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Xenopus Claudin-6 is required for embryonic pronephros morphogenesis and terminal differentiation

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ABSTRACT

Claudins are tetratransmembrane tight junction proteins and play important roles in regulating paracellular permeability of different nephron segments of the kidney. However, the roles of claudins in kidney development remain largely unknown. Here we studied the expression and functions of claudin-6 in *Xenopus* pronephros development. *Xenopus* claudin-6 is expressed in the developing pronephric tubule and duct but not glomus. Knockdown of claudin-6 by specific morpholino led to severe defects in pronephros tubular morphogenesis and blocked the terminal differentiation of the tubule cells. The claudin-6 morpholino targeted tubule cells showed failure of apical accumulation of actin and reduced lateral expression of tight junction protein Na/K-ATPase, suggesting an incomplete epithelization likely due to defected cell adhesions and apical-lateral polarity. Our work uncovered a novel role for claudin-6 in embryonic kidney development.

1. Introduction

The vertebrate kidney is a complex organ that performs important homeostatic tasks including reabsorption of nutrients, water balance and waste products excretion. During kidney development in high vertebrates, three successive renal structures form via inductive processes: the pro-, meso- and metanephros. Although there are profound differences in anatomical structure between these three forms, they all contain nephron as their basic functional unit, which consists of three components: the glomerulus, tubule, and duct [1,2]. Claudins are tetratransmembrane tight junction proteins and play important roles in regulating paracellular permeability in the kidney and maintaining cell polarity in epithelial and endothelial cells [3,4]. There are at least 26 members of the claudin family in mammals, which are differentially expressed in the nephron segments, conferring different permeability properties to them [4]. For example, claudin-2 is related to cation-reabsorption in the proximal tubule [5], and claudin-4, -7, and -8 are determinants of chloride permeability of the collecting duct [4,6,7]. Claudins are crucial for kidney function and mutations of claudins would lead to various kidney diseases [8–10]. However, the roles of claudins in kidney development remain largely unknown.

Xenopus pronephros has been a good model to reveal the molecular network underlying kidney development [1,2]. The pronephros of the frog *Xenopus laevis* consists of a single giant nephron, with its structural and functional segmentation compares well with that of mammalian nephrons [1,11,12]. The development of *Xenopus* pronephros involves a series of complex processes including induction, mesenchymal epithelial transition, patterning and differentiation, accompanied by complicated lumenization and morphogenesis processes [2,11,13]. The pronephric anlagen are first induced from the intermediate mesoderm lateral to somites 3–5 at about stage 12.5 by surrounding tissues, as indicated by the expression of the transcription factors Lim1 and Pax8 [14–16]. Once segregated from the intermediate mesoderm, the proximal condensate elongates in the dorsal-ventral direction, shaping the future pronephric tubule. Initiated at about stage 24, the cells undergo mesenchymal epithelial

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transition to become epithelial tubules. Later, the pronephric tubules elongate and become folded into a highly coiled structure while the pronephric duct grows caudally and fuses with the cloaca.

Claudin-6 (Cldn6) has been reported to play an important role during mouse embryonic epithelium formation [17] and the development of endodermal tissues [18]. Cldn6 has also been suggested to be a surface marker for mouse pluripotent stem cells [19] and overexpression of Cldn6 is able to trigger epithelial morphogenesis in mouse stem cells [20]. There are 2 Cldn6 genes reported in *Xenopus*, Cldn6.1 (also known as Cldn4L2) and Cldn6.2 (also known as Cldn4L1), both of which are expressed in the developing pronephros with similar patterns [21]. Here we showed that *Xenopus* Cldn6 is required for terminal differentiation of pronephros. Knockdown of XCldn6 also interfered with pronephros morphogenesis and the maintaining of the apical-basolateral polarity of the tubule cells. We suggest that XCldn6 regulates pronephros development likely through its roles in tight junction formation.

2. Materials and methods

2.1. Cloning of Xenopus Cldn-6 and plasmids construction

X. laevis Cldn6 cDNAs were cloned into the pCS2 or pBluescript vectors by PCR according to published (*XCldn6.2a*: NM_001088863; *XCldn6.1b*: NM_001088861) [21] or GenBank sequences (*XCldn6.1a*: NM_001093287; *XCldn6.2b*: NM_001088604).

2.2. Xenopus embryos, whole-mount in situ hybridization and microinjection

In vitro fertilization, embryo culture, and whole mount *in situ* hybridization of *Xenopus* embryos were carried out as described [22]. For *in situ* hybridization, the probes for *Lim1, Pax2, Pax8, PDZK1 and CLC-K* were used as described [14,15,23]. The probe for *XCldn6* was prepared using a vector containing the full open reading frame of *Cldn6.1a*. For microinjection, the embryos were injected at 8-cell stage into 1 or 2 V2 cells, targeting the presumptive pronephros regions. An antisense morpholino oligo (MO) was designed against a fragment at the 5′ untranslated region conserved in all 4 forms of *XCldn6* with the following sequence: 5′-TGGAGACCAGTAGAAGCCATCTTTT-3′, which was obtained from Gene Tools (OR) and was injected at the dose of 24 ng per embryo. For rescue experiments, capped mRNAs for microinjection were synthesized with the SP6 mMessage mMachine Kit (Ambion).

2.3. Immunohistochemistry

For immunohistochemistrical analysis, the embryos were sectioned at 10 μm. Antigen retrieval was performed with 0.01 M citrate buffer (pH6.0). The sections were blocked with 2.5% BSA for 1 h, incubated with the primary antibodies at 4 °C overnight, washed and incubated with secondary Alexa Fluor-488 or Alexa Fluor-594 conjugated donkey anti-rabbit IgG or anti-mouse IgG (Invitrogen). The samples were washed, mounted and imaged using an Olympus (FV1000) laser scanning confocal microscope. The first antibodies used were: monoclonal antibody to GFP (Proteintech), rabbit anti-GFP (Abcam), rabbit anti-β-actin (Sigma), monoclonal antibody to Na/K-ATPase (Abcam), rabbit anti-PKCζ (Santa cruz), rabbit monoclonal antibody to Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (Cell Signaling Technology). A rabbit antibody against *Xenopus* Cldn6 was prepared by Abmax (Beijing) against a peptide at its C-terminal: SQPRSDYPSKNYV, which was antigen affinity purified.

3. Results and discussion

Due to the tetraploid nature of *X. laevis*, there are 2 alleles of each gene, yielding 4 *Cldn6* genes in this species (Supplemental Fig. 1), which are hereafter named *Cldn6.1a*, *Cldn6.1b*, *Cldn6.2a* and *Cldn6.2b* respectively (Supplemental Fig. 1A). Genomic analysis revealed that *Cldn6.1* and *Cldn6.2* genes are clustered tandemly in reverse directions (Supplemental Fig. 1B)

We checked by *in situ* hybridization the expression of *XCldn6* at different embryonic stages. As reported previously, *XCldn6* was first detected in pronephric anlage at stage 24–25 (Fig. 1A). At stage 34–35, *XCldn6* was detected strongly in the pronephric tubule and duct but not the glomus (Fig. 1B). We then studied the expression of XCldn6 protein during *Xenopus* pronephros development using an antibody against a peptide at the C-terminal of XCldn6.1b. Although there are 1–3 amino acids different in this fragment among the XCldn6s (Supplemental Fig. 1A), the antibody recognizes all forms of XCldn6 when overexpressed in mammalian cells (data not shown), and is hereafter referred to as anti-XCldn6. Staining of sections of stage 25–39 embryos with the anti-XCldn6 antibody revealed specific signals in the pronephros tubules (Fig. 1C–F) and duct (data not shown), consistent with the results by *in situ* hybridization. At stages 25–29, XCldn6 was detected all around the plasma membrane of the pronephric epithelial cells (Fig. 1C and D). At stage 34, its staining became weak at the basal surface (Fig. 1E). With the development of pronephros, XCldn6 expression was no longer detected at the apical surface of the tubule cells at stage 39 (Fig. 1F).

To confirm the specificity of the antibody, we knocked down the expression of endogenous XCldn6 using an antisense morpholino oligonucleotide (MO). In XCldn6-MO injected embryos, the immunostaining for XCldn6 decreased substantially in the pronephric tubule cells (Fig. 2A). By western blot, a specific protein band of 22 kDa was detected in control whole embryo lysate, which was dramatically reduced in embryos injected with the XCldn6-MO (Fig. 2B). These data suggest that the antibody was specific and that the XCldn6-MO effectively blocked the expression of endogenous XCldn6 protein.

We then studied the roles of XCldn6 in pronephros development by injecting the XCldn6-MO into the V2 cells at 8-cell-stage, from which the pronephros anlagen derives [24]. Such embryos developed pronounced pericardial edema at stage 43 (20/25; Fig. 2C), indicative of pronephros dysfunction [24]. On sections, the pronephric tubules on injected sides became enlarged and the epithelia cells became more columnar shaped compared with the square shaped cells in the control tubules (Fig. 2A).

Next, we studied the expression of pronephros-specific marker genes in the XCldn6-MO injected embryos. Lim1, Pax8 and Pax2 are transcription factors crucial for pronephros development and are turned on early during pronephros induction at about stage 12.5 [14–16]. In the *XCldn6* morphants, the expression levels of the three genes have no clear changes (Fig. 3A), suggesting the process of pronephros induction and specification likely occurred properly, consistent with the relative late expression of *XCldn6* in the developing pronephros. However, knockdown of XCldn6 clearly led to defects in pronephros tubulogenesis (Fig. 3A and B). In control embryos of stage 36, three characteristic nephrostomes form as can be seen by *in situ* hybridization with *Pax2*, *Lim1*, or *Pax8* (Fig. 3A). In the *XCldn6* morphants, however, the three nephrostomes were no longer seen, which appeared to fuse into a single one. This phenotype can be largely rescued by co-injection of *XCldn6.1a* mRNA (Fig. 3A and B).

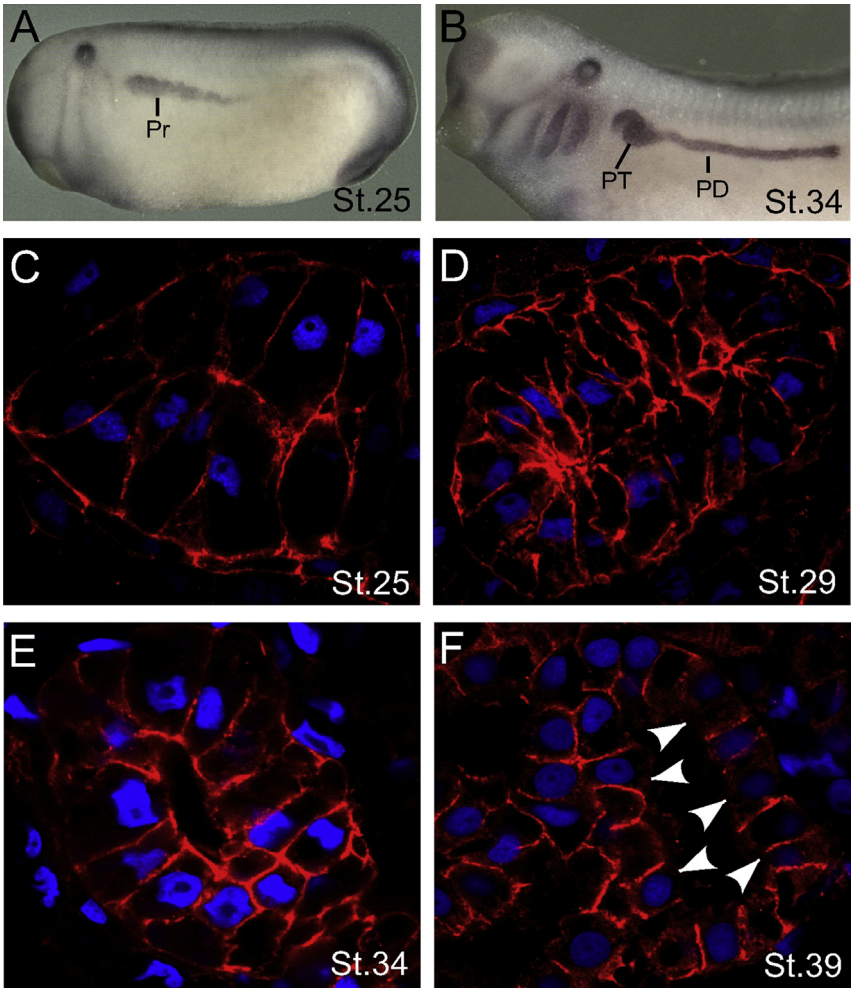

Fig. 1. Specific expression of XCldn6 in developing pronephros. (A and B) Expression of *XCldn6* in the embryos at stage 25 (A) and 34 (B) revealed by whole-mount *in situ* hybridization. Pr, pronephros; PT, pronephric tubule; PD, pronephric duct. (C–F) Expression of XCldn6 protein in the pronephric tubule cells in embryos at indicated stages revealed by immunostaining. XCldn6 is no longer detected at the apical surface of the tubule cells at stage 39 (arrowheads in F).

When the expression of differentiated tubular cell marker *PDZK1* and duct marker *CLC-K* [23,25] were examined, both of them were found dramatically reduced in the XCldn6-MO injected embryos, which were clearly rescued by co-injected *XCldn6.1a* mRNA (Fig. 3C and D). These data indicate that XCldn6 is not required for the induction and specification of the pronephros, but is probably involved in the tubulogenesis process and the terminal differentiation of pronephros.

As claudins are tight junction proteins, we next studied the potential roles of XCldn6 in the regulation of the polarity and cytoskeleton of the tubule cells. PKCζ is a member of the Par atypical polarity complex that labels the apical face of pronephric

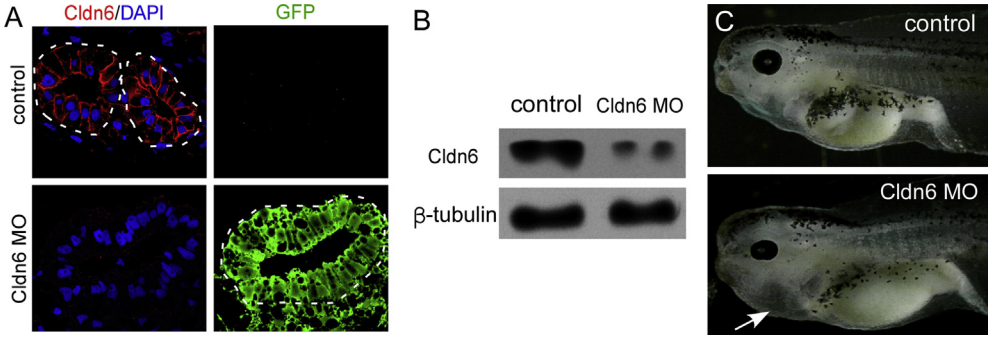

Fig. 2. The XCldn6 antibody and XCldn6-MO are specific. (A) Injection of the XCldn6 morpholino completely abolished the staining with the XCldn6 antibody in the pronephric tubule (lower panel), compared with the control (upper panel). (B) Endogenous XCldn6 level was dramatically reduced in embryos injected with the XCldn6-MO as revealed by western bolt using the XCldn6 antibody. (C) Embryos injected with the XCldn6-MO developed pericardial edema (arrowhead) at stage 43.

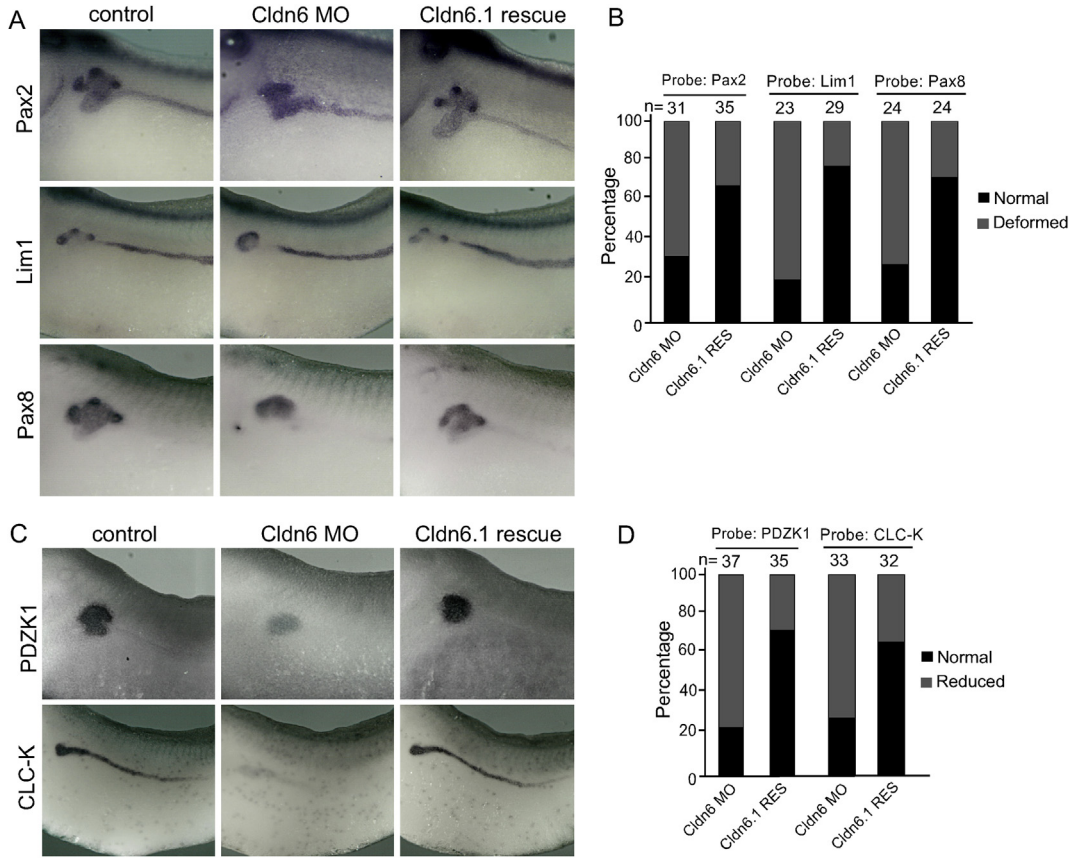

Fig. 3. Xcldn6 is required for pronephros development. (A and B) Expression patterns of *Pax2*, *Lim1* and *Pax8* in control embryos and embryos injected with XCldn6-MO or together with rescuing *XCldn6.1a* mRNA at stage 36 revealed by whole-mount *in situ* hybridization. The percentages of embryos with deformed pronephric tubules are shown in (B). (C and D) Injection of XCldn6-MO severely reduced the expression of *PDZK1* and *CLC-K* in stage 36 embryos which were rescued by co-expression of *XCldn6.1a* mRNA. The statistical data are shown in (D).

tubule cells [26,27] (Fig. 4A). Knockdown of XCldn6 led to abnormal apical surface of the tubule cells, which were loose and protruded into the lumen (Fig. 4B and B'). In such cells, PKCζ remained correctly localized at the apical region, suggesting that the general polarity of the cells were established correctly (Fig. 4B and B'). Ezrin, a member of the ERM (Ezrin/Radixin/Moesin) family, is a microvillus marker which localized to the apical surface of renal epithelia [28]. Activated Ezrins are phosphorylated and are involved in the actin filament/plasma membrane interaction. In control embryos, staining with an antibody against phosphorylated ERM (p-ERM) yielded a distinct signal at the apical surface of tubule epithelial cells (Fig. 4C). In the XCldn6 morphants, the apical staining of p-ERM became weaker which now appeared also at the lateral surface of the tubule epithelial cells (Fig. 4D and D'), likely suggesting a failure of the cells to maintain the apical-lateral polarity. In the pronephros tubes in which only part of the cells were targeted, the apical staining of p-ERM became weaker in the targeted cells which protruded into the tubular lumen (Fig. 4E and E'). Strong apical accumulation of actin was also observed in control pronephric tubule cells at stage 36, which was dramatically reduced in XCldn6-MO injected cells (Fig. 4F), supporting a failure of terminal differentiation of the these cells. In addition to its role as a sodium–potassium pump, Na/K-ATPase is also required for the formation of tight junctions and induction of polarity in epithelial cells [29,30]. In control tubule cells, Na/K-ATPase was detected on the basolateral surface (Fig. 4G). In the XCldn6-MO injected tubule cells, however, the expression levels of Na/K-ATPase were severely reduced (Fig. 4G). As Na/K-ATPase is widely considered as a marker for tight junction [31], these data suggest a failure of tight junction formation among the tubule cells.

As the planar cell polarity (PCP) pathway plays a key role in the regulation of pronephros morphogenesis [12], we also examined the relationship between XCldn6 and PCP signaling. Blocking PCP signaling in pronephros by injection of Xdd1 (a PCP-specific dominant-negative form of dishevelled-2) [12] has no effect on the localization or expression of XCldn6 (data not shown). Also, knockdown of XCldn6 did not affect the expression level of the core PCP genes *Prickle1* and *Celsr1* (data not shown). These data suggest that XCldn6 likely does not work through the PCP pathway.

Our data support XCldn6 as an essential modulator of tubulogenesis and terminal differentiation of the pronephros tubule, although it is not involved in the early induction and specification of the pronephros. XCldn6 likely works through modulating tight junction formation and thus has a role in maintaining the cell adhesion and apical-lateral polarity. Indeed, the XCldn6-MO targeted cells lose apical actin and p-ERM, with greatly reduced expression of the tight junction protein Na/K-ATPase. Such cells frequently protrude into the lumen (Fig. 4E), implicating impaired apical architecture and loss of surface tension. The failure of terminal differentiation of the tubule cells when XCldn6 was knocked-down is likely a secondary effect of incomplete epithelization due to defected cell adhesions and polarity. Consistent with our data, overexpression of Cldn6 in mouse stem cells was shown able to induce the expression of other tight-junction and microvillus

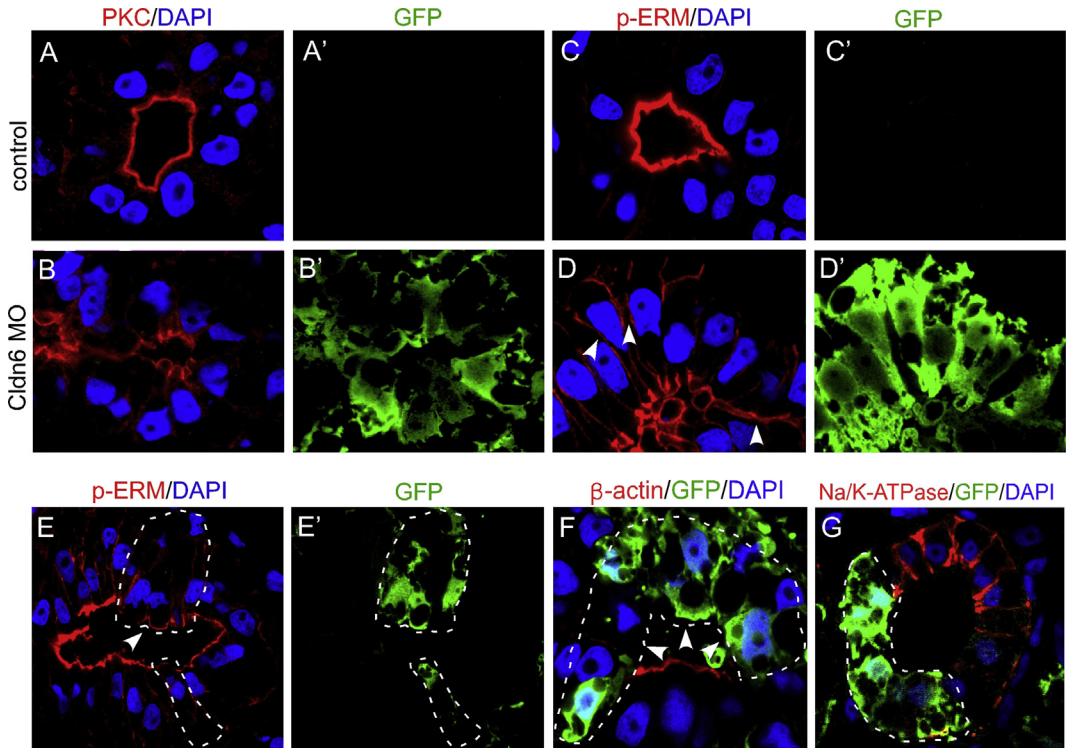

Fig. 4. Effects of XCldn6 knockdown on the polarity, cytoskeleton and junction protein expression in pronephric tubule cells. (A and B) Immunostaining of PKCζ (red) in pronephric tubule cells of control embryos and embryos injected with XCldn6-MO. The targeted areas were labeled green by tracing GFP (B'). (C and D) Immunostaining of p-ERM (red) in pronephric tubule cells of control embryos and embryos injected with XCldn6-MO. The targeted areas were labeled green by tracing GFP (D'). (E) Immunostaining of p-ERM (red) in a pronephric tube in which part of the cells were targeted with XCldn6-MO. The targeted areas are outlined which were labeled green by tracing GFP (E′). (F, G) Immunostaining of β-actin and Na/K-ATPase (red) in pronephric tubes in which part of the cells were targeted with XCldn6-MO. The targeted areas labeled by tracing GFP (green) are outlined. All embryos were at stage 36. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecules and triggers epithelial morphogenesis [20]. Collectively, our work uncovered a novel role for XCldn6 in embryonic kidney development.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.065.

Transparency document

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