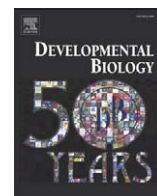


Contents lists available at [ScienceDirect](http://www.sciencedirect.com)

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiologyA reverse genetic screen in the zebrafish identifies *crb2b* as a regulator of the glomerular filtration barrierLwaki Ebarasi^a, Liquan He^b, Kjell Hultenby^c, Minoru Takemoto^d, Christer Betsholtz^b, Karl Tryggvason^a, Arindam Majumdar^{a,*}^a Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Scheeles väg 2, Plan 4 B1, SE-171 77 Stockholm, Sweden^b Laboratory of Vascular Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 17177 Stockholm, Sweden^c Department of Pathology, Karolinska University Hospital/Huddinge, Karolinska Institutet, Stockholm, Sweden^d Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan

ARTICLE INFO

Article history:

Received for publication 27 October 2008

Revised 26 March 2009

Accepted 16 April 2009

Available online 23 April 2009

Keywords:

Podocyte

Pronephros

Glomerulus

Glomerular filtration barrier

Nephrin

Zebrafish

Crumbs

Slit diaphragm

Differentiation

Cell polarity

ABSTRACT

The glomerular filtration barrier is necessary for the selective passage of low molecular weight waste products and the retention of blood plasma proteins. Damage to the filter results in proteinuria. The filtration barrier is the major pathogenic site in almost all glomerular diseases and its study is therefore of clinical significance. We have taken advantage of the zebrafish pronephros as a system for studying glomerular filtration. In order to identify new regulators of filtration barrier assembly, we have performed a reverse genetic screen in the zebrafish testing a group of genes which are enriched in their expression within the mammalian glomerulus. In this novel screen, we have coupled gene knockdown using morpholinos with a physiological glomerular dye filtration assay to test for selective glomerular permeability in living zebrafish larvae. Screening 20 genes resulted in the identification of *ralgaps1*, *rapgef2*, *rabgef1*, and *crb2b*. The *crumbs* (*crb*) genes encode a family of evolutionarily conserved proteins important for apical–basal polarity within epithelia. The *crb2b* gene is expressed in zebrafish podocytes. Electron microscopic analysis of *crb2b* morphants reveals a gross disorganization of podocyte foot process architecture and loss of slit diaphragms while overall polarity is maintained. Nephrin, a major component of the slit diaphragm, is apically mis-localized in podocytes from *crb2b* morphants suggesting that *crb2b* is required for the proper protein trafficking of Nephrin. This report is the first to show a role for *crb* function in podocyte differentiation. Furthermore, these results suggest a novel link between epithelial polarization and the maintenance of a functional filtration barrier.

© 2009 Published by Elsevier Inc.

Introduction

The kidney glomerulus filters the blood by passage of plasma across a selectively permeable and multi-layered filter, termed the glomerular filtration barrier, made of fenestrated endothelial cells, glomerular basement membrane (GBM), and epithelial podocytes, first described in detail by Farquhar et al. (1961). Proper filtration is dependent upon the combined integrity of these three layers and results in the retention of plasma proteins within the blood and passage of low molecular waste products and metabolites into the filtrate.

Chemically induced damage or genetic lesions which compromise filtration barrier integrity result in the leakage of blood plasma proteins into the urinary space termed proteinuria (Tryggvason et al., 2006). The presence of regularly spaced and interdigitated podocyte foot processes with their associated slit diaphragms is an essential

component of the filtration barrier. At the ultrastructural level, proteinuria is usually associated with the initial effacement and simplification of podocyte foot processes followed by the loss of foot process contacts with the GBM. Effacement is accompanied by profound changes in the podocyte cytoskeletal architecture and the loss of slit diaphragms. Proteinuria occurs in almost all glomerular diseases and is a common patho-mechanism in the progression to further glomerular damage.

The molecular cloning of human glomerular disease loci and targeted gene knock out in mouse have identified several important components of the filtration barrier assembly and also drawn attention to the central role of podocytes in glomerular function. Nephrin, a transmembrane protein belonging to the Ig superfamily, has been one focal point of research efforts. The Nephrin protein is a principle component of the slit diaphragm, and the human *NPHS1* gene is mutated in congenital nephrosis of the Finnish type (Beltcheva et al., 2001; Kestila et al., 1998). Nephrin, and several physically interacting proteins, participate in signaling functions to regulate the coupled processes of foot process arborization and slit diaphragm formation (Huber et al., 2003). Nephrin functions within a signaling pathway to

Abbreviations: GBM, glomerular basement membrane; dpf, days post fertilization; *crb*, *crumbs*.

* Corresponding author. Fax: +46 08 31 34 45.

E-mail address: Arindam.Majumdar@ki.se (A. Majumdar).

regulate foot process architecture, at least in part, through locally organizing the cytoskeleton (Huber et al., 2001; Jones et al., 2006; Verma et al., 2006). Studies of podocytes/endothelial interactions through the VEGF signaling axis have demonstrated the importance of cell–cell communication in the maintenance of the barrier in differentiated glomeruli (Eremina et al., 2007).

The filtration barrier is a complex and dynamic structure and its regulation involves the integration of multiple signaling pathways between endothelial, mesangial and podocyte cells. A complementary approach to studying individual genes or pathways described above has been molecular profiling of isolated glomeruli, for example using microarrays, in both healthy and disease states (Betsholtz et al., 2007).

GlomBase is a glomerular transcript bioinformatics database generated from the large scale sequencing of ESTs from normalized cDNA libraries made from isolated newborn and adult mouse glomeruli (Takemoto et al., 2006). Transcriptional profiling by microarray analysis has identified over 300 GlomBase genes or 'glomerular genes', which are expressed at over two-fold higher levels in the glomerulus in comparison to non-glomerular kidney tissue. In a complementary approach, EST mining was employed to identify glomerulus enriched genes in these libraries relative to whole kidney libraries (He et al., 2007). Importantly, many novel and known genes were also identified whose developmental or physiological roles in the glomerulus have not been explored and therefore await functional studies in animal model systems. GlomBase therefore represents a unique informational knowledgebase from which to explore new aspects of glomerular biology and disease.

Due to its several experimental advantages, the zebrafish has become an excellent vertebrate model system for studying gene function and generating human disease models for studies of disease mechanisms. Forward genetic screens, coupled with molecular cloning of the mutated loci, have been a powerful gene discovery approach and have led to the elucidation of new regulatory pathways in development and disease. A complementary approach takes advantage of morpholinos, sequence specific antisense oligonucleotide molecules, which have become extremely popular in rapidly and reliably testing the function of a known gene (Heasman, 2002; Nasevicius and Ekker, 2000).

The zebrafish embryonic kidney (pronephros) is a genetically tractable vertebrate experimental system for studying nephrogenesis (Drummond, 2004). The pronephros is made of two nephrons joined by a single glomerulus which is vascularized by capillaries made of fenestrated endothelia. Podocytes and endothelial cells are assembled into a filtration barrier with podocyte foot processes, slit diaphragms, and a GBM resembling the mammalian filtration barrier at the ultrastructural level (Drummond, 2004; Kramer-Zucker et al., 2005). Zebrafish glomerular filtration begins by 48 h post fertilization (hpf) and continues throughout adult life.

In this study, we have applied the zebrafish pronephros as an experimental system to conduct a novel, in vivo functional screen of GlomBase genes for glomerular function. We have coupled morpholino knockdown with a physiological glomerular filtration assay that can be performed in living zebrafish larvae. Using this screen, we have identified several genes which are required for the selective permeability of the glomerular filtration barrier. We have focused our attention on *crb2b*, a member of the Crumbs protein family implicated in the regulation of epithelial polarity. We demonstrate a role for *crb* genes in podocyte differentiation and suggest a novel link between epithelial polarization and podocyte foot process formation.

Materials and methods

Zebrafish husbandry

Zebrafish were grown following standard protocols (Westerfield, 1993).

Morpholino injections

Morpholinos (Gene Tools LLC, Philomath, Oregon) are listed in Table S1. Morpholino were injected at 250 μ M in 0.3 \times Danieau's buffer with phenol red as a tracer. For the rescue experiments, wildtype *crb2b* mRNA was co-injected with *crb2b*-ATG or *crb2b*-SP at approximately 100 pg/embryo. Injections were done using Harvard Apparatus borosilicate glass capillaries (GC100TF-10).

Histological analysis

Larvae were fixed in Bouin's (Polysciences) overnight at 4 °C. Samples were dehydrated through an ethanol series and processed for embedding in Polysciences JB-4. A Leica RM 2155 microtome was used to cut 6 μ m sections. Sections were stained in hematoxylin/eosin and mounted in Pertex (Mediate GmbH) for light microscopy.

Molecular cloning

Zebrafish cDNA sequences were cloned from 2–4 dpf random primed cDNA by PCR using primers listed in Table S3. PCR products were amplified using Invitrogen's Generacer Superscript III RT module and cloned using Invitrogen's TA TOPO cloning kit. RTPCR products were amplified using the forward primer 5' TGCATCCCTTCAATACAGCA 3' and the reverse primer 5'GATTCCATTGGAGGAGCGTA 3' spanning *crb2b* exon 9.

Glomerular dye filtration assay

Zebrafish larvae aged 3.5–4 dpf were anaesthetized with Tricaine prior to injections. Lysine fixable 1% 500 kDa FITC dextran/1% 10 kDa tetramethylrhodamine dextran (Molecular Probes) in 0.2 M KCl was injected under the pectoral fin into the CCV which connects directly to the cardiac inflow tract. The dyes were allowed to circulate for 8 h and the larvae were fixed in 4% PFA/1 \times PBS and processed for plastic sectioning in JB-4. The 10 kDa dye was used as an internal control for successful injection and the presence of tubules in morphants. Due to the size selectivity of the glomerular filter, the 500 kDa dextran dye is not able to pass through the filtration barrier resulting in tubules which are negative for FITC labeled endosomes. However, if the glomerular filter is damaged, the 500 kDa dextran dye flows across the filtration barrier and into the pronephric tubules where they are endocytosed into epithelial cells. Sections were cut at a thickness of 6 μ m and mounted in Vectashield (Vector Labs).

In situ hybridization

In situ hybridizations were done as described (Thisse et al., 2004). Templates were linearized prior to in vitro transcription: *nephrin* Eco RV/SP6, *podocin* Not I/SP6, *wt1* Not I/T7, *crb2a* Bam HI/T7 and *crb2b* Eco RV/SP6.

α -Nephrin antibody

A rabbit polyclonal antibody against zebrafish Nephrin was designed and produced at Innovagen (Lund, Sweden). A synthetic peptide of the carboxy terminal amino acids 163–176 [(NH₂-) CRDLDLPF-ELRGELV (-CONH₂)] was synthesized and coupled to KLH carrier via the cysteine residue (underlined). Antibodies were produced following Innovagen's standard protocol. The antiserum was affinity-purified with the synthetic peptide using the SulfoLink Kit (Pierce).

Cell transfections and Western blotting

The *nephrin* full length cDNA was cloned into pcDNA3.1. HEK-293 cells were cultured in Dulbecco's modified Eagle medium supple-

mented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (Invitrogen) at 37 °C in a CO₂ incubator. Transfection of cells was performed with the Lipofectamine 2000 Reagent (Invitrogen). Cells were solubilized in lysis buffer (0.1 M DTT, 2×SDS Loading buffer (pH6.8) and protease inhibitors (Roche Diagnostics), boiled, and sheared with a 27 gauge needle. 50 µg protein was separated on NUPAGE Novex Bis-Tris 4–12% Gel under reducing conditions and transferred to PVDF membrane (Amersham). The membrane was incubated with α-Nephrin antibody (0.9 µg/ml) and then incubated with rabbit Ig HRP (Amersham) HRP activity was detected using ECL (Amersham).

Immunofluorescence

The rabbit α-panCrb (gift from Dr. J. Malicki) and α-Nephrin polyclonal antibodies were used to stain frozen sections at a dilution of 1:250 and 1:200, respectively. Goat α-rabbit Alexa 488 (Molecular Probes) was used as a secondary at 1:2000 dilution. The α-6F monoclonal antibody (Developmental Studies Hybridoma Bank) was used for staining of Dent's fixed embryos at 1:100 dilution. α-ZO-1 mAb (Zymed) was used at 1:1000 to stain frozen sections. α-acetylated Tubulin (Sigma) was used to stain Dents fixed embryos as described in Sullivan-Brown et al. (2008).

Electron microscopy

Larvae were fixed in 2% glutaraldehyde/0.5% paraformaldehyde/0.1 M cacodylate/0.1 M sucrose/3 mM CaCl₂ and washed in 0.1 M cacodylate buffer pH 7.4 prior to staining in 2% OsO₄ in cacodylate buffer for 1 h at room temperature. Samples were dehydrated and en bloc staining was performed in 2% uranyl acetate in absolute ethanol for 1 h at room temperature. Samples were then taken through an Epon 812/acetone series and embedded at 60 °C in pure Epon 812. Thin sections of 70 nm thickness were made on a Leica EM UC6 ultratome and mounted on formvar coated copper slot grids. Post-staining was done with 2% aqueous uranyl acetate pH 3.5 and Venable and Coggesall's lead citrate. Grids were analyzed on a JEOL 1230 electron microscope.

Immuno-electron microscopy

Larvae were fixed in 3% paraformaldehyde/0.1 M phosphate buffer pH7.4, dehydrated into absolute methanol at low temperature, and embedded in Lowicryl K11 M (Chemische Werke Lowi GmbH). Ultrathin sections of 40 nm thickness were cut with a diamond knife and mounted on carbon/formvar nickel grids. The grids were blocked in 2% BSA/2% Gelatin/0.1 M phosphate buffer at pH7.4 for 2 h at room temperature followed by an overnight incubation in α-Nephrin antibodies at a 1:20 dilution at 4 °C. After rinsing in 0.1% BSA/0.1% Gelatin/0.1 M phosphate buffer pH7.4, protein A conjugated with 10 nm colloidal gold (Biocell) diluted 1:50 was added and incubated for 2 h at room temperature. The grids were then rinsed in 0.1 M phosphate buffer and post-fixed in 2% Glutaraldehyde/0.1 M cacodylate/0.1 M sucrose for 15 min. Grids were post-stained with 4% uranyl acetate (12 min) followed by Reynold's lead citrate (4 min). Samples were examined in a LEO 906 microscope at 80 kV.

Results

We designed morpholinos against selected GlomBase genes in order to test their requirements in glomerular function (Table S1). Gene targets were chosen using a combination of criteria. Genes were chosen based on their expression ranking where those genes which had a higher glomerulus to non-glomerulus expression ratio were favored. In addition, genes for which 5' untranslated sequences

surrounding the ATG start codon were available for the design of translation blocking morpholinos were prioritized. Thirdly we chose genes for which no previously reported role had been found in glomerular function.

We injected gene specific morpholinos into 1–2 cell stage zebrafish embryos and allowed the embryos to grow up to 84 hpf. By this time, fenestrated endothelia, GBM, regularly spaced podocyte foot processes, and slit diaphragms are all present indicating that the glomerular filtration barrier is relatively mature (Kramer-Zucker et al., 2005). We anticipated that loss of glomerular function would be associated with pericardial edema, based on reported knockdown of the *nephrin* and *podocin* genes in zebrafish and the analysis of zebrafish morphants and mutants (Kramer-Zucker et al., 2005; Majumdar and Drummond, 2000).

In the screen, several of the gene knockdowns resulted in pericardial edema and pronephric cyst phenotypes consistent with the inability to osmoregulate. The penetrance of these phenotypes varied from 21% to 78% depending on the targeted gene and the efficacy of the morpholino (Table S1). A standard control morpholino was injected and served as a baseline for the occurrence of spontaneous pericardial edema.

Pronephric morphants

Overall, 67% of the 20 knockdowns were associated with a pericardial edema and pronephric cyst phenotype. The pronephric phenotypes recovered may result from defects that span a developmental to functional spectrum. Morpholino knockdown of *arfgap*, *hlx1*, *nostrin*, *emp2*, and *rab3b*, resulted in severe pericardial and yolk edema by 48 hpf and an absence of blood circulation precluding dye filtration studies. Histological analysis of these morphants showed abnormal pronephric anatomy in these morphants. Since our goal was to perform a screen for genes necessary for the function of the glomerular filtration barrier, we pursued those morphants which developed pericardial edema at 3 dpf or later and had blood circulation.

Glomerular filtration screen

Proteinuria, as a read out of glomerular damage, has not been well defined in fish. In order to determine the integrity of the filtration barrier, a qualitative and molecular glomerular dye filtration assay was used. We have approximated a proteinuria assay by assessing the passage of fluorescently labeled dextran dyes through the pronephric glomerular filter using a glomerular dye filtration assay. The dye filtration assay has been used previously as a qualitative, yet molecular, test for glomerular permeability and the integrity of the filtration apparatus (Majumdar and Drummond, 2000). Knockdown of the slit diaphragm associated proteins Nephrin, Podocin, and CD2AP result in leakage of dextran dyes into the pronephric tubules paralleling the proteinuria phenotypes found in patients carrying mutations in these genes (Hentschel et al., 2007; Kramer-Zucker et al., 2005).

In the *ralgps1*, *rabgef1*, *rapgef2*, and *crb2* morphants, the 500 kDa FITC dye is found in the pronephric tubules indicating passage of this dye through the glomerular filter and that glomerular permeability is compromised (Fig. 1 and Table 1). In contrast, *sema3g* and *ga17* morphants do not show defective glomerular permeability even though they showed clear defects in glomerular histology. The dye filtration assay therefore implicates *ralgps1*, *rabgef1*, *rapgef2*, and *crb2* in filtration barrier function.

The *crb2b* gene is important for assembly of the filtration barrier

We chose to pursue the role of *crb2* in the glomerulus for several reasons. We observed a morphant phenotype in over 50% of injected

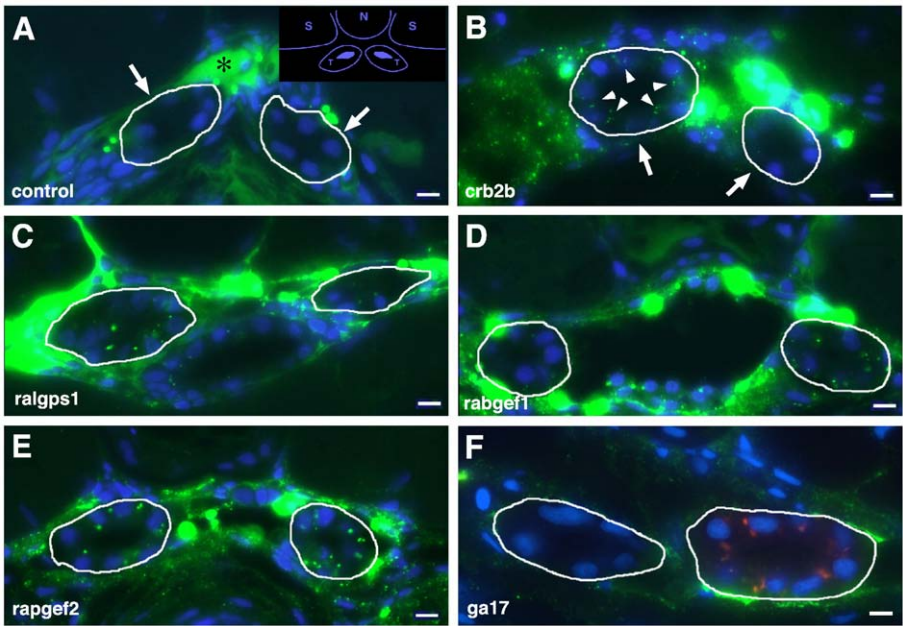


Fig. 1. Glomerular dye filtration assays on morphants. (A) Control morpholino injected 4 dpf larvae show no uptake of the 500 kDa FITC dextran dye into pronephric tubules (circled by white line). Dye is present within the dorsal aorta lumen (asterisk). In the inset, a schematic of a transverse section shows the relative positions of the somites (S), notochord (N), and pronephric tubules (T). (B) In *crb2b* morphants, the 500 kDa FITC dye is taken up into endosomes in the pronephric tubule epithelial cells (arrowheads). Similar uptake of the 500 kDa FITC dextran tracer is observed in *ralgps1* (C), *rabgef1* (D), *rapgef2* (E) morphants, but not in *ga17* morphants (F), which tested negative in this assay. Nuclei are labeled with DAPI. Scale bar, 20 μ m.

embryos and observed robust results in the dye filtration assay. Crumbs proteins are important in the establishment of epithelial apical–basal polarity (Richard et al., 2006). Since podocytes are highly polarized epithelial cells, exploring the possible role of *crb2* in podocytes might provide an entrance point into studying cell polarity in podocytes, a largely unexplored area. Starting from BLAST searches against the Ensembl database, we cloned two *crb2* paralogs from zebrafish cDNA and confirmed their identities with the published *crb2a* and *crb2b* sequences (Hsu et al., 2006; Omori and Malicki, 2006). We confirmed the *crb2b* morpholino targeted the *crb2b* locus by alignment of the morpholino with the Ensembl sequence and by RTPCR and sequence analysis (Fig. 2).

In situ hybridization revealed that *crb2b* is expressed within the pronephric glomerulus beginning at 48 hpf and continues through 96 hpf, correlating with the differentiation of podocytes (Fig. S1). In contrast to the results of Omori and Malicki (2006) *crb2b* mRNA was not detectable in tubular or duct segments of the pronephros. In agreement with the in situ experiments, Crb protein was detected within glomerular cells using a published α -panCrb antibody directed against the cytoplasmic PDZ binding domain (Fig. S1F). *crb2a* mRNA expression was not detectable in the pronephros during embryonic or larval stages. Because *crb2b* is the only *crb* gene expressed within the glomerulus during these developmental stages, we conclude that the α -panCrb immunoreactivity is due specifically to *crb2b*. Attempts to

determine the sub-cellular localization of Crb2b protein in podocytes by immuno-electron microscopy were unsuccessful.

The *crb2b* morpholino used in the screen was designed as a splice blocking (SP) morpholino predicted to cause the deletion of exon 9 and the fusion of exon 8 with 10 resulting in a 554 bp deletion (Figs. 2B, C). Conceptual translation of the splice product predicts that the open reading frame is thrown out of frame and after 42 bp of intron sequence contains a premature stop codon at amino acid 959. Both of the morphant mRNA species result in premature truncation of translation and are predicted to produce a Crb2b protein harboring the first 15 EGF-like repeats but lacking transmembrane, FERM and PDZ binding domains. The pericardial edema and pronephric cyst phenotypes were confirmed using an additional morpholino (Fig. 2A). Knockdown of *crb2b* using either *crb2b*-ATG MO or the *crb2b*-SP MO morpholino results in similar dye filtration defects and is associated with loss of α -panCrb glomerular expression (Figs. 2D–G). In addition, tubular α -panCrb staining persists in *crb2b* morphants indicating that the expression of other Crb proteins, but not Crb2b, within the tubules is not affected in morphants (data not shown). Pericardial edema and pronephric cyst phenotypes were rescued by co-injection with wildtype full length *crb2b* mRNA demonstrating that the phenotypes were due to loss of *crb2b* function only (Fig. 2A). We refer to *crb2b* morphants hereafter as *crb2b* MO.

Histological examination of *crb2b* morphants revealed an abnormal glomerular morphology and an expanded Bowman's space (Figs. 3C, D). In order to determine whether podocytes were differentiating in *crb2b* MO, we conducted in situ hybridization with *wt1*, *podocin*, and *nephrin* antisense probes which are markers of podocyte differentiation. In wildtype and control 48 hpf larvae, podocytes are found in two clusters underneath the notochord on either side of a midline capillary tuft (Figs. 3G, I, K). In *crb2b* MO, *nephrin*, *wt1*, and *podocin* positive cells are found indicating that podocytes are differentiating in *crb2b* MO (Figs. 3H, J, L).

We next sought to determine whether podocytes in *crb2b* MO differentiate foot processes and slit diaphragms. Podocytes are highly polarized cells with their apical cell bodies in the Bowman's space while basal membranes form elaborate, branched and regularly

Table 1
Penetrance of dye filtration phenotypes.

Gene targeted	Penetrance (%)	N
<i>crb2l-2</i>	100	11
<i>crb2l-atg</i>	69	13
<i>ga17</i>	8	12
<i>rabgef1</i>	33	3
<i>ralgps1</i>	67	9
<i>rapgef2</i>	80	5
<i>sema3g</i>	0	7
Standard control	0	10

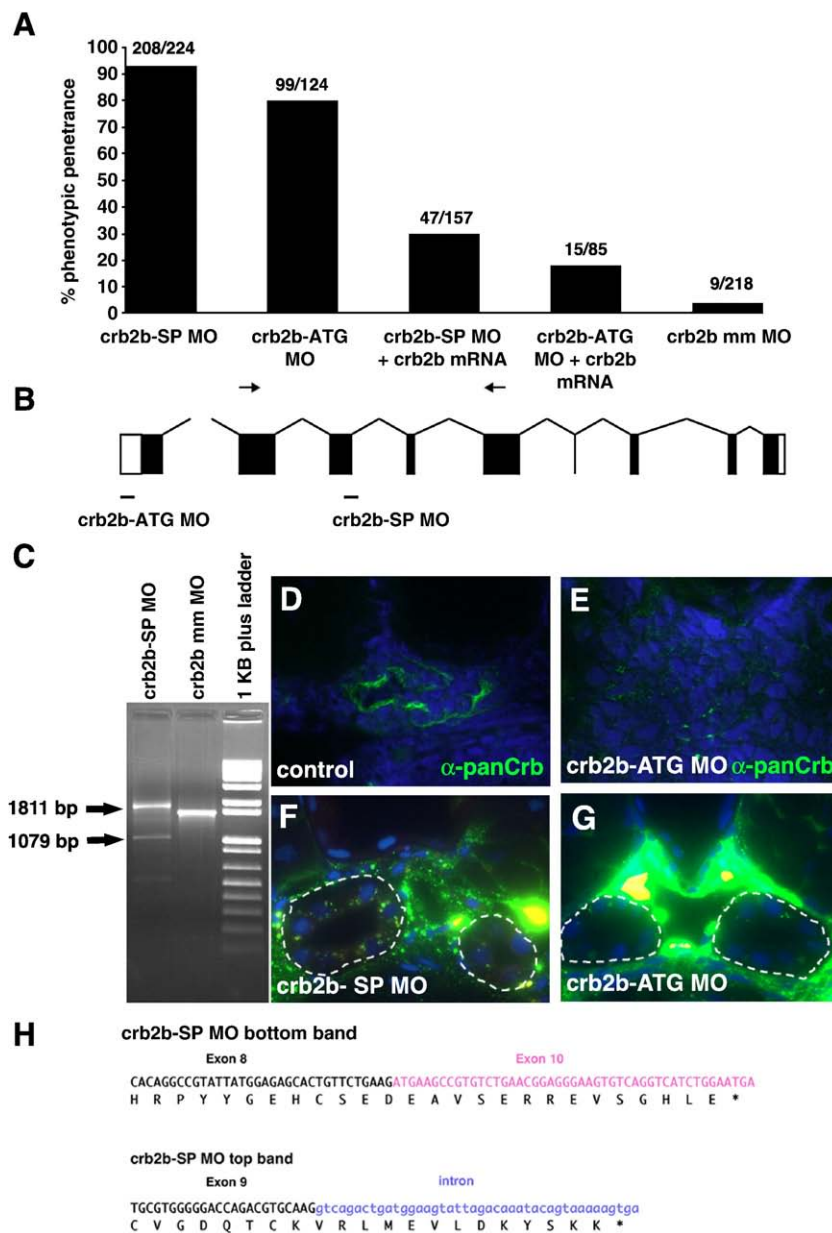


Fig. 2. Morpholino knockdown. (A) Penetrance of morphant phenotypes using *crb2b*-ATG and *crb2b*-SP morpholinos. Co-injection with wildtype *crb2b* mRNA rescues the morphant defects. A mismatch morpholino based on *crb2b*-SP does not induce pericardial edema or pronephric cyst phenotypes. Sample sizes are shown above the bars. (B) Exon structure of *crb2b* and position of *crb2b*-ATG and *crb2b*-SP morpholinos. PCR primers are shown (arrows). (C) RT-PCR of *crb2b* mRNA from mismatch control injected embryos yields the expected 1569 bp product. RT-PCR of *crb2b* mRNA from *crb2b*-SP embryos yields an expected 1079 bp band, resulting from the deletion of Exon 9, and an additional product of 1811 bp. Glomerular α -panCrb staining observed in mismatch injected embryos (D) is lost in embryos injected with *crb2b*-ATG (or *crb2b*-SP) (E). (F, G) *crb2b*-ATG and *crb2b*-SP induce glomerular permeability defects as assayed by the re-uptake of 500 kDa FITC dextran dye into pronephric tubule endosomes. (H) The lower 1079 band results from the deletion of exon 9 and the fusion of exon 8 (black) to exon 10 (pink). The 1811 bp upper band resulted from the insertion of 242 bp of intronic sequences (blue) downstream of the morpholino target sequence. Translation of intronic sequences results in premature stop codons (asterisk).

patterned, interdigitated foot processes on the GBM (Fig. 4A). Between the cell body and the foot processes the microtubule enriched primary, or major, processes are found. Slit diaphragms bridge foot processes from adjacent podocytes and are visible as a thin membrane close to the site of podocyte GBM contact. Tight and adherens junctions are found in immature podocytes, but are replaced by slit diaphragms in fully differentiated cells.

In *crb2b* MO, podocytes are found on the outer aspect of endothelial capillary loops and make contact with a morphologically normal GBM (Fig. 4B). In contrast to controls, *crb2b* MO podocytes show long stretches of cell membrane in close contact with the GBM and morphologically identifiable foot processes were not visible. Morphants showed 0.8 ± 0.3 slits/ μ m in comparison to controls which

showed 5.4 ± 0.8 slits/ μ m indicating reduced foot process formation. In some areas, tight junctions were observed while slit diaphragms were completely absent (Fig. S2). Consistent with our dye filtration results, we observed electron dense precipitate in the Bowman's space which likely results from the leakage of plasma proteins across the filtration barrier. Serial reconstruction of individual podocytes in *crb2b* morphants showed that podocytes extend protrusions from the apical membrane into Bowman's space ($n=8$ podocytes reconstructed in *crb2b* MO, $n=6$ podocytes in controls) (Fig. S3). These membrane protrusions appear disorganized and contact nearby GBM or parietal epithelium.

In order to assess overall polarity within *crb2b* MO podocytes, we have used α -acetylated Tubulin antibody as a molecular marker of

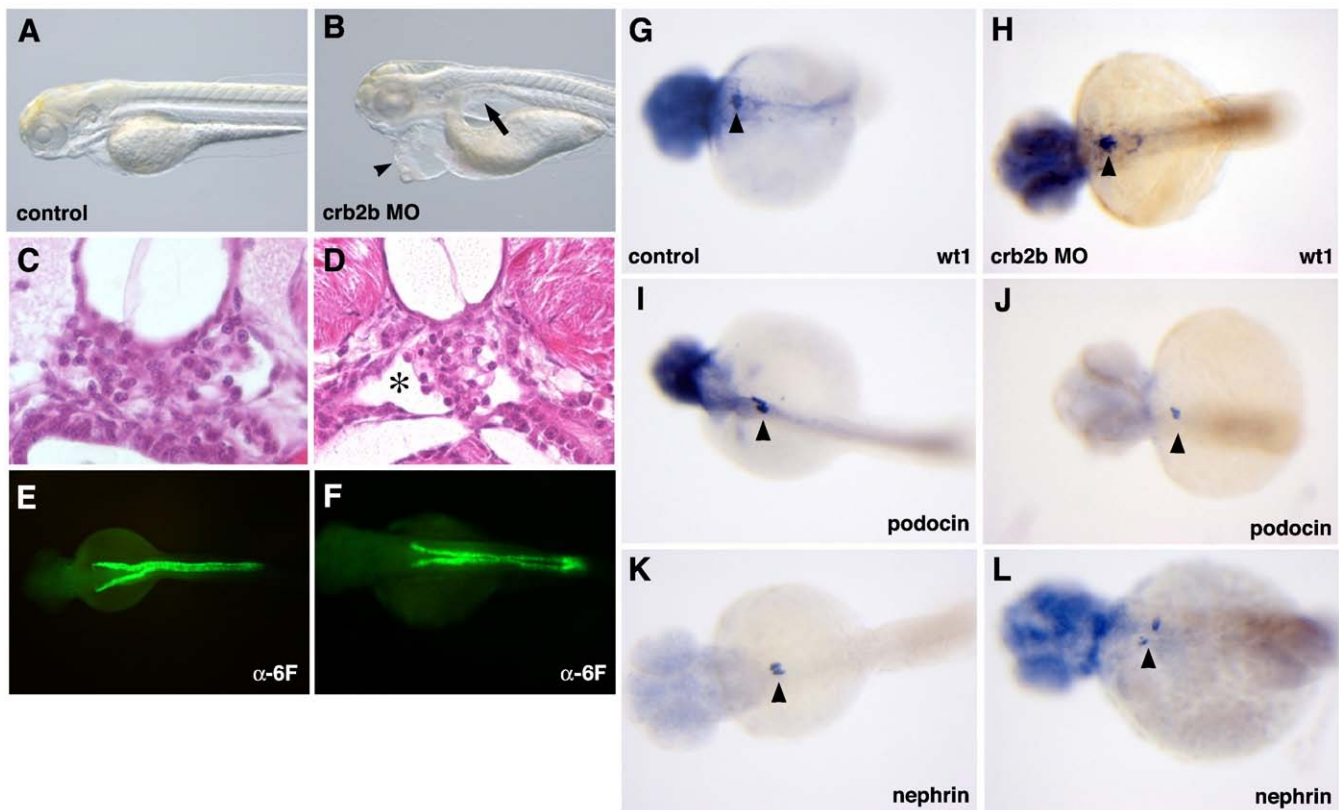


Fig. 3. Phenotype of the *crb2b* morphants. (A) Control mismatch and (B) *crb2b*-ATG injected 2.5 dpf larvae. Note the pericardial edema (arrowhead) and pronephric cysts (arrow). (C, D) Histological sections at the level of the glomerulus in control (C) and *crb2b*-ATG MO (D). Note the expanded Bowman's space (asterisk) in D. (E, F) Staining with α -Na⁺/K⁺ ATPase in the pronephric tubules and ducts is not generally affected in *crb2b* MO. (G–L) In situ hybridization on 2 dpf wildtype (G, I, K) or *crb2b*-ATG (H, J, L) with *wt1* (G, H), *podocin* (I, J), or *nephrin* (K, L) antisense probes. Cells expressing *wt1*, *nephrin*, and *podocin* are present in *crb2b* morphants (black arrowheads).

podocyte major, or primary, processes (Drenckhahn and Franke, 1988; Pavenstadt et al., 2003). Primary processes lie lateral and basal with respect to the nucleus/cell body in wildtype podocytes but apical to the foot processes and GBM. The relative orientation of α -acetylated Tubulin staining to the nucleus and basement membrane therefore serves as a readout of polarity within podocytes. We found that α -acetylated Tubulin staining is present in between the nucleus and GBM in *crb2b* MO, similar to wildtype, indicating that primary processes were present and general aspects of cell polarity are maintained (Fig. S4).

In order to molecularly define the podocyte foot process defect, we sought to determine the localization of the slit diaphragm protein Nephrin in *crb2b* morphants. A rabbit polyclonal antibody was made against the carboxy terminus of zebrafish Nephrin. On Western blots, the α -Nephrin antibody recognizes a single band in HEK293 cells transfected with a plasmid expressing the full length zebrafish *nephrin* cDNA (Fig. S5A) (Kramer-Zucker et al., 2005). In immuno-electron microscopy, α -Nephrin conjugated gold particles localize to the inner aspect of the slit diaphragm but not apical membranes (Fig. S5B). In control embryos injected with the mismatch morpholino, Nephrin protein is present along the basal aspect of podocyte cell bodies (Fig. 4C). Notably, in *crb2b* MO, Nephrin protein is mis-localized and present in punctate structures throughout the podocytes (Figs. 4D, E). These results suggest that *crb2b* is required for the correct targeting of Nephrin protein to slit diaphragms within podocytes. In order to determine whether the mis-localized Nephrin protein was present in podocyte apical membrane projections in *crb2b* MO, we performed immuno-electron microscopy using the α -Nephrin antibody. In *crb2b* MO podocytes, Nephrin protein was found associated with the plasma membrane in apical projections (Figs. 4F, G). These results demonstrate that

Nephrin protein is apically mis-targeted in podocytes lacking *crb2b* function.

Discussion

A screen for glomerular function in the zebrafish

Forward genetic screens in the zebrafish combined with molecular cloning of the mutated loci have been successful in gaining insight into developmental and disease mechanisms and for gene discovery. In our screen, we have tested known and novel genes for roles in glomerular function with the expectation that the gene targets identified will be relevant to normal mammalian glomerular function and perhaps disease. This approach has allowed us to explore new aspects of glomerular biology that would be too impractical to attempt by traditional gene knock out in mouse. From a glomerular perspective, the screen is novel and the first of its kind because it combines an *in vivo* glomerular permeability physiological assay with functional genetics in a vertebrate system.

Crb2b is a regulator of podocyte maturation

Relatively little is known about how Crb proteins function in development. *Drosophila crumbs* (*crb*) was first identified as a regulator of epithelialization and apical membrane differentiation during cellularization in the blastoderm stage (Tepass et al., 1990). Overexpression of *crb* causes the expansion of apical membrane at the expense of basolateral membrane (Wodarz et al., 1993). *crb* genetically interacts with *stardust* (*std*) and *bazooka* (*baz*), two other members of a signaling pathway important in establishing apical-basal polarity (Tanentzapf and Tepass, 2003; Tepass and Knust, 1993).

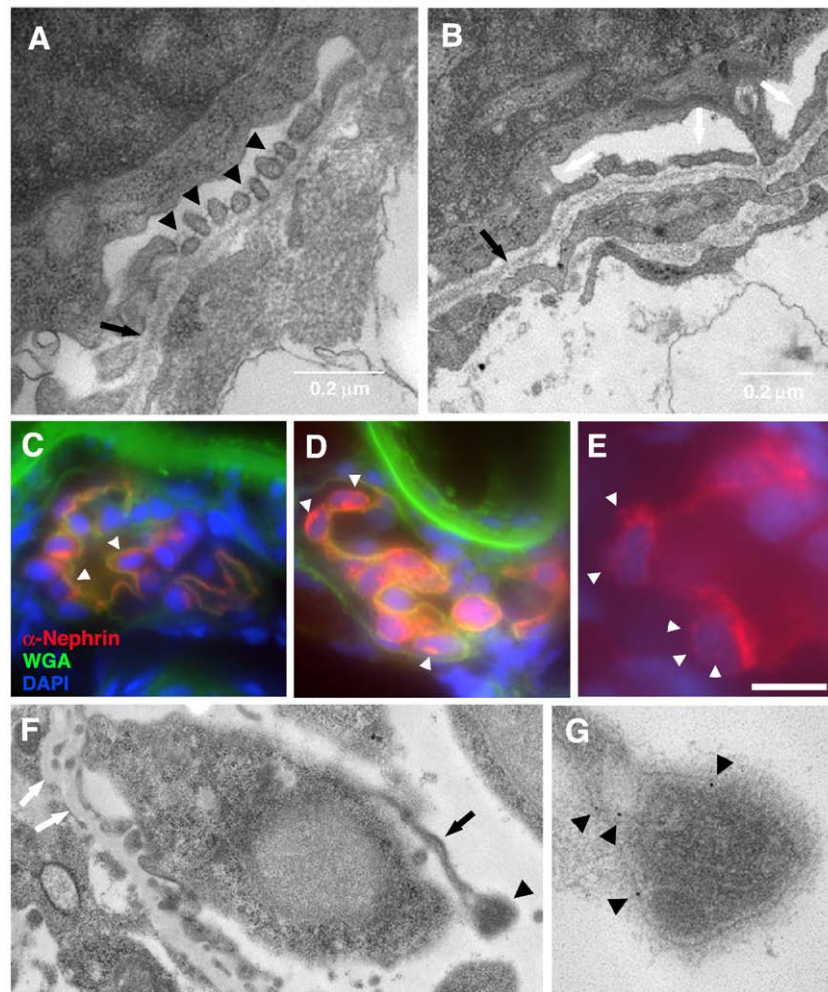


Fig. 4. *crb2b* morphants show defects in podocyte morphology. (A) Electron micrograph of a podocyte from control 4 dpf larvae shows the regular patterned array of foot processes along the GBM; GBM (black arrow) and slit diaphragms (black arrowheads). (B) In *crb2b* morphants, podocytes are attached to a GBM (black arrow), but the organization of regularly spaced foot processes is lost (white arrows show foot process fusion). Slit diaphragms are absent. Endothelial fenestrations are reduced in frequency. Nephtrin protein is apically mis-localized in *crb2b* morphant podocytes. (C) Staining with α -Nephtrin antibody shows that Nephtrin protein is present (arrowheads) only along the surface of the GBM (visualized with WGA FITC). (D, E) In *crb2b* morphants, Nephtrin protein is present throughout the podocytes with punctate staining (arrowheads) distal to the GBM. Nuclei visualized with DAPI; scale bar, 5 μ m. (F) Immuno-electron microscopy with the α -Nephtrin antibody in *crb2b* MO shows a podocyte with an apical membrane projection. White arrows indicate the GBM. (G) A magnification of the tip of the membrane projection indicated by the arrowhead in D. Nephtrin protein is associated with the inner aspect of the plasma membrane (gold particles indicated by black arrowheads in E) within ectopic projections extending from podocyte apical membranes.

Since then, the Crb family of proteins have been discovered in vertebrates and functions within an evolutionarily conserved Crb/Pals1/Patj multi-protein complex to regulate the establishment of apical–basal polarity in epithelia and the formation of apical membrane features (Margolis and Borg, 2005; Roh et al., 2003; Roh et al., 2002; Shin et al., 2006).

We found that *crb2b* is required for the morphological differentiation of podocyte foot processes. Foot processes were grossly simplified in morphant podocytes and the presence of slit diaphragms was greatly reduced or even absent thus providing an explanation for the isolation of *crb2b* in our glomerular filtration screen. The phenotypes we observe are more severe than those observed in the *Nephtrin* knock out mouse or *nephtrin* morphant zebrafish, where slit diaphragms are absent, but foot processes still form (Kramer-Zucker et al., 2005; Putaala et al., 2001). Defects in apical–basal differentiation are not observed in *nephtrin* loss of functions podocytes. In *crb2b* MO podocytes, morphological and molecular defects were observed in both apical and basal membranes indicating a wider role for *crb2b* in podocyte biology. In addition, these results stand in contrast to the photoreceptor defects reported in *crb2b* morphants, where the apical inner segment is specifically shortened, but cellular structures

basolateral to the outer limiting membrane are not affected (Omori and Malicki, 2006). Likewise, *crb2b* appears to have a wider role in podocytes than those proposed for *crb2b* or *crb3* as regulators of ciliogenesis in renal tubular epithelia. In both of these cases, the cilia, specialized structures of the apical membrane, are specifically affected without defects in basolateral membranes (Fan et al., 2004; Omori and Malicki, 2006).

However, some basic aspects of cell polarity are present within *crb2b* MO podocytes. Significantly, podocytes lacking *crb2b* function synthesize and make contact with an apparently normal GBM. The GBM arises through a fusion of the podocyte and capillary endothelial basement membranes during glomerulogenesis, and the presence of a GBM indicates that podocytes in *crb2b* MO are able to make a basement membrane, which is a fundamental early manifestation of polarity in epithelial cells. In addition, *crb2b* MO podocytes maintain the relative orientation of the cell body/nucleus, primary processes, and GBM consistent with the retention of overall polarity. These observations would suggest that *crb2b* is not required in establishing the early apical–basal polarity axis in podocytes.

What makes podocytes unique among epithelial cells is their highly branched cellular architecture and interdigitated foot processes

and their specialized cellular junctions, the slit diaphragms. Though the maintenance of foot processes has long been recognized as crucial for filtration barrier function and a pathological target, the molecular and genetic mechanisms regulating their formation and elaboration from larger processes has been largely unknown. Our analysis of the *crb2b* phenotype favors specific roles in podocyte foot process arborisation and membrane differentiation as opposed to a more general role in early fundamental decisions of apical–basal polarity. These results provide a way of understanding foot process formation within the larger context of apical–basal cell differentiation.

Crb2b and Nephrin

In *crb2b* morphants, the membrane projections could result from an expanded production of the apical membrane domain at the expense of foot processes. However, the presence of Nephrin protein in the projections is inconsistent with an apical character. Furthermore, we observed that these projections were competent to form foot processes when they encountered GBM suggesting that they may harbor the molecular machinery for elaborating foot processes when given the correct environmental stimulus. It will be important to determine whether other slit diaphragm components are similarly mis-targeted in the absence of *crb2b* function.

The presence of Nephrin protein in the projections gives the projections a basal or foot process character. It's possible that the projections represent ectopic micro-domains of basolateral membrane embedded within the podocyte apical membrane, making the podocyte plasma membranes in *crb2b* morphants a mosaic of apical and basal membranes. This interpretation is consistent with the expanded basolateral domains observed in *Drosophila crb* mutants and leads to two possible functions for *crb2b* in podocytes. Crb proteins have been proposed to be required for the formation of tight junctions and tight junctions are known to function as a fence whereby they prevent the mixing of apical and basolateral membrane domains (Roh et al., 2003; Shin et al., 2006). Therefore, the podocyte defects observed in *crb2b* morphants could arise from an inability of morphant podocytes to form tight and adherens junctions which could, in turn, result in a random mixing of apical and basolateral membranes. However, in *crb2b* MO podocytes, tight junctions were observed in electron micrographs while slits diaphragms were dramatically reduced, indicating that *crb2b* is not strictly required for tight junction formation, but may be involved in the maturation of tight/adherens junctions into slits. In fact, because Nephrin is a slit diaphragm but not a tight junction component, the persistence of tight junctions in morphant podocytes may be a consequence of the mis-targeting of Nephrin in the absence of *crb2b*.

Alternatively, Crb2b protein may play a more direct role in the synthesis of different membrane domains during epithelial differentiation by, for example, regulating the formation of lipid rafts or lipid sub-domains through vesicular trafficking events. It is known that Nephrin is recruited to lipid raft domains during slit diaphragm formation. Thus, it is conceivable that Nephrin mis-targeting observed in *crb2b* morphants reflects a primary defect in the organization of lipid raft micro-domains (Benzing, 2004; Huber et al., 2003). The mis-localized Nephrin may then regulate cytoskeletal organization to effect process branching. Nephrin signals through the Nck adaptor protein and CD2AP to modulate actin cytoskeletal rearrangements (Jones et al., 2006; Lehtonen et al., 2002; Verma et al., 2006; Yuan et al., 2002). Clearly, such roles for Crb proteins in the assembly of membrane domains will need to be tested as molecular and biochemical markers become available. Lastly, the correct targeting of Nephrin may occur through direct or indirect association with Crb2b, and this possibility is currently being tested. The sub-cellular localization of Crb2b is an important issue here, but unfortunately, our α -panCrb antibody does not allow us to unambiguously determine where Crb2b protein resides in podocytes and new antibodies are

needed. Nevertheless, these results lead us to conclude that *crb2b* is required for the correct trafficking of a specific junctional protein, Nephrin, to a specific structure, the slit diaphragm, unique to terminally differentiated podocytes. These results must be confirmed with genetic loss of function mutations in *crb2b* in zebrafish. Given the apparent conservation of *crb* function in a variety of epithelial cell types and model organisms, the analysis of specific protein trafficking events may provide a means towards a general mechanistic understanding of *crb* function.

Our observations implicate potential roles for cell polarity signaling pathways in foot process arborization. Several other members of cell polarity signaling complexes have been recovered as loss of function mutants in the zebrafish, and they show phenotypes in epithelial tissues. A mutation in zebrafish Pals1, *nagie oko* (*nok*) shows profound defects in polarity within the entire retinal neuroepithelium (Wei and Malicki, 2002). A mutation in aPKC λ has also been reported, *heart and soul* (*has*), which is required for the maintenance of epithelial polarity in the retinal photoreceptor layer and the establishment of polarity in the esophagus, intestine, and swim bladder (Horne-Badovinac et al., 2001). Conditional inactivation of aPKC lambda/iota in mouse podocytes results in foot process defects and associated proteinuria (Huber et al., 2009). Crb proteins also directly bind to the FERM domain protein Moe through the FERM binding domain located in the Crb intracellular tail (Hsu et al., 2006; Laprise et al., 2006). In zebrafish, Moe is mutated in the *mosaic eyes* (*moe*) mutant while in the fruit fly the *yurt* mutation occurs in the fly ortholog (Jensen and Westerfield, 2004; Laprise et al., 2006). Of special interest, podocyte foot process formation and slit diaphragm assembly are also affected in homozygous mutant *moe* embryos suggesting that *moe* and *crb2b* may be participating in a common membrane synthesis pathway in podocyte maturation (Kramer-Zucker et al., 2005).

Perhaps somewhat surprisingly, and in stunning contrast to other renal epithelial cell types, very little attention has focused on the apical–basal polarization of podocytes and how it related to their final differentiated state. These results suggest that other regulators of epithelial polarity may likewise be involved in the establishment and maintenance of foot process architecture. A corollary of this proposition is that the mis-regulation of apical–basal polarity signaling pathways within podocytes may be a common theme in podocyte pathologies.

Acknowledgments

We thank several people for their contributions to this work: Matalena Parikka, Yukino Nishibori, Timo Pikkarainen, Elisabeth Raschperger, Berit Rydlander, Johan Dixelius, Barbro Larsson, and Jaakko Patrakka for technical support and discussions. Special thanks to Susan Warner, Ulla Wargh, and Sajila Kisana from the KI fish facility. We are grateful to Jarema Malicki for providing the *crb2b* cDNA and α -panCrb antibody and for valuable comments on the manuscript. We thank Iain Drummond for the *nephrin* cDNA clone. We acknowledge the Zebrafish Information Resource Center (ZIRC) for providing fish and much helpful advice on fish husbandry. A.M. acknowledges financial support from the Swedish Research Council (Vetenskapsrådet) and the Knut and Alice Wallenberg Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.04.017.

References

- Beltcheva, O., Martin, P., Lenkkeri, U., Tryggvason, K., 2001. Mutation spectrum in the nephrin gene (*NPHS1*) in congenital nephrotic syndrome. Hum. Mutat. 17, 368–373.

- Benzing, T., 2004. Signaling at the slit diaphragm. *J. Am. Soc. Nephrol.* 15, 1382–1391.
- Betsholtz, C., He, L., Takemoto, M., Norlin, J., Sun, Y., Patrakka, J., Tryggvason, K., 2007. The glomerular transcriptome and proteome. *Nephron. Exp. Nephrol.* 106, e32–6.
- Drenkhahn, D., Franke, R.P., 1988. Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man. *Lab. Invest.* 59, 673–682.
- Drummond, I.A., 2004. Zebrafish kidney development. *Methods Cell. Biol.* 76, 501–530.
- Eremina, V., Baelde, H.J., Quaggin, S.E., 2007. Role of the VEGF—a signaling pathway in the glomerulus: evidence for crosstalk between components of the glomerular filtration barrier. *Nephron. Physiol.* 106, p32–7.
- Fan, S., Hurd, T.W., Liu, C.J., Straight, S.W., Weimbs, T., Hurd, E.A., Domino, S.E., Margolis, B., 2004. Polarity proteins control ciliogenesis via kinesin motor interactions. *Curr. Biol.* 14, 1451–1461.
- Farquhar, M.G., Wissig, S.L., Palade, G.E., 1961. Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. *J. Exp. Med.* 113, 47–66.
- Heasman, J., 2002. Morpholino oligos: making sense of antisense? *Dev. Biol.* 243, 209–214.
- He, L., Sun, Y., Patrakka, J., Mostad, P., Norlin, J., Xiao, Z., Andrae, J., Tryggvason, K., Samuelsson, T., Betsholtz, C., Takemoto, M., 2007. Glomerulus-specific mRNA transcripts and proteins identified through kidney expressed sequence tag database analysis. *Kidney Int.* 71, 889–900.
- Hentschel, D.M., Mengel, M., Boehme, L., Liebsch, F., Albertin, C., Bonventre, J.V., Haller, H., Schiffer, M., 2007. Rapid screening of glomerular slit diaphragm integrity in larval zebrafish. *Am. J. Physiol. Renal. Physiol.* 293, F1746–F1750.
- Horne-Badovinac, S., Lin, D., Waldron, S., Schwarz, M., Mbamalu, G., Pawson, T., Jan, Y., Stainier, D.Y., Abdelilah-Seyfried, S., 2001. Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr. Biol.* 11, 1492–1502.
- Hsu, Y.C., Willoughby, J.J., Christensen, A.K., Jensen, A.M., 2006. Mosaic Eyes is a novel component of the Crumbs complex and negatively regulates photoreceptor apical size. *Development* 133, 4849–4859.
- Huber, T.B., Kottgen, M., Schilling, B., Walz, G., Benzing, T., 2001. Interaction with podocin facilitates nephrin signaling. *J. Biol. Chem.* 276, 41543–41546.
- Huber, T.B., Simons, M., Hartleben, B., Sernetz, L., Schmidts, M., Gundlach, E., Saleem, M.A., Walz, G., Benzing, T., 2003. Molecular basis of the functional podocin–nephrin complex: mutations in the *NPHS2* gene disrupt nephrin targeting to lipid raft microdomains. *Hum. Mol. Genet.* 12, 3397–3405.
- Huber, T.B., Hartleben, B., Winkelmann, K., Schneider, L., Becker, J.U., Leitges, M., Walz, G., Haller, H., Schiffer, M., 2009. Loss of podocyte aPKC[lambda]/[iota] causes polarity defects and nephrotic syndrome. *J. Am. Soc. Nephrol.* 20, 798–806.
- Jensen, A.M., Westerfield, M., 2004. Zebrafish mosaic eyes is a novel FERM protein required for retinal lamination and retinal pigmented epithelial tight junction formation. *Curr. Biol.* 14, 711–717.
- Jones, N., Blasutig, I.M., Eremina, V., Ruston, J.M., Bladt, F., Li, H., Huang, H., Larose, L., Li, S.S., Takano, T., Quaggin, S.E., Pawson, T., 2006. Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature* 440, 818–823.
- Kestila, M., Lenkkeri, U., Mannikko, M., Lamerdin, J., McCready, P., Putaala, H., Ruotsalainen, V., Morita, T., Nissinen, M., Herva, R., Kashtan, C.E., Peltonen, L., Holmberg, C., Olsen, A., Tryggvason, K., 1998. Positionally cloned gene for a novel glomerular protein–nephrin—is mutated in congenital nephrotic syndrome. *Mol. Cell* 1, 575–582.
- Kramer-Zucker, A.G., Wiessner, S., Jensen, A.M., Drummond, I.A., 2005. Organization of the pronephric filtration apparatus in zebrafish requires Nephrin, Podocin and the FERM domain protein Mosaic eyes. *Dev. Biol.* 285, 316–329.
- Laprise, P., Beronja, S., Silva-Gagliardi, N.F., Pellikka, M., Jensen, A.M., McGlade, C.J., Tepass, U., 2006. The FERM protein Yurt is a negative regulatory component of the Crumbs complex that controls epithelial polarity and apical membrane size. *Dev. Cell* 11, 363–374.
- Lehtonen, S., Zhao, F., Lehtonen, E., 2002. CD2-associated protein directly interacts with the actin cytoskeleton. *Am. J. Physiol. Renal. Physiol.* 283, F734–F743.
- Majumdar, A., Drummond, I.A., 2000. The zebrafish floating head mutant demonstrates podocytes play an important role in directing glomerular differentiation. *Dev. Biol.* 222, 147–157.
- Margolis, B., Borg, J.P., 2005. Apicobasal polarity complexes. *J. Cell. Sci.* 118, 5157–5159.
- Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene ‘knockdown’ in zebrafish. *Nat. Genet.* 26, 216–220.
- Omori, Y., Malicki, J., 2006. *oko meduzy* and related *crumbs* genes are determinants of apical cell features in the vertebrate embryo. *Curr. Biol.* 16, 945–957.
- Pavenstadt, H., Kriz, W., Kretzler, M., 2003. Cell biology of the glomerular podocyte. *Physiol. Rev.* 83, 253–307.
- Putaala, H., Soininen, R., Kilpelainen, P., Wartiovaara, J., Tryggvason, K., 2001. The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. *Hum. Mol. Genet.* 10, 1–8.
- Richard, M., Roepman, R., Aartsen, W.M., van Rossum, A.G., den Hollander, A.I., Knust, E., Wijnholds, J., Cremers, F.P., 2006. Towards understanding Crumbs function in retinal dystrophies. *Hum. Mol. Genet.* 15 (Spec. No. 2), R235–R243.
- Roh, M.H., Makarova, O., Liu, C.J., Shin, K., Lee, S., Laurinec, S., Goyal, M., Wiggins, R., 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.
- Roh, M.H., Fan, S., Liu, C.J., Margolis, B., 2003. The Crumbs3–Pals1 complex participates in the establishment of polarity in mammalian epithelial cells. *J. Cell. Sci.* 116, 2895–2906.
- Shin, K., Fogg, V.C., Margolis, B., 2006. Tight junctions and cell polarity. *Annu. Rev. Cell. Dev. Biol.* 22, 207–235.
- Sullivan-Brown, J., Schottenfeld, J., Okabe, N., Hostetter, C.L., Serluca, F.C., Thiberge, S.Y., Burdine, R.D., 2008. Zebrafish mutations affecting cilia motility share similar cystic phenotypes and suggest a mechanism of cyst formation that differs from *pkd2* morphants. *Dev. Biol.* 314, 261–275.
- Takemoto, M., He, L., Norlin, J., Patrakka, J., Xiao, Z., Petrova, T., Bondjers, C., Asp, J., Wallgard, E., Sun, Y., Samuelsson, T., Mostad, P., Lundin, S., Miura, N., Sado, Y., Alitalo, K., Quaggin, S.E., Tryggvason, K., Betsholtz, C., 2006. Large-scale identification of genes implicated in kidney glomerulus development and function. *EMBO J.* 25, 1160–1174.
- Tanentzapf, G., Tepass, U., 2003. Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. *Nat. Cell. Biol.* 5, 46–52.
- Tepass, U., Knust, E., 1993. Crumbs and stardust act in a genetic pathway that controls the organization of epithelia in *Drosophila melanogaster*. *Dev. Biol.* 159, 311–326.
- Tepass, U., Theres, C., Knust, E., 1990. *crumbs* encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell* 61, 787–799.
- Thisse, B., Heyer, V., Lux, A., Alunni, V., Degraeve, A., Seiliez, I., Kirchner, J., Parkhill, J.P., Thisse, C., 2004. Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. *Methods Cell. Biol.* 77, 505–519.
- Tryggvason, K., Patrakka, J., Wartiovaara, J., 2006. Hereditary proteinuria syndromes and mechanisms of proteinuria. *N. Engl. J. Med.* 354, 1387–1401.
- Verma, R., Kovari, I., Soofi, A., Nihalani, D., Patrie, K., Holzman, L.B., 2006. Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. *J. Clin. Invest.* 116, 1346–1359.
- Wei, X., 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., 1993. In: Westerfield, M. (Ed.), *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio)*. Eugene, OR.
- Wodarz, A., Grawe, F., Knust, E., 1993. CRUMBS is involved in the control of apical protein targeting during *Drosophila* epithelial development. *Mech. Dev.* 44, 175–187.
- Yuan, H., Takeuchi, E., Salant, D.J., 2002. Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton. *Am. J. Physiol. Renal. Physiol.* 282, F585–F591.