Elucidation of megalin/LRP2-dependent endocytic transport processes in the larval zebrafish pronephros

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Summary
Megalin/LRP2 is an endocytic receptor in the proximal tubules of the mammalian kidney that plays a central role in the clearance of metabolites from the glomerular filtrate. To establish a genetic model system for elucidation of molecular components of this retrieval pathway, we characterized orthologous transport processes in the zebrafish. We show that expression of megalin/LRP2 and its co-receptor cubilin is conserved in the larval zebrafish pronephros and demarcates a segment of the pronephric duct that is active in clearance of tracer from the ultrafiltrate. Knock-down of megalin/LRP2 causes lack of Rab4-positive endosomes in the proximal pronephric duct epithelium and abrogates apical endocytosis. Similarly, knock-down of the megalin/LRP2 adaptor Disabled 2 also blocks renal clearance processes. These results demonstrate the conservation of the megalin/LRP2 retrieval pathway between the larval zebrafish pronephros and the mammalian kidney and set the stage for dissection of the renal endocytic machinery in a simple model organism. Using this model system, we provide first genetic evidence that renal tubular endocytosis and formation of endosomes is a ligand-induced process that crucially depends on megalin/LRP2 activity.

Key words: gp330, Cubilin, Disabled 2, Kidney, Endocytosis, PRKCiota, Mpp5

Introduction
The zebrafish pronephric kidney is the essential organ for blood ultrafiltration, clearance of metabolites from the ultrafiltrate and osmoregulation during larval stages (Drummond et al., 1998; Drummond, 2004). In contrast to the meso- and metanephros of adult forms or higher vertebrates, the zebrafish pronephros is a simplified organ composed of two laterally positioned nephrons with two glomeruli fused at the midline. Two bilateral pronephric tubules link the glomeruli with the pronephric ducts that pass on the ultrafiltrate to the outside.

Zebrafish genetics and embryology allow for a functional analysis of pronephric epithelial cell development, differentiation, and physiology. Renal uptake processes that occur within the pronephric duct can be visualized by injection of tracer dyes into the circulatory system. By 56 hours post fertilization (hpf), injection of 10 kDa Rhodamine-dextran (10kDa-RD) results in its filtration through the glomerulus into the lumen of the pronephric duct from where it is cleared into apical membrane vesicles (Drummond et al., 1998). Thus, renal clearance mechanisms appear to be in place within zebrafish larvae at this early stage. However, the molecular machinery involved in renal clearance pathways has not been investigated in zebrafish.

In the mammalian kidney, bulk clearance of solutes and metabolites that have been filtered through the glomerulus occurs within the proximal convoluted tubule (PCT). This nephron segment is composed of a simple columnar epithelium characterized by abundant apical microvilli forming a brush border. The extensive enlargement of the apical cell membrane is crucial to the function of the epithelial cells in reabsorption of ligands from the ultrafiltrate. PCT cells retrieve many filtered metabolites by virtue of endocytic receptors expressed on the apical brush-border membrane. Central to the endocytic machinery of the proximal tubular epithelium is a giant, 600 kDa cell surface receptor designated megalin/LRP2 (Christensen and Willnow, 1999; Christensen and Verroust, 2002). Megalin/LRP2 is a member of the low-density lipoprotein (LDL) receptor gene family of endocytic receptors that act as scavenging proteins in many tissues (Nykjaer and Willnow, 2002). Like other family members, megalin/LRP2 harbors all the structural features required to perform receptor-mediated endocytosis. These features include luminal protein domains for binding of ligands in the extracellular space and for their discharge in endosomes, as well as motifs in the cytoplasmic tail enabling internalization via coated-pits. In the PCT, megalin/LRP2 associates with the peripheral membrane protein cubulin, forming a dual receptor complex on the apical cell surface that binds a multitude of diverse ligands either by association with the megalin/LRP2 or the cubulin polypeptide (Burmeister et al., 2001; Christensen and Verroust, 2002). Internalization of the receptor-ligand complexes is governed by the intracellular tail of megalin/LRP2 and requires association with Disabled 2 (Dab2), a cytoplasmic adaptor protein with phosphotyrosine binding domain (Morris et al., 2002). Metabolites cleared by the megalin/LRP2-cubulin-Dab2 pathway include serum albumin, hormones such as insulin and parathyroid hormone, as well as complexes of vitamins A, D₃
and B12 with their plasma carriers (Orlando et al., 1998; Hilpert et al., 1999; Christensen and Willnow, 1999; Birn et al., 2000a; Birn et al., 2002; Leheste et al., 2003). In zebrafish, megalin/LRP2 is expressed within the embryonic and larval pronephric duct epithelium where it localizes to the apical membrane (McCarthy et al., 2002). Neither the role of megalin/LRP2 in pronephric duct clearance processes nor whether a functional segmentation of the zebrafish pronephric duct depends on megalin/LRP2 activity has previously been investigated. Potentially, the zebrafish provides a simple genetic model system with which to further dissect the molecular components of the megalin/LRP2 endocytosis pathway in vivo.

A genome-wide analysis of human kinases in endocytosis has implicated protein kinase C zeta (PRKCi) in the correct distribution of transferrin-positive endosomes in a cell culture assay (Pelkmans et al., 2005). PRKCi and protein kinase C iota PRKCI are essential regulators of cell polarity and epithelial integrity in diverse systems (Ohno, 2001). Typically associated with the tight junctions of epithelial cells, PRKCs are components of an evolutionarily conserved complex assembled around the PDZ-domain-containing Par3/ASIP-Par6 proteins that are physically linked to the Crumbs-MAGUK p55 subfamily member 5 (Mpp5) protein scaffold (Roh et al., 2002; Hurd et al., 2003; Wang et al., 2004). Zebrafish heart and soul (Has)/PRKCi is expressed in the kidney where it localizes to apical membranes of pronephric duct epithelial cells (Horne-Badovinac et al., 2001). Loss of Has/PRKCi and Nagie oko (Nok)/Mpp5 results in severe defects of different embryonic epithelia including the neural retina and the lateral plate mesoderm (Horne-Badovinac et al., 2001; Peterson et al., 2001; Wei and Malicki, 2002; Horne-Badovinac et al., 2003).

Here, we show that megalin/LRP2 and Dab2 define a segment of the early zebrafish kidney that is involved in endocytic uptake of metabolites. Loss of megalin/LRP2 and Dab2 abolishes endocytosis within pronephric duct epithelial cells as indicated by the complete lack of Rab4-positive early endosomes. Candidate gene approaches with has/prkci and its scaffolding partner nok/mpp5 provide first evidence that renal tubular endocytosis and formation of Rab4-positive endosomes is a ligand-induced process that crucially depends on megalin/LRP2 activity. Our results provide proof of concept for the applicability of the zebrafish model to genetically dissect renal endocytosis in vivo and they present an entry point for analysis of components of the megalin/LRP2 retrieval pathway.

Results

Expression of components of the megalin/LRP2 retrieval pathway in the zebrafish pronephric duct

We used a PCR-based approach with primers deduced from the zebrafish genome sequence assembly version Zv4.0 published on the Sanger Institute website to clone a central part of cubilin (GenBank accession number 68404433) as well as sequence encoding the transmembrane and part of the intracellular domain of megalin/lrp2 (GenBank accession number 68436945). Owing to the size and incomplete annotation of the zebrafish genomic sequence, we were unable to isolate the sequence encoding most of the megalin/LRP2 N-terminal extracellular domain. BLAST and SSAHA searches of the identified megalin/lrp2 and cubilin sequences against the Zv4.0 database of the Sanger Institute failed to identify additional homologs of these genes. Therefore, zebrafish megalin/lrp2 and cubilin are most probably the only homologs of each receptor type present within the zebrafish genome based on the Zv4.0 database, which covers most of the genomic sequence.

Sequence alignments using the isolated domains of the zebrafish receptors against their human homologs demonstrate the high sequence conservation of megalin/LRP2 and cubilin (Fig. 1).

In addition, we focused on additional known components of the megalin/LRP2 endocytic machinery in mammals and tested

![Fig. 1. Sequence comparison of zebrafish megalin/LRP2 and cubilin with human orthologs. (A) Aligned protein structures of human megalin/LRP2 and zebrafish LRP2 and human cubilin and zebrafish cubilin. (B) Sequence alignments of zebrafish megalin/LRP2 and human megalin/LRP2. (C) Sequence alignments of zebrafish cubilin and human cubilin.](image-url)
their conservation in zebrafish. Mammalian Dab2 is an adaptor protein that associates with a binding motif in the intracellular tail of megalin/LRP2 and that is essential for recycling of the receptor in the endocytic compartments of PCT cells (Morris et al., 2002; Nagai et al., 2005). We isolated the respective coding sequence by reverse transcription (RT)-PCR based on the NCBI sequence (GenBank accession number 32442447). BLAST and SSAHA homology-search tools indicate that zebrafish dab2 is most probably the only homolog present in this species.

First, we characterized the expression of megalin/lrp2 and cubilin, as well as dab2 within the developing pronephric duct. Formation of the pronephric duct and nephron primordia is initiated during early somitogenesis. At these stages, presumptive kidney intermediate mesoderm cells that contribute to the anterior pronephric duct express pax2.1 and nephron primordial cells are positive for wilms tumor 1 (wt1) (Krauss et al., 1991; Drummond et al., 1998; Serluca and Fishman, 2001). Epithelialization of the pronephric duct occurs throughout somitogenesis and is completed by 24 hpf. At 20-24 hpf, megalin/lrp2 and dab2 displayed overlapping expression patterns within the anterior third of the pronephric duct (Fig. 2A,C,F,H). Whereas expression of cubilin was not detectable at 20 hpf, it was largely overlapping with megalin/lrp2 and dab2 at 24 hpf (Fig. 2B,G). By 48 hpf, the bilateral nephron primordia integrate to form central pronephric glomeruli that express wt1 and tubules that express pax2.1. At this stage, megalin/lrp2, dab2 and cubilin are expressed in an expanded proximal portion of the pronephric duct but not in pronephric tubules (Fig. 2L-N). Finally, by 72 hpf, megalin/lrp2, dab2, and cubilin were expressed in a proximal portion of the pronephric duct and a small distal portion of the tubule epithelium (Fig. 2Q-S). Two-color in situ hybridization with megalin/lrp2 and dab2, as well as megalin/lrp2 and cubilin at 72 hpf confirmed the complete overlap of both gene expression patterns in the pronephric duct (Fig. 3), except for some extra-renal tissues including the otic vesicle (Fig. 3, arrows). However, at 48 hpf, dab2 displayed a wider extra-renal expression pattern that was partially overlapping with megalin/lrp2 within the otic vesicle and regions of the central nervous system (data not shown). This expression indicated a possible involvement of dab2 in megalin/LRP2-mediated endocytosis in pronephric duct tubular cells, as well as in extra-renal tissues during early development. We conclude that the expression of zebrafish megalin/lrp2, dab2, and cubilin defines a proximal part of the nephron, corresponding to the distal tubule and proximal duct epithelium.

Renal clearance of metabolites is restricted to the proximal pronephric duct

In zebrafish, the vascularization of glomeruli by ingrowing endothelial cells and the onset of blood filtration are completed by 48 hpf (Drummond et al., 1998). To further characterize those regions of the pronephric duct involved in renal clearance of metabolites, we injected tracer molecules of defined molecular mass into the cardinal vein of 72 hpf wt embryos as previously described (Drummond et al., 1998). 70 kDa fluorescein-dextran (70kDa-FD) was used as a fluid phase marker and was taken up into the proximal third of the pronephric duct and the distal tubule within minutes after injection (Fig. 4A). To define whether the region involved in renal clearance of metabolites corresponds to the megalin/LRP2 expression domain

Fig. 2. Expression of megalin, cubilin, dab2, wt1 and pax2.1 in the developing pronephros. Dorsal view of wild-type embryos at 20 hpf (A-E), 24 hpf (F-K), 48 hpf (L-P), and 72 hpf (Q-U) analyzed for expression of megalin/lrp2, cubilin, dab2, wt1 and pax2.1. Combinatorial gene expression patterns are indicated in the model. Arrows indicate the tubular expression of megalin/lrp2 (Q), arrowheads point at the pronephros expression domain of pax2.1 (R-U). gl, glomerulus; np, nephric primordium; pd, pronephric duct; pt, pronephric tubule. Bar, 100 μm.
Cy2-RAP was rapidly cleared from the pronephric duct and internalized into apical vesicles of the pronephric duct tubular cells in a pattern identical to that observed for 70kDa-FD (Fig. 5C). Thus, 70kDa-FD visualizes megalin/LRP2-dependent uptake processes and was used as a marker for further tests.

Many of the vesicles positive for 70kDa-FD co-stained with an antibody directed against the early endosome protein Rab4, indicating clearance via endocytic pathways (Fig. 5E-G) (Daro et al., 1996). Vesicles positive for 70kDa-FD that did not co-stain for Rab4 represented probably distal compartments of the endocytic pathway, such as late endosomes or lysosomes harboring the tracer. Thus, megalin/LRP2 demarcates an endocytic active region within the pronephric duct that is involved in the clearance of filtered model ligands (Cy2-RAP) and fluid-phase markers (70kDa-FD) via endocytic pathways.

Zebrafish megalin/LRP2 and Dab2 are essential for tubular clearance of metabolites by receptor-mediated endocytosis

To investigate the relevance of megalin/lrp2 and dab2 in zebrafish tubular-clearance mechanisms, we performed morpholino antisense oligonucleotide (MO)-mediated knockdown of these genes (Heasman et al., 2000; Nasevicius and Ekker, 2000). For megalin/lrp2, we designed two independent splice donor site MOs targeting the exon that encodes the transmembrane domain (megMO1) or the immediate upstream exon of megalin/lrp2 (megMO2, see Materials and Methods). Targeting of either exon results in the loss of the membrane anchor. This approach has been used before to inactivate the murine megalin/lrp2 gene (Leheste et al., 2003). Sequence analysis of the RT-PCR products confirmed that parts of the cDNA corresponding to the targeted exons were missing, resulting in premature stop of translation in the respective morphants (Fig. 6A; sequence data not shown). The efficacy of both splice MOs to abrogate megalin/LRP2 expression was demonstrated by western blot analysis in membrane extracts of 48 hpf morphants (Fig. 6B). Extracts from wild-type embryos displayed a prominent immunoreactive band of approximately 250 kDa, which probably represented the major proteolytic breakdown product of the receptor described before (Orlando and Farquhar, 1993; Bachinsky et al., 1993), and which was completely absent from the morphants. Successful ablation of megalin/LRP2 expression was also confirmed by immunofluorescence microscopy on whole-mount embryos using an alternative antiserum (Fig. 7F,G). To examine a

Fig. 3. Co-expression of megalin/lrp2 with dab2 or cubilin within the developing pronephros. Dorsal view of wild-type embryos double-labeled by two-color in situ hybridization at 72 hpf. Single staining for megalin/lrp2 is red and for dab2 and cubilin is blue, overlapping expression results in a brownish color precipitate. Arrows indicate the non-overlapping megalin/lrp2 gene expression within the otic vesicle. Within the pronephric duct, expression of all three genes overlaps. Bar, 200 μm.

(McCarthy et al., 2002), we co-visualized the receptor on whole-mounts with an anti-rat megalin/LRP2 antibody. Indeed, renal reuptake of 70kDa-FD occurred in a proximal-to-distal gradient of intensity that was entirely enclosed within the megalin/LRP2 expression domain (Fig. 4A-C). Transverse sections of these embryos showed apical megalin/LRP2 localization and confirmed that the 70kDa-FD molecules were distributed as punctuate vesicles in the pronephric duct epithelial cells (Fig. 4D-F).

To confirm that the tubular uptake is dependent on glomerular filtration, we compared the clearance of 10kDa-RD with 500 kDa fluorescein-dextran (500kDa-FD), which should not pass the ultrafiltration barrier (Drummond et al., 1998). Whereas 10kDa-RD was cleared from the proximal third of the pronephric duct and the distal tubule within minutes after injection (Fig. 5A) and was distributed as punctuate vesicles on transverse sections (Fig. 5B), no uptake of 500kDa-FD was observed confirming that glomerular ultrafiltration prevents passage of this large molecular mass tracer into the pronephric duct (Fig. 5D).

To visualize in vivo the reuptake of a megalin/LRP2 substrate, we injected Cy2-labeled rat receptor-associated protein (Cy2-RAP) into the circulation of 72 hpf wt embryos. RAP is an endoplasmatic reticulum-resident protein that physically associates with megalin/LRP2 and that can be used as a model ligand when applied exogenously (Willnow et al., 1996; Birn et al., 2000b). Cy2-RAP was rapidly cleared from the pronephric duct and internalized into apical vesicles of anterior pronephric duct tubular cells in a pattern identical to that observed for 70kDa-FD (Fig. 5C). Thus, 70kDa-FD visualizes megalin/LRP2-dependent uptake processes and was used as a marker for further tests.
possible function for dab2 in tubular endocytosis, we designed an ATG-directed MO (dab2MO) to block translation of maternal and zygotic transcripts. Knock-down of megalin/lrp2 or dab2 did not affect formation of the pronephric duct and nephron as demonstrated by in situ hybridization with pax2.1, wt1 and cubilin as differentiation markers (data not shown). The overall embryonic and larval anatomy appeared unchanged for the entire observation period up to 7 days post fertilization (dpf) (Fig. 6C). Moreover, Acridine-Orange vital embryonic staining failed to reveal increased apoptotic cell death in megalin/lrp2 or dab2 morphants compared with controls at 3.5 dpf (data not shown). Thus, we conclude that the formation and patterning of the pronephric duct and nephron is normal in megalin/lrp2 and dab2 morphant embryos.

Next, we performed 10kDa-RD, 70kDa-FD and Cy2-RAP injections into the circulation of megalin/lrp2 and dab2 morphants to assess the efficiency of tubular reabsorption of these molecules. Inline with a crucial role of megalin/LRP2 in pronephric duct clearance, both independent megalin/lrp2 morphants completely lacked receptor immunoreactivity (Fig. 7F,G) and exhibited an almost complete failure of renal uptake of tracers, as shown by fluorescence microscopy on whole mounts (Fig. 7B,C,K,L; Fig. 8A) and by quantifications thereof (Fig. 7N). Knock-down of Dab2 did not affect megalin/LRP2 expression (Fig. 7H) but clearance of 10kDa-RD, 70kDa-FD and Cy2-RAP was nevertheless completely blocked (Fig. 7D,M,N). The loss of renal uptake in dab2 morphants was not caused by a mis-localization of megalin/LRP2, which was correctly localized at the apical cell membrane (Fig. 8B). To verify the specificity of the dab2 morphant phenotypes, we performed mRNA rescue by co-injecting full-length dab2 transcripts with silent sequence alterations that rendered the mRNA resistant to the dab2MO (Fig. 7N).

Surprisingly, absence of megalin/LRP2 and Dab2 expression not only resulted in an inability to clear metabolites from the pronephric duct lumen but also caused an apparent loss of the Rab4-positive early endosome fraction in the pronephric duct epithelium of 72 hpf morphants (Fig. 9). However, Rab4-positive endosomes were still present in extra-renal tissues such as the retina (data not shown). These findings not only confirm a conserved function for megalin/LRP2 as clearance receptor in the zebrafish renal tubular epithelium but also highlight the crucial role of this protein in proper formation of early endosomes in this cell type. Moreover, zebrafish megalin/LRP2 apparently depends on the adaptor Dab2 for receptor-mediated endocytosis of metabolites from the pronephric duct. Thus, the molecular players required for receptor-mediated endocytosis are functionally conserved between the zebrafish larval pronephros and the adult mammalian kidney.

**Fig. 5.** Renal clearance of tracers occurs within the proximal pronephric duct. Wild-type embryos (72 hpf) were injected with 10kDa-RD (red), 70kDa-FD (green), Cy2-RAP (green) or 500kDa-FD (green), and renal clearance of tracer into the pronephric-duct tubular cells was evaluated 1.5 hours later on (A) whole mounts or (B-G) transversal sections by confocal fluorescence microscopy. There is no tubular uptake of the 500kDa-FD tracer (D). Partial colocalization of 70kDa-FD and Rab4 (red) can be seen in endosomes of tubular cells of the pronephric duct that were immunostained for the early endosomal marker Rab4 (E-G). White dotted lines demarcate the position of the pronephric ducts in (B-D) or the outline of the pronephric duct lumen (E-G). Bars, 5 μm.

**Fig. 6.** Molecular characterization of the megalin/lrp2 knock-down. Wild-type embryos were injected with buffer (WT) or with two different megalin/lrp2 splice-variant MOs (megMO1 or megMO2). Embryo extracts were generated at 48 hpf and analyzed by (A) RT-PCR or (B) western blotting of membrane fractions. megMO1 and megMO2 resulted in aberrant splicing of the megalin/lrp2 transcript as evidenced by truncated PCR products of the (A) cDNA encoding the transmembrane and intracellular portions of the receptor and (B) the absence of megalin/LRP2 immunoreactivity compared to controls. (C) Morphology of wild-type, megalin/lrp2 and dab2 morphants at 72 hpf. No gross abnormalities of the different morphants were apparent.
Evidence that megalin/LRP2-mediated endocytosis is ligand-dependent in the zebrafish embryonic pronephric duct

Various endocytic processes are regulated by kinase signaling events (Conner and Schmid, 2003; Pelkmans et al., 2005). A functional screen based on high-throughput RNA interference of human kinases has implicated PRKCz in endocytic control, which led us to explore the relevance of a zebrafish homologous kinase which is expressed in the kidney, Has/PRKCi, and its apical scaffolding partner Nok/Mpp5 for renal endocytic processes (Pelkmans et al., 2005). Owing to severe cardiac malformations, hasm567 and noks305 mutants lack circulation, which precludes cardinal vein injections of tracer molecules for reuptake assays (Yelon et al., 1999; Rohr et al., 2006). We circumvented this problem by using transgenic lines of zebrafish that express either wild-type Has/PRKCi or Nok/Mpp5 under the control of the cardiac myosin light chain 2 (cmlc2) promoter region (Huang et al., 2003) within all renal endocytic processes. Expression of megalin/LRP2 is unaffected in dab2 morphants (H). (N) Wild-type, megalin/lrp2 morphant, dab2 morphant and dab2 rescued embryos at 72 hpf were injected with the indicated fluorescent tracers and the number of embryos exhibiting tubular accumulation of tracers evaluated by fluorescence microscopy. Data of all animals injected are given in percent. 70kDa-FD: wild type, 45/55 embryos (81.8%); meg MO1, 2/24 embryos (8.3%); meg MO2, 3/29 embryos (10.3%); dab2 rescue, 26/48 embryos (54.2%). 10kDa-RD: wild type, 46/52 embryos (88.5%); meg MO1, 4/27 embryos (14.8%); meg MO2, 5/29 embryos (17.2%); dab2 MO, 5/39 embryos (12.8%). Cy2-RAP: wild type 42/46 embryos (91.3%); meg MO1, 3/25 embryos (12.0%); meg MO2, 3/22 embryos (13.6%); dab2 MO, 3/31 embryos (9.7%). Bar, 100 μm.

Fig. 7. Tubular-clearance defects in megalin/lrp2 morphants and quantification thereof. (A,E,I) Wild-type embryos, (B,C,F,G,K,L) megalin/lrp2 morphants, (D,H,M) dab2 morphants at 72 hpf were injected with 70kDa-FD (green). Renal clearance of tracer into the pronephric duct tubular cells was evaluated after fixation and immunostaining against megalin/LRP2 (red) of whole mounts by confocal fluorescence microscopy. Renal clearance of tracer occurs in (A,I) wild-type but not in (B,C,K,L) megalin/LRP2- and (D,M) Dab2-deficient embryos. Expression of megalin/LRP2 is unaffected in dab2 morphants (H). (N) Wild-type, megalin/lrp2 morphant, dab2 morphant and dab2 rescued embryos at 72 hpf were injected with the indicated fluorescent tracers and the number of embryos exhibiting tubular accumulation of tracers evaluated by fluorescence microscopy. Data of all animals injected are given in percent. 70kDa-FD: wild type, 45/55 embryos (81.8%); meg MO1, 2/24 embryos (8.3%); meg MO2, 3/29 embryos (10.3%); dab2 rescue, 26/48 embryos (54.2%). 10kDa-RD: wild type, 46/52 embryos (88.5%); meg MO1, 4/27 embryos (14.8%); meg MO2, 5/29 embryos (17.2%); dab2 MO, 5/39 embryos (12.8%). Cy2-RAP: wild type 42/46 embryos (91.3%); meg MO1, 3/25 embryos (12.0%); meg MO2, 3/22 embryos (13.6%); dab2 MO, 3/31 embryos (9.7%). Bar, 100 μm.

Fig. 8. Lack of Cy2-RAP uptake in megalin/lrp2 morphants and of 70kDa-FD in dab2 morphants. Confocal fluorescence microscopy on (A) whole mounts and (B) transversal sections through the pronephic ducts at 72 hpf wild-type and megalin/lrp2 morphants injected with (A) Cy2-RAP or (B) dab2 morphants injected with 70kDa-FD. megalin/lrp2 morphants fail to clear Cy2-RAP from the pronephric duct (A). Loss of renal uptake of 70kDa-FD despite correctly localized megalin/LRP2 at the apical membrane of dab2 morphants (B). Bars, 100 μm (A) and 5 μm (B).
myocardial cells \([Tg(\text{cmlec2:prkci}) \text{ or } Tg(\text{cmlec2:nok})]\), respectively \((\text{Rohr et al., 2006})\). In these \(Tg(\text{cmlec2:prkci})\) and \(Tg(\text{cmlec2:nok})\) transgenic embryos (that have been introduced into the \(\text{hasm}^{m567}\) and \(\text{nok}^{305}\) mutant backgrounds) cardiac morphogenesis and peripheral circulation is significantly restored while producing the complete range of epithelial defects characteristic of the mutants \((\text{Rohr et al., 2006})\). Whereas, \(\text{hasm}^{m567}\) mutants injected with 70kDa-FD completely lacked uptake of tracer molecules from the pronephric duct. \(\text{nok}^{305}\) mutants showed robust presence of endocytic vesicles filled with tracer, albeit at weaker levels than their wild-type siblings \((\text{Fig. 10A,D,G})\). In addition, we used an anti-Rab4 antibody to assess the presence of early endosomes in pronephric-duct epithelial cells. Whereas wild-type and \(\text{nok}^{305}\) mutants had significant amounts of Rab4-positive endosomes, \(\text{hasm}^{m567}\) mutants lacked clearly recognizable amounts of this vesicle type \((\text{Fig. 10B,E,H})\). These findings implicated Has/PRKCi but not Nok/Mpp5 in the megalin/LRP2 retrieval pathway, which is essential to the presence of Rab4-positive endosomes within the pronephric duct.

To identify the molecular mechanism underlying the tubular uptake defect in \(\text{hasm}^{m567}\) mutants, we analyzed the integrity of the pronephric duct epithelium and the subcellular distribution of megalin/LRP2 in \(\text{hasm}^{m567}\) and \(\text{nok}^{305}\) embryos. Similar to its localization in the wild type, megalin/LRP2 localized to the apical membrane of proximal pronephric duct epithelial cells in both mutants \((\text{Fig. 10P,R,S})\). In addition, we characterized membranous actin to assess the mono-layered organization and shapes of renal cells in both mutants and found that all pronephric duct cells displayed the correct mono-layered organization and apical enrichment of actin within adherens junctional belts \((\text{data not shown})\). Therefore, apical-basal polarity of pronephric duct cells or apical localization of megalin/LRP2 appeared not to be affected in \(\text{hasm}^{m567}\) mutants.

An explanation for the loss of endocytic activity in \(\text{hasm}^{m567}\) mutants could be the absence of ligands due to defective glomerular filtration. Alternatively, Has/PRKCi might have a direct regulatory role in tubular endocytic processes. To discriminate between both possibilities, we performed a mosaic clonal analysis of \(\text{hasm}^{prkci}\) function for endocytic activity. We made use of a transgenic line of zebrafish that expresses membrane-tethered GFP \((\text{lynGFP})\) under control of the epithelial claudin B promoter \((\text{clhdb:GFP})\) to genetically mark pronephric duct epithelial cells \((\text{Petra Haas and Darren Gilmour, unpublished results})\). We injected transgenic animals with \(\text{hasMO}\) and used them as donors for transplantations into unmarked wild-type hosts. The efficiency and specificity of the \(\text{hasMO}\) used in this experiment has been demonstrated in several studies \((\text{Horne-Badovinac et al., 2001; Rohr et al., 2006})\). Subsequently, \(\text{hasm}^{prkci}\) morphant pronephric duct clones within otherwise wild-type hosts were easily identified by GFP expression. In these transplants, \(\text{hasm}^{prkci}\) morphant pronephric duct clones showed robust uptake of 10kDa-RD \((\text{Fig. 11})\). This result excludes a direct role of Has/PRKCi in tubular endocytosis. Rather, lack of renal uptake in \(\text{hasm}^{m567}\) mutants most probably indicates defective glomerular filtration and, as a consequence, absence of ligands in the duct lumen. We suggest that the failure of Rab4 early endosomes to assemble in the absence of megalin/LRP2 activity \((\text{as in } \text{hasm}^{m567}\text{ mutants})\) indicates that ligand-induced activation of the receptor is an important trigger in the formation of Rab4 early endosomes in this cell type.

**Discussion**

**Proximal-distal patterning of the zebrafish pronephric duct**

Currently, it remains important to what extent proximal-distal patterning and physiologically active regions of larval aquatic vertebrate kidneys are comparable to the more complex adult mammalian organs. Moreover, one of the least explored problems of kidney development revolves around the question how epithelial specializations result in distinct transport functions along proximal-to-distal segments of the nephron. Expression mapping of transport proteins involved in

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**Fig. 9.** Loss of megalin/LRP2 and Dab2 abolishes Rab4-positive endosomes within the pronephric duct. Confocal fluorescence microscopy on transversal sections through the pronephric ducts of 72 hpf wild-type \((\text{A,E,I})\), megalin/lrp2 morphants \((\text{B,C,F,G,K,L})\), or \(\text{dab2}\) morphants \((\text{D,H,M})\) injected with 70kDa-FD \((\text{A-D,I-M})\) and immunostained for the early endosomal marker Rab4 \((\text{E-M})\). megalin/lrp2 morphants \((\text{K,L})\) and \(\text{dab2}\) morphants \((\text{M})\) are devoid of tubular Rab4-positive endosomes and lack uptake of 70kDa-FD compared with controls \((\text{I})\). Bar, 20 \(\mu\text{m}\).
osmoregulation in the *Xenopus* larval pronephric kidney predicts the presence of several distinct transport domains involved in renal clearance along the proximal-distal axis suggesting a segmented complexity that is similar to that of mammalian nephrons (Eid and Brandli, 2001; Zhou and Vize, 2004).

The zebrafish larval pronephric duct provides a simple linear system for the study of proximal-distal nephron patterning. Our results demonstrate that the expression domains of *megalin/lrp2*, *dab2* and *cubilin* define a segment of the zebrafish pronephric duct that is involved in renal clearance processes as indicated by tracer uptake studies. These data provide the first functional evidence for a segmental subdivision of the zebrafish pronephric duct epithelium. The *megalin/lrp2* expression domain comprises a sharply defined segment within the proximal half of the duct epithelium and a short distal segment of the pronephric tube. This segment probably corresponds to the PCT, a nephron segment that is central to the tubular resorption of solutes in the mammalian kidney. Mapping of the *megalin/lrp2* and *cubilin* expression domains in the zebrafish pronephros largely parallels the distinct expression of the receptors in the PCT of the developing mouse kidney (our unpublished observations), a pattern that is prototypic for most mammalian species. The situation is somewhat different in the rat embryo, where expression of the receptors can also be detected in podocytes in the glomerulus (Assémat et al., 2005).

As well as in the kidney, expression of mammalian *megalin/lrp2* and *cubilin* shows significant overlap in extra-renal tissues, including the retina and the CNS (Assémat et al., 2005). We were unable to detect significant expression of *cubilin* in zebrafish extra-renal tissues but because *cubilin* is generally expressed at lower levels than *megalin/lrp2*, not all expression domains may have been detected. However, the significant overlap of *megalin/lrp2* and *dab2* expression in extra-renal tissues at 48 hpf suggests that Dab2 has a crucial function in megalin/LRP2-mediated endocytosis and also in extra-renal cell types.

**Fig. 10.** Lack of renal clearance and Rab4-positive early endosomes in *has*<sup>ε567</sup> mutants. Transverse sections of 72 hpf wild-type embryos (A-C,K-L), *nok*<sup>ε305</sup> mutants (D-F,N-P), and *has*<sup>ε567</sup> mutants (G-I,Q-S) injected with 70kDa-FD (green) and immunostained for the early endosomal marker Rab4 (B,C,E,F,H,I) or for megalin/LRP2 (L,M,O,P,R,S). Whereas, *nok*<sup>ε305</sup> mutants show a robust presence of Rab4-positive endocytic vesicles filled with the tracer (D-F), *has*<sup>ε567</sup> mutants completely lack uptake of the tracers and Rab4-positive vesicles (G-I). The apical localization of the megalin/LRP2 receptor is not affected in *nok*<sup>ε305</sup> and *has*<sup>ε567</sup> mutants (O,P,R,S). Bars, 5 μm.

**Fig. 11.** Mosaic clonal analysis of *has/prkci* function in tubular endocytic processes. Confocal fluorescence microscopy on whole mounts of wild-type host embryos (non-GFP) that contain pronephric duct clones of *has* morphant tubular cells [genetically marked with *Tg(cldnB:GFP)*] that were injected with 10kDa-RD. (C) Endocytic punctate vesicles filled with the tracer are present within *has/prkci* morphant cell clones (arrowhead) and wild-type host neighboring cells (arrow). Bar, 40 μm.
It is not known how the expression of megalin/LRP2, cubilin and dab2 is controlled but proximal-distal patterning of the pronephric duct is most probably involved. The field of the pronephric kidney has attributes of a pre-patterned tissue based on partially overlapping expression patterns of wt1, pax2.1 and the basic helix-loop-helix factor simple minded 1 (sim1) that are consistent with lineage relationships of segments of pronephric progenitor cells (Serluca and Fishman, 2001). We have shown that the expression domains of megalin/LRP2 and cubilin are overlapping with different combinatorial codes of wt1 and pax2.1 expression (Fig. 2). However, the expression patterns of wt1 and pax2.1 are independent of megalin/LRP2 and dab2 function. Conversely, it remains to be seen whether the combinatorial codes of these transcription factors affect the expression of megalin/LRP2-cubilin-dab2 and thereby the functional specialization of the pronephric tubule.

Conservation of megalin/LRP2-dependent clearance mechanisms in the larval zebrafish pronephros

Since visualization of receptor-mediated endocytosis is feasible within the zebrafish pronephros, we explored the possibility to establish the fish as a simple model organism to dissect the molecular components of this endocytic pathway. As a proof of concept, we initially focused on known components of the megalin/LRP2 endocytic machinery in mammals and tested their relevance in zebrafish. In line with a central role of megalin/LRP2 in renal uptake processes, knock-down of this scavenger receptor or its adaptor Dab2 interfered with endocytic clearance of metabolites into the pronephric duct, which demonstrates functional conservation of this endocytic pathway across species. The ease with which the endocytic process can be manipulated in the zebrafish larva should allow for the systematic characterization of additional components required for megalin/LRP2 trafficking and function in vivo. To this end, a number of cytoplasmic adaptors that interact with the megalin/LRP2 intracellular tail have been identified by yeast two-hybrid screening (Oleinikov et al., 2000; Rader et al., 2000; Gotthardt et al., 2000; Petersen et al., 2003). Apart from Dab2, the significance of these adaptors for receptor function in vivo remains unclear. Where applicable, the relevance of these proteins for receptor function may now be identified using the respective morphants.

Intriguingly, knock-down of megalin/lrp2 and dab2 in the zebrafish pronephros not only impairs tubular clearance of receptor ligands (such as RAP) but also abolishes the uptake of fluid-phase markers or the formation of Rab4-positive endosomes. These findings might reflect the fact that other endocytic receptors contribute only insignificantly to renal clearance processes in the absence of megalin/LRP2 activity. Alternatively, megalin/LRP2 function might be directly required for the establishment of an endocytic apparatus in this cell type, a hypothesis strongly supported by previous observations in megalin-deficient mice. In these animals, lack of the receptor also results in complete absence of detectable endocytic structures, including endosomes and dense apical tubules (recycling membrane vesicles), as shown by morphological analysis using electron microscopy (Nykjaer et al., 1999; Christensen and Willnow, 1999).

A central role for the megalin/LRP2 receptor in the formation of endocytic structures was also demonstrated by inactivation of has/prkci. Initially, we considered the tight-junction-associated kinase Has/PRKCi and its scaffolding partner Nok/Mpp5 possible candidates to be directly involved in the regulation of endocytic uptake processes in the larval pronephros. As it turned out, inactivation of the kinase does impair tubular clearance processes and formation of early endosomes. Surprisingly however, this defect is not due to an abnormal epithelial cell polarity of the pronephros or an inability of megalin/LRP2 to perform endocytosis as indicated by the correct localization of the receptor to the apical membrane and of actin into adherens junctional belts (Fig. 10). Rather, our finding that has/prkci mutants fail to form Rab4-positive endosomes in pronephric-duct epithelial cells and fail to clear tracers, suggests that glomerular filtration is impaired in these mutants and that reduced availability of ligands for megalin/LRP2 prevents formation of early endocytic vesicles. This conclusion was confirmed by clonal analysis of morphant cells in otherwise wild-type hosts (with normal glomerular filtration) demonstrating that megalin/LRP2 is active in Has/PRKCi-deficient tubules (Fig. 11). We cannot formerly rule out the possibility that the presence of un-ligated receptor accelerates the kinetic of dissociation of Rab4 with endosomes thus reducing the amount of Rab4 that can be detected by immunofluorescence. However, complete absence of endosomal structures in megalin-deficient mouse PCT (Nykjaer et al., 1999) strongly supports the concept that the absence of receptor activity in the larval pronephros also impairs the formation of a proper endocytic machinery in this tissue.

Taken together, our findings highlight the evolutionary conservation of renal tubular clearance mechanisms from fish to mammals and the central role played by ligand-induced megalin/LRP activity in this process. The experimental model system established in this study provides a framework for detailed approaches to dissect the molecular components involved in this important endocytic receptor pathway.

Materials and Methods

Fish maintenance and stocks

Fish of the Zebrafish (AB background) were maintained and raised at 28.5°C under standard conditions (Westerfield, 1994) and staged according to Kimmel et al. (Kimmel et al., 1995). Generation of the Tg(cmlc2:nok) transgenic lines has been described elsewhere (Rohr et al., 2006). The construct for the Tg(idh1b:GFPER) transgenic line was generated by fusing the 8 kb promoter sequence upstream of the ATG of zebrafish claudinB (Kollmar et al., 2001) to a fusion construct encoding membrane-tethered GFP (lynGFP). Embryos were kept in egg water and 0.003% 1-phenyl-2-thiourea (PTU, Sigma) was used to suppress pigmentation. Whole embryos were observed using a dissecting stereomicroscope (Leica MZ12, Leica, Germany) equipped with a digital camera (Spot Insight, Visitron, USA).

Morpholino injections

Morpholino antisense oligonucleotides (MOs) were purchased from Gene Tools, LLC. Sequences were chosen to target an exon splice donor site of megalin (Zebrafish AB background) with several silent mutations within the expression site containing the ATG of zebrafish claudinB (Kollmar et al., 2001) to a fusion construct encoding membrane-tethered GFP (lynGFP). Embryos were kept in egg water and 0.003% 1-phenyl-2-thiourea (PTU, Sigma) was used to suppress pigmentation. Whole embryos were observed using a dissecting stereomicroscope (Leica MZ12, Leica, Germany) equipped with a digital camera (Spot Insight, Visitron, USA).

The effects of has/prkci knockdown were visualized using the respective MOs. MOs were synthesized by Gene Tools, LLC, which were injected at a final concentration of 500 nM. The expression of the Tg(cmlc2:nok) transgenic lines was analyzed using confocal imaging and the formation of Rab4-positive endosomes in pronephric-duct epithelial cells and failure to clear tracers was observed.
Dye filtration and reuptake experiments
Solutions (1 μg/μl) of lysozyme-fluorescein Dextramine (Mw 10,000; Molecular Probes) and lysozyme-fluorescein dextramine (Mw 70,000 and 500,000; Molecular Probes) were prepared in PBS. Reconstitute His-tagged receptor-associated protein (RAP; Mw 39,000) was custom-labeled with the Cy3®/Cy5® bisfunctional reactive dye kit (Amersham) according to the manufacturer’s instructions. The tracers were introduced into the common cardinal vein (CCV) of embryos aseptically with 0.2 mg/ml tricine (3-amino benzoic acid ethyl ester, Sigma) solution in egg water (Westferier, 1994). Uptake of tracer dyes by duct cells was evaluated at 1-1.5 hours after injection on whole mounts by using a fluorescent dissecting stereomicroscope (Leica MZ16F, Leica, Germany).

Plastic- and cryo-sections
For plastic sections, specimens were embedded inTechnovit 7100 (Heraeus Kulzer, Germany) according to the manufacturer’s instructions. Briefly, specimens were fixed in 4% PFA for 1 hour at room temperature and subsequently incubated in 70% ethanol/PBS, 96% ethanol/PBS and 100% ethanol for 2 hours each at room temperature. Samples were then pre-infiltrated with 50% ethanol/Technovit 7100 for 2 hours at room temperature. After that, samples were infiltrated with Technovit 7100 containing 1% (w/v) hardener I for 2 hours at room temperature. Finally specimens were embedded in Technovit 7100 containing 1% hardener I and 6% (v/v) hardener II. After polymerisation, samples were cut at 5 μm on a rotary microtome (Leica RM 2155, Leica, Germany).

For cryosections, the embryos were fixed for 1 hour at room temperature in 4% PFA and incubated in 30% sucrose/PBS overnight. The next day, embryos were transferred to Tissue-Tek® OCT (Sakura, USA) and cooled-down in ethanol-microtome (Leica RM 2155, Leica, Germany).

Whole-mount in situ hybridization
Digoxigenin-UTP-labeled riboprobes were transcribed from linearized plasmids using T7, T3 or Sp6 RNA polymerase and the DIG RNA labeling kit (Roche, Germany). Riboprobes for hepatic promoters were a gift from N. Hastie (MRC, Edinburgh). Whole-mount in situ hybridization was performed as previously described (Jowett and Lettice, 1994).

Antibody and Acridine-Orange stainings
Antibody stainings were performed on cryosections following the protocol by Horne-Badovinac et al. (Horne-Badovinac et al., 2001). Confocal images were processed with LSM image browser 5 software (Zeiss, Germany). The following antibodies were used: rabbit anti-Rab4 (1:400, Abcam, UK), rabbit anti-rat megalin/LRP2 (1:400 kindly provided by Dr. Herz, UTSS Medical Center, Dallas), donkey anti-rabbit Alexa Fluo 555 (1:1,000, Invitrogen). Rhodamine-phalloidin was processed with LSM image browser 5 software (Zeiss, Germany). The following antibody stainings were performed on cryosections following the protocol by Birn, H., Fyfe, J. C., Jacobsen, C., Mounier, F., Verroust, P. J., Orskov, H., Willnow, T. E., Mootrep, S. K. and Christensen, E. I. (2000a). Antibody stainings were performed on cryosections following the protocol by Birn et al. (2000b).

Cell transplantsations
For cell transplantsations, Tg(cldlB:FEGFP) transgenic eggs were injected with hesMO (100 μM) and used as donors at blastula stages. Several hundred cells were transplanted into unmarked blastula-stage wild-type host embryos according to Birn et al. (2000a). Antibody stainings were performed on cryosections following the protocol by Birn et al. (2000b).

Receptor-associated protein is important for normal processing of megalin in kidney proximal tubules. J. Am. Soc. Nephrol. 11, 191-202.

Birn, H., Willnow, T. E., Nielsen, R., Norden, A. G., Bonsch, C., Moestrup, S. K., Nexo, E. and Christensen, E. I. (2002). Megalin is essential for renal proximal tubule reabsorption and accumulation of transalbumin-B(12). Am. J. Physiol. Renal Physiol. 282, F408-F416.

Birn, H., Fyfe, J. C., Jacobsen, C., Mounier, F., Orskov, H., Willnow, T. E., Mootrep, S. K. and Christensen, E. I. (2000a). Antibody stainings were performed on cryosections following the protocol by Birn et al. (2000b).

Birn, H., Vorum, H., Verrout, P. J., Moestrup, S. K. and Christensen, E. I. (2000b).

References
Assézian, E., Chatelet, E., Chandellier, J., Commo, F., Cases, O., Verrout, P. and Kozyraki, R. (2005). Overlapping expression patterns of the multigland endocytic receptors cubulin and megalin in the CNS, sensory organs and developing epithila of the rodent embryo. Gene Expr. Patterns 6, 69-78.

Barbato, D. R., Zheng, G., Niles, J. L., Jowett, T. and Lettice, L. (1994). Detection of two forms of GFP330. Their role in Heymann nephritis. Am. J. Pathol. 143, 598-611.

Birn, H., Verrout, P. J., Moestrup, S. K. and Christensen, E. I. (2000a). Antibody stainings were performed on cryosections following the protocol by Birn et al. (2000b).
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Ohno, S. (2001). Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr. Opin. Cell Biol.* **13**, 641-648.

Oleinikov, A. V., Zhao, J. and Makker, S. P. (2000). Cytosolic adaptor protein Dab2 is an intracellular ligand of endocytic receptor gp600/megalin. *Biochem. J.* **347**, 613-621.

Orlando, R. A. and Farquhar, M. G. (1993). Identification of a cell line that expresses a cell surface and a soluble form of the gp330/receptor-associated protein (RAP) Heymann nephritis antigenic complex. *Proc. Natl. Acad. Sci. USA* **90**, 4082-4086.

Orlando, R. A., Rader, K., Authier, F., Yamazaki, H., Posner, B. I., Bergeron, J. J. and Farquhar, M. G. (1998). Megalin is an endocytic receptor for insulin. *J. Am. Soc. Nephrol.* **9**, 1759-1766.

Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E. and Zerial, M. (2005). Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature* **436**, 78-86.

Petersen, H. H., Hilpert, J., Miliot, D., Zandler, V., Jacobsen, C., Roebroek, A. J. and Willnow, T. E. (2003). Functional interaction of megalin with the megalin binding protein (MegBP), a novel tetratrico peptide repeat-containing adaptor molecule. *J. Cell Sci.* **116**, 453-461.

Peterson, R. T., Mahdy, J. D., Chen, J. N. and Fishman, M. C. (2001). Convergence of distinct pathways to heart patterning revealed by the small molecule concentramide and the mutation heart-and-soul. *Curr. Biol.* **11**, 1481-1491.

Rader, K., Orlando, R. A., Lou, X. and Farquhar, M. G. (2000). Characterization of ANKRA, a novel ankyrin repeat protein that interacts with the cytoplasmic domain of megalin. *J. Am. Soc. Nephrol.* **11**, 2167-2178.

Roh, M. H., Makarova, O., Liu, C. J., Shin, K., Lee, S., Laurinec, S., Goyal, M., Wiggins, R. and Margolis, B. (2002). The MAGUK protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. *J. Cell Biol.* **157**, 161-172.

Rohr, S., Bit-Avragim, N. and Abdelilah-Seyfried, S. (2006). Heart and soul/PRKCi and Nagie oko/Mpp5 regulate myocardial coherence and remodeling during cardiac morphogenesis. *Development* **133**, 107-115.

Serluca, F. C. and Fishman, M. C. (2001). Pre-pattern in the pronephric kidney field of zebrafish. *Development* **128**, 2233-2241.

Wang, Q., Hurd, T. W. and Margolis, B. (2004). Tight junction protein Par6 interacts with an evolutionarily conserved region in the amino terminus of PALS1/stardust. *J. Biol. Chem.* **279**, 30715-30721.

Wei, X. and Malicki, J. (2002). *nagie oko*, encoding a MAGUK-family protein, is essential for cellular patterning of the retina. *Nat. Genet.* **31**, 150-157.

Westerfield, M. (1994). *The Zebrafish Book* (ed. M. Westerfield). Eugene, OR: University of Oregon Press.

Willnow, T. E., Rohlmann, A., Horton, J., Otani, H., Braun, J. R., Hammer, R. E. and Herz, J. (1996). RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors. *EMBO J.* **15**, 2632-2639.

Yelon, D., Horne, S. A. and Stainier, D. Y. (1999). Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev. Biol.* **214**, 23-37.

Zhou, X. and Vize, P. D. (2004). Proximo-distal specialization of epithelial transport processes within the Xenopus pronephric kidney tubules. *Dev. Biol.* **271**, 322-338.