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*Original Article*

# Xwig1, a novel putative endoplasmic reticulum protein expressed during epithelial morphogenesis and in response to embryonic wounding

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**ABSTRACT In a subtractive differential screening, we identified a novel gene with interesting characteristics, termed *Xenopus* wounding induced gene 1 (*Xwig1*). *Xwig1* encodes a novel protein of 912 amino acids containing 13 putative transmembrane segments and an evolutionarily conserved carboxy-terminal domain. Protein localization studies revealed that Xwig1 is anchored in cytoplasmic structures, presumably the endoplasmic reticulum. Expression is largely confined to epithelial cells in regions that undergo morphogenetic processes, such as blastopore closure, hindgut closure, dorsal closure and optic vesicle invagination. Interestingly, *Xwig1* transcription is activated in response to embryonic epidermal wounding. The wounding-induced transcription occurs downstream of the transient phosphorylation of extracellular signal-regulated protein kinases and is in part mediated by Elk-1, but independent of dissection-induced FGF signalling. Thus, *Xwig1* provides a molecular link between epithelial morphogenesis and wound healing.**

KEY WORDS: *Xenopus laevis, wound healing, morphogenesis, endoplasmic reticulum.*

## Introduction

During embryonic development cell-cell communication is of crucial importance for pattern formation and morphogenetic events. The vertebrate embryonic body plan is set-up during gastrulation by intercellular signals, originating in the Spemann-Mangold or gastrula organizer (Harland and Gerhart, 1997; Nieto, 1999). Most of these intercellular signals act as growth factor antagonists, such as Chordin and Noggin which are BMP inhibitors, Frzb-1 which inactivates Wnt ligands and Cerberus, which acts as a multivalent growth factor antagonist, inhibiting BMP, Wnt and Nodal-related ligands (Nieto, 1999; Bouwmeester, 2000). Through this extracellular inhibitory mechanism cells in all three germ layers acquire their fate and corresponding morphogenetic behaviour.

Most of the downstream target genes identified so far encode a variety of transcriptional regulators that act as signal-induced effectors. So far, very few structural or other components have been identified that act cell autonomously, downstream of the signal-induced effectors, to co-ordinate cellular processes, such as cell shape changes, cell polarity or cell repair mechanisms.

Here we present the molecular cloning and characterization of a novel putative endoplasmic reticulum (ER) protein, termed Xwig1, an acronym for *Xenopus* wounding induced gene 1. *Xwig1* encodes a multipass membrane spanning protein with an evolutionarily conserved carboxy-terminal domain. *Xwig1* is primarily expressed in epithelial cells in structures that undergo morphogenetic changes, such as the rim of the blastopore lip. Furthermore, we demonstrate that *Xwig1* is activated in response to embryonic epithelial wounding, downstream of extracellular signal-regulated protein kinase (ERK) activation. The wounding-induced up-regulation is transient and is in part mediated by Elk/Ets proteins, but not by FGF-induced signal transduction. Thus, *Xwig1* provides a convenient molecular marker to study the parallels between wound healing and morphogenetic events (Martin, 1997; Grose and Martin, 1999).

*Abbreviations used in this paper:* BMP, bone morphogenetic protein; ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinase; EST, expressed sequence tag; FGF, fibroblast growth factor; GFP, green fluorescent protein; JNK, jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; SRE, serum response element; *Xwig1*, *Xenopus* wounding induced gene 1.

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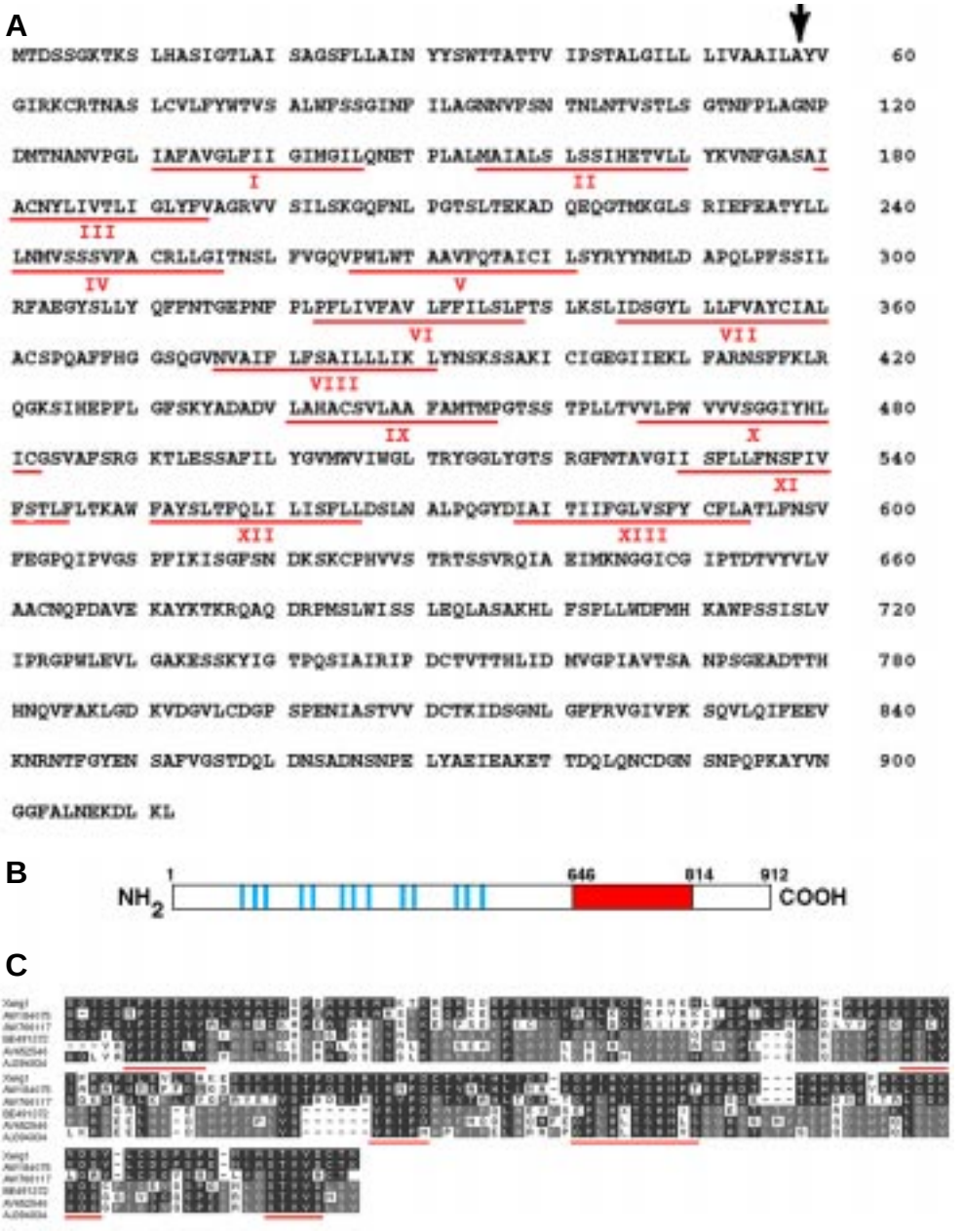


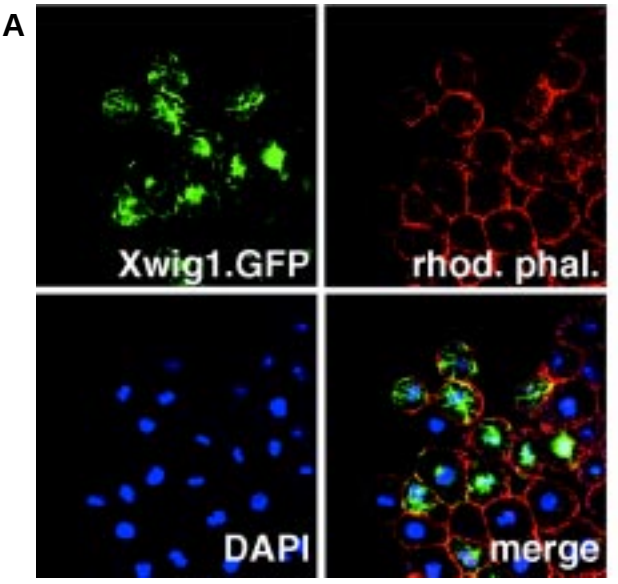

**Fig. 1. Predicted amino acid sequence of Xwig1 and homology with putative proteins encoded by different vertebrate ESTs. (A)** *Amino acid sequence of Xwig1. The black arrow indicates the presumptive signal peptide cleavage site and underlined in red are the numbered hydrophobic segments.* **(B)** *Schematic representation of Xwig1. Indicated are the putative transmembrane regions (blue) and the evolutionarily conserved carboxy-terminal domain (red).* **(C)** *Amino acid sequence alignment between the carboxy-terminus of Xwig1 (amino acids 646-814), zebrafish EST AW184075, human EST AV652546, chick EST AJ394934 and Xenopus ESTs AW766117 and BE491372. Underlined in red are conserved amino acids between all proteins. The genebank accession number for Xwig1 is AF310008.*

## Results

### *Molecular cloning and characterization of* Xenopus wig1

In our continuing effort to isolate novel genes expressed in the Spemann-Mangold organizer we identified in a subtractive differential screening a partial 3'-UTR fragment, that showed an interesting novel expression profile. A full-length cDNA of 2988 bp was assembled from a 2.5 kb partial cDNA, isolated from a tailbud cDNA library, and a 5'-RACE extension fragment. The resulting contig contains a long open reading frame that is preceded by an in frame termination codon, indicating that it is indeed full-length. This is furthermore supported by Northern analysis which revealed the presence of a single transcript of ~3.1 kb (data not shown). Based on the finding that this novel gene was activated in response to embryonic wounding we termed it *Xenopus* wounding induced gene 1 or *Xwig1*. *Xwig1* encodes a putative protein of 912 amino acids with a predicted molecular weight of 99 kDa (Fig. 1A). The deduced amino acid sequence contains 13 hydrophobic segments that are predicted to be transmembrane domains, suggesting a 13-pass membrane spanning topology (Fig. 1 A,B). Prosite motif predictions indicated the presence of a cleavable signal peptide (cleavage site at position 58) although this was not confirmed by SMART analysis (Schultz et al., 2000). Xwig1 contains multiple consensus protein kinase C and casein kinase 2 phosphorylation sites and 13 potential N-myristoylation sites. Besides the hydrophobic segments it does not contain any obvious biological motif.

Blast searches against the nr database revealed weak homology (probability score 1e-16) to the beta-1,4-exocellulose E6 precursor (celF) from *Thermobifida fusca* and other bacteria and to the *Sua5* gene product from *Saccharomyces cerevisiae* (Na et al., 1992). Dbest searches revealed strong homology to a putative protein encoded by zebrafish EST AW184075 (77% identity from aa 647-836). In addition, weaker, but significant homology was detected to human EST AV652546 (30% identity from aa 651-826) and chick EST AJ394934 (29% identity from aa 643-833) (Fig. 1C). Two other *Xenopus* EST sequences were also hit, corresponding to distinct, but related genes. EST AW766117 showed 51% identity (from aa 623-813) and EST BE491372 showed only 27% identity (from aa 642-811). Interestingly, human EST AV652546 and chick EST AJ394934 are more closely related to the most divergent *Xenopus* EST BE491372 than to *Xwig1*, suggesting that these might constitute an orthologous group (Fig. 1C; grey shading). Blast search against the *Drosophila* genomic database revealed 25% identity to genomic scaffold 1420000133866047 (section 1 of



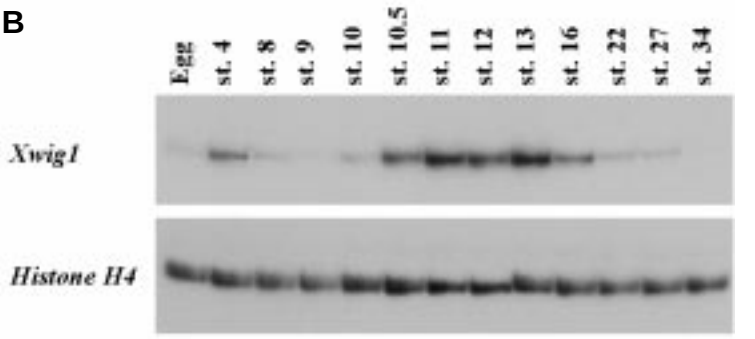




**Fig. 2. (A) Subcellular localization of Xwig1.GFP fusion protein in animal cap explants.** *Confocal images showing Xwig1.GFP distribution (green channel), cortical actin visualized by rhodamin phalloidin (red channel), nuclei visualized by DAPI staining (blue channel) and the overlay. Individual channels were scanned and each image is the merge of 4 optical sections. Xwig1.GFP is detected in cytoplasmic structures, presumably the endoplasmic reticulum.* **(B)** *RT-PCR analysis of Xwig1 expression during embryonic development (stages according to Nieuwkoop and Faber, 1967). Histone H4 serves as an internal loading control.*

52). The identity was again confined to the conserved carboxy-terminal motif from aa 641-857 (data not shown). Thus, Xwig1 is the founding member of a small evolutionarily conserved gene family that is defined by a novel domain of unknown generic function.

To gain insights into the biological function of Xwig1 we determined the subcellular localization of a carboxy-terminally-tagged Xwig1.GFP fusion protein. Contrary to Prosite predictions, Xwig1.GFP staining was not detected in the outer cell membrane after ectopic expression in animal cap explants. Instead however, we found the Xwig1.GFP chimera to be localized in intracellular cytoplasmic structures, which presumably correspond to the endoplasmic reticulum (Fig. 2A). Similar results were obtained with the Xwig1.HA chimera (data not shown). In line with the observation that Xwig1 is not secreted, we did not observe proteolytic processing of the Xwig1 precursor in an *in vitro* rabbit reticulocyte lysate translation system after addition of microsomal membranes, even though other control proteins were efficiently processed (data not shown). Thus, Xwig1 might be a resident endoplasmic reticulum protein.

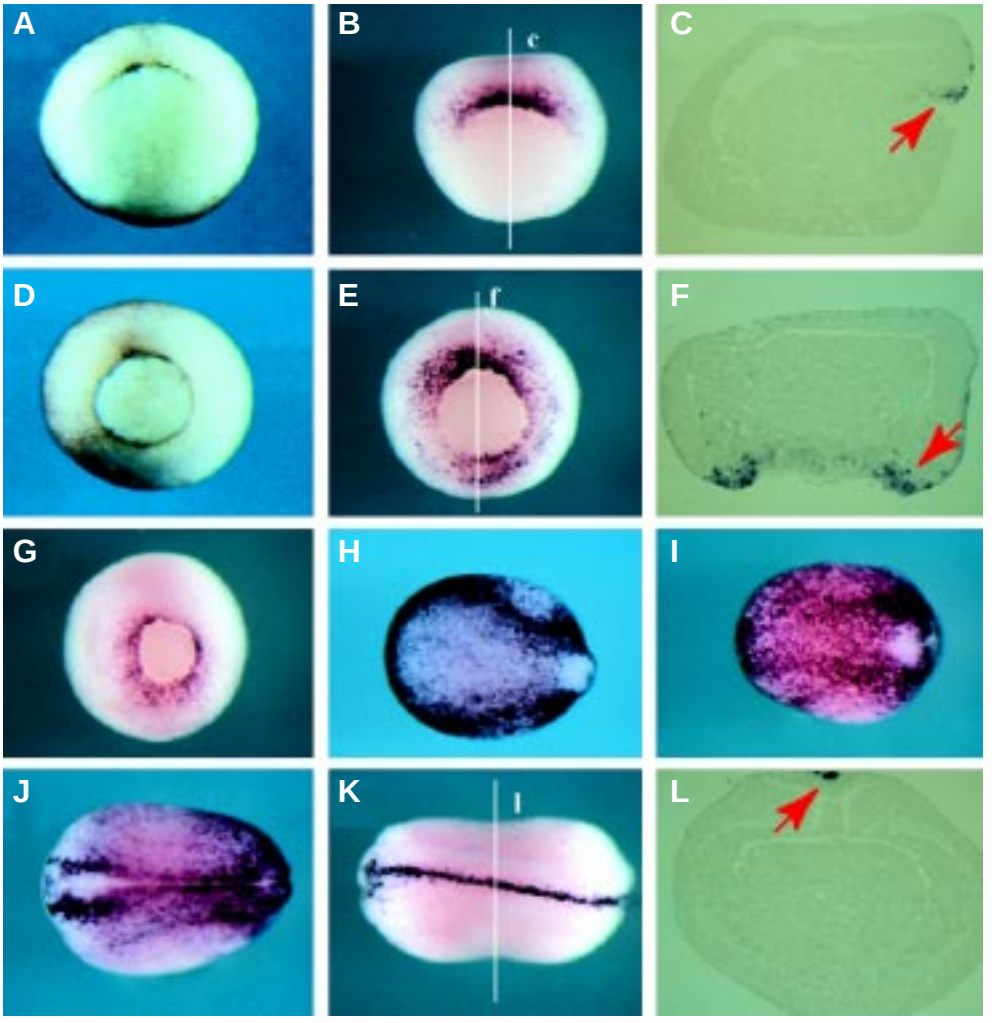



**Fig. 3. *Xwig1* is expressed in epithelial cells of the Spemann-Mangold organizer.** *Whole-mount in situ hybridization analysis was used to determine the spatio-temporal expression of Xwig1 during early* **(A-C)**, *mid* **(D-F)**, *late* **(G)** *gastrulation and neurulation* **(H-L)**. *A and D are wild-type pigmented embryos to show the blastopore rim. During gastrulation, expression is confined to epithelial cells of the blastopore rim (red arrows in C,F). At the end of neurulation expression is observed in a dorsal axial row of cells (red arrow in L). All whole embryos are vegetal views, except H-K which are dorsal views with anterior left.*

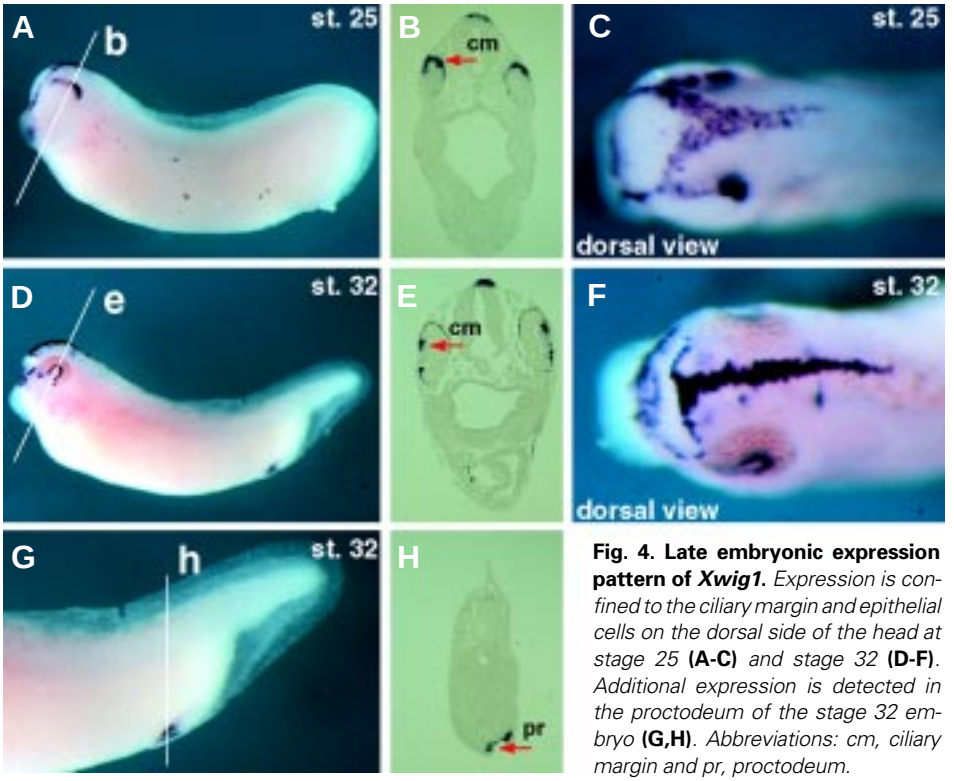



**Fig. 4. Late embryonic expression pattern of *Xwig1*.** *Expression is confined to the ciliary margin and epithelial cells on the dorsal side of the head at stage 25* **(A-C)** *and stage 32* **(D-F)**. *Additional expression is detected in the proctodeum of the stage 32 embryo* **(G,H)**. *Abbreviations: cm, ciliary margin and pr, proctodeum.*

### Xwig1 *is differentially expressed during* Xenopus *embryonic development*

The spatio-temporal expression profile of *Xwig1* was determined by RT-PCR analysis and whole mount *in situ* hybridization. *Xwig1* is weakly expressed maternally and transiently activated during embryonic development (Fig. 2B). Zygotic transcription is initiated at the early gastrula stage in superficial epithelial cells of the dorsal blastopore rim of the Spemann-Mangold or gastrula organizer (Fig. 3 A-C). As the blastopore rim extends ventral-laterally *Xwig1* expression is likewise extended around the epithelial cells of the blastoporal circumference (Fig. 3 D-F). Expression remains strongest on the dorsal side and appears punctuate, presumably nuclear staining reflecting *de novo* synthesis (Fig. 3E). During the course of gastrulation *Xwig1* expression remains associated with epithelial cells of the blastopore rim (Fig. 3G). At the end of gastrulation/onset of neurulation expression increases throughout the entire ectoderm with slightly elevated levels at the non-neural/neural plate border (Fig. 3 H,I). This pattern is observed throughout neurulation, although the generic ectodermal staining decreases over time. At the end of neurulation expression is detected only in a single row of dorsal axial cells that seal off the ectoderm/ neural tube along the entire anterior-posterior axis (Fig. 3 K,L). At tailbud stages *Xwig1* transcripts are detected in the dorsal aspect of the optic vesicle, where infolding of the retina is initiated, and on the dorsal side of the developing cranium (Fig. 4 A-C). During late eye morphogenesis *Xwig1* expression is maintained in the ciliary margin that lines the inner surface of the closing retina and in a stereotypical pattern on the head (Fig. 4 D-F). In addition, expression is maintained in the proctodeum (Fig. 4 G,H). In summary, *Xwig1* expression is primarily associated with epithelial cells that form constrictions during different morphogenetic processes, such as blastopore closure and optic vesicle invagination.

### Xwig1 *expression is activated in response to embryonic wounding*

During the course of the whole-mount *in situ* hybridization analyses we frequently observed ectopic patches of *Xwig1* expression in the periphery of ectodermal wounds, which were presumably inflicted by the dechorionation procedure. To further investigate this striking observation we systematically lesioned gastrula animal cap ectoderm with a fine needle and analyzed *Xwig1* expression in response to this wounding. In all cases (n=42) we observed that wound infliction in uncommitted gastrula ectoderm resulted in weak to strong activation of *Xwig1* expression around the periphery of the embryonic wound (Fig. 5A). Ectopic activation in many cases was prominent in the nuclei of wounded cells reflecting *de novo* synthesis (Fig. 5 B,C; red arrow indicates the wound). Embryonic wounding is known to evoke a transient dual phosphorylation of the extracellular signal-regulated protein kinases (ERK) (LaBonne and Whitman, 1997; Christen and Slack, 1999). Therefore, we compared the temporal kinetics of *Xwig1* transactivation to the rapid transient post-translational modification of ERK-1 and –2. To do so, we lesioned differentiated epidermal cells in the abdominal flank of the tailbud embryo and subjected wounded embryos after defined incubation spans to either anti-dpERK immunostaining or *Xwig1 in situ* hybridization analyses. As previously shown, ERK-1 and –2 are biphosphorylated within 5'-10' after epidermal wounding in an ephemeral fashion (LaBonne and Whitman, 1997; Christen and Slack, 1999; Fig. 5D; left panel). In contrast, the transcriptional activation of *Xwig1* is first observed 20'-30' after wound infliction and is strongest after 60', when phosphorylated ERKs are no longer detectable (Fig. 5D; right panel). In summary, the temporal correlation suggests that *Xwig1* is an immediate-early component of the transcriptional programme that is activated in response to embryonic epidermal wounding in *Xenopus*.

### *Dissection-induced* Xwig1 *activation is partially dependent on Ets/Elk factors*

Dissection of animal cap explants results in the spurious activation of immediate-early target genes, such as *Xfos* and *Xegr-1* (Krain and Nordheim, 1999). To compare the expression kinetics of wounding-induced *Xwig1* with *Xfos* and *Xegr-1* activation we performed a time course on dissected cap explants and analyzed gene expression by RT-PCR. All three genes are activated within 30' of incubation (Fig. 6A). Whereas, *Xfos* and *Xegr-1* expression is transient, declining after 3 hr, *Xwig1* transcripts accumulate till maximum levels after 3 hr and can be detected for a prolonged period till 6 hr before declining. The expression of the wounding-induced immediate-early target genes, *Xegr-1* and *Xfos* is to different extent dependent on Ets/Elk transcription factors that bind to serum response elements (SRE) (Krain and Nordheim, 1999). Ets/Elk transcriptional regulators are

substrates for the FGF-induced Ras/Raf/ERK signal transduction module (Treisman, 1996). To test if *Xwig1* likewise depends on Elk activity and/or on FGF signalling we injected animal blastomeres with mRNA coding for dominant negative forms of Elk (dnElk-1) and the FGF receptor (dnFGFR) and subjected explanted animal caps to RT-PCR analysis. Dissection-induced *Xwig1* expression is partially downregulated by dnElk-1, to the same extent as ectopic *Xfos* activation (Fig. 6B). Wounding-induced *Xegr-1* transcription is completely abolished to control levels (0 hr), in agreement with data shown by Krain and Nordheim, 1999. The inhibition of FGF signal transduction has no significant effect on the dissection-induced activation of any of the three genes tested, even though FGF-dependent mesoderm formation was completely inhibited in control embryos (data not shown).

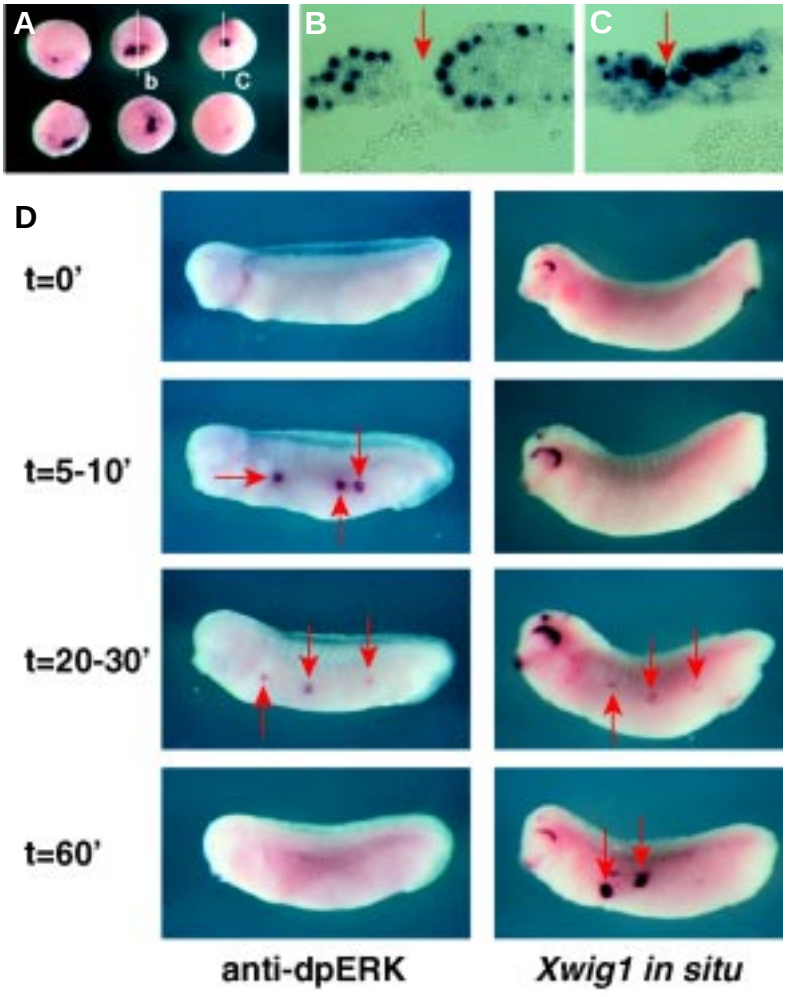



**Fig. 5. *Xwig1* transcription is activated in response to embryonic epithelial wounding. (A)** *Wounding-induced Xwig1 expression in gastrula embryos.* **(B,C)** *Transverse sections of embryos displayed in* **(A)**. **(D)** *Time course (0'-60') of Xwig1 activation compared to the transient phosphorylation of ERK-1 and –2, visualized by anti-dpERK staining, in response to lateral epidermal wounding of stage 27 embryos. Red arrows indicate the embryonic wounds. Xwig1 transcription is activated downstream of ERK activation.*

## Discussion

In this study we have presented the molecular cloning and spatio-temporal expression characteristics of a novel multipass membrane spanning protein, termed *Xenopus* wig1. *Xwig1* expression is mainly observed in epithelial cells during diverse morphogenetic processes, such as blastopore closure and optic vesicle invagination. In addition, we have shown that *Xwig1* transcription is activated in response to embryonic ectodermal wounding. The wounding-induced activation occurs downstream of extracellular signal-regulated protein kinase (ERK) activation and is partially mediated by Elk/Ets factors.

*Xwig1* encodes a novel protein with 13 potential membrane-spanning segments and an evolutionarily conserved carboxy-terminal domain. Limited homology is observed with the beta-1,4-exocellulose E6 precursor (celF) from bacteria and SUA5, a *Saccharomyces cerevisiae* protein required for growth (Na et al., 1992). In addition, several putative proteins encoded by vertebrate ESTs show extensive amino acid sequence identity with the carboxy-terminus. Despite the evolutionary conservation no functional link is apparent between these gene products.

What could be the role of Xwig1 during embryonic development and wound healing? Ectopic expression of *Xwig1* mRNA in different lineages (up to 2 ng/blastomere) has no detectable morphological effect on embryonic development, neither on cell shape of dissected animal cap ectoderm cells (data not shown). Xwig1 appears to be anchored in the endoplasmic reticulum. Xwig1 does not contain a bonafide ER membrane retention motif, but does carry a KKXX/KDEL-like retention/retrieval motif (KDLKL) at the extreme carboxy-terminus. Xwig1 could be involved in the secretion of growth factors or other membrane proteins akin to the role of the evolutionarily conserved *porcupine* gene product in the processing of Wg/Wnt ligands (Tanaka et al., 2000). Porcupine is also a resident ER protein with multipass transmembrane topology. Xwig1 could be involved in the post-translational modification of secreted proteins, although this awaits further biochemical characterization. Despite the ER retention in animal cap cells, it is still conceivable that signal peptide processing might be a regulatory step in the biogenesis of Xwig1 protein in different cellular contexts.

How is *Xwig1* expression regulated? Dissection-induced *Xwig1* up-regulation is similar to *Xfos* activation, as both are only partially down-regulated by dnElk-1. *Xwig1* kinetics are slightly different from both *Xfos* and *Xegr-1*, as peak levels are first observed between 3-6 hr, when expression of the other two genes is down-regulated. However, the sustained peak expression might in part be due to endogenous up-regulation at the onset of neurulation (see Fig. 3). *Xfos* expression requires Elk-1 and p38 MAPK-induced effectors for optimal activation in particular stress conditions (Price et al., 1996). This observation suggests that wounding-induced *Xwig1* activation requires additional factors too besides an Ets/Elk component. These could be effectors activated by the related JNK/SAPK or p38 MAPK cascades or may be different in nature. Epithelial wounding after physical damage is known to result in $Ca2^{+}$ influx, which in turn may trigger alterations in gene expression (Martin, 1997; Grose and Martin, 1999). In contrast to *Xwig1* and *Xfos*, wounding-induced *Xegr-1* expression is solely dependent on SRE binding Ets/Elk factors. The endogenous *Xegr-1* expression in the marginal zone mesoderm is likewise dependent on Elk-1 activity and is mediated by multiple serum response elements (Panitz et al., 1998). The endog-

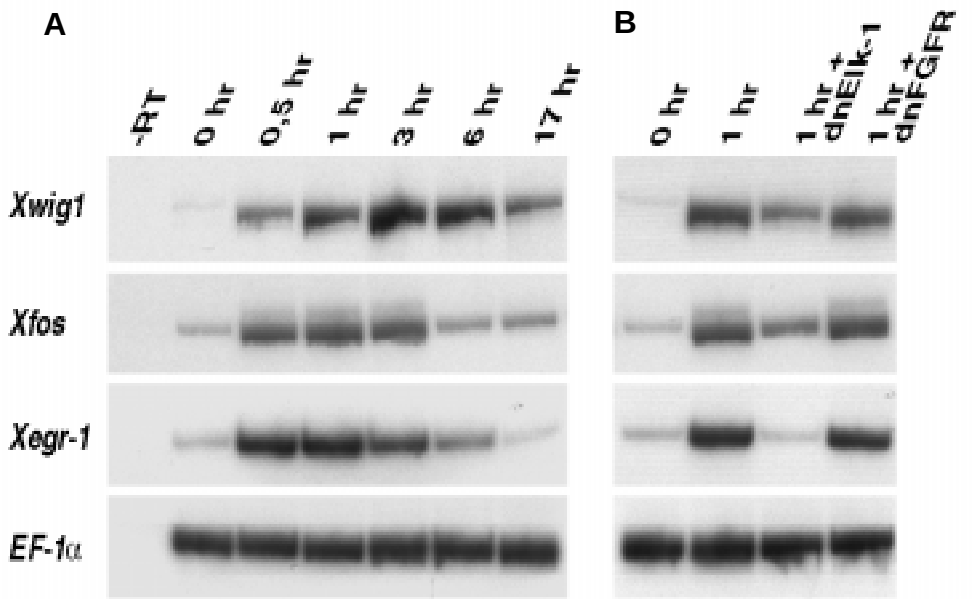



**Fig. 6. Wounding-induced activation of *Xwig1* is partially dependent on Elk-1, but independent of FGF signalling. (A)** *Time course of Xwig1, Xfos and Xegr-1 activation in response to ectodermal wounding in dissected gastrula animal cap explants.* **(B)** *Xwig1, Xfos and Xegr-1 activation in explanted control caps (0 and 1 hr) and caps injected with dominant-negative Elk-1 (1 hr+dnElk-1) and dnFGFR (1 hr+dnFGFR). EF-1α serves as loading control.*

enous expression patterns of *Xwig1* and *Xegr-1* bear no similarity also arguing for diverse regulatory control elements.

In future it will be worth dissecting in detail the regulatory network that controls the dissection-induced activation and the endogenous expression pattern of *Xwig1* to better understand the common elements between embryonic wound healing and certain morphogenetic events, such as blastopore closure and optic vesicle invagination in *Xenopus*. In addition, morpholino-mediated translational knock-down experiments will allow to determine the function of Xwig1 during embryonic development and during the process of wound healing.

## Materials and Methods

### *Molecular cloning of* Xwig1 *and derived plasmids*

A partial 330 bp *Xwig1* fragment was initially identified in a subtractive differential screening for novel organizer genes (unpublished). A full-length cDNA was cloned and assembled from a 2.5 kb partial cDNA, isolated from a directional tailbud cDNA library, and a 0.5 kb 5'-RACE extension fragment (SMART kit, Clontech). Carboxy-terminal tagged pCS2$^+$-*Xwig1.GFP* and pCS2$^+$-*Xwig1.HA* chimeras were generated by in frame fusion of the PCR-amplified *Xwig1* ORF with pCS2$^+$-*GFP* and pCS2$^+$-*HA*, respectively. Alignments were performed with the DNAMan and DNAStar software packages.

### *Embryo manipulations,* in situ *hybridization and microinjection*

RT-PCR analysis, whole mount *in situ* hybridization, animal cap assays and histology were done as described (Bouwmeester et al., 1996; Fetka et al., 2000).

*Xwig1* was amplified by the following primer pair,
(F) 5'-CTATGTTCTAGTGGCTGCTTGC-3';
(R) 5'-GTGAGTAGTAACAGTGCAGTC-3', yielding an amplicon of 303 bp; 28 cycles.

*Xegr-1* was amplified by:
(F) 5'-GAGATGTTAGCCTTGTATCTGC-3';
(R) 5'-GTACTGTTGATAGTCTTGAGGTCC-3' (Panitz et al., 1998)

*Xfos* was amplified by:
(F) 5'-CTCTGTACACATCAGAATGG-3';
(R) 5'-AATGTCCTTCAGCATTACAG-3' (Krain and Nordheim, 1999).

Digoxygenin labelled antisense mRNA was synthesized from a 2.2 kb partial cDNA, pCRII-*Xwig1*, linearized with *Nde*I and transcribed with T3. Embryos were wounded with a thin needle at stage 10.5 and stage 27 and fixed after defined time points in MEMFA. Anti-dpERK (Sigma) immunostaining was performed as described (Christen and Slack, 1999). For microinjection, pCS2$^+$-*dnELK-1* was linearized with *Nsi*I and transcribed with Sp6 (Panitz et al., 1998), p*XFD* (dnFGFR) with *Eco*RI and Sp6 (Amaya et al., 1991) and pCS2$^+$-*Xwig1*, pCS2$^+$-*Xwig1.GFP* and pCS2$^+$-*Xwig1.HA* with *Nsi*I and Sp6.

### Confocal microscopy

*Xwig1.GFP* mRNA was injected into 2 animal blastomeres of 2-4 cell embryos. Animal caps were explanted at stage 9-10, fixed in 4% PFA/PBS for 1 hr and processed. Caps were first stained with rhodamin phalloidin (Molecular Probes) at 1:500 dilution for 2-3 h, followed by DAPI staining (Boehringer) at 1:2000 dilution for 5 min. Caps were mounted upside down in Mowiol and fluorescence signals were visualized using a LEICA TCS NT laser-scanning confocal microscope. Single channels were separated by NIH Image 1.60 software and images were processed and assembled in Adope Photoshop 5.5 software. Similar results were obtained with a *Xwig1.HA* chimera. In this case caps were first incubated with anti-HA (BabCO: 1:1000) in PBSt/10% goat serum O/N at 4ºC followed by 1 h incubation in donkey anti-mouse FITC-conjugated secondary antibody (Jackson ImmunoResearch: 1:250).

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