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Short communication

Kidins220/ARMS interacts with Pdzrn3, a protein containing multiple binding domains

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ABSTRACT

We report the identification of a novel partner of Kidins220/ARMS (Kinase D-interacting substrate of 220 kDa/Ankyrin Repeat-rich Membrane Spanning) an adaptor of neurotrophin receptors playing crucial roles during neurogenesis. Screening a phage display library of brain cDNA products we found that *D. rerio* Pdzrn3, a protein containing RING-finger and PDZ-domains, interacts with Kidins220/ARMS through its first PDZ-domain. Both zebrafish proteins share high homology with the corresponding mammalian proteins and both genes are developmentally expressed in neural districts where early neurogenesis occurs. The interaction was also confirmed by biochemical assays and by co-localization at the tips of growing neurites of PC12 cells induced with nerve growth factor.

Pdzrn3 is a scaffold protein that was first identified in silico by homology analysis to other members of the Pdzrn-family, characterized by the presence of a RING-Finger motif, frequently associated to ubiquitin ligase activity, and one or more PDZ-domains [1]. PDZ (PSD-95/Discs-large/ZO-1) domains are very abundant in adaptor proteins and are generally responsible for contacting and linking specific targets at their carboxyl-termini. Pdzrn3 expression has been characterized in mammalian muscle tissues, in murine C2C12 cells differentiated in myotubes or osteoblasts and more recently in the central nervous system of zebrafish embryos, where it could play a role in neurogenesis [2–5].

A useful approach to assess Pdzrn3 functions in different tissues is to dissect the protein interaction network mediated by its binding domains. Therefore, searching for Pdzrn3 binding partners, we exploited the modular structure of the protein and the high level of conservation between zebrafish and human Pdzrn3, in particular at level of the PDZ-domains. We screened a human brain cDNA library displayed by phage T7 using as baits the isolated PDZ-domains of Pdzrn3 expressed in bacteria as GST (glutathione S-transferase) fusions. Following affinity selection, while GST-PDZ2 or GST alone only selected out-of-frame cDNA translated products, GST-PDZ1 bound two independent phage-clones, displaying similar peptide sequences of different length (Fig. 1A). Database analysis revealed a correspondence with the C-terminal region of Kidins220 (Kinase D-interacting substrate 220 kD), a crucial mediator of signal transduction in neural tissues [6]. Kidins220 is also named ARMS (Ankyrin Repeat-Rich Membrane Spanning) due to the presence of ankyrin repeats and transmembrane domains together with other binding motifs [7]. Amino acid sequence of Kidins220/ARMS (hereafter called Kidins220) is evolutionarily conserved. In particular, 100% identity among different species was found in the last nine C-terminal residues including the PDZ-binding motif (Fig. 1A,B).

In order to assess the physiological relevance of Kidins220 and Pdzrn3 association during embryonic development, we compared the expression patterns of the two genes in zebrafish embryos. Whole mount *in situ* hybridizations were performed using as probes the cDNA of Pdzrn3 (GenBank ID: JN108761) and a cDNA fragment coding for the C-terminal 186 residues of Kidins220 (GenBank ID: CA471696) (Fig. 1). The results demonstrate that the two genes have overlapping expression domains in hindbrain, ventral retina and motor neurons. In particular, the small patch of retinal precursors that express both genes at stage 32 hpf (hours post-fertilisation) (Fig. 1E,F) corresponds to the first postmitotic cells that differentiate to form ganglion cells with axons extending towards the optic fissure and dendrites branching in the inner retina [8]. It has been recently reported that Kidins220 is involved in mechanisms of neuritogenesis and axon/dendrite determination [9–11]. Our findings suggest an interesting correlation of the two genes and potential common functions.

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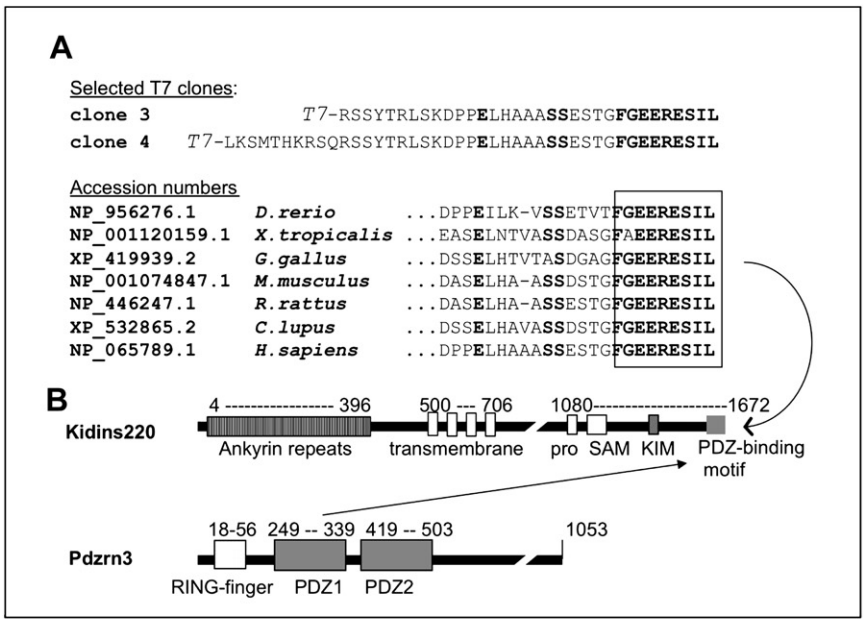
A
Selected T7 clones:
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clone 4 T7-LKSMTHKRSQRSSYTRLSKDPPELHAAASSESTGFGEERESIL
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NP_001120159.1 X.tropicalis ...EASELNTVASSDASGFAEERESIL
XP_419939.2 G.gallus ...DSSELHTVTASDGAGFGEERESIL
NP_001074847.1 M.musculus ...DASELHA-ASSDSTGFGEERESIL
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4 396 500 706 1080 1672
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Pdzrn3
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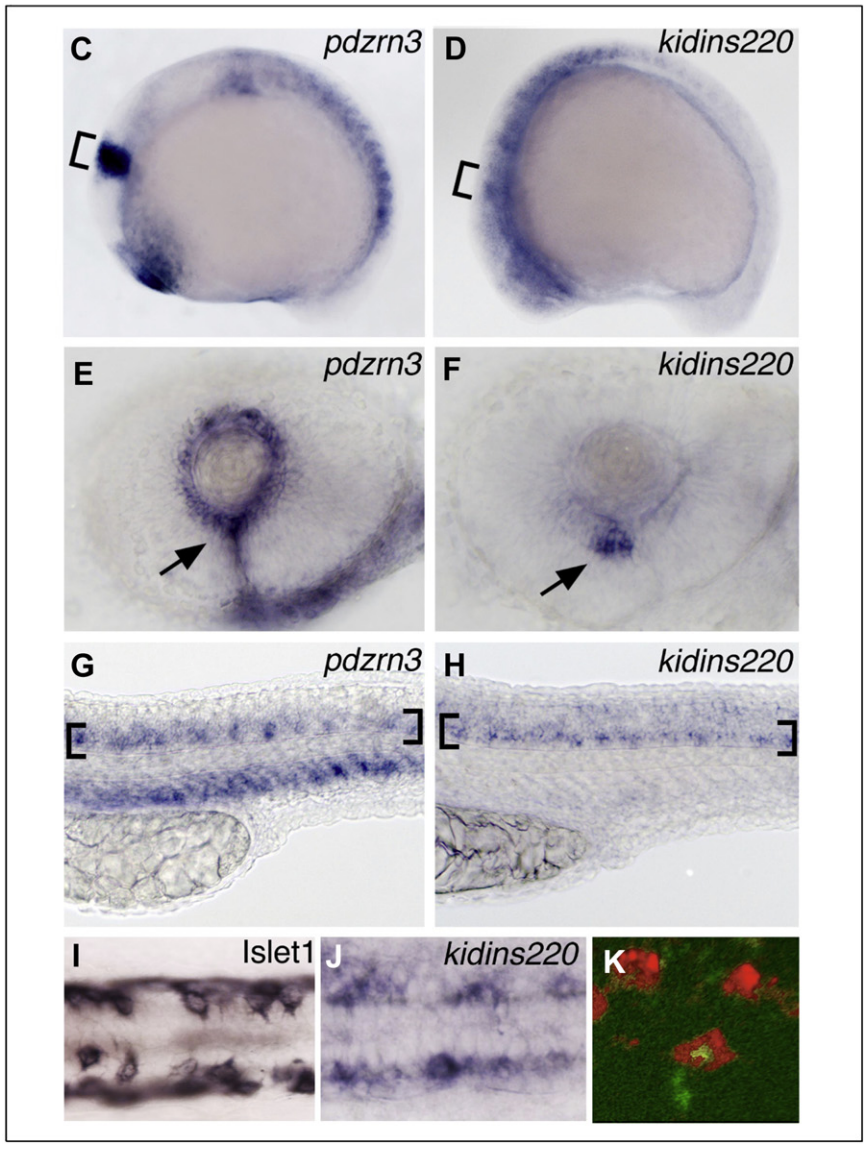
PDZ2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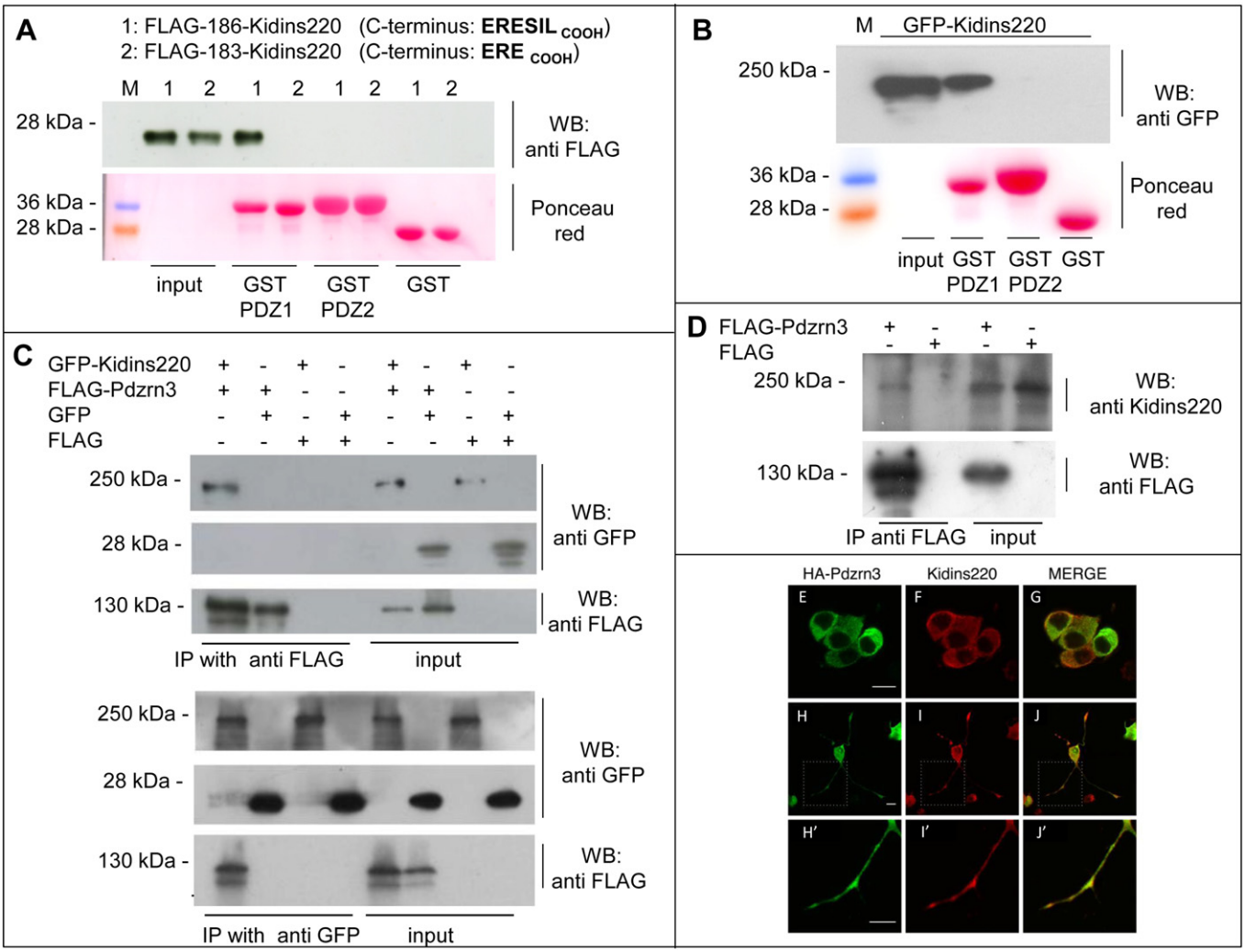

Fig. 2. Analysis of Pdzrn3–Kidins220 interaction. (A) Pull-down assay from HEK293T (human embryonic kidney) cell extracts expressing FLAG-C-teminal constructs of zebrafish Kidins220. "FLAG-186-Kidins220" construct, including the terminal 186 residues and deriving from IMAGE clone 6791960 was subcloned in p3XFLAG-CMV-10 vector. A deletion mutant "FLAG-183-Kidins220", which terminates with ERE (glutamic-arginine-glutamic), was generated by PCR amplification with specific primers to eliminate last three residues SIL (serine-isoleucine-leucine) from FLAG-186-Kidins220. The coding regions of the PDZ-domains were generated by PCR using full-length cDNA of zebrafish Pdzrn3, cloned in pYEX vector and sequenced using standard procedures [15]. Maintenance of HEK293T and biochemical assays were performed as previously described [15]. Extracts from transfected HEK293T were incubated with immobilized GST-PDZ fusions proteins or GST alone. Bound proteins were detected by immunoblotting with anti-FLAG; GST-baits were revealed by Ponceau staining. Input : 5% of the transfected extract. (B) Confirmation of the interaction with GST-PDZ1 in the context of full-length Kidins220 protein. The coding region of zebrafish Kidins220b was subcloned in pEGFPC1 and transiently expressed in HEK293T. Pull-down with GST-PDZs and GST was performed as above. (C) Co-immunoprecipitation of full-length GFP-Kidins220 and FLAG-Pdzrn3. FLAG-full length Pdzrn3 was obtained from Pdzrn3 cDNA subcloned in p3XFLAG-CMV-10. Lysates from HEK293T transfected cells were immunoprecipitated with anti-FLAG-M2 affinity gel (top panels) or with anti-GFP sepharose resin (bottom panels) and subjected to immunoblot with anti-GFP and anti-FLAG antibodies. (D) Co-immunoprecipitation of endogenous Kidins220 with FLAG-Pdzrn3. PC12 cells were transfected with FLAG-Pdzrn3 or control FLAG-vector. Cell lysates were immunoprecipitated with anti-FLAG-M2 gel and subjected to immunoblot with anti-Kidins220 and anti-FLAG antibodies. Input: 2% of the extract. (E–J): Co-localization of Kidins220 and Pdzrn3. An HA-tagged-construct of Pdzrn3 was transiently expressed in PC12 cells, where Kidins220 is constitutively expressed [6,7]. Cells were double stained with anti-Kidins220/anti-rabbit alexa fluor-red and with anti-HA/anti-mouse alexa fluor-green. Maintenance of PC12 cells, NGF induction and immunocytochemistry were performed as previously described [15]. Confocal images were acquired using a Leica TCS SP2 confocal microscope equipped with an argon laser for excitation at 488 nm (E–G) Undifferentiated PC12 cells (H–J) PC12 incubated for 72 h with 100 ng/ml NGF to induce a neuronal phenotype. (H′-J′) High magnification of the regions outlined by the boxes in the above panels, in order to highlight the overlap in the neurite protrusions. Scale bar, 10 μm. All experiments were always performed at least three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 1. (Top panel) the PDZ-binding motif of Kidins220 is target of Pdzrn3. (A) Peptide sequences of T7-phage clones isolated by the first PDZ of Pdzrn3 and comparison among Kidins220 C-terminal sequences of different species. The box highlights conserved residues at the C-terminus (in bold). T7Select 10-3b human brain cDNA library screening was performed as suggested in T7Select phage display system Manual (Novagen). Briefly, bound phages were recovered by incubating at 37 °C with *E.coli* BLT5615 induced with 1 mM IPTG for 30 min before phage addition. Following amplification and further selection rounds, the resulting phages were analysed by a plaque assay [13]. Positive plaques were isolated, phage suspensions were analysed by PCR using primers to the regions flanking the insert and fragments were sequenced. (B) Schematic representation of Pdzrn3 and Kidins220 binding domains. Numbers refer to the deduced proteins from zebrafish genes; Kidins220 (Genbank-ID: BC061450) and Pdzrn3 (Genbank-ID: JN108761). Pro: proline rich domain; SAM: sterile alpha-like domain; KIM: Kinesin1 interaction domain. (Bottom panel) co-expression of Pdzrn3 and Kidins220 genes during zebrafish embryogenesis. Embryo generation, staging and whole-mount *in situ* hybridization were performed as previously described [14]. The probes used are indicated in oblique. (C,D) fourteen-somite stage: lateral view, anterior to the left. Brackets indicate the position of rhombomere 1, specifically labelled by Pdzrn3. (E,F) 32-hpf: detail of the eye, lateral view, dorsal to the top. Arrows point to the ventral region, which includes the first postmitotic retinal ganglion cells. (G,H) 32-hpf: detail of the trunk-tail, lateral view, dorsal to the top. The brackets delimit the row of motor neurons in the spinal cord. (I,K) 32-hpf: detail of the trunk, dorsal view, showing common expression of Kidins220 with Islet1, a typical motor neuron marker. (J) Fluorescent *in situ* hybridization and antibody labelling performed with Kidins220 probe (green-staining) developed with Tyramide signal amplification Systems and anti-Islet1 antibody (red-staining), visualised with cyanine3 550 and Alexa Fluor 488-conjugated secondary antibody, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To confirm the interaction identified by phage display screening, we used tagged recombinant proteins expressed in cultured cells to perform biochemical assays. The fragment encoding the last 186 residues of Kidins220 was cloned in P3X-FLAG-CMV-10 expression vector. HEK293T (human embryonic kidney) cells were transfected with such construct (FLAG-186-Kidins220) and the protein extracts were subjected to GST pull down using as bait GST-PDZ1, GST-PDZ2 and GST alone. In parallel, in order to determine the residues responsible for the binding, we constructed a deletion mutant (FLAG-183-Kidins220) missing the PDZ-binding motif. Specific interaction occurred only with wild-type FLAG-186-Kidins220 construct, while removal of the last three residues completely impaired the binding (Fig. 2A). Prior to our study, a non-canonical interaction between the first PDZ of Pdzrn3 and murine receptor tyrosine kinase MuSK was described to occur at neuromuscular junctions [3]. In this case, the last three amino acids at the carboxy-tail of MuSK were not necessary to mediate the PDZ-interaction, since an internal portion of the kinase domain was required. In contrast, the last three residues of Kidins220 C-terminus are crucial determinants for Pdzrn3 binding. The same residues also control the association with alpha-syntrophin at the neuromuscular junctions [12]. However, finding different targets of the same motif is not unusual and it is not surprising considering that alternative interaction networks can be formed in distinct tissues.

Pdzrn3 binding was also analysed in the context of the full-length Kidins220. Actually two isoforms of different length have been reported in *D. rerio* (http://zfin.org). Kidins220a (1009 residues) missing large part of the C-terminus and a long isoform Kidins220b (1672 residues) that corresponds to the full-length human Kidins220 (GenBank ID: Q9ULH0). We expressed the coding region of isoform Kidins220b (GenBank ID: BC061450) as a GFP (green fluorescent protein) fusion in HEK293T cells, confirming specific interaction with the first PDZ of Pdzrn3 (Fig. 2B).

Next, we examined by co-immunoprecipitation whether such interaction could also occur in living cells expressing both full-length proteins. In this case, GFP-Kidins220 and FLAG-full-length Pdzrn3 were coexpressed in HEK293T cells. Protein extracts were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-GFP or anti-FLAG. GFP–Kidins220, but not GFP alone, was co-immunoprecipitated with FLAG-Pdzrn3 and not with FLAG-alone used as control. Opposite immunoprecipitation with anti-GFP sepharose confirmed the result (Fig. 2C).

Kidins220 has been at first described in rat pheochromocytoma PC12 cells, where it is constitutively expressed and specifically localized at membrane level and in intracellular vesicular compartments. Following induction with NGF (nerve growth factor), PC12 extend neurite protrusions, where Kidins220 is transported [6,7]. Although Pdzrn3 is not endogenously expressed in PC12, as revealed by RT-PCR analysis (data not shown), we verified that ectopically expressed Pdzrn3 associates with the endogenous Kidins220. PC12 were transfected with FLAG-Pdzrn3 or with FLAG vector and the extracts were immunoprecipitated with anti-FLAG antibody. We found that only small amounts of endogenous Kidins220 were bound to FLAG-Pdzrn3, as revealed by immunoblot with a specific polyclonal anti-Kidins220 antibody, directed against the C-terminus of the protein (Fig. 2D). However, the intracellular co-localization of both proteins was confirmed in PC12 cells (Fig. 2E–G) or in PC12 cells incubated with NGF to induce a neuronal phenotype (Fig. 2H–L) by immunocytochemistry. For this experiment, we transfected a full-length Pdzrn3 HA-tagged construct in order to detect the recombinant protein with a monoclonal anti-HA antibody (green-staining). Its co-localization with the endogenous Kidins220 (red-staining) was observed either in the cell body or in the tips of the neurites (Fig. 2G,J,J′).

In conclusion, all these results provide evidence of Pdzrn3–Kidins220 association. We also tried to co-immunoprecipitate both endogenous proteins from C2C12 cells, where either Kidins220 or Pdzrn3 have been independently described [2,12]. However, in our hands, a stable association of the endogenous proteins was undetectable (data not shown), probably because we used a commercial anti-Pdzrn3 (Sigma HPA038822) that weakly crossreacts with the murine protein or because the interaction occurring between these two proteins may be transient or dynamic and controlled by regulatory mechanisms. To further understand the interaction effects and the involvement of the two genes during neurogenesis and retinal ganglion cell differentiation, it will be important to extend our study *in vivo* by exploiting the zebrafish model for gain or loss of function experiments.

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