene. The *Dxbp-1* transcript was submitted to an "unconventional" splicing, which generated a processed *Dxbp-1s* transcript encoding a DXbp-1 protein isoform, as is the case for yeast, *Caenorhabditis elegans* and vertebrate *hac1/xbp-1* transcripts following UPR activation. However, in the absence of exogenously induced ER stress, the *Dxbp-1s* transcript was also detectable not only throughout embryonic and larval development, but also in adults with a high level of accumulation in the male sexual apparatus and, to a lesser extent, in the salivary glands of third instar larvae. Using a *Dxbp-1:GFP* transgene as an *in vivo* reporter for *Dxbp-1* mRNA unconventional splicing, we confirmed that *Dxbp-1* processing took place in the salivary glands of third instar larvae.

The *Dxbp-1* gene appears thus to play an essential role during the development of *Drosophila*, hypothetically by stimulating the folding capacities of the ER in cells committed to intense secretory activities.

Key words: *Drosophila*, *xbp-1*, unconventional splicing, UPR signalling, secretion

Introduction

The X-box binding protein-1 (Xbp-1) was first characterized as a basic domain-Leucine Zipper ("bZIP") containing transcription factor that binds to the *cis*-acting X box sequence present in the promoter region of genes of the human major histocompatibility complex class II (Liou et al., 1990), and in the *tax*-dependent enhancer of HTLV1 (Yoshimura et al., 1990). Mammalian and *Caenorhabditis elegans xbp-1* genes were further shown to be the orthologues of the yeast *hac1* gene, a key player in the Unfolded Protein Response (UPR) (Cox and Walter, 1996; Mori et al., 2000; Yoshida et al., 2001; Shen et al., 2001, for a review see Schroder and Kaufman, 2005). UPR is an evolutionarily conserved signalling pathway that is activated by an over-accumulation of either misfolded or unfolded proteins in the Endoplasmic Reticulum (ER) lumen as the result of mutations, drug treatments or environmental stresses. UPR activation elicits several adaptative responses to maintain ER function, in particular by restricting the import of novel proteins and by stimulating the degradation of abnormal proteins accumulated in the ER lumen. To restore ER function, UPR activation induces: (1) an up regulation of genes encoding ER chaperones, folding enzymes, and proteins involved in the ER-associated degradation (ERAD) system and (2) an inactivation of eIF4α mediated by PEK/PERK kinase, resulting in a global inhibition of the translational machinery to limit the import of novel proteins (for a review see Schroder and Kaufman, 2005). However, when these two processes are not sufficient to restore ER homeostasis, UPR activation eventually commits cells to apoptosis through activation of the Jun-N-Term-Kinase (JNK) pathway (Urano et al., 2000b; Nakagawa et al., 2000; Rao et al., 2001). Xbp-1 and ATF-6 are two transcription factors containing a "bZIP" domain, and key players in the UPR (Yamamoto et al., 2004). ATF6 is a transmembrane ER protein, which is comprised of a lumenal sensing domain and a

cytosolic "bZIP" containing region (Haze et al., 1999; Lee et al., 2002; Shen et al., 2005). In an early stage of UPR signalling, ATF-6 is activated by S2P-mediated cleavage of its cytosolic domain, which is then transported to the nucleus (Yoshida et al., 1998; Ye et al., 2000; Lee et al., 2002, Shen et al., 2005). Activation of Xbp-1 relies upon a completely different, and, as far as we know, specific post-transcriptional mechanism. The native unprocessed *xbp-1* mRNA, *xbp-1u*, encodes a transcription factor, Xbp-1u, which displays poor transcriptional activities and low stability (Yoshida et al., 2006; Tirosh et al., 2006). It has been shown that, following ER stress and UPR activation, the *xbp-1u* mRNA is cleaved by the site-specific endoribonuclease Ire-1 at two sites separated by 26 nucleotides (nt) (in vertebrates) or 23 nt (in *C. elegans*), and then re-ligated by a tRNA ligase (Sidrauski et al., 1997; Yoshida et al., 2001; Lee et al., 2002). This unconventional "splicing" of 23 (or 26) nt takes place approximately in the middle of the Xbp-1u ORF. This induces a frame-shift in the resulting processed transcript, *xbp-1s,* which encodes a novel protein that displays the same N-terminal region as Xbp-1u, including the "bZIP" domain, but a distinct C-terminal region that contains a potent trans-activation domain (Yoshida et al., 2001). In the nucleus, activated ATF6 and Xbp-1s bind to the UPR (UPRE) and ER stress (ERSE) response elements found in the promoter region of genes encoding ER resident proteins such as chaperone proteins, folding enzymes and ERAD proteins, and activate their transcription (Yoshida et al., 1998; Lee et al., 2003; Yamamoto et al., 2004).

In *C. elegans*, *xbp-1* RNAi-depleted worms are highly sensitive to ER stress, but these mutants are viable under physiological conditions, thus showing that this gene does not play an essential role during development (Shen et al., 2001). Nevertheless, *pek-1(ok275); xbp-1(RNAi)* double mutant worms arrest their development at or prior to the L2 larval stage, suggesting that the two genes mediate redundant pathways that are essential for survival. In contrast, Reimold et al. (2000) and Zhao et al. (2003) have shown that the functions of vertebrate *xbp-1* are crucial during development and that it is essential for the viability of embryos both in mice and frogs. In mouse, *xbp-1* is required for cardiac myocyte survival (Masaki et al., 1999), liver development (Reimold et al., 2000), skeletal formation and terminal differentiation of immature plasma cells into immunoglobulin secreting B-lymphocytes (Iwakoshi et al., 2003a; 2003b, for a review see Wu and Kaufman, 2006). In addition, these data suggest that *xbp-1* does not play a crucial role during early embryonic development in mammals. On the other hand, in *Xenopus*, *xbp-1* is essential during the early stages of embryo development. It forms a regulatory loop with BMP-4, which is required for mesoderm and neuroderm differentiation (Zhao et al., 2003; Cao et al., 2006). All together, these data suggest that *xbp-1*

has been recruited during vertebrate evolution to play several essential roles during development, particularly in cells committed to intense secretory activities in mammals and in the BMP-4 pathway in frogs.

Here, we report the first study of the function of *Dxbp-1,* the *Drosophila xbp-1* orthologue. Our results show that, like in vertebrates, this gene plays an essential role during development. We observed that, in the absence of exogenously induced ER stress, the unconventional splicing of *Dxbp-1* takes place throughout development and in adult tissues, particularly in secretory organs such as the larval salivary glands and the male accessory glands. Moreover, using an *UAS-Dxbp-1:GFP* transgene as a reporter for *Dxbp*-1 processing, we confirmed that putative Ire-1 dependent unconventional splicing took place in the salivary glands of third instar larvae. All together, our data support the hypothesis that, as in mammals, *Dxbp-1* plays an essential role during the development of Drosophila, particularly in tissues that show high levels of secretory activity.

Materials and methods

Drosophila stocks

Drosophila melanogaster stocks were raised on a standard cornmeal, yeast, agar medium at 25°C. The *Dxbp-1^{k13803}*, *Df(2)Exel^{6042}*, *Df(2)^{CC2}, da-Gal4, en-Gal4* and *puc^{e69}-lacZ* lines are described in FlyBase and relevant fly stocks are available from the Bloomington Stock Centre at Indiana University. The *pDI:GFP* protein trap line and the *lio-Gal4* driver line were kindly provided by Alain Debec (Bobinnec et al., 2003) and Jean-Maurice Dura (Taillebourg et al., 2005), respectively. *Dxbp-1^{k13803}* has been shown to contain an insertion of the *P[w^+; lacZ]* enhancer trap transposon (Bier et al., 1989) in the 5'-UTR region of the *Drosophila xbp-1* gene. In all experiments, the *w^{1118}* stock was used as a wild-type control.

UAS-Dxbp-1:GFP construct

In the *UAS-Dxbp-1:GFP* construct, a genomic DNA fragment corresponding to the 5' region of the *Dxbp-1* gene and extending 488 bp downstream from the unconventional intron and thus containing the entire *Dxbp-1u* ORF, was amplified by PCR with additional 5' BamHI and 3' KpnI restriction sites and cloned as an XbaI-KpnI fragment into the *pUAS_P-GFP* vector (Januschke et al., 2002) in frame with the ORF of the *Dxbp-1u* RNA. Transcription of the *UAS-Dxbp-1:GFP* construct, which was verified by sequencing, was predicted to encode a fluorescent DXbp-1s:GFP protein after removal of the 23 bp intron.

We generated *UAS-Dxbp-1:GFP* transgenic lines as previously described, using a *w^{1118}* strain as a recipient stock (Rubin and Spradling, 1982).

RNA in situ *hybridisation*

For *in situ* hybridisation, RNA probes were synthesized with T7 or T3 RNA polymerase and a *Dxbp-1* fragment as template, extending from position + 511 to + 1380. We amplified this DNA fragment by PCR, using two pairs of primers designed to include either a T7 promoter sequence within the 3' backward oligonucleotide or a T3 promoter within the 5' forward primer.

Total and polyA$^+$ RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen) and polyA$^+$ RNA was further purified using the Oligotex mRNA kit (Qiagen). For RT-PCR experiments, 10 μg of total RNA or 1 mg of polyA$^+$ RNA were reverse transcribed with random hexamers and the First Strand cDNA Synthesis Kit for RT-PCR (Roche). To visualise the processing of the 23 bp intron, we carried out PCR amplifications of the newly synthesized cDNA templates using the forward primer 5'-CAGATGCATCAGCCAATCCAAC-3' and the backward primer 5-GAGTGAGACCTTTCAACAC-3', which were predicted to amplify fragments of 191 bp (*Dxbp-1u* RNA) or 168 bp (the *Dxbp-1s* RNA). PCR products were separated by electrophoresis in 4% Metaphor (FMC) gels. All RNA samples were tested for a possible contamination by genomic DNA by RT-PCR by using a couple of primers flanking the 64 bp conventional intron common to both *Dxbp-1* RNA transcripts.

Results

1°/ Structure of the *Dxbp-1* gene and transcripts

The structure of the *Drosophila xbp-1* gene was first deduced from the annotated *Drosophila* genome sequence determined by the Berkley *Drosophila* genome Project (release 3.2 of the annotated *Drosophila melanogaster* genome) and from the partial or complete sequencing of numerous EST clones carried out by the Berkley *Drosophila* Genome Project. However, the predicted structure of *Dxbp-1* transcripts did not fit with the sequence of several EST clones. The annotated *Drosophila* genome sequence predicted two *Dxbp-1* RNA species, *Dxbp1-RA* and *Dxbp1-RB*, as a result of the alternative splicing of a 53 nucleotides (nt) intron in *Dxbp1-RB*. Although sequencing data from 16 EST clones confirmed the structure of the *Dxbp1-RA* transcript, none of the sequenced EST corresponded to the predicted *Dxbp1-RB* transcript isoform. Three EST corresponded to an alternatively spliced transcript distinct from the predicted *Dxbp1-RB* by the splicing of 23 nt instead of 53 (Fig. 1a). Because of this inconsistency, we sequenced a set of PCR fragments encompassing the complete *Dxbp-1* genomic region and we performed RT-PCR experiments using several couples of primers flanking the predicted or observed introns and RNA extracted from various tissues or at various developmental stages. The amplified RT-PCR products were also sequenced to determine intron lengths and sequences with precision.

Our data confirmed that the *Dxbp-1* gene gave rise to the transcription of two RNA isoforms. The major transcript, which corresponded to the predicted *Dxbp1-RA* RNA, was composed of two exons, 591 and 1552 nt in length, separated by a 64 nt intron flanked by canonical 5', GUAAGU, and 3', CAG, consensus sequences. The predicted translation product of this transcript was a 307 aa protein, whose amino-terminal region contained a "bZIP" domain, a 35 aa region rich in basic amino acids followed by 7 evenly spaced Leucine residues forming a "Leucine zipper" dimerisation domain (see Fig. s1 and Fig. 1a).

The second *Dxbp-1* RNA differed from the major transcript only by the absence of a 23 nt sequence located 304 nt downstream from the common 64 nt intron. The predicted 498 aa protein encoded by this transcript displayed the same 188 aa long amino-terminal region as the protein encoded by the unprocessed *Dxbp1-RA* transcript, but showed a distinct carboxy-terminal domain because of the frame shift induced by the splicing of this 23 nt intron (see Fig. s1 and Fig. 1a). This 23 nt intron was not flanked by canonical splice site consensus sequences and visual and *in silico* analyses revealed the presence of complex secondary structures in the flanking sequences. These included a long stem and two evolutionarily conserved stem-loop structures showing strong structure and sequence similarities with the Ire-1 endoribonuclease cleavage sites identified in worms and vertebrates (see Fig. s2). Thus, although the *Dxbp-1* gene did not display any significant sequence similarities with the fish, mammal or worm *xbp-1* genes outside the region coding for the basic domain (64% identity and 80% similarity) (Fig. 1c), the presence of these conserved features indicates that this gene is the *Drosophila xbp-1* orthologue. These data also suggested that the *Dxbp-1* transcript was submitted to an Ire-1-mediated unconventional "splicing" similar to that of the yeast *hac1* and worm and vertebrate *xbp-1* transcripts following UPR signalling. In agreement with the nomenclature used for worms and vertebrates, we will refer to *Dxbp-1u* as the unprocessed transcript and to *Dxbp-1s* as the spliced *Dxbp-1* transcript.

We showed that the level of *Dxbp-1s* was increased in flies fed for 24 hours with tunicamycine, a drug that inhibits protein glycosylation and folding and consequently induces UPR signalling (see Fig. s3). Because we also detected *Dxbp-1s* in the absence of exogenously induced ER stress (see Fig. s3), we determined the stage and tissue specificities of the two *Dxbp-1* transcripts by RT-PCR, using either total or polyA$^+$ RNA extracted from embryos at various developmental stages, first, second and third instar larvae, dissected third instar larval salivary glands and adult ovaries and testes. Both transcripts were detected in all samples analyzed, but the levels of accumulation

of the *xbp-1s* transcript compared to those of the *xbp-1u* form differed in the various stages and tissues analyzed. While levels of *Dxbp-1s* were low or barely detectable when compared to those of *Dxbp-1u* in embryos and in ovaries, its relative accumulation was significantly increased in the salivary glands of third instar larvae and, even more so, in the sexual apparatus of adult males (Fig. 1b).

2°/ *Dxbp-1* has an essential function during development

The *l(2)^{k13803}* line (Torok et al., 1993) has been shown to contain an insertion of the *P[w; lacZ]* enhancer-trap transposon (Bier et al., 1989) in the 5'-UTR region of the *Dxbp-1* gene (Fig. 1a). We used PCR experiments to confirm that this line contains an insertion of the *P[w; lacZ]* transposon at position + 156 respective to the predicted start site of transcription of the *Dxbp-1* gene. The lack of complementation between *l(2)^{k13803}* and *Df(2)CC_2* or *Df(2)Exel_{6042}*, two deficiencies that delete *Dxbp-1,* was a first indication that the recessive lethal phenotype in line *l(2)^{k13803}* was due to the *P[w; lacZ]* insertion in the gene. Then, we generated 100 *l(2)^{k13803}* ; *w^-* revertant lines following *D[2-3]* transposase-induced mobilisation of the transposon and we isolated 43 homozygous viable and fertile lines. In all these lines, we mapped the excision event by PCR analysis and subsequent sequencing of the amplified fragments. Precise excision of the transposon was detected in 28 of the 43 lines, therefore demonstrating: (1) that the recessive lethality of line *l(2)^{k13803}* was due to the insertion of the *P[w; lacZ]* transposon leading to a mutation of the *Dxbp-1* gene and (2) that line *l(2)^{k13803}* did not contain a second recessive lethal or sterile mutation. We therefore refer to this mutation as *Dxbp-1^{k13803}*.

We observed that 95% of *Dxbp-1^{k13803}* homozygous animals hatched and, at that time, were indistinguishable from control. However, as individuals aged, they remained at the first instar stage, became sluggish and sick before dying after two to four days. A very similar phenotype was observed in hemizygous *Dxbp-1^{k13803}* individuals obtained with either *Df(2)CC_2* or *Df(2)Exel_{6042}*, showing that *Dxbp-1^{k13803}* is either an amorphic or a strong hypomorphic mutation.

All together, these data suggest that the *Dxbp-1* gene has an essential function during development.

3°/ Detection *in situ* of *xbp-1* transcripts during oogenesis and embryogenesis

The pattern of accumulation of *Dxbp-1* mRNA during oogenesis and embryogenesis was established by whole-mount *in situ* hybridisation. Data showed that *Dxbp-1* transcripts accumulated almost ubiquitously throughout embryogenesis and oogenesis (data not shown). However, we detected significantly higher levels of *Dxbp-1* RNAs superimposed on this ubiquitous pattern of expression: (1) during oogenesis, in follicle cells that are committed to the synthesis of the respiratory appendages (Fig. 2a), (2) during embryogenesis, in the labial segment of stage 11 embryos (Fig. 2b) and (3) in the salivary glands of stages 14 to 17 embryos (Fig. 2c). In addition, a segmented pattern of *Dxbp-1* transcript accumulation was detectable in stage 10-11 embryos (Fig. 2b).

4°/ Unconventional splicing of *Dxbp-1s* occurs in salivary glands of third instar larvae

Because the structure of the two *Dxbp-1* transcripts made it impossible to design a *Dxbp-1s*-specific probe to establish the pattern of accumulation of this RNA isoform by *in situ* hybridisation, we constructed an *UAS-Dxbp-1:GFP* transgene as a tool to visualise *Dxbp-1* unconventional splicing. In this construct, a genomic fragment encompassing the 5'-UTR of the *Dxbp-1* gene, the entire *Dxbp-1u* ORF, including the unconventional intron and 488 bp of 3' downstream sequences, was cloned upstream from the reporter GFP gene in the *pUASp:GFP* vector (Januschke et al., 2002). The construct was designed to reveal unconventional splicing through the production of a fluorescent DXbp-1s:GFP fusion protein following the splicing of the 23 nt intron (see Fig. 3a and Materials and Methods). A similar transgenic model has been designed in the mouse for monitoring endoplasmic reticulum stress (Iwawaki et al., 2004). We first tested various ubiquitous Gal4 drivers, such as *act5C-Gal4, arm-Gal4* or *da-Gal4* as well as more specific drivers, such as *en-Gal4*. However, none of these drivers allowed us to detect fluorescent cells at any stage, even in larvae fed with drugs known to induce the UPR pathway such as tunicamycine, DTT or thapsigargin. Because the *Dxbp-1s* transcript appeared to accumulate at a relatively high level in salivary glands of third instar larvae (Fig. 1b), we analysed the expression of the *UAS-Dxbp-1:GFP* construct using the *lio-Gal4* driver line (Taillebourg et al., 2005), which leads to high levels of expression of the Gal4 transcription factor in the salivary glands of third instar larvae. In *lio-Gal4; UAS-Dxbp-1:GFP* third instar larvae, we observed a strong GFP fluorescent staining in the salivary glands of third instar larvae (Fig. 3b). Consistent with the fact that the DXbp-1u and DXbp-1s proteins are transcription factors, this GFP staining was restricted to salivary gland nuclei. Moreover, an increase in the GFP staining was observed in larvae fed for 24 hours with tunicamycine. This demonstrates that putative Ire-1-dependent splicing does indeed occur in salivary glands (Fig. 4). These results, which are also consistent with our RT-PCR data, confirmed that unconventional splicing of *Dxbp-1* occurred at relatively high levels in salivary glands of third instar larvae.

Discussion

In this study, we have analyzed the structure, expression pattern and requirement during development of a novel *Drosophila* gene encoding a "bZIP" protein. This gene was identified as being the *Drosophila* orthologue of the *xbp-1* gene, as deduced from: (1) strong sequence similarities of the basic domain of the encoded protein with that of vertebrate and nematode Xbp-1 protein; (2) conservation of both structure and sequences of putative Ire-1 cleavage sites in the *Dxbp-1* transcript; (3) unconventional splicing of a 23 nt intron, which so far has been detected in *xbp-1* transcripts only.

The UPR pathway and its major components such as the *per/perk*, *ire-1* and *hac1/xbp-1* genes have been conserved during evolution from yeast to mammals (for a review see Ma and Hendershot, 2001), but in higher eukaryotes the *xbp-1* gene has also been shown to possess functions that are essential during development, independently from pathological or drug-induced ER stresses (for a review see Wu and Kaufman, 2006).

1°/ Conserved requirement for *xbp-1* in secretory cells

In mammals, the first indication of a possible link between the *xbp-1* gene and secretory activities was provided by an *in situ* hybridisation analysis that revealed an abundant transcription of the gene in exocrine glands and in osteoblasts during skeleton formation (Clauss et al., 1993). It was further shown that, at later stages, *xbp-1* is required for terminal differentiation of plasma cells into antibody-secreting B cells (Reimold et al., 2001; Gass et al., 2002; Iwakoshi et al., 2003a; 2003b). Indeed, while *xbp-1⁻/⁻* hematopoietic cells underwent normal B-cell activation, germinal centre differentiation and class-switch recombination, they failed to secrete immunoglobulins, which is the final step in B-cell differentiation (Reimold et al., 2001). In addition, an increased accumulation of *xbp-1s* mRNA has been observed upon induction of the differentiation of the mouse B cell lymphoma CH12 cells into antibody-secreting B cells by lipopolysaccharide (LPS). The level of *xbp-1s* transcript started to increase after 8-12 h of stimulation and then peaked at 32 h, while a sharp increase in the accumulation of Xbp-1s protein was detected after 24 h of stimulation, just prior to an increased translation of Ig chains (Gass et al., 2002). These data seem to corroborate the hypothesis that *xbp-1* plays a key role during the very last stage of lymphocyte B-cell differentiation, that is, Ig secretion (Gass et al., 2002). More recently, Lee et al. (2005) designed *xbp-1⁻/⁻* mouse embryos whose early lethality, which is due to severe impairment of hepatocyte development (Reimold et al., 2000), was rescued by targeting expression of an *xbp-1* transgene in liver cells. These mice showed defects that were restricted to secretory organs such as exocrine pancreas and salivary glands, and they died at an early post-natal stage due to a lack of pancreatic digestive enzymes (Lee et al., 2005). These data all argue in favour of the postulate that *xbp-1* plays a key role in secretory organs in mammals.

Our results also suggest that *Dxbp-1* plays an important role in cells showing an intense secretory activity in *Drosophila*. Firstly, *in situ* hybridisation data revealed that this gene is expressed in two groups of 50 follicle cells that will secrete in less than two hours the large quantity of proteins required for the synthesis of the two respiratory appendages at late stages of oogenesis. However, because it is not possible to design a *Dxbp-1s*-specific probe to detect *Dxbp-1s* by *in situ* hybridisation, we were not able to establish which of the *Dxbp-1* mRNA isoforms, *Dxbp-1u* or *Dxbp-1s*, had accumulated in these follicle cells. Secondly, our RT-PCR experiments revealed that the *Dxbp-1s* transcript is comparatively more abundant in two types of tissue: (1) the paragonia, which are two accessory glands of the male sexual apparatus that secrete a large quantity of proteins, which are transferred with the sperm during copulation and, (2) to a lesser extent, in the salivary glands of third instar larvae. Thirdly, in agreement with this latter observation, we have confirmed with the *Dxbp-1:GFP* transgene used to detect *Dxbp-1* unconventional splicing, that significant levels of presumptive Ire-1-dependent processing of the *Dxbp-1* transcript take place in the salivary glands of third instar larvae, a tissue which is committed to the synthesis of large quantities of glue proteins right at the end of the third larval instar. These data suggest that, like the mouse gene, the *Dxbp-1* gene is active in secretory cells.

Because secretory cells display intense translational and post-translational activities such as covalent modifications and protein folding, their requirement for an active Xbp-1 protein likely reflects their important needs in ER resident proteins, such as chaperones proteins and modifying enzymes, which are encoded by genes directly activated by the Xbp-1s protein. In this context, it would be interesting to determine whether ATF-6, the second "bZIP" transcription factor of the UPR pathway, also plays a role in secretory cells. It could be proposed that this "physiological" aspect of the UPR pathway, i.e. the maintenance of ER homeostasis in secretory cells, is the ancestral function of the *xbp-1/hac1* and *ire-1* genes.

2°/ *xbp-1* plays an essential role during development

During the early stages of *Xenopus* development, both the xXBP-1 protein, and the BMP-4 protein are components of a regulatory loop that is essential for mesoderm and neuroderm differentiation (Zhao et al., 2003; Cao et al., 2006). During this process, xXBP-1 acts both as an activator of *BMP-4* and a repressor of *Xvent-2* genes through direct binding to their promoter sequences. This dual activity

of xXBP-1 depends upon its association with various co-factors such as c-Jun, p300 and Smad1 to form transcriptional activator complexes, or with Smad6 and Smad7 in the case of inhibitory complexes (Cao et al., 2006).

Mouse XBP-1$^{-/-}$ embryos died between E12.5 and E13.5 after organogenesis was completed and showed growth retardation, liver hypoplasia, and reduced blood cell production (Reimold et al., 2000). The lethality in these mutant embryos could be rescued by targeting expression of *xbp-1* in hepatocytes only (Lee et al., 2005). Thus, the absolute requirement of xXBP-1 during early stages of embryonic development in frogs, appears to be absent in mammals. This suggests that *xbp-1* was recently recruited in amphibians to play a role in early patterning of the embryo. Alternatively, this function of *xbp-1* in early frog development might have disappeared or became masked during mammal evolution by the function of an as yet unidentified gene. In this context, it would be interesting to use morpholino-mediated gene knockdown to determine whether *xbp-1* also plays an essential role during early embryonic development in fishes such as the zebrafish.

In *Drosophila*, the segmented pattern of expression of *Dxbp-1* in fully elongated germ-band embryos suggested a role for this gene during embryonic development, particularly in embryo patterning. However, the lack of detectable phenotype of *Dxbp-1* homozygous or hemizygous mutant embryos did not support this hypothesis. Alternatively, this lack of embryonic phenotype might be due to either residual, albeit sufficient, *Dxbp-1* function in available mutants or from maternal *Dxbp-1* contribution, which provided sufficient *Dxbp-1* function for the completion of embryonic development. All attempts to isolate a molecularly null allele of *Dxbp-1,* which would allow us to perform germ line clone analysis and generate embryos devoid of maternal *Dxbp-1* contribution, have failed so far. Whether *Dxbp-1* is essential for embryonic development thus remains to be established.

Whereas *Dxbp-1* mutants hatched normally, homozygous or hemizygous larvae hardly grew at all and did not moult. They lived for two to four days as sluggish L1s and then died. Thus, in contrast to *C. elegans* in which *xbp-1* null mutants were fully viable and fertile (Shen et al., 2001; Calfon et al., 2002), our data demonstrate an essential requirement for *Drosophila xbp-1* during larval development. Because [^{35}S] Methionine incorporation experiments suggested that protein synthesis was not significantly decreased in mutant larvae (data not shown), this larval lethality might be due to impaired secretory processes. The growth defects might reflect a lack, or at least a severe reduction, in the secretion of digestive enzymes and the moult inhibition is possibly the consequence of defects in ecdysone secretion.

Whatever the function of *Dxbp-1* during embryogenesis, this gene appears to play an essential and evolutionarily conserved role in *Drosophila*, hypothetically by stimulating the folding capacities of the ER in cells committed to intense secretory activities.

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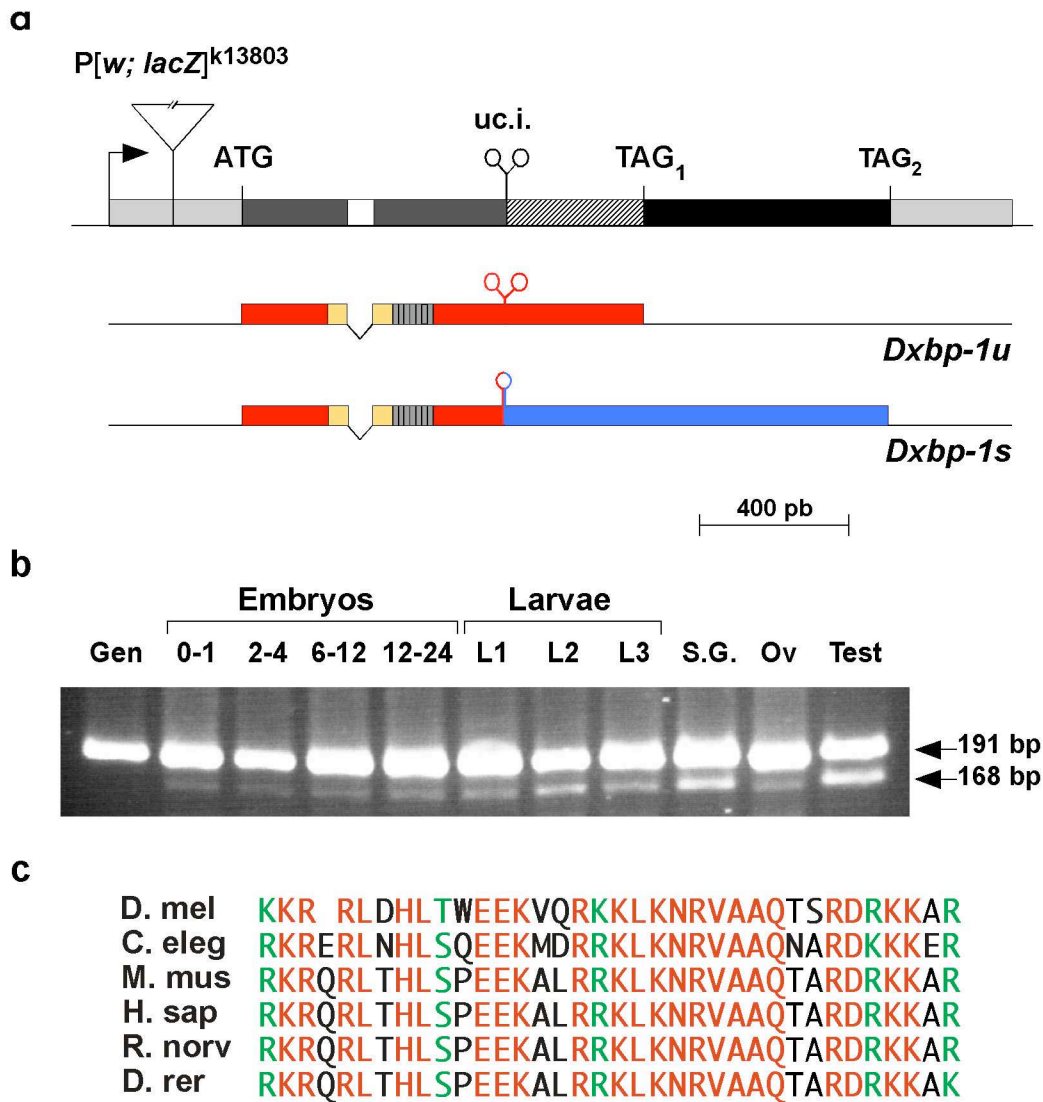
Figure 1. Structure and expression of *Dxbp-1* transcripts.

(a) The P[*lac; w*]^K13803 transposon is shown inserted into the 5'-UTR-region of the *Dxbp-1* gene. Intergenic sequences are depicted as single lines. Untranslated exonic sequences are represented by light grey boxes and open reading frames are shown as dark grey (common *Dxbp-1u*/*Dxbp-1s* ORF), black (*Dxbp-1s*-specific ORF) and striped (*Dxbp-1u/Dxbp-1s* overlapping ORFs) boxes. The intron is represented as an empty box and the unconventional intron (uc. i.) is indicated by two convergent stem-loop structures. Arrow shows the direction of transcription. The translation initiation codon (ATG) and the two termination codons (TAG$_1$ and TAG$_2$) are indicated above the *Dxbp-1* transcribed region. The structure of the *Dxbp-1u* and *Dxbp-1s* transcripts is shown below. Untranslated sequences are represented by single lines. The *Dxbp-1u* ORF and that shared by the *Dxbp-1u* and *Dxbp-1s* transcripts are indicated as red boxes, with the basic domain coloured in yellow, and the "Leucine zipper" as a hatched box. The *Dxbp-1s*-specific ORF is shown as a blue box. Unprocessed and spliced unconventional introns are depicted by two convergent stem-loops and by a single stem-loop, respectively. (b) RT-PCR analysis of the expression of the *Dxbp-1u* and *Dxbp-1s* transcripts. PCR amplifications were performed using genomic DNA (Gen) as control or reverse-transcribed polyA$^+$ RNA extracted from 0 to 1 h, 2 to 4 h, 6 to 12 h and 12 to 24 h embryos or total RNA extracted from first (L1), second (L2) and third (L3) instar larvae or dissected third instar larval salivary glands (S. G.), ovaries (Ov) or male sexual apparatus (Test), as templates. The length of the two RT-PCR products was determined by direct sequencing of the amplified fragments. (c) Sequence alignment of the basic domain of *Drosophila melanogaster* (D. mel), *Caernorhabditis elegans* (C. eleg), *Mus Musculus* (M. mus), *Homo Sapiens* (H. sap), *Rattus norvegicus* (R. norv), and *Brachydanio rerio* (D. rer) Xbp-1 proteins.
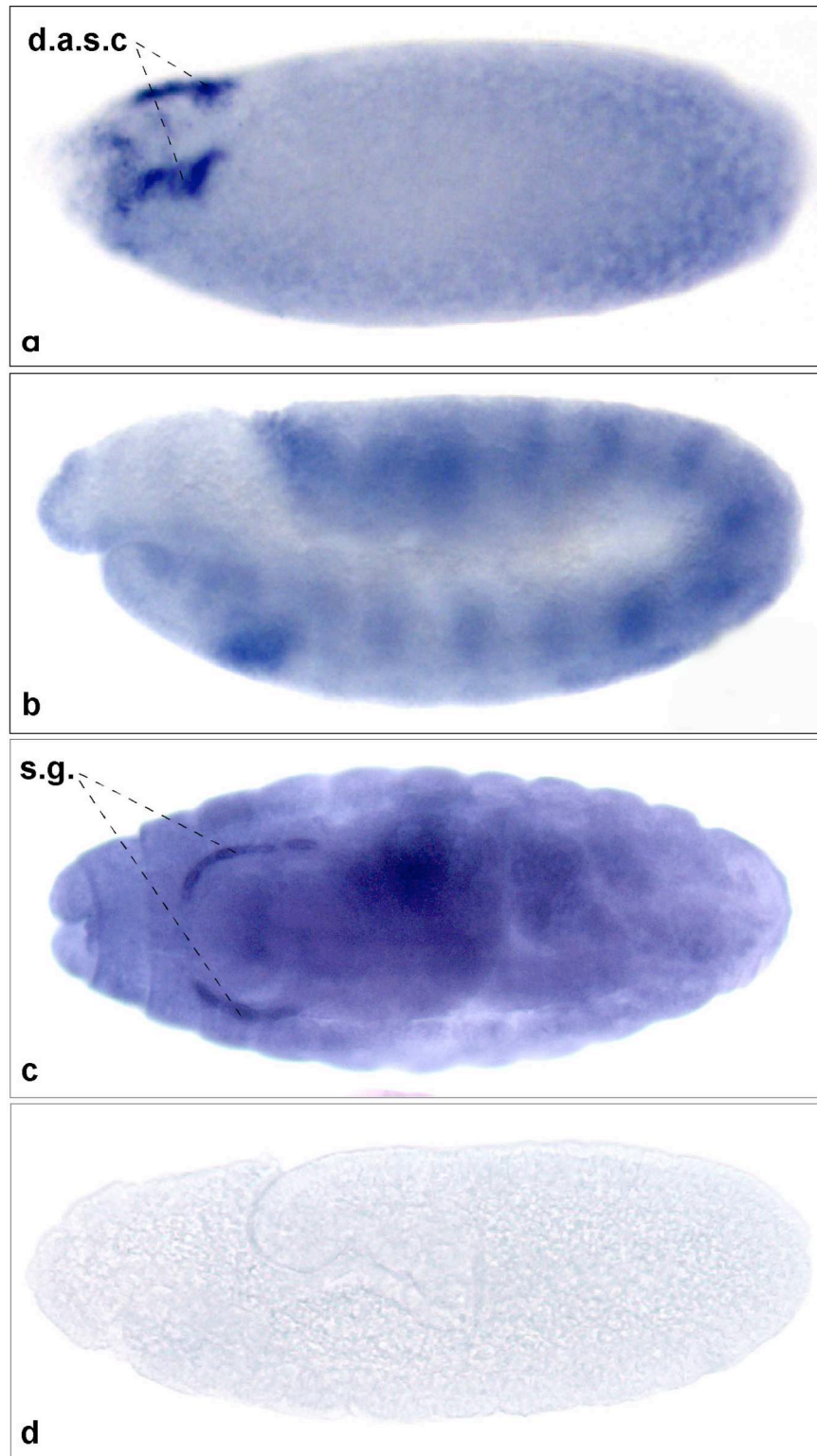
Figure 2. Accumulation pattern of the *Dxbp-1* transcript during oogenesis and embryogenesis.
(a-c) Whole-mount *in situ* hybridisations with a *Dxbp-1* anti-sense RNA probe on: (a) early stage 14 ovarian follicle, (b) stage 11 and (c) stage 17 embryos. (d) Whole-mount *in situ* hybridisation with a *Dxbp-1* sense RNA probe on a stage 11 embryo. Note that the intensely labelled cells observed in the labial segment of stage 11 embryo likely correspond to the salivary gland anlagen. Dorsal appendage secreting cells (d. a. s. c.), salivary glands (s. g.). Anterior is to the left.
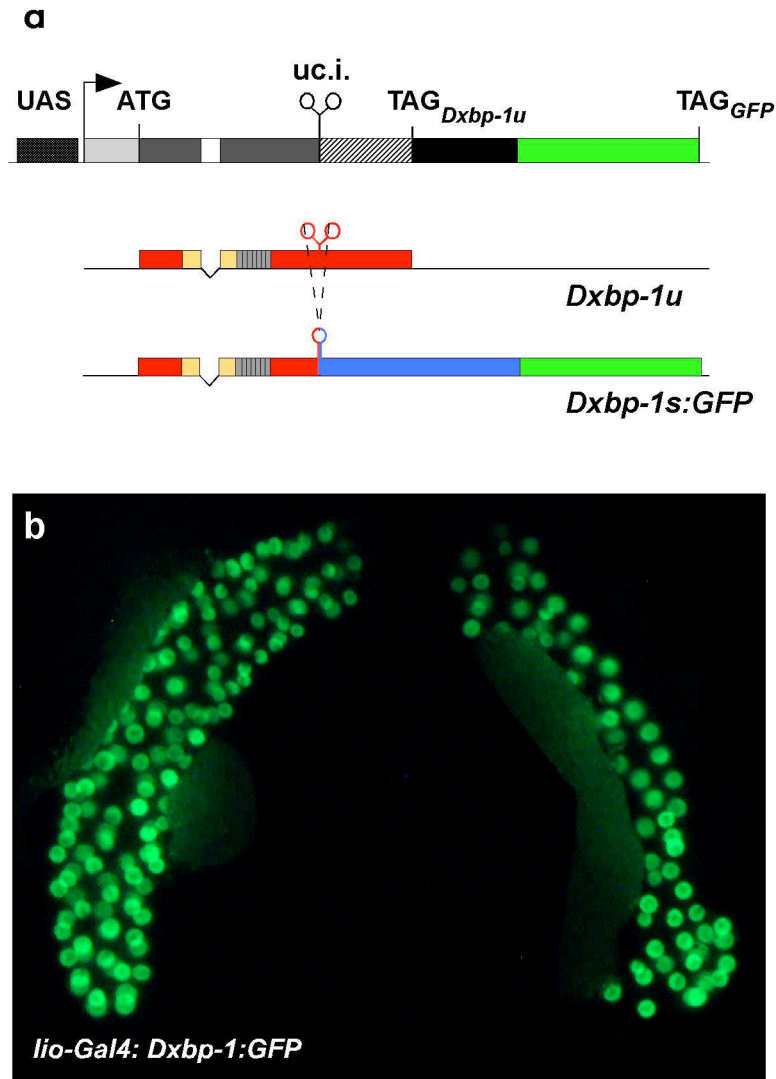
Figure 3. *In vivo* **detection of** *Dxbp-1* **RNA processing.**
(a) Structure of the *UAS-Dxbp-1:GFP* transgene. *Dxbp-1* sequences shown as light grey (5'-UTR), dark grey (*Dxbp-1u* and *Dxbp-1s* shared ORF), empty (common intron), hatched (*Dxbp-1u/Dxbp-1s* overlapping ORFs) and black (3'-truncated *Dxbp-1s* specific ORF) boxes, were inserted between *UAS* sequences (dotted box) and the GFP-encoding gene (green box) in the *pUAS$_P$-GFP* vector (Januschke et al., 2002) in phase with the *Dxbp-1u* ORF. Arrow points in the direction of transcription. Translation initiation (ATG) and termination (TAG$_{Dxbp-1u}$ and TAG$_{GFP}$) codons are indicated above the transcribed sequence. Underneath is depicted the predicted structure of the encoded transcripts. While the native RNA should encode the *bona fide* DXbp-1u protein (colours as in Fig. 1a), the processed *Dxbp-1s:GFP* transcript resulting from the splicing of the unconventional intron (uc. i.) is predicted to encode a fluorescent DXbp-1s:GFP fusion protein (colours as in Fig. 1a with the GFP encoding sequences indicated as a green box). (b) Salivary glands of *lio-Gal4; UAS-Dxbp-1:GFP* third instar larvae showing strong specific nuclear fluorescent staining.

Figure 4. ER stress increases processing of *Dxbp-1:GFP* transcripts

Feeding *lio-Gal4; UAS-Dxbp-1:GFP* larvae tunicamycine increases fluorescent labelling of salivary gland nuclei. All salivary glands were photographed in a single shot in order to fully visualise the increased fluorescent staining of the salivary glands of *lio-Gal4; UAS-Dxbp-1:GFP* third instar larvae fed with 50 μg/ml of tunicamycine for 24 hours (left) compared to that of *lio-Gal4; UAS-Dxbp-1:GFP* control larvae (right).

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

MAPTANTVLITVPRTAITSNNLPKLRPAPLTAALLKVSATPSASPTPSSS
GYASSSNMDDDNMAASQPKAKKRRLDHLTWEEKVQRKKLKNRVAAQTSRD
RKKARMEEMDYEIKELTDRTEILQNKCDSLQAINESLLAKNHKLDSELEL
LRQELAELKQQQQHNTRCISQSNASAGAEGCASTNLGSAAGYTTGGHTVV
SASAGGAAEEQQEPGLTLESCGPLPTLQDMLGVDEEFDVKRLEELAESLL
ADITADLETGAGASSPAAAQDAGNAERLPGPMVGPAAERLESDGHRANGL
NVEQEQETEHKVSLNVQMLKINGNPQHTTAAPASRTATITATAAASQLQA
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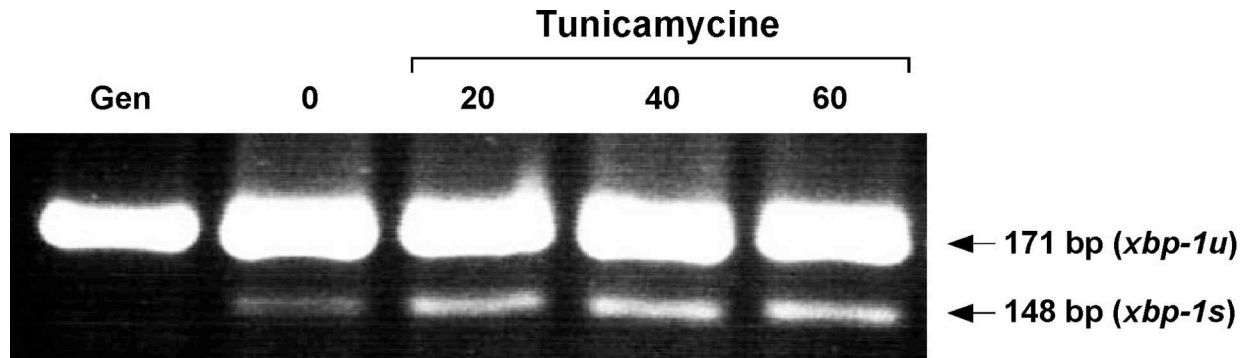   **DXbp-1s**

Figure s1. Deduced sequences of DXbp-1u and DXbp-1s proteins.
Amino acids of the amino-terminal region shared by the two proteins are depicted in black. Within this region, the basic domain is underlined and the Leucine residues constituting the "Leucine zipper" are indicated in green. Residues of the carboxy-terminal specific domains of the DXbp-1u and DXbp-1s proteins are represented in blue and red, respectively.
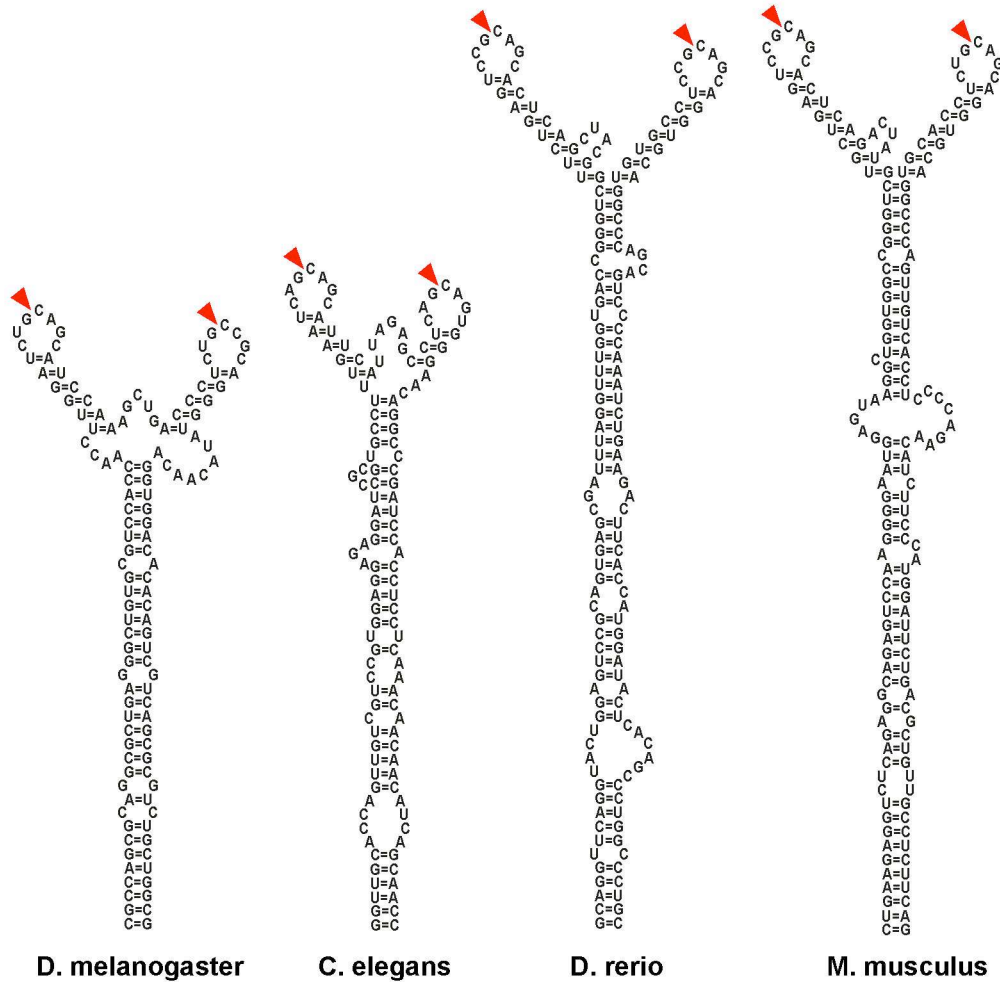
Figure s2. Hypothetical structures of Ire-1 cleavage sites.
Secondary structure of Ire-1 cleavage sites (red arrow heads) and adjacent sequences, of *Drosophila melanogaster* (D. melanogaster)*, Caernorhabditis elegans* (C. elegans)*, Brachydanio rerio* (D. rerio) and *Mus musculus* (M. musculus) *xbp-1* transcripts, as deduced from visual and *in silico* examinations of *xbp-1* sequences.

Figure s3. ER stress induces putative Ire-1 dependent processing of *Dxbp-1* RNA.
RT-PCR analysis of *Dxbp-1s* accumulation following activation of the UPR pathway in adults. PCR amplifications were performed using genomic DNA (Gen) or reverse transcribed RNA extracted from non-treated adults (0) or from flies fed with 20, 40 or 60 µg/ml tunicamycine for 24 hours.