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IRE1β is required for mesoderm formation in *Xenopus* embryos

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ABSTRACT

IRE1 is an atypical serine/threonine kinase transmembrane protein with RNase activity. In the unfolded protein response (UPR), they function as proximal sensor of the unfolded proteins in the endoplasmic reticulum (ER). Upon activation by ER stress, IRE1 performs an unconventional cytoplasmic splicing of *XBP1* pre-mRNA and thus allows the synthesis of active XBP1, which activates UPR target genes to restore the homeostasis of the ER. IRE1/XBP1 signaling is hence essential for UPR but its function during embryogenesis is yet unknown. The transcripts of the two isoforms of IRE1 in *Xenopus*, *xIRE1α* and *xIRE1β* are differentially expressed during embryogenesis. We found that xIRE1β is sufficient for cytoplasmic splicing of *xXBP1* pre-mRNA. Although gain of xIRE1β function had no significant effect on *Xenopus* embryogenesis, overexpression of both, *xIRE1β* and *xXBP1* pre-mRNA, inhibits activin A induced mesoderm formation, suggesting that an enhanced activity of the IRE1/XBP1 pathway represses mesoderm formation. Surprisingly, while loss of XBP1 function promotes mesoderm formation, the loss of IRE1β function led to a reduction of mesoderm formation, probably by action of IRE1 being different from the IRE1/XBP1 pathway. Therefore, both gain and loss of function studies demonstrate that IRE1 is required for mesoderm formation in *Xenopus* embryos.

1. Introduction

Endoplasmic reticulum (ER) in eukaryotic cells is responsible for the early steps in the maturation of most proteins in the secretory pathway, such as folding of the newly synthesized polypeptide chains and posttranslational modifications that are essential for protein function (Schröder and Kaufman, 2005). Nascent polypeptides are translocated to the ER lumen in an unfolded state, where they are processed for folding. However, the function of ER will be disrupted when the inflow of unfolded polypeptide chains exceeds the folding or processing capacity of the ER. This ER stress in turn leads to the activation of a series of adaptive pathways known as unfolded protein response (UPR) to maintain ER homeostasis (Schröder and Kaufman, 2005; Xu et al., 2005; Wu and Kaufman, 2006; Boyce and Yuan, 2006). The UPR signaling pathways are transduced by three ER resident transmembrane proteins IRE1[3], PERK and ATF6 upon activation (Schröder and Kaufman, 2005), among which IRE1 functions as an endoribonuclease (RNase) to process XBP1 pre-mRNA to a mature form. In an inactive state, IRE1 is associated with BiP (Bertolotti et al., 2000; Liu et al., 2003), a chaperone that can distinguish folded and unfolded proteins. As unfolded proteins accumulate in the ER, BiP is competitively released from IRE1 by the huge excess of unfolded proteins (Bertolotti et al., 2000). Consequently, IRE1 is activated and transduces the unfolded protein signal across

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[3] *Abbreviations used:* IRE, inositol requiring; XBP, X-box binding protein.
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the ER membrane. IRE1 is an atypical type I transmembrane protein with serine/threonine kinase (Cox et al., 1993) and RNase activities. It is composed of a luminal domain at the N-terminus responsible for sensing the ER stress signal and C-terminal cytoplasmic kinase and site-specific RNase domains (Schröder and Kaufman, 2005). Upon activation, IRE1 oligomerizes and phosphorylates itself and subsequently activates the RNase domain, which removes 23 or 26 bp, respectively, from *XBP1* pre-mRNA in metazoans (Shamu and Walter, 1996; Yoshida et al., 2001; Shen et al., 2001; Calfon et al., 2002) or 252 bp from Hac1 pre-mRNA in yeast (Cox and Walter, 1996; Kawahara et al., 1997). Subsequent ligation of RNA by a tRNA ligase generates a frameshift in the resulting mRNA encoding a functionally active bZIP transcription factor, which subsequently stimulates the expression of UPR target genes (Schröder and Kaufman, 2005). Noteworthy, we recently have shown that during gastrula and early neurula stages of *Xenopus* embryogenesis this frame shift is generated by an alternative mechanism, i.e., the removal of a complete exon (exon 4) that is probably deleted by the conventional splicing machinery within the nucleus (Cao et al., 2006a). The activated isoforms of these proteins either help to restore ER homeostasis and hence the physiological function of cells by increasing the folding capacity of cells, or induce cells to undergo apoptosis when the effort for restoration of ER homeostasis is in vain. Previous studies using induced ER stress conditions underlined that the IRE1/XBP1/Hac1 signaling pathway plays an essential role for the UPR in eukaryotic organisms such as yeast (Cox and Walter, 1996; Kawahara et al., 1997), *Caenorhabditis elegans* (Shen et al., 2001; Calfon et al., 2002), *Drosophila* (Ryoo et al., 2007; Plongthongkum et al., 2007) and mammals. However, little information is available about the function of the IRE1/XBP1 pathway during embryogenesis, although loss of function studies revealed that IRE1 or XBP1 are absolutely required for embryonic development of *C. elegans* (Shen et al, 2001), *Drosophila* (Souid et al., 2007) or mouse (Reimold et al., 2000; Urano et al., 2000; Lee et al., 2002). During *Xenopus* early embryogenesis, XBP1 is involved in the formation of the mesodermal germ layer (Zhao et al., 2003; Cao et al., 2006a). We therefore have investigated the function of the IRE1/XBP1 pathway and the relationship between the passive and active isoforms of XBP1 in embryonic development. In the present study, we show that the *IRE1* homologues in *Xenopus*, *xIRE1α* and β, are differentially expressed during embryogenesis. Both, gain and loss of function analyses revealed that xIRE1β is required for cytoplasmic splicing of *xXBP1* pre-mRNA, reflecting the conservation of IRE1/XBP1 pathway in *Xenopus*. In animal cap assays, we found that overexpression of *xIRE1β* and *xXBP1* pre-mRNA was able to inhibit mesoderm formation induced by activin A. However, functional knockdown of xIRE1β also blocked mesoderm formation, suggesting that xIRE1β is required for mesoderm formation.

2. Results

2.1. *Differential expression of IRE1α and IRE1β in Xenopus*

Two forms of IRE1 genes, *IRE1α* and *IRE1β*, exist in mammals (Tirasophon et al., 1998; Iwawaki et al., 2001). Search of *Xenopus laevis* protein sequence databases revealed an IMAGE clone (Genbank Accession No.: BC073092) coding for a protein of 958 amino acids. Further database searches led to three overlapping ESTs (Accession Nos.: BU908941, BJ064380 and BJ050438) being related but not identical to the IMAGE clone. RT-PCR was performed using primers designed from the EST sequences. The amplified cDNA (1963 bp) contains a partial open reading frame coding for a polypeptide of 653 amino acids. This peptide shares only 50% identity to the *Xenopus* IMAGE clone, reflecting a more distant relationship. Instead, it exhibits 75% identity to human IRE1α (hIRE1α) (Tirasophon et al., 1998) and only 42% to the human IRE1β (hIRE1β) (Iwawaki et al., 2001). While the amino acid sequence encoded by the *Xenopus* IMAGE clone is 55% identical to hIRE1α and 52% to hIRE1β (Fig. 1A and B), a phylogenetic analysis based on sequence similarities showed that the partial cDNA clone and hIRE1α were grouped together and that the IMAGE clone and hIRE1β were genetically closer (Fig. 1C). We therefore assigned the partial cDNA as *Xenopus IRE1α* and the IMAGE clone as *Xenopus IRE1β* (*xIRE1β*).

IRE1 proteins consist of conserved domains that are critical for their function (Tirasophon et al., 1998; Iwawaki et al., 2001). At the amino-terminus and the middle of the proteins there are two short regions that represent the putative signal peptide and the transmembrane region, separately (Fig. 1A). Between the two regions is the ER-luminal domain that locates in the lumen of endoplasmic reticulum. The remaining part between the transmembrane region and the C-terminus is predicted to be the cytoplasmic domain, which is composed of kinase and RNase subdomains (Fig. 1A).

We have examined the temporal and spatial expression patterns of *xIRE1α* and *xIRE1β* during *Xenopus* embryogenesis. Real-time RT-PCR demonstrated that both, *xIRE1α* and *xIRE1β* were already detected in fertilized eggs, showing that they are maternally expressed (Fig. 2A). Both transcripts persist during early cleavage stages. From the start of zygotic transcription, they gradually diminish until hatching tadpole stages, when their expression level is slightly increased (Fig. 2A). These results show that the temporal expression patterns of *xIRE1α* and *xIRE1β* are very similar. We next asked how the *IRE1* genes are spatially expressed during embryogenesis. Whole mount in situ hybridization revealed that *xIRE1α* and *xIRE1β* are expressed in a similar pattern from egg to gastrulation. Transcripts of both, *xIRE1α* and *xIRE1β*, were detected in the animal half of fertilized eggs and early cleavage stage embryos (Fig. 2B, C, H and I). During gastrulation, both transcripts are present in the animal and equatorial region but are excluded from the yolk plug (Fig. 2D and J). However, from the onset of neurulation the spatial expression patterns of the two genes differ completely. At stage 15, *xIRE1α* is localized to the neural plate (Fig. 2E). In tailbud embryos, *xIRE1α* was detected in the forebrain, midbrain, hindbrain, eyes, otic vesicles and neural tube (Fig. 2F and G). Additional expression domains were observed in the brachial arches, pronephric duct and a domain that is probably representing the dorsal pancreas anlage (Fig. 2F and G). Abundant expression of *IRE1α* has also been reported for the mammalian pancreas (Tirasophon et al., 1998). In the case of *xIRE1β*, transcription is only observed in the hatching gland and cement gland anlagen during neurula stage (Fig. 2K). These expression domains become more prominent in the hatching embryos and persist until the hatched tad-