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Expression analysis of *epb41l4a* during *Xenopus laevis* embryogenesis

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Abstract

Epbl41l4a (*e*rythrocyte *p*rotein *b*and *4.1-l*ike *4a*, also named Nbl4) is a member of the band 4.1/Nbl4 (novel band *4*.1-*l*ike protein *4*) group of the FERM (*4*.1, *e*zrin, *r*adixin, *m*oesin) protein superfamily. Proteins encoded by this gene family are involved in many cellular processes such as organization of epithelial cells and signal transduction. On a molecular level, band 4.1/Nbl4 proteins have been shown to link membrane-associated proteins and lipids to the actin cytoskeleton. Epbl41l4a has also recently been identified as a target gene of the Wnt/β-catenin pathway. Here, we describe for the first time the spatiotemporal expression of *epbl41l4a* using *Xenopus laevis* as a model system. We observed a strong and specific expression of *epb41l4a* in the developing somites, in particular during segmentation as well as in the nasal and cranial placodes, pronephros, and neural tube. Thus, *epbl41l4a* is expressed in tissues undergoing morphogenetic movements, suggesting a functional role of epbl41l4a during these processes.

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Author contribution
KCS and FC identified *epb41l4a* as a potential castor target gene and cloned and sequenced the corresponding cDNA clone. YG analyzed the temporal and spatial expression pattern in *Xenopus*. SG analyzed the synteny of *epb41l4a*. SG and MK analyzed data and wrote the manuscript.

Keywords

Xenopus laevis; Epb41l4a; Nbl4; Erythrocyte protein band 4.1-like 4a

Introduction

Members of the FERM (*4*.1, *e*zrin, *r*adixin, *m*oesin) protein superfamily are important regulators of embryonic development. They form a group of membrane-associated proteins involved in regulating cytoskeletal rearrangement, intracellular transport, and signal transduction. Mutations in several FERM protein-coding genes are associated with human diseases including cancers and blood cell disorders (Bretscher et al. 2002; Tepass 2009; Delaunay 2002). FERM domain proteins are characterized by a specific domain called the FERM domain, which is usually located at the N-terminus. The FERM domain itself is a composite of three subdomains, F1, F2, and F3, that fold independently. They are closely associated with each other, forming a cloverleaf structure. Each of these subdomains has some limited homologies to ubiquitin (F1), acyl-Co-binding protein (F2), and the pleckstrin homology/phosphotyrosine binding domain (F3), respectively (Tepass 2009). All three subdomains of the FERM domain interact with various molecules such as cytoskeletal proteins, intracellular signaling proteins, ion channels, cell adhesion molecules, and lipids such as phosphatidyl-4,5-bisphosphate. In addition, several consensus sequences for myristoylation and phosphorylation have been identified in the FERM domain (Bretscher et al. 2002; Takeuchi et al. 1994; Manno et al. 2005). Furthermore, FERM-containing proteins can interact intramolecularly, thereby regulating their own activity (Bretscher et al. 2002).

Data from the human genome indicate the presence of approximately 50 FERM domain-containing proteins of which 22 are also found in the *Drosophila* genome (Tepass 2009). Based on sequence homologies and the presence of additional protein domains, the FERM domain protein superfamily can be subdivided into distinct groups including the band 4.1/Nbl4 (*n*ovel *b*and 4.1-*l*ike protein *4*) family [also called the FERM-FA (*F*ERM-*a*ssociated) family], the ERM (*e*zrin, *r*adixin, and *m*oesin) family, and the myosin family. Other family members are represented by merlin or talins. Janus kinases and focal adhesion kinase also contain a FERM domain.

The first member of the band 4.1/Nbl4 family to be identified was the band 4.1 protein, which was originally isolated from red blood cells. In erythrocytes, band 4.1 is important for maintaining the shape and stability of cells by linking transmembrane proteins of the outer cell membrane such as glycophorin A and C and CD44 (*c*luster of *d*ifferentiation) to the cytoskeleton by interacting with actin and spectrin (Marfatia et al. 1995; Anderson and Lovrien 1984; Nunomura et al. 1997). The N-terminal FERM domain is required for the binding to transmembrane proteins, and the spectrin/actin binding domain is located more C-terminally. Proteins belonging to this subgroup also contain a FA domain (*F*ERM-*a*ssociated) near the FERM domain. Different studies have been conducted to explore the function of FERM-FA containing proteins during embryonic development and tissue organization to elucidate their developmental importance. In zebrafish, the gene *Mosaic eyes* belongs to the band 4.1/Nbl4 subfamily of the FERM superfamily and is the homolog of *Drosophila* Yurt and human EPB41L4B (Tepass 2009). Mosaic eyes is required for retinal lamination and integrity of the retinal pigment epithelium (Jensen and Westerfield 2004). *Drosophila* Yurt is found in a complex with Crumbs and atypical protein kinase C, and this interaction is required for proper apical–basal polarity of epithelial cells (Hsu et al. 2006). During mouse embryogenesis, the close homolog EPB41L5 is required for the organization of the neural plate and for epithelial–mesenchymal transitions in the primitive streak (Lee et al. 2007).

Epbl41l4a (*e*rythrocyte *p*rotein *b*and *4.1-l*ike *4a*; also named Nbl4) is closely related to protein band 4.1 and also belongs to the FERM-FA family (Takeuchi et al. 1994). It has been identified in different organisms such as mouse, human (Shimizu et al. 2000), and zebrafish (Kelly and Reversade 1997). Recently, *epbl41l4a* was shown to be a target gene of the Wnt/β-catenin signaling pathway (Ishiguro et al. 2000). Expression and functional data have not been described for any *epbl41l4a* homologs. Therefore, we here describe the isolation and spatio-temporal expression of *Xenopus laevis epbl41l4a* during early embryogenesis as an important prerequisite for later functional studies.

Results and discussion

A search in the database revealed a full-length clone of *X. laevis epbl41l4a* (Acc. No. NM_001091331) that codes for 666 amino acid residues. By synteny analyses, we show that the gene loci of *epbl41l4a* and the neighboring genes are highly conserved between *Homo sapiens*, *Mus musculus*, and *Xenopus tropicalis* (Fig. 1). Similar to the other members of the Protein 4.1 superfamily, *Xenopus* epbl41l4a contains a FERM domain including the F1, F2, and F3 subdomains in its N-terminal half and a FA domain as well (Fig. 2a and Suppl. Fig. 1). The predicted protein sequence of epbl41l4a is highly conserved among diverse species (Fig. 2b and Suppl. Fig. 1).

As a first step, we examined the temporal expression pattern of *epbl41l4a* in *X. laevis* during embryonic development. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that *epbl41l4a* is expressed at nearly the same level throughout whole embryogenesis (Fig. 2c). The spatial expression pattern of *epbl41l4a* during *Xenopus* embryonic development was then analyzed by whole-mount in situ hybridization (Figs. 3 and 4). For this purpose, we cloned a cDNA of *Xenopus epbl41l4a* into the pCRII TOPO vector and synthesized an antisense probe. Very early in development, *epbl41l4a* was detected in the animal hemisphere (Fig. 3a, b). Please note that this does not exclude expression of *epb41l4a* in the vegetal half of the embryo as it is well known that the high lipid content of the vegetal half of the embryo prevents proper probe accessibility. At stage 13, *epb41l4a* was expressed in the paraxial mesoderm on the dorsal side of the embryo (Fig. 3c). At stages 15 and 20, *epbl41l4a* transcripts were also seen in the cardiac progenitor cell population adjacent to the cement gland and weakly in the anterior neural plate and the closing neural tube (Fig. 3a–g). The expression in the developing somites and the cardiac progenitor population at stage 20 was confirmed by sectioning (Fig. 4a–c). As development proceeded, the expression of *epbl41l4a* in the somites became restricted to the posterior part of the embryo where segmentation and rotation of the somite blocks take place (Figs. 3h, j–l and 4f, h, i). At stage 24, *epbl41l4a* was strongly expressed in the nasal placode and the neural tube, whereas expression in the cardiac region disappeared (Figs. 3h, i and 4d, e). Furthermore, endogenous *epbl41l4a* was observed throughout all developing pronephric tubules, the profundal ganglion (gPr), the trigeminal ganglion (gV), and the facial epibranchial ganglion (egVII) beginning at stage 29 (Figs. 3j–l and 4f, g, k, m, n). Transverse sections revealed that *epbl41l4a* was expressed in the floor plate and in a subpopulation of neurons likely representing ventral interneurons (Fig. 4j). *Epbl41l4a* expression was also detected in the lens of stage 36 embryos (Fig. 4l)

In zebrafish, *epbl41l4a* is maternally supplied and is present throughout embryogenesis (Kelly and Reversade 1997) similar to our results in *Xenopus* embryos. In adult zebrafish, *epbl41l4a* transcripts are found in the ovary, eye, heart, and brain (Kelly and Reversade 1997). Human *epbl41l4a* transcripts show high levels of expression in adult brain, liver, thymus, and peripheral blood leukocytes and low levels of expression in heart, kidney, testis, and colon (Kawasoe et al. 2000). Thus, the investigation of epbl41l4a during embryonic

development in other organisms would be of interest to draw any conclusion of a conserved expression pattern or function across species.

In summary, we here describe for the first time the expression pattern of *epbl41l4a* during embryonic development of *X. laevis*. Epbl41l4a showed tissue-specific expression during *X. laevis* development. The expression of *epbl41l4a* in the rotating somites, the forming neural tube, and the developing pronephros implies that it may contribute to morphogenetic movements in these tissues during early development. *Epbl41l4a* was previously described as a target gene of the Wnt/β-catenin pathway, which is also involved in somitogenesis, neural tube formation, and pronephros development (Logan and Nusse 2004; Gessert and Kuhl 2010; Aulehla et al. 2003, 2008; Saulnier et al. 2002; Tetelin and Jones 2010). It is tempting to speculate that some of the effects of Wnt/β-catenin signaling on morphogenesis in these tissues might be mediated through epb41l4a. Other proteins of the FERM-FA family have also been implicated in establishing epithelial polarity, a process that is important during neural tube and pronephric tubule formation. Based on these findings, we predict that epbl41l4a is required for proper morphogenesis of these tissues, a hypothesis that requires future analysis with epbl41l4a gain- and loss-of-function studies.

Experimental procedures

Xenopus embryos

Xenopus embryos were obtained and staged according to Nieuwkoop and Faber (1994).

Cloning of *X. laevis* Nbl4

Epbl41l4a was identified in a chromatin immunoprecipitation (ChIP) sequence screen for potential transcriptional targets of the zinc finger transcription factor Castor (information available upon request). *Epbl41l4a*-specific PCR primers were designed based on the identified ChIP-sequencing product and predicted sequence obtained from putative ESTs; 5-′: CGAGCCAGGATCAAACAATG and 3-′: CTGCACTGTGCTGTGTATCT. Primers were used to amplify and clone a 874 bp *epbl41l4a* specific product from a mixed stage 19–26 cDNA pool (information available upon request) and cloned into Invitrogen pCRII TOPO vector. Insert and orientation were confirmed by 4× sequence coverage.

Whole-mount in situ hybridization and sectioning of stained embryos

Digoxigenin-labeled *epbl41l4a* antisense RNA probe were synthesized by digesting with Xhol I (NEB) and transcribing with Sp6 RNA polymerase (Roche). Embryos of different stages were fixed in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, and 4% formaldehyde) overnight at 4°C and subsequently dehydrated in 100% methanol. Whole-mount in situ hybridization was performed according to a standard protocol as previously described (Gamse and Sive 2000; Gessert et al. 2007). BM-Purple (Roche) was used for staining. Stained embryos were bleached with 30% H_2O_2. For sections of stained embryos, *Xenopus* embryos were embedded in gelatine/albumin overnight at 4°C, sectioned on a Vibratome at a thickness of 25 μm, coverslipped, and imaged on an Olympus BX60 microscope.

RNA isolation and RT-PCR assays

Total RNA was isolated from *Xenopus* embryos at different stages with peqGOLD RNAPure reagent (peq-Lab) according to the manufacturer's protocol. First strand cDNA primed with random hexamers was synthesized with SuperTranscript II reverse transcriptase (Invitrogen). PCR was performed with the following primers: Epbl41l4a_for: 5′-AAAAACCACACAGGCCTCAC-3′; Epbl41l4a_rev: 5′-ATCAGTCCCTGTCCAACCAG-3′; GAPDH (*g*lycer*a*ldehyde-3-*p*hosphate

*de*hydrogenase) for: 5′-GCCGTGTATGTGGAATCT-3′; GAPDH_rev: 5′-AAGTTGTCGTTGATGACCTTTGC-3′; annealing was done at 55°C.

Protein alignment and synteny analyses

The ClustalW2 program was used for amino acid sequence alignment and homology calculation. For amino acid sequence comparisons, the following sequences were used: human EPBL41L4A: Acc. No. NM_022140; mouse Epbl41l4a: NM_013512; zebrafish epbl41l4a: NM_131223; *X. laevis* epbl41l4a: NM_001091331. For the synteny analyses, genomic structure and chromosomal organization of *epbl41l4a* in human, mouse, and *X. tropicalis* were compared using NCBI and Xenbase G Browse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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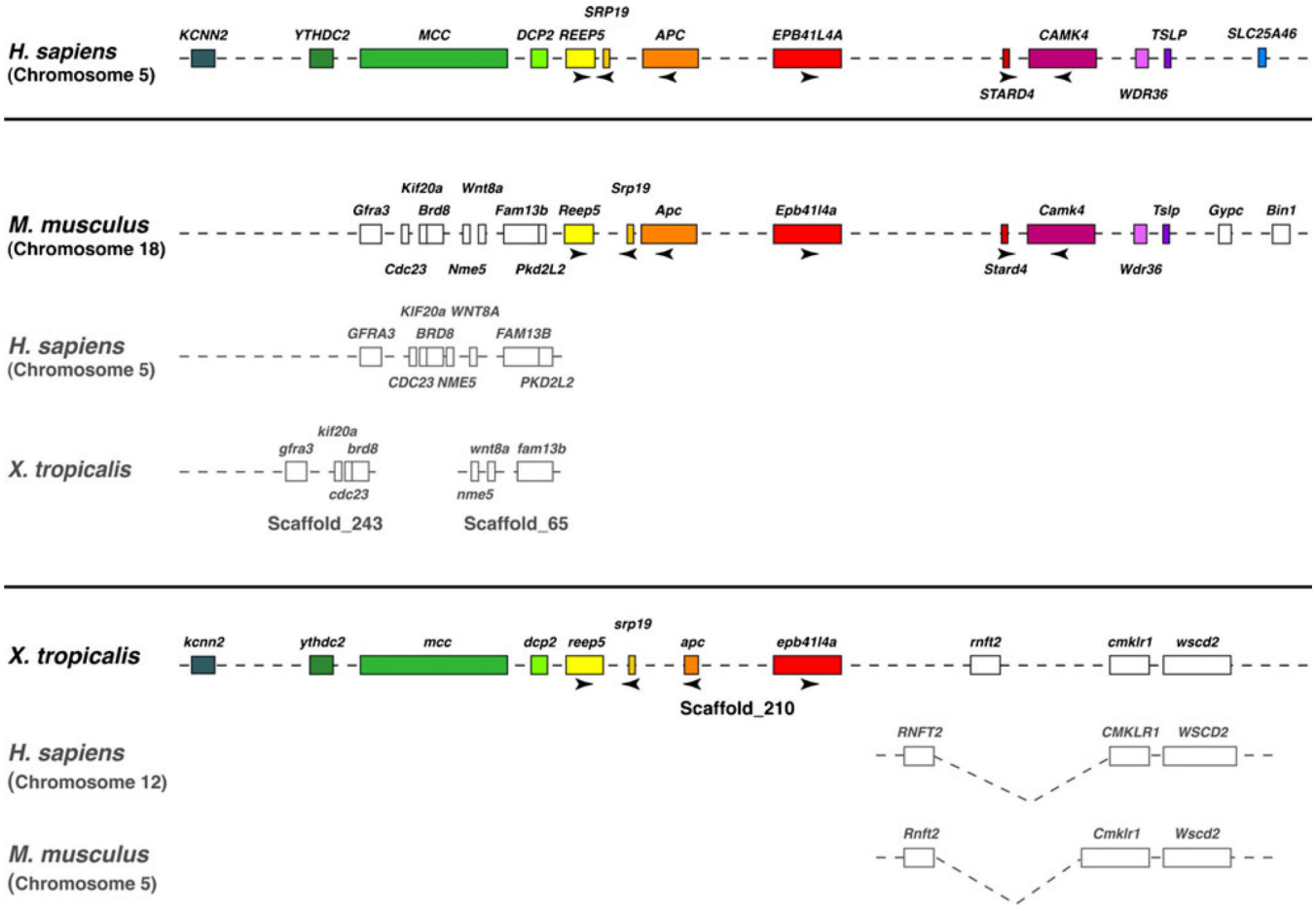
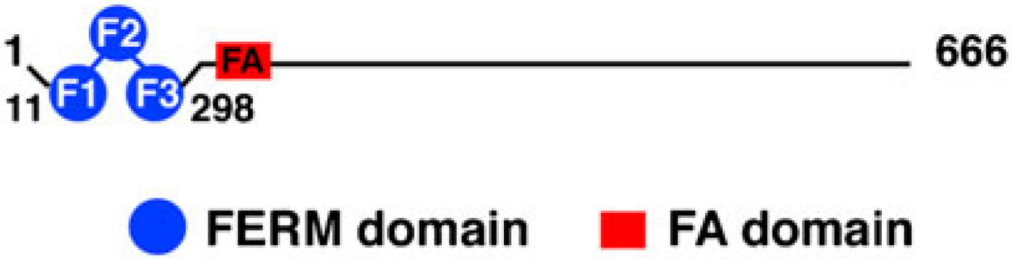


Fig. 1.

Synteny analysis of *epbl41l4a* in *Homo sapiens*, *Mus musculus*, and *Xenopus tropicalis*. A schematic illustration shows the comparison of the *epbl41l4a* gene and neighboring gene loci in *H. sapiens* (chromosome 5), *M. musculus* (chromosome 18), and *X. tropicalis* (scaffold 210). The orientation of open reading frames of some genes is indicated by *arrowheads*. The length of genes and distances between them are not drawn to scale. Conserved genes are indicated by a color code. Nonconserved genes are indicated with *open boxes* and chromosomal localization for those in other species is indicated as well. *apc* Adenomatous polyposis coli, *bin1* bridging integrator 1, *brd8* bromodomain containing 8, *camk4* calcium/calmodulin-dependent protein kinase 4, *cdc23* cell division cycle 23 homolog, *cmklr1* chemokine-like receptor 1, *dcp2* decapping protein 2, *epb41l4a* erythrocyte protein band 4.1-like 4a, *fam13b* family with sequence similarity 13, member b, *gypc* glycophorin C, *kcnn2* potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2, *kif20a* kinesin family member 20a, *nme5* non-metastatic cell 5, *mcc* mutated in colorectal cancers, *pkd2l2* polycystic kidney disease 2-like 2, *reep5* receptor accessory protein 5, *rnft2* ring finger protein, transmembrane 2, *slc25a46* solute carrier family 25, member 46, *stard4* StAR-related (START) domain containing 4, *srp19* signal recognition particle 19, *tslp* thymic stromal lymphopoietin, *wdr36* WD repeat domain 36, *wscd2* wsc domain containing 2, *ythdc2* YTH domain containing 2

A



B

Species	aa	Overall	FERM	FA
H. sapiens	686	100	100	100
M. musculus	686	93	96	90
X. laevis	666	77	83	69
D. rerio	619	72	85	58

C

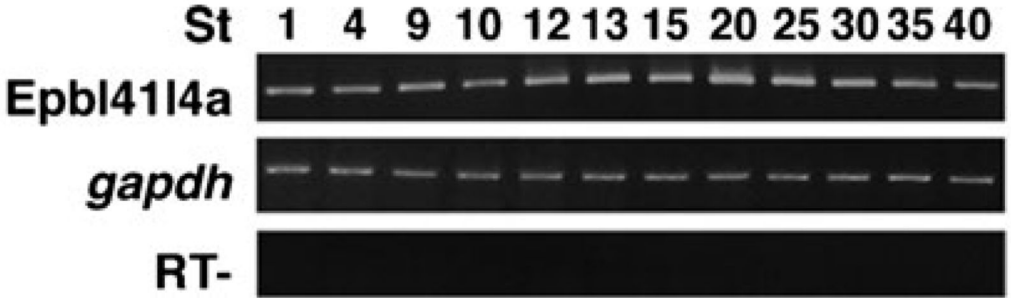


Fig. 2.

Epbl41l4a in *Xenopus*. **a** Schematic representation of *Xenopus* epbl41l4a. The cloverleaf-shape region (*blue*) indicates the FERM (*4*.1, *e*zrin, *r*adixin, *m*oesin) domain including F1, F2, and F3 subdomains, the *red box* the FA (*F*ERM-*a*ssociated) domain. N-terminal is to the left. **b**. Homology in percent between epbl41l4a proteins among different species. In addition to overall similarity, the degree of conservation of FERM and FA domains is shown, respectively. *aa* length in number of amino acids. **c** Temporal expression pattern of *Xenopus laevis* epbl41l4a analyzed by semiquantitative RT-PCR. *Epbl41l4a* transcripts are

present throughout whole embryogenesis but are slightly increased during neurula stages. *Gapdh* was used as loading control

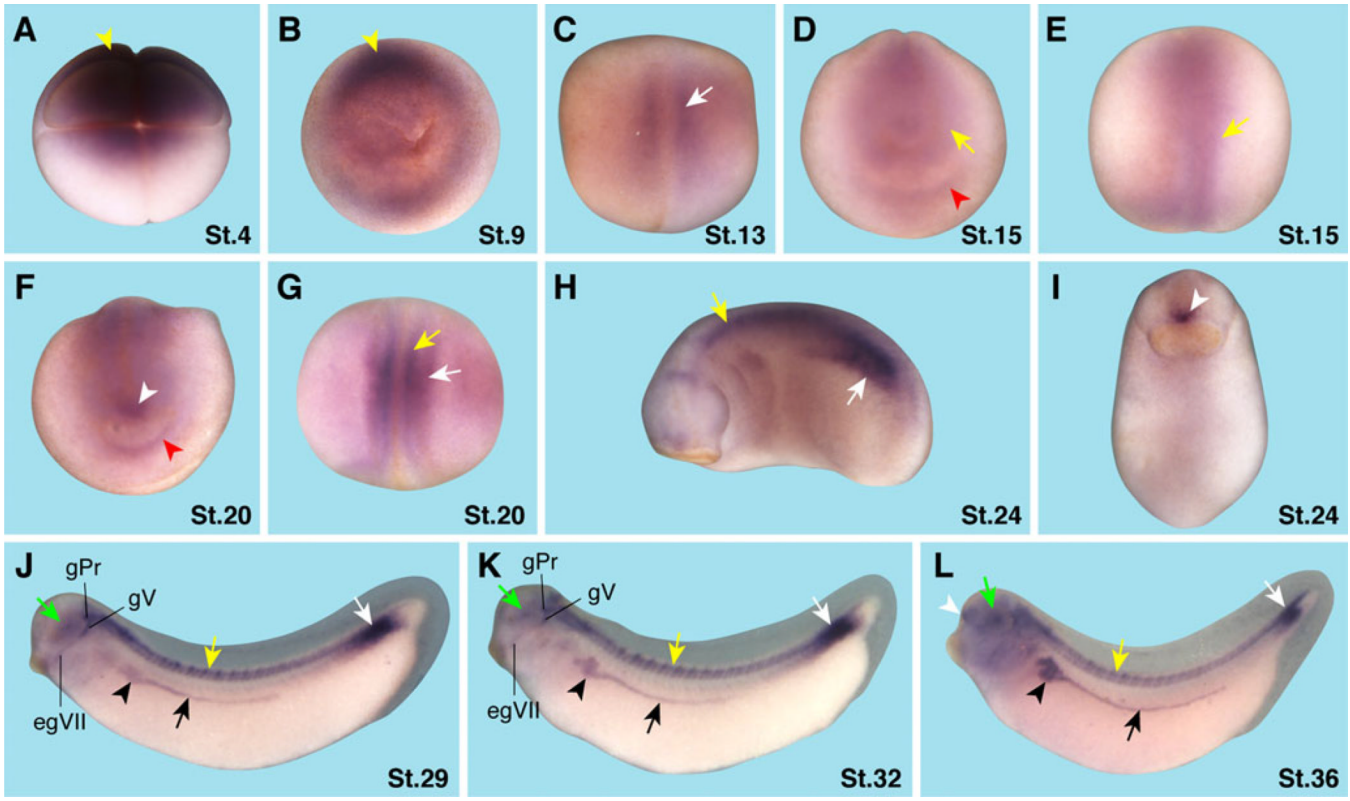


Fig. 3.

Spatial expression pattern of *epbl41l4a* mRNA during *Xenopus laevis* embryonic development. The spatio-temporal expression of *epbl41l4a* is visualized by whole-mount in situ hybridizations. Embryonic stages are indicated in each panel. **a** Lateral view of an embryo at stage 4. *Epbl41l4a* is expressed in the animal pole of the embryo (*yellow arrowhead*). **b** Animal view of a stage 9 embryo. *Epbl41l4a* transcripts are detected in the animal half of the embryo (*yellow arrowhead*). **c** Dorsal view of an embryo at stage 13. *Epbl41l4a* transcripts are visualized in the paraxial mesoderm representing somitic precursor cells (*white arrow*). **d** Anterior view. *Epbl41l4a* mRNA is expressed in the cardiac progenitor cell population (*red arrowhead*) as well as slightly in the anterior neural plate (*yellow arrow*). **e** Dorsal views of a stage 15 embryo. *Epbl41l4a* transcripts are detected in the dorsal neural tube and somite anlage (*yellow arrow*). **f** An embryo at stage 20 from the anterior side. Endogenous *epbl41l4a* is detectable in the cardiac crescent (*red arrowhead*) and in the nasal placode (*white arrowhead*). **g** Dorsal view of a stage 20 embryo. *Epbl41l4a* is expressed in the neural tube (*yellow arrow*) and the forming somites (*white arrow*). **h** An embryo at stage 24 shown from the lateral side with the head to the left. *Epbl41l4a* transcripts are visualized in the neural tube (*yellow arrow*) and the posterior, unsegmented part of the somites (*white arrow*). **i** Ventral view of an embryo at stage 24 with the head to the top. *Epbl41l4a* is expressed in the nasal placode (*white arrowhead*). **j–l** Lateral views of embryos at indicated stages with anterior to the left. *Epbl41l4a* starts to be expressed in the pronephric tubules (*black arrow* and *black arrowhead*) at stage 29 and the expression becomes stronger at later stages. It can also be visualized in the neural tube (*yellow arrow*), the eye (*green arrow*), the nasal placode (*white arrow*), the profundal ganglion (*gPr*), the trigeminal ganglion (*gV*), and the facial epibranchial ganglion (*egVII*) as indicated

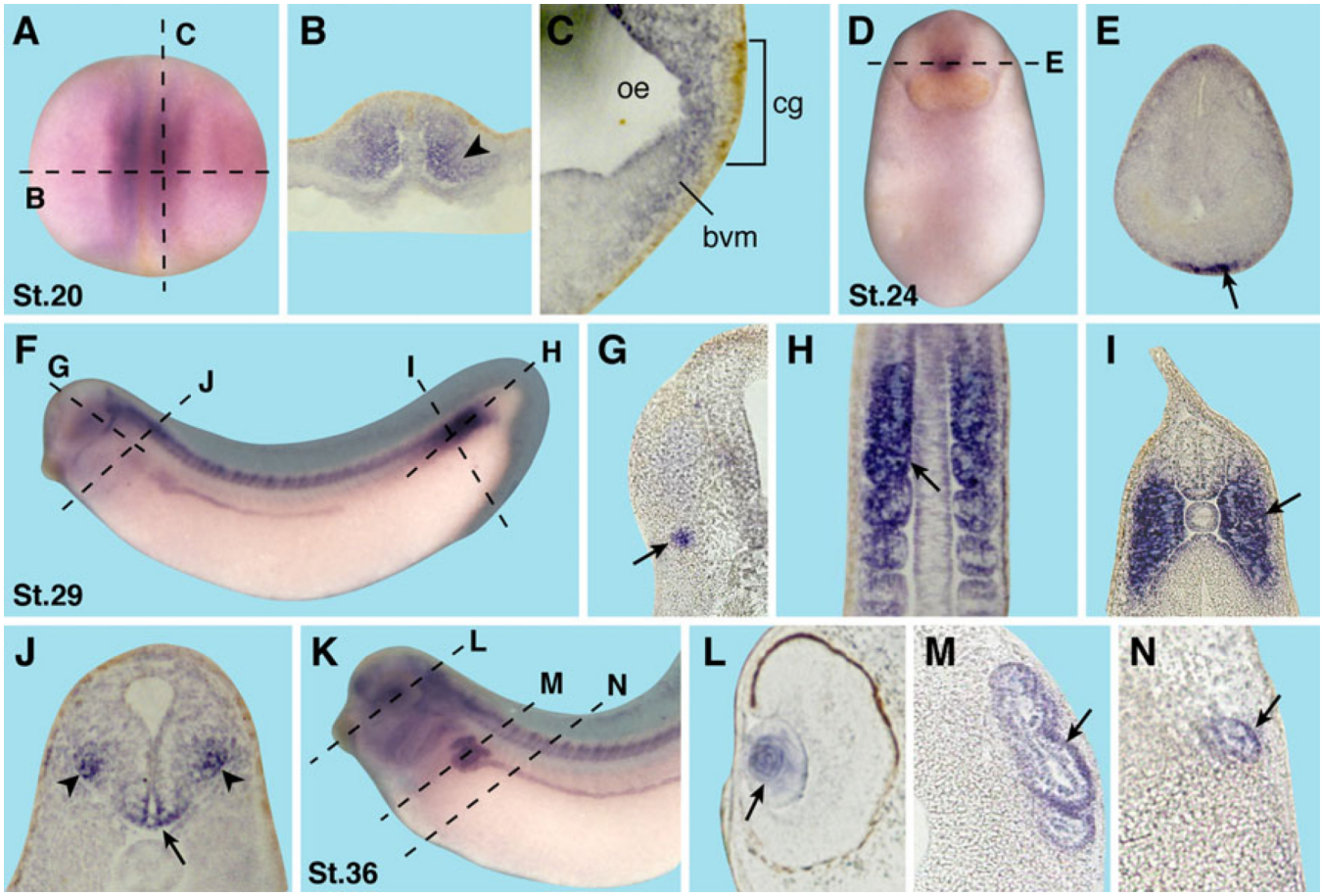


Fig. 4.

Epbl41l4a expression on *Xenopus* sections. Sections of stained *Xenopus* embryos at different stages. *Dashed lines* indicate levels of sections. **a** Dorsal view of a stage 20 embryo. Levels of sections as shown in Fig. 3b and c are given. **b** A transverse section shows the expression of *epbl41l4a* in the paraxial mesoderm (*arrowhead*). **c** *Epbl41l4a* is expressed in the border of ventral mesoderm (*bvm*) which represents the cardiac progenitor cell population posterior to the cement gland (*cg*) and ventral to the oral evagination (*oe*) as shown by a parasagittal section. **d** Ventral view of an embryo at stage 24. Level of section as shown in (*E*) is indicated by a *dashed line*. **e** The transverse section demonstrates *epbl41l4a* expression in the nasal placode (*arrow*). **f** Lateral view with anterior to the left. Levels of sections as shown in (*G–J*) are indicated by *dashed lines*. **g** *Epbl41l4a* transcripts are visualized in the trigeminal ganglion (*arrow*) shown by a horizontal section. Anterior is to the top. **h** Horizontal section through the somites on the level of rotating somites. Posterior is to the top. *Epbl41l4a* is expressed in the somites before and during rotation (*arrow*) and becomes downregulated in the anterior segmented and rotated somites. **i** *Epbl41l4a* transcripts are visualized in the paraxial mesoderm before somite segmentation (*arrow*). **j** Transverse section demonstrates *epbl41l4a* expression in the neural tube (*arrow* indicates the floor plate, *arrowheads* show a discrete population of ventral interneurons). **k** Lateral view of a stage 36 embryo with anterior to the left. Levels of sections as shown in (*L–N*) are indicated by *dashed lines*. **l–n** Transverse sections of a stage 36 embryo. Endogenous *epbl41l4a* is observed in the lens (**l**), proximal and intermediate tubules (**m**), and distal tubules (**n**) as indicated by *arrows*