

SHORT THESIS FOR THE DEGREE OF DOCTOR OF  
PHILOSOPHY (PHD)

**Amino acid variants in human transglutaminase 2 and their  
biological relevance**

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The Examination took place at Department of Human Genetics, University of Debrecen at 12:00 p.m., on 04<sup>th</sup> of November, 2015.

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# **1. INTRODUCTION**

## **1.1. Comparative genomics as a tool to study evolution and disease**

Comparative genomics is a large-scale powerful approach to identify similarities and difference between genomes and to understand biology of the individual genomes by comparing two or more genomes. It offers insights in to genome structure including genome rearrangement at both DNA and gene level. It is also applied in analyzing the coding regions including the protein content and noncoding regions including the prediction of regulatory elements. In this way it helps to identify genes pertaining to inborn errors and defense mechanisms that have protected us and our ancestors from extinction. In the past decade, development in whole-genome comparative analysis and associated tools, and advances in sequencing has revolutionized the field of comparative genomics. Genome-to-genome comparisons and large scale analysis of individual genomes have become feasible due to the public availability of whole genome sequences of numerous organisms. Human, mouse and other mammals diverged about 75 million years ago. Mouse is a premier genetic model organism for probing human biology and disease because of extensive genetic studies, availability of scores of inbred strains, and techniques to genetically modify them. In both organisms, comparative genomics helps in mapping the genes involved in a common pathway or diseases. Comparative primate genomics is gaining ground and by studying non-human primate genomes, origin, history and mechanism of human genome can be reconstructed and eventually human diseases can be analyzed.

The positively selected genes involved in brain development and cognitive abilities may offer valuable molecular clues about the evolution of our species and the selective pressure that drives it. Positively selected genes can also play a potential role in human medicine as many diseases are specific for humans and the therapeutic strategies based on animal models fail in humans. This is due to the differences between the humans and other species and genes under positive selection may be fundamental for some of these dissimilarities. These gene variants in humans may participate directly in the disease pathogenesis. Rapid population growth in the last few thousand years coupled with inefficient and weak natural selection lead to excessive damaging rare variants in our genome. In the recent decade considerable efforts are underway in human genetics research to study the allelic variations in human genes and relate it to evolutionary forces which shape these variations.

## **1.2. Genomic polymorphism and ongoing evolution in the human genome**

After the completion of human genome project it has become evident that vast majority of our proteins are polymorphic to single nucleotide variations (SNV). Exome sequencing has become a powerful approach for discovering rare variations and has facilitated the genetic dissection of unsolved Mendelian disorders and the study of human evolutionary history. Multi-center efforts like NHLBI-GO Exome Sequencing Project (ESP), and Exome aggregation consortium (ExAC) have made data from exome projects easily accessible for analysis. Single nucleotide variation research aids in understanding the genetics of human phenotype variation and complex diseases. The non-synonymous single nucleotide variants (nsSNVs) lead to change in the amino acid sequence which can affect protein function. Out of 13,000 exonic variants present per person, 58 % are non-synonymous. The nsSNVs along with variants in regulatory region have highest impact on the phenotype and former is implicated in both Mendelian and complex diseases. Moreover, protein misfolding or instability caused by nsSNVs also influence protein-protein interactions.

The human genome creates mutations within its sequence and preserves the genetic variation in order for human adaptability. Continuous genetic changes caused by mutations will lead to increase in allele frequencies and thus evolution of the human genome. Allele frequencies are an important indicator of evolutionary changes as increase in allele frequency of a gene is favored and decrease in allele frequency is selected against. Increase in mutated allele frequency rate signifies ongoing selection, given the selection is recurrent and positive selection acts on protein-coding genes. Loads of rare variation is generated by recent rapid population growth and there is a growing body of evidence that rare SNVs with < 5 % minor allele frequency (MAF) might play an important role in common human disease etiology.

## **1.3. The transglutaminase family**

First transglutaminase (TG; EC 2.3.2.13) was identified and reported in 1957 by Clarke and co-workers from guinea pig liver extracts. Since then, proteins exhibiting transglutaminase activity have been described in unicellular organisms, invertebrates, amphibians, fish, plants and mammals. Mammalian transglutaminases seem to be derived from papain-like superfamily of cysteine proteases with a good structural homology. Nine TGs are present in humans of which 8 are catalytically active (FXIII, TG1-TG7) and one is inactive (erythrocyte membrane protein band 4.2). These structurally and functionally related enzymes catalyze variety of  $\text{Ca}^{2+}$ - and thiol-dependent post-translational modifications like crosslinking, amine

incorporation, acylation (all transamidation reactions), esterification and hydrolysis either by deamidation or isopeptide cleavage (isopeptidase activity). Apart from this, TG2, TG4, and TG5 bind and hydrolyze GTP. The most studied and important activity is the crosslinking reaction leading to formation of isopeptide bonds, highly resistant to mechanical stress and proteolytic degradation. Functions of TGs include blood coagulation, fibrin clot stabilization, wound healing, bone synthesis (Factor XIII), skin barrier formation, extracellular matrix assembly (TG1 and TG3), formation of cornified envelopes (TG1, TG3, and TG5), prostate function (TG4), apoptosis and extracellular-matrix assembly (TG2). The functions of TG6 and TG7 are still unclear. In addition to their structural role, enzymatic and non-enzymatic functions of TGs regulate plethora of biological processes. Due to the involvement in many essential processes, human transglutaminase mutations are linked to many deficiencies and disease pathologies. TG1 mutations are associated with lamellar ichthyosis, characterized by abnormal cornification of the epidermis. Missense mutations in TG2 are implicated in early onset Type 2 diabetes. TG5 and TG6 inactivating mutations result in skin peeling syndrome and spinocerebellar ataxia 35 respectively. Mutations in F13a lead to bleeding syndrome and impaired wound healing. When compared to other members of the transglutaminase family, human transglutaminase 2 protein coding sequence has low polymorphism in the population with lowest variability.

#### **1.4. Transglutaminase 2**

The Transglutaminase 2 (TG2) is a unique member of the transglutaminase family with several enzymatic, non-enzymatic activities and interacting partners. Human TG2 gene is localized in chromosome 20q11-12 with a 37 kb exon. The molecular mass of TG2 protein is 77.3-kD protein, consisting of 686 amino acids and abundant in cytosol but also present in nucleus, mitochondria and recycling endosomes. It is also present on the cell surface and by non-classical mechanisms gets secreted to the extracellular matrix (ECM). TG2 structure could explain the uniqueness and multifunctionality exhibited by this enzyme. GDP- and GTP-bound crystal forms of human TG2 and four different irreversible inhibitors bound human TG2 have been characterized. The crystal structure of TG2 reveals four sequential and structurally distinct domains: N-terminal  $\beta$ -sandwich, catalytic core, and C-terminal  $\beta$ -barrel 1, and  $\beta$ -barrel 2. The binding of calcium ion is necessary for the catalytic crosslinking activity of TG2. This provides a conformational diversity with GTP/GDP bound form renders TG2 in a closed conformation (catalytically inactive) and upon  $\text{Ca}^{2+}$  binding TG2 acquires an open conformation (catalytically active). Five potential  $\text{Ca}^{2+}$  binding sites were identified by

mutagenesis studies but the crystal structure of  $\text{Ca}^{2+}$  bound form of TG2 is still unresolved. So under physiological conditions the crosslinking activity of TG2 is tightly modulated by  $\text{Ca}^{2+}$ , guanine nucleotides, and redox potential. Because of its ubiquitous presence in various cellular compartments and due to its diverse enzymatic and non-enzymatic functions TG2 has been implicated in versatile biological roles such as cell growth, differentiation, adhesion, migration, apoptosis, angiogenesis, ECM stabilization, wound healing, receptor signaling and signal transduction.

#### **1.4.1. Importance of the catalytic activities of transglutaminase 2 – the neglected isopeptidase activity**

At various cellular locations, distinct biochemical functions are mediated by TG2. Because of its physiological importance diverse biochemical activities are correlated with its cellular functions. The  $\text{Ca}^{2+}$  dependent transamidation reaction is an acyl-transfer reaction involving the crosslinking of  $\gamma$ -carboxamide group of glutamine residue and either the  $\epsilon$ -amino group of a lysine residue or primary amines like histamine or polyamines. The previously formed  $\text{N}^\epsilon$ -( $\gamma$ -glutamyl) lysine as well as  $\gamma$ -glutamylamine derivatives are hydrolyzed by the isopeptidase activity of TG2. Endogenous GTPase activity of TG2 is involved in the regulation of transmembrane signaling and it is shown to function as a G-protein. Furthermore TG2 can act as protein disulphide isomerase, DNA hydrolase and a regulator of mitochondrial function. Enzymatic interactions of TG2 with its substrate proteins are known to affect its activity and allow it to perform an array of diverse biological functions in the cell. The level of intracellular  $\text{Ca}^{2+}$  is elevated in certain cell types favoring TG2 mediated transamidation. Apart from this TG2 transamidation activity is reported to play a role in many other physiological and pathological processes.

So far, no enzyme has been reported to split the proteinase resistant isopeptide bonds in proteins formed by transamidation reaction as discussed above. Factor XIIIa, a member of the transglutaminase family, was reported to reverse crosslinking of  $\alpha 2$ -plasmin inhibitor to fibrinogen and fibrin potentially regulating the fibrinolytic processes. Parameswaran and coworkers proposed that due to its similarity to papain superfamily, transglutaminases could play a vital role in  $\text{N}^\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide bond cleavage and confirmed their claim using peptides cross-linked with small primary amines. Recently it was shown that TG2 has affinity to mucin 2 CysD2 domain and can catalyze the crosslinking of this network. The isopeptidase activity of TG2 was utilized to show the cleavage of the reduction-insensitive

bonds. This raised the possibility that isopeptidase activity of TG2 could also play important roles in regulation of biological processes. Like deubiquitinases, the isopeptidase activity of TG2 might also impact critical cellular functions, but due to the lack of protein based methods for its detection and extensive research, potential applications of TG2 isopeptidase activity remains unexplored.

#### **1.4.2. Transglutaminase 2 in disease; its role in neurodegenerative disorders**

Progressive neuronal loss, formation of insoluble aggregates and the aggregation of disease specific pathogenic proteins are characteristic features of neurodegenerative disorders. TG2 is highly expressed in human central nervous system and TG2 transamidating activity plays a role in covalently crosslinking pathogenic proteins leading to formation of protein polymers. TG2 is implicated in neurodegenerative disorders like Alzheimer's, Huntington's, Parkinson's and amyotrophic lateral sclerosis as well as in nervous system injuries. Several factors like energy depletion, loss of calcium homeostasis, low GTP levels, role of autophagy and increase in reactive oxygen species connect TG2 to neurodegenerative disorders.

TG2 involvement in Huntington's disease (HD) is extensively investigated. HD is an age related neurodegenerative disorder caused by a poly-glutamine repeat expansion in huntingtin protein (HTT) resulting in formation of protein aggregates (neuronal intranuclear inclusions) (NII). The activity, expression and amounts of TG2 is increased in HD and the conditions favouring the activation of this enzyme like high  $\text{Ca}^{2+}$  concentration and low GTP levels have also been observed. Misfolded proteins are either cleared by the ubiquitin-proteasome or by autophagy lysosome system and impairment of both the pathways are reported in many neurodegenerative disorders. TG2 is involved in autophagosome maturation and in clearance of ubiquitinated proteins by interacting with autophagy cargo protein nucleoporin 62. Taken together, the role of TG2 in HD is still not clear with contradictory results from numerous studies. The role of isopeptidase activity in the clearance of protein aggregates and the reversal of crosslinked proteins in intraneuronal inclusions in pathological conditions remains to be elucidated.

## **2. AIM OF THE STUDY**

- 1) By using comparative genomics and protein structural analysis to study the impact of evolutionary changes in TG2 on its structure and function.**
- 2) To screen the large-scale public databases for transglutaminase genomic variants and to compare TG2 variants with those appearing in other members of the family**
- 3) To study the impact of TG2 non-synonymous SNVs on its structure, function and biochemical activities**
- 4) To separate the transamidase and isopeptidase activities of TG2 by producing mutants and to study the possible role of both these activities on cell aggresome system**
- 5) To develop a real-time kinetic, protein based method for monitoring the isopeptidase activity of TG2.**



### **3. MATERIALS and METHODS**

#### **3.1. Materials**

All other materials were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

#### **3.2. Methods**

##### **3.2.1. Databases and Bioinformatics tools**

UniProt database sequences served as the source for the comparative genomics study. Eukaryotic Linear Motif (ELM) resource was used to identify short linear motifs (SLiMs). Exome Aggregation Consortium browser (ExAC) (beta version) was used for single nucleotide variation data. Secondary structures of wild type and mutant human and mouse TG2 proteins were predicted by GORIV method. FoldX program was applied for stability analyses using default parameters of the program.

##### **3.2.2. Expression and purification of proteins**

QuikChange Site-Directed Mutagenesis Kit II Manual (Stratagene, La Jolla, CA, USA) was used to construct TG2 mutants and variants and were checked by restriction analysis and DNA sequencing.

##### **3.2.3. Large scale production of the crosslinked FLpepT26-S100A4 (GST)**

In the presence of 5 mM Ca<sup>2+</sup>, mixture of 5 μM FLpepT26, 12.8 μM S100A4 (GST) and 5 nM TG2 was incubated for 1 hour in the reaction buffer 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 5 mM DTT and 0.01% Tween 20. EDTA (10 mM final concentration) was added to stop the reaction and to prevent unwanted modification of the crosslinked molecules during their separation. Centrifugal concentrator filter (Amicon ultra, 10kDa, Millipore, Billerica, MA, USA) was used to purify FLpepT26-S100A4 (GST) with unmodified S100A4 (GST) from the free, unbound FLpepT26 peptide. 20 mM MOPS buffer, pH 6.8 containing 0.5 mM EDTA, 150 mM NaCl, 5 mM DTT, 0.01% Tween 20 was used for isopeptidase activity. The isopeptidase assay products were confirmed by SDS-PAGE analysis and Mass spectrometric analysis.

##### **3.2.4. Activity assays**

Kinetic amine incorporation assay and real time fluorescence anisotropy assay was used to measure transamidase activity. For isopeptidase activity, Zedira assay was used which is based on the cleavage of isopeptide bond by transglutaminase in the synthesized substrate releasing the dark quencher (2,4-dinitrophenyl) linked to the cadaverine spacer followed by the increase of fluorescence from the N-terminally attached fluorophore 2-aminobenzoyl (2-Abz). Real-time fluorescence method using novel crosslinked protein-peptide substrate was used to measure isopeptidase activity. To compare the nucleotide binding 500 nM BODIPY-FL-GTPγS GTP analog was used. The fibronectin-binding property of the variants was tested using a published direct ELISA assay.

##### **3.2.5. Production of Lenti-viral TG2 constructs, cell lysate collection and western blot analysis**

Lentiviral Gateway cloning expression system (Invitrogen, Carlsbad, CA, United States) was used to produce lentiviral plasmid constructs for transfection. The cells were lysed in RIPA lysis buffer (Radioimmunoprecipitation assay buffer) and quantified by the Bradford assay. Equal amount of proteins were subjected to SDS-PAGE (7.5 % - 12 % Tris-Glycine gel) and wet blot was performed.

## 4. RESULTS

### 4.1. Comparative genomics reveals novel amino acid clusters of potentially functional effects in transglutaminase 2

For the comparative sequence alignment of human, primate and rodent (mouse) TG2 sequences from UniProt database was used. Chimpanzee and human TG2 differ only in two amino acid residues (D84E and G366E), with 99.7 % sequence identity. Similarity between human and mouse TG2 sequences is 84.1 %. The similarity in case of F13a (86.7 %), TG6 (85.6 %) and TG5 (81.7 %) is in the same range but in others it is either higher as in TG1 (89.9 %) or lower as in TG3 (76.4 %), TG7 (69 %) and TG4 (53.7 %). Human and mouse TG2 differ in 108 residues, and the differences are distributed in various domains of human TG2: 3.6 % in the  $\beta$ -sandwich, 4.3 % in the catalytic core, and 4 % each in both  $\beta$ -barrel 1 and  $\beta$ -barrel 2. Six novel amino acid clusters containing minimum of three to four amino acids next to one another appeared in human TG2 of which four are triplets and two of them are quartets.  $\beta$ -sandwich and the catalytic core domains contain one novel clusters each and  $\beta$ -barrel 1 and  $\beta$ -barrel 2 contains two clusters each. Chimpanzee, gorilla, northern white-cheeked gibbon have all the six human amino acid clusters and rhesus macaque, sumatran orangutan, olive baboon and green monkey have five clusters. Other TG2 species like horse, dog, pig, and zebrafish does not contain these amino acid clusters. As compared to mice, other human transglutaminase family members also contain novel amino acid clusters but they are different from the ones in TG2 and also from one another.

#### 4.1.1. Impact of novel amino acid clusters on structure and stability of TG2

The human TG2 sequence was modified by replacing the clusters with the mouse sequences in order to check the effect of amino acid clusters on secondary structure. By the presence of the mouse residues, conformation of all, but one (502-504) cluster was affected. The mouse clusters could destabilize the structure of human TG2 as in most cases regular secondary structural elements were shifted towards coils ( $\alpha$ -helices to  $\beta$ -strands). Although modified amino acid clusters were mostly located in loop regions and were exposed to the solvent, the secondary structure predictions indicate that the novel clusters increased the stability of human TG2. Thus by incorporating the mouse sequences at cluster sites, *stability* changes were computed for both the opened and closed forms of human TG2. Human TG2 was found to be destabilized by all the mouse sequences, especially the 527-530 cluster, where a charge inversion took place (K527E and Y528D). Considerable destabilization was observed in case

of clusters 502-504 and 614-616 in both opened and closed conformations but some of the individual changes (e.g. E15Q and A616P) were slightly stabilizing. To find an optimal packing for the mouse residues, individual residue contributions to cluster stability might implicate conformational rearrangements in the loop regions. By producing mouse homology models, stability changes for mouse TG2 in both opened and closed conformation were also computed. Except cluster <sup>323</sup>IQGD<sup>326</sup> in the open form, all other human amino acid clusters stabilized the mouse TG2 when mouse sequence was replaced with human amino acid clusters. Overall the results indicate a convergence and strong selection pressure on TG2 structure, supported by the increase in stability from mouse to human TG2 during evolution.

#### **4.1.2. Impact of novel amino acid clusters on functional elements**

Except for one non-canonical Ca<sup>2+</sup>-binding site, the novel amino acid clusters are in regions outside the known functional sites in human TG2. S3B Ca<sup>2+</sup>-binding site 326-333 present within cluster <sup>323</sup>IQGD<sup>326</sup> might lead to modified Ca<sup>2+</sup>-regulation of TG2 in humans. Cluster <sup>657</sup>LHM<sup>659</sup> is part of the newly described human specific nuclear export signal motif <sup>657</sup>LHMGLHKL<sup>664</sup> and also coincides with the celiac epitope residue M659 raising the possibility that in pathologic conditions compared to mouse protein, human TG2 is more prone for the development of autoantibodies. Post-translational modifications and protein-protein interactions are mediated by low complexity short peptide regions (3–20 residues) called SLiMs. Mostly protein segments that lack well-defined tertiary structure termed as IDRs embed SLiMs. Some IDRs and amino acid clusters are located in proximity to each other: Cluster <sup>14</sup>LET<sup>16</sup> is part of IDR 14-21, cluster <sup>614</sup>PVA<sup>616</sup> is next to IDR 597–602, and cluster <sup>657</sup>LHM<sup>659</sup> is located near IDR 626-647. The conformational diversity of TG2 can be modulated by novel amino acid clusters via the affected IDRs. Compared to the rodents, human TG2 has gained several new SLiMs since SLiMs were disabled/removed when mouse sequence replaced the human TG2 clusters. This indicates that human TG2 has gained functional motifs during evolution. Compared to rodents, human TG2 has gained two phosphorylation sites Y503 and Y528 with the appearance of the related clusters. But humans lost the S325 phosphorylation and N326 N-glycosylation sites as a consequence of the inserted cluster <sup>323</sup>IQGD<sup>326</sup>.

## **4.2. Genomic polymorphism/variants in genes of the human transglutaminase family**

Total number of entries for transglutaminases in ExAC was 5,766 SNV of which 3,623 SNVs fall under synonymous, non-synonymous or loss-of-function categories in exons. In case of each family, non-synonymous SNVs constitute 4.5-6 % of the total entries with TG2, TG4, TG5 having the lowest and TG6 the highest numbers. The number of loss-of-function (LOF; including frameshift, splice acceptor and stop gained) variants for TGs are: 22 in F13a, 19 in TG1, 29 in TG2, 20 in TG3, 40 in TG4 and TG5, while 39 in TG6 and 28 in TG7.

### **4.2.1. Evolutionary constraint on the transglutaminase genes**

For non-synonymous variations of each gene in the ExAc dataset, Z scores were calculated in order to determine the constraint of a particular gene. Positive Z scores indicate increased constraint and negative Z scores indicate decreased constraint. TG1, TG2 and F13a had fewer variants than expected and are under more constraint as specified by highest and positive Z scores. But other members had more variants than expected and are under less constraint with negative Z scores. Recently by gene damage index scores (GDI), load of disease causing mutations and mutational damage on protein-coding human genes was estimated. Amidst transglutaminases, TG2 and TG1 are under more selective pressure with lowest values and cannot tolerate damaging mutations but other transglutaminases, particularly TG4 and F13a with highest GDI scores tolerate damaging mutations. SIFT analysis revealed that F13a and TG6 have lowest and highest ratios of damaging nsSNVs, respectively. According to PolyPhen analysis TG2 and F13a have lowest while TG1 and TG5 have the highest ratios of damaging nsSNVs. The damaging nsSNVs in TG2 is around 45 % and out of these 17 % are concentrated in  $\beta$ -sandwich domain and 54 % in the catalytic core domain.

### **4.2.2. Effect of damaging nsSNVs on TG2 stability and secondary structures**

The influence of PolyPhen or SIFT predicted damaging nsSNVs on protein stability, secondary structure and functional sites including novel amino acid clusters, IDRs, SLiMs and LC3 (microtubule-associated protein light chain) interacting regions (LIRs) were analyzed. Relative stability ( $\Delta\Delta G$ ) of the opened and closed conformation was equally influenced by the mutations. Accordingly, 24.4 % of nsSNVs in the opened conformation and 28.6 % in the closed conformation were found to be destabilizing ( $\Delta\Delta G > 1$  kcal/mol), 26.7 % and 23.5 % were highly destabilizing ( $\Delta\Delta G > 3$  kcal/mol) in opened and closed conformation respectively. Eight damaging nsSNVs had a minor impact on the secondary structure

propensities based on GORIV predictions. Stability of the human TG2 structure is underscored by the persistence of secondary structures after generation of nsSNVs.

#### **4.2.3. Occurrence of damaging nsSNVs of TG2 at functional and interaction sites**

Active site residues (W241, C277, H335, and D358) and novel amino acid clusters (described in section 4.1) does not contain damaging nsSNVs. There is no damaging nsSNV at residues crucial for isopeptidase (W332) or transamidase (W278) activity. GDP binding sites are slightly influenced by the damaging nsSNVs S171L, R476Q and R478C but all the non-canonical calcium binding sites are affected. Six nsSNVs are part of Syndecan-4 interaction sequence of TG2. Similarly, interaction sites of various proteins in TG2 enclose damaging nsSNVs. Cellular TG2 functions like transmembrane signaling, cell adhesion, migration, Ca<sup>2+</sup> regulation of transamidation, cell death induction and protein turnover might be influenced due to the proximity of nsSNVs to the sites targeted by the multiple interacting partners.

In TG2 gene, three heterozygous missense mutations associated with early-onset type 2 diabetes in a small disease cohort were reported and one of those nsSNV, M330R has damaging scores. But in larger diabetes patient cohorts, the association of TG2 mutations and dysfunction has not been confirmed so far and relative to wild-type littermates, TG2 KO mice have no impairment in glucose-stimulated insulin secretion by pancreatic islets. By generating immunogenic gliadin peptides through deamidation and because of the appearance of disease specific anti-TG2 antibodies, TG2 plays a crucial role in celiac disease pathogenesis. The so far identified celiac epitopes, namely epitope 1 which is composed of Lys30, Arg116, His134 and epitope 2 consisting of Arg 19, Glu153, Glu154, Met 659 does not have any damaging nsSNV.

#### **4.2.4. Occurrence of damaging nsSNVs of TG2 at intrinsically disordered regions and short linear motifs**

We looked for the presence of damaging nsSNVs in recently reported 13 IDRs embedding 39 SLiMs in humans.  $\beta$ -sandwich domain houses a damaging nsSNV located in the IDR 65-74 and a SLiM motif recognized by SH3 domains. Series of six nsSNVs are located in IDR 208-217 and the SLiMs motifs involved in proline directed phosphorylation signaling pathways in the catalytic core. IDR 411–414 embeds SLiMs important for actin binding (WH2 domains), GSK3 and PLK phosphorylation to which four nsSNVs are co-localized. Therefore, nsSNV mutations can influence IDRs and their SliMs of TG2 with possible functional consequences.

Role of selective autophagy mediated by the so-called selective autophagy receptors (SARs) is rapidly emerging. Short linear sequence motifs called LIRs mediate interaction between SARs and proteins of the Autophagy-related protein 8 (Atg8) family. Involvement of TG2 in autophagy, prompted us to check for LIRs in TG2. The xLIR motif <sup>352</sup>EGWQAL<sup>357</sup> is targeted by two damaging nsSNVs and nine of them target WxxL motifs. Given the role of TG2 in autophagosome maturation and its interaction with autophagy cargo proteins, nsSNVs present within LIRs might have functional implications in autophagic process.

### **4.3. Heterozygous and homozygous occurrence of TG2 variants**

#### **4.3.1. Population occurrence of non-synonymous SNVs in transglutaminases**

The population distribution of nsSNVs is crucial to determine the impact of nsSNVs on human health and disease. TG3 and TG4 had the highest (over 0.2 million) nsSNV allele counts in the population covered in the ExAc dataset and TG2 and TG1 had the lowest (2601 and 5174 respectively). Common nsSNVs are present in F13a, TG3, TG4, and TG6, but TG1 and TG7 have only rare nsSNVs. The nsSNVs in TG2 are rare with allele frequency values less than 0.5 %.

#### **4.3.2. Biochemical analysis of homozygous TG2 variants**

Occurrence of damaging TG2 nsSNVs at some functional sites of the protein prompted us to screen databases to see whether the homozygous form is tolerated when compared to nsSNV variants of the other transglutaminase family members. Besides, by biochemical assays we wanted to test the function of the protein products of TG2 nsSNVs alleles found in homozygotes. The number of such homozygous individuals are highest for TG3, TG4, and TG6 (in the range of 47 to 73 thousands), while lowest for TG2, TG1, and TG7 (12, 25 and 170, respectively). TG2 and TG1 have the lowest number of nsSNV alleles in homozygous form. All together 12 individuals in various populations in the World, contain only six TG2 nsSNVs in homozygous form, by far the lowest number in the TGM family. Amidst these, probably damaging PolyPhen scores were predicted for R222Q, V542F and P612T, and P612T is highly destabilizing in both closed and open conformations, whereas V542F and D671N mostly affect the closed form.

We analyzed the functional impact of TG2 homozygous nsSNVs since they can be associated with various diseases. By site directed mutagenesis all the six homozygous occurring TG2 variants containing the respective nsSNVs were produced and tested in biochemical assays. Using two previously published kinetic assays transglutaminase activity of the variants was

measured. The transamidase activity of the variants was comparable in both the assays except R222Q, which was completely active in amine incorporation assay but inactive in the protein crosslinking assay. Compared to the wild type enzyme the variant R76H displayed increase in transamidase activity and variant V542F showed 40 % less transamidase activity. P612T variant exhibited 40 % less activity in the amine incorporation assay but only 18 % less activity in the protein crosslinking assay compared to the wild type. Interestingly PolyPhen/SIFT damaging scores were predicted for both V542F and P612T variants. Based on the amine incorporation assay calcium dependence of the transamidase reaction was also measured. At both measured  $\text{Ca}^{2+}$  concentrations, R76H exhibited high transamidase activity and variants V542F, P612T had less activity than wild type. Compared with wild type at 0.25 mM  $\text{Ca}^{2+}$  concentration variants R433Q, D671N manifested a several fold increase in the transamidase activity. At 0.25 mM calcium concentration R222Q showed the lowest efficiency, but increasing of which restored activity. Transglutaminase reactions are reciprocally regulated by  $\text{Ca}^{2+}$  and nucleotides. By using BODIPYFL-GTP $\gamma$ S analog the nucleotide binding of the variants were examined. Compared to the wild type 18 % higher GTP binding was observed for R76H and D671N and 24 % low GTP binding in case of P612T at 250 nM enzyme concentration.

By using a commercially available small chemically produced substrate the isopeptidase activity of the six variants were measured. The R222Q variant was 15 % active and the variants V542F and P612T displayed less isopeptidase activity compared to wild type. While compared to the wild type, variants R76H, D671N showed increase in isopeptidase activity. The fibronectin-binding property of the variants was tested by an ELISA method. Around 15 % less fibronectin binding was observed for the variants V542F, P612T but other variants bound fibronectin similarly to the wild-type enzyme.

Novel clusters, functional sites, and predicted IDRs were not influenced by homozygous variants R76H and D671N with benign or tolerated scores. R433Q variant is part of the  $\text{Ca}^{2+}$  binding site S5 (432-440) and IDR (428-473) embedding SLiMs like USP7 binding motif, TRAF2 binding motif, and CK1 and CK2 phosphorylation motif. V542F variant located within the MOD CK1 phosphorylation site and MOD PLK site phosphorylated by polo like kinases has damaging PolyPhen score and exhibits decreased activity. Damaging P612T variant is near to an IDR (597-602) and a novel amino acid cluster PVA (613-615) (described in section 4.1) and displays less activities and GTP binding. The C-terminal class 3 PDZ-

binding motif contains the homozygous variant D671N. The PolyPhen damaging variant R222Q with very low transamidase activity at physiological  $\text{Ca}^{2+}$  concentration and low isopeptidase activity falls in the catalytic core domain near the  $\text{Ca}^{2+}$  binding site S1 (228-236) and SLiM STAT5 SH2 domain binding motif.

#### **4.3.3. Heterozygous and homozygous LOF variants of transglutaminases**

The damaging nsSNVs of TG2 were rare and those found in homozygous individuals did not lose the biochemical function completely (one displayed reduced activity). This prompted us to investigate the population frequency of TG2 LOF variants to explore the relationship between TG2 and human diseases. Based on the available data we compiled the LOF variants of transglutaminase family members from different populations. As part of the pilot phase of the 1000 Genomes Project exon sequencing data from 185 individuals was analyzed and 2951 LOF variants, rare and likely deleterious LOF alleles, including 26 known and 21 predicted severe disease-causing variants were reported. A homozygous TG6 LOF variant is present in this dataset. Recently, in the Icelandic population a list of autosomal genes with rare LOF mutations have been published. The sequence variants identified in whole genome sequencing of 2,636 Icelanders were imputed into a set of 101,584 additional chip-genotyped Icelanders. Because of rare loss-of-function variants, 1,171 genes in 8,041 individuals (7.7 %) are completely knocked out. Icelandic dataset has homozygous LOF variants belonging to transglutaminase family members TG1, TG4, and TG5. Recently, 1111 rare gene knockouts in 781 genes were identified in 3222 exomes of consanguineous British population of Pakistani heritage. Single homozygous TG4 LOF variant was identified in this dataset. In an aim to improve the understanding and research of autism spectrum disorders a project called Simons Simplex Collection was established. A homozygous TG4 LOF variant was identified in this dataset. In Atherosclerosis Risk in Communities cohort study samples, heterozygous LOF variants for all the transglutaminases were identified. Loss-of-function heterozygotes were identified for all the members of the family in ExAC dataset but in homozygous form only for TG4 and TG6.



## **4.4. Separation and comparison of transamidase and isopeptidase activity of transglutaminase 2 and their biological relevance**

### **4.4.1. In silico considerations for the separation of transamidase and isopeptidase activity of TG2**

Crystal structures of human transglutaminases compared with corresponding papain structures reveals that the active site of both are surrounded by bulky hydrophobic residues, and by being buried in the core domain they are isolated from ambient water. The active site is quite exposed to water in the presence of substrate (inhibitor) as indicated by space-filling models. But in substrates absence the hydrophobic tunnels isolated by annealing hydrophobic side chains, such as W241-W332, W278-F334 repel water from the catalytic cavity. Active site is also shielded from the intrusion of water by W180, W337 and Y510 residues. Reaction catalysis of the human TG2 not only depends on the hydrophobicity of the amino acids around the active site and in the substrate binding area. It also depends on the complexity of the surrounding Tryptophan residues (space filling property, aromatic carbon atom ring containing nitrogen), which have important effect on stabilisation of the transition states of transglutaminase activities. Studies showed W332F mutation in rat TG2 lead to loss of transamidase activity but isopeptidase activity was not reported. In relation to this, Keillor et al targeted selected amino acids around the active site in guinea pig TG2 and identified Trp332 which when replaced by Phe or Tyr conferred very low transamidation activity on the enzyme but this study did not target Trp278 (W278F). Replacement of one or two of the seven residues (W241-W332, W278-F334, W180, W337 and Y510) by in silico modeling suggested that these residues would make the catalytic core “leaky” to the solvent water molecules without disrupting secondary domain structures. The catalytic site exposed to ambient water is expected to divert transglutaminase activity towards hydrolysis of the transglutaminase reaction products. Amino acids with shorter side chains were designed to prevent the disruption of the active site and to save the hydrophobic character. Based on this consideration we designed 12 single mutants. But only mutants W278F, W332F, inactive conserved Trp mutant W241F (control) and active site mutant C277S (control) relative to this dissertation are discussed here.

### **4.4.2. Characterisation of transglutaminase 2 mutants with dominant isopeptidase or transamidase activity**

By site directed mutagenesis two special mutants, one deficient in transamidase activity with higher isopeptidase activity (W332F) and another with opposite properties (W278F) were

found and characterised. Based on the genetic studies (e. g. 1000 Genomes) 224 position in TG2 contains valine but TG2 clone used by laboratories in the transglutaminase research field had Gly amino acid at 224 position. But in 2013, we reported structural significance of 224 position in TG2 and biochemical differences between Val<sup>224</sup> and Gly<sup>224</sup> containing variants. So we produced the Val<sup>224</sup> containing wildtype and mutants and compared the transamidase and isopeptidase activities with the Gly<sup>224</sup> containing variant. Compared to the Gly<sup>224</sup> variant, Val<sup>224</sup> wildtype TG2 displayed 33 % higher transamidase activity. As expected the Gly<sup>224</sup> and Val<sup>224</sup> W332F mutants (TG2-I) were deficient in transamidase activity. The Gly<sup>224</sup> and Val<sup>224</sup> W278F mutants (TG2-T) had similar transamidase activity compared to Gly<sup>224</sup> variant and Val<sup>224</sup> wildtype respectively. Regarding isopeptidase activity, Val<sup>224</sup> wildtype TG2 showed 45 % higher activity than Gly<sup>224</sup> variant. The Val<sup>224</sup> W332F and W278F mutants showed two fold higher isopeptidase activity than Gly<sup>224</sup> variant mutants respectively. Compared to Val and Gly 224 variants, the corresponding W332F TG2 mutants presented 1.5 and 1.3 times higher isopeptidase activity respectively. But Gly and Val 224 W278F mutant showed very less isopeptidase activity compared to the wildtype and W332F mutant. The C277S active site mutant was completely inactive in all measurements but Val<sup>224</sup> W241F mutant showed detectable transamidase but no isopeptidase activity

#### **4.4.3. Role of transamidase and isopeptidase activities on cell aggresome system in rat huntington cell model**

The above characterized transamidase (W278F, TG2 T) and isopeptidase mutant (W332F, TG2 I) along with wild-type and C277S active site mutant was used to determine the putative role of TG2 in aggregate formation and mutant huntingtin crosslinking. The *in situ* effect of TG2 on aggregate formation was studied in PC12 neuronal rat cells, which express tetracycline inducible GFP-tagged exon 1 fragment of htt gene with 23 (control) or 74 (prone to aggregate formation) glutamine repeats and stably transfected with human wild type TG2 and TG2 mutants described above. We analyzed whether TG2 can modify exon 1 fragment of huntingtin with 74 glutamine repeats. The antibody against GFP identified few bands around 55 kDa, but the band slightly above 55-kDa is likely to be the exon-1 fragment of mutant huntingtin and it was confirmed by 1C2 antibody against polyglutamine repeats. There was no significant difference in the GFP tagged exon1 fragment of huntingtin (band around 57 kDa) in the presence or absence of TG2. But high molecular weight polymers are observed only in PC12 Q74 cells transfected with wild type TG2. Without tetracycline addition these crosslink polymers were not observed.

It is well known that aggregates are cleared either by ubiquitin-proteasome or autophagy lysosomal system. In line with this, we performed autophagy and proteasome inhibition experiments to study the effect of TG2 on huntingtin aggregates. For inhibiting autophagy we used  $\text{NH}_4\text{Cl}$ , which hampers the activation of the lysosomal enzymes, hence blocking the degradation process. After 48 hrs of tetracycline addition,  $\text{NH}_4\text{Cl}$  was added for 18 hrs with fresh addition of tetracycline. Pathological huntingtin polymers were more pronounced in cells expressing Wt TG2 and TG2 T mutant compared to cells without TG2 or expressing either TG2 I or TG2 C277S mutant. In next experiments the cells were subjected to proteasome inhibition using commercially available proteasome inhibitor MG132. After 48 hrs of tetracycline addition, MG132 was added for 18 hrs with fresh tetracycline. Compared to autophagy inhibition, less crosslink polymers were observed when proteasome was blocked. After proteasome inhibition only one band was observed around 55 kDa compared to three in absence of the same or in the presence of autophagy inhibitor. Moreover the control cells with Q23 repeats induced for exon 1 fragment of huntingtin and transfected with Wt TG2 did not show any crosslink polymers. There was also no difference in the endogenous full length huntingtin levels in Q74 cells transfected with TG2 Wt and I mutant.

#### **4.5. Real time kinetic assay to study the isopeptidase activity of transglutaminase 2**

The biological significance of isopeptidase activity has not been revealed yet mainly because of the lack of protein based method for its characterization. Recently to measure the transamidase activity of TG2 a real-time fluorescence polarization assay was published. It is based on increase in anisotropy of the enzymatically crosslinked product formed after TG2 mediated crosslinking of fluorescently labelled TG2 specific dodecapeptide (FLpepT26) into bovine serum albumin (BSA). We hypothesize that with an appropriate lysine donor substrate, after the crosslinking reaction, isopeptidase activity of TG2 (cleavage of the isopeptide bond) can be monitored using the same feature. We suppose it can be attained by following the decrease in anisotropy, similar to assays measuring protease activity, for example cleavage of isopeptide bond between ubiquitin and its substrate protein by deubiquitinating enzymes. In a recent study, S100A4 turned out to be a good amine donor substrate of TG2. The product formed by crosslinking the fluorescently labelled FLpepT26 dodecapeptide and S100A4 was purified and used as a substrate in the isopeptidase assay.

#### **4.5.1. Design and implementation of the fluorescence anisotropy assay**

S100A4 was expressed with GST-tag in order to get a higher anisotropy change, better fluorescence polarization signal. We confirmed that GST did not effect TG2-catalyzed transamidation reaction as TG2 did not crosslink GST alone into FLpepT26. In order to avert unwanted further modification of the crosslinked molecules during their separation from free FLpepT26 peptide the reaction was stopped by the addition of 10 mM EDTA (final concentration). SDS-PAGE was used to confirm the efficiency of the separation and at ~42 kDa the presence of the crosslinked, fluorescently labelled FLpepT26-S100A4(GST) molecule was confirmed. Furthermore, disappearance of the 1885 Da FLpepT26 peptide which normally appears in the gel as a 10 kDa diffuse band was also confirmed.

Before it can be used as a substrate for TG2 isopeptidase activity measurement, the purified crosslinked product was validated. SDS-PAGE clearly displayed the cleavage of isopeptide bond in FLpepT26-S100A4 (GST) by TG2. During the enzymatic reaction there was a decrease in amount of substrate while the amount of released fluorescein-conjugated peptide showed a marked increase. The isopeptidase activity was validated by applying the product of the isopeptidase reaction to a Jupiter 300 C5 RP-HPLC column (Phenomenex) and HPLC-ESI-MS/MS (tandem mass spectrometry) was further used to analyse the peak corresponding to the deamidated FLpepT26 peptide. Compared to FLpepT26 (1886.1 Da) the released peptide was a single compound with a molecular mass of 1884.9 Da with slightly higher retention time. To confirm the location of the mass difference both peptides were subjected to collision induced dissociation. Complete set of mainly *b* and *y* type fragment ions were the result of MS/MS sequencing of the triply protonated parent ions ( $m/z$  629.3 and 629.7). Sequence of FLpepT26 correlate with the identified peptide fragments and the Gln to Glu transformation in the released peptide was unequivocally confirmed based on the mass differences of the *N*-terminal peptide fragments.

#### **4.5.2. Characterisation of the developed protein based isopeptidase assay**

After successfully demonstrating the TG2 mediated hydrolysis of FLpepT26-S100A4 we checked its connection with decreased anisotropy. Anisotropy is expected to decrease over time during the isopeptidase reaction as opposed to crosslinking reaction where there is an increase of anisotropy. Indeed, there was a decrease in anisotropy signal over time with increasing TG2 concentration. Reaction rates were calculated in the range of 10-600 nM TG2 concentration by using linear regression based on the first phase (9-10 minutes) of the decrease. The isopeptidase activity could be detected at 10 nM TG2 concentration, using 5  $\mu$ g

substrate (which corresponds to 0.5  $\mu\text{M}$  concentration) while the reaction rate was linearly dependent on TG2 concentration in the 10-300 nM range. We further characterized the kinetic parameters of isopeptidase reaction. At fixed TG2 level, (0.3  $\mu\text{M}$  TG2, 5 mM  $\text{Ca}^{2+}$ ) the effect of increasing FLpepT26-S100A4 (GST) substrate concentration on the reaction rate was measured.  $V_{\text{max}}$  was calculated to be  $57.94 \pm 0.92$   $\text{mr}/\text{min}/\mu\text{M}$  TG2 and  $K_{\text{m}}$  to be  $53.91 \pm 4.4$  nM for FLpepT26-S100A4(GST). Glycine methyl ester was present in the assay solutions as it was shown to facilitate isopeptidase reaction by aiding aminolysis. The fluorescence polarization was comparable in the presence or absence of glycine methyl ester (and the transamidated peptide product was not identified by MS analysis).

Influence of calcium on isopeptidase activity of TG2 was checked at different  $\text{Ca}^{2+}$  concentrations. For isopeptidase bond cleavage, 4-5 mM  $\text{Ca}^{2+}$  concentration was required to attain saturation with  $\text{EC}_{50}$  value of  $0.96 \pm 0.17$  mM (calculated by fitting a dose-response curve). In intracellular environment  $\text{Ca}^{2+}$  and nucleotides reciprocally regulate the TG2 activity. By a stable GTP compound,  $\text{GTP}\gamma\text{S}$  the inhibitory effect was tested on TG2 isopeptidase activity. The inhibitory effect of  $\text{GTP}\gamma\text{S}$  on isopeptidase activity of TG2 was similar in the protein-based and Zedira A102 substrate assay. Due to TG2 involvement in various pathologies, pharmacological intervention to regulate transglutaminase activity by inhibitors is gaining ground. Effect of a cell permeable, irreversible active site directed inhibitor of TG2, Z-DON (Zedira) was also tested on the newly developed method. Around 0.3  $\mu\text{M}$  Z-DON was enough to inhibit the isopeptidase activity completely with an  $\text{IC}_{50}$  value of 30.7 nM. We also characterised the effect of earlier described transamidase mutant (TG2 T, W278F) and isopeptidase mutant (TG2 I, W332F) (described in section 4.4) using the new method. In the protein-based assay, TG2 T mutant showed 46.6 % and TG2 I mutant showed 78.6 % lower activity than wild type TG2 respectively. Compared to the previously discussed Zedira deamination assay (described in section 4.4) W278F mutant showed higher while W332F mutant showed lower activity compared to the wild type. The isopeptidase activity of six homozygous nsSNVs of TG2 (Discussed in section 4.3.2) was also determined using the newly developed real-time kinetic assay. The relative activity was calculated for all the variants compared to the wild type TG2. Except R222Q, all the other variants showed similar activity like the wild type TG2.

## **5. KEYWORDS**

transglutaminase 2; transamidase activity; isopeptidase activity; mutants; non-synonymous single nucleotide variants; novel amino acid clusters; protein stability; real-time kinetic method; huntington's disease; crosslinking.

## 6. SUMMARY

- Novel amino acid clusters in human TG2 were identified and computational predictions revealed that these peptide sequences contribute to increasing stability of human TG2 and could potentially regulate vital functions.
- Based on the information from exome databases, TG2 non-synonymous single nucleotide variants were rare and under selective evolutionary constraint compared to other members of transglutaminase family.
- The damaging non-synonymous single nucleotide variants destabilize the protein structure and can influence vital functions. Six existing homozygous non-synonymous single nucleotide variants of TG2 were experimentally characterized and only one, the R222Q variant was shown to influence TG2 transamidase and isopeptidase activities.
- The transamidase and isopeptidase activities of TG2 were successfully separated by site-directed mutagenesis. TG2 transamidase activity was shown to be involved in the formation of covalently cross-linked protein polymers and the potential role of isopeptidase activity in reversing the protein crosslinks was also demonstrated.
- A kinetic real-time protein based method to monitor the isopeptidase activity of TG2 was successfully developed.

## 7. PUBLICATIONS



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Registry number: DEENK/264/2016.PL  
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### List of publications related to the dissertation

1. **Thangaraju, K.**, Király, R., Mótyán, J. A., Ambrus, V. A., Fuxreiter, M., Fésüs, L.: Computational analyses of the effect of novel amino acid clusters of human transglutaminase 2 on its structure and function.  
*Amino Acids*. [Epub ahead of print], 2016.  
DOI: <http://dx.doi.org/10.1007/s00726-016-2330-0>  
IF: 3.196 (2015)
2. **Thangaraju, K.**, Biri, B., Schlosser, G., Kiss, B., Nyitray, L., Fésüs L., Király R.: Real-time kinetic method to monitor isopeptidase activity of transglutaminase 2 on protein substrate.  
*Anal Biochem*. 505, 36-42, 2016.  
DOI: <http://dx.doi.org/10.1016/j.ab.2016.04.012>  
IF: 2.243 (2015)
3. Király, R., **Thangaraju, K.**, Nagy, Z., Collighan, R., Nemes, Z., Griffin, M., Fésüs, L.: Isopeptidase activity of human transglutaminase 2: disconnection from transamidation and characterization by kinetic parameters.  
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## 8. SUBMITTED MANUSCRIPT'S

1. **Kiruphakaran Thangaraju**, Róbert Király, Máté A. Demény, János András Mótyán, Mónika Fuxreiter, László Fésüs. Genomic variants reveal differential evolutionary constraints on human transglutaminases and point towards unrecognized significance of transglutaminase 2. PLoS One. Under review.

## 9. CONFERENCE PAPERS, PRESENTATIONS AND POSTERS

**Kiruphagaran Thangaraju, Monika Fuxreiter and Laszlo Fésüs.** Significance of evolutionary changes in human transglutaminases. Abstracts presented at the 14<sup>th</sup> International Congress on Amino acids, Peptides and Proteins. **Amino acids**. 2015 Aug; 47(8): page 1629. (Abstract).

### Oral presentations

**‘Role of transglutaminase 2 on cell aggresome system and its implications on neurodegenerative disorders’** Marie Curie Initial Training Network (ITN) – ‘TRANSPATH’, Mini-conference, 2015, Covalab, Lyon, France

**‘Comparative and population genomics data reveal strong evolutionary pressure on structure and functions of transglutaminase 2’**, 9<sup>th</sup> Winter Symposium 2016, Molecular, Cellular and Immune Biology Doctoral School, University of Debrecen

**‘Influence of Transglutaminase 2 activities on cell aggresome system’**, 8<sup>th</sup> Winter Symposium 2015, Molecular, Cellular and Immune Biology Doctoral School, University of Debrecen

**‘Measurement of isopeptidase activity of TG2 on protein substrates by kinetic fluorescence anisotropy assay’** 7<sup>th</sup> Winter Symposium 2014, Molecular, Cellular and Immune Biology Doctoral School, University of Debrecen

**‘Role of transglutaminase 2 on cell aggresome system and its implications on neurodegenerative disorders’** Marie Curie Initial Training Network (ITN) – ‘TRANSPATH’, 2013, Aston University, United Kingdom

**‘Transglutaminase variants in large human population’**, 6<sup>th</sup> Winter Symposium 2013, Molecular, Cellular and Immune Biology Doctoral School, University of Debrecen

### Poster presentations

**‘Influence of transglutaminase 2 activities on aggresomes and formation of covalently cross-linked protein polymers in a cellular model of Huntington’s disease’** Annual meeting of the Hungarian Biochemical Society 2016, Szeged, Hungary

**‘Development of protein and cell based systems to study isopeptidase activity of Transglutaminase 2’** Hungarian Molecular and Life Science Conference 2015, Eger, Hungary

**‘Comparative population genomics and protein structural analysis to reveal novel functions of human Transglutaminase 2’**, Gordon Research Conference on Transglutaminases in Human Disease Processes 2014, Lucca, Italy and at Annual meeting of the Hungarian Biochemical Society 2014, Debrecen, Hungary

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