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Transglutaminase 2 (TG2) is a ubiquitously expressed multi-functional member of the transglutaminase enzyme family. It has been implicated to have roles in many physiological and pathological processes such as differentiation, apoptosis, signal transduction, adhesion and migration, wound healing and inflammation. Previous studies revealed that TG2 has various intraand extracellular interacting partners, which contribute to these processes. In this study, we identified a molecular co-chaperone, DNAJA1 as novel interacting partner of human TG2 using a GST pull down assay and subsequent mass spectrometry analysis and further confirmed this interaction via ELISA and SPR measurements. Interaction studies were also performed with domain variants of TG2 and results suggest that the catalytic core domain of TG2 is essential for the TG2-DNAJA1 interaction. Crosslinking activity was not essential for the interaction since DNAJA1 was also found to interact with the catalytically inactive form of TG2. Further, we have showed that DNAJA1 interacts with the open form of TG2 and regulates its transamidation activity both in vitro and in situ conditions. We also found that DNAJA1 is a glutamine donor substrate of TG2. Since DNAJA1 and TG2 are reported to regulate common pathological conditions such as neurodegenerative disorders and cancer, the findings in this paper open up possibilities to explore molecular mechanisms behind TG2 regulated functions.

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Identification of DNAJA1 as a novel interacting partner and substrate of human transglutaminase 2

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Running Title: Transglutaminase 2 interacts with DNAJA1

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ABSTRACT

Transglutaminase 2 (TG2) is a ubiquitously expressed multi-functional member of the transglutaminase enzyme family. It has been implicated to have roles in many physiological and pathological processes such as differentiation, apoptosis, signal transduction, adhesion and migration, wound healing and inflammation. Previous studies revealed that TG2 has various intra- and extracellular interacting partners, which contribute to these processes. In this study, we identified a molecular co-chaperone, DNAJA1 as novel interacting partner of human TG2 using a GST pull down assay and subsequent mass spectrometry analysis and further confirmed this interaction via ELISA and SPR measurements. Interaction studies were also performed with domain variants of TG2 and results suggest that the catalytic core domain of TG2 is essential for the TG2-DNAJA1 interaction. Crosslinking activity was not essential for the interaction since DNAJA1 was also found to interact with the catalytically inactive form of TG2. Further, we have showed that DNAJA1 interacts with the open form of TG2 and regulates its transamidation activity both *in vitro* and *in situ* conditions. We also found that DNAJA1 is a glutamine donor substrate of TG2. Since DNAJA1 and TG2 are reported to regulate common pathological conditions such as neurodegenerative disorders and cancer, the findings in this paper open up possibilities to explore molecular mechanisms behind TG2 regulated functions.

INTRODUCTION

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 posttranslational 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 [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 wound healing [12], whereas outside the cell TG2 modulates cell-ECM adhesion, cell migration and outside-in signaling, which is 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].

It is amazing how a single protein can participate in such diverse functions, which has long been a matter of intense investigation. In our recent review article we suggested several crucial factors such as the presence of compartment specific interacting partners of TG2, short linear motifs (SLiMs) and intrinsically disordered regions (IDRs) in TG2 sequence, which most likely contribute to its functional diversity [15, 16]. It is well known that SLiMs and IDRs facilitate highly specific protein-protein interactions with moderate affinities, therefore they are often exploited in signaling pathways [17-19]. Numerous SLiMs and 13 IDRs have been revealed in the TG2 structure. However, we could report only 6 known interacting partner binding regions namely integrins, syndecan-4, SUMO1, 14-3-3, BAX/BAK and α1 adrenoreceptor, overlapping these regions, suggested that probably there are large number of interacting partners, which needs to be explored [16].

Therefore, in this paper we have explored novel interacting partners of TG2 and investigated how they could regulate TG2 mediated functions. Besides some of the already known interacting partners

we could identify a co-chaperone, DNAJA1 as a novel interacting partner of TG2. DNAJ family of proteins is the largest and most diverse family of co-chaperones that works in collaboration with chaperones HSP70 and HSP90. However, they also include members, which can work independently from these chaperons [20]. DNAJ proteins are involved in several important cellular functions including the suppression of protein aggregation, folding of nascent and damaged proteins, translocation of proteins into cellular compartments and the targeting of proteins for degradation [21]. Furthermore, they play important roles in several pathological processes such as neurodegenerative disorders [22, 23] and cancer [24]. Particularly, DNAJA1 itself has been reported to participate in various pathological conditions such as autoimmune diseases [25], neurodegenerative diseases [26], and cancer [27, 28].

Like DNAJA1, TG2 is also reported to regulate cancer cell migration [29], apoptosis [30] and neurological disorders [31]. We speculated that DNAJA1 and TG2 might work in collaboration in regulating these cellular processes and pathological states. In this paper we have performed several biochemical and biophysical analysis to confirm DNAJA1 and TG2 interactions. By using deletion mutants of TG2 we determined, which domains of TG2 interact with DNAJA1. Further, we have also explored the biological relevance of this interaction under cellular conditions and investigated whether DNAJA1 is a potential novel substrate of TG2.

MATERIALS AND METHODS

Expression, purification and analysis of recombinant proteins

Human TG2 was cloned into pET 30 EK/LIC (Novagen) and pTRIEX 4 Neo mammalian expression vectors (Novagen) as described previously [15]. DNAJA1 cDNA was subcloned into pET 30 EK/LIC via ligation independent cloning according to manufacturers' protocol. TG2 was purified as described previously [15] and same protocol was used to purify DNAJA1. For western blots, CUB7402 antibody 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) were used. Full length and domain deleted variants of TG2 in pGEX 2T vector (GE Healthcare) were available in the lab [32]. GST tagged full length TG2 was a valine 224 variant while GST tagged domain variants of TG2 had glycine instead of valine at the 224^{th} position. The constructs were expressed in Rosetta 2 DE3 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 hr 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 via SDS PAGE.

Cell culture

General cell culture reagents were purchased from Sigma unless otherwise stated. Human embryonic kidney cells (HEK 293T AD) (Agilent Technologies, US) were cultured in DMEM and NB4 cells (DSMZ GmbH) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Sigma and GIBCO® life technologies, respectively), L-glutamine and penicillin/streptomycin antibiotics. Cells were grown in 5% CO_2 containing humidified atmosphere at 37^{o}C.

Generation of stable cell lines of HEK 293T AD cells overexpressing human TG2 were carried out as described before [15]. For the downregulation of DNAJA1, TG2 overexpressing HEK 293T AD cells were transfected with DNAJA1 specific Silencer Select Pre-designed siRNA (Ambion) using Lipofectamine® 2000 (Invitrogen) and scrambled RNA transfected cells were used as control. NB4 cells were differentiated for 72 hours by adding 1 µM all trans retinoic acid (ATRA) (Sigma, R2625) to express endogenous TG2 [33]. These differentiated cells were used for experimental analysis.

GST Pull Down Assay

GST pull down experiment was performed with differentiated NB4 cells. NB4 cells were collected and washed in PBS and subsequently resuspended in RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.5% Triton and protease inhibitor cocktail). The cells were lysed for 30 min at 4^{o}C and then centrifuged at 10.000 g for 20 min. The clear lysate was estimated via Bradford assay. Glutathione resin was incubated with 1 mg/ml of 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 resins were washed 4-5 times with 1 ml of RIPA buffer and then boiled in Laemmli sample buffer and loaded on SDS PAGE. DNAJA1 was detected with anti-DNAJA1 antibody. GST Pull down experiment was also repeated with recombinant purified GST-TG2 and DNAJA1. 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. During digestion first a reduction was

performed using DTT followed by alkylation with iodoacetamide. 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 4 µl of sample was used for LC-MS/MS analysis. 4000 Q TRAP (ABSCiex) - nanoHPLC (Bruker) LC-MS/MS System was used for data acquisition. 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, $CaCl_2$, $CaCl_2$ + Z-DON (zedira), or GTP for overnight at 4^{o}C. 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^{o}C at 40 mA and different conformers of TG2 were visualized by Page blue protein staining solution (Thermo Scientific).

ELISA

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 via ELISA according to standard protocol described previously [34]. 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 diluted (1:1000) in TBS-T for 1 hour at RT. After 3 washes, wells were incubated with HRP conjugated anti mouse IgG (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. Assays were carried out in triplicates.

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 (GE

Healthcare) was immobilized on sensor chip CM5 (BR-1000-12; Biacore™, GE Healthcare) using the amine coupling method as recommended by the manufacturer. On the sensor chip surfaces full-length GST-TG2 and GST tagged domain variants of TG2 (GST-TG2Δβ-barrel1, GST-TG2Δβ-barrel2, GST-TG2ΔCAT, GST-TG2Δβ-sandwich and 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 level of GST-fusion proteins was 500-1000 RU according to their molecular weight. The various concentrations of DNAJA1 protein were injected over the different TG2 variants coated surfaces and control for 7 min at 5 µl/min flow rate. The evaluation of the sensorgrams was carried out with BIAevaluation 3.1 software.

Immunocytochemistry

TG2 overexpressing HEK 293T AD cells were cultured on glass coverslips and fixed with 4% paraformaldehyde 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) in 5% goat serum in PBS-T for 2 hours at 25^{o}C. 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

Measurement of BPA incorporation into surface bound N,N-dimethylated casein (DMC) was performed as described previously by Slaughter et al [35] with some modifications [15]. In case of the the kinetic TG2 activity assay, monodansylcadaverine (Dansyl-Cd, a fluorometric-labeled cadaverine) (Sigma) incorporation into DMC was monitored. 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 min. The increase in flourescence intensity was followed using BioTek Synergy H1 Multi-Mode Reader (Bio Tek US; Ex/Em: 360/530 nm). For precise determination of Km and Kcat values monodansylcadaverine fluorescence standard curve was prepared.

Analysis of DNAJA1 as a substrate of TG2

In order to determine if DNAJA1 is a substrate of TG2, Maxisorp (Nunc) microtiter plate was coated with 0.2-1 µg DNAJA1 and BPA incorporation into DNAJA1 was measured as described above in TG2 activity assay. In another confirmation experiment described by Ruoppolo et al [36], 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.

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 t test (parametric) by using Graph Pad Prism version 5.0. The $p \leq 0.05$ value was considered significant.

RESULTS

1. Screening for TG2 interacting proteins in differentiated NB4 cells

TG2 interacting proteins play a very crucial role in modulating TG2 functions. We have focused on identifying novel interacting partners of TG2 and used human promyelocytic leukemia cell line (NB4). NB4 cells upon differentiation with all trans retinoic acid (ATRA) express a very high level of TG2, which leads to a massive upregulation of several thousand genes involved in many physiological and pathological processes. Knocking down TG2 in ATRA treated NB4 cells leads to downregulation of the above reported genes [33]. Therefore NB4 cells were considered as an excellent cell model to explore TG2 interacting partners and the related functions they modulate. GST Pull down experiments and subsequent mass spectrometry analysis of differentiated NB4 cells lead to the identification of human Glutathione S Transferase (hGST-P1), Tubulin α, Histone H2A and DNAJA1 as a potential binding partner of TG2 (Figure 1A). Since all of the above proteins except DNAJA1 have already been reported either to interact with TG2 or as a substrate of TG2, DNAJA1 could be 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 in a Western Blot on GST Pull down samples (Figure 1B). Further we expressed and purified recombinant TG2 and DNAJA1 and performed GST Pull down experiments to confirm their direct interactions (Figure 1C).

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

ELISA and SPR measurements were carried out to verify physical interaction of TG2 with DNAJA1. Interaction of DNAJA1 with domain variants of TG2 has also been investigated to determine DNAJA1 binding domain of TG2. The results shown in Figure 2 demonstrate that DNAJA1 interacts with full length TG2 and domain variants of TG2 including GST-TG2Δβ-barrel2, GST-CAT (catalytic domain), GST-TG2Δβ-sandwich, GST-TG2Δβ-barrel1 and GST-TG2ΔCAT. Dissociation constants (Kd) for these interactions were calculated from three independent experiments and given in Figure 2D. We observed the highest binding affinity for DNAJA1 with GST-TG2Δβ-barrel2 (which contains the β sandwich, the catalytic and the β barrel 1 domains) with dissociation constant of $5.87x10^{-8}$ μM whereas GST-TG2ΔCAT variant showed the least interaction with dissociation constant of $5.3x10^{-7}$ μM. There is a strong interaction of DNAJA1 with GST-CAT domain itself (Kd: $6.885x10^{-8}$ μM), which is almost the same as its interaction with full length TG2 (Kd: $6.095x10^{-8}$ μM). These results suggest that catalytic domain of TG2 is required for TG2-DNAJA1 interaction and β sandwich domain together with β barrel 1 domain stabilize this interaction.

3. DNAJA1 interacts with the open conformer of TG2

As mentioned before, TG2 can adopt mainly two distinct conformations depending on the type of binding effectors. In agreement with earlier reports, electrophoresis under non-denaturing 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 3A). 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 3A). 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 3A). 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 Figure 3B, TG2-DNAJA1 interaction was significantly higher in the presence of EDTA and 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 interaction is not dependent on crosslinking activity of TG2.

4. DNAJA1 facilitates and stabilizes in vitro crosslinking activity of TG2

Among several activities of TG2, the most prominent is its transamidase activity which has been implicated in the progression of a number of diseases [29, 37]. To explore the significance of DNAJA1 and TG2 interaction we firstly wanted to determine whether DNAJA1 could modulate the crosslinking activity of TG2. As we can see in the Figure 4A, 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. Increase in the crosslinking reaction was observed on increasing the DNAJA1 concentration. 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 up to 30 min. Nevertheless, after 30 – 60 min an increase in transamidation activity of TG2 was observed (Figure 4B 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.

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

To explore how DNAJA1 influenced the crosslinking activity of TG2 in cells, 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 un-transfected cells as control. Downregulation of DNAJA1 was demonstrated via Western blot (Figure 5A). 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 5A and B). We also used TG2 un-transfected HEK cells and there was no crosslinking activity in these cells (Figure S1). 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 [15]. 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 5A) 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 S2).

6. Co-localization of TG2 with DNAJA1 in cytoplasm of TG2 overexpressing HEK cells

In order to determine whether TG2 co-localize with DNAJA1 in an intact mammalian cellular system TG2 overexpressing human embryonic kidney cells (HEK-TG2) were used since NB4 cells gave very high background. 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 compartment with low expression in nuclei (Figure 6). Co-localization of TG2 and DNAJA1 in the cytosolic region indicates a potential association of the two molecules with physiological processes in vivo.

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 amine 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 7A 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 7C).

DISCUSSION

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 the NB4 acute promyelocytic leukemia (APL) cell line since treating this cell line with all trans retinoic acid (ATRA) results in enhanced expression of TG2 as well as massive changes in the expression of other genes [33]. 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 bind 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 its 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 [38]. We have used various biochemical and biophysical methods to confirm DNAJA1 and TG2 interactions.

GST Pull down experiments with NB4 cell lysate and subsequent Western blot analysis of pulled down samples confirmed TG2 and DNAJA1 interactions. Moreover, we confirmed the direct interactions further by using recombinant TG2 and recombinant DNAJA1. TG2 adopts an open conformation in the presence of calcium and is catalytically active, while in the presence of GTP it is predominantly in the closed conformation and inactive. Our data in this paper demonstrate that DNAJA1 interacts mainly with open conformer of TG2. 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 proposition that TG2 can be present in open conformation and in active state in the cell cytoplasm [15, 39] which is against the prevailing view that under physiological conditions TG2 cannot be active as a transamidase inside the cells owing to low Ca^{2+} and high GTP concentrations [40, 41]. DNAJA1 was also found to interact with the active site mutant of TG2 thereby suggesting 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 interacted with TG2 domain variants with different affinities. DNAJA1 could interact with full length TG2 as expected. The most significant observation was that TG2 showed the least interaction with the core domain deleted variant. However, it interacted with the core domain alone suggesting that the core domain of TG2 is the most important domain in this interaction and

since the core domain alone 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 observe an increase in the TG2 activity on increasing DNAJA1 concentrations in an end point assay. 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 gave contrasting results compared to *in vitro* assays. We observed an increase in TG2 activity in DNAJA1 downregulated HEK-TG2 cells compared to scrambled and un-transfected 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 the 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 becomes 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.

In this paper, we could successfully identify yet another interacting partner which can interact with TG2 non-enzymatically as well as also functions as its substrate. Other proteins, which have been reported to have such dual functions, include fibronectin, BCR, angiocidin and retinoblastoma protein. As mentioned previously, DNAJ family of proteins and particularly DNAJA1 play important roles in certain types of cancer and neurodegeneration and since TG2 is also involved in these processes, it will be interesting to see whether DNAJA1 and TG2 interaction regulates these pathological conditions. Our future attempt will be to explore the significance of this interaction via using different cancer and neurological cell models.

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FIGURE LEGENDS

Figure 1. TG2-DNAJA1 interaction via GST-pull down assay and mass spectrometry. A. 1 mg/ml 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: hTG2 (100 kDa), GST (24 kDa), hGSTP1 (24 kDa), putative tubulin like protein alpa (45 kDa), hDNAJA1 (45 kDa) and BSA (70 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.

Figure 2. 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.

Figure 3. 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.

Figure 4. 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 (0.2-1 µg) of DNAJA1 (Control: 1 µg TG2). Reaction mixtures were incubated for 30 minutes at 37^{o}C **B.** Kinetic transamidation assay was carried out monitoring monodansyl cadaverine (0.2 mM) incorporation into DMC (16 µM) with (red line) and without (blue line) DNAJA1 (3 µM). **C.** Slopes of each line in figure B were used for calculation 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)

Figure 5. 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 un-transfected 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 Ca^{2+}-Ionophore, A23187 for 1 hour. Cell lysates (20 µg) were used in western blotting. **B.** ELISA. ** $p<0.05$ between the groups.

Figure 6. Immunoflourescent images of TG2 and DNAJA1 in TG2 overexpressing HEK 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 co-localization of TG2 and DNAJA1 in the cytoplasm of HEK 293T AD cells transfected stably with TG2.

Figure 7. 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.

Figure 1.

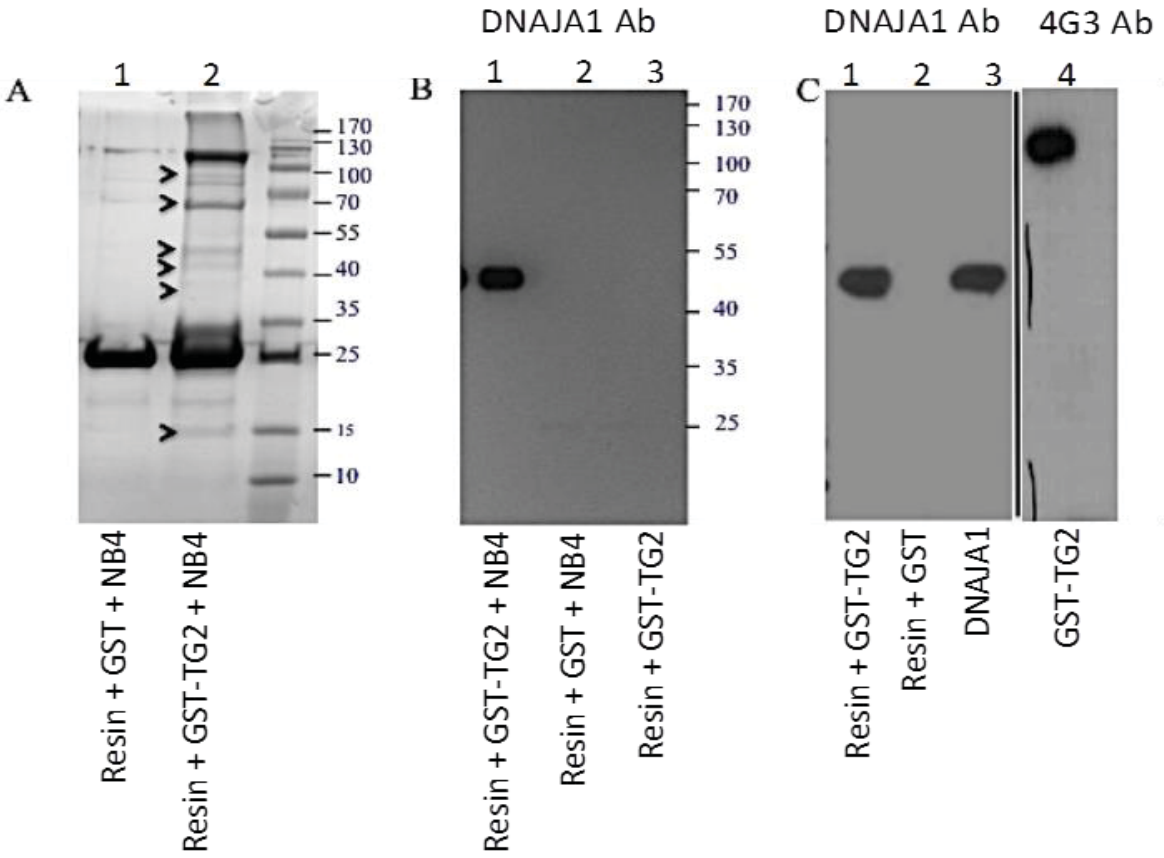

A
1
2
170
130
100
70
55
40
35
25
15
10
Resin + GST + NB4
Resin + GST-TG2 + NB4
B
DNAJA1 Ab
1
2
3
170
130
100
70
55
40
35
25
Resin + GST-TG2 + NB4
Resin + GST + NB4
Resin + GST-TG2
C
DNAJA1 Ab
4G3 Ab
1
2
3
4
Resin + GST-TG2
Resin + GST
DNAJA1
GST-TG2

Figure 2.

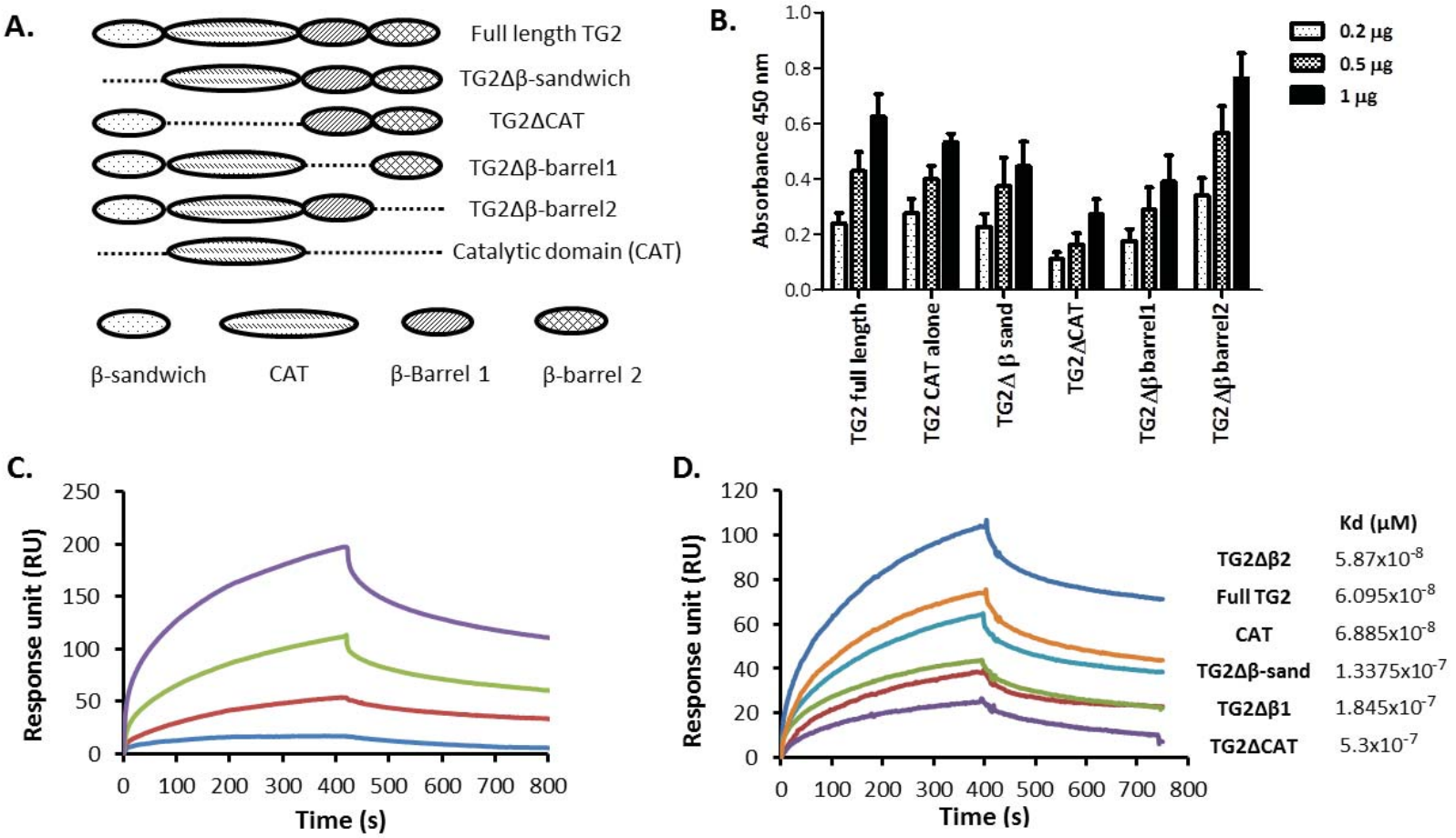

A.
Full length TG2
TG2Δβ-sandwich
TG2ΔCAT
TG2Δβ-barrel1
TG2Δβ-barrel2
Catalytic domain (CAT)
β-sandwich
CAT
β-Barrel 1
β-barrel 2
B.
Absorbance 450 nm
0.2 µg
0.5 µg
1 µg
TG2 full length
TG2 CAT alone
TG2 Δ β sand
TG2 ΔCAT
TG2 Δβ barrel1
TG2 Δβ barrel2
C.
Response unit (RU)
Time (s)
D.
Response unit (RU)
Time (s)
Kd (µM)
TG2Δβ2 5.87x10-8
Full TG2 6.095x10-8
CAT 6.885x10-8
TG2Δβ-sand 1.3375x10-7
TG2Δβ1 1.845x10-7
TG2ΔCAT 5.3x10-7

Figure 3.

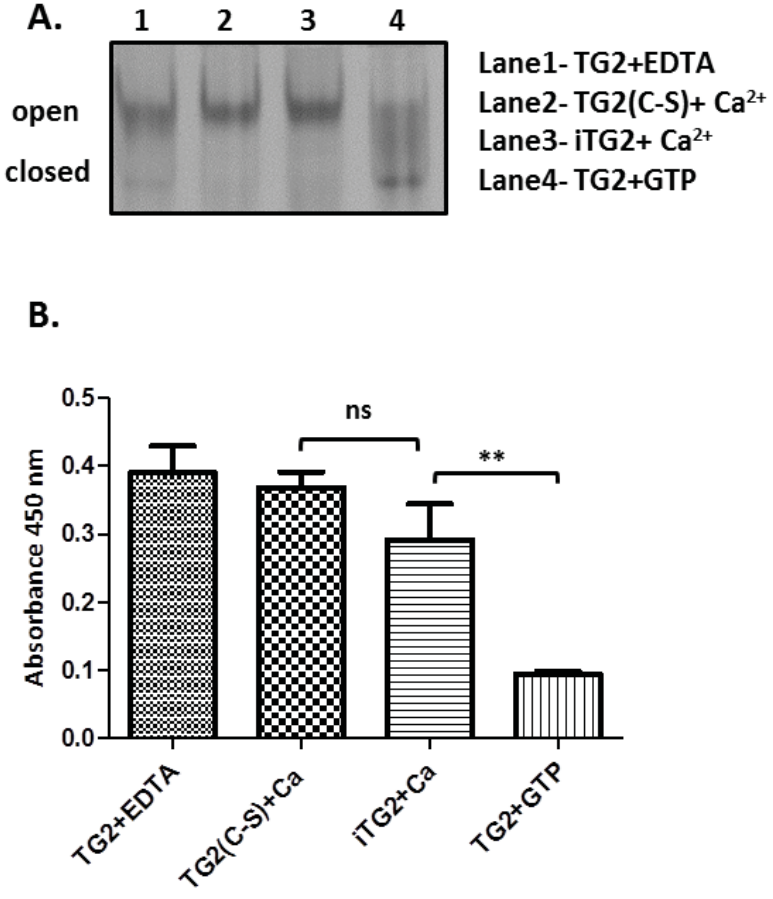

A.
1
2
3
4
open
closed
Lane1- TG2+EDTA
Lane2- TG2(C-S)+ Ca2+
Lane3- iTG2+ Ca2+
Lane4- TG2+GTP
B.
Absorbance 450 nm
0.5
0.4
0.3
0.2
0.1
0.0
ns
**
TG2+EDTA
TG2(C-S)+Ca
iTG2+Ca
TG2+GTP

Figure 4.

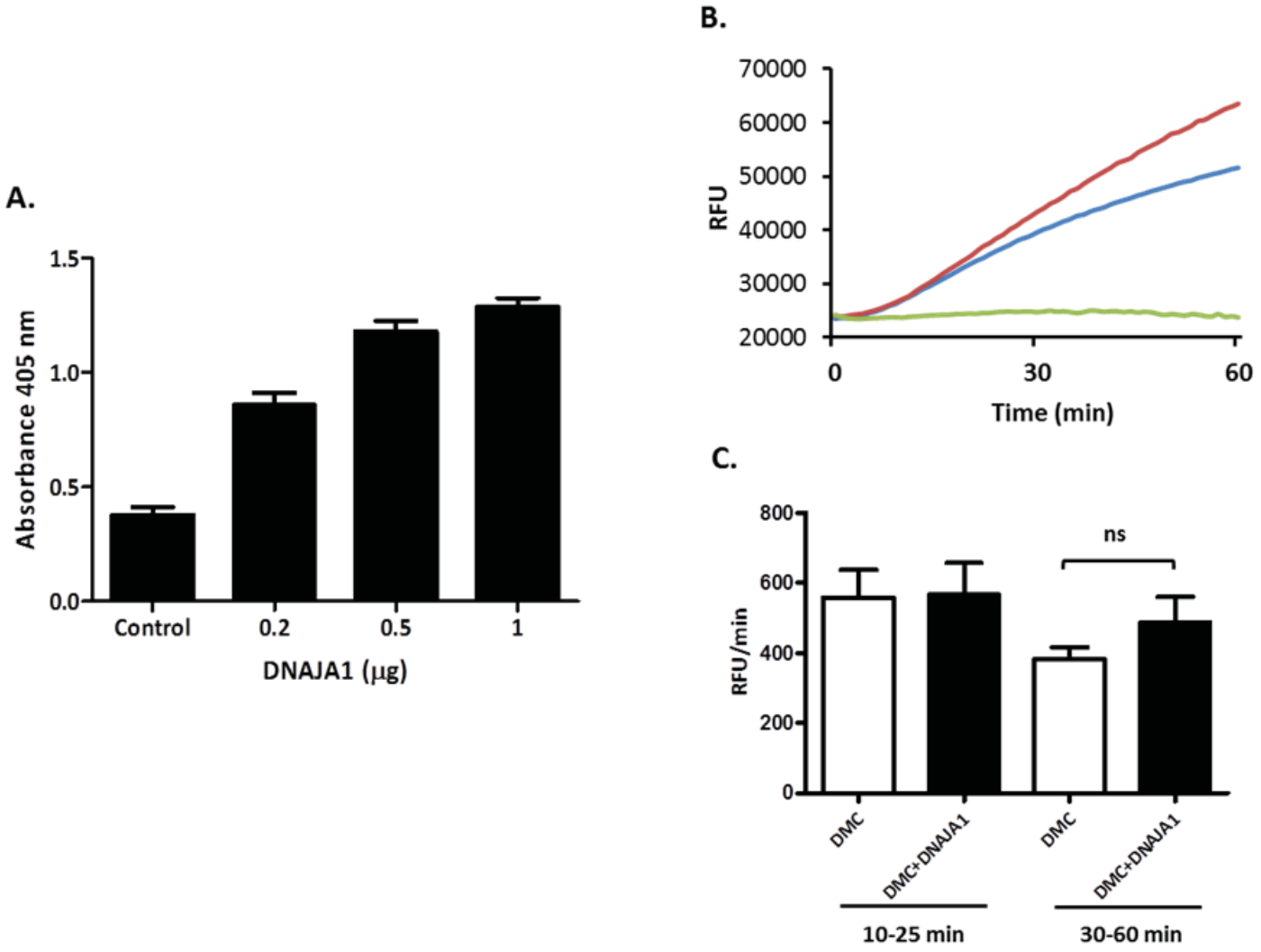

A.
Absorbance 405 nm
1.5
1.0
0.5
0.0
Control
0.2
0.5
1
DNAJA1 (μg)
B.
RFU
70000
60000
50000
40000
30000
20000
0
30
60
Time (min)
C.
RFU/min
800
600
400
200
0
ns
DMC
DMC+DNAJA1
DMC
DMC+DNAJA1
10-25 min
30-60 min

Figure 5.

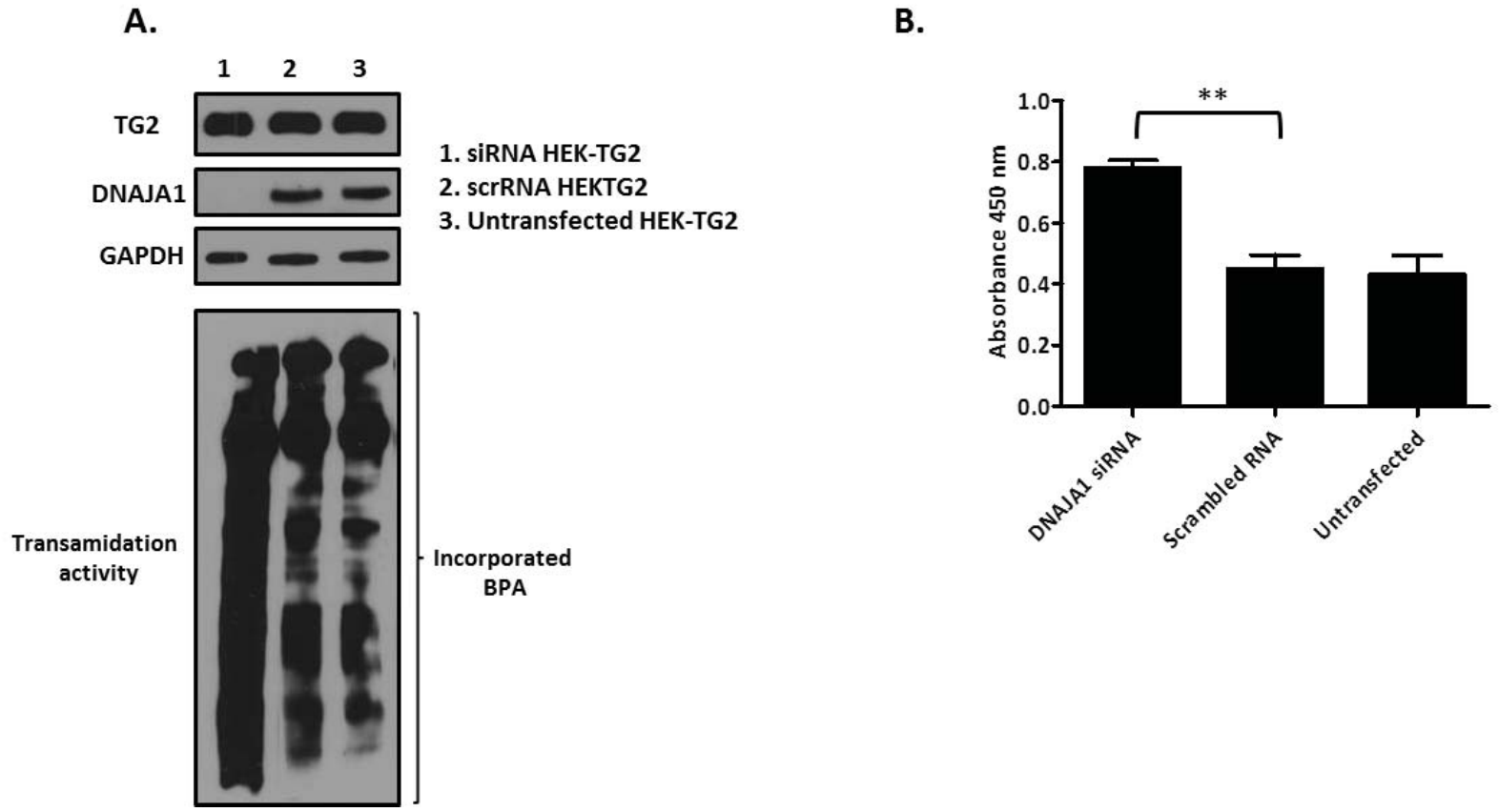

A.
1
2
3
TG2
DNAJA1
GAPDH
1. siRNA HEK-TG2
2. scrRNA HEKTG2
3. Untransfected HEK-TG2
Transamidation activity
Incorporated BPA
B.
**
Absorbance 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
DNAJA1 siRNA
Scrambled RNA
Untransfected

Figure 6.

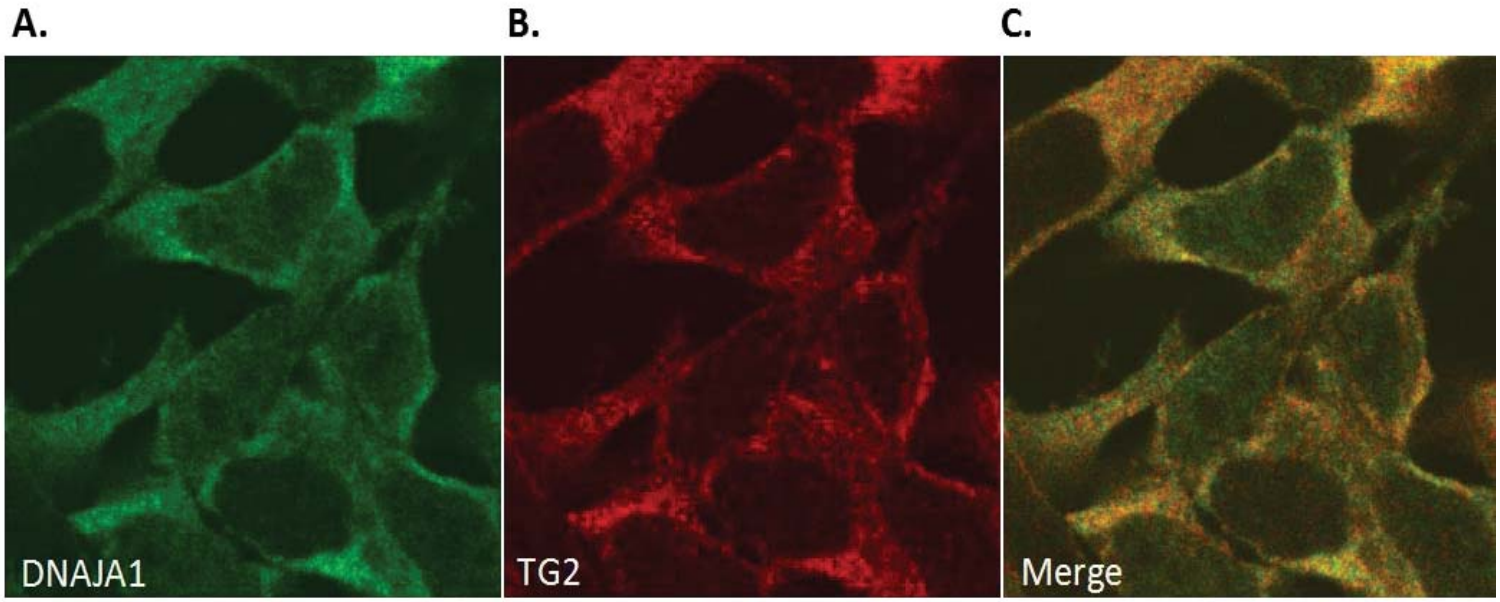
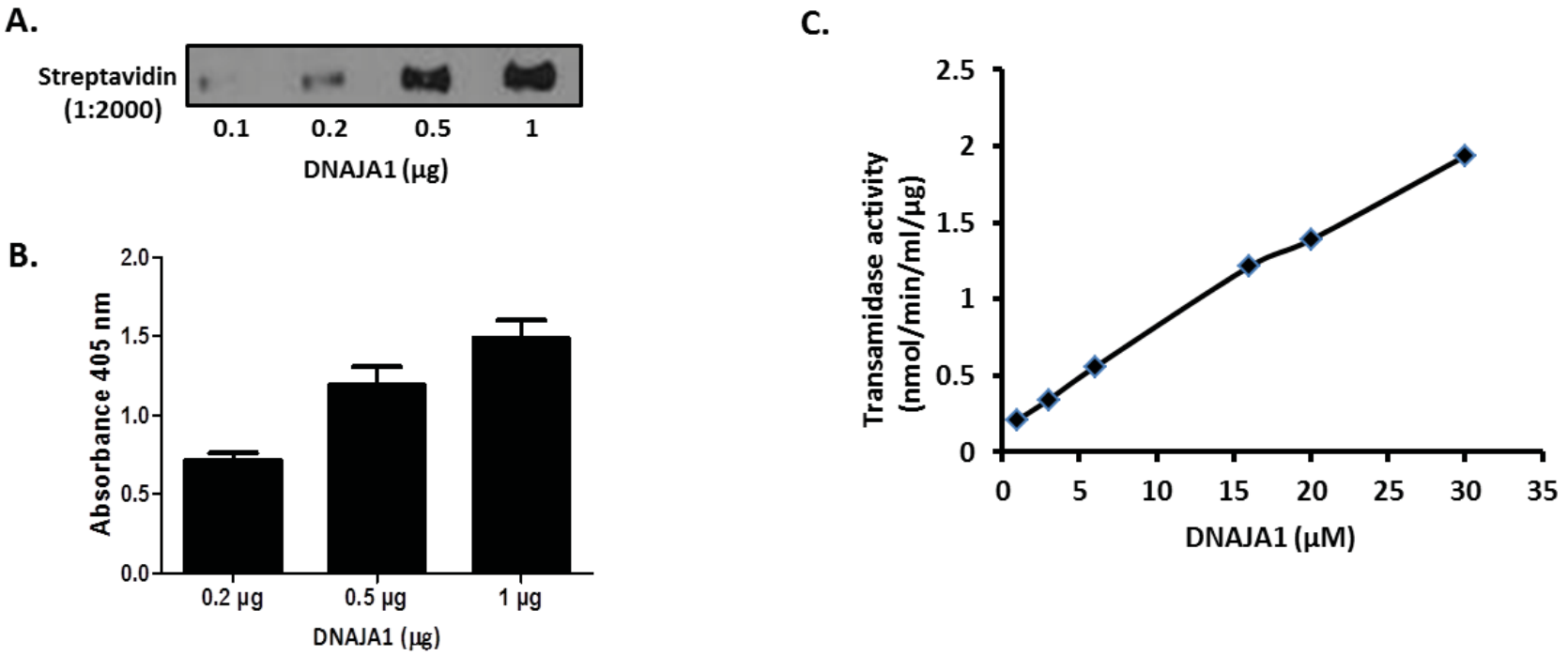


Figure 7.


A.
Streptavidin
(1:2000)
0.1
0.2
0.5
1
DNAJA1 (µg)
B.
Absorbance 405 nm
2.0
1.5
1.0
0.5
0.0
0.2 µg
0.5 µg
1 µg
DNAJA1 (µg)
C.
Transamidase activity
(nmol/min/ml/µg)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
DNAJA1 (µM)