Identification of mechanistic CKD biomarkers in a rat SNx kidney fibrosis model by transcriptomics and proteomics detectable in biofluids

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Supplementary Methods

Subtotal Nephrectomy rat model generation

Animals were housed in open-top cages, in a specific-pathogen-free facility according to the Federation of European Laboratory Animal Science Associations recommendations. Typically, animals were housed in groups of four or five. Food (RM1; SAFE, Paris, France) and water (untreated mains tap water) were available ad libitum, and the light cycle was 12/12 hours light and dark. Room temperature and humidity were 21℃ and 45%, respectively. Nest building material was provided, and all cages contained enrichment such as a red translucent house, a wheel and a tunnel.

Prior to surgery, male Wistar rats (200-300g) were acclimatised to the facility for one week. They were then dosed with pre-operative analgesia (0.01mg/kg Buprenorphine) and anaesthetised with isoflurane. The left flank was then shaved, the animals dosed with pre-operative carprofen (5mg/kg) and the animals transferred to the surgery suite. The animals were placed on an isoflurane nose-cone, covered with a sterile drape, and eye ointment was applied to prevent drying. The surgical site was cleaned and disinfected, and an incision made through the skin, and then blunt dissection was used to part the muscle later. The left kidney was decapsulated and the renal blood vessels clamped. The poles of the kidney were ligated using 3-0 mersilk sutures, and then excised using a scalpel. 3M tissue glue was applied to the cut ends. The clamp was then removed, and the kidney replaced into the abdomen. The muscle was sutured using 5-0 Vicryl in a continuous pattern, and the skin was sutured using 5-0 Vicryl in a sub-cuticular continuous pattern. Local anaesthetic (marcain (0.5mg/kg) was applied to the surgical site. Animals were allowed to recover on a warming rack and monitored for welfare. Animals received post-operative analgesia for 4 days.

One week later, the animals were prepared for surgery as above, and the right kidney decapsulated and clamped. The right renal blood vessels were ligated using 3-0 mersilk, and the entire right kidney removed. Animals were sutured as above, and allowed to recover as above, with post-operative analgesia for 4 days.

Animals were tail-pricked or bled (30 µL) for twice-weekly serum creatinine measurements and were placed in metabolic cages bi-weekly for proteinuria measurements. The animals were allocated to a study group in a random manner using a random number generator once they reached the threshold of 2-fold increase in serum creatinine and 3-5x fold increase in proteinuria compared to non-operated sham control. Animals were anaesthetised, bled by cardiac puncture, terminated by cervical dislocation, death confirmed, and the kidney remnant removed, cut into quarters, and either fixed in formalin or snap frozen in liquid nitrogen.

Analysis was performed in a blinded manner, with the analyst and the interpreting researcher unaware of which animals were sham or subtotal nephrectomy-operated.

UUO mouse model generation

UUO surgery was performed on male C57Bl6/J mice of between 20-30g body weight.

Prior to surgery, all animals are dosed with 100 µL pre-operative Buprenorphine (0.05mg/kg Mice / 0.01mg/kg Rats). Mice are anaesthetised with isoflurane and a small patch of skin shaved just below the ribs on the left flank. Mice are then dosed with pre-operative carprofen (5mg/kg). The mouse is laid on the operating table on a sterile drape under isoflurane anaesthesia. The surgical site is disinfected with chlorhexidine and the animal covered by a sterile drape. A small incision is made below the ribcage, parallel to the spine, the muscle is exposed by blunt dissection and a small incision made. The opening in the muscle is widened using blunt dissection. The ureter is located and ligated using 2x 3-0 Mersilk (Ethicon). The ureter is replaced, and the muscle sutured using 5-0 Vicryl (Ethicon). The skin is then sutured in a sub-cuticular pattern with 5-0 Vicryl (Ethicon). The wound is cleaned with chlorhexidine and levobupivacaine administered to the site (0.5mg/kg). Surgical duration is approximately 15-20 minutes, and animals are monitored throughout for surgical anaesthetic plane. Animals were returned to their home cage and received post-operative analgesia for 4 days (Buprenorphine and Carprofen at the doses above). All mice were housed under specific pathogen-free conditions and maintained on a 12- hour light/dark cycle with free access to food and water.

Animals were monitored daily for welfare scoring and body weight. At Day 21 animals were terminated by a schedule 1 method and tissues harvested.

Adriamycin mice model generation:

The Adriamycin model was performed on male BalbC mice of between 20-30g body weight.

A dose of 10.5mg/kg Adriamycin was administered via the tail vein of unanaesthetised mice. Animals were returned to their home cage and monitored daily for welfare scoring and body weight. Urine was collected prior to termination using a metabolic cage. Termination was performed by a schedule 1 method at day 14 and tissues collected. All mice were housed under specific pathogen-free conditions and maintained on a 12- hour light/dark cycle with free access to food and water. 24-hour urine samples were collected prior to injection of Adriamycin and termination, so there were 2 urine collections per animal.

Collagen Picrosirius Red (PSR) Staining

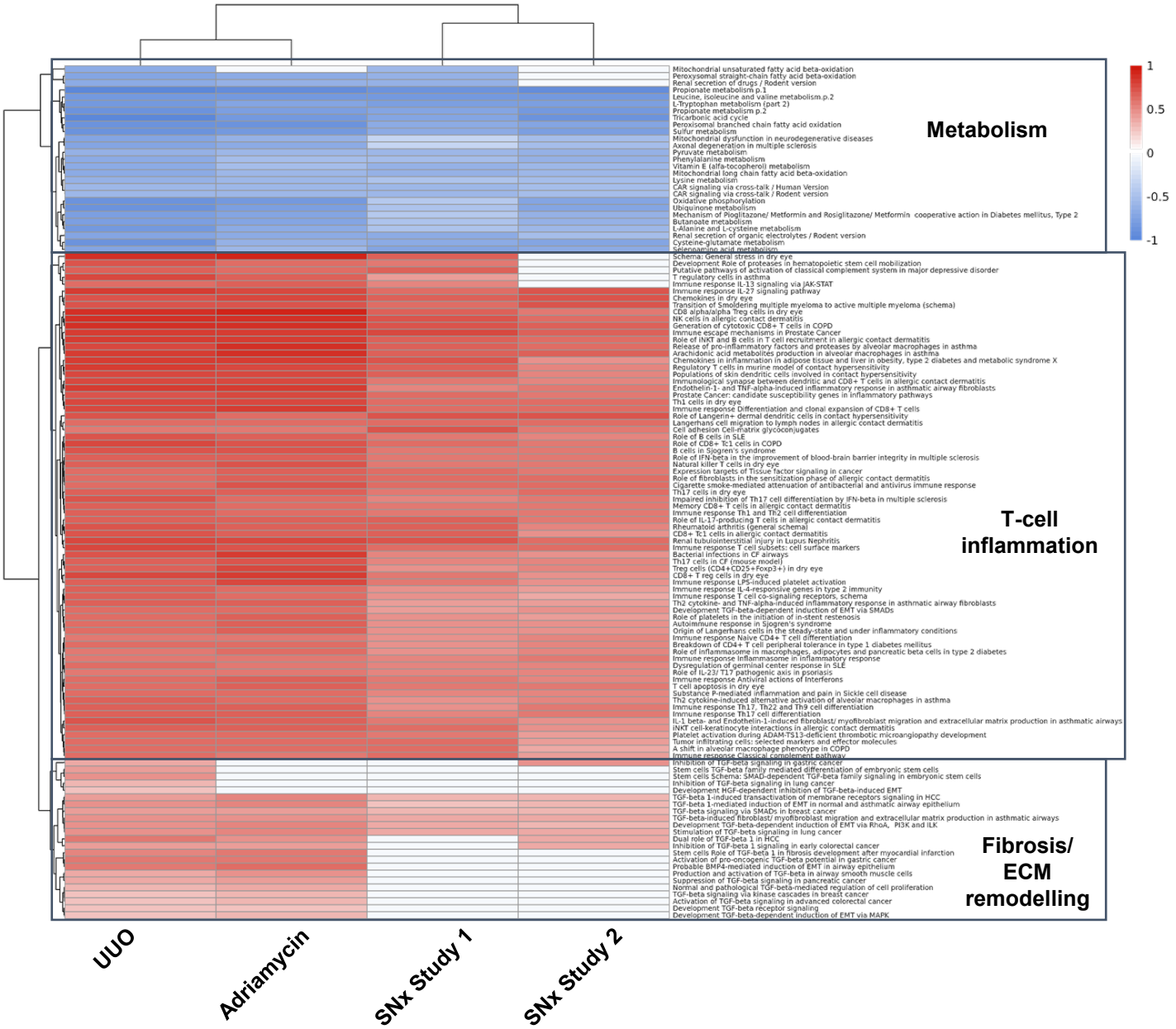
Kidney samples were harvested and fixed in 10% formalin for 24 hours. The samples were coded for blinding and placed in a labelled cassette, which entails further formalin fixation, dehydration in increasing concentrations of alcohol, clearing in xylene and impregnation with paraffin wax. After this, samples were orientated in paraffin wax with the cassette placed on top of the embedding moulds. The kidney samples were sectioned at 3 µm using a Leica Histocore multicut. These sections were floated onto a warm water bath and were placed onto labelled Superfrost Plus Gold slides for staining. The slides were de-waxed and hydrated.

Nuclei were stained with Weigert's haematoxylin for 8 minutes, and then the slides washed for 10 minutes in running tap water. The slides were stained in picro-sirius red solution for one hour, and then washed in two changes of acidified water. Water was removed, and the slides dehydrated in 100% alcohol. The slides were then cleared with xylene, and coverslips applied. Whole slides were then scanned using an OlyVIA slide scanner (Olympus) and given codes for the blinding of the image analysis.

Image Analysis

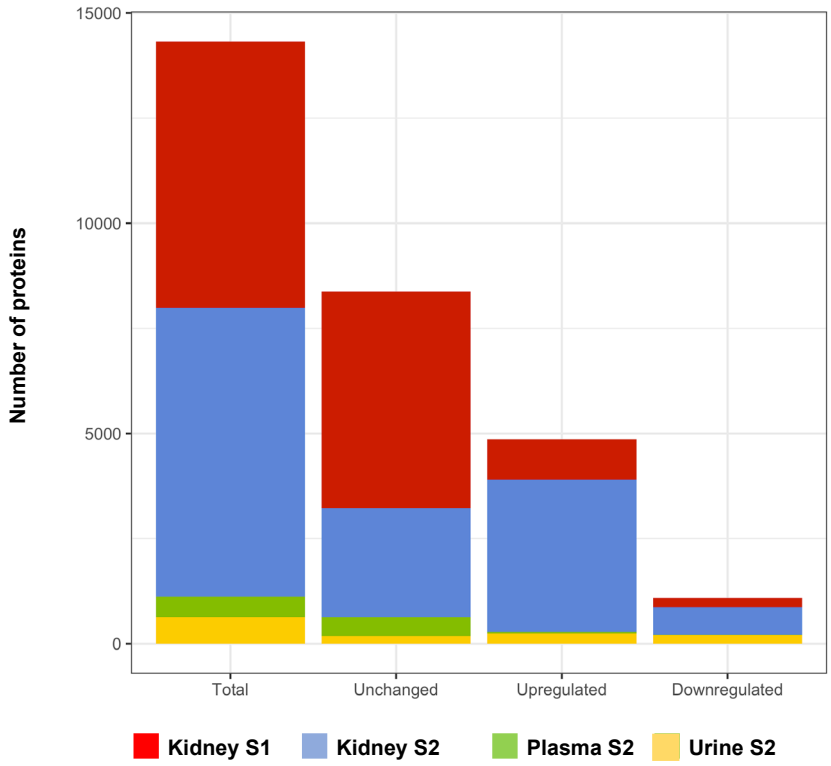
Whole slide scans were uploaded to VisioPharm Image analysis software. In a blinded manner, the cortices of the kidneys were manually annotated, excluding the medulla from analysis. Thresholds were set for detecting red-stained collagen and the software allowed to run this detection over the entire cortex of each kidney. Data were outputted as the percentage area of the renal cortex that stained positive for collagen / PSR staining.

Supplementary Figure 1



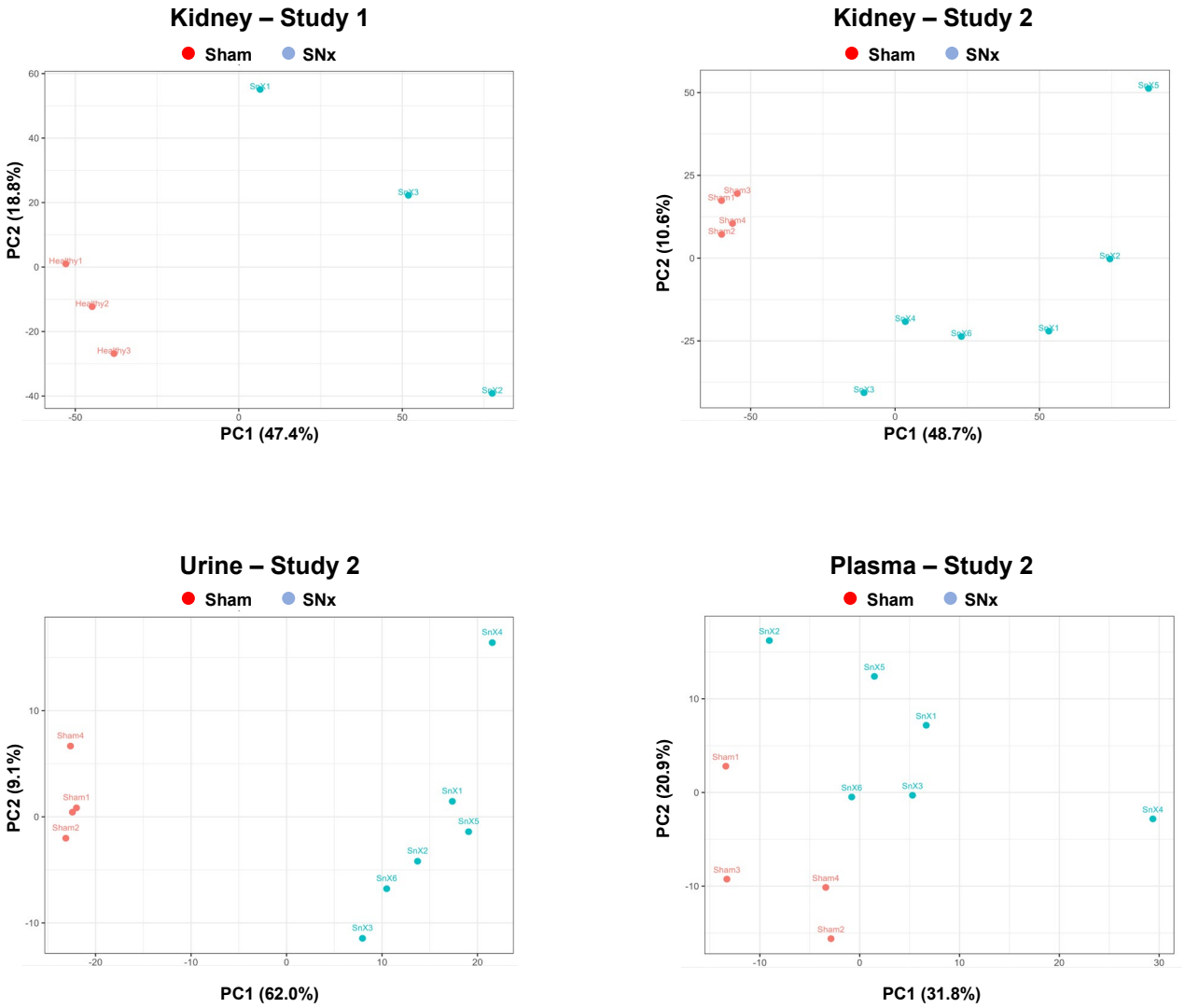
Supplementary figure 1: Comparison of transcriptomics profiles of rat SNx with other CKD models.

Supplementary Figure 2



Supplementary figure 2: Summary of protein counts

Supplementary Figure 3



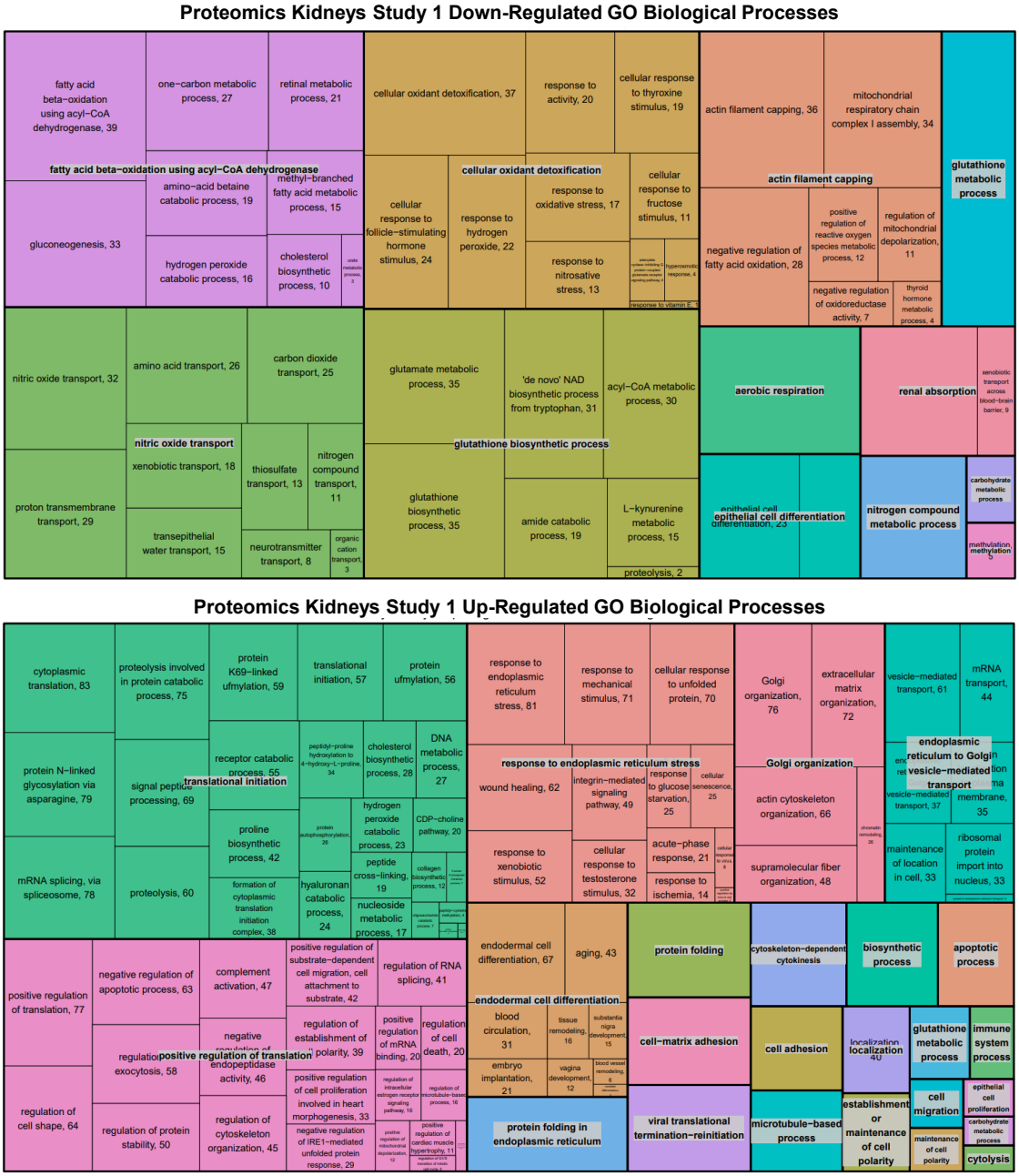
Supplementary Figure 3: PCA analysis of proteomic data.

Supplementary Figure 4

Supplementary figure 4: TreeMaps Biological Processes GO enriched in unsupervised cluster analysis – DAVID – KEGG of proteomic data

Supplementary Figure 4a

a

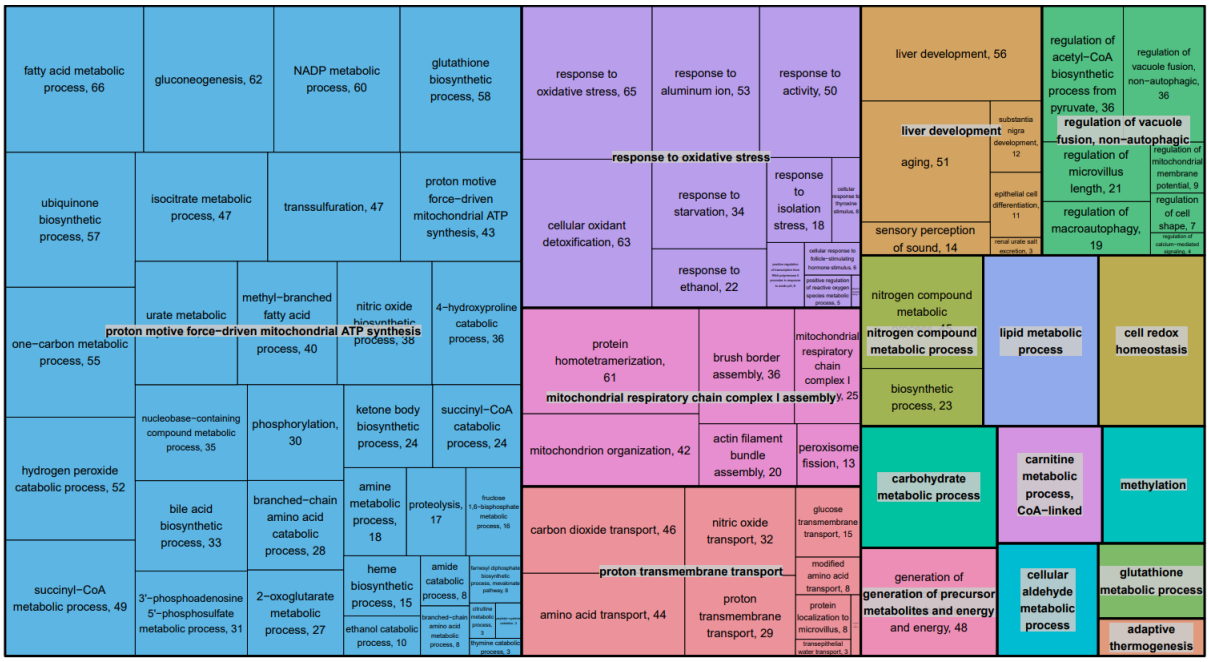


Supplementary figure 4a: TreeMaps of down- and up-regulated proteins enriched in Kidney Study 1

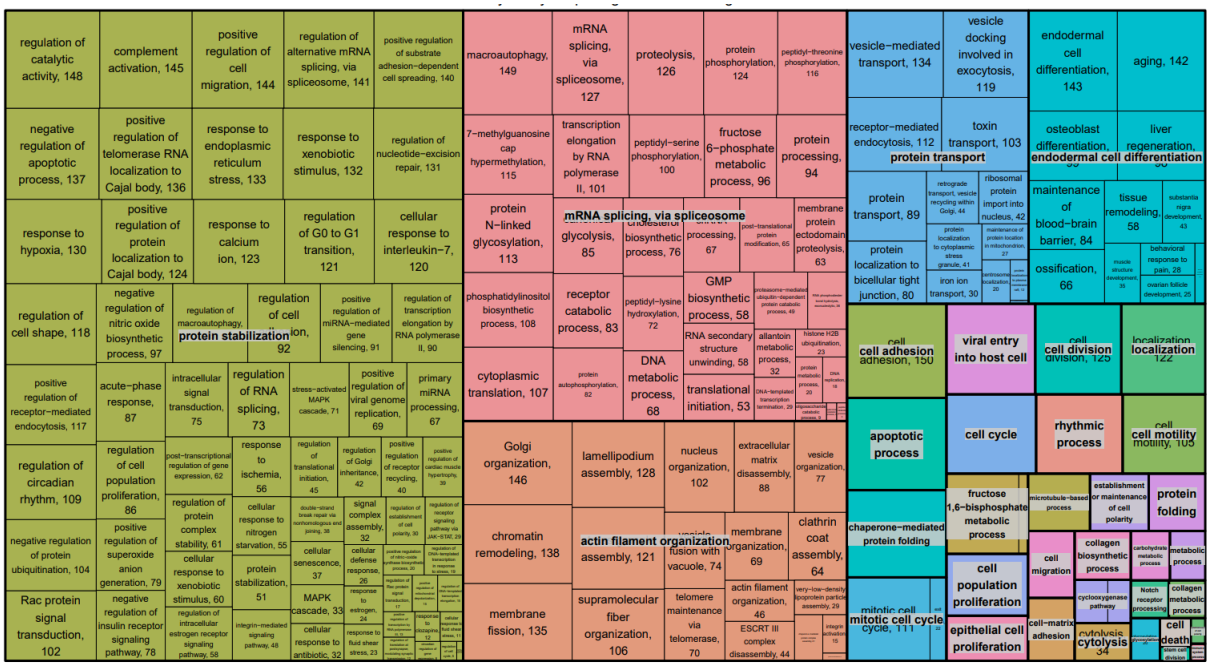
Supplementary Figure 4b

b

Proteomics Kidneys Study 2 Down-Regulated GO Biological Processes



Proteomics Kidneys Study 2 Up-Regulated GO Biological Processes

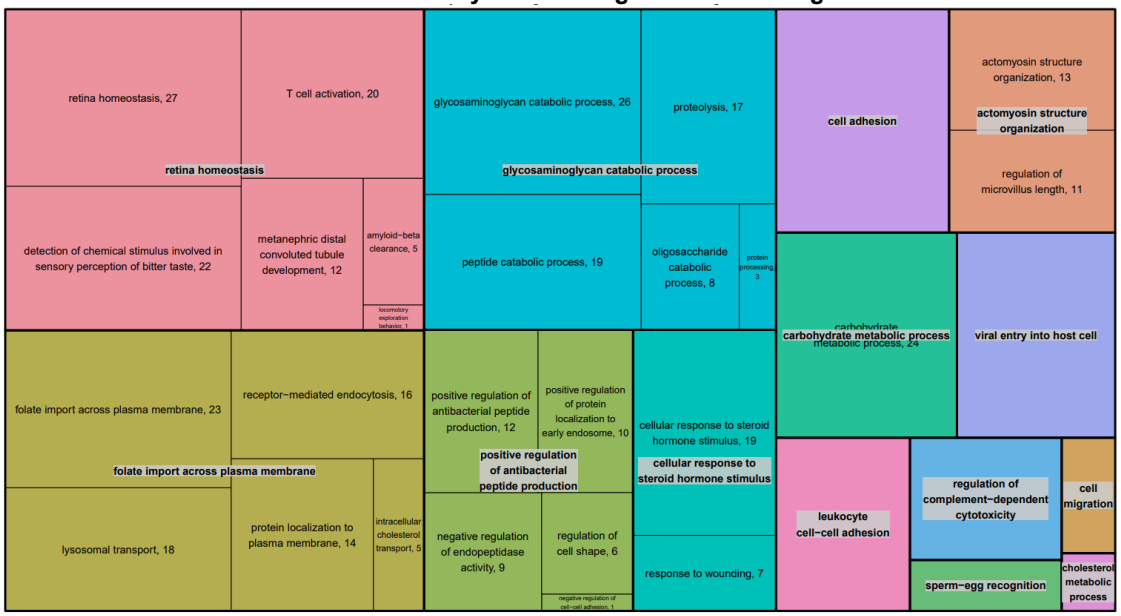


Supplementary figure 4b: TreeMaps of down- and up-regulated proteins enriched in Kidney Study 2

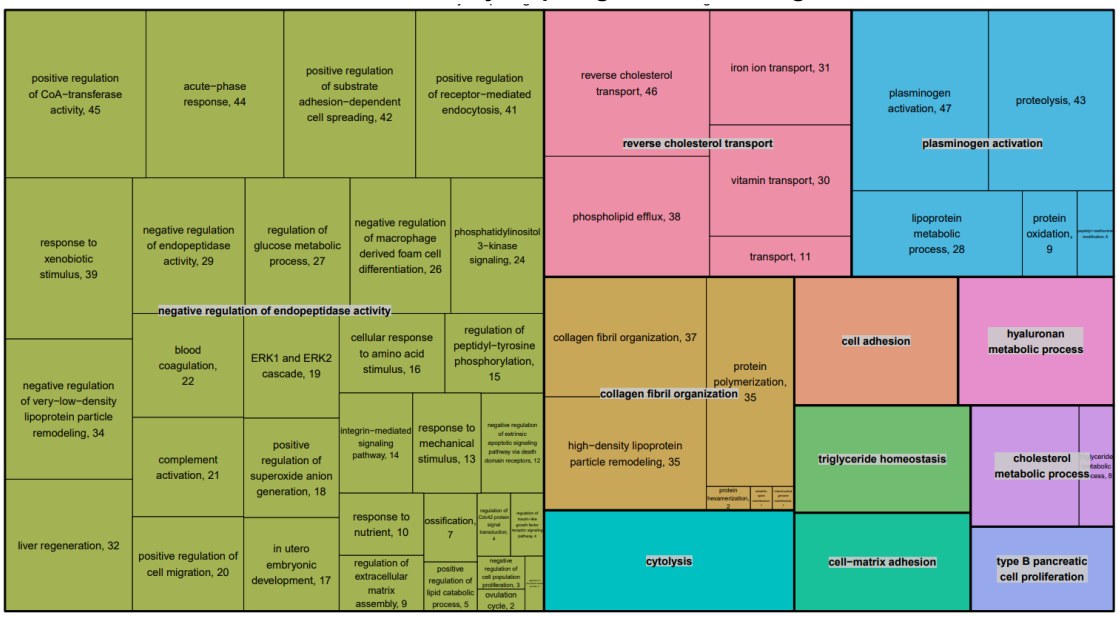
Supplementary Figure 4c

c

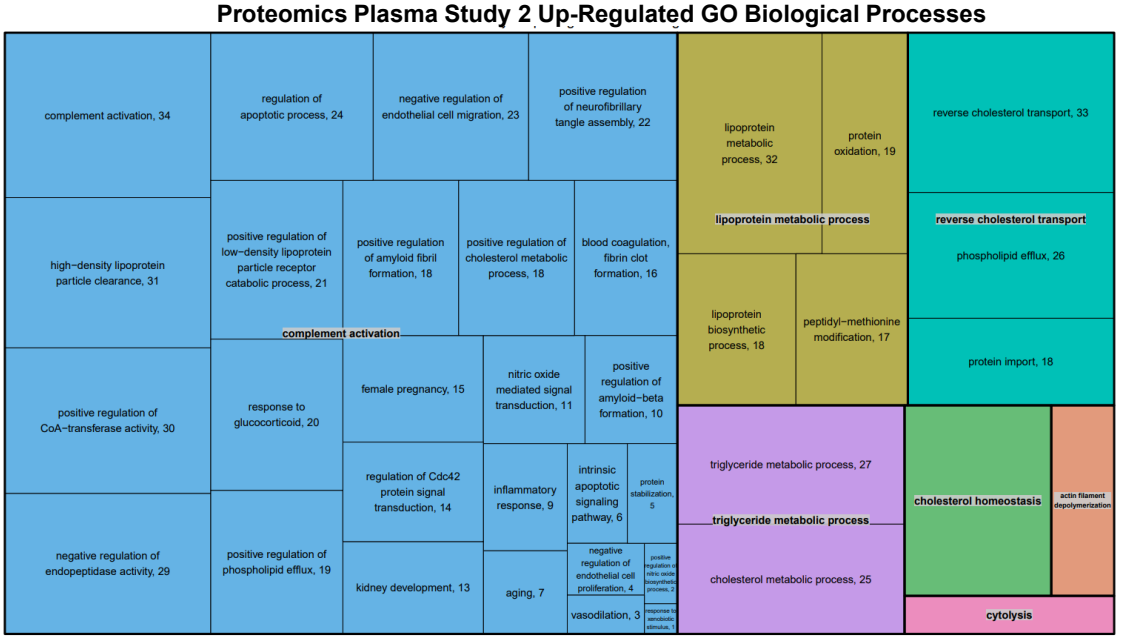
Proteomics Urine Study 2 Down-Regulated GO Biological Processes



Proteomics Urine Study 2 Up-Regulated GO Biological Processes

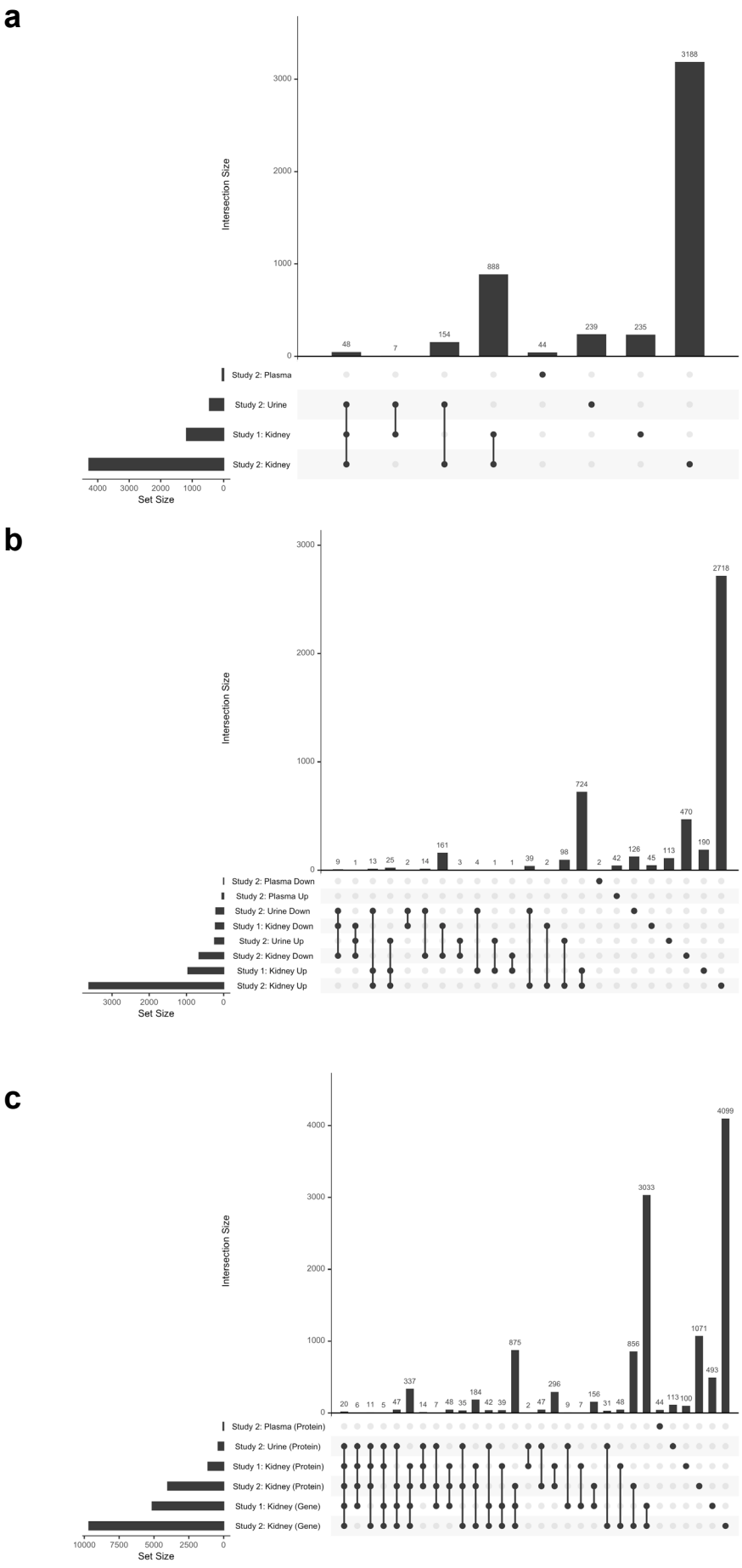


Proteomics Plasma Study 2 Up-Regulated GO Biological Processes



Supplementary figure 4c: TreeMaps of down- and up-regulated proteins in Study 2 Plasma and Urine

Supplementary Figure 5



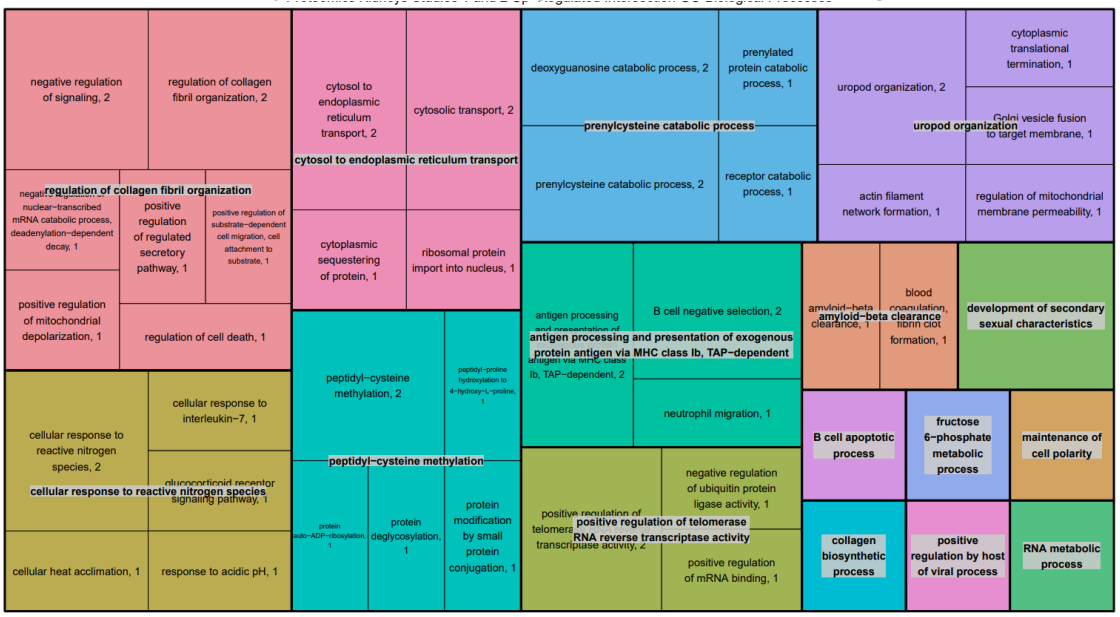
Supplementary figure 5: Upset plots comparing different tissues or analyses. a) Proteomics intersections in Kidneys, Plasma and Urine (total numbers). **b)** Proteomics intersections in Kidneys, Plasma and Urine (significantly changed proteins). **c)** Proteomics and transcriptomics intersections in Kidneys, Plasma and Urine (total numbers).

Supplementary Figure 6

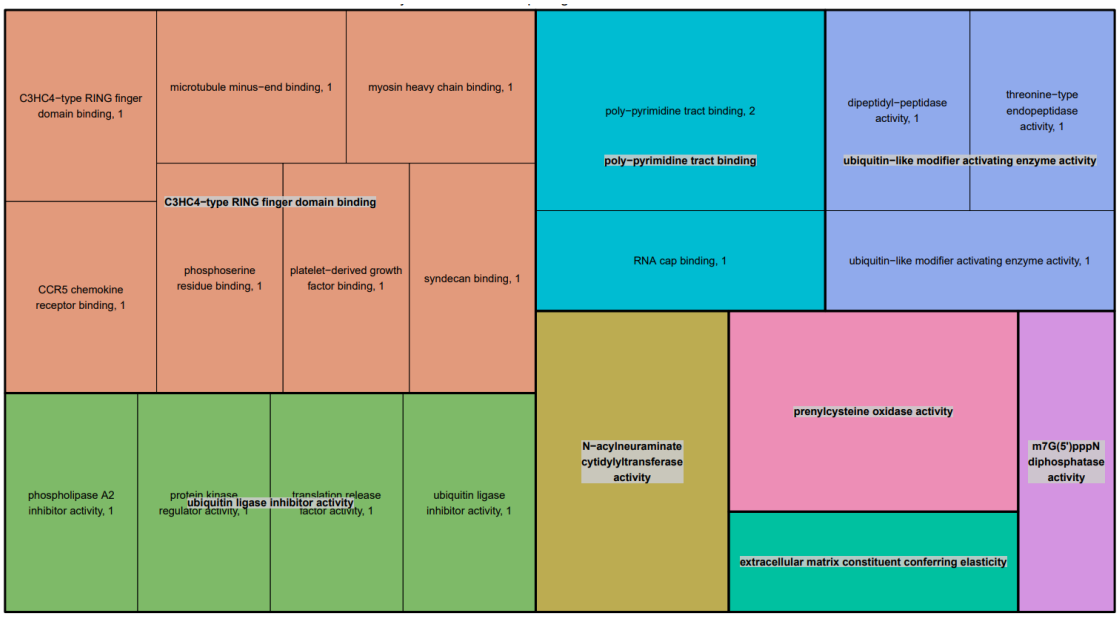
Supplementary Figure 6: TreeMaps GO (Biological processes, Molecular Function and Cellular Component) enriched in unsupervised cluster analysis – DAVID in proteomic and transcriptomic datasets. a) GO enriched in up-regulated proteins in both Kidney Studies 1 and 2. **b)** GO enriched in down-regulated proteins in both kidney Studies 1 and 2. **c)** GO enriched in up-regulated proteins in both Study 2 kidney and urine. **d)** GO enriched in down-regulated proteins in both Study 2 kidney and urine. **e)** GO enriched in down-regulated proteins and genes in both Study 1 kidney proteomic and transcriptomics datasets. **f)** GO enriched in down-regulated proteins and genes in both Study 2 kidney proteomic and transcriptomics datasets. **g)** GO enriched in down-regulated proteins and genes in both Studies 1 and 2 kidney proteomic and transcriptomics datasets.

a

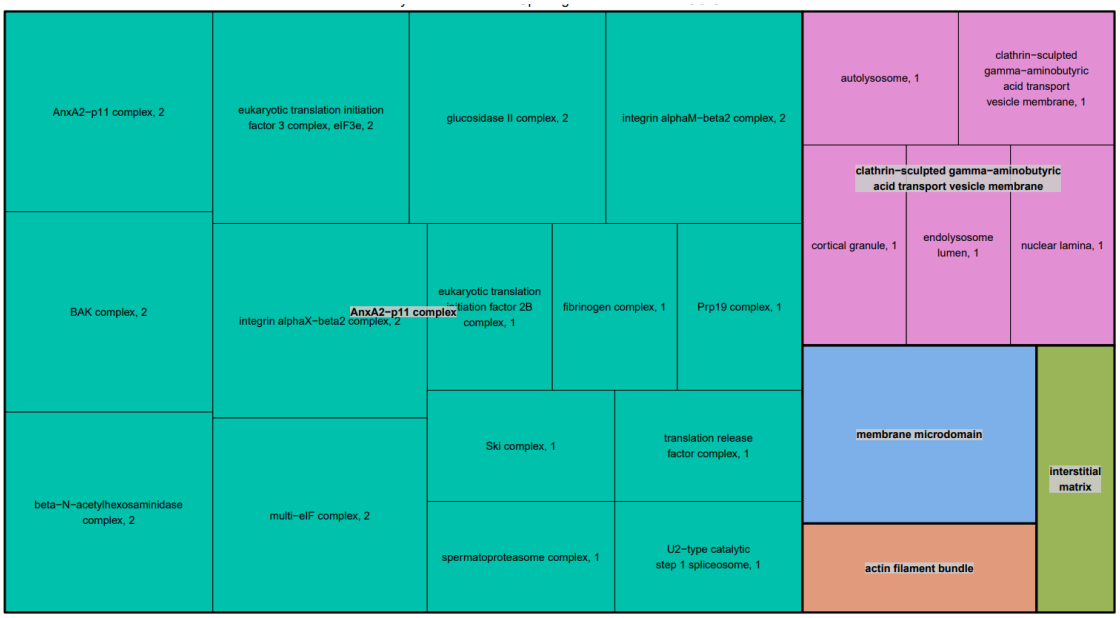
Proteomics Kidneys Studies 1 & 2 Up-Regulated Intersection GO Biological Processes



Proteomics Kidneys Studies 1 & 2 Up-Regulated Intersection GO Molecular Function

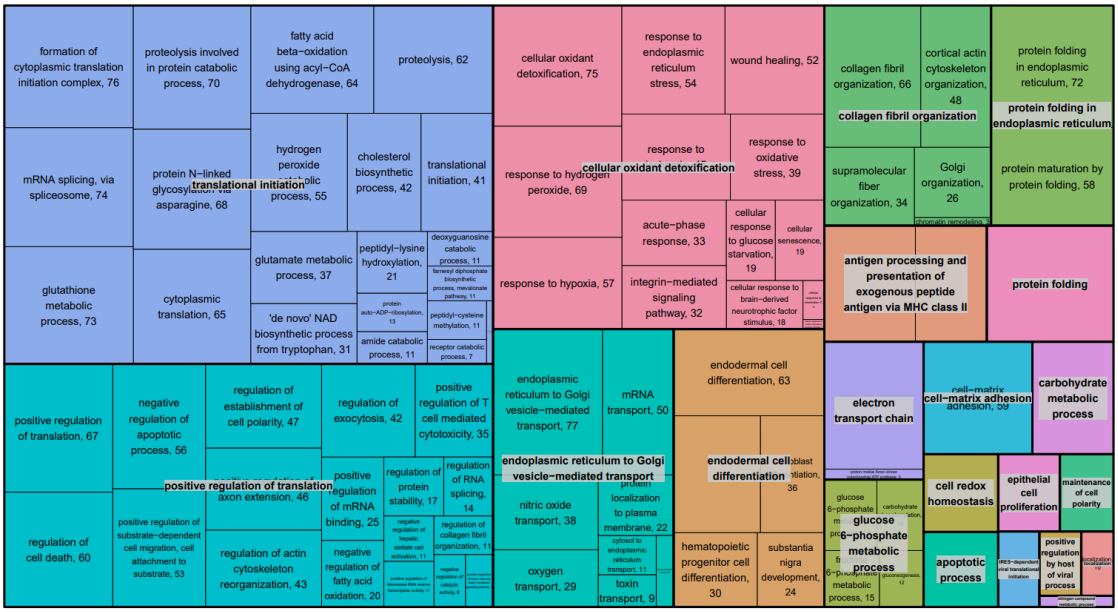


Proteomics Kidneys Studies 1 & 2 Up-Regulated Intersection GO Cellular Component



b

Proteomics Kidneys Studies 1 & 2 Down-Regulated Intersection GO Biological Processes



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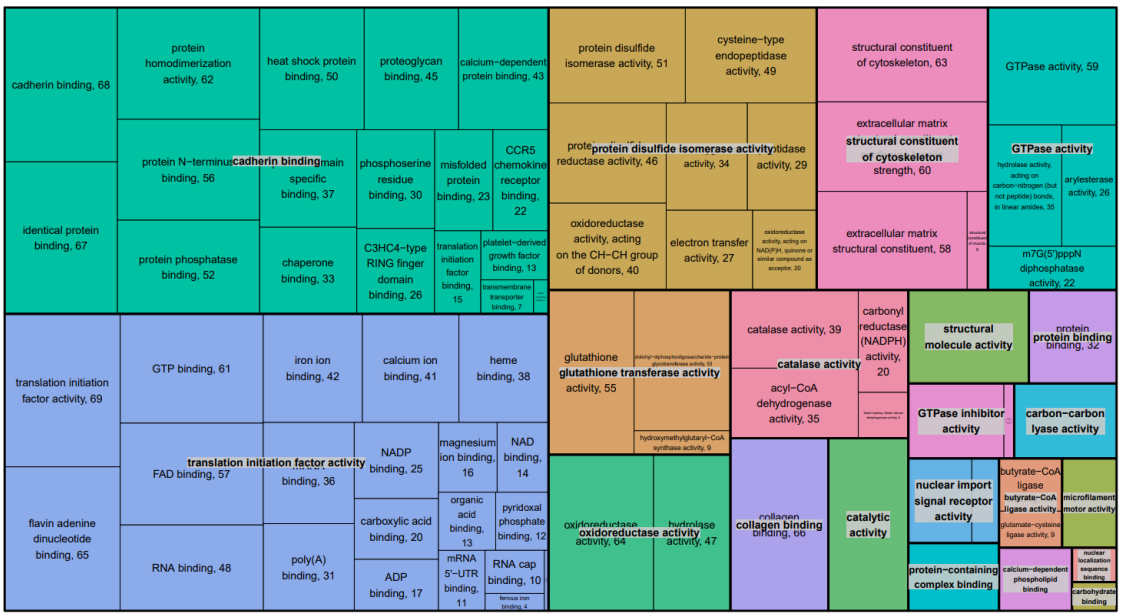

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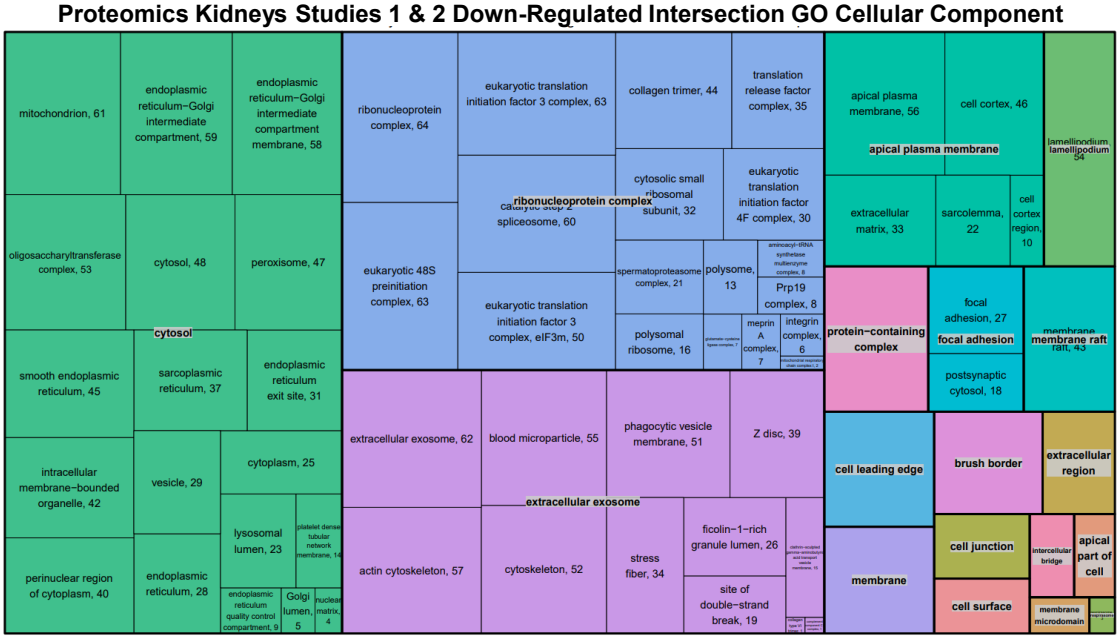

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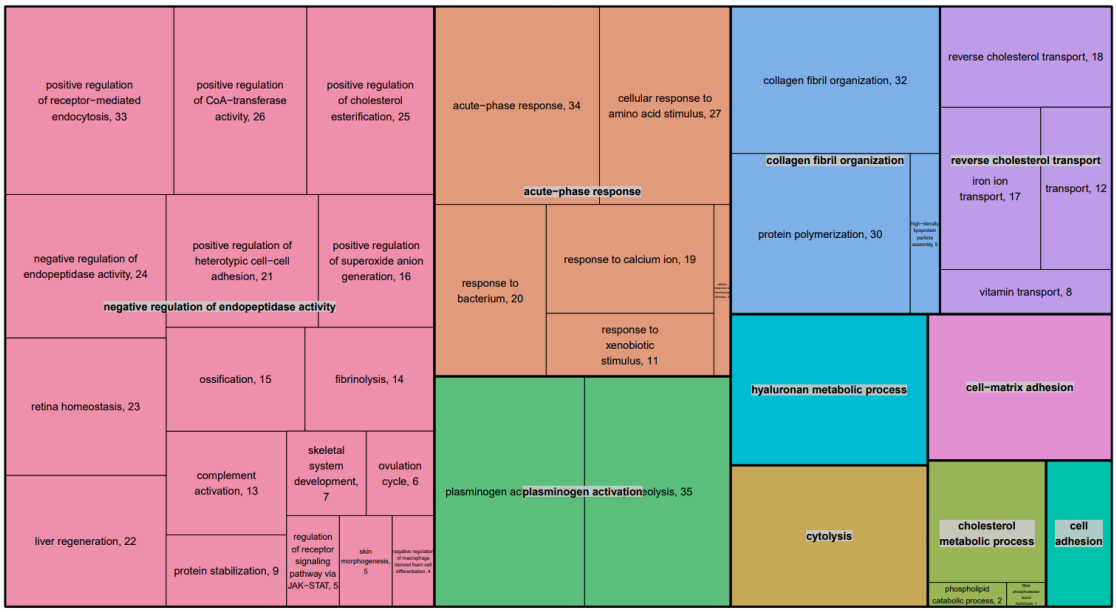
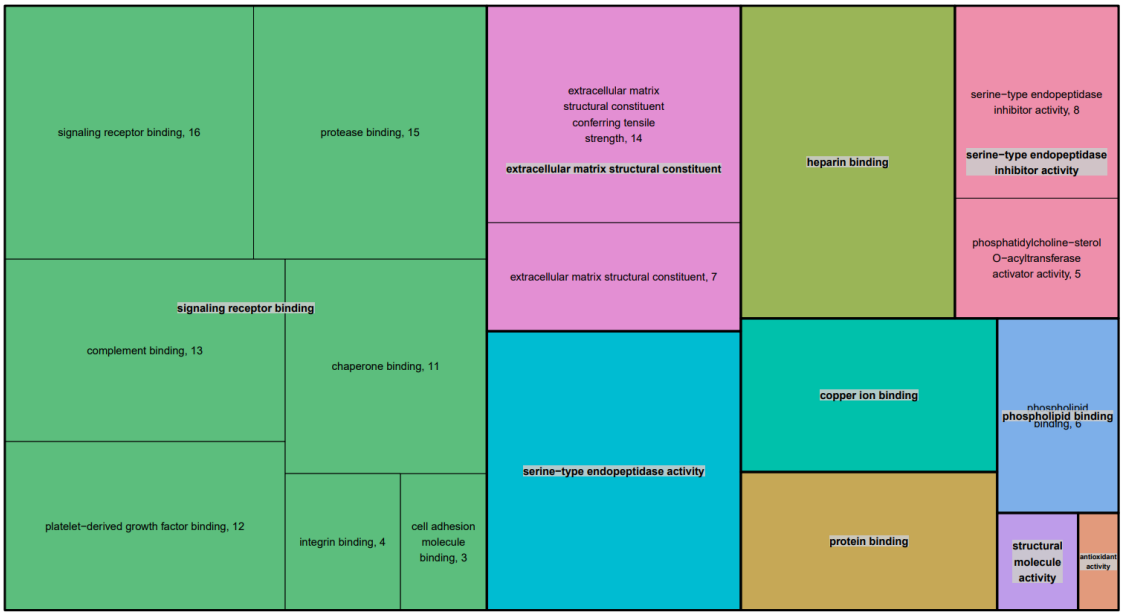
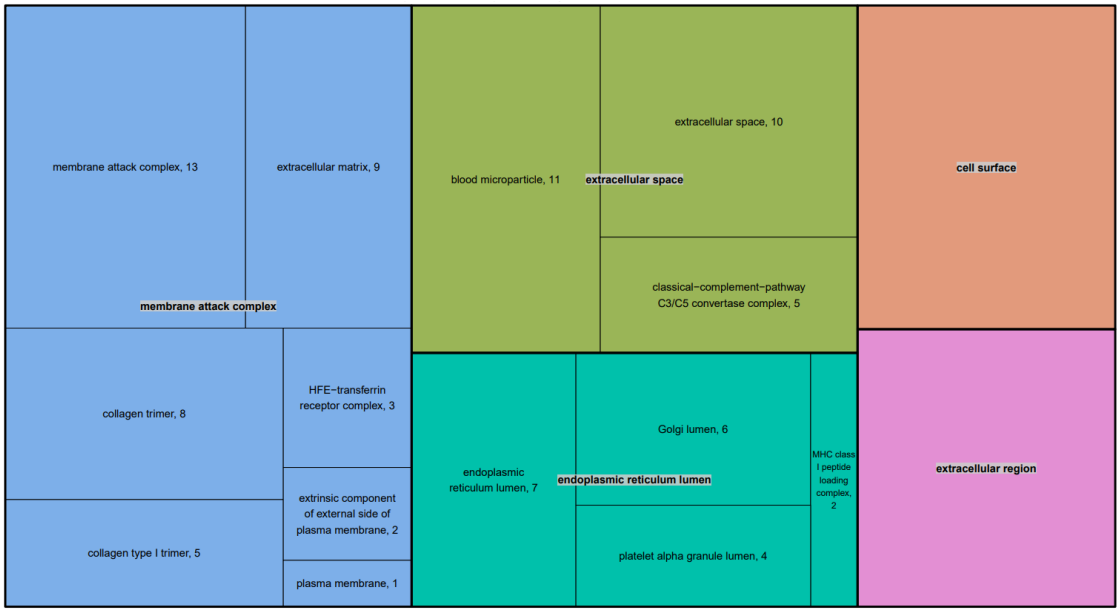
Proteomics Kidney & Urine Study 2 Up-Regulated Intersection GO Biological Processes



Proteomics Kidney & Urine Study 2 Up-Regulated Intersection GO Molecular Function



Proteomics Kidney & Urine Study 2 Up-Regulated Intersection GO Cellular Component



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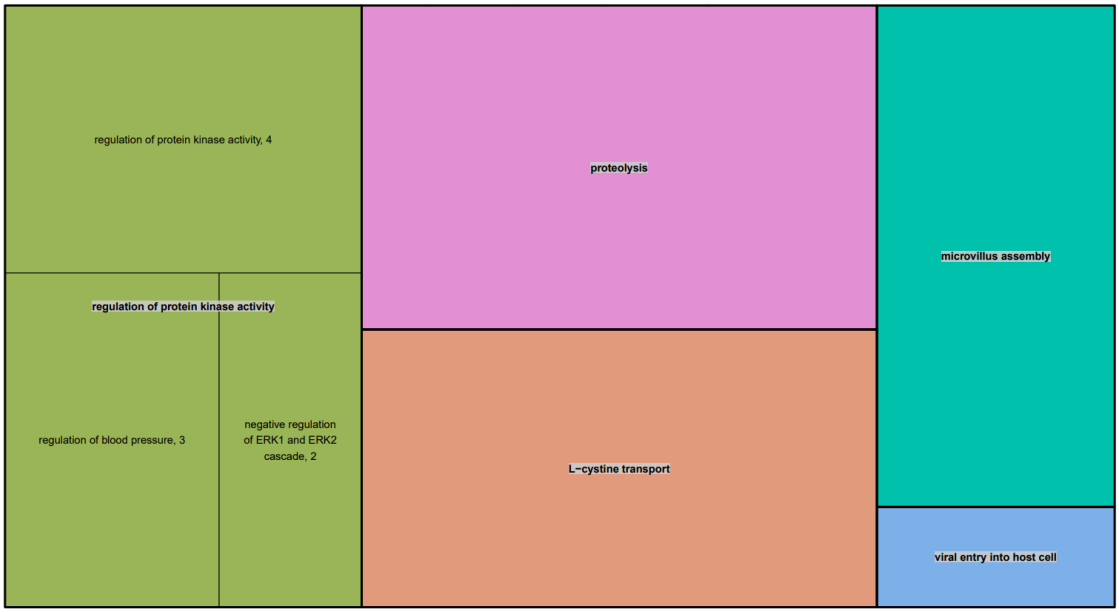
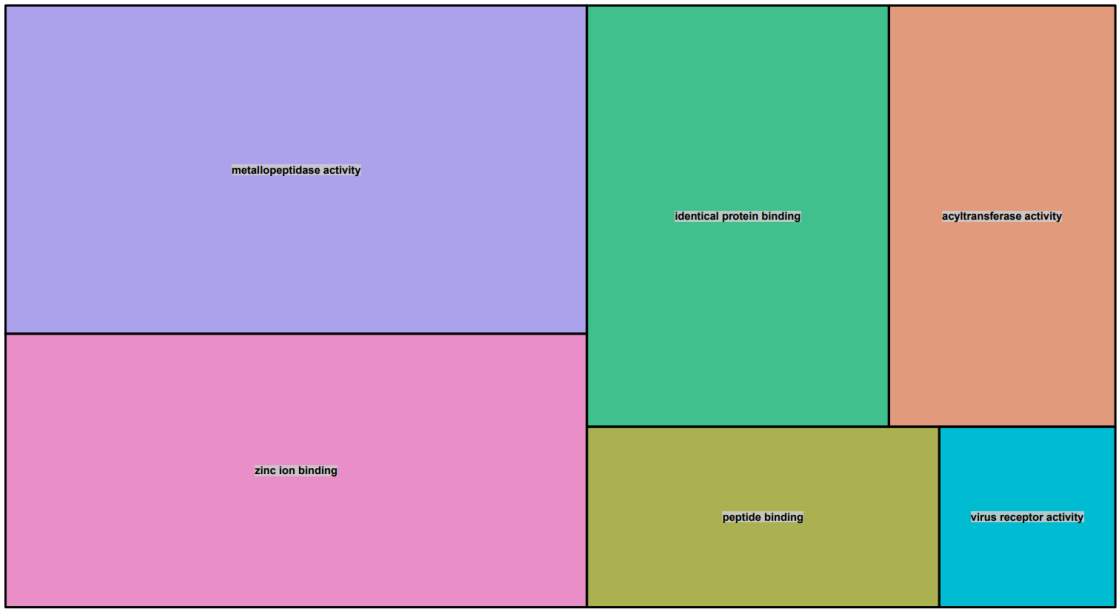
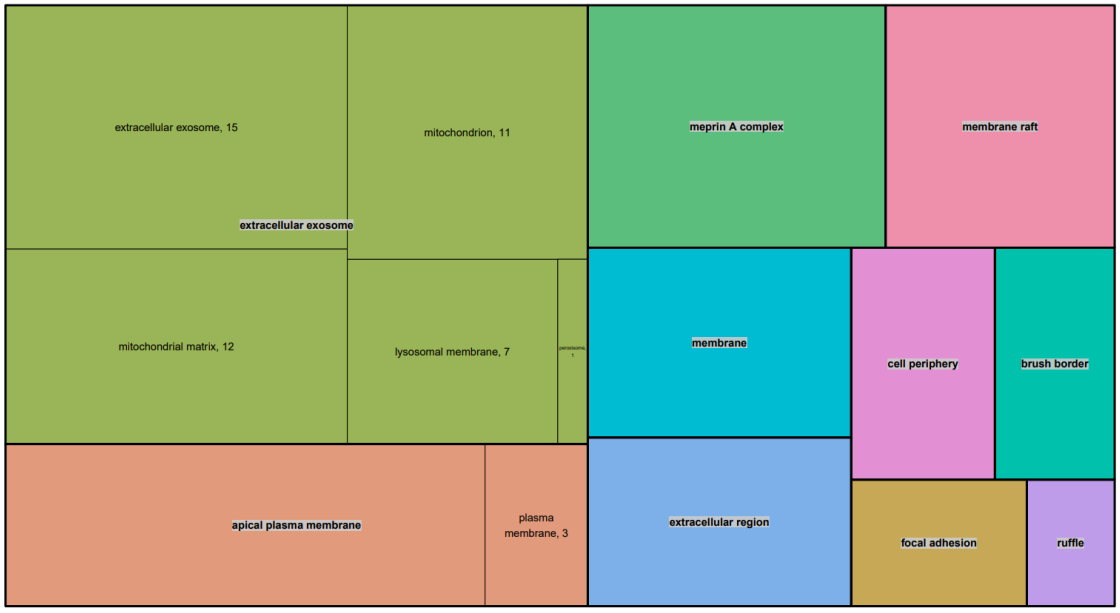
Proteomics Kidney & Urine Study 2 Down-Regulated Intersection GO Biological Processes



Proteomics Kidney & Urine Study 2 Down-Regulated Intersection GO Molecular Function



Proteomics Kidney & Urine Study 2 Down-Regulated Intersection GO Cellular Component



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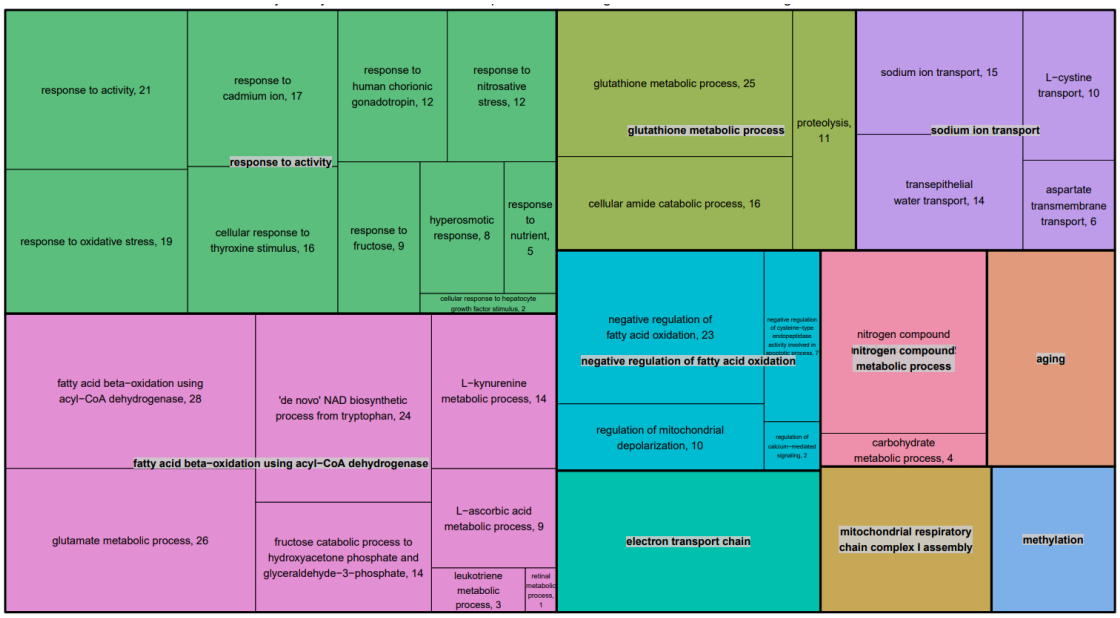
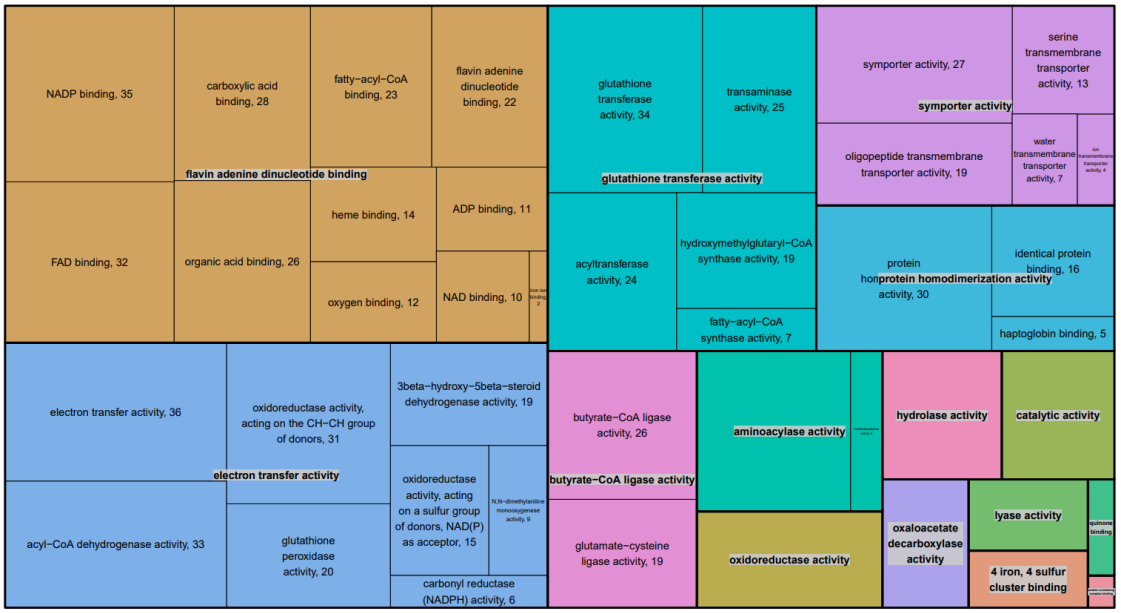
Kidney Study 1 Proteomics and Transcriptomics Down-Regulated Intersection GO Biological Processes



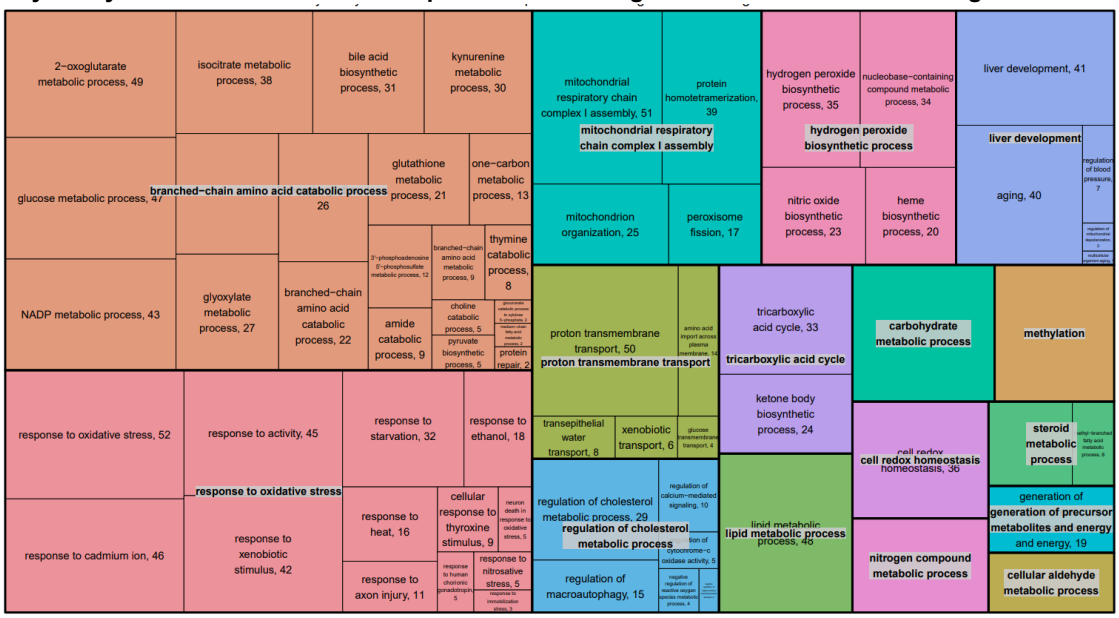
Kidney Study 1 Proteomics and Transcriptomics Down-Regulated Intersection GO Molecular Function



Kidney Study 1 Proteomics and Transcriptomics Down-Regulated Intersection GO Cellular Component

f

Kidney Study 2 Proteomics and Transcriptomics Down-Regulated Intersection GO Biological Processes



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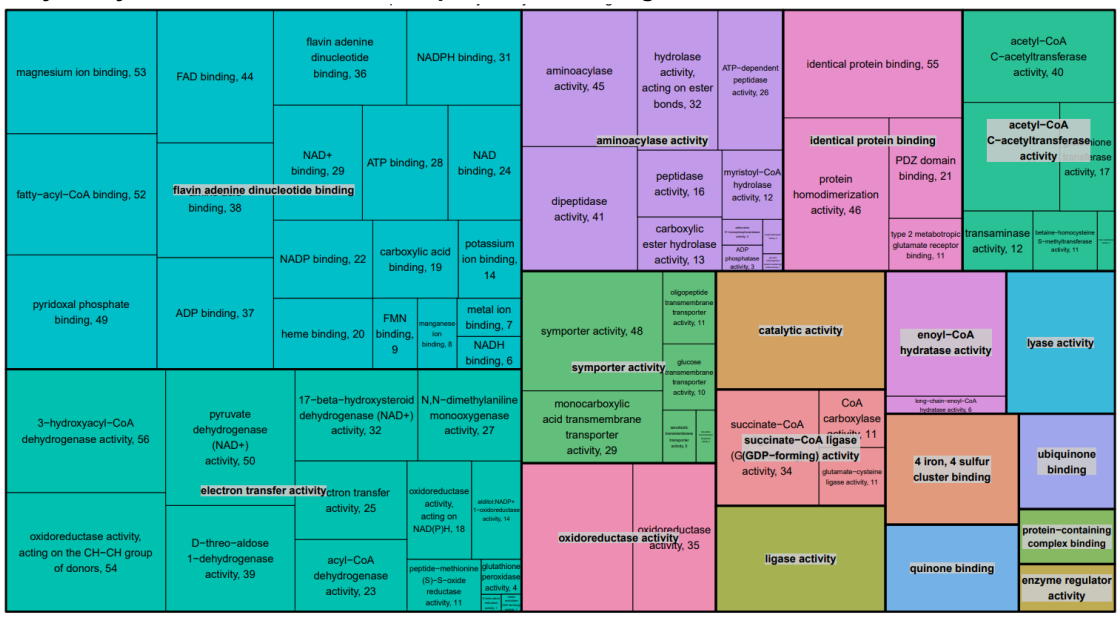
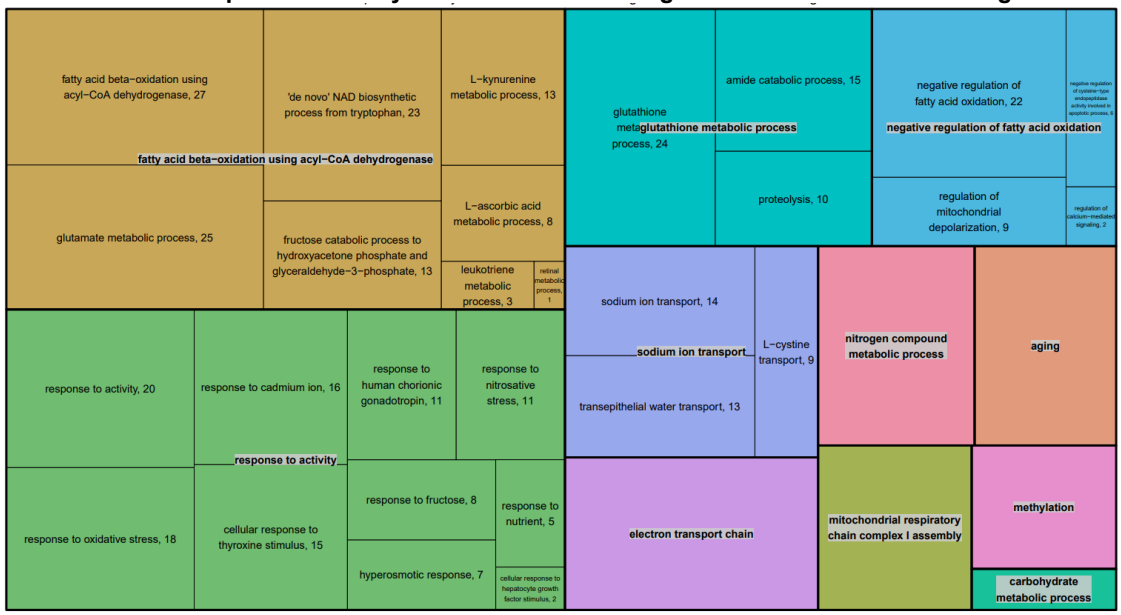


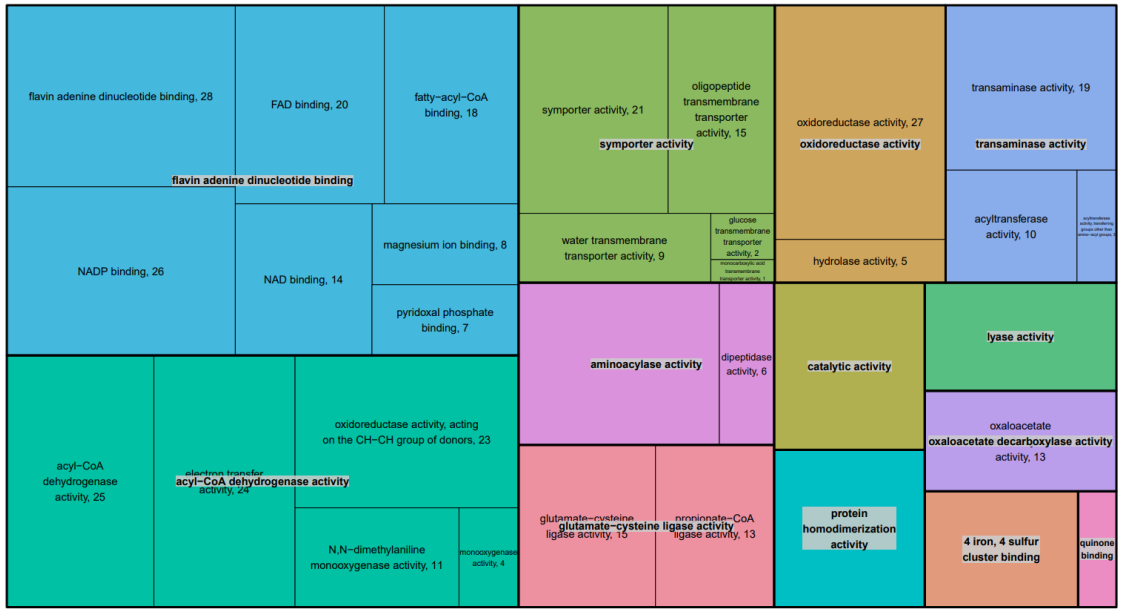
Kidney Study 2 Proteomics and Transcriptomics Down-Regulated Intersection GO Cellular Component

g

Proteomics and Transcriptomics Kidney Studies 1&2 Down-Regulated Intersection GO Biological Processes



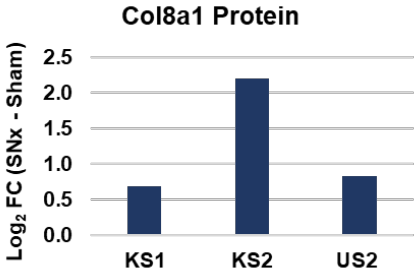
Proteomics and Transcriptomics Kidney Studies 1&2 Down-Regulated Intersection GO Molecular Function



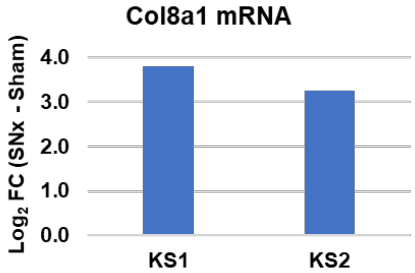
Proteomics and Transcriptomics Kidney Studies 1&2 Down-Regulated Intersection GO Cellular Component

Supplementary Figure 7: Protein and gene expression of proteins in common in all proteomic and transcriptomic datasets. **a)** Additional up-regulated proteins (plasma proteins excluded). **b)** Down-regulated proteins.

a



Tissue	log2FC	P-Value	FDR
KS1	0.68	6.97E-03	4.36E-02
KS2	2.19	7.25E-04	2.85E-03
US2	0.84	3.67E-03	6.68E-03



	log2FC	P-Value	FDR
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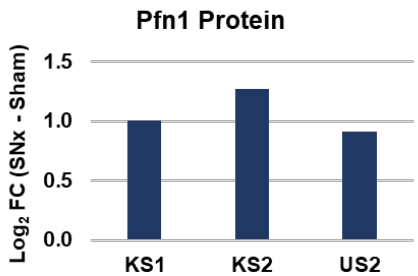

Tissue	log2FC	P-Value	FDR
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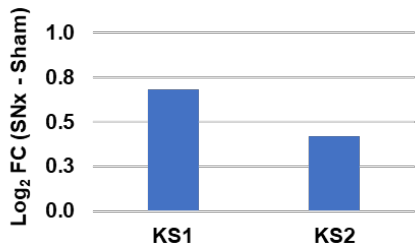
US2	0.91	3.03E-02	4.36E-02



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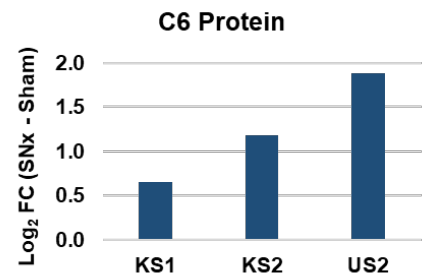

Tissue	log2FC	P-Value	FDR
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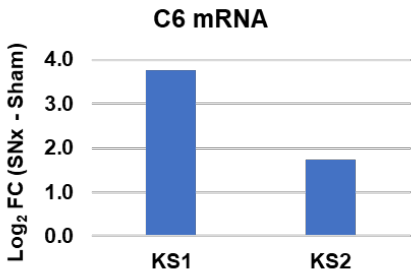
KS2	1.18	4.77E-05	2.88E-02
US2	1.88	2.62E-05	3.17E-03



Tissue	log2FC	P-Value	FDR
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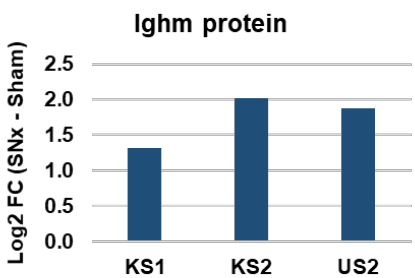
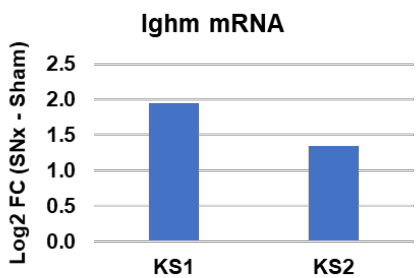


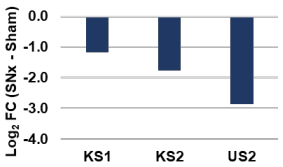
Tissue	log2FC	P-Value	FDR
KS1	1.31	3.28E-04	1.17E-02
KS2	2.03	9.46E-04	3.40E-03
US2	1.88	2.62E-05	3.17E-03



Tissue	log2FC	P-Value	FDR
KS1	1.95	1.68E-03	7.56E-03
KS2	1.35	2.62E-02	3.71E-02

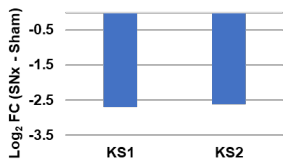
b

Tmlhe Protein



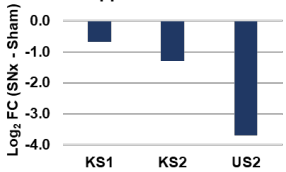
Tissue	log2FC	P-Value	FDR
KS1	-1.17	5.78E-03	4.04E-02
KS2	-1.77	1.57E-02	2.88E-02
US2	-2.86	1.50E-03	3.17E-03

Tmlhe mRNA



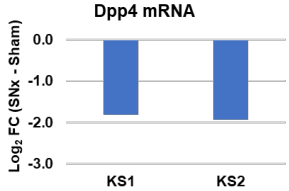
Tissue	log2FC	P-Value	adj.P-Val
KS1	-2.71	2.44E-05	7.32E-04
KS2	-2.62	5.55E-08	1.31E-06

Dpp4 Protein



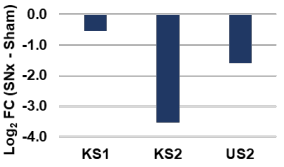
Tissue	log2FC	P-Value	FDR
KS1	-0.70	4.27E-03	3.54E-02
KS2	-1.31	1.96E-02	3.43E-02
US2	-3.70	2.26E-07	1.93E-06

Dpp4 mRNA



Tissue	log2FC	P-Value	adj.P-Val
KS1	-1.81	1.80E-05	6.67E-04
KS2	-1.92	6.07E-09	4.81E-07

Slc7a13 Protein

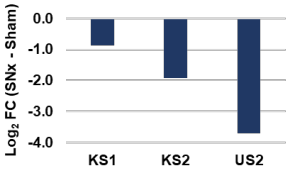


Tissue	log2FC	P-Value	FDR
KS1	-0.56	2.18E-03	2.67E-02
KS2	-3.54	1.83E-06	1.37E-04
US2	-1.60	1.30E-03	2.80E-03

Slc7a13 mRNA

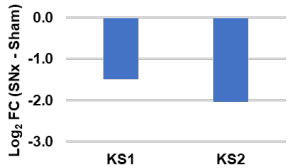
Tissue	log2FC	P-Value	adj.P-Val
KS1	-4.01	1.56E-03	7.19E-03
KS2	-5.75	5.35E-06	2.45E-05

Abhd14b Protein



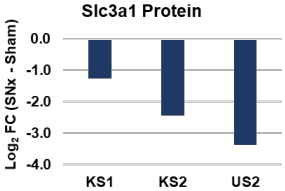
Tissue	log2FC	P-Value	FDR
KS1	-0.86	2.60E-03	2.85E-02
KS2	-1.93	1.47E-02	2.72E-02
US2	-3.69	3.97E-04	1.04E-03

Abhd14b mRNA



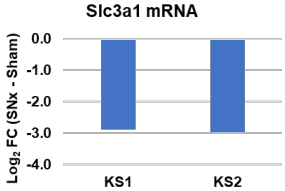
Tissue	log2FC	P-Value	adj.P-Val
KS1	-1.49	1.21E-03	5.99E-03
KS2	-2.05	6.50E-07	5.54E-06

Slc3a1 Protein



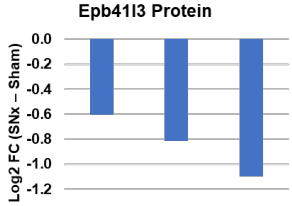
Tissue	log2FC	P-Value	FDR
KS1	-1.25	1.99E-03	2.55E-02
KS2	-2.44	9.81E-03	1.99E-02
US2	-3.36	1.64E-05	7.19E-05

Slc3a1 mRNA



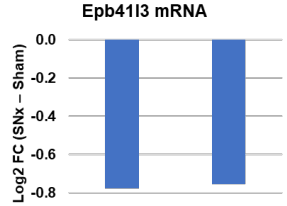
Tissue	log2FC	P-Value	adj.P-Val
KS1	-2.88	2.12E-04	1.99E-03
KS2	-2.98	4.84E-07	4.67E-06

Epb41l3 Protein



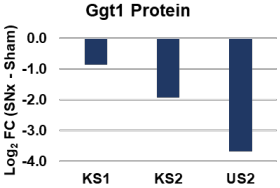
Tissue	log2FC	P-Value	FDR
KS1	-0.61	6.61E-03	4.28E-02
KS2	-0.81	7.79E-05	7.11E-04
US2	-1.10	9.23E-03	1.52E-02

Epb41l3 mRNA



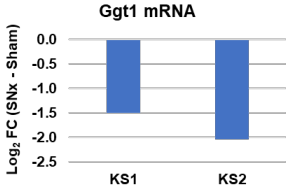
Tissue	log2FC	P-Value	FDR
KS1	-0.78	9.77E-04	5.20E-03
KS2	-0.76	1.01E-02	1.56E-02

Ggt1 Protein



Tissue	log2FC	P-Value	FDR
KS1	-0.86	2.60E-03	2.85E-02
KS2	-1.93	1.47E-02	2.72E-02
US2	-3.69	3.97E-04	1.04E-03

Ggt1 mRNA



Tissue	log2FC	P-Value	adj.P-Val
KS1	-1.49	1.21E-03	5.99E-03
KS2	-2.05	6.50E-07	5.54E-06

Supplementary Figure 8: Next Bio Human Kidney Disease gene expression database repository. Lumican and Col3a1 gene expression in kidneys compared to controls in diseased patients.