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Kidins220/ARMS is dynamically expressed during *Xenopus laevis* development

SILVIA MARRACCI*, MARIANNA GIANNINI, MARIANNA VITIELLO, MASSIMILIANO ANDREAZZOLI and LUCIANA DENTE

Department of Biology, University of Pisa, Pisa, Italy

ABSTRACT Kidins220 (Kinase D interacting substrate of 220 kDa)/ARMS (Ankyrin Repeat-rich Membrane Spanning) is a conserved scaffold protein that acts as a downstream substrate for protein kinase D and mediates multiple receptor signalling pathways. Despite the dissecting of the function of this protein in mammals, using both *in vitro* and *in vivo* studies, a detailed characterization of its gene expression during early phases of embryogenesis has not been described yet. Here, we have used *Xenopus laevis* as a vertebrate model system to analyze the gene expression and the protein localization of Kidins220/ARMS. We found its expression was dynamically regulated during development. *Kidins220/ARMS* mRNA was expressed from neurula to larval stage in different embryonic regions including the nervous system, eye, branchial arches, heart and somites. Similar to the transcript, the protein was present in multiple embryonic domains including the central nervous system, cranial nerves, motor nerves, intersomitic junctions, retinal ganglion cells, lens, otic vesicle, heart and branchial arches. In particular, in some regions such as the retina and somites, the protein displayed a differential localization pattern in stage 42 embryos when compared to the earlier examined stages. Taken together our results suggest that this multidomain protein is involved in distinct spatio-temporal differentiative events.

KEY WORDS: *PDZ binding protein, neural system, glial cell, intersomitic region, heart*

Introduction

Kidins220 (Kinase D interacting substrate of 220 kDa)/ARMS (Ankyrin-Repeat-rich Membrane-Spanning), hereafter referred as Kidins220, is a transmembrane protein highly conserved in the evolution (Iglesias *et al.*, 2000; Kong *et al.*, 2001). Sequence analysis of Kidins220 predicts 11 ankyrin-repeats within the N-terminal region, whereas the C-terminal segment contains a proline-rich stretch, a SAM-like domain and a PDZ (PSD-95, Dlg, ZO-1)-binding motif, raising the possibility that Kidins220 may interact with PDZ proteins (Luo *et al.*, 2005; Andreazzoli *et al.*, 2012). Kidins220 acts as a downstream substrate for protein kinase D (Sanchez-Ruiloba *et al.*, 2006). Furthermore it interacts with multiple membrane receptors, mediating different signalling pathways (Neubrand *et al.*, 2012). Kidins220 is preferentially expressed in neural cells. In cultured hippocampal neurons, Kidins220 is more abundant during early stages of *in vitro* differentiation compared to later stages and it is mainly localized at the tips of extended neurites (Higuero *et al.*, 2010). Perturbation of its expression led to aberrant dendrite formation and extension of multiple aberrant axons (Higuero 2010). Transgenic studies showed that Kidins220$^{-/-}$ embryos displayed early embryonic lethality, and adult Kidins220$^{+/-}$ mice showed defects in dendritic growth and branching (Wu *et al.*, 2009; Cesca *et al.*, 2012). Other studies have shown that Kidins220 localization at the neuromuscular junction (NMJ) enhances EphA4 signaling, through α-syntrophin regulation, suggesting a role in NMJ postsynaptic organization (Luo *et al.*, 2005).

Recently, it has been demonstrated that Kidins220 interacts with VEGFR, suggesting a potential role during vascular development (Cesca *et al.*, 2012). Although the functional assays carried out both *in vivo* and *in vitro* are informative about the versatile roles played by Kidins220, an exhaustive characterization of its expression pattern during early embryogenesis is missing so far. Here, we used *Xenopus laevis*, a vertebrate model which, thanks to its external development, allows easy manipulation and observation of very early embryonic stages that are difficult to analyze in mammals. We present a detailed characterization of *Kidins220* spatio-temporal gene expression and protein localization.

Abbreviations used in this paper: ARMS, ankyrin repeat-rich membrane spanning; CNS, Central Nervous System; kidins220, kinase D interacting substrate of 220 kDa; NMJ, neuromuscular junction; st, stage.

***Address correspondence to:** Silvia Marracci, Department of Biology, via Abetone e Brennero 4, University of Pisa, 56127 Pisa, Italy. Fax 050-2211495
E-mail: smarracci@biologia.unipi.it

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Results and Discussion

Spatio-temporal localization of kidins220 *mRNA during* Xenopus *embryogenesis*

Transcripts encoding *Xenopus kidins220* are first detected in the initial neural tube stage embryos (st. 19), localized in neural plate, neural crests and in a region corresponding to the first segregated anterior somites (Fig. 1 A,B). In early tail bud stage (st. 22), *kidins220* mRNA is detected in different regions of the head, including central nervous system (CNS) and branchial arches, and of the trunk including somites (Fig. 1C). At this stage, 9-10 somites are segregated from paraxial mesoderm in a cranial-caudal direction. As development proceeds (st. 27), *kidins220* is visible at the level of hindbrain, in the anterior portion of spinal cord, in somites and in ophtalmic and maxillomandibular branches of the primordium of the trigeminal nerve (Fig. 1D), where it continues to be expressed also in later hybridized stages (Fig. 1E).

The trigeminal neurons are among the first neurons to develop. Around stage 26 the growth cones of their neuritis progress and fasciculate with each other till they form a nerve bundle before they reach the target region (Davies *et al.*, 1982). Early expression of *kidins220* in developing trigeminal branches at stage 27 suggests a potential role of this gene in events of differentiation of the trigeminal nerve, in agreement with data reported in mammals (Cesca *et al.,* 2012). In stage 33-34 embryos, the presence of *kidins220* mRNA can be observed in forebrain, midbrain, and hindbrain, eye vesicle, branchial arches and somite region, (Fig.1 E,F), as highlighted using *mastr* gene, a typical somitic marker (Fig. 1G) (Meadows *et al.*, 2008). *kidins220* expression is detected in heart region and in otic vesicle at stage 35-36 (Fig. 1H) and in the latest analysed stages (st. 37-38) in dorsal fin, as well as in the somite region (Fig. 1I). Histological sections of hybridized st. 37-38 embryos show a specific signal in retinal ganglion cells (RGCs) and in lens (Fig.1J).

Localization pattern of Kidins220 protein during Xenopus *embryogenesis*

Immunolocalization analysis allowed us to obtain a detailed pattern of the spatio-temporal expression of Kidins220 protein during *Xenopus* development. We found that RNA and protein expression in general coincide, but at early embryonic stages, the expression of Kidins220 protein is delayed compared to mRNA expression (st. 19). The first, very weak, immunopositive signal was detectable only in st. 22 embryos, through DAB whole-mount immunostaining experiments (data not shown). The signal is more distinct in st. 28 embryos (Fig. 2A) at the level of the first segmented somites, as highlighted by Hoechst-counterstain showing the typical stacked arrangement of the nuclei of myoblasts in somites (Fig. 2B). No signal was detectable in unsegmented paraxial mesoderm (Fig. 2 A,B, red square brackets, and data not shown). Somite staining is maintained and extended in developmental stages 33-34 (Fig. 2 C,E,G). A specific signal is present at level of the intersomitic junctions (Fig. 2 E,G,H), differently from the signal obtained by using a muscle-specific antibody that labeled the entire myoblast cytoplasm (Fig. 2 D,F). Transverse sections show the expression in motor neurons that at stage 33-34 are elongating towards the somites (Fig. 2H) as highlighted by the acetylated tubulin immunolabeling (Fig. 2I). Interestingly, the expression of Kidins220 protein is dynamically regulated in *Xenopus* during the development of this embryonic region. Sagittal sections of the immunostained stage 42 embryos show that the intensity of the Kidins220 signal significantly decreases in the somites (Fig. 2 J,K) bringing to light the presence of immunostained spinal motor nerves in the somites (Fig. 2J arrowheads).

Kidins220 protein resulted expressed in other developing regions of *Xenopus* embryos. Sections of the immunolabeled st. 33-34 embryos confirmed and extended the *in situ* hybridization data revealing that Kidins220 is expressed in hypothalamus and thalamus nervous fibers (Fig. 3A), in ophtalmic, maxillary and mandibulary branches of trigeminal nerve (cranial nerve V) (Fig. 3 B,C), otic vesicle (Fig. 3D) and branchial arches (Fig. 3E).

This pattern is maintained in later stages (37-38): Kidins220 is expressed in the cranial nerves (VII, IX, X) innervating the 2nd, 3th, 4th branchial arch, respectively (Fig. 3F).

In stage 37-38 embryos, a Kidins220-immunopositive

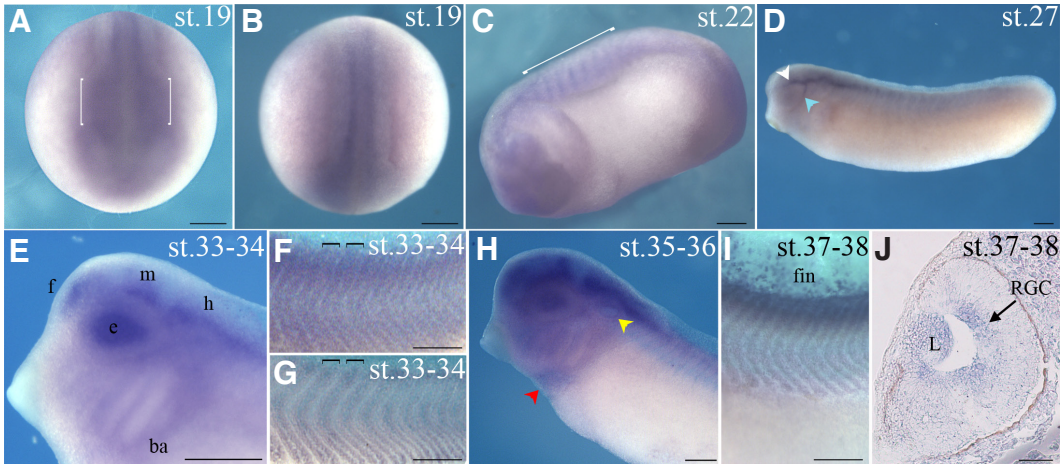

Fig. 1. Spatio-temporal expression pattern of kidins220 mRNA in Xenopus laevis embryos. *The developmental stages (st.) are indicated in each panel* **(A,B)** *Initial neural tube stage embryos (st. 19),* **(A)** *frontal view, dorsal to the top and* **(B)** *dorsal view, anterior to the bottom, respectively.* **(C)** *Early tail bud stage (st. 22) embryo showing* kidins220 *expression at the level of the head and in somites. In (A) and (C) the square brackets indicate the forming somites).* **(D)** *st. 27 embryo in which the labeled ophtalmic and maxillomandibular branches of the primordium of the trigeminal nerve are highlighted by white and light blue arrowheads, respectively.* **(E)** *Magnified view of st.33-34 embryo head, showing signal in forebrain (f), midbrain (m) hindbrain (h), eye vesicle (e), branchial arches (ba). Magnification of the somite region in stage 33-34 embryos, hybridized with* Kidins220 **(F)** *and* mastr, *respectively* **(G)**. *The square brackets indicate individual somites.* **(H)** *Anterior portion of st. 35-36 embryo showing signal in the heart region (red arrowhead) and in otic vesicle (yellow arrowhead).* **(I)** *Magnification of somites and dorsal fin of stage 37-38 hybridized embryos.* **(C-I)** *Lateral views, anterior to the left, dorsal to the top.* **(J)** *Transverse microtome section of hybridized st. 37-38 embryo showing a specific signal in retinal ganglion cells (rgc) and in lens (l). Scale bars: (A-I) 250 μm; (J) 50 μm.*

signal is visible in the developing heart (Fig. 3G), consistently with the observation that in mammals this protein is required for a correct development of heart. Indeed, Kidins220 knockout mice showed defects primarily in development of cardiovascular as well as nervous system (Cesca *et al.*, 2012). Additionally, presence of Kidins220-positive cells is revealed by whole-mount immunolabeling in the abdominal region of stage 42 embryos (Fig. 3 H,J). Sectioning of these embryos show that these labeled cells are hypodermically localized around the intestine (Fig. 3J, red arrowheads) as well as in intestinal wall (Fig. 3J, black arrowheads). Finally, Kidins220-positive signal is present at the edges of a cranial muscle (Fig. 3I).

A peculiar pattern consisting in spotted-like Kidins220 signals, organized in irregularly parallel rows to the sides of the junctions intersomitic is detectable in stage 42 DAB-stained embryos (Fig. 4 A-C). To better investigate this pattern, we performed whole-mount immunofluorescence analyses, taking advantage of the fact that stage 42 embryos are more transparent than earlier stages (Fig. 4 D,F,G,I).

The location of Kidins220-positive spots might correspond to the specific sites where spinal motor nerves reach the intermyotomal regions, including myotendinous and neuromuscular junctions (Somasekhar and Nordlander, 1995, see references therein). This finding is in agreement with observation of murine Kidins220 in developing muscle at the level of neuromuscular junctions (Luo *et al.*, 2005). On the other hand a straightforward fluorescent Kidins220-positive signal is detected at level of motor neurons innervating somites (Fig. 4 G-I) as well as in nervous fibers innervating dorsal fin (Fig. 4 J-O) and abdominal muscles (Fig. 4 P-R). In these experiments we used an anti-acetylated tubulin antibody for labeling axons of nerve fibers (Fig. 4 E,F,H,I,K,L,P,R). We can observe clusters of Kidins220 signal located over the axons, along their length (Fig. 4 J-L) as shown by absence of overlapping of the Kidins220 signal with the acetylated tubulin signal (Fig. 4L). This pattern suggests a possible expression of Kidins220 in a type of neural crest-derived cells, the glial cells. Detection of cells, double-labeled for Kidins220 and GFAP, located near some nerve fibers of the dorsal fin (Fig. 4 M-O), supports this hypothesis. Furthermore it is in agreement with the observation of an abnormal myelination of the CNS including all cortical layers, striatum, hippocampus and the underlying white matter in Kidins220/ARMS$^{+/-}$mice (Duffy *et al.*, 2011).

Immunofluorescent staining of cryosections of stage 42 embryos double-labeled for Kidins220 and acetylated tubulin reveal a Kidins220-positive signal in dorsal and ventral regions of the spinal cord (Fig. 5 A-C) as well as in motor neurons (Fig. 5 A-C, D-F). In earlier developmental stages (st. 33-34), a Kidins220 signal was detected in the lateral regions of the spinal cord, containing nerve fibers (white matter), as demonstrated by absence of Hoechst-stained nuclei (Fig. 2E, arrows). Furthermore, Kidins220 is expressed in eye with a developmentally regulated pattern (Fig. 5 G-J). At stage 33-34 Kidins220 is present in RGCs and in lens anlagen (Fig. 5G), while in stage 42 embryos Kidins220 expression is extended also to other different eye regions, including photoreceptor layer, and optical nerve (Fig. 5 H-J). RGC cells are the first to differentiate during *Xenopus* retinal development: axon protrusions initiate at

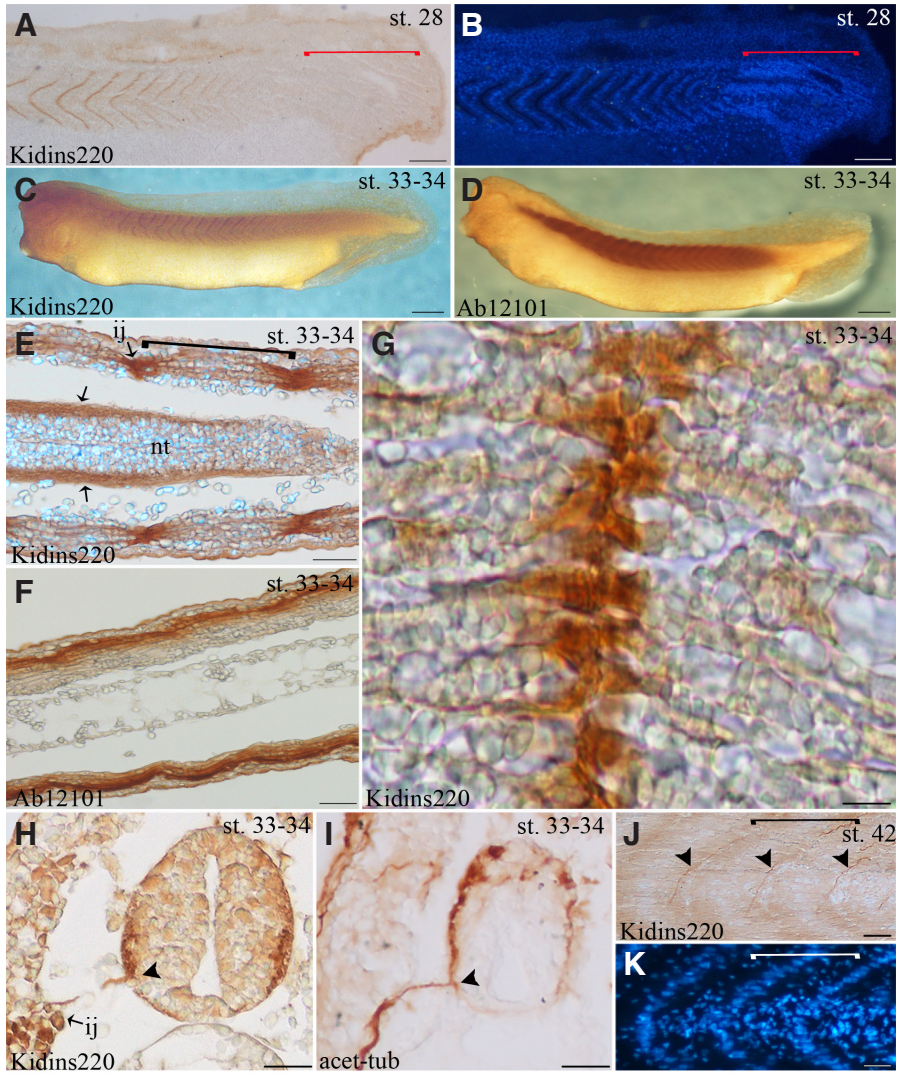

Fig. 2. Kidins220 localization pattern in somites and spinal neurons of DAB-stained embryos. *Lateral views, anterior to the left, dorsal to the top* **(A-D)**. *Sagittal section of a Kidins220-immunostained embryo* **(A)** *and Hoechst-counterstaining* **(B)**. *The red square brackets indicate paraxial mesoderm. Whole-mount ab34790 anti-Kidins220-immunostaining* **(C)** *and Ab12101 anti muscle-immunostaining* **(D)**. *Coronal sections of Kidins220 (Hoechst-counterstained) and Ab12101–stained embryos, respectively* **(E,F)**. *The arrows in (E) show the Kidins220 signal in lateral regions of spinal cord. High magnification of a Kidins220-positive intersomitic junction* **(G)**. *Transverse sections of Kidins220 and acetylated tubulin-immunostained embryos, respectively* **(H,I)**: *the arrowheads show the exit point of a motor neuron that in (H) resulted broken because of sectioning. Sagittal section of a stage 42 embryo* **(J)** *and Hoechst-counterstaining* **(K)**. *The arrowheads in (J) indicate motor neurons. The square brackets in (E,J,K) span somite length. Acet. tub.= acetylated tubulin; nt= neural tube; ij= intersomitic junction. Scale bars: (A,B,H,I) 50 μm; (C,D) 250 μm; (E,F) 25 μm; (G,J,K) 10 μm.*

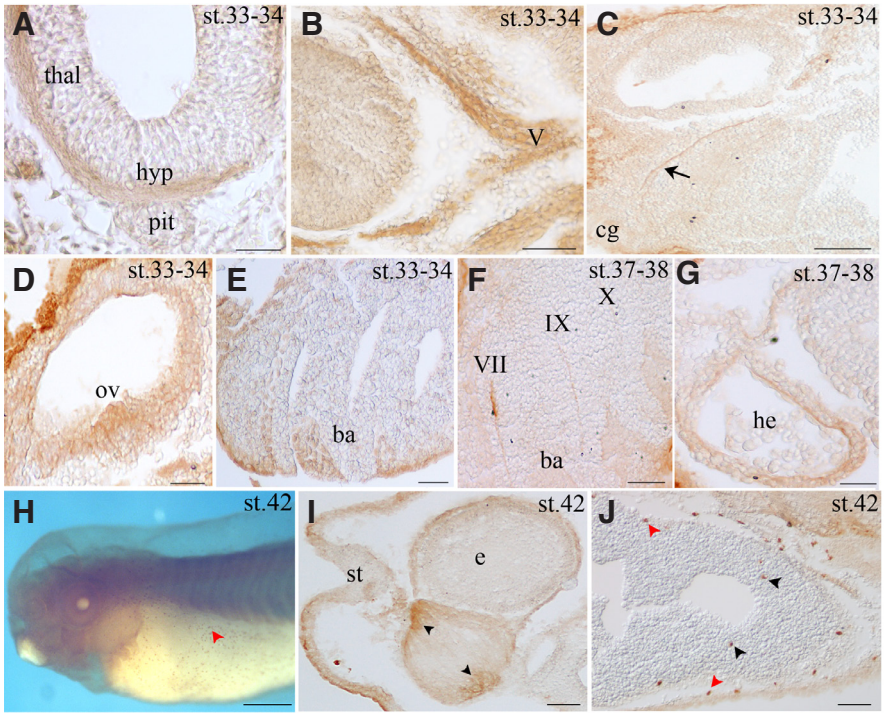

Fig. 3. Kidins220 expression in other different developmental regions of DAB-stained embryos. *Coronal and transverse sections showed that Kidins220 was expressed in: hypothalamus and thalamus* **(A)**, *trigeminal nerve (V)* **(B)**, *the mandibular branch (arrow) of trigeminal nerve innervating the cement gland* **(C)**, *otic vesicle* **(D)**, *branchial arches* **(E)**, *VII, IX, X cranial nerves* **(F)**, *heart* **(G).** *Lateral view of the anterior portion of a whole-mount Kidins220-immunolabeled stage 42 embryo* **(H)**. *Sagittal sections showing a view of head* **(I)** *and abdomen* **(J)**. *(H-J) Anterior to the left, dorsal to the top. The red arrowheads in (H,J) indicate Kidins220-immunopositive cells around the intestine. The black arrowheads in (I) show the Kidins220 signal in the edges of a cranial muscle. The black arrowheads in (J) indicate a Kidins220-immunopositive cell localized in intestine wall. ba, branchial arches; cg, cement gland; e, eye; he, heart; hyp, hypothalamus; ov, otic vesicle; pit, pituitary; thal, thalamus; st, stomodeum. Scale bars: (A,B,D,G) 25 μm; (C) 100 μm; (E,F,I,J) 50 μm; (H) 250 μm.*

stage 28, exit retina via the optic nerve and synapse on the optic tectum in the brain. Dendrite protrusions begin to differentiate at stage 30/31 extending into the inner plexiform layer containing the amacrine and bipolar cell nuclei. Both types of processes elongate and contact their respective targets by stage 40 RGCs. (MacFarlene and Lom, 2011). The finding of Kidins220 signal in RGCs from stage 33-34 is in agreement with previous observations on mammalian cells that suggested the involvement of this protein in events of neurite differentiation and/or optic nerve formation (Higuero *et al.*, 2010).

A Kidins220 signal is visible also in nerve fibers innervating epidermis as demonstrated by the overlapping of Kidins220 and acetylated tubulin signal (Fig. 5 I-K, yellow arrowheads).

In summary, our data on RNA and protein local-

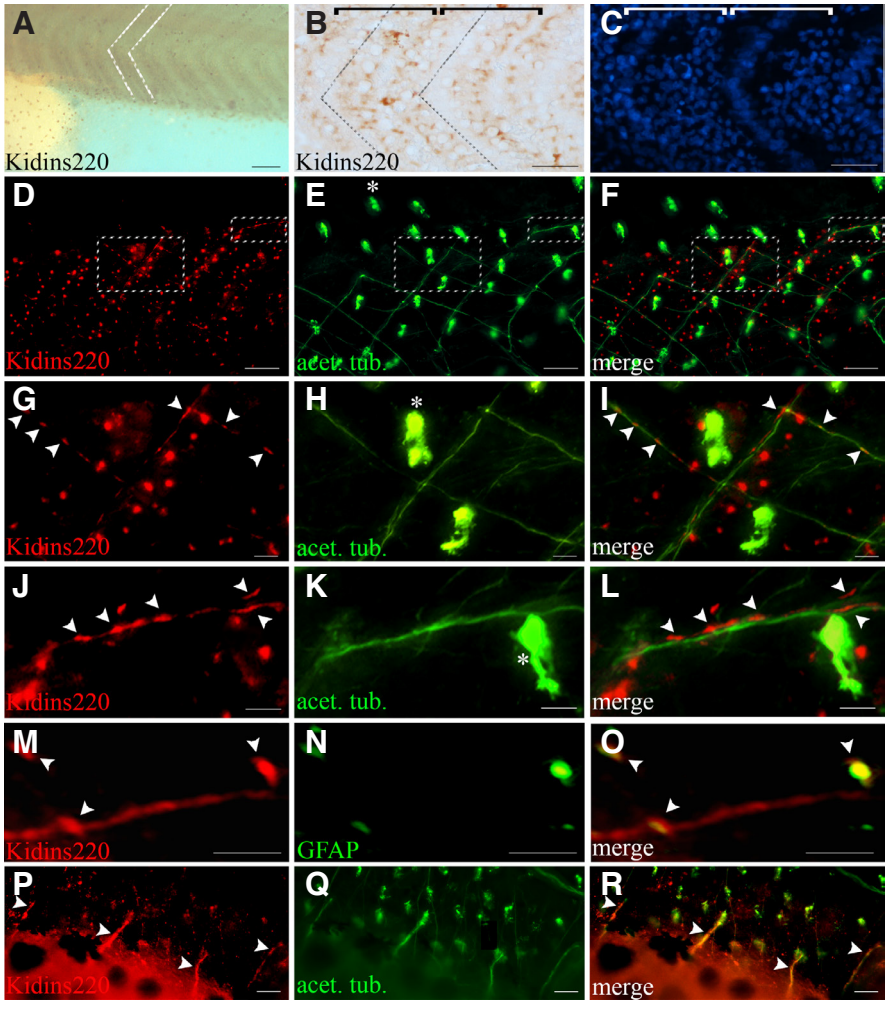

Fig. 4. Whole mount immunohistochemistry experiments on stage 42 embryos. *Lateral view of a posterior portion of DAB-stained embryo for Kidins220* **(A)**. *Parasagittal section performed laterally between the skin and the somites of an immunostained embryo* **(B)** *and Hoechst-counterstaining* **(C).** *Dashed lines in (A,B) highlight the pattern of the Kidins220 signal, organized in irregularly parallel rows to the side of intersomitic junctions. The square brackets in (B,C) span somite length. Lateral view of somite region of whole-mount double immunofluorescent staining for Kidins220 (red signal) and acetylated tubulin (green signal)* **(D-F)**. *Magnified views of neurons in somite region* **(G-I)**, *dorsal fin* **(J-L; M-O)**, *abdominal region* **(P-R)**. *The largest dashed box in (D) indicates the region magnified in (G-I); the smallest one, the region magnified in (J-L). White arrowheads in (G,I,J,L,M,O) highlight the spotted signal of kidins220 at some neural fiber. White arrowheads in (M,O) show specific cells in dorsal fin, positive for both Kidins220 and GFAP. The asterisks in (E,H,K) denote the acetylated tubulin-positive but Kidins220-negative ciliated cells. Scale bars: (A) 125 μm; (B-F,J-O) 50 μm; (G-I,P-R) 10 μm.*

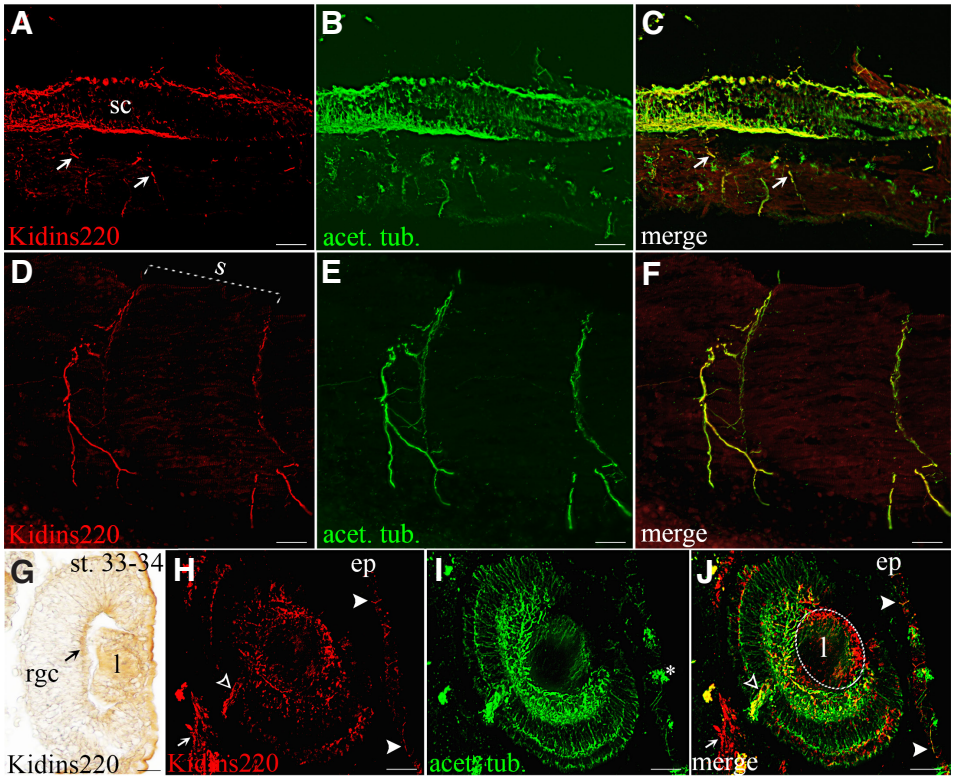

Fig. 5. Sagittal cryosections of stage 42 embryos double-labeled for Kidins220 (red) and acetylated tubulin (green) by immunofluorescent staining. *Kidins220-positive signal was present in dorsal and ventral regions of the spinal cord as well as in motor neurons (arrows)* **(A)** *as shown by the acetylated tubulin staining* **(B,C)**. *Magnification of motor neurons double-stained by Kidins220* **(D)**, *acetylated tubulin* **(E)** *and merged* **(F)**. *The dashed bar in* **(D)** *spans the somite length. Kidins220 protein in eye of st. 33-34 DAB-stained embryos* **(G)** *and st. 42 embryos double-immunolabeled for Kidins220 and acetylated tubulin* **(H-J).** *The open arrowhead in (H,J) indicates the Kidins220 signal in the optic nerve. The dashed circle indicates the position of lens in the eye. The white arrow in (H,J) denotes the presence of Kidins220 signal in a facial muscle near the eye. The white arrowheads in (H,J) indicate Kidins220 and acetylated tubulin-positive nerve fibers present in epidermis. ep, epidermis; l, lens; rgc, retinal ganglion cell; s, somite; sc, spinal cord; *, ciliated cell. Scale bars: (A-C) 50 μm; (D-F,G-J) 25 μm.*

ization developmental patterns indicate that Kidins220 expression is dynamically regulated during *Xenopus* early embryogenesis. Overall, transcript and protein expression were revealed in the same districts. In particular, a detailed description of the protein expression was achieved through immunostaining of specific cells. Furthermore, we report a precise Kidins220 localization at early stages, at the level of intersomitic junctions, which was not previously observed in mammals. Taken together our results suggest crucial developmental functions of Kidins220. This multidomain protein might play different roles in distinct embryo regions, which will be worthwhile to further investigate.

Materials and Methods

Whole-mount in situ *hybridization*

Xenopus embryos were obtained as described in D'Autilia *et al.* (2010). Whole-mount *in situ* hybridization was performed according to standard protocols (Harland *et al.,* 1991) with minor modifications (D'Autilia *et al.*, 2010). As probe we used a *Xenopus laevis* cDNA, clone IMAGE: 6959220 (GeneBank Acc. CD300871) 5300 bp long, including part of the coding sequence (from the KAP_NTPASE until the C-terminus), and a 3'untranslated region 1733 bases long. Sense and antisense transcripts were prepared by plasmid (pCMV-SPORT6) linearization with *Xho*I (sense) or *Sal*I (antisense) and *in vitro* transcription using SP6 and T7 RNA polymerase (Roche), respectively. *Mastr* cDNA clone was linearized with SalI and transcribed with T7 RNA polymerase. Probes were visualized with anti-DIG antibody as described in Dente *et al.,* 2011.

Whole-mount immunostaining

For immunochemistry experiments, embryos were treated as in Somasekhar and Nordlander (1995). The following primary antibodies were used: a rabbit polyclonal anti-Kidins220 antibody (ab34790, Abcam, 1:1000) directed against a synthetic peptide, corresponding to the carboxyterminal region of human Kidins220, that is highly conserved in *Xenopus* Kidins220 and in homologous proteins of other vertebrates (Andreazzoli *et al.*, 2012; Kong *et al.*, 2001); a muscle-specific monoclonal antibody (12/101, Hybridoma Bank, 1:30) directed against a skeletal muscle marker, 102kDa; a monoclonal anti-acetylated tubulin (clone 6-11B-1, Sigma-Aldrich, 1:300); a monoclonal anti-GFAP (Glial Fibrillary Acidic Protein) (clone G-A-5, Sigma-Aldrich, 1:500) used as a marker of glial cells. After incubation o/n at 4°C, embryos were washed in PBTr (PBS 1X + 0,5% TritonX) and then incubated o/n at 4°C with the appropriate secondary antibodies: Oregon Green 488 goat anti-mouse IgG (Molecular probes, 1:500), Rhodamine Red goat anti-rabbit IgG (Molecular probes, 1:500), goat anti-rabbit IgG peroxidase-conjugated (Sigma, 1:500); goat anti-mouse IgG peroxidase-conjugated (Sigma, 1:500). DAB (Roche, 3, 3'-diaminobenzidine) was used for the colorimetric reaction. Nuclei were stained with Hoechst H33258 (Sigma, 1:1000).

Paraffin sectioning

Whole-mount hybridized embryos and DAB-immunostained embryos were paraffin-embedded and cut at a thickness of 12-15 μm with a Reichert-Jung Autocut 2040 Microtome. After Hoechst-counterstaining, the sections were washed and mounted in Aqua Poly/Mount (Polysciences Inc.).

Double immunostaining on cryosections

For immunostaining experiments on sections (12 μm), embryos were fixed in 4% paraformaldehyde at room temperature for 2 hrs, cryoprotected in 30% sucrose in PBS o/n at 4°C and stored at –70°C until cryosectioning. Immunostaining was performed using the polyclonal anti-Kidins220 (Abcam Ab34790, 1:1000) and a monoclonal anti-acetylated tubulin clone 6-11B-1 (Sigma, 1:300) and a monoclonal anti-GFAP (Glial Fibrillary Acidic Protein) (clone G-A-5, Sigma, 1:500). Sections were washed three times in PBST (PBS 1X + 0,1% Tween20) for 5 min (min= minutes) and three times for 20 min, and were then incubated with both Oregon Green 488 goat anti-mouse IgG (Molecular probes, 1:500) and Rhodamine Red goat anti-rabbit IgG (Molecular probes, 1:500), for 2 hrs. The sections were washed again in PBST and mounted. Finally Hoechst-counterstaining was carried out.

Microscopy and image analysis

Light micrographs were taken using a stereoscopic microscope Nikon SMZ-1500 and a microscope Nikon Y-IM for imaging whole-mounts and

sections, respectively. In both cases the image acquisition was made using NIS-elements F-3.0 software (Nikon). Immunofluorescent staining was imaged using a Nikon Eclipse-T*i* microscope and the image acquisition software was NIS-elements AR 3.2. Fluorescent images are projections of deconvolved *Z*-series.

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