Increased expression of rififylin in a $< 330$ kb congenic strain is linked to impaired endosomal recycling in proximal tubules

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INTRODUCTION

The composition of plasma membranes of virtually all eukaryotic cells is established, maintained, and remodeled by exocytosis, endocytosis, and a process of membrane recycling facilitated by endosomes. Cells are estimated to internalize their cell surface equivalent one to five times per hour (Steinman et al., 1983). This rapid removal of membrane from the cell surface is balanced by endosomal recycling pathways, which return most of the endocytosed proteins and lipids back to the plasma membrane (Maxfield and McGraw, 2004). Thus, a stringent regulation of recycling is essential to maintain the balance between endocytic uptake and recycling pathways. Disruptions in endocytosis and recycling are known to adversely affect diverse cellular processes (Yamamoto et al., 2000; Hryciw et al., 2006; Golachowska et al., 2010; Stendel et al., 2010).

Cell surface proteins are internalized into the cell through endocytosis and either degraded within lysosomes or recycled back to the plasma membrane. While perturbations in endosomal internalization are known to modulate renal function, it is not known whether similar alterations in recycling affect renal function. Rififylin is a known regulator of endocytic recycling with E3 ubiquitin protein ligase activity. In this study, using two genetically similar strains, the Dahl Salt-sensitive rat and an S.LEW congenic strain, which had allelic variants within a $< 330$ kb segment containing rififylin, we tested the hypothesis that alterations in endosomal recycling affect renal function. The congenic strain had 1.59-fold higher renal expression of rififylin. Transcriptome analysis indicated that components of both endocytosis and recycling were upregulated in the congenic strain. Transcription of Atp1a1 and cell surface content of the protein product of Atp1a1, the alpha subunit of Na$^+$K$^+$ATPase were increased in the proximal tubules from the congenic strain. Because rififylin does not directly regulate endocytosis and it is also a differentially expressed gene within the congenic segment, we reasoned that the observed alterations in the transcriptome of the congenic strain constitute a feedback response to the primary functional alteration of recycling caused by rififylin. To test this, recycling of transferrin was studied in isolated proximal tubules. Recycling was significantly delayed within isolated proximal tubules of the congenic strain, which also had a higher level of polyubiquitinated proteins and proteinuria compared with S. These data provide evidence to suggest that delayed endosomal recycling caused by excess of rififylin indirectly affects endocytosis, enhances intracellular protein polyubiquitination and contributes to proteinuria.

Keywords: carp-2, kidney disease, hypertension, rat, linkage mapping, gene, rffl, proteinuria

Kidneys reabsorb $>95\%$ of all proteins filtered through the glomerular apparatus (Nielsen, 1993). Proteinuria is one of the markers of renal dysfunction. Within the apical membranes of proximal tubule cells in the kidney, an extensive endocytic apparatus plays a key role in the reabsorption and degradation of glomerular-filtered albumin and other proteins (Marshansky et al., 2002) and in the recycling of many functionally important membrane transporters (Brown and Stow, 1996). We hypothesized that any alterations in endosomal recycling disrupts cellular homeostasis and thereby could affect renal function. The current study was designed to test whether altered endosomal recycling facilitated by a congenic segment previously mapped on rat chromosome 10 containing rififylin (Gopalakrishnan et al., 2011) can affect renal molecular and cellular physiology and thereby contribute to the extent of
protein excretion in a rat model of cardiovascular and renal disease.

MATERIALS AND METHODS

ANIMALS

All of the animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and as per approved protocols by the institutional animal care and use review committee of the University of Toledo College of Medicine and Life Sciences. The congenic strain used in the current study was constructed in our laboratory using S and LEW rats. The strain is designated as S.LEW (10) × 12 × 2 × 3 × 5 and the construction of this congenic strain is detailed elsewhere (Gopalakrishnan et al., 2011).

cDNA ANALYSES

mRNA from kidneys of neonates and 53 days old rats were extracted using TRIzol Reagent (Life Technologies). cDNA was obtained by reverse transcription with SuperScript III (Invitrogen) using an Oligo dT primer. Using genomic sequence data for rat Rfl gene available at the Ensembl website, sense (5′CAGCTGAAGGAGATCCTGGC3′) and antisense (5′CCATGCAAATCTTACACAGGTTC3′) primers were designed to amplify exons 4–6 of the Rfl transcript by PCR. The resultant cDNA product was confirmed by sequencing using services provided by MWG Biotech Inc. DNA alignments were done using the sequence analysis software Sequencher from GeneCodes Corporation. Transcript expression of Rfl was analyzed by Real-Time PCR (BioRad) and expression levels relative to Gapdh were calculated by the 2−ΔΔCT method (Livak and Schmittgen, 2001).

IMMUNOBLOT ANALYSES

Protein lysates were prepared as described previously (Gopalakrishnan et al., 2011) and subjected to Tricine/SDS-PAGE, transferred to PVDF membrane, incubated with specific primary antibodies followed by secondary antibodies and processed by ECL. Membranes were re-probed with monoclonal anti-Gapdh. The immunoblots were analyzed by densitometric scanning using Image J software. Sources of primary antibodies: Cell Signaling Technology (anti-Gapdh), Abcam (anti-Rfl), the Developmental studies hybridoma bank at the University of Iowa (monoclonal antibody against the Na+K+ATPase α-1 subunit, clone α6F), Santa Cruz Biotechnology (Donkey anti-rabbit IgG-HRP conjugate).

EARLY ENDOSONE ISOLATION AND WESTERN BLOT ANALYSIS OF NA+K+ATPASE α1 SUBUNIT

Early endosome (EE) fractions (Eea-1 and Rab5 positive) were isolated from renal proximal tubules by sucrose flotation centrifugation as previously described (Liu et al., 2011). The enrichment of EE fractions was assessed by the EE marker Eea-1. Equal amount of total proteins (25µg) from the EE fraction of each sample was precipitated with tricloroacetic acid for subsequent western blot analysis.

1 www.ensembl.org

FIGURE 1 | (A) Schematic diagram of the congenic strain used in the study. The <330 kb region spanned by the congenic strain S.LEW (10) × 12 × 2 × 3 × 5 is shown alongside the physical map of rat chromosome 10. The basepairs delineating the ends of the congenic segment and the gene annotations were obtained from Ensembl.org. (RGSC 3.4) RNO10, Rat chromosome 10; Mb, Megabases. (B) Expression of Rfl transcript in the kidneys at 53 days of age as detected by RTPCR. (C) Quantification of Rfl transcripts relative to S rats by real-time PCR using whole kidney samples from 53-day-old rats (n = 6 animals per group). Immunoblot of Rfl in (D) whole-cell lysates from S (n = 3) and congenic (n = 3) rat kidneys at 53 days of age. (E) proximal tubules from S (n = 3) and congenic (n = 3). RFLP INP_0010717368, 2aa-99aa, 36.41 kDa) partial recombinant protein was used as positive control and Gapdh was the loading control. Quantification of Rfl protein ± SEM is shown alongside.
### Table 1 | Differentially expressed transcripts in the clathrin-mediated endocytosis network.

| Affymetrix ID | Fold change | p-Value | Symbol | Entrez gene name |
|---------------|-------------|---------|--------|-----------------|
| 1369733_at    | 2.201       | 0.0258  | Ctnnb1 | Catenin (cadherin-associated protein), beta 1, 88 kDa |
| 1393288_at    | 1.987       | 0.0366  | Rab5b  | RAB5B, member RAS oncogene family |
| 1398825_at    | 1.802       | 0.0434  | Rab11b | RAB11B, member RAS oncogene family |
| 1371113_a_at  | 1.787       | 0.0411  | Tfc    | Transferrin receptor (p90, CD71) |
| 1368762_at    | 1.749       | 0.0232  | Ubd    | Ubiquitin D |
| 1399153_at    | 1.715       | 0.0356  | Rab5b  | RAB5B, member RAS oncogene family |
| 1369998_at    | 1.708       | 0.0268  | Arf6   | ADP-ribosylation factor 6 |
| 1372513_at    | 1.63        | 0.0268  | Rac1   | Ras-related C3 botulinum toxin substrate 1 |
| 1388022_a_at  | 1.459       | 0.018   | Dnmt1  | Dynamin 1-like |
| 1388104_at    | 1.436       | 0.0225  | Igr4   | Leucine-rich repeat containing G protein-coupled receptor 4 |
| 1370672_a_at  | 1.416       | 0.0422  | Dnmt3  | Dynamin 3 |
| 1374232_at    | 1.416       | 0.0166  | Pik3ca  | Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide |
| 1384101_at    | 1.414       | 0.0362  | Wasp   | Wiskott–Aldrich syndrome-like |
| 1370081_a_at  | 1.409       | 0.0236  | Vegfa  | Vascular endothelial growth factor A |
| 1384750_at    | 1.392       | 0.037   | Numb   | Numb homolog (Drosophila) |
| 1395648_at    | 1.378       | 0.0331  | Eps15  | Epidermal growth factor receptor pathway substrate 15 |
| 1392643_at    | 1.355       | 0.0355  | Rab5b  | RAB5B, member RAS oncogene family |
| 1387170_at    | 1.238       | 0.0473  | Csnk2a1 | Casein kinase 2, alpha 1 polypeptide |
| 1368096_at    | -1.291      | 0.0321  | Rab7/1 | RAB7, member RAS oncogene family |

Statistical analyses of the microarray data were performed with RMA, robust multiarray averaging; BH, Benjamini and Hochberg adjustment using the R statistical package (version 2.8.1). The complete microarray data is available to the reviewers at the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hryjdweamuioidi&acc=GSE30770.

### WHOLE GENOME TRANSCRIPTIONAL PROFILING
RNA was isolated from the kidneys of concomitantly raised, male, 53-day-old S, and congenic rats (n = 6 per group) using TRIzol and purified by RNeasy kit (Qiagen). RNA from two animals was pooled. Three such pooled RNA samples from S and congenic rats were hybridized to Affymetrix Rat Expression Arrays 230 2.0. The arrays were scanned at 488 nm. Transcripts were selected from the list of probes that were significantly altered (p < 0.05, BH-adjusted) between S and Congenic.

### ISOLATION AND PRIMARY CULTURE OF RAT PROXIMAL TUBULE CELLS
Primary rat proximal tubule (PRT) cells were isolated from cortices of rat kidneys from S and congenic rats as described previously (Liu et al., 2011). Isolated PRT cells were seeded at 5 × 10⁴ cells/cm² and allowed to intercalate a fluorescent derivative of transferrin (AlexaFluor 647-Tf, Molecular Probes) for 90 min at 37°C and washed three times with ice cold PBS. Recycling was induced by warming the cells to 37°C in a serum free medium containing 0.1% BSA and a 100-fold excess of unlabelled holotransferrin (Sigma) and monitored by live imaging using a Leica TCS SP5 laser scanning confocal microscope. Just before monitoring, DRAQ5 was added to the cell suspension to visualize the nuclei. Cells were imaged using a 488 and 433 laser line in the XY plane with scanning set at 30 s intervals for 30 min. Paired time lapse studies were performed in triplicate using the same gain, offset, and laser power settings to ensure that there were no intensity differences due to the acquisition settings between S and Congenic. Mean fluorescence intensity was measured in Image J at individual time points of the acquired images.

### TRANSFERRIN RECYCLING
Transferrin recycling was studied as described previously (Gopalakrishnan et al., 2011). In brief, isolated proximal tubules were maintained at 37°C with 5% CO₂ and allowed to internalize a fluorescent derivative of transferrin (AlexaFluor 647-Tf, Molecular Probes) for 90 min at 37°C and washed three times with ice cold PBS. Recycling was induced by warming the cells to 37°C in a serum free medium containing 0.1% BSA and a 100-fold excess of unlabelled holotransferrin (Sigma) and monitored by live imaging using a Leica TCS SP5 laser scanning confocal microscope. Just before monitoring, DRAQ5 was added to visualize the nuclei. Cells were imaged using a 488 and 433 laser line in the XY plane with scanning set at 30 s intervals for 30 min. Paired time lapse studies were performed in triplicate using the same gain, offset, and laser power settings to ensure that there were no intensity differences due to the acquisition settings between S and Congenic. Mean fluorescent intensity was measured in Image J at individual time points of the acquired images.

### POLYUBIQUITINATED PROTEINS
Polyubiquitin-modified proteins were isolated from kidneys using the Pierce Ubiquitin Enrichment Kit as per previously published procedures (Gopalakrishnan et al., 2011).

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1. [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hryjdweamuioidi&acc=GSE30770](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hryjdweamuioidi&acc=GSE30770)
2. [www.ingenuity.com](http://www.ingenuity.com)
URINARY PROTEIN EXCRETION
Urinary Protein Excretion (UPE) determination was done as previously described (Kumarasamy et al., 2011). Briefly, at 53 days of age, rats fed with low salt (0.3% NaCl) was housed individually in metabolic cages and urine was collected over a 24-h period. Urinalysis was conducted using services provided by the University of Toledo Medical Center. The pyrogallol based QuanTtest Red Total Protein Assay from Quantimetrix (Redondo Beach, CA, USA) was used to determine protein concentrations of the urine samples. A VERSAmax microplate reader from Molecular Devices (Sunnyvale, CA, USA) was used to determine absorbance at 600 nm. Protein concentrations were determined by reading against the absorbance of the QuanTtest human protein standards (25–200 mg/dL). UPE data is presented as mg/mg creatinine over a 24-h period.

STATISTICAL ANALYSES
All phenotypic data obtained from the two groups (congenic and S rats) were statistically analyzed by Student t-test. A p-value of <0.05 was considered statistically significant. Statistical analyses of the microarray data were performed with robust multiarray averaging and Benjamini and Hochberg adjustment using the R statistical package (version 2.8.1).

RESULTS
The rat strains chosen as tools for this study were the Dahl S rat and a > 99% genetically identical strain, the S.LEW congenic strain, which has a < 330 kb of the LEW rat genome introgressed onto the genome of the S rat (Figure 1A). At 52 days of age, the systolic blood pressure of the congenic strain measured by the telemetry method was 138 ± 2 mmHg compared with that of the S, 132 ± 2 mmHg, p < 0.01 (Gopalakrishnan et al., 2011). The introgressed segment contained the gene rififylin, overexpression of which is known to cause a delay in endosomal recycling in cardiomyocytes (Gopalakrishnan et al., 2011). Rififylin was also transcribed in the kidneys of both the S and the congenic strain (Figure 1B), however, kidneys of congenic rats had a 1.56-fold higher mRNA of rififylin compared with that of the S (p < 0.001; Figure 1C). Protein levels of rififylin were also higher both in the kidney and within the proximal tubules of congenic rats compared with S (Figures 1D,E).

To study the alterations in the renal transcriptome between the S and the congenic strain with increased expression of rififylin, a whole genome renal transcriptome analysis was conducted. A total of 1082 probes representing 838 genes and 244 ESTs were upregulated in the congenic strain compared with S. Similarly, a total of 785 probes representing 423 genes and 362

FIGURE 2 | Illustration of the IPA network analysis of the differentially expressed transcripts associated with Clathrin-mediated endocytosis and recycling. Transcripts shown in red were upregulated and green were down-regulated in the congenic strain compared with S. The fold changes of the corresponding Affymetrix probes are given in Table 1.
FIGURE 3 | Illustration of the IPA networks of transcripts associated with cell morphology and renal function. (A) network 1 with Atp1a1 and (B) network with Rab proteins. Transcripts shown in red were upregulated and transcripts shown in green were down-regulated in the congenic strain compared with S. The fold changes of the corresponding Affymetrix probes are given in Table A1 in Appendix.
ESTs were down-regulated in the congenic strain compared with S (GSE30770). Among these transcripts, the highest differential expression of 5.33-fold was observed with Atp1a1, which was upregulated in the congenic strain compared with S (Table A1 in Appendix). Notably, a number of transcripts coding for proteins either directly or indirectly related to the sorting of endosomes were upregulated in the congenic strain compared with S. The relative changes in gene expression of differentially expressed genes are in Table 1. The networks of these gene products that facilitate clathrin-coated membrane invagination and endocytosis are depicted in Figure 2. The other genes differentially expressed belonged to two prominent networks related to cellular morphology and renal associated function (Figures 3A,B). While Atp1a1 featured in the network represented in Figure 3A, several transcripts coding for Rab proteins including Rab5 which regulates transport from plasma membrane to EEs and Rab11 involved in endocytic recycling (Trischler et al., 1999) featured in the network represented in Figure 3B. The fold changes of all the transcripts within these two additional networks are given in the Table A1 in Appendix.

Next, we assessed the content of the protein product of the most differentially expressed gene, Atp1a1. Within the proximal tubules, the total protein content of the alpha subunit of Na\(^{+}\)K\(^{+}\)ATPase (referred to hereafter as alpha 1) was not different between S and the congenic strain (data not shown). Protein levels of alpha 1 were not different between the early endosomal fractions isolated from the proximal tubules of the congenic strain and the S (data not shown). However, surface biotinylation experiments indicated that the content of alpha 1 was notably higher on the cell membranes from the congenic strain compared with S (Figure 4). Total polyubiquitinated proteins were also significantly higher in the congenic strain compared with S (Figure 5).

To assess the extent of endosomal recycling in the kidney of the congenic strain with increased expression of Riffl, recycling of fluorescently labeled transferrin was monitored in individual proximal tubules. As shown in Figures 6A,B, recycling of transferrin was significantly delayed in the congenic strain compared with S.

These observations, coupled with the fact that rififylin residing within the congenic segment is a regulator of cellular protein recycling, suggested that the primary delay in recycling of endosomes caused membrane proteins to accumulate intracellularly within the proximal tubules from the congenic strain. Because similar defects in membrane traffic and enhanced degradation of proteins are known to cause proteinuria (Marshansky et al., 2002), we tested the urine composition of the two rat strains at a very young age of 53 days. The total protein excretion was significantly higher by 31% in the congenic strain compared with the S (8.26 ± 0.08 mg/mg creatinine/day, p = 0.016; Figure 7). The other urinary parameters analyzed, i.e., urea nitrogen, glucose, and creatinine excretion were not significantly different between the S and the congenic strain (data not shown).

**DISCUSSION**

Hypertension in the Dahl S rat is accompanied with proteinuria (Sustarsic et al., 1981; Sterzel et al., 1988; Garrett et al., 2003). Compared to the S rat, both blood pressure (Gopalakrishnan et al., 2011) and UPE are further increased in the congenic strain reported in the current study. We have previously demonstrated that overexpression of rififylin in the neonatal cardiomyocytes of this congenic strain is linked to short QT-interval and hypertension (Gopalakrishnan et al., 2011). While alterations in QT-interval can contribute to the development of hypertension (Baumert et al., 2011), it does not independently explain the observed increase in UPE of the congenic strain. Because rififylin is also reported to be expressed in other tissues (Coumailleau et al., 2004), we suspected that the fundamental cellular mechanism altered by the overexpression of rififylin could be operational.
in the kidney wherein rififylin is expressed at higher levels in the congenic strain compared with S. The present study provides evidence to suggest that upregulation of rififylin in the congenic strain compared with S is not limited to the heart, but is also observed at least in one additional organ, the kidney. Functional analysis of rififylin revealed that endocytic recycling is delayed within the proximal tubules. The renal transcriptome signature is reminiscent of perturbations in the endosomal sorting and transport pathways, alterations in which are reported to lead to proteinuria (Nielsen, 1994; Nielsen and Christensen, 2010).

Several structural proteins and GTPase regulators are indispensable for recycling endosomes (Grant and Donaldson, 2009; Schweitzer et al., 2011). Rififylin, also known as Carp-2, is a recent addition to the growing list of proteins associated with the cellular recycling machinery. Coumailleau et al. (2004) described that overexpression of rififylin represents a novel means to inhibit recycling. Using deletion mutants, they demonstrated that the amino-terminal region of rififylin is critical for the recruitment of Rffl to recycling endocytic membranes and for the inhibition of recycling. The current study of delayed recycling in proximal tubules caused by increased renal expression of Rffl along with a previous similar report on cardiomyocytes from our group (Gopalakrishnan et al., 2011) represent the first two in vivo validations of the in vitro studies on HeLa cells reported by Coumailleau et al. (2004).

Transcriptome profiling demonstrates that there are numerous changes in gene transcript levels in the kidneys of S versus the congenic strain. According to the IPA network analysis, genes upregulated were in networks including cellular assembly and organization, cellular function and maintenance and cell morphology,
that an additional factor contributing to the increased blood strain (Gopalakrishnan et al., 2011). The current study indicates attributed partly to increased heart rate observed in the congenic strain.

polyubiquitinated proteins in the congenic strain relative to the S, 2011). Therefore, it is possible that the increased accumulation of known E3 ubiquitin ligase and we have previously demonstrated with S point to the latter, i.e., upregulation of the cellular degrada-
tion machinery. This is not surprising because rififylin is also a particular only represent <1.5% of the total variance in albuminuria observed in human populations. Therefore a large number of loci causing or contributing to renal function disorders in humans remain unidentified. Genome-wide studies have identified single nucleotide polymorphisms around the gene coding for rififylin in humans to QT-intervals (Newton-Cheh et al., 2009; Pfuefer et al., 2009), but not to any renal phenotypes. Through the discovery of a link between endosomal recycling, enhanced degradation, and a resultant altered trafficking of proteins within the proximal tubules, the present study provides the basis for evaluating rififylin as a novel candidate gene for renal disease characterized by proteinuria in humans.

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all of which are processes known to involve endocytic recycling (Schweitzer et al., 2011). Two lines of evidence further point to impaired endocytic recycling: (1) upregulation of transcripts in the clathrin-mediated endocytosis and recycling pathways and (2) delayed recycling of transferrin.

Additionally, Coumailleau et al. (2004) have reported that rififylin per se does not affect endocytosis. Therefore any alteration in endocytosis is perhaps a representation of the concerted cellular feedback response to the primary defect in recycling in order to maintain cellular homeostasis.

A defect in recycling should either demonstrate an increased accumulation of cargo within the endosomes or trigger degrada-
tion of proteins. Evidence from increased polyubiquitinated proteins within the proximal tubules of the congenic strain compared with S point to the latter, i.e., upregulation of the cellular degra-
dation machinery. This is not surprising because rififylin is also a known E3 ubiquitin ligase and we have previously demonstrated similar increased cellular polyubiquitination of proteins within the cardiomyocytes of the congenic strain used in the current study compared with S (Gopalakrishnan et al., 2011). Increased accumu-
lation of polyubiquitination leads to cellular stress, which is known to adversely affect proteinuria (Meyer-Schwesinger et al., 2011). Therefore, it is possible that the increased accumulation of polyubiquitinated proteins in the congenic strain relative to the S, at least in part, contributes to the observed increased in proteinuria of the congenic strain.

The increase in blood pressure of this strain has been previously attributed partly to increased heart rate observed in the congenic strain (Gopalakrishnan et al., 2011). The current study indicates that an additional factor contributing to the increased blood pressure of the congenic strain could be due to the compensatory mechanism of increased transcription and availability of the Na\(^+\) K\(^+\) ATPase at the surface of cells within the proximal tubules, which may cause increased sodium retention and thereby increase blood pressure.

Overall, three main reasons lead us to conclude that overexpres-
sion of rififylin within the congenic strain compared with S is a contributor to the observed alterations in kidney function as noted by alterations in proteinuria – (1) the two strains com-
pared were genetically identical except for the very short <330 kb congenic segment harboring rififylin; (2) two known functional consequences of delayed endocytic recycling and accumulation of polyubiquitinated proteins (Coumailleau et al., 2004, 2005) as a result of overexpression of rififylin were recapitulated in the congenic strain; and (3) Rffl is a candidate gene within the congenic interval that is reported to affect both recycling and polyubiqui-
tination. Despite these compelling arguments, it remains to be determined using future mapping studies to further dissect the <330 kb congenic segment as to whether additional factors within the congenic interval also contribute to the reported phenotypes.

Given that alpha1 is not within the congenic segment, it is also reasonable to conclude that the primary physiological perturbations that may have lead to the observed increase in transcription of alpha1 and the increased alpha 1 content on the plasma mem-
brane is a compensatory mechanism. Of course, we would expect increased blood pressure as one of the consequences to this com-
ponsatory mechanism and the congenic strain indeed has higher blood pressure at a very young age of 52 days. Further, a pro-
longed cellular stress as a result of accumulation of excess proteins marked for degradation could be viewed as being highly detrimen-
tal because the congenic strain is reported to have a decreased life span compared with S (Gopalakrishnan et al., 2011).

Genome-wide association and linkage studies in humans and model organisms point to a number of candidate genes for chronic renal disease and/or albuminuria (Liu and Freedman, 2005; Krolewski et al., 2006; Turner et al., 2006; Arar et al., 2007, 2008; Garrett et al., 2007, 2010; Hwang et al., 2007; Iyengar et al., 2007; Leon et al., 2007; Martinez et al., 2010; Sterken and Kiryluk, 2010). The genome-wide association studies in particular only represent <1.5% of the total variance in albuminuria observed in human populations. Therefore a large number of loci
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## Table A1 | Differentially expressed transcripts in networks (Figures 3A,B).

| Affymetrix ID       | Fold change | p-Value   | Symbol | Entrez gene name                                      |
|---------------------|-------------|-----------|--------|-------------------------------------------------------|
| 1371108_a_at        | 5.3380      | 0.0334    | Atp1a1 | ATPase, Na\(^+\)/K\(^+\) transporting, alpha 1 polypeptide |
| 1380533_at          | 4.2845      | 0.0180    | App    | Amyloid beta (A4) precursor protein                   |
| 1369152_at          | 3.3827      | 0.0319    | Ppp3r1 | Protein phosphatase 3, regulatory subunit B, alpha     |
| 1388948_at          | 3.1624      | 0.0383    | Msln   | Moesin                                               |
| 1370503_s_at        | 2.9373      | 0.0422    | Epb41l3 | Erythrocyte membrane protein band 4.1-like 3          |
| 1390525_a_at        | 2.7000      | 0.0397    | Stra6   | Stimulated by retinoic acid gene 6 homolog (mouse)     |
| 1378015_at          | 2.6839      | 0.0206    | Ccl21   | Chemokine (C–C motif) ligand 21                        |
| 1388744_at          | 2.6075      | 0.0051    | Mbd2    | Methyl-cpg binding domain protein 2                    |
| 1383899_at          | 2.5896      | 0.0335    | Nedd4   | Neural precursor cell expressed, developmentally down-regulated 4 |
| 1368948_at          | 2.5624      | 0.0327    | Actr3   | APR3 actin-related protein 3 homolog (yeast)          |
| 1374002_at          | 2.5213      | 0.0303    | Myh9    | Myosin, heavy chain 9, non-muscle                     |
| AFFX_Rat_beta-actin_5_at | 2.4164     | 0.0236    | Actb    | Actin, beta                                          |
| 1389681_at          | 2.4077      | 0.0157    | Slc16a1 | Solute carrier family 16, member 1 (monocarboxylic acid transporter 1) |
| 1328161_at          | 2.3050      | 0.0359    | Gls     | Glutaminase                                          |
| 1369733_at          | 2.2009      | 0.0258    | Ctnnb1  | Catenin (cadherin-associated protein), beta 1, 88 kDa |
| 1370288_a_at        | 2.1568      | 0.0201    | Tpm1    | Tropomyosin 1 (alpha)                                |
| 1398822_at          | 2.1499      | 0.0202    | Gna12   | Guanine nucleotide binding protein (G protein) alpha 12 |
| 1369227_at          | 2.0847      | 0.0284    | Gd2     | GDP dissociation inhibitor 2                         |
| 1370789_a_at        | 2.0256      | 0.0137    | Chm     | Choroideremia (Rab escort protein 1)                  |
| 1392406_at          | 2.0290      | 0.0370    | Ipp     | Intracisternal A particle-promoted peptidyl          |
| 1370789_a_at        | 2.0256      | 0.0435    | Prf     | Prolactin receptor                                   |
| 139312_a_at         | 2.0143      | 0.0280    | Cank1a1 | Casein kinase 1, alpha 1                             |
| 1371028_at          | 2.0125      | 0.0372    | Tgcn2   | Trans-golgi network protein 2                         |
| 1371139_at          | 2.0094      | 0.0475    | Pfs3    | Plastin 3                                           |
| 1387810_at          | 2.0016      | 0.0177    | Keap1   | Kelch-like ECH-associated protein 1                   |
| 1396267_at          | 1.9907      | 0.0392    | Pak2    | p21 protein (Cdc42/Rac)-activated kinase 2           |
| 1368537_at          | 1.9863      | 0.0225    | Dcn4    | Dynactin 4 (p62)                                     |
| 1383263_at          | 1.9628      | 0.0177    | Ogn     | Osteoglycin                                         |
| 1387392_at          | 1.9581      | 0.0276    | Mlt4    | Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 4 |
| 1369979_at          | 1.9506      | 0.0099    | Myo1d   | Myosin ID                                           |
| 1375137_at          | 1.9173      | 0.0366    | Arpc2   | Actin-related protein 2/3 complex, subunit 2, 34 kDa  |
| 1379452_at          | 1.9090      | 0.0372    | Gas2    | Growth arrest-specific 2                            |
| 1371239_s_at        | 1.9033      | 0.0379    | Tpm3    | Tropomyosin 3, gamma                                |
| 1393288_at          | 1.8966      | 0.0366    | Rab5b   | RAB5B, member RAS oncogene family                    |
| 1370141_at          | 1.8715      | 0.0280    | Mcl1    | Myeloid cell leukemia sequence 1 (BCL2-related)       |
| 1394965_at          | 1.8507      | 0.0450    | Clint1  | Claatrinh interactor 1                              |
| 1387952_a_at        | 1.8175      | 0.0221    | Cd44    | CD44 molecule (Indian blood group)                   |
| 1398825_at          | 1.8107      | 0.0434    | Rab11b  | RAB11B, member RAS oncogene family                   |
| 1396797_at          | 1.7912      | 0.0321    | Eif4a2  | Eukaryotic translation initiation factor 4A2         |
| 1371113_a_at        | 1.7874      | 0.0411    | Tfrc    | Transferrin receptor (p90, CD71)                     |
| 1367651_at          | 1.7835      | 0.0464    | Ctsd    | Cathepsin D                                         |
| 1398311_a_at        | 1.7425      | 0.0411    | Kidins220 | Kinase d-interacting substrate, 220 kDa              |
| 1369197_at          | 1.7379      | 0.0268    | Apat1   | Apoptotic peptidase activating factor 1               |
| 1388808_at          | 1.7327      | 0.0282    | Cap1    | CAP, adenylate cyclase-associated protein 1 (yeast)   |
| 1368838_at          | 1.7273      | 0.0388    | Tpm4    | Tropomyosin 4                                        |
| 1396214_at          | 1.7272      | 0.0205    | Kitg    | KIT ligand                                          |
| 1369319_at          | 1.7265      | 0.0333    | Arf6p5   | ADP-ribosylation-like factor 6 interacting protein 5 |
| 1388251_at          | 1.7156      | 0.0368    | Prkci   | Protein kinase C, iota                              |
| 1396072_at          | 1.6782      | 0.0364    | Appbp2  | Amyloid beta precursor protein (cytoplasmic tail) binding protein 2 |
| 1382878_at          | 1.6562      | 0.0224    | Sfpr1   | Secreted frizzled-related protein 1                  |

(Continued)
| Affymetrix ID     | Fold change | p-Value  | Symbol | Entrez gene name |
|-------------------|-------------|----------|--------|-----------------|
| 1391543_at        | 1.6489      | 0.0266   | Ripk1  | Receptor (TNFRSF)-interacting serine-threonine kinase 1 |
| 1382615_at        | 1.6466      | 0.0460   | Sec61a1| Sec61 alpha 1 subunit (S. cerevisiae) |
| 1397843_at        | 1.6417      | 0.0473   | Wdr44  | WD repeat domain 44 |
| 1370662_a_at      | 1.6351      | 0.0421   | Ap2b1  | Adaptor-related protein complex 2, beta 1 subunit |
| 1369720_at        | 1.6345      | 0.0257   | Myo1b  | Myosin IB |
| 1377769_at        | 1.6329      | 0.0402   | Ap1s1  | Adaptor-related protein complex 1, sigma 1 subunit |
| 1372513_at        | 1.6297      | 0.0268   | Rac1   | Ras-related C3 botulinum toxin substrate 1 |
| 1387844_at        | 1.6240      | 0.0175   | Lasp1  | LIM and SH3 protein 1 |
| 1396250_at        | 1.6206      | 0.0311   | Coro1c | Coronin, actin binding protein, 1C |
| 1368832_at        | 1.6121      | 0.0317   | Akt2   | v-akt murine thymoma viral oncogene homolog 2 |
| 1369557_at        | 1.6047      | 0.0093   | Casp7  | Caspase 7, apoptosis-related cysteine peptidase |
| 1397395_at        | 1.5994      | 0.0392   | Zeb2   | Zinc finger E-box binding homeobox 2 |
| 1368821_at        | 1.5950      | 0.0180   | Fst1   | Follistatin-like 1 |
| 1378207_at        | 1.5947      | 0.0439   | Tik1   | Tousled-like kinase 1 |
| 1378816_a_at      | 1.5832      | 0.0221   | osbp   | Oxysterol binding protein |
| 1369234_at        | 1.5818      | 0.0282   | Sic20a2| Solute carrier family 20 (phosphate transporter), member 2 |
| 1368395_at        | 1.5704      | 0.0307   | Gpc3   | Glypican 3 |
| 1382230_at        | 1.5600      | 0.0327   | Jub    | Jüb, ajuba homolog (Xenopus laevis) |
| 1370266_at        | 1.5554      | 0.0327   | Parva  | Parvin, alpha |
| 1395132_at        | 1.5543      | 0.0221   | Utm    | Utrophin |
| 1367974_at        | 1.5493      | 0.0342   | Anxa3  | Annexin A3 |
| 1387420_at        | 1.5445      | 0.0202   | Clic4  | Chloride intracellular channel 4 |
| 1367890_at        | 1.5417      | 0.0180   | Casp3  | Caspase 3, apoptosis-related cysteine peptidase |
| 1397200_at        | 1.5319      | 0.0277   | Chd4   | Chromodomain helicase DNA binding protein 4 |
| 1390706_at        | 1.5289      | 0.0323   | Sptbn1 | Spectrin, beta, non-erythrocytic 1 |
| AFFX_Rat_Hexokinase_3_at | 1.5278  | 0.0388   | Hk1    | Hexokinase 1 |
| 1370599_a_at      | 1.5255      | 0.0292   | Ptnrs  | Protein tyrosine phosphatase, receptor type, S |
| 1388762_at        | 1.5226      | 0.0374   | Igkap1 | IQ motif containing GTPase activating protein 1 |
| 1369248_a_at      | 1.5212      | 0.0442   | Xiap   | X-linked inhibitor of apoptosis |
| 1367939_at        | 1.5168      | 0.0362   | Rbp1   | Retinol binding protein 1, cellular |
| 1384938_at        | 1.5126      | 0.0470   | Arhgap1| Rho GTPase activating protein 1 |
| 1369879_a_at      | 1.4963      | 0.0208   | Tmbim6 | Transmembrane BAX inhibitor motif containing 6 |
| 1378287_at        | 1.4835      | 0.0421   | Rdx    | Radixin |
| 1371127_at        | 1.4768      | 0.0137   | Bmp1   | Bone morphogenetic protein 1 |
| 1371056_at        | 1.4595      | 0.0335   | Neo1   | Neogenin 1 |
| 1378842_at        | 1.4550      | 0.0465   | Gabarap1| GABA(A) receptor-associated protein like 1 |
| 1379889_at        | 1.4488      | 0.0321   | Lamin2 | Laminin, gamma 2 |
| 1367891_a_at      | 1.4455      | 0.0175   | Casp2  | Caspase 2, apoptosis-related cysteine peptidase |
| 1396742_at        | 1.4423      | 0.0137   | Ipo5   | Importin 5 |
| 1388557_at        | 1.4420      | 0.0404   | Tubb2c | Tubulin, beta 2C |
| 1367981_at        | 1.4338      | 0.0436   | Rabep1 | Rabaptin, Rab GTPase binding effector protein 1 |
| 1393055_at        | 1.4328      | 0.0399   | Pkn2   | Protein kinase N2 |
| 1369085_s_at      | 1.4298      | 0.0323   | Snrpn  | Small nuclear ribonucleoprotein polypeptide N |
| 1371103_at        | 1.4287      | 0.0268   | Rab6a  | RAB6A, member RAS oncogene family |
| 1384005_at        | 1.4212      | 0.0355   | Dr1    | Down-regulator of transcription 1, TBP-binding (negative cofactor 2) |
| 1394077_at        | 1.4202      | 0.0337   | Rnd3   | Rho family GTPase 3 |
| 1370130_at        | 1.4201      | 0.0393   | Rhoa   | Ras homolog gene family, member A |
| 1379345_at        | 1.4034      | 0.0457   | Col15a1| Collagen, type XV, alpha 1 |
| 1373473_a_at      | 1.3975      | 0.0307   | Nap111 | Nucleosome assembly protein 1-like 1 |
| 1375528_at        | 1.3927      | 0.0399   | Vcl    | Vinculin |
| 1384187_at        | 1.3826      | 0.0287   | Ap1s2  | Adaptor-related protein complex 1, sigma 2 subunit |
| 1369816_at        | 1.3815      | 0.0369   | Rab3a  | RAB3A, member RAS oncogene family |

(Continued)
Table A1 | Continued

| Affymetrix ID | Fold change | p-Value | Symbol | Entrez gene name |
|---------------|-------------|---------|--------|-----------------|
| 1368218_at    | 1.3797      | 0.0424  | Ralbp1 | ralA binding protein 1 |
| 1395648_at    | 1.3777      | 0.0331  | Eps15  | Epidermal growth factor receptor pathway substrate 15 |
| 1385797_at    | 1.3757      | 0.0377  | Actc1  | Actin, alpha, cardiac muscle 1 |
| 1382402_at    | 1.3687      | 0.0321  | Ulk1   | Unc-51-like kinase 1 (C. elegans) |
| 1371059_at    | 1.3669      | 0.0302  | Prkar2a | Protein kinase, cAMP-dependent, regulatory, type II, alpha |
| 1383531_at    | 1.3571      | 0.0342  | C5orf41 | Chromosome 5 open reading frame 41 |
| 1381509_at    | 1.3482      | 0.0341  | Nbr1   | Neighbor of BRCA1 gene 1 |
| 1383701_at    | 1.3447      | 0.0327  | Laptm5  | Lysosomal protein transmembrane 5 |
| 1383531_at    | 1.3470      | 0.0434  | Map2k4  | Mitogen-activated protein kinase kinase 4 |
| 1382402_at    | 1.3469      | 0.0373  | Tgfbr2  | Transforming growth factor, beta receptor II (70/80 kDa) |
| 1391390_at    | 1.3447      | 0.0236  | Tns1   | Tensin 1 |
| 1386066_at    | 1.3434      | 0.0470  | Map1lc3b | Microtubule-associated protein 1 light chain 3 beta |
| 1381509_at    | 1.3427      | 0.0472  | Rhoc    | Ras homolog gene family, member C |
| 1393639_at    | 1.3411      | 0.0341  | C5orf41 | Chromosome 5 open reading frame 41 |
| 1373240_at    | 1.3385      | 0.0373  | Tgfbr2  | Transforming growth factor, beta receptor II (70/80 kDa) |
| 1380993_at    | 1.3332      | 0.0411  | Rock1   | Ras homolog gene family, member C |
| 1386655_at    | 1.3322      | 0.0411  | Myo1c   | Myosin IC |
| 1370097_at    | 1.3318      | 0.0411  | Ccx4    | Chemokine (C-X-C motif) receptor 4 |
| 1397592_at    | 1.3315      | 0.0421  | Yeats4  | YEATS domain containing 4 |
| 1384186_at    | 1.3313      | 0.0334  | Edem1   | ER degradation enhancer, mannosidase alpha-like 1 |
| 1370087_at    | 1.3310      | 0.0327  | Rab2a   | RAB2A, member RAS oncogene family |
| 1388932_at    | 1.3286      | 0.0437  | Ugg1    | UDP-glucose glycoprotein glucosyltransferase 1 |
| 1388490_at    | 1.3191      | 0.0212  | Cd14    | CD14 molecule |
| 1392174_at    | 1.3189      | 0.0470  | Pdec4   | Programmed cell death 4 (neoplastic transformation inhibitor) |
| 1380993_at    | 1.3177      | 0.0441  | Myo10   | Myosin X |
| 1370097_at    | 1.3168      | 0.0374  | Ccx4    | Chemokine (C-X-C motif) receptor 4 |
| 1399639_at    | 1.3165      | 0.0421  | Yeats4  | YEATS domain containing 4 |
| 1384186_at    | 1.3133      | 0.0334  | Edem1   | ER degradation enhancer, mannosidase alpha-like 1 |
| 1370087_at    | 1.3130      | 0.0327  | Rab2a   | RAB2A, member RAS oncogene family |
| 1388932_at    | 1.3126      | 0.0437  | Ugg1    | UDP-glucose glycoprotein glucosyltransferase 1 |
| 1388490_at    | 1.3119      | 0.0212  | Cd14    | CD14 molecule |
| 1392174_at    | 1.3109      | 0.0470  | Pdec4   | Programmed cell death 4 (neoplastic transformation inhibitor) |
| 1380993_at    | 1.3177      | 0.0441  | Myo10   | Myosin X |
| 1370097_at    | 1.3168      | 0.0374  | Ccx4    | Chemokine (C-X-C motif) receptor 4 |
| 1399639_at    | 1.3165      | 0.0421  | Yeats4  | YEATS domain containing 4 |
| 1384186_at    | 1.3133      | 0.0334  | Edem1   | ER degradation enhancer, mannosidase alpha-like 1 |
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| 1380993_at    | 1.3177      | 0.0441  | Myo10   | Myosin X |
| 1370097_at    | 1.3168      | 0.0374  | Ccx4    | Chemokine (C-X-C motif) receptor 4 |
| 1399639_at    | 1.3165      | 0.0421  | Yeats4  | YEATS domain containing 4 |
| 1384186_at    | 1.3133      | 0.0334  | Edem1   | ER degradation enhancer, mannosidase alpha-like 1 |
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| 1370097_at    | 1.3168      | 0.0374  | Ccx4    | Chemokine (C-X-C motif) receptor 4 |
| 1399639_at    | 1.3165      | 0.0421  | Yeats4  | YEATS domain containing 4 |
| 1384186_at    | 1.3133      | 0.0334  | Edem1   | ER degradation enhancer, mannosidase alpha-like 1 |
| 1370087_at    | 1.3130      | 0.0327  | Rab2a   | RAB2A, member RAS oncogene family |
| 1388932_at    | 1.3126      | 0.0437  | Ugg1    | UDP-glucose glycoprotein glucosyltransferase 1 |
| 1388490_at    | 1.3119      | 0.0212  | Cd14    | CD14 molecule |
| 1392174_at    | 1.3109      | 0.0470  | Pdec4   | Programmed cell death 4 (neoplastic transformation inhibitor) |
| 1380993_at    | 1.3177      | 0.0441  | Myo10   | Myosin X |

(Continued)
### Table A1 | Continued

| Affymetrix ID  | Fold change | p-Value | Symbol | Entrez gene name |
|----------------|-------------|---------|--------|-----------------|
| 1372467_at     | $-1.3411$   | 0.0425  | Hs6st1 | Heparan sulfate 6-O-sulfotransferase 1 |
| 1387898_at     | $-1.3285$   | 0.0234  | Hspb6  | Heat shock protein, alpha-crystallin-related, B6 |
| 1397224_at     | $-1.3272$   | 0.0287  | Atp2b1 | ATPase, Ca$^{++}$ transporting, plasma membrane 1 |
| 1395499_at     | $-1.3089$   | 0.0335  | Eps8   | Epidermal growth factor receptor pathway substrate 8 |
| 1372265_at     | $-1.3030$   | 0.0459  | C14orf153 | Chromosome 14 open reading frame 153 |
| 1373494_at     | $-1.2980$   | 0.0259  | Bcr    | Breakpoint cluster region |
| 1379413_at     | $-1.2966$   | 0.0378  | Nmnat1 | Nicotinamide nucleotide adenyllytransferase 1 |
| 1367812_at     | $-1.2966$   | 0.0334  | Sptbn2 | Spectrin, beta, non-erythrocytic 2 |
| 1378198_at     | $-1.2959$   | 0.0333  | Ophn1  | Oligophrenin 1 |
| 1367977_at     | $-1.2942$   | 0.0406  | Snca   | Synuclein, alpha (non-A4 component of amyloid precursor) |
| 1368774_a_at   | $-1.2666$   | 0.0399  | Espn   | Espin |
| 1372638_at     | $-1.2656$   | 0.0434  | Arhgef7 | Rho guanine nucleotide exchange factor (GEF) 7 |
| 1385785_a_at   | $-1.2595$   | 0.0406  | Ptx2   | Paired-like homeodomain 2 |
| 1382055_at     | $-1.2588$   | 0.0316  | Rtkn   | Rhotekin |
| 1387124_at     | $-1.2526$   | 0.0499  | Inha   | Inhibin, alpha |
| 1376041_at     | $-1.2509$   | 0.0321  | Epn3   | Epsin 3 |
| 1396392_at     | $-1.2474$   | 0.0446  | Dctn6  | Dynactin 6 |
| 1384319_at     | $-1.2451$   | 0.0436  | Ttk2   | Tousled-like kinase 2 |
| 1373146_at     | $-1.2379$   | 0.0323  | Ssx2ip | Synovial sarcoma, X breakpoint 2 interacting protein |
| 1374444_at     | $-1.2367$   | 0.0424  | Ptkn1  | Plexin B1 |
| 1391915_at     | $-1.2313$   | 0.0411  | Hspa9  | Heat shock 70 kDa protein 9 (mortalin) |
| 1385526_at     | $-1.2223$   | 0.0441  | Atg5   | ATG5 autophagy related 5 homolog (S. cerevisiae) |
| 1381190_at     | $-1.2181$   | 0.0444  | Lmo7   | LIM domain 7 |
| 1387656_at     | $-1.2131$   | 0.0463  | Slc4a1 | Solute carrier family 4, anion exchanger, member 1 |

Statistical analyses of the microarray data were performed with RMA, robust multiarray averaging; BH, Benjamini and Hochberg adjustment using the R statistical package (version 2.8.1). The complete microarray data is available to the reviewers at the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hryjdweamuoid&acc=GSE30770