

## **Insights on the heparan sulphate-dependent externalisation of transglutaminase-2 (TG2) in glucose-stimulated proximal-like tubular epithelial cells**

Giulia Furini<sup>1</sup>, Izhar Burhan<sup>1</sup>, Linghong Huang<sup>2</sup>, Maria Pia Savoca<sup>1</sup>, Adeola Atobatele<sup>1</sup>, Tim Johnson<sup>2</sup>, Elisabetta Verderio<sup>1,3\*</sup>

<sup>1</sup> School of Science and Technology, Centre for Health, Ageing and Understanding of Disease, Nottingham Trent University, Nottingham NG11 8NS, UK

<sup>2</sup> University of Sheffield, Academic Nephrology Unit, Medical School, Sheffield, S10 2RZ, United Kingdom

<sup>3</sup> BiGeA, University of Bologna, Bologna 40126, Italy

\*Corresponding author

[\\*elisabetta.verderio-edwards@ntu.ac.uk](mailto:elisabetta.verderio-edwards@ntu.ac.uk)

## **Abstract**

The extracellular matrix crosslinking enzyme transglutaminase 2 (TG2) is highly implicated in tissue fibrosis that precedes end-stage kidney failure. TG2 is unconventionally secreted through extracellular vesicles in a way that depends on heparan sulphate (HS) proteoglycan syndecan-4 (Sdc4), the deletion of which reduces experimental kidney fibrosis as a result of lower extracellular TG2 in the tubule-interstitium. Here we establish a model of TG2 externalisation in NRK-52E tubular epithelial cells subjected to glucose stress. HS-binding TG2 mutants had reduced extracellular TG2 in transfected NRK-52E, suggesting that TG2-externalisation depends on an intact TG2 heparin binding site. Inhibition of N-ethylmaleimide sensitive factor (NSF) vesicle-fusing ATPase, which was identified in the recently elucidated TG2 kidney membrane-interactome, led to significantly lower TG2-externalisation, thus validating the involvement of membrane fusion in TG2 secretion. As cyclin-G-associated kinase (GAK) had emerged as a further TG2-partner in the fibrotic kidney, we investigated whether glucose-induced TG2-externalisation was accompanied by TG2 phosphorylation in consensus sequences of cyclin-dependent kinase (CDK). Glucose stress led to intense TG2 phosphorylation in serine/threonine CDK-target. TG2 phosphorylation by tyrosine kinases was also increased by glucose. Although the precise role of glucose-induced TG2 phosphorylation is unknown, these novel data suggest that phosphorylation may be involved in TG2 membrane-trafficking.

## **Keywords**

Transglutaminase 2; Heparan sulphate (HS) proteoglycan; Syndecan-4 (Sdc4); Extracellular vesicles; Glucose stress; Phosphorylation; Chronic kidney disease (CKD).

# 1. Introduction

Transglutaminase 2 (TG2) is a member of a family of  $\text{Ca}^{2+}$ -dependent transamidases. It catalyses the reaction between a glutamyl residue and a lysine residue of the same or different proteins forming a stable and proteolytic resistant  $\epsilon$ -( $\gamma$ -glutamyl)-lysine isodipeptide crosslink. GTP can block the enzyme activity by stabilizing the enzyme in its closed conformation and inhibiting  $\text{Ca}^{2+}$  binding to the enzyme [1], furthermore an oxidising environment limits the accessibility of the Cys active site for transamidation [2-4]. In reducing conditions,  $\text{Ca}^{2+}$  binding lowers the enzyme affinity for GTP/GDP, leading to an enzymatically active “open” conformation (recently reviewed in [5]). Therefore, TG2 is regarded to be predominantly inactive intracellularly in physiologically “normal” conditions and active outside the cells, where it targets several extracellular matrix (ECM) proteins leading to ECM remodelling and contributing to the scarring process. TG2 has been implicated in a number of fibrotic diseases such as tissue fibrosis in liver [6, 7], lung [8, 9], heart [10, 11], kidney [12-15], and in atherosclerosis [16, 17]. Its pathological role in the kidney has been extensively studied [reviewed in [13]]. In tubular epithelial cells, increased extracellular release of TG2 causes extensive ECM accumulation by accelerating collagen deposition as well as by stabilising the ECM against the action of matrix metalloproteinases [18]. Furthermore, it acts as a rate-determining factor in latent transforming growth factor- $\beta$  (TGF- $\beta$ ) activation by crosslinking the large latent TGF- $\beta$  into the ECM, thus priming it for activation, as observed in OK and NRK-52E proximal-like tubular epithelial cells (TEC) [19, 20]. Extracellular TG2 and TG2 activity are increased in experimental models of chronic kidney disease (CKD) [14, 20, 21] and TG2 deletion results into lower levels of active TGF- $\beta$  [22, 23]. Both extracellular TG2 and its crosslinking products are also reported to positively correlate with scores of fibrosis evaluated in human kidneys with different types of CKD [24], and modulation of TG2 activity results in decreased kidney scarring and kidney function preservation in both diabetic [25] and

non-diabetic [26] models of experimental CKD. Targeting TG2 externalisation is therefore an important strategy to control the scarring process.

Unlike most exported proteins, TG2 does not possess a signal peptide, and how TG2 is externalised into the extracellular environment has been object of intense scrutiny. Recently, the presence of clusters of exosomal proteins in the TG2-membrane interactome has led to the demonstration that TG2 can be externalised via extracellular membrane vesicles by TEC during CKD progression, in a way which is dependent on the heparan sulphate (HS) proteoglycan syndecan-4 [22]. TG2 affinity for the HS chains has led to the mapping of the TG2 heparin binding site, showing that heparin binding is lost when TG2 is in the linear (“active”) conformation [27-29] [recently reviewed in [30]]. Vesicular secretion of TG2 has been proposed also in other cell systems [31], and the N-ethylmaleimide (NEM), inhibitor of membrane fusion of NSF ATPase, has been shown to interfere with TG2 secretion in NIH/3T3 [31]. Moreover, TG2 has been shown to be present in cancer shed microvesicles [32], externalised from smooth muscle cells in via microvesicles in a transamidation dependent way [33], and in stressful conditions such as those seen in abnormal proteostasis. Unconventional secretion mechanisms can be distinguished in vesicular and non-vesicular mechanisms [34], the latter characterised by direct translocation through the plasma membrane. A well described example is that of FGF2 secretion which involves binding to HS proteoglycans [35, 36], in common with TG2 externalisation, and also plasma membrane phosphoinositides (PIPs). According to this model, FGF2 binds PIPs on the inner side of the plasma membrane leading to oligomerisation and formation of a hydrophilic pore. HS chains of the HSPG on the outer side would trap FGF2 facilitating its secretion [37], which would require the folded conformation and FGF2 phosphorylation by a Tec non-receptor tyrosine kinase [38]. In addition, ATP1A1, an  $\alpha$ 1-chain of sodium/potassium-ATPase has been shown to be a crucial factor for the export of FGF2 [39]. Although our *in vivo* proteomic approach combined with *in*

*vitro* investigations have identified a HS-dependent vesicular transport for TG2, we do not know if this is the unique pathway, and investigations of phosphorylation events in connection with TG2 secretion have never been carried out. Moreover, we do not know if an intact TG2 heparin binding site is required. Here we give mechanistic insights on the externalisation of TG2 in NRK-52E TEC induced by glucose exposure. We show that glucose-stimulated release of TG2 requires an intact HS binding site and membrane fusion events. Furthermore, we show that glucose stimulates targeting of TG2 by cyclin-G-associated kinase (GAK).

## **2. Experimental Material and Methods**

### **2.1 Cell lines**

NRK-52E proximal-like tubular epithelial cells (TEC) were obtained from the European Cell Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK) supplemented with 5% heat-inactivated foetal calf serum (FCS) (Biosera, UK), 2 mM L-glutamine (Lonza, UK), 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma, UK). All cultures were maintained at 37°C and in a humidified atmosphere of 5% CO<sub>2</sub>. EGFP-TG2 NRK-52E stably transfected clone [22] was cultured in medium supplemented with G418 (400 µg/ml).

### **2.2 Construction of pEGFP-N1-TG2(M1c) and pEGFP-N1-TG2(M3) plasmids**

TGM2 cDNA TG2(M1c) and TG2(M3) cDNA (produced by site directed mutagenesis [27], were amplified by PCR using Pwo-DNA polymerase (Roche) and the *EcoRI*-modified primers 5'-ATCAGAATTCATGGCCGAGGAGCTGGTCTTAGAGAGG-3' and 5'-ATCAGAATTCCGGCGGGGCCAATGATGACATTCCGG-3', and then subcloned into the *EcoRI*-site of pEGFP-N1 plasmid (BD Biosciences). The obtained pEGFP-N1-TG2 plasmid expresses TG2 fused in frame to the N-terminus of EGFP.

### **2.3 Transient transfection of NRK-52E cells**

Transient transfection of NRK-52E TEC with plasmid expressing wild type (WT) or mutated TGM2 cDNA were performed using 5µg of either pEGFP-N1-TG2 [22], pEGFP-N1-TG2(M1c) or pEGFP-N1-TG2(M3). Transfections were carried out by electroporation on an Amaxa Nucleofactor™ device using Nucleofactor™ Solution Kit V (Lonza, UK) and program T-027.

## **2.4 Glucose induction and cell treatments**

For glucose stress simulation, NRK-52E cells were grown in DMEM containing 6 mM D-Glucose for 7 days. After this time, cells were cultured in the same medium supplemented with increasing concentration of D-Glucose (6, 12, 18, 24 and 30 mM) for up to 96 hours [40]. In order to cleave heparan sulphate chains or block HS binding, cells cultured in 24 mM D-Glucose were incubated with either 30 mU/ml Heparinase I (Sigma, UK) or 12 µmol/L Surfen [*bis*-2-methyl-4-amino-quinolyl-6-carbamide, a gift from J.D. Esko (University of California, San Diego)], at 37°C for an hour.

For N-ethylmaleimide (NEM) treatment, cells were cultured in 24 mM glucose containing medium as until 90% confluent. At this point, medium was supplemented with 0.6 mM NEM (E3876, Sigma; from a 12.5 mg/ml freshly prepared in aqueous solution) and cells were incubated for 30 min at 37°C as previously described [31].

## **2.5 Knock Down of Sdc4 expression in NRK-52E cells and clones by siRNA transfection**

In order to knock down the expression of Sdc4 in NRK-52E cells or EGFP-TG2 expressing clones, transient transfection using Sdc4-targeting siRNA was performed.  $3 \times 10^5$  cells per well were plated in a 6-well plate in 5% FCS supplemented medium without antibiotics, containing 24 mM D-Glucose. The day after cells were transfected with either 100 nM Rat Sdc4-targeting siRNA or non-targeting scrambled control siRNA, using DharmaFECT-1 solution (Dharmacon, UK) as transfection reagent and antibiotic-free complete medium. Cells were allowed to grow 24 hrs after transfection. Furthermore, the efficiency of knock down was assessed by quantitative RT-PCR (qRT-PCR) using specific primers against rat Sdc4 (5'-GAGTCGATTCGAGAGACTGA-3' and 5'-AAAAATGTTGCTGCCCTG-3'). Cyclophilin

A was amplified from the same cDNA using appropriate primers (5'-ACGCCGCTGTCTCTTTTC-3' and 5'-CTTGCCACCAGTGCCATTAT-3'), and was used as an housekeeping gene. After amplification, threshold cycle (Ct) values were obtained and averaged among replica. The  $2^{-\Delta\Delta C_t}$  relative quantification method [41] was employed to calculate the level of expression of the target gene.

## **2.6 Immunoprecipitation**

Cell lysates were produced in 500  $\mu$ l of IP Lysis/Wash buffer (Thermo Scientific, UK). For the detection of phosphorylation 1% (v/v) protease inhibitors cocktail (P8340 Sigma), 1% (v/v) phosphatase inhibitors [phosphatase inhibitor cocktail 2 (P5726 Sigma, UK) and phosphatase inhibitor cocktail 3 (P0044 Sigma, UK)] were added to the lysate to inhibit dephosphorylation. EGFP-immunoprecipitation was performed in equal amounts of proteins (750  $\mu$ g) with 2.5  $\mu$ g of rabbit polyclonal anti-GFP antibody (Ab290, Abcam, UK), using the Pierce™ Crosslink Magnetic IP/Co-IP Kit (Thermo Scientific, UK), following the manufacturer's instruction. As a negative control, the same procedure was performed in the absence of antibody (referred to as "beads only" control).

## **2.7 Western Blotting**

Equal amount of proteins were separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Bio-Rad, UK). The membrane was blocked with 5% (w/v) non-fat dried milk and 0.1% (v/v) TWEEN® 20 in TBS, and then immunoprobed using each primary antibody at 4°C for 15 hours. To investigate protein phosphorylation the following antibodies detecting phosphotyrosine or phosphoserine/threonine at specific sites were used: polyclonal anti-phosphotyrosine (BD Transduction



laboratories, 1:250); monoclonal Phospho-(Ser/Thr)AMPK Substrate (P-S/T2), monoclonal Phospho-AKT Substrate (RXRXXS/T) (110B7E), monoclonal Phospho-PKA Substrate (RRXS/T), monoclonal Phospho-(Ser/Thr)ATM/ATR Substrate (P-S3-101), monoclonal Phospho-(Ser) PKC Substrate (P-S3-100), monoclonal Phospho-(Ser) CDKs Substrate (1:1000 Cell Signalling Technology, UK) and polyclonal anti-GFP (1:2500 ab290, Abcam UK). In each blot, equal volumes of GFP-precipitates and “beads only”- precipitates were analysed for both treatments. Primary antibody binding was revealed with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence using ECL detection kit (Geneflow, UK) and blots were imaged using LAS4000 imaging system (GE Healthcare, UK). Densitometric analysis was performed using AIDA (Advanced image Data Analyser) image analyser software.

## **2.8 Extracellular TG2 activity assay**

Extracellular TG2 activity was measured through the enzyme’s ability of incorporating biotinylated cadaverine (BTC) (Molecular Probes, The Netherlands) into fibronectin (Sigma, UK) as previously described [42]. This assay was performed using  $4 \times 10^4$  cells per well, and cells were incubated in the presence of BTC for 1 hour at 37° C.

## **2.9 Extracellular TG2 and GFP immunofluorescent staining**

For immunofluorescent staining of extracellular TG2 or GFP, cells were plated in 8-well chamber slides and cultured overnight prior to staining. Cells were fixed with 3% paraformaldehyde (PFA) for 8 minutes at room temperature and blocked with 3% (w/v) BSA in PBS for 30 minutes at 37° C. Extracellular TG2 was detected by incubation with a 1:50 dilution of mouse monoclonal anti-TG2 antibody IA12 [43] in blocking solution, overnight at 4°C, followed by a 1 hour incubation at room temperature with secondary goat anti-mouse

antibody Alexa Fluor® 598. Extracellular EGFP was detected by incubating with a 1:500 dilution of rabbit polyclonal anti-GFP antibody in blocking solution, overnight at 4°C, followed by a 1 hour incubation at room temperature with 1:500 dilution of Alexa Fluor® 568-conjugated donkey anti-rabbit antibody in blocking solution. Slides were mounted with Vectashield® mounting medium containing DAPI. **Images were obtained by acquiring 1 µm sections that were then merged to project the staining from the apical to the basal side of cells (20 µm specimens).**

### **2.10 Lactate dehydrogenase (LDH) bioassay**

LDH assay was performed using Pierce LDH Cytotoxicity Assay kit (Thermo Scientific, UK) following the manufacturer's protocol.

### **2.11 Statistical analysis and software**

Data is shown as mean  $\pm$  SEM. Data analyses were performed using unpaired T-test with Welch's correction. A probability of 95% ( $p < 0.05$ ) was taken as significant.

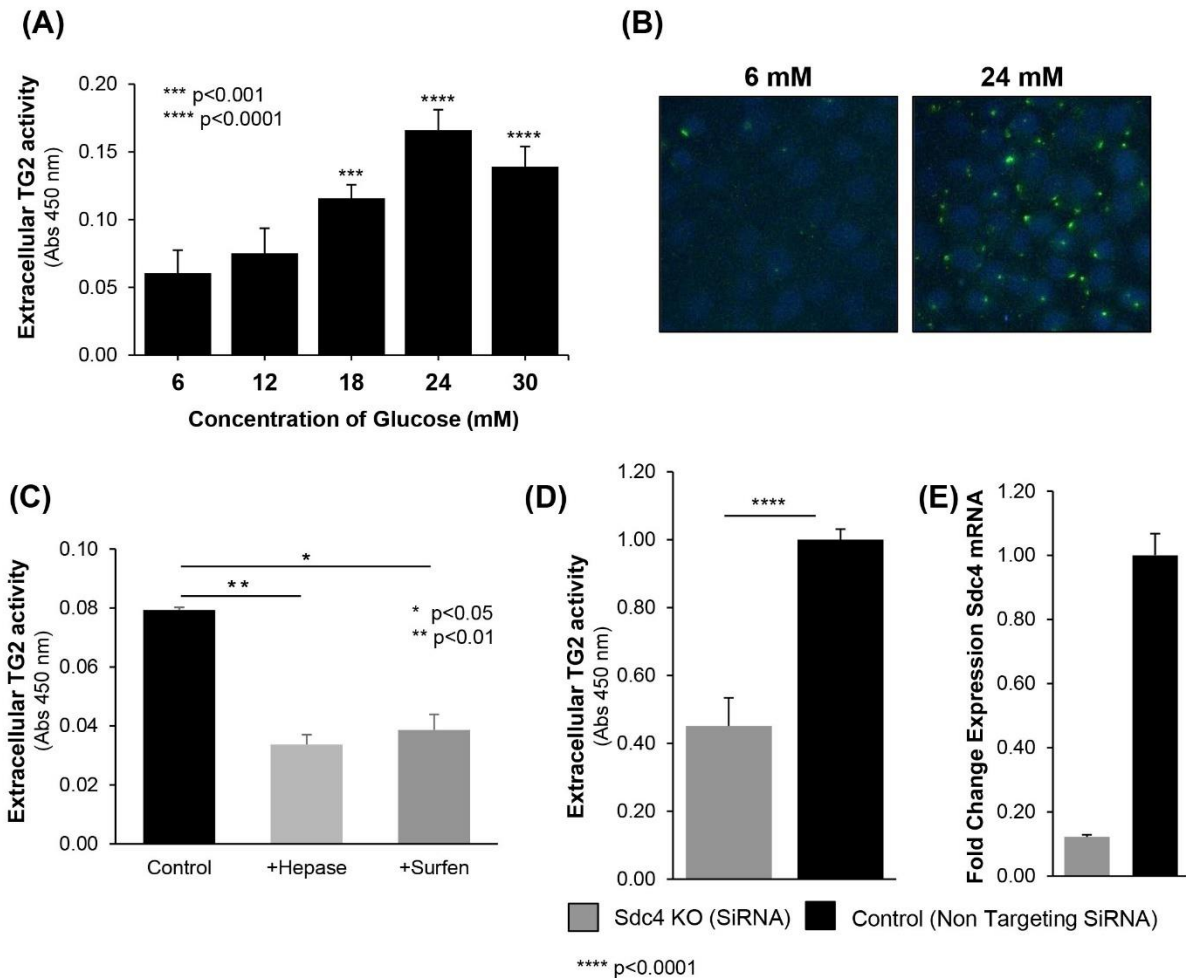
Putative phosphorylation sites of *Homo sapiens* TG2 protein sequence (EC:2.3.2.13) were analysed using NetPhos online tool (<http://www.cbs.dtu.dk/services/NetPhos>).

### 3. Results

#### 3.1 TG2 export depends on cell surface Sdc4/HS in NRK-52E tubular epithelial cells

Since extracellular TG2 was elevated in experimental and human diabetic nephropathy, glucose is believed to be one of the stimuli that can cause cellular release of TG2. To optimise the glucose concentrations that trigger TG2 translocation on the cell surface, a rat renal proximal epithelial-like tubular cell line (NRK-52E) [44] was treated with increasing concentrations of glucose for 96 hours, starting from 6 mM glucose, which may be representative of a normoglycemic environment, to 24 mM and 30 mM glucose concentrations, representative of extreme hyperglycemia. Increased cell surface TG2 activity was detected in cells cultured in glucose concentration above 18 mM ( $p < 0.001$ ) (**Fig 1A**). TG2 immunofluorescence revealed a punctate staining which was visibly enhanced by higher levels of glucose, suggesting that the increased cell surface TG2 activity resulted from elevated cellular release of TG2 (**Fig 1B**). The punctate pattern was consistent with possible extracellular vesicles containing TG2, as we previously described [22].

To test the possibility that TG2 export depends on cell surface HS, cells were pre-treated with Hepase, which selectively cleaves HS chains, or Surfen, an antagonist of HS. Hepase or Surfen significantly abolished the increase in extracellular TG2 activity (**Fig 1C**). To test whether Sdc4 was the HS proteoglycan responsible for TG2 externalisation, its level was modulated by siRNA silencing. Knockdown of Sdc4 led to over 50% reduction of cell surface TG2 (**Fig 1D-E**), comparable to that achieved by Hepase digestion and Surfen, suggesting that it is the main HS proteoglycan involved in this cell line.

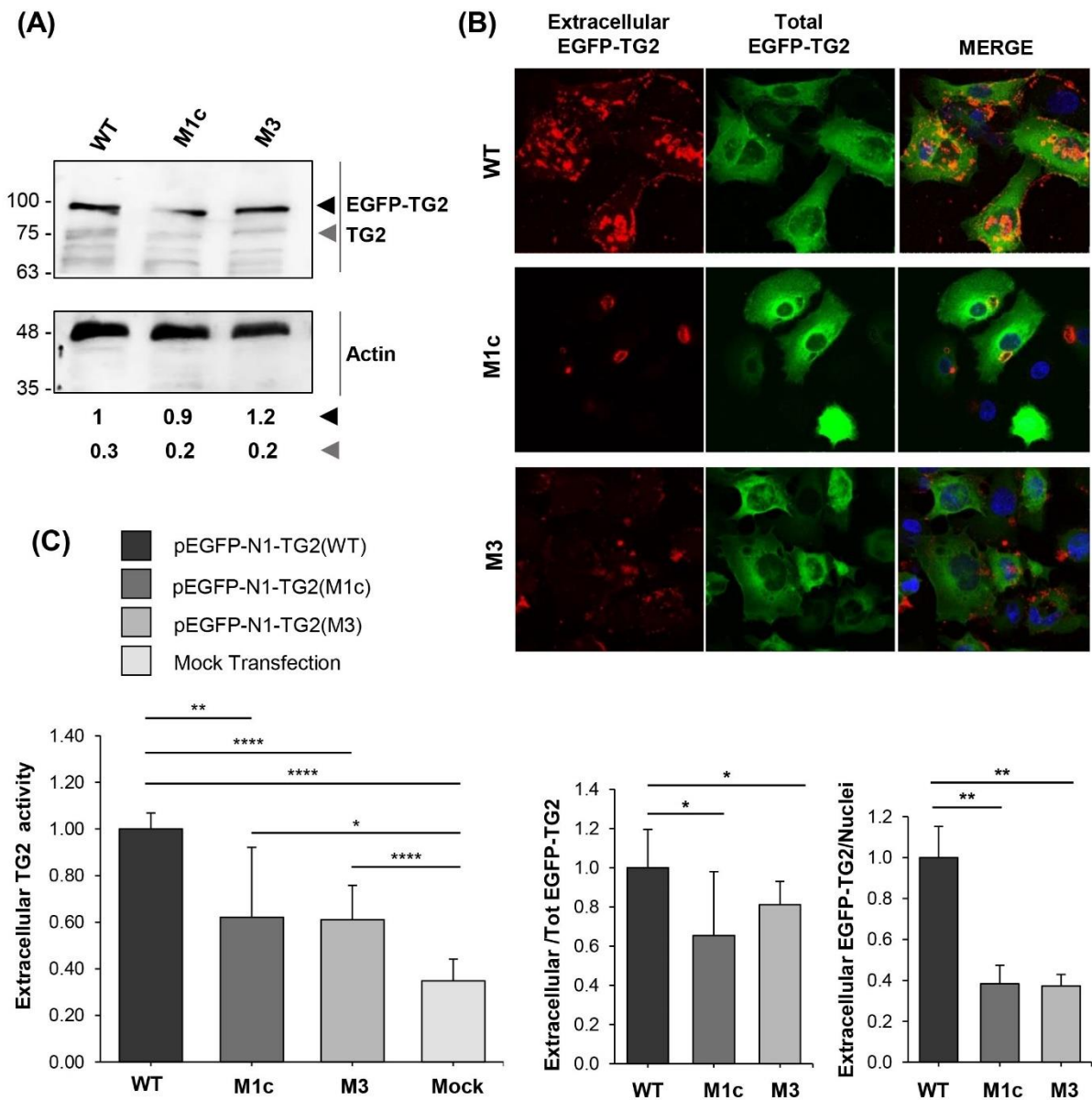


**Fig 1: Glucose induces HS and syndecan-4 dependent externalisation of TG2.** (A,B) NRK-52E cells were cultured in different concentrations of glucose for 96 hours. (A) The extracellular activity of TG was detected through the enzyme's ability of incorporating BTC in FN by living cells. Data represent mean absorbance (Abs) (450 nM)  $\pm$  SEM, n = 3 independent experiments undertaken in triplicates. Statistically significant changes are expressed relative to the values at 6 mM Glucose. (B) TG2 immunofluorescent staining was performed in cells growing in 6 mM glucose and 24 mM glucose (magnification 63X). (C) Cells growing in 24 mM glucose were treated with 30 mU/ml Hepase or 12  $\mu$ M Surfen, which was followed by measurement of extracellular TG2 activity as described above. Data represent mean absorbance (450 nM)  $\pm$  SEM, n = 3 independent experiments undertaken in triplicates. (D,E) NRK-52E cells were transfected with 100 nM Rat Sdc4 – targeting siRNA for 24 hours. The same cells transfected with 100 nM non-targeting scrambled siRNA were used as control. (D) Extracellular TG2 activity was measured as described above. Data represent mean absorbance (450 nM), normalised for the control,  $\pm$  SEM, n = 3 independent experiments undertaken in triplicates. (E) The relative expression of Sdc4 mRNA after siRNA transfection was analyzed via qRT-PCR, using the  $\Delta\Delta C_t$  method. Cyclophilin A was employed as housekeeping gene. Differences in mRNA expression are here shown as fold change from the control ( $2^{-\Delta\Delta C_t}$ )  $\pm$  SEM.

### 3.2 The heparin binding domain of TG2 is required for TG2 cell surface availability

To test whether the interaction of HS with the heparin binding site of TG2 is responsible for its cell surface location, NRK-52E cells were transiently transfected with previously characterised TG2 heparin binding mutants [27], and the level of cell surface TG2 measured. Mutant cDNAs were selected **based on enzymatic activity and thermal shift analysis described in our previous work** [27], as they expressed mutant TG2s with similar enzymatic activity and stability to the WT enzyme; moreover, they could be modulated by  $\text{Ca}^{2+}$  and GTP like the wild type enzyme [27, 30]. Mutant M1c disrupted the 261-LRRWK-265 sequence while M3 the 598-KQKRK-602 TG2 sequence of TG2. Both clusters are central for heparin binding as they form a composite heparin binding site when TG2 is in the closed conformation. Their mutation completely blocks heparin binding of TG2 when measured by surface plasmon resonance [27]. M1c and M3 were expressed as EGFP-tagged proteins by subcloning in pEGFP-N1 and transfection of the constructs into NRK-52E cells. **Immunoblotting of total cell lysates revealed that the wild type and mutant forms of EGFP-tagged TG2 (100 kDa), were expressed at a comparable level and several times more than the endogenous TG2 (75 kDa) (Fig 2A)**. To verify whether the transfection of either WT (pEGFP-N1-TG2) or mutant TG2 (pEGFPN1-TG2(M1c) and pEGFP-N1-TG2(M3)) was also accompanied by cellular release of TG2, the level of externalised TG2 was detected by an anti-EGFP antibody in not permeabilised cells, and intensities values normalised per transfection efficiency (monitored via EGFP fluorescence) (**Fig 2B**). Cells transfected with WT TG2 **displayed a marked punctate pattern of extracellular EGFP-TG2** (denoted by the red fluorescence), which was significantly reduced in cells transfected with TG2 heparin binding mutants, **when the extracellular red staining was quantified by image analysis and normalised for either total EGFP-TG2 (green) or cell nucleus (blue)**. This quantification suggests that the TG2 heparin binding site plays a significant role in TG2 export. **Heparan sulphate chains are characteristic of syndecan transmembrane receptors,**

which are present at discrete locations on the cell surface but also in extracellular vesicles, and of extracellular matrix proteoglycans such as perlecan, all interacting partners of TG2 [22]. Mutation of the heparin binding site however did not completely abolish TG2 externalisation. This data was confirmed when availability of cell surface TG2 was measured enzymatically (**Fig 2C**): cells transfected with both TG2 heparin binding mutants (M1c and M3) displayed lower cell surface TG2 activity than cells transfected with wild type TG2 (WT), however higher than that of mock transfected cells, given the considerable overexpression of the exogenous TG2 forms. This data suggests that mutation of the heparin binding site is important for the availability of TG2 outside the cells, but it does not completely abolish TG2 externalisation.



**Fig 2: The heparin binding site of TG2 is required for TG2 secretion. (A,B) NRK-52E cells cultured in 24 mM glucose for 96 hours** were transiently transfected by electroporation with 5  $\mu$ g of pEGFP-N1-plasmid containing either TG2(WT), TG2(M1c) and TG2(M3). A mock transfection was performed as a control. **(A) Post-transfection, the expression of wild type and mutant EGFP-TG2 was monitored by western blotting of total cell lysates using anti-TG2 antibody and anti-actin antibody to reveal the loading control actin; densitometric analysis of the transfected EGFP-TG2 (black arrowhead) and the endogenous TG2 (grey arrowhead) of a typical experiment is shown (values are normalised per loading control).** **(B) Extracellular EGFP-TG2 chimera was detected by immunofluorescent staining on fixed (2% PFA) but not permeabilised cells by a rabbit polyclonal anti-GFP antibody followed by a goat anti-**

rabbit Alexa 568 secondary antibody. Nuclei were stained with DAPI. Representative pictures at 100X magnification are here shown. Cell surface and matrix bound EGFP-TG2 was quantified by ImageJ intensity analysis (8 non overlapping images per section) and presented either as mean relative intensity of red over green (total EGFP-TG2)  $\pm$  SEM, or red over blue (nuclei)  $\pm$  SEM, and expressed relative to the TG2(WT) transfected cells (equalised to 1). (C) The cell surface activity of TG2 was detected through the enzyme's ability of incorporating BTC in FN by living cells. Data represent mean absorbance (450 nM), normalised for the control,  $\pm$  SEM, n = 3 independent experiments undertaken in triplicates.

### 3.3 Importance of membrane fusion for TG2 externalisation in NRK-52E cells

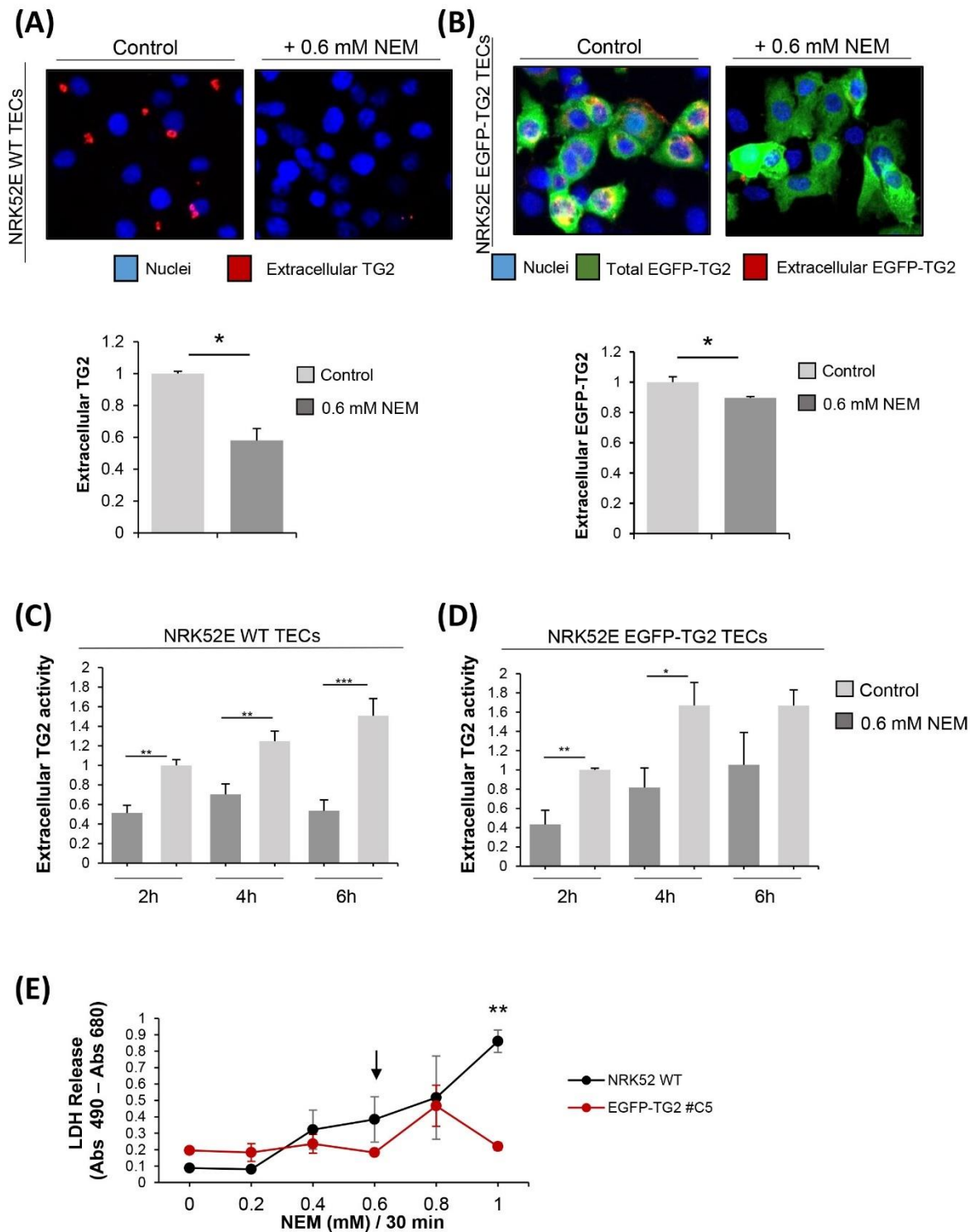
NSF vesicle-fusing ATPase is required for intracellular **SNARE (Soluble NSF Attachment protein Receptor)**-mediated membrane fusion events, including exocytosis. Interestingly, together with other proteins with vesicular transport function, it was identified in the recently elucidated TG2 interactome, as specifically associated with TG2 membranes isolated from normal kidneys [22]. NEM, a specific inhibitor of NSF ATPase [45] was shown to interfere with TG2 secretion before [31]. Therefore, we evaluated consequences of NSF ATPase inhibition on TG2 release from NRK-52E at 24 mM glucose stimulation.

NRK-52E WT cells showed a punctate pattern of extracellular TG2 antigen staining that was significantly reduced upon NEM treatment when the intensity of extracellular TG2 (red) over the nuclei (blue) was expressed relative to the control untreated cells (**Fig 3A**). Similarly, also EGFP-TG2 stably expressing clone [22] displayed a level of extracellular EGFP-TG2 (red) when quantified over the total EGFP-TG2 (green), although at a lower degree (**Fig 3B**). It is possible that TG2 overexpression slightly exceeds the endogenous trafficking systems in the transfected cells.

Next, the implication of NSF ATPase on the availability of cell surface TG2 activity was explored. Cells displayed an increase in cell surface extracellular TG2 activity which was



dependent on cell adhesion time on fibronectin (**Fig. 3C-D**). At each time point, pre-incubation with NEM resulted in a significant reduction of cell surface TG2 in NRK-52E WT cells (**Fig 3C**), and up to 4 hours post-seeding in the EGFP-TG2 transfected clone (**Fig 3D**). Unspecific release of cytosolic components by NEM in the employed conditions was ruled out by a LDH assay (**Fig 3E**). These data imply that membrane fusion events such as exocytosis drive the cell surface trafficking of TG2, consistent with the location of TG2 in exosomes [22, 31, 32].



**Fig 3: Inhibition of NSF vesicle-fusing ATPase leads to a reduction in extracellular TG2.** (A,B) NRK-52E WT TEC and EGFP-TG2 overexpressing NRK-52E, cultured in 24 mM glucose as described previously, were incubated with 0.6  $\mu$ M (NEM) for 30 minutes. (A) Immunofluorescent staining of extracellular TG2 was performed in fixed (3% PFA) but not permeabilised NRK-52E WT cells using a

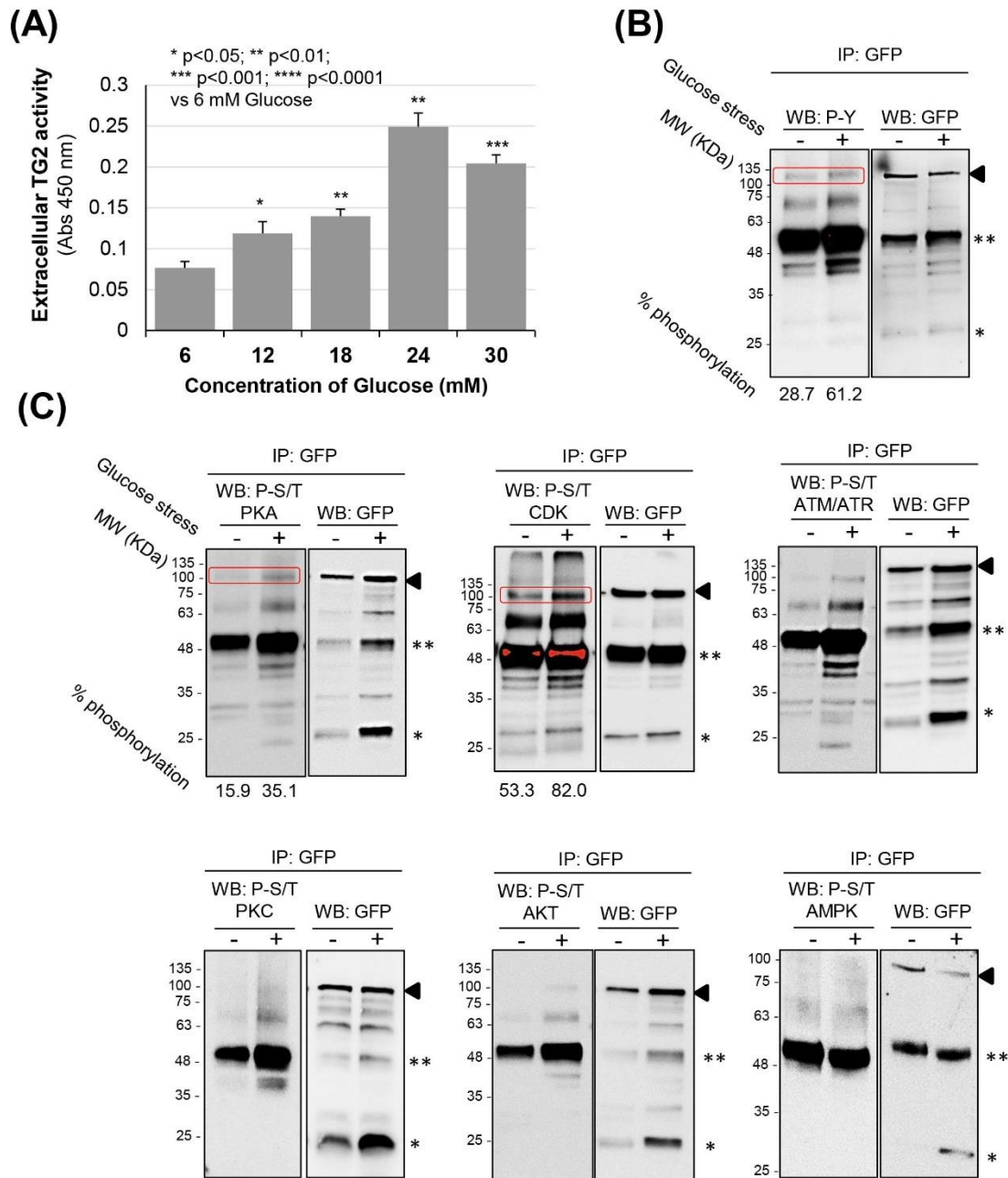
mouse monoclonal anti-TG2 antibody (IA12) followed by a secondary goat anti-mouse antibody Alexa Fluor® 598. Extracellular TG2 was quantified by ImageJ intensity analysis on at least 8 non-overlapping fields per treatment and expressed as mean relative intensity of red over blue (nuclei), normalised to the control cells without NEM (equalised to 1)  $\pm$  SEM. (B) Extracellular EGFP-TG2 chimera was detected on fixed (3% PFA) but not permeabilised cells using a Rabbit, UK polyclonal anti-GFP antibody (Abcam, UK) followed by a donkey anti-rabbit Alexa Fluor® 568 antibody, with red fluorescence. Nuclei were stained with DAPI. Extracellular EGFP-TG2 (B) was quantified as described in A and expressed as mean relative intensity of red over green (total EGFP-TG2), normalised to the control cells without NEM (equalised to 1)  $\pm$  SEM. Significance of the differences between treatments was determined by unpaired t-test: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ . Representative pictures at 200X magnification are shown. (C) WT or (D) EGFP-TG2 overexpressing NRK-52E TEC were pre-treated with 0.6 mM NEM for 30 minutes and seeded in a 96-well plate (20,000 cells/well) in quadruplicates and extracellular TG2 was detected as described in the Methods, for 2, 4 and 6 hours. Non-treated cells were used as a control. The values are average absorbances (450 nm) of three independent experiments, each undertaken in quadruplicates, normalised for the relative control (equalised to 1)  $\pm$  SD, N=3. (E) WT or EGFP-TG2 overexpressing NRK-52E TEC were incubated with different concentrations (0 to 1  $\mu$ M) of N-ethylmaleimide (NEM) for 30 minutes. After incubation, cell viability was measured by Lactose Dehydrogenase bioassay. Data are expressed as spontaneous LDH Release (Abs 490 nm – Abs 680nm) at each concentration for each cell line  $\pm$  SD. \*\* represents significant difference ( $p < 0.01$ ) in LDH release between NRK52WT and EGFP-TG2 transfected clone.

### 3.4 Glucose induces TG2 phosphorylation in NRK-52E cells

Data so far convincingly point at a Sdc4-dependent vesicular secretion of TG2 upon glucose stimulation, which is predominantly responsible for TG2 externalisation, however not all extracellular TG2 may be controlled by this pathway. As protein phosphorylation drives the HS-dependent translocation of FGF2 [35, 36], possible phosphorylation of TG2 upon glucose stimulation was explored. Firstly, putative phosphorylation sites of *Homo sapiens* TG2 protein (EC:2.3.2.13) were predicted using NetPhos (**Supplementary Table 1**). Among the residues with higher phosphorylation potential, tyrosine (Y) phosphorylation sites (Y-369, Y-583 and Y-503) had a high score, but tyrosine and threonine phosphorylation sites were lower in number comparing to serine residues in TG2. Then we explored TG2 phosphorylation in EGFP-tagged

TG2 immunoprecipitated from cell lysates via beads conjugated to anti-EGFP antibody. Specificity of immunoprecipitation was tested using “beads only” controls which showed no EGFP immunoreactivity (**Supplementary Figure 1**).

In NRK-52E cells expressing EGFP-tagged TG2, glucose stimulation increased TG2 externalisation (**Fig 4A**). To explore glucose-induced TG2 secretion and phosphorylation, cells were cultured in medium with high glucose (30 mM) and the phosphorylated EGFP-TG2 100 kDa band, which was detected after precipitation, was visibly increased (**Fig 4B-C**). Glucose induced general tyrosine phosphorylation of TG2 (**Fig 4B**) and also serine/threonine phosphorylation (**Fig 4C**). In particular, glucose induced protein kinase A (PKA)-mediated phosphorylation and cyclin-dependent kinase (CDK) phosphorylation (**Fig 4C**). Interestingly, cyclin-G-associated kinase appeared to target TG2 also in cells cultured in low glucose, compared to the other serine/threonine kinases; moreover, CDK was identified as significantly associated with TG2 in the TG2-interactome of fibrotic kidneys in the murine urinary ureteric obstructions (UUO) model [22], corroborating this finding that TG2 is a CDK target. Instead, RAD3-related kinase (ATM/ATR), protein kinase C (PKC), protein kinase B (Akt), and 5' AMP-activated protein kinase (AMPK) did not appear to phosphorylate the enzyme as no convincing band was seen at 100 kDa (black arrowhead) when EGFP-precipitates were probed for these kinase's -dependent phosphorylation (**Fig 4C**). Therefore, for the first time we have shown that glucose concentrations which trigger TG2-release into the extracellular environment from tubular epithelial cells also induce TG2 phosphorylation. Whether the two events are linked remains to be investigated.



**Fig 4: Glucose induced externalisation and phosphorylation of EGFP-TG2**

(A) EGFP-TG2 overexpressing NRK-52E clone was cultured in different concentrations of glucose for 96 hours. Extracellular activity of TG was measured as described in Methods. Data represent mean absorbance (Abs) (450 nm)  $\pm$  SEM, n = 3 independent experiments undertaken in triplicates. Statistically significant changes are expressed relative to the values at 6 mM Glucose. (B-C) NRK-52E EGFP-TG2 overexpressing clones were subjected to glucose stress to simulate a pro-fibrotic condition (5 mM glucose = - / 30 mM glucose = +) and EGFP-TG2 chimera was immunoprecipitated from equal amounts of cell lysate (750  $\mu$ g) with a rabbit polyclonal anti-GFP antibody (Abcam, UK). Equal volumes (40  $\mu$ l) of GFP-immunoprecipitated proteins (IP: GFP) were screened for the presence of

phosphorylated tyrosine, serine or threonine by Western blotting using specific antibodies as described in the Methods. Immunoreactive bands were detected by enhanced chemiluminescence (Geneflow, UK) after incubation with appropriate HRP-conjugated secondary antibody. The blot was then stripped and probed with rabbit polyclonal anti-GFP (Abcam, UK), followed by goat anti-rabbit IgG conjugated to HRP (Dako) to confirm the immunoprecipitation (WB: GFP). The EGFP-TG2 chimera expected MW is 100 kDa (arrowhead). The figure shows representative blots for each antibody employed. Asterisk represent co-eluted IgGs (\*\* = Heavy Chain; \* = Light Chain). Red circles identify phosphorylated EGFP-TG2. **(B)** Rabbit polyclonal anti-phosphotyrosine; **(C)** Rabbit monoclonal Phospho-PKA; Rabbit monoclonal Phospho-(Ser) CDKs; Rabbit monoclonal Phospho-(Ser/Thr) ATM/ATR; Rabbit monoclonal Phospho-(Ser) PKC; Rabbit monoclonal Phospho-AKT; Rabbit monoclonal Phospho-(Ser/Thr) AMPK. Degree of phosphorylation of the 100 kDa TG2 band was quantified by densitometric analysis and expressed as percentage of the unphosphorylated (values under each blot).

## 4. Discussion

Abnormal ECM accumulation causing expansion of glomerular and tubulointerstitial basement membranes, increased mesangial matrix and interstitial deposition is a common feature of CKD with different aetiology. TG2 plays a key role in this process by accelerating ECM deposition, stabilising ECM [18] and activating the fibrogenic cytokine TGF- $\beta$  [19]. Regulation of extracellular TG2 is beneficial to CKD by slowing down the progression of the disease [25, 26]. Therefore, it is important to understand how TG2 is externalised from renal cells, and targeting key factors involved in TG2 cell surface localisation and export may affect kidney fibrosis without the lethal side effects brought by the pan-TG inhibition.

Recent progress using an *in vivo* proteomic approach has led to the identification of TG2 interactome in kidney membranes, a powerful tool which in combination with primary and immortal tubular cell models has revealed that TG2 is externalised via extracellular vesicles. Among the TG2 partners, Sdc4 is one of the better characterised components of the TG2 interactome. Firstly, there is a direct association of Sdc4 and TG2 [46]; secondly, when the kidney was subjected to subtotal nephrectomy or damage caused by aristolochic acid [47], increased extracellular TG2 was detected to be colocalised with HS chains [20, 43]; and finally, the protective effect of Sdc4 deletion on kidney fibrosis was evident in experimental CKD models by lowering levels of extracellular TG2 and TG2 activity [43]. It has been recently hypothesized that TG2 may be recruited as an HS binding cargo and targeted to small vesicles by Sdc4 [22].

However, not all the 217 proteins directly or indirectly associated with TG2 in the UUO or sham kidney interactome were validated or understood [22]. In this study, glucose, a reliable stimulus among several renal stress factors (eg. hypoxia, acidosis, albumin overload and hemodynamic change etc) that up-regulates extracellular TG2 expression [40] was used to boost cell surface TG2 in NRK-52E TEC. **Glucose concentrations simulating the clinical**

hyperglycemic status induced TG2 externalisation, which was low and hardly detectable in the normoglycemic-like conditions. Here, we have shown for the first time that the heparin binding site of TG2 [27] is required for the availability of TG2 at the surface of NRK-52E TEC, and that HS/Sdc4 is needed for TG2 cell surface trafficking in TEC.

Another less characterised partner of the TG2 kidney interactome [22] is NSF ATPase, a protein necessary for most intracellular vesicular trafficking and membrane fusion events [45, 48-50] including exosome secretion by multivesicular body fusion [51]. Here we have validated the involvement of NSF ATPase in the externalisation of TG2 stimulated by glucose stress in NRK-52E TEC, showing that inhibition of NSF ATPase leads to a significant reduction in both TG2 cell surface antigen and activity. These data are consistent with a vesicular pathway of secretion for TG2.

Based on the TG2 interactome, cyclin-G-associated kinase GAK is a specific partner of TG2 in the UUO kidney at a point where TG2 is specifically released from TEC in the tubular interstitial space [22]. Here we show that TG2 is phosphorylated at serine/threonine consensus sequences which are target for cyclin-G-associated kinase GAK. TG2 phosphorylation has been previously reported at serine-216 by PKA [52], however to our knowledge phosphorylation and partnership with GAK is a novel finding. This finding is consistent with TG2 vesicular trafficking, given GAK's relation with vesicle-mediated transport, particularly the uncoating of clathrin [53, 54].

Work by the group of Aeschlimann [55] has proposed an alternative TG2 secretion pathway by direct translocation, involving the pore-formation function of purinergic P2X7 receptor induced by extracellular ATP in the context of inflammation. Absence of P2X7 in the TG2 interactome in kidney suggested that this pathway may not be shared [22], however we cannot exclude its involvement in this cell line under appropriate stimulus.



HS binding is a hallmark of TG2 secretion that has also been involved in the secretion of FGF-2, moreover TG2 has affinity for phosphoinositides [31] and we now know it is phosphorylated upon secretory stimulus. Our previous work showed the need for an intact fibronectin (FN) N-terminal binding site for TG2 secretion from TEC, which is crucial for a correct tertiary structure [56]. Although there are common factors in the export of TG2 and FGF2, further sophisticated investigations would be required to test whether these elements constitute a pathway and TG2 can also be secreted in a similar way by inducing lipidic membrane pores [38]. Diabetes mellitus is the main cause of end-stage kidney disease. Hyperglycemia significantly affects the renal tubules, with increase in proximal tubule reabsorption via Na<sup>+</sup>-glucose cotransporters (consequent to increased glucose glomerular filtration), changes in cell proliferation and pathways which are linked to the fibrotic process [57]. Here we have shown that vesicular HS-dependent externalisation of TG2 and TG2 phosphorylation triggered by glucose stress, are part of the molecular signature of TEC in diabetic kidney.

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