A mouse model for inherited renal fibrosis associated with endoplasmic reticulum stress

Sian E. Piret1, Eric Olinger2, Anita A. C. Reed1, M. Andrew Nesbit1,3, Tertius A. Hough4, Liz Bentley4, Olivier Devuyst2, Roger D. Cox4 and Rajesh V. Thakker1,*

ABSTRACT
Renal fibrosis is a common feature of renal failure resulting from multiple etiologies, including diabetic nephropathy, hypertension and inherited renal disorders. However, the mechanisms of renal fibrosis are incompletely understood and we therefore explored these by establishing a mouse model for a renal tubular disorder, referred to as autosomal dominant tubulointerstitial kidney disease (ADTKD) due to missense uromodulin (UMOD) mutations (ADTKD-UMOD). ADTKD-UMOD, which is associated with retention of mutant uromodulin in the endoplasmic reticulum (ER) of renal thick ascending limb cells, is characterized by hyperuricemia, interstitial fibrosis, inflammation and renal failure, and we used targeted homologous recombination to generate a knock-in mouse model with an ADTKD-causing missense cysteine to arginine uromodulin mutation (C125R). Heterozygous and homozygous mutant mice developed reduced uric acid excretion, renal fibrosis, immune cell infiltration and progressive renal failure, with decreased maturation and excretion of uromodulin, due to its retention in the ER. The ER stress marker 78 kDa glucose-regulated protein (GRP78) was elevated in cells expressing mutant uromodulin in heterozygous and homozygous mutant mice, and this was accompanied, both in vivo and ex vivo, by upregulation of two unfolded protein response pathways in primary thick ascending limb cells from homozygous mutant mice. However, this did not lead to an increase in apoptosis in vivo. Thus, we have developed a novel mouse model for renal fibrosis, which will be a valuable resource to decipher the mechanisms linking uromodulin mutations with ER stress and renal fibrosis.

KEY WORDS: Kidney, Fibrosis, ER stress, Mouse model, Endoplasmic reticulum

INTRODUCTION
Renal fibrosis is an integral factor in progression to end-stage renal failure (ESRF), regardless of the primary etiology, which may include diabetic nephropathy, hypertension or monogenic disorders (Eddy, 2014). However, mechanisms of renal fibrosis are incompletely understood, and there are currently no therapies to reverse or slow down the progression of renal fibrosis (Eddy, 2014). Autosomal dominant tubulointerstitial kidney disease (ADTKD), a monogenic cause of renal fibrosis, is characterized by urine concentrating defects, interstitial nephropathy with immune cell infiltration and glomerulosclerosis, and progressive loss of renal function leading to ESRF (Eckardt et al., 2015). Mutations in four genes have been identified to cause ADTKD, with the most common being in the UMOD gene, encoding uromodulin (Eckardt et al., 2015; Hart et al., 2002; Turner et al., 2003), referred to as ADTKD-UMOD. ADTKD-UMOD was previously referred to as familial juvenile hyperuricemic nephropathy type 1 (FJHN1; OMIM #162000), medullary cystic kidney disease type 2 (MCKD2; OMIM #603860) and glomerulocystic kidney disease (GCKD; OMIM #609886) (Eckardt et al., 2015; Rampoldi et al., 2003). In addition to the general clinical characteristics of ADTKD, patients with ADTKD-UMOD present with elevated serum urate concentrations due to low fractional excretion of uric acid (FEUA).

Uromodulin, a 640 amino acid glycosylphosphatidylinositol (GPI) anchored protein, is only expressed in the thick ascending limb (TAL) of the loop of Henle (Rampoldi et al., 2011). Uromodulin is synthesized within the endoplasmic reticulum (ER), trafficked to the apical plasma membrane, cleaved into the tubule and excreted in the urine, where it is the most abundant protein (Rampoldi et al., 2011). Uromodulin likely contains: four epidermal growth factor (EGF)-like domains, of which the second and third are calcium-binding (cb) EGF domains; a cysteine-rich region, which includes a domain of eight cysteines; and a zona pellucida (ZP) domain (Bokhove et al., 2016) (Fig. S1). Several functions have been postulated for uromodulin, including: regulating sodium transport in the TAL by modulating the activity of the apical transporters NKCC2 and ROMK (renal outer medullary potassium channel) (Trudu et al., 2013); inhibiting renal stone formation; preventing bacterial colonization; and modulating immune responses (Bates et al., 2004; Durisipudi et al., 2012; Mathey and Naftalin, 1992; Mo et al., 2004a,b, 2007; Raffi et al., 2009, 2005; Säemann et al., 2005a,b).

Over 90% of the >55 reported ADTKD-UMOD mutations are missense, suggesting a dominant negative disease mechanism, and >60% cause loss or gain of a cysteine residue (Moskowitz et al., 2013), implicating a role for protein misfolding in ADTKD-UMOD. Most mutations are clustered in the EGF domains and cysteine-rich region, which are likely involved in formation of most of the 24 disulfide bridges in uromodulin (Rampoldi et al., 2011), and uromodulin missense mutations cause altered intracellular trafficking, with retention of immature, core-glycosylated uromodulin in the ER, resulting in reduced or absent expression at the plasma membrane with little or no secretion (Bernacone et al., 2006; Choi et al., 2005; Dahan et al., 2003; Jennings et al., 2007; Rampoldi et al., 2003; Vylet’al et al., 2006; Williams et al., 2009).
However, the role of uromodulin mutations in causing renal interstitial fibrosis, glomerulosclerosis and immune cell infiltration is not fully understood.

ER accumulation of mutant proteins can cause ER stress, which activates the unfolded protein response (UPR), which may be adaptive or apoptotic in different tissues (Tsang et al., 2010), and consists of three pathways: the activating transcription factor-6α (ATF6α), inositol-requiring enzyme-1 (IRE1) and RNA-activated protein kinase-like ER kinase (PERK) pathways. These act to upregulate chaperones and ER-associated degradation (ERAD) components, and reduce global protein translation to decrease the ER protein load. Under prolonged and unresolved ER stress, cells may also undergo apoptosis (Tsang et al., 2010). The ER stress-induced chaperone 78 kDa glucose-regulated protein [GRP78; also known as binding immunoglobulin protein (BiP)] is upregulated in renal biopsies from patients harboring mutations in UMOD (Adam et al., 2012); however, mechanisms of ER stress and the UPR pathways have not been studied further in ADTKD.

Two Umod mouse knock-out models have been developed, which have revealed roles for uromodulin in: defense against bladder and urinary tract bacterial infections (Bates et al., 2004; Mo et al., 2004b; Raffi et al., 2009, 2005); prevention of renal stone formation (Liu et al., 2010; Mo et al., 2004a, 2007); and water impermeability and solute handling in the TAL (Bachmann et al., 2005; Mutig et al., 2011; Renigunta et al., 2011; Wolf et al., 2013). However, Umod knock-out mice up to 3 years of age do not develop any of the histological features or renal failure characteristic of ADTKD (Raffi et al., 2006). A transgenic mouse model carrying a human UMOD transgene containing the ADTKD-UMOD-causing mutation Cys148Thr (C148W) in addition to the two mouse Umod alleles was not reported to have renal impairment or abnormal renal histology (Takue et al., 2008a,b) (Table S1). However, a second transgenic mouse expressing mouse Umod with the C148W mutation developed several features of ADTKD-UMOD, including mild renal failure, urinary concentrating defects, and interstitial inflammation and fibrosis, but decreased uric acid excretion was not reported (Bernascone et al., 2010) (Table S1). Thus, two similar transgenic models demonstrated two different phenotypes and, since in both models uromodulin was overexpressed compared to in non-transgenic mice, these models require cautious interpretation. An N-ethyl-N-nitrosourea (ENU)-generated uromodulin mutant [Ala227Thr (A227T)] mouse has been described to develop azotemia, impaired urine concentrating ability and reduced excretion of uric acid, but not renal fibrosis (Kemter et al., 2009), whilst a second ENU mutant, Cys93Phe (C93F), developed renal failure, urinary concentrating defects, and interstitial inflammation and fibrosis, but decreased uric acid excretion was not reported (Bernascone et al., 2010) (Table S1). This mutation was therefore selected as being representative of the majority of disease-causing uromodulin mutations.

**RESULTS**

**Introduction of C125R mutation into the mouse germline**

A targeting vector containing 7.6 kb of the mouse Umod gene, with the C125R mutation in exon 3, a thymidine kinase (TK) cassette at the 5’ end and a neomycin resistance (NeoR) cassette in intron 2, was used to introduce the C125R mutation into the mouse genome by homologous recombination in ES cells (Fig. S1). Of 272 ES cell clones that were selection resistant, 5 clones, termed B6, C2, D11, E10 and H10, were shown to have undergone homologous recombination at the 5’ and 3’ ends of the targeting construct by PCR/restriction assay (data not shown) and Southern blot analysis (Fig. 1A). Blastocyst injection of clone C2 resulted in the birth of chimeras with a high degree of chimerism (>90%). Male chimeras were bred with C57BL/6J females and the mutation was shown to enter the germline by genotyping of mice using PCR and BsoBI restriction digest (Fig. 1B). Germline mutant mice were bred with β-actin-Cre mice to excise the NeoR cassette. Heterozygous (Umod+/125R) and homozygous (Umod125R/125R) mutant mice were viable and fertile, with appearances and body weights (Table 1) similar to wild-type (Umod+/+).

**Plasma biochemistry in 8-week- and 6-month-old Umod+/+, Umod125R+/+ and Umod125R/125R mice**

Male (P<0.001) and female (P=0.01) Umod125R/125R mice had plasma urea levels ~2-fold greater than those of Umod+/+ and Umod125R/125R littersates at 8 weeks of age (Table 1). By 6 months of age, plasma urea levels were increased ~1.5-fold and ~2.5-fold in Umod125R/125R (male P<0.0001; female P<0.01) and Umod125R/125R mice (male P<0.0001; female P<0.0001) mice, respectively, compared to Umod+/+ littersates (Table 1). Plasma urea increased between 8-week- and 6-month-old Umod125R/125R mice by ~1.8-fold (P<0.0001) in males and ~1.2-fold (P<0.05) in females, demonstrating progressive renal failure (Table 1). Plasma creatinine was also significantly raised, by ~1.5-fold and ~1.8-fold, in 6-month-old Umod125R/125R (P<0.05) and Umod125R/125R males (P<0.001), respectively, and by ~1.2-fold in Umod125R/125R females (P<0.05) compared to age and sex matched Umod+/+ littersates (Table 1). Male and female 6-month-old Umod125R/125R mice also had significantly raised plasma corrected calcium concentrations (P<0.01) compared to Umod+/+ littersates, which, in conjunction with a significantly raised plasma alkaline phosphatase (ALP) activity (males P<0.001; females P<0.01), is consistent with the likely onset of tertiary hyperparathyroidism (Table 1). However, this hypercalcemia in association with raised plasma ALP activity in the Umod125R/125R mice, which has also previously been reported in two ENU-induced mutant mouse models with A227T and C93F Umod mutations (Kemter et al., 2009, 2013) (Table S1), has not been observed to occur in ADTKD-UMOD patients who develop renal failure. A plausible explanation for this occurrence of hypercalcemia, likely due to tertiary hyperparathyroidism, in Umod mutant mice but not ADTKD-UMOD patients is that the earlier detection of renal failure and associated secondary hyperparathyroidism, with appropriate treatment using vitamin D analogs, phosphate binders and calcimetics, will prevent the
progression to tertiary hyperparathyroidism in patients. Plasma uric acid levels were not increased in Umod+/-125R or Umod125R/125R mice at 8 weeks or 6 months, likely due to expression of the hepatic enzyme uricase in mice (Wu et al., 1992). Plasma sodium and potassium concentrations were similar in mutant and wild-type mice (data not shown).

Urine biochemistry in 8-week- and 6-month-old Umod+/-, Umod+125R and Umod125R/125R mice

Male and female Umod125R/125R mice excreted ~2–4-fold increased volumes of urine (corrected for body weight) (P<0.0001), and this urine was more dilute with ~2–4-fold decreased urine osmolality (8-week-old males and 6-month-old males and females P<0.0001;

Fig. 1. Uromodulin maturation, excretion and trafficking. (A) Homologous recombination in ES cell clones assessed by Southern blot analysis: DNA from 5 clones (B6, C2, D11, E10, H10) and a wild-type (WT) control underwent SpeI digestion, generating products of ~8.3 kb [wild-type (W) allele] and ~9.6 kb [mutant (m) allele]. The probe for detection spanned Umod exon 6. (B) Genotyping of mice: the C125R mutation introduced an additional BsoBI site, which was used to digest PCR products of part of exon 3, leaving the wild-type (W) 144 bp product uncut, and cleaving the mutant product into 116 bp (m) and 38 bp (not shown) fragments. Digests from two each of wild-type (+/+), heterozygous (+/125R) and homozygous mutant (125R/125R) mice are shown. (C) Western blot analysis of whole kidney lysates from Umod+/-, Umod+125R and Umod125R/125R male mice aged 8 weeks (top) and 6 months (bottom). Umod+/- mice had only the mature form (M) of uromodulin (U), but Umod+125R and Umod125R/125R mice had varying amounts of the precursor form (P), and high molecular mass uromodulin that may be dimers of the precursor. Tubulin (T) was used as a control. For technical reasons, in the lower panel of C, the same lysates were blotted for uromodulin and tubulin on two different gels. (D) Representative western blots of urine from males aged 8 weeks (upper blot) and 6 months (lower blot), with densitometric analysis. Umod+/- and Umod+125R samples with 1/20 dilution; Umod125R/125R samples undiluted. Densitometric measurements were plotted relative to mean of Umod+/- densities (set to 100%). Umod+/-: black bars; Umod+125R: gray bars; Umod125R/125R: white bars. Values shown as mean+s.e.m.; n=8 for 8-week-old females, n=7 for 8-month-old females, n=8 for 8-week-old Umod+/- females, n=12 for 6-month-old Umod+/- females, n=6 for 6-month-old Umod125R/125R males, n=10 for 6-month-old Umod125R/125R females. *P<0.001, **P<0.0001 versus Umod+/- littermates of the same age and sex. $P<0.05, $$$P<0.00001 versus 8-week-old mice of the same sex and genotype (Student’s unpaired 1-tailed t-test). (E) Immunohistochemical analysis of uromodulin showing apical expression in Umod+/- mice, partial intracellular retention in Umod+125R and Umod125R/125R mice, and dense intracellular deposits in Umod125R/125R mice. Nuclei counterstained with hematoxylin; scale bars: 25 µm. (F) Co-immunofluorescent labeling for uromodulin (red) and calnexin (green), showing lack of colocalization in Umod+/- kidneys, and partial and almost total colocalization in Umod125R/125R kidneys, respectively. Nuclei counterstained with DAPI; scale bars: 25 µm.
compared to wild-type mice of the same age and sex.

### Table 1. Plasma biochemistry in 8-week- and 6-month-old mice

| Parameter | Male | Female |
|-----------|------|--------|
| Umod+/+ | Umod+125R | Umod125R/125R |
| 8 weeks | | |
| n | 8 | 8 | 8 |
| Body weight (g) | 25.8±0.3 | 26.1±0.4 | 26.5±0.6 |
| Urea (mmol/l) | 15.9±0.6 | 11.9±0.6 | 19.1±1.8<sup>7</sup> |
| Creatinine (umol/l) | 3.4±0.1 | 3.4±0.2 | 3.3±0.2 |
| Pi (mmol/l) | 150.9±9.5 | 143.3±5.7 | 146.8±6.7 |
| Corr.Ca (mmol/l) | 2.85±0.03 | 2.83±0.03 | 2.77±0.05 |
| Uric acid (umol/l) | 345.9±3.6 | 347.±2.6 | 353.6±1.7 |
| Osm (mOsm/kg) | 32.6±0.9 | 32.4±0.6 | 32.9±1.1 |
| 6 months | | |
| n | 10 | 12 | 6 |
| Body weight (g) | 32.6±0.9 | 32.4±0.6 | 32.9±1.1 |
| Urea (mmol/l) | 13.6±0.9 | 21.5±1.4<sup>8,11</sup> | 36.4±2.6<sup>8,11</sup> |
| Creatinine (umol/l) | 17.6±0.9 | 25.9±4.4<sup>4</sup> | 31.3±4.1<sup>4</sup> |
| Pi (mmol/l) | 150.9±9.5 | 143.3±5.7 | 146.8±6.7 |
| Corr.Ca (mmol/l) | 2.85±0.03 | 2.83±0.03 | 2.77±0.05 |
| Uric acid (umol/l) | 345.9±3.6 | 347.±2.6 | 353.6±1.7 |
| Osm (mOsm/kg) | 32.6±0.9 | 32.4±0.6 | 32.9±1.1 |

Values are mean±s.e.m.; +/+, wild types; +/125R, heterozygotes; 125R/125R, homozygotes; * denotes number of mice; ALP, alkaline phosphatase; Corr.Ca, calcium corrected for albumin; Osm, osmolality; Pi, inorganic phosphate. *P<0.05; †P<0.01; ††P<0.001 compared to wild-type mice of the same age and sex. †P<0.05; ††P<0.001 compared to 8-week-old mice of the same sex and genotype.

Eight-week-old females (P<0.05) compared to Umod+<sup>+/+</sup> littmates (Table 2). In addition, male Umod+<sup>+/+125R</sup> mice aged 8 weeks (P<0.05) and male and female Umod+<sup>125R/125R</sup> mice aged 6 months (P<0.05) had reduced urine osmolality, and 6-month-old male Umod+<sup>125R</sup> mice also excreted 1.5-times more urine (P<0.05) compared to Umod+<sup>+/+</sup> littmates (Table 2). In 6-month-old Umod+<sup>125R/125R</sup> males and females (P<0.01), and Umod+<sup>+/125R</sup> males (P<0.05), this large excretion of dilute urine caused plasma osmolality to rise and, in other mutant mice, plasma osmalities were inappropriately normal (Table 1). Thus, Umod+<sup>125R</sup> and Umod+<sup>125R/125R</sup> mice have a urine concentrating defect, which is similar to that reported in ADTKD-UMOD patients (Rampoldi et al., 2003; Scolari et al., 2004).

Table 2. Urinary parameters in 8-week- and 6-month-old mice

| Parameter | Male | Female |
|-----------|------|--------|
| Umod+<sup>++</sup> | Umod+<sup>++125R</sup> | Umod+<sup>125R/125R</sup> |
| 8 weeks | | |
| n | 8 | 8 | 8 |
| 24 h vol/bw (µl/g) | 99.5±5.0 | 102.3±10.7 | 173.1±7.1<sup>8</sup> |
| Osm (mOsm/kg) | 2212±64 | 1960±78<sup>9</sup> | 1322±90<sup>9</sup> |
| Cr clear (ml/min) | 0.43±0.03 | 0.40±0.02 | 0.40±0.03 |
| FEUA (%) | 0.7±0.02 | 0.7±0.07 | 0.6±0.11 |
| 24 h UA (mmol) | 1165±7.0 | 1071±8.2 | 709±3.4<sup>9</sup> |
| 24 h Ca (µmol) | 3.47±3.0 | 4.12±4.07 | 4.46±5.58 |
| pH | 6.67±0.07 | 6.53±0.13 | 6.27±0.06<sup>9</sup> |
| 6 months | | |
| n | 10 | 12 | 6 |
| 24 h vol/bw (µl/g) | 76.3±7.6 | 98.8±6.7<sup>8</sup> | 170.1±21.0<sup>9</sup> |
| Osm (mOsm/kg) | 1581±118 | 1198±75<sup>9</sup> | 675±42<sup>9</sup> |
| Cr clear (ml/min) | 0.31±0.04 | 0.25±0.04 | 0.16±0.06<sup>8</sup> |
| FEUA (%) | 1.52±0.08 | 0.75±0.17<sup>10,11</sup> | 0.54±0.20<sup>10,11</sup> |
| 24 h UA (mmol) | 1024±140 | 426±477<sup>9</sup> | 213±67<sup>9</sup> |
| 24 h Ca (µmol) | 2.92±0.40 | 4.52±0.25<sup>10</sup> | 4.28±0.80<sup>9</sup> |
| pH | 6.51±0.08 | 5.71±0.07<sup>9</sup> | 5.79±0.09<sup>9</sup> |

Values are mean±s.e.m.; +/<sup>+</sup>, wild types; +<sup>125R</sup>, heterozygotes; <sup>125R/125R</sup>, homozygotes; * denotes number of mice; 24 h vol/bw, 24 h urine volume (vol) corrected for body weight (bw); Osm, osmolality; Cr clear, creatinine clearance; FEUA, fractional excretion of uric acid; 24 h UA, 24 h uric acid excretion; 24 h Ca, 24 h calcium excretion. ‡P<0.05 because two of the urine samples were below the lower limit of detection for uric acid concentration. †P<0.05; ††P<0.005; ‡P<0.001 compared to wild-type mice of the same age and sex.
Umod<sup>125R/125R</sup> male and female mice had a significantly (P<0.05 to P<0.005) reduced FEUA, compared to Umod<sup>+/+</sup> littermates (Table 2). This specific reduction in uric acid excretion in Umod<sup>+/125R</sup> and Umod<sup>125R/125R</sup> mice is analogous to the human ADTKD-UMOD phenotype, in which >90% of patients have a decreased FEUA (Williams et al., 2009).

Urinary calcium excretion was elevated by ~1.6-fold (P<0.05) and ~1.8-fold (P<0.005) in 8-week-old female Umod<sup>125R</sup> and Umod<sup>125R/125R</sup> mice, respectively, compared to Umod<sup>+/125R</sup> and Umod<sup>+/125R</sup> mice, and elevated by ~1.5-fold in Umod<sup>+/125R</sup> (P<0.005) and Umod<sup>125R/125R</sup> (P<0.05) 6-month-old males, and ~1.6-fold (P<0.0001) and ~1.8-fold (P<0.0001) in Umod<sup>125R</sup> and Umod<sup>125R/125R</sup> 6-month-old females, respectively, when compared to Umod<sup>+/125R</sup> littermates (Table 2). This hypercalciuria of Umod mutant mice, which has not been described in human ADTKD-UMOD patients, was associated with urinary acidification. Thus, urinary pH was significantly reduced in 8-week-old Umod<sup>125R/125R</sup> mice, corresponding to ~1.5-fold (P<0.005) and ~2.5-fold (P<0.0001) increases in urinary H<sup>+</sup> ion concentrations in males and females, respectively, compared to Umod<sup>+/125R</sup> littermates (Table 2). Six-month-old Umod<sup>+/125R</sup> and Umod<sup>125R/125R</sup> mice also had a decreased urinary pH corresponding to up to ~5.3-fold (P<0.0001) and ~7-fold (P<0.005) increases in urinary H<sup>+</sup> ion concentrations in males and females, respectively, compared to Umod<sup>+/125R</sup> littermates (Table 2). This urinary acidification helps to prevent renal stone formation, and is a likely renal response to hypercalciuria (Renkema et al., 2009).

Uromodulin maturation, excretion and trafficking in 8-week- and 6-month-old Umod<sup>+/125R</sup> and Umod<sup>125R/125R</sup> mice

The C126R uromodulin mutation results in defective maturation, trafficking and secretion of uromodulin in vitro (Williams et al., 2009), and we therefore carried out western blot analysis of kidney lysates and urine, and immunohistochemistry, to study this in Umod mutant mice. Kidney lysates from Umod<sup>+/125R</sup> mice showed the presence of uromodulin at ~100 kDa as previously described (Bates et al., 2004), corresponding to mature, fully glycosylated uromodulin (Fig. 1C). Kidney lysates from Umod<sup>+/125R</sup> mice at 8 weeks and 6 months of age had mature uromodulin and a lower molecular mass form of uromodulin, likely the ER-resident precursor, which has core glycosylations only (Williams et al., 2009). Kidney lysates from Umod<sup>125R/125R</sup> mice almost entirely lacked mature uromodulin, with a predominance of the precursor form of uromodulin. Higher molecular mass bands were detected in kidney lysates from Umod<sup>125R/125R</sup> and Umod<sup>125R/125R</sup> mice at 8 weeks and 6 months, at approximately double the molecular mass of the precursor uromodulin; these may represent dimers of lower molecular mass of the precursor uromodulin. Dual-labeling for uromodulin and the ER marker calnexin showed that the intracellular, and, in Umod<sup>125R/125R</sup> kidneys, uromodulin was partly apical and partly intracellular, and, in Umod<sup>125R/125R</sup> kidneys, uromodulin was almost exclusively located in dense intracellular deposits. Dual-labeling for uromodulin and the ER marker calnexin showed that the intracellular uromodulin in Umod<sup>125R</sup> and Umod<sup>125R/125R</sup> kidneys was colocalized with the ER (Fig. 1F), as previously demonstrated both in vitro (Williams et al., 2009) and in ADTKD-UMOD patients (Bernacone et al., 2010), and consistent with the presence of the lower molecular mass precursor detected by western blotting (Fig. 1C). In Umod<sup>+/125R</sup> mouse kidneys, uromodulin colocalized with calnexin and was present at the apical membrane.

Renal fibrosis and immune cell infiltration in 8-week- and 6-month-old Umod<sup>+/125R</sup> and Umod<sup>125R/125R</sup> mice

Masson’s trichrome staining, to study renal damage and fibrosis, of kidney sections from 8-week- and 6-month-old mice revealed that, at 8 weeks, kidneys of Umod<sup>+/125R</sup> mice did not have any abnormalities, but that kidneys of Umod<sup>125R/125R</sup> mice had thickened basement membranes and some intratubular fibrous deposits, similar to those reported in a renal biopsy from a patient with a UMOD mutation (Nasr et al., 2008). Glomeruli appeared normal in both Umod<sup>+/125R</sup> and Umod<sup>125R/125R</sup> mouse kidneys at 8 weeks of age (Fig. 2A). At 6 months of age, Umod<sup>+/125R</sup> mouse kidneys had mild interstitial fibrosis, and Umod<sup>125R/125R</sup> mouse kidneys had large areas of fibrosis, with some renal tubules appearing to become detached from the basement membrane. Furthermore, some glomeruli in Umod<sup>125R/125R</sup> mouse kidneys also had increased fibrotic material, consistent with glomerulosclerosis (Fig. 2A). No cysts were observed in Umod<sup>+/125R</sup> or Umod<sup>125R/125R</sup> mouse kidneys at 8 weeks or 6 months of age.

During analysis of uromodulin-labeled and trichrome-stained kidney sections, interstitial cell infiltrates were noted. To determine the nature of these infiltrates, kidney sections were labeled for T-cell (CD3) and macrophage (F4/80) markers. Umod<sup>+/125R</sup> mouse kidneys at 8 weeks of age had no detectable immune cell infiltration; however, Umod<sup>125R/125R</sup> mouse kidneys showed small areas of interstitial infiltrates, consisting of T-cells and macrophages (Fig. 2B,C). At 6 months of age, Umod<sup>+/125R</sup> mouse kidneys showed small areas of interstitial T-cells and macrophages, but Umod<sup>125R/125R</sup> mouse kidneys had multiple, large areas of interstitial infiltrates, consisting of both T-cells and macrophages (Fig. 2B,C). These infiltrates occurred mainly in the medullary region, and often in close proximity to TAL cells expressing mutant uromodulin.
Upregulation of ER stress and UPR pathways in mutant TALs

Accumulation of mutant proteins in the ER may cause ER stress, triggering an adaptive pathway called the unfolded protein response (UPR). Members of the chaperone family of GRPs, and in particular GRP78, are upregulated by the UPR in response to ER stress. We therefore used immunohistochemistry of serial kidney sections to investigate GRP78 expression in TAL cells expressing mutant uromodulin. In \textit{Umod}^{+/+} kidneys, GRP78 was expressed at a very low level in non-TAL tubular epithelial cells, and at a slightly elevated level in TAL tubular cells (Fig. 3A,B), a finding that was confirmed in kidneys from an unrelated mouse line (data not shown). However, in kidneys from both 8-week- and 6-month-old \textit{Umod}^{125R} and \textit{Umod}^{125R/125R} mice, GRP78 expression was upregulated specifically in TAL cells expressing mutant uromodulin, and not in other tubular segments (Fig. 3A,B).

We sought to determine which of the three UPR pathways may be active in TAL cells expressing mutant uromodulin, by immunohistochemistry to detect expression of a member of each of the three pathway, which are: ATF6α (ATF6α pathway), spliced (active) XBP1 (XBP1S; IRE1 pathway) or ATF4 (PERK pathway). None of the three pathways were upregulated in TAL cells in \textit{Umod}^{+/+} mice when compared to non-TAL cells (Fig. 4A,B). No activation of the ATF6α pathway was detected in \textit{Umod}^{+/125R} or \textit{Umod}^{125R/125R} mice at either 8 weeks or 6 months of age, as there was no increase in nuclear ATF6α expression in TAL cells (Fig. 4A,B). By contrast, both XBP1S and ATF4 were upregulated in TAL cells of \textit{Umod}^{+/125R} and \textit{Umod}^{125R/125R} mice compared to \textit{Umod}^{+/+} littermates at both 8 weeks and 6 months of age (Fig. 4A,B), demonstrating upregulation of the IRE1 and PERK pathways, respectively, in response to retention of mutant uromodulin in the ER.

In order to quantify the upregulation of these ER stress and UPR pathways, we microdissected TALs from \textit{Umod}^{+/+} and \textit{Umod}^{125R/125R} mice. Quantitative real-time PCR (qRT-PCR) analysis for nephron segment-specific genes was undertaken to demonstrate the purity of the obtained TALs (data not shown), followed by qRT-PCR and western blot analyses for UPR components. qRT-PCR
Confirmation of CHOP protein upregulation and Atf6α for uromodulin showed that, as in TUNEL assay and co-staining however, analysis of kidney sections using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and co-staining showed that CHOP protein was significantly upregulated by ∼4.2-fold (P < 0.001) in Umod125R/+ TALs compared to Umod+/+ TALs, consistent with regulation of GRP78 expression at the translational level (Gulow et al., 2002) (Fig. 5A). Interestingly, ATF6α was significantly downregulated by ∼1.8-fold (P < 0.05) in Umod125R/125R TALs compared to Umod+/+ TALs (Fig. 5B,C). Xbp1S and ATF4 were significantly upregulated by ∼13.6-fold (P < 0.001) and ∼4.5-fold (P < 0.01), respectively, in Umod125R/125R TALs compared to Umod+/+ TALs, confirming the results obtained by immunohistochemical analysis.

Upregulation of the PERK pathway via ATF4 is associated with suppression of general protein translation but induction of expression of specific UPR target genes, such as CCAAT/enhancer-binding protein homologous protein (CHOP) (Hetz, 2012). qRT-PCR analysis of isolated TALs showed that Umod mRNA was downregulated by ∼6.6-fold (P < 0.001) in Umod125R/125R TALs compared to Umod+/+ TALs (Fig. 5A). Chop mRNA was upregulated by ∼2.2-fold (P < 0.001) in Umod125R/125R TALs compared to Umod+/+ TALs (Fig. 5A), and immunohistochemical analysis of serial kidney sections confirmed that CHOP protein was upregulated in TAL cells in Umod125R/125R and Umod125R/125R mice compared to Umod+/+ littermates at both 8 weeks and 6 months of age, as shown by accumulation of CHOP in the nuclei of TAL cells (Fig. 6A,B). Increased CHOP expression may lead to apoptosis (Hetz, 2012); however, analysis of kidney sections using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and co-staining for uromodulin showed that, as in Umod+/+ littermates, there were no TUNEL-positive TAL cells in Umod125R/125R and Umod125R/125R mouse kidneys at either 8 weeks or 6 months of age (Fig. 6A,B). However, occasional TUNEL-positive cells were detected in other segments, demonstrating that the assay was functional. Thus, mechanisms other than apoptosis are likely responsible for tubular damage in Umod+/125R and Umod125R/125R mice.

**DISCUSSION**

We have generated a mouse model with an ADTKD disease-causing UMOD mutation, that displays: defective uric acid excretion; urinary concentrating defects; renal failure; defective uromodulin trafficking, maturation and secretion; renal fibrosis; interstitial immune cell infiltration; and ER stress with upregulation of the UPR in the TAL. This model is representative of ADTKD-UMOD patients, who develop hyperuricemia, low FEUA and gout, chronic renal failure, tubulointerstitial nephropathy, glomerulosclerosis and lymphocytic infiltration (Lhotta et al., 1998; Turner et al., 2003). Thus, this mouse provides an in vivo model to study mechanisms of renal fibrosis and ER stress.

A major feature of ADTKD-UMOD in humans is hyperuricemia, which was not present in Umod mutant mice. This is not surprising, as mice express the hepatic enzyme uricase (urate oxidase), which catalyses low solubility uric acid to the high solubility allantoin; uricase has been evolutionarily silenced in humans and other primates (Wu et al., 1992). However, Umod+/125R and Umod125R/125R mice, similar to ADTKD-UMOD patients, still demonstrated significant decreases in both FEUA and 24 hour uric acid excretion. Interestingly, in female Umod125R/125R mice, this decreased uric acid excretion was detected after the onset of renal failure, as measured by a significantly increased plasma urea, since 8-week-old Umod125R/125R females had an elevated plasma urea, but a similar uric acid excretion compared to Umod+/+ females. The role of plasma uric acid levels in the pathogenesis of ADTKD-UMOD has been the subject of much debate, particularly whether drugs that lower plasma uric acid can slow the progression of ADTKD-UMOD in patients (Bleyer and Hart, 2003; Fairbanks et al., 2002, 2004; Puig et al., 2006; Puig and Torres, 2003, 2004).
Our model demonstrates that hyperuricemia is a secondary consequence of the other phenotypes of ADTKD-UMOD (e.g. decreased uric acid excretion, renal failure and kidney histopathology), and is not required to cause the renal pathophysiology, since our model develops these other phenotypes without hyperuricemia. However, hyperuricemia may help to propagate or enhance the disease progression in humans, and this hypothesis could be tested in our model by using uricase inhibitors.

The mechanism of decreased uric acid excretion is not fully understood, but may be a consequence of the urine concentrating defect. In the normal TAL, the water impermeability is likely due to the presence of uromodulin in a gel-like structure at the apical plasma membrane, which is permeable to ions but not to water (Mattey and Naftalin, 1992). In Umod<sup>+-/-</sup> and Umod<sup>125R/125R</sup> mice, retention of the mutant uromodulin protein in the ER of the TAL cells would decrease the amount of uromodulin at the apical plasma membrane, and may compromise this water impermeability. This may lead to a reduction in the normally high osmolality of the medullary interstitium, thus inhibiting water reabsorption in the collecting duct (CD), and resulting in a more dilute urine (Devuyst et al., 2005). The previously reported Umod<sup>-/-</sup> knock-out mice also have a urine concentrating defect (Bachmann et al., 2005). This defect in urine concentration due to alterations of TAL characteristics may be analogous to patients treated with loop diuretics, such as those with hypertension, who also develop secondary hyperuricemia (Ramsay et al., 1994). Loop diuretics (e.g. furosemide) specifically inhibit the TAL Na+:K+:2Cl<sup>-</sup> (NKCC2) transporter, decreasing water reabsorption with a consequent increase in urine volume (Gamba and Friedman, 2009). During loop diuretic treatment, the resulting decrease in extracellular fluid (ECF) volume causes increased sodium reabsorption by the proximal convoluted tubule (PCT), which likely drives increased uric acid reabsorption, hence causing the hyperuricemia which is often associated with these drugs (Weinman et al., 1975). A possible compensatory mechanism for excess water loss has been described in Umod<sup>-/-</sup> knock-out mice by upregulation of several critical TAL ion transporters, including the target of loop diuretics, NKCC2, which is usually activated by uromodulin (Bachmann et al., 2005). Thus, similar mechanisms may

Fig. 4. Analysis of UPR pathways in Umod<sup>+-</sup>, Umod<sup>125R</sup> and Umod<sup>125R/125R</sup> kidneys. Immunohistochemical staining of serial sections for uromodulin, ATF6α, ATF4 and spliced (active) XBP1 (XBP1<sup>S</sup>) in (A) 8-week-old and (B) 6-month-old mice, showing upregulation of ATF4 and XBP1<sup>S</sup> specifically in TAL cells expressing mutant uromodulin in Umod<sup>125R</sup> and Umod<sup>125R/125R</sup> kidneys, but no upregulation of ATF6α. Nuclei counterstained with hematoxylin; scale bars: 50 µm.
be responsible for hyperuricemia in loop diuretic-treated hypertensive patients and ADTKD-UMOD patients; this requires further investigation in Umod+/–/125R and Umod+/25R/125R mice.

The urine concentrating defect described in Umod+/–/ knock-out mice (Bachmann et al., 2005) and Umod+/–/125R and Umod+/125R/125R mice in this study is likely due to the absence of uromodulin at the apical plasma membrane of the TAL. However, Umod+/–/ knock-out mice do not develop any histological characteristics of ADTKD-UMOD, or renal failure (Raffi et al., 2006). Thus, mutant uromodulin protein must be present to cause the renal pathology associated with ADTKD-UMOD, and we have shown that histopathological changes characteristic of human ADTKD-UMOD patients, such as interstitial fibrosis and immune cell infiltration, are present in our TgUmod+/125R and Umod+/125R/125R knock-in mice. Uromodulin is the most abundant protein in the urine, and is expressed only by the TAL (Scolari et al., 2015), indicating a high rate of uromodulin expression in TAL cells. Thus, even though cells possess mechanisms to degrade misfolded proteins, such as ERAD, the high expression rate of mutant uromodulin over time may overwhelm such mechanisms. Uromodulin can also stimulate the innate and adaptive immune systems (Säemann et al., 2005b; Su et al., 1997). Thus, it has been postulated that kidney damage may compromise its integrity and allow uromodulin to be exposed to the immune system, which may cause antibody production (Säemann et al., 2005b), as well as inflammation and/or immune cell infiltration, which can result in fibrosis of the kidney (Eddy, 2014).

Umod+/125R and Umod+/125R/125R mice have several phenotypic characteristics that are similar to those reported for the C147W uromodulin transgenic (TgUmodC147W) mouse model, including renal failure, urinary concentrating defects, fibrosis, immune cell infiltration and intracellular uromodulin retention. However, Umod+/125R and Umod+/125R/125R mice had a selective defect in renal uric acid handling that was not studied in TgUmodC147W transgenic mice (Bernascone et al., 2010), whereas TgUmodC147W mice had renal cysts, which were not detected in Umod+/125R or Umod+/125R/125R mice (Table S1). The reason for these differences between Umod+/125R mice and TgUmodC147W mice remains to be elucidated, but there are several possible explanations. First, these differences may involve strain-specific factors, as the TgUmodC147W mice were generated on an FVB background, whilst the Umod+/125R mice were on a C57BL/6 background. Second, the TgUmodC147W mice are a transgenic model that express Umod at higher levels than wild-type mice without the transgene, whereas, in Umod+/125R mice, the mutant Umod directly replaces the wild-type allele. Third, TgUmodC147W mice express mutant uromodulin with a different mutation compared to Umod+/125R mice. Patients with different mutations have marked variations in some phenotypic features, such as rate of progression to ESRF (Moskowitz et al., 2013); however, this does not seem to be true of hyperuricemia, which is present in >90% of patients (Williams et al., 2009). The presence or absence of cysts is the most variable phenotype associated with ADTKD-UMOD in humans and may only be found in <15% of patients (Williams et al., 2009). Indeed, different members of the same family may have variable cyst formation (Rampoldi et al., 2003; Vylet et al., 2006); this variation may be due to other genetic differences or environmental factors rather than to different UMOD mutations. Cysts have not been reported in

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**Fig. 5. Expression of ER stress and UPR pathway components in isolated Umod+/+ (WT) and Umod+/125R/125R (hom) TALs.** (A) qRT-PCR analysis of Grp78, Atf6α, spliced (active) Xbp1 (Xbp1s), Atf4, Chop and Umod. Gene expression changes are relative to Gapdh and expressed as a fold change over WT expression (mean set to 1). Individual TAL preparations (n=6-11) from 2-4 mice per group are represented as single points; mean±s.e.m. are displayed to the right of each data set; **P<0.01, ***P<0.001 versus WT, using 2-tailed Mann-Whitney U-test. (B) Representative western blots and (C) quantification of GRP78, ATF6α, XBP1s and ATF4. Protein was normalized for loading using β-actin and quantification is expressed as a fold change over WT expression (mean set to 1). Individual TAL preparations (n=6-11) from 3-4 mice per group are represented as single points; mean±s.e.m. are displayed to the right of each data set; *P<0.05, **P<0.01, ***P<0.001 versus WT, using 2-tailed Mann-Whitney U-test.

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**A**

|        | WT | hom |
|--------|----|-----|
| Grp78  | 2  | 1   |
| Atf6α  | 3  | 1   |
| Xbp1s  | 1  | 1   |
| Atf4   | 1  | 1   |
| Chop   | 1  | 1   |
| Umod   | 1  | 1   |

**B**

|        | WT | hom |
|--------|----|-----|
| GRP78  | 2  | 1   |
| ATF6α  | 3  | 1   |
| XBP1s  | 1  | 1   |
| ATF4   | 1  | 1   |
| β-actin| 1  | 1   |

**C**

|        | WT | hom |
|--------|----|-----|
| Grp78  | 2  | 1   |
| Atf6α  | 3  | 1   |
| Xbp1s  | 1  | 1   |
| Atf4   | 1  | 1   |
| Umod   | 1  | 1   |
families with either the C126R or C148W mutations (Lhotta et al., 1998; Rampoldi et al., 2003), and the basis for this variation in cyst formation between different families, family members and mouse models requires further investigation. Two ENU-mutagenized mouse models, carrying A227T or C93F uromodulin mutations on the same backgrounds, have recently been described to have differing rates of disease onset and progression, with C93F mutant mice having the more severe phenotype (Kemter et al., 2013). The ENU mutant mice both displayed decreased uric acid excretion that was present in $Umod^{125R}$ mice but absent in $Tg^{UmodC147W}$ mice, tubulocystic changes that were present in $Tg^{UmodC147W}$ mice but absent in $Umod^{125R}$ mice, and glomerulocystic changes that were absent in both $Tg^{UmodC147W}$ and $Umod^{125R}$ mice (Bernascone et al., 2010; Kemter et al., 2013) (Table S1). These differences between the germline $Umod^{125R}$, transgenic $Tg^{UmodC147W}$, and ENU A227T and C93F mice with different mutations highlight the advantages of having multiple mouse models that provide the opportunity of elucidating further the relationships between protein, uromodulin mutation, genetic modifiers, phenotype and mechanism in ADTKD-UMOD.

Our results provide mechanistic insights by showing that ER stress is present in TAL cells expressing mutant uromodulin, and that two of the three UPR pathways (PERK and IRE1) are active, leading to upregulation of CHOP. However, this did not lead to apoptosis of TAL cells, demonstrating that other mechanisms may be responsible for TAL damage, and consequent fibrosis and immune cell infiltration, in this model. CHOP is a transcription factor typically thought to upregulate pro-apoptotic pathways; however, it may also be protective under certain conditions in different cell types. For example, mice with autosomal dominant retinitis pigmentosa due to ER retention of mutant rhodopsin have retinal damage that is associated with UPR and CHOP upregulation; however, deletion of CHOP led to increased retinal damage in these mice, suggesting a protective role for CHOP (Nashine et al., 2013). Several studies have implicated ER stress, leading to CHOP expression and apoptosis, in models of renal disease. However, these studies have primarily investigated the renal response to toxins (e.g. high glucose in diabetic nephropathy or high albumin levels to mimic proteinuria), drugs (e.g. acetaminophen) or acute renal injury (e.g. using unilateral ureteric obstruction), or have focused on glomerular injury (Dickhout and Krepinsky, 2009). Relatively little work has been carried out to understand the role of chronic ER stress in renal tubule cells endogenously expressing an ER-retained mutant protein, and our mouse model for ADTKD-UMOD will be an important resource for further studies in this area. Active XBP1 protein was also upregulated in TAL cells in $Umod^{125R}$ mice.
demonstrating upregulation of the IRE1 pathway, and this may also be an adaptive or apoptotic response to ER stress (Ghosh et al., 2014). During prolonged ER stress, PERK signaling is maintained whilst IRE1 signaling is attenuated, which may lead to a switch from adaptive to apoptotic signaling (Lin et al., 2007). Consistent with this hypothesis, prolonged IRE1 signaling enhanced cell proliferation and prevented apoptosis when human embryonic kidney (HEK)293 cells were exposed to the UPR inducers tunicamycin and thapsigargin (Lin et al., 2007, 2009). In Umod<sup>125R</sup> mice, both the PERK and IRE1 pathways appeared to be active even at 6 months of age, and the presence of both of these pathways may explain the absence of the apoptotic response in TAL cells in these mice. Studies of the transgenic Tg<sup>Umod147W</sup> mice also showed an absence of apoptosis by a lack of active caspase-3 staining; thus, apoptosis does not appear to play a significant role in tubule damage in ADTKD-UMOD (Bernascone et al., 2010).

In conclusion, we have generated a mouse model for a monogenic cause of renal fibrosis carrying an ADTKD-UMOD disease-causing mutation, C125R, in the endogenous mouse Umod gene. Umod<sup>+/125R</sup> and Umod<sup>125R/125R</sup> mice demonstrate several of the phenotypes of human ADTKD-UMOD patients, including progressive renal failure, urine concentrating defects, decreased uric acid and uromodulin excretion, retention of uromodulin in the ER, and pathological changes in the kidney such as fibrosis, immune cell infiltration and ER stress. The PERK and IRE1 UPR pathways were found to be specifically upregulated in TAL cells of Umod<sup>+/125R</sup> and Umod<sup>125R/125R</sup> mice, which was associated with upregulation of CHOP; however, this did not lead to apoptosis. This novel knock-in model will be a valuable resource to study the mechanisms of pathogenesis and progression of renal fibrosis, ESRF and ADTKD-UMOD, and to test novel therapeutic agents for these diseases.

**MATERIALS AND METHODS**

**Generation of knock-in targeting vector and mutant mice**

Exon 3 of mouse Umod was amplified by PCR and used to screen a mouse PAC library (MRC Geneservice, Cambridge, UK). A PAC containing mouse Umod was then used as a template in a long-range PCR reaction to amplify an ∼7.6 kb product between the 5′ upstream region and intron 5 of Umod. Exon and intron-exon boundary sequences were verified by direct DNA sequencing as described (Nesbit et al., 2004). After insertion into pGEM-T (Promega, Southampton, UK), site-directed mutagenesis (SDM) was performed to introduce the C125R mutation as described (Nesbit et al., 2004); during this procedure, a novel BsoBI restriction site was also generated. SDM was also used to introduce a HindIII site into intron 2. Following each round of SDM, the sequences of exons and intron-exon boundaries were verified by direct DNA sequencing. A neomycin resistance (Neo<sup>+</sup>) cassette flanked by two <i>loxP</i> sites was excised from plasmid pmmneofox8 and cloned into intron 2 of <i>Umod</i> using XbaI and HindIII. Plasmid pBSSTK, containing a thymidine kinase (TK) cassette, was mutagenized by SDM to introduce a SfiI restriction site, and the TK cassette was inserted into the targeting vector at the 5′ end of the <i>Umod</i> gene using SfiI and SacI (Fig. S1). The vector was linearized using SfiI, electroporated into the R1 embryonic stem (ES) cell line, and cells selected using 300 µg/ml G418 (Geneticin, Gibco, Paisley, UK) and 0.2 µM gancyclovir (Calbiochem, Manchester, UK). Cells in which homologous recombination had occurred at both the 5′ and 3′ arms were identified by PCR/restriction digest and Southern blot analysis (Harding et al., 2009) as follows: 5′ PCR: forward primer in the 5′ region outside the vector and reverse primer within the Neo<sup>+</sup> cassette generated a 2.8 kb product in recombined clones only; 3′ PCR: forward primer within exon 3 and reverse primer within intron 5 (outside the vector) generated a 5.3 kb fragment in all clones, which, when digested with BsoBI, generated an extra fragment in recombined clones; 5′ Southern blot: digestion with PstI and a probe within the 5′ upstream region detected an ∼11.6 kb <i>Umod</i> wild-type fragment and an ∼7.4 kb <i>Umod</i> mutant fragment; 3′ Southern blot: digestion with SpeI and a probe corresponding to exon 6 of <i>Umod</i> detected an ∼8.3 kb <i>Umod</i> wild-type fragment and an ∼9.6 kb <i>Umod</i> mutant fragment (Fig. 1A). ES cells heterozygous for the mutation were injected into C57BL/6 blastocysts at embryonic day 2.5, which were transferred into the uterus of pseudopregnant C57BL/6 females. Male chimeras were mated with C57BL/6 females and the offspring tested for germline transmission by extraction of DNA from ear biopsy material in DNA extraction buffer (50 mM Tris pH 8.5, 1 mM EDTA, 0.5% Tween-20, with 50 µg Proteinase K), followed by PCR and BsoBI digestion. The Neo<sup>+</sup> cassette was excised between <i>loxP</i> sites by mating with β-actin-Cre mice, and the removal of the Neo<sup>+</sup> cassette was verified by PCR assay. Mice were backcrossed to the C57BL/6J background strain and mice from backcross (BC3)-BC5 were used for studies in 6-month-old mice, and from BC4-BC7 for studies in 8-week-old mice. Animals were maintained according to local welfare guidelines, and all procedures were carried out under UK Home Office personal and project license restrictions.

**Metabolic cage analysis**

Eight-week- and 6-month-old <i>Umod<sup>+/+</sup></i>, <i>Umod<sup>+/125R</sup></i> and <i>Umod<sup>125R/125R</sup></i> littersmates were investigated in individual metabolic cages (Tecniplast) for 24 h with free access to food and water. Body weight, water intake and urine output were measured and urine was collected under mineral oil. Mice were euthanized by terminal anesthesia, blood collected via the jugular vein, and plasma obtained by centrifugation at 800 g for 10 min. Plasma and urine biochemical analysis was performed on a Beckman Coulter AU680 semi-automated clinical biochemistry analyzer as described (Piret et al., 2012), and plasma calcium was corrected for albumin (Corr.Ca) using the formula: Corr.Ca=Ca(mmol/l)−([Alb(g/l)−30]×0.017) (Piret et al., 2012). Osmolalities were measured using a Model 3320 Osmometer (Advanced Instruments Inc., Norwood, MA, USA) and urinary pH was measured using a Microprocessor pH meter (Hanna Instruments, Leighton Buzzard, UK).

**Western blot analysis of whole kidney lysates and urine**

Uromodulin maturation and excretion in <i>Umod<sup>+/+</sup></i>, <i>Umod<sup>+/125R</sup></i> and <i>Umod<sup>125R/125R</sup></i> mutant mice were studied by western blot analysis of kidney lysates and urine samples. Kidneys were homogenized in β-octylglucoside lysis buffer [20 mM Tris-HCl pH 8; 150 mM NaCl; 60 mM β-octylglucoside (Bernascone et al., 2010)] with a protease inhibitor cocktail (Roche, Burgess Hill, UK). Insoluble material was removed by centrifugation, total protein concentration of the soluble fraction determined using the micro BCA Protein Assay (Pierce, USA) (Bernascone et al., 2010; Williams et al., 2009), and lysates loaded at 20-50 µg total protein per lane. Urine samples were normalized to urinary creatinine such that, for <i>Umod<sup>+/+</sup></i> and <i>Umod<sup>+/125R</sup></i> mutant samples, a urine volume corresponding to 0.5 nmol creatinine was loaded and, for <i>Umod<sup>125R/125R</sup></i> mutant samples, a urine volume corresponding to 10 nmol was loaded. Western blotting and densitometric analysis were performed as previously described, using sheep anti-uromucoid antibody (Biodimension International, Leco, ME, USA), or rabbit anti-tubulin antibody (Abcam ab15246, 1:500), followed by horseradish peroxidase (HRP)-conjugated mouse anti-sheep (Sigma, Poole, UK) or HRP-conjugated goat anti-rabbit (Bio-Rad, Watford, UK) antibodies, respectively (Williams et al., 2009). To control for variances between blots of urinary uromodulin, at least two <i>Umod<sup>+/+</sup></i> samples were loaded on each gel, a standard ‘remove background’ tool was used prior to quantification by densitometry, and mean densities of <i>Umod</i><sup>+/+</sup> bands were set to 100% for comparison with other bands on the same blot.

**Histological and immunohistochemical analysis**

Kidneys were dissected and fixed in 10% neutral buffered formalin for 24 h, then dehydrated, paraffin embedded and sectioned at 5 µm thickness. Trichrome staining was carried out according to standard protocols. For immunohistochemical labeling, sections were rehydrated, then subjected to antigen unmasking either by incubation in 20 µg/ml proteinase K for 5-15 min, or by heat-mediated antigen retrieval in either pH 6, pH 9 or pH 10. Immunohistochemistry was performed as described (Piret et al., 2012), using a Mouse monoclonal Uromodulin antibody (Biodesign International, Saco, ME, USA) or rabbit anti-Uromodulin antibody (Abcam ab25475, 1:500) followed by horseradish peroxidase (HRP)-conjugated mouse anti-rabbit (Sigma, Poole, UK) or HRP-conjugated goat anti-rabbit (Bio-Rad, Watford, UK) antibodies, respectively (Williams et al., 2009). To control for variances between blots of urinary uromodulin, at least two <i>Umod<sup>+/+</sup></i> samples were loaded on each gel, a standard ‘remove background’ tool was used prior to quantification by densitometry, and mean densities of <i>Umod</i><sup>+/+</sup> bands were set to 100% for comparison with other bands on the same blot.
10 buffers. Sections were blocked in pre-immune serum, and probed with primary antibodies: sheep anti-uromucoid (Biodesign International, 1:500); rabbit anti-calcnexin (Abcam ab22595, 1:50); rabbit anti-CD3 (Abcam ab16669, 1:50); rat anti-F4/80 (Serotec clone A3-1, 1:50); rabbit anti-GRP78 (Abcam ab21685, 1:1000); rabbit anti-ATF4 (Abcam ab105383, 1:500); rabbit anti-ATF6α [Santa Cruz Biotechnology (Santa Cruz, CA, USA) sc-22799, 1:50]; goat anti-XBP1 (spliced) (Abcam ab85546, 1:100); or rabbit anti-CHOP (Santa Cruz sc-575, 1:50). Secondary detection was achieved by: HRP-conjugated donkey anti-sheep antibody (Jackson ImmunoResearch, 1:500) visualized using the 3,3′-Diaminobenzidine (DAB) reaction kit (Vector Labs, Petersborough, UK) for uromodulin; the DAKO Envision+ System DAB kit for rabbit primary antibodies; the Santa Cruz ABC staining system for goat primary antibodies; or fluorescently conjugated donkey secondary antibodies (Jackson ImmunoResearch or Invitrogen) for dual-labeling. TUNEL assay was performed using the ApopTag Fluorescein kit (Millipore, Hertfordshire, UK) according to the manufacturer’s instructions. DAB-stained slides were counterstained with hematoxylin, and fluorescently labeled slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were examined by light or UV microscopy using an Eclipse E400 microscope (Nikon, Japan), and images were captured using a DXM1200C digital camera, with an original magnification of 20× or 40×, and NIS-Elements BR2.30 software (both Nikon). Immunohistochemistry was performed on 2 kidney sections from each of 4 mice per group.

**Isolation of mouse TALs, and qRT-PCR and western blot analyses**

Mouse TALs were isolated following a previously described protocol (Glaudemans et al., 2014) with minor modifications. Briefly, 2- to 3-month-old male Umodβ/β and UmodΔz/Δz mice were sacrificed by cervical dislocation. Kidneys were removed, decapsulated and placed in ice-cold dissection solution (Hanks balanced salt solution with 15 mM HEPES, 10 mM D-glucose, 5 mM glycine, 1 mM L-alanine, adjusted to dissection solution (Hanks balanced salt solution with 15 mM HEPES, 10 mM D-glucose, 5 mM glycine, 1 mM L-alanine, adjusted to 325 mMOS/kgH₂O with mannitol and pH 7.4 with NaOH). Each kidney was cut into 2 equal parts by a midsagittal section and then into thin transverse slices that were further cut into radial corticomedullary stripes. Kidney pieces were digested for 30 min in dissection solution supplemented with 245 U/ml type 2 collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 96 µg/ml soybean trypsin inhibitor (Sigma). Remaining undigested kidney fragments were digested for 10 min in fresh collagenase solution. TALs were manually selected under a light microscope using a glass pipet connected to a micromanipulator. TALs were microdissected using RNAqueous® Total RNA Isolation Kit (Millipore, Hertfordshire, UK) and 96 µg/ml soybean trypsin inhibitor (Sigma). Kidney pieces were digested for 30 min in dissection solution supplemented with 1567-1579.

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