Activated renal tubular Wnt/β-catenin signaling triggers renal inflammation during proteinuria

Dickson W. L. Wong[1,2], Wai Han Yiu[1,2], Kam Wa Chan[1,2], Ye Li[1], Bin Li[1], Sarah W.Y. Lok[1], Makoto M. Taketo[3], Peter Igarashi[4], Loretta Y. Y. Chan[1], Joseph C. K. Leung[1], Kar Neng Lai[1] and Sydney C. W. Tang[1,2*]

[1]Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong

[2]The University of Hong Kong Shenzhen Institute of Research and Innovation, China

[3]Division of Experimental Therapeutics, Graduate School of Medicine, Kyoto University, Yoshida-Konoé-cho, Sakyo, Kyoto 606-8501, Japan

[4]Department of Medicine, University of Minnesota Medical School, Minneapolis, Minnesota 55416, USA

*Corresponding author

Sydney C.W. Tang, Division of Nephrology, Department of Medicine,

The University of Hong Kong, Queen Mary Hospital,

102 Pokfulam Road, Hong Kong.

Phone: +852-22553879; Fax: +852-28162863; Email: scwtang@hku.hk

Running title: Activated Wnt/β-catenin in proteinuric nephropathy

Word Count: 227 (abstract), **4007** (text)

Figures: 10; Table: 1 Keywords: Wnt/β-catenin, renal inflammation, proteinuric nephropathy

ABSTRACT

Imbalance of Wnt/β-catenin signaling in renal cells is associated with renal dysfunction, yet the precise mechanism is poorly understood. Previously we observed activated Wnt/β-catenin signaling in renal tubules during proteinuric nephropathy with an unknown net effect (rescue *vs.* damage?). To identify the definitive role of tubular Wnt/β-catenin, we generated a novel transgenic "Tubcat" mouse, which conditionally expresses stabilized β-catenin specifically in renal tubules after tamoxifen administration. Four weeks after tamoxifen injection, uninephrectomized Tubcat mice displayed proteinuria and elevated BUN levels comparing to non-transgenic mice, implying a detrimental effect of the activated signaling. This was associated with tubulointerstitial infiltration predominantly by M1 macrophages and overexpression of the inflammatory chemocytokines CCL-2 and RANTES. Induction of overload proteinuria by low-endotoxin BSA injection, after uninephrectomy, for 4 weeks aggravated proteinuria and increased BUN levels to a significantly greater extent in Tubcat mice. Renal dysfunction correlated with the degree of M1 macrophage infiltration in the tubulointerstitium and renal cortical up-regulation of CCL-2, IL-17A, IL-1β, CXCL1 and ICAM-1. There was overexpression of cortical TLR-4 and NLRP-3 in Tubcat mice, independent of BSA injection. Finally, there is no fibrosis formation or activation of epithelial-mesenchymal-transition or non-canonical Wnt pathways being observed in the Tubcat kidney. In conclusion, conditional activation of renal tubular Wnt/β-catenin signaling in a novel transgenic mouse model demonstrates that this pathway enhances intrarenal inflammation via the TLR-4/NLRP-3 inflammasome axis in overload proteinuria.

INTRODUCTION

Persistent albuminuria is a surrogate marker of progressive chronic kidney disease (CKD) and its amelioration has been consistently associated with improved renal outcomes in both diabetic and non-diabetic CKD.[1] Excessive protein trafficking across the glomerular filtration barrier causes tubulotoxicity and drives progressive nephropathy[2] by triggering renal inflammation and subsequent fibrosis leading to eventual organ failure.[3]

In health, albumin is reabsorbed via receptor-mediated endocytosis by the proximal tubule followed by lysosomal degradation.[4, 5] In CKD patients, excessive albumin trafficking overwhelms the albumin handling capacity of renal tubules and mandates an array of detrimental intracellular signaling cascades in tubular cells,[6-9] including the hyperproduction of numerous chemocytokines that confer a pro-inflammatory microenvironment to enhance the transmigration of immune cells.[10] In particular, the macrophage was postulated to be the origin of myofibroblast accumulation that perpetuates excessive extracellular matrix protein deposition in the renal tubulointerstitium.[11]

Wnt/β-catenin signaling is highly conserved during embryonic development[12] and regulates different organogenesis.[13] There are several non-canonical Wnt pathways[14] which may cross talk with the Wnt/β-catenin signaling but the mechanisms are still rudimentary. Reports on the role of aberrant canonical Wnt/β-catenin signaling in different nephropathies have yielded contradicting results,[15-18] such that a consensus has not been reached.[19] Both podocyte-specific β-catenin overexpression or knock-out[15] and tubule-specific β-catenin knock-out mice[20] developed more severe renal dysfunction. Therefore, it has been postulated that an imbalance of Wnt/β-catenin signaling gives rise to nephropathy.[21]

65   Activated Wnt/β-catenin signaling is defined by stabilization and nuclear translocation of β-

66   catenin followed by transcription of Wnt target genes. To avoid excessive accumulation of β-

67   catenin in the cell, the serine/threonine sites on β-catenin, encoded by exon 3 within the *Catnb*

68   allele,[22] are phosphorylated which will trigger ubiquitination and proteasomal degradation.[23]

69   Recent data support a reciprocal link between renal fibrosis and activated canonical Wnt/β-

70   catenin signaling pathway in renal tubular cells.[24, 25] In this regard, we previously reported

71   dynamic renal tubular Wnt/β-catenin signaling in proteinuric nephropathy.[26] Activated signaling

72   occurred during the early onset of disease but regressed during the later phase. This signified that

73   suppressed Wnt/β-catenin activity is associated with heightened tubular cell death and

74   exacerbates proteinuria. Regardless of the fibrogenesis, activated Wnt/β-catenin signaling

75   participates in a range of inflammatory diseases.[27-29] In addition, renal inflammation was

76   observed in the early phase of proteinuric nephropathy in mice[30] and rats.[9, 31]

77   To provide a definitive role of Wnt/β-catenin signaling in kidney inflammation associated with

78   heavy proteinuria, we generated a transgenic mouse strain which could be induced with

79   tamoxifen to overexpress β-catenin exclusively in renal tubules, namely the Tubcat mouse.

80   Tubcat mice were subjected to protein overload which recapitulates many features of CKD. We

81   demonstrated that over-activated Wnt/β-catenin signaling in renal tubules exacerbates renal

82   dysfunction. Importantly, the activated signaling *per se* is sufficient to propagate macrophage

83   infiltration and increase chemocytokine levels in the kidney. In the protein overload model,

84   Tubcat mice demonstrated further renal dysfunctions with more intense renal inflammation.

85

86

87 **RESULTS**

88 *Generation and characterization of a conditional Tubcat mouse (Ksp-CreER^{T2}-Catnb$^{lox(ex3)/wt}$)*

89 We successfully generated the first mouse strain that conditionally overexpresses β-catenin in

90 renal tubules upon tamoxifen administration. As shown in Supplementary Figure 1A, mice

91 carrying *loxP* sites flanking the exon 3 sequence of *Catnb* with (Tubcat) or without the *Ksp-Cre*

92 allele (Cre$^{-/-}$; non-transgenic) were genotyped. Four weeks after tamoxifen administration,

93 genomic DNA was extracted from the kidney, heart, spleen and liver. An additional PCR product

94 corresponding to the recombined *Catnb* allele (~500bp) was evident in the genomic DNA of

95 Tubcat mice from the kidney (Supplementary Figure 1B), but not in the tissue of heart, spleen

96 and liver. This smaller size of the PCR product indicated the absence of exon 3 in the *Catnb*

97 allele (*Catnb$^{\Delta exon3}$*) only in the kidney of Tubcat mice. Real-time PCR also showed that the

98 expression of *Catnb exon 3* mRNA was significantly less in the kidney cortex of Tubcat mice

99 versus Cre$^{-/-}$ mice (Figure 1A). Using immunoblotting assay, we detected the mutant form of β-

100 catenin in the renal cortex of Tubcat mice (Figure 1B). Notably, colocalization by

101 immunohistochemistry (IHC) staining of β-catenin and sodium chloride cotransporter (NCC) on

102 the kidney sections (Figure 1C) revealed that up-regulated β-catenin expression was observed in

103 renal tubules (both proximal and distal) of Tubcat mice. These results confirmed the success of

104 the conditional overexpression of β-catenin specifically in the renal tubules of Tubcat mice.

105 *Renal dysfunction in uninephrectomized Tubcat mice*

106 To accelerate the renal injury in the animal model, six- to eight-week-old Tubcat mice and Cre$^{-/-}$

107 mice underwent uninephrectomy followed by tamoxifen injection (Figure 2A). Four weeks after

108 transgenic induction, uninephrectomized (UNX) Tubcat mice expressed significantly more

109 nuclear β-catenin in renal cortex (Figure 2B). UNX-Tubcat mice displayed significantly higher

110 urine albumin-to-creatinine ratio (UACR; 130 ± 29 mg/ug vs. 45 ± 5.7 mg/ug) and blood urea

111 nitrogen (BUN; 37.1 ± 3.2 mg/dL vs. 27.7 ± 1.9 mg/dL) compared to UNX-Cre^{-/-} mice (Figure

112 2D), while there was no difference at baseline before uninephrectomy and tamoxifen injection

113 (Figure 2C). Notably, we observed significant proteinuria and increase in serum BUN level in

114 Tubcat mice, *de novo* to the transgenic effect (Supplementary Figure 2A&B). Acute kidney

115 injury (AKI) markers including *Kim1*, *Cystatin C* and *Ngal* were all up-regulated in the kidney

116 cortex of UNX-Tubcat mice vs. UNX-Cre^{-/-} mice (Figure 2E). Periodic acid-Schiff (PAS)

117 staining revealed glomerular damage in UNX-Tubcat mice, as reflected by diffuse mesangial

118 expansion (Figure 2F) with a higher glomerular damage score (Figure 2G). The glomerular

119 histology associates with significant less nephrin expression, both in gene and protein manner

120 (Figure 2H&I), in the UNX-Tubcat mice. UNX-Tubcat mice displayed significant higher urinary

121 β2-microglobulin level (65.4 ± 11 ng/ml vs. 37.3 ± 1.7 ng/ml), a renal tubular damage marker,

122 compared to uninephrectomized Cre^{-/-} mice (Figure 2J). Collectively, these data demonstrated

123 that enhanced β-catenin expression in renal tubules is detrimental and impairs renal function.

124 ***Overexpression of tubular β-catenin enhanced tubulointerstitial macrophage infiltration***

125 Overexpression of tubular β-catenin in UNX-Tubcat mice resulted in 3-fold increase in

126 macrophage infiltration (CD68+ cells) versus Cre^{-/-} mice (Figure 3A). M1 macrophage markers

127 (*Ly6c* and *Ccr2*) were significantly elevated in the kidneys of UNX-Tubcat mice compared to

128 UNX-Cre^{-/-} mice, while there was no difference in the M2 macrophage markers (*Cd163* and

129 *Cd106*; Figure 3B). Enhanced tubulointerstitial macrophage infiltration in UNX-Tubcat mice

130 was accompanied by up-regulated chemocytokine expression including CCL-2 and RANTES

131 (Figure 3C). IHC staining of CCL-2 and β-catenin on serial kidney sections from UNX-Tubcat

132   mice demonstrated the colocalization of CCL-2 and overexpressed β-catenin in renal tubules

133   (Figure 3D). In line with this proinflammatory phenotype, the expression of TLR-4 and NLRP-3

134   was also increased in the renal cortex of UNX-Tubcat mice at both the gene and protein levels

135   (Figure 3E&F).

136   ***Overexpressed β-catenin does not feedback or activate the non-canonical Wnt pathways***

137   We investigated whether overexpression of β-catenin will impact other non-canonical WNT

138   pathways. The non-canonical Wnt signaling related gene expression including *Camkii*, *Daam1*,

139   *Plcb1*and *Ptk7* were investigated. We speculated *Camkii* and *Damm1* expression was

140   significantly down-regulated in UNX-Tubcat kidney (Figure 4A). In Figure 4B, immunoblotting

141   assays showed the phosphorylation level of several kinases involved in the non-canonical Wnt

142   pathways, including c-Jun N-terminal kinase (JNK) and Ca^{2+}/calmodulin-dependent protein

143   kinase II (CaMKII). We observed less phosphorylated CaMKII in the UNX-Tubcat kidney

144   comparing to UNX-Cre$^{-/-}$ mice. In addition, we did not observe changes in phosphorylation of

145   glycogen synthase kinase-3β (GSK-3β), an upstream regulator of β-catenin in UNX-Tubcat

146   kidney (Figure 4C). Taking together, these data showed the overexpressed tubular β-catenin

147   could not activate non-canonical Wnt pathways or induce feedback regulation of the canonical

148   pathway.

149   ***Uninephrectomized Tubcat mice do not activate epithelial-mesenchymal-transition (EMT) or***

150   ***develop fibrosis in the kidney***

151   To date, a number of articles has asserted the correlation between heightened tubular β-catenin

152   expression and fibrogenesis during kidney injury. Treatment of β-catenin inhibitors could

153   successfully ameliorate the EMT/fibrotic gene expression and extracellular matrix protein (ECM)

154 deposition in kidney and thereby we studied the development of renal fibrosis via direct

155 overexpression of tubular β-catenin in our mouse model. In Figure 5A, we examined the fibrotic

156 markers including fibronectin, α-smooth muscle actin (α-SMA) and collagen I in experimental

157 kidneys using immunoblotting assay. Surprisingly, no enhanced level of these fibrotic markers

158 was detected in UNX-Tubcat mice comparing to UNX-Cre$^{-/-}$ mice. Moreover, there was no

159 change in expression of two EMT markers, vimentin and E-cadherin, in the experimental

160 kidneys (Figure 5B) which was in line with the fibrotic level.

161 *Tubcat mice developed severe proteinuria during protein overload*

162 To recapitulate the impact of protein overload in the renal tubules of CKD patients, an overload

163 proteinuria model was established. After uninephrectomy and tamoxifen injection, high-dose of

164 BSA (10mg/g body weight) or an equal volume of saline was injected intraperitoneally into

165 UNX-Tubcat and UNX-Cre$^{-/-}$ mice for four weeks (Figure 6A). At baseline, all experimental

166 mice had similar UACR and serum BUN level (Figure 6B). Four weeks after protein overload,

167 BSA injected UNX-Cre$^{-/-}$ mice exhibited significantly higher UACR, serum BUN level

168 compared to saline injected UNX-Cre$^{-/-}$ mice (Figure 6C). Importantly, BSA injected UNX-

169 Tubcat mice displayed even higher UACR (1,500 ± 111 ug/mg vs. 689 ± 184 ug/mg), serum

170 BUN levels (58.0 ± 3.7 mg/dL vs. 43.0 ± 5 mg/dL). Similar trend was also observed in urinary

171 β2-microglobulin level (440 ± 74 ng/ml vs. 236 ± 40 ng/ml), suggesting more significant renal

172 damage in UNX-Tubcat mice injected with BSA.

173 *Enhanced tubular β-catenin triggers renal inflammation*

174 Intrarenal expression of the *Catnb* allele (exon 3) was up-regulated in the BSA-injected Cre$^{-/-}$

175 mice compared to control group (Figure 6E), indicating that the enhanced transcriptional activity

176   of the *Catnb* allele was due to BSA administration. As a result of Cre-lox recombination, its

177   expression was significantly lower in Tubcat mice compared to Cre$^{-/-}$ mice when both were

178   injected with BSA. Loss of exon 3 of *Catnb* allele stabilized β-catenin in the cell; thus

179   conceivably more β-catenin could translocate into the nucleus and mediate its transcriptional

180   function. Immunoblotting assay indicated the nuclear β-catenin level in the kidney was up-

181   regulated in BSA-injected Cre$^{-/-}$ mice compared to the control, while BSA-injected Tubcat mice

182   expressed the highest level of nuclear β-catenin (Figure 6F). Heightened β-catenin expression

183   was mainly localized to the renal tubules of BSA-injected Cre$^{-/-}$ mice (Figure 6G). In Figure 6H,

184   PAS staining revealed the renal architecture of experimental mice. We observed injured, dilated

185   renal tubules and diffuse mesangial expansion in the kidney of BSA injected UNX-Cre$^{-/-}$ mice.

186   Importantly, BSA injected UNX-Tubcat mice displayed further renal damage such as more

187   dilated tubule with cast formation in the renal cortex.

188   IHC staining revealed increased tubulointerstitial infiltration by CD68+ macrophages after BSA

189   injection in UNX-Cre$^{-/-}$ mice, and this was further enhanced in BSA injected UNX-Tubcat mice

190   (Figure 7A). Real-time PCR confirmed that these infiltrating macrophages were of the M1 rather

191   than the M2 phenotype (Figure 7B).

192   ***Up-regulated TLR-4/NLRP-3 and inflammatory chemocytokines in proteinuric Tubcat mice***

193   Gene expression of *Tlr4*, *Ccl2*, *Il1b* and *Cxcl1* were all up-regulated in the renal cortex of BSA

194   injected UNX-Tubcat mice compared to UNX-Cre$^{-/-}$ control animals (Figure 8A).

195   Immunoblotting assay demonstrated that the protein levels of inflammatory chemocytokines

196   (CCL-2, IL-17A and ICAM-1) were also significantly up-regulated in BSA injected UNX-

197   Tubcat mice (Figure 8B), which was not observed in BSA injected UNX-Cre$^{-/-}$ mice. Finally,

198   renal cortical protein levels of TLR-4 and NLRP-3 were significantly increased in BSA injected

199 UNX-Tubcat mice (Figure 8C). Collectively, these data demonstrated that activation of Wnt/β-

200 catenin signaling in renal tubules promoted an inflammatory response during proteinuric

201 nephropathy, possibly via the TLR-4/NLRP-3 inflammasome pathway.

202 **DISCUSSION**

203 CKD affects approximately 8-16% of the population worldwide. Proteinuria has long been

204 known to be tubulotoxic and is an established biomarker that predicts progression and prognosis

205 in various forms of diabetic and nondiabetic CKD. Wnt/β-catenin signaling, among a myriad of

206 intracellular signal transduction pathways, has recently been studied in different renal cell types.

207 Yet, reports on its ectopic activation in renal tubules and association with renal inflammation

208 remain scarce.

209 We recently reported that Wnt/β-catenin signaling in renal tubules fluctuates during the different

210 phases of experimental proteinuric nephropathy,[26] being evidently activated at the early phase.

211 To characterize the role of tubular Wnt/β-catenin signals during overload proteinuria, we

212 generated a mouse strain, the "Tubcat", which could conditionally overexpress β-catenin

213 specifically in the renal tubules. Not only are Tubcat mice tissue-specific, the temporal Cre/lox

214 recombination circumvents any potential untoward consequences arising from overexpression in

215 the early embryonic life. We characterized the Tubcat mice by several approaches, including

216 PCR detection of *Cre* allele/ *loxP* sites in genomic DNA and the mutant *Catnb* allele among

217 different organs. This transgenic recombination deleted exon 3 from *Catnb* allele, thereby

218 stabilized β-catenin exclusively in the renal tubules of Tubcat mice and enhanced β-catenin

219 expression in the tubular nuclei.

220   In addition to its overarching importance in renal fibrosis, Wnt/β-catenin signaling also mediated

221   renal inflammation. Here, Tubcat mice displayed aggravated renal dysfunction compared to Cre[-]

222   [/-] mice and the renal injury could be accelerated by uninephrectomy surgery, as evident by the

223   significant glomerular and tubular damage. Moreover, the expression of AKI markers (*Kim1,*

224   *Cystatin C and Ngal*) was up-regulated in the renal cortex of uninephrectomized Tubcat mice,

225   indicating that activated Wnt/β-catenin signaling in renal tubules is detrimental to the kidney.

226   Our data lend support to studies on transgenic knock-out/overexpression of β-catenin in

227   podocytes and renal tubules.[15, 20] These studies confirmed that ectopic activation of tubular

228   Wnt/β-catenin signaling triggers different renal pathophysiological events during CKD

229   progression.

230   It is substantial that overexpressed tubular β-catenin correlates with fibrogenesis in CKD animal

231   models, and treatment of Wnt inhibitors has yielded a promising therapeutic effect on renal

232   function. However, animal data regarding direct overexpression of tubular β-catenin on

233   fibrogenesis is sparse. Additionally, the cross-talk between canonical and non-canonical Wnt

234   pathways in kidney deserves further investigation. From our Tubcat mice, we did not speculate

235   formation of fibrosis or enhanced EMT markers, or activation of non-canonical Wnt pathways.

236   We hypothesized the overexpressed tubular β-catenin induces renal fibrosis partially via

237   recruitment of resident macrophage into the kidney which potentially trans-differentiate to

238   myofibroblast.[32] It has been documented elevated intracellular Ca^{2+} level is followed by β-

239   catenin translocation into nucleus in several cell lines.[33] Interestingly, we also observed

240   suppressed CaMKII phosphorylation in UNX-Tubcat kidney which may indicate the activation

241   of Wnt/β-catenin in renal tubules could interfere the Wnt/Ca^{2+} pathway.

242   Previous studies indicated that activated Wnt/β-catenin signaling could induce inflammatory

243   cytokine production such as CCL-2 in breast cancer cells[34] and various organs.[35, 36] Particularly,

244   β-catenin could interact with CCL-2 promoter and regulates its transcription level. Our study is

245   the first to demonstrate the activation of the TLR-4/NLRP-3 inflammasome axis in the kidney by

246   Wnt/β-catenin signaling in renal tubules. The expression of TLR-4, NLRP-3 and the downstream

247   chemokines CCL-2[37] and RANTES[38] were all up-regulated in the kidney of UNX-Tubcat mice.

248   Such inflammatory responses coincided with enhanced macrophage infiltration in the

249   tubulointerstitium, a hallmark of renal inflammation. The pleiotropic function of polarized

250   macrophages in the kidney has been well characterized in different nephropathies.[39] Ample

251   evidence shows that M1 macrophages induce renal inflammation while M2 macrophages play a

252   role in tissue repair. In UNX-Tubcat mice, we observed tubulointerstitial infiltration by M1

253   macrophages which is in line with the overexpression of various proinflammatory

254   chemocytokines. We postulate the tubulointerstitial inflammation is influenced by

255   overexpression of tubular β-catenin *per se*, then subsequently leading to proteinuria as an

256   aftermath of tubular and glomerular damage.

257   Tubcat mice subjected to overload proteinuria recapitulate changes observed during CKD

258   pathogenesis. We observed proteinuria and elevated BUN levels in non-transgenic mice

259   challenged with protein overload. Notably, BSA injected UNX-Tubcat mice displayed further

260   renal dysfunction and increased expression of tubular injury marker. In addition, tubular β-

261   catenin expression was enhanced in the BSA injected UNX-Cre^{-/-} mice but to a lesser extent

262   compared to UNX-Tubcat mice. We suggest that the protein overload induces tubulointerstitial

263   inflammation via induction of tubular β-catenin expression and such inflammatory response

264   could further augment the glomerular and tubular injury. As a result, it perpetuates the vicious

265   cycle of the heavy proteinuria formation during CKD progression.

266   In agreement with previous findings,[40] our BSA injected mice displayed renal inflammation as

267   evident by M1 macrophage infiltration and activated TLR-4/NLPR-3 inflammasome axis in

268   renal cortices. Numerous studies have demonstrated the inflammatory role of TLR-4 in CKD,[41]

269   and the TLR-4 antagonist CRX-526 exerts reno-protective effects by ameliorating renal

270   inflammation.[42] Here, we observed that TLR-4 gene expression correlated with the β-catenin

271   level and enhanced expression of TLR-4/NLRP-3 in the renal cortex of Tubcat mice. BSA-

272   injected Tubcat mice further overexpressed inflammatory chemocytokines (CCL-2, ICAM-1)

273   which may conceivably accelerate renal inflammation. Furthermore, IL-17A[43] and *Cxcl1*[44]

274   expression which is enhanced in the inflamed kidney of AKI models, was also up-regulated in

275   the proteinuric Tubcat kidney. These findings support the notion that tubular Wnt/β-catenin

276   signaling confers a pro-inflammatory state in the kidney during proteinuric nephropathy.

277   In summary, we demonstrated a proinflammatory role of Wnt/β-catenin signaling in renal

278   tubules during overload proteinuria. Activated tubular Wnt/β-catenin signaling could induce

279   TLR-4/NLRP-3 related chemocytokines in the kidney and orchestrate the subsequent chemotaxis

280   of macrophages into the tubulointerstitium.

281   **METHODS**

282   Animal model

283   The animal experiments were approved by the Committee on the Use of Live Animals in

284   Teaching and Research (Laboratory Animal Unit, The University of Hong Kong) and followed

285   stringently with the National Institute of Health Guide for the Care and Use of Laboratory

13

286   Animals. Mice were housed in 12-hour light/12-hour dark cycle, ad libitum to food and water.

287   Ksp-CreER^{T2} mice were generated by Dr. Peter Igarashi (The UT Southwestern O'Brien Kidney

288   Research Core Center, Dallas, US).[45] The CreER^{T2} fusion protein was expressed exclusively

289   under the kidney specific Ksp-cadherin (cadherin 16) gene promoter, thereby restricted in the

290   renal tubules. The CreER^{T2} fusion protein consists of a Cre recombinase that carries an in-frame

291   modified human estrogen receptor which could be activated by 4-hydroxytamoxifen.

292   The Catnb$^{lox(ex3)/ lox(ex3)}$ mice were generated and characterized by Professor Makoto Mark Taketo

293   (Kyoto University, Kyoto, Japan).[46] The Catnb$^{lox(ex3)/ lox(ex3)}$ mice carry two loxP sites

294   sandwiching the exon 3 sequence of *Catnb* alleles where the serine/threonine phosphorylation

295   sites of β-catenin were transcribed. These phosphorylation sites regulate the degradation of β-

296   catenin thus deletion of the exon 3 sequence could stabilize β-catenin in the desirable tissue cells.

297   ***Generation and characterization of Tubcat mice (Ksp-CreER^{T2}-Catnb$^{lox(ex3)/wt}$)***

298   We generated the Tubcat mouse littermate which carried Ksp-CreER^{T2} and Catnb$^{lox(ex3)/wt}$ alleles

299   by cross-breeding the Ksp-CreER^{T2} mice and Catnb$^{lox(ex3)/lox(ex3)}$ mice. Those mice without Ksp-

300   CreER^{T2} allele served as negative control. All mice were administered 4-hydroxytamoxifen or

301   tamoxifen dissolved in corn oil (Sigma-Aldrich, St Louis, MO, USA; 1mg/g body weight) for 5

302   consecutive days via intraperitoneal (i.p.) injection to induce the Cre-lox recombination. For

303   protein overload model, Tamoxifen was given an hour before BSA injection during the first

304   week to Tubcat and Cre$^{-/-}$ mice. Specifically, the activated Cre recombinase in the renal tubular

305   cells excised the exon 3 sequence of *Catnb* allele (Catnb$^{\Delta(ex3)}$) leading to β-catenin stabilization

306   in the renal tubules.

307   ***Genotyping of Ksp-CreER^{T2} transgenic mice and Catnb $^{lox(ex3)/ lox(ex3)}$ mice***

14

308   Genomic DNA was extracted from ear biopsies collected from mice and PCR based genotyping

309   was performed with the HotStart Mouse Genotyping Kit (Kapa Biosystems, Woburn, MA, USA),

310   followed by separation of the PCR products by agarose gel (Invitrogen, Carlsbad, CA, USA).

311   *Cre* allele was confirmed by using the *forward primer 5'-AGGTTCGTGCACTCATGGA-3'* and

312   *reverse primer 5'-TCGACCAGTTTAGTTACCC-3'* (235bp). Catnb *lox P* sites were verified by

313   using *forward primer 5'-AGGGTACCTGAAGCTCAGCG-3'* and *reverse primer 5'-*

314   *CAGTGGCTGACAGCAGCTTT-3'* (645bp: Catnb$^{lox(ex3)}$ and 412bp: Catnb^{wt}).

315   To verify the successful Cre-lox recombination after tamoxifen administration, genomic DNA

316   extracted from various organs was analyzed by PCR with the *forward primer 5'-*

317   *CATTGCGTGGACAATGGCTACTCA-3' and reverse primer 5'-GGCAAGTTCCGGTCATCC-3'*

318   (867bp: Catnb^{wt} and ~500bp: Catnb$^{\Delta(ex3)}$). The *Catnb* mRNA expression (exon 3) was verified by

319   quantitative real time PCR (qPCR). Protein expression of the normal β-catenin and the mutant β-

320   catenin in kidney cortices was confirmed by immunoblotting assay.

Murine protein overload nephropathy model

322   The Tubcat mice and non-transgenic mice (Cre$^{-/-}$) were subjected to protein overload animal

323   model as previously described.[26] Urine and blood serum were collected for the baseline before

324   experiments. Male mice at 6-8 weeks of age were uninephrectomized under anesthesia and

325   followed by intraperitoneal injection of low-endotoxin BSA (A-9430, Sigma-Aldrich) weekly for

326   5 consecutive days, up to 4 weeks. Equal volume of saline was injected to the disease control

327   mice. After 4-week BSA injection, 24-hour urine and blood serum samples were collected for

328   analysis. Tissues include kidney, spleen, heart and liver were harvested and snap frozen in liquid

329   nitrogen and stored at -70°C. Half of the kidney was fixed with 10% formalin and embedded in

330   paraffin for immunohistochemistry analysis.

Quantitative real-time PCR (qPCR) and immunoblotting assay

Cortical protein and total RNA were extracted from the frozen kidney tissues using NucleoSpin®

Triprep (Macherey-Nagel, Duren, Germany). Nuclear and Cytosolic protein was fractionized by

NE-PER® extraction kit (Thermo Scientific). The RNAs were reversely transcribed to cDNAs

(High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Carlsbad, CA, USA)

followed by qPCR analysis. qPCR was performed using StepOnePlus™ Real-Time PCR

Systems (Applied Biosystems) with SYBR Green reagent (Applied Biosystems) and specific

primers (Table 1). Relative gene expression was calculated after normalization with β-actin

expression. All experimental groups were compared to their respective control group using

StepOne™ software v2.3 (Applied Biosystems). Equal amount of protein lysate was resolved in

Bolt™ 4-12% gel and transferred to 0.2μm PVDF membrane. Membranes were blotted with 5%

non-fat milk and followed by overnight incubation of primary antibody against β-catenin, E-

cadherin (BD Transduction Laboratories™, San Jose, CA, USA), α-SMA, CCL-2, collagen I,

RANTES, TLR-4, Vimentin (Santa Cruz Biotechnology, Santa Cruz, CA), NLRP-3, p-CamKII

(Novus Biologicals, Littleton, CO), Histone H3, p-JNK, Total JNK, p-GSK-3β, total GSK-3β

(Cell Signaling Technology, Beverly, CA, USA), nephrin (R&D Systems, Minneapolis, MN,

USA), p-CamKII (Abcam, Cambridge, UK), fibronectin (Sigma-Aldrich), and β-actin (Thermo

Scientific). After washing, the membranes were then incubated with matched horse radish

peroxidase (HRP) conjugated secondary antibodies (Dako, Carpinteria, CA, USA) and visualized

by ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA) after incubation with Clarity™

Western ECL Substrate (Bio-Rad) for 5 minutes. Densitometries of protein bands were

353   quantified by Image Lab™ software (Bio-Rad). Relative expression of target protein was

354   normalized with β-actin or Histone H3 expression.

Immunohistochemistry staining

356   Paraffin-embedded kidney tissue was sectioned (4µm) and de-paraffinized then followed by

357   rehydration with concentration gradient of ethanol. The sections were then proceeded to

358   microwave-based antigen retrieval step in 10mM citrate buffer and quenched by 3% hydrogen

359   peroxide for 10 mins. Primary antibody against β-catenin (BD Transduction Laboratories™),

360   CCL-2 and CD68 (Santa Cruz Biotechnology) diluted in IHC diluent (Enzo Life Sciences,

361   Farmingdale, NY, USA) was applied on the sections overnight and subsequently with the

362   secondary peroxidase conjugated antibody (Dako). The sections were developed by DAB

363   substrate (Dako) then counterstained with CAT hematoxylin (Biocare Medical, Walnut Creek,

364   CA, USA), followed by dehydration with ethanol/xylene solution before mounting. Following

365   co-immunohistochemical staining with β-catenin and Thiazide sensitive sodium chloride

366   cotransporter (NCC; Millipore, Bedford, MA, USA the sections were developed by the

367   MultiView® (mouse-HRP/rabbit-AP) IHC kit (Enzo Life Sciences) according to the kit manual.

368   CD68+ cells were counted in at least 10 non-overlapping tubulointerstitial area (400 X; excluded

369   glomeruli) and average for individual sample.

Histology

371   Paraffin-embedded kidney section was processed as mentioned followed by staining with

372   periodic acid-Schiff solution (Sigma-Aldrich) to evaluate glomerular and renal tubular injury. At

373   least 20 glomeruli from the kidney section were randomly selected and scored to evaluate

374   glomerular damage. Glomerular damage index was graded as followings: 0, no sclerosis; 1, 10-

25% mesangial expansion/sclerosis; 2, 25-50% mesangial expansion/sclerosis; 3, 50-75%

mesangial expansion/sclerosis; 4, 75-100% mesangial expansion/sclerosis; The final score for

each section was calculated as followings: Final scores = 0 x (% of grade 0 glomeruli) + 1 x (%

of grade 1 glomeruli) + 2 x (% of grade 2 glomeruli) + 3 x (% of grade 3 glomeruli) + 4 x (% of

grade 4 glomeruli).

Urine and serum biochemistry

Mouse urinary albumin was measured by Albuwell M (Exocell Inc., Philadelphia, PA, USA).

Urinary or serum creatinine was measured by enzymatic assay: Creatinine LiquiColor Test

(Stanbio Laboratory, Boerne, TX, USA). Serum blood urea nitrogen (BUN) was measured by

QuantiChromTM Urea Assay Kit (BioAssay Systems, Hayward, USA). Urinary β2-

microglobulin level was measured by ELISA kit (Lifespan Biosciences, Seattle, WA, USA).

Statistics

All data were expressed as mean ± SEM. One-way ANOVA with Tukey's multiple comparison

test or two-sided t-test were employed to calculate the difference between experimental groups in

GraphPad Prism v.6 (GraphPad Software, San Diego, CA). $p < 0.05$ was considered statistically

significant.

DISCLOSURE: None.

REFERENCES

1.   Heerspink HJ, Kropelin TF, Hoekman J, et al. Drug-Induced Reduction in Albuminuria Is
       Associated with Subsequent Renoprotection: A Meta-Analysis. *J Am Soc Nephrol.*
       2015;26**:**2055-2064.

2.   Baines RJ, Brunskill NJ. Tubular toxicity of proteinuria. *Nat Rev Nephrol.* 2011;7**:**177-
       180.

399

400 3. Li RX, Yiu WH, Tang SC. Role of bone morphogenetic protein-7 in renal fibrosis. *Front*
401   *Physiol.* 2015;6**:**114.

402
403 4. Saito A, Sato H, Iino N, et al. Molecular mechanisms of receptor-mediated endocytosis in
404   the renal proximal tubular epithelium. *J Biomed Biotechnol.* 2010;2010**:**403272.

405
406 5. Birn H, Christensen EI. Renal albumin absorption in physiology and pathology. *Kidney*
407   *Int.* 2006;69**:**440-449.

408
409 6. Coombes JD, Mreich E, Liddle C, et al. Rapamycin worsens renal function and
410   intratubular cast formation in protein overload nephropathy. *Kidney Int.* 2005;68**:**2599-
411   2607.

412
413 7. Nagai J, Yamamoto A, Yumoto R, et al. Albumin overload induces expression of
414   hypoxia-inducible factor 1alpha and its target genes in HK-2 human renal proximal
415   tubular cell line. *Biochem Biophys Res Commun.* 2013;434**:**670-675.

416
417 8. Cao W, Zhou QG, Nie J, et al. Albumin overload activates intrarenal renin-angiotensin
418   system through protein kinase C and NADPH oxidase-dependent pathway. *J Hypertens.*
419   2011;29**:**1411-1421.

420
421 9. van Timmeren MM, Bakker SJ, Vaidya VS, et al. Tubular kidney injury molecule-1 in
422   protein-overload nephropathy. *Am J Physiol Renal Physiol.* 2006;291**:**F456-464.

423
424 10. Tang S, Leung JC, Abe K, et al. Albumin stimulates interleukin-8 expression in proximal
425   tubular epithelial cells in vitro and in vivo. *J Clin Invest.* 2003;111**:**515-527.

426
427 11. Nikolic-Paterson DJ, Wang S, Lan HY. Macrophages promote renal fibrosis through
428   direct and indirect mechanisms. *Kidney International Supplements.* 2014;4**:**34-38.

429
430 12. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell.* 2012;149**:**1192-1205.

431
432 13. Moon RT, Kohn AD, De Ferrari GV, et al. WNT and beta-catenin signalling: Diseases
433   and therapies. *Nature Reviews Genetics.* 2004;5**:**689-699.

434
435 14. Kestler HA, Kuhl M. From individual Wnt pathways towards a Wnt signalling network.
436   *Philos Trans R Soc Lond B Biol Sci.* 2008;363**:**1333-1347.

437

438 15. Kato H, Gruenwald A, Suh JH, et al. Wnt/beta-catenin pathway in podocytes integrates
439   cell adhesion, differentiation, and survival. *J Biol Chem.* 2011;286**:**26003-26015.

440

441 16. Lancaster MA, Louie CM, Silhavy JL, et al. Impaired Wnt-beta-catenin signaling
442   disrupts adult renal homeostasis and leads to cystic kidney ciliopathy. *Nat Med.*
443   2009;15**:**1046-1054.

444

445 17. Rooney B, O'Donovan H, Gaffney A, et al. CTGF/CCN2 activates canonical Wnt
446   signalling in mesangial cells through LRP6: implications for the pathogenesis of diabetic
447   nephropathy. *FEBS Lett.* 2011;585**:**531-538.

448

449 18. Zhou L, Li Y, Zhou D, et al. Loss of Klotho contributes to kidney injury by derepression
450   of Wnt/beta-catenin signaling. *J Am Soc Nephrol.* 2013;24**:**771-785.

451

452 19. Kawakami T, Ren SY, Duffield JS. Wnt signalling in kidney diseases: dual roles in renal
453   injury and repair. *Journal of Pathology.* 2013;229**:**221-231.

454

455 20. Zhou D, Li Y, Lin L, et al. Tubule-specific ablation of endogenous beta-catenin
456   aggravates acute kidney injury in mice. *Kidney Int.* 2012;82**:**537-547.

457

458 21. Zhou D, Tan RJ, Fu H, et al. Wnt/beta-catenin signaling in kidney injury and repair: a
459   double-edged sword. *Lab Invest.* 2016;96**:**156-167.

460

461 22. De Langhe SP, Reynolds SD. Wnt signaling in lung organogenesis. *Organogenesis.*
462   2008;4**:**100-108.

463

464 23. Li VSW, Ng SS, Boersema PJ, et al. Wnt Signaling through Inhibition of beta-Catenin
465   Degradation in an Intact Axin1 Complex. *Cell.* 2012;149**:**1245-1256.

466

467 24. Hao S, He W, Li Y, et al. Targeted inhibition of beta-catenin/CBP signaling ameliorates
468   renal interstitial fibrosis. *J Am Soc Nephrol.* 2011;22**:**1642-1653.

469

470 25. He W, Dai C, Li Y, et al. Wnt/beta-catenin signaling promotes renal interstitial fibrosis. *J
471   Am Soc Nephrol.* 2009;20**:**765-776.

472

473 26. Wong DW, Yiu WH, Wu HJ, et al. Downregulation of renal tubular Wnt/beta-catenin
474   signaling by Dickkopf-3 induces tubular cell death in proteinuric nephropathy. *Cell
475   Death Dis.* 2016;7**:**e2155.

476

477   27.   Pereira CP, Bachli EB, Schoedon G. The Wnt pathway: A macrophage effector molecule
478   that triggers inflammation. *Current Atherosclerosis Reports.* 2009;11**:**236-242.

479
480   28.   Liu Y, Almeida M, Weinstein RS, et al. Skeletal inflammation and attenuation of Wnt
481   signaling, Wnt ligand expression, and bone formation in atherosclerotic ApoE-null mice.
482   *American Journal of Physiology-Endocrinology and Metabolism.* 2016;310**:**E762-E773.

483
484   29.   George SJ. Wnt pathway: a new role in regulation of inflammation. *Arterioscler Thromb*
485   *Vasc Biol.* 2008;28**:**400-402.

486
487   30.   Wu HJ, Yiu WH, Li RX, et al. Mesenchymal Stem Cells Modulate Albumin-Induced
488   Renal Tubular Inflammation and Fibrosis. *PLoS ONE.* 2014;9**:**e90883.

489
490   31.   Liu FY, Li Y, Peng YM, et al. Norcantharidin ameliorates proteinuria, associated
491   tubulointerstitial inflammation and fibrosis in protein overload nephropathy. *Am J*
492   *Nephrol.* 2008;28**:**465-477.

493
494   32.   Nikolic-Paterson DJ, Wang S, Lan HY. Macrophages promote renal fibrosis through
495   direct and indirect mechanisms. *Kidney Int Suppl (2011).* 2014;4**:**34-38.

496
497   33.   Thrasivoulou C, Millar M, Ahmed A. Activation of intracellular calcium by multiple Wnt
498   ligands and translocation of beta-catenin into the nucleus: a convergent model of
499   Wnt/Ca2+ and Wnt/beta-catenin pathways. *J Biol Chem.* 2013;288**:**35651-35659.

500
501   34.   Mestdagt M, Polette M, Buttice G, et al. Transactivation of MCP-1/CCL2 by beta-
502   catenin/TCF-4 in human breast cancer cells. *Int J Cancer.* 2006;118**:**35-42.

503
504   35.   Silva-Garcia O, Valdez-Alarcon JJ, Baizabal-Aguirre VM. The Wnt/beta-catenin
505   signaling pathway controls the inflammatory response in infections caused by pathogenic
506   bacteria. *Mediators Inflamm.* 2014;2014**:**310183.

507
508   36.   Reuter S, Beckert H, Taube C. Take the Wnt out of the inflammatory sails: modulatory
509   effects of Wnt in airway diseases. *Lab Invest.* 2016;96**:**177-185.

510
511   37.   Vilaysane A, Chun J, Seamone ME, et al. The NLRP3 inflammasome promotes renal
512   inflammation and contributes to CKD. *J Am Soc Nephrol.* 2010;21**:**1732-1744.

513
514   38.   Hu CY, Ding HY, Li YY, et al. NLRP3 deficiency protects from type 1 diabetes through
515   the regulation of chemotaxis into the pancreatic islets. *Proceedings of the National*
516   *Academy of Sciences of the United States of America.* 2015;112**:**11318-11323.

517

518 39. Tian S, Chen SY. Macrophage polarization in kidney diseases. *Macrophage (Houst).*
519 2015;2.

520

521 40. Wu HJ, Yiu WH, Li RX, et al. Mesenchymal stem cells modulate albumin-induced renal
522 tubular inflammation and fibrosis. *PLoS One.* 2014;9**:**e90883.

523

524 41. Yiu WH, Lin M, Tang SC. Toll-like receptor activation: from renal inflammation to
525 fibrosis. *Kidney Int Suppl (2011).* 2014;4**:**20-25.

526

527 42. Lin M, Yiu WH, Li RX, et al. The TLR4 antagonist CRX-526 protects against advanced
528 diabetic nephropathy. *Kidney Int.* 2013;83**:**887-900.

529

530 43. Xue L, Xie K, Han X, et al. Detrimental functions of IL-17A in renal ischemia-
531 reperfusion injury in mice. *J Surg Res.* 2011;171**:**266-274.

532

533 44. Chung AC, Lan HY. Chemokines in renal injury. *J Am Soc Nephrol.* 2011;22**:**802-809.

534

535 45. Patel V, Li L, Cobo-Stark P, et al. Acute kidney injury and aberrant planar cell polarity
536 induce cyst formation in mice lacking renal cilia. *Hum Mol Genet.* 2008;17**:**1578-1590.

537

538 46. Harada N, Tamai Y, Ishikawa T, et al. Intestinal polyposis in mice with a dominant stable
539 mutation of the beta-catenin gene. *EMBO J.* 1999;18**:**5931-5942.

540

541 **ACKNOWLEDGMENTS**

553

554

FIGURE LEGENDS

556 **Figure 1   Tubcat mice overexpress β-catenin specifically in renal tubules after**

557 **tamoxifen induction**

558   (A) qPCR showed less mRNA expression of *Catnb* allele (exon 3) in the renal cortex of Tubcat

559 mice after transgenic modification (n=5). (B) Expression of the native and mutant forms of β-

560 catenin in renal cortical cells by immunoblotting. (C) Consecutive kidney sections revealed β-

561 catenin overexpression in the renal tubules of Tubcat mice. Upper panels show the IHC staining

562 of β-catenin (brown) and the lower panels show the co-staining of β-catenin (brown) and Na^+-Cl^-

563 cotransporter (NCC, tubule marker; red). Red arrows indicate tubular β-catenin expression in

564 $Cre^{-/-}$ and Tubcat mice, respectively. Bar scale = 250 µm. Results were expressed as means ±

565 SEM. A two-sided t-test was used for comparison. G, glomerulus. T, renal tubules. M, DNA

566 ladder. *p<0.05

567 **Figure 2   Renal dysfunction in uninephrectomized Tubcat mice 4 weeks after**

568 **tamoxifen induction**

569 (A) Schema of transgenic induction in Tubcat mice. (B) Expression of nuclear β-catenin in renal

570 cortices of experimental mice (n=5). Representative immunoblots are shown. Urine albumin-to-

571 creatinine ratio (UACR) and blood urea nitrogen (BUN) were measured at (C) baseline (n=5)

572 and (D) 4 weeks after tamoxifen administration (n=5). (E) Renal cortical expression of AKI

573 markers (n=5) in Tubcat and $Cre^{-/-}$ mice at experimental endpoint. (F) Representative periodic

574 acid-Schiff (PAS) staining of kidney section from UNX-Tubcat and UNX-$Cre^{-/-}$ mice. Asterisks

575 indicates the mesangial expansion. Bar scale = 250 µm. (G) Glomerular damage scoring of the

576 kidney sections from the experimental mice (n=5). (H) Gene expression of *Nphs1* in renal

577   cortices of uninephrectomized Tubcat and Cre^{-/-} mice (n=5) 4 weeks after tamoxifen

578   administration by qPCR. (I) Renal cortical protein expression of nephrin in the experimental

579   mice (n=5) by immunoblotting assay. (J) Urinary β2-macroglobulin was measured in

580   experimental mice 4 weeks after tamoxifen administration (n=5). Results were expressed as

581   means ± SEM. A two-sided t-test was used for comparison. *p<0.05, **p< 0.01, ***p<0.0001

**Figure 3   Renal inflammation in uninephrectomized Tubcat mice with enhanced
                  cortical chemocytokine expression**

584   (A) Immunohistochemical staining of CD68+ cells (red arrows) on kidney tissues. Average

585   CD68+ cell counts on tubulointerstitial area (400X magnification) of Tubcat and Cre^{-/-} mice

586   (n=5). (B) Gene expression of M1 (*Ly6c* and *Ccr2*) and M2 (*Cd163* and *Cd206*) macrophage

587   markers in renal cortices of Tubcat and Cre^{-/-} mice (n=5) 4 weeks after tamoxifen administration

588   by qPCR. Renal cortical expression of (C) RANTES, CCL-2, and (E) TLR-4 and NLRP-3 in

589   experimental mice by immunoblottings (n=5). (D) Immunohistochemistry staining of CCL-2 and

590   β-catenin on serial kidney sections from a Tubcat mouse by using different primary antibodies as

591   annotated. Red arrows indicate the colocalization of CCL-2 and β-catenin detected in the same

592   tubular cells. Bar scale = 250 µm. (F) Gene expression of TLR-4 and NLRP-3 by qPCR (n=5).

593   Results were expressed as means ± SEM. A two-sided t-test was used for comparison. *p<0.05,

594   **p< 0.01, ***p<0.0001

**Figure 4   Expression of non-canonical Wnt pathway regulators in uninephrectomized
                  Tubcat mice for 4 weeks**

597   (A) Expression of non-canonical Wnt signaling related genes in renal cortices of

598   uninephrectomized Tubcat and non-transgenic mice (n=5) by qPCR. Phosphorylation level of (B)

JNK, CaMKII and (C) GSK-3β in renal cortices of uninephrectomized Tubcat and non-transgenic mice (n=5) by immunoblotting assay. Results were expressed as means ± SEM. A two-sided t-test was used for comparison. **$p < 0.01$

Figure 5 **Fibrosis and EMT markers in UNX-Tubcat mice for 4 weeks**

(A) Expression of fibrotic markers (fibronectin, α-SMA and collagen I) in renal cortices of UNX-Tubcat and UNX-Cre^{-/-} mice (n=5) by immunoblotting assay. (B) Renal cortical expression of EMT markers (vimentin and E-cadherin) of uninephrectomized Tubcat and non-transgenic mice (n=5) by immunoblotting assay. Results were expressed as means ± SEM.

Figure 6 **Induction of protein overload in uninephrectomized Tubcat and Cre^{-/-} mice for 4 weeks**

(A) Schema of UNX-Tubcat mice in protein overload model. UACR and BUN levels of experimental mice at (B) baseline (n=5) and (C) 4 weeks after BSA injection (n=5). (D) Urinary β2-macroglobulin measured in BSA injected UNX-Cre^{-/-} mice and UNX-Tubcat mice at the experimental endpoint (n=5). (E) Renal cortical mRNA expression from *Catnb* allele (exon 3) by qPCR (n=5). (F) Both mutant and wild-type forms of nuclear β-catenin expression in the renal cortical lysate by immunoblotting (n=5). (G) Immunohistochemical staining of β-catenin on kidney tissues. Red arrows indicate β-catenin expression in tubules. Bar scale = 125 µm. (H) Representative periodic acid-Schiff (PAS) staining of kidney section from BSA injected UNX-Tubcat and UNX-Cre^{-/-} mice. Yellow asterisks indicate the mesangial expansion and red asterisks indicate tubular injury. Bar scale = 250 µm. A One-way ANOVA was used for comparison. Results were expressed as means ± SEM. *$p<0.05$, **$p< 0.01$, ***$p<0.0001$

Figure 7   **Increased tubulointerstitial macrophage infiltration in protein overloaded Tubcat mice**

(A) CD68+ cells (red arrows) in kidney sections of protein overloaded animals and quantification (n=5). (B) Renal cortical expression of M1 and M2 macrophage markers in Tubcat and Cre$^{-/-}$ mice (n=5) by qPCR. Bar scale = 250 µm. Results were expressed as means ± SEM. A One-way ANOVA was used for comparison. *$p<0.05$, **$p< 0.01$, ****$p<0.0001$

Figure 8   **Induction of inflammatory chemocytokines and TLR-4/NLRP-3 in BSA-injected Tubcat mice**

(A) Cortical gene expression of inflammatory chemocytokines (*Cxcl1*, *Tlr4*, *Ccl2* and *IL1b*) in BSA-injected mice by qPCR (n=5). Protein levels of (B) CCl-2, ICAM-1, IL-17A and (C) TLR-4 and NLRP-3 by immunoblotting of kidney cortical lysates (n=5). Results were expressed as mean ± SEM. A One-way ANOVA was used for comparison. *$p<0.05$, **$p< 0.01$, ***$p<0.001$

Supplementary Figure 1   Genotyping of Tubcat and non-transgenic mice

Genotyping of ear biopsies from Cre$^{-/-}$ and Tubcat mice was performed. (A) All mice carried the heterozygous floxed *Catnb* alleles, indicated by two PCR bands (645bp: *Catnb*$^{lox(ex3)}$ and 412bp: *Catnb*^{wt}) and Tubcat mice carried an additional Ksp-CreER^{T2} allele (235 bp: Ksp-CreER^{T2}). (B) PCR (*Catnb* allele) analysis on genomic DNA extracted from kidney of Cre$^{-/-}$ and Tubcat mice 4 weeks after tamoxifen induction. An extra band (~500bp) indicated the *Catnb* allele lacking exon 3 (Catnb$^{\Delta exon3}$), which was absent in the DNA genome of the heart, spleen and liver of Tubcat mice.

Supplementary Figure 2   Renal function of sham operated Tubcat and non-transgenic

mice for 4 weeks

(A) UACR and (B) serum BUN levels of Tubcat mice and Cre^{-/-} mice without uninephrectomy

(Sham) at experimental endpoint (n=4). Results were expressed as means ± SEM. A two-sided t-

test was used for comparison. $*p< 0.05$

646 **Table 1. Custom primers for quantitative real-time PCR**

Gene	Primers
Actb	Forward: *5'-TCCATCATGAAGTGTGACGT-3'*
	Reverse: *5'-GAGCAATGATCTTGATCTTCAT-3'*
Catnb (exon 3)	Forward: *5'-GACGCTGCTCATCCCACTAA-3'*
	Reverse: *5'-CCACCTGGTCCTCATCGTTT-3'*
Camkii	Forward: *5'-ACCACTTCCTTCCACCACTT-3'*
	Reverse: *5'-TGAGATACAGCATTCCATACAAGA-3'*
Ccl2	Forward: *5'-CTCTTCCTCCACCACCAT-3'*
	Reverse: *5'-CTCTCCAGCCTACTCATTG-3'*
Ccr2	Forward: *5'-ATCCACGGCATACTATCAACATC-3'*
	Reverse: *5'-CAAGGCTCACCATCATCGTAG-3'*
Cd163	Forward: *5'-TGCTGTCACTAACGCTCCTG-3'*
	Reverse: *5'-TCATTCATGCTCCAGCCGTT-3'*
Cd206	Forward: *5'-AAGGCATGCGTTGCACATAC-3'*
	Reverse: *5'-ATTCTGCTCGATGTTGCCCA-3'*
Cystatin C	Forward: *5'-TGTTTGCACCAGGAGACAGT-3'*

	Reverse: *5'-AATTGAGCAAGGCATGGCAG-3'*
Cxcl1	Forward: *5'-TTGACGCTTCCCTTGGACAT-3'*
	Reverse: *5'-CTTTGAACGTCTCTGTCCCGA-3'*
Daam1	Forward: *5'-CACCTGCGAGTTGGTAACCT-3'*
	Reverse: *5'-GCTAATGAATGAGTGCGGCG-3'*
Il1b	Forward: *5'-TTCAGGCAGGCAGTATCA-3'*
	Reverse: *5'-CCAGCAGGTTATCATCATCA-3'*
Kim1	Forward: *5'-AAACCAGAGATTCCCACACG-3'*
	Reverse: *5'-GTCGTGGGTCTTCCTGTACTC-3'*
Ly6c	Forward: *5'-GCAGTGCTACGAGTGCTATGG-3'*
	Reverse: *5'-ACTGACGGGTCTTTAGTTTCCTT-3'*
Ngal	Forward: *5'-ACAACCAGTTCGCCATGGTA-3'*
	Reverse: *5'-AAGCGGGTGAAACGTTCCTT-3'*
Nlrp3	Forward: *5'-AGAGCCTACAGTTGGGTGAAATG-3'*
	Reverse: *5'-CCACGCCTACCAGGAAATCTC-3'*
Nphs1	Forward: *5'-CCCAACACTGGAAGAGGTGT-3'*
	Reverse: *5'-CTGGTCGTAGATTCCCCTTG-3'*
Plcb1	Forward: *5'-TCAAACCCAATCCGATATGTCAAT-3'*
	Reverse: *5'-AGCCTCCTTCTTTACTTCCTCTT-3'*
Ptk7	Forward: *5'-AATGCTGGAACCCTACACTTTG -3'*
	Reverse: *5'-CCCTGTGGCTCGTTGGAT-3'*
Tlr4	Forward: *5'-CATCCAGGAAGGCTTCCACA-3'*
	Reverse: *5'-GGCGATACAATTCCACCTGC-3'*

647