

# **A CRUCIAL SEQUENCE FOR TRANSGLUTAMINASE TYPE 2 EXTRACELLULAR TRAFFICKING IN RENAL TUBULAR EPITHELIAL CELLS LIES IN ITS N-TERMINAL B-SANDWICH DOMAIN.**

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**Transglutaminase type 2 (TG2) catalyses the formation of an  $\epsilon$ -( $\gamma$ -glutamyl)-lysine isopeptide bond between adjacent peptides or proteins including those of the extracellular matrix (ECM). Elevated extracellular TG2 leads to accelerated ECM deposition and reduced clearance that underlies tissue scarring and fibrosis. The extracellular trafficking of TG2 is crucial to its role in ECM homeostasis, however the mechanism by which TG2 escapes the cell is unknown as it has no signal leader peptide and therefore cannot be transported classically. Understanding TG2 transport may highlight novel mechanisms to interfere with TG2s extracellular function as isoform specific TG2 inhibitors remain elusive. Mammalian expression vectors were constructed containing domain deletions of TG2. These were transfected into 3 kidney tubular epithelial cell lines (TEC) & TG2 export assessed to identify critical domains. Point mutation was then used to highlight specific sequences within the domain required for TG2 export. The removal**

**of  $\beta$ -sandwich domain prevented all TG2 export. Mutations of Asp<sup>94</sup> and Asp<sup>97</sup> within NH<sub>2</sub>-terminal of  $\beta$ -sandwich domain were identified as crucial for TG2 externalisation. These form part of a previously identified fibronectin binding domain (<sup>88</sup>WTATVVDDQDCTLSLQLTT<sup>106</sup>). However siRNA knockdown of fibronectin failed to affect TG2 export. The sequence <sup>88</sup>WTATVVDDQDCTLSLQLTT<sup>106</sup> within the  $\beta$ -sandwich domain of TG2 is critical to its export in TEC. The extracellular trafficking of TG2 is independent of fibronectin.**

## **INTRODUCTION**

Elevated tissue transglutaminase (transglutaminase type 2 or TG2) activity is associated with abnormal wound healing (1). This includes liver (2), pulmonary (3), heart (4) and kidney fibrosis, (5) as well as atherosclerosis (6). The process of scarring and fibrosis is linked to the increased synthesis of TG2 and most importantly raised export of TG2 to the interstitial space. Once

outside the cell TG2 is able to crosslink extracellular matrix (ECM) proteins such as fibronectin and collagen (7) by the incorporation of an  $\epsilon(\gamma\text{-glutamyl})$  lysine di-peptide bond (8). Studies have shown that this can accelerate the deposition of available ECM components, while at the same time conferring resistance to proteolytic clearance by the MMP system (9-10). Taken together this causes an accumulation of ECM proteins and thus scar tissue (9). Further TG2 has an emerging role in the activation of latent TGF- $\beta$ 1 in the scarring process (11) and has also been associated with interleukin-6 (12) and tumour necrosis factor- $\alpha$  activation pathways (13).

Inhibition of TG2 *in vitro* decreases extracellular matrix levels (14) while cells derived from TG2 knockout mice have lower levels of mature ECM (9). *In vivo* application of TG2 inhibitors in models of chronic kidney disease reduce the development of glomerulosclerosis and tubulointerstitial fibrosis preserving renal function (15-16). Similar benefits are seen in the TG2 knockout mouse subjected to unilateral ureteric obstruction (17).

The pathological role of TG2 in fibrosis is tightly associated with its trafficking to the extracellular environment (5,10,18) where it has both enzymatic (post translational ECM modification and activation of cytokines (19-20)) and non enzymatic roles (cell migration, adhesion (21), and growth (1)). However the mechanism by which TG2 is trafficked to the extracellular space remains unknown. The normal secretory pathway for proteins through the endoplasmic reticulum, Golgi apparatus and plasma membrane to the extracellular environment (22) requires the protein to have a leader sequence. TG2 has no leader sequence, therefore, cannot be exported via the Golgi apparatus (23-24). Recent studies have

highlighted several molecules such as fibroblast growth factor-1 and -2 (25) that like TG2 are exported without a leader sequence. Therefore TG2 must be trafficked to the cell surface by an alternative (or unconventional) export mechanism. This mechanism would likely rely on specific motifs within the TG2 molecule that are essential to this process.

The aim of this study is to use deletion and point mutation studies to identify crucial elements within the TG2 molecule required for its export from renal tubular epithelial cells that may give an insight to the export pathway. This will provide new targets for TG2 specific interventional strategies in treating scarring & fibrosis.

## MATERIALS AND METHODS

### *cDNA & vectors*

All constructs were prepared by PCR using the human TG2 cDNA as a template (26) employing high fidelity Pfu turbo polymerase (Stratagene, UK). Primers for the domain deletion and point mutation constructs are shown in Table 1. PCR products were initially inserted in the pENTR Directional TOPO vector (Invitrogen, UK) before transfer to the pCDNA<sup>TM</sup> 6.2/nTC-Tag-DEST tetra cysteine tag Vector (Invitrogen, UK). The pFC14K CMV Flexi Halotag vector and HaloTag® Biotin Ligand were obtained from Promega (UK). The identity of all cDNA constructs was confirmed by DNA sequencing (Core Genomic Facility of the University of Sheffield) using a T7 Promoter primer and TK poly A Reverse primer.

### *Cell culture*

OK cells (27) and rat NRK-52E tubular epithelial cells (28) were obtained from the European Cell Culture Collection. Canine MDCK II cells were a gift from N. Simmons (University of Newcastle,

Newcastle, UK). All cells were grown in complete DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100µg/ml streptomycin at 37°C, 5% CO<sub>2</sub> atmosphere. To determine basolateral or apical TG2 trafficking, cells were grown in 6-well culture inserts (0.4 µm pores, Transparent PET Membrane, Falcon, UK). The culture medium in the inserts represents apical TG2 secretion and outside the insert represents basolateral TG2 secretion.

#### *Cell transfection*

Constructed cDNA were transfected in renal epithelial cells using Amaxa Nucleofector (Amaxa, Cologne, Germany). All transfections were performed using the Ingenio electroporation solution (Geneflow, UK). Optimised programs were A20 for Opossum proximal tubule cells (OK), L-05 for MDCK II and X-01 for NRK-52E cells with 5µg of selected vectors. This approach ensured similar levels of transfected TG2 constructs were delivered to each cell line and thus comparable increases in TG2 seen in each cell line. The cells were grown in a 6-well plate post transfection and incubated at 37°C for 48 hours before analysis. 5µg of vector was selected for transfection as it produced TG2 levels within the linear range of TG2 assays (Supplementary Figure 1A) Stable transfectants were selected by blasticidin resistance (Invitrogen)

#### *Preparation of cell lysates*

Cells were trypsinised, centrifuged at 300g for 2 mins and homogenised by sonication in 100µl of STE buffer (0.32M sucrose, 5mM Tris, 2mM EDTA, pH 7.5) containing protease inhibitors [1 mM leupeptin, 1 mM benzamidine and 1 mM phenylmethylsulphonyl fluoride (PMSF)].

#### *Preparation of conditioned medium*

48 hours after transfection, medium was then

replaced with 1ml of serum free medium. The medium was collected after 4 hour incubation and centrifuged at 10,000g. The supernatant was used for analysis.

#### *Total transglutaminase activity in cell lysate and medium*

Total TG2 activity was measured by the incorporation of Biotinylated TVQQEL peptide (a unique TG2 substrate) into casein using a modified ELISA described by Trigwell (29). 96 well plates were precoated with 1mg/ml casein in 50mM sodium carbonate (pH 7.5), blocked with 250µL of blocking solution [0.1% BSA in 50mM sodium carbonate] at 37°C for 1 hour. The reaction started by mixing 50 µl of cell lysates or medium with 150µL of reaction buffer (13.3mM dithiothreitol, 6.7mM CaCl<sub>2</sub>, 10µM biotin-TVQQEL in 100mM Tris-HCl pH 8.5) at 37°C for 1 hour. 200µl of diluted Extravidin-peroxidase (1:5000) in blocking solution was added and incubated for 1 hour at 37°C. The colour was revealed with 200 µl of TMB solution. The colour development was stopped with 50µl of 2.5M H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450nm in a Labsystems Multiskan Ascent plate reader using Genesis Software (version 3.05). The TG activity was calculated in units of TG activity from a TG2 vs absorbance at 450nm standard curve (Supplemental Figure 1B) obtained from Guinea pig liver Transglutaminase (T006, Zedira).

#### *Immunoblotting*

The protein concentrations of cell lysates and medium was determined using the bicinchoninic acid (BCA) assay. Lysates (20µg) or 20µl of medium were diluted with reducing buffer, incubated in a boiling water bath for 5 min, electrophoresed on 5% (fibronectin) or 7.5% (TG2) SDS-polyacrylamide gels, transferred to nitrocellulose and probed with either anti-tTG

(cub7402, ab2306, abcam, UK) or anti-fibronectin (ab6328, abcam, UK) antibodies.  $\beta$ -actin (abcam, UK) was used as a loading control. Antibody binding was revealed using a polyclonal anti-mouse horseradish peroxidase-conjugated antibody (P0447, Dako) and developed using enhanced chemiluminescence (ECL) (BD, UK). For the measurements of TG2 in culture medium, the film was exposed for at least 1 hour

#### *Assay of Extracellular transglutaminase activity*

Extracellular TG activity was measured by the incorporation of biotinylated cadaverine into fibronectin as previously described (30). Briefly, cells were harvested after 48 hours post transfection using EDTA in PBS.  $8 \times 10^4$  cells were resuspended in 100 $\mu$ l serum-free DMEM containing 0.1 mM biotinylated cadaverine (Molecular Probes, Netherlands) & placed into a 96 well plate precoated with fibronectin at 37°C for 1 hour. The reaction was stopped by 5 mM EDTA in PBS. The cells were lysed using 100 $\mu$ l of 0.1% (w/v) sodium deoxycholate in PBS with 5 mM EDTA. The wells are blocked with 3% (w/v) BSA, followed incubation with Extravidin-peroxidase (1:5000). Incorporated biotinylated cadaverine was revealed using TMB. The resulting colour was then read in an ELISA plate reader at 450nm as above and represents the extracellular/cell surface TG2 activity. Assay linearity was demonstrated by a TG2 standard curve produced by applying rhTG2 to wells containing no cells (Supplementary Figure 1C).

#### *Detection of extracellular-matrix bound TG2 antigen*

Extracellular TG2 antigen was measured using a modified quantitative ELISA adapted from Achyuthan et al (31) based on the ability of TG2 to adhere to fibronectin.  $8 \times 10^4$  cells per well were plated into a fibronectin coated 96-well plate.

Cells were incubated in serum-free DMEM for 2 hours at 37°C and lysed with 0.1% deoxycholate in 5 mM EDTA. The remaining fibronectin layer containing TG2 was blocked with 3% BSA, followed by incubation with anti-TG2 monoclonal antibody (cub7402, abcam 1:500) at 4°C overnight. After 0.1% Tween 20 wash, the wells were incubated with HRP conjugated goat anti-mouse IgG (1:1000, Dako, Denmark) at 37°C for 1 hour. The colour was revealed by TMB and read at 450nm and represents the extracellular/cell surface TG2 antigen Assay linearity and range was shown using a standard curve generated by the addition of rhTG2 to wells containing no cells (Supplementary Figure 1D).

#### *Fibronectin knock-down by siRNA*

The cells were cotransfected with 5 $\mu$ g DNA with 300nM of nonsense siRNA or anti-fibronectin 1 siRNA (Applied Biosystems, UK) with TG2 cDNA using the Amaxa Nucleofector. The TG activity of the cell lysate, extracellular TG activity, antigen and TG activity in the culture medium were measured 48 hours after transfection and the knock down of fibronectin in the cell lysate was determined by 7.5% SDS-PAGE gels in reducing buffer followed by western blotting for fibronectin.

#### *Tracking TG2 with the FIAsh-EDT2 reagent and co-localisation analysis*

To visualise TG2 transport, NRK52E cells were grown on sterilised microscope slide coverslips and incubated with 2.5 $\mu$ M FIAsh-EDT<sub>2</sub> reagent (Invitrogen, UK) or CellMask plasma membrane staining as required (C10046, invitrogen). The coverslip was rinsed in PBS and fixed with 4% paraformaldehyde. For co-localisation dual immunofluorescent staining was performed using the following antibodies at a 1:300 dilution; monoclonal anti-human collagen IV (C1926, Sigma, UK); rabbit polyclonal human calnexin

(endoplasmic reticulum) (C4731, Sigma, UK); monoclonal anti-Golgi 58K protein (G2404, Sigma, UK) & monoclonal anti-LAMP2 (lysosomes) (SAB1402250, Sigma, UK). Secondary antibodies were polyclonal anti-rabbit IgG-TRITC (T5268, Sigma, UK) and anti-mouse IgG-TRITC at 1:500 (T5393, Sigma, UK). Images were acquired on an Olympus BX-61 microscope using Cell-F software. Where applicable images were deconvolved using AutoQuant X2 (Media Cybernetics, Silver Spring, MD, USA) and 3D-rendered using Imaris (Bitplane AG, Zurich, Switzerland).

#### *Site-directed mutagenesis*

The QuickChange Site-Directed Mutagenesis kit with Pfu turbo polymerase (Stratagene, UK) was used as per manufacturer's instructions. The forward and reverse primers were designed as previously described (32). PCR was performed for 12 cycles for 1 point mutation and 18 cycles for two point mutations.

#### *Halotag protein measurements*

Halotag protein was measured by ELISA according to manufacturer's instructions. Briefly, 50µl of cell lysate or culture medium was plated into 96-well plate and incubated at 37°C for 1hr. After three washes of 1% of BSA, anti-Halotag pAb (1:500 G9281, Promega, UK) was added in the well and incubated for 1 hr at 37°C. Following three washes of 1% BSA, anti-rabbit immunoglobulins/HRP (1:1000) was added and incubated for 1hr at 37°C. The anti-Halotag protein pAb and anti-rabbit immunoglobulins/HRP binding was revealed by TMB and read at 450nm.

#### *Statistical analysis*

The results were presented as mean±SEM and analysed using one-way analysis of variance followed by a Bonferroni post hoc test. A p value

less than 0.05 was considered statistically significant.

## **RESULTS**

We engineered five constructs expressing domain deletion mutants of TG2 (3D construct images are shown in Supplemental Figure 2): TG2 lacking the β-sandwich domain (-bsw); the β-barrel 2 domain (-b2); both β-barrel domains (-b1b2); the β-sandwich and β-barrel 2 domains (-bswb2); the β-sandwich, β barrel 1 & 2 domains leaving just the TG2 core domain (core). Each construct was transiently transfected in three different tubular epithelial cell lines (TEC) (OK, NRK-52E, MDCK-II) and the externalisation of the mutated TG2 quantified. The standard curve for each assay used in this study and TG load according to different amount of vector transfected are shown in Supplement Figure 1.

#### *Deletion analysis - Total TG2 activity & antigen*

Post transfection, western blotting for TG2 in cell lysates demonstrated comparable increases in TG2 with all constructs as well as confirming appropriate sized proteins were being produced (Figure 1 A-C). TG activity in the cell lysates (Figure 1 D-F) also increased equally for all constructs within each cell line. This indicated comparable transfection efficiency and that all domain deletions retained TG activity. Subsequently TG2 transport could be tracked using extracellular TG activity assays.

#### *Deletion analysis – Extracellular TG activity and TG2 antigen*

Extracellular TG activity increased between 3 & 4 fold in all 3 cell lines transfected with full length TG2 (Figure Legends

### **Figure 1. Intra and extra-cellular TG2 levels following domain deletion**

OK, NRK52E and MDCK II renal epithelial cells

were transfected with the following constructs 1) full length tissue transglutaminase (TG2), 2) TG2 with  $\beta$ -sandwich domain deleted (-bsw), 3) TG2 with the 2 barrel deleted (-b2), 4) TG2 with  $\beta$ -barrel 1 and  $\beta$ -barrel 2 domains deleted (-b1b2), 5) TG2 with both  $\beta$ -sandwich and  $\beta$ -barrel 2 domains deleted (-bwb2) and finally 6) TG2 catalytic core (core). The expression of TG2 is shown by western blots (WB) in cell lysates 48 hrs post transfection (A-C). Total TG activity in cell lysates (D-F), extracellular TG activity (G-I), WB for TG2 in culture medium (J-L), and TG activity in culture medium (M-O) was measured, wt indicates non-transfection control. Data represents mean values  $\pm$ SEM, n=5, \*= p<0.05.

**Figure 2. Affect of TG2 export of removing the amino acid 1-7 fibronectin binding site**

OK, NRK52E and MDCK II renal epithelial cells were transfected with full length TG2 (tg) or mutant TG2 lacking the first 7 amino acid of  $\beta$ -sandwich domain missing that constitute a fibronectin binding site (tg-7a.). Western blots (WB) for TG2 in cell lysates post transfection are shown in A. Total TG activity in cell lysates (B), extracellular TG activity (C), WB for TG2 in culture medium (D) and TG activity in culture medium (E) was measured, wt indicates non-transfection control. Data represents mean values  $\pm$  SEM n=5, \*: p<0.05.

**Figure 3. Intra and extra-cellular TG2 levels following point mutation**

OK, NRK52E and MDCK II renal epithelial cells were transfected with either 1) full length TG2 (tg), 2) single point mutation of Asp<sup>94</sup> to Ala (f1), 3) single point mutation of Asp<sup>97</sup> to Ala (f2), or 4) a two point mutation of TG2 at Asp<sup>94</sup> & Asp<sup>97</sup> to Ala (f3). TG2 expression is shown by Western blots

(WB) for TG2 in cell lysates post transfection (A-C). Total TG activity in cell lysates (D-F), extracellular TG activity (G-I), WB for TG2 in culture medium (J-L) and TG activity in culture (M-O) medium was measured. Wt indicates non-transfection control. Data represents mean values  $\pm$ SEM. n=5, \*\* p<0.001, \*p<0.05)

**Figure 4. Intra and extra-cellular TG2 levels in NRK52E cells stably transfected with mutant and full length TG2**

NRK52E renal epithelial cells were selected by blasticidin resistance after transfection with either wild type TG2 (tg), TG2 with  $\beta$ -sandwich domain deleted (-bsw) or a two point mutation of TG2 at Asp<sup>94</sup> & Asp<sup>97</sup> to Ala (f3). Western blots for TG2 in cell lysates are shown in A and TG2 in culture medium in B. Total TG activity in cell lysate (C), extracellular TG activity (D) and TG activity in medium (E) were measured.

**Figure 5. Intra and extra-cellular TG2 levels following fibronectin knockdown**

OK, NRK52E and MDCK II renal epithelial cells were co-transfected with TG2 plus either anti-fibronectin siRNA (tg-f) or non-sense siRNA (tg-ns). Western blots (WB) was used to show basal fibronectin level in the cell lysate 48 hours after transfection (A) with  $\beta$ -actin as a loading control. Value in parenthesis shows volume density measurements as a percentage of the fibronectin knockdown. Total TG activity in cell lysates (B), extracellular TG activity (C), extracellular TG2 antigen (D) and TG activity in culture medium (E) was measured, wt indicates non-transfection control. n=5, \*: p<0.05.

**Figure 6. Wild type TG2 and mutant TG2 fluorescent and cell organelle imaging**

NRK52E cells were transfected using a tetra cysteine -Tag vector carrying either full length TG2 (TG2) or mutant TG2 with  $\beta$ -sandwich domain deleted (-bsw). Wild-type with Cellmask plasma membrane stain (A, wt), tetra cysteine tagged TG2 (B, +tg2) and TG2 with  $\beta$ -sandwich missing (C, +tg2-bsw) was labeled post transfection with FAsH reagent staining the proteins green and plasma membrane red. White arrows show intracellular TG2 staining and yellow arrows show extracellular TG2 staining. To determine the direction of TG2 export, TG2 transfected cells were co-stained with FAsH and collagen IV (red). The 3D images were generated and apical (C), side (D) and basal views (E) acquired. To assess co-localization with cell organelles TG2 transfected cells were co-stained with organelle (red) for endoplasmic reticulum (ER) (F) Golgi apparatus (G) and lysosomes (H). Organelles are indicated by red arrows. In panel G, the green arrow shows peripheral TG2 (green) and orange arrow shows TG2 around the cell nucleus that is co-localised with ER (orange). Cell nuclei are stained blue with DAPI.

**Figure 7. TG2 is exported across the basolateral membrane.**

OK, NRK52E & MDCK II tubular epithelial cells were transfected with TG2 and plated in cell culture well inserts. TG activity of the culture medium below the insert (b, basolateral) and within the insert (a, apical) was measured in the culture medium. Data represents mean TG activity in Units per mg of protein  $\pm$ SEM. \*= $p < 0.05$ , n=5.

**Figure 8. Can the TG2 export motif target other proteins for cell export?**

The TG2  $\beta$ -sandwich domain (bsw), TG2 core domain (core) and the TG2 export motif 88-106

(motif) were placed into the halotag vector pFC14K CMV. 48 hours post transfection the amount of halotag labeled protein found in the culture medium and in cell lysates was assessed by anti-Halotag pAb. L:cell lysate, M:culture medium. Data represents mean halotag levels  $\pm$  SEM. n=5.

**Figure 9. Possible TG2 extracellular trafficking pathways and protein structure of  $\beta$ -sandwich domain**

(A) Cartoon showing the known unconventional protein transmembrane transport routes. 1: Lysosome route, 2: protein channel / flip protein, 3: Endoplasmic reticulum budding, 4: membrane blebbing. ER: endoplasmic reticulum, = protein for export). (B) Protein structure of the TG2  $\beta$ -sandwich domain showing the TG2 export motif (amino acids 88-106) lightly shaded with  $\beta$ 5-strand and  $\beta$ 6-strands labeled. Black indicates Asp<sup>94</sup> and Asp<sup>97</sup>.

**Table 1. List of primers sequences.**

Forward and reverse primers used to generate TG2 deletions and point mutations. The constructs were shown in 3D images.

**Figure 1** G-I) indicating TG2 is readily trafficked to the extracellular environment after transfection in TECs. Similarly, cells transfected with TG2 cDNA with  $\beta$ -barrel 2 (-b2) and both  $\beta$ -barrel 1 &  $\beta$ -barrel 2 domains missing (-b1b2) had the same levels of extracellular TG activity to that of full length TG2 in all 3 cell lines suggesting that  $\beta$ -barrel domains played no role in TG2 externalisation. Measurement of extracellular TG2 antigen in the media by western blotting confirmed constructs with  $\beta$ -barrel 2 (-b2) and both  $\beta$ -barrel 1 &  $\beta$ -barrel 2 domains missing (-b1b2) can be exported comparably to full length TG2 (Figure 1 J-L).

However, when cells were transfected with constructs in which the  $\beta$ -sandwich domain was missing (i.e. -bsw, -bwb2, and just core), extracellular TG activity was similar to that in cells that had not been transfected. This suggests that the transfected deletion mutant of TG2 is not being trafficked & that  $\beta$ -sandwich domain is crucial for TG2 transport.

As it is possible that removing a domain may not be affecting TG2 transport, but simply its ability to localise to the cell surface or ECM which the extracellular TG activity assay used predominantly measures, we repeated measurements of TG activity and antigen in the culture media to ensure TG2 was not “floating” in the medium post transport. In all 3 tubular epithelial cells, the TG2 fraction found in the medium mirrored measurements of extracellular TG activity both in terms of activity (Figure Legends

**Figure 1. Intra and extra-cellular TG2 levels following domain deletion**

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with the 2 barrel deleted (-b2), 4) TG2 with  $\beta$ -barrel 1 and  $\beta$ -barrel 2 domains deleted (-b1b2), 5) TG2 with both  $\beta$ -sandwich and  $\beta$ -barrel 2 domains deleted (-bwb2) and finally 6) TG2 catalytic core (core). The expression of TG2 is shown by western blots (WB) in cell lysates 48 hrs post transfection (A-C). Total TG activity in cell lysates (D-F), extracellular TG activity (G-I), WB for TG2 in culture medium (J-L), and TG activity in culture medium (M-O) was measured, wt indicates non-transfection control. Data represents mean values  $\pm$ SEM, n=5, \*= p<0.05.

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Forward and reverse primers used to generate TG2 deletions and point mutations. The constructs were shown in 3D images.

**Figure 1 M-O)** and antigen (Figure 1 J-L) confirming that none of the constructs with  $\beta$ -sandwich domain missing can be detected in the medium.

#### *Mutation analysis*

A previous report by Griffin and colleagues (33) on TG2 trafficking in transfected COS-7 cells had suggested that a fibronectin site encompassing the first 7 amino acids of the TG2  $\beta$ -sandwich domain was crucial for TG2 export. To validate this in TEC we deleted this site, transfected the construct and assessed TG2 export as above. The construct (tg-7a) was transfected with equal efficiency to full length TG2 (tg) and retained full activity (Figure 2 A & B). However the loss of the fibronectin binding site failed to prevent TG2 export (Figure 2, C-E) suggesting that it is not important in TG2 export in tubular epithelial cells.

Examination of the literature revealed a second fibronectin binding site ( $^{88}\text{WTATVVDQDCTLSLQLTT}^{106}$ ) in the  $\beta$ -sandwich domain of TG2 (32), although this had not been implicated in TG2 trafficking. Subsequently we introduced 2 point mutations (Asp<sup>94</sup> & Asp<sup>97</sup>) in this binding domain that had previously been shown to disable its binding to fibronectin (32). Constructs containing either 1 or both mutations were equally expressed (f1, f2 & f3) and had the same level of TG activity in the cell lysate to full length TG2 (Figure 3 A-F). However single mutation of either Asp<sup>94</sup> or Asp<sup>97</sup> into Ala significantly decreased extracellular TG activity and antigen by 50% ( $p < 0.05$ ), while two point mutation of Asp<sup>94</sup> and Asp<sup>97</sup> totally prevented TG2 export ( $p < 0.05$ ) (Figure 3 G-I) including TG2 in the media (Figure 3 J-O). This indicates this second fibronectin binding domain is critical for TG2 trafficking in TECs.

#### *Affect of deletion and mutation of the TG2 export*

#### *sequence in stably transfected NRK 52E cells*

To confirm the results from deleting and mutation analysis, cloned, stably transfected NRK52E cells containing full length TG2, TG2 with  $\beta$ -sandwich domain missing (-bsw) and 2 point mutations (Asp<sup>94</sup> & Asp<sup>97</sup>, f3) were selected using blasticidin resistance. The expression of TG2 antigen (Figure 4A) and activity (Figure 4B) in cell lysates shows stable and comparable incorporation of all constructs. Cells expressing TG2 with the  $\beta$ -sandwich domain missing (-bsw) and 2 point mutations (f3) had no detectable TG2 antigen or activity outside the cell (Figure 4 C-E) whereas cells expressing full length TG2 did. This data is wholly consistent with the transient transfections used previously.

#### *TG2 Co-transport with fibronectin*

To assess if TG2 is simply co-transported with fibronectin given the crucial export sequence has been reported as a novel fibronectin binding site, we knocked down fibronectin using co-transfection of anti-fibronectin siRNA with full length TG2 (tg-f) in to the cells. Fibronectin knockdown was confirmed by western blot analysis (Figure 5A). Volume densitometry measurements indicated that fibronectin was decreased by over 80% compared to fibronectin levels in cells co-transfected with nonsense siRNA and TG2 cDNA (tg-ns) in all three cell lines used. Measurement of extracellular TG activity, antigen and TG activity in culture medium was identical irrespective of fibronectin knockdown (Figure 5 B-E). Therefore, TG2 externalisation appears to be independent of cell-secreted fibronectin and thus TG2 is not co-transported with fibronectin.

#### *Possible TG2 externalisation pathways*

Transfection with tetracysteine tagged TG2 (nTC-Tag-DEST) allowed the use of FIAsh staining to colour TG2 green under fluorescence so its

movement could be tracked in the cell. This was used in combination with Cellmask (red) to define the plasma membrane. TG2 in cells transfected with full length TG2 and TG2 lacking the  $\beta$ -sandwich can clearly be seen inside the cell possibly in vesicles (Figure 6 B & C white arrow) with nothing visible in non transfected cells (Figure 6A). Following transfection of full length TG2, green staining can also be seen between the cells clearly in the extracellular space (Figure 6 B, yellow arrow) as well as in the plasma membrane which appears orange which some intense yellow spots due to the co-localisation of green and red fluorescents. Neither of these are seen in with the  $\beta$ -sandwich domain deleted. This imaging is consistent with the biochemical measurements of TG activity and antigen both in and out of the cell. To visualise the direction of TG2 transport, we used collagen IV (red) as a basolateral marker. Following deconvolution microscopy and 3D rendering, TG2 (green) was not visible from above (Figure 6D, apical view), just visible at the bottom of the cells from the side (lateral view, Figure 6E), while basal views from underneath clearly show TG2 in the same plane as collagen IV (Figure 6F). TG2 is therefore being exclusively exported to basolateral side of the cells with the most abundant secretion of TG occurring directly under the nucleus.

Co-localisation of TG2 (yellow, orange arrow) with endoplasmic reticulum (ER, red arrow) (Figure 6 G) showed that there was some co-localisation of ER with TG2 around the cell nucleus (orange arrow), however no peripheral TG2 (green arrow) was co-localised with ER and no ER at all was seen near the periphery of these cells. Overall 28% of the transfected TG2 was co localised with the ER. There was only 0.3% co-localisation of TG2 (green) with Golgi apparatus

(red) (Figure 6H) and only 0.2% co-localisation with lysosomes red (Figure 6I) suggesting that TG2 is not externalised through Golgi or lysosomes dependant routes.

#### *Direction of TG2 secretion*

To confirm if TG2 was being transported basolaterally (ie towards the tubular basement membrane) rather than apically (i.e. into the lumen), cells were grown in cell culture well inserts. Culture medium inside the insert represented apical secretion of TG2 and had a TG activity below the detection limit of the assay. In contrast, TG activity was 4 mIU/mg of protein in the culture medium outside the cell culture insert indicating basolateral TG secretion to the basement membrane (Figure 7).

#### *Transport Motif*

To access if the motif 88-106 itself is enough to target proteins for externalisation in tubular epithelial cells, we inserted this motif, the  $\beta$ -sandwich domain and the core domain (not transported) in to a HaloTag vector and measured HaloTag protein levels in the cell lysate and culture medium (Figure 8). The HaloTag ligand in the cell lysates was similar between all 3 constructs indicating similar transfection efficiency and production. In contrast, when the halotag ligand was measured in culture medium, halotag linked to the TG2 export motif 88-106 and the TG2  $\beta$ -sandwich domain were significantly higher than that of core domain ( $p < 0.05$ ) construct suggesting the motif 88-106 is capable of targeting proteins for externalisation independently of the rest of the  $\beta$ -sandwich or TG2.

## **DISCUSSION**

In this study, we found that the N-terminal  $\beta$ -sandwich domain and in particular the amino acid sequence 88-106 within the  $\beta$ -sandwich domain is

crucial for TG2 externalisation in TECs. This finding is consistent across all three cell lines used with extracellular transglutaminase assessed using three different assays. Therefore one can conclude this is not a cell line specific affect or an artefact of a particular assay. As the identified sequence is not found in any other TG, targeting this may provide a novel approach to interfere with the extracellular trafficking of TG2 seen in renal fibrosis.

In performing these studies we initially expected that the cells transfected with TG2 mutants that were not exported would lead to higher TG levels in the cell lysate. However, practically no difference in total TG activity/ antigen in the cell lysate was found despite the clear differences in TG2 outside the cell. This data initially appeared contradictory, however by calculating the total amounts of TG2 in intracellular and extracellular compartments (Supplementary Figure 3) rather than per mg of protein it became evident that extracellular TG2 is only about 5% of total TG in the cell lysate and thus such small relative changes in intracellular TG2 levels would unlikely to be detectable. However when total intracellular and extracellular TG2 per well was calculated, 2 of the 3 cell lines do report higher mean intracellular values when transfected with non-exported TG2 constructs, although the differences remain too small to reach statistical significance despite the multi-fold changes in extracellular TG2.

We chose to track TG2 by both antigen and activity routes to ensure robust data. Central to this was ensuring the mutants did not have altered TG activity by measuring the activity in cell lysates. To confirm this we also produced a TG2 core protein which displayed identical activity to wild type TG2 confirming that even with all other domains removed the TG2 core retained its

activity.

Visualisation of cells transfected with a non-exported mutant of TG2 showed some potential qualitative differences in addition to those shown in the figures which require further study. For example when TG2 was not exported the intracellular TG2 tended to accumulate in larger and more dense vesicle type structures closer to the nucleus. In addition prior to confluency cells transfected with non-exported mutants do not flatten to the same extent as those with wild type TG2 and thus appear to have a smaller diameter. This likely relates to TG2s extracellular role in cell adhesion which is well known to cause this affect on morphology..

In the literature, four possible pathways had been proposed for un-conventional protein secretion (Figure 9A) (25). Proteins that are passed to the extracellular environment can be secreted through 1) lysosomes, 2) a direct molecular trap, 3) exosomes derived from multivesicular bodies and 4) plasma membrane blebbing. Interleukin 1 $\beta$  is one of the unconventional secretory proteins linked to intracellular vesicles (34). The vesicles are positive for Lamp-1 and cathepsin D that are both classical markers for lysosomes (35). In co-localisation studies, TG2 was not co-localised with lysosomes and therefore lysosome transport is unlikely to be involved in TG2 externalisation. Increase sodium tolerance protein (Ist2) is another protein that passes through the plasma membrane without ER-to-Golgi transport (36). The newly synthesised Ist2 can be visualised by fluorescent microscopy and localised with ER at peripheral patches near the plasma membrane (37). We found that 30% of the ER stain was co-localised with TG2 (Figure 6 G), however this was around the nucleus and consistent with TG2 synthesis at the ribosomes on the ER membrane, most likely in ER

bound polyribosomes. Neither ER (Figure 9A-3) nor lysosomes (Figure 9A-1) were co-localised with TG2 in vesicles around the sub-plasma membrane area. This coupled with a lack of TG2 being seen in plasma membrane blebs (Figure 9A-4) or exovesicles by fluorescent microscopy. TG2 externalisation is mostly like linked to a direct molecular trap mechanism (Figure 9A-2),

Fibroblast growth factor (FGF-2) is a protein without a classical N-terminal signal peptide, its transmembrane transport is dependent on the cell surface heparan sulphate proteoglycans (HSPGs) and the transport involves a direct molecular trap on the cell surface (38). TG2 externalisation has been recently related to the presence of cell surface HSPGs such as syndecan-4 (39) in primary skin fibroblasts as TG2 is known to bind heparin (40) and heparan sulphate (39). It remains to be established if this pathway is relevant to TEC and whether amino acids 88-106 that make up the TG2 trafficking motif are critical for heparan sulphate binding (41). The accumulation of TG2 under the plasma membrane in the  $\beta$ -sandwich deletion and TG2 trafficking motif mutants would be consistent with such an export mechanism as would the identification of the TG2 export motif which is an absolute requirement for its export. Subsequently identifying the proteins interacting with this export motif will be essential in determining the precise nature of the molecular trap mechanism and confirming this as a route for TG2 export.

Gaudry and colleagues (33) studied the interaction of fibronectin and TG2 using confocal microscopy co-localisation studies. These experiments were performed in TG2 transfected Cos-7 cells with cell surface TG2 determined using a  $\beta$ -galactosidase assay. They found that the first 7 amino acids in the N-terminal  $\beta$ -sandwich domain were important

for cell surface TG2 with no binding to fibronectin if removed. In contrast, we report in TEC that the removal of first 7 amino acid of the TG2  $\beta$ -sandwich domain did not stop TG2 externalisation. These discrepancies could simply mean that TEC handle TG2 export differently to fibroblasts. Of relevance both Gaudry and ourselves have identified perceived fibronectin binding sites as important in TG2 trafficking (even though fibronectin does not appear crucial for export). However later work by Hang et al (32), showed that the removal of 1-16 amino acid of N-terminal  $\beta$ -sandwich domain did not affect TG2 binding to fibronectin. The 86 to 106 amino acid motif we have identified as crucial for TG2 export is an interesting part of the  $\beta$ -sandwich forming a  $\beta 5/\beta 6$  hairpin that is exposed on the surface of TG2. Asp<sup>94</sup> and Asp<sup>97</sup> are crucial for the  $\beta 5/\beta 6$  hairpin to form (Figure 9B) and subsequently the 3 dimensional folding here is also critical TG2 for export. By adding a single mutation that would partially collapse the loop we could partially prevent export relating export to structure. Simply attaching this amino acid sequence to the C terminal of another protein (e.g. Halotag) seems to target it for export which confirms that it is the part of TG2 required for TG2 trafficking. There have been several hypotheses suggesting that TG2 could be co transported with fibronectin, however our 80% knockdown of fibronectin by siRNA had no effect on TG2 extracellular trafficking. This not only contradicts these hypothesis but also implies that the amino acid sequence 88-106 must have additional binding partners and/or properties. It is likely that this TG2 export motif can bind to other proteins that contain a fibronectin like domain and that this can act as a transmembrane binding partner.

Using bio-informatics we have confirmed that the

sequence <sup>88</sup>WTATVVVDQQDCTLSLQLTT<sup>106</sup> is specific to TG2. Two very similar sequences were identified in two mammalian cell membrane proteins. The first is the P2X purinoceptors family (15 of 19 amino acids in common with TG2). These are cell membrane ion channels and are activated by ATP (42-44). The other is Semaphorin/CD100 antigen (16 of 19 amino acids in common with TG2). This belongs to a large family of secreted and transmembrane proteins that function as repellent signals during axon guidance (45-47). Semaphorin, in particular is interesting as it can be secreted and thus may have some common elements with TG2 export. However little is known about Semaphorin export. Perhaps unsurprisingly, we found that TG2 is transported basolaterally into what would be the tubular basement membrane *in vivo*. This finding is consistent with TG2s function in cell adhesion process and its role in ECM deposition and stabilisation (9) that becomes pathological in

fibrosis (15). In terms of understanding the process of TG2 trafficking then this may help if differences can be identified between the basal and apical membranes in terms of potential transmembrane proteins or channels (9).

In conclusion, TG2 is a protein without a leader sequence. Its transport to the extracellular environment is important in the development of renal fibrosis. This export mechanism is restricted to the basolateral membrane and wholly dependent on the 88-106 amino acid sequence within NH3-terminal  $\beta$ -sandwich domain. Even though this has motif has fibronectin binding properties, export is independent of fibronectin. These findings are consistent across all tubular epithelial cells tested. Targeting this motif pharmacologically could “lock” TG2 in the cell preventing its detrimental functions in chronic kidney disease.

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## Figure Legends

### Figure 1. Intra and extra-cellular TG2 levels following domain deletion

OK, NRK52E and MDCK II renal epithelial cells were transfected with the following constructs 1) full length tissue transglutaminase (TG2), 2) TG2 with  $\beta$ -sandwich domain deleted (-bsw), 3) TG2 with the 2 barrel deleted (-b2), 4) TG2 with  $\beta$ -barrel 1 and  $\beta$ -barrel 2 domains deleted (-b1b2), 5) TG2 with both  $\beta$ -sandwich and  $\beta$ -barrel 2 domains deleted (-bwb2) and finally 6) TG2 catalytic core (core). The expression of TG2 is shown by western blots (WB) in cell lysates 48 hrs post transfection (A-C). Total TG activity in cell lysates (D-F), extracellular TG activity (G-I), WB for TG2 in culture medium (J-L), and TG activity in culture medium (M-O) was measured, wt indicates non-transfection control. Data represents mean values  $\pm$ SEM, n=5, \*= p<0.05.

### Figure 2. Affect of TG2 export of removing the amino acid 1-7 fibronectin binding site

OK, NRK52E and MDCK II renal epithelial cells were transfected with full length TG2 (tg) or mutant TG2 lacking the first 7 amino acid of  $\beta$ -sandwich domain missing that constitute a fibronectin binding site (tg-7a.). Western blots (WB) for TG2 in cell lysates post transfection are shown in A. Total TG activity in cell lysates (B), extracellular TG activity (C), WB for TG2 in culture medium (D) and TG activity in culture medium (E) was measured, wt indicates non-transfection control. Data represents mean values  $\pm$  SEM n=5, \*: p<0.05.

### Figure 3. Intra and extra-cellular TG2 levels following point mutation

OK, NRK52E and MDCK II renal epithelial cells were transfected with either 1) full length TG2 (tg), 2) single point mutation of Asp<sup>94</sup> to Ala (f1), 3) single point mutation of Asp<sup>97</sup> to Ala (f2), or 4) a two point mutation of TG2 at Asp<sup>94</sup> & Asp<sup>97</sup> to Ala (f3). TG2 expression is shown by Western blots (WB) for TG2 in cell lysates post transfection (A-C). Total TG activity in cell lysates (D-F), extracellular TG activity (G-I), WB for TG2 in culture medium (J-L) and TG activity in culture (M-O) medium was measured. Wt indicates non-transfection control. Data represents mean values  $\pm$ SEM. n=5, \*\* p<0.001, \*p<0.05)

### Figure 4. Intra and extra-cellular TG2 levels in NRK52E cells stably transfected with mutant and full length TG2

NRK52E renal epithelial cells were selected by blasticidin resistance after transfection with either wild type TG2 (tg), TG2 with  $\beta$ -sandwich domain deleted (-bsw) or a two point mutation of TG2 at Asp<sup>94</sup> & Asp<sup>97</sup> to Ala (f3). Western blots for TG2 in cell lysates are shown in A and TG2 in culture medium in B. Total TG activity in cell lysate (C), extracellular TG activity (D) and TG activity in medium (E) were measured.

### Figure 5. Intra and extra-cellular TG2 levels following fibronectin knockdown

OK, NRK52E and MDCK II renal epithelial cells were co-transfected with TG2 plus either anti-fibronectin siRNA (tg-f) or non-sense siRNA (tg-ns). Western blots (WB) was used to show basal fibronectin level in the cell lysate 48 hours after transfection (A) with  $\beta$ -actin as a loading control. Value in parenthesis shows volume density measurements as a percentage of the fibronectin knockdown. Total TG activity in cell lysates (B), extracellular TG activity (C), extracellular TG2 antigen (D) and TG activity in culture medium (E) was measured, wt indicates non-transfection control. n=5, \*: p<0.05.

### Figure 6. Wild type TG2 and mutant TG2 fluorescent and cell organelle imaging

NRK52E cells were transfected using a tetra cysteine -Tag vector carrying either full length TG2 (TG2) or mutant TG2 with  $\beta$ -sandwich domain deleted (-bsw). Wild-type with Cellmask plasma membrane stain (A, wt), tetra cysteine tagged TG2 (B, +tg2) and TG2 with  $\beta$ -sandwich missing (C, +tg2-bsw) was labeled post transfection with FIASH reagent staining the proteins green and plasma membrane red. White arrows show intracellular TG2 staining and yellow arrows show extracellular TG2 staining. To determine the direction of TG2 export, TG2 transfected cells were co-stained with FIASH and collagen IV (red). The 3D images were generated and apical (C), side (D) and basal views (E) acquired. To assess co-localization with cell organelles TG2 transfected cells were co-stained with organelle (red) for endoplasmic reticulum (ER) (F) Golgi apparatus (G) and lysosomes (H). Organelles are indicated by red arrows. In panel G, the green arrow shows peripheral TG2 (green) and orange arrow shows TG2 around the cell nucleus that is co-localised with ER (orange). Cell nuclei are stained blue with DAPI.

### Figure 7. TG2 is exported across the basolateral membrane.

OK, NRK52E & MDCK II tubular epithelial cells were transfected with TG2 and plated in cell culture well inserts. TG activity of the culture medium below the insert (b, basolateral) and within the insert (a, apical) was measured in the culture medium. Data represents mean TG activity in Units per mg of protein  $\pm$ SEM.  $^* = p < 0.05$ , n=5.

### Figure 8. Can the TG2 export motif target other proteins for cell export?

The TG2  $\beta$ -sandwich domain (bsw), TG2 core domain (core) and the TG2 export motif 88-106 (motif) were placed into the halotag vector pFC14K CMV. 48 hours post transfection the amount of halotag labeled protein found in the culture medium and in cell lysates was assessed by anti-Halotag pAb. L:cell lysate, M:culture medium. Data represents mean halotag levels  $\pm$  SEM. n=5.

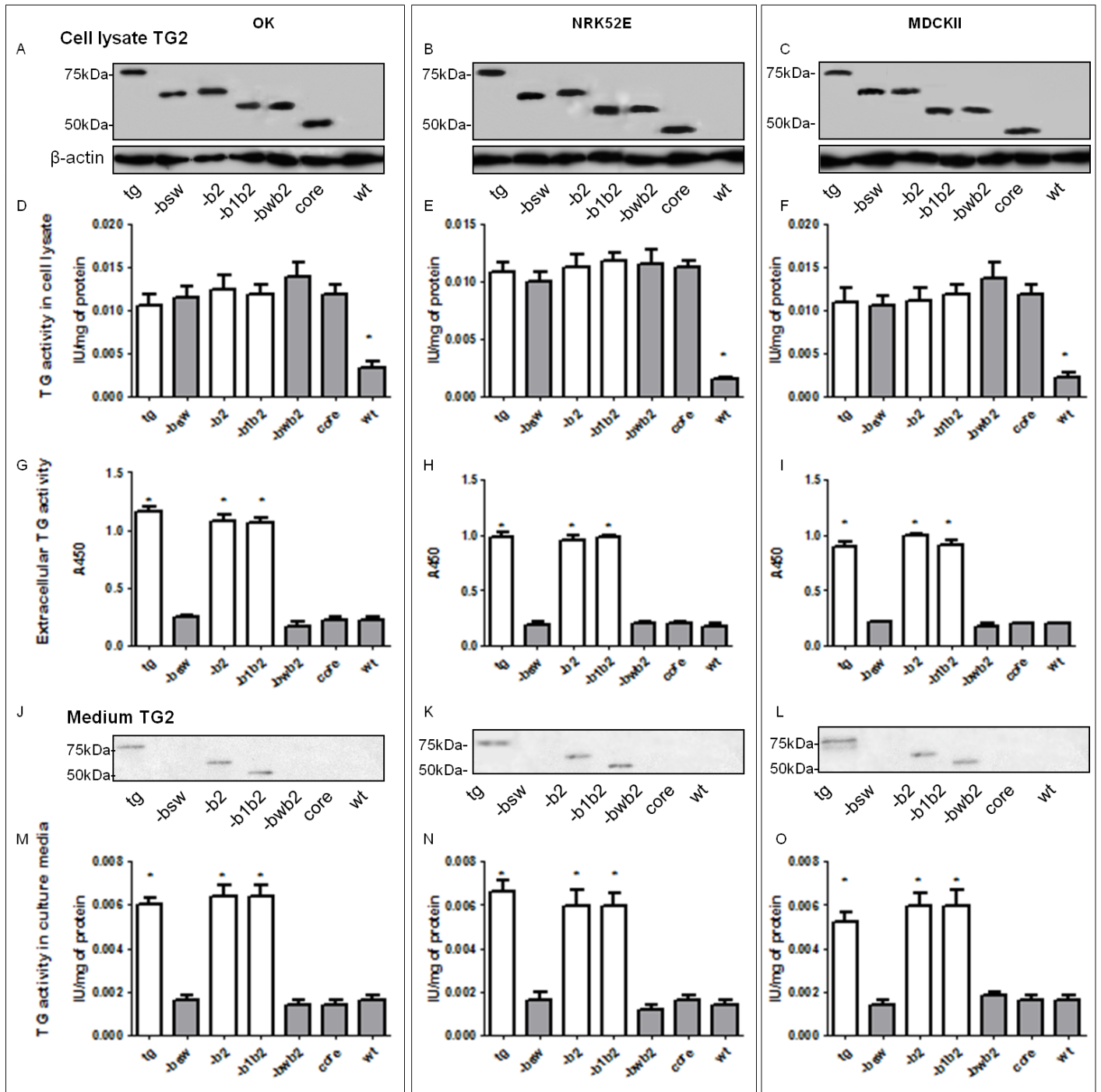
### Figure 9. Possible TG2 extracellular trafficking pathways and protein structure of $\beta$ -sandwich domain

(A) Cartoon showing the known unconventional protein transmembrane transport routes. 1: Lysosome route, 2: protein channel / flip protein, 3: Endoplasmic reticulum budding, 4: membrane blebbing. ER: endoplasmic reticulum, = protein for export). (B) Protein structure of the TG2  $\beta$ -sandwich domain showing the TG2 export motif (amino acids 88-106) lightly shaded with  $\beta$ 5-strand and  $\beta$ 6-strands labeled. Black indicates Asp<sup>94</sup> and Asp<sup>97</sup>.

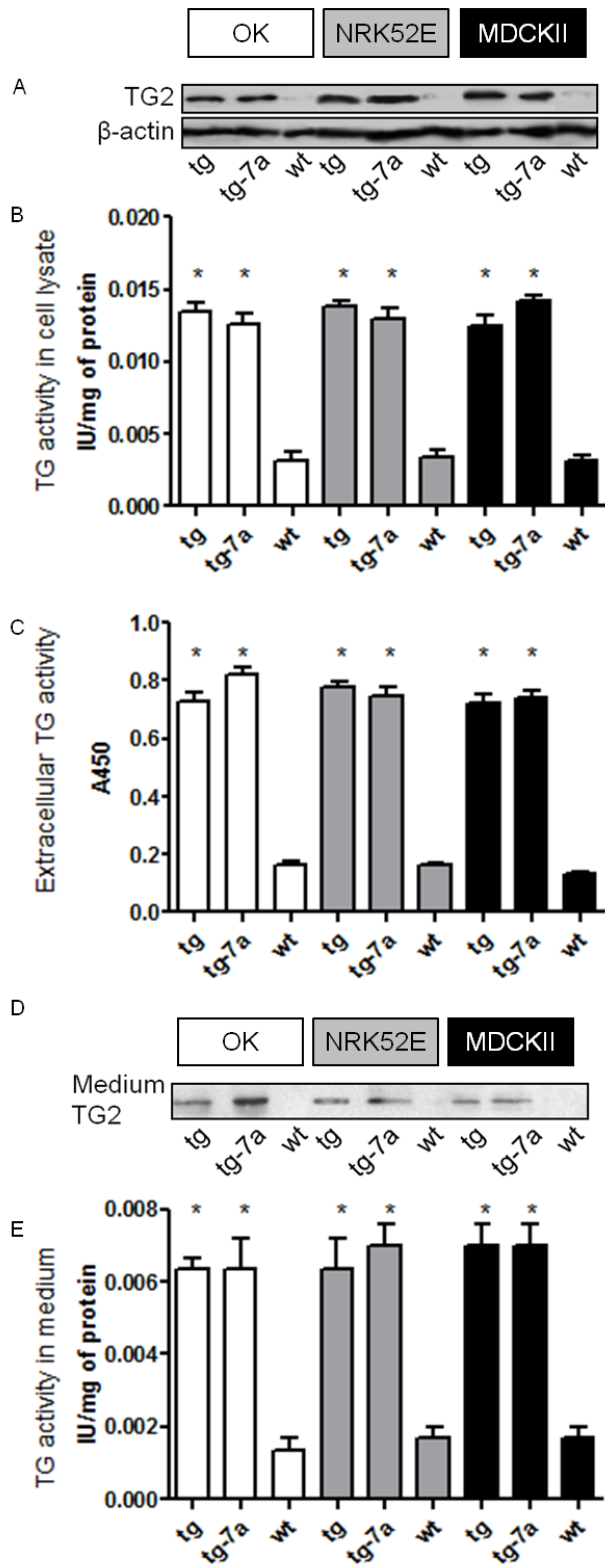
### Table 1. List of primers sequences.

Forward and reverse primers used to generate TG2 deletions and point mutations. The constructs were shown in 3D images.

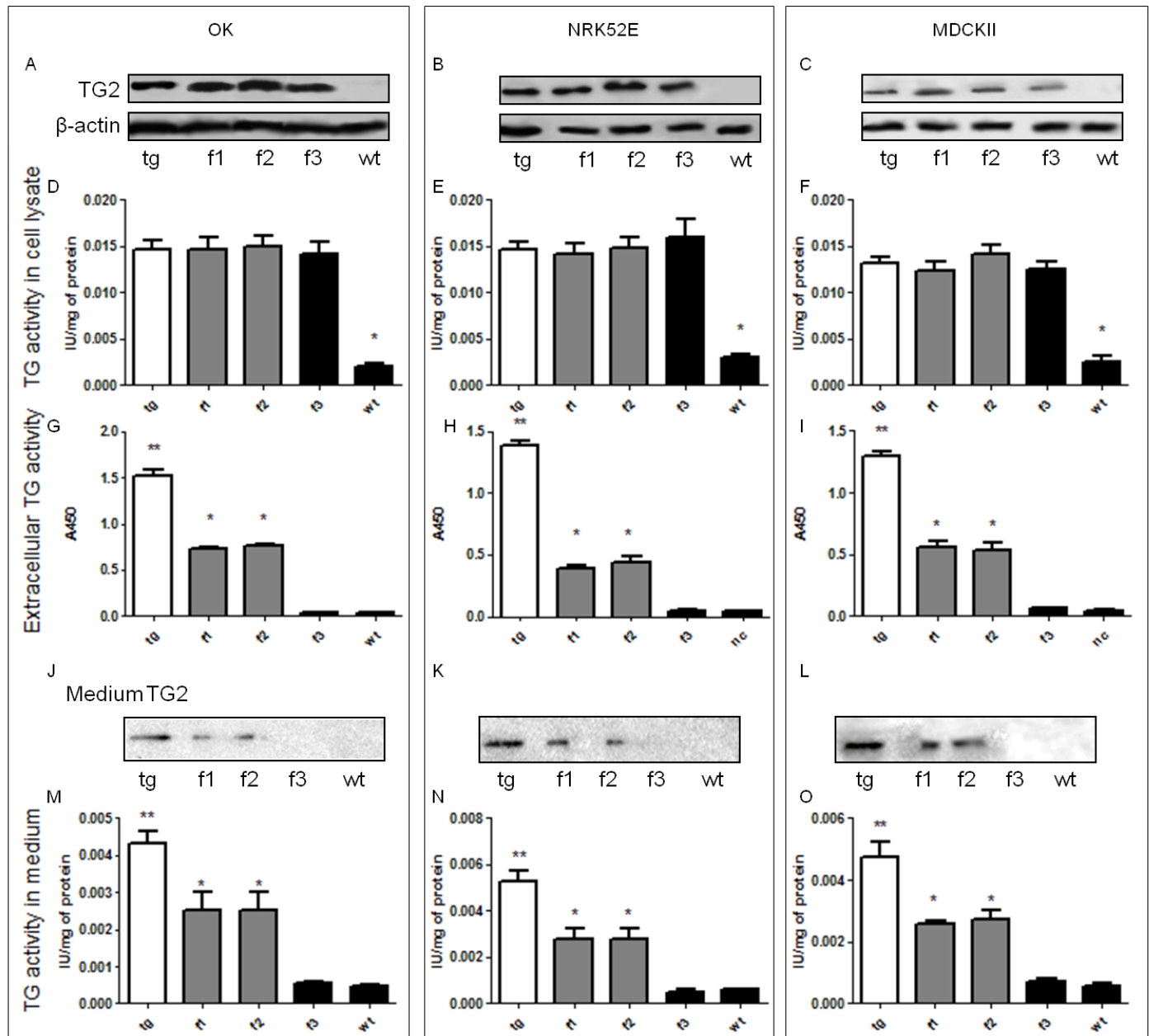
**Figure 1. Intra and extra-cellular TG2 levels following domain deletion**



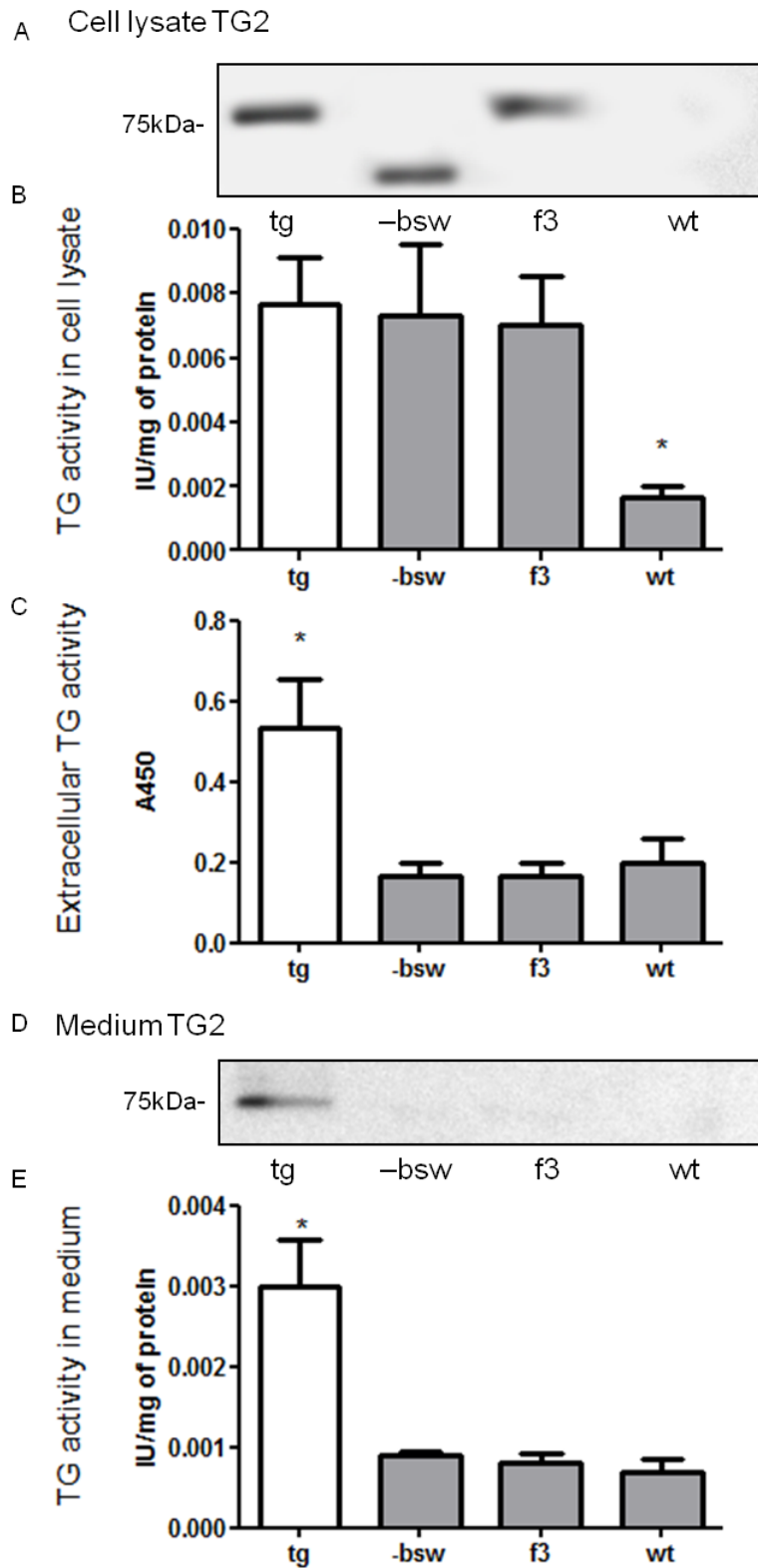
**Figure 2. Affect of TG2 export of removing the amino acid 1-7 fibronectin binding site**



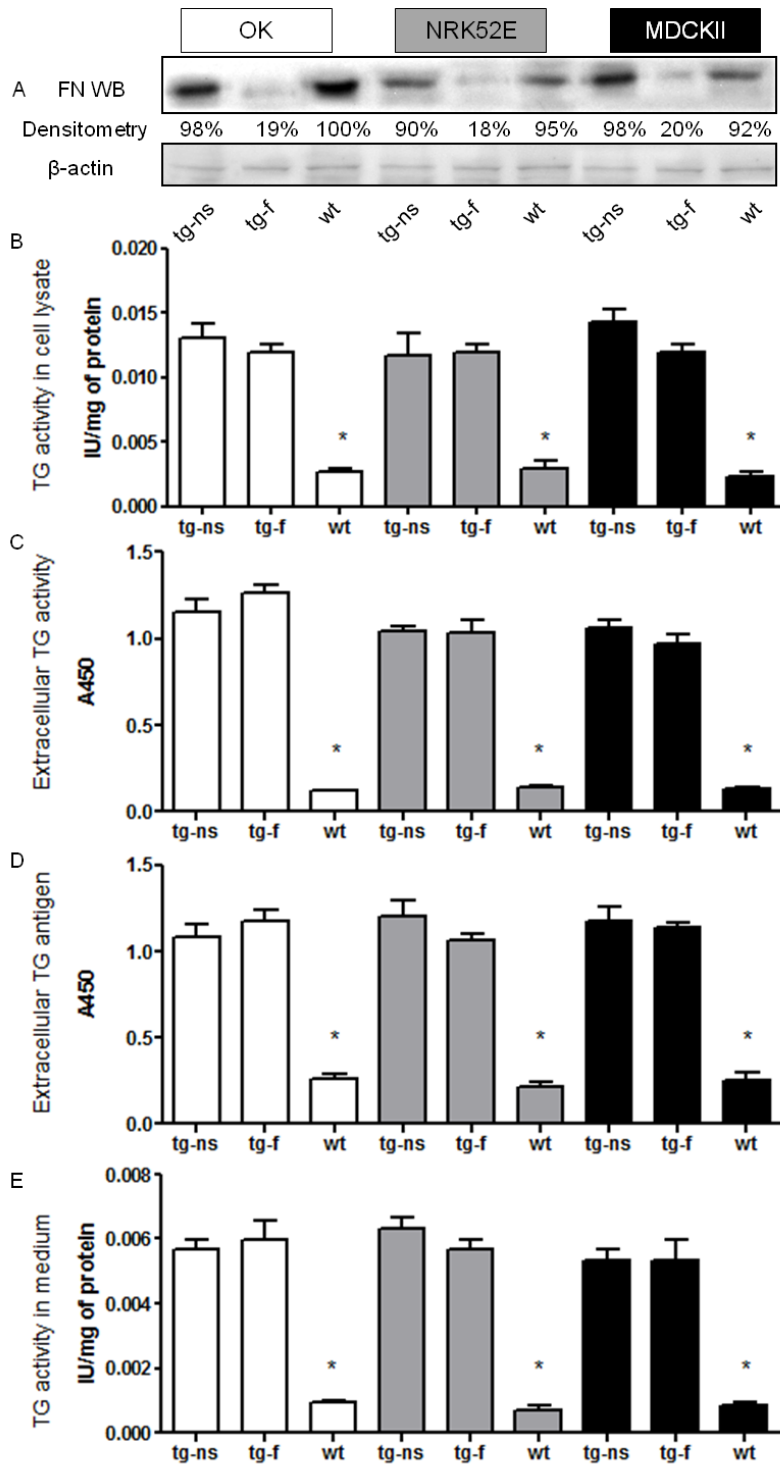
**Figure 3. Intra and extra-cellular TG2 levels following point mutation**



**Figure 4. Intra and extra-cellular TG2 levels in stable NRK52E cells stably transfected with mutant and full length TG2**



**Figure 5. Intra and extra-cellular TG2 levels following fibronectin knockdown**





**Figure 6. Wild type TG2 and mutant TG2 fluorescent and cell organelle imaging**

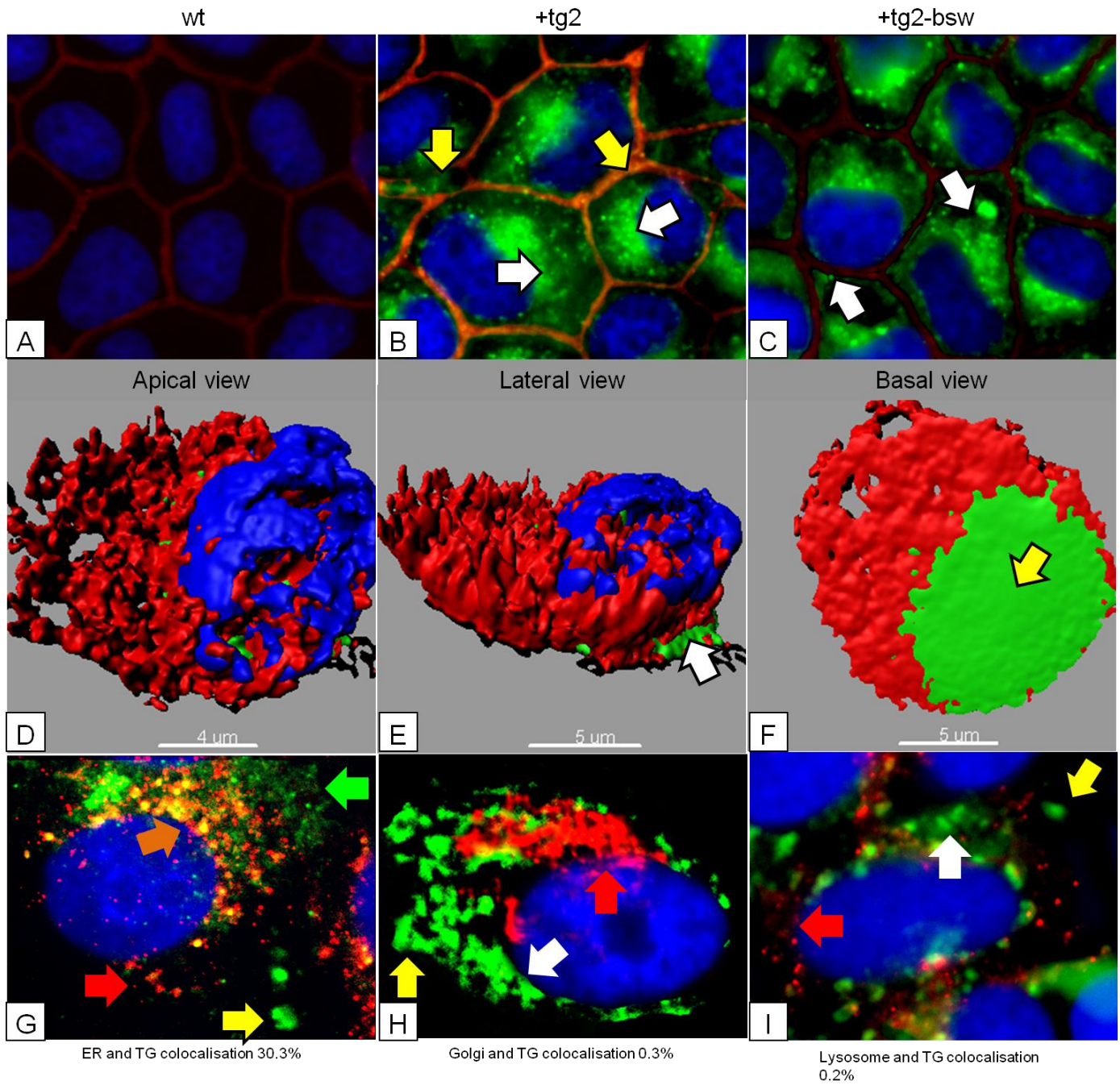


Figure 7. TG2 is exported across the basolateral membrane.

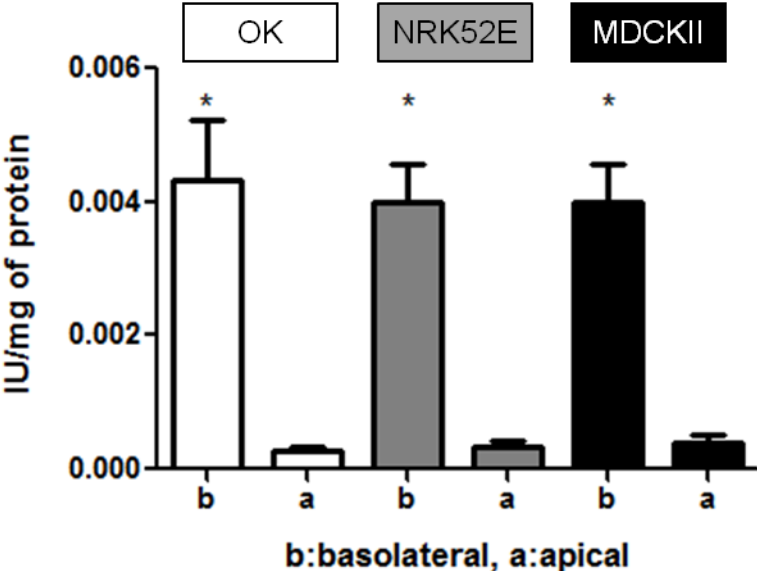
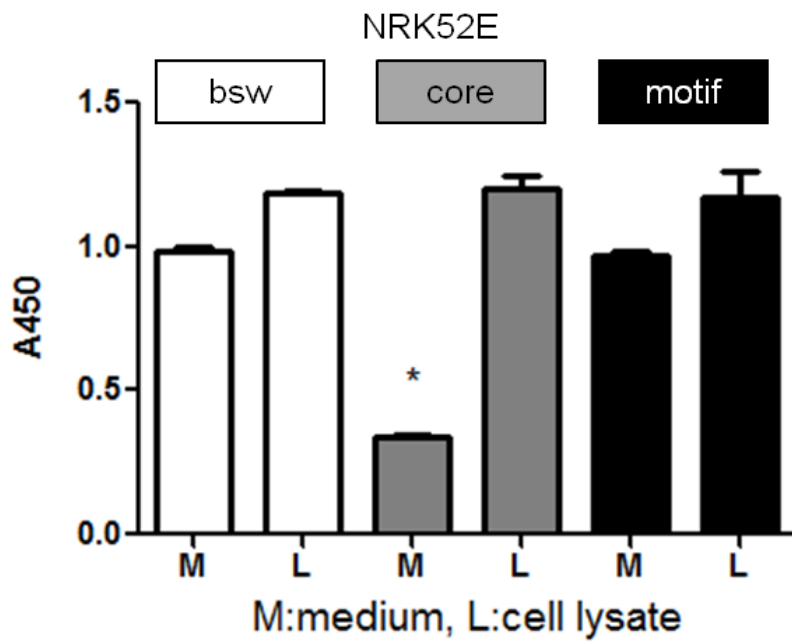
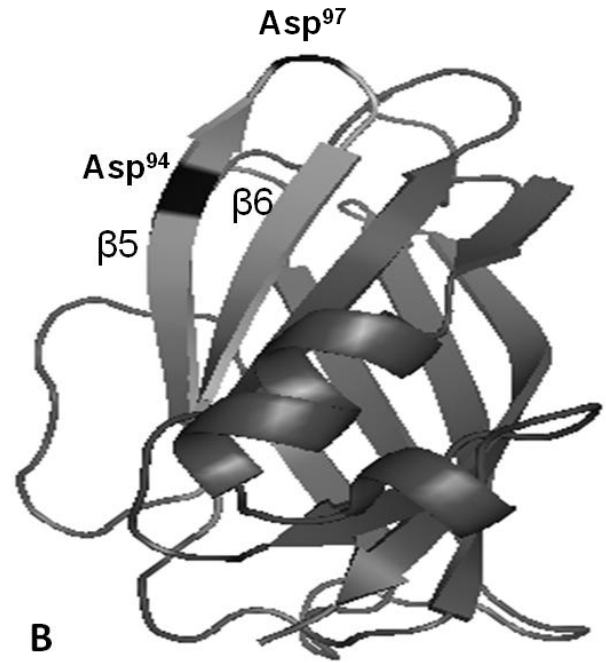
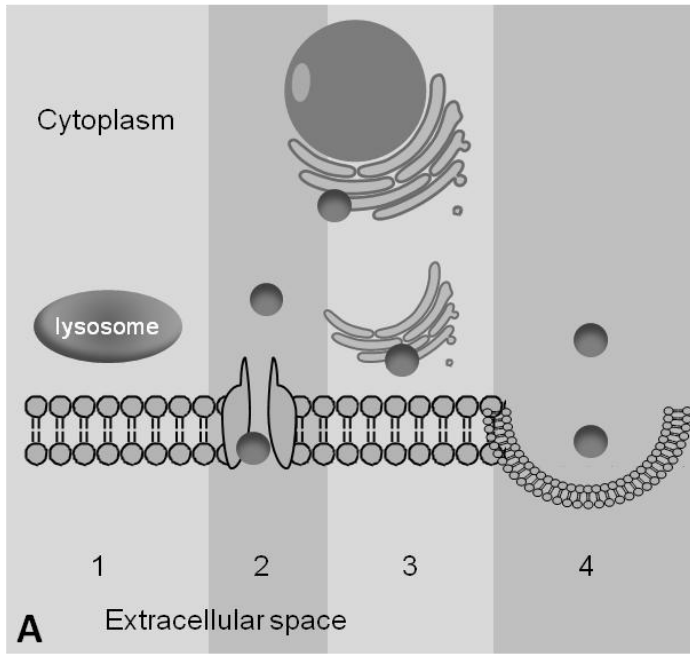


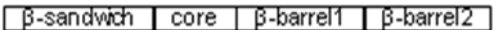
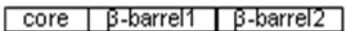
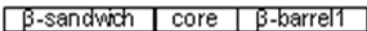
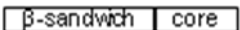
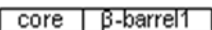

Figure 8. Can the TG2 export motif target other proteins for cell export?



**Figure 9. Possible TG2 extracellular trafficking pathways and protein structure of  $\beta$ -sandwich domain**



**Table 1. List of primers sequence**

	Construct	Forward primers	Reverse primers
tg		CACCATGGCCGAGGAGCTGGTCTT	AAATCCGCCCCGGTTACTACTGT
-bsw		CACCATGCTGTGTACCTGGACTCG	AAATCCGCCCCGGTTACTACTGT
-b2		CACCATGGCCGAGGAGCTGGTCTT	AAAGTAGAGGTCCCTCTCAGCCA
-b1b2		CACCATGGCCGAGGAGCTGGTCTT	AAAGTTCGCCCTTGTGAAGGCCT
-bwb2		CACCATGCTGTGTACCTGGACTCG	AAAGTAGAGGTCCCTCTCAGCCA
core		CACCATGCTGTGTACCTGGACTCG	AAAGTTCGCCCTTGTGAAGGCCT
f1	<sup>88</sup> WTATVVAQQDCTLSLQLTT <sup>106</sup>	CAGCCACCGTGGTGGCCAGAAAGACTGCA C	GTGCAGTCTTGCTGGGCCACCACGGTGGCTG
f2	<sup>88</sup> WTATVVDDQQAQACTLSLQLTT <sup>106</sup>	TGGTGGACCAGCAAGCCTGCACCCTCTCGCT	AGCGAGAGGGTGCAGGCTTGCTGGTCCACCA
f3	<sup>88</sup> WTATVVAQQACTLSLQLTT <sup>106</sup>	CAGCCACCGTGGTGGCCAGCAAGCCTGCA CCCTCTCGCT	AGCGAGAGGGTGCAGGCTTGCTGGGCCACCA CGGTGGCTG