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Original Article

The role of *XTRAP-γ* in *Xenopus* pronephros development

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ABSTRACT We isolated and characterized the *Xenopus* translocon-associated protein *XTRAP-γ*, one of four subunits of the translocon-associated protein complex. TRAP has been proposed to aid the translocation of nascent polypeptides into the lumen of the endoplasmic reticulum, but this has not been demonstrated until now. *XTRAP-γ* was specifically expressed in the pronephros tubules of *Xenopus laevis* from stage 25 during kidney development. Antisense morpholino oligonucleotide-mediated knockdown of *XTRAP-γ* suppressed tubulogenesis and decreased expression of the pronephros marker genes *Pax-2* and *Wnt-4*. *XTRAP-γ* morpholinos also inhibited differentiation of the pronephros in activin/retinoic acid-treated animal caps. We conclude that *XTRAP-γ* plays an important role in the process of pronephros differentiation.

KEY WORDS: *XTRAP-γ, Xenopus laevis, pronephros, Pax-2, Wnt-4*

Introduction

During vertebrate development a succession of embryonic kidneys, known as the pronephros, mesonephros and metanephros, regulate fluid balance, osmolarity and disposal of metabolic waste products. The embryonic kidneys develop from the intermediate mesoderm, following a precise temporal and spatial sequence. Each successive kidney is formed as a result of an inductive interaction with the previous form. The pronephros, mesonephros and metanephros share a common basic functional unit, the nephron, but increase in complexity with respect to the number and organization of nephrons (Vize *et al.*, 1995). The pronephros is the first to be established and is the simplest excretory organ in vertebrates. The simple tubule and excretory system of the pronephros provides an ideal model for studying kidney organogenesis. The precursors of the pronephros, derived from the intermediate mesoderm, are determined by developmental stage 13 in *Xenopus* (Vize *et al.*, 1995). By stage 21, the primordium of the pronephros thickens and becomes morphologically distinct. By stage 27, this is separate from the lateral plate and the pronephros precursor cells are sorted in a radial pattern. Formation of the lumina is initiated in the anterior portion of the pronephric primordium by stage 28 and extends until stage 30. From stage 35/36, further organization of the collecting tubes and the nephrotomes proceeds and the pronephros begins functioning as a kidney. The mechanism regulating development of the pronephros has been extensively investigated, resulting in the identification of many pronephros-specific genes and the characterization of the temporal and spatial patterns of expression of these genes (Carroll and Vize, 1999, Carroll *et al.*, 1999, Onuma *et al.*, 2000, Sato *et al.*, 2000, Wallingford *et al.*, 1998). Many pronephros-specific genes are also expressed during the development of the mesonephros and metanephros, including *Lim-1, Pax-2, Pax-8, Wnt-4 and WT-1* (Chan *et al.*, 2000, McLaughlin *et al.*, 2000, Saulnier *et al.*, 2002, Seville *et al.*, 2002). The regulatory networks of genes and proteins involved in pronephros development remain poorly defined and the initial events in kidney organogenesis are not yet fully understood. The aim of the present study was to use whole-mount *in situ* hybridization to identify novel genes expressed during pronephros development in *Xenopus* following loss-of function experiments. Using this approach, we identified *XTRAP-γ*, which encodes the translocon-associated protein (XTRAP). *XTRAP-γ* is one of four subunits that makes up the translocon-associated protein complex. It is believed that this complex plays a role in the translocation of nascent polypeptides, but until now this had not been demonstrated. The translocation process is initiated by when the nascent polypeptide binds to a signal recognition particle (SRP) (Rapoport, 1992), which enables the complex to target a signal sequence receptor (Walter and Lingappa, 1986) on the ER membrane at specific

Abbreviations used in this paper: ER, endoplasmic reticulum; MO, morpholino; SRP, signal recognition peptide; XTRAP, Xenopus translocon-associated protein.

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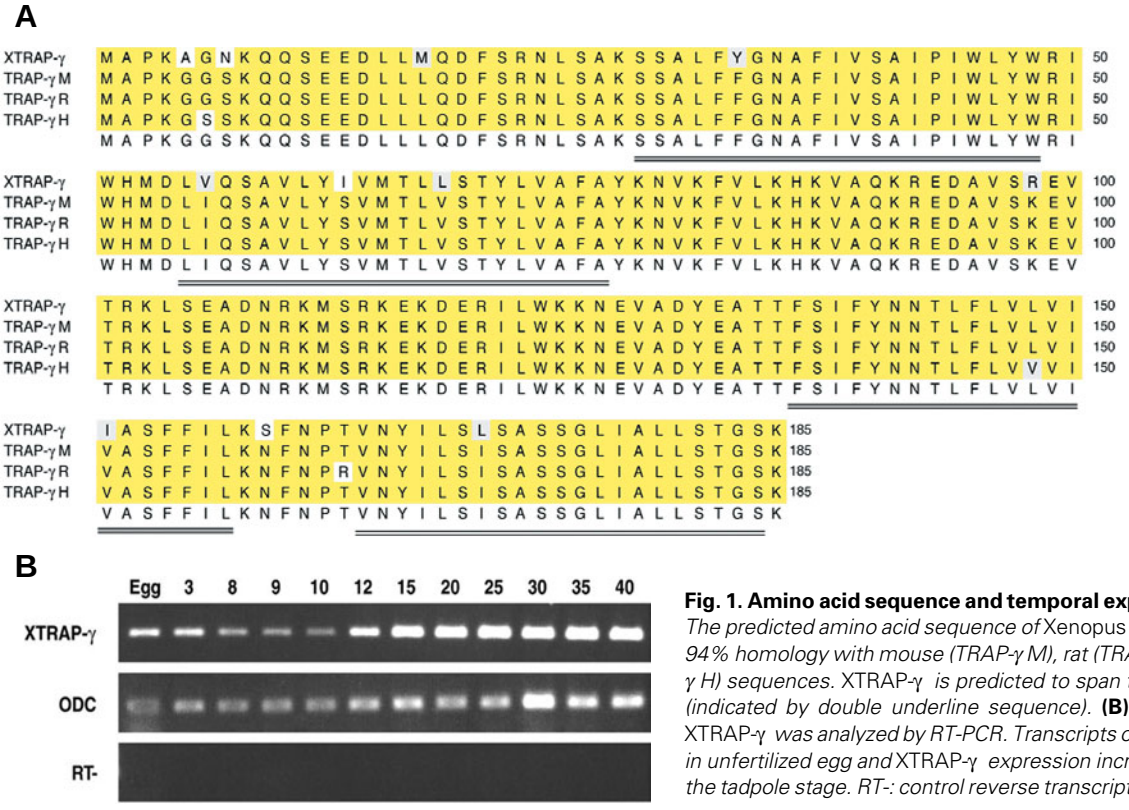

Fig. 1. Amino acid sequence and temporal expression of *XTRAP-γ*. (A) *The predicted amino acid sequence of* Xenopus TRAP-γ *(XTRAP-γ) shares 94% homology with mouse (TRAP-γ M), rat (TRAP-γ R) and human (TRAP-γ H) sequences.* XTRAP-γ *is predicted to span the membrane four times (indicated by double underline sequence).* **(B)** *Temporal expression of* XTRAP-γ *was analyzed by RT-PCR. Transcripts of* XTRAP-γ *were detected in unfertilized egg and* XTRAP-γ *expression increased from stage 12 until the tadpole stage. RT-: control reverse transcription without transcriptase.*

sites or translocons. The translocon core is composed of a Sec61p complex that is thought to form a protein-conducting channel through the ER membrane to allow transport of polypeptide into the ER lumen (Rapoport *et al.*, 1996),(Beckmann *et al.*, 1997). However, the localization of the translocon-associated protein and its interaction with Sec61p has not been fully defined. The present study highlights the importance of TRAP in protein translocation during development. Although it was previously assumed that TRAP was ubiquitously expressed, the present study clearly shows that during Xenopus development *XTRAP-γ* is specifically expressed in the otic vesicles, pronephros tubules, eye and liver from stage 25 onward, coinciding with the time cells are condescending and differentiating into pronephric tubules. Therefore, we hypothesized that *XTRAP-γ* may play a role in pronephric tubulogenesis. In addition, the function of *XTRAP-γ* in pronephros development was further analyzed by loss-of-function experiments using morpholino antisense oligonucleotides injection. Pronephric tubulogenesis was inhibited by *XTRAP-γ* morpholinos and associated with a decrease in expression of the *Pax-2* and *Wnt-4* marker genes. These results suggest *XTRAP-γ* is essential for pronephros development.

Results

Temporal and spatial expression of XTRAP-γ *in* Xenopus *development*

The *Xenopus* TRAP subunit γ (*XTRAP-γ)* was isolated by screening a cDNA library constructed from the lateral mesoderm of *Xenopus* neurulae. *XTRAP-γ* was selected for further study as it was specifically expressed in *Xenopus* pronephros tubules. The TRAP complex, previously called the signal sequence receptor complex, consists of four integral membrane subunits (α, β, γ, δ) (Hartmann *et al.*, 1993). The *XTRAP-γ* subunit was speculated to span the membrane of ER four times and is thought to be responsible for interaction of the other subunits of the TRAP complex. The deduced amino acid sequence of *XTRAP-γ* (GenBank accession number AB058505) contains 153 amino acids, with 93% and 94% identity to human and rat *TRAP-γ*, respectively (Fig. 1A). The temporal expression of *XTRAP-γ* was analyzed by RT-PCR. *XTRAP-γ* mRNA transcripts were detected in all stages of development, from the unfertilized egg to the tadpole stage (Fig. 1B). Although *XTRAP-γ* mRNA expression could be detected at all stages, specific localization of *XTRAP-γ* transcripts could only be observed by whole-mount *in situ* hybridization from stage 12 onward. Expression of *XTRAP* -γ was detected in the notochord during the late gastrula stage (Fig. 2A) and in the anterior neural fold during the neural stage (Fig. 2B). From the late neurula stage onward, expression of *XTRAP-γ* was observed in the cement gland (Fig. 2C) and neural folds (data not

TABLE 1

RESULTS OF *XTRAP-γ* MO AND *5-MIS-XTRAP-γ* MO MICROINJECTION OR CO-INJECTION WITH *5-MIS-XTRAP-γ* mRNA

XTRAP-γ MO	+	+	-
5-mis-*XTRAP-γ* MO	-	-	+
5-mis-*XTRAP-γ* mRNA	-	+	-
Pronephros tubule defect	25 (44.6%)	5 (10.4%)	0
Normal tubules	31 (55.4%)	43 (89.6%)	52 (100%)
Total number	56	48	52

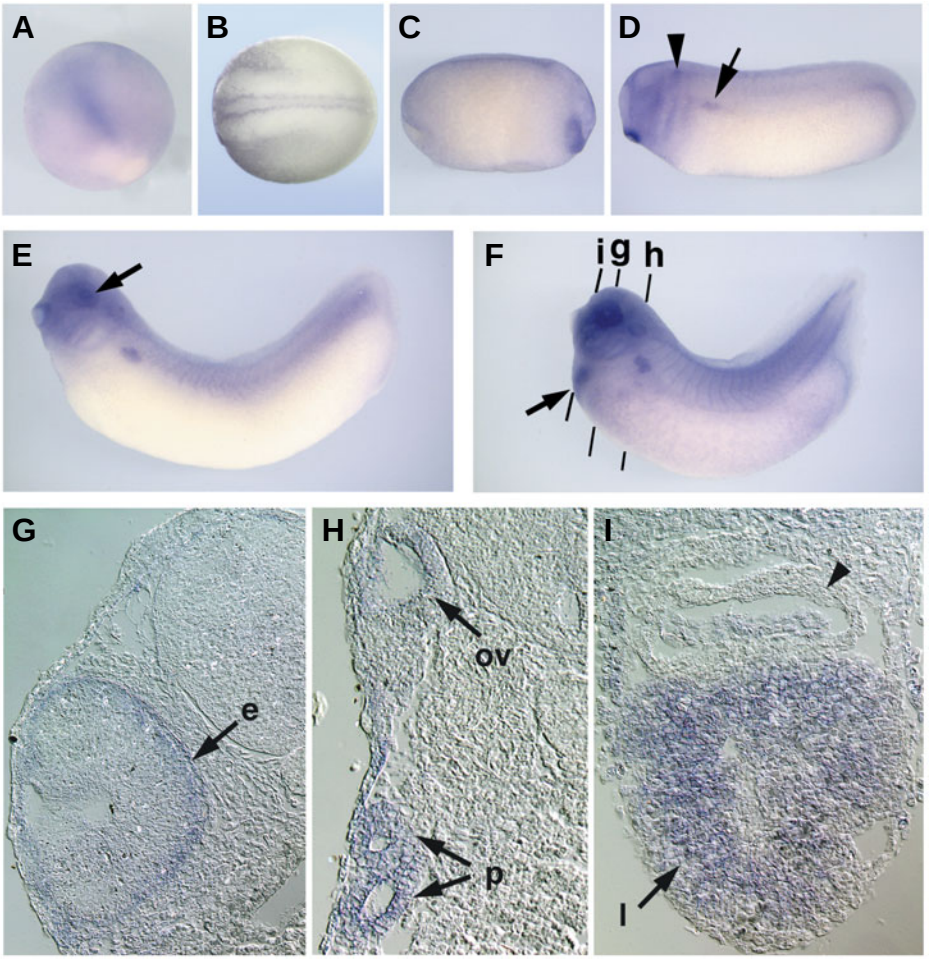

Fig. 2. Whole-mount *in situ* hybridization analyses demonstrate the localization of *XTRAP-γ* transcripts. *The expression of* XTRAP-γ *was weakly ubiquitous during all stages of development.* **(A-F)** *Localization of* XTRAP-γ *was observed from stage 12 onward.* **(D)** *Condensed* XTRAP-γ *signals were detected in the otic vesicles (arrowhead) and pronephric tubules (arrow) from stage 25 onward.* **(E)** *In the early tadpole stages,* XTRAP-γ *transcripts were localized in the eyes (arrow).* **(F)** *By stage 33/34,* XTRAP-γ *was expressed in liver (arrow).* **(G-I)** *Histology section of the* Xenopus *embryo from Fig. 2F. e, eye; ov, otic vesicle; p, pronephros; l, liver.*

shown). *XTRAP-γ* signals were also strongly detected in the otic vesicles and pronephric tubules from stage 25 onward (Fig. 2D). In the early tadpole stages, *XTRAP-γ* transcripts were detected in the eyes (Fig. 2E) and by stage 33/34, *XTRAP-γ* was expressed in the liver (Fig. 2F). Stage 33/34 *Xenopus* embryo had specific localization of *XTRAP -γ* expression in the eye, otic vesical, pronephros and liver (Fig. 2G, 2H, 2I).

Morpholino-mediated knockdown of XTRAP-γ *causes failure of tubulogenesis during pronephros development*

XTRAP-γ was over-expressed in *Xenopus* embryos to analyze the role of *XTRAP-γ* in *Xenopus* embryogenesis. This yielded no apparent phenotype (data not shown). To further investigate the role of *XTRAP-γ*, loss-of-function experiments using two *XTRAP-γ* antisense morpholinos were performed. The *XTRAP-γ* morpholino (*XTRAP-γ* MO) was used for translational inhibition of internal *XTRAP-γ* and the 5-mis-*XTRAP -γ* MO was designed to incorporate five mispairs relative to *XTRAP-γ* MO. HA-tagged *XTRAP-γ* mRNA was synthesized to demonstrate the effect and specificity of the *XTRAP-γ* MO. The HA-tagged *XTRAP-γ* was translated *in vivo* and its translation is blocked by *XTRAP-γ* MO. However, the 5-mis-*XTRAP-γ* MO did not affect the translation of HA-tagged *XTRAP-γ* (Fig. 3A).

Whole-mount *in situ* hybridization revealed that *XTRAP-γ* expression is predominantly localized in the pronephric tubules from stage 25. To investigate the role of *XTRAP-γ* in normal pronephros tubulogenesis, *XTRAP-γ* MO was injected into one side of the lateral marginal zone of 32-cell-stage embryos. Both the C-2 and C-3 blastomeres of embryos were injected with morpholinos, corresponding to cells of the presumptive anterior somites (C-2) and posterior somites, pronephros and lateral plate (C-3), respectively (Dale and Slack, 1987). Only phenotypically normal embryos with β-galactosidase activity in the region of the pronephros were scored for the effects of morpholinos on pronephric development (Table 1). Formation of pronephric tubules was inhibited significantly on the side injected with *XTRAP-γ* MO. Tubule branches were shorter and reduced in number (Fig. 3B), compared with the non-injected-side (Fig. 3C). To determine whether the failure of pronephros tubulogenesis formation was due to secondary effects of morpholinos, one side of the embryos were injected with 5-mis-*XTRAP -γ* MO. Pronephros development was normal in 5-mis-*XTRAP -γ* MO injected embryos (Fig. 3D). The phenotype caused by injection of *XTRAP-γ* MO was rescued by co-injection with 5-mis-*XTRAP-γ* mRNA (Fig. 3E). These observations also suggest that depletion of *XTRAP-γ* causes direct suppression of tubulogenesis. It was also important to deter-

TABLE 2

***XTRAP-γ* MO AFFECTS THE EXPRESSION OF PRONEPHROS MARKER GENES**

	Pax-2		*Wnt-4*		*Lim-1*	
	XTRAP-γ MO	5-mis-*XTRAP-γ* MO	*XTRAP-γ* MO	5-mis-*XTRAP-γ* MO	*XTRAP-γ* MO	5-mis-*XTRAP-γ* MO
++	0	0	0	0	18	0
+	34	49	33	51	29	38
-	24	0	22	0	0	0
Total Number	58	49	55	51	47	38

Notes: ++ The expression pattern is enhanced; + Normal; - Repressed.

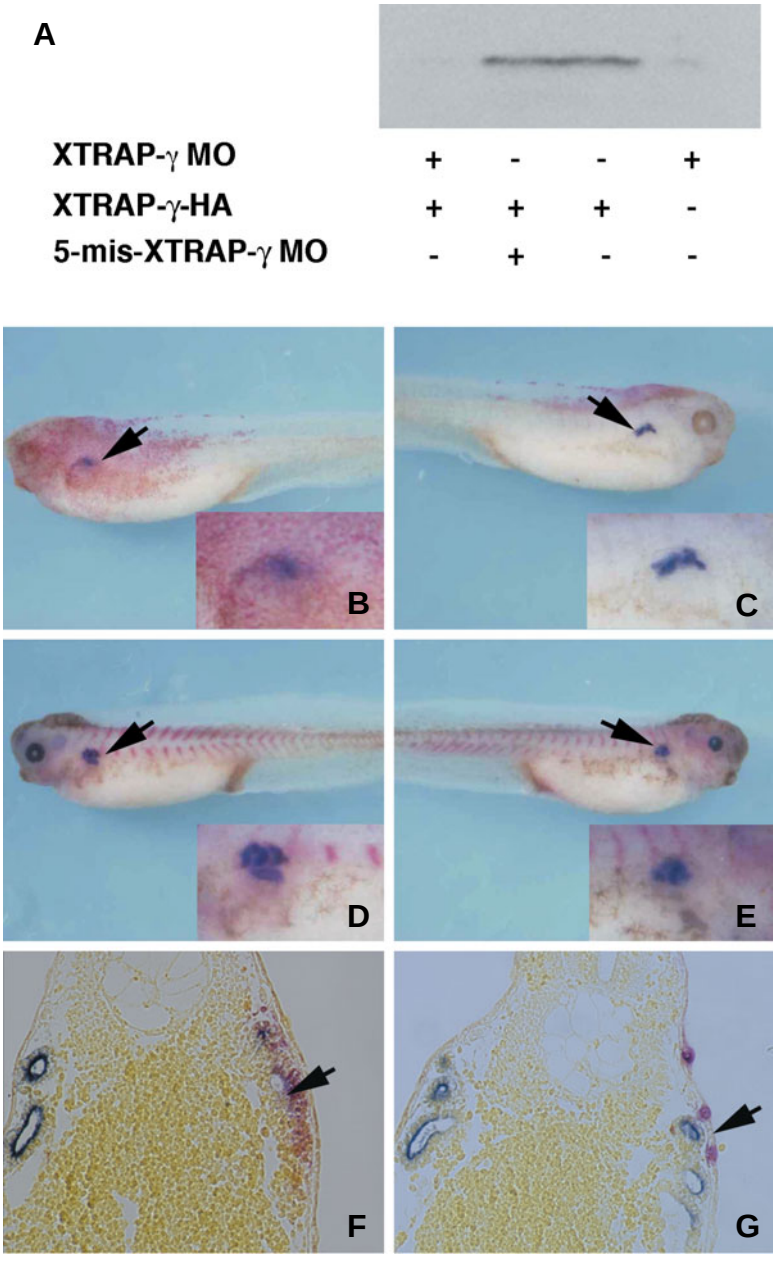

Fig. 3. *XTRAP-γ* MO affects pronephric tubulogenesis. (A) XTRAP-γ *MO specificity* in vitro. *Western-blotting analysis showed significant inhibition of* XTRAP-γ- *HA (100 pg/embryo) protein synthesis by* XTRAP-γ *MO (40 ng/embryo). Protein synthesis was not affected with 5-mis-*XTRAP-γ *MO (40 ng/embryo).* **(B-G)** *Whole-mount immunohistochemistry analysis on pronephros.* **(B)** XTRAP-γ *MO (40 ng),* **(D)** *5-mis-*XTRAP-γ *MO (40 ng) and* **(E)** XTRAP-γ *MO (40 ng) plus 5-mis-*XTRAP-γ *mRNA (1 ng) were injected into one side of the lateral marginal zone of 16-cell-stage embryos. Embryos were fixed at stage 40. Development of the pronephros was analyzed by staining with the monoclonal antibody 3G8.* **(B)** *Pronephric tubule formation was inhibited in* XTRAP-γ *MO-injected embryos (arrow in B) compared with the non-injected-side (arrow in C) and the embryos injected 5-mis-*XTRAP-γ *MO (arrow in D). The phenotype of the pronephric tubules was rescued by co-injection of* XTRAP-γ *MO with 5-mis-*XTRAP-γ *mRNA* **(E)**. **(F,G)** *Histological analysis of* XTRAP-γ *MO injected embryo and 5-mis-*XTRAP-γ *MO injected embryos. Pronephros tubules in* XTRAP-γ *MO-injected embryos showed severe developmental defects (arrow in F) compared with that of the 5-mis-*XTRAP-γ *MO-injected embryos (arrow in G).*

mine whether *XTRAP-γ* MO inhibited the formation of pronephric tubules by indirect methods, such as interfering with the development of other structures such as anterior somites, which are necessary for pronephros differentiation. Gross examination and histology (Fig. 3F, 3G) show that all structures except the pronephros tubules were normal.

XTRAP-γ *is also required for pronephros development in activin/retinoic acid-treated explants*

Pronephros differentiation can be induced by activin/retinoic acid treatment (Nakamura *et al.*, 1992), (Uochi *et al.*, 1997). To further examine the effect of *XTRAP-γ* MO specifically on pronephros and to avoid any secondary effects of *XTRAP-γ* MO on pronephros development, *XTRAP-γ* MO was injected at the 2-cell stage and animal caps were isolated at stage 9 of development for analysis. After activin/retinoic acid treatment, examination of sections revealed that pronephros differentiation was markedly inhibited by *XTRAP-γ* MO (Fig. 4A). Electron microscopy was used to determine the effect of *XTRAP-γ* MO on the differentiation of pronephric and other cell types. This confirmed the absence of typical pronephric cells with microvilli and demonstrated condensation of mesodermal cells and a radial arrangement of cells with a basal membrane, characteristic of epithelium (Fig. 4C, D), compared to the normal structure described by Osafune (Osafune *et al.*, 2002). Muscle differentiation was normal (Fig. 4E). These observations suggest that depletion of *XTRAP-γ* led to suppression of tubulogenesis.

The depletion of XTRAP-γ *at the later stages of development had an effect on the expression of other pronephric genes*

To investigate whether *XTRAP-γ* affected the differentiation of pronephric tubules by inhibiting marker genes, the effect of depleting *XTRAP-γ* expression on other pronephros-specific genes was assessed. The expression of the pronephros-specific genes *Lim-1*, *Pax-2, Pax-8 and Wnt-4* in *XTRAP-γ* MO injected embryos was analyzed by whole-mount *in situ* hybridization. Only phenotypically normal embryos with β-galactosidase activity in the region of the pronephros were scored (Table. 2). The expression of *Pax-2* and *Wnt-4* was noticeably suppressed (Fig. 5E, I); expression of *Lim-1* increased (Fig. 5A); and expression of *Pax-8* was unchanged (Fig. 5M). In stage 22 embryos, *Pax-2 and Wnt-4* expression was not strongly affected by injection of *XTRAP-γ* MO (Fig. 5Q, S) or 5-mis-*XTRAP-γ* MO (Fig. 5R, T). As *XTRAP-γ* is not strongly expressed in pronephros until after stage 25, the pronephric tubule markers tested were not affected by perturbation of *XTRAP-γ* expression at stage 22. After stage 25, the expression of pronephric genes was directly inhibited by *XTRAP-γ* MO. *Pax-2* and *Wnt-4* were co-injected with *XTRAP-γ* MO to rescue the *XTRAP-γ*- depleted phenotype. Embryos injected with *XTRAP-γ* MO were compared with the co-injected and the non-injected control embryos. Pheno-

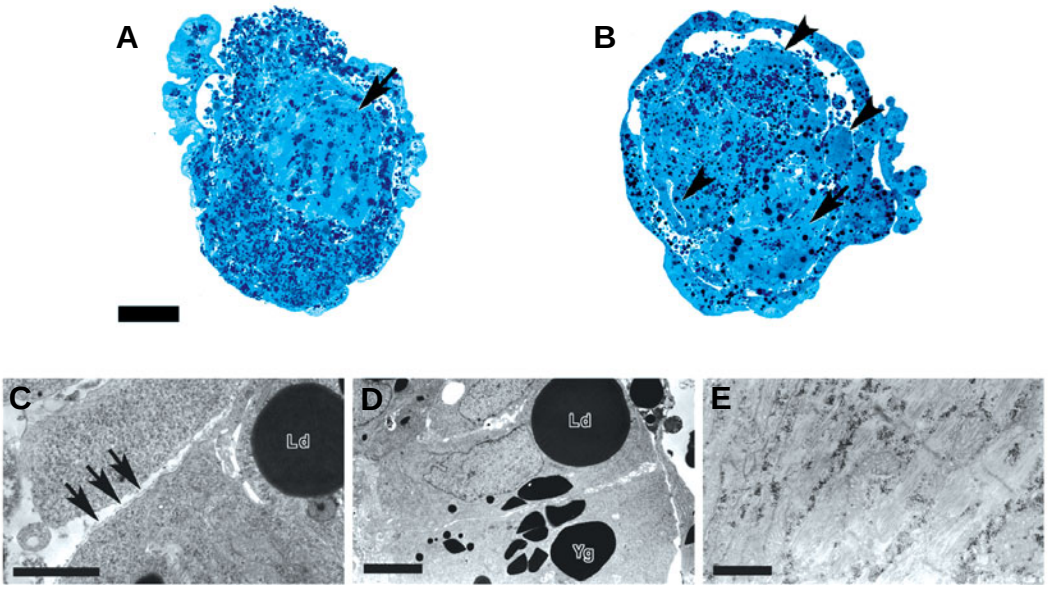

Fig. 4. Induction experiments in *XTRAP-γ* MO-injected animal caps. *Two-cell stage embryos were injected with 40 ng/embryo* XTRAP-γ *MO. All animal caps were treated with activin/retinoic acid for 3 h.* **(A,B)** *For light microscopy, semi-thin sections were stained with 0.1% toluidine blue solution.* **(A)** *Pronephros differentiation was markedly inhibited by* XTRAP-γ *MO (arrow indicates muscle tissues) compared with* **(B)** *animal caps of the control (arrowhead indicates pronephros tissues).* **(C,D,E)** *For electron microscopy, ultra-thin sections were double stained with uranyl acetate and lead citrate. Only samples injected with* XTRAP-γ *MO are shown (C,D). The results show the condensation of mesodermal cells and a radial arrangement of the cells with the basal membrane (arrows), however the unique pronephric cells with microvillus was invisible.* **(E)** *Muscle differentiation was normal. Ld, lipid droplet; Yg, yolk granule. Scale bar of (A,B) is 100 μm; scale bar of (C,D) is 1 μm; scale bar of (E) is 5 μm.*

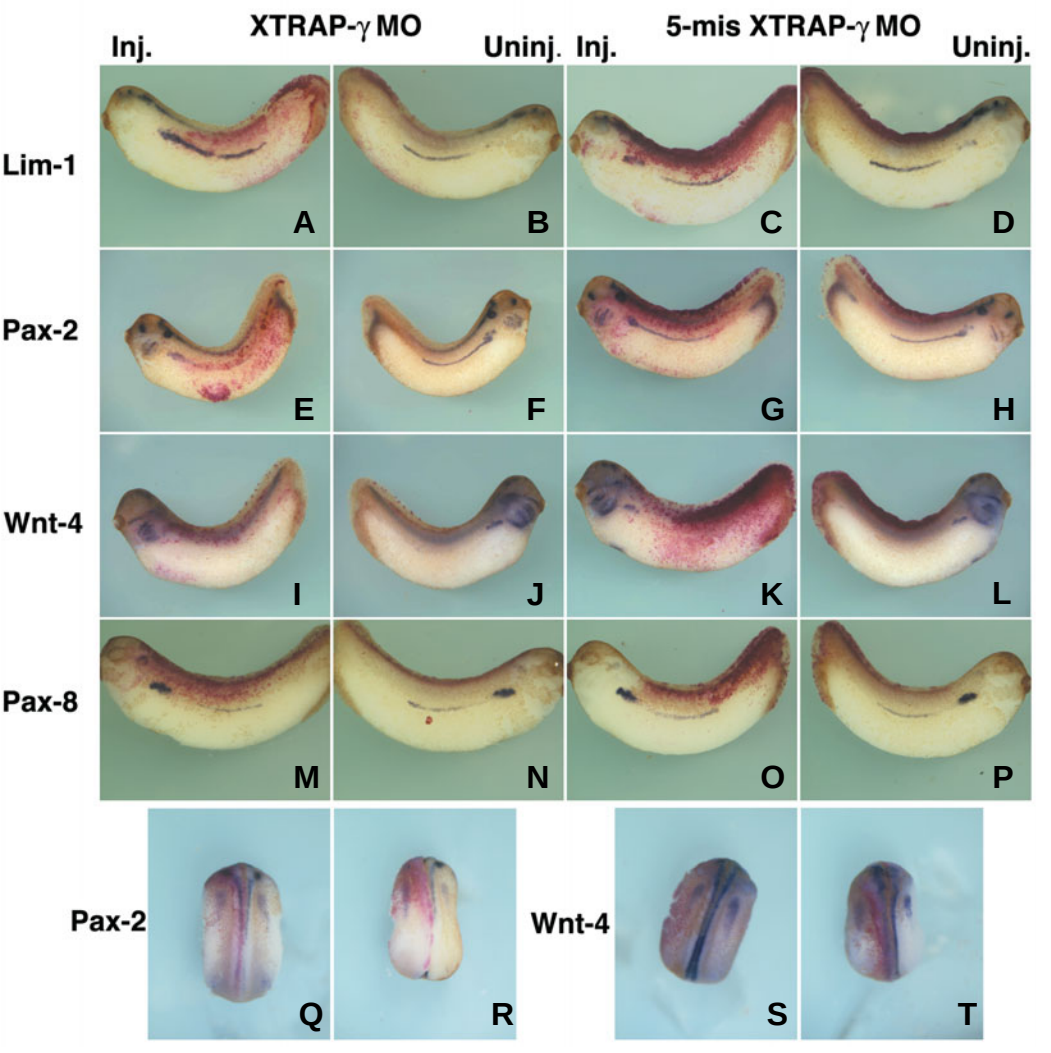

Fig. 5. Whole-mount *in situ* hybridization shows the effect on expression of pronephros marker genes *XTRAP-γ* MO injection. *Embryos were injected with* XTRAP-γ *MO (40 ng) or 5-mis-*XTRAP-γ *MO (40 ng) and synthetic of β-galactosidase mRNA (250 ng), then fixed at stage 30/31 (A-P) and stage 22/23* **(Q-T)**. *Expression of* Lim-1 *was increased in the side of embryos injected with* XTRAP-γ *MO* **(A)**, *compared the non-injected side* **(B)**. *Expression of* Pax-2 *and* Wnt-4 *was inhibited by injection of* XTRAP-γ *MO* **(E,I)**, *compared with non-injected side* **(F,J)**. *For those embryos fixed at stage 22/23, expression of* Pax-2 **(Q)** *and* Wnt-4 **(S)** *in embryos fixed at stage 22/23 was not affected by injection of* XTRAP-γ *MO or the control 5-mis-*XTRAP-γ *MO* **(R,T)**. *Expression of* Pax-8 *was not affected by* XTRAP-γ *MO* **(M,N)**. *The expression of* Lim-1 **(C,D)**, Pax-2 **(G,H)**, Wnt-4 **(K,L)** *and* Pax-8 **(O,P)** *was unchanged in embryos injected with 5-mis-*XTRAP-γ *MO.*

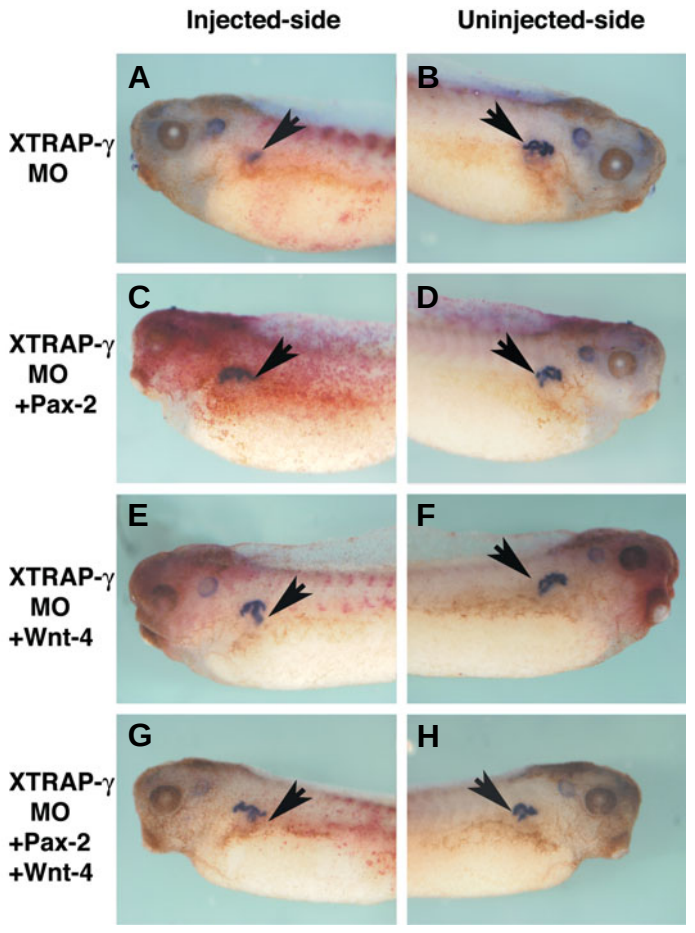

Fig. 6. The phenotype of pronephric tubules was rescued by co-injection with *Pax-2, Wnt-4,* or *Pax-2* and *Wnt-4*. (A) XTRAP-γ *MO (40 ng),* **(C)** XTRAP-γ *MO (40 ng) plus* Pax- *2 (100 pg),* **(E)** XTRAP-γ *MO (40 ng) plus* Wnt-4 *(100 pg),* **(G)** XTRAP-γ *MO (40 ng) plus* Pax-2 *(100 pg) and* Wnt-4 *(100 pg) were injected into one side of the lateral marginal zone of 16-cell-stage embryos. Embryos were fixed at stage 40. Development of the pronephros was analyzed by staining with the monoclonal antibody 3G8.* **(A)** *Pronephric tubule formation was inhibited in the side of embryos injected with* XTRAP-γ *MO (arrow in A) compared to non-injected-side (arrow in B).* **(C)** *Pronephric tubule formation was significantly rescued in embryos injected with* XTRAP-γ *MO (40 ng) plus* Pax-2 *(100 pg) (arrow in C) compare to the* XTRAP-γ *MO-injected embryos (arrow in A). Although the dorsal branches of the pronephric tubules were shorter in the injected side than in the non-injected side (arrow in D), three dorsal branches of the pronephric tubules were still visible. Injection of* **(E)** XTRAP-γ *MO (40 ng) plus* Wnt-4 *(100 pg), or* **(G)** XTRAP-γ *MO (40 ng) +* Pax-2 *(100 pg) and* Wnt-4 *(100 pg) almost completely rescued pronephric tubule formation, with relatively normal pronephric tubules being observed.*

types of embryos co-injected with either *XTRAP-γ* MO and *Pax-2*, *XTRAP-γ* MO and *Wnt-4,* or *XTRAP-γ* MO with *Pax-2* and *Wnt-4,* were also significantly rescued (Fig. 6C, E, G; Table 3).

Discussion

The aim of this study was to identify novel genes involved in pronephros development. We isolated, localized and analyzed the *Xenopus* translocon-associated protein-γ (*XTRAP-γ*), which is believed to aid in the translocation of nascent polypeptides into the ER lumen. The results of this study indicate that *XTRAP-γ* is necessary for pronephros development. We believe that *XTRAP-γ* may be involved in the translocation of proteins that are important for pronephros tubulogenesis in *Xenopus laevis*. TRAP was first identified as an integral glycoprotein of the ER membrane and was provisionally termed signal sequence receptor (SSR) (Wiedmann *et al.*, 1987). SSR was subsequently localized to the translocation site and was therefore renamed 'translocon-associated protein' (TRAP). The TRAP complex consists of four integral membrane subunits and the *TRAP-γ* subunit is predicted to span the membrane four times and may be responsible for interacting with the other subunits of the complex (Hartmann *et al.*, 1993). However, the localization of TRAP on the ER membrane and the role of *XTRAP-γ* in the translocation of nascent polypeptides had not yet been elucidated until now. Recently, the TRAP complex was shown to function in protein transport in a substrate-specific manner (Fons *et al.*, 2003). As *XTRAP-γ* expression was found to localize to pronephros tubules, a series of analyses of pronephric tubulogenesis were undertaken. Using loss-of-function analyses, we identified a clear role for *XTRAP-γ* in pronephric tubulogenesis. Overexpression of *XTRAP-γ* yielded no apparent phenotype. One possible explanation for this is that the essential translocation machinery currently (the Sec61p complex, SR and TRAM) are able to mediate a basal level of translocation for many substrates (Gorlich and Rapoport, 1993),(Voigt *et al.*, 1996), even if the translocation of some proteins required for pronephros development also require TRAP. If, however, the expression of only *XTRAP-γ* is increased, the translocation of these specific proteins cannot also be increased. Depletion of *XTRAP-γ* by antisense morpholino resulted in shortened proximal tubules and a shortening or absence of the distal tubule of the pronephros. Transverse sections of embryos also showed inhibition of pronephros tubule differentiation on the side injected with morpholinos, but other structure had differentiated normally. Electron microscopy of animal caps treated with activin and RA showed the condensation of mesodermal cells and a radial arrangement of the cells with the basal membrane, which was characteristic of epithelium. However, the characteristic pronephric cell with microvilli was not apparent and muscle cells differentiated normally. Together, these results demonstrated that *XTRAP-γ* MO inhibited pronephric tubulogenesis directly and not through secondary effects. Pronephros marker gene analysis revealed a decrease in the expression of *Pax-2* and *Wnt-4* and an increase in the expression of *Lim-* 1 in the sides of embryos injected with *XTRAP-γ* MO compared to the non-injected sides. At stage 22, however, neither *XTRAP-γ* MO nor 5-mis *XTRAP-γ* MO injected embryos were able to affect the expression of *Pax-2* and *Wnt-4*. The process of pronephric tubule formation requires changes

TABLE 3

RESULTS OF *XTRAP-γ* MO MICROINJECTION OR CO-INJECTION WITH *PAX-2* AND/OR *WNT-4*

XTRAP-γ MO	+	+	+	+	-
Pax-2	-	+	-	+	-
Wnt-4	-	-	+	+	-
Pronephros tubule defect	25 (44.6%)	6 (13.9%)	9 (18.7%)	5 (11.1%)	0
Normal tubules	31 (55.4%)	37 (86.1%)	39 (81.3%)	40 (88.9%)	50 (100%)
Total number	56	43	48	45	50

in cell shape and extensive cell rearrangements, which must be accompanied by changes in gene expression patterns. *Lim-1* is one of the earliest marker genes of pronephros: its expression is first detected during late gastrulation at stage 12/13 (Taira *et al.*, 1994). This corresponds precisely with the occurrence of pronephric tubule lineage specification (Brennan *et al.*, 1998). Expression of *Wnt-4* was detected as early as stage 19, where it was weakly expressed throughout the pronephric mesoderm. *Wnt-4* expression increased in the developing pronephric tubule until the tailbud stages (Ungar *et al.*, 1995). *Pax-2* was expressed at very low levels in the pronephric mesoderm at stage 22. However, its expression strengthened progressively throughout the tailbud stages (Heller and Brandli, 1997). The onset of expression of *XTRAP-γ* occurred at stage 25, so the pronephric tubule markers tested were not affected by perturbation of *XTRAP-γ* expression at stage 22. At later stages, progression of pronephric tubule differentiation was affected by depleting *XTRAP-γ* gene function. Therefore, we proposed that the defective of pronephric tubules in *XTRAP-γ*MO-injected embryos was caused by a decrease in the expression of *Pax-2* and *Wnt-4* at a later stage. In *Xenopus* pronephros development, *Lim-1* expression decreased at the later stages and was confined to the distal tips by the late tadpole stage. However, depletion of *XTRAP-γ* inhibited presumptive pronephric cells from differentiating into tubules and kept these cells at an earlier stage of tubulogenesis, so that *Lim-1* expression was stronger in the injected side compared to the non-injected side. Based on these results, we propose that *XTRAP-γ* is necessary for pronephric tubulogenesis and that it plays a role in the differentiation of pronephros tubules by affecting the expression of marker genes *Pax-* 2 and *Wnt-4* in the pronephros. Recently, the TRAP complex was demonstrated to function in protein transport in a substrate-specific manner (Fons *et al.*, 2003). We determined that *XTRAP-γ* may promote the translocation of unknown factors that are responsible for the regulation of *Lim-1*, *Pax-2* and *Wnt-4* and for the differentiation of pronephric tubules. Further analyses are required to reveal the role of *XTRAP-γ* in the translocation of nascent polypeptides necessary for pronephros differentiation. Although the molecular mechanisms for TRAP regulation remain unclear, this study has demonstrated a role for *XTRAP-γ* in vertebrate embryogenesis in pronephros development of *Xenopus laevis.*

Materials and Methods

Eggs and embryos

Eggs were obtained by the injecting of human chorionic gonadotropin into *Xenopus laevis* (Gestron: Denka Seiyaku, Japan). Staging of embryos was performed according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). The jelly coat was removed with Steinberg's solution containing 3% cysteine hydrochloride (pH 7.8).

Construction of the cDNA library and screening

A cDNA library from the lateral mesoderm of *Xenopus* neurulae was constructed as reported by Sato *et al* (Sato *et al.*, 2000). Subtraction and whole-mount *in situ* hybridization was used to identify clones expressed in the pronephric region. Using this process, *XTRAP-γ* was selected for further analysis.

Morpholino nucleotide and microinjection

Morpholino oligonucleotides were obtained from Gene Tools LLC. The sequence of *XTRAP-γ* antisense morpholinos were: *XTRAP-γ* MO, 5'-GCTTGTTGCCAGCTTTCGGAGCCAT-3'; 5-mis-*XTRAP-g* MO, 5'-GCATGTTGCCTGCTATCGCAGCGAT-3'. Genbank searches failed to detect significant homologues of the morpholinos in the *Xenopus* genome. Microinjection was carried out according to the method described by Chan *et al.* (Chan *et al.*, 2000).

In vitro *translation of* **XTRAP-γ** *and Western blotting*

The *XTRAP-γ* ORF was amplified by PCR and cloned into pCS2+HA (Nitta *et al.*, 2004) to generate the *XTRAP-γ*-HA fusion protein. *XTRAP-γ* proteins with HA tags were detected using the 3F10 monoclonal antibody (Santa Cruz). The 5-mis-*XTRAP-γ* mRNA was constructed according to Yabe *et al* (Yabe *et al.*, 2003). For the secretion assay, the mRNAs were injected into defolliculated ooctyes and Western blotting was performed as described previously (Kaneko *et al.*, 2003).

Preparation of test solution

The test solution contained 10 ng/ml activin A (kindly provided by Dr. Yuzuru Eto, Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan), 10^{-4} mol/l retinoic acid and 0.1% BSA in Steinberg's solution.

Animal cap assay

The vitellin membrane of the blastula embryos was removed with fine forceps and the presumptive ectoderm was isolated using tungsten needles. Both presumptive mesodermal and endodermal cells were removed from the isolated ectoderm. Ectoderm sheets were immediately transferred into Steinberg's solution and cultured at 20ºC in a 24-well plate (Sumilon MS-8024; Sumitomo Bakelite, Tokyo, Japan). Explants were fixed after a four-day incubation for histological examination.

Light and electron microscopy

Ectoderm sheet explants were fixed in prefixative solution (3% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer at pH 7.4) overnight at room temperature. After several buffer washes, the specimens were post-fixed in 1% OsO4 and 0.1 M cacodylate buffer at pH 7.4 for 2 h, then washed, dehydrated with alcohol and acetone and embedded in epoxy resin. Semi-thin sections (approximately 1 μm) were prepared for light microscopy and stained with 0.1% toluidine blue solution. For electron microscopy, ultra-thin sections (80-90 nm) were stained with uranyl acetate and lead citrate, then examined under an electron microscope (JEM-1010 or JEM-200CX; JEOL, Tokyo, Japan).

RT-PCR

Total RNA isolation and RT-PCR were performed as described previously (Chan *et al.*, 2000). The following primer sets were used:

XTRAP-γ: sense: 5'-AGGACAAGGCAGTTGTAGGC-3',
antisense: 5'-TGAGGAGAGTCATTCAGCG-3';

ODC (ornithine decarboxylase):
sense: 5'-GTCAATGATGGAGTGTATGGATC-3',
antisense: 5'-TCCATTCCGCTCTCCTGAGCAC-3'.

Whole mount **in situ** *hybridization*

Single probe *in situ* hybridization was performed according to Harland (Harland, 1991). Antisense digoxygenin (DIG)-labeled RNA probes were transcribed from full-length *XTRAP-γ, Lim-1, Pax-8, Pax-2* and *Wnt-4* constructs. DNA was linearized and transcribed with RNA polymerase to generate antisense transcripts. A series of albino embryos encompassing a range of developmental stages were hybridized with the labeled probes. The color reaction was performed using NBT/BCIP.

Immunohistochemistry and histological examination

Histological analyses were performed as described previously (Chan *et al.*, 2000). For immunohistochemistry, speciments were fixed in MEMFA for 1.5 h at room temperature, assayed for β-galactosidase activity, refixed for 30 min, washed in PBS and then dehydrated in methanol overnight. Embryos were rehydrated in PBS and then incubated in primary antibody, a pronephric tubule specific antibody 3G8, at 4ºC overnight. Binding of an alkaline phosphatase-conjugated secondary

antibody, Goat anti-mouse IgG (BIOSOURCE), to 3G8 was visualized using NBT/BCIP. After staining, specimens were fixed in MEMFA and dehydrated in methanol overnight. Wild-type embryos were bleached in 10% H2O2 for 6 h. Specimens were transferred to ethanol and xylene and then embedded in paraffin. Embedded specimens were sectioned at 10 μm.

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