Int. J. Dev. Biol. 55: 989-993
doi: 10.1387/ijdb.113437ld

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Cloning and developmental expression of zebrafish pdzrn3

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ABSTRACT Pdzrn3, a member of the PDZRN/SEMCAP/LNX protein family containing a RING finger and two PDZ domains, has been implicated in myoblast and osteoblast differentiation. However, its spatio-temporal expression pattern during embryonic development has not been defined. Here, we describe the cloning and expression pattern of *pdzrn3* during zebrafish development. We found that in addition to being expressed in several mesodermal structures, this gene displays specific expression in the central nervous system including rhombomere 1, ventral retina, thalamus and motor neurons, indicating a novel function during neural development. In particular, the absence of expression of *pdzrn3* in the ventral retina of *noi* mutant fish suggests a possible role for this gene in regulating fasciculation and/or navigation of retinal ganglion cell axons.

KEY WORDS: *PDZRN protein, PDZ domain, RING finger, zebrafish development, ventral retina*

Mechanisms involved in development and tissue differentiation are generally based on molecular recognition and formation of networks of functional interactions. The identification of genes specifically expressed in restricted regions of a tissue can help to identify interacting proteins that are determinants for given structures or functions. During the course of a large scale *in situ* hybridization screen for genes developmentally regulated in zebrafish embryos (Kudoh *et al.*, 2001), we selected a gene displaying a peculiar expression in the central nervous system. Analysis of the deduced translated sequence revealed it encodes for Pdzrn3, a protein that was originally identified in silico by homology analysis to LNX1 or SEMCAP (Katoh and Katoh, 2004). PDZRN- proteins contain a RING finger and one or more PDZ domains and belong to the family of RING proteins that are generally involved in mechanisms of cell cycle progression, development, signal transduction, ubiquitination and apoptosis (Joaizero and Weissman, 2000). RING (Really Interesting New Gene) fingers are specialized structures containing a sequence motif with conserved cysteine and histidine residues related to zinc fingers (Katoh and Katoh, 2004). PDZ (PSD-95/Discs-large/ZO-1) domains are small protein-protein interaction modules, which are often present in duplicate or in multiple copies, allowing recognition and clustering of signaling molecules for the organization of protein networks (Feng and Zhang, 2009).

Current information about Pdzrn3 mainly derives from studies in muscle tissues. Human *pdzrn3* was isolated from a heart cDNA library and its expression was characterized by northern analysis in various adult human and murine tissues. It was mainly detected in heart, skeletal muscle and liver (Ko *et al.*, 2006). The amount of *pdzrn3 RNA* decreases during muscle postnatal development and increases during adult muscle regeneration following injury, suggesting that the gene is developmentally regulated (Ko *et al.*, 2006; Lu *et al.*, 2007). Furthermore, PDZRN3 is involved in the process of differentiation of C2C12 murine mesenchymal progenitor cells into myotubes or into osteoblasts (Ko *et al.*, 2006; Honda *et al.*, 2010). Finally, a role as an E3-ubiquitin ligase has been proposed for PDZRN3 at the level of neuromuscular junctions for the regulation of the surface expression of MuSK (Muscle-Specific receptor tyrosine Kinase; Lu *et al.*, 2007).

Here, we describe the cloning of zebrafish *pdzrn3* and its expression pattern during embryonic development. This analysis highlights a previously undescribed dynamic expression of *pdzrn3* in specific domains of the developing central nervous system of zebrafish.

These data, together with the repression of *pdzrn3* expression in

Abbreviations used in this paper: aa, aminoacids; ac, accession number; nt, nucleotides; PDZ, PSD-95/Discs-large/ZO1; RING, really interesting new gene.

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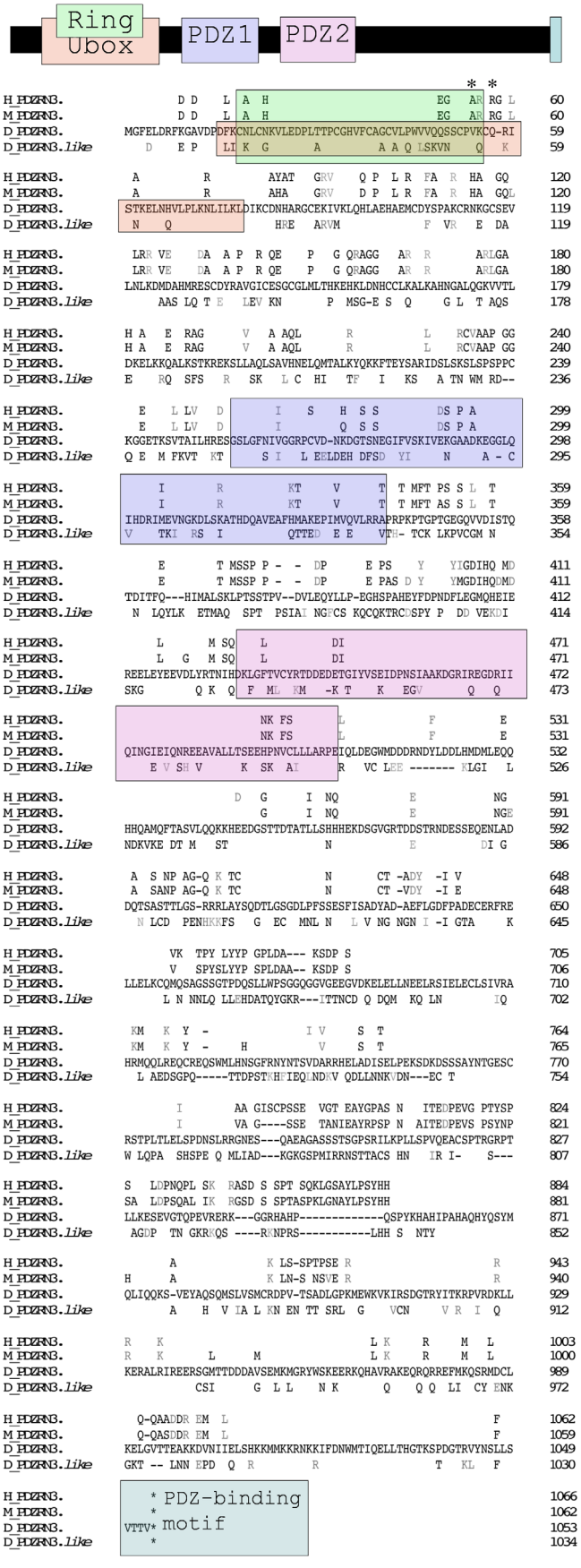
Accepted: 16 November 2011. *Final, author-corrected PDF published online:* 15 December 2011.

ISSN: Online 1696-3547, Print 0214-6282



the ventral eye region of zebrafish mutants affecting eye development, suggest that this gene might be involved in controlling neuritogenesis and in particular axon outgrowth of retinal ganglion cells.

Results

Cloning of zebrafish pdzrn3

The *pdzrn3* gene was selected for its peculiar expression in the developing eye of zebrafish in the course of a large scale *in situ* hybridization screen (Kudoh *et al.*, 2001). A complete coding clone of 3162 nucleotides (nt), was identified by 5' RACE and end-to-end PCR (GenBank accession no. JN108761). Sequence analysis revealed an open reading frame encoding a predicted protein of 1053 aminoacids (aa) (Fig. 1). A database search provided precise alignment with a predicted zebrafish *pdzrn3* sequence (GenBank accession no. XM_001344515), derived from assembled genomic sequences and supported by EST evidence. The two sequences share 99% identity, diverging in 14 nucleotides that determine 6 different amino acids (H/Q_{154}; H/R_{324};V/M_{543}; V/M_{554}; L/Q_{989}; A/D_{998}). The *pdzrn3* gene is localized on zebrafish chromosome 6 (http://www.ncbi.nlm.nih.gov/gene/100005518). The zebrafish genome often displays more alleles of genes that are present in single copy in mammals. In this case, a related gene, named *pdzrn3-like* has been reported on chromosome 23 (http://www.ncbi.nlm.nih.gov/gene/100149029). The corresponding protein Pdzrn3-like (XP_001921128.2), deduced from translated genomic and EST sequences, diverges from our Pdzrn3 sequence in multiple positions sharing only 54% identity. Comparison with human and murine PDZRN3 proteins revealed higher homology with zebrafish Pdzrn3 protein (72%) than with Pdzrn3-like (50%) (Fig. 1). Homology with murine PDZRN4 (XP_997312) is even lower: 51% for Pdzrn3 and 37% for Pdzrn3-like. Only in the zebrafish proteins a potential U box overlapping the Ring finger motif is present. *The residues* V_{54} *and* Q_{57} are determinants for U box prediction according to SMART (http://smart.embl-heidelberg.de/). U boxes are domains deriving from RING fingers, which miss the full complement of Zn^{2+}-binding ligands. It has been proposed that U box scaffold has evolved from a RING finger domain by appropriation of a new set of residues required to stabilise its structure through a system of salt-bridges and hydrogen bonds (Aravind and Koonin, 2000). U-box proteins have been reported to function as ubiquitin-protein ligases (E3s) and to interact with molecular chaperones. The presence of both RING and U-box domains strengthens the hypothesis that Pdzrn3 is an ubiquitin ligase involved in the degradation of unfolded or misfolded proteins.

Developmental expression pattern of pdzrn3

The *pdzrn3* spatio-temporal expression pattern was analyzed

Fig. 1. Sequence and structure of zebrafish Pdzrn3. *Predicted domain composition of Pdzrn3 (top) and comparison of amino acid sequences: H_PDZRN3 (human, ac.Q9UPQ7); M_PDZRN3 (murine, ac.Q69ZS0): D_PDZRN3 (zebrafish, translated from ac. JN108761) and D_PDZRN3.like (predicted by automated computational analysis, ac.XP_001921128.2). The amino acid sequence deduced from the pdzrn3 cDNA we have cloned is completely shown. Similar amino acids of the aligned proteins are indicated in gray, different aminoacids are in black, whereas identical residues of the listed proteins are shown only in the zebrafish Pdzrn3 sequence. Asterisks indicate the critical residues involved in the U-box domain structure. Predicted domains deriving by SMART analysis (http://smart.embl-heidelberg.de) are boxed.*

using wholemount *in situ* hybridization. *pdzrn3* transcripts are initially detected at the 3-somite stage in two areas of the presumptive hindbrain and in the notochord (Fig. 2A). By 6-somite stage the two hindbrain sites of expression fuse medially and at 10-somite stage *pdzrn3* begins to be expressed in the ventral region of the optic primordia (Fig. 2B and data not shown). These expression sites are maintained in the following stages (Fig. 2C-E). Prolonged staining at 20-somite stage reveals expression also in somites and in posterior notochord (Fig. 2F). At 24 hpf, *pdzrn3* displays a clear expression in the ventral retina and optic stalk, and, at a lower level, in the dorsal region of the retina (Fig. 2H). A comparison with *pax2.1a* expression at 24 hpf highlights the absence of *pdzrn3* expression in more medial regions of the optic stalk, which are labeled by *pax2.1a* (Fig. 2 G,H). Double *in situ* hybridizations with *pax2.1a* and *krox20* (also known as *egr2b*), markers of the midbrain-hindbrain boundary and of rhombomere 3 and 5, respectively, show that the hindbrain expression domain of *pdzrn3* corresponds to the lateral regions of rhombomere 1 (Fig. 2 I,J). At 24 hpf, prolonged staining reveals expression in ventral spinal cord neurons and in cells of the ventral somites (Fig. 2K). By 32hpf *pdzrn3* expression in the eye lines the choroid fissure, and now also shows expression in ganglion cells. At this stage, *pdzrn3* begins to be expressed also in the thalamus and in the posterior tuberculum, as well as in head mesenchyme, pectoral fins and pronephric duct (Fig. 2 L-N). The same expression sites are present at 48 hpf when the neural expression in trunk-tail becomes more clearly restricted to motor neurons (Fig. 2O and data not shown).

Analysis in zebrafish mutants

As a preliminary analysis to identify factors regulating *pdzrn3* expression in the central nervous system, we took advantage of zebrafish mutants affecting different genes involved in retina and brain development. *pdzrn3* expression does not appear to be particularly affected in *you-too (yot)*, *acerebelar* (*ace*), *chameleon* (*con*), and *cyclops* (*cyc*) mutants, and only slightly affected in *smoothened* (*smo*) mutants (Fig. 3 C,D and data not shown; Hatta *et al.*, 1994; Brand *et al.*, 1996; Barresi *et al.*, 2000). On the contrary, a more specific effect is observed in *no isthmus* (*noi*) mutants that show a regionally restricted lack of *pdzrn3* expression in the ventral retina and distal optic stalk (Fig. 3 G,H). Notably, in *noi* mutants optic axons display fasciculation and pathfinding defects in these positions (Macdonald *et al.*, 1997).

Discussion

In this work, we describe the cloning of pdzrn3 and show that this gene displays a dynamic expression during zebrafish development. Although some studies have addressed the function of this gene, in particular in the context of murine muscle differentiation/regeneration and osteogenesis, its spatio-temporal expression pattern during embryogenesis was not previously described. We found that *pdzrn3* is expressed in several mesodermal and neuroectodermal sites. By *in situ* hybridization, the earliest detected signal in somites appears at the 20-somite stage, much later than the onset of expression of the muscle determinants myoD and myogenin (Weinberg *et al.*, 1996). This is in keeping with functional studies of mouse *Pdzrn3* by Ko *et al.*, (2006), indicating that this gene may act downstream or independently of *MyoD* and *Myogenin*. An early mesodermal site of expression is the developing notochord. As development proceeds, *pdzrn3* expression is progressively restricted to more posterior regions of the notochord. Further mesodermal expression sites become evident only at 24 hpf and include cells in the ventral

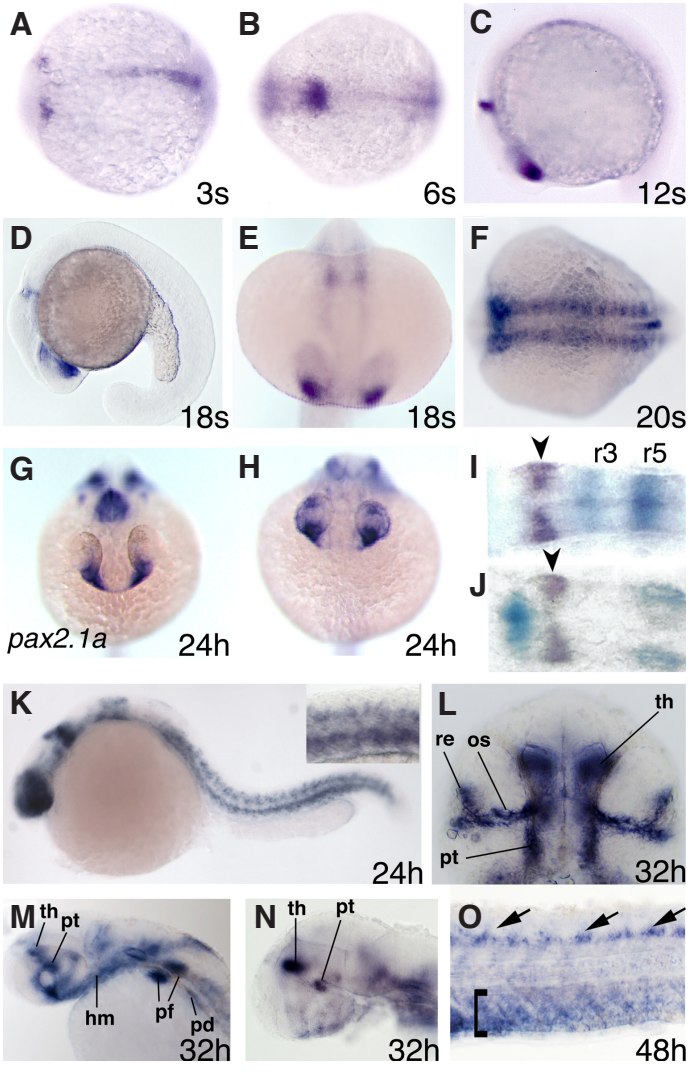


Fig. 2. Expression pattern of *pdzrn3*. (A,B) *Three-somite (A) and six-somite (B) stage: dorsal views, anterior to the left.* **(C-E)** *twelve-somite (C) and eighteen-somite (D) stage: lateral views, anterior to the left. (E) Eighteen-somite stage: dorsal view, anterior to the bottom.* **(F)** *Twenty-somite stage embryo stained for prolonged time: dorsal view, anterior to the left.* **(G,H)** *Comparison between the anterior expression of* pax2.1a *(G) and* pdzrn3 *(H) at 24-hpf stage: frontal views, dorsal to the top.* **(I,J)** *Comparison between the rhomboencephalic expressions of* pdzrn3 *(I, J; purple staining indicated by arrowheads),* krox20 *(I, turquoise staining) and* pax2.1a *(J, turquoise staining); dorsal view, anterior to the right.* **(K)** *24-hpf stage embryo stained for prolonged time: lateral view, anterior to the left. The inset in the top right corner shows a magnification of somite expression in the trunk.* **(L)** *Head expression in 32 hpf embryo: ventral view anterior to the top; os, optic stalk; pt, posterior tuberculum; re, retina; th, thalamus.* **(M,N)** *32 hpf stage: lateral view, anterior to the left; hm, head mesenchyme; pd, pronephric duct; pf, pectoral fin. To analyze internal brain expression sites the eyes of the embryo shown in N were removed.* **(O)** *Trunk and tail expression at 48 hpf: dorsal to the top, anterior to the left. Arrows point to the motor neurons of the spinal cord. The bracket delimits* pdzrn3 *expression in the ventral region of somites.*

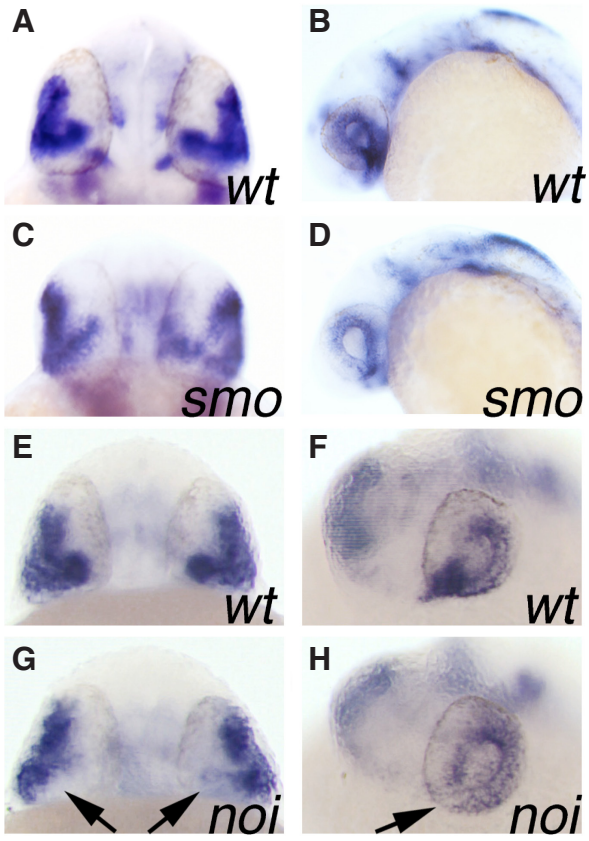


Fig. 3. Retinal expression of *pdzrn3* in selected zebrafish mutants. *(A-H) Retinal expression of* pdzrn3 *at 24-hpf stage in wild type* **(A,B,E,F)**, smo **(C,D)** *and* noi *mutants* **(G,H)**. *(A,C,E,G) ventral view, anterior to the top. (B,D) Lateral view, anterior to the left (F,H) latero-anterior view, dorsal to the top. Arrows in (G,H) point to the lack of* pdzrn3 *expression in the ventral retina of* noi *mutants.*

somites, head mesenchyme and pronephric duct.

A previously undescribed feature of *pdzrn3* expression, highlighted by this study, is its expression in the central nervous system. In fact, *pdzrn3* is expressed in presumptive rhombomere 1 starting from 3-somite stage and in the ventral optic primordia starting from 10-somite stage and, later on, in thalamus and motor neurons. Because of the early expression in the presumptive ventral retina, *pdzrn3* is potentially involved in the initial dorso-ventral patterning of this organ. As *pdzrn3* was shown to counteract the effect of BMP2 in osteogenesis, it is tempting to speculate that it may play the same role in the retina antagonizing the BMP signal, a key determinant of retina dorso-ventral polarity (French *et al.*, 2009; Honda 2010). At 24 hpf, *pdzrn3* is expressed in the ventral retina and at the optic nerve head potentially including the first ganglion cells that are generated at this stage in the ventral retina (Stenkamp, 2007). At 32 hpf, *pdzrn3* expression is extended to retinal ganglion cells as well as cells lining the choroid fissure and to the thalamus. Notably, among the analyzed mutants, the *noi* mutant displays the most specific repression of *pdzrn3* expression. This may indicate that *pdzrn3* acts downstream of *pax2.1a* (the gene mutated in *noi*), which encodes a transcription factor controlling ventral retina morphogenesis and choroid fissure closure, optic stalk differentiation and axon fasciculation (Macdonald *et al.*, 1997).

A role for *pdzrn3* in axon development is possible as Semcap1, another member of the pdzrn/LNX protein family was shown to interact with Semaphorin 4C (Wang *et al.*, 1999).

Materials and Methods

Vectors and clones

A partial pdzrn3 cDNA clone was initially isolated from a 10-somite zebrafish cDNA library by randomly picking isolated colonies in the course of an *in situ* hybridization screening (Kudoh *et al.*, 2001). 5' RACE PCR was performed using the SMART RACE cDNA AMPLIFICATION KIT (Clontech) and the following pdzrn3 specific primer: 8RACE-1 GTCACCACTGCCAAGGGTGTCCTG. A complete coding pdzrn3 cDNA was obtained by end-to-end PCR performed with primers EE-F ATAATGGGATTTGAGTTGGATCGTTTC and EE-R GAGCCTACACTGTAGTCACAGAGAGTAGGGA. The resulting cDNA was cloned in pGEM-T easy vector (Promega).

Zebrafish embryo manipulation and in situ hybridization

Embryo generation, staging and *in situ* hybridization were performed as described in Andreazzoli *et al.,* (2001). BM purple and BCIP (Roche) were used as alkaline phosphatase substrates.

Acknowledgements

We thank Giuseppina Barsacchi for helpful suggestions and Elena Landi, Marzia Fabbri and Donatella De Matienzo for technical assistance. We also gratefully acknowledge Maria Elena Terreni for her help with some in situ hybridization experiments.

This work was supported by the Intramural Research Program of the National Institute of Child Health and Human Development, NIH, by a grant from MIUR to M.A. and by grants from Telethon and MRC to G.G.

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