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The transcriptional coactivators *Yap* and *TAZ* are expressed during early *Xenopus* development

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ABSTRACT The *Yap* and *TAZ* genes encode highly conserved domains which bind various transcription factors. Yap and TAZ act as transcriptional coactivators to modulate transcriptional activity. The activities of Yap and TAZ are negatively regulated by Hippo signaling *via* direct phosphorylation. In this study, we describe the expression patterns of *Yap* and *TAZ* during the development of *Xenopus tropicalis*. The *Xenopus tropicalis Yap (xtYap)* and *Xenopus tropicalis TAZ (xtTAZ)* genes are expressed maternally. *xtYap* is widely expressed throughout embryogenesis, particularly in the facial connective tissues, branchial arch, midbrain-hindbrain boundary, otic vesicle, pronephros, notochord, hindgut and tailbud. *xtTAZ* expression occurs predominantly in the presomitic mesoderm, facial connective tissues, brain, branchial arch, trunk neural crest cells and migrating hypaxial myoblasts. In the muscle lineage, *xtTAZ* expression is transient and restricted to proliferating cells, the presomitic mesoderm and the edges of the hypaxial myoblasts, with no expression detected in mature muscle cells. These results provide insights into the functions of Yap and TAZ and their regulation by Hippo signaling during early development in *Xenopus*.

KEY WORDS: *Yes-associated protein, Yap, TAZ, WWtr1, Xenopus*

The Hippo pathway was originally identified in the fly as a controller of organ size, and its components are evolutionarily conserved in vertebrates (reviewed in Wang *et al*., 2009; Badouel *et al*., 2009). The core components of Hippo, Sav, Wts, and Mats in the fly are conserved in mammals as Mst1/2, WW45, LATS1/2, and Mob1, respectively. Protein interaction screenings in *Drosophila* revealed that Hippo signaling negatively regulates Yorkie through direct phosphorylation. In the absence of Hippo signaling, Yorkie can promote transcription together with binding partners. The Yes-associated protein (Yap) and transcriptional coactivator with PDZ-binding motif (TAZ) are vertebrate homologs of Yorkie. Yap and TAZ show conservation of multiple functional domains, i.e., the TEF/TEAD binding domain, 14-3-3 binding motif, WW domain, and transactivation domain. In similarity to Yorkie, Yap and TAZ bind to transcription factors and act as transcriptional coactivators.

The overexpression of *Yap* and *TAZ* induces cell proliferation, epithelial-mesenchymal transition, and anti-apoptosis (Overholzer *et al*., 2006; Lei *et al*., 2008). Gene ablation of *Yap* results in embryonic lethality, with defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in the mouse (Morin-Kensicki *et al*., 2006). The injection into zebrafish of *Yap*-specific morpholino oligonucleotides results in the phenotype of small head with smaller eyes, no tail, and cardiac edema (Jiang *et al*., 2009). *TAZ*-knockout mice show partial lethality; survivors have renal cysts and slightly shorter skeletons (Hossain *et al*., 2007; Tian *et al*., 2007; Makita *et al*., 2008). *TAZ* morphants of zebrafish show cardiac edema, ventral curvature, and lack of ossification (Hong *et al*., 2005; Tian *et al*., 2007). These studies indicate that the functions of Yap and TAZ and their regulation by Hippo signaling are essential for the development of multiple tissues, and that Yap and TAZ play distinct roles in normal

Abbreviations used in this paper: orf, open reading frame; Yap, Yes-associated protein.

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vertebrate development.

Previous studies have demonstrated that Tead, Runx, Tbx5, Pax3, and other transcriptional factors are binding partners of Yap and TAZ (reviewed in Wang *et al.*, 2009). These transcriptional factors are differentially expressed and function in several developmental processes. Yap and TAZ may also be required as transcriptional coactivators in each process. Elucidating the spatio-temporal expression patterns of *Yap* and *TAZ* in vertebrate embryogenesis is important for understanding how Hippo signaling is involved in differentiation, tissue size control, and cell proliferation. In the present study, we reveal the expression patterns of *Xenopus tropicalis Yap* and *TAZ* genes, thereby providing insights into the functions of Yap and TAZ in early development.

Results

Molecular cloning of the xtYap *and* xtTAZ *genes*

The sequences of the open reading frames (ORFs) of *xtYap* and *xtTAZ* were obtained from the genome databases. We performed PCR-based cloning using the cDNA species derived from stage 10.5 embryos and adult testes, respectively. The full-length ORFs of *xtYap* and *xtTAZ* (GenBank accession numbers AB551789 for *xtYap* and AB551790 for *xtTAZ*) encode 456 and 390 amino acids, respectively. The deduced amino acid sequences were aligned and compared with their homologs in other vertebrates (Fig. 1-3). The xtYap protein showed 76.4%, 78.0%, and 75.4% identities with the human Yap (hYAP), mouse Yap (mYap), and zebrafish Yap (zYap), respectively. The xtTAZ protein showed 76.2%, 74.0%, and 62.2% identities with hTAZ, mTAZ, and zTAZ, respectively. Although the functional domains of these proteins are conserved across species, a proline-rich motif that is conserved in the N-terminal regions of the mYap and hYAP proteins is not present in the xtYap and zYap proteins.

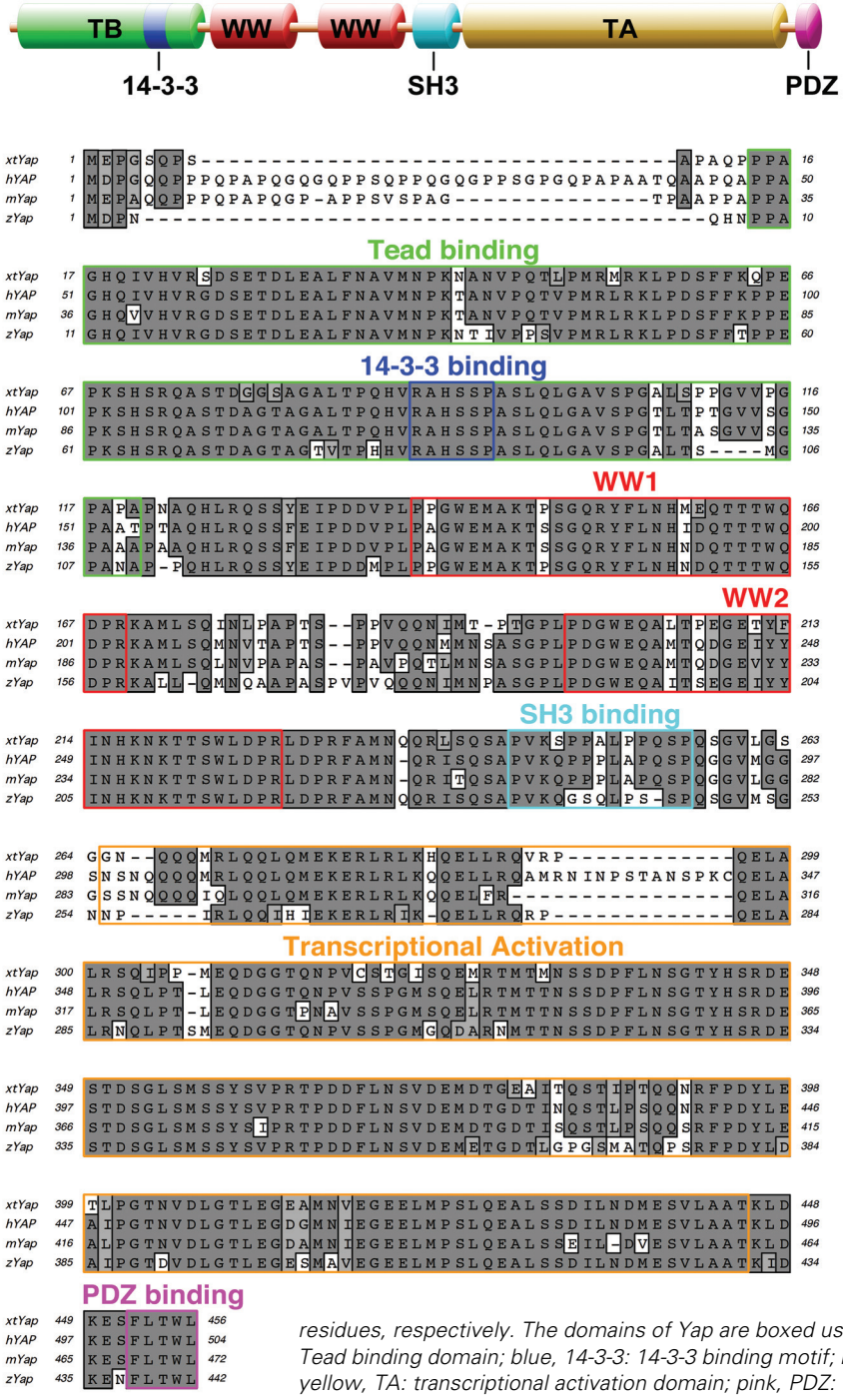

Fig. 1. Comparisons of the Yap protein sequences of *Xenopus tropicalis* and other vertebrates. *The scheme of xtYap is shown on the top. The amino acid sequence of xtYap was aligned with those of human Yap (hYAP; accession no. NP_001123617), mouse Yap (mYap; NP_033560), and zebrafish Yap (zYap; NP_001132952). The dark or light background highlights identical or similar residues, respectively. The domains of Yap are boxed using the following colors and abbreviations; light-green, TB: Tead binding domain; blue, 14-3-3: 14-3-3 binding motif; red, WW: WW domain; light-blue, SH3: SH3 binding motif; yellow, TA: transcriptional activation domain; pink, PDZ: PDZ binding motif.*

Expression patterns of xtYap *and* xtTAZ

We performed semi-quantitative RT-PCR analyses of embryos and adult tissues (Fig. 4). During embryogenesis, *xtYap* transcripts were detected continuously from the unfertilized egg to the tadpole stages. The levels of *xtTAZ* transcripts were low from the unfertilized egg to gastrula stages, but gradually increased from the neurula stage to tadpole stage (Fig. 4A). In the adult tissues,'*xtYap* transcripts were found to be abundant in the gut, ovary and testis. In addition, *xtYap* was expressed at moderate levels in the heart and liver, whereas it was weakly expressed in the eyes and skin. The levels of *xtTAZ* transcripts were high in the gut, heart, skin, and testes. *XtTAZ* was also expressed at moderate levels in the eyes, liver and ovaries, whereas it was weakly expressed in the muscles (Fig. 4B).

To examine the spatial expression patterns of these genes during the early development of *Xenopus*, we performed whole-mount *in situ* hybridiza-

tion analysis. The *xtYap* transcripts were localized to the animal hemisphere until the early gastrula stage (Fig. 5 A-C). During gastrulation, the embryo was covered with *xtYap*-expressing ectodermal cells (Fig. 5D). At the neurula stage, *xtYap* was mainly expressed in the center of the neural plate and in the cranial neural crest cells (Fig. 5 E,F,J). From the tailbud stage to the tadpole stage, *xtYap* expression was evident in the facial connective tissues, branchial arch, midbrain-hindbrain boundary, otic vesicle, pronephros, notochord, hindgut (cloaca), and tailbud (Fig. 5 G-I). Histologic sectioning of a stage 30 embryo revealed that *xtYap* was expressed in the ectodermal and mesodermal derivatives (Fig. 5K).

The expression of *xtTAZ* was initially detected in the paraxial mesoderm at the late neurula stage (Fig. 6 A,B,D). Thereafter, *xtTAZ* expression was detected in the hindbrain and presomitic mesoderm from the early tailbud stage (Fig. 6 C,E,K,L). It is noted that xtTAZ was not detected in the matured somite (Fig. 6 E,J). In a transverse section of the tailbud stage, *xtTAZ* expression was observed in the heart, hindbrain, and the trunk neural crest cells (Fig. 6 H-L). At the late tailbud stage, *xtTAZ* expressed was detected in the facial connective tissues, midbrain-hindbrain boundary, hindbrain, branchial arch, and trunk neural crest cells. From embryonic stage 35, *xtTAZ* was expressed in the migrating hypaxial myoblasts, including the facial musculature (Fig. 6 F,G,M,N).

Discussion

The *Xenopus tropicalis* Yap and TAZ proteins contain conserved domains, which include not only transcriptional factor binding sites, such as the Tead binding domain and WW domain, but also other functional domains (Fig. 1,2). However, a proline-rich region that is conserved in the N-terminal regions of the chick, murine, and human Yap proteins is not present in the *Xenopus* or zebrafish Yap proteins. It has been reported that this proline-rich region interacts with the SH3 domain of c-Yes and other proteins (Sudol, 1994).

The expression patterns of *Yap* and *TAZ* during embryogenesis have been studied in mice and zebrafish. *Yap* transcripts have been detected at the 1-cell stage of mouse development (Nishioka *et al.*, 2009). The murine *Yap* gene is expressed in almost all tissues, with the exceptions of the visceral and definitive endoderm. In particular,Ä*mYap* is strongly expressed in the extraembryonic ectoderm and epiblast at E6.5. In addition to these regions, *mYap* is expressed in the extraembryonic mesoderm and chorion at E7.5, and at the distal tip of the allantois at E8.5 (Morin-Kensicki *et al.*, 2006). The *mYap* expression patterns at later developmental stages have not been analyzed. In zebrafish, *zYap* transcripts exist maternally. Zygotic *zYap* is mainly expressed in the notochord, brain, eyes, branchial arches, and pectoral fins (Jiang *et al.*, 2009). In the present study, we show that *xtYap* is maternally expressed and is widely expressed throughout the developmental process, with strong expression in the center of neural plate and in the cranial neural crest cells at the neurula stage. We also demonstrate that *xtYap* is strongly expressed in the facial connective tissues, branchial arch, midbrain-hindbrain boundary, otic vesicle, pronephros, notochord, hindgut, and tailbud at the tailbud stage.

TAZ is expressed from the 1-cell stage of the mouse embryo (Nishioka *et al.*, 2009). *TAZ* is localized to the forebrain, hindbrain, and somites between E9.0 and E10.5 of mouse embryogenesis (Di Palma *et al.*, 2008). In the present study, we show that *xtTAZ* is maternally expressed and is expressed in the hindbrain and

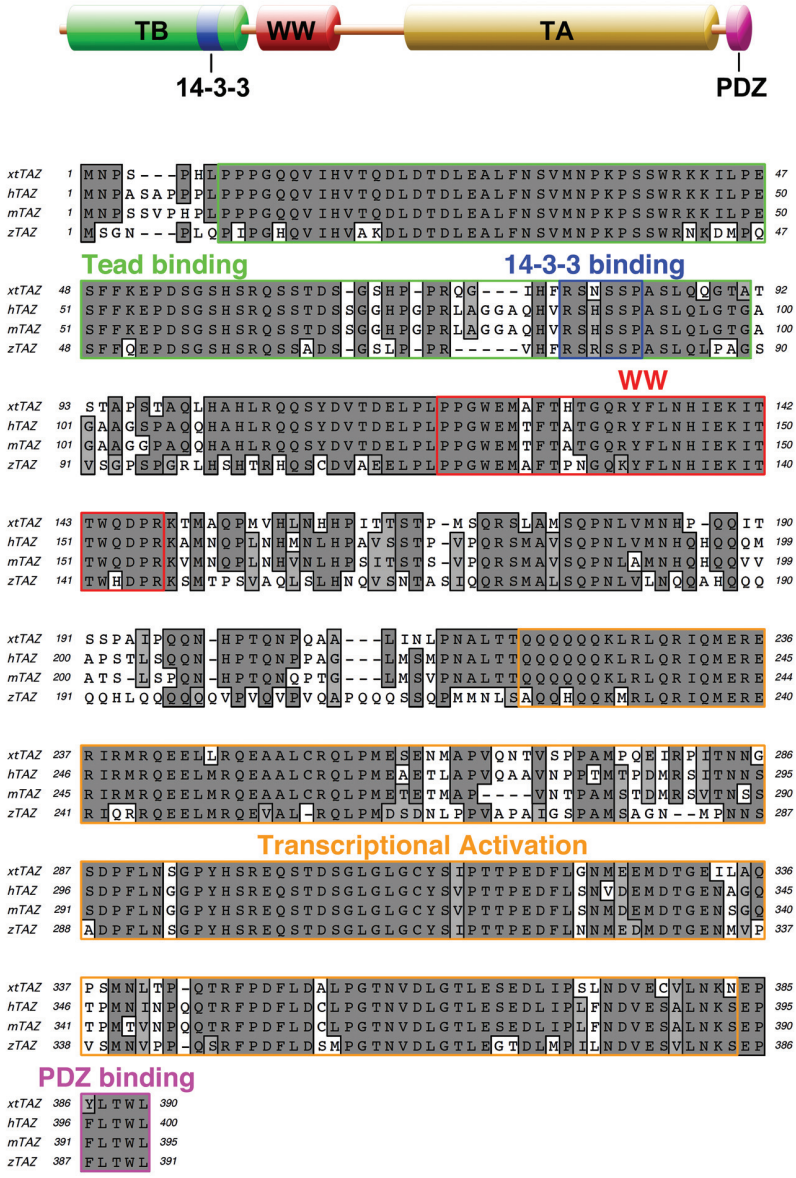

Fig. 2. Comparisons of the TAZ protein sequences of *Xenopus tropicalis* and other vertebrate TAZ. *The scheme of xtTAZ is shown on the top. The amino acid sequence of xtTAZ was aligned with those of human TAZ (hTAZ; accession no. NP_056287), mouse TAZ (mTAZ; NP_598545) and zebrafish TAZ (zTAZ; NP_001032785). The dark or light background highlights identical or similar residues, respectively. The domains of TAZ are boxed using the following colors and abbreviations; light-green, TB: TEAD binding domain; blue, 14-3-3: 14-3-3 binding motif; red, WW: WW domain; yellow, TA: transcriptional activation domain; pink, PDZ: PDZ binding motif.*

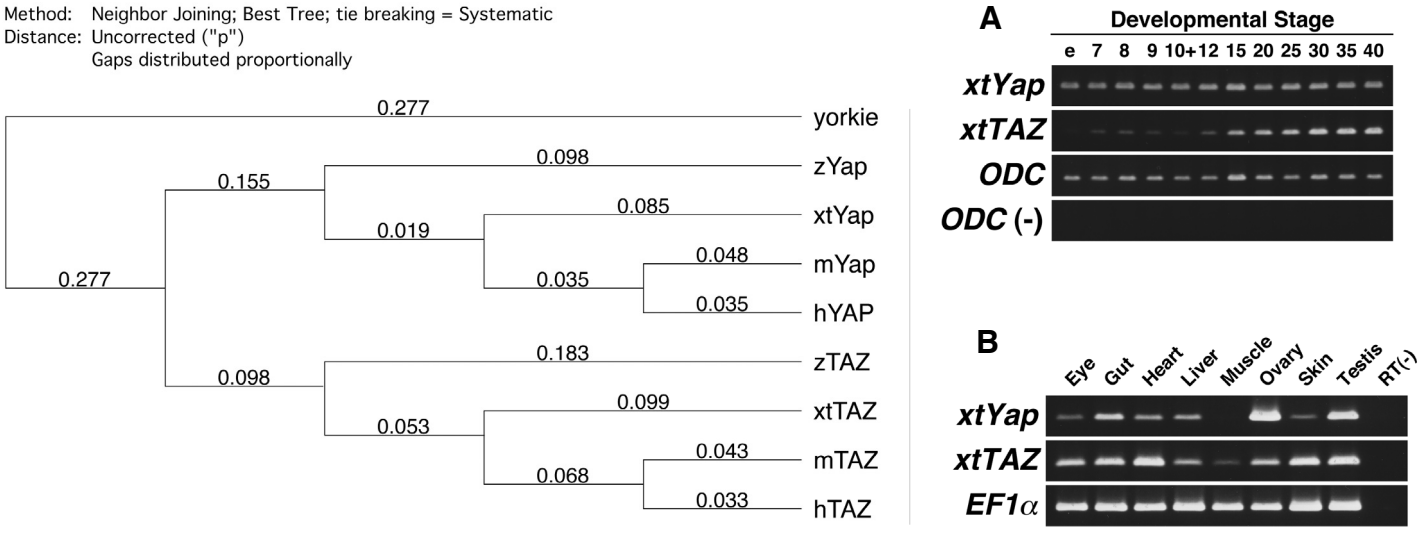

Fig. 3 (Left). Rooted phylogeny of the Yap, TAZ and Yorkie amino acid sequences. *The following amino acid sequences were used in the analysis: human Yap (hYAP; accession no. NP_001123617); mouse Yap (mYap; NP_033560);* Xenopus tropicalis *Yap (xtYap; AB551789); zebrafish Yap (zYap; NP_001132952); human TAZ (hTAZ; NP_056287); mouse TAZ (mTAZ; NP_598545);* Xenopus tropicalis *TAZ (xtTAZ; AB551790); and zebrafish TAZ (zTAZ; NP_001032785); Yorkie (NP_001036568).*

Fig. 4 (Right). Comparison of the *xtYap* and *xtTAZ* expression patterns. (A) *Temporal expression profiles of* xtYap *and* xtTAZ. *RNA samples were extracted from embryos at various developmental stages, as indicated above each lane. e, Unfertilized egg.* ODC *(-) is a negative control for* ODC. **(B)** *Expression profiles of* xtYap and xtTAZ *in adult* Xenopus *tissues. RNA samples were extracted from* Xenopus *tissues, as indicated above each lane. RT (-), RT-PCR without reverse transcriptase in the testis cDNA synthesis reaction.*

presomitic mesoderm from the neurula stage to the tailbud stage. We also show that *xtTAZ* is expressed in the facial connective tissues, the midbrain-hindbrain boundary, the hindbrain, the branchial arch, and in the trunk neural crest cells at the tadpole stage. Furthermore, we demonstrate that *xtTAZ* is expressed in the presomitic mesoderm and hypaxial myoblasts, but not in the differentiated somite. These observations suggest that *xtYap* and *xtTAZ* are expressed widely, and that in some tissues (e.g., the center of the neural plate and cranial neural crest cells for *xtYap*, and the hindbrain and presomitic mesoderm and hypaxial myoblasts for *xtTAZ*), their expression levels are high. It has been reported that the large amounts of Yap and TAZ produced by overexpression cannot be inhibited by the endogenous Hippo signaling in *Drosophila* and mammalian cells (Dong *et al.*, 2007). There is no information as to when Hippo signaling is active during *Xenopus* development. The region highly expressing *Yap* or *TAZ* may be resistant to be the Hippo signaling.

Yap and TAZ have been shown to function as transcriptional

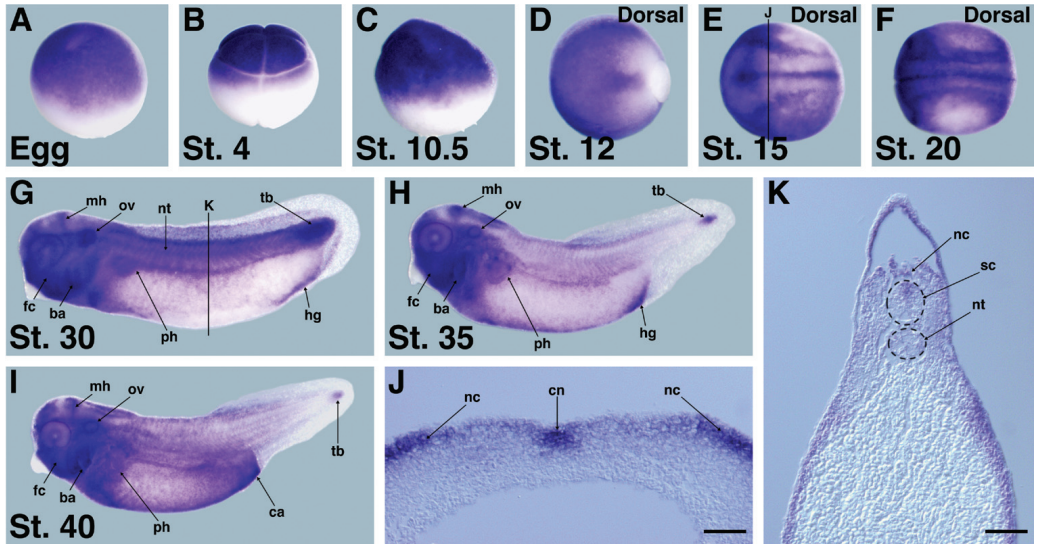

Fig. 5. Spatial expression pattern of *xtYap* during the early development of *Xenopus tropicalis*. (A-K) *Spatial expression of* xtYap *was analyzed by whole-mount* in situ *hybridization. Anterior is at the left of each panel.* **(A)** *Unfertilized egg,* **(B)** *8-cell stage,* **(C)** *early gastrula,* **(G)** *tailbud stage,* **(H)** *late tailbud stage, and* **(I)** *tadpole stage in lateral view.* **(D)** *Late gastrula,* **(E)** *neurula stage, and* **(F)** *late neurula stage in dorsal view.* **(J)** *Transverse section of* **(E)**. **(K)** *Transverse section of* **(G)**. *The line position is indicated in* **(E,G)**. *Scale bars, 50 μm. Abbreviations: ba, branchial arch; ca, cloaca; cn, center of neural plate; fc, facial connective tissues; hg, hindgut; mh, midbrain-hindbrain boundary; nc, neural crest; nt, notochord; ov, otic vesicle; ph, pronephros; sc, spinal cord; tb, tailbud.*

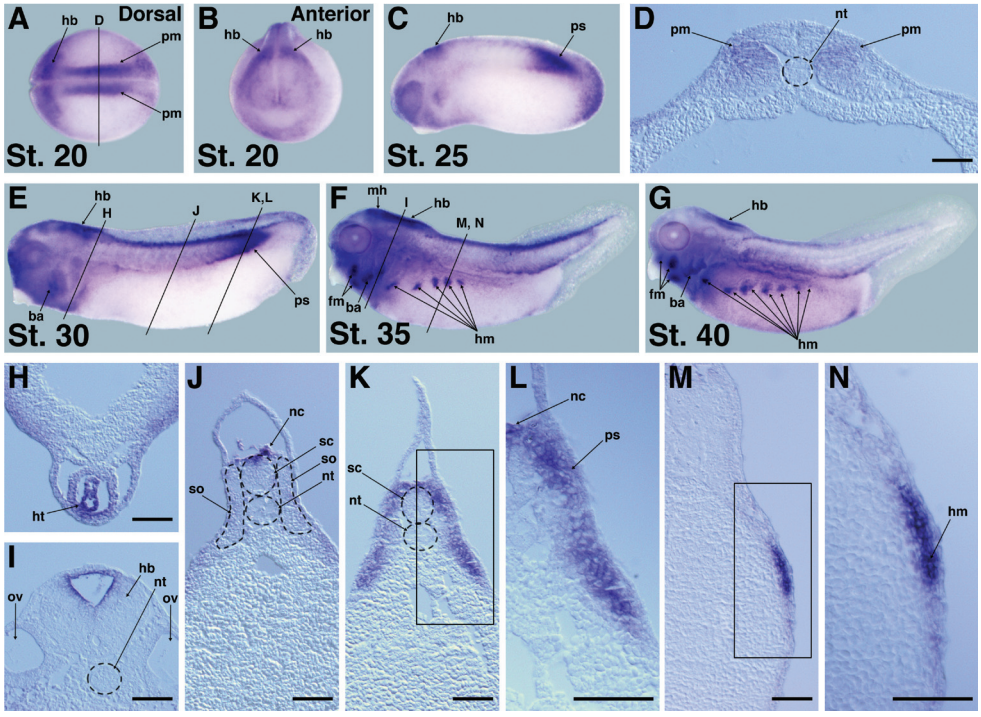

Fig. 6. Spatial expression pattern of *xtTAZ* during the early development of *Xenopus tropicalis*. **(A-N)** *Spatial expression of* xtTAZ *was analyzed by whole-mount* in situ *hybridization. Late neurula stage in* **(A)** *dorsal view and* **(B)** *anterior view,* **(C)** *early tailbud stage,* **(E)** *tailbud stage,* **(F)** *late tailbud stage, and* **(G)** *tadpole stage in lateral view.* **(D)** *Transverse section of* **(A)**. **(H, J, K)** *Transverse section of* **(E)**. **(I, M)** *Transverse section of* **(F)**. *The line position is indicated in* **(E-G)**. **(L)** *Higher magnification of* **(K)**. **(N)** *Higher magnification of* **(M)**. *The box position is indicated in* **(K,M)**. *Scale bars, 50 μm. Abbreviations: ba, branchial arch; fm, facial musculature; hb, hindbrain; ht, heart; hm, hypaxial myoblasts; mh, midbrain-hindbrain boundary; nt, notochord; pm, paraxial mesoderm; ps, presomitic mesoderm; sc, spinal cord; so, somite.*

coactivators for Tead, Tbx5, and Pax3. Tead family members are major transcriptional factors that interact with Yap and TAZ and induce cell proliferation and epithelial-mesenchymal transition (Vassive *et al.*, 2001, Mahoney *et al.*, 2005, Wang *et al.*, 2009). The mammalian *Tead* family has four members, i.e., *Tead1-4* (*NTEF-1*, *ETEF-1*, *DTEF-1* and *RTEF-1*, respectively). However, inÄ*Xenopus* only two *Tead* genes have been identified: *xTead1* (*xNTEF-1*) and *xTead3* (*xDTEF-1*); a previous study has characterized the expression patterns of *xTead1* and *xTead3* (Naye *et al.*, 2007). Some of the expression domains of *xtYap* and *xtTAZ* overlap with those of *xTead1* and *xTead3*. The *xTead1* and *xTead3* transcripts were predominantly detected in the eyes, embryonic brain, somites, and heart. Therefore, xtYap and TAZ can act as binding partners for xTead1 and xTead3 in these tissues. In other regions, in which *xtYap* or *xtTAZ* but not the *Tead* genes are expressed, these coactivators may function together with other transcriptional factors. For example, in the present study, we show that *xtTAZ* is expressed at the edge of the migrating hypaxial muscle. *Pax3* is expressed in *Xenopus* hypaxial muscle at the same stage, and functions in abdominal muscle formation in the mouse (Martin and Harland, 2001; Tajbakhsh *et al.*, 1997; Tremblay *et al.*, 1998). It has been reported that the transcriptional activity of Pax3 is coactivated by TAZ (Murakami *et al.*, 2006). Therefore, TAZ may act as a coactivator of Pax3 at the edges of proliferating and migratory hypaxial muscles to cancel signaling by the Hippo pathway.

Materials and Methods

Plasmid construction

Using the BLAST search algorithm (Altschul *et al.*, 1990), we identified from JGI *Xenopus tropicalis* v4.1 the genomic clones that correspond to *Xenopus tropicalis Yap* and *TAZ*. The full-length ORFs of *xtYap* and *xtTAZ* were amplified by PCR from the corresponding cDNAs of *Xenopus tropicalis* embryos and adult testes, respectively. The following primer sets (forward and reverse, respectively) were used:

xtYap 5'-ATGGAGCCCGGATCCCAG-3'
5'-CTATAACCACGTGAGGAAACTTTC-3';
xtTAZ, 5'-ATGAATCCGAGCCCACATCTCC-3' and
5'-CTACAGCCAGGTGAGGTAGGGCTC-3'. Each clone was subcloned into the pGEM-T-easy vector, and *xtYap* was subcloned into the pCS2p (+) vector. pCS2p-*xtYap* and pGEM-*xtTAZ* were used for synthesis of the *in situ* hybridization probes.

Alignment of sequences and construction of a phylogenic tree

Multiple protein sequence alignments and the generation of a phylogenic tree were performed using the CLUSTALW tool of the MacVector software (MacVector Inc.). The following sequences were used: hYAP (accession no. NP_001123617); mYap (NP_033560); zYap (NP_001132952); hTAZ (NP_056287); mTAZ (NP_598545); zTAZ (NP_001032785); and Yorkie (NP_001036568).

Semi-quantitative RT-PCR

Total RNA samples were isolated from various developmental stage embryos and adult tissues using RNAiso (Takara Bio Inc.), and 2 μg of total RNA was used as the template for first-strand cDNA synthesis using SuperScript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen). The cDNA (1 μl) was used as a template in the PCR. The following primer sets (forward and reverse, respectively) were used:

xtYap 5'-TCACTCCAGAGACGAAAGCACTGAC-3'
5'-CGTTGAGGATGTCGGAACTGAGAG-3';
xtTAZ 5'-GGCATTCACACATACGGGACAGAG-3'
5'-GCAGGGCTGGAAGTTATTTGTTGG-3';

ornithine decarboxylase (*ODC*) and *elongation factor 1* α (*EF1α*) were used as internal controls. The primer sets for ODC and EF1α were described in Sekizaki *et al.* (2004).

Whole-mount in situ *hybridization and histology of embryos*

Xenopus tropicalis embryos were obtained by artificial fertilization according to the method of Showell and Conlon (2009) with modifications,

cultured in 10% Steinberg's solution, and staged according to the scheme of Nieuwkoop and Faber (1994). Whole-mount *in situ* hybridization analysis was performed as described previously (Harland, 1991). Stained embryos were re-fixed in Bouin's solution overnight, dehydrated, embedded in paraffin, and sectioned at a thickness of 10 μm.

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