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MOLECULAR AND GENOMIC PHYSIOLOGY

Gene expression analysis defines the proximal tubule as the compartment for endocytic receptor-mediated uptake in the *Xenopus* pronephric kidney

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Abstract Endocytic receptors in the proximal tubule of the mammalian kidney are responsible for the reuptake of numerous ligands, including lipoproteins, sterols, vitamin-binding proteins, and hormones, and they can mediate drug-induced nephrotoxicity. In this paper, we report the first evidence indicating that the pronephric kidneys of *Xenopus* tadpoles are capable of endocytic transport. We establish that the *Xenopus* genome harbors genes for the known three endocytic receptors megalin/LRP2, cubilin, and amnionless. The *Xenopus* endocytic receptor genes share extensive synteny with their mammalian counterparts. In situ hybridizations demonstrated that endocytic receptor expression is highly tissue specific, primarily in the pronephric kidney, and did not occur prior to neurulation. Expression was strictly confined to proximal tubules of the pronephric kidney, which closely resembles the situation reported in mammalian kidneys. By immunohistochemistry, we demonstrated that *Xenopus* pronephric tubule epithelia express high amounts of the endocytic receptors megalin/lrp2 and cubilin in the apical plasma membrane. Furthermore, functional aspects of the endocytic receptors were revealed by the vesicular localization of retinol-binding protein in the proximal tubules, probably representing endocytosed protein. In summary, we provide here the first comprehensive report of endocytic receptor expression, including amnionless, in a nonmammalian species. Remarkably, renal endocytic receptor expression and function in the *Xenopus* pronephric kidney closely mirrors the situation in the mammalian kidney. The *Xenopus* pronephric kidney therefore represents a novel, simple model for physiological studies on the molecular mechanisms underlying renal tubular endocytosis.

Keywords *Xenopus* · Endocytosis · Proximal tubule · Renal function · Gene expression · Kidney · Morphology

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Introduction

The development of the vertebrate kidney involves the progressive formation of three distinct kidneys: the pronephros, the mesonephros, and the metanephros [1]. The pronephros or pronephric kidney, while vestigial in higher vertebrates, is the functional embryonic kidney of amphibians. It consists of a single nephron and therefore represents the simplest vertebrate excretory organ [2, 3]. Due to its simple organization and the amenability of *Xenopus* embryos to experimental manipulation, the *Xenopus* pronephric kidney has emerged as an attractive model for studying human kidney development and function [4, 5]. Using gain- and loss-of-function approaches, several genes involved in pronephric kidney development and differentiation have been identified and characterized including key players such as wnt4, bicaudal-c, fgf8, notch, and irx3 [6–

10]. Despite the long-known fact that functional pronephric kidneys are essential for survival of amphibian larvae [11], the range of physiological functions performed by the pronephros and their analogies to metanephric renal physiology still remain poorly characterized. Renal endocytosis, for example, has to our knowledge not been studied in *Xenopus* to date.

Megalin/LRP2 and cubilin are large endocytic receptors, 600 and 460 kDa, respectively, that are highly expressed in the apical endocytic apparatus and in apical microvilli in the mammalian proximal tubule [12]. Both receptors have also been localized to the proximal part of the pronephros in zebrafish where also fluid phase and receptor-mediated endocytosis were shown to take place [13]. The two receptors appear to be responsible for proximal tubular uptake of the majority of proteins filtered in mammalian glomeruli [12] including the carrier proteins retinol-binding protein (RBP) [14], vitamin D-binding protein [15, 16], transcobalamin [17], transferrin [18], and albumin [19–21], which illustrate the importance of renal endocytic receptors for minimizing urinary losses of vitamins and other solutes. It is interesting to note that many ligands bind to both receptors [12]. Megalin/LRP2 appears also to be involved in aminoglycoside nephrotoxicity by its ability to bind and mediate uptake of different aminoglycosides including gentamicin [22, 23]. Cubilin is a peripheral membrane protein, and several data suggest that the internalization of cubilin and its ligands at least in part is carried out by megalin/LRP2 [18, 24, 25]. Finally, amnionless (AMN) is a 45–50-kDa transmembrane protein apparently involved in cellular processing of cubilin and endocytosis of cubilin and its ligands [26–29]. The importance of AMN for endocytosis of cubilin and its ligands in megalin/LRP2-expressing cells is, however, unknown.

Megalin/LRP2 knockout mice [30] and conditional renal megalin/LRP2 knockout mice [31, 32] have been invaluable tools in revealing the megalin/LRP2 function in the kidney. Conditional knockout mice present with low-molecular-weight proteinuria, while the conventional knockouts display in addition developmental abnormalities including holoprosencephalic syndrome [30]. Recently, mutations in the megalin/LRP2 gene have been identified in families with Donnai–Barrow syndrome and facio-oculo-acoustico-renal syndrome [33].

A strain of dogs carrying an amnionless (amn) gene mutation have been instrumental in unraveling the function of cubilin [28, 34]. Cubilin is also responsible for the intestinal uptake of intrinsic factor-vitamin B12 complexes. The AMN deficiency results in a lack of apical expression of cubilin in the proximal tubule and small intestine, and as a consequence, the affected dogs develop megaloblastic anemia 1 and low-molecular-weight proteinuria [16, 35]. Human counterparts are represented by the Imerslund–Gräsbeck syndrome, where Finnish families carry mutations in the cubilin gene [36], and families from Norway and Turkey have mutations in the AMN gene [37].

The *Xenopus* pronephros is a bilateral excretory organ that consists of a single nephron composed of three basic components: (1) the glomus or glomerulus, which is the site of blood filtration, (2) the tubules, where filtrate resorption occurs, and (3) the duct, which carries the urine to the cloaca [2, 38]. Recent studies have demonstrated that the organization of the pronephric nephron is more complex than previously anticipated. Distinct domains and subdomains within the tubule and duct compartments have been defined based on the localized expression of selected membrane transporters and ion channels [39, 40]. On the basis of a large-scale gene expression pattern screen, a comprehensive model of the segmental organization of *Xenopus* pronephric kidney was recently reported that suggests remarkable functional correlations to the segments of the mammalian kidney [7]. For example, the proximal tubule is divided into three segments (PT1, PT2, and PT3), which largely correspond to the S1, S2, and S3 segments of the mammalian proximal tubule. Taken together, the characteristic hallmarks of vertebrate nephron organization—the presence of distinct segmented tubular compartments—can be delineated already at the level of the *Xenopus* pronephric nephron.

To date, it is not known whether endocytic receptors are expressed in *Xenopus* pronephric kidney and whether this simple excretory organ is capable of renal tubular endocytosis. In this paper, we examined the *Xenopus tropicalis* genome and screened expressed sequence tag (EST) databases for *Xenopus laevis* and *X. tropicalis* complementary deoxyribonucleic acids (cDNAs) encoding AMN, cubilin, and megalin/LRP2. We show that all three genes are coexpressed in the pronephric kidney, where their expression domains are confined to the proximal tubule. In immunocytochemical studies, we localized cubilin and megalin/LRP2 expression to the apical plasma membrane of proximal tubules, where they are likely to be engaged in active endocytic protein transport as evidenced by the presence of RBP localizing to intracellular vesicles. The highly conserved renal expression domains of *Xenopus* and mammalian endocytic receptor genes suggest that the *Xenopus* embryo could serve as an alternative, simple animal model to study the physiology of endocytic receptor function.

Materials and methods

Nomenclature for *Xenopus* genes

The standard gene nomenclature suggested by Xenbase (http://www.xenbase.org/gene/static/geneNomenclature.jsp) and adopted by the National Center for Biotechnology

Information (NCBI) for *X. laevis* and *X. tropicalis* genes is utilized rather than the original gene names to maximize compatibility with data available from other model systems. *Xenopus* gene names are written in lower case. Where possible, *Xenopus* gene names are the same as the human orthologs. When a gene is duplicated in *Xenopus* relative to mammals the duplicated genes are tagged with ".1" and ".2."

Genome analysis

Synteny maps of endocytic receptor genes found in the human, mouse, and *X. tropicalis* genomes were retrieved from the Ensembl genome browser (http://www.ensembl.org; release 45) by virtue of gene name search and basic local alignment search tool (BLAST) sequence similarities.

Identification and sequencing of *Xenopus* cDNAs encoding endocytic receptors

The predicted *X. tropicalis* cubilin sequence (protein ID 178868) was retrieved from the *X. tropicalis* genome assembly (version 4.1) website (http://genome.jgi-psf.org/Xentr4) of the Joint Genome Institute (JGI). None of transcripts predicted by Ensembl encoded the complete open reading frame (ORF) of cubilin. They lacked the sequences encoding the signal peptide and the CUB domains 25, 26, and 27. Predicted nucleotide sequences of *X. tropicalis* gene transcripts encoding AMN and megalin/lrp2 were retrieved from the Ensembl *X. tropicalis* genome browser (http://www.ensembl.org/Xenopus_tropicalis; release 45). In cases where multiple transcripts were predicted, the longest transcript was selected. The Ensembl transcript IDs of the *X. tropicalis* sequences are as follows: AMN (ENSXETT00000027872) and megalin/lrp2 (ENSXETT00000035400). *X. tropicalis* AMN transcript harbored, however, only a partial ORF. Similarly, the longest annotated *X. tropicalis* megalin/lrp2 transcript (ENSXETT00000035400) lacked the N terminus. The predicted megalin/lrp2 gene model (protein ID 353026), which was retrieved from JGI *X. tropicalis* genome assembly (version 4.1), contained the missing 12 N-terminal amino acids. They were identified as "MHFNDIQFTFAA."

Screening of nonredundant and EST nucleotide databases for *X. laevis* cDNAs encoding endocytic receptors was performed at the NCBI BLAST website (http://www.ncbi.nlm.nih.gov/BLAST). The *X. tropicalis* amino acid sequences for AMN, cubilin, and megalin/lrp2 were used as protein queries in TBLASTN searches, which compare a protein sequence to the six-frame translations of a nucleotide database. *X. laevis* cDNAs encoding the complete ORF of AMN (GenBank acc. no. NM_001092600) and several *X. laevis* EST cDNAs encoding partial ORFs of megalin/lrp2 (CF522099, CD302505) and cubilin (DT081913, CB2008401) were identified. Only one (GenBank CB208401; IMAGE:6881221) of the two partial *X. laevis* cubilin cDNAs identified could be verified by sequencing. In addition, *X. tropicalis* EST cDNAs encoding partial ORFs of cubilin (CX902985, CX982421) were retrieved.

The corresponding *Xenopus* cDNAs were obtained from the RZPD German Resource Center for Genome Research/imaGenes. The following cDNAs were verified by DNA sequencing: *X. tropicalis* cubilin (CX982421) and *X. laevis* AMN (NM_001092600), cubilin (CB208401), and megalin/lrp2 (CD302505). Double-stranded sequencing of the *X. tropicalis* cubilin (CX982421) and *X. laevis* megalin/lrp2 (CD302505) EST cDNAs was performed by Microsynth AG (Balgach, Switzerland). The resulting 3,137-bp nucleotide sequence of *X. tropicalis* cubilin encoded 994 residues of the C terminus of the protein encompassing part of CUB domain 19 and all the CUB domains from 20 to 27. The nucleotide sequence of *X. laevis* megalin/lrp2 had a length of 1,976 bp and encoded the C-terminal 572 residues of megalin/lrp2 starting upstream of the LDL-R class B repeat 35 in the extracellular domain and encompassing the transmembrane and cytoplasmic domains. The partial *X. laevis* megalin/lrp2 protein shared greater than 90% amino acid identity with *X. tropicalis* megalin/lrp2. In contrast to the predicted *X. tropicalis* megalin/lrp2 transcript, the partial *X. laevis* megalin/lrp2 transcript contained also the EGF repeats 16 and 17. The nucleotide sequences were deposited with GenBank: *X. laevis* megalin/lrp2 (GenBank acc. no. EU124653) and *X. tropicalis* cubilin (EU127292).

Sequence and phylogenetic analysis

The references sequences of human, mouse, and chicken endocytic receptors were retrieved from GenBank. The deduced human AMN, cubilin, and megalin/LRP2 proteins were used as queries in TBLASTN database searches to identify the corresponding *Drosophila* homologs. Analysis of nucleotide and protein sequences was performed using the DNAStar Lasergene software package (version 6.0). Signal peptides and transmembrane domains were predicted using web-based software at the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and the DAS-TMfilter server (http://mendel.imp.ac.at/sat/DAS/DAS.html), respectively. Amino acid sequence alignments were generated with MegAlign (DNAStar) using the Clustal W algorithm and the identity residue weight table. The alignments served as a basis to compute phylogenetic trees with the neighbor-joining algorithm [41].

The GenBank accession numbers of the sequences used for phylogenetic analysis are as follows: chicken AMN, XM_421379; chicken cubilin, XM_001235155; chicken megalin/LRP2, XM_422014; *Drosophila* CG11592, NM_134671; *Drosophila* CG32702, NM_167193; *Drosophila* CG12139,

NM_132335; human AMN, NM_030943; human cubilin, NM_001081; human Megalin/LRP2, NM_004525; mouse Amn, NM_033603; mouse cubilin, NM_001081084; mouse megalin/Lrp2, NM_001081088; *X. laevis* amn, NM_001092600; *X. laevis* megalin/lrp2, EU124653; and *X. tropicalis* cubilin, EU127292. The predicted amino acid sequences for *X. tropicalis* amn (Ensembl peptide ID: ENSXETP00000027872) and megalin/lrp2 (ENSXETP00000035400) were retrieved from the Ensembl genome browser. The *X. tropicalis* megalin/lrp2 sequence was amended to contain the complete N terminus as described above. The *X. tropicalis cubilin* sequence (protein ID 178868) was retrieved from the *X. tropicalis* genome assembly version 4.1.

Embryo manipulations, in situ hybridizations, and mapping of gene expression

In vitro fertilization, culture, and staging of *X. laevis* embryos were performed as previously described [42, 43]. In situ probe synthesis, whole-mount in situ hybridization, and bleaching of *Xenopus* embryos were carried out according to [8, 43, 44]. The *X. laevis* cubilin cDNA (CB208401) was amplified by polymerase chain reaction (PCR) using the Expand High Fidelity PCR System (Roche Diagnostics), subcloned into the pGEM-TEasy (Promega) vector, and confirmed by DNA sequencing. Digoxigenin-labeled probes were generated from linearized plasmids encoding *X. tropicalis* cubilin (GenBank acc. no. EU127292), and *X. laevis* amn (NM_001092600), cubilin (pGEM-cubn), and megalin/lrp2 (EU124653). Sense controls were tested negative by in situ hybridization. Digital photographs of stained embryos were taken with an AxioCam Color camera mounted on a Zeiss SteREO Lumar.V12 stereoscopic microscope. Pronephric gene expression patterns of endocytic receptors were mapped onto the contour model of the stage 35/36 pronephric nephron using unambiguous morphological landmarks as described previously [7]. In brief, these included the nephrostomes, the characteristically broad proximal tubule domain known as PT3, and the looped part of the pronephric nephron, which consists of IT1, IT2, and DT1.

Electron microscopy and immunohistochemistry

For electron microscopy (EM) and immunohistochemistry, *Xenopus* embryos (stage 35/36 and 40) were fixed in 3.5% paraformaldehyde (PFA) for 2 h and then transferred to 1% PFA. For cryosectioning, the embryos were subsequently frozen. For EM or paraffin immunohistochemistry, PFA-fixed embryos were either embedded in low-temperature lowicryl [45] or paraffin. For EM morphological studies, PFA-fixed embryos were postfixed in 1% glutaraldehyde, 1% OsO_4, and embedded in epon. Semithin (0.8 μm) or ultrathin (70–90 nm) cryosections were obtained at −100°C with an FCS Reichert Ultracut S cryo-ultramicrotome (Leica). Ultrathin, 60-nm lowicryl sections were obtained with an FCS Reichert Ultracut S ultramicrotome. Paraffin sections were cut at 2 μm. For light microscopy immunolabeling, the sections were preincubated in phosphate-buffered saline containing 5 mM glycine and 1% bovine serum albumin and then incubated with the primary antibodies (Sheep α-rat megalin/LRP2 [46], 1:5,000; rabbit α-rat cubilin [47], 1:1,000; rabbit α-RBP [Dako A/S, Glostrup, Denmark], 1:2,000) at room temperature for 1 h. The sections were subsequently incubated with the relevant peroxidase-conjugated secondary (rabbit α-sheep and goat α-rabbit) antibodies (Dako). Peroxidase was visualized with diaminobenzidine, and the sections were subsequently counter stained with Meier's stain for 2 min. The sections were examined with a Leica DMR microscope equipped with a Leica DFC320 digital camera. For EM immunolabeling, the sections were incubated with primary antibodies (α-megalin/LRP2, 1:2,000; α-cubilin, 1:200) at 4°C overnight followed by incubation at room temperature for 1 h with 10-nm gold particles coupled to the relevant immunoglobulins (BioCell, Cardiff, UK). The cryosections were embedded in methylcellulose containing 0.3% uranyl acetate, and the lowicryl sections were stained with uranyl acetate and lead. The sections were studied with a FEI CM100 electron microscope.

Computer graphics

All digital images were processed in Adobe Photoshop 8.0. Composite figures were assembled and labeled either with Adobe Illustrator CS2 or Adobe InDesign CS2. Schematic figures were drawn using Adobe Illustrator CS2.

Results

Isolation of *Xenopus* sequences encoding endocytic receptors

We used the human reference sequences of AMN, cubilin, and megalin/LRP2 as baits to survey public repositories of non-redundant nucleotide, EST, and genomic data to identify *X. laevis* and *X. tropicalis* sequences. For both *Xenopus* species, we identified distinct sequences encoding the three endocytic receptors (Supplementary Figs. S1–S3), which will be discussed in detail below.

Xenopus amnionless

Querying the *X. tropicalis* genome assembly (Ensembl, release 45), we found that the amn gene (ENSEXTG00000012751) was located on scaffold 332. For *X. laevis*,

a full-length AMN cDNA (GenBank acc. no. NM_001092600) encoding a deduced protein of 483 amino acids was identified. The two *Xenopus* amn gene products shared 89% identity at the nucleotide level and 85% at the amino acid level. A comparison with other AMN proteins revealed that the *X. laevis* protein shared 44% amino acid identity with the chicken and 39% with its mammalian counterparts. Supplementary Fig. S1 shows an alignment of the predicted amino acid sequences of human and the *Xenopus* AMN proteins. The structural features conserved among these species included the predicted signal peptide cleavage site, 12 cysteine residues in the mature extracellular domain with nine clustered in the cysteine-rich domain (CRD), a single transmembrane domain, and a cytoplasmic domain harboring two amino acid sequences conforming to a consensus motif (F/Y)XNPX(F/Y) for ligand-independent endocytosis via clathrin-coated pits [27]. The N-terminal portion, which includes the CRD, is the most conserved region of vertebrate AMN proteins.

Xenopus cubilin

Homology searches of Ensembl's *X. tropicalis* genome assembly resulted in the identification of a single cubilin gene (ENSXETG00000007453) located on scaffold 437. The predicted complete cubilin ORF (PID 178868) was retrieved from the *X. tropicalis* genome assembly (Version 4.1) of the JGI. The *X. tropicalis* cubilin ORF has a length of 10,617 bases and encodes an amino acid sequence of 3,538 residues. Comparison of the predicted full-length *X. tropicalis* cubilin protein with other vertebrate cubilin proteins revealed highest overall amino acid sequence identity with chicken cubilin (62%; 3,729 residues) and lower values with the human (57%; 3,624 residues) and mouse cubilin (53%; 3,624 residues) proteins.

As its human counterpart, *X. tropicalis* cubilin encodes a peripheral membrane protein with a signal peptide, a conserved cleavage site for the endopeptidase furin and a 97-amino acid domain containing a single cysteine residue. This is followed by a cluster of eight contiguous epidermal growth factor (EGF) repeats, each ~40 amino acids in length and harboring six conserved cysteine residues, and a large cluster of 27 CUB domains, each about 100–110 amino acids in length (Supplementary Fig. S2). Most CUB domains contain signature glycine and phenylalanine residues and four conserved cysteine residues, which probably form two disulfide bridges (C1–C2; C3–C4) [48, 49]. The following exceptions were found (Supplementary Fig. S2). CUB domains 6 and 15 contained only three instead of the four cysteine residues, suggesting that they form only one disulfide bridge. It is interesting to note that the CUB domain 6 of human cubilin is also predicted to contain only one disulfide bridge [50]. CUB domain 13 lacks, as was described for mammalian cubilin proteins, the first two cysteine residues that are suggested to form the upstream first disulfide bond. Finally, the predicted *X. tropicalis* cubilin ORF contains a large deletion in CUB domain 27.

Mutations in the human cubilin gene cause hereditary megalobastic aneamia 1 (OMIN 261100) of the Finnish type [36]. The majority of patients carry a missense mutation changing proline to leucine (P1297L) in CUB domain 8. It interesting to note that this proline residue and the flanking amino acids are highly conserved as they are also found in the CUB domain 8 of *X. tropicalis* cubilin (Supplementary Fig. S2).

Xenopus megalin/lrp2

We identified a single megalin/lrp2 gene (ENSXETG00000016214) located on scaffold 236 in the *X. tropicalis* genome assembly. The complete amino sequence of *X. tropicalis* megalin/lrp2 of 4,507 residues was assembled in silico (see "Materials and methods"). The overall amino acid identity shared between *X. tropicalis* megalin/lrp2 with its chicken counterpart was 79%, whereas the values were slightly lower with human (69%) and mouse (68%) megalin/LRP2 proteins. Amino acid sequence alignments revealed that the overall domain structure of *X. tropicalis* megalin/lrp2 and its human counterpart was well conserved (Supplementary Fig. S3). The large, 4,285-residue extracellular domain is comprised of the characteristic fourfold duplicated low-density lipoprotein receptor (LDL-R) extracellular domain consisting of three types of proteins folds: the ligand-binding LDL-R class A repeats, the EGF repeats, and the LDL-R class B repeats containing the YWTD motif [51, 52]. In line with human megalin/LRP2, *X. tropicalis* megalin/lrp2 contained 36 LDL-R class A repeats and 37 LDL-R class B repeats. It is interesting to note, however, that only 15 out of the 17 EGF repeats were present in the predicted *X. tropicalis* megalin/lrp2 protein. Sequences encoding EGF repeats 16 and 17 were absent due to a small deletion in the predicted nucleotide sequence. A single transmembrane domain and a comparatively short cytoplasmic domain containing two (F/Y)XNPX(F/Y) motifs, which are important for endocytosis, followed the large extracellular domain.

Human megalin/LRP2 gene mutations were reported to cause Donnai–Barrow and facio-oculo-acoustico-renal syndromes [33]. Affected individuals showed nonsense, splice junction, frameshift, or missense mutations that are believed to be functionally null. The only missense mutation known to date leads to the substitution of a tyrosine for a histidine residue (Y2522H) in the LDL-R class B repeat 27. This tyrosine residue is evolutionary conserved and present also in *X. tropicalis* megalin/lrp2 (Supplementary Fig. S3).

Genomic synteny and molecular phylogeny of endocytic receptor genes

We examined next the synteny maps between human, mouse, and *X. tropicalis* to confirm the identity of the *Xenopus* endocytic receptor genes. The synteny maps were derived from the Ensembl genome browser (Release 45—June 2007). Diagrammatic representations of the synteny maps including the gene orientation are presented in Figs. 1, 2, and 3. The flanking genes were remarkably conserved between the amn genes in all three genomes examined here (Fig. 1a). The conserved syntenic regions consisted of the Rcor1, Traf3, Amn, and Cdc42bpb genes. A similar situation was observed for the cubilin genes, where the core of the syntenic region was comprised of the following genes: Pter, C1ql3, Rsu1, Cubn, and Trdmt1 (Fig. 2a). While the synteny blocks flanking the megalin/LRP2 genes were completely conserved in the human and mouse genomes, the situation for megalin/lrp2 in the *X. tropicalis* genome was more complex (Fig. 3a). The *Xenopus* megalin/lrp2 gene is located on scaffold 236. The syntenic cluster of genes downstream of the mammalian megalin/LRP2 genes comprised of Bbs5, Kbtbd10, and Fastkd1 was, however, not located on scaffold 236 but on scaffold 105. Furthermore, col28a1 is located downstream of megalin/lrp2 in the *X. tropicalis* genome. The synteny block immediately upstream of megalin/LRP2 consists in mammalian genomes of G6pc2, Abcb11, and Dhrs9. Similarly, the megalin/lrp2 gene is linked upstream to the abcb11 and dhrs9 genes, which are, however, present as a tandem duplicated clusters in the *X. tropicalis* genome.

We performed also phylogenetic analysis of endocytic receptor proteins to assess the orthology assignments by protein sequence conservation (Figs. 1b, 2b, and 3b). Besides human, mouse, chicken, and *Xenopus* proteins, we also included those *Drosophila* proteins showing the highest amino acid identities to human endocytic receptors proteins. Queries of the *Drosophila* genome identified the

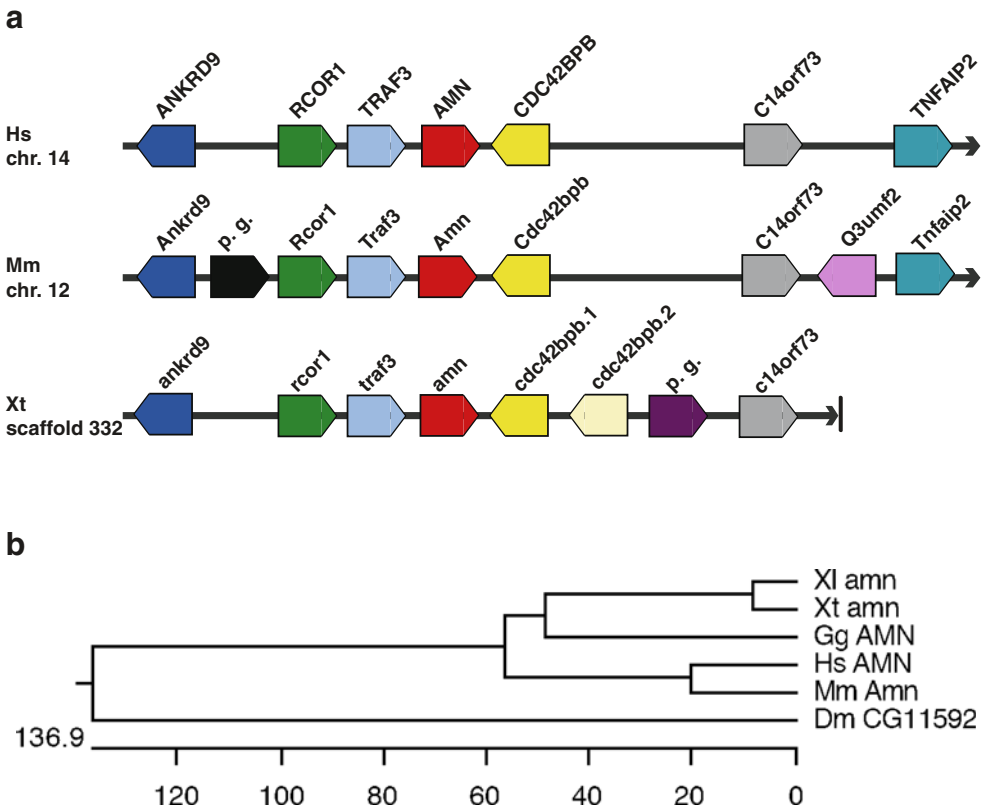

Fig. 1 Comparison of synteny and phylogenetic analysis of AMN genes. **a** Schematic representations of the synteny blocks containing amn genes in the human (*Hs*), mouse (*Mm*), and *X. tropicalis* (*Xt*) genomes. The gene organization flanking the human AMN gene was used as basis for the comparison. The chromosomal localization is indicated on the *left*. For *X. tropicalis*, the scaffold number is given. Each gene is represented as a *broad arrow* indicating the orientation of the transcription unit. The relative spacing between the genes is not shown because it does not influence the assignment of synteny. Orthologous genes are shown in the *same color*. Predicted genes (*p.g.*) that are species-specific are indicated. Note that the *X. tropicalis* genome contains to two cdc42 bpb genes (*cdc42bpb.1*, *cdc42 bpb42.2*), which probably arose by segmental duplication. **b** Phylogenic analysis of AMN proteins. The *scale bar* measures the distance between the sequences. Units indicate nucleotide substitution events. The accession numbers of the amino acid sequences are listed in "Materials and methods." Abbreviations: *Dm Drosophila melanogaster*, *Gg Gallus gallus*, *Hs Homo sapiens*, *Mm Mus musculus*, *Xl X. laevis*, *Xt X. tropicalis*

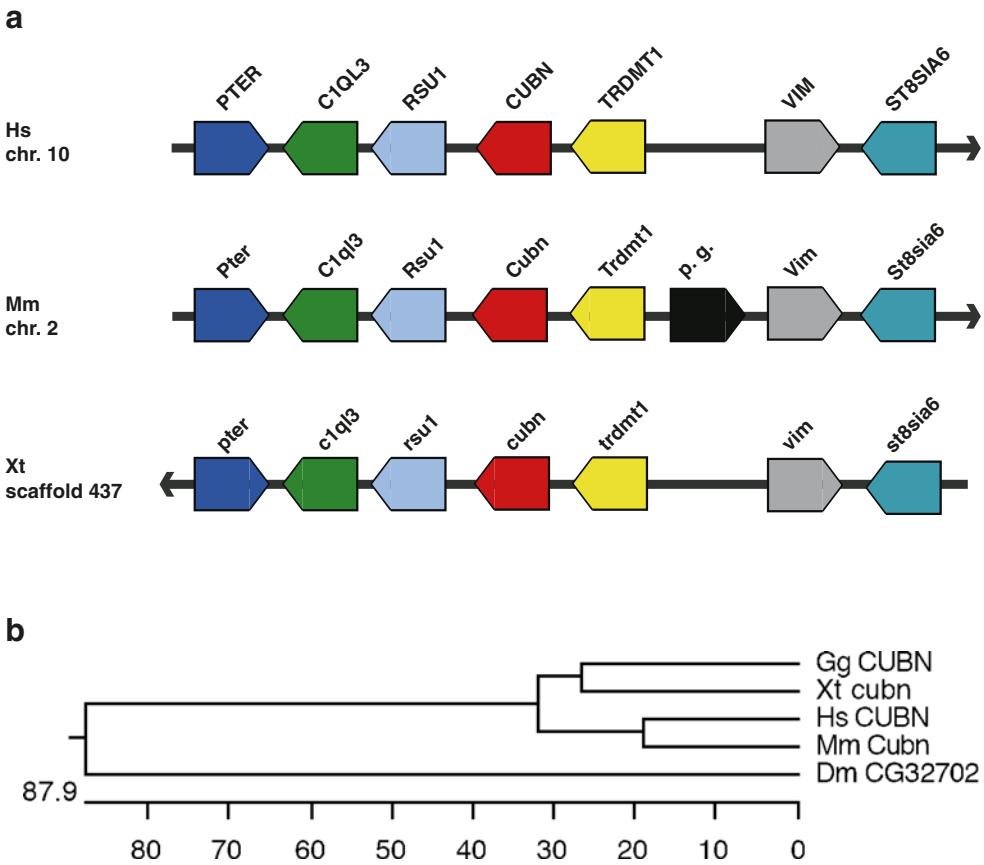

Fig. 2 Comparison of synteny and phylogenetic analysis of cubilin genes. **a** Schematic representations of the synteny blocks containing cubilin genes in the human (*Hs*), mouse (*Mm*), and *X. tropicalis* (*Xt*) genomes. The gene organization flanking the human *CUBN* gene was used as basis for the comparison. **b** Phylogenic analysis of cubilin proteins. The accession numbers of the amino acid sequences are listed in "Materials and methods." For abbreviations, see legend to Fig. 1

following genes: *Drosophila* CG11592 represents an AMN homolog [53, 54] sharing amino acid identities ranging from 16% to 19% with its vertebrate counterparts. CG32702 encodes a *Drosophila* cubilin homolog with 28% overall amino acid identity to vertebrate cubilin proteins. CG12139 was identified as a *Drosophila* megalin/LRP2 homolog sharing amino acid identities of 42–44% with vertebrate megalin/LRP2 proteins. The phylogenetic analysis revealed that the *Xenopus* endocytic receptor proteins are most closely related to their chicken orthologs. Overall, the calculated molecular phylogenies agreed well with the widely accepted evolutionary relationships between *Drosophila* and vertebrate species [55, 56]. Taken together, the assigned orthology of the *Xenopus* endocytic receptor genes was confirmed by conserved gene synteny and molecular phylogeny.

Developmental expression of *Xenopus* amnionless, cubilin, and megalin/lrp2 genes

We performed whole-mount in situ hybridizations to examine the expression of endocytic receptor genes during embryogenesis of *X. laevis*. Embryos ranging from stage 18 (20 h postfertilization) to stage 40 (66 h postfertilization) were analyzed to cover the time period between neural plate stage to the establishment of functional cardiovascular and excretory organ systems (Fig. 4; Supplementary Fig. S4). Transcription of megalin/lrp2 was already evident at stage 18 in the developing nervous system with highest expression in the brain (Supplementary Fig. S4). In contrast, no regionalized gene expression could be detected for amn and cubilin prior to embryos reaching tailbud stages. At stage 29/30, expression of amn and megalin/lrp2 became apparent in the developing pronephric anlage (Fig. 4a,c). This was followed by the onset of pronephric cubilin expression at stage 32 (Fig. 4b). Pronephric expression of all three endocytic receptor genes persisted at least until stage 40 (Fig. 4a–c,g). In addition, megalin/lrp2 expression also occurred in the developing brain, eyes, and otic vesicles (Fig. 4c). It is interesting to note that all three endocytic receptor genes were coexpressed in a proximal region of the nephron, whereas no expression could be detected in more distal parts. Using morphological landmarks (see "Materials and methods"), we mapped the proximal expression domains in the stage 35/36 pronephric nephron to cover PT1, PT2, and PT3 (Fig. 4d–f).

Ultrastructural analysis of the proximal tubules of *Xenopus laevis*

Given that the expression of endocytic receptors was restricted to proximal tubules, we performed EM to study

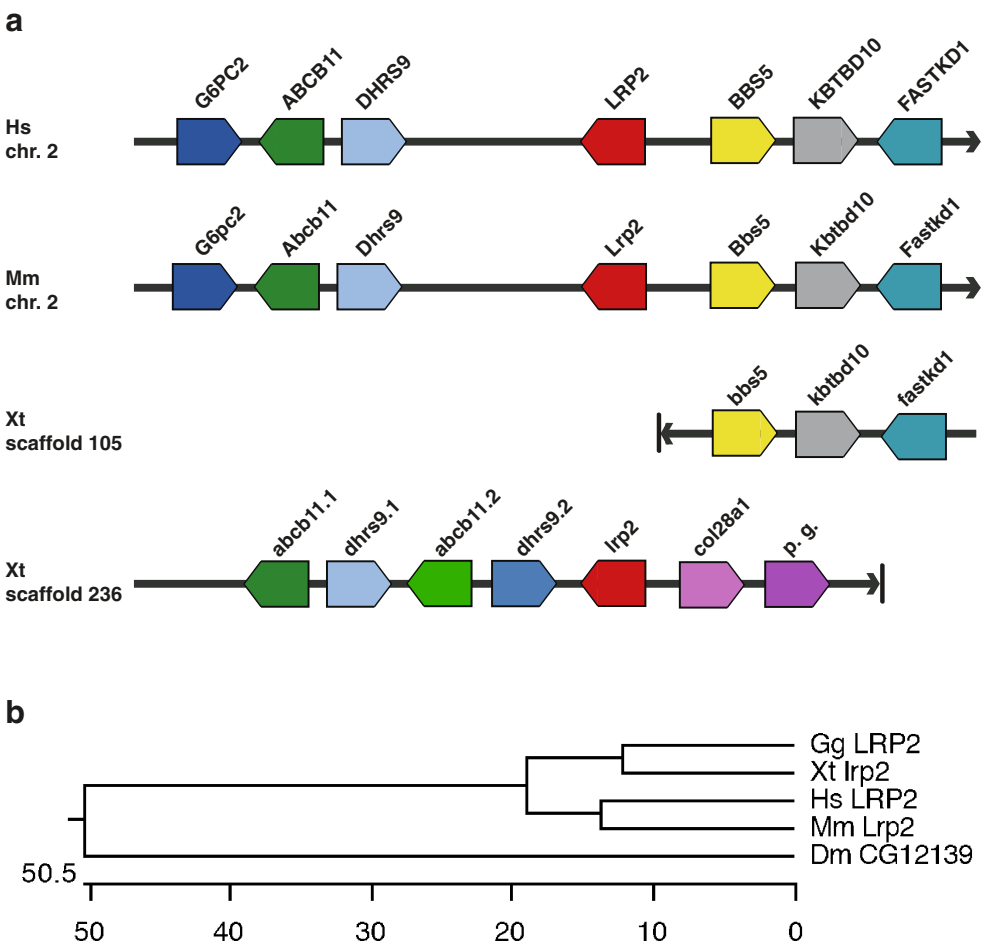

Fig. 3 Comparison of synteny and phylogenetic analysis of megalin/*LRP2* genes. **a** Schematic representations of the synteny blocks containing megalin/LRP2 genes in the human (*Hs*), mouse (*Mm*), and *X. tropicalis* (*Xt*) genomes. The gene organization flanking the human megalin/LRP2 gene was used as basis for the comparison. In *X. tropicalis*, this region is represented by two distinct contigs. Note that the tandem arrangement of the two clusters of abcb11 and dhrs9 genes in the *X. tropicalis* genome suggests that they arose by a duplication event. **b** Phylogenic analysis of megalin/LRP2 proteins. The accession numbers of the amino acid sequences are listed in "Materials and methods." For abbreviations, see legend to Fig. 1

the ultrastructure. The proximal tubules in the pronephric kidney at stage 40 presented with a very-well-developed brush border and an extensively developed apical endocytic apparatus including coated pits, endosomes, recycling dense apical tubules, and lysosomes (Fig. 5a–d). In addition, most cells had large lipid droplets in the basal cytoplasm (Fig. 5a,b). The cells were cuboidal and did not show the extensive interdigitating lateral processes containing a large number of mitochondria that is characteristic for mammalian proximal tubules [57]. Instead, these cells have small folds or microvilli that project into the lateral intercellular spaces (Fig. 5a–c). This indicates that the *Xenopus* proximal tubule in the stage 40 embryo has limited fluid absorption ability.

Subcellular expression of megalin/lrp2 and cubilin proteins

Immunocytochemistry was performed to determine the subcellular localization of endocytic receptors in proximal tubule epithelia at the light microscope and electron microscope level. The analysis included cubilin and megalin/lrp2 proteins but not AMN for which no antibodies were available. The subcellular expression of megalin/lrp2 and cubilin proteins was examined in stage 35/36 and stage 40 embryos. Despite robust expression of megalin/lrp2 and cubilin transcripts at stage 35/36 (Fig. 4a,c), only sporadic immunolabeling was observed (not shown). Both receptors were, however, clearly expressed in the proximal tubules at stage 40 (Fig. 6). Megalin/lrp2 expression was highly localized to the apical brush border membrane (Fig. 6a,b). Similarly, the expression of cubilin was found predominantly apically in cross-sectioned proximal tubules (Fig. 6c). Robust cubilin labeling was also apparent in intracellular vesicles. Immuno-gold labeling demonstrated the presence of both receptors at apical microvilli, apical coated pits, coated vesicles, and small and large endosomes (Fig. 7). In addition, dense apical tubules, known to be responsible for receptor recycling in mammalian proximal tubule cells, were intensively labeled (Fig. 7).

Presence of retinol-binding protein in proximal tubules

In mammals, tubular uptake of RBP is mediated by megalin/LRP2 [58]. We therefore probed transverse sections of stage 40 proximal tubules for the presence of RBP immunoreactivity. Labeling for RBP revealed intense

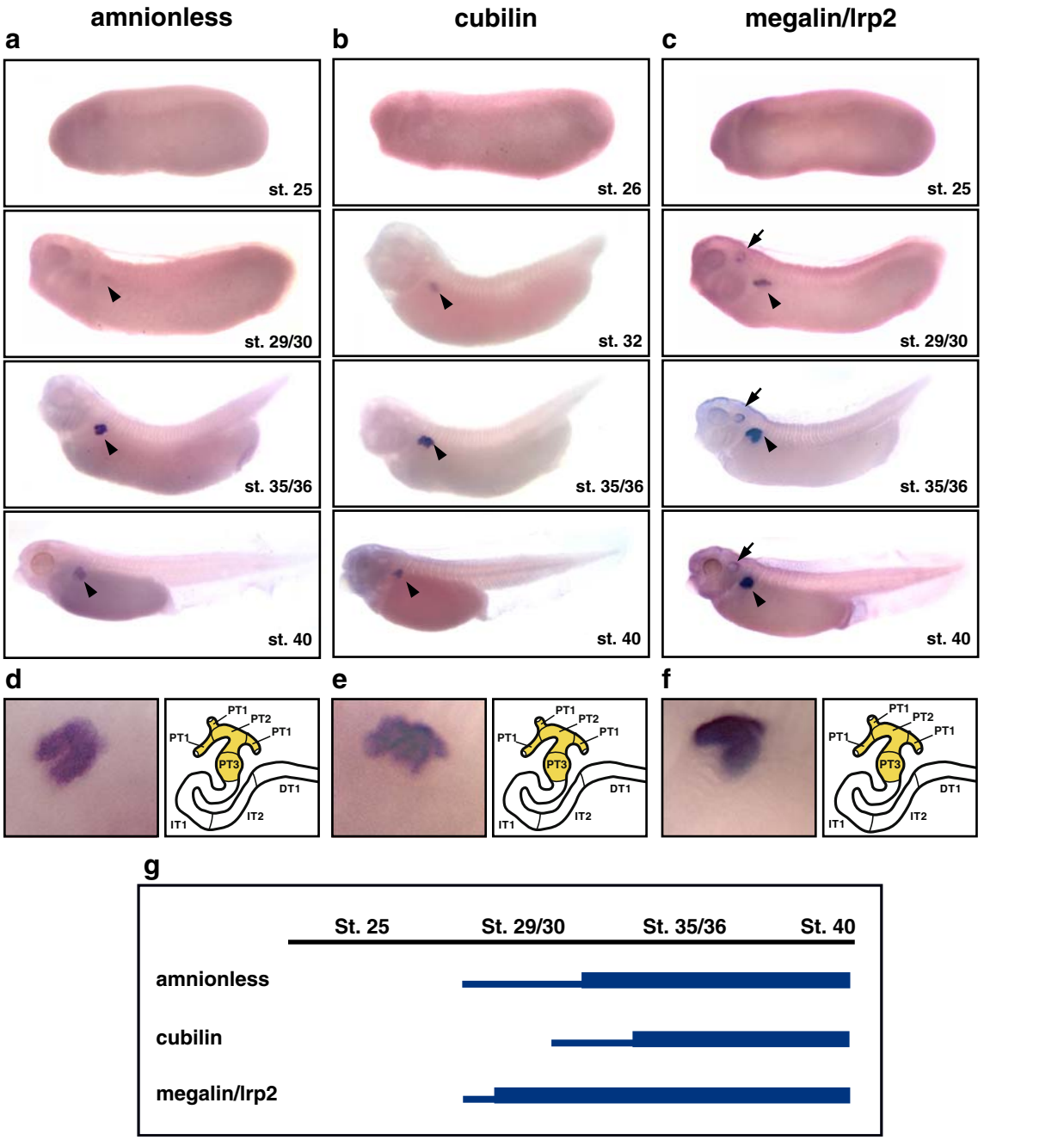

Fig. 4 Pronephric expression of endocytic receptor genes is confined to proximal tubules. **a**–**c** Expression patterns of AMN (**a**), cubilin (**b**), and megalin/lrp2 (**c**) during *X. laevis* embryogenesis as determined by whole-mount in situ hybridization. Lateral views of whole embryos are shown. *Arrowheads* indicate pronephric expression. Expression of megalin/lrp2 is also found in the developing otic vesicle (*arrows*). **d**–**f** Pronephric expression of AMN (**d**), cubilin (**e**), and megalin/lrp2 (**f**) in stage 35/36 *Xenopus* embryos. Enlargements of the pronephric region (*left panels*) and color-coded schematic representations of the segment-restricted expression domains (*right panels*) are shown. The segmental organization of the stage 35/36 pronephric nephron is drawn according to Reggiani et al. [7]. Note that expression of all three genes is confined to the proximal tubule (PT1, PT2, and PT3). Abbreviations: *PT1* proximal tubule segment 1, *PT2* proximal tubule segment 2, *PT3* proximal tubule segment 3, *IT1* intermediate tubule segment 1, *IT2* intermediate tubule segment 2, *DT1* distal tubule segment 1. **g** Summary of the temporal expression profiles of endocytic receptor genes during pronephric kidney development. The embryonic stages of *X. laevis* are indicated. High and low levels of gene expression are illustrated with *thick* and *thin lines*, respectively

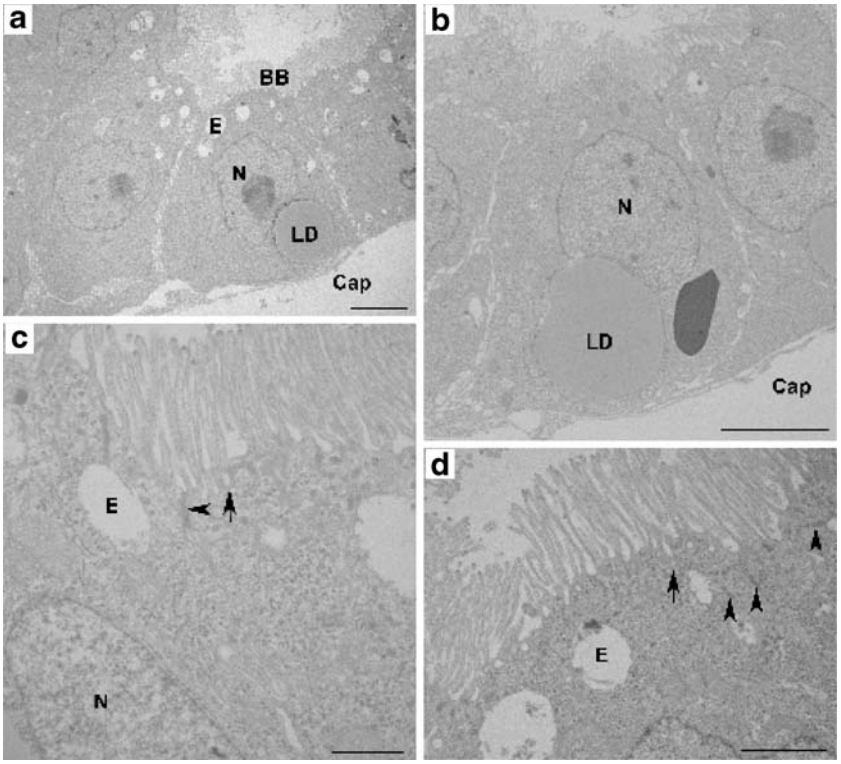

Fig. 5 Electron micrographs of proximal tubules in stage 40 *Xenopus* embryos. **a** Low magnification of proximal tubule cuboidal cells revealing an extensively developed apical brush border, an apical endocytic apparatus, and large lipid droplets in the basal cytoplasm. **b** Higher magnification illustrating the very large lipid droplets and the well-defined lateral border of the cells with small folds or microvilli projecting in the lateral intercellular space. **c** Micrograph of apical cytoplasm demonstrating the microvilli of the brush border, a junctional complex (*arrowhead*), an apical coated pit, and endosomes. **d** Apical cytoplasm containing a large number of recycling dense apical tubules (*arrowheads*). The following abbreviations are used to indicate specific structures: *BB* brush borders, *N* nuclei, *E* endosomes, *LD* lipid droplets, *Cap* capillaries. *Arrows* are used to indicate coated pits. *Scale bars*: **a**, **b**, 5 μm; **c**, 1 μm; **d**, 2 μm

punctuate staining in proximal tubule cells (Fig. 6d). This suggests that the *Xenopus* pronephric kidney is capable of endocytic uptake of endogenous proteins from the tubular lumen.

Discussion

We provide here first evidence on the basis of database searches and phylogenetic analysis that the *X. tropicalis* genome harbors distinct genes encoding the three endocytic receptors AMN, cubilin, and megalin/lrp2. Comparisons of the genome organization flanking the endocytic receptor genes demonstrated remarkable synteny between *Xenopus* and mammals. The predicted amino acid sequences suggest that the *Xenopus* endocytic receptors cubilin and megalin/lrp2 genes encode for large cell surface proteins. It is interesting to note that we found that amino acid residues mutated in human patients with deficiencies in endocytic receptor function [33, 36] were found to be invariant in the *Xenopus* proteins indicating that these residues are likely to be of functional importance also in amphibians.

To date, the expression and function of endocytic receptors during embryonic development has been best studied in rodents. In the mouse, expression of endocytic receptors is detected during the earliest stages of embryogenesis, and targeted gene disruptions demonstrate distinct roles in the developing embryo. Homozygous mutant mice deficient for either amn or cubilin die during gastrulation due to impaired primitive streak assembly and functionally defective visceral endoderm [53, 59]. AMN and cubilin are both expressed in the yolk sac visceral endoderm, where they appear to be required for endocytosis and/or transcytosis of high-density lipoproteins and possibly other factors necessary for proper growth of the embryo [29, 59]. In contrast, mutations in cubilin or AMN have no effect on embryonic development in humans or dogs [28, 36, 60], which suggests species-specific differences in visceral endoderm and yolk sac functions. The visceral endoderm also expresses megalin/Lrp2 [61], but its function does not seem to be essential for early embryogenesis. Megalin/Lrp2-deficient mice die perinatally owing to respiratory insufficiency and suffer from malformations of the forebrain and face structures consistent with features

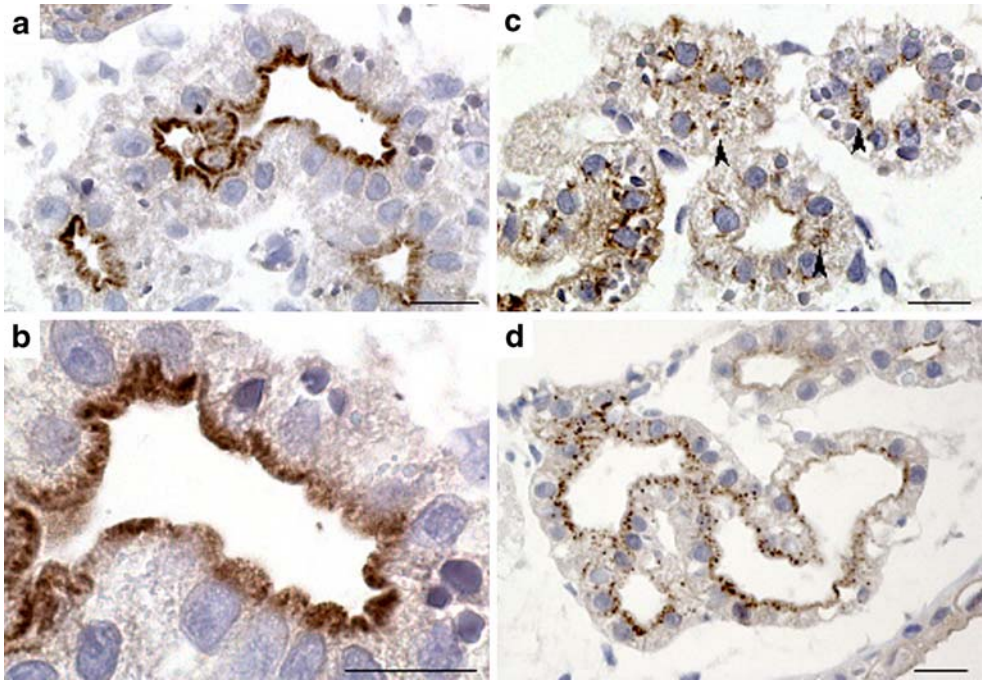

Fig. 6 Immunocytochemical localization of megalin/lrp2, cubilin, and RBP to proximal epithelia of the pronephric kidney. Transverse sections of *Xenopus* embryos cut at the level to the pronephric kidneys were treated with primary polyclonal antibodies to megalin/lrp2, cubilin, or RBP followed by treatment with peroxidase-coupled secondary antibodies. **a**, **b** Subcellular expression of megalin/lrp2. Panel **b** is an enlargement of **a** illustrating in greater detail apically restricted expression of megalin/lrp2. **c** Subcellular expression of cubilin. Note that cubilin expression is to a larger extent present in intracellular vesicles (*arrowheads*). **d** Extensive labeling of RBP in proximal tubule. *Scale bars*=20 μm

observed in holoprosencephalic syndrome [30]. Similarly, fetuses carrying homozygous mutations in megalin/Lrp2 go to term but present with multiorgan defects resulting in high mortality [33]. In the postimplantation rodent embryo, cubilin and megalin/Lrp2 share very similar expression patterns in sensory organs, the central nervous system, and various developing epithelia including those of the respiratory system and kidney [30, 62, 63]. Finally, expression of

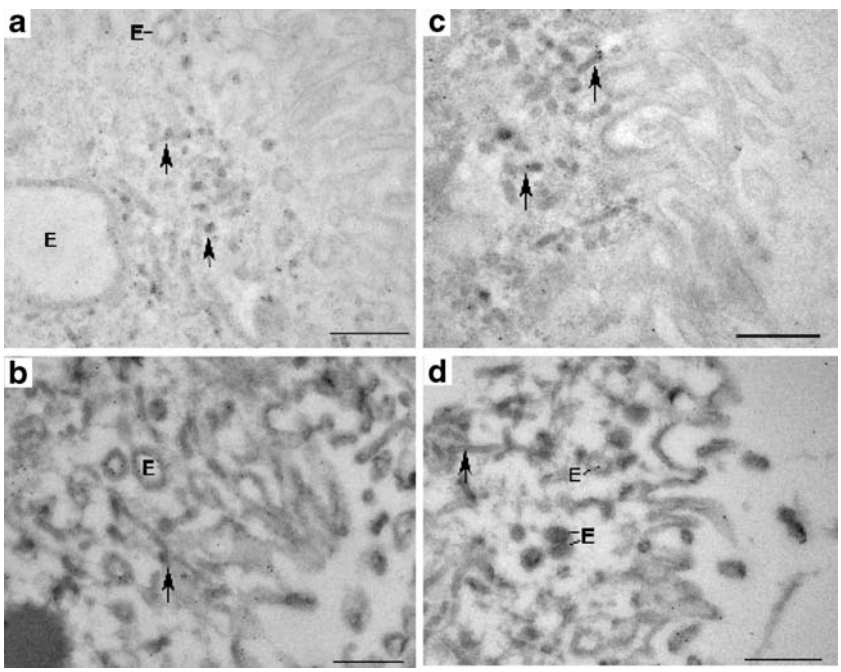

Fig. 7 Subcellular localization of megalin/lrp2 and cubilin by immunelectron microscopy. Lowicryl (**a**, **c**) sections or cryosections (**b**, **d**) of *Xenopus* embryos were cut at the level of the pronephric kidneys and treated with primary polyclonal antibodies to megalin/lrp2 (**a**, **b**) and cubilin (**c**, **d**) followed by treatment with secondary antibodies coupled to 10-nm gold particles. *Arrows* indicate dense apical tubular labeling. Endosomal (**e**) labeling is also shown. Labeling of microvilli is especially seen in **c** and **d**. *Scale bars*: **a**–**d**, 0.5 μm

AMN during fetal development in the mouse is more restricted with transcripts only detected in the kidney and small intestine [29].

In zebrafish embryos, megalin/LRP2 expression was prominently detected in the developing nervous system (forebrain, midbrain, midbrain–hindbrain border, and neural tube), orofacial regions (frontonasal and maxillary processes), and selected sensory organs (optic and otic vesicles) [64]. Furthermore, expression of cubilin and megalin/LRP2 in the developing pronephric kidney was reported recently [13]. In *Xenopus* embryos, the expression of megalin/lrp2 in the developing nervous system, brain, sensory organs, and the pronephros mirrors the situation reported for zebrafish [64] and mouse embryos [62, 63]. In contrast, AMN and cubilin were coexpressed with expression domains confined to the developing pronephric kidney. While AMN expression in zebrafish has not been reported to date, the renal expression of *Xenopus* AMN was comparable to the situation in mouse embryos [29]. Different to mouse, we could not detect any intestinal AMN expression in *Xenopus*. This apparent discrepancy is likely due to the fact that differentiation of the intestinal tract occurs only after stage 40 [65], which was the last stage analyzed in the present study. For cubilin, we also failed to observe any significant extrarenal expression in *Xenopus* embryos, which is a feature *Xenopus* shares with zebrafish [13]. These findings are in contrast with the broader range of developing tissues expressing cubilin in rodent embryos [62]. Coexpression of cubilin and AMN in *Xenopus* embryos is consistent with the role of AMN as a coreceptor of cubilin [27, 29]. Furthermore, it appears that the developing pronephros represents the primary organ system with endocytic receptor expression in the *Xenopus* embryo.

Initiation of pronephric endocytic receptor expression starting first with AMN and megalin/lrp2 at stage 29/30 and followed by cubilin at stage 32 correlated well with the maturation phase of the pronephric development leading to a functional excretory organ [2]. In the mammalian metanephric kidney, all three endocytic receptors are coexpressed in epithelia of the proximal tubules [12, 27, 29]. Mapping of the AMN, cubilin, and megalin/lrp2 expression domains in *Xenopus* was performed on the basis of our model of pronephric nephron segmentation [7], which incorporates analogies to the mammalian metanephric nephron. In the stage 35/36 pronephric kidney, we detected that the *Xenopus* endocytic receptor genes were coexpressed in all three segments of the proximal tubule. At the subcellular level, megalin/lrp2 and cubilin were localized to in the apical plasma membrane of proximal tubule epithelia, and ultrastructural studies revealed an extensively developed endocytic apparatus, expressing the endocytic receptors megalin/lrp2 and cubilin. In zebrafish, megalin/LRP2 and cubilin were coexpressed in the distal tubule and proximal duct epithelium [13]. It is interesting to note that it was recently shown that this domain of the zebrafish pronephros corresponds to the mammalian proximal tubule [66]. Uptake studies in zebrafish and the accumulation of the RBP in the *Xenopus* proximal tubules indicate that the pronephric kidneys in both species are capable of endocytic transport.

Most of our insights in endocytic receptor functions have been gained from studies in mammalian animal models and from patients carrying inherited mutations in endocytic receptor genes. Our studies in *Xenopus* demonstrate unequivocally that simple excretory organs such as the pronephric kidneys are endowed with a fully functional system of endocytic receptors that includes also AMN. Remarkably, endocytic receptor expression was compartmentalized to the proximal part of the pronephric nephron highly reminiscent to the situation in the mammalian metanephric kidney. The renal tubular clearance mechanisms have therefore remained highly conserved during vertebrate evolution underscoring their fundamental importance for excretory organ function and physiology. The framework of molecular tools established in the present study lays the foundations to dissect in detail endocytic receptor functions in *Xenopus* embryos taking advantage of the ability to perform rapidly gain- and loss-of-function approaches in this animal model. Given that endocytic receptors are generally not expressed prior to pronephric kidney development suggests that the renal functions of endocytic receptors could be studied more directly in *Xenopus* embryos than in mice, where the requirement of endocytic receptor functions in early embryogenesis has hampered the analysis of renal defects.

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