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Anna Ghilardi, Alberto Diana, Laura Prosperi, Luca Del Giacco

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TITLE

Expression pattern of the *small muscle protein, X-linked (smpx)* gene during zebrafish embryonic and larval developmental stages

AUTHOR NAMES AND AFFILIATIONS

Anna GHILARDI, Alberto DIANA, Laura PROSPERI, Luca DEL GIACCO

Università degli Studi di Milano, Department of Biosciences, via Celoria 26, 20133 – Milano, ITALY

AUTHORS EMAIL ACCOUNTS

anna.ghilardi@unimi.it; alberto.diana@unimi.it; lau.prosperi@gmail.com; luca.delgiacco@unimi.it

CORRESPONDING AUTHOR

Luca DEL GIACCO, Università degli Studi di Milano, Department of Biosciences, via Celoria 26, 20133 – Milano, ITALY

email: luca.delgiacco@unimi.it

ph: +39 02 50314807

HIGHLIGHTS

- Zebrafish *smpx* is expressed during development in a spatio-temporal fashion similar to higher Vertebrates

- Zebrafish *smpx* is expressed in the Kupffer's vesicle (KV), in the somites, in the muscles, in the heart, and in the pronephric ducts

- Zebrafish *smpx* is also expressed in the ciliated cells of the embryonic inner ear, and mutations in human *SMPX* are known to produce hearing impairment

ABSTRACT

The *small muscle protein, X-linked (SMPX)* gene encodes a cytoskeleton-associated protein, highly expressed in both cardiac and skeletal muscles, as well as in fetal inner ears, with suggested roles as mechanotransductor. Recently, several mutations in the *SMPX* gene have been associated with X-chromosomal progressive deafness in human. However, very little information is known concerning the roles of SMPX, and no *in-vivo* models are currently available. Therefore, we characterized the zebrafish ortholog of *SMPX* to pave the way towards the establishment of a biotool for future functional studies. Despite the genome duplication occurred in the ancestry of teleosts, zebrafish retain only one copy of *smpx* which shares a high degree of similarity with the mammalian counterpart in terms of genomic organization, syntenic map, and encoded protein. RT-PCR, as well as whole-mount *in-situ* hybridization and immunohistochemistry analyses, revealed that *smpx* is expressed in several embryonic areas starting from the 4-somite stage. Specifically, *smpx* mRNA marked the Kupffer's vesicle (KV), the somites, the myocardium, the hair cells of the anterior and the posterior macula of the inner ear, the pronephric ducts, and the muscles of the branchial arches, eyes and pectoral fins. According to our data, zebrafish *smpx* expression pattern closely resembles that observed in mouse and human, supporting the notion that zebrafish might represent a suitable *in-vivo* model to disclose the cellular and molecular mechanisms underlying the involvement of SMPX in development and disease.

1.INTRODUCTION

The *small muscle protein, X-linked (SMPX)* gene is evolutionarily conserved across mammals (Palmer et al., 2001). The gene encodes a 9 kD proline-rich cytoskeleton-associated protein with a CKII (protein kinase CK2) target sequence, suggestive of potential modulation activity through phosphorylation, and a PEST consensus sequence, a region rich in P (proline), E (glutamate), S (serine), and T (threonine), which is often associated to rapid protein degradation; no known functional domains have been identified so far (Patzak et al., 1999; Schraders et al., 2011). SMPX also contains a potential nuclear localization signal but, besides a single study reporting the accumulation of the protein in the nucleus of C2C12 cells in culture (Kemp et al., 2001), other authors showed clear evidence of exclusion of Smpx from the nuclei also *in vivo*, both in normal and hypertrophic muscle (Eftestøl et al., 2014 and reference therein). The gene is highly transcribed in mammalian fetal and adult skeletal muscle, as well as in the developing and adult heart, and a role as a mechanotransductor in sensing changes in mechanical loads has been suggested (Schindeler et al., 2005). Despite this pattern of expression, *Smpx* knockout in mouse (Palmer et al., 2001) and *Smpx* overexpression in rat (Eftestøl et al., 2014) did not produce obvious signs of muscular dysfunction. On the other hand, *in-vitro* studies suggested *Smpx* potential roles in regulating muscle fibers size via the Rac1/p38 and IGF-1 pathways (Palmer et al., 2001; Schindeler et al., 2005) and indicated NOR-1 - a nuclear receptor involved in the differentiation of human skeletal myoblast to myotubes and associated with hypertrophy of heart and skeletal muscle - as a positive regulator of *SMPX* expression (Ferrán et al., 2016).

In the last few years, mutations in the human *SMPX* gene have been associated with progressive hearing loss, as well as congenital hearing impairment, with more severe phenotypes in hemizygous males (Huebner et al., 2011; Schraders et al., 2011; Weegerink et al., 2011; Abdelfatah et al., 2013; Stanton et al., 2014; Niu et al., 2017; Niu et al., 2018; Deng et al., 2018). Remarkably, Schraders and colleagues reported the expression of *SMPX* in both fetal and adult inner ear (Schraders et al., 2011). Moreover, mouse *Smpx* has been identified as a novel gene potentially associated with

hair cell differentiation, being expressed in cochlear and vestibular hair cells (Yoon et al., 2011). Given the association of Smpx with the cytoskeleton, its responsiveness to mechanical force, and its presence in mouse hair cells, Huebner and colleagues suggested that SMPX might protect the stereocilia of the sensory epithelium from the continuous physical stress they are usually exposed during ear functioning (Huebner et al., 2011). Therefore, lack of functional SMPX due to nonsense gene mutations might cause the early deterioration of stereocilia and, by consequence, the onset of hearing loss. This wealth of data stimulates further analyses to elucidate potential *Smpx* contribution in muscle development, maintenance, and function, as well as its role in inner ear hair cells development and/or maintenance.

In this scenario, we propose zebrafish as a novel model to elucidate Smpx roles. Here, we analyzed zebrafish *SMPX* ortholog expression employing RT-PCR and Western blot techniques, *in-situ* hybridization and immunohistochemistry on whole embryo and paraffin sections. The analysis has been performed from the 1-cell stage to 5 days post fertilization.

2.RESULTS AND DISCUSSION

This study is focused on the characterization of the zebrafish *SMPX* ortholog providing an extensive analysis of the pattern of expression of the gene over the course of development up to 120 hpf, with particular emphasis on *smpx* expression in the vestibular and auditory components of the inner ear.

2.1. Zebrafish SMPX ortholog identification

Blast analysis of the ENSEMBL zebrafish assembly version 9 (Zv9) using mouse *Smpx* full-length mRNA sequence returned one positive hit on chromosome 24 (Figure 1) mapping at location 25,467,465-25,500,712. Therefore, despite the so-called teleost-specific genome duplication (TSD) (Meyer and Schartl, 1999), zebrafish retain only one copy of *smpx*. The matching mRNA sequence, identified by the GenBank accession number NM_001002185.1, encodes a protein sharing a high degree of similarity (71%) with the mammalian counterpart. It is also interesting to note that the zebrafish *smpx* gene is located within a region that is highly synthenic with the region of the human X-chromosome encompassing *SMPX* (Figure 1A).

2.2. Zebrafish smpx expression pattern analysis in embryos and larvae

Temporal *smpx* expression during early stages of zebrafish development has been analyzed by means of RT-PCR. The assay has been performed from 1-cell stage to 72 hours post fertilization (hpf). *Smpx* started to be expressed from the 4-somite stage (Figure 1B), with the *smpx*-specific primers revealing two amplicons of 188 and 275 bp, respectively, corresponding to alternative splicing isoforms (Figure 1B,C), as confirmed by DNA sequencing (data available on request from the authors). The two mRNAs encoded a short (54 amino acid residues, identified by the GenBank accession number KU880689.1) and a long (83 amino acid residues) form of the protein (Figure 1C). Interestingly, even though no Smpx short form was reported in any protein database when we performed the analysis, a more in-depth bioinformatic inquiry revealed that such splicing event does occur also in higher vertebrates. Indeed, the BLAST/tblastn analysis of the NCBI Expressed Sequence Tags (ESTs) database returned - besides some positive

hits concerning Smpx short form in other fish species *(i.e. Oncorhynchus mykiss* and *Salmo salar)* - also positive hits regarding the presence of the mRNA encoding such form in the two mammals *Heterocephalus glaber* and *Sus scrofa*. At this juncture, the 2 forms of the protein have been revealed in several other species, from fish to human. Later on, only the 275 bp *smpx* amplicon is detectable in all the stages analyzed (Figure 1B). Such splicing switch might be functionally relevant being the two isoforms specifically associated to different stages during zebrafish embryonic development, thus deserving a future more in-depth analysis. We were not able to identify any other splicing form involving other exons of the gene.

The expression of *smpx* at the 4-somite stage has been also revealed by whole mount *in-situ* hybridization (WISH). At this stage, *smpx* mRNA specifically marks the Kupffer's vesicle (KV) (Figure 2A). From the 10-somite stage *smpx* is expressed at the level of each somite (Figure 2B-D), painting few rows of the most adaxial cells adjacent to the notochord (Figure 2B'). At 24 hpf the signal is still present in the somites, labeling both the fast and the slow muscle fibers, and starts to be visible in the heart (Figure 2D and inset). By 36 hpf, *smpx* mRNA labels the heart and is also detectable at the level of the ear (Figure 2E). The images at 48 hpf show *smpx* persistent expression in the heart and display the specific signal in the ear (Figure 2F), precisely in the hair cells of the anterior and the posterior maculae (Figure 2I-J'). At 72 hpf, *smpx* transcripts are visible in both the ventricular and the atrial myocardium (Figure 2K), in the ear, in the musculature of the branchial arches, in the eye muscles and in the musculature of the pectoral fins (Figure 2G,H).

We were also able to detect the presence of the Smpx protein employing a commercial antibody raised against mouse Smpx. After confirming the specificity of the antibody by means of Western blot (data not shown), the analysis was extended to the whole embryo, as well as to tissue sections, specifically covering the 48 hpf and 120 hpf stages. In particular, our immunofluorescence (IF) analysis focused on the inner ear, being mutations in human *SMPX* responsible for congenital and progressive forms of hearing loss (Huebner et al., 2011; Schraders et al., 2011; Weegerink et al., 2011; Abdelfatah et al., 2013; Stanton et al., 2014; Niu et al., 2017; Niu et al., 2018; Deng et al., 2018). The expression data obtained by WISH have been fully

confirmed by IHC, with clear signals at the level of the maculae (48 hpf, Figure 3 A,B) and cristae (120 hpf, Figure 3 C-E), the organs responsible for both hearing and sensing linear acceleration and gravity. According to our whole mount IHC experiments, Smpx localizes at the level of the hair cell apical plasma membrane (Figure 3). To increase the resolution of the analysis, we next performed IHC on histological sections. Once again, the antibody differentially decorated the hair cells, indicating that Smpx is mainly localized at the level of the actin-based cuticular plate (CP, Figure 4), a specialized structure found at the apical surface of such cells and essential for their mechanotransduction activity (Du et al., 2019 and reference therein), as pointed out by the actin/Smpx signals partial overlap (Figure 4 B,B'). On the other hand, Smpx/acetylated tubulin co-labeling showed two signals in close proximity to each other, but did not reveal evidence of co-localization, nor with the kinocilium neither with the somatic tubulin underlying the CP (Figure 4 C,C').

Interestingly, the IHC analysis showed the presence of the protein in the cells of the pronephric duct and tubules, as clearly visible in cross sections at 48hpf (Figure 5), thus representing a novelty compared to mammalian kidney, in which the expression of the gene has never been reported. The zebrafish pronephros is populated by two different types of ciliated cells, one responsible for transport, the other specialized in fluid propulsion (Liu et al., 2007). Therefore, we suggest that the Smpx signal observed in the pronephric structures might reflect the expression of the gene in one or both ciliated cell types.

3.EXPERIMENTAL PROCEDURES

3.1. Zebrafish strains

Zebrafish embryos and larvae of the AB strain were obtained through natural spawning of wild type adult fish, raised at 28℃ in the presence of 0.002% Methylene Blue and 0.003% PTU (1-phenyl-2-thiourea), and staged as previously described (Kimmel et al., 1995).

Our facility strictly complies with the relevant Italian laws, rules and regulations (Legislative Decree No. 116/92), as confirmed by the authorization issued by the municipality of Milan (PG 384983/2013). The procedures were carried out in accordance with the relevant guidelines and regulations.

3.2. smpx identification and cDNA cloning

Zebrafish chromosome 24 region spanning the *smpx* gene was identified through *in-silico* search of the ENSEMBL zebrafish assembly version 9 (Zv9) using mouse *Smpx* full-length cDNA as a bait. Three gene specific primers (smpx_F0: 5'-TTGACATTTGTGTCTGACACCA-3', smpx_F1: 5'-ACTGCACACAATGTCAAAACA-3' and smpx_R1: 5'-CACCTGAGCCAATTTAGTCTT-3') have been designed and used to amplify the *smpx* cDNA fragment starting from reverse transcribed total RNA purified from 24 hpf embryos. PCR on cDNA has been performed using smpx_F0 and smpx_R1 primers, then smpx_F1 and smpx_R1 primers have been used for a seminested reaction. The identity of the 188 and 275 bp amplicons (see Results and Discussion section) has been confirmed by DNA sequencing.

3.3. RT-PCR expression analysis

RT-PCR was performed on total RNA from embryos at 11 different developmental stages (1–2 cells, oblong, 30% epiboly, 90% epiboly, tailbud, 2 somites, 4 somites, 8 somites, 24 hpf, 48 hpf and 72 hpf) extracted with the SV Total RNA Isolation System (Promega) and reverse transcribed with the ImProm-II™ Reverse Transcriptase kit (Promega), according to manufacturers' instructions.

The RT-PCRs have been performed as described above. The β - actin primers have been used to control the quality of the RNA/cDNA preparations (β - actin_F: 5'-TGTTTTCCCCTCCATTGTTGG-3' and β - actin_R: 5'-TTCTCCTTGATGTCACGGAC-3').

3.4. Whole-Mount In-Situ Hybridization

For the riboprobes preparation (sense and antisense), the 275 bp amplicon (smpx_F1/smpx_R1 primers) was cloned using the TOPO™ TA cloning kit (Thermo Fisher) then transcribed with digoxigenin modified nucleotides (Roche). Whole mount *in-situ* hybridization (WISH), was carried out as previously described (Thisse et al., 1995) on embryos fixed overnight in 4% paraformaldehyde/PBS (phosphate buffered saline), rinsed with PBS-Tween®-20, dehydrated in 100% methanol and stored at -20℃ until processed for WISH. Embryos were imaged by Digital Camera Leica DFC310 FX and the Leica Application Suite (LAS) software (Leica) on a Leica MZ10 F stereomicroscope.

For histological sections, stained embryos were dehydrated, wax embedded and sectioned by a microtome (Leitz 1516) into 7 μm slices. Images were acquired with a Leica DFC450C digital camera and the Leica Application Suite (LAS) software (Leica) on a Leitz DM RB microscope.

3.5. Immunofluorescence and phalloidin staining

Whole mount immunofluorescence (IF) assays were performed according to routine protocol (Imperatore et al., 2019). Fresh embryos were fixed overnight in 4% paraformaldehyde/PBS, washed with PBS/ Tween®-20/Triton™X-100 and then incubated with 5% BSA (Bovine Serum Albumin – Merck) and primary antibodies, anti-acetylated α-tubulin (T7451, Merck) and SPMX-polyclonal antibody (PA3-070, Thermo Fisher); Goat anti-mouse Alexa fluor™ 488 (Thermo Fisher) and goat anti-rabbit Alexa fluor™ 555 (Thermo Fisher) are used as fluorescent secondary antibodies. Cell nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI). Phalloidin staining was performed on embryos fixed overnight in 4% paraformaldehyde/PBS and subsequently washed with PBS. Samples were stained with a 50 g/ml fluorescent

Phalloidin-Atto 550 (19083, Sigma-Aldrich) conjugate solution in PBS for overnight incubation.

For histological sections, fixed embryos were dehydrated, wax embedded and sectioned (7 μm) by a microtome (Leitz 1516). Immunofluorescence procedures were executed according to Zhang et al. (2017) protocol. The antibodies used are the same of the whole mount immunofluorescence assays.

All the immunofluorescent samples images were acquired by a Nikon A1 laser-scanning confocal microscope (Nikon Instruments Inc.).

4.CONCLUSION

In recent years, mutations in *SMPX* gene have been associated with progressive non-syndromic hearing loss, as well as congenital non-syndromic hearing impairment in human, with more severe phenotypes in hemizygous males. The expression analyses of the zebrafish orthologue point out several similarities with the mouse model and humans, suggesting the possible conservation of the function of the protein through evolution. Particularly, the expression of the *smpx* in the inner ear of the zebrafish larva may serve as the foundation for the employment of the model to undertake the study of the cellular and molecular mechanisms behind the genetic susceptibility to hearing loss in *SMPX*-mutated patients.

5.CONFLICTS OF INTEREST

The authors have no conflicts of interest to be declared.

6.FINANCIAL SUPPORT

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8.FIGURE LEGENDS

Figure 1. ***Genomic organization and RT-PCR* expression profile of *smpx* during zebrafish development. (A)** Schematic representation of the synteny between zebrafish chromosome 24 region hosting *smpx* and human *SMPX* locus on the X chromosome. The orthologs and their reciprocal orientation are indicated. Gene abbreviation: *POLA1 - DNA polymerase alpha 1, catalytic subunit; PCYT1BA - phosphate cytidylyltransferase 1, choline, beta a; PDK3 - pyruvate dehydrogenase kinase 3; PTCHD1 - patched domain containing 1; PHEX - phosphate regulating endopeptidase homolog X-linked; MBTPS2 – membrane bound transcription factor peptidase, site 2; SMPX – small muscle protein X-linked; KLHL34 - kelch like family member 34; CNKSR2 – connector enhancer of kinase suppressor of Ras 2; SH3KBP1 – SH3 domain containing kinase binding protein 1; MAP3K15 – mitogen-activated protein kinase kinase kinase 15; PDHA1 – pyruvate dehydrogenase E1 subunit alpha 1.* **(B)** Ethidium bromide-stained gel of the semi-nested RT-PCR performed with the *smpx*_F1 and *smpx*_R1 primers using the *smpx*_F0/*smpx*_R1 reaction as template (see *Experimental procedures* for details). The 188 and 275 bp bands represent the two products of alternative splicing of the *smpx* RNA. The gene is expressed starting from the 4-somite stage up to 72 hpf (upper panel). RT-PCR employing β-actin specific primers has been performed as control for RNA integrity and cDNA synthesis. **(C)** *smpx* genomic organization and alternative splicing forms of the RNA (intron/exon boundaries are indicated). Zebrafish *smpx*, as well as the human *SMPX*, spans five exons (ex1 - ex5) on chromosome 24, three of which encoding a polypeptide of 83 amino acid residues (long form). When intron 2 donor site (gt) and intron 3 acceptor site (ag) interact, exon 3 is spliced out, resulting in a smaller transcript encoding a 54 amino acid residues protein (short form). The portion of the long form encoded by exon 3 is depicted in blue.

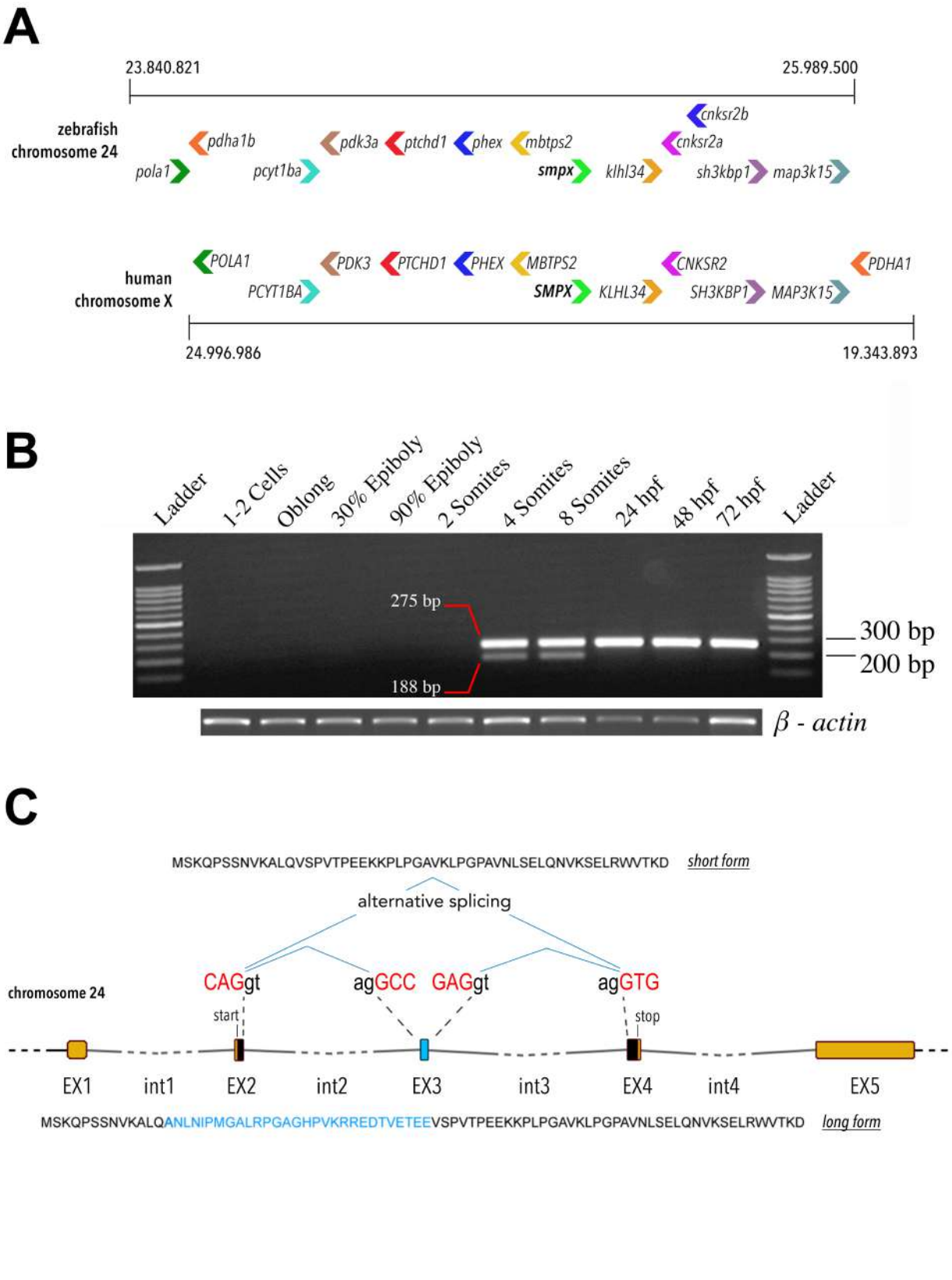
Figure 2. ***smpx embryonic expression pattern analyzed by in-situ hybridization.*** **(A)** At first *smpx* signal appears at 4-somite stage at the level of the Kupffer's vesicle (white arrowhead, frontal view), as also shown in the inset (lateral view). **(B-D)** From 10 to 24 hpf the gene is abundantly expressed in each somite. **(B')** Cross-section of a 10 somites embryo showing *smpx* mRNA labeling the most adaxial cells next to the notochord. **(D)** The 24 hpf stage marks the onset of *smpx* expression in the developing heart (white arrowhead); *smpx* now labels both fast (white asterisk) and slow (black arrowhead) muscle fibers, as shown by the cross-section in the inset. **(E-G)** While *smpx* expression decreases in the trunk, the signal at 36, 48, and 72 hpf persists in the heart (black arrowhead), labeling both ventricle and atrium **(K)** and is now visible in the forming ear (white arrow), specifically in the territories corresponding to the anterior **(I,I')** and posterior **(J,J')** maculae. **(G,H)** Larvae at 72 hpf; besides heart and ear, *smpx* is also expressed in the muscles of the branchial arches (bracket), of the eye (white arrowheads) and of the pectoral fins (black arrowhead).
B,C,D,E,F,G: Lateral views, anterior to the left. H: Dorsal view, anterior up. n, notochord; nt, neural tube; am, anterior macula; pm, posterior macula; v, ventricle; a, atrium. Scale bars = 200 mm in A,B; 20 mm in B'; 150 mm in C; 500 mm in D,E,F,G; 25 mm in I,J; 10 mm in I',J',K.

Figure 3. ***Smpx embryonic expression pattern analyzed by immunohistochemistry.*** **(A)** Image of the anti-Smpx antibody staining the ear of a 48 hpf embryo and counterstained with phalloidin (F-actin) to visualize the cytoskeleton. Smpx signal is restricted to the region of the anterior and posterior maculae. **(B,C)** At 120 hpf Smpx is confined to the anterior, lateral and posterior cristae, labeling the apical membrane of the hair cells, below the kinocilia stained with the antibody against acetylated tubulin (B) and the stereocilia bundle painted with phalloidin (C). **(D,E)** Close-up views of the zebrafish ear at 120 hpf highlighting the lateral crista and the anterior macula; co-labeling of the apical membrane of the hair cells with the antibody against Smpx and with phalloidin; the nuclei (DAPI) are located in the basal portion of the cells.

Images are all lateral views, anterior to the left, of confocal Z-stacks taken from whole mount embryos and larvae. am, anterior macula; pm, posterior macula; ac, anterior crista; lc, lateral crista; pc, posterior crista. Scale bars = 20 mm in A; 50 mm in B,C; 10 mm in D,E.

Figure 4. ***Smpx localizes to the cuticular plate of the inner ear hair cells.*** **(A)** Schematic representation of the hair cell apical membrane of the zebrafish inner ear; the kinocilium, with the structural tubulin organized in microtubules (in blue), and the bundle of F-actin-based mechanosensitive stereocilia (in brown) are depicted. The cuticular plate and the somatic tubulin (white asterisk) are also shown. **(B,C)** Paraffin sections co-labeled with antibodies against Smpx and phalloidin (B) and with antibodies against Smpx and acetylated tubulin (C), with the nuclei stained with DAPI. **(B',C')** 5X magnifications of B and C; Smpx is located in the region corresponding to the cuticular plate, in between the stereociliary F-actin-based bundle above (B') and the somatic tubulin below (C'); F-actin (red) and Smpx (green) signals co-localize in the uppermost part of the cuticular plate (yellow in B').
Images are all lateral views, anterior to the left, of confocal Z-stacks taken from paraffin sections from embryos and larvae. am, anterior macula; lc, lateral crista; CP, cuticular plate; SB, stereociliary bundle; k, kinocilium. Scale bars = 30 mm.

Figure 5. ***Smpx localizes to the ciliated cells of the zebrafish pronephros.*** **(A)** Cross-section of the trunk of a 48 hpf embryo; the pronephric ducts are labeled in green with the antibody against Smpx. **(A')** 5X magnifications of the area enclosed in the rectangle in A; Smpx labels the apical membrane of the ciliated cells facing the lumen of the duct, as also confirmed **(B)** by the co-labeling with antibodies against Smpx and acetylated tubulin, where the lumen is populated exclusively by the acetylated tubulin-based kinocilia (red) sprouting from the apical membrane and devoid of Smpx (green).
Images are all confocal Z-stacks taken from paraffin sections. pd, pronephric duct; n, notochord; nt, neural tube. Scale bars = 50 mm in A; 20 mm in C.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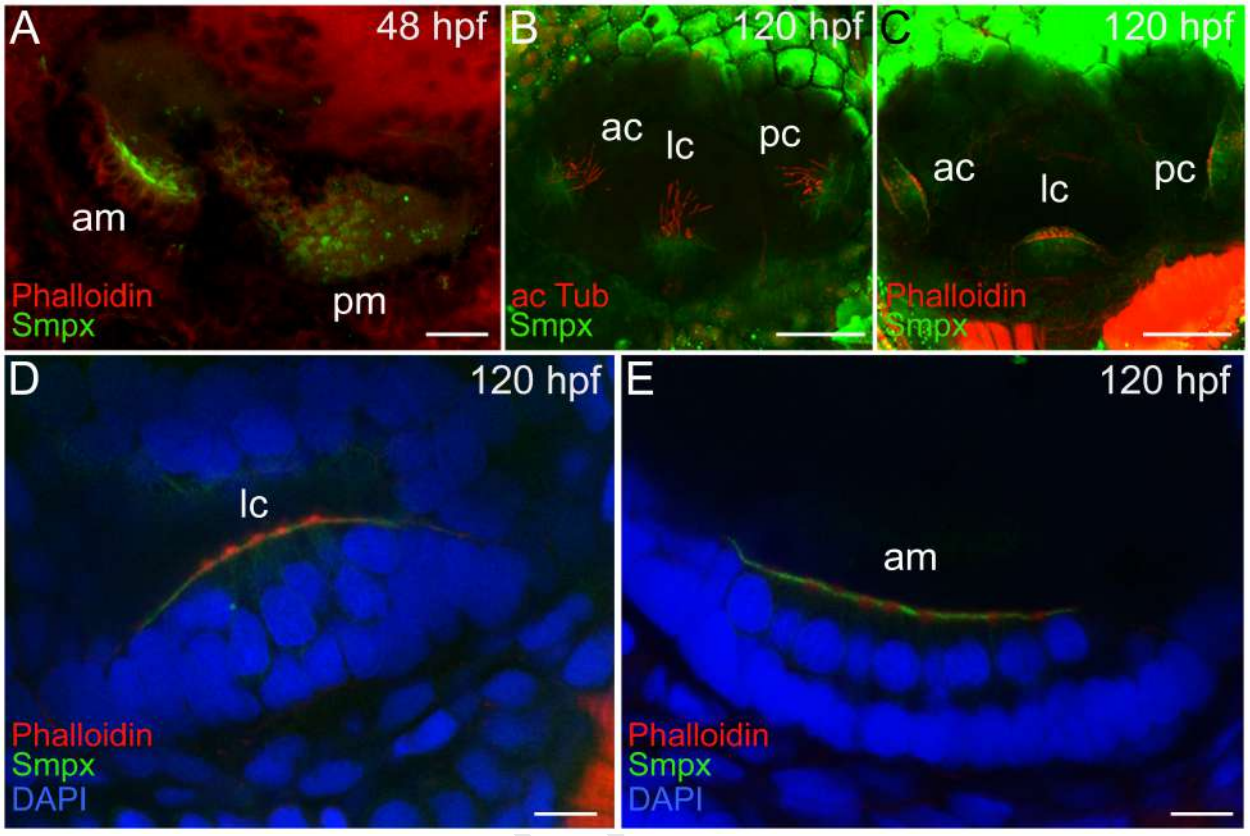

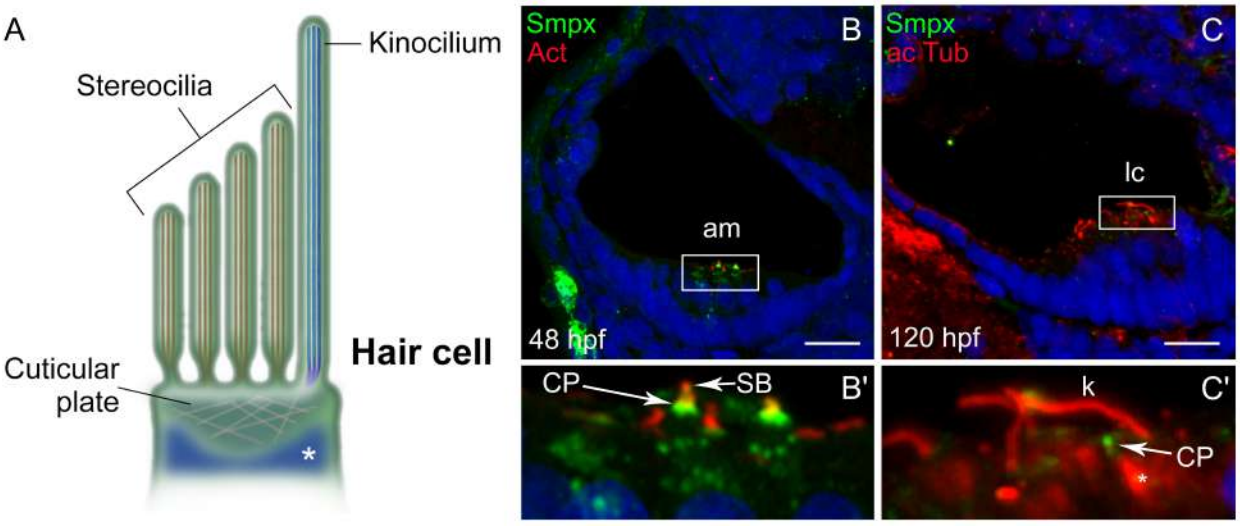

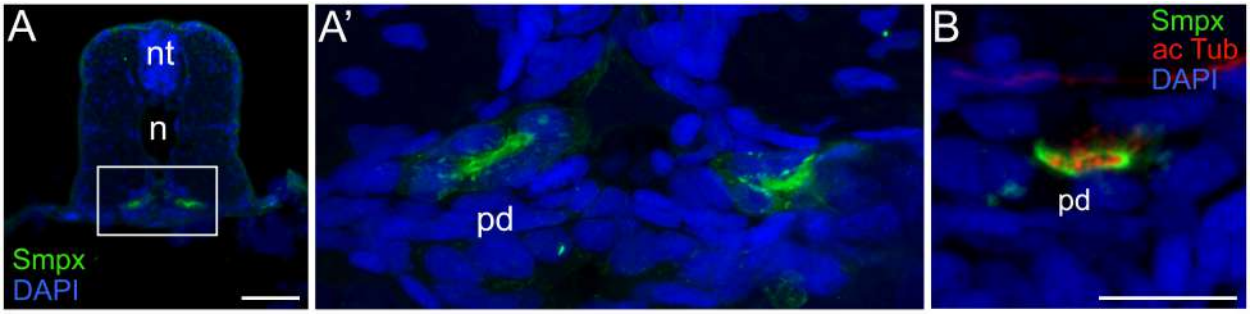

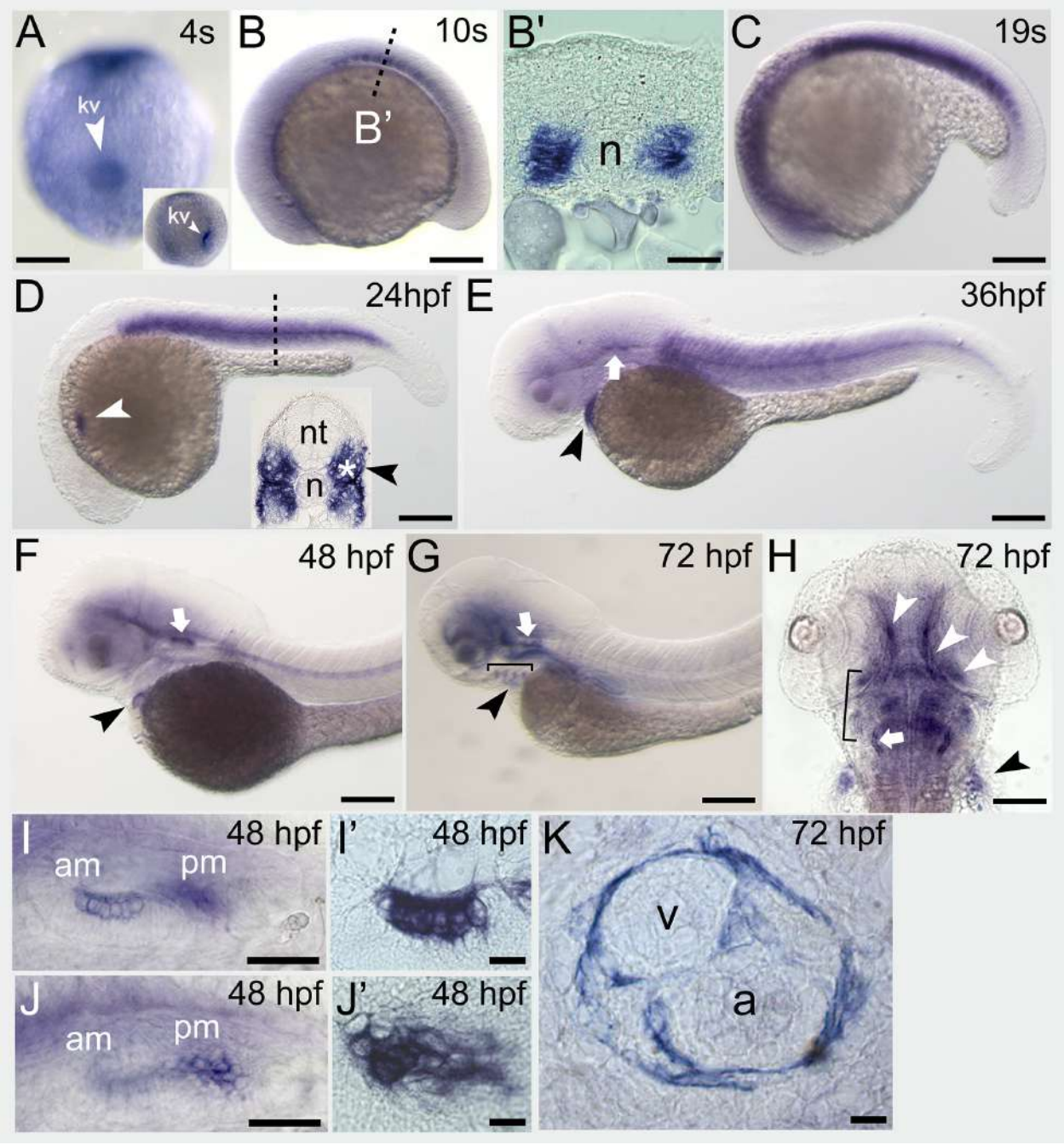
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