Significance and novel roles of the multifunctional transglutaminase 2 protein in the biology and pathobiochemistry of human cells.

NKFI 105046

Final report. Written according to the Aims presented in the original version of the submitted Research Plan.

1. Systematic computational analysis of the human TGM2 to reveal novel functional motifs, interaction domains, posttranslational modification sites to interpret evolutionary constraints on its structure supporting other modules of the research project.

i) Unlike its enzymatic interactions, the significance of TGM2's non-enzymatic regulation of its activities has recently gained importance. We summarized all the partners that directly interact with TGM2 in a non-enzymatic manner and analyzed how these interactions could modulate the crosslinking activity and cellular functions of TGM2 in different cell compartments. We have found that TGM2 mostly acts as a scaffold to bridge various proteins, leading to different functional outcomes. We have also studied how specific structural features, such as intrinsically disordered regions (which we could reveal) and embedded short linear motifs contribute to multi-functionality of TGM2. Conformational diversity of intrinsically disordered regions enables them to interact with multiple partners, which can result in different biological outcomes. Indeed, ID regions in TGM2 were identified in functionally relevant locations, indicating that they could facilitate conformational transitions towards the catalytically competent form. We reason that these structural features contribute to modulating the physiological and pathological functions of TGM2 and could provide a new direction for detecting unique regulatory partners. *Kanchan et al. Cellular and Molecular Life Sciences 2015.*

ii) With comparative genomic and computational analysis we have revealed phylogenetic changes of transglutaminase 2 (TGM2) resulting in novel amino acid clusters in humans and other primates, which may impact secondary structure and increase protein stability. These clusters are located in intrinsically disordered regions and via short linear motifs influence interactions with TGM2 partners directly, or through post-translation modification (phosphorylation and *N*-glycosylation sites). Our data shed new light on the structural background and evolution of TGM2 multi-functionality and points to so far unrevealed biological roles of the enzyme. *Thangaraju et al. Amino Acids 2017.*

iii) We compared data on non-synonymous single nucleotide variations (nsSNVs) and loss of function (LOF) variants on TGM1-7 and F13a from the Exome aggregation consortium (ExAC) dataset, and used computational and biochemical analysis to reveal the roles of damaging nsSNVs of TGM2. TGM2 and F13a display rarer damaging nsSNV sites than other TGMs and sequences of TGM2, F13a and TGM1 are evolutionary more constrained. TGM2 nsSNVs, which destabilize protein structure influence Ca^{2+} and GTP regulation, and non-enzymatic interactions, but none coincide with conserved functional sites. We have experimentally characterized the six TGM2 allelic variants detected so far in homozygous form, out of which only one, p.Arg222Gln, has decreased activities. Published exome sequencing data from various populations have not uncovered individuals with homozygous LOF for TGM2, TGM3

and TGM7. Thus it can be concluded that human transglutaminases differ in harboring damaging variants and TGM2 is under purifying selection suggesting that it may have so far not revealed physiological functions. *Thangaraju et al. PLOS One 2017*.

2. Development of novel methods and tools for TGM2 research to detect Ca2+-activated TGM2 conformation in compartments of living cells, and to find interacting partners and substrates.

i) We have attempted to crystalize TGM2 in a Ca^{2+}-binding, transamidase active form in collaboration with the group of Beata Vértessy in Budapest but obtained only low resolution crystals so far. Other groups, including our competitors in the transglutaminase field, have been not successful either.

ii) We identified a molecular co-chaperone, DNAJA1, as a novel interacting partner of human TMG2 protein using a GST pull-down assay and subsequent mass spectrometry analysis, and further confirmed this interaction via ELISA and surface plasmon resonance measurements. Interaction studies were also performed with domain variants of TGM2 and results suggest that the catalytic core domain of TGM2 is essential for the TGM2–DNAJA1 interaction. Cross-linking activity was not essential for the interaction since DNAJA1 was also found to interact with the catalytically inactive form of TGM2. Furthermore, we have showed that DNAJA1 interacts with the open form of TGM2 and regulates its transamidation activity under both in vitro and in situ conditions. We also found that DNAJA1 is a glutamine donor substrate of TGM2. Since DNAJA1 and TG2 are reported to regulate common pathological conditions such as neurodegenerative disorders and cancer, the findings in the present paper open up possibilities to explore molecular mechanisms behind TGM2-regulated cellular functions. *Ergulen et al. Biochemical Journal 2016.*

iii) We developed a novel kinetic method for measuring isopeptidase activity of human TGM2 (*Thangaraju et al. Analytical Biochemistry 2016*) by monitoring decrease in the fluorescence polarization of a protein substrate previously formed by crosslinking fluorescently labeled glutamine donor FLpepT26 to S100A4 at a specific lysine residue revealed in our earlier study (*Biri et al. Biochem J. 2015*). The developed method could be applied to test mutant enzymes and compounds that influence isopeptidase activity of TG2.

3. To investigate whether active TGM2 can promote mitochondrial calcium uptake and influence energy homeostasis of cells.

i) Timed overexpression of both the wild type (wt) and the cross-linking mutant of TGM2 induced apoptosis in Jurkat T cells, the wt being more effective. Part of TGM2 molecules in the cells co-localised with mitochondria. WtTGM2-induced apoptosis was characterized by enhanced mitochondrial Ca(2+) uptake. Ca(2+)-activated wtTGM2 cross-linked RAP1, GTP-GDP dissociation stimulator 1, an unusual guanine exchange factor acting on various small GTPases, to induce a yet uncharacterized signaling pathway that was able to promote the Ca(2+) release from the endoplasmic reticulum via both Ins3P and ryanodine sensitive receptors leading to a consequently enhanced mitochondrial Ca(2+)uptake. TGM2 might act as a Ca(2+) sensor to amplify endoplasmic reticulum-derived Ca(2+) signals to enhance mitochondria Ca(2+) uptake. Since enhanced mitochondrial Ca(2+) levels were previously shown to sensitize mitochondria for various apoptotic signals, our data demonstrate a novel

mechanism through which TGM2 can contribute to the induction of apoptosis in certain cell types. *Hsieh et al. PLOS One 2013*.

ii) The threshold level of calcium needed for endogenous and recombinant TGM2 activity has been controversial, the former being more sensitive to calcium than the latter. In the present study we address this question by identifying a single amino acid change from conserved valine to glycine at position 224 in recombinant TGM2 compared with the endogenous sequence present in the available genomic databases. Substituting a valine residue for Gly224 in the recombinant TGM2 increased its calcium-binding affinity and transamidation activity 10-fold and isopeptidase activity severalfold, explaining the inactivity of widely used recombinant TGM2 at physiological calcium concentrations. ITC (isothermal titration calorimetry) measurements showed 7-fold higher calcium-binding affinities for TGM2 valine residues which could be activated inside cells. The two forms had comparable substrate- and GTP-binding affinities and also bound fibronectin similarly, but coeliac antibodies had a higher affinity for TGM2 valine residues. Structural analysis indicated a higher stability for TGM2 valine residues and a decrease in flexibility of the calcium-binding loop resulting in improved metal-binding affinity. The results suggest that Val224 increases TGM2 activity by modulating its calcium-binding affinity enabling transamidation reactions inside cells. *Kanchan et al. Biochemical Journal 2013.*

iii) A novel strategy was described to identify and characterize the signal-specific functional enhancer set of TGM2 in mice by integrating genome-wide datasets and measuring the production of enhancer specific RNA molecules (*Sándor et al. BBA 2016*). Previous studies from our laboratory have shown that in dying thymocytes the expression of TGM2 is induced by external signals derived from engulfing macrophages, such as retinoids, transforming growth factor (TGF)-β and adenosine, the latter triggering the adenylate cyclase signaling pathway. The existence of TGF-β and retinoid responsive elements in the promoter region of TGM2 has already been reported, but the intergenic regulatory elements participating in the regulation of TGM2 have not yet been identified. We used publicly available results from DNase I hypersensitivity analysis followed by deep sequencing and chromatin immunoprecipitation followed by deep sequencing against CCCTC-binding factor (CTCF), H3K4me3, H3K4me1 and H3K27ac to map a putative regulatory element set for TGM2 in thymocytes. By measuring eRNA expressions of these putative enhancers in retinoid, rTGF-β or dibutiryl cAMP-exposed thymocytes we determined which of them are functional. By applying ChIP-qPCR against SMAD4, retinoic acid receptor, retinoid X receptor, cAMP response element binding protein, P300 and H3K27ac under the same conditions, we identified two enhancers of TGM2, which seem to act as integrators of the TGF-β, retinoid and adenylate cyclase signaling pathways in dying thymocytes. Our study described a novel strategy to identify and characterize the signal-specific functional enhancer set of a gene by integrating genome-wide datasets and measuring the production of enhancer specific RNA molecules.

iv) Our data revealed a novel crosstalk between macrophages and apoptotic cells, in which apoptotic cell uptake-related adenosine production contributes to the appearance of TGM2 in the dying thymocytes (*Sándor et al. Amino acids 2017)*. TGM2 has been known for a long time to be associated with the in vivo apoptosis program of various cell types, including T cells. Though the expression of the enzyme is strongly induced in mouse thymocytes following apoptosis induction in vivo, no significant induction of TGM2 can be detected, when

thymocytes are induced to die by the same stimuli in vitro indicating that signals arriving from the tissue environment are required for the proper in vivo induction of the enzyme. Previous studies from our laboratory have demonstrated that two of these signals, transforming growth factor-β (TGF-β) and retinoids, are produced by macrophages engulfing apoptotic cells. However, in addition to TGF-β and retinoids, engulfing macrophages produce adenosine as well. We could show that in vitro adenosine, adenosine, and retinoic acid or adenosine, TGF-β and retinoic acids together can significantly enhance the TMG2 mRNA expression in dying thymocytes. The effect of adenosine is mediated via adenosine A2A receptors (A2ARs) and the A2AR-triggered adenylate cyclase signaling pathway. In accordance, loss of A2ARs in A2AR null mice significantly attenuates the in vivo induction of TGM2 following apoptosis induction in the thymus indicating that adenosine indeed contributes in vivo to the apoptosis-related appearance of the enzyme. We also demonstrate that adenosine is produced extracellularly during engulfment of apoptotic thymocytes, partly from adenine nucleotides released via thymocyte pannexin-1 channels.

4. Understanding, with the help of human TGM2 knock out and gene edited cell lines, how TGM2 regulates gene expression for myeloid differentiation and - as an extension of these studies to - learn whether TGM2 is involved and how in differentiation of mesenchymal stem cells toward various cell types.

i) The most efficient therapy of promyelocytic leukemia is treatment of patients with all trans retinoic acid retinoic acid (ATRA) which induces differentiation of the leukemia cells to neutrophils and eventually apoptosis. However, the synchronous differentiation of the large mass of leukemia cells leads to differentiation syndrome with a massive release of pro-inflammatory cytokines often leading to death. One of the prominent genes induced by retinoic acid is TGM2 and we presumed that it may have a casual role in the induction of the inflammatory phenotype of the differentiating leukemia cells.

We could establish a TGM2 knock-out human NB4 promyelocytic leukemia cell line using the TALEN technology and added this to the previously produced TGM2 knock-down cell line for our studies. In the experimental protocols we used the following cell lines: NB4 (wild-type), NB4 TGM2-C (virus control), NB4 TGM2-KD (TMG2 knock-down with the help of viral antisense construct – *Csomós et al. Blood 2010*) , NB4 TGM2-ha (TGM2 heterozygous allele), NB4 TGM2-KO (TG2 knock-out). The following results were obtained:

From the nitroblue-tetrazolium test of NB4 cell lines we concluded the TGM2 accelerates microbial killing ability of differentiating NB4 cell.

While full TGM2 expression in NB4 WT cells accelerated a large amounts of PMA induced ROS production at the early stages of ATRA induced differentiation, in the absence of TGM2 in TGM2-KO cells, only a small portion of this was generated. The ATRA-differentiated TGM2-expressing NB4 WT cells produced 20-times larger amount of ROS, than the ATRA-differentiated, TG2-deficient NB4 TGM2-KO cells

As a result of the ATRA treatment, the cell surface CD11b and CD11c integrins were activated on NB4 cell lines and the amount of which were not further enhanced by the PMA stimulus, indicating the total activated state of those cells.

Depending on the amount of TGM2, "resting" differentiated NB4 cells lines expressed and secreted inflammatory cytokines and chemokines such as TNFα Il-β and MCP-1 to varying degrees. While high TGM2 expression was accompanied by elevated mRNA and protein expression of cytokines (TNFα Il-β) and MCP-1 chemokine in NB4 WT, TGM2-C cells, at low or deficient expression of TGM2 in NB4 TGM2-KD, TGM2-ha and TGM2-KO cells all of them remained low.

Our data show that these cytokines / chemokines and the GP91PHOX, component of NADPH oxidase, are expressed in NF-κB dependent manner. Thus, NB4 cell lines containing the NF-κB response element with lucuferase reporter-gene were prepared from the NB4 WT, TGM2-C, TGM2-KD, TGM2-ha and TGM2-KO cells, and by using them we could demonstrate that when TGM2 expression was reduced or abolished (TGM2-KD, TGM2-ha and TGM2-KO cell), the NF-κB activation was minimal, however, depending on the amount of TGM2, the rate of NF-κB activation was increasing in differentiating cell. It could be concluded that TGM2 induces typical pro-inflammatory cytokines and chemokine expression through NF-κB and transcriptional activation.

ELISA-based Raybiotech 200 Human Biomarker Testing Service defined the secreted cytokines of supernatants of NB4 WT, TGM2-KD, and TGM2-KO cell lines. The amount of cytokines varied depending on the amount of TGM2: 44 were expressed in a TGM2-dependent manner from which 19 were identified as NF-κB transcription factor target genes.

Based on the results above it can be assumed that when retinoic acid treatment of promyelocytic leukemia cells leads differentiation the cells produce pro-inflammatory cytokines and chemokine in a TGM2-dependent manner through the NF-κB pathway and this can explain why the differentiation syndrome develops in leukemia patients receiving retinoid therapy. Adding TGM2 inhibitors to the therapeutic protocol may be an efficient approach to prevent the serious consequences of the differentiation syndrome.

Jambrovics et al. Manuscript has been submitted.

ii) Neutrophil extracellular trap (NET) ejected from activated dying neutrophils is a highly ordered structure of DNA and selected proteins capable to eliminate pathogenic microorganisms. Studying the formation of human NETs we have observed that polyamines were incorporated into the NET. Inhibition of myeloperoxidase, which is essential for NET formation and can generate reactive chlorinated polyamines through hypochlorous acid, decreased polyamine incorporation. Addition of exogenous primary amines that similarly to polyamines inhibit reactions catalyzed by transglutaminases has similar effect. Proteomic analysis of the highly reproducible pattern of NET components revealed cross-linking of NET proteins through chlorinated polyamines and $\varepsilon(\gamma$-glutamyl)lysine as well as bis-γ-glutamyl polyamine bonds catalyzed by TGM1 detected in neutrophils. Competitive inhibition of protein cross-linking by monoamines disturbed the cross-linking pattern of NET proteins, which resulted in the loss of the ordered structure of the NET and significantly reduced capacity to trap bacteria. Our findings provide explanation of how NETs are formed in a reproducible and ordered manner to efficiently neutralize microorganisms at the first defense line of the innate immune system. *Csomós et al. Cell Death and Disease 2016*.

iii) During cold-exposure ’beige’ adipocytes with increased mitochondrial content are activated in white adipose tissue (WAT). These cells, similarly to brown adipose tissue (BAT), dissipate stored chemical energy in the form of heat with the help of uncoupling protein 1 (UCP1). We

investigated the effect of TGM2 ablation on the function of adipose tissues in mice. Although $TGM2^{+/+}$ and $TGM2^{-/-}$ mice had the same amount of WAT and BAT, we found that $TGM2^{+/+}$ animals could tolerate acute cold exposure for 4h, whereas $TGM2^{-/-}$ mice only for 3h. Both $TGM2^{-/-}$ and $TGM2^{+/+}$ animals used up half of triacylglycerol content of subcutaneous WAT (SCAT) after 3h treatment; however, $TGM2^{-/-}$ mice still possessed markedly whiter and higher amount of gonadal WAT (GONAT) as reflected in the larger size of adipocytes and lower free fatty acid levels in serum. Furthermore, lower expression of 'beige' marker genes such as UCP1, TBX1 and TNFRFS9 was observed after cold exposure in GONAT of $TGM2^{-/-}$ mice, paralleled with a lower level of UCP1 protein and a decreased mitochondrial content. The detected changes in gene expression of Resistin and Adiponectin did not provoke glucose intolerance in the investigated $TGM2^{-/-}$ mice. Our data suggest that TGM2 has a tissue-specific role in GONAT function and browning, which becomes apparent under acute cold exposure. *Mádi et al. BBA. Molecular and Cell Biology of Lipids 2017.*

iv) We could obtain evidence and publish that browning is induced by clozapine in human adipocytes (*Kristóf et al. Translational Psychiatry 2016*).

v) Laser-scanning cytometry is presented as a tool allowing population scale analysis of ex vivo human brown adipogenic differentiation. It combines texture analysis and detection of Ucp1 protein content in single brown adipocytes of mixed cell populations with gene expression pattern and functional characteristics of browning. Using this method we could validate mouse data in human samples demonstrating the effectiveness of irisin to induce "beige" differentiation of subcutaneous white adipocytes. *Kristóf et al. Sci Rep 2015.*

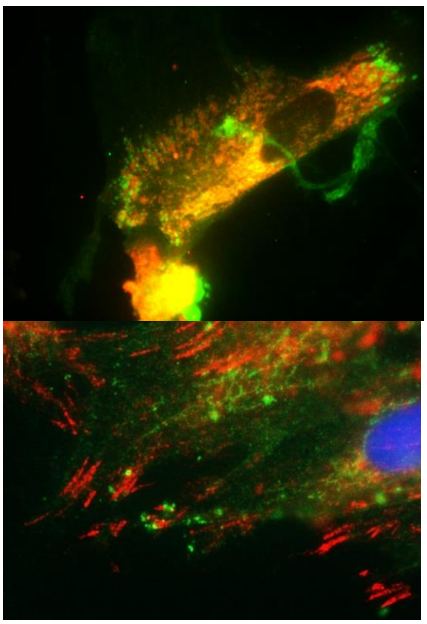
vi) One of the prominent functions of transglutaminase 2 (TG2) is to ensure effective clearance of apoptotic cells. Increasing amount of evidence indicate that defective clearance of dying cells can lead to the development of chronic systemic inflammatory diseases, and loss of TG2 seems to sensitize for various chronic inflammatory diseases, such as atherosclerosis and autoimmunity. Obesity is also characterized by chronic low- grade inflammation and by accumulation of apoptotic cells and inflammatory macrophages in the adipose tissue. It is recognized that this chronic inflammatory state is involved in the pathogenesis of obesity-related insulin resistance, metabolic syndrome and type 2 diabetes. In our experiments, we investigated the effects of the loss of TG2 and thus impaired clearance of apoptotic cells on the development of obesity, insulin resistance and obesity related inflammation. We performed a 17-week long feeding experiment in which TG2 KO mice and their wild type counterparts were fed with control, low fat/high sucrose or high fat/high sucrose diet. Our results indicate that TG2 deficient mice - kept on high fat/high sucrose diet - are characterized by enhanced apoptotic cell accumulation (with enhanced Bid and Bim expression) in the adipose tissue, and enhanced insulin, adipokine and macrophage derived inflammatory cytokine production as well as pre-diabetic insulin resistance and early hepatosteatosis as compared to their wild type counterparts. Inflammation, and hepatosteatosis were related to the loss of TG2 in bone marrow-derived cells. The symptoms induced by high fat diet could be prevented if mice were exposed to an LXR agonist that enhances clearance of apoptotic cells by both wild type and TG2 null macrophages, but not that of the hepatosteatosis. Our data describe a new phenotype of transglutaminase 2 null mice. *Manuscript in preparation.*

5. To find novel TGM2-related cell signalling processes and their abnormalities studying primary endothelial cells of healthy individuals and celiac patients.

Endothelial cells express high levels of TGM2 and also externalize it into their matrix, where it contributes to cellular adhesion. The role of extracellular TGM2 is well characterized in the literature in the RGD-independent adhesion. Fibronectin, syndecan 4, collagen VI and XVIII are known matrix proteins to provide anchor points by binding TGM2. The protein's protein cross-linking catalytic activity probably also contributes to this process. The role and relative importance of intracellular TGM2 is less clear and more difficult to evaluate. Celiac disease with high production of anti-TGM2 autoantibodies in response to ingestion to cereals gluten proteins is a good model to evaluate functions of TGM2 in endothelial cells. Interestingly, celiac anti-TGM2 antibodies of the VH3 framework tend to confer a gain of function for the cross-linking activity while VH5-framed antibodies might inhibit it. Previous studies from our group (*Caja et al, 2010*) found that celiac anti-TGM2 antibodies influence spreading and differentiation of normal endothelial cells and this process involves the elevation of RhoA. Defective angiogenesis is a feature of celiac disease and it is thought to lead to villous atrophy in the small bowel as target organ. Now we collected and isolated human endothelial cells (HUVEC) from umbilical cords of neonates from risk families where one or both of the parents, or a sibling were suffering of celiac disease. These cells were successfully maintained in culture and we investigated them in comparison with endothelial cells obtained by the same methods from the normal Hungarian population. During follow-up, the risk children were regularly investigated for the presence of anti-TGM2 antibodies and when seroconverted, a small intestinal biopsy was performed to prove the presence of clinical celiac disease. In this way, we obtained 10 celiac cell lines of which 6 were successfully immortalized for more extensive studies (utilizing pBABE vector-based telomerase method). All these cells carry HLA-DQ2 or DQ8, and some of the controls also do have these normal variation alleles.

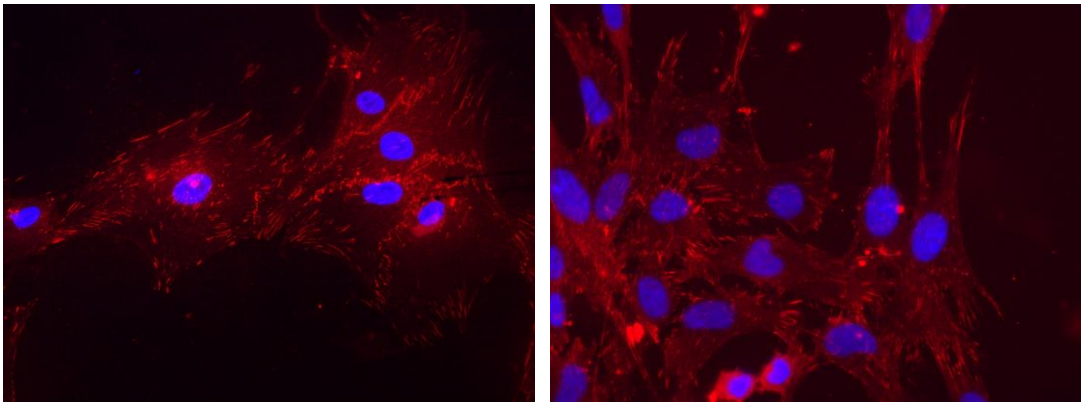
During the current is research we found that the total IgA fraction of celiac serum samples which contains celiac antibodies of VH3 (90%) and VH5 (10%) framework disturbs the adhesion of normal endothelial cells in a dose–dependent manner (*Nadalutti et al. 2013*). This process is coupled with the increased cross-linking extracellular activity of TGM2 as cell-impermeable TGM2 inhibitors were able to reverse this effect. Also agents interfering with the maintenance of TGM2 activity interfered with the action of celiac antibodies. One such example was thioredoxin which prevents the decay of extracellular TGM2 by its reducing effect. Thioredoxin inhibitor PX12 was able to reverse the effect of TGM2 antibodies to similar extent as TGM2 inbihitors. Further, we also have shown that celiac anti-TGM2 antibodies (purified from human serum) also inhibit migration and polarization of the cells (*Nadalutti et al. 2014*). These functions are also related to the RhoA pathways in several ways. Although increased Rho A expression can lead to higher levels of focal adhesion kinase (FAK), the relation of FAK and TGM2 was not yet demonstrated earlier. Usual TGM2 detection methods by antibodies in immunohistochemistry failed to recognize TGM2 intracellularly in adhesion complexes in paraformaldehyde-fixed cells. A long optimization of the staining process was needed to find the procedures suited for different adhesion components. In cells using MES-methanol-based preservatives or acetone and overnight incubation with primary antibodies and 3-4 hours incubation with secondary antibodies we have shown that intracellular TGM2 is directly

segregating on the surface of FAK already in the perinuclear cytoplasmic area and the two proteins maintain a contact even after transport to the adhesion complexes.



TGM2 (green) and FAK (red) closely associate intracellularly (top) before transported to adhesion complexes (bottom)

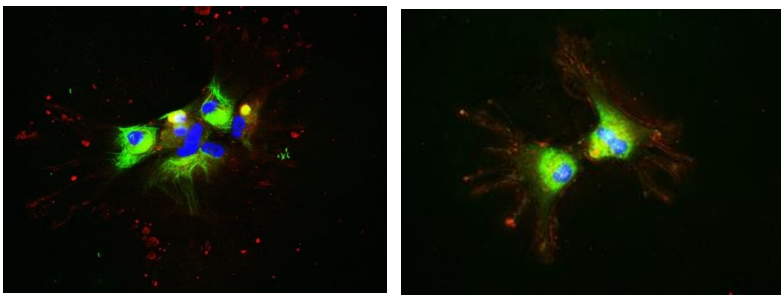
Further, a co-localization of TGM2 with other adhesion proteins, such as vinculin, actinin and lipoma-preferred protein (Lpp) was demonstrated. When comparing celiac and non-celiac cells, there was no significant difference netiher in the expression pattern of any of these proteins, nor in that of TGM2.



Expression of LPP in normal (left) and celiac cells (right prepared from umbilical cord

However, handling of adhesion complexes and dynamics of the cytoskeleton changed rapidly and even on the same slide wide variations could be seen. When the cells were induced to move rapidly by the addition of higher quantities of growth factors to their medium, focal adhesions were simply cleaved and left behind. In this respect, celiac cells tended to show more often larger cytoplasmic fragments which had the appearance of exosomes. In order to evaluate the

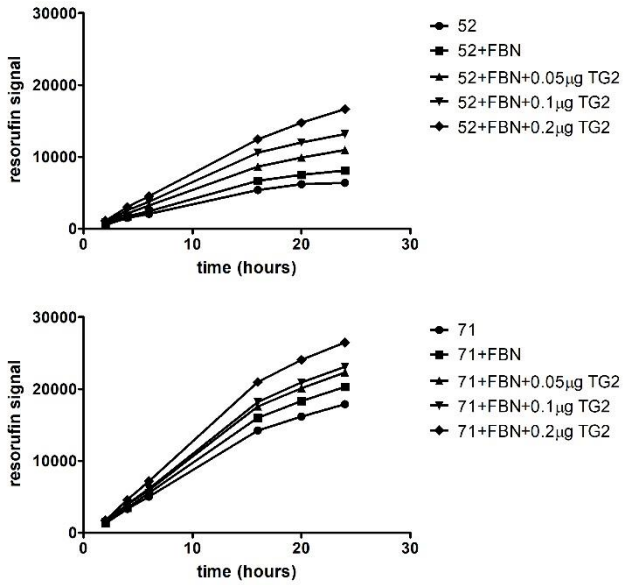
dynamic changes in talin, we transfected cells with fluorescent green protein-coupled talin (Cell-Light™Talin). Although these reagents worked very well in muscle cells prepared from the umbilical cords, only very few endothelial cells in culture (<1%) took the vectors up and expressed the protein despite similar conditions and also when trying to adjust incubation time and density.



FAK (green) and talin-1 staining (red) in normal (left) and celiac cells (right prepared from umbilical cord

In functional studies measuring cellular adhesion by real-time viability (cell number) assay by resorufin metabolism, fibronectin and TGM2 increased adhesion, but celiac cells constantly showed reduced values compared to normal cells and also compared to cells from risk children who did not develop the disease until the age of 6 years.

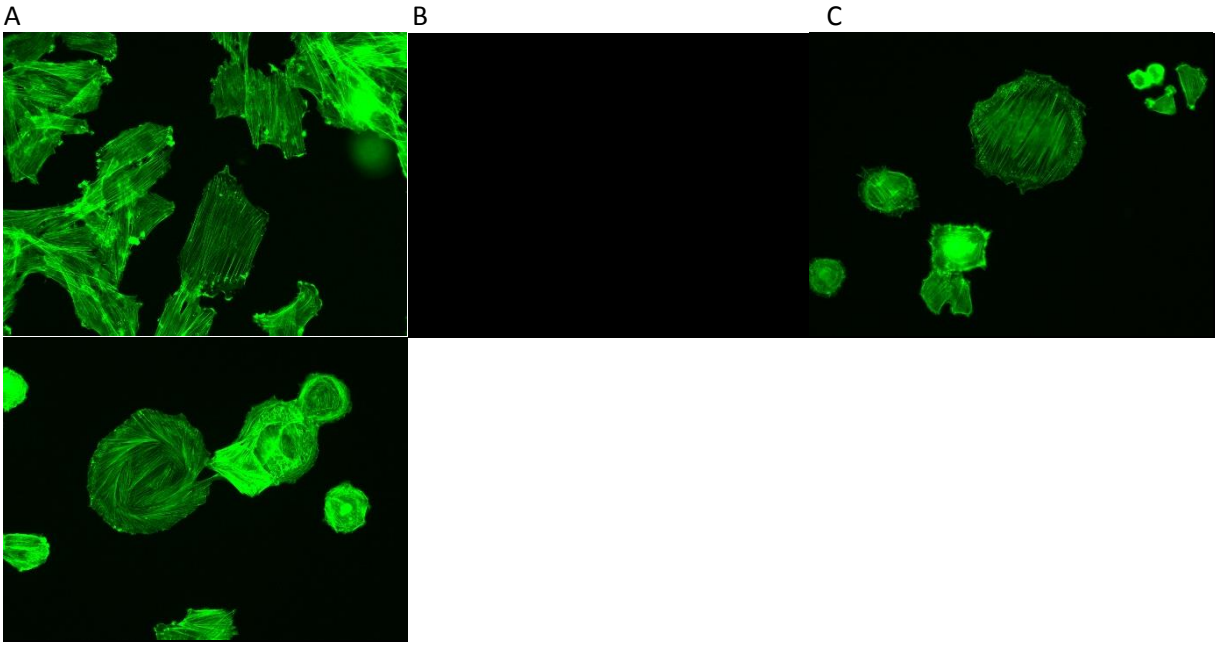
A B



Investigation of cellular adhesion in risk cells with later celiac disease (A) or heatlhy status (B)

These results indicate that even in the absence of gluten compounds and celiac antibodies, cells from subjects with forthcoming celiac disease are more vulnerable to external factors affecting cellular adhesion. When these cells were exposed to celiac antibodies, they were more affected than normal cells, but the difference only reached significance after starving the cells in 1% fetal blood serum and utilizing plastic surface without fibronectin and adding antibody

preparations rich in VH3 antibodies an binding to TGM2 celiac epitope 2. In contrast, celiac antibodies with VH5 framework and acting via a different epitope (celiac epitope 1) had negligible effect. However, when at least one additional adhesion protein (e.g. Lpp) was knocked down, celiac cells reacted more to the same conditions than normal cells. A selective increase in FAK was observed as well (Toth B. et al. manuscript in preparation).
We also explored how modulation of Rho influences the TGM2 and the behavior of cells. In the presence of Rho activator CN04 (0.5 ug/ml) there was only slight non-significant decrease in adhesion, but cells became sticky, rounded and their cytoskeletal arrangement changed. Often a twisting movement was observed in life cells imaging experiments. These changes were more severe in celiac cells.



Cytoskeleton changes of cells after addition of Rho activator CN04. A.Basal condition, B. non-celiac cells and C. celiac cells after CN04.

Investigation of the effect of celiac antibodies was also planned by using mouse cells and utilizing TGM2-/- mouse cells as controls. However, we found that mouse TGM2 does not possess the epitope for VH3 celiac antibodies and only VH5 antibodies (which represent only some 10% of the serum antibodies) can bind. However, as shown above in human experiments, VH5 antibodies did not decrease cellular adhesion in our model. In accordance with previous results, the N-terminal domain of TGM2 was responsible for the VH3 epitope and chimeras consisting of the N-terminal domain of human TGM2 and the core-barrels domains of mouse TGM2 were sufficient to restore binding. In further studies, we identified individual amino acids in the mouse protein where changes to the human homologues also could restore the epitope *(Elek et al, manuscript in preparation*).

6. To reveal the contribution of TGM2 to intracellular protein inclusions and aggregates in cellular models of neurodegenerative disease.

i) TGM2 has a poorly studied isopeptidase activity cleaving these bonds. We have developed and characterised TGM2 mutants which are significantly deficient in transamidase activity while have normal or increased isopeptidase activity (W332F) and vice versa (W278F). The W332F mutation led to significant changes of both the K m and the V max kinetic parameters of the isopeptidase reaction of TGM2 while its calcium and GTP sensitivity was similar to the wild-type enzyme. The W278F mutation resulted in six times elevated amine incorporating transamidase activity demonstrating the regulatory significance of W278 and W332 in TGM2 and that mutations can change opposed activities located at the same active site. The further application of our results in cellular systems may help to understand TGM2-driven physiological and pathological processes better and lead to novel therapeutic approaches where an increased amount of crosslinked proteins, particularly in neurodegenerative diseases with aggregates, correlates with the manifestation of degenerative disorders. *Király et al. Amino Acids 2016.*

ii) Huntington's disease (HD) is a dominant genetic neurodegenerative disorder. The pathology affects principally neurons in the basal ganglia circuits and terminates invariably in death. Despite the high number of studies on HD still there is no hypothesis which clearly describes the HD pathogenesis. In rat PC12 neuronal cell line, expressing physiological or pathological exon-1 fragment of huntingtin protein we observed TGM2 related covalent modification of endogenous huntingtin protein which became apparent after inhibition of autophagy. After introducing loss of function mutant of TGM2 enzymes the amount of these covalent products could be reduced. *Unpublished data.*

iii) Formerly, we reported that ubiquitin was attached to α-synuclein and Hsp27 within SDS-insoluble protein aggregates in Alzheimer's disease affected human brains through N'-ε-(*γ-glutamyl*)-lysine crosslinks typical of transglutaminases (Nemes et al. FASEB J. 2004). We could place this observation in a new light and propose that this type of attachment, novel and non-canonical for ubiquitin, might subserve a signaling role and can represent an extension to the 'ubiquitin code'. This can be effective in the context of neural inclusions or other potential settings. To inspire studies into this hypothesis we attempted to uncover the direct molecular mechanism behind it. We tried to identify the TGMs, which accept ubiquitin for substrate, but using purified factors, unexpectedly, found that *in vitro* neither mono-Ub, nor Lys48 or Lys63-linked homotypic poly-Ub chains are substrates of TGMs found in the central nervous system (CNS). We demonstrated that CNS TGMs *in vitro* can bind ubiquitin neither to monomeric SNCA nor to SNCA polymerized into amyloid fibrils and this inert behavior is unchanged on lipid surfaces known to modify the substrate specificity of the enzymes. The major neuronal TGM isoform, TGM2, was equally unable to generate adducts of ubiquitin and $SNCA_{A53T}$ aggregated in neurons differentiated from SH-SY5Y neuroblastoma cells. The apparent discrepancy between our previous and present observations are likely due to factors only present in the ailing brains and missing from our test tube experiments. This draws attention to the neglected factors determining how TGMs select and recognize their substrates. We have elaborated the rational and mechanistic parallelisms by virtue of which TGM-generated isopeptide crosslinks may be one among the emerging non-canonical ubiquitin linkages. *Demény and Fésüs, has been submitted to PLOS One 2018.*

IMPACT

Beyond publishing 23 papers which generated citations, the project leader of this grant was asked to write a chapter in the book on “Transglutaminases” (of which he is one of the editors) published by Springer:

Demény M, Korponay-Szabó I, Fésüs L: ***Structure of transglutaminases: Unique features serve diverse functions***, in "Transglutaminases. Multiple Functional Modifiers and Targets for New Drug Discovery." Eds: Kiyotaka Hitomi, Soichi Kojima, László Fésüs. SPRINGER pp 1-41, 2015

In addition, the project leader organized a transglutaminase conference in Debrecen which was attended by most of the leading scientists of the field providing the opportunity to present results of this project and editing an Abstract book:

Debrecen University Symposium on “Transglutaminases in Medicine” August 3-5, 2017
http://tgase.unideb.hu/