THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

DNAJA1 as a novel interacting partner of transglutaminase 2

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CONTENTS

Abbreviations

aa	Amino acid
Akt	v-Akt Murine Thymoma Viral Oncogene
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAK	Focal adhesion kinase
GST	Glutathione S-transferase
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
IL	Interleukin
KD	Knock down
LC-MS/MS	Liquid chromatography tandem mass spectrometry
NF-κB	Nuclear factor kappa B
OD	Optical density
PBS	Phosphate buffer saline
PDI	Protein disufide isomerase
PFA	Paraformaldehyde
PMSF	Phenylmethane sulfonyl fluoride or phenylmethylsulfonyl fluoride
RPMI	Roswell Park Memorial Institute
RU	Response unit
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TBS	Tris buffer saline
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Z-DON	Z-(D)-DON-Val-Pro-Leu-OMe

1. INTRODUCTION

1.1. Transglutaminase 2; a multifunctional protein crosslinking enzyme

Transglutaminase 2 (TG2) is a widely expressed multifaceted enzyme with distinct biochemical activities that functions both inside and outside the cell [1]. TG2 was primarily described with its transamidation activity which results in postranslational amine incorporation into proteins or the formation of proteolytically resistant γ-glutamyl-ε-lysine isopeptide bonds between γ-carboxamide group of a protein bound glutamine and ε-amino group of a peptide bound lysine residues [2]. TG2, as a transglutaminase, is regulated allosterically by calcium and GTP/GDP; that is TG2 is found in open conformation and activated when bound to calcium whereas GTP/GDP binding keeps the enzyme in closed conformation which in turn results in its inactivation [3]. In addition to its transamidating activity, TG2 acts as G protein, protein disulphide isomerase (PDI) [4], protein kinase [5] and DNA hydrolase [6] which distinguish TG2 from other members of the family. Besides regulating enzymatic activities, TG2 also has certain non-enzymatic roles such as functioning as adaptor protein, cell surface adhesion mediator [7] and forming protein scaffolds [8].

Since TG2 has such diverse catalytic activities and non-enzymatic functions, it regulates plethora of physiological and pathological conditions. In the intracellular environment, TG2 participates in signaling events [9] and thus regulates cell survival particularly in response to hypoxia [10], oxidative stress [11], and cell wounding [12], whereas outside the cell TG2 modulates cell-ECM adhesion, cell migration and outside-in signaling which are largely linked to its interaction with members of the integrin family and fibronectin [13]. Furthermore, TG2 has been implicated in wide range of pathological conditions such as tissue fibrosis, inflammation, cardiovascular and neurodegenerative diseases, cancer progression and metastasis [14].

1.1.1. Different isoforms of Transglutaminase 2

The first transglutaminase, now designated TG2, was discovered in 1957 as liver enzyme catalyzing the incorporation of amines into proteins [15]. Since then, other members of transglutaminase family with this activity have been identified from unicellular organisms, invertebrates, fish, mammals, and plants. Only one single transglutaminase gene has been found in invertebrates whereas nine evolutionary related genes encoding TG1–7, blood coagulation FXIIIa, and the inactive epb42 have evolved in vertebrates by successive duplications [16]. These proteins are implicated in diverse functions including protein scaffolding, maintaining membrane integrity, cell adhesion, and modulation of signal transduction. Keratinocyte TG (TG1) is expressed in the stratified squamous epithelia of the skin and upper digestive tract and in the lower female genital tract and its catalytic activity is induced by proteolytic cleavage, increased Ca^{2+} level, and interaction with tazarotene-induced gene 3 (TIG3). Epidermal TG (TG3) is expressed in hair follicles, epidermis, and brain. It catalyzes the crosslinking of trichohyalin and keratin intermediate filaments which is essential for hair fiber morphogenesis. Prostate TG (TG4) is found in the prostate gland, prostatic fluids, and seminal plasma but its exact function in humans is not known. Transglutaminase 5 (TG5) is mainly present in foreskin keratinocytes, epithelial barrier lining, and skeletal muscle and crosslinks loricrin, involucrin, and SPR3 in epidermis contributing to hyperkeratosis in ichthyosis and psoriasis patients. In addition to their transamidation activity, TG2, TG3, TG4 and TG5 can also bind and hydrolyse GTP [1, 14]. Transglutaminase 6 (TG6) is localized in the human testes and lungs, and in the brain of mice. Its activity is induced following proteolytic cleavage of the proenzyme. TG7 is expressed in testes, lungs, and brain but its function is not yet known. Plasma TG (FXIIIa) is found in platelets, plasma, astrocytes, macrophages, dermal dendritic cells, the placenta, chondrocytes, synovial fluid, the heart, the eyes, and in cells of osteoblast lineage and is essential component of blood coagulation

cascade. It is activated by thrombin-dependent cleavage and in the presence of Ca^{2+} it catalyzes crosslinking of fibrin molecules to stabilize fibrin clots. FXIII-A also functions as adaptor protein in ECM and facilitate cell-matrix interactions. Band 4.2 is a unique enzymatically inactive member of the family. It is major component of the erythrocyte membrane cytoskeleton and involved in maintenance of membrane integrity and regulation of cell stability. Band 4.2 is mainly expressed in erythrocytes, bone marrow, fetal liver, and spleen [1, 14].

1.1.2. Structural features of Transglutaminase 2

TG2 is the most extensively studied and most widely distributed member of the transglutaminase family. Human TG2 is a 76-kD protein consisting of 687 amino acids divided into 4 distinct domains including an N-terminal β-sandwich domain, the catalytic core and two C-terminal β-barrel domains [17]. The catalytically active site of TG2 is composed of cysteine 277 (C277), histidine 335 (H335) and aspartate 358 (D358) residues which are critical for transamidation activity (Figure 1). Additionally, two conserved tryptophan residues (W241 and W332) are essential for transamidation activity stabilizing the enzyme-thiol intermediate that forms during catalysis [18]. Human TG2 was first cloned and sequenced in 1991 [19]. Until now, major research groups working in the transglutaminase field used this recombinant TG2 as a reference sequence for most of the biochemical, cellular and structural analysis [17, 20]. This sequence however, has glycine at position 224 that differs from the TG2 gene in the NCBI, Ensembl, and ESP (exon sequencing project) databases, which show valine at this position. According to exon data and also the Uniprot database, TG2 with glycine 224 likely arised from a cloning error [21]. All so far available human exon sequencing data show that valine is found at position 224 of TG2 which is highly

conserved across species and all transglutaminases presumed to work in cells. Importantly, Val224 is located in the catalytic core domain of the enzyme.

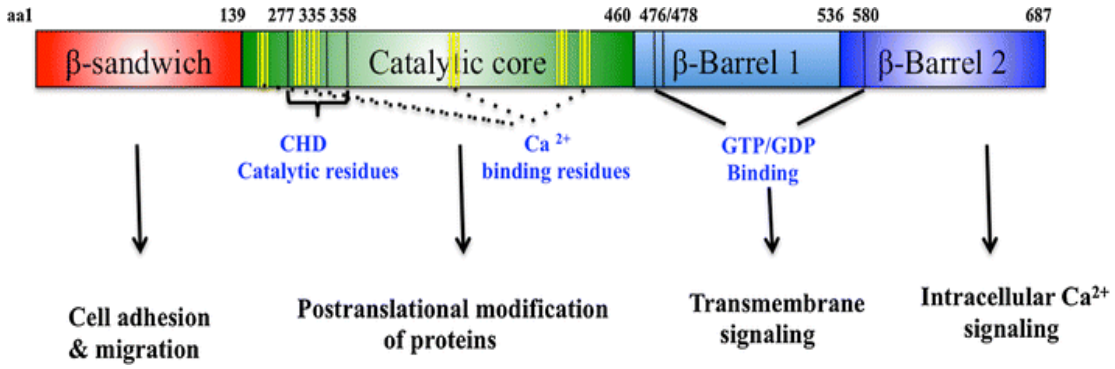

Figure 1. Scheme of TG2 with its domains along with its proposed functions. TG2 domains are *color coded*. Domain boundaries have been indicated by *amino acid numbers*. Calcium binding residues, catalytic residues, and GTP/GDP binding residues have also been indicated. Amino acid numbers have been provided for the catalytic residues and GTP/GDP binding residues [21].

1.1.3. Regulation of Transglutaminase 2 expression

TG2 is widely expressed in almost all cells and tissues and can be localized in different compartments of the cell [22]. Several regulatory elements of human TG2 including retinoic acid response elements (1.7 kb upstream of the transcription start site), glucocorticoid response element (1399 bp upstream), an interleukin 6 response element (1190 bp upstream), TGF- β1 response element (900 bp upstream), two AP2-like response elements (634 bp and 183 bp upstream of the start site), hypoxia response element (367 bp upstream), and 4 Sp1 binding motifs (54 bp and 43 bp upstream and 59 bp and 65 bp downstream of the start site) are located in the TG2 promoter. TG2 expression is tightly regulated by various external signals including retinoids, vitamin D, IL-6, TGF-β1, EGF, TNF and transcription factors including RAR:RXR, NF-κB, Sp1 and hypoxia inducible factor (HIF) [23].

1.1.4. The activation of Transglutaminase 2

The transamidation reactions result in either the formation of isopeptide bonds between acyl-acceptor and acyl-donor peptides or modification of proteins by the addition of primary amines which alters the properties of proteins. Polyamines as substrates can also mediate an indirect crosslink formation between polypeptides [24]. When the thioester bond between enzyme and acyl donor peptide is attacked by a water molecule then the reaction is called deamination that results in the conversion of the acyl-donor glutamine residue to a glutamate residue [25]. TG2 activity is tightly regulated under physiological conditions by interaction with cofactors (Figure 2). It can bind up to six Ca^{2+} which keep the enzme in open conformation and expose the catalytic core domain for the substrate [26]. On the other hand, TG2 contains a unique guanine-binding site between the catalytic core and the first β-barrel. GTP/GDP binding leads to considerable interaction between the catalytic domain and two β-barrel domains, which renders TG2 in a closed conformation and results in the inhibition of its activity [15]. In its GTP/GDP bound form TG2 was shown to act as a G protein in α1 adrenergic receptor signaling, carrying the signal to phospholipase C-δ1 [27]. Additionally, TPα thromboxane A2 receptor [28] and oxytocin receptor [29] were also implicated to exploit TG2 as a G-protein.

TG2 inside living cells is believed to be predominantly in catalytically-inactive GDP/GTP bound form due to the low Ca^{2+} concentrations [30]. Interestingly, extracellular TG2 was shown to be inactive despite high calcium and low GTP levels [31]. In the extracellular environment, TG2 is present in two different states including oxidized (inactive) and reduced (active) forms (Figure 2). The triad of cystein residues including Cys370, Cys371, and Cys230, have very high redox potential and oxidizing conditions lead to the formation of

interstrand disulfide bonds between these residues which in turn inactivate the transamidation activity of TG2 [32].

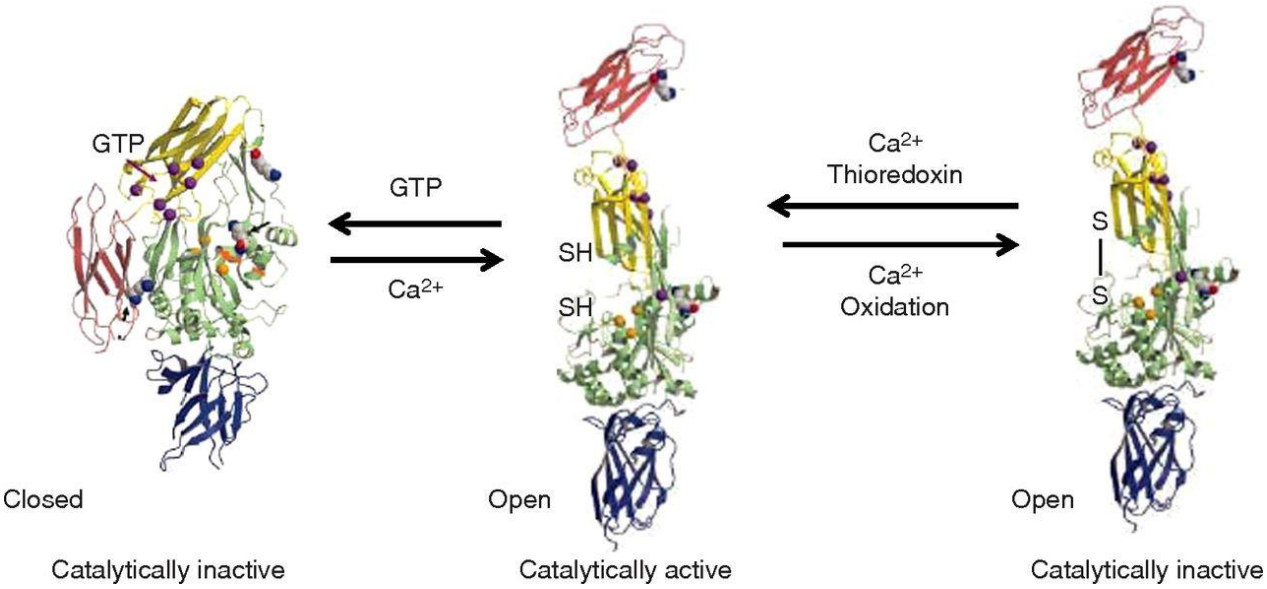

Figure 2. Different TG2 conformations. GTP/GDP-bound closed and catalytically inactive conformation (left). The putative Ca^{2+}-bound (catalytically active) open conformation (middle). The oxidized state (open, catalytically inactive) which can be prevented by treatment with thioredoxin (right). Functional domains: NH_2-terminal β sandwich domain (blue), catalytic core domain (green) and COOH-terminal β1 barrel domain (yellow) and β2 barrel domain (red) [1].

1.2. Cellular functions of transglutaminase 2

In addition to its transamidation and GTPase functions, TG2 has been reported to exhibit protein kinase acitivity and be able to phosphorylate insulin-like growth factor-binding protein-3 (IGFBP-3) [33], histones [34], p53 [35] and retinoblastoma protein [36]. Weak phosphorylation of TG2-cross-linked IGFBP-3 polymers in the presence of Ca^{2+} revealed that protein kinase activity of TG2 was inhibited by Ca^{2+}. Surprisingly, an inhibitor of the TG2 transamidating function, cystamine, was also able to block its protein kinase activity [33]. TG2 itself appeared phosphorylated by protein kinase A (PKA) resulting in reduced

transamidating activity but increased kinase activity of the protein [37]. It was also reported in several studies that TG2 can function as protein disulfide isomerase. In one of these studies, denatured RNAse A was converted into active enzyme by TG2 through the formation of correct disulphide bonds. It was revealed that PDI activity of TG2 is independent from its transamidation activity and is not affected by the presence of calcium and nucleotides [4]. Mitochondrial complex I, II and V [38] and a mitochondrial protein, the adenine nucleotide translocator 1 (ANT1) are the other proteins which were suggested to be substrates for PDI activitiy of TG2 [39].

TG2 is also involved in various nonenzymatic functions which are dependent on direct noncovalent interactions of this protein with a number of interacting partners localized in different cell compartments. These interactions implicate TG2 in a plethora of adapter/signaling functions both inside and outside of cells [40].

1.3. Interacting partners of Transglutaminase 2 outside and inside of cells

Numerous studies indicate TG2 in a wide range of pathophysiological conditions including tissue fibrosis, regeneration and response to injury, inflammation, cardiovascular and neurodegenerative diseases, and cancer progression and metastasis [15]. So far, various TG2 interacting partners have been identified and their contribution in TG2 related pathological and physiological processes have been well documented. TG2 is able to promote cell-matrix adhesion, cell migration, and signaling through its interaction with fibronectin in ECM [8]. TG2 binds with high affinity to the gelatin-binding region of fibronectin via its N-terminal β-sandwich domain. Cell surface TG2 was also found to collaborate with integrins (β1/β3/β5) in cell adhesion via both the first and fourth domains of the protein. TG2 serves as a bridge between integrins and fibronectin and enhances the interaction of cells with

fibronectin [40]. It was reported that TG2 also has a strong affinity for another ECM protein, heparin (heparan sulfate proteoglycan) and this binding does not affect the catalytic activity of TG2 but protects it from thermal unfolding and proteolytic degradation [41]. Cell-surface HS was also implicated to be essential in TG2-mediated RGD-independent cell adhesion [42]. HSPGs are divided into 3 subfamilies; the membrane-spanning proteoglycans (namely syndecans, betaglycan and CD44v3), the glycophosphatidylinositol (GPI)-linked proteoglycans (namely glypicans), and the secreted extracellular matrix (ECM) proteoglycans (namely agrin, collagen XVIII and perlecan) and are major components of extracellular matrices [43]. Among the subfamilies of HSPGs, syndecan-4 (S4) is a possible candidate receptor for TG2 in cell adhesion and migration [44].

TG2 was also found to interact with PTEN (a phosphatase and tensin homologue deleted on chromosome 10, also called MMAC1 or TEP1), a tumor suppressor protein in pancreatic ductal adenocarcinoma cell lines (PDAC). PTEN is involved in cell growth, invasion, migration, and focal adhesion functions by negatively regulating the PI3K/AKT pathway [45]. Since the expression of TG2 in PDAC cells leads to constitutive activation of focal adhesion kinase and its downstream PI3K/AKT signaling pathway it was proposed that TG2 could affect FAK/PI3K/AKT pathways by regulating PTEN expression and function [46,47].

Furthermore, TG2 has been reported to interact with several other proteins inside the cells including Bcr and Abr, Retinoblastoma (Rb) protein, Angiocidin, PLC δ1, Calreticulin, Importin α3, HIF1β, Calmodulin, Bax and Bak. All of these proteins listed above could bind to either an open (e.g., Bcr and Abr) or closed (e.g., calreticulin) form of TG2. Some of the above listed interacting partners of TG2 including Rb protein, Bcr, fibronectin and Angiocidin also serve as substrate for transamidation activity of TG2 [21]. The bioinformatic analysis to determine the role of unique structural features on TG2 function revealed several intrinsically disordered (ID) regions along with short linear motifs (SLiMs)

in the TG2 sequence. Some of the interacting protein binding sites overlapped these ID regions indicating the importance of these regions in mediating additional protein–protein interaction and its role in regulating diverse protein functions [21].

1.4. Molecular chaperons

Molecular chaperons are essential group of proteins in the cells working as guardians of protein homeostasis. These proteins are involved in several key cellular functions such as protein folding, unfolding, translation, translocation, and degradation under both physiological and stressful conditions [48]. In addition to these functions, most chaperones also participate in the regulation of protein conformation and stability, protein transport and protein–protein interactions [49]. The heat shock proteins (HSP) are the largest and most well-described group of molecular chaperones [50]. HSP classes include the well-characterised HSP90 (HSPC) and HSP70 families (HSPA), and the less-well studied HSP40 group (DNAJ) which has the greatest number of members [51,52]. Most chaperones do not work alone and their function is mediated by chaperone-regulating proteins known as co-chaperones [53]. These proteins either stimulate the chaperons or recruit other chaperons into functional multi-chaperon complexes [54].

1.4.1. Structure and function of DNAJ/HSP40 family

DNAJ family of proteins mostly work as co-chaperons and are the largest and most diverse group of co-chaperons. In humans, DNAJ family consists of 49 members and is categorized into three subclasses (Figure 3) including type I (DNAJA), type II (DNAJB) and type III (DNAJC) [51]. Type I DNAJ (DNAJA, 4 members) similar to E. coli DnaJ, is composed of an

N-terminal J-domain, a glycine/phenylalanine (G/F) rich region, a cysteine repeat region and a largely uncharacterized C-terminus. Type II DNAJ (DNAJB, 13 members) lack the cysteine repeat region and consists of the other 3 domains. However, Type III DNAJ (DNAJC, 32 members) lack both the G/F and Cys-repeat regions and the J-domain might be positioned anywhere along the protein [55]. The 70 amino acid J-domain is highly conserved across all organisms and pivotal for the interaction with, and stimulation, of the molecular chaperone HSP70. Highly conserved histidine–proline–aspartic acid [HPD] motif of J-domain is essential for the stimulation of ATP hydrolysis of HSP70 [56]. The G/F rich region is also thought to support the interaction between the J-domain and the ATPase domain of HSP70 facilitating the formation of a stable chaperone complex [57]. However, G/F rich region is not essential for stimulation of HSP70, as the selected type III DNAJ (DNAJC) which lacks the G/F rich region can also stimulate ATP hydrolysis [58]. The Cys-repeat region, also known as the Zinc finger, forms part of the substrate binding domain of DNAJ and is important for the presentation of peptides to HSP70. This domain consists of Cys-X-X-Cys motif repeated four times, where X represents any amino acid [59]. By this way and particularly due to the presence of 49 DNAJ and only 13 HSP70s, DNAJ can modulate the specificity of HSP70 action on different client substrates [60]. Although the C-terminal region of the DNAJ family takes part in the substrate binding domain of type I and II DNAJ and is important for efficient co-chaperone functioning, it remains largely uncharacterized [61]. Some DnaJ family members contain additional domains which may contribute to their functional diversity [62]. For example, the protein disulfide isomerase-like domain of the mammalian DnaJ protein ERdj5/JPD1 promotes the formation of appropriate disulfide bonds of endoplasmic reticulum (ER) proteins [63, 64].

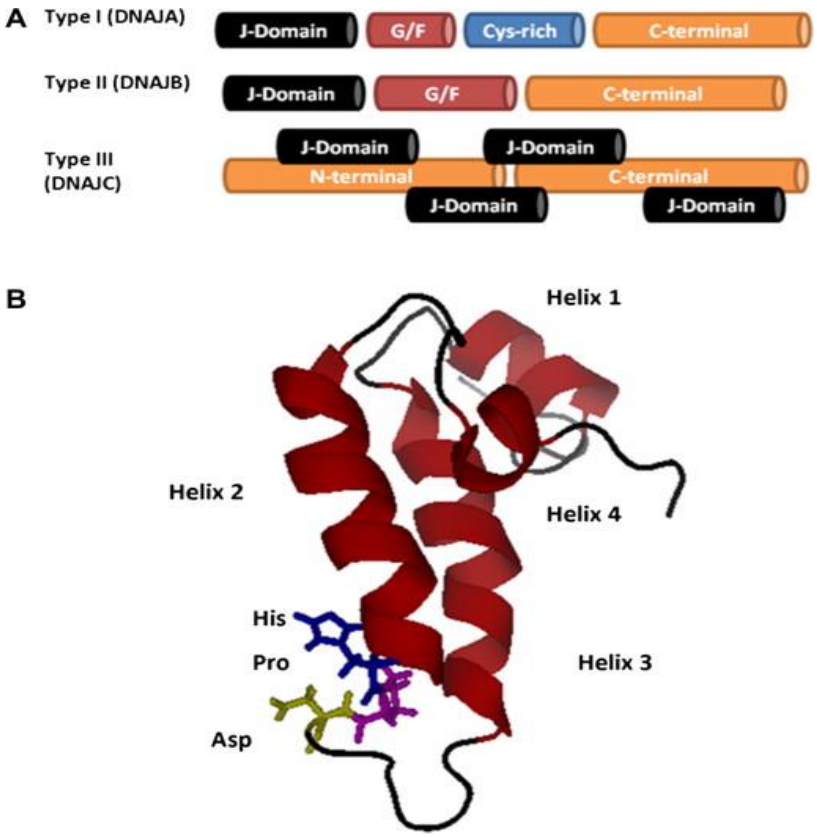

Figure 3. Classification and functional domains of DNAJ. (A) DNAJ may be classified according to the presence or absence of three domains, namely the J domain, a glycine/phenylalanine rich region (G/F) and the cysteine repeat motif (Cys-repeats) together with a C terminal domain that is largely uncharacterized. (B) The three dimensional J-domain structure (*E. coli* J-domain; 1BQ0) that is currently used to define the DNAJ family. The figure illustrates the structure of the J domain that resembles a 'protruding finger' (helix 2 and 3) containing the highly conserved HPD (His–Pro–Asp) motif located on the loop between helix 2 and 3. This HPD motif is important for stimulation of Hsp70 ATPase activity [64].

1.4.2. Human protein DnaJ subfamily A member 1 (DNAJA1)

Some of the members of DNAJ family including DNAJA1A, DNAJB1, DNAJB6b and DNAJB8 may work as chaperons and suppress the aggregation of client proteins independently of HSP70 [65]. In addition, DNAJA1 has been indicated to regulate androgen receptor signaling and spermatogenesis in mice [66], and to contribute to the radioresistance

of glioblastomas [67]. Genomics analysis of pancreatic cancer cells has revealed that DNAJA1 was downregulated 5-fold in these cells relative to normal healthy cells and cells undergoing pancreatitis [68]. In another study, overexpression of DNAJA1 resulted in the suppression of the stress response capabilities of the oncogenic transcription factor, c-Jun, and the decrease in cell survival [69]. Additionally, DNAJA1 seems to play role in importing proteins into the mitochondria [70].

2. AIMS OF THE STUDIES

1. Characterization and comparison of transamidation activities and calcium sensitivities of TG2 Val “wild type” and TG2 Gly “artificial” variants of transglutaminase 2.
2. Identification of novel interacting partners of human TG2.
3. Confirmation of DNAJA1 as a novel interacting partner of human TG2.
4. Determination of the DNAJA1 binding domain of TG2.
5. Determination of DNAJA1 binding conformer of TG2.
6. Identification of the role of DNAJA1 on in vitro and in situ crosslinking activity of TG2.
7. To explore if DNAJA1 is a substrate of TG2.

3. MATERIALS AND METHODS

Preparation and analysis of recombinant proteins

For the expression of recombinant human TG2 and DNAJA1 proteins in bacterial system, cDNAs for these proteins were subcloned into pET 30 EK/LIC (Novagen) via ligation independent cloning according to manufacturers' procedure. For the mammalian cell expression, TG2 was subcloned from pET 30 EK/LIC vector into pTRIEX 4 Neo vector (Novagen) using the BamH I and Xho I sites of the vector and the Bgl II and Xho I sites of the TG2 clone in the pET vector. Clones were checked by in house DNA sequencing facility (ABI PRISM; Capillary sequencing runs were performed by Genomic Medicine and Bioinformatics Core Facility at University of Debrecen) and the constructs prepared for bacterial expression were transformed into Rosetta 2 E. coli cells (Novagen) by standard protocol. The cells were grown at 30 ^{o}C until OD600 reached 0.6 and induced at 18^{o}C with 100 μM isopropyl β-D-thiogalactoside (IPTG) overnight. The cells were then collected by centrifugation at 4°C and lysed by sonication in 50 ml of lysis buffer [binding buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM ß-mercaptoethanol, 5 % glycerol, 5 mM imidazole), 1 mM PMSF, protease inhibitor cocktail tablets (Roche)]. The cells were further lysed by adding 1 % Triton-X100 into lysis buffer and incubating the lysate at 4 ^{o}C for 30 min and then centrifuged at 20,000 x g for 30 min. Clear supernatant was incubated with HisPurTm Ni-NTA metal-affinity resin (Thermo Scientific) for 2 hours at 4 ^{o}C. Resins were washed several times with buffer A (binding buffer + 150 mM NaCl) and buffer B (binding buffer + 15 mM imidazole). Protein was eluted with buffer C (binding buffer + 250 mM imidazole). The purity of the protein was determined by staining of SDS polyacrylamide gels with Page blue protein staining solution (Thermo Scientific) and Western blotting with mouse monoclonal antibody (CUB7402 for TG2 (1:2000); ThermoFisher, MA5-12745 antibody for

DNAJA1 (1:1000)) and secondary antibody, horseradish peroxidase (HRP) conjugated anti mouse IgG (Covalab) (1:10.000). The bands were detected by Chemiluminescence ECL Detection System (Millipore). Full length and domain deleted variants of TG2 subcloned into pGEX 2T vector (GE Healthcare Life Sciences) to produce recombinant GST fusion proteins [71] were provided for the binding experiments. The constructs were expressed in Rosetta 2 E. coli cells (Novagen). The cells were induced at 25^{o}C with 100 μM isopropyl β-D-thiogalactoside (IPTG) for 6 hours. Cell lysis was performed as described above using binding buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 5 % glycerol) and supernatant was incubated with Pierce Glutathione Superflow Agarose Resin (Thermo scientific) for 2 hours at 4^{o}C . After 2 washes with binding buffer (without glycerol), fractions were collected with the binding buffer containing 10 mM reduced L-Glutathione (Sigma) and analysed as described before.

Cell culture

Human embryonic kidney (HEK 293T AD) (purchased from Agilent Technologies, US) cells were grown in DMEM (Sigma) medium and NB4 cells (purchased from DSMZ GmbH) were grown in RPMI (Sigma) medium both supplemented with 10 % fetal calf serum (FCS, Sigma F7524 and GIBCO® life technologies, respectively), L-glutamine (300 mg/mL) (Sigma G7513) and penicillin/streptomycin antibiotics (Sigma, P 433). All cell lines were incubated in 5 % CO_2 containing humidified atmosphere at 37^{o}C. HEK 293T AD cells were stably transfected for the overexpression of human TG2 and both stable and transient transfections were carried out using Lipofectamine® 2000 (Invitrogen) according to the manufacturers' instructions. Stable cell lines were generated by using 500 μg/mL of neomycin and they were maintained by using 250 μg/mL of neomycin. TG2 overexpressing HEK 293T AD cells were transfected with DNAJA1 specific Silencer Select Pre-designed siRNA (Ambion) for the downregulation of DNAJA1, and with scrambled RNA as control using

Lipofectamine® 2000 (Invitrogen). 1 μM all trans retinoic acid (ATRA) (Sigma, R2625) was used for differentiation of NB4 cells for 72 hours to express endogenous TG2 [72]. These differentiated cells were used for experimental analysis.

GST Pull-Down Assay

Differentiated NB4 cells were used for GST Pull down experiment. Firstly, the cells were collected and washed in PBS and then resuspended in lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.5 % Triton and protease inhibitor cocktail). Cells were incubated in lysis buffer for 30 min at 4^{o}C and then centrifuged at 10,000 g for 20 min. Bradford assay was performed to estimate the clear lysate and 1 mg/ml lysate was taken for GST Pull down experiments. Pierce Glutathione Superflow Agarose Resin (Thermo scientific) were washed in RPMI medium and incubated with cell lysate and 100 μg of purified recombinant GST tagged TG2 for 1 hour. Equal amount of GST was used as a control. Later, the beads were washed 4-5 times with 1 ml of lysis buffer and then boiled in laemli sample buffer and loaded on SDS PAGE. Immunoblotting was also performed with the same samples and DNAJA1 was detected using anti-DNAJA1 antibody (ThermoFisher, MA5-12745). Recombinant purified GST-TG2 and DNAJA1 were also used for GST Pull down experiment. Equal amount of GST-TG2/GST and DNAJA1 (100 μg) were combined in a single Eppendorf tube with glutathione beads and left for interaction for 1 hour at 4^{o}C. Rest procedure was the same as described above.

Mass Spectrometry (LS MS/MS)

GST Pull down assay samples were given for mass spectrometry analysis. Only those protein bands which were unique to TG2 pulled down samples compared to GST control were excised from the SDS PAGE. The gel slices were in-gel digested with trypsin [73]. During digestion first a reduction was performed using DTT followed by alkylation with

iodoacetamide (Bio-Rad). The overnight trypsinization was performed using stabilized MS grade bovine trypsin (ABSciex) at room temperature and the digested peptides were extracted and lyophilized. The peptides were redissolved in 10 µl 1 % formic acid and used for LC-MS/MS analysis. 4 µl of sample was used in each case for mass spectrometry analysis. 4000 Q TRAP (ABSCiex) - nanoHPLC (Bruker) LC-MS/MS System was used for data acquisition. Prior to mass spectrometry analysis the peptides were separated using a 90 min. water/acetonitril gradient and 300 nl/min flow rate on an EasynLC II (Bruker) nano HPLC. The separation was done on a reverse phase Zorbax 300SB-C18 analytical column (Agilent). In order to eliminate the carryover wash steps were administrated after each sample. The aquired LC-MS/MS data were used for protein identification with the help of ProteinPilot 4.0 (ABSciex) search engine searching the SwissProt database and using the Biological modification table included in the ProteinPilot 4.0. The proteomic analyses were done in the Proteomics Core Facility, University of Debrecen.

Nondenaturing polyacrylamide gel electrophoresis

Recombinant His-TG2 and active site mutant of His-TG2 (TG2 C277S) were incubated in reaction buffers (50 mM Tris/HCl pH 7.4, 150 mM NaCl and 0.1 % Tween 20) including EDTA, $CaCl_2$, Z-DON (zedira), $CaCl_2$ + Z-DON and GTP for overnight at 4^oC. Nondenaturing electrophoresis was carried out in 8 % polyacrylamide gel in 25 mM Tris and 192 mM glycin including buffer pH 8.3, for 2 hours at 4^oC at 40 milliamper and different conformers of TG2 were visualized by Page blue protein staining solution (Thermo Scientific).

ELISA

The ELISA measurements were performed in triplicates according to standard protocol described previously [74]. Interaction of His-DNAJA1 with full length GST-TG2 and domain deleted variants of TG2 (GST-TG2Δβ-barrel1 and GST-TG2Δβ-barrel2, GST-TG2ΔCAT, GST-TG2Δβ-sandwich, GST-CAT) were analysed. Briefly, TG2 and domain variants (1 μg) diluted in coating buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA) were used to coat the wells of Maxisorp (Nunc) microtiter plate for overnight at 4^{o}C and wells were blocked with 5 % (w/v) milk powder in TBS-T (0.05 M Tris/HCl pH 7.5, 0.15 M NaCl, 0.01 M EDTA, 0.1 % Tween 20) for 1 hour at RT. The plate was then incubated with 0.5 μg DNAJA1 in TBS-T including 5 mM $CaCl_2$ for 1 hour at RT and binding was detected with an anti-DNAJA1 monoclonal antibody (ThermoFisher, MA5-12745) diluted (1:1000) in TBS-T for 1 hour at RT. After 3 washes, wells were incubated with HRP conjugated anti mouse IgG (Covalab) (1:5000) in TBS-T and reaction was detected by adding 3,3',5,5'-tetramethylbenzidine and measuring the absorbance at 450 nm. GST coated wells were used to measure nonspecific binding and subtracted from the values observed in the TG2 and domain variants coated wells.

Surface plasmon resonance measurements

Surface plasmon resonance (SPR) measurements were performed in a Biacore 3000 instrument (Biacore, Uppsala, Sweden). Binding assays were performed at 25^{o}C. Anti-GST antibody (Biacore™, GE Healthcare) was immobilized on sensor chip CM5 (BR-1000-12; Biacore™, GE Healthcare) using the amine coupling method as recommended by the manufacturer. Surface activation was performed by an injection of 35 μl EDC/NHS solution (200 mM EDC and 50 mM NHS). The anti-GST antibody was diluted to 30 μg/ml in 10 mM Na-acetate (pH 5.0) (immobilization buffer) and injected over the surface for 7 min at 5

µl/min flow rate. Excess reactive sites were subsequently blocked by injection of 1 M ethanolamine (pH 8.5). On the sensor chip surfaces full-length GST-TG2 and GST tagged domain variants of TG2 (GST-TG2Δβ-barrel1 and GST-TG2Δβ-barrel2, GST-TG2ΔCAT, GST-TG2Δβ-sandwich, GST-CAT) were immobilized in running buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA and recombinant GST was used as reference. The immobilization levels of GST-fusion proteins were 500-1000 RU according to their molecular weight. The various concentrations of DNAJA1 protein were injected over the control and the different TG2 variants coated surfaces. After each binding analysis, the sensor surfaces were regenerated and the sensor surfaces were recoated with fresh protein solutions for the next binding studies. The evaluation of the sensorgrams was carried out with BIAevaluation 3.1 software (Biacore 3000).

Immunocytochemistry

Co-immunostaining experiments were performed to determine the localization of TG2 and DNAJA1 in TG2 overexpressing HEK 293T AD cells. Firstly, the cells were cultured on glass coverslips and fixed with 4 % PFA for 15 min at 25^{o}C. After that, the cells were treated with NH_4Cl-PBS for 10 min at 25^{o}C to quench free aldehyde groups and permeabilized with 0.1 % Triton-X 100 for 10 min at 25^{o}C. After washing with PBS-T (phosphate buffered saline, pH 7.4, with 0.1 % Tween-20), they were blocked first with 5 % goat serum in PBS-T for 30 min and with 5 % milk powder for 1 hour at 25^{o}C. The coverslips were then incubated with anti TG2 (1:500, polyclonal rabbit IgG, Santa Cruz Biotechnology) and anti DNAJA1 antibody (1:500, monoclonal mouse IgG, ThermoFisher, MA5-12745) in 5 % goat serum in PBS-T for 2 hours at RT. They were blocked once more with 5 % goat serum for 10 min and treated with secondary goat antibodies coupled to Alexa Fluor® 488 (anti mouse IgG) and Alexa Fluor® 568 (anti rabbit IgG) (1:5000) in 5 % goat serum. The nuclei were stained by DRAQ5

(1:1000). Finally, the coverslips were mounted with DAPCO-Mowiol (1:50) and visualized by confocal microscopy (Olympus FluoView FV1000).

TG2 activity assays

To compare the transamidation activities of TG2 glycine224 and valine224 variants, kinetic spectrophotometric UV assay was used. Briefly, the deamidation of a glutamine substrate releasing ammonia was measured. The released ammonia was absorbed by α ketoglutarate in the coupled reaction generating glutamate with oxidation of NADPH to NAD [75]. The assay was performed as described before [76].

Microtiter plate assay, based on the incorporation of 5-(biotinamido) pentylamine (BPA) into immobilized N,N-dimethylated casein was used as described before by Slaughter et al [77]. This assay was used for comparison of TG2 glycine224 and valine224 activities and also to determine the role of DNAJA1 on TG2 valine224 activity. Briefly, Maxisorp (Nunc) microtiter plate was coated with 1 mg/ml N,N-dimethylated casein (DMC; Sigma) in coating buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA) overnight at 4 ^{o}C and washed twice with PBS-T (phosphate buffered saline with 0.1% tween-20) pH 7.4. Wells were then blocked with 5 % (w/v) milk powder in PBS-T for 1 hour at RT. After washing with PBS-T, the reaction mixture including 0.1 M Tris/HCl pH 7.5, 1 mM BPA (5-(Biotinamido)pentylamine), 1 mM DTT, 5 mM $CaCl_2$, 1 μg recombinant His-TG2 (to detect the effect of DNAJA1, different concentrations (0.2-1 μg) were added along with TG2) were added to the wells and incubated for 30 minutes at 37 ^{o}C. Subsequently, streptavidin-alkaline phosphatase in PBS-T was added to each well and incubated for 1 hour at 25^{o}C. After adding the substrate solution (p-nitrophenyl phosphate), BPA incorporation was quantitated measuring the absorbance at 405 nm in ELISA microplate reader.

In kinetic assay, incorporation of monodansylcadaverine (Dansyl-Cd, a fluorometric-labeled cadaverine) (Sigma) into DMC (N,N-dimethylated casein) was measured. The reaction mixture comprised 50 mM Tris/HCl pH 7.5, 0.2 mM Dansyl-Cd, 16 μM DMC, 1 mM DTT, 5 mM $CaCl_2$, and 100 nM His-TG2 with or without 3 μM DNAJA1. The reaction mixture was incubated at 37 ^{o}C for 60 minutes. The increase in flourescence intensity was followed using BioTek Synergy H1 Multi-Mode Reader (Bio Tek US; Ex/Em: 360/530 nm).

In situ TG2 activity assay was performed as described previously by Zhang et al [78] with some modifications. The HEK 293T AD cells overexpressing TG2 (HEK-TG2) were first treated with 1 mM BPA for 1 hour and then with calcium ionophore A23187 (Sigma) for 1 hour to activate the TG2. The cells were then collected and washed with PBS. The cell pellets were resuspended in lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.5 % Triton and protease inhibitor cocktail) and further lysed by sonication. The protein in the supernatant was quantified with Bradford. Western blot was performed on the cell lysate with streptavidin conjugated HRP and HRP substrate. For quantification, 30 μg of the cell lysate was coated to a Maxisorp (Nunc) microtiter plate and then detected as described before [77].

Analysis of DNAJA1 as a substrate of TG2

Maxisorp (Nunc) microtiter plate was coated with 0.5 μg DNAJA1 and BPA incorporation into DNAJA1 was measured as described above in TG2 activity assay.

Another confirmation experiment was performed as described by Ruoppolo et al [79]. Recombinant His-TG2 (0.5 μg) was incubated with various concentrations of DNAJA1 (0.1-1 μg) in reaction buffer including 0.1 M Tris/HCl pH 7.5, 1 mM BPA as acyl donor, 1 mM DTT and 5 mM $CaCl_2$ for 1 hour at 37 ^{o}C. The reaction products were directly analysed by

immunoblotting, using streptavidin peroxidase and the monoclonal antibodies against TG2 and DNAJA1.

Quantitative RT–PCR (qRT–PCR)

Trizol reagent was used for the isolation of total RNA. Reverse transcription was performed at 25°C for 10 min, 37°C for 2 h and 85°C for 5 min from 1μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qPCR was performed in the LightCycler® 480 II (Roche) instrument using TaqMan® (Thermo Fisher Scientific) probes for transglutaminases (TG1-7 and FIIIa). Thermocycling conditions used for qPCR were 1 cycle (95°C, 10 min), 45 cycles (95°C, 10 s; 60°C, 8 s; 72°C, 15 s) and 1 cycle (95°C, 1sn; 65°C; 15 s). The comparative Ct method was used to quantify TG transcripts and normalization was performed with the GAPDH housekeeping gene.

Statistical Analysis

Experiments were repeated three times (stated in the figure legend) with three parallels and the data reported are as mean ± SEM from the representative experiment. Statistical significance was determined by two tailed paired Student's t test (parametric) by using Graph Pad Prism version 5.0. The $p \leq 0.05$ value was considered significant.

4. RESULTS

4.1. Characterization and comparison of transamidation activity and calcium sensitivity of Gly224 and Val224 variants.

4.1.1. Valine 224 strongly influences transamidation activity of TG2 at low Ca2+ concentrations

We investigated the biochemical and structural characteristics of the TG2 Val variant and compared them to those of TG2 Gly. We analysed the transamidation activities of these variants and observed that TG2 Val showed 20% increase in transamidase activity compared to TG2 Gly variant when microtiter plate or radioactive assays were used (data not shown). Since both these assays were end point measurements we resorted to kinetic assays to monitor real time activities. Using kinetic UV-test, TG2 Val showed ten-fold higher transamidase activity as compared to TG2 Gly at low Ca^{2+} concentrations (Figure 4). The activity difference between the two variants decreased with increasing Ca^{2+} concentration but did not disappear. This suggests that the Ca^{2+} sensitivity deviates in the two variants. Indeed, EC50 values for Ca^{2+}-dependence / sensitivity in case of transamidase activities were 0.36 ± 0.18 mM for TG2 Val and 2 ± 0.14 for TG2 Gly, respectively, indicating a higher binding affinity for Ca^{2+} for TG2 Val as compared to TG2 Gly.

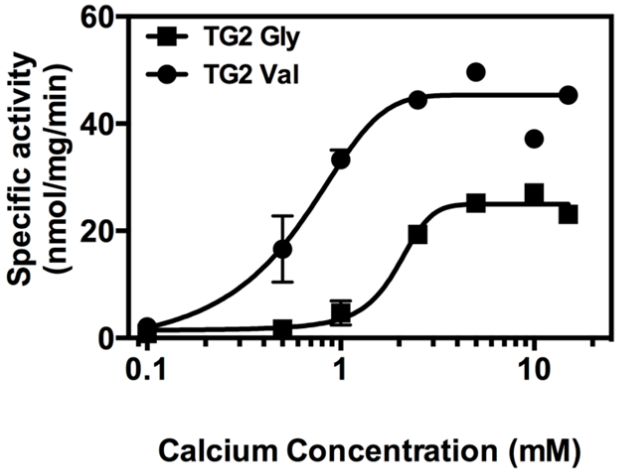

Figure 4. Effect of Ca^{2+} on transamidation activity of recombinant TG2 Gly and TG2 Val from *E. coli* cells. TG2 transamidation activity at different calcium concentrations measured by UV assay. EDTA was used in the blank and was subtracted. Kinetic assay was carried out using peptide substrate (5 mM), ethyl amine (0.75 mM), α-ketoglutarate (7.5 mM), NADPH (0.7 mM), ADP and glutamate dehydrogenase (22.5U/ml) and 120 mM HEPES buffer contining 50 mM DTT, n=2, p = 0.0027. Values were plotted on LOG scale to calculate the EC50 values. EC50 2 ± 0.14 mM (TG2 Gly), EC50 0.36 ± 0.18 mM (TG2 Val); p = 0.0027.

4.1.2. Effect of V224 on transamidation activity of TG2 in cells

To eliminate confounding factors due to expression of human TG2 in bacterial cells, the activity measurements were repeated using HEK 293T cell lysate overexpressing TG2 variants (Figure 5A, B). In accord to the results obtained by enzymes produced in *E.coli*, TG2 Val showed a higher transamidation activity than TG2 Gly.

Since *in vitro* results showed a clear difference in the biochemical properties of the two enzyme variants, we further wanted to confirm the results via cellular assays. We used BPA, a cell permeable amine substrate for transglutaminases, to determine the *in situ* TG2 activity in 293T AD cells stably transfected with the TG2 variants. Untransfected cells showed no crosslinking activity either with or without BPA (data not shown). Cells transfected with TG2

Val showed a significant increase in incorporation of BPA into intracellular proteins while TG2 Gly showed a negligible increase when compared to control (Figure 5C). On treating the samples with calcium ionophores, which had been used previously to activate TG2 in cells, both variants showed an increase in crosslinking activity. However, this increase was much more pronounced in case of TG2 Val compared to TG2 Gly (Figure 5C, D). The crosslinking activity could be inhibited when the cells were pre-incubated with 100 μM of TG2 active site inhibitor ZDON confirming the TG2 specific reaction (Figure 5D). The expression levels of TG2 were identical in all the samples confirming that the difference in activity was not due to different amounts of TG2 (Figure 5D). Both Western blot and ELISA were used for visualizing the crosslinking reaction but Western blot was more sensitive in detecting the difference in TG2 activities with TG2 Val containing cells consistently showing higher transamidation activity compared to TG2 Gly transfected cells. TG2 Val has been used in the rest of the studies.

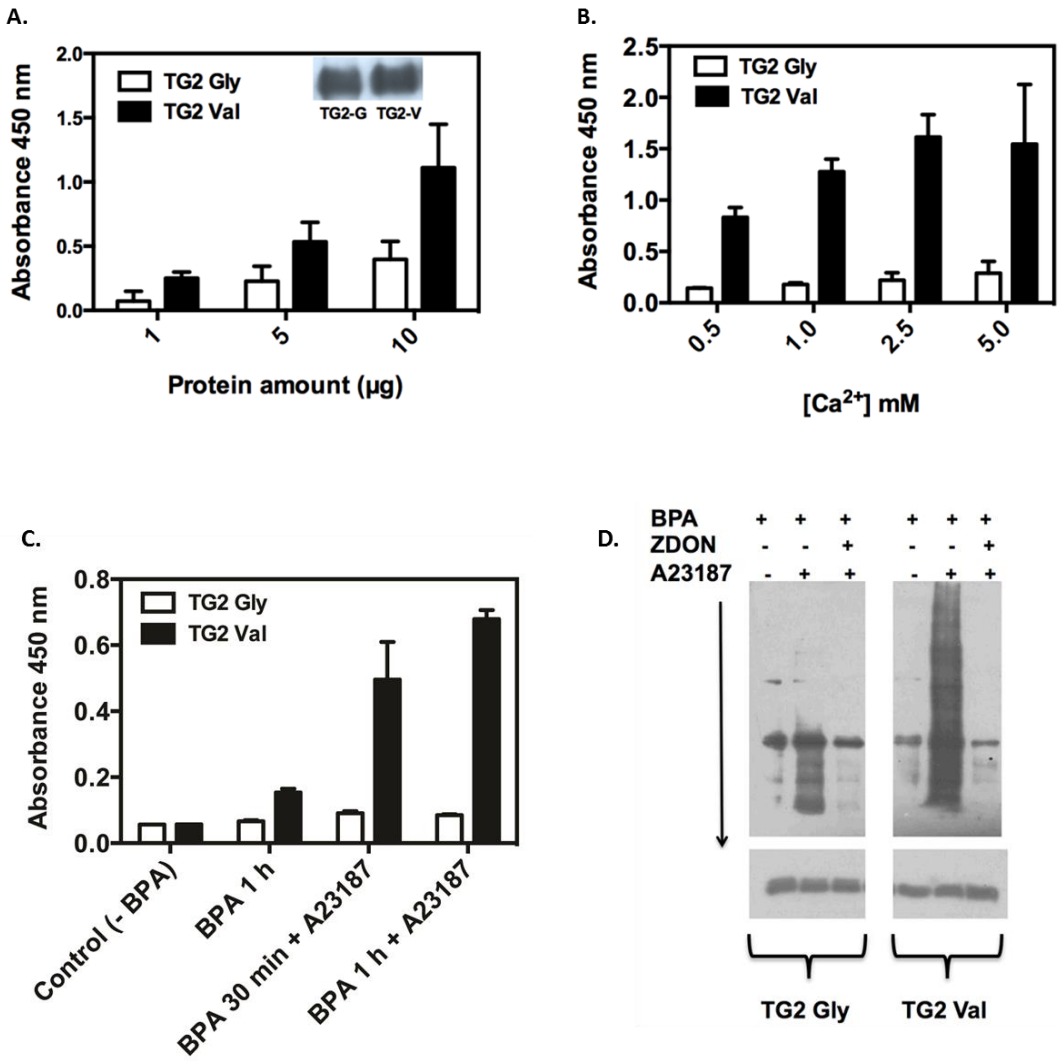

Figure 5. Comparison of TG2 Gly and TG2 Val transamidation activities *in vitro* and *in cells.* A. Recombinant TG2 was stably expressed in 293T AD cells and lysates were used for activity measurements at different protein concentrations via microtiter plate assay in the presence of 5 mM CaCl2. Inset: western blot of the proteins (0.5 µg), n=3; **B.** Activity measurements with TG2 transfected 293T AD cell lysate (10 µg) at different calcium concentrations, n=2; **C.** ELISA measurements of BPA treated samples, 293T AD cells stably transfected with TG2 variants were treated with 2 mM BPA for 1 hour. The cells were then activated with calcium ionophore A23187 for another 1 hour. Samples were lysed and supernatant (30 µg) was used to coat the microtiter plates overnight at 4°C and then detected with HRP conjugated streptavidin and HRP substrate for quantification, n=3. **D.** Western blot analysis of TG2 variants. The cells were treated with BPA overnight and then treated with or without TG2 active site inhibitor ZDON for 1 hour. The cells were activated with A23187 for another 1 hour. The BPA labeled products were observed by HRP conjugated streptavidin, n=3. Lower panel shows the identical expression levels of the TG2 variant in each sample. Arrow indicates the direction of the electrophoresis.

4.2. DNAJA1 as a novel interacting partner of transglutaminase 2

4.2.1 Screening of TG2 interacting proteins in differentiated NB4 cells

TG2 interacting proteins play a very crucial role in modulating TG2 functions. It is well documented that the same interacting partner can modulate TG2 functions inversely depending upon its cellular location [21]. Therefore we have mainly focused on identifying novel interacting partners of TG2 using human promyelocytic leukemia cell line (NB4). NB4 cell line upon differentiation with all trans retinoic acid (ATRA) expresses a very high level of TG2, which leads to a massive upregulation of genes involved in many physiological and pathological processes [72]. Therefore NB4 cells were considered as an excellent cell model to explore TG2 interacting partners and the related functions they modulate. Secondly this cell line has also never been used before for such kind of studies making it an attractive model for our analysis. GST Pull down experiments and subsequent mass spectrometry analysis of differentiated NB4 cells lead to identification of human Glutathione S Transferase (hGST-P1), Tubulin α, Histone H2A and DNAJA1 as potential binding partners of TG2 (Figure 6A). Since all of the above proteins except DNAJA1 have already been reported either to interact with TG2 or is a substrate of TG2 therefore DNAJA1 was considered as a novel interacting protein. Mass spectrometry identified peptides of DNAJA1 with 90 % confidence. We confirmed the specific interaction by using anti-DNAJA1 antibody on GST Pull down samples (Figure 6B). Further we expressed and purified recombinant TG2 and DNAJA1 and performed GST Pull down experiments to confirm their direct interaction (Figure 6C). Since DNAJA1 and TG2 has been reported to be involved in similar pathological conditions such as autoimmune diseases [80], neurodegenerative diseases [81], and cancer [82] we selected DNAJA1 to investigate further the functional significance of this protein in association with TG2.

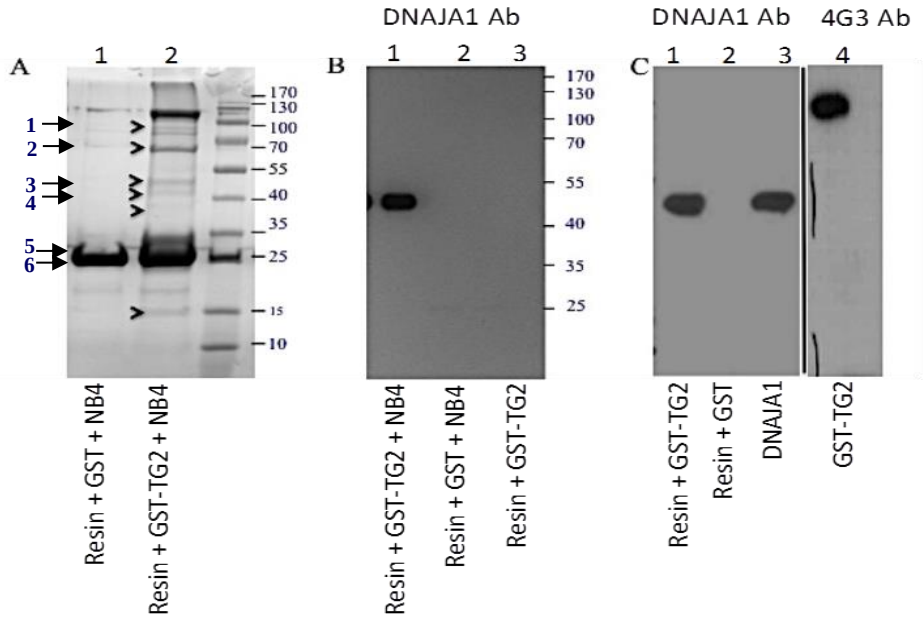

Figure 6. TG2-DNAJA1 interaction via GST-pull down assay and mass spectrometry. A. 1 mg/ml (500 µl) NB4 cell lysate was incubated with 100 µg of purified GST tagged TG2 (GST-TG2) and GST. The bands marked with arrow were excised and analysed via LC MS/MS. Peptides identified were as follows: **1.** hTG2 (100 kDa), **2.** BSA (70 kDa), **3.** putative tubulin like protein alpa (45 kDa), **4.** hDNAJA1 (45 kDa), **5. and 6.** GST (24 kDa) and hGSTP1 (24 kDa). **B.** Interaction of GST-TG2 and DNAJA1 was confirmed by using anti DNAJA1 monoclonal antibody on GST-Pull down samples of NB4 cells. GST + NB4 cell lysate and purified GST-TG2 alone were used as control. **C.** Purified recombinant GST-TG2/GST and DNAJA1 (100 µg each) were used to detect interaction confirmed by anti-DNAJA1 antibody. DNAJA1 as well as GST-TG2 alone were used as positive controls for western blot (lane 3 and 4). Anti-TG2 antibody (4G3) was used for identification and confirmation of the integrity of TG2.

4.2.2. TG2 directly interacts with DNAJA1 mainly through its catalytic domain

Physical interaction of TG2 with DNAJA1 was confirmed via ELISA and SPR measurements. Domain deleted variants of TG2 were also used in these experiments to determine DNAJA1 binding domain of TG2. In both assays, we observed that TG2-DNAJA1 interaction increased with higher concentrations of DNAJA1. The results shown in Figure 7 demonstrate that

DNAJA1 interacts with full length TG2 and domain variants of TG2 including GST-TG2Δβ-barrel2, GST-CAT, GST-TG2Δβ-sandwich, GST-TG2Δβ-barrel1 and GST-TG2ΔCAT. Highest binding affinity for DNAJA1 was observed with GST-TG2Δβ-barrel2 whereas GST-TG2ΔCAT variant showed the least interaction with DNAJA1 suggesting that catalytic domain of TG2 is essential for TG2-DNAJA1 interaction and β sandwich domain together with β barrel 1 domain futher improve this interaction.

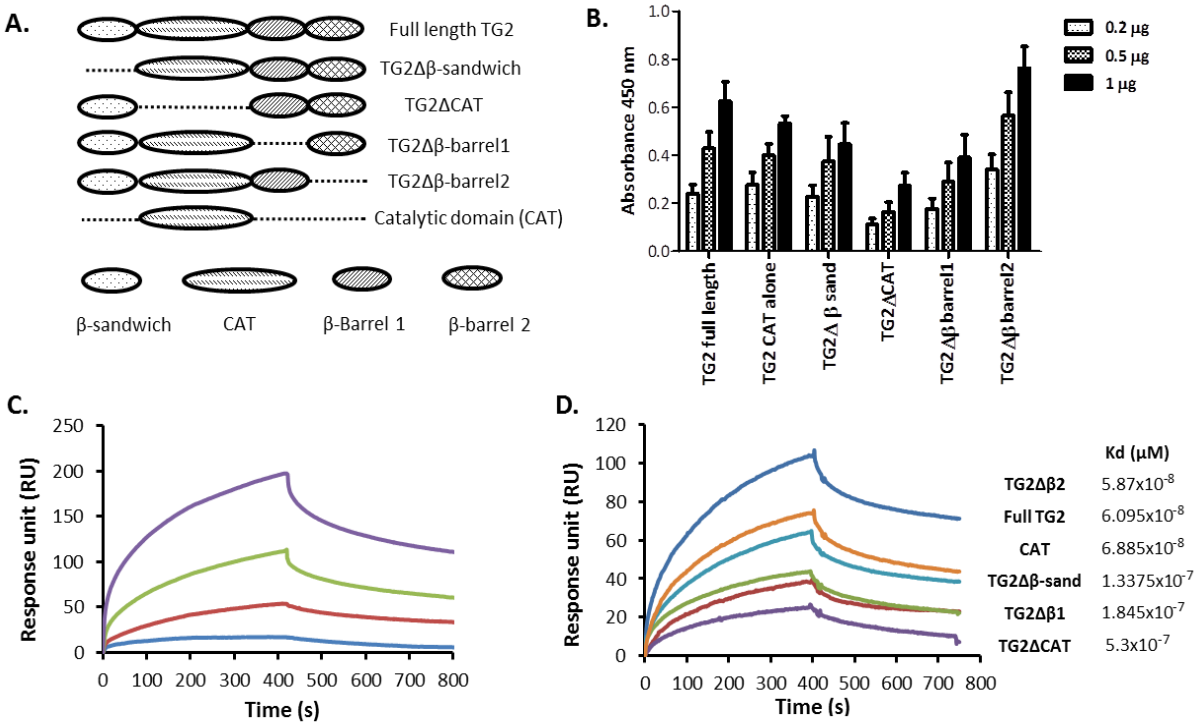

Figure 7. Confirmation of TG2-DNAJA1 interaction and identification of DNAJA1 binding domains of TG2 via ELISA and SPR measurements. **A.** Schematic representation of full length and domain deleted variants of TG2. **B.** 1 µg of GST-TG2 and domain variants of TG2 were used to coat the microtiter plate and incubated with different concentrations of DNAJA1 (0.2, 0.5, and 1 µg) to detect the interaction via ELISA. Recombinant GST was used as control and its absorbance was subtracted. **C.** In SPR measurements binding of DNAJA1 to TG2 was monitored using 2.5 µM full length TG2 with DNAJA1 protein at (from top to bottom) 4, 2, 1 and 0.5 µM concentrations. **D.** Interaction of domain variants of TG2 with DNAJA1 was also determined using 2.5 µM full length TG2 and domain variants of TG2 with 1 µM DNAJA1 (Domain variants of TG2 (from top to bottom);

GST-TG2Δβ-barrel2, full length TG2, GST-CAT, GST-TG2Δβ-sandwich, GST-TG2Δβ-barrel1, GST-TG2ΔCAT). Recombinant GST was used as a reference. Similar results were obtained in each of three experiments. Dissociation constants (Kd) of DNAJA1 from full length and domain variants of TG2 were also calculated and represented in the figure.

4.2.3. DNAJA1 interacts with the open conformer of TG2

TG2 mainly adopts two different conformations depending on the type of binding effectors. In agreement with earlier reports, electrophoresis under nondenaturing conditions revealed that recombinant human TG2 produced in E.coli adopts an open conformation in the presence of Ca^{2+} whereas GTP induces a closed conformation (Figure 8A). Since TG2 undergoes self-crosslinking in the presence of calcium, we have treated TG2 with ZDON (referred to as iTG2), which binds irreversibly to the cysteine present in the active site of TG2 thereby inhibiting its crosslinking activity (Lane 3, Figure 8A). We also checked TG2 conformer in the presence of EDTA, which was used to remove residual calcium from the bacterial expression system. However, instead of observing a closed conformation, majority of TG2 existed in open conformation in the presence of EDTA (Figure 8A). This is most likely because TG2 binds very strongly to the calcium present in the bacterial cell lysate and by mere addition of EDTA was not enough to chelate / dissociate this bound calcium from TG2. Also, there are probably some effectors derived from expression system that help in keeping the TG2 in open conformation and was not completely removed during the purification and by the addition of EDTA either. To explore whether DNAJA1 binds to the open or closed form of TG2 we performed interaction studies via ELISA in the presence of aforementioned effectors. As indicated in Fig.8B, TG2-DNAJA1 interaction was significantly higher in the presence of EDTA or Ca^{2+} in combination with Z-DON compared to GTP including conditions which suggest that DNAJA1 interacts mainly with the open conformer of TG2. DNAJA1 also showed interaction with transamidation inactive mutant of TG2 (TG2 C277S),

which indicates that TG2-DNAJA1 interaction is not dependent on crosslinking activity of TG2.

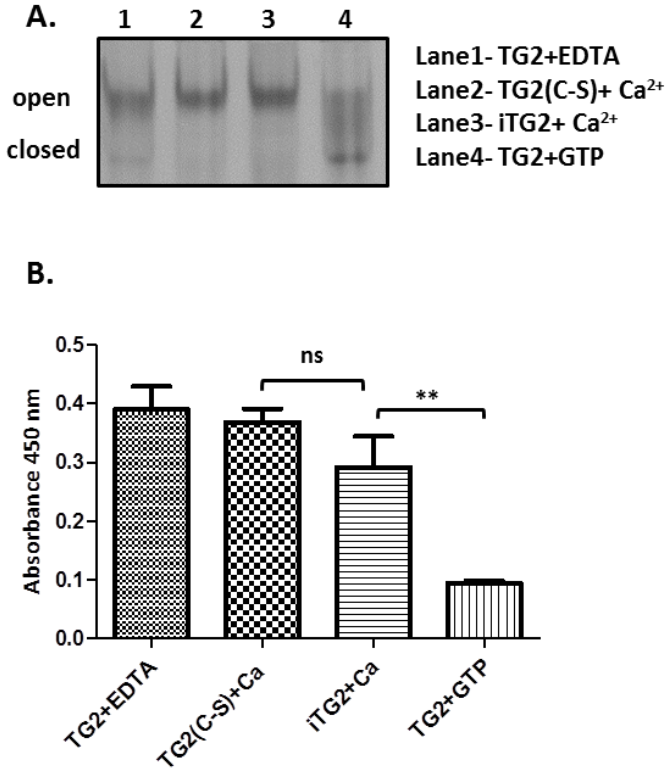

Figure 8. Separation of open and closed conformers of TG2 and determination of DNAJA1 binding conformer of TG2. A. TG2 conformations were examined by non-denaturing polyacrylamide gel electrophoresis with 5 mM EDTA, 5 mM $CaCl_2$ or 1 mM GTP. Active site inhibitor of TG2 (Z-DON) was used in Ca^{2+} including sample to avoid self-crosslinking of TG2 (iTG2). **B.** ELISA was performed to detect the DNAJA1 binding conformer of TG2. 1 µg TG2 and active site mutant of TG2, TG2 (C-S) were immobilized on the surface of the plate in the presence of effectors at 37^{o}C for 1 hour and then incubated with 1 µg DNAJA1 prepared in the same reaction mixtures at 4 ^{o}C overnight. TG2 uncoated wells were used as control and results were normalized. Similar results were obtained in three independent experiments. **, $p<0.05$ between the groups.

4.2.4. DNAJA1 facilitates and stabilizes *in vitro* crosslinking activity of TG2

To explore the significance of TG2-DNAJA1 interaction, we firstly wanted to determine the effect of DNAJA1 on crosslinking activity of TG2. As shown in the Figure 9A, BPA incorporation into surface bound glutamine donor substrate N,N-dimethylated casein (DMC) by TG2 in an endpoint assay was higher in the presence of DNAJA1 as compared to control. The amount of crosslinked product was further increased with higher DNAJA1 concentrations. This indicated that DNAJA1 either modulated or stabilized the crosslinking activity of TG2 *in vitro*. Kinetic measurement of Dansyl-cadaverine incorporation into DMC by TG2 in the presence of DNAJA1 in a fluid phase system didn't show any effect on TG2 crosslinking activity when the measurements were taken until first 25 min. Nevertheless, after 30 – 60 min an increase in transamidation activity of TG2 was observed. However, this increase in activity had a p value greater than 0.05 (Figure 9B and C). The kinetic parameters of TG2 for DMC substrate in the presence and absence of DNAJA1 were estimated by Michaelis Menten and Lineweaver-Burk plots. Km was calculated as 17.6 μM, Vmax as 8.9 μM/min and Kcat as 90 min^{-1} for TG2 without DNAJA1. In the presence of DNAJA1 we observed a decrease in Km value, which was calculated as 13 μM, Vmax as 8 μM/min and Kcat value as 78 min^{-1}. These results suggest that DNAJA1 increases the substrate affinity of TG2 thereby increasing its enzymatic activity as observed in the endpoint activity measurements and later half of the kinetic activity measurements.

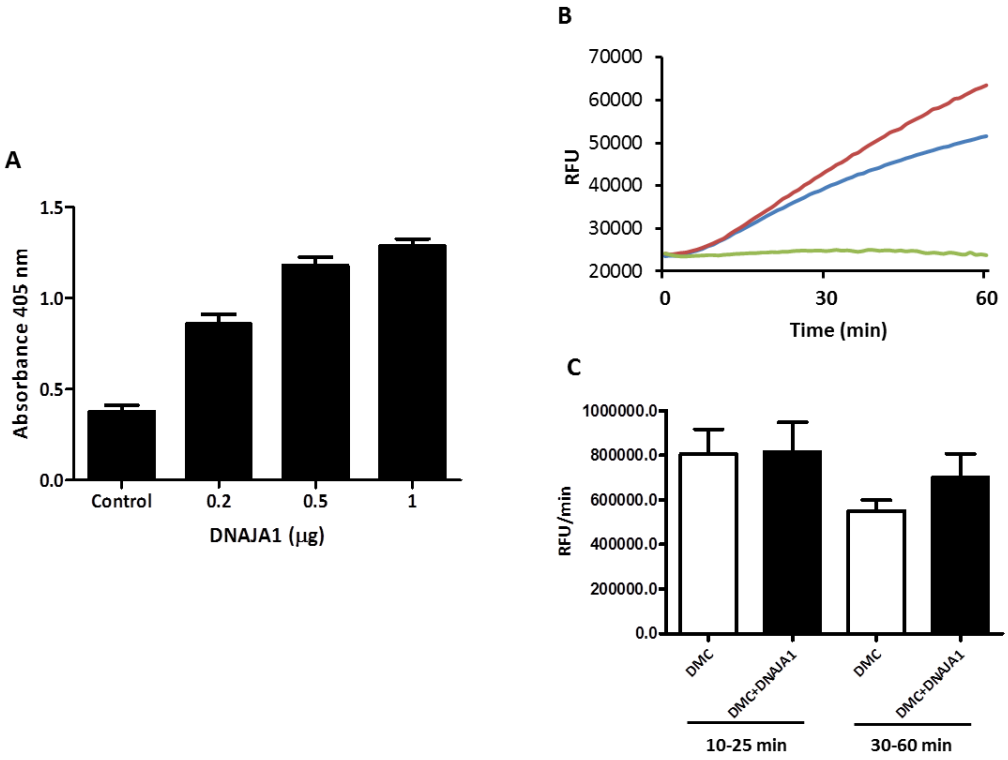

Figure 9. The role of DNAJA1 on crosslinking activity of TG2 via in vitro transamidation assays. A. In vitro BPA incorporation assay was performed using 1 µg TG2 and increasing concentrations of DNAJA1 (Control: 1 µg TG2). Reaction mixtures were incubated for 30 minutes at 37°C **B.** Kinetic transamidation assay was also carried out and 0.2 mM monodansyl cadaverine incorporation into 16 µM DMC with (red line) and without (blue line) of 3 µM DNAJA1 was monitored. **C.** Slopes of each line in figure B were calculated between 10-25 minutes and 30-60 minutes. DNAJA1 (3 µM) was used as control (green line) and results were normalized. Similar results were obtained in three independent experiments. (p: 0.0551 for 30-60 minutes)

4.2.5. Downregulation of DNAJA1 results in increased BPA incorporation in TG2 overexpressing HEK cells

To confirm the role of DNAJA1 on crosslinking activity of TG2, we further performed cellular experiments wherein we used BPA, a cell permeable amine substrate for transglutaminases, to determine the in situ TG2 activity in DNAJA1 downregulated HEK cells stably transfected with human TG2 (HEK-TG2). DNAJA1 siRNA were used to

knockdown the expression of DNAJA1 and scrambled RNA transfected and untransfected cells were used as control. Downregulation of DNAJA1 was demonstrated via Western blot (Figure 10A). Both ELISA and Western blot were performed to visualize the crosslinking reaction initiated by adding Ca^{2+}-ionophore to the cells. The results indicated surprisingly that there was a significant increase in crosslinking activity of TG2 in cells with downregulated DNAJA1 compared to the controls (Figure 10A and B). We also used TG2 un-transfected HEK cells and there was no crosslinking activity in these cells (Figure 10C). In our previous study, it was also demonstrated that 100 μM of TG2 active site inhibitor Z-DON could inhibit the crosslinking activity in HEK-TG2 cells confirming the TG2 specific reaction [83]. These results suggest that DNAJA1 has the ability to regulate the crosslinking activity of TG2 in the cells. To ascertain whether downregulation of DNAJA1 has any impact on the expression of TG2 in HEK-TG2 cells, Western blot (Figure 10A) was carried out and there was no difference in the amount of TG2 protein. To explore the changes in the gene expression levels of other transglutaminases upon DNAJA1 downregulation, we performed QPCR analysis. None of the other protein members of transglutaminase family were expressed in HEK-TG2 either with or without downregulation of DNAJ1 (Figure 10D).

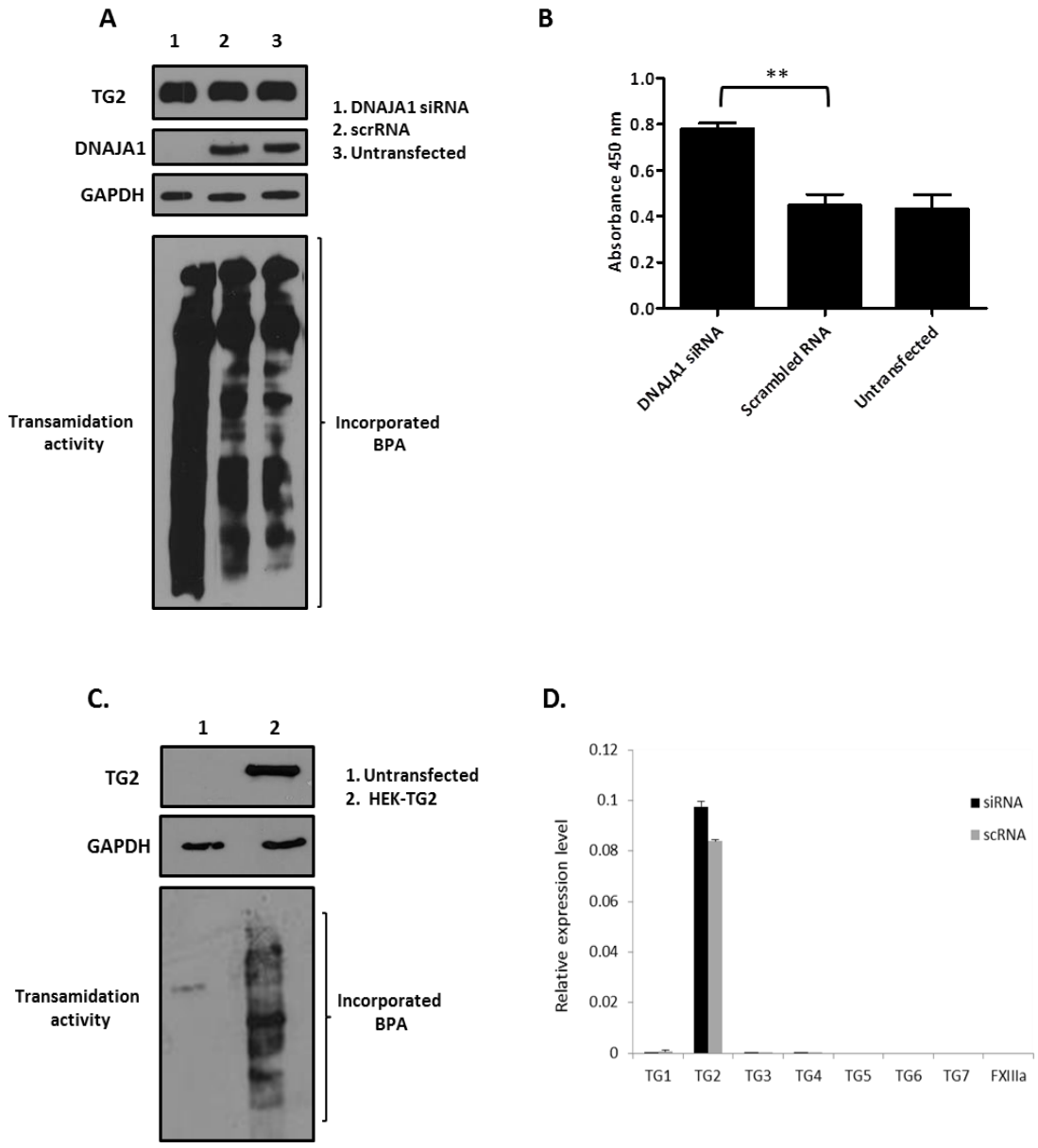

Figure 10. The effect of DNAJA1 on *in situ* crosslinking activity of TG2. A. DNAJA1 siRNA was used for the downregulation of DNAJA1 in HEK-TG2 cells and the expressions of TG2 and DNAJA1 were detected via western blotting in DNAJA1 downregulated cells, scrambled RNA transfected control cells and in untransfected cells. In situ BPA incorporation experiment was performed using these cells. Cells first treated with 1 mM BPA for 1 hour and then incubated with 2 μM calcium ionophore A23187 for 1 hour as described in materials and methods. Cell lysates were used in western blotting **B.** ELISA. **, $p<0.05$ between the groups. **C.** In situ BPA incorporation experiment was also performed using TG2 un-transfected HEK cells and compared to TG2 overexpressing HEK cells (HEK-TG2). **D.** RNA samples were collected from DNAJA1 downregulated and scrambled RNA transfected control HEK-TG2 cells on 3rd day of transfection and used for RT-qPCR analysis. All

qPCR results were normalized to the expression of GAPDH. Similar results were obtained in three independent experiments.

4.2.6. Colocalization of TG2 with DNAJA1 in cytoplasm of TG2 overexpressing HEK cells

HEK-TG2 cells were used to determine the localization of TG2 and DNAJA1 in an intact mammalian cellular system. Co-localization experiments with NB4 cells were also performed but gave very high background therefore HEK-TG2 cells were used for the experiments. Dual immunohistochemistry staining of HEK-TG2 cells was performed with anti-TG2 and anti-DNAJA1 antibodies. Both TG2 and DNAJA1 were observed to localize predominantly in cytosolic fraction with low expression in nuclei (Figure 11). Co-localization of TG2 and DNAJA1 in the cytosolic region indicates a potential association of the two molecules with physiological processes in vivo.

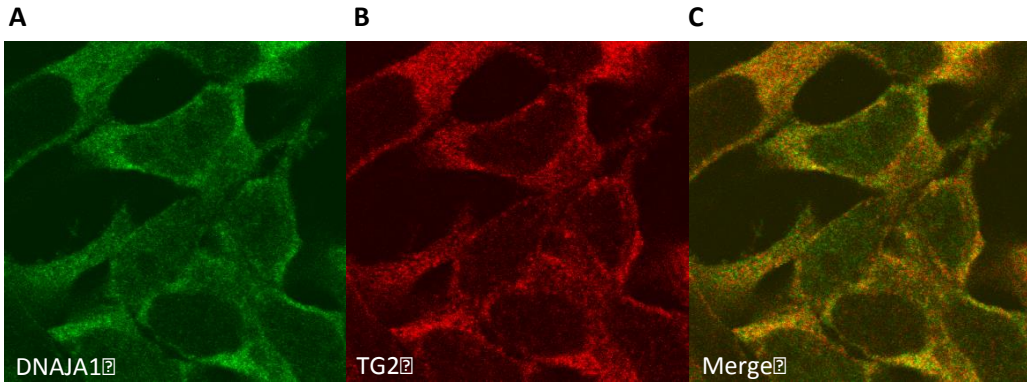

Figure 11. Immunoflourescent images of TG2 and DNAJA1 in TG2 overexpressing HEK 293T AD cells. The cells were stained with secondary goat anti rabbit antibody for TG2 (A, red) and goat anti mouse antibody for DNAJA1 (B, green) following the treatment of cells by specific polyclonal anti-TG2 and monoclonal anti-DNAJA1 antibodies. Superimposition of the images indicates the colocalization of TG2 and DNAJA1 in the cytoplasm of HEK 293T AD cells (TG2 was transfected stably into HEK 293T AD cells).

4.2.7. DNAJA1 is a glutamine donor substrate of TG2

Since DNAJA1 was found to interact with catalytic core domain of TG2 we speculated that it can also serve as a TG2 substrate. To explore this hypothesis, DNAJA1 was incubated with the enzyme in the presence of an acyl donor BPA. Reaction products were analysed by SDS/PAGE, immunoblotting and ELISA and results showed that BPA was crosslinked to DNAJA1. The amount of incorporation was increased with higher concentrations of DNAJA1 (Figure 12A and B). Incorporation did not occur in the absence of TG2 or calcium confirming that BPA incorporation into DNAJA1 is mediated by transamidating activity of TG2 (data not shown). We also performed the kinetic transamidation assay replacing dimethylated casein with DNAJA1 and monitored the incorporation of Dansyl-Cd into increasing concentrations of DNAJA1. We observed a linear increase in the amount of incorporated Dansyl-Cd at increasing concentrations of DNAJA1 confirming that it is a glutamine donor substrate of TG2 (Figure 12C).

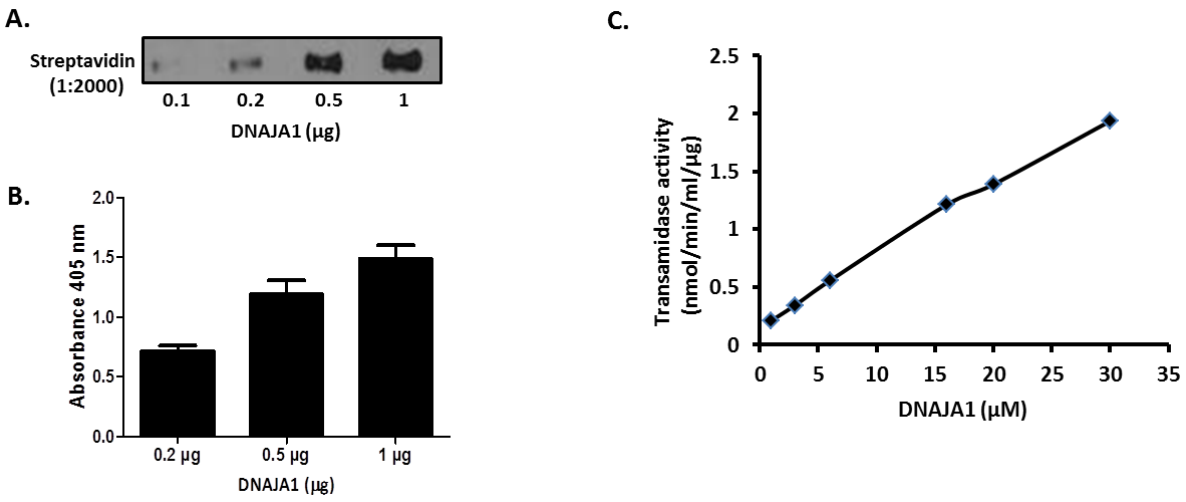

Figure 12. TG2 mediated amine incorporation into DNAJA1. **A.** Various concentrations of DNAJA1 (0.1-1 µg) were incubated with TG2 and BPA in the presence of Ca^{2+}. BPA incorporation into DNAJA1 was detected via immunoblotting using streptavidin antibody. **B.** ELISA was performed for further confirmation and BPA incorporation into DNAJA1 coated surface by TG2 was monitored. BSA coated wells were used as control and results were normalized. Similar results were obtained in three independent experiments. **C.** Monodansylcadaverine (0.2 mM) incorporation into increasing concentrations of DNAJA1 (1-30 µM) was monitored via the kinetic transamidation assay using 1 µg TG2. Blanks comprised the above mixture without transglutaminase were applied for calculating the results.

5. DISCUSSION

The recent publication on the polymorphisms of human TG2 [84] reported that the sequence at the position 224 is Valine instead of Glycine in all the known human genomes. During the last two decades, artificial variant of TG2 (TG2 224Gly) has been used in various laboratories [17, 20]. Hence, it was important to first characterize the biochemical and structural relevance of TG2 Val "wild type" enzyme before working further on TG2 regulated processes. We performed several experiments to compare the transamidase activities and calcium sensitivity of these TG2 variants and found that TG2 Val shows 10 folds increase in both transamidation activity and calcium sensitivity. These results explain why high Ca^{2+} concentration had to be used in earlier studies for activation of TG2 [85]. According to the previous views, TG2 does not exhibit activity inside the cell at physiological conditions due to low calcium concentrations and high GTP concentrations, which is inhibitory for transamidation activity of TG2 [86]. Therefore, calcium ionophores have been used in most cellular experiments to activate TG2 [78, 87]. In our study, crosslinking activity of TG2 Val was detected in the absence of Ca^{2+}-ionophore and it could be increased further by adding A23187. All these results suggest that TG2 can be activated inside the cells at physiological conditions and therefore reassessment of its potential cellular activity should be considered.

TG2 shows broad substrate specificity and it also non-enzymatically interacts with numerous proteins inside and outside the cells which may account for its multiple biological functions. For this reason, we have chosen to focus on identification of novel interacting partners of human TG2 using NB4 acute promyelocytic leukemia (APL) cell line. We identified a novel intracellular interacting partner of human TG2 which is a molecular heat shock protein, namely DNAJA1. Previous reports suggest that there are few other heat shock proteins, which binds to TG2 and play important roles in various physiological and pathological processes in

cooperation with TG2. For example, TG2 overexpression upon excitotoxic stress and thereby interaction with Hsp20 leads to modulation of anti-apoptotic function of Hsp20/27 complex and reduction in the activity of caspase 3 ultimately protecting the cells from apoptotic damage [7]. TG2 was also shown to interact with HSP70 in HELA and MDAMB231 breast carcinoma cell, which ultimately regulated cancer cell migration [88].

From the above examples it is evident that TG2 interacting partners play a crucial role in regulating TG2 mediated functions, which ultimately accounts for its multifunctional nature. TG2 has quite a lot of interacting partners as summarized in a recent review article [21]. Additionally, it was reported in the same review article that the presence of newly described features such as short linear motifs (SLiMs) and intrinsically disordered regions (IDRs) in TG2 sequence are known to facilitate highly specific protein-protein interactions with moderate affinities therefore they are often exploited in signaling pathways [89, 90, 91]. Surprisingly, numerous SLiMs and 13 IDRs were identified in TG2 sequence. However, only 6 known interacting partner binding regions namely integrins, syndecan4, SUMO1, 14-3-3, BAX/BAK and α1 adrenoreceptor, overlapping these regions could be reported which suggest that probably there are more number of unknown interacting partners [21].

Our finding that DNAJA1 could interact with TG2 opened new avenues to be explored since DNAJA1 is not only involved in chaperon activity but it has also been reported to regulate several other TG2 mediated cellular functions such as neurodegenerative disorders and cancer As we know that like DNAJA1, TG2 is also reported to regulate cancer cell migration [92], apoptosis [93] and neurological disorders [94], we speculate that DNAJA1 and TG2 might work in collaboration in regulating these cellular processes and pathological states.

Our data demonstrate that DNAJA1 interacts mainly with open conformer of TG2. As mentioned before, TG2 adopts open conformation in the presence of calcium and is found in

catalytically active state. After we observed strong interaction of DNAJA1 with open conformer of TG2, we could also observe a co-localization of TG2 and DNAJA1 in the cell cytoplasm. This observation reconfirms our previous justifications that TG2 can be present in open conformation and in active state in the cell cytoplasm [83, 95] which is against the prevailing view that, under physiological conditions, TG2 cannot be active inside the cell owing to low Ca^{2+} and high GTP concentrations [96, 97]. DNAJA1 was also found to interact with the active site mutant of TG2, which suggests that transamidation activity of TG2 is not essential for DNAJA1 and TG2 interaction.

When we used different constructs of TG2, each one lacking one or more domains of TG2, we observed that DNAJA1 interacts with core domain itself as well as it does with full length TG2. However, domain deleted variant lacking core domain shows the least interaction with DNAJA1 suggesting that core domain of TG2 is the most important domain in this interaction and since core domain has been shown to be catalytically inactive [10] this interaction also confirms that catalytic activity of TG2 is not required for TG2-DNAJA1 interaction.

The role of DNAJA1 on TG2 crosslinking activity was also investigated and we could see an increase in the TG2 activity in the end point assay measured via ELISA on increasing DNAJA1 concentrations. However, TG2 activity measurements via kinetic assay didn't show any significant increase in enzymatic activity in the early phase of kinetic reaction but some increase was seen in the later stage i.e. after 30-60 min. Statistical analysis on the kinetic assay confirmed that the increase in the activity in the later half was non-significant. We also determined the kinetic parameters to understand how DNAJA1 regulated TG2 activity. Addition of DNAJA1 shifted the Km values of TG2 from 17.6 μM to 13 μM while Vmax remained unchanged. Kinetic parameters were calculated only for the first half of the reaction i.e until 30 min. During the later half, the reaction was non-linear therefore the kinetic parameters could not be calculated. Our results suggest that, DNAJA1 stabilizes the TG2

active state conformation thereby increasing its substrate specificity and its transamidation activity. Though in the early phase of the kinetic measurement we didn't observe any change in the activity, we could observe an increase in the activity in the later half suggesting that DNAJA1 stabilizes TG2 active state conformation in longer time period. Surprisingly, *in situ* BPA incorporation assay results indicated that enzyme activity was somehow enhanced in DNAJA1 downregulated HEK-TG2 cells compared to scrambled and untransfected controls. There might be several possible explanations for this result. One of the possible reasons could be that DNAJA1 interaction keeps the enzyme in a multiple protein complex which together masks the catalytic site of TG2 inside cells thereby preventing catalysis. We have shown here that DNAJA1 binds to the catalytic domain of TG2 which corroborates with this observation. TG2 catalytic site is freely available for substrate binding on downregulating DNAJA1 therefore enhanced crosslinking activity is seen subsequently. Another possible reason could be that, DNAJA1 might compete with other substrates of TG2 and limits its substrate specificity since we also found that DNAJA1 is a glutamine donor substrate of TG2. In other words, we can conclude that compared to *in vitro* conditions, the effect of DNAJA1 on TG2 activity in cells shows difference most likely due to the presence of several other factors. As mentioned before, the crosslinking activity of TG2 plays significant roles in several important pathological processes and its *in vivo* inhibition has gained great importance for therapeutic treatments of human diseases. Our results therefore suggest that DNAJA1 could be considered as an important protein target with therapeutic potential. Since we showed that the downregulation of DNAJA1 improves the activity of TG2 in cells, it will be interesting to observe whether its overexpression inhibits TG2 activity in normal cells and in different cell models of diseases.

We could successfully identify yet another interacting partner which can interact with TG2 non-enzymatically as well as also function as a substrate. Other proteins which have been

reported to have such dual functions include fibronectin, BCR, Angiocidin and Retinoblastoma. As mentioned before, DNAJ family of proteins and particularly DNAJA1 play important roles in certain types of cancer and neurodegeneration, it will be interesting to see whether DNAJA1 and TG2 interaction regulates these pathological conditions. We already performed several cell migration assays using differentiated NB4 cells and we downregulated TG2 and also DNAJA1 and compared the migration of these cells to un-transfected controls. Using these cells we could not detect any difference in cell migration between TG2 KD and un-transfected cells and also DNAJA1 downregulated cells showed the similar result compared to un-transfected NB4 cells. Our future attempt will be to explore the significance of TG2-DNAJA1 interaction via using different cancer and neurological cell models.

6. SUMMARY

Transglutaminase 2 (TG2) is a multifunctional enzyme which participates in posttranslational modification of proteins, such as catalyzing calcium dependent crosslinking of proteins, incorporation of primary amines and deamidation of proteins. In addition, TG2 also acts as a G protein, has protein disulphide isomerase, protein kinase activities and plays non-enzymatic roles such as functioning as an adaptor protein, cell surface adhesion mediator and forming protein scaffolds. In our study, we first compared the transamidation activity and calcium sensitivities of Gly224 and Val224 variants of TG2 and found that the Val variant, which is the actual wild type variant in the human genome, exhibits significantly higher activity and calcium sensitivity compared to Gly variant which was used in many previous studies in other laboratories. In the rest of the project we used Val224 "wild type" variant of TG2. Our main aim in this study was to identify the novel interacting partners of human TG2. For this aim, we used NB4 promyelocytic leukemia (APL) cell line which overexpresses TG2 and also other APL related genes upon differentiation by ATRA. Among several other interacting partners, we identified DNAJA1 as a novel interacting partner of TG2. We further comfirmed this interaction via several in vitro experiments such as ELISA and SPR measurements. The same experiments were performed to determine the DNAJA1 binding domain of TG2 and results suggested that core domain of TG2 is the essential domain for TG2-DNAJA1 interaction. Furthermore, DNAJA1 was found to interact mainly with open conformer of TG2. To explore the effect of DNAJA1 on crosslinking activity of TG2 we carried out some *in vitro* and *in situ* experiments and observed that DNAJA1 inversely regulates the activity of TG2. Moreover, DNAJA1 was also identified as a novel substrate of TG2. DNAJA1 and TG2 have been independently reported to regulate similar cellular and pathological processes, such as cellular transport, apoptosis, neurodegenerative disorders and cancer. We propose that both the proteins regulate these overlapping functions via intermolecular interactions.

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8. Publications, Conferences

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List of publications related to the dissertation

1. **Ergülen, E.**, Bécsi, B., Csomós, I., Fésüs, L.*, Kanchan, K.*: Identification of DNAJA1 as a novel interacting partner and substrate of human transglutaminase 2.
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CONFERENCES

Oral Presentations:

Ergulen E. **Functional relevance of DNAJA1, as a novel interacting partner of human transglutaminase 2.** 6nd Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 8-11 , 2013

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