

Inositol-requiring enzyme 1 α is required for gut development in *Xenopus laevis* embryos

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Abstract

AIM: To investigate the role of inositol-requiring enzyme 1 α (IRE1 α) in gut development of *Xenopus laevis* embryos.

METHODS: *Xenopus* embryos were obtained with *in vitro* fertilization and cultured in 0.1 \times MBSH. One and half nanogram of IRE1 α , 1 ng of IRE1 α -GR mRNA, 1 ng of IRE1 α Δ C-GR mRNA, and 50 ng of IRE1 α morpholino oligonucleotide (MO) or XBP1(C)MO were injected into four blastomeres at 4-cell stage for scoring the phenotype and marker gene analysis. To rescue the effect of IRE1 α MO, 1 ng of IRE1 α -GR mRNA was co-injected with 50 ng of MO. For the activation of the GR-fusion proteins, dexamethasone was prepared as 5 mmol/L stock solutions in 100% ethanol and applied to the mRNA injected embryos at desired stages in a concentration of 10 μ mol/L in 0.1 \times MBSH. Embryos were

kept in dexamethasone up to stage 41. Whole-mount *in situ* hybridization was used to determine specific gene expression, such as IRE1 α , IRE1 β , Xbra and Xsox17 α . IRE1 α protein expression during *Xenopus* embryogenesis was detected by Western blotting.

RESULTS: In the whole-mount *in situ* hybridization analysis, xenopus IRE1 α and IRE1 β showed quite different expression pattern during tadpole stage. The relatively higher expression of IRE1 α was observed in the pancreas, and significant transcription of IRE1 β was found in the liver. IRE1 α protein could be detected at all developmental stages analyzed, from stage 1 to stage 42. Gain-of-function assay showed that IRE1 α mRNA injected embryos at tailbud stage were nearly normal and the expression of the pan-mesodermal marker gene Xbra and the endodermal gene Xsox17 α at stage 10.5 was not significantly changed in embryos injected with IRE1 α mRNA as compared to uninjected control embryos. And at tadpole stage, the embryos injected with IRE1 α -GR mRNA did not display overt phenotype, such as gut-coiling defect. Loss-of-function assay demonstrated that the IRE1 α MO injected embryos were morphologically normal before the tailbud stages. We did not observe a significant change of mesodermal and endodermal marker gene expression, while after stage 40, about 80% of the MO injected embryos exhibited dramatic gut defects in which the guts did not coil, but other structures outside the gastrointestinal tract were relatively normal. To test if the phenotypes were specifically caused by the knockdown of IRE1 α , a rescue experiment was performed by co-injection of IRE1 α -GR mRNA with IRE1 α MO. The data obtained demonstrated that the gut coiling defect was rescued. The deletion mutant of IRE1 α was constructed, consisting of the N-terminal part without the C-terminal kinase and RNase domains named IRE1 α Δ C, to investigate the functional domain of IRE1 α . Injection of IRE1 α Δ C-GR mRNA caused similar morphological alterations with gut malformation by interfering with the function of endogenous xIRE1 α . In order to investigate if IRE1 α /

XBP1 pathway was involved in gut development, 50 ng of XBP1 MO was injected and the results showed that knockdown of XBP1 resulted in similar morphological alterations with gut-coiling defect at tadpole stage.

CONCLUSION: IRE1 α is not required for germ layer formation but for gut development in *Xenopus laevis* and it may function *via* XBP1-dependent pathway.

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Key words: Inositol-requiring enzyme 1 α ; XBP1; *Xenopus laevis*; Gut; Development

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INTRODUCTION

The endoplasmic reticulum (ER) plays a central role in the synthesis and modification of secretory and membrane proteins in all eukaryotic cells^[1-3]. Under normal conditions, these proteins are correctly folded and assembled in the ER. However, when cells are exposed to disturbed environment, such as overproduction of ER proteins, viral infection, and glucose deprivation, these proteins accumulate as unfolded or misfolded forms in the ER lumen and, consequently, cause ER stress. To maintain cellular homeostasis, cells induce some adaptive responses to ER stress. One of them is the unfolded protein response (UPR), which up-regulates the transcription of various genes to increase the protein-folding and protein-degradation activity in the ER^[4-7]. Inositol requiring enzyme-1 (IRE1) is an ER-located type I transmembrane protein with a kinase domain and RNase domain in the cytosolic region and has a unique function of relieving ER stress in cells. When the amino-terminal luminal region senses perturbations in the ER environment, *via* trans-autophosphorylation and activation of its RNase domain, IRE1 induces unconventional splicing of mRNA coding, a specific transcription factor for activating the UPR^[8-10]. IRE1 is highly conserved from yeast to humans, and two IRE1 paralogues have been reported in mammals: IRE1 α and IRE1 β ^[11-13]. Upon activation by ER stress, IRE1 performs an unconventional cytoplasmic splicing of XBP1 pre-mRNA, thus allowing the synthesis of active XBP1, which activates UPR target genes to restore the homeostasis of the ER^[10,14,15]. The spliced XBP1 mRNA is translated into a functional transcription factor to up-regulate gene expression for ER quality control. IRE1 is also reported to activate proapoptotic JNK signaling under ER stress conditions^[16].

IRE1 α is also known to be expressed ubiquitously in fetal and adult mice and to be essential for mammalian

developmental processes^[17]. Therefore, IRE1 α inactivation results in widespread developmental defects, leading to embryonic death after 12.5 d of gestation in mice^[18]. However, the cause of this embryonic lethality is not fully understood. These lines of evidence suggest that IRE1 α has a unique function in mammalian developmental processes, but it has been hitherto unclear in which tissues and how IRE1 α functions during embryogenesis.

Xenopus laevis is an excellent model system for studying organ development^[19]. 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 is absolutely required for embryonic development of *C. elegans*^[20], *Drosophila*^[21] or mouse^[22]. In *Xenopus*, although the transcripts of two isoforms of IRE1, IRE1 α and IRE1 β show similar spatial expression in pre-neurula embryos, they are also differentially expressed following the onset of neurulation^[23]. IRE1 α is localized to the nervous system and mesoderm or endoderm-derived organs, such as pronephros and pancreas. However, the role of IRE1 α in organogenesis is still unclear. In the present study, both gain and loss of function analyses revealed that IRE1 α is required for gut development in *Xenopus*.

MATERIALS AND METHODS

Embryos

Wild type *Xenopus laevis* eggs were obtained by injecting 1000 IU of human chorionic gonadotrophin into the dorsal lymph sacs of adult females 6-8 h before egg collection. Eggs were fertilized *in vitro* with minced testes, dejellied with 2% cysteine hydrochloride (pH 7.8-8.0) 30 min after fertilization, and cultured in 0.1 \times MBSH (8.8 mmol/L NaCl, 0.24 mmol/L NaHCO₃, 0.1 mmol/L KCl, 0.082 mmol/L MgSO₄, 0.041 mmol/L CaCl₂, 0.033 mmol/L Ca(NO₃)₂, 1 mmol/L HEPES, pH 7.4). Staging of *Xenopus laevis* embryos was according to Nieuwkoop and Faber (1967).

Plasmids and constructs

IRE1 α ORF was amplified from a cDNA pool consisting of st.1, st.8, st.10, st.15, st.20 and st.28 cDNAs and subcloned to pCS2⁺ vector. The construct was named pCS2⁺-IRE1 α . To make expression constructs, complete coding region and the N-terminal region (aa 1-479) were amplified from the pCS2⁺-IRE1 α using PCR and subcloned to the BamHI-EcoRV sites on pCS2⁺-GR. The resulting constructs were designated as pCS2⁺IRE1 α -GR and pCS2⁺IRE1 α ΔC-GR.

In vitro RNA synthesis, antisense morpholino oligonucleotides and microinjection

Plasmids pCS2⁺IRE1 α , pCS2⁺IRE1 α -GR and pCS2⁺IRE1 α ΔC-GR were linearized with *Not*I. Capped mRNA for microinjection was synthesized with SP6 mMessage mMachineTM kit (Ambion) and cleaned up with RNeasy kit (Qiagen). The antisense morpholino

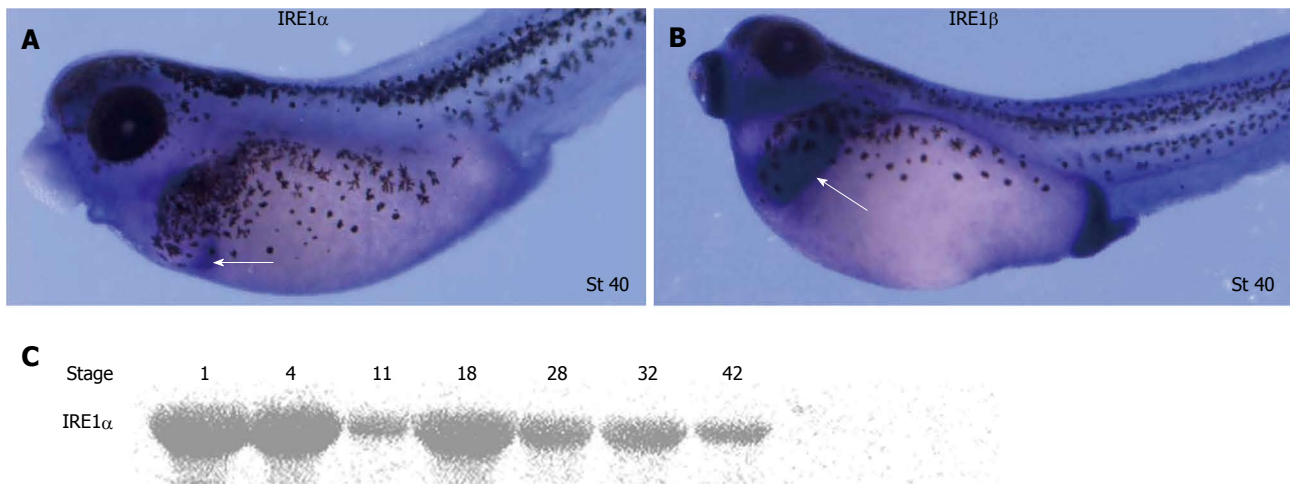


Figure 1 Expression pattern of xIRE1 in *Xenopus laevis* during development. A, B: Whole-mount *in situ* hybridizations revealed relatively high expression of inositol-requiring enzyme (IRE) 1 α in pancreas (white arrow in A) and relatively high expression of IRE1 β in liver (white arrow in B); C: Western blotting revealed temporal expression of IRE1 α during *Xenopus* embryogenesis.

oligonucleotide (Gene Tools) used for IRE1 α functional knockdown (IRE1 α MO) was: 5'-AAGAGAACCGC-CAGAGGC GCCATG T-3'; and an antisense morpholino oligonucleotide XBP1(C) MO designed to inhibit the cytoplasmic splicing of xXBP1 was: 5'-GACATCT-GGGCCTGCTC CTGC TGCA-3'. One and half nanogram of IRE1 α , 1 ng of IRE1 α -GR mRNA, 1 ng of IRE1 α Δ C-GR mRNA, and 50 ng of IRE1 α MO or XBP1(C)MO were injected into four blastomeres at 4-cell stage for scoring the phenotype, whole-mount *in situ* hybridization and marker gene analysis. For the activation of the GR-fusion proteins, dexamethasone (Sigma) was prepared as 5 mmol/L stock solutions in 100% ethanol and applied to the control and mRNA injected embryos at desired stages in a concentration of 10 μ mol/L in 0.1 \times MBS. Embryos were kept in dexamethasone up to stage 41. To rescue the effect of blocking xXBP1 splicing by IRE1 α MO, 1 ng of IRE1 α -GR mRNA was co-injected with 50 ng of MO.

***In vitro* translation**

In vitro protein translation was performed with TNT coupled Reticulocyte Lysate Systems (Promega) to test the efficiency of IRE1 α MO for blocking protein translation. One μ g of pCS2⁺IRE1 α plasmid was used either alone or together with 20 μ g IRE1 α MO for *in vitro* translation. Translation products were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and chemiluminescent detection.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed according to the protocol described elsewhere^[24]. The digoxigenin-labeled antisense probes were prepared as follows: pDrive-IRE1 α cut with *Hind*III, transcribed with T7 RNA polymerase; pCS2⁺XIRE1 β cut with *Xba*I, transcribed with T3 RNA polymerase; pBS-Xbra cut with *Sa*I, transcribed with T7 RNA polymerase; and pCS2⁺XSox17 α cut with *Cl*aI, transcribed with T7 RNA

polymerase.

Protein extraction and Western blotting

Embryos were homogenized in RIPA lysis buffer (20 mmol/L Tris, pH 8; 2 mmol/L EDTA, pH 8; 0.5% NP-40; 25 mmol/L β glycerophosphate; 100 mmol/L NaF; 100 mmol/L PMSF and phosphatase inhibitor cocktail). Lysates were centrifuged at 4 $^{\circ}$ C, 15 000 $\times g$, for 20 min, and the supernatants were added to 5 \times SDS loading buffer. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Millipore, Billerica, MA). Immunoblotting membranes were blocked with 5% milk in TBST. After several washes in TBST, membranes were incubated overnight with the first antibody [anti-IRE1 α antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:500]. Loading controls of presumably constantly expressed proteins such as β -actin were used; however, their variability and increase in development precluded their use^[25]. Detection was then done with HRP-labeled secondary antibodies and enhanced chemiluminescence (ECL).

RESULTS

Expression of xIRE1 α in *Xenopus laevis* during development

Two forms of IRE1 genes, IRE1 α and IRE1 β , exist in *Xenopus laevis*. Whole-mount *in situ* hybridization revealed that IRE1 α and IRE1 β were expressed in a similar pattern from egg to gastrulation. In tailbud embryos, IRE1 α was detected in a domain that probably represented the dorsal pancreas anlage. IRE1 β was only observed in the hatching gland and cement gland until the hatched tadpole stage^[23]. To further explore the spatial expression patterns of IRE1 α and IRE1 β in *Xenopus* embryos at later stages, whole-mount *in situ* hybridizations were carried out. During tadpole stages, relatively high expression of IRE1 α was observed in the pancreas (Figure 1A), and significant transcription of IRE1 β was observed in the

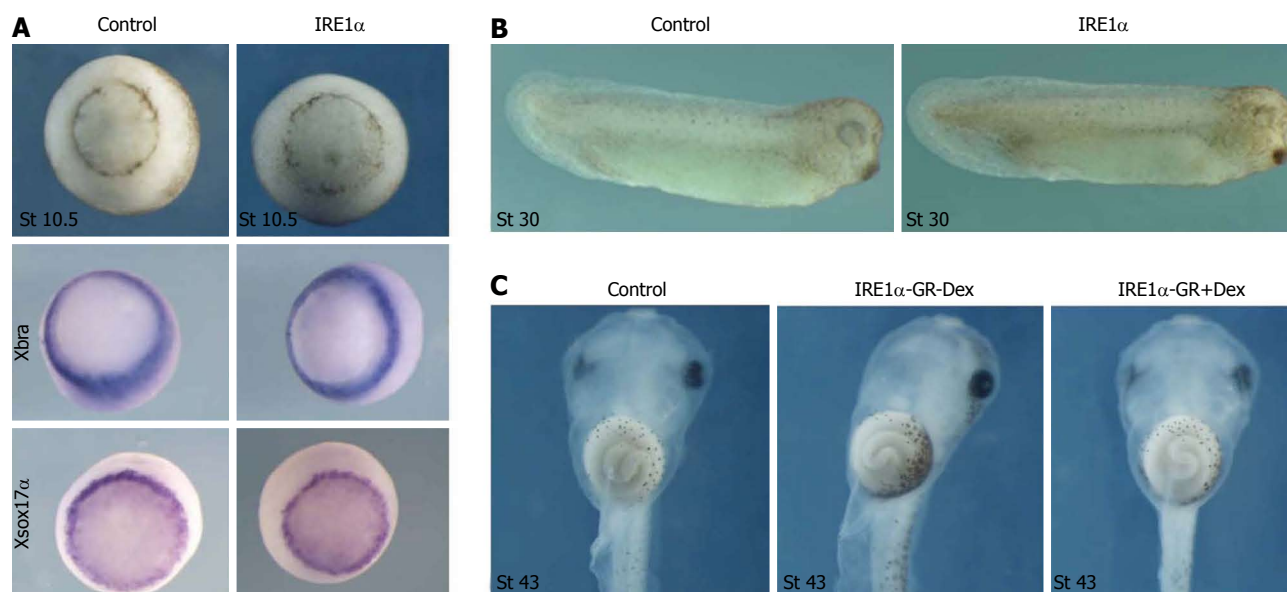


Figure 2 Overexpression of inositol-requiring enzyme 1 α affected neither mesoderm and endoderm formation nor gut development. A: Overexpression of inositol-requiring enzyme (IRE) 1 α did not change the phenotype and the expression of Xbra and Xsox17 α at stage 10.5; B: IRE1 α mRNA injected embryos at tailbud stage were nearly normal; C: Embryos injected with IRE1 α -GR mRNA did not display any defect until tadpole stage.



Figure 3 Inositol-requiring enzyme 1 α morpholino oligonucleotide blocks inositol-requiring enzyme 1 α translation in an *in vitro* transcription/translation assay. In this assay, xIRE1 α was effectively transcribed/translated from a pCS2+xIRE1 α construct. However, translation was dramatically decreased by addition of IRE1 α morpholino oligonucleotide (MO). IRE: Inositol-requiring enzyme.

liver (Figure 1B). IRE1 α protein could be detected at all developmental stages analyzed (Figure 1C).

IRE1 α gain of function

Different doses of IRE1 α mRNA were injected into all blastomeres at 4-cell stage. Even at a dose of 1.5 ng, injected embryos at tailbud stage were nearly normal (Figure 2B). Whole-mount *in situ* hybridization of embryos injected with IRE1 α mRNA revealed that the expression of the pan-mesodermal marker gene Xbra and the endodermal gene Xsox17 α at stage 10.5 was not significantly changed as compared to uninjected control embryos (Figure 2A). To test the function of IRE1 α in the later stage of development, the mRNA encoding a dexamethasone inducible variant of IRE1 α , referred to

as IRE1 α -GR, was injected into all four blastomeres at 4-cell stage *Xenopus* embryos. For the activation of the GR-fusion proteins, dexamethasone treatment was administered at embryonic stage 27. No apparent change of phenotype was observed even at tadpole stage (Figure 2C). Therefore, gain of function of IRE1 α did not lead to an apparent change of phenotype and we did not observe a significant change of mesodermal and endodermal marker gene expression.

IRE1 α MO blocking IRE1 α translation

To further explore the function of xIRE1 α during embryonic development, we performed a loss of function (LOF) analysis using an antisense morpholino oligonucleotide (IRE1 α MO) directed against xIRE1 α . *In vitro* protein translation was performed with TNT coupled Reticulocyte Lysate Systems to test the efficiency of IRE1 α MO for blocking protein translation. As shown in Figure 3, IRE1 α MO totally blocked IRE1 α translation.

IRE1 α loss of function

The IRE1 α MO-injected embryos were morphologically normal before the tailbud stages (Figure 4B) and we did not observe a significant change of mesodermal and endodermal marker gene expression (Figure 4A), but after stage 40, about 80% of the MO (50 ng)-injected embryos exhibited dramatic gut defects in which the guts did not coil, while other structures outside the gastrointestinal tract developed normally (Figure 4). To test if the phenotypes were specifically caused by the knockdown of IRE1 α , a rescue experiment was performed by co-injection of IRE1 α -GR mRNA with MO. The results indicated that the phenotype could be rescued (Figure 5). Taken together, these data indicate that IRE1 α is re-

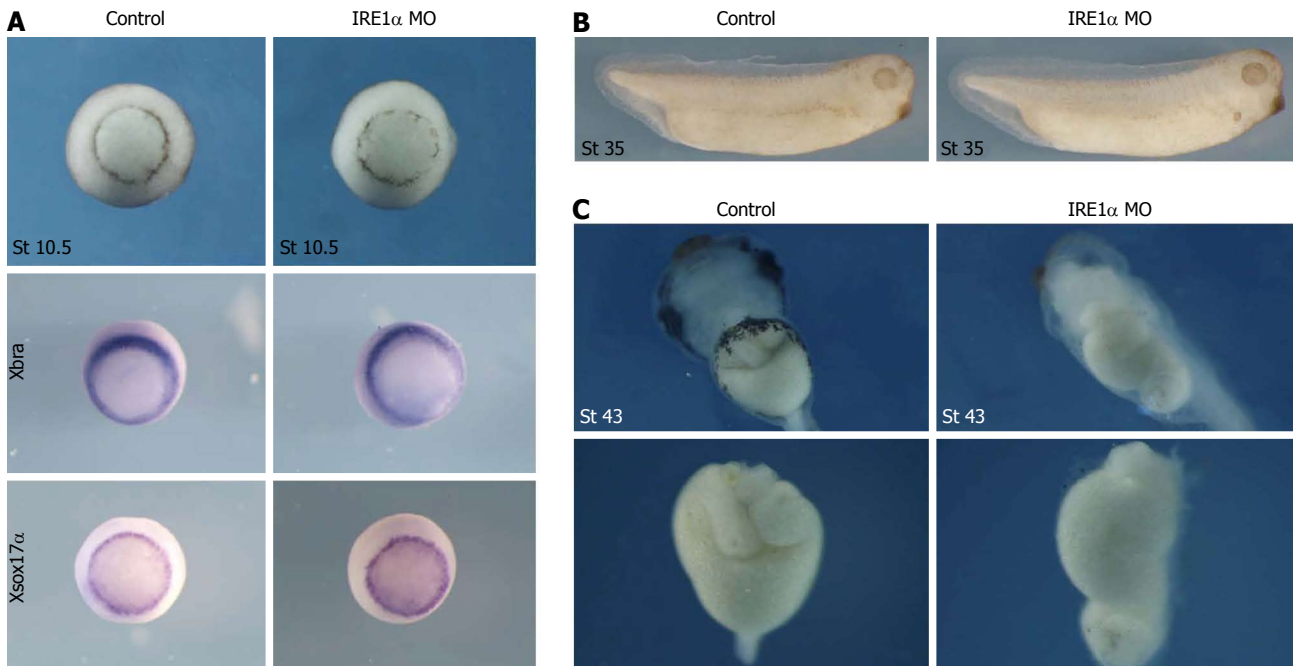


Figure 4 *Xenopus* inositol-requiring enzyme 1 α is required for gut development. A: Knockdown of x IRE1 α did not change the phenotype and the expression of Xbra and Xsox17 α at stage 10.5; B: The inositol-requiring enzyme (IRE) 1 α morpholino oligonucleotide (MO)-injected embryos were morphologically normal before the tailbud stages; C: IRE1 α knockdown upon injection of 50 ng of MO resulted in a gut defective phenotype. Surgically-resected guts from embryos were shown in C under panel. Coiled structure of gut was not detected in IRE1 α MO-injected embryos at stage 43. MO injection was repeated 5 times in a total of 278 embryos.



Figure 5 Rescue of xIRE1 α knockdown with IRE1 α -GR mRNA. Gut defective phenotype caused by 50 ng of morpholino oligonucleotide (MO) could be rescued by co-injection of 1 ng of inositol-requiring enzyme (IRE) 1 α -GR mRNA. Co-injection was done 3 times in a total of 258 embryos.

quired for proper gut development in *Xenopus*.

Deletion mutant of IRE1 α

IRE1 α is a Ser/Thr protein kinase and endoribonuclease that, upon activation, initiates the unconventional splicing of the mRNA of XBP1^[12]. The deletion mutant was constructed consisting of the N-terminal part without the C-terminal kinase and RNase domains (Figure 6A). Injection of IRE1 α Δ C-GR mRNA caused similar morphological alterations with gut malformation by interfering with the function of endogenous xIRE1 α (Figure 6B-D). These data further confirmed the notion that IRE1 α Δ C represents a dominant-negative form of IRE1 α .

XBP1 loss of function

IRE1 α is the most evolutionarily conserved branch of the UPR, and upon activation, initiates the unconventional splicing of the mRNA encoding the transcriptional

factor XBP1 to attenuate ER stress by mediating UPR. In other words, IRE1 α mediates XBP1 splicing. IRE1 α MO could knockdown IRE1 α expression, and then XBP1 splicing would be repressed. To test if knockdown of XBP1 might cause similar phenotype with IRE1 α knockdown, 50 ng of XBP1(C)MO was injected into four blastomeres at 4-cell stage for scoring the phenotype. As shown in Figure 7, injection of XBP1(C)MO caused similar phenotype with gut-coiling defect.

DISCUSSION

In eukaryotic cells, ER 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 post-translational modifications that are essential for protein function^[26,27]. Nascent polypeptides are translocated to the ER lumen in an unfolded state, where

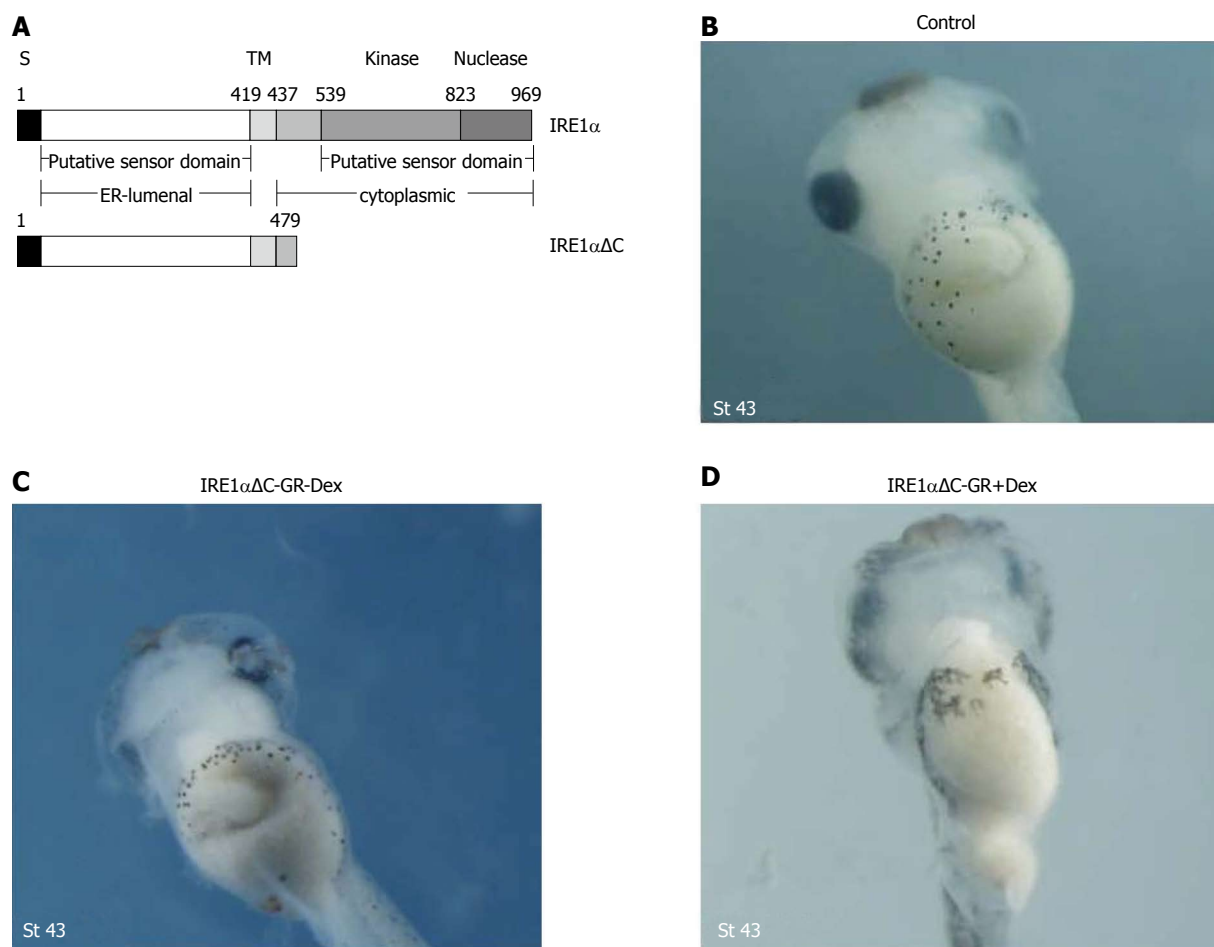


Figure 6 Injection of IRE1 α Δ C-GR mRNA caused morphological alterations with gut malformation. A: Diagram depicting the construction of deletion mutants. S and TM denote the signal peptide and the transmembrane domain; B-D: Gut defects were also observed with a dominant-negative mutant, IRE1 α Δ C, lacking the cytoplasmic kinase and RNase domains.



Figure 7 XBP1 knockdown affected gut development. Injection of XBP1(C) morpholino oligonucleotide (MO) caused similar phenotype with gut-coiling defect at stage 43.

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^[28,29]. This ER stress in turn leads to the activation of a series of adaptive pathways known as UPR to maintain ER homeostasis^[30]. The adaptive process, UPR,

has at least two distinct components^[4]. The first consists of the rapid and transient attenuation of new protein synthesis and can be considered to be an attempt on the part of the cell to limit the load on the folding apparatus in the ER. The second component consists of the upregulation of expression of genes whose products promote protein folding in the ER and degradation of malformed proteins. The latter UPR signaling pathways are transduced by three ER resident transmembrane proteins IRE1, PERK and ATF6 upon activation^[14], among which IRE1 functions as an endoribonuclease (RNase) to process XBP1 pre-mRNA to a mature form. Spliced XBP1 is a transcriptional activator that plays a fundamental role in the activation of a wide variety of UPR target genes^[31,32]. Two mammalian homologues of yeast IRE1 have been identified: IRE1 α and IRE1 β . IRE1 α is expressed in most cells and tissues, with highest levels of expression in the pancreas and placenta^[17]. IRE1 β expression is prominent only in intestinal epithelial cells^[13].

Accumulation of malformed proteins in the ER occurs under many pathophysiological conditions, in addition, it also takes place during embryonic development. During embryogenesis of vertebrates, such as *Xenopus*, germ layer induction, pattern formation, and morphoge-

netic movement are known to be mediated by secreted proteins, including fibroblast growth factors (FGFs)^[33], TGF- β /nodal/BMPs, and Wnts^[34,35]. Therefore, dysfunction of the ER should also interfere with the secretion of these proteins and consequently disrupt early embryonic development^[36].

Although the effect of ER stress on cellular physiology has been extensively investigated, little is known so far about how it affects early embryonic development. The previous study has verified the conservation of IRE1/XBP1 pathway in *Xenopus* embryos and the importance of IRE1 β for mesoderm formation in *Xenopus* embryos^[23]. In this study, we demonstrated that (1) IRE1 α mainly expressed in pancreas at tadpole stages and IRE1 α protein could be detected at all developmental stages analyzed (Figure 1); (2) although it did not cause overt phenotypes upon overexpression (Figure 2), specific knockdown of IRE1 α (Figure 4) or XBP1 (Figure 7) led to a gut-coiling defect, and injection of xIRE1 α Δ C-GR mRNA caused similar morphological alterations with gut malformation (Figure 6); and (3) IRE1 α -GR mRNA can rescue IRE1 knockdown phenotypes (Figure 5).

In conclusion, our loss and gain of function data support the notion that IRE1 α is required for gut development in *Xenopus* embryos. Our results demonstrate that homeostasis of ER and xIRE1 α functions are required for gut development in *Xenopus* embryos. We infer from our results that IRE1 regulates gut development through IRE1 α -XBP1 pathway.

COMMENTS

Background

Inositol-requiring enzyme 1 α (IRE1 α) is an endoplasmic reticulum (ER)-located type I transmembrane protein with a kinase domain and RNase domain in the cytosolic region. IRE1 α induces the unconventional splicing of XBP1 mRNA under ER stress condition. However, a XBP1-independent IRE1 α function also exists. IRE1 α is expressed ubiquitously in fetal and adult mice and is essential for mammalian developmental processes. However, the function of IRE1 α in specific organs and tissues remains incompletely understood.

Research frontiers

IRE1 α is confirmed to be essential during mammalian development. However, the IRE1 α conventional knockout mice showed embryonic lethality, and it has been reported that during development IRE1 α is required for B-cell differentiation, placental development and embryonic viability. And the function of IRE1 α in specific organs and tissues deserves to be illustrated.

Innovations and breakthroughs

Compared with previous studies, this study used the *Xenopus laevis* as animal model to study the function of IRE1 α during their development. After overexpressing and knockdown of the IRE1 α , the phenotype could be analyzed. And the results showed that at early stage, IRE1 α did not play a significant role in germ layer formation, however, at the stage of organogenesis, knockdown of IRE1 α or XBP1 caused gut-coiling defect. The results suggested that IRE1 α is not required for germ layer formation, but for gut development in *Xenopus laevis* and it may function via XBP1-dependent pathway.

Applications

The results in this study showed that IRE1 α does play a role in organogenesis, however, two questions remain to be answered: which organs or tissues in gut are affected and what is the underlying mechanism.

Peer review

This is an interesting article. The authors show in a systematic manner that the role of IRE1 α in the gut development of *Xenopus*. They also show that it acts through the XBP1 processing.

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