NKFIH K 129139

EXPOSING CELLULAR INTERACTOME OF MULTIFUNCTIONAL HUMAN TRANSGLUTAMINASE 2 FOR UNDERSTANDING ITS FUNCTIONS

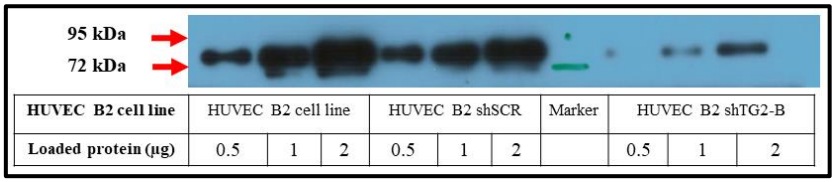
The transglutaminase 2 (TG2) functional and interactome experiments have been planned and carried out using a newly developed HUVEC (Human Umbilical Vein Endothelial) and an already-established NB4 cell model. This closing report describes the results obtained along these two approaches. The HUVEC cell model has revealed new potential interacting partners of TG2 involved in the endothelial cell related biological functions and we firstly observed RNA-binding feature of TG2. In the acute promyelocytic leukaemia (APL) NB4 cell model, novel TG2 dependent pathways with TG2 interacting partners have been identified.

A. The HUVEC system

Development of endogenous TG2 knocked down and transgene 3xFLAG-TG2 expressing endothelial cell model

In order to generate a sufficient amount of protein sample for mass spectrometry based interactome determination, five stable immortalised human endothelial cell lines were established by retroviral gene delivery of telomerase gene using pBABE-neo-hTERT retroviral vector (Addgene #8454). We have set up and optimized the virus packaging in HEK-293FT cells using pCMV-VSV-G (Addgene #8454) and pUMVC (Addgene #8449) because the earlier used packaging cell line had low efficiency. The overexpression of telomerase protein helps to maintain the endothelial cells preventing their early senescence while keeping their original properties and making feasible sample production for latter MS analysis to identify interacting partners. **Immortalised HUVEC cell lines maintained their original morphological properties and the expression of von Willebrand factor and PECAM-1 endothelial markers.** One of the immortalized cell lines (HUVEC B2) was applied to test the effect of irisin-stimulated adipokine release on HUVEC cells (*Shaw A et al. 2021. Front. Cell Dev. Biol.*).

As a next step, to characterise the role of TG2 in the HUVEC cells a TG2 knock-down model has been developed using an earlier published pLKO-puro-shRNA-TG2-A vector based viral gene delivery (Sigma#TRCN0000000239=A). After the viral infection and 4 µg/ml puromycin selection Western blot showed that, unfortunately, in HUVEC cells the 3'-UTR targeting silencing sequence was less efficient than earlier in NB4 cells. We have replaced the silencing sequence with a slightly modified one (Sigma#TRCN0000272816=B) which also targeted the 3'-UTR of the TG2 mRNA. This pLKO-puro-shRNA-TG2-B construct resulted in more efficient silencing than the previous one but using the CUB7402 monoclonal mouse antibody TG2 was still detected by Western blot (Figure 1). Long culturing of the cell lines revealed that TG2 silencing takes a longer time (2-3 weeks) to down-regulate TG2 protein level.



HUVEC B2 cell line	HUVEC B2 cell line			HUVEC B2 shSCR			Marker	HUVEC B2 shTG2-B		
Loaded protein (µg)	0.5	1	2	0.5	1	2		0.5	1	2

Figure 1. Detection and comparison of TG2 down-regulation between unmodified, scramble and pLKO-puro-shRNA-TG2-B transduced HUVEC B2 cell lines. Various protein amounts were examined by Western blot using CUB7402 monoclonal mouse antibody.

In contrast to published results (lentiviral silencing of TG2 in mouse neurons or siRNA-based silencing of TG2 in endothelial cells), in our experiments, HUVEC cells did not show significantly decreased

viability using a resorufin-based viability assay compared to the scramble infected control cells. We assume that the reason is the incomplete silencing of TG2. For the functional study of the developed cell lines, the application of a protein-based, anisotropy changing transglutaminase activity assay was tested. It turned out that the assay is applicable for the characterization of another transglutaminase in serum samples (*Csobán-Szabó Z et al. 2020 Anal Biochem*).

To increase the efficiency of TG2 down-regulation, another approach has been developed. Tet-inducible lentiviral vectors (Tet-pLKO-puro-shRNA-TG2-A/B) were also constructed for inducible, tuneable TG2 silencing (Empty vector: Addgene#21915) using both previously applied interfering sequences. Application of Tetracycline inducible knockdown could help to separate the effect between the selection media after pLKO-puro-shRNA-TG2 lentiviral infection and the TG2 down-regulation, particularly on cell viability. After optimisation of doxycycline induction, in the presence of 4 µg/ml doxycycline, a more efficient TG2 protein silencing was observed in the case of the B sequence (TRCN0000272816) compared to the earlier published ones (A). Long-term induction of the silencing also confirmed that TG2 down-regulation needs a relatively long time, probably due to its long half-life time in endothelial cells.

Before the detailed characterization of the TG2 knocked-down cell line, we have complemented the TG2 level with a Flag-tagged transgene protein. The applied silencing sequences target the endogenous TG2 mRNA's 3'-UTR regions, making possible the complementation of the endogenous TG2 down-regulated cells by an exogenous N-terminal 3xFlag-tagged TG2 lentiviral construct. The presence of a 3xFLAG tag results in high affinity towards anti-FLAG antibodies which later have been beneficial in co-immunoprecipitation experiments. For high cloning efficiency a GATEWAY cloning system was selected. First, into the pENTR4-FLAG plasmid (Addgene#17423) two other FLAG-tag sequences were inserted by site-directed mutagenesis. Then, TG2 cDNA was cloned into the 3xFLAG containing vector in reading frame with the N-terminal tag. The entry vector was recombined with a PGK promoter containing destination vector (Addgene#19065) which could have expressed the transgene TG2 at a low but stable level. Unfortunately, this was unsuccessful, probably due to spontaneous recombination in the applied destination vector. Then, we have made the recombination with a Tet inducible pCW57.1 destination plasmid (Addgene#41393; pLKO-based all-in-one Tet-On plasmid: pLKO Tet-On). The replication-incompetent virus particles were produced in HEK-293FT cells using psPAX2 and pMD2.G packaging plasmids (Addgene#12260 and 12259, respectively). Both immortalised and TG2 knocked down HUVEC B2 cell lines were infected with the produced virions and selected in the presence of 6 µg/ml puromycin. Applying the earlier determined optimal 4 µg/ml doxycycline for induction (replaced by every 48 hours) the **downregulation of endogenous TG2 and expression of tagged, transgene 3xFLAG-TG2 was confirmed by RT-qPCR and Western blot** (Figures 2. and 3.; Poster presentation and abstract on the Hungarian Molecular Life Sciences Congress 2021. *Csaholczi et al. Development and characterisation of a transglutaminase 2 knocked down endothelial cell model.*).

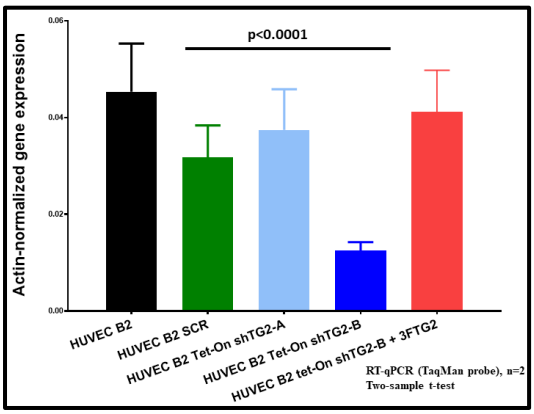
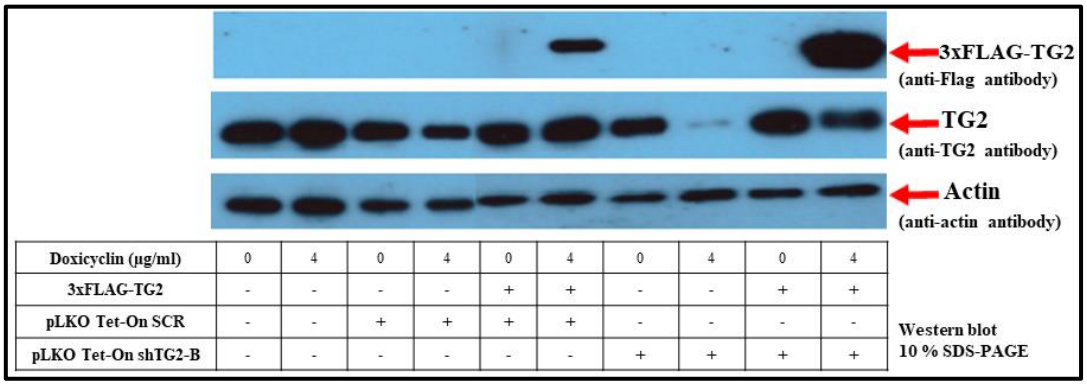


Figure 2. The shTG2-B sequence decreased the level of endogenous TG2 mRNA, which was compensated by the introduction of the transgenic TG2 to a level similar to the HUVEC B2 cell line.



Doxicyclin (µg/ml)	0	4	0	4	0	4	0	4	0	4
3xFLAG-TG2	-	-	-	-	+	+	-	-	+	+
pLKO Tet-On SCR	-	-	+	+	+	+	-	-	-	-
pLKO Tet-On shTG2-B	-	-	-	-	-	-	+	+	+	+

Figure 3. Monitoring of endogenous TG2 protein silencing and 3xFLAG-TG2 transgene expression by Western blot (representative picture based on four independent experiments)

Functional consequences of TG2 knockdown in HUVEC cells

As a next step, we have completed the characterization of the established immortalized HUVEC cell model which was transduced by a Tet inducible lentiviral vector (Tet-pLKO-puro-shRNA-TG2-B) and a Tet inducible 3xFLAG tagged TG2 vector. First, we have confirmed that **decreased TG2 level was not compensated by increased expression of other transglutaminases** (Figure 4).

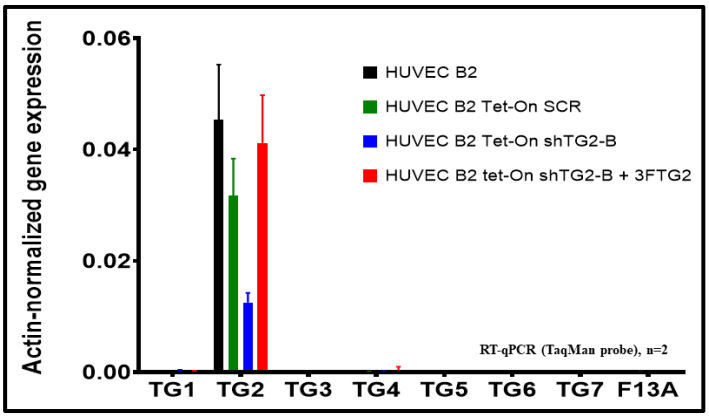


Figure 4. Silencing of TG2 does not lead to increased expression of other transglutaminase family members in HUVEC B2 cells.

In accordance with other laboratories, we have also observed that the **TG2 silenced cell line shows a higher apoptotic rate than the controls** testing by Annexin-V and propidium iodide staining (FACS) (Figure 5.). The delivered **transgene 3xFLAG-TG2 was able to compensate for the increased cell death rate** observed in the endogenous TG2 silenced cell line. Our results also suggest that TG2 plays a role in the regulation of HUVEC cell death and survival. We participated in a study which published that Caspase-9 is a regulator of necroptosis (*Molnár et al. 2021. FEBS J*) and we can use later the applied methodological approaches in the endothelial cell systems.

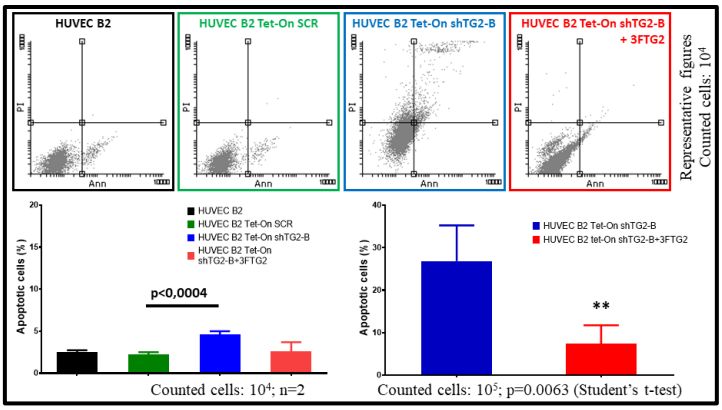


Figure 5. TG2 silenced endothelial cells demonstrated a significantly higher cell death rate which was diminished by the expression of transgene 3xFlag-tagged TG2. Annexin-V / PI (propidium iodide) staining was analysed by flow cytometry; *p<0.05, **p<0.01).

Compared to earlier published data about TG2 silencing in HUVEC using siRNA, **in our cell model we observed only non-significant decreased adhesion property in the case of silenced endogenous TG2 level**, and the transgene was not able to compensate for this feature. Based on the literature, doxycycline, used for the induction, could influence the gene expression pattern of endothelial cells by increasing the expression of VEGF, which can diminish the differences between the adhesion properties of the tested cell lines (Figure 6.). The continuously silenced HUVEC B2 shTG2-B cell line was also tested and showed decreased adhesion and morphological changes.

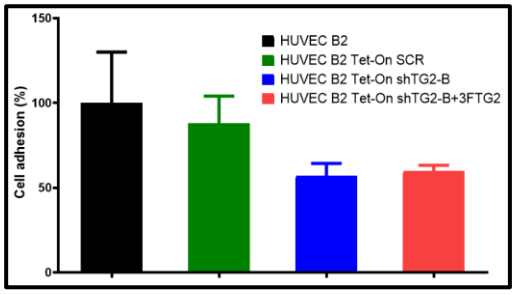


Figure 6. Cell adhesion of HUVEC B2 cell lines after 1-hour incubation was assayed using a cell viability reagent (Promega) to compare the adhered cell number in 96 well plates.

Based on the literature, TG2 plays a role in the inflammatory response of the endothelial cells. The difference between the cytokine production of HUVEC B2 and HUVEC B2 shTG2-B was tested using the Proteome Profiler Human XL Cytokine Array (R&D Systems) upon 6 hours of LPS induction. Increased expression of ST-2, MCP-1, IL-8, ICAM-1, Pentraxin-3, MIF, IL-17A was observed **upon LPS treatment in HUVEC B2**, but **there was no detectable difference in our TG2 knocked down cell line**, at least under this assay conditions and short LPS stimulation.

Based on the insignificant changes in cell adhesion properties upon TG2 knocked down, to monitor the efficiency of silencing, the amount of TG2 protein content was determined in the established cell lines using a recently developed commercial kit (CovalAb, Transglutaminase-2 ELISA Kit #kit0001). Figure 7. shows that **continuous or Tet-induced silencing caused approximately 63% or 71.5% decrease in TG2 amount compared to the HUVEC B2 cell line**. Our results suggest that the remaining TG2 protein is still enough to maintain physiological functions with relatively small changes. The major conclusion is that we have to develop a TG2-KO cell line to reveal the functional significance of TG2 in HUVEC cells. (Poster presentation and abstract on the Hungarian Molecular Life Sciences Congress 2021. *Csaholczi et al. Development and characterisation of a transglutaminase 2 knocked down endothelial cell model.)*

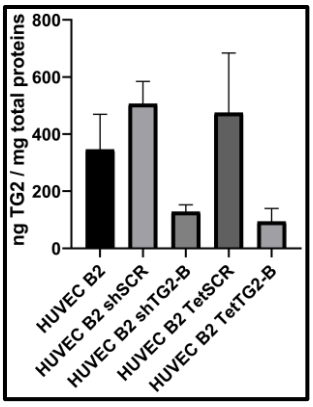


Figure 7. Determination of TG2 protein amount in the scramble and TG2 silenced cell lines using CovalAb Transglutaminase-2 ELISA Kit (300 mg cell lysate was applied)

Development of a TG2 KO HUVEC cell line

In order to develop the TG2-KO HUVEC cell line CRISPR/Cas9 based technology was selected. First, in collaboration, human TG2 N-terminal specific gRNA was designed and cloned into the pSpCas9(BB)-2A-Puro (PX459) vector (Addgene#48138). After the first transformation and selection, it turned out, that there was a mutation in the puromycin resistance gene of the plasmid that required a very low amount of puromycin during the selection. HUVEC cells are hard to transfect cells, the transfection was repeated with several settings using Lipofectamin 2000 and 3000 (Thermo). After an extremely long selection and cloning period, we finally got some potential TG2-KO HUVEC cell lines in which the detection of TG2 was not possible by Western blot using CUB 7402 or N-terminal domain specific monoclonal antibodies. From isolated genomic DNA, cloning of the gRNA target region into pTOPO plasmids and subsequent **sequencing confirmed the deletion of one nucleotide on both TGM2 alleles**. This frameshift makes any TG2 protein expression impossible due to the generation of an altered amino acid sequence and early stop codon.

Culturing the **HUVEC B2 TG2-KO cell line** immediately turned out that it **has a lower cell proliferation rate compared to the wild type** while the **size of the cells is bigger than the normal HUVEC** cells. The detailed characterization of the TG2-KO cells is still in progress due to their slow proliferation, but the latest results showed the complete **absence of any transglutaminase activity** using the microtiter plate method. The adhesion property of the TG2-KO cells was also tested. Interestingly, the fluorescent signal, which is related to the living cell numbers in the wells after 1 hour adhesion time, is 3 times higher in the case of TG2-KO HUVEC cells than the wild type (Figure 8.). After repeating the adhesion test in a transparent plate, the visualization of the living cells with MTT reagent demonstrated in the wells approximately equal cell numbers in both wild type and TG2-KO HUVEC cell lines. This suggests that **the absence of TG2 initiates a mitochondria-related compensatory mechanism in enzyme systems involved in reductive processes**. The RNAseq analysis of the cells is in progress, this could reveal the TG2-dependent gene expression changes in endothelial cells. In addition, we have tested their migration properties using scratch assay and the **TG2-KO cell showed significantly lower migration speed** compared to the wild-type endothelial cells (Csaholczi et al. Functional consequences of TG2 silencing in HUVEC cells, manuscript under preparation).

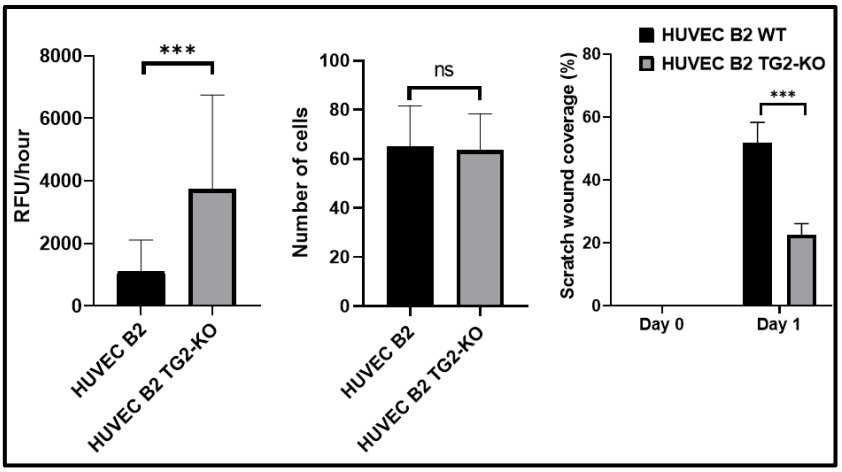


Figure 8. Comparison of cell adhesion and migration between HUVEC B2 wild type and TG2 KO cell lines. Left: Adhesion assay using CellTiter-Blue Cell Viability Assay to compare adhered cell numbers. Middle: Adhesion assay using MTT Assay reagent to visualize living cells for manual counting. Right: Scratch assay

The observation that TG2 KO mice have metabolic disturbance (*Lénárt K et al, 2020 Int J Mol Sci*) has underlined the importance of revealing the molecular interactions of transglutaminase 2. RNAseq analysis of a human cell differentiation model (*Tóth BB et al, 2020 Cells*) has provided further clues about molecular pathways influenced by TG2.

The presence of another transglutaminase, TG4, in tumour cells showed a correlation with higher invasiveness and increased adhesion properties. In collaboration, we performed the biochemical characterization of recombinant human TG4 (*Csobán-Szabó Z et al. 2021. IJMS*). In addition, we revealed that human TG4 is present in extracellular vesicles and can covalently modify secreted immunoglobulins in the saliva potentially participating here in immunological processes. This is also supported by the identified salivary TG4 interacting partners (the manuscript is still preparation due to the maternity leave of the first author).

Identification of TG2 interactome in HUVEC cells

We intended to use the developed endogenous TG2 silenced and N-terminally FLAG-tag fused transgene expressing HUVEC cell line model for revealing new interacting partners in the presence of NC9 and LDN-27219 inhibitors, which facilitates the open and closed conformation of TG2, respectively. The **triple FLAG tag makes possible the high-affinity binding of the cellularly developed protein complexes by anti-FLAG antibody during coimmunoprecipitation**.

First, we started to optimize the conditions of coimmunoprecipitation from HUVEC cells. We intended to identify weak interacting partners using photoactive amino acids, but this approach is not feasible due to the observed long half life-time of TG2 protein.

We selected another possibility to fix weak interactions in the living cells and tried to optimize the application (time and concentration) of dithiobis(succinimidyl propionate (DSP) chemical crosslinker reagent. DSP forms stable amide bonds crosslinking interacting proteins. It is cell penetrable but has low solubility, and reducing conditions can cleave the developed crosslinks. However, several conditions were tested, but the chemical crosslinker did not generate a significant amount of crosslinked 3xFLAG-TG2 from the feasible amount of HUVEC cell culture based on Western blot analysis.

The optimization of co-immunoprecipitation experiments for TG2 interacting partner search has taken a longer time than we expected. We had to increase the sample amount and decrease washing stringency based on the performed testing LC-MS/MS analysis which has also taken time. The variability between the LC-MS/MS analysis was also high because either the number of identified proteins was various or the number of overlapping proteins between independent experiments was low.

In parallel, **another approach has been optimised for the determination of TG2 interactome in HUVEC cells using a site specifically biotin-labelled recombinant TG2** mixed with HUVEC cell protein extract.

The potential conformation dependency of the interactions with TG2 was explored by using cell-permeable NC9 and LDN-27219 inhibitor treatments, which can stabilize TG2 in its open or closed conformation, respectively. In normal cellular conditions, both conformations could be present and can influence protein-protein interactions of TG2. Based on a recent publication, which demonstrated that in living cells LDN-27219 treatment can convert only a small portion of TG2 protein into the closed conformation, GTPγS was selected to force the closed conformation of TG2. To reveal open TG2 related interacting partners the developed transgene 3xFLAG-TG2 expressing cell line was pretreated with 15 µM NC9 for 24 hours before cell lysis, while recombinant protein was also pretreated with 100 µM NC9 before the coimmunoprecipitation (Figure 9). The GTPγS, which can not penetrate into the cells, was applied during the lysis and coimmunoprecipitation in 1 mM concentration.

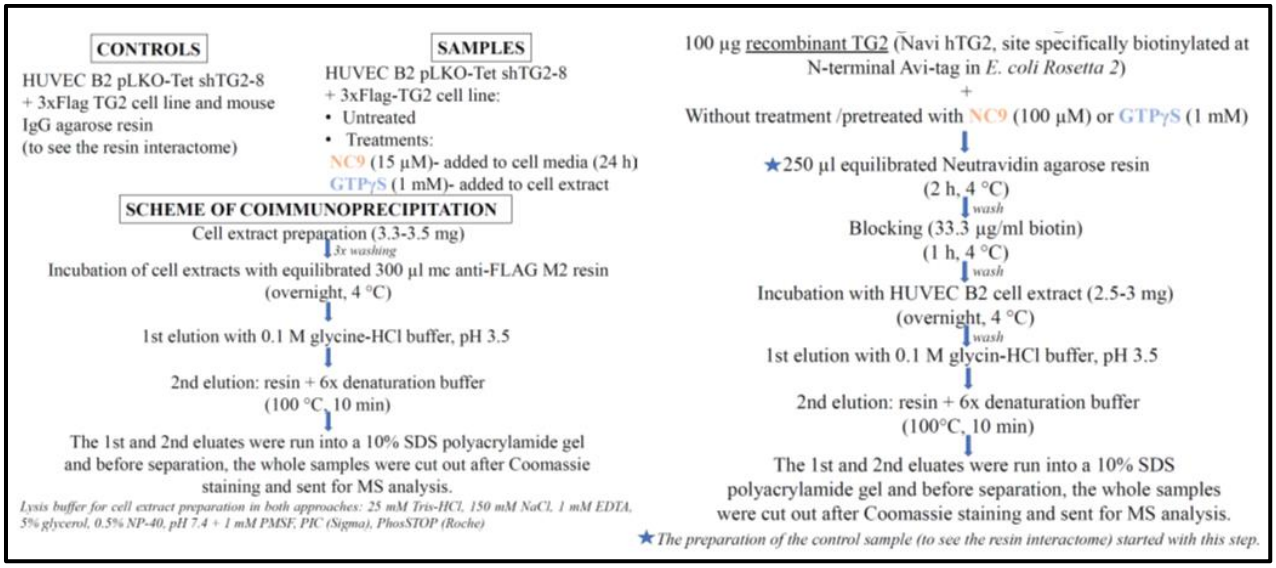
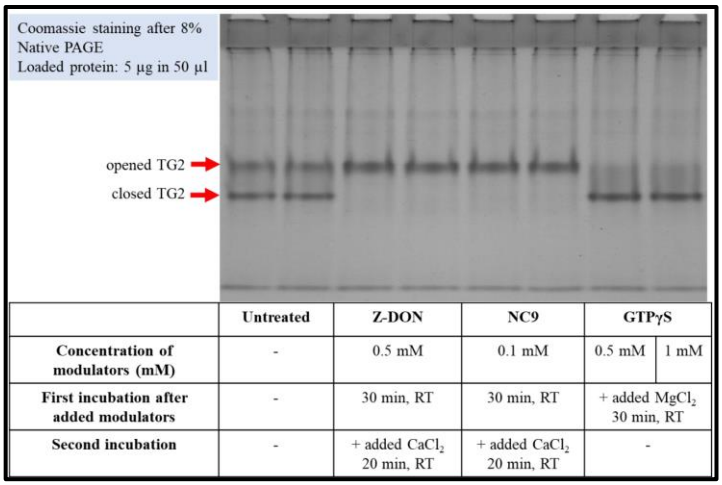


Figure 9. Scheme of the coimmunoprecipitation experiments. Left: Identification of the conformation-dependent interaction partners of 3xFlag-TG2 in the developed HUVEC B2 cell line using anti-FLAG M2 resin. Right: Identification of the conformation-dependent interaction partners of specifically biotinylated recombinant TG2 from HUVEC B2 cell line using Neutravidin agarose resin

During the optimisation, there was no significant change in TG2 level in the presence of the NC9 inhibitor and the inhibitor did not affect either the stability or the nuclear and cytosolic distribution of TG2 in the cells. Native, conformation-sensitive electrophoresis was used to check whether both the applied NC9 and GTPγS converted the majority of the TG2 in open or closed form during incubation (Figure 10.). These data have provided us with a solid basis for the upcoming interactome experiments when the induced conformational change will be utilized to reveal new interacting partners of TG2 in HUVEC cells.



	Untreated	Z-DON	NC9	GTPγS	
Concentration of modulators (mM)	-	0.5 mM	0.1 mM	0.5 mM	1 mM
First incubation after added modulators	-	30 min, RT	30 min, RT	+ added $MgCl_2$ 30 min, RT	
Second incubation	-	+ added $CaCl_2$ 20 min, RT	+ added $CaCl_2$ 20 min, RT	-	

Figure 10. Effect of inhibitors on recombinant N-terminally biotinylated TG2 conformation determined by native gel electrophoresis.

After the optimization of co-immunoprecipitation experiments in our developed HUVEC cell line **we have identified 342 potential TG2 interacting proteins using anti-Flag agarose resin** (in three independent experiments). Several **already known TG2 interacting partners were recognized in HUVEC cells, for example, fibronectin, integrin-beta, calreticulin, myoferlin, talin, DNAJA1, peroxiredoxin 1, tubulin A1A, BRCA1, vinculin, zyxin, and topoisomerase-2** which have confirmed the appropriate experimental settings. We have identified a lot of new potential interacting proteins which should be confirmed by independent methods like biolayer interferometry, ELISA, or thermophoresis experiments. To select biologically relevant proteins from the potential interacting partners, pathway analyses were performed using STRING, Panther, and Reactome databases. Based on these in silico analyses, **most of the TG2 interacting partners are involved in translational, nuclear and vesicular transport, focal adhesion and cytoskeleton organization processes**. The adhesion-related interacting partners can either explain while TG2 silencing negatively influences cell adhesion. The nuclear and extracellular transport of TG2 is also poorly known. Due to the large number of potential new TG2 interacting partners we applied another approach to select later the most likely new interacting partners for detailed analysis.

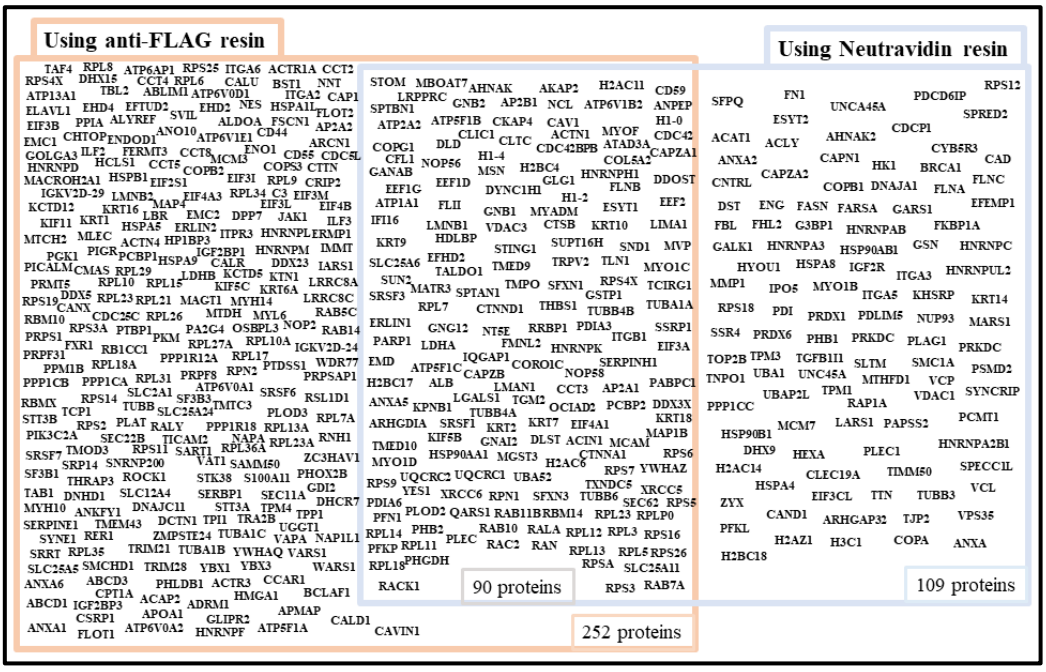


Figure 11. Potential interaction partners of TG2 in HUVEC cells identified using two different approaches

To confirm our result by another approach, a recombinant TG2, site-specifically biotinylated at its N-terminal fusion tag and purified from E. coli Rosetta 2 (DE3) bacterial cells was also developed and applied for co-immunoprecipitation from immortalized HUVEC cell extract using Neutravidin agarose (four independent experiments). Here, **199 proteins were identified as total interacting proteins with approximately 45% overlapping with anti-Flag precipitated proteins** (Figure 11.). The in silico analysis also resulted in the enrichment of similar biological processes compared to the 3xFlag tagged TG2 (Figures 12 and 13.; Poster presentation and abstract at the Annual Meeting of the Hungarian Biochemical Society, 2022. Pécs, *Csaholczi et al. Identification of potential interaction partners of transglutaminase 2 in endothelial cells.)*

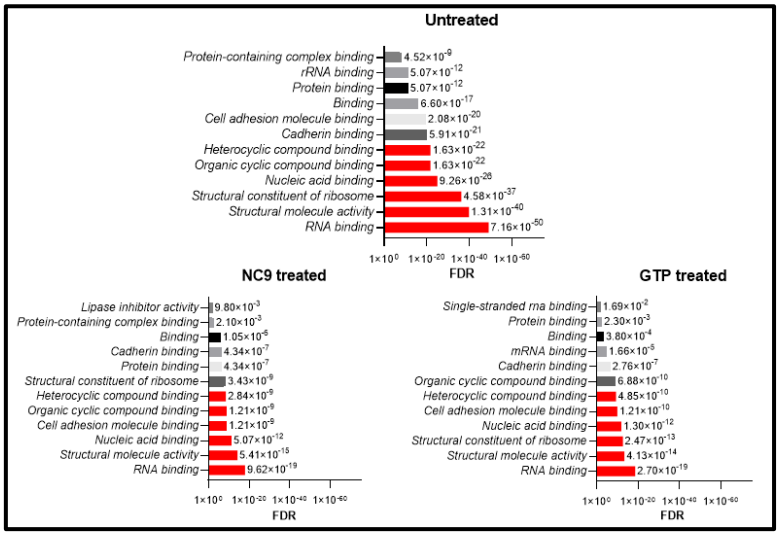


Figure 12. Most enriched GO Biological Functions (STRING) of the identified potential interaction partners of 3xFLAG-TG2 in HUVEC cells

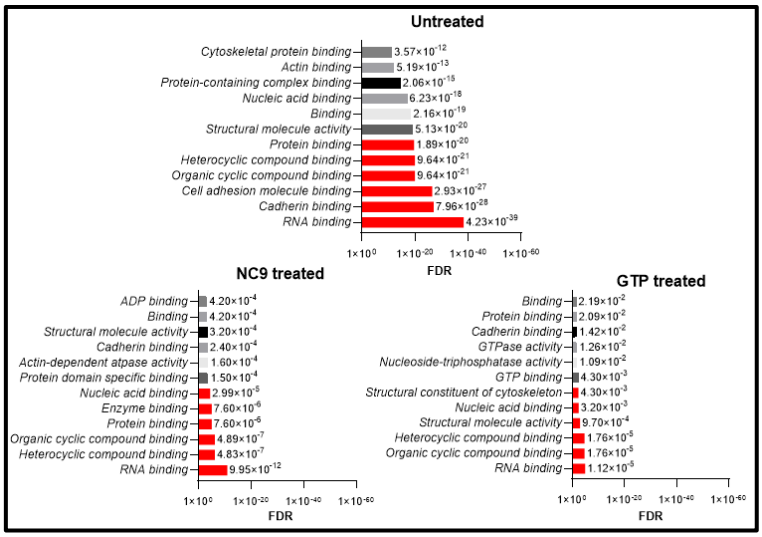


Figure 13. Most enriched GO Biological Functions (STRING) of the identified potential interaction partners of recombinant N-terminally biotinylated TG2 in HUVEC cells

The coimmunoprecipitation upon inhibitor treatment revealed 115 potential open TG2 interacting proteins in the presence of NC9 inhibitor including 13 new proteins compared to the untreated sample. In the presence of GTPγS 107 closed TG2 interacting proteins were identified with 8 new potential partners, including histones and translation-related proteins. Identification of histones as interacting partners of TG2 by both approaches supports that TG2 also could modify gene expression in HUVEC cells. Based on our data, **stabilization of one conformational state of the TG2 significantly decreases**

the number of potential interacting proteins, but here the number of hits was also large, requiring the application of further in silico analysis (Figures 12. and 13.).

Based on the STRING analysis the identified interacting proteins are involved in various biological functions. “Cell adhesion molecule binding” and “Structural molecule activity” are among the most enriched GO Biological Processes. The involvement of TG2 in cell adhesion and migration is already known and confirmed by our functional study. Our most interesting new finding was, that a still unknown biological function regarding TG2 was **the most enriched GO Biological Process in the case of each coimmunoprecipitation setting: “RNA binding”**. Several similarly enriched processes are in connection with RNA binding, like “Heterocyclic compound binding”, “Organic cyclic compound binding”, and “Nucleic acid binding” raising up a new, still unknown function of TG2. Instead of selecting almost randomly a few proteins from the potential interacting partners, in the last period of the project, we tried to clarify the role of TG2 in RNA binding (Poster presentation and abstract at the Hungarian Molecular Life Sciences Conference, 2023. Eger, *Csaholczi et al. Conformation dependency of human transglutaminase 2 interactome upon modulator treatments in endothelial cells.*).

Discovery of RNA-binding feature of TG2

In order to confirm the involvement of TG2 in RNA binding or regulation of RNA binding proteins, after UV-crosslinking (254 nm) of RNA-binding proteins with RNA, orthogonal organic phase separation was applied. Interestingly, the TG2 level showed a significant increase in the organic phase after a combined sonication, denaturation and RNase treatment compared to the previous, last washing fraction (Figure 14. left). This suggests, that **TG2 is present in the RNA binding complexes**, either it could be bound to an RNA binding protein with high affinity or itself can serve as an RNA binding protein. The presence of TG2 in the RNA-binding protein containing fraction was confirmed by LC-MS/MS analysis. To reveal whether TG2 has an RNA binding property, RNA pull-down experiments were performed using biotinylated RNA and Streptavidin magnetic bead (Pierce). Indeed, **we have found an RNA sequence which demonstrated the RNA binding property of TG2** (Figure 14. right). The click chemistry-assisted *RNA*-interactome capture technique, which could identify other TG2-bound RNA sequences, is under optimization (Csaholczi et al. RNA-binding as a newly revealed property of transglutaminase 2 based on its interactome analysis in HUVEC cells, manuscript under preparation).

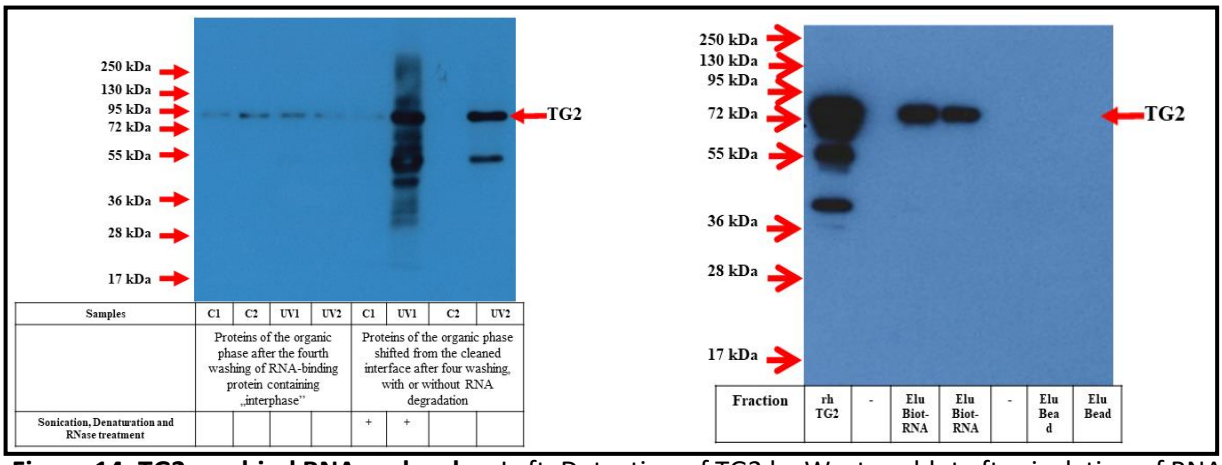


Figure 14. TG2 can bind RNA molecules. Left: Detection of TG2 by Western blot after isolation of RNA binding proteins from wild-type HUVEC using Orthogonal Organic Phase Separation. Right: RNA pull-down experiment using biotinylated RNA and recombinant TG2.

B. The NB4 model

Acute promyelocytic leukemia (APL), a type of acute myeloid leukemia (AML), is identified by the growth of clones of promyelocytic precursor cells that are less able to mature into neutrophil granulocytes. The NB4 APL cell line can be used to model the all-trans retinoic acid (ATRA)-induced differentiation therapy of APL, as the administration of ATRA substantially changes gene expression in these cells. One gene that shows the greatest change is transglutaminase 2 (TG2), a gene that is **not normally expressed in neutrophil granulocytes**. ATRA-induced differentiation therapy leads to differentiation syndrome (DS), which can be fatal in 2.5% to 30% of cases. DS is characterized by large numbers of inflammatory differentiating leukemic cells in the bloodstream, releasing chemokines and cytokines in a so-called cytokine storm, which shifts endothelial cell function from normal toward inflammatory processes. DS is also characterized by the manifestation of unexplained fever, respiratory distress, pleural and pericardial effusions, pulmonary oedema, episodic hypotension, and vascular capillary leakage, which may lead to acute renal failure. Although glucocorticoid treatment leads to recovery in most patients within 12 hours and resolution of symptoms within 24 hours, **the condition is fatal in 1% to 5% of patients. Dexamethasone treatment will not inhibit the induction of chemokines in differentiating APL cells**. TG2 expression silencing in NB4 cells (NB4 TG2 knockdown, TG2-KD) has revealed functional TG2 participation in modulation of gene expression, reactive oxygen species (ROS) generation, cytokine expression, adhesion, and migration, and phagocytic capacity of differentiated neutrophil granulocytes (*Csomós et al. 2010. Blood*). Thereafter, the experimental techniques employed in the following study involved the utilization of various cell lines, namely NB4 (wild-type), NB4 TG2-C (virus control), NB4 TG2-KD (TG2 knock-down achieved through the use of a viral antisense construct), NB4 TG2-ha (TG2 heterozygous allele), and NB4 TG2-KO (TG2 knock-out).

Transglutaminase 2 programs differentiating acute promyelocytic leukemia cells in all-trans retinoic acid treatment to inflammatory stage through NF-kB activation

Here, we evaluated the pathophysiological function of TG2 in the context of immunological function and disease outcomes as DS, using both an NB4 TG2-KO cell line and a TG2 inhibitor, NC9, that inhibits both the transamidase and guanosine triphosphate (GTP) binding activities of TG2. We found that ATRA enhanced the cell surface expression of CD11b and CD11c, while also inducing their **high-affinity states**. Atypical expression of TG2 in NB4 wild-type (WT) cells activated the NF-κB pathway, which drives pathogenic processes with an inflammatory cascade through the expression of numerous inflammatory cytokines. We showed a link between the different expression levels of TG2 protein in NB4 WT, NB4 TG2-KD, and NB4 TG2-KO cell lines and the same level of NF-κB promoter-driven luciferase reporter activity; this could not be increased by adding additional tumor necrosis factor alpha (TNF-α). Our findings using differentiated NB4 APL cells also showed that TG2 induces a **dose-dependent expression of several proteins**, including MCP-1 (CCL2), I-309 (CCL-1), IP-10 (CXCL10), MIP-3alpha (CCL20), MPIF-1 (CCL23), IL-10, ICAM-1, MCSF, IL-1ra, MDC (CCL22), PAI-1, MIP-1α (CCL3), MIP-1b (CCL4), IL-8, IL-9, CCL28, OPN (SPP1), MCP-3 (CCL7), MCSF R, TNF RII, GDF-15, Angiogenin, VEGF R1, PECAM-1, lymphotactin (XCL1), CXCL16, Cathepsin S, TNF RI, Resistin, IL-2 Rb, Eotaxin-3 (CCL26) Eotaxin-2 (CCL24), GP91PHOX, the NRF2 transcription factor (which regulates the expression of approximately 250 genes), and the transcription factor NF-κB (which regulates the expression of hundreds of genes), **suggesting that inhibition of atypically expressed TG2 is a promising treatment target for APL leukemia** (*Jambrovics et al. 2019. Haematologica)***.**

Benefits of Combined All-Trans Retinoic Acid and Arsenic Trioxide Treatment of Acute Promyelocytic Leukemia Cells and Further Enhancement by Inhibition of Atypically Expressed Transglutaminase 2

Randomized trials with patients diagnosed with APL have shown that giving a combination therapy of ATRA and arsenic trioxide (ATO) works better than giving either therapy alone. This combined treatment approach can significantly reduce mortality rates in APL treatments; however, the molecular underpinnings responsible for the efficacy of the ATRA and ATO combination therapy remain unclear. Our findings demonstrated that cells treated with ATRA or ATO exhibited comparable levels of superoxide production, but these **levels were subsequently reduced by approximately 66% by the combined treatment**. The levels of secreted inflammatory biomarkers, namely monocyte chemoattractant protein-1 (MCP-1), interleukin-1 beta (IL-1β), and TNF-α, **exhibited significant decreases, and these decreases required the reduced expression of TG2**. The quantity of TNF-α secreted into the supernatant was approximately 50 times less for NB4 TG2-KO cells than for ATRA-treated differentiated NB4 WT cells. Treatment with NC9, the irreversible inhibitor of TG2, decreased reactive oxygen species production by 28-fold while also causing 8-fold, 15-fold, and 61-fold decreases in MCP-1, IL-1β, and TNF-α, respectively, in the combined ATRA + ATO-treated wild-type NB4 cell culture. We hypothesized that **the atypical expression of TG2 induces inflammation, and therefore inhibition of it represents a promising possibility for the mitigation of differentiation syndrome** (*Jambrovics et al. 2020, Cancers (Basel)*)**.**

Cancer, Transglutaminase 2, and protein-protein interactions

Transglutaminase 2 associated with PI3K and PTEN in a membrane-bound signalosome platform blunts cell death

The American Cancer Society predicted the occurrence of about 609,360 cancer deaths and 1.9 million new cancer cases in the United States in 2022, and over 4 million new cancer cases and 1.9 million cancer-related deaths were estimated in Europe for 2020. Previous research on almost 3,000 solid tumors reported that TG2 expression was linked to a worse prognosis in terms of overall survival, shorter disease-free and recurrence-free survival, and an almost doubled hazard ratio. These findings confirmed that atypical TG2 expression is a poor prognostic sign for people with many types of cancer. The pathology of certain leukemias, including acute myeloid leukemia (AML), may involve the modulation or enhanced activation of cellular signaling pathways, such as the phosphatidylinositol-3-kinase (PI3K)-AKT and mammalian/mechanistic target of rapamycin (mTOR) pathways that regulate the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor. The most frequent mutations connected with human cancer are activating mutations in the proto-oncogene PI3K and inactivating mutations in PTEN. Using APL as a model, we addressed the “advantages” possessed by cancer cells expressing TG2 and examined whether atypical expression of TG2 alters the signal transduction pathways in an NB4 APL cell line.

We showed that the survival of APL leukemic cells depends on an active PI3K-AKT-mTORC pathway and a transcriptionally inactive FOXO3 signaling axis. An active mTORC2 complex was needed to phosphorylate phospho-AKT T308 on S473 to create the fully active AKT form that, in turn, phosphorylated FOXO3 and retained it in the transcriptionally inactive form. In the absence of TG2 expression, mTOR-S2481 and AKT S473 were dephosphorylated following the ATO treatment, leading to transcriptionally active FOXO3. However, the phosphorylation of mTOR on S2481 was TG2-dependent, while phospho-AKT S473 production depended directly on TG2 levels and inversely on the ATO concentration.

TG2 is known to modulate gene expression, and our findings showed that it can reciprocally regulate PI3K and PTEN. However, the differences in enzyme activities in the presence or absence of TG2 expression did not explain the nearly 180,000-fold difference in phosphatidylinositol-3, 4, 5-triphosphate (PIP3) levels, suggesting that TG2 also enhances PIP3 production through some other mechanism that does not involve gene expression differences. PIP3 was present in minute amounts in the membranes of apoptotic cells, suggesting that the PI3K-AKT survival signaling pathway was inactivated in dying cells, whereas live Annexin-V-negative cells contained high PIP3 levels. Our examination of TG2 in Annexin-V negative live cells and Annexin-V positive apoptotic cells showed that the amount of TG2 was below the detection limit in Annexin-V positive apoptotic cells, but was measurable in Annexin-V negative cells. The synthesis of PIP3 was localized to the cell membrane and appeared to depend on TG2, prompting the question of how TG2, which is commonly considered a cytoplasmic protein, might form an association with the plasma membrane.

Mass spectrometry analysis of the plasma membrane fraction of NB4 cells identified several hundred proteins. Of these, **the CD18 integrin protein, which is a component of the activated integrin receptors CD11b and CD11c,** was pulled down in a complex that was immunoprecipitated with a TG2 antibody. TG2 also co-immunoprecipitated with CD18; therefore, we concluded that **CD18 forms plasma membrane-associated protein complexes with TG2** in differentiated NB4 cells. TG2 was detected in a membrane-associated form, leading us to suspect that TG2 might cooperate with other proteins, such as c-SRC, PI3K/110, PTEN, and mTOR, which lie downstream in the PI3K-AKT-mTOR signal transduction cascade and participate in protein-protein interactions. Co-immunoprecipitation experiments with the polyclonal TG2 antibody detected interactions between TG2, CD18, c-SRC, phosphorylated mTOR (S2481, S2448) from mTORC1 and mTORC2, phospho-AKT (S473, T308), PI3K/p110, and PI3K phospho-p85. Concomitantly, antibodies to mTOR, AKT, CD18, p85, PI3K/p110, PTEN, and c-SRC also co-immunoprecipitated with TG2 in the reciprocal co-immunoprecipitation experiments. Treatment of NB4 cells for 5 days with either the **NC9 TG2 inhibitor or the PP2 c-SRC inhibitor abolished all interactions between TG2 and its interacting proteins (Figure 15).** The reciprocal co-immunoprecipitation revealed the interacting binding partners of cell membrane-localized TG2; therefore, we applied a modified far-western blot analysis using disuccinimidyl suberate (DSS) to stabilize the protein-protein interaction in situ on the membrane. Bait proteins (TG2, mTOR, AKT, p85, p110, CD18, PTEN, and SRC) were co-immunoprecipitated from the plasma membrane, renatured, and then incubated with plasma membrane lysates. The bound prey proteins were detected with a primary antibody to identify the position of the bait proteins.

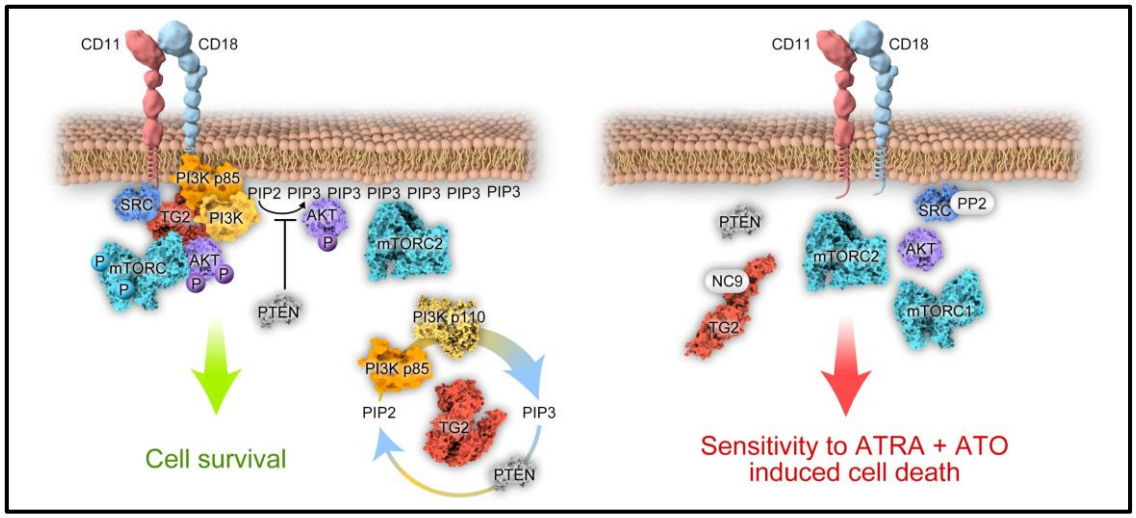


Figure 15. TG2-dependent signalosome formation and its inhibition by NC9 or PP2.

We revealed that plasma membrane-anchored TG2 forms a signaling platform (Figure 15), establishing a signalosome with CD18, c-SRC, PI3K/p110, PI3K, p85, phospho-AKT T308, S473, phospho-mTOR S2481, and S2448 proteins, leading to PI3K hyperactivity and PTEN inactivation. We demonstrated that NC9 (a TG2 inhibitor) and PP2 (a c-SRC inhibitor) break down platforms into their components. We propose that TG2 acts as a hub for signal transduction activation in APL, with gain-of-function PI3K and loss-of-function/tumor suppressor PTEN consequences.

We also used the same experimental approach and found that, after two days of differentiation, only the rudimentary requirements for the creation of the signalosome were present in NB4 WT cells, suggesting **a time dependence for the assembly of the signalosome.** Finally, the far-western blot analysis with CD18-/- cells showed that TG2 bound to all co-immunoprecipitated proteins except CD18 and SRC and that the CD18 antibody could not detect the TG2 and SRC bait proteins. **These experiments demonstrated that TG2 could support cell survival *in vitro* by this previously unknown capability of TG2 to form signalosome platforms by interacting with CD18** (*Jambrovics et al., 2023, Cell Death Dis)***.**

TG2 Interacting Protein

To investigate the potential interacting proteins, we performed LC-MS/MS analyses from the collected plasma membrane fractions, followed by the co-Immunoprecipitation method with a polyclonal TG2 antibody. From the analyzed samples, we could identify 392 proteins, out of which the main interacting proteins were mentioned before, however, several other target or potential interacting partners were also detectable. These proteins were clustered by their localization inside the cell, the chemical reaction they catalyzed, or the pathway they were involved in (unpublished data).

From the identified proteins, we could cluster **mitochondrial enzymes**: [ATP synthase subunit beta; ATP synthase subunit d; ATP synthase subunit gamma; ATP synthase subunit O; ATP-binding cassette sub-family B member 10]. Calcium-binding mitochondrial carrier protein, Citrate synthase, Cytochrome b-c1 complex subunit 1, Cytochrome c1, heme protein, Dihydrolipoyllysine-residue succinyl transferase component of the 2-oxoglutarate dehydrogenase complex, Leucine-rich PPR motif-containing protein, Mitochondrial import receptor subunit TOM70, Mitochondrial proton/calcium exchanger protein, NAD(P) transhydrogenase, mitochondrial, Pyruvate dehydrogenase E1 component subunit beta, Serine hydroxymethyltransferase, Stomatin-like protein 2, Succinate dehydrogenase [ubiquinone] flavoprotein subunit], **important regulatory enzymes** involved in cell proliferation and cell survival pathways [DnaJ homolog subfamily C member 11, Isoform 4 of DNA-directed RNA polymerases I, II, and III subunit RPABC3, TAR DNA-binding protein 43, Isoform 4 of tyrosine-protein phosphatase non-receptor type 6, Phosphatidylinositol-3,4,5-Trisphosphate 3-Phosphatase and Dual-Specificity Protein Phosphatase PTEN, Serine/threonine-protein phosphatase 2A, 65 kDa regulatory subunit A alpha, Serine/threonine-protein phosphatase PP1-alpha catalytic subunit, Ras-related protein Rab-5C, Ras GTPase-activating-like protein IQGAP2, Cyclin-dependent kinase 1] and **voltage-dependent channel proteins** [Voltage-dependent anion-selective channel protein 1, Voltage-dependent anion-selective channel protein 2 (Figure 16).

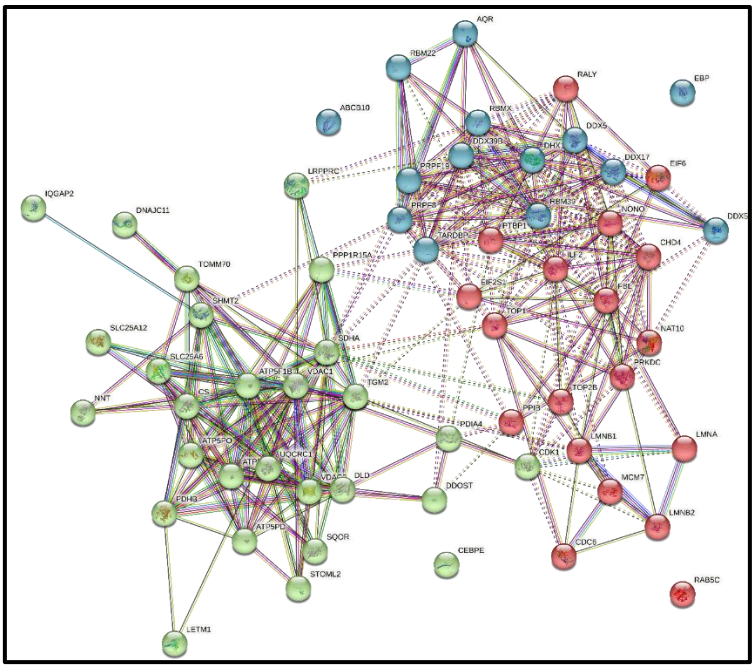


Figure 16. Multiprotein interaction STRING analyses of identified potential interaction partners of TG2 in the plasma membrane. STRING analyses were carried out using LC-MS/MS protein data, selectively focusing on a "multiprotein interaction cluster" profile involving two clusters based on the function of the proteins.

As the second target of the study, we examined the nuclear TG2 interaction potential as well. First, we isolated a pure nuclear fraction from 5-day ATRA-differentiated NB4 WT cells, followed by the Co-IP method with a polyclonal TG2 antibody. The eluted fractions were analyzed by the LC-MS/MS method, from which approximately 324 proteins were identified as potential interaction partners of the nuclear TG2. Clustering the results, we found several **histones** [Histone H4, Histone H2A, Histone H2A type 1-C, Histone H2B type 1-M, Histone H2B type 1-J, Histone H2AX, Histone H3.1, Histone H1.5, Histone H2A.Z, Histone H1.2, Histone H1.3, Histone H1.4, Core histone macro-H2A.1, Histone-binding protein RBBP4], **RNA and DNA binding factors [**Spliceosome RNA helicase DDX39B] ATP-dependent RNA helicase A, nucleolar RNA helicase 2, ATP-dependent RNA helicase, Probable ATP-dependent RNA helicase DDX5, Probable ATP-dependent RNA helicase DDX17, Isoform 2 of glycine-tRNA ligase, ATP-dependent RNA helicase DHX15, Phenylalanine-tRNA ligase beta subunit, rRNA 2'-O-methyltransferase fibrillarin, RNA-binding motif protein, X chromosome, Phenylalanine-tRNA ligase alpha subunit, Pre-mRNA-processing-splicing factor 8, RNA cytidine acetyltransferase, Pre-mRNA-processing factor 19, RNA-binding protein 39, Isoform 4 of DNA-directed RNA polymerases I, II, and III subunit RPABC3, RNA-binding protein Raly, DNA-directed RNA polymerases I and III subunit RPAC1, ATP-dependent RNA helicase DDX54**] Heat shock proteins [**Heat shock protein HSP 90-beta, ,Heat shock protein HSP 90-alpha, Heat shock cognate 71 kDa protein, Heat shock 70 kDa protein 1A, Heat shock 70 kDa protein 4**] and nuclear enzymes**. [Spliceosome RNA helicase DDX39B, ATP-dependent RNA helicase A, Nucleolar RNA helicase 2, ATP-dependent RNA helicase, Probable ATP-dependent RNA helicase DDX5, Probable ATP-dependent RNA helicase DDX17, Isoform 2 of glycine-tRNA ligase, ATP-dependent RNA helicase DHX15, Phenylalanine-tRNA ligase beta subunit, RRNA 2'-O-methyltransferase fibrillarin, RNA-binding motif protein, X chromosome, Phenylalanine-tRNA ligase alpha subunit, Pre-mRNA-processing-splicing factor 8, RNA cytidine acetyltransferase, Pre-mRNA-processing factor 19, RNA-binding protein 39, Isoform 4 of DNA-directed RNA polymerases I, II, and III subunit RPABC3, RNA-

binding protein Raly, DNA-directed RNA polymerases I and III subunit RPAC1, ATP-dependent RNA helicase DDX54] (Figure 17., unpublished data).

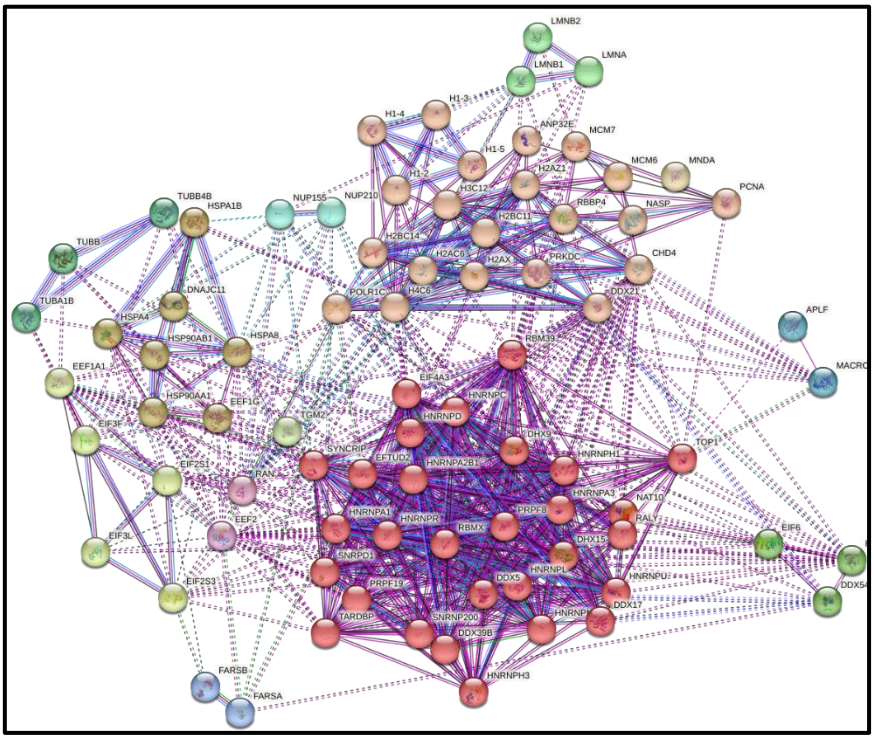


Figure 17. Multiprotein interaction STRING analyses of identified potential interaction partners of TG2 in the nucleus. STRING analyses were carried out using LC-MS/MS protein data, selectively focusing on the "multiprotein interaction cluster" profile.

Arsenic Trioxide (ATO) initiates TG2 breakdown.

ATO Increases ROS Production and Apoptosis of Cells by Enhancing Calpain-Mediated Degradation of the Cancer Survival Protein TG2

ATO demonstrates cytotoxic properties through various mechanisms, including the initiation of oxidative stress, genotoxicity, disruption of signal transduction pathways, and potential epigenetic alterations. Our findings demonstrated that the administration of ATO increased the generation of reactive oxygen species (ROS) as well as apoptosis rates in NB4 leukemia cells that had undergone differentiation with ATRA. We also found further amplification of these cellular responses in the absence of TG2. The combined administration of ATRA and ATO elevated the levels of the nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that serves as an adaptive regulator of the cellular response to oxidative stress. **The combined ATRA and ATO treatment also promoted an increase in calpain proteolytic activity, which resulted in the degradation of TG2.** Consequently, the survival of NB4 WT leukemia cells was diminished. The expression of the TG2 protein was also induced and subsequently degraded in the MCF-7 epithelial cell line and primary peripheral blood mononuclear cells following treatment with ATO. This degradation process rendered these cell types more susceptible to apoptotic signals (*Jambrovics et al., 2023, Int J Mol Sci)*.

Vitamin D3 can be a crucial component in the therapeutic approach for acute promyelocytic leukemia (APL)

Administration of 25-dihydroxyvitamin D3 could be a remedy for DS

In our recent study, we investigated the potential protective effects of 25-dihydroxyvitamin D3 (25-(OH)D3) in ATRA-differentiated NB4 cells, with the objective of elucidating the underlying mechanism involved in this process. The administration of 25-(OH)D3 did not result in any significant alterations in cell viability; however, the transcriptional activity of the NF-kB pathway was attenuated in the ATRA-differentiated NB4 cells, leading to a significant decrease in the levels of mRNA (IL-1β, TNF-α, MCP-1) and secreted proteins associated with inflammatory cytokines and chemokines (IL-1β, TNF-α, MCP-1). Our investigation revealed a hitherto undiscovered and noteworthy role of 25-(OH)D3 in attenuating the functioning of the NF-κB pathway and thereby breaking the emergence of sporadic unconventional reactions, such as cytokine storms, in standard ATRA therapy for APL. This finding may represent a potential avenue for impeding the advancement of DS through the administration of 25-(OH)D3 and perhaps a new drug analogue of NC9 (*Jambrovics et al., unpublished manuscript, 2023)*.