Novel roles for mucin 1 in the kidney

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Purpose of the review.

Recent studies in the kidney have revealed that the well-characterized tumor antigen mucin 1 (MUC1/Muc1) also has numerous functions in the normal and injured kidney.

Recent findings.

Mucin 1 is a transmembrane mucin with a robust glycan-dependent apical targeting signal and efficient recycling from endosomes. It was recently reported that the TRPV5 calcium channel is stabilized on the cell surface by galectin-dependent cross-linking to mucin 1, providing a novel mechanism for regulation of ion channels and normal electrolyte balance.

Our recent studies in mice show that mucin 1 is induced after ischemia, stabilizing HIF-1α and β-catenin levels, and transactivating the HIF-1 and β-catenin protective pathways. However, prolonged induction of either pathway in the injured kidney can proceed from apparent full recovery to chronic kidney disease. A very recent report indicates that aberrant activation of mucin1 signaling after ischemic injury in mice and humans is associated with development of chronic kidney disease and fibrosis. A frame-shift mutation in MUC1 was recently identified as the genetic lesion causing Medullary Cystic Kidney Disease type 1, now appropriately renamed MUC1 Kidney Disease (MKD).

Summary.

Studies of mucin 1 in the kidney now reveal significant functions for the extracellular mucin-like domain and signaling through the cytoplasmic tail.

Key words.

Mucin 1, MUC1, Muc1, MUC1 Kidney Disease, acute kidney injury, chronic kidney disease, TRPV5, inflammation

Key points (3-5)

1. Mucin1 membrane trafficking insures its apical localization, and galectin binding to mucin 1 glycans enhances crosslinking and surface stability of channels such as TRPV5.
2. A global frame-shift mutation in mucin 1 causes only kidney disease, while mice lacking mucin 1 are seemingly normal but more susceptible to stressors.
3. Mucin1 induced during ischemia-reperfusion injury of the kidney stabilizes HIF-1α and β-catenin, thereby enhancing the associated protective pathways.

4. Transient increases in mucin 1 enhance recovery from acute kidney injury, but prolonged increases lead to chronic kidney disease and fibrosis.
5. Mucin 1 is a cell surface barrier to bacteria and viruses but also limits inflammation by regulating the NLRP3 inflammasome and TLR signaling.

Introduction

Mucin 1, known as MUC1 in humans and Muc1 in other mammals, is a multi-functional protein expressed on the apical surface of most epithelial cells (for review, see [1-4]). The numerous biological activities attributed to MUC1/Muc1 in promoting cell growth and survival, are sometimes overshadowed by its name and classification as a *tethered mucin*. MUC1/Muc1 does undergo autocatalytic cleavage early in its biogenesis yielding a large mucin-like subunit (>300 kDa) composed of a variable number of tandem repeats (VNTR, range 40-140) that provides cell surface protection from bacteria, viruses and other environmental insults [5-9]. MUC1 glycans on this subunit also bind to galectins which are associated with crosslinking cell surface proteins and thereby preventing endocytosis [10-12] (Fig. 1). The smaller transmembrane subunit (25 kDa) includes the 72-residue cytoplasmic tail with sites for (i) binding adaptors for endocytosis and recycling, (ii) phosphorylation by kinases, and (iii) docking proteins involved in either signaling or transcriptional transactivation by trafficking to the nucleus (Fig. 2) [1-3,13-15]. The cytoplasmic juxtamembrane sequence (CQCRRK) is implicated in nuclear targeting of mucin 1 by disulfide crosslinking of MUC1 dimers, thereby forming a non-conventional nuclear targeting sequence despite the reported dual Cys-palmitoylation of this CQC motif [13,16].

The highest expression of MUC1 is reported in the stomach>lung>kidney>esophagus>colon> pancreas>breast tissues, etc. based on gene expression (RPKM, reads per kilobase per million reads from *gtexportal.org*). Interestingly, MUC1 is also expressed in immune cells including B cells, T cells, monocytes, macrophages and dendritic cells [17-20]. However, MUC1 activities have been primarily characterized in tumor cells where high expression levels in tumors correlate with a poor prognosis for the patient due to its ability to promote cell growth and survival [21-23]. In fact, the National Cancer Institute has priority-ranked MUC1 at #2 on a list of cancer vaccine target antigens where major criteria were immunogenicity, oncogenicity and therapeutic function [24]. Epithelial tumors in Muc1 global knockout (KO) mice exhibit reduced growth when compared to congenic controls consistent with a role for mucin 1 in promoting tumor growth and survival [25].

Under normal conditions the Muc1 KO mice have no obvious phenotype except that they are more sensitive to bacterial infections [26,27]. For this reason, the studies of MUC1/Muc1 function in stomach and lung have focused primarily on infection of either cell lines or Muc1 KO and congenic control mice with *Helicobacter pylori* and *Pseudomonas aeruginosa*, respectively (discussed below). The function of Muc1/MUC1 in kidney has been more difficult to address as direct infection by bacteria and virus is much less common. While sepsis does stress the kidney primarily by indirect means, the most well characterized model of acute kidney injury is produced by clamping both the renal artery and vein, or the artery alone, to produce ischemia, and studying recovery of kidney function and morphology after return of blood flow for hours to days [28-30]. Using this approach, we found that Muc1 is protective in the kidney in a mouse model of ischemia-reperfusion injury by transactivation of the HIF-1 and β-catenin protective pathways [31,32].

Lessons learned from MUC1 Kidney Disease (MKD)

A frame-shift mutation in MUC1 (MUC1-fs) was recently identified as the genetic lesion causing Medullary Cystic Kidney Disease type 1 (MCKD1 variant of autosomal dominant tubule-interstitial kidney disease) [33]. As these patients rarely have cysts, the disease has been appropriately renamed autosomal dominant tubule-interstitial kidney disease due to MUC1 mutations, with an abbreviated name of Mucin 1 Kidney Disease (MKD) [34,35]. The most common causative mutation is a cytosine duplication in a string of seven cytosines within any one of the G-C-rich tandem repeats. This cytosine insertion results in a frameshift mutation, producing a chimeric protein with the N-terminus of normal MUC1 and a C-terminus with a unique highly basic repeating sequence rich in Cys and His [33]. The frame-shift in mutant MUC1 (MUC1-fs) also places a stop codon after the repeats. Although the chimera should be secreted, it is found as intracellular staining by imunohistochemistry in patient kidney tissue and as diffuse and/or fine granular intracellular staining by immunofluorescence microscopy with puncta co-localizing with wild-type MUC1 staining at the apical surface [33]. Patients with MKD present with asymptomatic elevation of serum creatinine, exhibit bland urinary sediment with minimal blood or protein, non-specific tubulointerstitial fibrosis, a gradual decline in glomerular filtration rate, and a need for dialysis between the third and eighth decade of life [35,36]. Most notably, the patients usually have a family history of chronic kidney disease and exhibit only renal disease despite the presence of MUC1-fs in multiple organs. The basis for the highly variable age of disease onset is an active area of research and could be influenced by both the reduced levels of normal MUC1 and the accumulation of mutant MUC1-fs within the cell.

A role for MUC1 in surface expression of transporters

Uromodulin (UMOD, also known as Tamm-Horsfall protein) is the most abundant protein in human urine and mutations in uromodulin are the most common genetic cause of autosomal dominant tubule-interstitial kidney disease. These patients exhibit reduced urinary UMOD due to its intracellular accumulation in the thick ascending limb (TAL), as well as hypouricosuric hyperuricemia, hypertension, renal fibrosis and progressive renal failure. Mice expressing UMOD with the corresponding human mutations also have reduced urinary UMOD but exhibit hypercalciura, renal calcium crystals and reduced immunofluorescence staining of the renal calcium channel TRPV5, which is localized to the distal convoluted tubule (DCT) and collecting ducts (CD) [37]. Studies in transiently transfected HEK293 cells revealed that current density of TRPV5 was enhanced by either co-expression with UMOD or by addition of exogenous UMOD, through a mechanism that reduced TRPV5 endocytosis and increased its cell surface expression. The data are consistent with a role for UMOD after shedding from the TAL in directly stabilizing TRPV5 in the distal segments of the tubule.

As MUC1 is also expressed in the TAL and found in the urine while the mutant MUC1 accumulates within cells as described for UMOD mutations, similar studies were carried out in HEK293 cells to determine if MUC1 can also enhance activity of the renal TRPV5 channel. Interestingly, urinary MUC1 was also reduced in patients with calcium nephrolithiasis, a common type of kidney stone, supporting the possibility that shed MUC1 could have a role in enhancing calcium reabsorption [37]. Transient expression of MUC1 in HEK293 cells revealed a MUC1 dose-dependent increase in TRPV5 surface currents associated with reduced TRPV5

endocytosis and stabilization at the cell surface [37]. Earlier studies revealed that TRPV5 surface expression is also enhanced by binding galectin-1 after Klotho-dependent removal of sialic acid from TRPV5, and by galectin-3 binding to the N-glycan of TRPV5 [38,39]. These more recent studies in HEK293 cells revealed that MUC1 enhancement of TRPV5 surface expression proceeds by galectin-3-dependent crosslinking of O-glycans on MUC1 with the N-glycan on TRPV5, whereas galectin-1 had no role [37].

MUC1 does have an exceptionally strong glycan-dependent apical targeting signal that can re-direct a basolaterally expressed protein to the apical cell surface in polarized epithelia; and the rate of MUC1 recycling from endosome to the cell surface is quite efficient and significantly higher than its rate of endocytosis (4-fold) [13,40]. It is therefore possible that the MUC1 mucin-like subunit directly enhances surface expression of additional channels by a similar mechanism of crosslinking and maintenance at the cell surface. For example, a large genome-wide association study focused on serum concentrations of cations revealed that the highest association with low serum magnesium levels (hypomagnesemia) was a very common genetic variant of MUC1 that adds nine amino acids to the extracellular N-terminus of the protein (*MUC1* SNP: rs4072037 with coded allele frequency of 0.46) [41]. A Single nucleotide polymorphism (SNP) in the magnesium transporter TRPM6 was also associated with low serum magnesium but to a lesser extent than the MUC1 variant [41]. Interestingly, the MUC1 SNP was associated with higher bone mineral density and lower fasting glucose levels which could proceed by a direct interaction of either the transmembrane MUC1 or shed MUC1 with transporters within the kidney tubule [41].

MUC1 has been previously implicated in enhancing glucose uptake and metabolism. Transfection of pancreatic tumor cells with MUC1 enhanced uptake of [^{3}H]2-deoxyglucose, while knockdown of MUC1 in liver tumor cells reduced uptake [42]. Injection of the pancreatic tumor cells into mice produced tumors with significantly higher uptake of dye-coupled 2-deoxyglucose than tumors lacking MUC1. The MUC1-expressing tumor cells had increased levels of the glucose transporter GLUT-1, as well as increased levels of HIF-1α and LDHA, and increased staining with Ki67, a marker of cell prolilferation. Studies using the same pancreatic tumor cells in culture were the first to reveal that MUC1 (small subunit) stabilizes HIF-1α by direct binding and enhances expression of downstream targets of HIF-1 by enhancing promoter occupancy of genes that shift metabolism in response to hypoxia [42]. Clearly, MUC1 can also modulate metabolism and solute transport by transcriptional means that alter the expression of enzymes and transporters. While Wang et al. [43] showed that intestinal uptake and absorption of cholesterol was significantly reduced in Muc1 KO mice compared with the wild-type mice by an undefined mechanism, Nath et al. [44] showed that MUC1 up-regulates multidrug resistance genes including the *ABCC1* gene that encodes a cholesterol efflux pump, by direct binding to the promoter region of the *ABCC1* gene in pancreatic cancer cells.

Lessons learned from Muc1 activities during acute kidney injury.
The mouse model of ischemia-reperfusion injury (IRI) results in significant damage to primarily the proximal tubule (PT) that is normally well-oxygenated by the extensive renal vasculature in the cortex. Deep sequencing of microdissected adult rat renal tubule segments revealed that

MUC1 was present in collecting duct (CD)> medullary loop of Henle> thick ascending limb (TAL)> distal convoluted tubule (DCT)>>>> PT (based on RPKM) [45]. We also found the highest levels of Muc1 in the DCT, CD and TAL in the kidneys of sham treated mice using immunohistochemistry (IHC), but we also found Muc1 staining at very low levels in the PT that was absent in the Muc1 KO mouse kidney [31]. Muc1 appeared in the cytoplasm of all tubule epithelia immediately after 19 min ischemia, and was found in the nuclei after 4 h recovery [31]. Total Muc1 levels as assessed by immunblotting kidney homogenates was increased 4.2-fold after 3 d recovery when Muc1 staining by IHC was observed on the apical surface of flattened cells in the recovering proximal tubule [31]. The appearance of Muc1 on the apical surface of the recovering PT at 3 d recovery is even more convincing using immunofluorescence microscopy and co-staining with antibodies against the organic ion transporter 1 (OAT1) localized on the basolateral surface (Fig. 3). Published studies indicate that mucin 1 is expressed on the apical surface of polarized epithelial cells in the proximal tubule during development and potentially after injury, such that our findings in this mouse model is consistent with previous reports [46,47].

A role for Muc1 in effective recovery from ischemic injury in the proximal tubule is supported by the fact that mice do not efficiently recover kidney function when Muc1 is absent [31]. When we followed sCr levels in mice for 3 d after 19 min ischemia, we found that sCr peaked after 24 h in both Muc1 knockout (KO) and congenic control mice and returned to normal in control mice, but not Muc1 KO mice, at 3 d recovery. The appearance of regenerating flatten cells in the proximal tubule after 3 d was also absent in Muc1 KO mice [31]. As MUC1 is known to co-immunoprecipitate with the transcription factors HIF-1α and β-catenin in tumor cell lines and prevent their degradation [42,48], we assessed levels of these two proteins in whole kidney homogenates and discovered that Muc1 stabilizes HIF-1α and β-catenin in the injured kidney [31,32].

HIF-1α was previously observed in the TAL and CD of sham-treated rats and appeared in the PT during IRI [49], tubule segments where we observed Muc1 expression during IRI [31]. Staining for HIF-1α was noticeably reduced in kidneys of Muc1 KO mice after 4 h recovery when compared to control mice, consistent with reduced HIF-1α levels (32%) measured by immunoblotting kidney tissue [31]. Induction of downstream targets of the HIF-1 protective pathway involved in a shift of metabolism from oxidative phosphorylation to glycolysis, were also aberrant in Muc1 KO kidneys during IRI [31]. MUC1 stabilization of HIF-1α and transactivation of HIF-1 target genes was previously reported in studies of pancreatic tumor cells [42]. In line with this, we found that levels of AMP-activated protein kinase (AMPK) were higher in Muc1 KO kidneys when compared to controls, and AMPK activation by phosphorylation (phosphor-Thr^{172} AMPK-α) was prolonged, indicating metabolic stress in the absence of Muc1 [31].

MUC1 is also reported to stabilize β-catenin in tumor cells by blocking its phosphorylation by glycogen synthase kinase (GSK) 3β [48]. Consistent with this, we found that induction of β-catenin during IRI was blocked in Muc1 KO mice while GSK3β activity was increased [32]. Targeting of β-catenin to the nucleus during IRI was also completely blocked in Muc1 KO mice,

as was induction of the β-catenin protective pathway including stimulation of prosurvival factors (activated Akt, survivin, transcription factor T cell factor 4 (TCF4) and downstream target cyclin D1), and repression of proapoptotoc factors (p53, active Bax and cleaved caspase-3) [32].

Both HIF-1α and β-catenin are induced during ischemic injury in the kidney tubule epithelial cells and transactivate complex protective pathways [50,51]. This was established in part by finding that kidney injury and recovery is worse in mice with tubule knockout of either HIF-1α or β-catenin [51-53]. However, prolonged induction of either HIF-1α or β-catenin in response to kidney injury can progress from apparent full recovery to chronic kidney injury with fibrosis. While studies in mice have shown that stabilization of HIF-1α expression improves the kidney's response to ischemia, prolonged HIF activation through knockout of the von Hippel-Lindau E3 ligase, promotes interstitial fibrosis [50,54]. In turn, genetic ablation of HIF-1α blocked development of fibrosis in a unilateral ureteral obstruction (UUO) model of injury [55]. Haase has also identified a significant correlation of percent of tubular cells expressing HIF-1α and the stage of kidney nephropathy in diabetic patients [56].

Xiao et al. [57] have now established that moderate ischemia (20 min) in a mouse model of IRI causes a transient induction of β-catenin and apparent full recovery of kidney morphology and function, while a more severe ischemia (30 min) produced a prolonged elevation of β-catenin and progression to fibrosis after just ten days. Using mouse models, cultured kidney cells, and human biopsy specimens, Gibier et al. [58] now report that prolonged aberrant activation of mucin 1 signaling is associated with the development and progression of chronic kidney disease (CKD) including fibrosis. In their mouse model, sustained activation of Muc1 is associated with induction of epithelial-to-mesenchymal transition (EMT) features such as fibronectin, type I collagen, and *Snail 1*, which have been previously reported to cause renal fibrosis in alternative mouse models of kidney injury [59-61]. Furthermore, there was a positive correlation between MUC1 expression and expression of EMT markers in human biopsies which also exhibit more interstitial fibrosis levels [58]. This correlation could be explained by the role of mucin 1 in prolonged transactivation of both the HIF-1 and β-catenin protective pathways. Altogether, the data indicate that an early and transient activation of mucin 1 signaling appears to be renoprotective by promoting repair and recovery of kidney function, while sustained activation of mucin 1 appears to promote renal fibrosis and accelerate AKI to CKD progression [58].

The promoter of the mucin 1 gene contains HIF-responsive elements and is induced by hypoxia in a HIF-1-dependent manner [62]. HIF-1 also induces β-catenin expression [63]. As mucin-1 stabilizes both HIF-1α and β-catenin [1,48], it is clear that mucin 1 is a key figure in the kidney's normal recovery from transient ischemia and aberrant response to chronic ischemia, progressing to EMT and fibrosis.

Lessons learned from Muc1 activities in other tissues.
Mucin 1 is highly expressed on the lung and gastric mucosal surfaces, and there is considerable evidence that mucin 1 provides both a physical barrier to potential pathogens, and modulates inflammatory and immune responses to bacterial infection. For example, Muc1 binds directly to

the flaggelins of *Pseudomonas aeruginosa* (PA) and to adhesins on H. pylori, common pathogens in lung and stomach, respectively [8,64]. However, mucin 1 protection of these organs is primarily through its anti-inflammatory actions. PA stimulates alveolar macrophages to release TNF-α, and TNF-α induces mucin 1 levels in airway epithelial cells (AEC). PA also stimulates AEC to secrete TGF-α that activates the EGF receptor (EGFR), the EGFR phosphorylates mucin 1, and mucin 1 then associates with toll like receptor (TLR) 5 [65]. Mucin 1 expressed on macrophages also regulates the host immune system during *H. pylori* infection and limits gastritis by negatively regulating NLRP3 inflammasome activity that promotes IL-1β production [66]. This protective effect of mucin 1 is mediated by its interaction with TLRs, effecting NF-κB signaling and inhibition of IRAK4 activation [66]. As mucin 1 is known to limit inflammation by regulating the NLRP3 inflammasome and TLR signaling, future studies will address this role during kidney injury.

CONCLUSION
Mucin 1 is essential for both normal renal function and recovery from injury. Galectin cross-linking of mucin 1 with transporters such as TRPV5 provides a novel mechanism for regulation of ion balance. In response to ischemia, mucin 1 levels increase and stabilize both HIF-1α and β-catenin to potentiate downstream protective pathways, although prolonged increases lead to chronic kidney disease with fibrosis. As mucin 1 is reported to regulate inflammation in other tissues and the immune system by suppressing the NLRP3 inflammasome and TLR signaling, these topics should be the focus of future studies of renal injury.

Conflict of interest
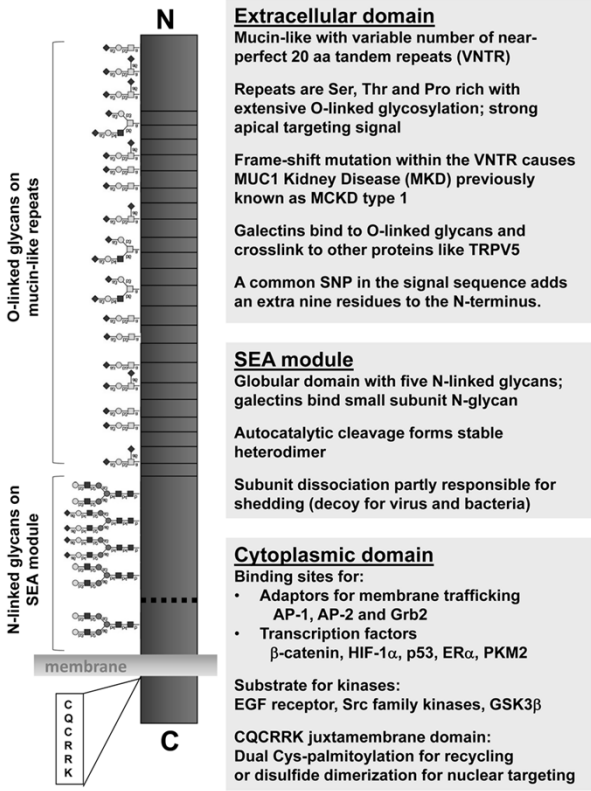The authors declare that they have no conflicts of interest.

FIGURE LEGENDS

Figure 1. Mucin 1 structure and functional domains. Mucin 1 is a type 1 transmembrane protein with a cleaved signal sequence and cytoplasmic C-terminus. The extracellular domain includes a SEA (sea urchin sperm protein, enterokinase and agrin) module with five N-linked glycans that exhibits autocatalytic cleavage yielding a heterodimeric structure (dashed line). The large mucin-like subunit also includes a variable number of tandem repeats (VNTR) that are rich in Pro and O-glycosylated Ser and Thr residues. The smaller subunit includes the transmembrane domain and cytoplasmic tail detailed in Figure 2. Functions attributed to each domain are noted with details in the text.

Figure 2. Functional activities of the mucin 1 cytoplasmic tail. The 72-residue cytoplasmic domain is numbered from the transmembrane domain due to the extracellular VNTR. MUC1 binding of Grb2 at $pY^{60}TNP$ and the clathrin adaptor AP-2 at $Y^{20}HPM$ is required for endocytosis, while binding of clathrin adaptor AP-1 at $Y^{20}HPM$ and dual palmitoylation of the CQC^3 motif is required for recycling from endosomes back to the cell surface [13,14]. Mucin dimerization through the CQC^3 motif yields a nuclear targeting signal based on the adjacent RRK [16]. Peptides based on the CQCRRK motif can block MUC1 activities in the nucleus [67]. The site of IKKβ interaction is indicated which initiates NF-κB activation [68]. β-catenin binding to a canonical $SXXXXXSSLS^{59}$ site is(i) enhanced by PKCδ phosphorylatin of Thr^{41} and Tyr^{46} phosphorylation by either the EGF receptor or Src family kinases, and (ii) blocked by Ser^{44} phosphorylation by GSK3β that binds at $SXXXS^{44}$ (for review, see [1-3]).

Figure 3. Mucin 1 is expressed in the proximal tubule during ischemia-reperfusion injury. Mice were subjected to 19 min ischemia and recovery for t=0 or 3 days. Kidneys were fixed in PFA, embedded in paraffin, subjected to antigen retrieval, and incubated with rabbit anti-OAT1 (organic anion transporter 1) antibodies (red) to label the basolateral surface of the proximal tubule cells (PT), and with Armenian hamster CT-2 anti-MUC1 cytoplasmic tail antibodies (green). Nuclei were stained blue. Muc1 appears on the apical surface of flatten cells in the recovering proximal tubule at 3 d recovery (yellow arrows). Glomeruli (G) are also indicated.
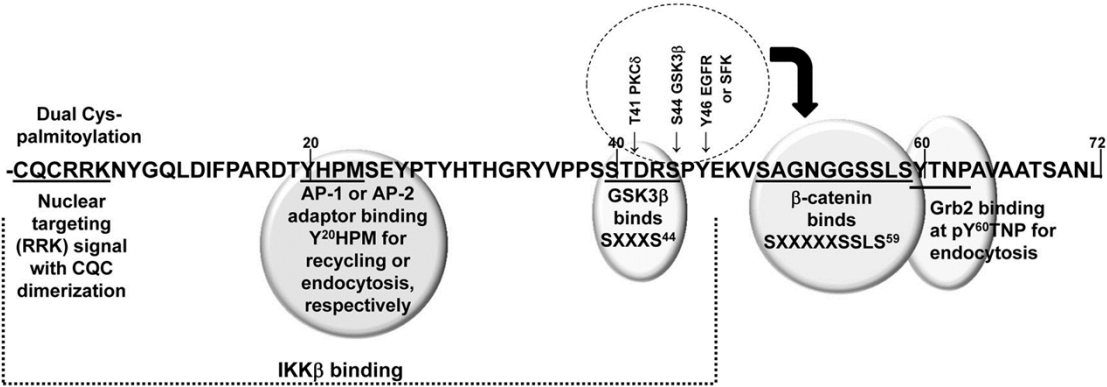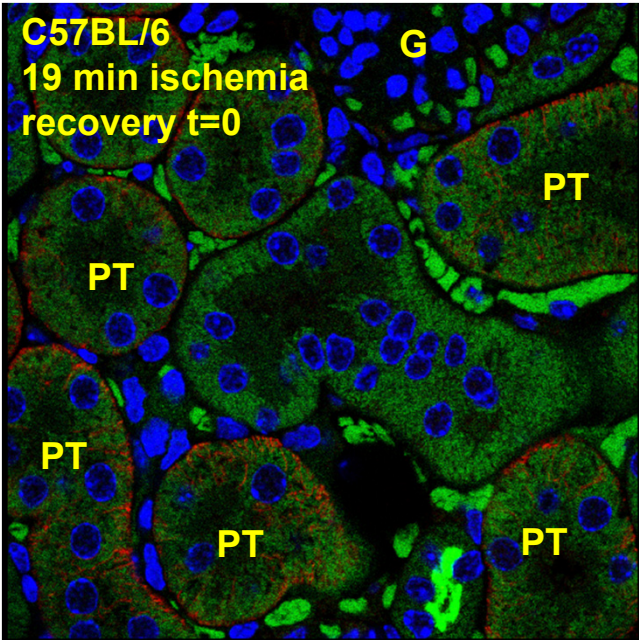
FIGURE 1

N

O-linked glycans on mucin-like repeats

N-linked glycans on SEA module

membrane

C
Q
C
R
R
K

C

<u>Extracellular domain</u>
Mucin-like with variable number of near-perfect 20 aa tandem repeats (VNTR)

Repeats are Ser, Thr and Pro rich with extensive O-linked glycosylation; strong apical targeting signal

Frame-shift mutation within the VNTR causes MUC1 Kidney Disease (MKD) previously known as MCKD type 1

Galectins bind to O-linked glycans and crosslink to other proteins like TRPV5

A common SNP in the signal sequence adds an extra nine residues to the N-terminus.

<u>SEA module</u>
Globular domain with five N-linked glycans; galectins bind small subunit N-glycan

Autocatalytic cleavage forms stable heterodimer

Subunit dissociation partly responsible for shedding (decoy for virus and bacteria)

<u>Cytoplasmic domain</u>
Binding sites for:
- Adaptors for membrane trafficking
  AP-1, AP-2 and Grb2
- Transcription factors
  β-catenin, HIF-1α, p53, ERα, PKM2

Substrate for kinases:
EGF receptor, Src family kinases, GSK3β

CQCRRK juxtamembrane domain:
Dual Cys-palmitoylation for recycling
or disulfide dimerization for nuclear targeting

FIGURE 2

Dual Cys-palmitoylation

Nuclear targeting (RRK) signal with CQC dimerization

T41 PKCδ
S44 GSK3β
Y46 EGFR or SFK

-CQCRRKNYGQLDIFPARDTYHPMSEYPTYHTHGRYVPPSSTDRSPYEKVSAGNGGSSLSYTNPAVAATSANL-

20

40

60

72

AP-1 or AP-2 adaptor binding $Y^{20}HPM$ for recycling or endocytosis, respectively

GSK3β binds $SXXXS^{44}$

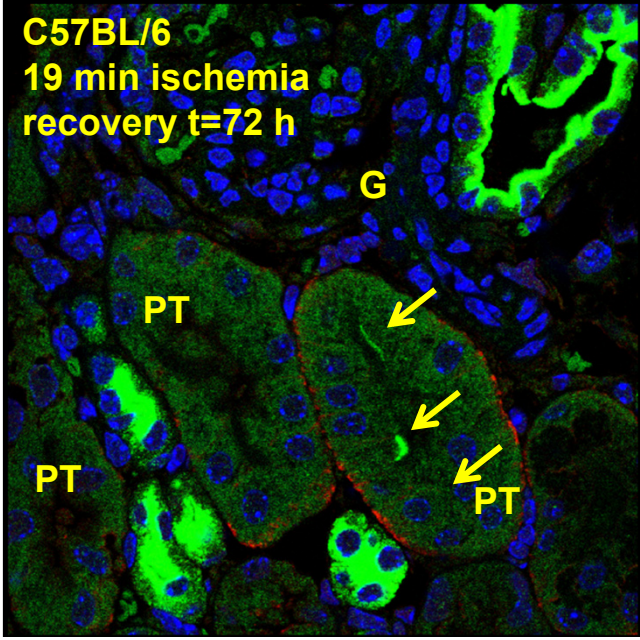β-catenin binds $SXXXXXSSLS^{59}$

Grb2 binding at $pY^{60}TNP$ for endocytosis

IKKβ binding

FIGURE 3

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* An earlier publication (Ref 10) reported that MUC1 large subunit interacted best with Gal-3 and Gal-9 while the small subunit interacted best with Gal-3. In this study, a chimera carrying MUC1 repeats with the apical targeting signal interacted best with Gal-9, which is essential in MDCK cells forming polarized monolayers. The functional significance of MUC1 binding Gal-3 is highlighted in Ref 37.

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