Circadian clock disruption and growth of kidney cysts in autosomal dominant polycystic kidney disease

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Running Title: Lack of BMAL1 causes rapid cyst growth in ADPKD.

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Abstract

Background: Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in the *PKD1* and *PKD2* genes, and often progresses to end stage renal disease (ESRD). ADPKD progression is not uniform among patients, suggesting that factors secondary to the *PKD1/2* gene mutation could regulate the rate of disease progression. Here we tested the effect of circadian clock disruption on ADPKD progression. Circadian rhythms are regulated by cell-autonomous circadian clocks composed of clock proteins. BMAL1 is a core constituent of the circadian clock.

Methods: To disrupt the circadian clock, we knocked out *Bmal1* gene in the renal collecting ducts of the *Pkd1*$^{RC/RC}$ (RC/RC) mouse model of ADPKD, to get *Pkd1*$^{RC/RC}$;*Bmal1*$^{f/f}$;*Pkhd1*^{cre} double knockout (DKO) mouse, and in *Pkd1* knockout mouse inner medullary collecting duct cells (*Pkd1*KO mIMCD3) to get *Pkd1Bmal1*KO cells.

Results: Human nephrectomy ADPKD kidneys and *Pkd1*KO mIMCD3 cells showed reduced *Bmal1* gene expression compared to normal controls. When compared to RC/RC kidneys, DKO kidneys showed increased cyst area, cyst number, kidney/body weight ratio, cell proliferation, apoptosis and renal fibrosis. Similarly, *in vitro Pkd1Bmal1*KO cells showed increased cell proliferation compared to *Pkd1KO* cells. Expression of clock genes was significantly altered, and lipogenesis and cholesterol synthesis-related genes was increased in DKO kidneys and *Pkd1Bmal1*KO cells compared to RC/RC kidneys and *Pkd1*KO cells respectively. Moreover, fatty-acid oxidation-related gene expression was reduced in *Pkd1Bmal1*KO cells compared to *Pkd1*KO cells.

Conclusion: Renal collecting duct specific *Bmal1* gene deletion disrupts the circadian clock and triggers accelerated ADPKD progression by altering lipid metabolism-related gene expression.

Key points:

- Lack of BMAL1, a circadian clock protein in renal collecting ducts disrupted the clock and increased cyst growth in an ADPKD mouse model.
- BMAL1 gene deletion increased cell proliferation, fibrosis, lipogenesis and reduced fatty acid oxidation related gene expression.
- Thus, circadian clock disruption could be a risk factor for accelerated disease progression in patients with ADPKD.

Introduction:

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease which is caused by mutations in the *PKD1* and *PKD2* genes. Progressive cyst growth in ADPKD kidneys is accompanied by inflammation and fibrosis, which often leads to loss of renal function and end stage renal disease (ESRD). However, disease progression to ESRD varies significantly among ADPKD patients, even within families with the same *PKD1* gene mutation [1]. Based on the Mayo-Irazabal classification for age-adjusted assessment of ADPKD progression, the predicted incidence of ESRD at 10 years ranges widely between 2.2 to 77.4% in older adults [2]. Thus, factors secondary to the *PKD1/2* gene mutation could regulate the rate of ADPKD progression to ESRD. Here we tested if disruption of the circadian clock is a trigger for accelerated ADPKD progression.

Circadian rhythms are intrinsic, cyclical ~24-hour oscillations in behavior and physiology that coordinate biological processes with the time of day. In mammals, circadian rhythms are regulated by cell-autonomous circadian clocks, which at the molecular level are comprised of clock proteins. The central clock, located in the suprachiasmatic nucleus in the hypothalamus, synchronizes peripheral clocks based on external timing cues. The canonical circadian clock involves heterodimerization of circadian proteins (transcription factors), CLOCK and BMAL1 (activators), which activate the gene expression of *Per1,2,3* and *Cry1,2* (repressors). PER and CRY proteins accumulate over time, repressing CLOCK and BMAL1 action, thereby inhibiting their own transcription. RORα/β and REV-ERBα/β function in an ancillary loop to mediate opposing action on BMAL1 transcription, thus driving rhythmic expression of BMAL1. These feedback loops result in ~24-hour oscillations of clock gene expression, and rhythmic expression of ~40% of protein-coding genes in mammals [3].

In mouse kidneys, ~13% of all genes are expressed in a circadian manner [4, 5]. Moreover, kidney functions including blood pressure rhythms, renal blood flow, glomerular filtration rate (GFR), and excretion of electrolytes such as sodium and potassium display circadian influence [6-9]. Chronic disruption of the circadian clock (chronodisruption) is a well-known risk factor for accelerated ageing, memory loss, metabolic defects, obesity, cancer, cardiovascular defects, and metabolic syndrome in humans [10, 11]. The role of chronodisruption in ADPKD pathogenesis is currently unclear.

BMAL1 is encoded by the *Arntl* (Aryl hydrocarbon receptor nuclear translocator-like protein-1) gene and is a core component of the molecular circadian clock. Together with other clock proteins, BMAL1 plays an essential role in cellular metabolic processes by regulating time-dependent utilization of nutrients and metabolites to support cell growth [12]. *Bmal1* is a master clock gene, and the only clock gene whose deletion alone leads to complete loss of circadian rhythms [13]. Here we examined the effect of collecting duct-specific gene deletion of *Bmal1* on disease progression in an ADPKD mouse model and *Pkd1* gene knockout cells.

Methods:

Human tissue samples: Tissues from de-identified human normal and APKD kidneys were "medical discard" nephrectomy samples from the Kansas PKD Research and Translational Center. IRB approval #5929 approval date 8/25/2020. The time of collection is unknown.

***Bmal1* gene knockout in WT and *Pkd1*KO mIMCD3 cells using Crispr-Cas12a:** Murine *Bmal1* (*Arntl* gene, ID 11865) was disrupted in mIMCD3 cells using a microhomology end joining-based strategy and Cas12a [14]. The **TTTG**GCGTATCTACCACAGGAACT sequence in exon 13 was targeted (PAM in bold). CRISPR sequence was, 5'/AltR1/rUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrGrCrGrUrArUrCrUrArCrCrArCrArGrGrArArCrU/AltR2/-3'. PCR products that showed deletion on PAGE were sequenced after alkaline phosphatase treatment (ExoSAP, # 78200, Applied Biosystems, Foster City, CA). *Bmal1*KO clone and *Pkd1Bmal1*KO had frame-shift deletions of 10bp and 16bp respectively.

Mouse model: RC/RC (p.R3277C) mice [15] were bred with *Bmal1*$^{f/f}$ mice (B6.129S4(Cg)-*Bmal1tm1Weit/J*, carrying loxP sites flanking exon 8 of *Bmal1* gene, Strain#007668, The Jackson Laboratory), and mice carrying Pkhd1^{cre} [16]. The resultant *Pkd1*$^{RC/RC}$;*Bmal1*$^{f/f}$;*Pkhd1*^{cre} mice (RC/RC-*Bmal1*KO) were compared with *Bmal1*$^{f/f}$ (wild type-WT), *Bmal1*$^{f/f}$;*Pkhd1*^{cre} (*Bmal1*KO) and RC/RC littermates. Male mice were used and sacrificed at 8 months of age, between 11:30 AM and 12:30 PM. Studies were approved by our Univerisity's IACUC committee.

Recombination specificity in *Bmal1*KO and DKO mice:

Bmal1 recombination was confirmed by PCR on genomic DNA of whole papilla from mouse kidneys [17, 18]. PCR reaction products run on a 2% agarose gel yielded a 431-bp band for floxed *Bmal1* allele and 572 bp band for recombination product.

Statistics: Values expressed as mean±SEM for *in vivo* and mean±SD for *in vitro* studies. Data were analyzed by two-tailed unpaired T-test with Welch's correction; or one-way ANOVA followed by Tukey's multiple comparison test and two-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism software (Version 9). $P \leq 0.05$ was considered significant.

Supplementary methods: We provide detailed methods for *in vitro* BrdU incorporation [19, 20], quantification of cyst [21], Western blot [22, 23], TUNEL staining [24, 25], immunohistochemistry/immunofluorescence (IHC/IF) staining [26] and quantitative real time PCR (QRTPCR) [27] in supplementary methods, and primer list for QRTPCR in Supplemental Table 1.

Results:

1. Renal collecting duct specific *Bmal1* gene deletion in ADPKD mouse kidneys.

Examination of *BMAL1* (*ARNTL* gene) expression in human nephrectomy ADPKD kidneys showed over 2-fold decrease in *BMAL1* mRNA levels, compared to normal human kidneys (Fig 1A). Consistent with this, the kidney interactive transcriptomics database [28] of single cell RNA-sequencing showed reduced *Bmal1* expression in most renal cells of human ADPKD kidneys compared to normal control kidneys (Fig 1B). To examine the effect of *Pkd1* gene deletion on *Bmal1*, we examined its mRNA levels in control and *Pkd1*$^{-/-}$ mouse renal inner medullary collecting duct cells (mIMCD3) [29] and mouse proximal tubular cells [16, 30]. Cells were synchronized using 100nM dexamethasone for 2h [31], and released for 12h before collection every 4h, for 48h. The control *Pkd1*$^{+/+}$ mIMCD3 cells and *Pkd1*$^{+/-}$ proximal tubule cells showed clear circadian oscillation of *Bmal1* mRNA, with ~20h interval between peaks, while *Bmal1* mRNA levels and circadian oscillation were significantly dampened in the corresponding *Pkd1*$^{-/-}$ cells (Fig 1C, D).

To determine the role of BMAL1 in ADPKD progression, we chose the *Pkd1* gene hypomorphic *Pkd1*$^{RC/RC}$ (RC/RC) mouse model of ADPKD. The RC/RC mice showed no significant difference in renal *Bmal1* levels at 4 or 8 months of age compared to wild type (WT) littermates (Supplemental Fig 1A,B,C,D,E). Renal collecting duct specific *Bmal1* gene knockout in WT mice- *Bmal1*$^{f/f}$;*Pkhd1*^{cre} (*Bmal1*KO) and RC/RC mice- RC/RC;*Bmal1*$^{f/f}$;*Pkhd1*^{cre} double knockout (DKO) mice were generated. To test the effect of *Bmal1* gene deletion, the DKO mice were compared with WT (*Bmal1*$^{f/f}$), RC/RC and *Bmal1*KO littermates. We detected recombination of exon-8 of the *Bmal1* gene (disrupted allele) in the *Bmal1*KO and DKO kidneys, while recombination was not detected in the WT or RC/RC kidneys (Figure 1E). BMAL1 expression was detected in the nuclei of tubular epithelial cells in WT and RC/RC mouse kidneys (Fig 1F).

In the *Bmal1*KO and DKO kidneys, BMAL1 was not detected in some tubular or cyst epithelial cells (Fig 1F). Most large cysts in DKO kidneys showed Dolichos biflorus agglutinin (DBA) staining, implying collecting ducts (Supplemental Fig 1F).

To determine the effect of Bmal1 gene deletion on the circadian clock, we measured clock gene expression. When compared to RC/RC kidneys, the DKO kidneys showed significantly increased mRNA levels of *Clock*, *Cry1*, *RevErba* and *Tef* (Fig 1G). RC/RC kidneys showed significant decreases in *Per1*, *Dbp* and *Tef*, and increases in *Clock* mRNA levels when compared to WT kidneys (Fig 1G). *Clock*, *Cry1* and *RevErba* genes were upregulated in both *Bmal1*KO and DKO kidneys compared to WT kidneys and RC/RC kidneys respectively (Fig 1G).

2. Renal collecting duct specific *Bmal1* gene deletion increased cyst growth in ADPKD mouse kidneys.

At 8 months of age, the DKO kidneys were larger and more cystic (Fig 2A, 2B), with significantly higher cyst number (Fig 2C), cystic index (Fig 2D), kidney weight (Fig 2E) and kidney to body weight ratio (Fig 2F) compared to RC/RC kidneys. No significant changes were observed in the *Bmal1*KO kidneys compared to WT kidneys (Fig 2A,E,F). The results show that gene deletion of *Bmal1* in collecting ducts accelerates renal cyst growth in an ADPKD kidney but has no overt effect on WT kidneys.

3. *Bmal1* gene deletion increased cell turnover and fibrosis in ADPKD mouse kidneys.

To examine the effect of *Bmal1* gene deletion on cell proliferation, kidney tissue sections were immunostained for Ki67 and quantified. DKO mouse kidneys showed significantly increased Ki67 stained nuclei compared to RC/RC kidneys (Fig 3A,B). Immunoblot of kidney tissue lysate showed increased cyclin D1 and reduced P53 protein levels in DKO kidneys compared to

RC/RC kidneys (Fig 3C,D,E). In DKO kidneys, pERK/ERK, pCREB/CREB, and pS6/S6 ratios were significantly increased, suggesting higher activity compared to RC/RC kidneys (Fig 3C,F,G,H). No change was observed in c-Myc levels (Supplemental Fig 2A). No significant difference was observed between *Bmal1*KO and WT kidneys in Ki67 staining (Supplemental Fig 2B, C), or in cyclin D1, pERK/ERK or pCREB/CREB protein levels (Supplemental Fig 2D,E). TUNEL staining of kidney tissue sections (Fig 4A,B) and mRNA levels of renal injury markers, *Kim1* and *Ngal1* (Fig 4C,D) were significantly increased in DKO kidneys compared to RC/RC kidneys. The increased cell proliferation and apoptosis indicate high turnover of cells in the DKO kidneys.

DKO mouse kidneys also showed increased renal fibrosis. Immunostaining for the myofibroblast marker αSMA and the extracellular matrix (ECM) protein, collagen1 were increased in the DKO kidneys compared to RC/RC kidneys (Fig 4A). Moreover, mRNA levels of ECM proteins, *collagen1a*, *collagen3a1* and *fibronectin* were significantly higher in the DKO kidneys compared to RC/RC kidneys (Fig 4E, F, G, H).

4. *Bmal1* gene knockout altered the expression of circadian clock genes and gene targets of BMAL1 in mIMCD3 cells.

To better understand the mechanism by which *Bmal1* gene knockout in the renal collecting ducts accelerates disease progression in RC/RC mice, we deleted *Bmal1* gene in WT and *Pkd1* gene knockout mIMCD3 cell lines using Crispr-Cas12a (Fig 5A, Supplemental Fig 3). WT, *Bmal1*KO, *Pkd1*KO, and *Pkd1Bmal1*KO cells were compared. Consistent with results in mouse kidneys, cell proliferation was significantly increased in *Pkd1Bmal1*KO cells compared to *Pkd1*KO cells, while *Bmal1*KO cells showed no significant difference compared to WT cells (Fig 5B). Examination of circadian clock gene expression showed significantly reduced *Per1*, *Per2*,

RevErba and *Rora*, and increased *Cry1* mRNA levels in *Pkd1Bmal1*KO cells when compared to *Pkd1*KO cells (Fig 5C). The *Pkd1*KO cells showed significant increase in *Per1, Per2*, *RevErba, Rora* and *Hlf* mRNA levels compared to WT cells (Fig 5C). Significant difference in *Per2*, *Cry1* and *RevErba*, members of the repressor arm of the clock were common to both *Bmal1*KO and *Pkd1Bmal1*KO cells when compared to WT and *Pkd1*KO cells respectively (Fig 5C). *Rorb* and *Dbp* were not detected.

We also examined BMAL1's prospective gene targets. BMAL1, a transcription factor binds to E-box elements on gene promoters [32]. The *Pkd1* gene promoter contains an E-box element [33], although it is unknown if *Bmal1* is a transcriptional regulator of *Pkd1*. Contrary to our expectation, the *Bmal1*KO cells had significantly increased *Pkd1* mRNA levels when compared to WT cells (Supplemental Fig 4A). We also examined gene expression of *Ets1*, *Sp1*, *Rxra*, *Cebpa*, *Hnf4a*, *Fli1*, *Hnf1a*, *Hnf1b*, *Hnf4a*, *Pax8* and *Tead4,* based on a previous Chip-seq analysis [34] that identified these genes as targets of BMAL1 in WT mouse kidneys [9]. Of the genes detected, *Cebpa, Ets1* and *Hnf1b* were significantly reduced in DKO kidneys and *Pkd1Bmal1*KO cells when compared to RC/RC kidneys and *Pkd1*KO cells respectively (Supplemental Fig 4A,B).

5. *Bmal1* gene knockout altered the lipid metabolism-related gene expression in *Pkd1Bmal1*KO mIMCD3 cells and DKO mouse kidneys.

Reduced fatty-acid oxidation and increased fatty acid synthesis enhance cyst growth in ADPKD [35-37]. Since *Bmal1* is known to regulate fatty-acid oxidation, lipogenesis and cholesterol metabolism [38-42], we examined genes regulating these processes. In *Pkd1Bmal1*KO cells, mRNA levels of fatty-acid oxidation-related genes, *Acox1*, *Acox2*, *Acadsb*, *Acadvl*, *Cpt1a*, *Cypt2*, *Fabp3*, and *Ppara* showed significant decreases, while *Pgc1a* was increased compared

to *Pkd1*KO cells (Fig 5D). *Pkd1*KO cells showed significantly reduced *Acox2*, *Ppara* and *Pgc1a* mRNA levels compared to WT cells (Fig 5D). *Acox2*, *Acadsb*, *Acadvl* and *Cpt1a* are common genes that were significantly decreased in both *Bmal1*KO and *Pkd1Bmal1*KO cells compared to WT and Pkd1KO cells respectively (Fig 5D).

Lipogenesis associated genes, *Acaca*, *Acss2*, *Dagla*, *Daglb*, *Fads1*, *Fads3*, *Scd1*, and Srebp1c were significantly increased in *Pkd1Bmal1*KO cells compared to *Pkd1*KO cells (Fig 5E). *Acss2*, *Fads3* and *Srebp1c* are common genes that were upregulated in *Pkd1Bmal1*KO cells, but downregulated in the *Bmal1*KO cells compared to WT and *Pkd1*KO cells respectively (Fig 5E). Compared to WT cells, *Pkd1*KO cells showed significantly increased *Srebp1c*, and reduced *Dagla* mRNA levels (Fig 5E). Genes related to cholesterol synthesis including *Hmgcs1*, *Hmgcr*, *Mvk* and *Mvd* were significantly increased in *Pkd1Bmal1*KO cells when compared to *Pkd1*KO cells (Fig 5F). When compared to WT cells, the *Pkd1*KO cells showed significantly reduced levels of *Hmgcs1*, *Hmgcsr*, *Mvk*, *Pmvk*, *Fdps* and *Mvd* (Fig 5F). Other fatty acid oxidation and lipogenesis-related genes that remained unchanged are shown in Supplemental Fig 5A,B.

Examination of lipogenesis-related genes in our mouse study showed significantly increased mRNA levels of *Acaca*, *Dagla*, *Fads1*, *Scd1*, *Srebp1c*, *Mag1* and *Lpcat* genes in DKO mouse kidneys compared to RC/RC kidneys (Fig 6A-H). Similarly, cholesterol metabolism-related *Mvk*, *Pmvk* and *Fdps6* genes were significantly increased in DKO kidneys compared to RC/RC kidneys (Fig 6I, J, K). Fatty-acid oxidation-related genes were not significantly different between DKO and RC/RC kidneys. However, RC/RC kidneys showed significantly reduced expression of *Acox1*, *Acadsb*, *Ppara*, *Acat1*, *Acat2* and *Acads* compared to WT kidneys (Supplemental Fig 6 A-N). Unchanged lipogenesis and cholesterol synthesis-related genes are shown in Supplemental Fig 7A-I.

Discussion

Our studies show that disruption of the circadian clock stimulates accelerated cyst growth in ADPKD. We found reduced *Bmal*1 gene expression in human nephrectomy ADPKD kidneys, and in *Pkd1* gene knockout mIMCD3 and proximal tubule cells, but not in *Pkd1* gene hypomorphic RC/RC mouse kidneys. Renal collecting duct specific gene deletion of *Bmal1* in RC/RC mice increased renal cell proliferation, apoptosis, fibrosis, and cyst growth. *Bmal1* gene deletion significantly altered the expression of other clock genes, reduced the expression of BMAL1 target genes and increased the expression of lipogenesis and cholesterol synthesis-related genes in *Pkd1*KO cells *in vitro*, and in RC/RC mouse kidneys. *Bmal1* gene deletion also reduced the expression of fatty-acid oxidation-related gene expression in *Pkd1*KO mIMCD3 cells.

Our finding that *Bmal1* gene knockout in the renal collecting ducts increased cyst growth in ADPKD mice is significant because it shows for the first time that chronodisruption accelerates ADPKD progression. Chronodisruption is disease inducing, and known to directly drive metabolic dysfunction, cause type-2 diabetes, obesity, various cancers and neurodegenerative conditions, and adversely affect fetal development [43-47]. Environmental or behavioral factors such as mistimed feeding, shift work or jet lag can induce chronodisruption. Shift work for instance, reduces the estimated GFR in humans and is a risk factor for CKD [48-50], cardiovascular disease [51], multiple cancers [52] and metabolic disorders [53]. Since chronodisruption by alterations in behavior or the environment are systemic effects, we used renal collecting duct specific *Bmal1* gene deletion as a model for genetic disruption of the molecular clock so as to focus on the kidney. In our study, *Bmal1* gene knockout altered the expression of clock genes including *Cry1*, *Per1*, *Per2*, and *RevErba* that are in the negative arm of the clock, and are known to be regulated by *Bmal1*.

We found that collecting duct-specific gene deletion of *Bmal1* dramatically increased cyst growth, cell proliferation and fibrosis in the RC/RC mouse model of ADPKD. In prior studies, *Bmal1* gene knockout reduced locomotor activity, and induced tendon calcification, sarcopenia, hypersensitivity to insulin-shock, fragmented sleep, and rapid ageing in mice [54, 55]. In the kidneys, nephron-specific *Bmal1* gene deletion caused Na^+ retention in response to a K^+-restricted diet [56], lowered blood pressure [56-58] and altered the urinary metabolome in mice [54]. However, the effect of altering *Bmal1* gene expression on kidney disease progression is context dependent, as shown in experimental mouse models [59]. In a mouse model of AKI, shRNA mediated *Bmal1* gene knockout alleviated AKI, while adenoviral overexpression of *Bmal1* accelerated renal injury [60]. Similarly in unilateral ureteral obstruction model of CKD, global gene knockout of *Bmal1* reduced fibrosis [61] or had no effect on fibrosis or disease progression [62]. In contrast to the above mentioned studies, and supporting our results, renal tubule specific *Bmal1* gene knockout worsened hyperglycemia, and accelerated renal hypertrophy in mice with streptozotocin-induced type-1 diabetes, compared to similarly treated WT mice [63]. Similarly, global or proximal tubule specific gene knockout of *Bmal1* worsened disease progression in the adenine induced model of CKD in mice [64,65]. We found that in the presence of *Pkd1* gene mutation, *Bmal1* gene knockout in renal collecting ducts increased cell proliferation indicated by increase in Ki-67 immunostaining, and pERK/ERK1/2, pCREB/CREB, pS6/S6 and CyclinD1 levels, all factors known to increase cell proliferation in ADPKD kidneys [24]. Our data is consistent with previous studies where *Bmal1* gene deletion or inactivation increases the proliferation rate of cancer cells [66, 67].

BMAL1 binds to E-box elements on promoters of rhythmically and non-rhythmically expressed genes [32]. Over 7 million E-box elements exist in the mouse genome, ~ 0.7% of which

are bound by CLOCK-BMAL1 or NPAS2-BMAL1 dimers in mouse peripheral tissues [34, 68]. Based on this published data, we examined 12 prospective gene targets of BMAL1 and identified *Ets1*, *Hnf1b* and *Cebpa* to be significantly reduced in *Bmal1* deficient *Pkd1*KO cells and RC/RC mouse kidneys. C/EBPα plays an essential role in embryonic development and supresses cell proliferation by stabilizing p21 and inhibiting *cdk2*, *cdk4* and *E2F* [69]. Inhibiting C/EBPα contributes to tumor progression in liver, pancreatic, breast and lung cancers [69]. Similarly, ETS1 and HNF1 transcription factors have oncogenic or tumor supressor roles in a context-dependent manner in human cancers [70, 71]. HNF1β and ETS1 are also thought to regulate *Pkd1* gene expression [33, 72, 73].

Fatty-acid metabolism is impaired in ADPKD and is thought to enhance renal cyst growth [35-37]. Previous studies in RC/RC mice have shown reduced renal mRNA levels of fatty-acid oxidation-related genes compared to WT mice, and treatment with fenofibrate, a PPARα agonist inhibited cyst growth in the RC/RC mice [35]. Similarly, pups born from female mice fed with a high fat diet had a significantly higher cystic index at P21 as compared to those fed with low fat diet [36]. *In vitro* studies have also shown that *Pkd1* gene mutation leads to defects in fatty-acid oxidation [36, 37]. Consistent with these studies, we found fatty-acid oxidation-related genes to be significantly reduced in *Pkd1*KO cells compared to WT cells, and in RC/RC mouse kidneys compared to WT mouse kidneys.

BMAL1 is known to regulate fatty-acid oxidation [38-42]. Bignon *et al* [42] showed that renal tubule specific *Bmal1* gene deletion in WT mice significantly reduced mRNA and protein expression of ACADS, ACADM, ACADL, and ACADVL which catalyze the rate-limiting step of β-oxidation, suggesting disrupted fatty-acid oxidation. However in our study, renal collecting duct specific *Bmal1* gene deletion in the *Bmal1*KO mice or DKO mice did not alter fatty-acid

oxidation-related genes when compared to WT or RC/RC mouse kidneys. The reason for this disparity is presently unclear. Our results from the mIMCD3 cells were different, with 4 of the 14 fatty acid oxidation-related genes showing significant decreases in the *Bmal1*KO and *Pkd1Bmal1*KO cells compared to WT and *Pkd1*KO cells respectively.

Unlike fatty-acid oxidation, our *in vitro* and *in vivo* results consistently showed increased lipogenesis and cholesterol metabolism-related genes in *Bmal1* gene deleted cells and mouse kidneys. The mRNA levels of 7 out of the 14 lipogenesis-related genes tested in *Pkd1Bmal1*KO cells and in DKO kidneys showed significant increase compared to WT cells and RC/RC kidneys. Of the lipogenesis-related genes, *Acaca*, *Fads1*, *Scd1* and *Srebp1c* were upregulated in both DKO mouse kidneys and in *Pkd1Bmal1*KO cells, suggesting regulation by *Bmal1.* The *Pkd1Bmal1*KO cells and DKO kidneys showed significant increases in several cholesterol synthesis-related genes. Mvk was a frequently upregulated genes in the cells and kidneys. Renal collecting duct specific *Bmal1* gene deletion in WT mice (*Bmal1*KO mice) did not alter any lipogenesis, cholesterol synthesis or fatty-acid oxidation-related genes when compared to WT mouse kidneys, suggesting that *Bmal1* gene knockout in the ADPKD kidneys is important for these changes.

Although *Bmal1* gene deletion altered the expression of multiple clock genes and BMAL1 regulated genes and lipid metabolism-related genes in the mIMCD3 cells and mouse kidneys, their changes were not always similar in the cells and kidneys. This could be because *Bmal1* is deleted only in the collecting duct cells of the mouse kidneys, while the IMCD3 cells had complete gene deletion of *Bmal1*. Moreover, the mice were all sacrificed around 12 PM, while the mIMCD3 cells were collected 24h after synchronization *in vitro*.

While patients with ADPKD often develop non-dipping hypertension, polyuria, polydipsia, and nocturnal polyuria [74-76], it had not previously been shown whether chronodisruption accelerates ADPKD. Our results show that *Bmal1* gene knockout in the renal collecting ducts disrupts the circadian clock and expression of key elements of cell proliferation, fibrosis and lipid metabolism, thereby accelerating cyst growth in our ADPKD models. It is possible, therefore, that chronodisruption in patients with ADPKD might be a cause of, or contribute to more rapid disease progression.

Disclosure Statement:

The authors have declared that no conflict of interest exists.

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Author contributions:

RR conceptualized and designed studies, analyzed the results, and wrote the paper. AJ performed experiments, analyzed results and wrote parts of the paper. VR, CJW and MMS performed some studies, analyzed results and edited the paper. MLG conceptualized studies and reviewed the paper. All authors read, edited and approved the paper.

Data sharing statements:

No proteomics, transcriptomics or GWAS data was generated in this paper. All in vivo studies using mouse models were done, and tissues analyzed at the University of

Kansas Medical Center. All data that was generated are provided in the figures in the main text or in Supplementary Material. The datasets used to examine Bmal1 expression levels in human samples are openly available on the kidney interactive transcriptomics database (https://humphreyslab.com/SingleCell/) and permission was obtained to use it. No proteomics, transcriptomics or GWAS data was generated in this paper. If any added information is required to re-examine or analyze the data shown in this paper, they will be readily available from the lead author. All requests for data will be processed based on institutional policies for noncommercial research purposes. Data sharing could require a data transfer agreement as determined by the University of Kansas Medical Center's legal department.

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Figure Legends:

Figure1. Renal collecting duct specific *Bmal1* gene knockout in WT and RC/RC mice. (A) *Bmal1* mRNA levels in normal human kidneys (NHK) and ADPKD kidneys measured by QRTPCR. ***P<0.001 by T-test (B) *Bmal1* gene expression in human normal control and ADPKD kidneys using kidney interactive transcriptomics database of single cell RNA-seq data. (C) Time course showing fold change in *Bmal1* mRNA relative to 18S mRNA in mouse inner medullary collecting duct cells (mIMCD3) (n=4), and (D) proximal tubule cells (n=3). ***P<0.001 by 2-way ANOVA for C and D. Cells were synchronized using dexamethasone (100nM, for 2h) and released into media with 5% FBS. After 12h, cell lysates were collected every 4 hours for 48h. (E) Agarose gel showing recombination PCR products of genomic DNA obtained from mouse kidney. The 431 bp band denotes the floxed *Bmal1* allele and the 572 bp band represents the recombination product. A single 431 bp band indicates absence of recombination. (F) Immunostaining for BMAL1 (brown) in kidney tissue sections of WT, *Bmal1*KO, RC/RC and RC/RC;*Bmal1*KO (DKO) mice (male, 8 months old) (Scale bar = 100μm). (G) QRTPCR analysis of mRNA levels of clock genes in whole kidney tissue lysates. *=*vs* WT, # =*vs Bmal1*KO and $=*vs* RC/RC. *P<0.05, **P<0.01, ***P<0.001 by One-way ANOVA. n=5 in WT and *Bmal1*KO, and n=7 in RC/RC and DKO.

Figure 2. Renal collecting duct specific *Bmal1* gene knockout increased cyst growth in RC/RC mice. (A) Images of H&E staining of kidney sections and (B) magnified images. Scale bar = 1 mm for A and 400μM for B. (C) Cyst number, (D) cystic index, (E) total kidney weight, and (F) two kidney to body-weight ratio. **P<0.01, ***P<0.001 by T-test for C and D, and One-way ANOVA for E and F.

Fig 3. Renal collecting duct specific *Bmal1* gene knockout increased cell proliferation in RC/RC mouse kidneys. (A) Immunostaining for Ki-67 in mouse kidney tissue sections (brown nuclear staining denotes Ki-67). Scale bar = 100 μm. (B) Quantitation of Ki-67-stained nuclei per kidney section. (C) Immunoblot on kidney tissue lysate and quantitation of band density for (D) Cyclin D1, (E) P53, (F) pERK/ERK ratio, (G) pCREB/CREB ratio, (H) pS6/S6 ratio. *P<0.05, **P<0.01, ***P<0.001 by T-test.

Fig 4. Renal collecting duct specific *Bmal1* gene knockout increased apoptosis and fibrosis in RC/RC mouse kidneys. (A) TUNEL staining (green) (Scale bar 400μm), and immunostaining for αSMA (red), and Collagen1 (green) (Scale bar 100μm) in mouse kidney tissue sections. (B) Quantitation of TUNEL staining per kidney tissue section. (C) QRTPCR for *Kim1*, (D) *Ngal1* (E) *αSMA*, (F) *Collagen 1a1*, (G) *Collagen 3a1,* and (H) *Fibronectin* in kidney tissue lysates. *P<0.05, **P<0.01, ***P<0.001 by T-test in B and One-way ANOVA in C, D, E, F, G and H.

Fig 5. *Bmal1* gene knockout increased cell proliferation and altered clock genes and altered lipid metabolism-related gene expression in *Pkd1*KO mIMCD3 cells. A) Western blot shows WT and *Bmal1*KO clones in WT and *Pkd1* gene knockout mIMCD3 cells. WT-*Bmal1*KO clone #4 and *Pkd1Bmal1*KO clone# 3 were used for further studies. (B) Cell proliferation assessed by BrdU incorporation assay. ***P<0.001 by One-way ANOVA. (C) QRTPCR analysis for mRNA levels of clock genes in cells. (D) mRNA levels of genes that regulate fatty acid oxidation, (E) lipogenesis, and (F) cholesterol synthesis. * = *vs* WT, # = *vs* *Bmal1*KO and $ = *vs Pkd1*KO. *P<0.05, **P<0.01, ***P<0.001 by One-way ANOVA. n=4 in C,D,E and F.

Fig 6. Renal collecting duct specific *Bmal1* gene knockout increased lipogenesis and cholesterol metabolism-related gene expression in RC/RC mouse kidneys. The mRNA levels of genes that regulate lipogenesis, (A) *Acaca*, (B) *Dagla*, (C) *Fads1*, (D) *Scd1*, (E) *Srebp1c*, (F) *Magl*, (G) *Lpcat*, (H) *Elovl6*; and genes that regulate cholesterol metabolism, (I) *Mvk*, (J) *Pmvk,* and (K) *Fdps*. *P<0.05, **P<0.01, ***P<0.001 by One-way ANOVA.

Supplemental Figure legends:

Supplemental 1. *Bmal1* expression in WT and RC/RC mouse kidneys: (A) *Bmal1* mRNA expression measured by QRTPCR in WT and RC/RC kidneys at 4 and 8 months of age. (B) Western blot for BMAL1 and (C) densitometry for 4 month old mouse kidneys (WT, n=2, RC/RC, n=4), and (D) Western blot and (E) densitometry for 8 month old mouse kidneys (WT, n=6, RC/RC, n=7). (F) Immunostaining for DBA (red) and DAPI (blue).

Supplemental 2. Cell proliferation in mouse kidneys: (A) Western blot for c-Myc. (B) Ki67 stained kidney section (Scale bar 100μm) and (C) Ki-67 quantitation (n=6) (D) Western blot and (E) densitometry for pro-proliferative proteins in WT and *Bmal1*KO mouse kidneys (n=6)..

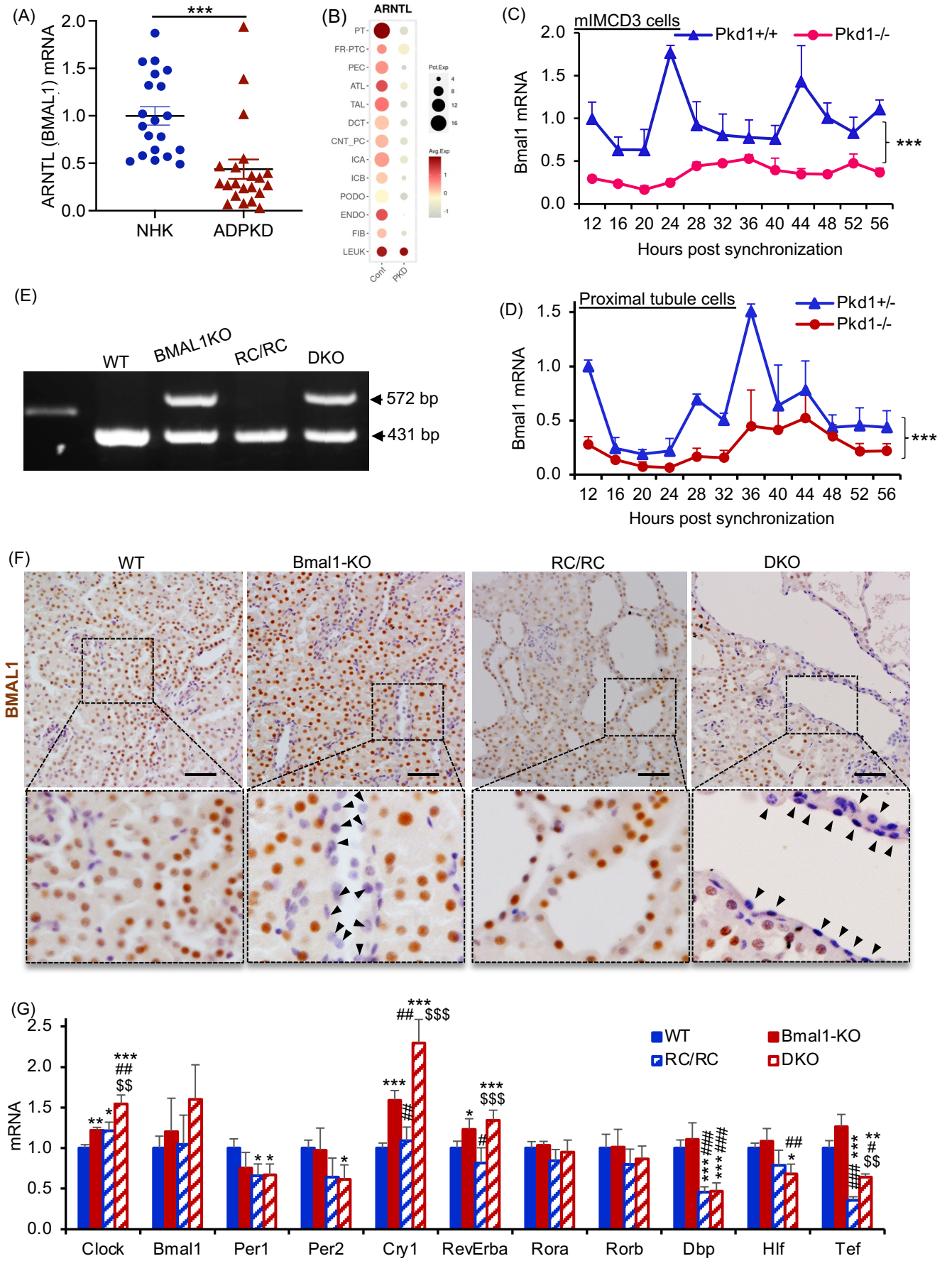
Supplement 3. *Bmal1* gene knockout clones of WT and *Pkd1*KO mIMCD3 cells: (A) Gene knockout of *Bmal1* by CRISPR/Cas12a in WT and *Pkd1* gene knockout mIMCD3 cells. Sequences of wild-type mIMCD3 (WT), and the selected clones #4 of WT and clone #3 of *Pkd1*KO mIMCD3 cells, validating the deletion of base pairs in exon 2.

Supplemental 4. BMAL1 regulated genes: (A) QRTPCR for mRNA levels of BMAL1 target genes in mIMCD3 cell lysates and (B) in mouse kidney tissue lysates. * = *vs* WT, # = *vs* *Bmal1*KO and $ = *vs* *Pkd1*KO. *P<0.05, **P<0.01, ***P<0.001 by by One-way ANOVA.

Supplemental 5. Fatty acid oxidation and lipogenesis-related gene expression in *Pkd1*KO mIMCD3 cells. (A) QRTPCR analysis for mRNA levels of genes related to fatty acid oxidation (n=4) and (C) lipogenesis (n=4). *P<0.05, **P<0.01, ***P<0.001 by by One-way ANOVA.

Supplemental 6. Fatty-acid oxidation-related gene expression in mouse kidneys. The mRNA levels of genes that regulate fatty-acid oxidation. *P<0.05, **P<0.01, ***P<0.001 by One-way ANOVA.

Supplemental 7. Lipogenesis and cholesterol metabolism-related gene expression in mouse kidneys. QRTPCR analysis for mRNA levels of genes that regulate lipogenesis and cholesterol metabolism in whole kidney lysates.


(A)
ARNTL (BMAL1) mRNA
***
NHK
ADPKD
(B)
ARNTL
PT
FR-PTC
PEC
ATL
TAL
DCT
CNT_PC
ICA
ICB
PODO
ENDO
FIB
LEUK
Cont
PKD
Pct.Exp
Avg.Exp
(C)
mIMCD3 cells
Pkd1+/+
Pkd1-/-
Bmal1 mRNA
Hours post synchronization
(D)
Proximal tubule cells
Pkd1+/-
Pkd1-/-
Bmal1 mRNA
Hours post synchronization
(E)
WT
BMAL1KO
RC/RC
DKO
572 bp
431 bp
(F)
WT
Bmal1-KO
RC/RC
DKO
BMAL1
(G)
WT
Bmal1-KO
RC/RC
DKO
mRNA
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Bmal1
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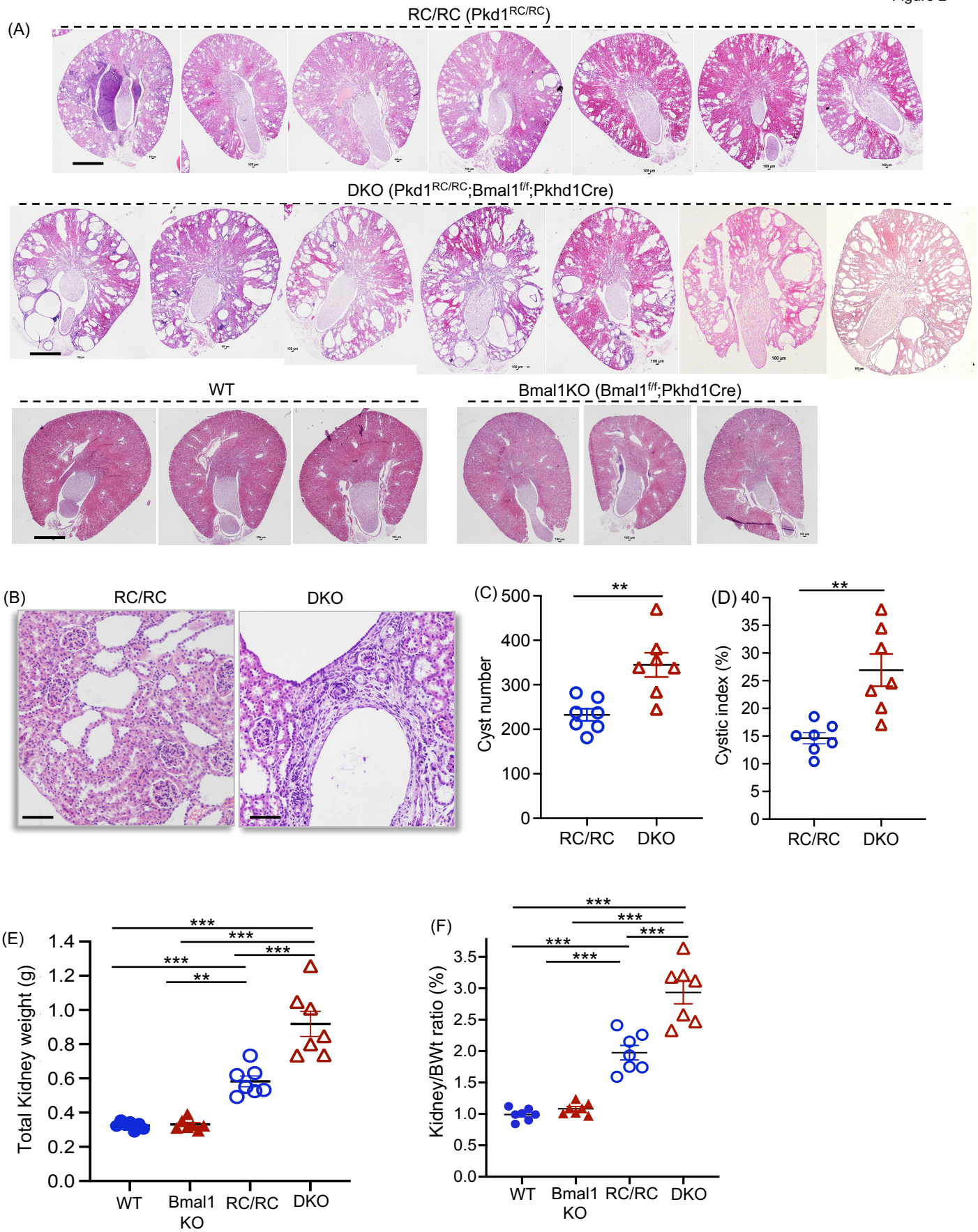
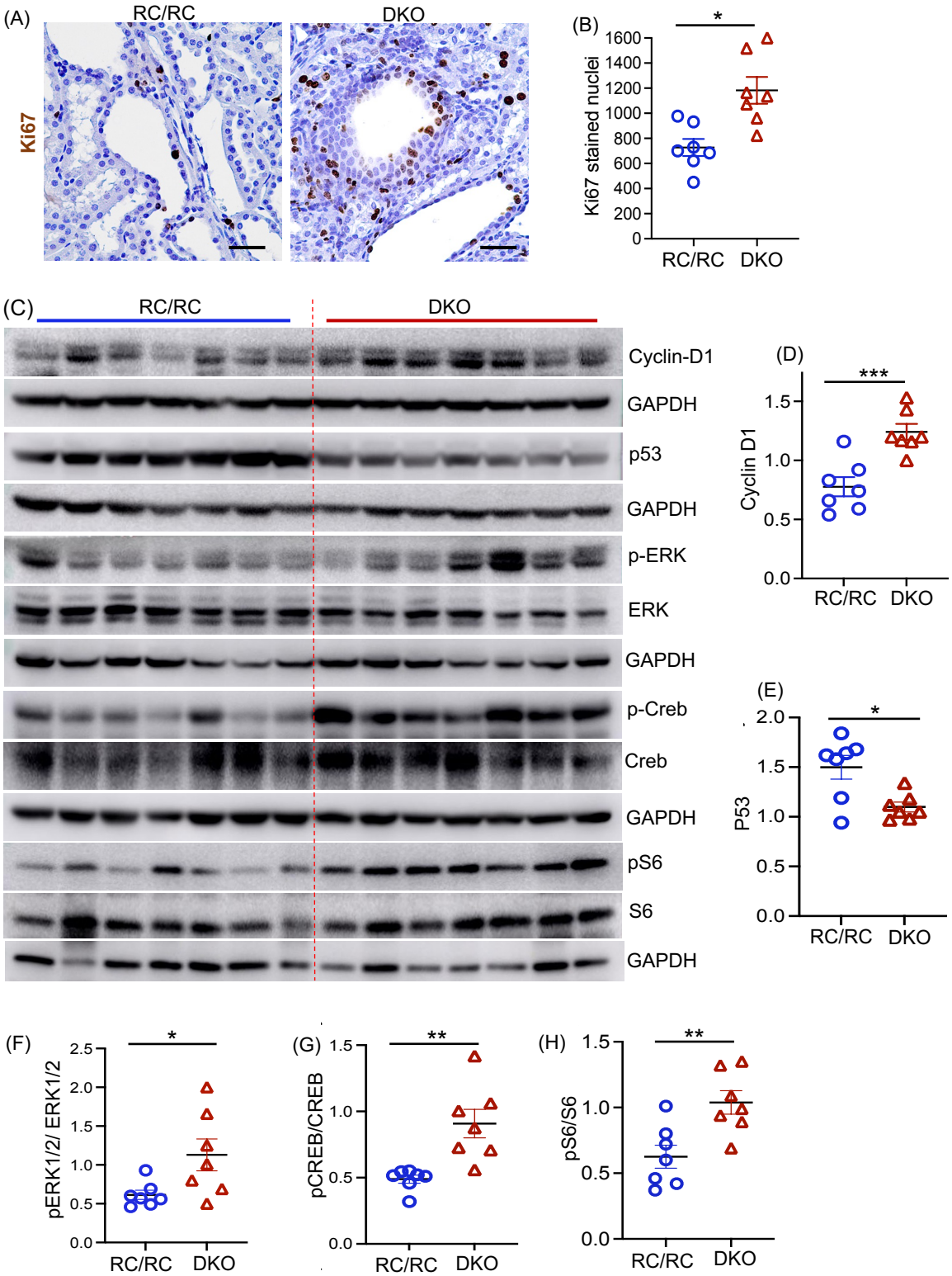
Per2
Cry1
RevErba
Rora
Rorb
Dbp
Hlf
Tef

Figure 2


(A)
RC/RC (Pkd1RC/RC)
DKO (Pkd1RC/RC;Bmal1f/f;Pkhd1Cre)
WT
Bmal1KO (Bmal1f/f;Pkhd1Cre)
(B)
RC/RC
DKO
(C)
Cyst number
RC/RC
DKO
(D)
Cystic index (%)
RC/RC
DKO
(E)
Total Kidney weight (g)
WT
Bmal1 KO
RC/RC
DKO
(F)
Kidney/BWt ratio (%)
WT
Bmal1 KO
RC/RC
DKO


(A)
RC/RC
DKO
Ki67
(B)
Ki67 stained nuclei
1600
1400
1200
1000
800
600
400
200
0
*
RC/RC
DKO
(C)
RC/RC
DKO
Cyclin-D1
GAPDH
p53
GAPDH
p-ERK
ERK
GAPDH
p-Creb
Creb
GAPDH
pS6
S6
GAPDH
(D)
Cyclin D1
1.5
1.0
0.5
0.0
***
RC/RC
DKO
(E)
P53
2.0
1.5
1.0
0.5
0.0
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(F)
pERK1/2/ ERK1/2
2.5
2.0
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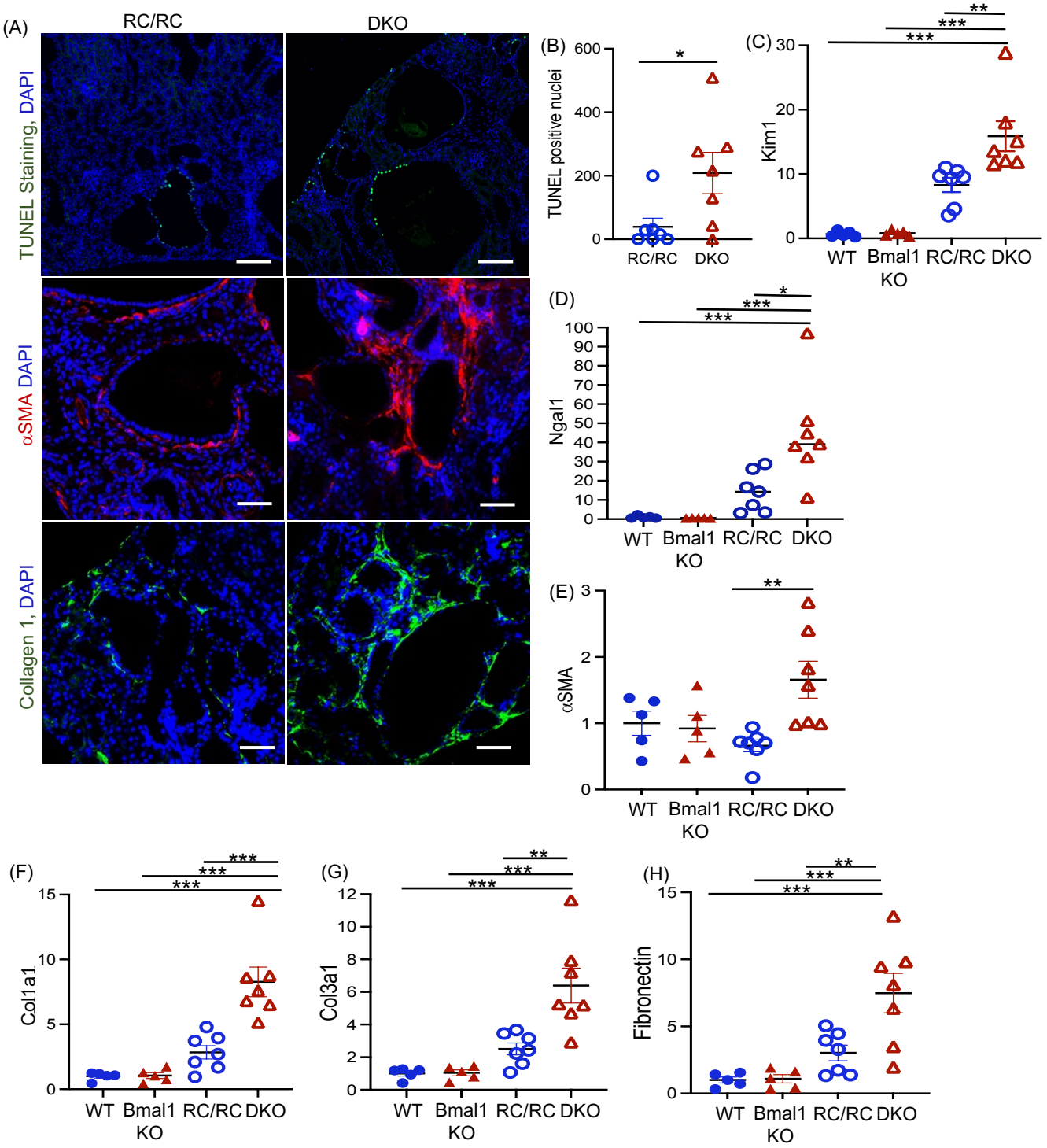
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RC/RC
DKO
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Collagen 1, DAPI
(B)
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RC/RC
DKO
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Kim1
WT
Bmal1 KO
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Col1a1
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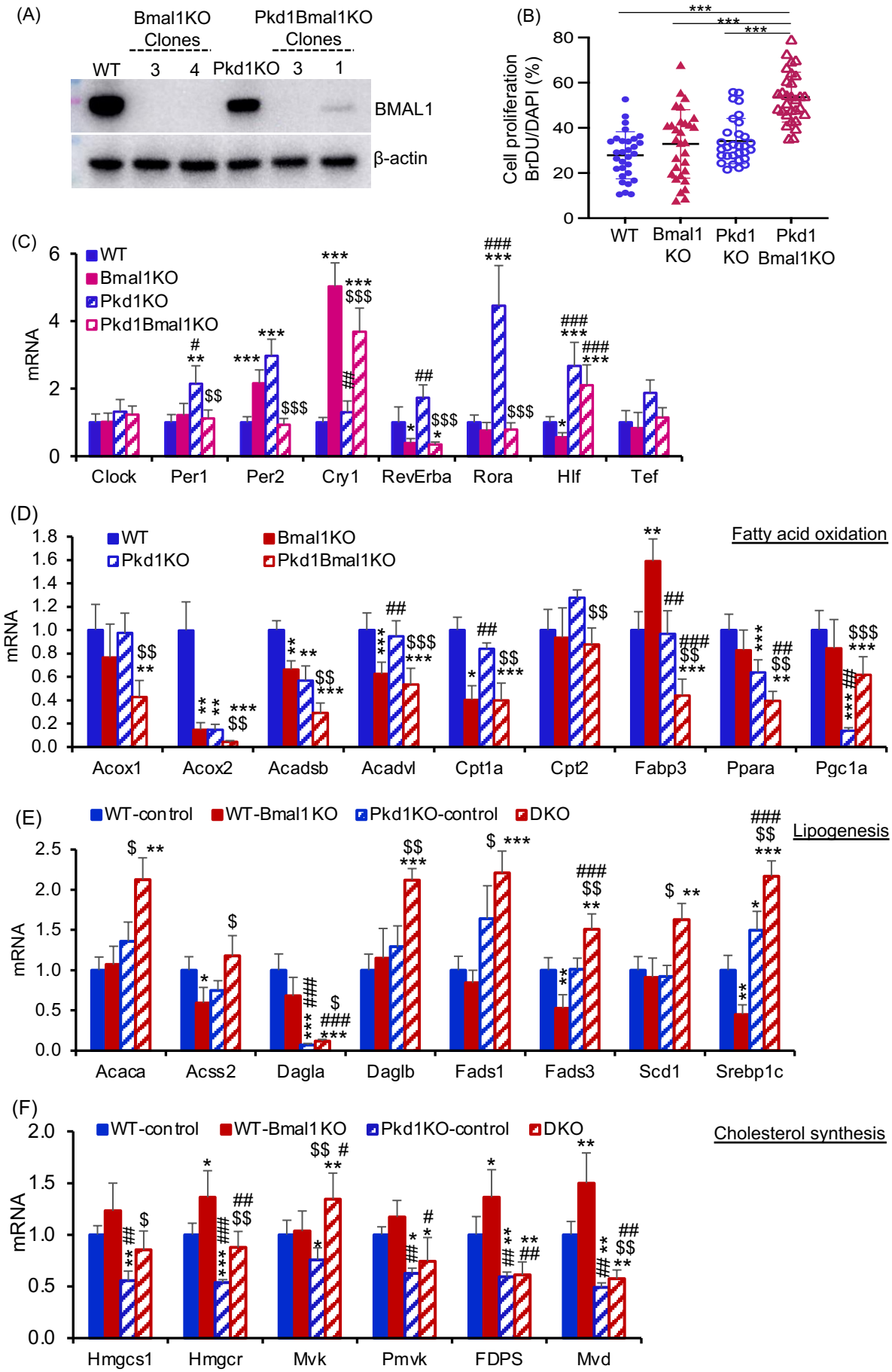
Col3a1
(H)
Fibronectin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Pkd1Bmal1KO Clones
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4
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BMAL1
β-actin
(B)
Cell proliferation BrDU/DAPI (%)
WT
Bmal1 KO
Pkd1 KO
Pkd1 Bmal1KO
(C)
mRNA
WT
Bmal1KO
Pkd1KO
Pkd1Bmal1KO
Clock
Per1
Per2
Cry1
RevErba
Rora
Hlf
Tef
(D)
Fatty acid oxidation
Acox1
Acox2
Acadsb
Acadvl
Cpt1a
Cpt2
Fabp3
Ppara
Pgc1a
(E)
WT-control
WT-Bmal1KO
Pkd1KO-control
DKO
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Dagla
Daglb
Fads1
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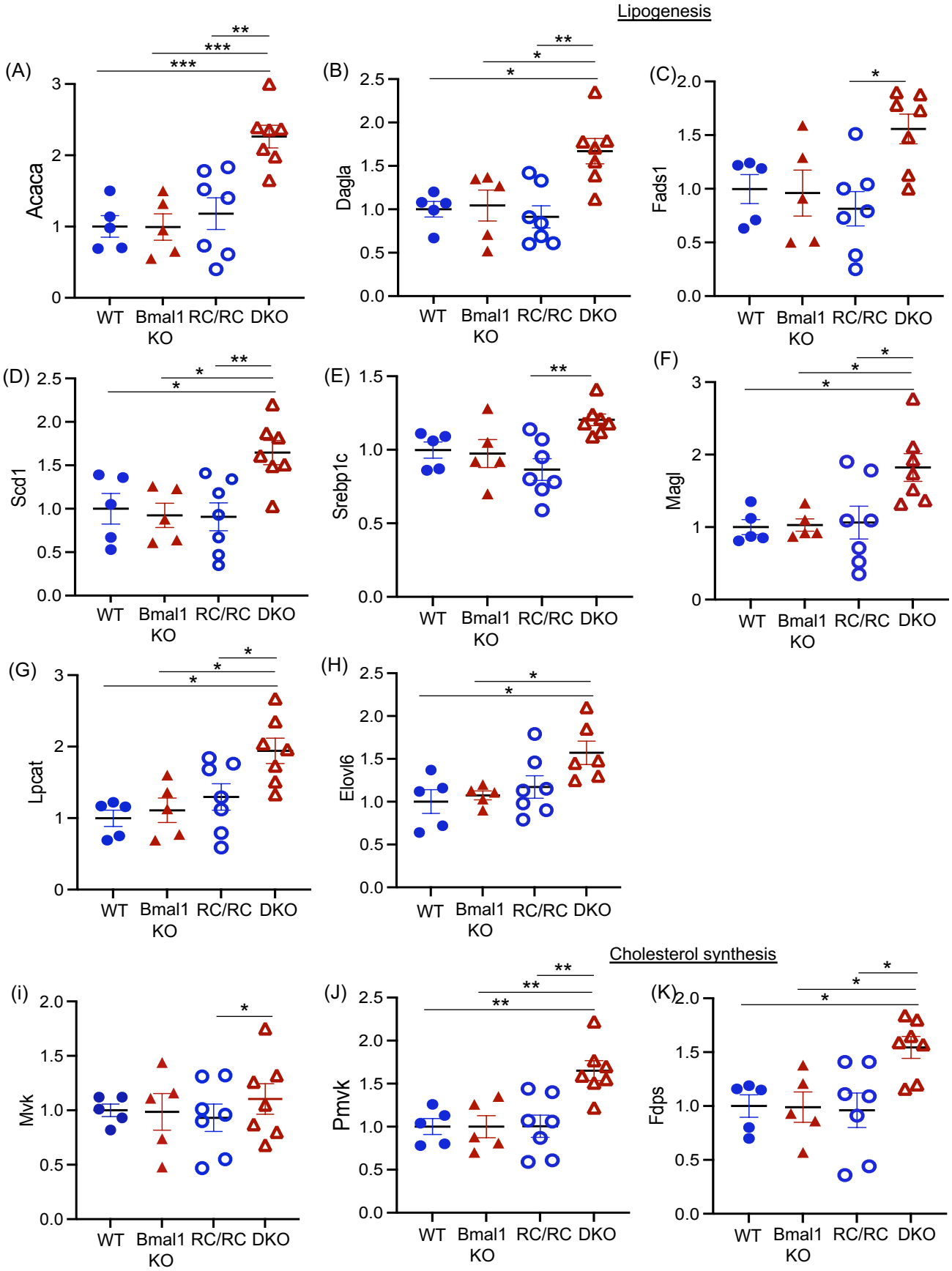
Figure 6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(i)
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