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Endoplasmic Reticulum Stress Induced by Tunicamycin Disables Germ Layer Formation in *Xenopus laevis* Embryos

Li Yuan,[†‡] Ying Cao,[†] and Walter Knöchel[*]

Maintenance of endoplasmic reticulum (ER) homeostasis is essential for correct protein targeting and secretion. ER stress caused by accumulation of unfolded or misfolded proteins leads to disruption of cellular functions. We have investigated the effect of ER stress on *Xenopus* embryogenesis. ER stress induced by tunicamycin (TM) treatment of embryos resulted in defects affecting germ layer formation. We observed up-regulation of ER stress response genes, enhanced cytoplasmic splicing of *xXBP1* RNA, and increased rate of apoptosis. In animal cap assays, TM treatment inhibited mesoderm formation induced by overexpression of *activin/nodal* RNA but did not affect mesoderm formation induced by functional activin protein, suggesting that dysfunction of ER caused a failure in activin/nodal processing and/or secretion. The observation that activin protein renders mesoderm formation under ER stress strengthens the role of activin/nodal for mesoderm induction. The results underline the functional significance of ER homeostasis in germ layer formation during *Xenopus* embryogenesis. *Developmental Dynamics 236:2844–2851, 2007.*

Key words: tunicamycin; activin/nodal; cap assay; endoplasmic reticulum; *Xenopus laevis*

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INTRODUCTION

In eukaryotic cells, endoplasmic reticulum (ER) is the site for biosynthesis of sterols, lipids, membrane-bound and secreted proteins, and glycoproteins (Kaufman, 2004). The 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 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). Dysfunction of ER affects many aspects of cell physiology, such as protein targeting and secretion. Accumulation of unfolded or misfolded proteins resistant to proteasomal degradation in the ER can completely disrupt ER and cellular function, resulting in apoptosis of cells (Boyce and Yuan, 2006). ER stress has been proven to be the cause of some human diseases (Xu et al., 2005). Moreover, UPR plays critical roles in differentiation and function of professional secretory cells, such as plasma cells, pancreatic β-cells, hepatocytes, and osteoblasts (Wu and Kaufman, 2006).

Although the effect of ER stress on cellular physiology has been exten-

Institute of Biochemistry, University of Ulm, Ulm, Germany
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[†]Li Yuan and Ying Cao contributed equally to this work.
[‡]Li Yuan's present address is Department of Biochemistry and Molecular Biology, Nanjing Medical University, 210029 Nanjing, China.
[*]Correspondence to: Dr. Walter Knöchel, Institut für Biochemie, Universität Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany. E-mail: walter.knoechel@uni-ulm.de

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sively investigated, little is known so far about how it affects early embryonic development. During embryogenesis of vertebrates, such as *Xenopus*, germ layer induction, pattern formation, and morphogenetic movements are known to be mediated by secreted proteins, including FGFs (Ornitz and Itoh, 2001; Bottcher and Niehrs, 2005), TGF-β/nodal/BMPs, and Wnts (Kimelman, 2006; Clevers, 2006). At the onset of gastrulation, a small part of the dorsal mesoderm, known as the Spemann organizer, is specialized for the secretion of many proteins that are required for embryonic patterning, neural induction, and self-regulation (De Robertis and Kuroda, 2005; De Robertis, 2006). Therefore, dysfunction of the ER should also interfere with the secretion of these proteins and consequently disrupt early embryonic development. In the present study, we investigated the effect of increased ER stress on *Xenopus* embryogenesis by treating the embryos or explants with the nucleoside antibiotic tunicamycin (TM), an inducer of ER stress, which structurally resembles UDP-N-acetylglucosamine and prevents the formation of the dolichol intermediate being necessary for N-linked protein glycosylation. A former study described the morphological alterations in *Xenopus* early embryos caused by TM treatment (Romanovsky et al., 1985). However, the underlying mechanisms were not addressed. We here found that TM treatment resulted in an increased cytoplasmic splicing of *xXBP1* RNA leading to the active form of transcription factor xXBP1 and in an up-regulation of ER stress related genes such as *BiP* or *CHOP*. TM-induced ER stress in whole embryos or vegetal-equatorial explants caused apoptosis, an inhibition of mesoderm and endoderm formation, and consequently a failure of morphogenetic movements. These defects in embryogenesis obviously correlate to the malfunction of ER that cannot secrete functional proteins. In animal cap assays, increased ER stress did not affect mesoderm formation induced by functional activin A protein but inhibited mesoderm formation induced by *activin/nodal* RNA injection. Therefore, maintenance of ER homeostasis is crucial for proper processing and secretion of activin/nodal ligands to induce mesoderm formation.

RESULTS AND DISCUSSION

Tunicamycin Treatment Induces Developmental Defects in *Xenopus* Embryos

In order to investigate the effect of increased ER stress on embryonic development, we used different concentrations of tunicamycin (TM) to induce ER stress in early embryos. After fertilization, embryos were cultured in 0.1 × MBSH containing TM until control embryos had reached stage 8.5. At concentrations higher than 3 μg/ml, treated embryos died before or during gastrulation. At a concentration of 2.5 μg/ml TM, embryos could survive but were severely affected. During neurulation, untreated embryos developed with normal blastopore closure and neural fold formation (Fig. 1A). TM-treated embryos showed no blastopore closure and no clearly visible neural fold formation. Consequently, the yolk plug exposed exteriorly due to failure in morphogenetic movements (Fig. 1B). With ongoing development, these embryos showed severe cytolysis, resulting in death of all treated embryos at early tailbud stages. Finally, we treated embryos with an even lower concentration of 2 μg/ml TM. Embryos showed less severe phenotypic alterations (Fig. 1C). However, the neural folds did not close and remained wide open at the end of neurulation. When control embryos reached tailbud stage, TM-treated embryos still exhibited open neural folds and developed with severe inhibition of anterior-posterior axis elongation and loss of head structure (Fig. 1D,E).

Tunicamycin Treatment Enhances Cytoplasmic Splicing of *xXBP1* and Induces Up-Regulation of ER Stress Response Gene Expression

ER stress in cells leads to an up-regulation of genes in response to ER stress, like *BiP* and *CHOP*. *BiP* encodes a chaperone, which when occupied by unfolded proteins, releases ER transmembrane proteins involved in the induction of UPR (Xu et al., 2005). *CHOP* codes for a bZIP transcription factor involved in the apoptosis pathway mediated by ER stress or DNA damage (Oyadomari and Mori, 2004; Marciniak et al., 2004). Moreover, CHOP also mediates the specification of body axis by inhibiting the Wnt/TCF signaling pathway (Horndasch et al., 2006). We have treated embryos with 2 μg/ml TM from stage 1 to 8.5, and isolated RNA from treated and untreated embryos at stages 11, 19, and 30, respectively, which was subjected to real time RT-PCR. At stage 11, *BiP* expression was not changed significantly in response to TM treatment. However, during neurula and tailbud stages, it was notably up-regulated (Fig. 2A). In contrast, *CHOP* was already up-regulated at stage 11. At stages 19 and 30, an even higher increase in *CHOP* expression was observed in embryos treated with TM (Fig. 2B). Moreover, the fold induction of *CHOP* is more dramatic than that of *BiP*, suggesting that *CHOP* transcription is more sensitive to ER stress than *BiP*. Since CHOP promotes apoptosis mediated by ER stress, the dramatic increase in its expression explains the death of embryos treated with different doses of TM. XBP1 is not only a key regulator in the unfolded protein response by promoting the synthesis of molecular chaperones required for ER (Lee et al., 2003) and by increasing the volume of ER (Sriburi et al., 2004; Ron and Hampton, 2004); it is also required for mesoderm specification in *Xenopus* (Zhao et al., 2003; Cao et al., 2006a). Unspliced *XBP1* mRNA encodes a protein that is functionally inactive. Under ER stress, *XBP1* mRNA undergoes an unconventional cytoplasmic splicing by IRE1, thereby generating a functional transcription factor (Yoshida et al., 2001). We, therefore, tested the effect of TM treatment on *xXBP1* splicing in *Xenopus* embryos. Embryos were treated in the same way as described above. RNA was isolated at different stages and subjected to semi-quantitative RT-PCR. As reported previously, in normal embryos at stage 11 the cytoplasmic splicing was replaced by conventional, probably nuclear, splicing (Cao et al., 2006a). However, the cytoplasmic splice form of *xXBP1* (*xXBP1(C)*) was

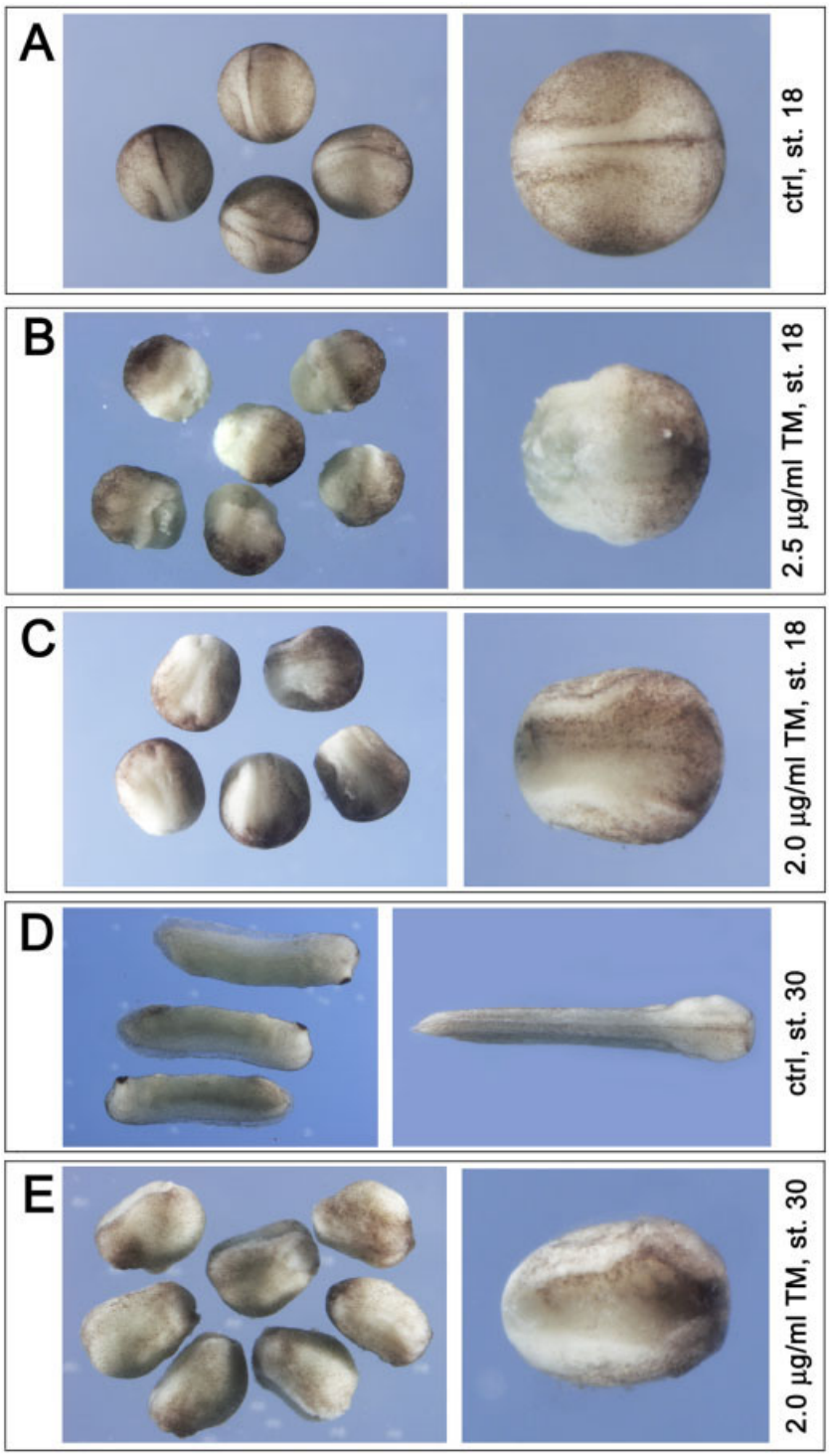

Fig. 1. Embryos treated with TM from stage 2 until stage 8.5 showed severe developmental defects. **A:** Control embryos at stage 18. **B:** At stage 18, embryos treated with 2.5 μg/ml TM developed with widely open blastopore, displayed no neural fold formation and died soon after neurulation. **C:** At stage 18, embryos treated with 2 μg/ml showed less severe phenotypes but still displayed open neural folds. **D:** Control embryos at stage 30. **E:** At stage 30, embryos treated with 2 μg/ml TM developed with open neural fold and body axis defects. Right panels show dorsal views of embryos. Anterior is to the right.

present in TM-treated embryos (Fig. 2C). During neurula and tailbud stages, when cytoplasmic splicing occurs in normal embryos, the expression of *xXBP1(C)* was significantly enhanced in TM-treated embryos, whereas the corresponding nuclear splice variant was decreased (Fig. 2C). The results demonstrated that ER stress induced by TM could primarily induce or promote cytoplasmic splicing of *xXBP1*.

TM Treatment Induces Apoptosis in Embryos

The dramatic increase of *CHOP* expression in embryos treated with TM led us to examine whether TM could indeed induce apoptosis in TM-treated embryos. Untreated embryos and embryos treated with 2 μg/ml of TM were collected at the neurula stage and TUNEL assay was carried out to detect apoptosis. In most of the untreated embryos, no apoptotic signal was detected. A few apoptotic cells were present in only a minor part of the embryos (15%, n = 41. Fig. 3A). In contrast, apoptotic cells were detected in most of the treated embryos (73%, n = 45. Fig. 3B), although the numbers of apoptotic cells varied in different embryos. In summary, the TUNEL assay demonstrated that increased ER stress causes apoptosis. This result may provide an explanation to the observation of cytolysis and subsequent death of treated embryos.

TM Treatment Inhibits Expression of Marker Genes for Germ Layer Formation

Since TM treatment of early embryos led to gastrulation defects, we examined if mesendoderm formation was disrupted in these embryos. Embryos treated with 2.5 μg/ml TM were collected and subjected to real-time RT-PCR to test the expression of genes marking the formation of germ layers. We found that expression of the pan-mesodermal gene *Xbra* was significantly repressed. Accordingly, ventral mesodermal genes such as *Xvent1, Xvent2*, and *Xwnt8*, and dorsal mesodermal genes such as *chordin* and *gsc*, were all inhibited (Fig. 4). Therefore, mesoderm formation was inhibited in response to TM treatment (Fig. 4). Furthermore, we observed that genes responsible for endoderm formation, like *Xsox17α* and *Mixer*, and additionally, two genes representing neuroectoderm, *Xsox2* and *Xsox3*, were also significantly inhibited in embryos

treated with TM (Fig. 4). In summary, these results reveal that TM treatment of early embryos resulted in an inhibition of marker genes for ectodermal, mesodermal, and endodermal germ layers.

TM Treatment Has No Effect on Activin A Induced Mesoderm Formation in Animal Caps But Inhibits Mesoderm Formation in Vegetal-Equatorial Explants

We have shown that TM-induced ER stress inhibits mesoderm formation during gastrulation. As mesoderm is induced by the activin/nodal signaling pathway (Asashima et al., 1990; Smith et al., 1990; Tiedemann et al., 1992; Agius et al., 2000), we further tested if TM treatment had any influence on the activity of activin A in mesoderm induction using animal cap assays. Embryos were treated with 2 μg/ml TM from 2-cell stage till stage 8.5. Animal caps were excised from TM treated or untreated embryos and then half of the caps were incubated in culture medium containing activin A. When control sibling embryos had reached neurula stage, naive animal caps without treatment had developed into epidermis (Fig. 5A). Those caps from TM-treated embryos showed no discernible morphological difference (Fig. 5C). We then examined the effect of TM treatment on mesoderm induction by activin A. As control, activin A induced animal caps from embryos without TM treatment to elongate because of mesoderm formation (Fig. 5B). Unexpectedly, caps treated with both TM and activin A did also elongate, suggesting that mesoderm formation induced by activin A was not altered by previous TM treatment (Fig. 5D). We postulated that the period for TM treatment might not have been sufficient. However, prolonged TM treatment until the time point, when activin A treatment was stopped, also did not lead to a discernible reduction of activin A activity (data not shown).

When control sibling embryos had reached stage 18, caps were subjected to real time RT-PCR to examine the expression of marker genes for mesoderm differentiation. In animal caps without treatment or with TM treatment alone, expression of *Xnot,* α-*actin*, and *XMyoD* was very low or barely detectable. In contrast, all these genes were dramatically induced in caps treated with activin A, irrespective of previous TM treatment of embryos or not (Fig. 5E). In conclusion, previous TM treatment of embryos does not block or interfere with the activity of activin A protein in inducing mesoderm differentiation in animal caps. The results also suggest that ER stress does not block the function of the activin signal transduction pathway in mesoderm induction.

We next examined how the vegetal-equatorial (VE) explants, where mesoderm and endoderm form, responded to increased ER stress. Embryos were treated with TM at 2 μg/ml starting from a 2-cell stage. At stage 8.5, VE explants were removed from treated and untreated embryos. When control embryos reached the neurula stage, VE explants from untreated embryos exhibited apparent elongation due to mesoderm differentiation (Fig. 6A). In contrast, the explants from TM-

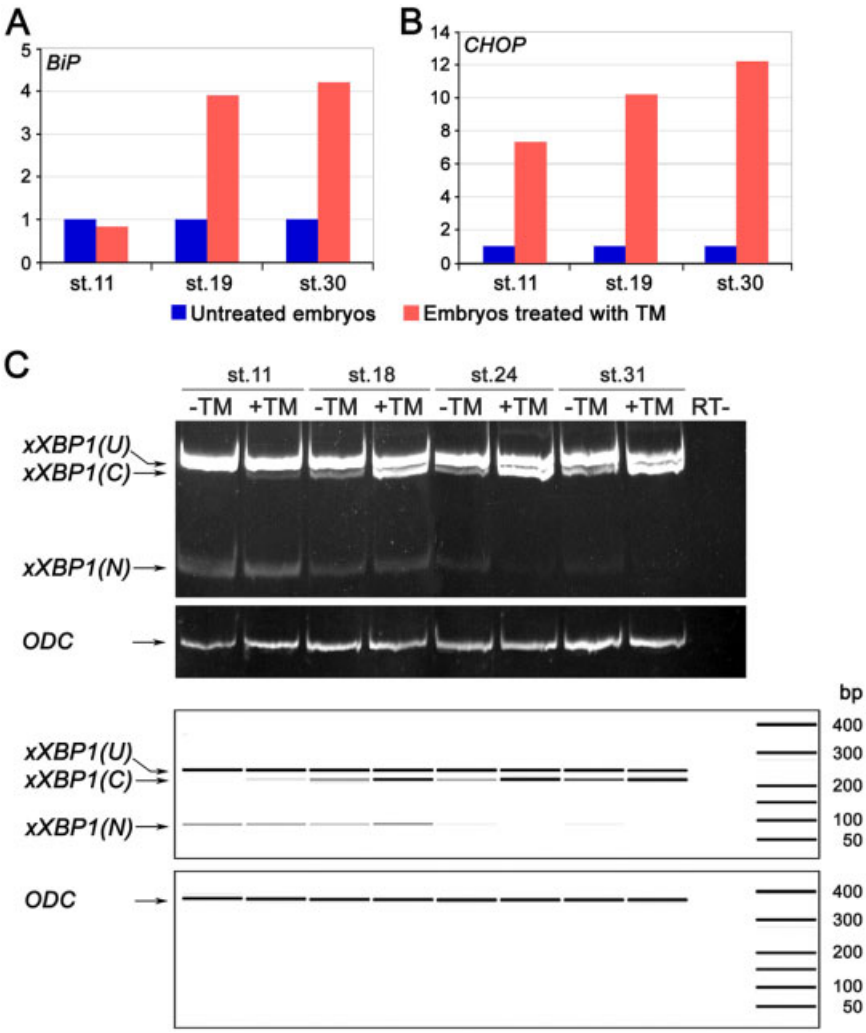

Fig. 2. Regulation of ER stress-mediated genes. **A:** Real time RT-PCR demonstrated that expression of *BiP* did not change significantly in TM-treated embryos at stage11, but increased at stages 19 and 30. **B:** Expression of *CHOP* showed strong up-regulation at all three stages examined. **C:** Cytoplasmic splicing of *xXBP1* was affected by TM treatment. RT-PCR detected stronger bands representing the cytoplasmic splice variant of *xXBP1* (*xXBP1(C)*) in TM-treated embryos (+TM) than in control embryos (−TM) at stages indicated. *ODC* (*ornithine decarboxylase*) served as a loading control. Photographic gel documentation is shown in the top panel; result obtained by a Bioanalyzer 2100 instrument (Agilent Biotechnologies) is shown in the bottom panel. It represents the migration of DNA bands run in a mini electrophoresis chip. Strength of bands (measurement of fluorescence signals) reflects the amount of DNA. *xXBP1(U)*, unspliced *xXBP1; xXBP1(N)*, nuclear spliced *xXBP1*.

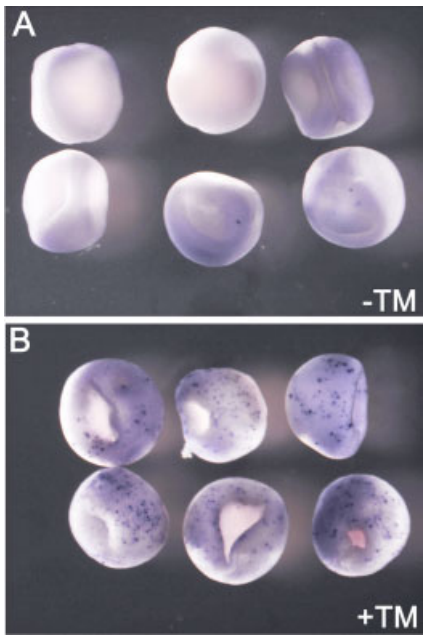

Fig. 3. TM treatment induced apoptosis in embryos. **A:** At stage 18, embryos without TM treatment showed very few signals representing apoptotic cells. **B:** The number of cells undergoing apoptosis increased dramatically in TM-treated embryos.

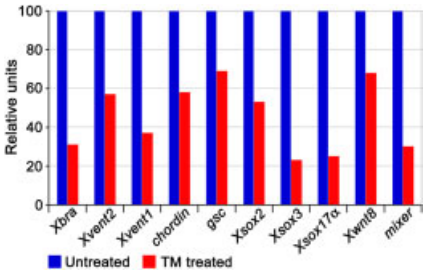

Fig. 4. Gene expression analysis on embryos treated with TM. Treated and untreated embryos were collected at stage 11 and subjected to real time RT-PCR. Expression of mesodermal, endodermal, and neuroectodermal genes were inhibited in TM-treated embryos.

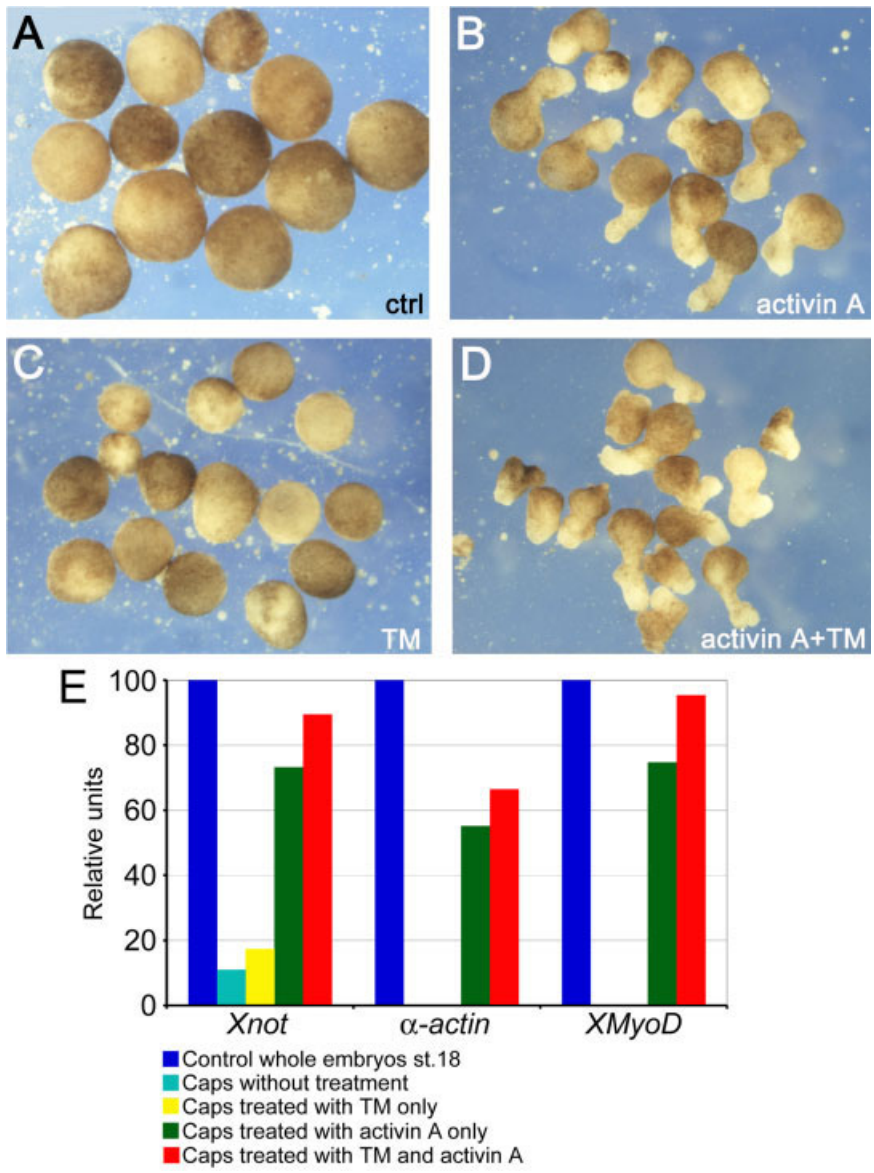

Fig. 5. TM treatment did not affect mesoderm differentiation induced by mature activin A. **A:** Naïve caps without treatment. **B:** Caps treated with activin A at 10 ng/ml. **C:** Caps from embryos treated with TM showing no difference from those in A. **D:** Caps from embryos treated with TM still showed an elongation induced by activin A. **E:** Real time RT-PCR confirmed no significant effect of previous TM treatment on expression of mesodermal genes in animal caps.

treated embryos did not elongate (Fig. 6B), suggesting that mesoderm differentiation was perturbed. We also examined the expression of mesodermal marker genes in these explants by real time RT-PCR (Fig. 6C). A significant reduction of *Xnot,* α-*actin*, and *XMyoD* was observed in VE explants of embryos treated with TM. This observation is in agreement with the finding that TM treatment inhibits mesodermal gene expression in whole embryos (Fig. 4). It is known that secreted proteins, notably nodal/activins, play essential roles in mesoderm differentiation. In whole embryos or VE explants, proper secretion of these factors is a prerequisite for their function. Under the condition of increased ER stress by TM treatment, ER function is disrupted. Consequently, the newly translated proteins cannot undergo correct folding or further posttranslational modification. Proteins generated in this way are thus nonfunctional and the pathways for mesoderm induction cannot be triggered. In the case of the animal cap assay, previous TM treatment has no significant influence on mesoderm differentiation, because the inducer activin A is already a mature and functional protein that, unlike the endogenous precursor protein, does not have to go through ER stress-sensitive posttranslational modification. In contrast to their precursors, both human and *Xenopus* wild-type mature activin proteins do not contain N-linked glycosylation sites. This could explain that functional activin A protein is sufficient to render inducing activity

under TM-induced ER stress condition. However, elongation of caps is still surprising, because the elongation movement is generally thought to be coupled with glycoproteins. We can only speculate that cells of the animal cap possess a higher maternal store of these proteins or are less sensitive to TM treatment than equatorial or vegetal cells. At least, the morphology of TM-treated embryos or explants suggests that presumptive ectodermal cells are less affected than presumptive mesoderm or endoderm (Figs. 1, 5, and 6).

TM Treatment Inhibited Mesoderm Formation in Animal Caps Injected With *activin/nodal* mRNA

To test whether deficiencies in the maturation of TGF-β like molecules can account for the failure of mesoderm induction under ER stress conditions, we have investigated overexpression of *Xnr5* mRNA in animal caps. *Xnr5* encodes a nodal-related protein in *Xenopus* that, when overexpressed, induces mesoderm formation in animal caps (Takahashi et al., 2000). Under the condition of increased ER stress, we expected that injected *Xnr5* mRNA would not be able to induce mesoderm formation effectively. We thus injected 50 pg of *Xnr5* into 2-cell stage embryos; one half of the embryos were treated with 2 μg/ml TM and the other half were left untreated. Animal caps were excised from uninjected and *Xnr5* injected embryos at stage 8.5 and cultured until the neurula stage. Without TM treatment, *Xnr5* injected caps revealed significant elongation (Fig. 7C). In contrast, caps isolated from

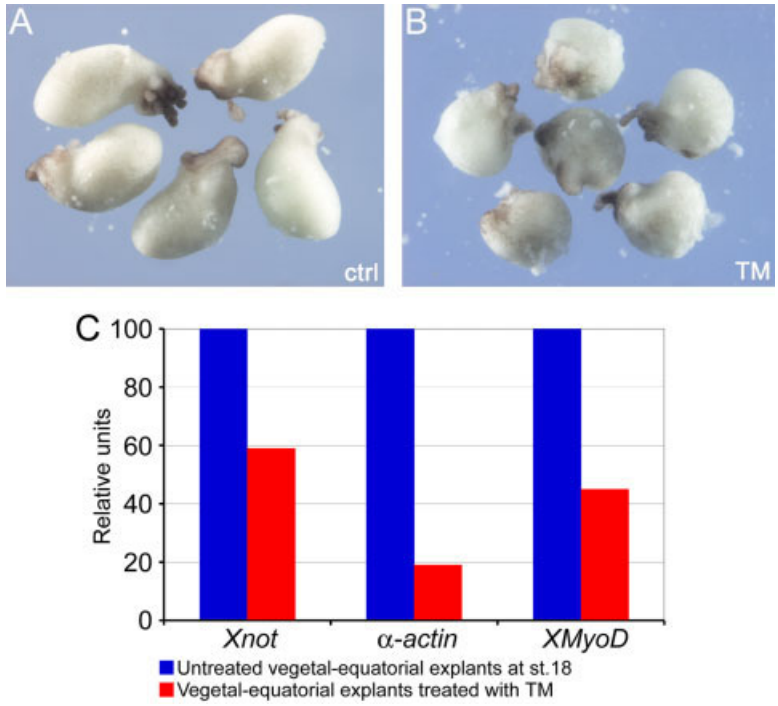

Fig. 6. TM treatment affected mesoderm differentiation in vegetal-equatorial explants. **A:** Untreated vegetal-equatorial explants at stage 18 showed elongation. **B:** Vegetal-equatorial explants from embryos treated with TM did not elongate. **C:** Real time RT-PCR showed inhibition of mesodermal gene expression in VE explants from embryos treated with TM.

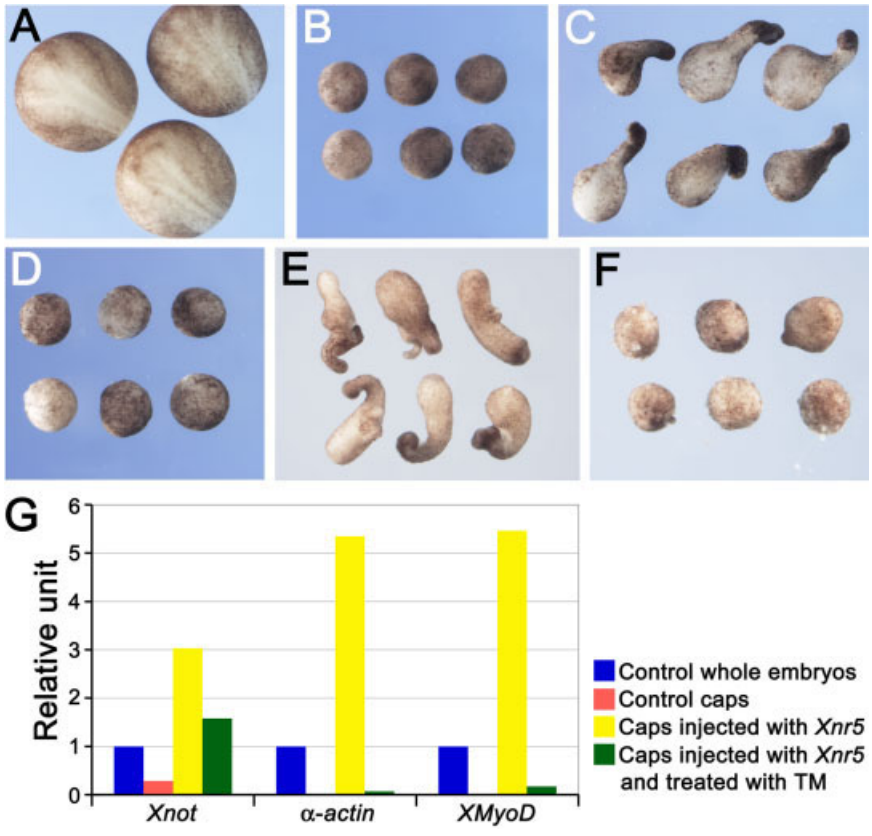

Fig. 7. TM treatment inhibited mesoderm formation in animal caps of embryos injected with *Xnr5/activin* mRNA. **A:** Control embryos. **B:** Control caps without RNA injection and TM treatment. **C:** Caps from embryos injected with *Xnr5* mRNA showing significant elongation. **D:** Caps from embryos injected with *Xnr5* mRNA and treated with TM showing no elongation anymore. **E:** Caps from embryos injected with *activin* mRNA elongate. **F:** Caps from embryos injected with *activin* mRNA and treated with TM do not elongate. **G:** Real-time RT-PCR revealed that TM treatment inhibited mesoderm formation induced by *Xnr5* injection.

TABLE 1. Primers and Conditions Used for Real Time RT-PCR

Primer pairs	Sequences (5′→3′)	Denaturation temperature (°C)	Annealling temperature (°C)/time(s)	Extension temperature (°C)/time(s)
BiP	F: CATCTAATCCAGAGAACACCGTGT R: GGTACTTGATGTCCTGCTGAACAG	95	55/5	72/5
CHOP	F: AAAGTGTCCCATTTGTTAGCTGAAA R: CCTGGTCTGCTCCACTTCCTT	95	55/5	72/5
Xvent2	F: TCTCCTCAGTAGAATGGCTTGCT R: GAATATCGCTGATCCACTTTGCT	95	55/5	72/5
Xvent1	F: TGAAGAGGCAGATACAAGACAAGC R: GGACAGGAAAGCCACCAGG	95	55/5	72/5

Xnr5-injected and TM-treated embryos did not elongate anymore, suggesting that mesoderm formation was blocked (Fig. 7D). Activins and Xnrs can both utilize type I receptors of the ALK family, but differ in cripto, a coreceptor of nodal signaling (Kumar et al., 2001; Yeo and Whitman, 2001). Fucosylation of cripto has been shown to be required for its ability to facilitate nodal signaling (Schiffer et al., 2001). Therefore, it might be argued that failure of elongation after *Xnr5* injection under ER stress is a specific effect for nodal. However, injection of *activin* mRNA led to the same result. Whereas animal caps of embryos injected with *activin* mRNA showed elongation, treatment with TM completely abolished this effect (Fig. 7E and F). We then examined the expression of mesodermal marker genes using real-time RT-PCR. In control whole embryos and caps from *Xnr5*-injected embryos without TM treatment, there was strong expression of *Xnot,* α*-actin*, and *XMyoD*. However, expression of these genes was severely repressed in caps from *Xnr5*-injected and TM-treated embryos (Fig. 7G). Therefore, mesoderm formation induced by *activin*/*nodal* mRNA overexpression was indeed blocked by increased ER stress in animal caps. In this context, it has to be mentioned that previous studies already suggested that TM treatment appeared to block the secretory exit of TGF-β1 and led to an increase of the cellular associated, nonglycosylated pro-TGF-β1 form (Sha et al., 1989). On the other hand, functional activin A protein is sufficient to render mesoderm formation under TM-induced ER stress condition. Due to the fact that various signaling pathways have been implicated in mesoderm formation, this result was rather unexpected and it strengthens the important role of the activin/nodal pathway for induction of mesodermal tissue.

EXPERIMENTAL PROCEDURES

Tunicamycin Treatment of Embryos

Tunicamycin (TM) (Sigma) was dissolved in DMSO to make a stock solution of 5 mg/ml. Fertilized eggs or 2-cell stage embryos were cultured in 0.1 × MBSH (1 × MBSH: 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 10 mM Hepes, pH 7.4) containing TM at the indicated concentrations. Embryos grown in culture media without TM were used as control. When control sibling embryos reached mid-blastula stage, treated embryos were transferred to fresh media without TM. Embryos or explants were collected at the desired stages and subjected either to microscopy or to RT-PCR.

In Vitro RNA Synthesis and Microinjection

To prepare *Xenopus activin* and *Xnr5* RNAs for microinjection, pSP64T-activin plasmid was linearized with EcoRI and transcribed with SP6 mMessage mMachine kit (Ambion); pNRRX-Xnr5 plasmid was cut with XbaI and transcribed with T7 mMessage mMachine kit (Ambion). *Activin* RNA was injected at a total dose of 0.3 pg per embryo, and *Xnr5* was injected at 50 pg per embryo.

Real Time RT-PCR and Semi-quantitative RT-PCR

Real time RT-PCR was performed and primers for α*-actin, Xbra, chordin, gsc, XMyoD, Xnot, Xsox2, Xsox3, Xsox17*α *Xwnt8*, and *mixer* were as described previously (Cao et al., 2006b). Additional primers are listed in Table 1. Semi-quantitative RT-PCR was used to detect unspliced, cytoplasmic splice, and nuclear splice variants of *xXBP1* transcript according to the method previously described (Cao et al., 2006a). In addition, PCR products were also electrophoretically separated using DNA 1000 kits (series II) on the Bioanalyzer 2100 (Agilent Biotechnologies).

Animal Caps and Vegetal-Equatorial Explants

TM treatment was performed in the same way as described above. Animal caps were excised from treated or untreated control embryos at stage 8.5. Caps were split into two parts; one part was cultured in 0.5 × MBSH containing no activin A, and the other was treated with 10 ng/ml activin A (Sigma) for 3 hr. To perform prolonged TM treatment, caps from TM-treated embryos were further cultured for an additional 3 hr in TM-containing solution. The remaining parts of embryos after removal of animal caps were used as vegetal-equatorial (VE) explants. VE explants were excised from untreated and TM-treated embryos.

TUNEL Assay

TUNEL assay on whole embryos was done using the protocol described (Hensey and Gautier, 1998).

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