Quantitative Proteomics By SWATH-MS reveals an Endosomal Transport hub of proteins which interact with TG2 in a Model of Experimental Kidney Fibrosis

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INTRODUCTION AND AIMS:

Increased synthesis and export of Transglutaminase 2 (TG2) by tubular epithelial cells (TEC) into the surrounding tubulo-interstitium is a significant feature of progressive kidney scarring (*Johnson et al., J Clin Invest, 1997; Johnson et al., J Am Soc Nephro, 2003*). Once outside the cell, TG2 accelerates the deposition of available extracellular matrix (ECM) components and confers ECM-resistance to proteases. Further, TG2 enhances TGF-β1 activation. Clinical development of anti-TG2 therapy is hampered by the lack of TG2 specific inhibitors. However, TG2 has a largely unknown unconventional export mechanism that we believe can be targeted to control extracellular TG2. In this study, to identify the molecular partners of TG2 leading to its export and extracellular function, we report a comprehensive and unbiased analysis of the membrane interactome of TG2 in kidneys subjected to Unilateral Ureteric Obstruction (UUO).

EXPERIMENTAL METHOD:

TG2-null and WT inbred C57BL/6J mice (n=12) were subjected to UUO or a sham operation, and harvested at 21-day post-surgery. UUO kidneys were positive to α-SMA and displayed a significantly higher level of active TGF-β1 (**Fig 2**). To identify TG2-associated proteins, we combined TG2-immunoprecipitation from whole kidney membrane preparations of WT and TG2-null kidneys with quantitative proteomics by DIA/SWATH™-MS (Gillet et al., Mol Cell Proteom, 2012) using AB SCIEX TripleTOF-5600. We performed 5 independent experiments, each based on a lysate of 2 kidneys, and employed only male mice. Differences between WT and TG2-null precipitated proteins (negative control) were established by a paired sample z-test (**Fig 4**). To focus on plasma membrane interactions, ribosomal, nuclear and mitochondrial proteins were subtracted from the list. A workflow is shown in **Fig 1**.

Workflow of the experimental procedure:

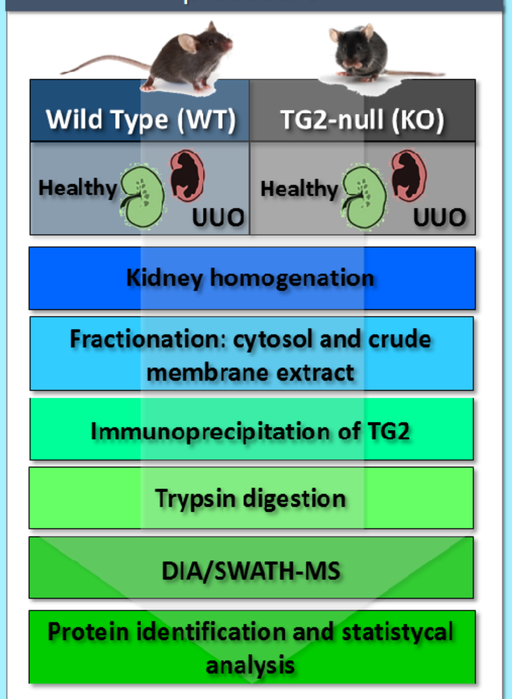


Figure 1: A proteomic approach for the analysis of TG2-associated proteins in healthy and fibrotic kidney using next generation high-resolution mass spectrometry (DIA/SWATH™-MS).

Validation of experimental fibrosis

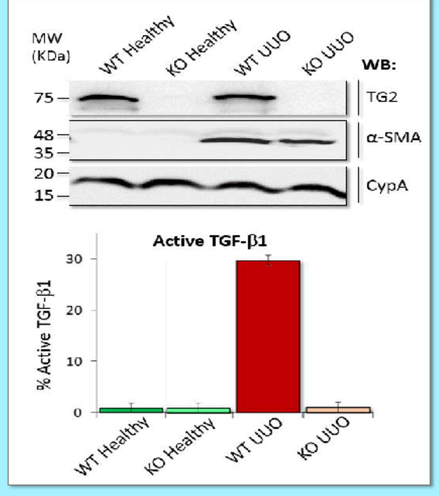


Figure 2: Assessment of organ fibrosis in healthy kidneys and kidneys subjected to UUO - Immunoblot of the total kidney extracts probed for TG2, the fibrosis marker α-Smooth Muscle Actin (α-SMA) and the loading control Cyclophilin A (CypA). Percentage of active TGFβ1 over the total was obtained by Mink Lung Epithelial Cells (MLEC) Luciferase Assay (*Abe et al., Anal Biochem,1994*). Values are the mean average of 3 kidneys +/- SD, each assessed in triplicate.

Validation of fractionation and immunoprecipitation

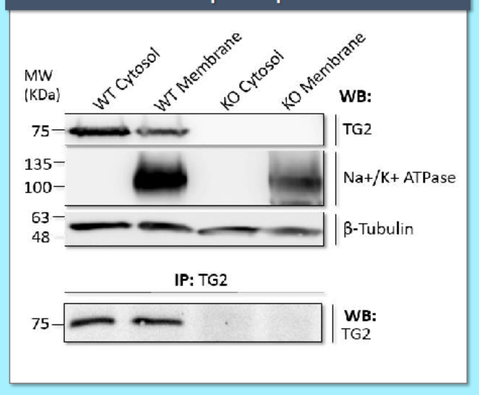


Figure 3: Validation of the fractionation and immunoprecipitation method - Immunoblot of the fractionated kidney extracts from WT and KO mice, probed for TG2, the membrane marker Na+/K+ ATPase and the loading control β-Tubulin. Immunoblot of TG2 immunoprecipitated from the same kidneys, probed for TG2.

Statistical analysis

(1) $Z_i = \frac{X_i - \mu}{\sigma}$

(2) $\Delta Z_i = Z_{WT,i} - Z_{KO,i}$

(3) $Z\ test_i = \frac{\Delta Z_i \ H_0}{SE}$

(4)

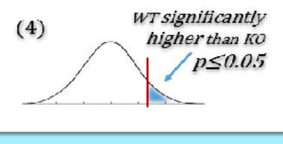
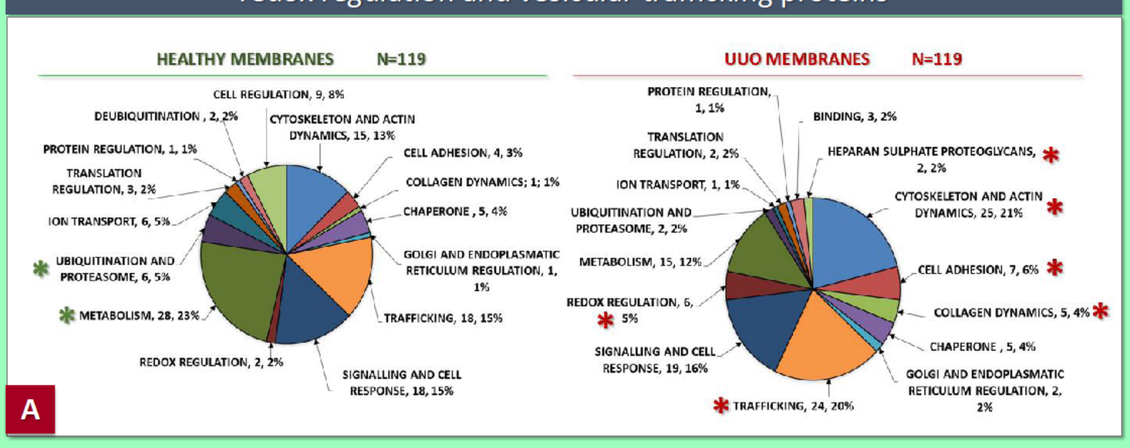


Figure 4: Application of *z*-test on the SWATH-MS output data: Differences between WT and TG2-null precipitated proteins were established by a paired sample *z*-test, according to *Haverland et al. (J Am Chem Soc, 2014).* **(1)** The raw peaks were normalised using a z-score (X_i= *experimental value,* μ = *mean ,* σ = *standard deviation of all values).* **(2)** ΔZ was calculated for each protein/experiment. **(3)** A *z*-test was performed (H_0=*hypotized mean,* $\overline{\Delta Z_i}$= *mean,* SE = *standard error).* **(4)** T test results were plotted on a normal distribution to obtain probability values.

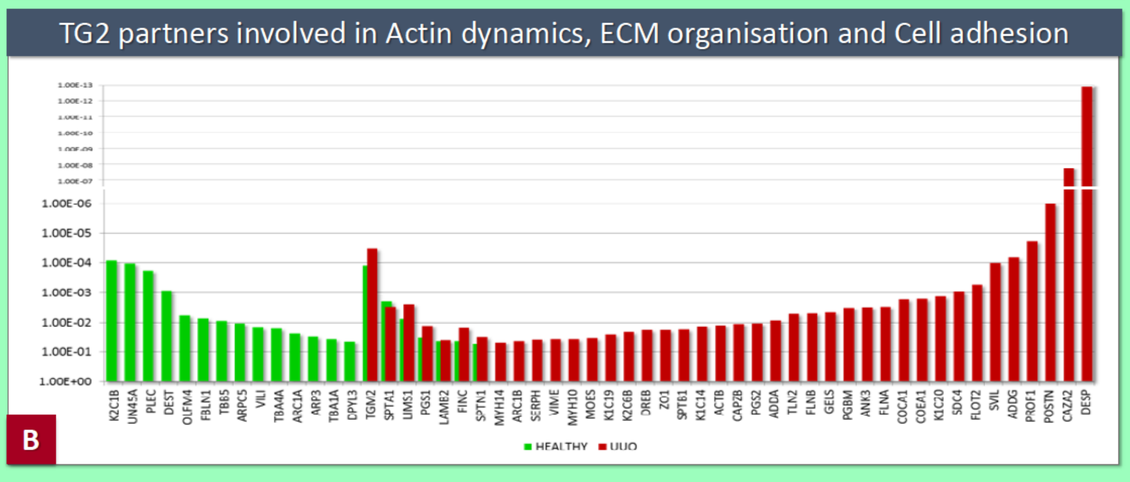
RESULTS:

205 membrane-associated proteins specifically interacting with TG2 were identified (p≤0.05, n≥4), of which 95 were specifically associated with TG2 in the UUO model. Gene Ontology terms overrepresented in TG2 partners (p≤0.05) compared to references proteomes revealed a predominance of cell adhesion, actin dynamics, redox regulation and a number of endosome-related proteins (**Figure 5**). Protein interaction networks were investigated using STRING 9.1 (confidence 0.35). A subnetwork of proteins (clathrin, adaptor protein 2, dynactin and sorting nexin proteins) responsible for endosomal transport were found to be associated with TG2 and formed significant "hubs" post UUO (**Figure 6**). Furthermore, the heparan sulphate (HS) proteoglycans syndecan-4 and perlecan were specifically associated with TG2 in the UUO model.

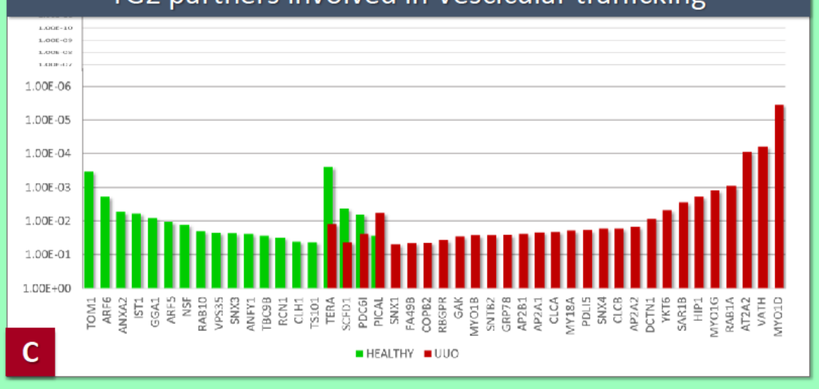
TG2-interactome in UUO kidney: predominance of cell adhesion, actin dynamics, redox regulation and vesicular trafficking proteins



TG2 partners involved in Actin dynamics, ECM organisation and Cell adhesion



TG2 partners involved in Vescicular trafficking



TG2 partners involved in Redox regulation

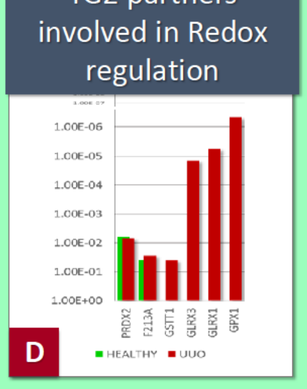
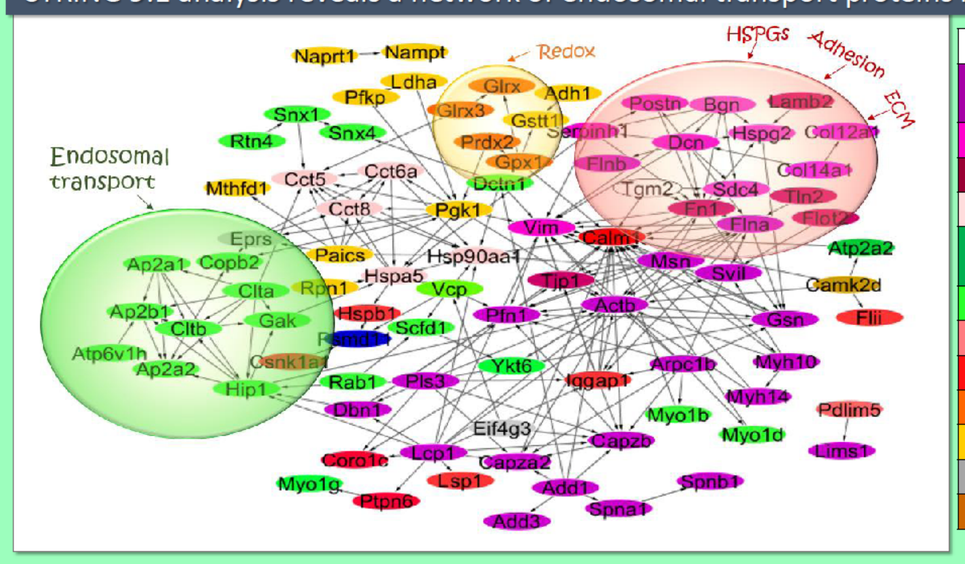


Figure 5: Candidate proteins significantly associated with TG2 in Healthy and UUO Kidney membranes clustered based on their general function: Uniprot Identification codes of each candidate protein significantly associated with TG2 (p ≤ 0.05, n≥4) in kidney membranes, associated to the corresponding proteins by manual search on the UniProtKB, SwissProt and Genecards Databases, are clustered based on their general functions. **A)** Piechart of the functions represented in healthy/UUO membranes and the number of proteins for each category. Over-represented categories are marked with an asterisk. **B,C,D)** TG2-associated proteins belonging to the specific categories overrepresented upon UUO, ordered by their p-values. Functional analysis using Gene Onthology (GO) references (Panther Database) and over-represented pathways analysis (KEGG pathways, DAVID bioinformatic resource) were also performed to confirm the results (data not shown).

STRING 9.1 analysis reveals a network of endosomal transport proteins in the TG2 interactome



General functions
Cytoskeleton and actin dynamics
ECM organisation
Cell adhesion
Chaperone
Endosomal Reticulum Regulation
Vesicular Trafficking
Protein binding
Signalling and cell response
Redox Regulation
Metabolism
Transcription regulation
Ion transport

Figure 6: STRING 9.1 analysis of known protein interactions: Network of interactions obtained for the significant TG2-associated proteins in UUO membranes using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) 9.1. Lines represent direct or indirect interactions between proteins at a confidence level higher that 0.35 (medium). Unconnected proteins were removed from the final picture. Color coding (table) is used to visualise clusters of proteins depending on their general function.

TG2 partially associates with Clathrin and Syndecan-4 in NRK52 TEC

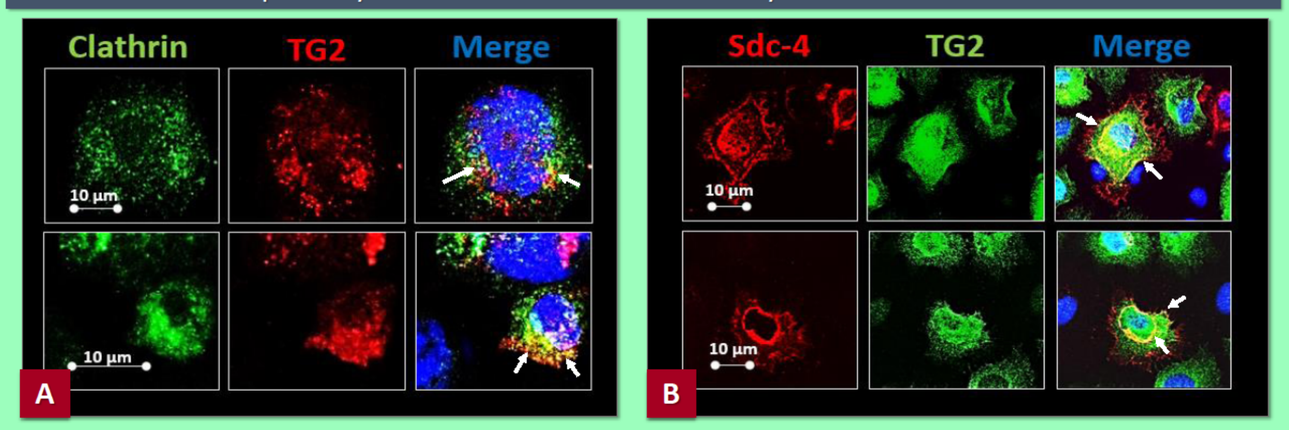


Figure 7: Immunofluorescent staining of TG2 and a selection of partners involved in its trafficking: A) Clathrin and TG2 dual staining. Fixed (2%PFA/10 mins) but not permeabilised NRK52 cells were probed for Clathrin using a rabbit polyclonal antibody followed by Donkey Anti-Rabbit IgG H&L Alexa Fluor® 486 (Green), and for TG2 using mouse monoclonal antibody followed by Goat Anti-Mouse IgG H&L Alexa Fluor® 596 (Red). **The arrows point at areas of TG2 localisation at perinuclear and peripheral Clathrin puncta**. B) Syndecan-4 (Sdc-4) and TG2 dual staining. Stable clones expressing GFP-TG2 were transiently transfected with pcDNA-HA-hSdc4 by electroporation. Fixed but not permeabilised cells were probed for cell surface HA-Sdc-4 using a rabbit polyclonal anti-HA antibody followed by Donkey Anti-Rabbit IgG H&L Alexa Fluor® 568 (Red). **The arrows point at areas of TG2-Sdc-4 co-localisation in perinuclear structures and at the plasma membrane.** Nuclei are stained with DAPI (Blue). Specimens (20µm-thick) were scanned by confocal microscopy at every um. The picture combines all the 20 levels observed.

CONCLUSIONS:

Our study shows that an immunoprecipitation approach combined with SWATH MS can be used to characterise the TG2 membrane interactome post UUO. A similar approach may be applied to uncover interacting partners of other fibrogenic proteins. Our data show that cell surface and extracellular HS proteoglycans associate with TG2 post UUO, consistent with previous evidence of their involvement in controlling TG2 export (*Scarpellini&Huang et al., J Am Soc Nephro, 2014*). The novel findings that UUO induces association of TG2 with endosomal proteins suggest their potential involvement in TG2-trafficking during fibrosis progression.

Kidney Research UK *Work supported by KRUK project grant RP25/2012 to EVE and TJ*