SALT STRESS IN THE RENAL TUBULES IS LINKED TO TAL SPECIFIC EXPRESSION OF UROMODULIN AND AN UPREGULATION OF HEAT SHOCK GENES.

Lesley A. Graham¹, Alisha Aman¹, Desmond D. Campbell¹, Julian Augley², Delyth Graham¹, Martin W. McBride¹, Niall, J. Fraser³, Nicholas. R. Ferreri⁴, Anna F. Dominiczak¹, and Sandosh Padmanabhan¹.

Running title: Salt stress transcriptomics

¹ University of Glasgow, College of Medical, Veterinary, and Life Sciences, Institute of Cardiovascular and Medical Sciences, 126 University Place, The British Heart Foundation Centre of Excellence, Glasgow, G12 8TA. ² University of Glasgow, Glasgow Polyomics, Wolfson Wohl Cancer Res Centre, Garscube Estate Switchback Rd, Bearsden G61 1QH. ³ University of Dundee, Ninewells hospital, James Arrott drive, Dundee, DD2 1SY. ⁴ New York Medical College, Department of Pharmacology, Basic Sciences Building, Valhalla, NY 10595.

Corresponding author: Dr Lesley Graham, the University of Glasgow, Institute of Cardiovascular and Medical Sciences, College of Medical and Veterinary Life Sciences, the British Heart Foundation Centre for Excellence, 126 University place, level 2, Glasgow, G12 8TA, United Kingdom. Lesley.Graham@glasgow.ac.uk

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Abstract (250 words max no abbreviations)

Previously, our comprehensive cardiovascular characterisation study validated *Uromodulin* as a blood pressure gene. Uromodulin is a glycoprotein exclusively synthesised at the thick ascending limb of the loop of Henle and is encoded by the *Umod* gene. *Umod*−/− mice have significantly lower blood pressure than *Umod*+/+ mice, are resistant to salt-induced changes in blood pressure, and show a leftward shift in pressure–natriuresis curves reflecting changes of sodium reabsorption. Salt stress triggers transcription factors and genes that alter renal sodium reabsorption. To date there are no studies on renal transcriptome responses to salt stress. Here we aimed to delineate salt stress pathways in tubules isolated from *Umod*+/+ mice (a model of sodium retention) and *Umod*−/− mice (a model of sodium depletion) ±300mOsmol sodium chloride (n=3 per group) performing RNA-Seq. In response to salt stress, the tubules of *Umod*+/+ mice displayed an up regulation of heat shock transcripts. The greatest changes occurred in the expression of: *Hspa1a* (Log2 fold change 4.35, p=2.48e-12) and *Hspa1b* (Log2 fold change 4.05, p=2.48e-12). This response was absent in tubules of *Umod*−/− mice. Interestingly, 7 of the genes discordantly expressed in the *Umod*−/− tubules were electrolyte transporters. Our results are the first to show that salt stress in renal tubules alters the transcriptome, increasing the expression of heat shock genes. This direction of effect in *Umod*+/+ tubules suggest the difference is due to the presence of *Umod* facilitating greater sodium entry into the tubule cell reflecting a specific response to salt stress.
**Introduction**

Blood pressure (BP) responses to salt intake are variable (50), however, epidemiologic, interventional, evolutionary and genetic studies in humans and animals have clearly demonstrated a link between salt intake and hypertension (HTN) (1, 2, 4, 7, 9, 10, 14, 22, 30, 40, 42, 51, 52). High salt sensitivity is estimated to be present in 51% of hypertensive and 26% of normotensive populations (20, 21). Although salt sensitivity is a well-recognised phenomenon in experimental and human HTN, the pathophysiological mechanisms are not fully elucidated (3). Recent systems approaches by means of metabolomics (31, 45) and proteomics (17, 32) have identified putative pathways that are affected by salt stress in plants and rats but to date studies in humans are lacking.

Osmoregulation, the cellular response to environmental changes of osmolarity and ionic strength, is important for the survival of living organisms. Large fluctuations in environmental osmolarity due to dietary salt may modulate stress responses across the nephron, affecting BP control. A family of proteins called heat shock factors (HSFs) are activated by the non-native proteins that accumulate in response to salt stress. Activated HSFs binds to heat shock elements (HSEs) and stimulate transcription of HSP70. The promoter of HSP70 is stimulated in response to hypertonicity due to the toxicity-responsive enhancer binding protein (TonEBP) binding. TonEBP, also known as nuclear factor of activated T cells 5 (NFAT5), is the master transcriptional regulator for the cellular accumulation of organic osmolytes in the renal medulla (34, 53). Once NFAT5 is activated by hyperosmotic stress it triggers increased expression of osmosensitive genes that alter Na⁺ reabsorption in the thick ascending limb of the loop of Henle (TAL) (18). The rate of NaCl transport in the TAL is an important determinant of medullary hypertonicity, occurs via the Na⁺/K⁺/Cl⁻ cotransporter (Nkcc2) a protein expressed exclusively in the apical membrane of the TAL and macula densa cells (13). Nkcc2 and NFAT5 are sequentially expressed in the TAL, and inhibition of Nkcc2 activity by furosemide reduces expression of NFAT5 and its target genes in the renal medulla (43), implicating an important functional link between Nkcc2, NFAT5, and Na⁺ reabsorption. Ferreri *et al* reported the Nkcc2 A isoform (Nkcc2A) contributes to the regulation of NFAT5 in primary cultures of medullary TAL cells exposed to hypertonic NaCl concentration (18, 19).

Uromodulin (Umod) is a kidney protein exclusively synthesised at the TAL and is encoded by the *Umod* gene. We have reported previously that altered *Umod* expression is causal of HTN (37) and have since validated *Umod* as a blood pressure gene in a comprehensive characterisation study in *Umod*⁻/⁻ mice (16). *Umod*⁻/⁻ mice display augmented Na⁺ excretion, thought to be a consequence of reduced expression of Nkcc2. This modulated Na⁺ reabsorption by reduced Nkcc2 leads to exaggerated natriuresis and lower arterial pressure in the *Umod*⁻/⁻ mice; furthermore, these mice are not sensitive to salt induced changes in BP consistent with findings in humans with salt wasting phenotypes and hypotension (41, 44, 49). In a complimentary set of experiments, Trudu *et al* demonstrated that uromodulin-transgenic mice over expressing *Umod* manifested salt-sensitive HTN, due to activation of the SPAK kinase and activating N-terminal phosphorylation of Nkcc2 (47). These studies combined imply a permissive role of *Umod* in the modulation of Na⁺ transport. The specific pathways involved in salt tolerance in the TAL are not well defined and to date there are no studies on transcriptome response(s) in the TAL to salt stress. Here we aimed to delineate salt stress pathways in TAL tubules to determine the changes in the TAL transcriptome associated with the expression of *Umod* and salt status.
Results
The study design is described in Figure 1. Previously we have performed cardiovascular characterisation studies in Umod+/+ and Umod−/− mice (±2% NaCl in the drinking water for 6 weeks). These studies have demonstrated that Umod+/+ mice are a model of sodium retention and Umod−/− mice are a model of sodium depletion (Figure 1A). Based on sodium status of each strain, the present study utilised RNA-seq technology to investigate the specific effects of TAL salt stress in vitro depending on Umod expression (Figure 1B).

Salt related differentially expressed genes (DEG) in TAL tubules.
In Umod+/+ mice tubules, of the 43629 transcripts detected, DESeq2 filtered and analysed 22355 transcripts out of which 14 DEGs were identified with a nominal significant difference in gene expression (FDR Q-value <0.1; 5 decreased and 9 increased) Figure 2 and Supplementary Figure 1A. In Umod−/− mice tubules, of the 43629 transcripts detected, DESeq2 filtered and analysed 15723 transcripts. Of the DEGs, 178 were identified with a nominal significant difference in gene expression (FDR Q-value <0.05; 27 decreased and 151 increased).

Supplementary Table 1 and Supplementary Figure 1B.
In Umod+/+ mice, the transcript count for Umod (ENSMUSG00000030963) were 538475, 498026, 777919 for +300mOsm NaCl Umod+/+ mice, and 353713, 425034, 256762 for no added NaCl Umod+/+ mice. The log 2-fold change for Umod was 1.10 with an FDR corrected P value of 0.17. In contrast, the Umod−/− mice showed lower Umod transcript counts - 7756, 6951, 9533 for +300mOsm NaCl Umod−/− mice, and 9920, 8916, 6782 for no added NaCl Umod−/− mice. The log 2-fold change for Umod in this dataset was 0.03 with an FDR corrected P value of 0.8.
In Umod+/+ mice, data analysis for biological interpretation of DEGs revealed among the up-regulated genes a cluster of transcripts involved in heat stress (Hspa1b, Hspa1a, Dnajb1, Dnaja4) (Figure 2) while, the downregulated genes did not demonstrate any obvious enrichment for pathways. Quantitative real-time polymerase chain reaction (qRT-PCR) validated the over expression of Hspa1b, Dnajb1, and Nr0b2 (Figure 2).
In the Umod−/− mice, DEGs revealed a multitude of upregulated genes across a range of pathways, but no single predominantly enriched pathway (Supplementary Table 1).
We compared the direction of effect of all the DEGs in tubules of Umod+/+ and Umod−/− mice (summarised in Figure 3). Genes significantly over-expressed in Umod−/− mouse TAL tubules showed log2 fold-change >3 and the corresponding expression levels in Umod+/+ mouse TAL tubules were substantially smaller but the directions of effect were similar. Of the significant DEGs in Umod−/− mouse TAL tubules, gene expression levels were generally higher compared to Umod+/+ mouse TAL tubules. However, 7 out of the 39 (Q<0.05) genes showed discordant directions of effect between Umod−/− and Umod+/+ mouse TAL tubules: Gm2026, Slc7a12, Cp, Akap12, Slpr1, Slc38a2, and Havcr1.

Gene Set Analysis
Gene Set Analysis (GSA) was used to identify Gene Ontology categories (of which there were a count of 2813 gene-sets for Umod+/+ mouse tubules and 2352 gene-sets for Umod−/− mouse tubules) that were enriched for genes that were differentially expressed across treatments (no salt vs. +300mOsomol NaCl). This GSA was performed using the R package Piano (48). Among the most significantly upregulated categories were heat shock and chaperone binding (Umod+/+ mouse tubules), while anion transporters predominate among the downregulated pathways in response to salt stress (Umod+/+ mouse tubules) Figure 4A and Supplementary
We and others have shown previously that heat shock proteins (Hsps) act by capturing an unfolded polypeptide by recognising exposed hydrophobic patches. This chaperone could be HSP70 itself or a J-domain co-chaperone (HSP40) that forms a complex with HSP70. Cohen et al. reported that salt induced stress increased the mRNA expression of HSP70 in cultured Madin-Darby canine kidney (MDCK) cells (8), consistent with our findings here in tubules from Umod+/+ mice. They postulated that HSP70 may play a role in the kidney to stabilise proteins in the face of the elevated and potentially denaturing ionic concentrations that accompany sudden changes in the medullary osmotic environment. Contrary to these findings Medina et al. reported neither the HSP70 message nor its protein product differed significantly in medulla tubules, possibly due to prolonged water diuresis that might have attenuated expression of HSP70 (33).

Nevertheless, in the past few years it has become increasingly clear that HSPs may contribute decisively to survival of medulla cells by conferring protection against the extremely high interstitial solute concentrations (36).

Here, our novel transcriptomic findings suggest a biological link between salt stress in the TAL and interactions between Umod and HSPs. HSPs are involved in salt stress pathways to protect renal cells to maintain normal Na+ homeostasis at this nephron segment. We observed a striking difference in the transcriptomic profile in Umod−/− mice TAL tubules in response to salt with HSPs not featuring in the set of 178 gene attaining experiment wide significance. The TAL tubules of Umod−/− mice showed a greater number of DEGs with substantially smaller effect sizes in terms of log2-fold change in contrast to that of tubules from Umod+/+ mice. The direction of effect of the HSP in Umod+/+ tubules suggest that the difference is due to the presence of Umod facilitating greater Na+ entry into the Umod+/+ tubule cell and thus reflecting a specific response to salt stress.

We and others have shown previously that Umod is associated with salt-sensitive HTN and this is likely through Umod-Nkcc2 interaction (16, 47). The genes that attained statistical significance in the TAL tubules of Umod−/−...
mice represented a wide spectrum of pathways with no single pathway showing a predominant contribution.

Many of the genes that appear elevated in the Umod\(^{-}\) TAL tubules are electrolyte transporters and glomerular proteins; Atp1a2 (ATPase, Na+/K+ transporting, alpha 2 polypeptide), Aqp4 (aquaporin 4), Slc12a1 (solute carrier family 12, member 1), Kcnk3 (potassium channel, subfamily K, member 3), Slc5a3 (solute carrier family 5 (inositol transporters), member 3), Hsd17b11 (hydroxysteroid (17-beta) dehydrogenase 11), Podxl (podocalyxin-like), Nphs1 (nephrosis 1, nephrin), Syt7 (synaptotagmin VII), Car3 (carbonic anhydrase 3), and Cyp4a12a (cytochrome P450, family 4, subfamily a, polypeptide 12a) indicating that these responses are related to increased luminal NaCl. Interestingly, Slc12a1 which encodes Nkcc2 is significantly differentially overexpressed in Umod\(^{-}\) mouse TAL tubules but shows no differential expression in Umod\(^{-}\)-/ mouse TAL tubules. It has been previously demonstrated that total Nkcc2 expression is increased in Umod\(^{-}\) mice due to an abundance of “inactive” intracellular Nkcc2 expressed in the subapical vesicles (35). Using a less conservative significance threshold we find that 18% of the genes that are differentially expressed in Umod\(^{-}\) mouse TAL tubules show an opposite direction of effect in Umod\(^{-}\)-/ mouse TAL tubules, but in general the majority of the genes show concordant direction of effect. Umod is co-expressed at the apical surface of the TAL with Nkcc2 and is reported to play a key role in Na\(^+\) homeostasis and BP control (16, 47). A positive correlation between urinary Umod and dietary salt intake revealed that in subjects with high salt sensitivity, there is a greater excretion of Umod in the urine compared low salt intake (46). Likewise, there is a direct relationship between high salt intake and Umod mRNA expression (16).

The accumulation of organic osmolytes is only one component of the adaptive process allowing medullary cells to survive in the harsh environment of salt stress. This effect is at least partially attributable to the chaperoning activities of HSP40/HSP70 complex (36). Ma et al have previously demonstrated the rescue of Umod to the apical surface of the TAL is mediated by the restoration of the level and subcellular localisation of cytosolic chaperone HSP70 during salt stress (29). It seems likely, we have identified a novel link between HSPs and salt stress pathways in the TAL, implicating potential biological mechanisms to be investigated in the management of salt sensitive HTN. There are some limitations to the current study. We have not measured apoptosis in the TAL tubules following the salt stress conditions; however, we speculate HSP70 is part of a protective mechanism, which is not present or needed in the Umod\(^{-}\) TAL. Future studies will focus on exacerbating apoptosis by blocking HSP70 and HSP pathways in TAL tubules of Umod\(^{-}\)-/ mice. This would allow the investigation of pathogenetic pathways that emerges from these studies.

In the context of human essential HTN, antibodies to HSP70 are commonly observed and polymorphisms in HSP70 gene expression have also been associated with HTN in specific populations (27, 39). Pons et al have shown that during salt stress, the increased expression of HSP70 in medulla region of the kidney causes an activation of T cells as a response, leading to salt sensitive hypertension (39). Pockley et al (38), reported IgG anti-HSP70 antibodies in the plasma of hypertensive patients, which is in concordance with preliminary studies from Pons et al (39). This prompted the exploration of the proliferative responses of peripheral blood lymphocytes in a small group of well-defined patients with essential hypertension to the antigenic peptide used in their experimental studies (39). The selection of patients was designed to limit the age range and exclude conditions that are known to be associated with anti-HSP70 antibodies and the results showed a clear separation between the proliferative response of the patients and the control subjects. These studies provide evidence that
autoimmunity plays a role in salt-sensitive hypertension and identifies HSP70 expressed in the kidney as one key antigen involved in the development of autoimmune reactivity in the kidney and thereby in the impairment of physiological mechanisms of sodium excretion that accompanies salt-sensitive hypertension.

Genome wide association studies in humans have shown SNPs in the promoter region of the Umod gene to be associated with renal function and hypertension (23-25, 37). Specifically, the allele associated with higher Umod levels are associated with higher blood pressure and lower eGFR. The results from this study indicate that the increase in BP in those with the Umod increasing genotype may be influenced by multiple mechanisms including those mediated by activation of HSP. A recent study showed global frequencies of the Umod alleles significantly correlated with pathogen diversity and prevalence of antibiotic-resistant urinary tract infections, but not with the latitudinal clines in the frequencies of variants associated with salt-sensitivity (15). The implication of this study is that Umod ancestral allele has been kept at a high frequency because of its protective effect against UTIs and salt-sensitive hypertension may be a maladaptive phenotype through increased sodium reabsorption. It is also possible that the Umod increasing allele may have arisen to preserve sodium in the salt-poor evolutionary past. Our findings of activation of heat shock pathway in response to salt suggests that heat shock proteins are activated as a protective measure against the influx of sodium into the tubule cells, however there is evidence that they may also facilitate salt-sensitive hypertension through other independent pathways and activate the immune system which may protect against infections. Further work will focus on elucidating the role of Umod and heat shock proteins on infection and salt sensitivity as these are critical to downstream efforts to modulate Umod for hypertension treatment.
Methods

Experimental animals

The Umod<sup>−/−</sup> mouse model was generated by Bates et al (Oklahoma University, USA) along with the Umod<sup>+/+</sup> strain and used throughout this study (5). These mice have been maintained as breeding colonies at Glasgow University since 2010. The mice were housed under controlled environmental conditions, fed standard rat chow (rat and mouse No. 1 maintenance diet, Special Diet Services containing 0.19% Na<sup>+</sup> and 0.32% Cl<sup>−</sup>), and water provided ad libitum. All animal procedures performed were approved by the Home Office according to regulations regarding experiments with animals in the United Kingdom. The genotype of the Umod<sup>+/+</sup> and Umod<sup>−/−</sup> mice was verified using end point PCR of tail genomic DNA with specific primers (forward: 5′ AGGGCTTTACAGGGGATGGTTG-3′ and reverse: 5′ GATTGCACTCAGGGGGCTCTGT 3′). Male mice of both strains were used in this study the experimental design is outlined in Figure 1.

Isolation of thick ascending limb of the loop of Henle tubules

Male Umod<sup>+/+</sup> and Umod<sup>−/−</sup> mice of 5-7 weeks old were used for medullary TAL tubule isolation. Isolations were performed as previously described (12). In brief, mice were anesthetised with isofluorane and the kidneys were perfused with sterile 0.9% saline solution via retrograde perfusion of the aorta. Once kidneys were excised they were cut along the corticopapillary axis, to expose the medulla. The inner strip of the outer medulla was dissected out and minced with a sterile blade in 0.1% (w/v) collagenase solution (collagen type IV collagenase prepared in Hanks Balanced Saline) (Sigma Aldrich, Poole, UK and Gibco<sup>®</sup>, Paisley, UK) that was gassed with 95% oxygen. The pulp was incubated for ten minutes at 37°C. The cell suspension was sedimented on ice and mixed with Hanks Balanced Saline (HBSS) containing 2% (w/v) BSA, and the crude suspension of tubules was collected. The remaining undigested tissue was collagenase treated a further three times. The combined supernatants were spun at a low speed (1000 RPM) for ten minutes, and resuspended in HBSS. The resuspension was passed over a 52µm nylon mesh membrane (Fisher Scientific, Loughborough, UK). The filtered solution was discarded and the tubules collected on the mesh were washed with HBSS and centrifuged for 5 minutes at a low speed (500 RPM). The supernatant was aspirated and the tubules were re-suspended with Clonetics™ REGM™ growth media supplemented with the growth factor BulletKit™ (CC-3190): hEGF, 0.5 ml; hydrocortisone, 0.5 ml; epinephrine, 0.5 ml; insulin, 0.5 ml; triiodothyronine 0.5 ml; transferrin, 0.5 ml; GA-1000, 0.5 ml; FBS, 2.5 ml. The tubule suspension was split into two separate 15ml falcone tubes and the media was supplemented with an additional 300mOsmol NaCl (salt stress group) or received no NaCl treatment (control group) (n=3 per group). The tubule suspensions were rotated for 4 hours at 37°C. After the incubation period, the suspensions were centrifuged at a low speed (1000 RPM) for ten minutes, the supernatant removed and the tubule pellets were taken forward for RNA isolation.

RNA extraction, quantification, and analysis

Total RNA was extracted using Qiagen column based miRNeasy Mini kits (QIAGEN, Manchester, UK). Tubules were homogenised with Qiazol homogenisation reagent included in the kit. RNA was eluted in 30µl RNase-free H<sub>2</sub>O. A Nanodrop<sup>®</sup> ND-1000 spectrophotometer (Thermo Scientific, Loughborough, UK), was used to measure RNA concentrations. This method is sensitive at measuring concentrations between 2-37000 ng/µl of double-stranded DNA. Absorbance ratios (260 nm/280 nm) of approximately 2.0 for RNA indicated that the
nucleic acid preparations were sufficiently free from protein contamination for downstream experiments.

Additionally, the ratio of absorption at 260 nm and 230 nm was used as an indicator of RNA purity; pure RNA has a ratio of 2.0 - 2.2. The concentration of the sample is calculated using the Beer-Lambert Law of absorption;

Concentration of RNA (μg/ml) = (A260 reading – A320 reading) x 40. RNA samples were stored at -80°C until time of RNA-Seq. Total RNA isolated was quality tested for degradation prior to RNA-Seq, via electrophoresis on the Agilent Bioanalyser 2100 and a Eukaryote Total RNA Nano Series II chip. The analysis was run at the Molecular Biology Support Unit at the University of Glasgow. Electrograms produced indicate defined bands for 18S and 28S ribosomal RNA (rRNA) species and a RNA integrity number (RIN).

**RNA-Seq dataset of renal TAL tubules from mice exposed to no salt and salt stress (+300mOsmol).**

500ng of total RNA was isolated and RNA-Seq performed using the NextSeq 500 platform operating 2*75bp paired end cycles generating approximately 42 million reads per sample. Raw read files were adapter trimmed and quality filtered using Cutadapt, to produce reads with mean quality score no less than 20 using Sanger quality scores. Read counts were obtained using Kallisto version 0.42.3 to pseudod align reads to the GRCm38 (mm10) transcriptome. RNA-Seq datasets of all samples exhibited similar distribution and quantity of read counts.

**Gene expression studies.** Quantitative real-time polymerase chain reaction of total RNA extracted from TAL tubule isolations from male Umod+/+ and Umod−/− mice of 7 weeks old was performed using Qiagen column based miRNeasy Mini kits (QIAGEN, Manchester, UK). Tubules were homogenised with Qiazol homogenisation reagent included in the kit. cDNA was prepared using Applied Biosystems 'TaqMan Reverse Transcription Reagents'. 1 μg of total RNA template was utilised for downstream applications. Determination of mRNA abundance of specific genes was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) (ThermoFisher, Paisley, UK). n=3 tubule isolations per group (no additional NaCl vs. +300mOsmol NaCl). Results were normalised to the housekeeper Gapdh (Mm03302249) Gene expression probes used: Mouse Taqman probe Hspa1b (Mm03038954_s1), Mouse Taqman probe Dnajb1 (Mm00444519_m1), and Mouse Taqman probe Nr0b2 (Mm00442278_m1) (ThermoFisher, Paisley, UK).

**Statistical analysis**

RNA-Seq analysis of differentially expressed genes (DEG) was conducted using the Bioconductor package DESeq2 (Stanford, California, USA) (28). DESeq2 assumes that the RNA-Seq counts are negative binomial distribution. DESeq2 utilises generalised linear modelling and variance-reduction techniques for estimated coefficients to test individual null hypotheses of zero log2-fold changes between high salt and low salt for each gene. It uses both an independent filtering method and the Benjamini-Hochberg procedure to improve power and control the false discovery rate (FDR). The default DESeq2 options were used and an FDR Q value < 0.1 threshold for Umod+/+ mice and FDR Q value < 0.05 for Umod−/− mice was used to identify DEG.

We performed an ontology enrichment analysis looking for differentially expressed mRNA levels in each of the 2813 gene-sets for Umod+/+ and 2352 gene-sets for Umod−/− mice of Gene Ontology for *Mus Musculus* which was obtained using the R package ‘BiomaRt’ (48). For this we used the R package *Piano* (48) which enables functional characterisation and interpretation through gene set analysis (GSA). We used *Piano* as recommended in the authors’ paper. In brief, *Piano* implements 12 GSA methods, taking as input either p-value plus direction
of effect, or t score, per gene and the fold change. Of these 24 combinations of the enrichment analysis method with the input statistic type, 16 are valid. For each GO term, a p-value for enrichment was obtained via each of these 16 method/input-type combinations. These p-values were estimated using 100,000 gene sampling permutations. For each of the 16 method/input-type combinations, the GO terms were ranked according to p-value. The ‘consensus score’ for each GO term was the median of these 16 ranks for the corresponding GO term. The ‘median p-value’ for each GO term was the median of the 16-corresponding p-values. Furthermore, the above was done for 5 different enrichment hypotheses. The central hypothesis tested (‘non-directional’), was whether a GO term is enriched for differentially expressed genes. Two further hypotheses concerned up regulation (i) enrichment for upregulation, ignoring any downregulation (‘mixed-directional’) (ii) enrichment for upregulation, penalised by any downregulation (‘distinct-directional’). Two corresponding down regulation hypotheses were also tested.

Statement of financial interest
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References


Our initial cardiovascular characterisation studies in \textit{Umod}^{+/+} and \textit{Umod}^{-/-} mice have shown that \textit{Umod}^{-/-} mice have reduced systolic blood pressure, have limited sodium reabsorption at the thick ascending limb of the loop of Henle (TAL), and are not sensitive to dietary salt intake (2%NaCl in the drinking water for 6 weeks) (16). The phenotype of each mouse strain is illustrated in panel (A). Salt status of \textit{Umod}^{+/+} and \textit{Umod}^{-/-} (in vivo) are as follows: \textit{Umod}^{+/+} (normal salt diet) represent salt repletion; \textit{Umod}^{+/+} under salt loading conditions (2%NaCl in the drinking water for 6 weeks) represent a model of excess salt reabsorption; \textit{Umod}^{-/-} mice (normal salt diet) represent a sodium deplete model; and \textit{Umod}^{-/-} mice under salt loading conditions (2%NaCl in the drinking water for 6 weeks) represent a model of salt resistance. Therefore due to the salt status and cardiovascular phenotype of each mouse strain, the current study utilised TAL tubules from each mouse under normal dietary conditions to take forward for \textit{in vitro} salt stress studies. (B) TAL tubules were isolated from both \textit{Umod}^{+/+} and \textit{Umod}^{-/-} mice and incubated in growth media for 4 hours at 37°C under the following salt stress conditions: either incubation media supplemented with an additional 300mOsmol NaCl (salt stress group) or received no additional NaCl treatment, normal incubation media (control group) (n=3 per group). Transcriptomic profiles using RNA-Seq were then determined in isolated TAL tubules in \textit{Umod}^{+/+} and \textit{Umod}^{-/-} mice (±300mOsmol NaCl), n=3 biological reps per group. Male \textit{Umod}^{+/+} and \textit{Umod}^{-/-} mice of 5-7 weeks of age were utilised in this \textit{in vitro} study.

Figure 2. Heatmap visualisation of RNA-seq transcriptome analysis of transcription levels for the most significantly differentially expressed genes in \textit{Umod}^{+/+} mice TAL tubules (±300mOsmol NaCl).

(A) The heatmap shows differentially expressed genes (DEG) significant for one pairwise test. Analysis was performed using DESeq2 with the read counts as input. The heatmap indicates the genes with significantly altered expression (FDR Q-value<0.1). The colours indicate the transform counts as log2(x+1), where x = read counts, with the red spectra indicating high expression while the blue indicating low expression. Gene details are depicted on the right along the vertical axis. NOSALTWT1, NOSALTWT2 and NOSALTWT3 are the control group (no additional salt treatment added to the incubation media) and SALW1T1, SALW1T2, SALW1T3 are the tubules treated with additional 300mOsmol NaCl in the incubation. Fold change is reported as the log2 value with its standard error of estimation. The final P values after FDR correction is given as the Q values. qRT-PCR analysis show an increased expression of (B) Hspa1b, (C) Dnajb1, and (D) Nr0b2 in \textit{Umod}^{+/+} TAL tubules when treated with an additional 300mOsmol NaCl. Data are shown as Mean RQ ± SEM (Cycle threshold values were normalised to Gapdh mRNA and expressed relative to \textit{Umod}^{+/+} tubule isolations under normal NaCl conditions), n=3 per group. Analysed by One way ANOVA followed by Tukey’s post Hoc multiple comparison test. ns=not significant. p<0.05 is deemed statistically significant.
Figure 3. Nominally significant genes in either Umod^+/+ or Umod^-/- isolated TAL tubules (+300mOsmol NaCl).

(A) Compares the log 2 fold change of the genes that are highly significant in Umod^+/+ mouse TAL tubules (q<0.1) and nominally significant in Umod^-/- mouse TAL tubules (p<0.1); of these five significant genes, three belong to the heat shock protein family (B) Compares the log 2 fold change of the genes that are highly significant in Umod^-/- mouse TAL tubules (q<0.05) and nominally significant in Umod^+/+ mouse TAL tubules (p<0.1); out of the 39 significant genes, 7 of them (in bold) are discordant in their direction of expression in both the groups.

Figure 4. Pathway enrichment analysis of (A) Umod^+/+ and (B) Umod^-/- mice TAL tubules post incubation with 300mOsmol NaCl.

Enrichment was performed using Piano with 100,000 permutations across the gene sets and subsequently ranked across five hypotheses based on the differential expression. Consensus ranks are depicted as a heatmap. Consensus scores and P-values were calculated using the median values of the ranks per hypothesis. Three directional classes have been specified in the enrichment analysis: non-directional, mixed-directional, and distinct-directional. In the non-directional class, information on the direction of differential expression is removed. Mixed-directional class contains information indicating whether the gene set is significantly affected by the DEG in either or both directions; two P values are assigned for each gene set under this class, one for up- and the other for downregulation. The distinct-directional class identifies gene sets that are significantly altered by the DEG only in a particular direction - two P values are reported for each gene set, one for each direction. The consensus scores for heat shock binding protein and chaperone binding are very low and hence are ranked highest in non-directional, mixed-directional up and distinct-directional up suggesting significant upregulation of the genes in those pathways under salt stress. The gradient of the heatmap depicts the consensus scores - the red spectra indicating the lowest consensus scores.
Figure 1
### Figure 2

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<td>1.89</td>
<td>0.12</td>
<td>Neuraglin 5</td>
</tr>
<tr>
<td>Slc9a3</td>
<td>3.43</td>
<td>0.21</td>
<td>Ser/Asp transporter 3</td>
</tr>
<tr>
<td>Sema4d</td>
<td>1.83</td>
<td>0.13</td>
<td>Sema4d, semaphorin D</td>
</tr>
<tr>
<td>Celpm1</td>
<td>2.56</td>
<td>0.36</td>
<td>Celpm1, cell migration inducible protein, myeloid</td>
</tr>
<tr>
<td>Atp1a1</td>
<td>2.47</td>
<td>0.15</td>
<td>Atp1a1, ATP synthase subunit a1</td>
</tr>
<tr>
<td>Acot2</td>
<td>1.96</td>
<td>0.29</td>
<td>Acot2, acyl-CoA thioesterase 2</td>
</tr>
<tr>
<td>Cmyc</td>
<td>1.60</td>
<td>0.37</td>
<td>Cmyc, cell migration inducible protein, myeloid</td>
</tr>
<tr>
<td>Slc1a6</td>
<td>1.94</td>
<td>0.32</td>
<td>Slc1a6, solute carrier organic anion transporter family member 1a</td>
</tr>
<tr>
<td>Srp40c</td>
<td>3.33</td>
<td>0.28</td>
<td>Serpine, serine peptidase inhibitor, clade H, member 1</td>
</tr>
</tbody>
</table>

**Relative Quantification (RQ)/Gapdh**

- **(B)**
  - Umod+/+ (Normal NaCl)
  - Umod+/+ (+300mOsmol NaCl)
  - Umod+/ (Normal NaCl)
  - Umod+/ (+300mOsmol NaCl)

- **(C)**
  - Umod+/+ (Normal NaCl)
  - Umod+/+ (+300mOsmol NaCl)
  - Umod+/ (Normal NaCl)
  - Umod+/ (+300mOsmol NaCl)

- **(D)**
  - Umod+/+ (Normal NaCl)
  - Umod+/+ (+300mOsmol NaCl)
  - Umod+/ (Normal NaCl)
  - Umod+/ (+300mOsmol NaCl)
Comparing DEG in Umod^+/+ and Umod^-/- (A) Top genes in Umod^+/+ which are nominally significant in Umod^-/- (P<0.1)

Comparing DEG in Umod^+/+ and Umod^-/- (B) Top genes in Umod^-/- which are nominally significant in Umod^+/+ (P<0.1)

Figure 3
Figure 4