Renal defects associated with improper polarization of the CRB and DLG polarity complexes in MALS-3 knockout mice

Olav Olsen,1 Lars Funke,1 Jia-fu Long,4 Masaki Fukata,1 Toshinari Kazuta,1 Jonathan C. Trinidad,3 Kimberly A. Moore,2 Hidemi Misawa,1 Paul A. Welling,3 Alma L. Burlingame,3 Mingjie Zhang,4 and David S. Bredt1,6

1Departments of Physiology, 2Cellular and Molecular Pharmacology, and 3Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94143
2Department of Biochemistry, Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong
3Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201
4Department of Integrative Biology, Eli Lilly and Company, Indianapolis, IN 46285

Kidney development and physiology require polarization of epithelia that line renal tubules. Genetic studies show that polarization of invertebrate epithelia requires the crumbs, partition-defective-3, and discs large complexes. These evolutionarily conserved protein complexes occur in mammalian kidney; however, their role in renal development remains poorly defined. Here, we find that mice lacking the small PDZ protein mammalian LIN-7c (MALS-3) have hypomorphic, cystic, and fibrotic kidneys. Proteomic analysis defines MALS-3 as the only known core component of both the crumbs and discs large cell polarity complexes. MALS-3 mediates stable assembly of the crumbs tight junction complex and the discs large basolateral complex, and these complexes are disrupted in renal epithelia from MALS-3 knockout mice. Interestingly, MALS-3 controls apico-basal polarity preferentially in epithelia derived from metanephric mesenchyme, and defects in kidney architecture owe solely to MALS expression in these epithelia. These studies demonstrate that defects in epithelial cell polarization can cause cystic and fibrotic renal disease.

Introduction

Polarity is a physical attribute of most eukaryotic cells that is indispensable for their function. Generation and maintenance of cell polarity requires the active segregation of molecular components and imparts distinct properties to subcellular domains. Polarization occurs along a major axis in epithelia, neurons, and asymmetrically dividing cells. In epithelia, polarization relative to the tight junction functionally separates the apical and basolateral domains; neuronal polarization allows for differential development of dendrites and axons. During asymmetric cell division, a polarity cue in the mother cell directs distribution of cell fate determinants in daughter cells. In all these cases, failure to establish cell polarity compromises tissue differentiation and function.

Sustained polarization of epithelial cells lining renal tubules is essential for kidney development and function (Campo et al., 2005). Tubules in the permanent kidney develop through reciprocal interactions between the ureteric bud and metanephric mesenchyme (Dressler, 2006). The ureteric bud invades the metanephros and undergoes a series of branching events that give rise to the collecting duct system. In response to signals released from ureteric bud tips, mesenchymal cells transform into polarized epithelia, which differentiate into the tubule cells along the remainder of the nephron. Epithelial cell polarization relies on tight junctions, which connect tubule cells, provide paracellular barriers to ion and fluid movement, and organize the membrane into apical and basolateral domains (Schneeberger and Lynch, 1992; Van Itallie and Anderson, 2004; Shin et al., 2006). Polarized expression of channels and transporters allows for vectorial transport of solutes along the nephron, resulting in physiological urine formation. Aberrations in epithelial cell polarity are implicated in the pathogenesis of renal cysts, renal fibrosis, and renal failure (Kalluri and Neilson, 2003; D.B. Lee et al., 2006); however, the molecular mechanisms underlying these complex diseases remain poorly understood.
The mammalian homologue of LIN-7 (MALS) can bind components from both the apical (Kamberov et al., 2000) and basolateral (Kaech et al., 1998; Lee et al., 2002) polarity complexes, suggesting an additional mode for interplay. Three MALS genes occur in mammals, and each is a small protein complex including LGL and SCRIB and the WD40 motif protein LGL (Bilder et al., 2000). Interplay between these basolateral polarity proteins and the apical polarity complexes establish and maintain cellular polarity. For example, LGL is phosphorylated by aPKC, thereby excluding LGL from the apical compartment (Betschinger et al., 2003; Plant et al., 2003). The CRB and PAR-3 polarity complexes were originally thought to act independently, but physical and functional interactions between these complexes establish cell polarity (Hurd et al., 2003). Genetic analyses of invertebrates also identified several basolateral proteins required for epithelial cell polarization. Discs large (dlg) (Woods and Bryant, 1991), lethal giant larvae (lgl) (Gateff and Schneiderman, 1969), and scribble (scrib) (Bilder and Perrimon, 2000; Bilder et al., 2000) were originally identified as tumor suppressor genes in Drosophila. Mutations in dlg, lgl, or scrib disrupt epithelial cell polarity and cause neoplastic overgrowth of tissues. DLG genetically interacts with the multi-PDZ protein SCRIB and the WD40 motif protein LGL (Bilder et al., 2000). Interplay between these basolateral polarity proteins and the apical polarity complexes is unknown. Biochemical and cell biological experiments suggest a variety of possible roles for MALS in mammalian epithelial cell lines. MALS localizes to the basolateral membrane of Madin-Darby canine kidney (MDCK) cells (Perego et al., 1999; Olsen et al., 2002). By analogy to the role of LIN-7 in Caenorhabditis elegans, the PDZ domain of MALS may anchor receptors to the basolateral domain of MDCK cells (Straight et al., 2001; Shelly et al., 2003). Alternatively, MALS may stabilize PALS and mediate tight junction formation (Straight et al., 2006). Other studies suggest that MALS interacts with β-catenin and mediates organization of adherens junctions (Perego et al., 2000). Finally, MALS can regulate endocytosis or endosomal sorting in epithelial cell lines (Perego et al., 1999; Straight et al., 2001). Despite these numerous suggestions, genetic analyses have yet to identify functions for MALS in mammalian epithelia.

We characterize MALS-3 knockout (−/−) mice and identify an essential role for MALS-3 in defining polarity of renal
epithelial cells. MALS-3−/− mice have hypomorph kidneys characterized by numerous cysts and fibrosis. These developmental defects owe to a loss of polarity specifically in epithelia derived from the metanephric mesenchyme. Immunoprecipitation proteomics analysis reveals MALS-3 binds to the CRB tight junction and DLG basal component complexes. Biochemical studies show the L27 domain of MALS-3 assembles and stabilizes these complexes. In proximal tubule cells from MALS-3 mutant mice, the CRB complex is lost from tight junctions and DLG mislocalizes to tight junctions. These studies demonstrate that MALS-3 organizes these two discrete polarity complexes and that disruption of epithelial cell polarity can result in renal agenesis, cysts, and fibrosis.

Results

Kidney pathology in MALS-3−/− mice

Kiddneys from neonatal and adult MALS-3−/− mice are noticeably smaller than those from heterozygous (+/−) littermates (Fig. 1A and B), and ~15% of MALS-3−/− display unilateral renal agenesis (unpublished data). Furthermore, MALS-3−/− kidneys are studded with numerous cysts (Fig. 1B; and Fig. S1A, available at http://www.jcb.org/cgi/content/full/jcb.200702054/DC1). Most cysts (8 of 11) fail to express tubule segment-specific markers, only 1 of 11 cysts stained with a lectin that labels proximal tubules (Fig. S1B). Kidneys from MALS-3−/− are dramatically reduced in weight relative to MALS-3+/− (Fig. 1C). Whereas the kidney is reduced by nearly 45%, only a ~10% reduction in the size of heart, spleen, and overall body weight is observed. Renal hypoplasia occurs in MALS-3−/− mice backcrossed to either the 129/Sv or C57BL/6 strain and is specific to MALS-3−/−; as kidneys from mice lacking both MALS-1 and MALS-2 are normal in size (Fig. 1D).

Microscopic analysis of adult and newborn MALS-3−/− kidneys reveals dramatic cellular abnormalities in kidney architecture (Fig. 1E; Fig. S1C). MALS-3−/− kidneys display marked tubulointerstitial changes including tubular dilatation and dedifferentiation/simplification (Fig. 1E). Trichrome staining reveals interstitial fibrosis (Fig. 1F; Fig. S1D), a condition characterized by the accumulation of extracellular matrix proteins and fibroblasts, which is a hallmark of end-stage renal disease (Kalluri and Neilson, 2003; J. M. Lee et al., 2006). Renal cysts and fibrosis in MALS-3−/− are accompanied by a loss of epithelial cell polarity. The Na+/K+ ATPase, which is normally localized basolaterally, becomes diffusely localized in dedifferentiated tubular epithelia (Fig. 1G).

MALS-3−/− mice also manifest defects in renal function (Table I). Compared with MALS-3+/−, MALS-3−/− mice exhibit a considerable increase in urine output and sodium excretion. Creatine clearance, a measure of glomerular function, was modestly decreased whereas blood urea nitrogen (BUN) was elevated (Table II). This is likely a consequence of extracellular fluid volume contraction resulting from the polystasis. Urine concentrating ability, measured as the response to 12-h water deprivation and vasopressin injection after a high water load, was also impaired; urine osmolality only increased 3 ± 0.9-fold (1,024 ± 490 mOsm/l change) in MALS-3−/− mice compared with 7.2 ± 0.9-fold (2,764 mOsm/l + 332 mOsm/l in MALS-3−/− (Fig. S1E)). Together with the histopathological observations, these results are reminiscent of nephropathy, the most common genetic disorder of progressive renal failure in children.

MALS-3 localizes to epithelial cells along the length of the nephron

To define the pathogenesis of these kidney defects, the cellular distribution of MALS-3 protein was examined in kidney sections. MALS-3 is present in epithelial cells of renal tubules but is absent from glomeruli (Fig. 2). In the kidney cortex, MALS-3 occurs in collecting duct epithelia that label for aquaporin-2 (Fig. 2B) and proximal tubule epithelia that stain intensely for phallolidin (Fig. 2C). In proximal tubule cells, MALS-3 localizes to the basolateral membrane and the tight junction (Fig. 2, C and D; Fig. S2A, available at http://www.jcb.org/cgi/content/full/jcb.200702054/DC1). In the inner medulla, MALS-3 is present in tubule cells of the loop of Henle and collecting duct (Fig. 2E). In collecting duct epithelia, MALS-3 is restricted to the basolateral membrane and is not detected at the tight junction (Fig. 2, F and G). As expected, MALS-3 staining is absent in MALS-3−/− mice (Fig. 2, B, C, E, and F; and Fig. S2A).

MALS-3 interacts with apical and basolateral polarity complexes

To define the proteins associated with MALS-3, we conducted immunoprecipitation proteomics. Immunoprecipitation of MALS-3 from adult MALS-3−/− kidney homogenates shows a series of specific protein bands that are absent from MALS-3+/− immunoprecipitates (Fig. 3A). As determined by mass spectrometry, MALS-3 interacts with several proteins found in complexes...
that determine cell polarity. MALS-3 immunoprecipitates contain DLG and CASK, proteins that localize to the basolateral surface, and PALS-1, PALS-2, PALS-4 (Mpp7), and PATJ, proteins that localize to tight junctions. Western blotting confirmed all of these interactions and demonstrated comunduprecipitation of CRB-3, the prototypical tight junction polarity protein, with similar recovery (Fig. 3 B).

To characterize the role of MALS-3 in these polarity complexes, we first assessed protein levels in MALS-3−/− kidneys. Strikingly, levels of all components of the CRB-3 tight junction complex are drastically diminished in MALS-3−/− kidneys (Fig. 3 C). CRB-3 itself is decreased by 60% and PALS-1 and PATJ are each decreased by ~80% (Fig. 3, C and D). In contrast, aPKCζ and PAR-3, members of a distinct tight junction polarity complex, were unchanged in MALS-3−/−. Levels of basolateral proteins, LGL, CASK, and DLG are also significantly decreased (Fig. 3, C and D). Structural proteins of the tight junction, claudin-7 and -8, are unaffected in MALS-3−/−, while ZO-2 is modestly decreased. E-cadherin and β-catenin of the adherens junctions are also unchanged (Fig. 3, C and D). MALS-2 protein is up-regulated in kidneys of MALS-3−/−, which has also been noted in brains of MALS mutants (Misawa et al., 2001).

Figure 2. MALS-3 localizes at the basolateral membrane and tight junction of renal epithelia. (A) Schematic depicting the arrangement of tubular segments in the kidney. ATL, ascending thin limb; CD, collecting duct; DCT, distal convoluted tubule; DTL, descending thin limb; G, glomerulus; LH, loop of Henle; PT, proximal tubule; TAL, thick ascending limb. (B) Low magnification fluorescent images from the cortex of kidney sections from a 6-wk-old mouse reveal that MALS-3 (red) primarily localizes to the basolateral membrane of tubular structures. MALS-3 staining is absent from MALS-3−/−. AQP2 (green) specifically labels collecting ducts. (C) High magnification of proximal tubules shows a prominent brush border (phalloidin, green); DAPI (blue) stains nuclei. In proximal tubules, MALS-3 (red) is at both the basolateral membranes (arrowheads) and the tight junctions (arrows). (D) Cartoon summarizing MALS-3 localization in proximal tubules. Brush border and tight junction ( TJ) are shown in green and yellow, respectively. (E and F) Low and high magnification images of the inner medulla show basolateral localization (arrows) of MALS-3 in collecting ducts (AQP2 positive tubules; green). (G) Cartoon depicting AQP2 (green) and MALS-3 (red) localizations in collecting ducts. Bars are 50 μm in B and E; 10 μm in C and F.

Figure 3. MALS-3 interacts with both apical and basolateral polarity complexes in the kidney. (A) Silver staining of kidney extracts immunoprecipitated with anti-MALS-3 antibody shows a series of proteins from MALS-3+/− homogenates that are absent from MALS-3−/− precipitates. (B) Western blotting of extracts immunoprecipitated with anti-MALS-3 antibody confirms specific association of CASK, DLG, PALS, PATJ, and CRB-3 with MALS-3. (C) Kidney homogenates from MALS-3−/− and MALS-3+/− mice immunoblotted for numerous proteins associated with MALS-3 and with epithelial cell junctions. (D) Quantification of protein levels in C. The number of blots quantified for each protein is in parentheses. In A–D, proteins of the CRB, PAR-3, and DLG complexes are shown in red, blue, and green, respectively. Asterisks denote P values < 0.05.
MALS-3 L27 domain mediates assembly of CRB and DLG complexes

Through L27 domain interactions, MALS-3 associates directly with CASK and PALS (Kaech et al., 1998; Kamberov et al., 2000; Lee et al., 2002), which in turn bind to DLG and PATJ (Lee et al., 2002; Roh et al., 2002), respectively. Interestingly, levels of DLG and PATJ, which do not directly bind to MALS-3, are reduced to a greater or similar extent as CASK and PALS (Fig. 3, C and D). This suggests that MALS-3 may mediate cooperative assembly of these L27 domain complexes.

To explore this biochemically, the L27 domains of CASK and DLG were expressed in the absence of the MALS L27 domain in bacteria. Under these conditions, the CASK/DLG binary complex is substantially degraded upon isolation and further deteriorated within 24 h at 4°C (Fig. 4 A). Inclusion of the MALS L27 domain promotes formation of a MALS/CASK/DLG L27 complex that is stable for several days at 35°C (Fig. 4 A). Furthermore, the MALS/CASK/DLG ternary complex was more resistant to urea denaturation than either the MALS/CASK or DLG/CASK binary complexes (Fig. 4 B). MALS also stabilized the MALS/PALS/PATJ L27 complex (unpublished data). The oligomeric nature of protein complexes containing four L27 domains has not been determined. NMR showed that the isolated CASK and DLG L27 domains form a “dimer of dimers” structure (Feng et al., 2004), which might imply high order oligomerization of L27 domain complexes containing three proteins. However, gel filtration analysis shows that the MALS/CASK/DLG complex elutes as a single peak corresponding to ~38 kD (Fig. 4 C), implying a 1:1:1 stoichiometry. Similarly, the tandem PALS L27 domains elute with MALS L27 and PATJ L27 as a single peak corresponding to a 1:1:1 stoichiometry (unpublished data).

The CRB-3/PATJ/PALS polarity complex requires MALS-3

We next asked whether loss of MALS-3 affects the apico-basal polarization of its interaction partners. PALS, PATJ, and CRB-3 are enriched at tight junctions of proximal tubule epithelia (Fig. 5, A–D). Consistent with Western blotting results, PALS and PATJ staining is lost from tight junctions in MALS-3−/− (Fig. 5, A, B, and D); CRB-3 staining is reduced, and the remaining protein concentrates abnormally in punctate vesicles in the subapical region (Fig. 5, C and D). These major defects are not observed in collecting duct. In collecting duct epithelia, PALS and PATJ staining is lost from tight junctions in MALS-3−/− (Fig. 5, A, B, and D); CRB-3 staining is reduced, and the remaining protein concentrates abnormally in punctate vesicles in the subapical region (Fig. 5, C and D). These major defects are not observed in collecting duct. In collecting duct epithelia, PALS and PATJ staining is reduced, but not lost, at the tight junction of the knockout, and CRB-3 localization appears normal (Fig. S3, A–D; available at http://www.jcb.org/cgi/content/full/jcb.200702054/DC1). Molecular compensation does not account for the localization of the CRB complex in MALS-3−/− collecting duct epithelia, as MALS-1 and -2 are not detected (Fig. S3 E).

Loss of MALS-3 specifically disrupts the CRB complex. In proximal tubule and collecting duct epithelia, aPKCζ, PAR-3, and ZO-1 remain at the tight junction (Fig. S3 H, Fig. S4, A, B, and D; and unpublished data). Other junction-associated proteins are also unaltered in renal epithelia from MALS-3−/− (Fig. S4, E and F).

Figure 4. The L27 domain of MALS mediates cooperative formation of a stable MALS/CASK/DLG ternary complex. (A) Purification of a recombinant binary complex containing the single L27 domain of DLG and the tandem L27 domains of CASK shows degradation (asterisk, lane 1) and further degrades over 24 h at 4°C (asterisk, lane 2). Co-expression of MALS L27 domain stabilizes the MALS/CASK/DLG complex (lane 3), which does not degrade after 3 d at 35°C. (B) Comparison of the urea-induced denaturation profile of the L27DLG/L27NL27CASK/L27MALS complex with those of the L27NL27CASK and L27N-NL27DLG complexes. The ellipticities of each spectrum at 222 nm were used to construct the denaturation curves. The MALS/CASK/DLG ternary complex is more stable than the binary L27 domain complexes. (C) The tandem L27 domains of CASK assemble MALS and DLG into a stable 1:1:1 MALS/CASK/DLG ternary complex with a molecular mass of ~38 kD over a wide concentration range. Cartoon inset shows stoichiometry and molecular oligomerization of the MALS/CASK/DLG complex.
MALS-3 mediates apical repulsion of DLG

The distribution DLG undergoes complex changes in MALS-3−/− (Fig. 6). Normally, DLG is predominantly distributed basolaterally in proximal tubule epithelia (Fig. 6, A, C, and D) and in more distal tubular segments of the nephron (Fig. 6, A and B; Fig. S3 F). Weaker tight junction and cytoplasmic staining is also observed in control animals (Fig. 6 C). In MALS-3−/−, DLG is lost from the basolateral surface and becomes abnormally concentrated at tight junctions of epithelia in proximal tubules (Fig. 6, A, C, and D; and Fig. S2 B) and in more distal tubules (Fig. 6, A and B; Fig. S3 F). Other basolateral polarity proteins, scribble and LGL, remain concentrated along the basolateral membrane (Fig. S4 C, Fig. S3 G; and unpublished data).

Kidney organogenesis in MALS-3−/−

The resemblance of the MALS-3 null phenotype to human nephronophthisis raises the possibility that MALS-3 may be involved in a common pathway with the six known nephronophthisis genes (NPHP1–6), which affect the organization and/or development of primary cilia (Torres et al., 2007). Consistent with this idea, CRB-3 localizes to cilia and C. elegans lacking crb2b, a CRB-3 homologue, exhibit renal hypoplasia, cysts, and tubular dilatation. Renal abnormalities in crb2b mutants are associated with malformation and dysfunction of primary cilia (Omori and Malicki, 2006). A conserved role for CRB-3 in cilia formation in mammalian epithelia has been demonstrated (Fan et al., 2004). Although CRB-3 is lost from the tight junction in renal epithelia lacking MALS-3, we found that CRB-3 localizes properly to primary cilia (Fig. 7 B), suggesting an alternative mechanism for targeting CRB-3 to cilia. Consistent with this hypothesis, PALS and PATJ are not detected in cilia (unpublished data) and the morphology of primary cilia appears unaltered in MALS-3−/− mice (Fig. 7 A). Analysis of EST databases (http://www.ncbi.nlm.nih.gov) revealed an alternatively spliced variant of CRB-3 (Fig. 7 C). The resulting protein product, CRB-3b, shares identical extracellular and transmembrane domains with canonical CRB-3, but has a divergent cytoplasmic tail. Importantly, the splicing variant results in a longer C-terminal tail that lacks the PDZ ligand (Fig. 7 C) required for PALS/PATJ interaction (Bhat et al., 1999; Roh et al., 2002). Interestingly, the CRB-3 antibody recognizes a doublet in kidney homogenates, with the larger product, likely CRB-3b, being more moderately reduced in MALS-3−/− (Fig. 3 C).

Normalcy of ciliary structure and CRB localization do not exclude that more specific aspects of ciliary function are compromised in these animals (Hildebrandt and Zhou, 2007).

Dysfunction of primary cilia and associated centrosomes can impair cell proliferation (Simons and Walz, 2006) and thereby reduce kidney size in MALS-3 mutants. To determine whether cell proliferation is altered in MALS-3−/− mice, mitotic cells in kidneys from E14.5 embryos were labeled using anti-phospho-histone3 antibody. We found no difference in cell proliferation between MALS-3−/− (0.388 ± 0.013) and MALS-3−/− (0.375 ± 0.013; P = 0.61) littermates (Fig. 8, A and B), excluding reduced cell division as the cause for the smaller kidney size.

Next, we examined whether changes in apoptosis/cell death contribute to this phenotype. Apoptotic cells are found in nephrogenic regions (Koseki et al., 1992) of E14.5 kidneys from both control and MALS-3−/− littermates (Fig. 8 C). However, abnormal cell death was detected in cells underlying the renal capsule in MALS-3−/− kidneys (Fig. 8, C and D), resulting in a
10-fold increase in apoptotic signal from MALS-3-/- (5.80 ± 0.76) as compared with MALS-3+/− kidneys (0.42 ± 0.19; P = 0.007). These changes are detected as early as E12.5 (unpublished data), indicating that increased apoptosis causes reduced kidney size in MALS-3-/- mice.

**MALS-3 polarizes epithelia derived from the metanephric mesenchyme**

The profound disruption of the CRB-3 complex in proximal tubule cells and the normalcy of PALS and CRB-3 localization in the collecting duct suggest that MALS-3 serves distinct functions in these two epithelial cell types. Proximal tubule and collecting duct cells derive from different tissue types (Dressler, 2006). During embryonic development, the ureteric bud extends from the Wolffian duct, invades the metanephric mesenchyme, and induces mesenchymal to epithelial cell transition. Tubules derived from metanephric mesenchyme extend from the glomerulus to the distal tubule, whereas the collecting duct derives from the ureteric bud (Fig. 9 A).

To assess the role of MALS-3 in each of these embryonic tissues, a new MALS-3 floxed allele was created (Fig. S5, A–C, available at http://www.jcb.org/cgi/content/full/jcb.200702054/DC1). Breeding these MALS-3 flox/flox mice to transgenics that express cre recombinase under the control of the HoxB7 or Pax3 promoter (Fig. 9 B) allows deletion of MALS-3 specifically from ureteric bud (MALS-3 flox/flox; HoxB7-Pro-cre) [UBKO] (Yu et al., 2002) or metanephric mesenchyme (MALS-3 flox/flox; Pax3-Pro-cre) [MMKO] (Li et al., 2000; Chang et al., 2004) (Fig. S5, D–F).

Levels of MALS-3 are differentially reduced in UBKO and MMKO mice (Fig. 9 C; Fig. S5, D–F). Importantly, gross abnormalities in kidney structure are only noted in the MMKO mice. Kidneys from MMKO mice are reduced in size (Fig. 9 D) with renal pathologies identical to MALS-3-/- mice (Fig. 9 E). These abnormalities are not observed in the UBKO mice (Fig. 9, D and E). Finally, in MMKO proximal tubules, but not UBKO, the CRB-3 and DLG complexes are affected (Fig. 9 F and unpublished data) in a manner similar to MALS-3-/-.

**Kidney organogenesis in MMKO**

Kidneys from adult MMKO mice phenocopy those of MALS-3-/- mice, indicating a shared developmental defect. Like the MALS-3-/-, kidneys from MMKO embryos (E14.5) show extensive apoptosis underlying the renal capsule (Fig. 10 A).
To determine whether defects in the CRB-3 and DLG polarity complexes underlie these developmental abnormalities, we examined the expression levels of these protein complexes in kidneys from MMKO embryos. Western blotting showed that both the CRB and DLG complexes were dramatically reduced (Fig. 10 B). These results show that MALS-3 is a critical component of the CRB and DLG complexes and that disruption of these complexes in the metanephric mesenchyme is associated with the renal defects in MALS-3−/− mice.

**Discussion**

This study shows that MALS-3 is essential for polarization of renal epithelia and for kidney development. Kidneys from...
MALS-3/− mice display tubule dysgenesis, interstitial fibrosis, and cysts. These pathologies arise from defective polarization of tubule epithelia derived from the metanephric mesenchyme. MALS-3 mediates polarization of tubular epithelia through interactions with the CRB and DLG polarity complexes. Loss of MALS-3 disrupts polarized expression of these two critical protein complexes, establishing MALS-3 as an essential component of each. Furthermore, these data demonstrate that primary abnormalities in epithelial polarization can cause renal dysgenesis, fibrosis, and cysts.

**MALS-3 is a component of epithelial CRB-3 and DLG polarity complexes**

LIN-7 was originally identified in *C. elegans* as a gene essential for vulval development (Simske et al., 1996). In vulval precursor cells, LIN-7 is one component of a heterotrimeric PDZ protein complex that includes LIN-2 and LIN-10 (Kaech et al., 1998). Assembly of the complex is mediated through LIN-7, which binds LIN-7 through their complementary L27 domains, and binds LIN-10 through a more N-terminal site (Kaech et al., 1998). Vulval development requires signaling by an EGF-like receptor (LET-23) that binds the PDZ domain of LIN-7 (Simske et al., 1996). The LIN-2/-7/-10 complex localizes LET-23 to the basolateral surface where it binds its ligand (Kaech et al., 1998). Mutations in *lin-2, -7, or -10* mislocalize LET-23 to the apical domain and block vulval induction.

The LIN-2 (CASK)/LIN-7 (MALS)/LIN-10 (MINT-1) complex is conserved in mammalian brain, where it occurs both pre- and post-synaptically (Butz et al., 1998; Borg et al., 1999; Jo et al., 1999). Presynaptically, CASK binds to liprin-α (Olsen et al., 2005a). Postsynaptically, the PDZ domain of MALS binds to the C-terminal tail of NMDA receptors (Jo et al., 1999). Altered neurotransmitter release in neurons lacking MALS is consistent with the MALS complex organizing the presynaptic active zone and vesicle cycling (Olsen et al., 2005a). Composition of the MALS complex in kidney differs fundamentally from that in *C. elegans* or brain, as kidney epithelium lacks MINT-1 (Okamoto and Sudhof, 1997). MINT-3 occurs in kidney, but it lacks the CASK interaction domain (Okamoto and Sudhof, 1997). MINT or liprin-α isoforms were not detected in MALS complexes isolated from kidney (Fig. 3).

Our work shows that MALS-3 occurs in two conserved epithelial cell polarity complexes and differentially colocalizes with these complexes in specific renal cell populations. In proximal tubule epithelia, MALS localizes to the tight junction—likely owing to its association with the CRB complex. *crb* was the first epithelial polarity gene characterized in *Drosophila* (Tepass et al., 1990). It is a transmembrane protein whose C-terminal tail interacts with the PDZ domain of stardust, SDT (PALS) (Roh et al., 2002), or dPATJ (PATJ) (Bhat et al., 1999). SDT is a membrane-associated guanylate kinase (MAGUK) that contains two L27 protein motifs (Kamberov et al., 2000). The first and second L27 domains of SDT bind to the L27 domains of dPATJ (Roh et al., 2002) and MALS (Kamberov et al., 2000), respectively.

Additionally, MALS associates with the basolateral DLG complex. In invertebrates, DLG mediates apico-basal polarization of epithelia and controls imaginal disc proliferation (Woods and Bryant, 1991). DLG is a MAGUK containing a single L27 domain that binds to the first of two L27 domains in CASK.
MALS-3 mutants suggests that MALS-3, through its interaction with PALS (Kamberov et al., 2000), assembles the tight junction complex. Our results are supported by studies in MDCK cells that show MALS-3 knockdown disrupts the localization of PALS and PATJ (Straight et al., 2006). The current study makes several important advances on the role of MALS-3 in regulation of the CRB-3 complex. First, we show that the loss of PALS and PATJ in proximal tubule epithelia lacking MALS-3 is accompanied by a specific loss of CRB-3 from the tight junction. Importantly, these changes are not conserved in collecting duct epithelia, from which MDCK cells derive. This may explain why previous studies of MALS in MDCK cells have not described changes in CRB-3 expression, localization, or function (Straight et al., 2006). We also show that disruption of the CRB-3 complex results from the instability of the unbound PALS L27C domain, but direct interactions between MALS-3 and PATJ or CRB-3 cannot be ruled out. Finally, we show that CRB-3 properly localizes to cilia in these MALS-3–deficient epithelia. An alternatively spliced CRB-3 variant, CRB-3b that lacks the PDZ ligand, likely accounts for these discrete localizations at the tight junction and cilia.

In addition to regulating assembly of the CRB complex, MALS-3 directs basolateral localization of DLG. The molecular mechanism underlying DLG mislocalization remains uncertain. MALS-3 associates with DLG through the MAGUK scaffolding protein CASK (Lee et al., 2002; Leonoudakis et al., 2004), suggesting that MALS-3 may directly control basolateral expression of DLG. In support of this, DLG and CASK communoprecipitate with MALS-3 from kidney homogenates. Alternatively, DLG/LGL complexes that cross the apico-basal boundary may be recruited to aPKC by MALS-3. This recruitment may result in phosphorylation of LGL and restriction of the DLG/LGL complex to the basolateral membrane. Alternatively, the CRB complex may be required to apically exclude DLG. Fitting with this model, overexpression of CRB can rescue DLG polarization in C. elegans (Segbert et al., 2004).

**Polarity defects in MALS-3** are restricted to specific renal epithelia

Why does MALS-3 differentially polarize in distinct epithelial cell populations? In proximal tubule epithelia derived from metanephric mesenchyme, MALS-3 is expressed at the tight junction and basolateral membrane, whereas in cells derived...
from ureteric bud, MALS-3 is exclusively detected along the basolateral membrane. In *Drosophila*, cell-specific mechanisms have been identified for the MALS homologue, DLIN-7 (Bachmann et al., 2004). Here, the PALS homologue (SDT) associates with DLIN-7 at epithelial junctions, whereas *Drosophila* DLG interacts with DLIN-7 at the neuromuscular junction (Bachmann et al., 2004). Ectopic expression of SDT or DLG mediates DLIN-7 delivery to epithelial and neuromuscular junctions, respectively. This suggested that junctional targeting of MALS is downstream of both SDT and DLG (Bachmann et al., 2004). Our results are not consistent with this model. Loss of MALS-3 in proximal tubule epithelia causes dissolution of the CRB complex and redistribution of DLG, demonstrating that MALS is not always downstream of either PALS or DLG. Instead, MALS may primarily determine polarization in specific cell populations. In such a model, the PDZ binding partner for MALS could serve as the tight junction anchor. Identification of physiological PDZ ligands for MALS may help understand its cell type-specific functions.

Does MALS determine polarity in tissues other than kidney? In *Drosophila* with mutations to components of the CRB complex, numerous ectodermally derived epithelia are disorganized and degenerated (Tepass et al., 2001). The viability and fertility of MALS-3 knockout mice indicate that many epithelial cell populations develop and function properly without MALS-3. The additional MALS isoforms may serve redundant functions; indeed, MALS-deficient mice die from respiratory failure (Olsen et al., 2005a). That these mice show grossly normal size and morphology (Olsen et al., 2005a) suggests that MALS are not essential for polarizing all cell populations. Future studies of MALS triple-knockout mice should help define which cell types require MALS for polarization and determine how other pathways compensate or work in parallel.

**Implications for renal development and disease**

Our studies reveal that renal defects arise solely from a loss of MALS-3 in epithelia derived from metanephric mesenchyme. MALS-3 is also present in epithelial cells derived from ureteric bud (Olsen et al., 2005b), but restricted ablation of MALS-3 in the ureteric bud neither causes changes in renal morphology nor disrupts CRB-3 polarization. By contrast, loss of MALS-3 in the metanephric mesenchyme is sufficient to recapitulate the renal pathologies observed in MALS-3−/− mice. Localization of MALS-3 to the tight junctions in renal epithelia of mesenchymal origin suggests that tight junction-associated MALS-3 underlies the renal defects observed in MALS-3−/−.

Mesenchymal to epithelial transition (MET) is a critical process underlying the development of numerous organs, including the kidney. Differentiation of epithelia from mesenchyme requires the establishment cell polarity. In MMKO mice, polarization cues originating from CRB and DLG complexes are disrupted. This likely leads to changes in the efficacy of epithelial cell polarization during MET, resulting in apoptosis of smaller kidneys in MALS-3 mutants. However, most renal epithelia in MALS-3−/− show normal cytoarchitecture. This indicates that redundant or parallel pathways compensate, albeit incompletely, for loss of the CRB and DLG complexes. Further analysis of MALS-3−/− embryos will be useful for determining the developmental processes regulated by these polarity complexes.

The converse of MET may underlie renal tubule simplification/dedifferentiation and fibrosis in MALS-3 mutants. Recent studies suggest that epithelial to mesenchymal transition (EMT) of tubule epithelia is a significant source of disease-related fibroblasts (Kalluri and Neilson, 2003; J.M. Lee et al., 2006). A critical step in EMT is a loss of cell polarity. Because MALS-3 epithelia are improperly polarized, uncontrolled EMT could account for the dedifferentiated tubular epithelia and fibrosis in MALS-3 mutants. Alternatively, renal epithelia lacking MALS-3 may be increasingly susceptible to external cues, such as injury or inflammation, which can trigger EMT (Kalluri and Neilson, 2003; J.M. Lee et al., 2006). As defects in epithelial polarization and associated MET/EMT play central roles in renal cysts and fibrosis, MALS-3−/− mice should provide an important new model for understanding and treating these common kidney diseases.

**Materials and methods**

**Antibodies**

Isoform specific and pan antibodies against MALS-1, -2, and -3 were generated in rabbits as described previously (Misawa et al., 2001). Anti-SAP-97 antibody was generated in rabbit (Topinka and Bredt, 1998), for immunohistochemistry an anti-SAP-97 antibody from Morgan Sheng (Massachusetts Institute of Technology, Cambridge, MA) was used. Rabbit anti-JAMS-α and anti-claudin antibodies were purchased from Invitrogen. PALS, PAL, and Crumbs-3 antibodies were gifts from Ben Margolis (University of Michigan, Ann Arbor, MI) and rabbit anti-LGL antibody was provided by Valeri Vasoukhin (Fred Hutchinson Cancer Research Center, Seattle, WA). Rabbit anti-PAX2 was from BAbCO. Goat anti-aquaporin-2 antibody and rabbit anti-mPKCγ were from Santa Cruz Biotechnology, Inc. Rabbit anti-PAR-3 and anti-phospho-histone3 antibodies were from Upstate Biotechnology and goat anti-scribble antibody was from GeneTex, Inc. Mouse anti-CASK, anti-ZO-2, anti-β-catenin, anti-E-cadherin, and anti-N-cadherin were all purchased from Transduction Laboratories. Alexa-conjugated Phalloidin and secondary antibodies were purchased from Molecular Probes and mouse anti-pancytokeratin was from Sigma-Aldrich.

**Mouse lines**

The following mouse lines were used in this study: STOCK Gt(Rosa)26SORTm1(Smo/EYFP)Amc/J, STOCK Tg(HoxB7-cre)13Amc/J, 129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym/J, C57BL/6, and 129/Sv were purchased from The Jackson Laboratory. Pax3-Pro-Cre mice were a gift from Valeri Vasoukhin (Fred Hutchinson Cancer Research Center, Seattle, WA). Rabbit anti-PAX2 was from BAbCO. Goat anti-aquaporin-2 antibody and rabbit anti-mPKCγ were from Santa Cruz Biotechnology, Inc. Rabbit anti-PAR-3 and anti-phospho-histone3 antibodies were from Upstate Biotechnology and goat anti-scribble antibody was from GeneTex, Inc. Mouse anti-CASK, anti-ZO-2, anti-β-catenin, anti-E-cadherin, and anti-N-cadherin were all purchased from Transduction Laboratories. Alexa-conjugated Phalloidin and secondary antibodies were purchased from Molecular Probes and mouse anti-pancytokeratin was from Sigma-Aldrich.

**Isolation of MALS-3 genomic DNA and construction of targeting vector**

Isolation of MALS-3 genomic DNA and construction of targeting vector is described previously (Olsen et al., 2005a). For construction of the targeting vector, a 1.5-kb region encoding the targeted exons (the fourth and fifth exon) of MALS-3 was PCR-amplified, digested with ClaI, and subcloned into the Clal site of pks2loxPFRTNT (a gift from Shinya Yamanaka, Kyoto University, Kyoto, Japan). A 1.4-kb region downstream from the targeted exons was PCR-amplified, digested with BamHI and EcoRI, and subcloned into the BamHI-EcoRI sites of pks2loxPFRTNT. Finally, a 5.8-kb genomic region upstream to the targeted exons was PCR-amplified, digested with KpnI and BamHI, and inserted into the KpnI-Sall sites of the pks2loxPFRTNT vector. Two loxP sites flank targeted exons and a neomycin cassette. Two FRT sites flank the neo cassette for its removal.

**Generation of MALS-3 loxP mice**

The targeting vector was linearized and electroporated into R1 ES cells. Clones resistant to G418 and gancyclovir were analyzed for recombination by PCR. To ensure proper homologous recombination, PCR-positive clones resistant to G418 and gancyclovir were analyzed for recombination by PCR. To ensure proper homologous recombination, PCR-positive...
clones were further analyzed by Southern blotting using probes containing genomic sequences outside of the targeting vector and with a neo probe. Properly targeted clones were injected into blastocysts from C57BL6 mice and transferred to surrogate mothers (Transgenic Facility, Stanford University, Stanford, CA). Male chimeras were mated with 129S4/SvJae-<sup>Slc2a6</sup>Sor-Gr<sup>ROSA26Sor-Tm1(1)Usp</sup>Dym-J (The Jackson Laboratory) females for transmission of the mutated allele through the germ line and for removal of the neomycin cassette from the targeted allele. Genotypes for MALS-3 and MALS-3<sup>loxp</sup> mice lacking the neomycin cassette were determined by Southern blotting or by PCR using the primers: 5'-GAAATGTGTCCTGTCGTTGTC-3' and 5'-ATGCTGTCACACTGTCGTGGCC-3', which yields a 280-bp product for the wild-type allele and a 350-bp product for the targeted allele. Presence of the cre-recombinase and EYFP transgenes was determined using the following primer pairs: 5'-GAAATGTGTCCTGTCGTTGTC-3' and 5'-ATTGCTGTCACACTGTCGTGGCC-3' for cre and 5'-CCTGGAAGGTACATGTCA-CC-3' and 5'-GGACTTGTACAGCTGTCATGC-3' for EYFP.

Protein expression and purification

Genes corresponding to the L27 domain of rat DLG (L27<sub>DLG</sub>, residues 1–65), the L27 domain of mouse MALS (L27<sub>MALS</sub>, residues 2–78), and the tandem L27 domain of rat CASK (L27<sub>N27/CASK</sub>, residues 329–460) were PCR amplified from the respective full-length cDNAs. The single-chain fusion protein, containing L27<sub>DLG</sub>, L27<sub>N27/CASK</sub>, and L27<sub>MALS</sub> connected with a thrombin-cleaveable segment (Leu-Val-Pro-Arg-Gly-Ser-Ser-Gly), was cloned into a modified version of the pET32a vector in which the S-tag and the thrombin recognition site were replaced by a sequence encoding a protease 3C cleavage site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro). Similarly, the single chain fusion protein, containing L27<sub>DLG</sub> and L27<sub>N27/CASK</sub>, connected with a thrombin-cleaveable segment, was PCR-amplified and inserted into the modified pET32a vector. Bacterial cells harboring the fusion protein expression plasmid were grown at 37°C, and protein expression was induced by IPTG at the same temperature for 3 h. The His-tagged, thioredoxin-containing protein was purified under native conditions using nickel-NTA agarose (QIAGEN) affinity chromatography. After protease 3C digestion the His-tagged thioredoxin were removed by passing the digestion mixture through an S-200 gel filtration column, then the single-chain protein was digested by thrombin and the L27 domain complex protein was further purified through the same size-exclusion column. The expression and purification of L27<sub>MALS</sub>/L27<sub>N27/CASK</sub> and L27<sub>N27/CASK</sub>/L27<sub>DLG</sub> complex proteins were described previously (Feng et al., 2004, 2005).

Kidney immunohistochemistry

Adult mice were anesthetized and perfused with 2% paraformaldehyde in PBS. Kidneys were removed, immersed in the same fixative for 2 h at 4°C, and then cryoprotected in PBS containing 30% sucrose overnight at 4°C. Frozen sections (10 μm) were rehydrated into PBS containing 1% Triton X-100 (PBSX) for 20 min and incubated for 1 h in blocking solution (PBSX containing 1% BSA). Primary and secondary antibodies were diluted in blocking solution and incubated overnight at 4°C and 2 h at room temperature, respectively. Antibody incubations were followed by three washes in PBSX and then incubated with secondary antibodies in Fluoromount-G (Southern Biotechnology Associates, Inc.). Images were taken with a confocal microscope (LSM 5 Pascal Axioplan 2; Carl Zeiss Microimaging, Inc.) under Zeiss 63× 1.4 or 40× 1.3 oil-immersion objectives or a Zeiss 10× 0.3 objective at room temperature and acquired using LSM 5 Pascal (v.3.2) software. Images were processed using Adobe Photoshop. To study primary molecular defects in MALS<sup>3/−</sup> mice, only kidney sections that showed normal arrangement of tubules with mild anatomical abnormalities were used for localization of pAFC/Crumbs/DLG complex proteins. More than three sections from six KO and six control littersmates were examined. Proximal tubules were identified by phalloidin staining of apical brush border, whereas collecting ducts were stained using aquaporin-2 antibody.

For hematoxylin and eosin or trichrome staining, perfused kidneys were immersed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated using an alcohol series and xylene, and embedded in paraffin. Paraffin sections (4 μm) were cut on a microtome and mounted on slides. All light images were taken using an AxioCam HRC camera mounted to a microscope (Eclipse E600; Nikon) with 10× 0.3 and 20× 0.5 objectives at room temperature. Images were acquired with AxioVision (v.4.6) software and processed in Adobe Photoshop.

Renal function

Mice were housed in metabolic cages on a 12 h light-dark cycle and fed ad libitum on normal chow. After 24 h, urine was collected under oil, and aliquots were analyzed on a JASCO J-720 spectropolarimeter at room temperature. The sample contained 15 μM protein in buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT).

Immunoprecipitations

Adult mice kidneys from MALS<sup>3/−</sup> or MALS<sup>3/−</sup> mice were homogenized in three volumes of STE buffer (320 mM sucrose, 20 mM Tris, pH 7.4, and 2 mM EDTA) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin and 200 μg/ml PMSF. Homogenates were centrifuged at 20,000 g for 1 h and pellets were resuspended in TET buffer (20 mM Tris, pH 8.0, 1 mM EDTA, and 1.3% Triton X-100) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin and 50 μg/ml PMSF. Lysates were pelleted at 100,000 g for 1 h. Precollected lysates were immunoprecipitated with 5 μg of MALS-3 antibody or control rabbit IgG overnight at 4°C. To collect immunoprecipitated protein complexes, 80 μl of a 50% protein A-Sepharose slurry was added to the lysates and incubated for 1 h at 4°C. Immunoprecipitates were washed extensively and loaded onto SDS-PAGE to separate the proteins. Gels were either silver stained or transferred to nitrocellulose for Western blotting.

Nano-LC-ESI-Qq-TOF tandem mass spectrometry analysis

Gel bands were reduced with 10 mM dithiothreitol (DTT) followed by alkylation with 55 mM iodoacetamide. Proteins were digested with trypsin and extracted with a 50% acetonitrile/5% formic acid solution. The peptides were dried down and rehydrated using an alcohol series and xylene, and embedded in paraffin. Paraffin sections (4 μm) were cut with a microtome and mounted on slides. All light images were taken using an AxioCam HRC camera mounted to a microscope (Eclipse E600; Nikon) with 10× 0.3 and 20× 0.5 objectives at room temperature. Images were acquired with AxioVision (v.4.6) software and processed in Adobe Photoshop. To study primary molecular defects in MALS<sup>3/−</sup> mice, only kidney sections that showed normal arrangement of tubules with mild anatomical abnormalities were used for localization of pAFC/Crumbs/DLG complex proteins. More than three sections from six KO and six control littersmates were examined. Proximal tubules were identified by phalloidin staining of apical brush border, whereas collecting ducts were stained using aquaporin-2 antibody.

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Cell death and proliferation

To detect dying cells, LysoTracker (Invitrogen) labeling of embryonic kidney

Kidney immunohistochemistry

Adult mice were analyzed by perfusion with 2% paraformaldehyde in PBS. Kidneys were removed, immersed in the same fixative for 2 h at 4°C, and then cryoprotected in PBS containing 30% sucrose overnight at 4°C. Frozen sections (10 μm) were rehydrated into PBS containing 1% Triton X-100 (PBSX) for 20 min and incubated for 1 h in blocking solution (PBSX containing 1% BSA). Primary and secondary antibodies were diluted in blocking solution and incubated overnight at 4°C and 2 h at room temperature, respectively. Antibody incubations were followed by three washes in PBSX and then incubated with secondary antibodies in Fluoromount-G (Southern Biotechnology Associates, Inc.). Images were taken with a confocal microscope (LSM 5 Pascal Axioplan 2; Carl Zeiss Microimaging, Inc.) under Zeiss 63× 1.4 or 40× 1.3 oil-immersion objectives or a Zeiss 10× 0.3 objective at room temperature and acquired using LSM 5 Pascal (v.3.2) software. Images were processed using Adobe Photoshop. To study primary molecular defects in MALS<sup>3/−</sup> mice, only kidney sections that showed normal arrangement of tubules with mild anatomical abnormalities were used for localization of pAFC/Crumbs/DLG complex proteins. More than three sections from six KO and six control littersmates were examined. Proximal tubules were identified by phalloidin staining of apical brush border, whereas collecting ducts were stained using aquaporin-2 antibody.

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Online supplemental material

Figure S1 shows renal cysts in kidney sections from adult and embryonic MALS−/− mice as well as urine concentrating ability in mutant mice. Figure S2 shows the colocalization of MAI5 and Dlg with the tight junction. Figure S3 shows partial disruption of polarity complexes in collecting ducts of MALS−/−. Figure S4 shows that localization of PAR-3, aPKC, and SCRIB does not require MALS−/−. Figure S5 depicts the generation and characterization of the MALS−/− floxed allele. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200702054/DC1.

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