

A new method of high-speed cellular protein separation and insight into subcellular compartmentalization of proteins

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Abstract Transglutaminase (TGM)-2 is a ubiquitous protein with important cellular functions such as regulation of cytoskeleton, cell adhesion, apoptosis, energy metabolism, and stress signaling. We identified several proteins that may interact with TGM-2 through a discovery-based proteomics method via pull down of flag-tagged TGM-2 peptide fragments. The distribution of these potential binding partners of TGM-2 was studied in subcellular fractions separated by density using novel high-speed centrifugation technology. Centrifugation is a compressed air-driven, low-temperature stepwise ultracentrifugation procedure where low extraction volumes can be processed in a relatively short time in non-denaturing separation conditions with high recovery yield. The fractions were characterized by immunoblots against known organelle markers. The changes in the concentrations

of the binding partners were studied in cells expressing short hairpin RNA against TGM-2 (shTG). Desmin, mitochondrial intramembrane cleaving protease (PARL), protein tyrosine kinase (NTRK3), and serine protease (PRSS3) were found to be less concentrated in the 8.5%, 10%, 15%, and 20% sucrose fractions (SFs) from the lysate of shTG cells. The Golgi-associated protein (GOLGA2) was predominantly localized in 15% SF fraction, and in shTG, this shifted to predominantly in the 8.5% SF and showed larger aggregations in the cytosol of cells on immunofluorescent staining compared to control. Based on the relative concentrations of these proteins, we propose how trafficking of such proteins between cellular compartments can occur to regulate cell function. Centrifugation is useful for elucidating biological function at the molecular level, especially when combined with traditional cell biology techniques.

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Abbreviations

ARHGAP	Rho GTPase activating protein
DMSO	Dimethyl sulfoxide
DES	Desmin
ERC	ELKS/RAB6-interacting/CAST family member
GRIK	Glutamate receptor ionotropic kainite
GOLGA	Golgi-associated protein
HCE	Human corneal epithelial
HIVP	Human immunodeficiency virus type I enhancer binding protein
ITGB	Integrin beta
LC	Liquid chromatography
MS	Mass spectrometry
NTRK3	Protein tyrosine kinase

PARL	Mitochondrial intramembrane cleaving protease
PBS	Phosphate-buffered saline
PRSS3	Serine protease
shRNA	Short hairpin ribonucleic acid
shTG	TGM-2 silencing short hairpin RNA
SF	Sucrose fraction
STXBP2	Syntaxin binding protein
TGM-2	Transglutaminase-2

Introduction

Transglutaminase (TGM)-2 is a ubiquitous Ca^{+} -dependent protein cross-linking enzyme that plays various important roles in wound healing, fibrogenesis, apoptosis, inflammation, cell cycle control, tissue formation, blood clotting, as well as protection from infection [1, 2]. It is a member of a large multi-gene TGM family of proteins that all have the ability to covalently cross-link substrates [3]. At a physiological level, TGM-2 functions as an extracellular matrix stabilizer through its protein cross-linking activity, as well as a cell adhesion molecule important for cell survival [4]. Unlike other TGMs, TGM-2 is secreted and deposited in the extracellular matrix and is also localized in the cytosol, plasma membrane, and nucleus of cells [5].

Previous studies on TGM-2 interactions and trafficking indicate that TGM-2 in different cell compartments is associated with different physiological functions [6]. Cytosolic TGM-2 is pro-apoptotic, whereas nuclear TGM-2 is anti-apoptotic [7]. However, the protein-binding partners of TGM-2, apart from integrins and matrix molecules such as fibronectin, have yet to be investigated. It is important to understand the specific binding partners and domains of TGM-2 in different parts of the cell as this may have an impact on the signaling pathways that are involved with cell migration or adhesion. For example, trafficking of molecules such as low-density lipoprotein receptor-related protein 1 (LRP1), associated with TGM-2 through different cell locations, may affect endocytosis and other important functions of the cell [8]. In order to fully understand the function of TGM-2, a method to separate the cellular proteins efficiently is a prerequisite.

Previously, TGM-2 binding partners, such as Bcl-2-associated X protein (Bax), which interacts with TGM-2, were discovered by immunoprecipitation [9]. However, the protein-interacting partners of specific domains of TGM-2 have not been published. We undertook a mass spectrometry-based method to elucidate the proteins, which may bind to TGM-2 by expressing fragments of flag-tagged TGM-2 and then pulling down the complexed by anti-flag immunoglobulins.

Traditionally, separation of proteins can be performed using gradient ultracentrifugation [10], including lipoproteins [11], enzymes [12], membrane vesicles [13], and

muscle proteins [14]. Ultracentrifugation has been used in several studies examining subcellular trafficking in various cell types [13, 15–17]. However, conventional ultracentrifugation has drawbacks such as requiring long extraction times and relatively large volumes as well as balancing of sample masses before separation. Centricollation is a novel method of protein separation based on a stepwise density extraction or flotation principle which provides uniform and specific separations by applying equal centrifugal force to the entire sample. The hallmark of this technique is that the sample stays at high centrifugal force for only 2–3 min for each fraction (samples could undergo rapid acceleration of 125,000g force within a very short time of 45 s) instead of multiple hours, allowing multiple extractions in a relatively short time. In contrast to ultracentrifugation, balancing of the sample is not required. Other advantages of using centricollation technique include the possible use of small samples with extraction volumes as low as 100 μL , use of predefined density extracting media with a range of increasing densities, non-denaturing separation conditions, and high recovery yield of more than 90%.

We aimed to discover a few TGM-2 interacting proteins with potential importance in cellular physiology and understand how these proteins are trafficked between cell compartments by using centricollation, a novel method of separating proteins by density, and immunoblotting of potential binding partners in cells in combination with RNA interference of TGM-2.

Materials and methods

Reagents and antibodies

The rabbit anti-human eukaryotic translation elongation factor 1 alpha 2 monoclonal antibody, anti-mitochondrial intramembrane cleaving protease (PARL) or H-300, anti-Trk C (NTRK3) polyclonal antibodies, anti-TGM-2 (Ab421), and the following goat polyclonal antibodies against mesotrypsinogen (PRSS3), Rho GTPase activating protein (ARHGAP)-17 or F-18, desmin (DES), Unc 18-2 (STXBP2), glutamate receptor ionotropic kainate (GRIK) 2, ELKS/RAB6 interacting/CAST family member (ERC) 2, Golgi-associated protein (GOLGA)2 or P-20, GLI family zinc finger (GLI)-1, golgin 160 (GOLGA3), integrin beta (ITGB) 4 or N-20, human immunodeficiency virus type I enhancer binding protein (HIVEP) 2 or H-20 were all from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The rabbit anti-human CAP350 polyclonal antibody was obtained from Novus Biologicals (Littleton, CO, USA). Peroxidase, fluorescein isothiocyanate, or Alexa-542 coupled anti-rabbit IgG (whole molecule) and anti-goat IgG

(whole molecule) antibodies were purchased from Sigma-Aldrich Pte. Ltd. (Saint Louis, MO, USA).

Cell culture

A human SV-40 immortalized corneal epithelial cell line (RCB 1384, HCE-T) at passages 88–98 was obtained from the Riken Bioresource Cell Bank in Ibaraki, Japan, courtesy of Kaoru Araki-Sasaki (Kinki Central Hospital, Hyogo, Japan) [18]. HCE-T cells stably expressing scrambled shRNA or TGM-2 silencing short hairpin RNA (shTG) have been previously described, and the latter has been shown to express significantly lower amounts of TGM-2 [19]. As reported, the biological effect of the shRNA against TGM-2 could be compensated for by overexpression of the wild-type TGM-2, showing specificity of the shRNA for TGM-2.

Cell lines were cultured in Dulbecco's modified Eagle's medium F12 supplemented with 5% fetal bovine serum. All cells were maintained in a 5% CO₂ incubator at 37°C and culture media were replaced every 2 days. Before experiments, cells were cultured in T175 cell culture flasks and lysed at 80–90% confluency.

Transfection of TGM-2 fragments

Cell cultures were grown to 70–80% confluency before experiments and 1.0×10^6 cells were seeded for each electroporation experiment. Transfection was carried out using the Neon 100 µL Transfection Kit (Invitrogen, Grand Island, NY, USA) and a MicroPorator-mini MP100 (DigitalBio, Seoul, Korea) adjusted at 1,300 V, 30 ms of voltage in single-pulse mode, where HCE-T cells were transfected with 1 µg of pIRES-hrGFP II plasmid vector that expressed flag-tagged peptides corresponding to each of the four domains of TGM-2: C terminal 1 (T1), C terminal 2 (T2), catalytic core (C), and N terminal (N) fragments from Stratagene (La Jolla, CA, USA). This was used to map the binding of potential interacting protein of TGM-2 domains.

Immunoprecipitation and mass spectrometry

Twenty-four hours after transient transfection, cell culture media were aspirated and discarded. After washing the cells with PBS, they were then scraped, collected, and centrifuged at 1,200 rpm for 5 min at 4°C. The bicinchoninic acid assay was carried out for the purpose of quantification of the proteins as described in the manufacturer's protocol obtained (Pierce Biotechnology Inc., Rockford, IL, USA). Immunoprecipitation was performed with anti-flag antibodies, where elutes were sent for tryptic digest and nano-liquid chromatography (LC)/LC mass spectrometry (MS)/

MS (refer to [Electronic supplementary material](#)). Details of nano-LC, peptide sequencing, and database search have been published elsewhere [20]. Negative controls include “immunoprecipitation” with pre-immune serum. A typical mass spectrogram is shown in Fig. 1. A short list of interacting partners was produced based on the frequency of finding the same interacting partners in independent repeat experiments and the known biological functions of the discovered proteins as documented in Gene Ontology (Fig. 2a). Proteins with functions clearly related to cell adhesion or trafficking were preferentially retained and the known relations of these proteins with TGM-2 plotted in a diagram (Fig. 2b).

Preparation for centricollation

After lysis of cells and centrifugation as described above, the supernatant was discarded and the cell pellets were resuspended in homogenizing buffer (1 mL of 8.5% GradiSpec sucrose solution with 2 µL of 0.5 M of EDTA and 10 µL of protease inhibitor). The cells were then homogenized with a 27-G needle and syringe. Homogenate aliquots were centrifuged again at 1,000 rpm for 5 min at 4 °C and the total post nuclear supernatant obtained for protein concentration assays. Higher protein concentrations were diluted with homogenizing buffer to match the samples with lower concentration.

Centricollation

The procedure was performed on control shRNA and shTG cell lysates with solutions of equal volumes and concentrations as illustrated in Fig. 3. Briefly, the centricollation of both cell lysates was firstly carried out by fractionating protein samples according to a set of standard sucrose densities (8.5%, 20%, 30%, 40%) using the Enhanced density gradient extraction (Edge™) 200 System (Prospect Biosystems, Inc., Newark, NJ, USA). This is a bench-top, air-driven temperature-controlled ultracentrifuge system that has a unique rotor design, driven by centrifugal force for a stepwise ordered extraction of particles. This system does not use vacuum or high-speed bearings, but uses an external air compressor supplying pre-dried low-temperature air.

Briefly, this involved spinning the sample supernatant in the DNase, RNase, and pyrogen-free 8.5% GradiSpec sucrose solution in the previous step for 5 min at the fixed speed of 125,000g force. The spinning was performed in a disposable liner which was available in sterilized sealed packs for easy loading. After spinning, the supernatant was carefully removed with a fine tip pipette. On the addition of 100 µL of GradiSpec sucrose solution with the next higher density to the pellet, the same procedure was

Fragmentation Evidence

SLPYQVSLNSGSH		
Residue	b	y
S	88.0393	1388.5805
L	791.1234	1301.5484
P	298.1761	1188.5644
Y	461.2395	1891.5116
Q	589.2980	928.4483
V	688.3665	800.3897
S	775.3985	701.3213
L	880.4825	614.2893
N	1002.5255	591.2952
S	1089.5575	387.1623
Q	1146.5790	300.1302
S	1233.6110	243.1088
H	1370.6999	156.0768

Spl: T1.12 (C Terminal 1)

Gene Symbol=PRSS3 Protease serine 4 isoform B

Conf: 97%

Sequence: SLPYQVSLNSGSH

m/z = 694.8339 (charge = +2)

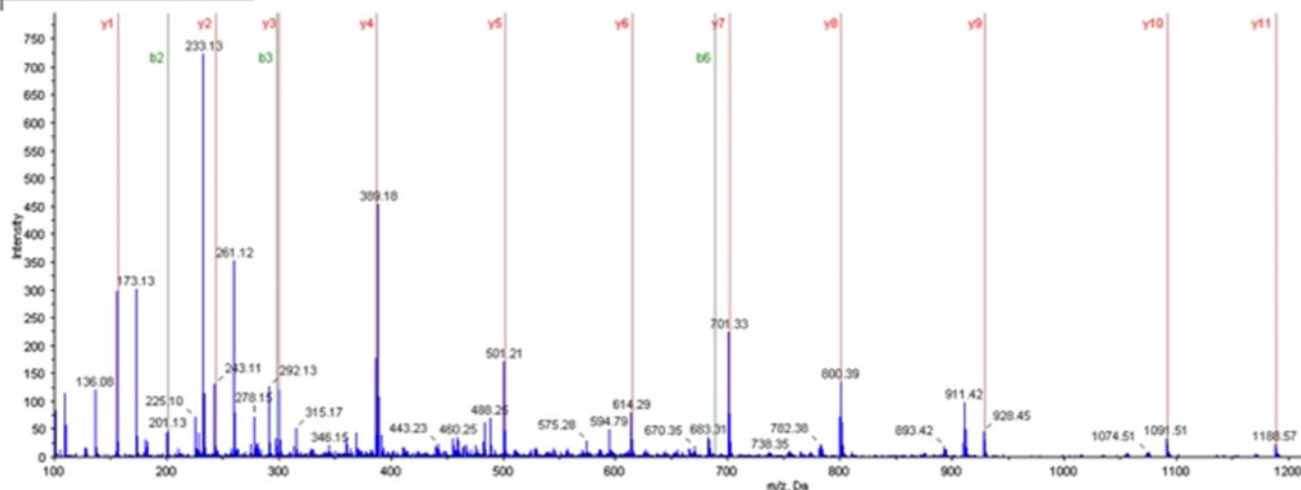


Fig. 1 Example of mass spectrogram output showing the detection of ion fragment with peptide sequence that matched serine protease (PRSS) 3

repeated until various supernatants (higher density sucrose fractions, SFs) were obtained. Gross fractionation into density fractions of 10%, 20%, 30%, and 40% was firstly carried out. Since most of the proteins of interest were localized in the 10% and 20% SFs, further fractionations were performed to get supernatants at 8.5%, 10%, 15%, and 20% SFs.

Western blots

The presence of TGM-2 interacting proteins in the various SF was determined by Western blots as previously described [21]. Briefly, this involved an optimized amount of the sample (7–30 μ L) combined with 30% loading buffer [sodium dodecyl sulfate (SDS) buffer with 20% beta-mercaptoethanol], which was briefly centrifuged and heated at 99 $^{\circ}$ C for 5 min. Samples were then pipetted into the wells of 4–20% pre-cast SDS-polyacrylamide gel electrophoresis gels and electrophoresed at 70 V for 30 min, then at 100 V for an hour. Gels were then transferred onto polyvinylidene fluoride membranes in 4 $^{\circ}$ C at 20 V overnight. Membranes were then incubated in 5% milk (blocking buffer) for an hour, washed, incubated in

1:200 primary antibody (diluted in 5% milk buffer), then washed and incubated in 1:7,000 secondary antibody (diluted in 5% milk buffer). Pico, Dura, or Femto substrate (Pierce Biotechnology) was applied to the washed membranes, and detection of luminescent substrates (HRP on the bound secondary antibody) was performed via exposure of X-ray films and subsequent development of the films.

Immunofluorescence staining

This procedure was performed as previously described [21]. Briefly, fixation was performed with 2% paraformaldehyde, washed in phosphate-buffered saline (PBS), and 0.15% of Triton X-100 was added. Slides were then incubated in blocking buffer (5% bovine serum albumin) before they were washed again with PBS. The diluted primary antibody (1:50) was pipetted onto the cells on each coverslip slide and was left overnight. Coverslip slides were washed again with PBS and the diluted secondary antibody was added for 45 min. Slides were washed with PBS before mounting of glass slides using 4',6-diamidino-2-phenylindole. Slides were then observed under a micro-

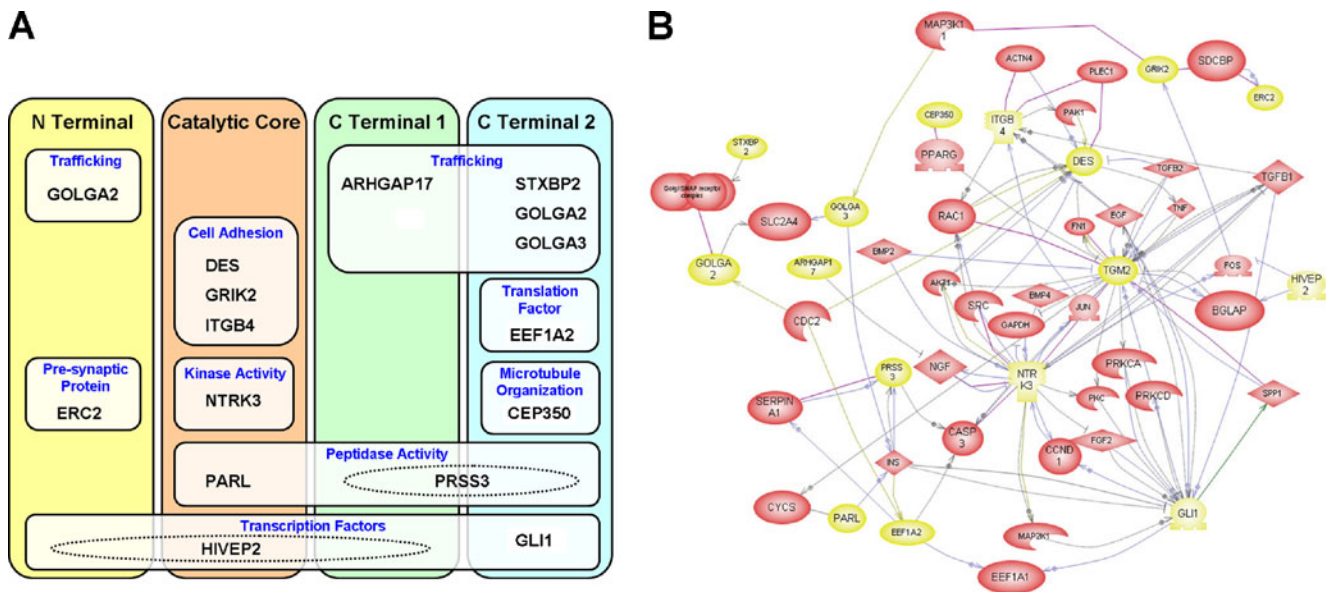


Fig. 2 **a** Schematic showing the proteins interacting with different domains of TGM-2 and their known biological functions. For example, the mass spectrometry results suggest that desmin (*DES*) interacts with the catalytic core of TGM-2. **b** Molecular network illustrating the interactions of TGM-2 and its related potential binding partners. Image was produced by Pathway Studio, version 5.0. *Yellow*

scope and fluorescent images were captured using the Axiovision software.

Statistical analysis

All experiments were performed at least three times to ensure reproducibility of the results, and in this paper, representative blots or images were shown.

Results and discussion

These interacting proteins were identified using mass spectrometry and a protein database search (Table 1). Although the scores for individual mass spectrometry experiment may not be very high, the fact that the same molecules were discovered in independent experiments (repeated immunoprecipitation) increases the confidence of the interaction. In addition, these proteins were found to be indirectly linked to TGM-2 in the existing scientific literature. One reason for the relatively low scores may also be related to the low concentration of TGM-2 ligands in the elutes. Subsequent identification of these proteins in the centrifugation fractions utilizes Western blots rather than mass spectrometry. They were also known to be involved in wound healing in some way. Using commercially available antibodies, we were able to perform Western blots which consistently detected seven of these proteins.

entities represent TGM-2 interacting proteins that were found in both mass spectrometry results and on MASCOT protein database to have some relationship with TGM-2 through indirect pathways. *Red* entities represent TGM-2 interacting proteins that were only found in previous studies to be related to TGM-2

Grouping and detection of potential TGM-2 binding proteins

The results were categorized into three groups according to how TGM-2 influenced their concentration and localization (Fig. 3). Group 1 consisted of proteins that were found to be consistently lower in shTG cells as compared to control shRNA cells in all fractions. This group included *DES*, mitochondrial intramembrane cleaving protease (*PARL*), protein tyrosine kinase (*NTRK3*), and serine protease (*PRSS3*). Group 2 consisted of proteins that were found to be lower in shTG cells in one SF compared to control, but higher in shTG than control in another SF, showing a reversal in the relative concentration between fractions. Proteins falling into this category were Golgi-associated protein (*GOLGA2*) and integrin beta-4 (*ITGB4*). Syntaxin binding protein (*STXBP2*) belonged to group 3 where proteins were found to be expressed in similar levels in both control and shTG cells in all fractions tested.

The characterization of the 8.5%, 10%, 15%, and 20% SFs for known organelles is shown in Fig. 4. Some organelles, for example recycling endosomes, endoplasmic reticulum, and mitochondria, were only present in particular fractions. Recycling endosomes were predominantly found in the 8.5–15% SF, the endoplasmic reticulum, in the 8.5% SF, and the mitochondria, in the 10% SF. On the other hand, other organelles such as early endosomes, late endosomes, secretory vesicles, and lysosomes were found

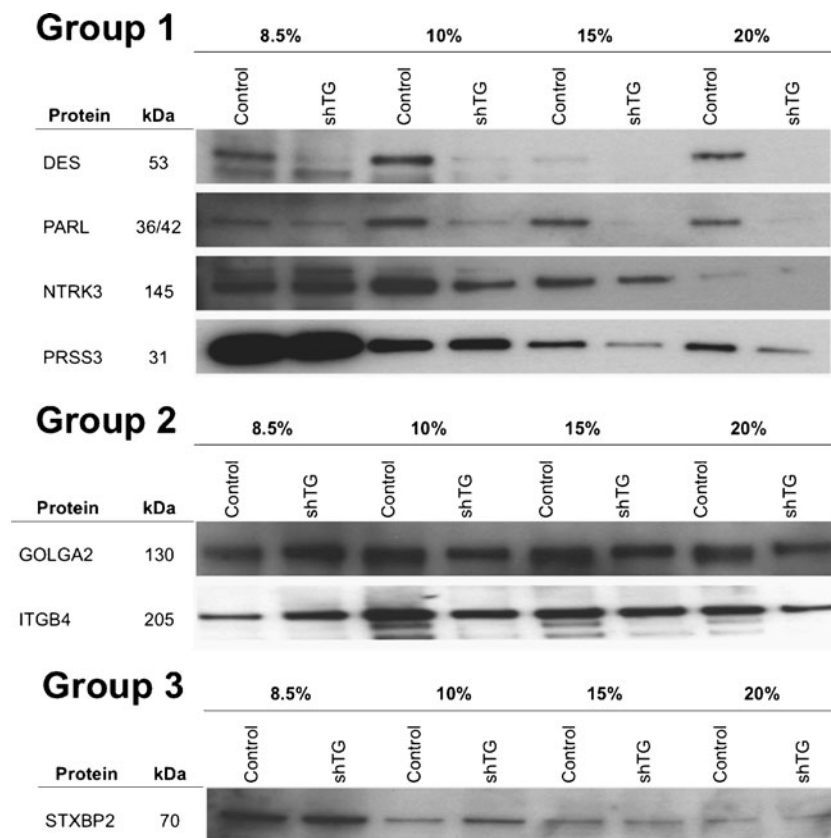


Fig. 3 Expression levels of TGM-2 ligands in centricollated HCE-T cell lysates according to 8.5%, 10%, 15%, and 20% density sucrose fractions determined by Western blotting. Results are categorized into three groups according to the localization expression patterns of shRNA control cells and shTG cells between fractions. Group 1 ligands: desmin (*DES*), mitochondrial intramembrane cleaving protease (*PARL*), protein tyrosine kinase (*NTRK3*), and serine protease (*PRSS3*). These are characterized by their lowered expression in shTG

as compared to control (shRNA) in all (*DES* and *PARL*) or in specific fractions only (*NTRK3* and *PRSS3*). Group 2 ligands: integrin beta-4 (*ITGB4*) and Golgi-associated protein (*GOLGA2*). These are characterized by their reduced expression in shTG, but showed an increased expression relative to control in the 8.5% fraction. Group 3 ligand: syntaxin binding protein (*STXBP2*), which is characterized by the similar expression patterns between shTG and control in all the fractions tested

Table 1 Interacting proteins identified using MS and a protein database search

Peptide	Fragment	<i>m/z</i>	Mr	Charge	Score
PRSS3	SLPYQVSLNSGSH	694.8339	1,389.6678	+2	23
	LSSPAVINAR	514.25	1,026.58	+2	18
DES	K.LQEELQLK.E	500.77	999.56	+2	20
GRIK2	-.CSHEMAAGILK.Q+Oxidation (M)	588.26	1,174.55	+2	21
ITGB4	R.LTAGVPDTPTR.L	564.29	1,126.60	+2	10
NTRK3	R.RDIVLKR.E	450.23	898.57	+2	20
ERC2/1	R.SPRLPR.S	363.19	724.43	+2	16
PARL	K.KGGGSNPRR.H	464.73	927.44	+2	16
HIVEP2	R.RDLSPRR.E	450.24	898.51	+2	19
GLI1	-.MHISRRLLR.S+Oxidation (M)	599.27	1,196.69	+2	17
ARHGAP17	R.SIFPEMHSDSASK.D	718.29	1,434.56	+2	17
STXBP2	K.ILSGVIRSVK.K	536.27	1,070.68	+2	16
GOLGA2	K.SELEEKLR.V	502.25	1,002.53	+2	14
GOLGA3	K.EQFLQK.V	460.72	919.48	+2	19
EEF1A2	K.IGGIGTVPVGR.V	513.28	1,024.60	+2	40
CEP350	R.LQEANKAAR.K	500.76	999.55	+2	17

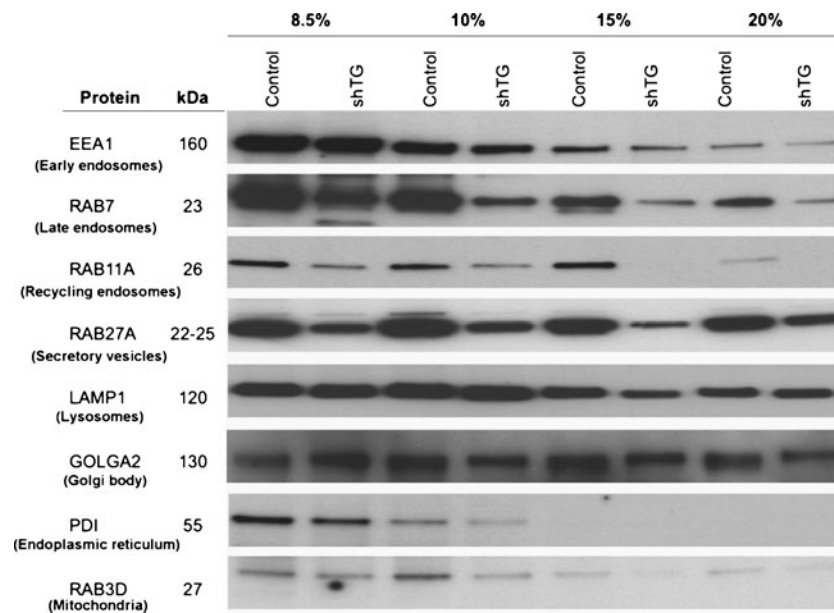


Fig. 4 Expression levels of organelle markers in centricollated HCE-T cells according to 8.5%, 10%, 15%, and 20% density SFs determined by Western blotting. Late endosome (*RAB7*), recycling endosome (*RAB11A*), and the secretory vesicle (*RAB27A*) marker expressions were reduced in shTG cells as compared to control (*shRNA*). Lysosomal (*LAMP1*), early endosome (*EEA1*), mitochondria (*RAB3D*), and endoplasmic reticulum (*PDI*) marker expressions were unaffected by TGM-2. Early endosomes, late endosomes, secretory vesicles, and lysosomes were found to be present in all fractions. The

intensity of their expression may have varied especially in the case of the early endosomal marker which was expressed predominantly in the 8.5–15% SF range. Recycling endosome, endoplasmic reticulum, and mitochondria markers indicate that these organelles were not present in all fractions. Recycling endosomes were mostly found in the 8.5–15% range. Endoplasmic reticulum was found in the 8.5% fraction only. Mitochondria were mostly found in the 15% fraction only

in all fractions tested, although with varying intensities of immunoreactivities. This was especially the case of the early endosomal marker which was expressed predominantly in the 8.5–15% range of SF.

The immunoreactivity against late endosomes (*RAB7*), recycling endosomes (*RAB11A*), and the secretory vesicles (*RAB27A*) were found to be lower in shTG cells compared to control, but integrity of lysosomes (*LAMP1*), early endosomes (*EEA1*), mitochondria (*RAB3D*), and endoplasmic reticulum (*PDI*) were not affected by TGM-2 status (Fig. 4).

GOLGA2 immunofluorescent staining results are shown in Fig. 5. As expected, the expression of TGM-2 (red) was reduced in shTG as compared to control cells. TGM-2 and GOLGA2 (green) were found to co-localize in particular structures within the cytoplasm of both control and shTG cells, although TGM-2 had a more widespread pattern, staining more diffusely in the cytoplasm, nucleus, and periphery of the cells. In addition, GOLGA2 was stained as coarser aggregates in the case of shTG compared to control cells, where GOLGA2 aggregates appeared to be finer or smaller.

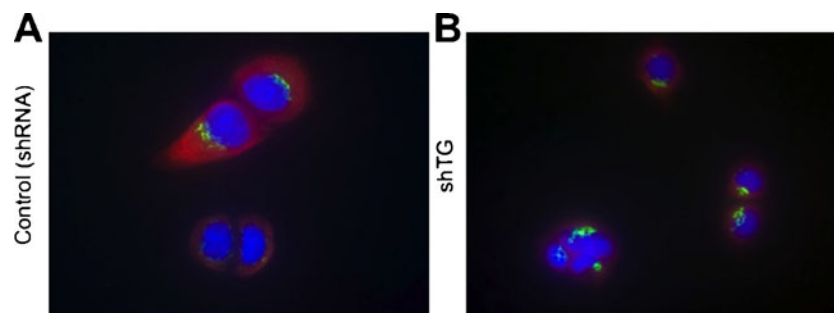


Fig. 5 Immunofluorescent co-localization of TGM-2 and GOLGA2 in control (*shRNA*) (a) compared to shTG cells (b). TGM-2 is stained in red, GOLGA2 stained in green, and nuclear staining in blue. Both TGM-2 and GOLGA2 are co-localized in both control and shTG cells.

It can be seen that when TGM-2 is suppressed, GOLGA2 expresses coarser aggregates with more intense staining, unlike control cells where GOLGA2 aggregates appear to be sparser with less intense staining

Comparison with previous studies

The ubiquitous TGM-2 protein present in different intracellular locations was responsible for different functions, depending on its subcellular location [6, 8, 22]. These studies were performed in non-ocular cell types, for example, neuroblastoma cells [6], human and mouse fibroblasts [8], Swiss 3T3 murine fibroblasts, and primary vascular endothelial cells [22]. Moreover, cell surface TGM-2 has been found to function differently from intracellular TGM-2 [5, 22]. Unlike the current study, the study of Lesort et al. [6] used subcellular fractionation techniques to separate neuroblastoma cells into only two fractions: the nucleus and cytoplasm. The study of Zemskov et al. [8] involved fractionation into three SFs: 5%, 35%, and 45%. In contrast, the current study carried out fractionations over a wider range of SF and with greater resolution between 5% and 20% SF. Our study evaluated a wide range of organelles, and the study of Zemskov et al. [8] would have included diverse organelles, except there was no systemic characterization of the SFs. Other studies only evaluate proteins from one particular organelle, such as the nucleus, cell membrane, or mitochondria [6, 23]. Apart from the distribution of LRP1 [8], the above studies did not examine how TGM-2 affects the trafficking of other molecules. In the current study, the relative distribution of TGM-2 interacting proteins between cellular compartments was reported.

Our results show that the 8.5% SF contained most of the organelles tested, except for mitochondria and the Golgi apparatus; the 10% SF contained all organelles tested, except for the Golgi apparatus and endoplasmic reticulum; the 15% SF contained the recycling endosomes, secretory vesicles, lysosomes, or Golgi apparatus; and the 20% SF contained secretory vesicles or lysosomes (Fig. 4). The localization of late endosomes and lysosomes using RAB7 and LAMP1, respectively, was consistent with a previous study [24]. In the current study, RAB7 was detected in the 8.5% and 10% SFs and LAMP1 was detected in the 8.5%, 10%, and 20% SFs (Fig. 4). Previously, RAB7 was detected in 6–11% SFs and LAMP1 in 6–16% SFs [24], therefore consistent with the current results.

Proposed hypothesis

Although the exact mechanism is still not clearly understood, we propose the following hypothesis on the role of TGM-2 and trafficking of its associated partners. Firstly, for group 1 proteins, DES, PARL, NTRK3, and PRSS3 that were detected at higher levels in the control than shTG for all the SFs tested (Fig. 3), it is proposed that TGM-2 may act as an adaptor protein for the interacting protein or an upstream regulator of interacting protein transcription. As an adaptor protein, TGM-2, when present in normal levels

in a particular cellular compartment, may sequester and bind its interacting protein within specific cell compartments (Fig. 3). When TGM-2 is deficient, this may allow non-differential accumulation or equal distributions of the interacting protein throughout the other cell compartments. This theory parallels a report by Park et al. [25] which states that TGM-2 acts as an adaptor for several proteins such as $\alpha 1$ and $\beta 1$ integrins in subcellular compartments. Alternatively, TGM-2 may also act as an upstream regulator that is eventually involved in transcription of the partner (Fig. 3). In this scenario, TGM-2, directly or indirectly, may cause the gene of the specific protein to be more transcriptionally active. The increase in expression of TGM-2 would cause the expression of the interacting protein to increase in all cell compartments of the cell, including any tested SF with TGM-2.

Group 2 proteins GOLGA2 and ITGB4 have been found to show a reversal in relative levels from 8.5% to 10% SF between control and shTG cells (Fig. 3). There appeared to be an accumulation of the TGM-2 interacting protein in the 8.5% SF with TGM-2 reduction and not in the 10% and higher SF. Instead, the specific protein levels were higher in the control cells in the 10% SF. We propose that TGM-2 acts as a carrier protein that is required for the translocation of GOLGA2 and ITGB4 from one cellular compartment “B” (8.5% SF) to another compartment “A” (10% and higher SF). Therefore, if TGM-2 is deficient, its associated ligands would not be moved away from compartment “B” (8.5% SF) and would accumulate in it (Fig. 3). Fibronectin has been found to act as a carrier protein for extracellular TGM-2 [26]. It would not be surprising if intracellular TGM-2 exhibits a similar type of relationship with an interacting partner.

Lastly, the trafficking of group 3 protein STXBP2 was most likely unaffected by TGM-2 as Western blots indicate similar immunoreactivities between control and shTG cells in all tested SFs. This suggests that STXBP is probably unaffected by the concentration of subcellular TGM-2 (Fig. 3).

Combining our proposed mechanism with the characterization of the SF (Fig. 4), TGM-2 may serve as an adaptor or scaffolding protein for DES, NTRK3, and especially PARL and PRSS3 (group 1 proteins) in most organelles. TGM-2 may translocate the cargos GOLGA2 and ITGB4 (group 2 proteins) between the Golgi apparatus or mitochondria (mainly 10% SF or higher) and the early, late or recycling endosomes, secretory vesicles, lysosomes, or endoplasmic reticulum (8.5% SF). In a situation with reduced TGM-2, Golgi bodies may be abnormally formed, explaining the larger aggregates on immunofluorescence (Fig. 5).

In our previous studies, we found TGM-2 to regulate the rate of wound closure (unpublished data). Taken in combination with the current findings, one can hypothesize that TGM-2 may affect wound healing via trafficking of critical molecules relevant for cell migration and adhesion.

The main limitation of centricollation technique here is that we could not separate entities based on particle size. Ideally, if centricollation is combined with a size exclusion technique or a technique based on particle diameter such as asymmetric field flow fractionation, one could obtain more purified fractions. Besides, the molecules discovered by mass spectrometry may not be definite ligands in living cells. After the lysis of cells, it is possible that nonspecific interactions between TGM-2 and other molecules may occur; this may apply to molecules that may have been previously sequestered and separated spatially but brought into close proximity after lysis of cells. Other limitations of the current study include the fact that HCE-T cells may not behave in the same way as native cells in tissues, and that we did not perform scratching of cultured cells or some method of simulated wounding.

Concluding remarks

Centricollation, a novel method of protein separation based on density, is useful for elucidating the biological function of specific molecules, especially when combined with traditional cell biology techniques. Here, we show that TGM-2 is a molecule that affects the subcellular distribution of many proteins which may regulate the biological and cellular functions relevant to wound healing processes [27]. We also characterized the fractions in terms of known organelles in the cells evaluated. The reduction of TGM-2 may have disturbed Golgi apparatus in view of the larger bodies that accumulated in shTG cells and the reduction of GOLGA2 on immunoblots of the corresponding cellular proteins.

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