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BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF ZEBRAFISH TRANSGLUTAMINASE 2 PROTEINS

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ABSTRACT

Transglutaminase 2 (TG2) is the most ubiquitous member of a family of proteins with the shared feature of catalysing the Ca^{2+}-dependent protein crosslinking. Its gene encodes for 77 kDa protein comprising 4 domains: a N terminal β-sandwich, responsible for the cell adhesion functions of TG2, a catalytic core, containing the Cys-His-Asp catalytic triad responsible for the crosslinking activity, and two C terminal β-barrels, important for the regulation of TG2 activity and for its involvement in cell signaling. Indeed, the enzymatic functions of TG2 are regulated by a conformational switch that brings the protein from an extended and active form, favored by high Ca^{2+} levels, to a closed inactive one, mainly driven by GTP/GDP, where the β-barrels are folded over the catalytic core, shielding the active site, and inhibiting the transamidase activity. Upon binding to GTP, TG2 exerts GTPase activity and mediates GPCRs signaling acting as an atypical G protein. TG2 has a widespread distribution in tissues, and it localizes both in the ECM with cell adhesion functions, and inside the cells, at the inner surface of the plasma membrane as a signal transducer, but also in the cytosol as well as in several organelles, i.e., the nucleus, where TG2 can regulate gene expression. Given its multiple functions and expression in several cell types, TG2 is involved in many physiological and pathological processes, such as bone development, wound healing, angiogenesis, but also fibrosis, celiac disease, and cancer. However, its actual role in biological events is often controversial, as TG2 has often been shown to have opposing effects in several processes, depending on its localization, cell type, or experimental condition. With the aim of laying the foundations of the use of the zebrafish (Danio rerio) as a valid tool to better characterize TG2 functions *in vivo*, we characterized the enzymatic and functional properties of the zebrafish TG2 proteins. Zebrafish is a well-established animal model for studying vertebrate biology that offers several advantages, such as high genetic conservation with humans, and the ability to spawn weekly hundreds of externally fertilized and optically clear embryos, characterized by rapid development and ease of manipulation. We confirmed the existence of 3 TG2 orthologues (zTGs2) in the zebrafish genome and their actual expression throughout the embryonic development. Then, we produced and purified the zTGs2 as recombinant protein and demonstrated that, like the human enzyme, zTGs2 catalyze a Ca^{2+}-dependent transamidation activity, which can be inhibited with the use of hTG2-specific inhibitors. We also showed in a cell model of human fibroblasts that zTGs2 in the extracellular environment are able to mediate RGD-independent cell adhesion, like hTG2. Ultimately, we transfected and selected zTGs2-overexperessing HEK293 cells, and demonstrated that in this cell line intracellular zTGs2 have a protective role in the apoptotic process, as already shown for hTG2. Overall, our findings suggest that zTGs2 behave in a very similar way as the human orthologue and pave the way for future *in vivo* studies of TG2 functions in the zebrafish.

RIASSUNTO DELLA TESI

La transglutaminasi 2 (TG2) è il membro più ubiquitario di una famiglia di proteine con la caratteristica comune di catalizzare il *crosslinking* Ca^{2+}-dipendente di substrati proteici. Il suo gene codifica per una proteina di 77 kDa formata da 4 domini: un *β-sandwich* N-terminale, responsabile delle funzioni di adesione cellulare della TG2, un *core* catalitico, contenente la triade catalitica Cys-His-Asp responsabile dell'attività di *crosslinking*, e due *β-barrels* C-terminali, importanti per la regolazione dell'attività della TG2 e per il suo coinvolgimento nella segnalazione intracellulare. Infatti, l'attività enzimatica della TG2 è regolata da un cambio conformazionale che porta la proteina da una forma estesa ed attiva, favorita da alti livelli di Ca^{2+}, a una forma chiusa ed inattiva, promossa principalmente dalla presenza di GTP/GDP, in cui i due domini C-terminali sono ripiegati sul nucleo catalitico, schermando il sito attivo e inibendo l'attività di transamidazione. Quando si lega al GTP, la TG2 esercita anche attività di GTPasi e media la segnalazione dei *GPCRs*, agendo come una proteina G non canonica. La TG2 ha una distribuzione diffusa nei tessuti e si localizza sia nella ECM, dove ne regola la composizione e media adesione cellulare, sia all'interno delle cellule, dove può posizionarsi sulla superficie interna della membrana plasmatica e mediare la trasduzione di segnali, ma anche nel citosol e in diversi organelli, come ad esempio nel nucleo, dove la TG2 può regolare l'espressione genica. Date le sue molteplici funzioni e la sua espressione in diversi tipi di cellule, la TG2 è coinvolta in molti processi fisiologici e patologici, come lo sviluppo osseo, la guarigione delle ferite e l'angiogenesi, ma anche la fibrosi, la celiachia e il cancro. Tuttavia, il preciso ruolo svolto dalla TG2 in questi eventi biologici è spesso controverso. Infatti, è stato dimostrato come la TG2 possa avere effetti opposti in diversi processi, a seconda della sua localizzazione, del tipo di cellula o della condizione sperimentale. Con l'obiettivo di gettare le basi per l'utilizzo dello zebrafish (Danio rerio) come valido strumento per caratterizzare meglio le funzioni di TG2 *in vivo*, abbiamo studiato le proprietà enzimatiche e funzionali delle tre TG2 dello zebrafish. Lo zebrafish è un modello animale consolidato per lo studio della biologia dei vertebrati che offre diversi vantaggi, come l'elevata conservazione genetica con l'uomo e la capacità di generare settimanalmente centinaia di embrioni fertilizzati esternamente e trasparenti, caratterizzati da un rapido sviluppo e facilità di manipolazione. Abbiamo confermato l'esistenza di 3 ortologhi della TG2 umana nel genoma di zebrafish (zTGs2) e la loro effettiva espressione durante lo sviluppo embrionale. Abbiamo poi prodotto e purificato le zTGs2 come proteine ricombinanti e abbiamo dimostrato che le zTGs2 possiedono un'attività Ca^{2+}-dipendente analoga all'attività di transamidazione della TG2 umana, che può essere inibita con l'uso di inibitori TG2-specifici. Abbiamo anche dimostrato in un modello cellulare di fibroblasti che le zTGs2 nell'ambiente extracellulare sono in grado di mediare adesione cellulare RGD-indipendente, come l'enzima umano. Infine, dopo aver trasfettato e selezionato cellule HEK293 per la sovraespressione delle zTGs2, abbiamo trattato queste cellule con H_2O_2 e dimostrato che in questa linea cellulare le zTGs2 hanno un effetto anti-apoptotico, come già dimostrato per l'enzima umano. Nel complesso, i nostri risultati suggeriscono che le zTGs2 si comportano in modo molto simile all'ortologo umano, e aprono la strada a futuri studi *in vivo* sulle funzioni delle TG2 nello zebrafish.

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1. INTRODUCTION

1.1 Introduction on transglutaminases

The pioneering work of Clarke and colleagues in the late 1950s identified and described for the first time an enzymatic activity responsible for catalysing amine incorporation into protein substrates [1]. They hypothesized and demonstrated that this reaction is carried out by a protein present in the soluble fraction of guinea pig liver extract and that Ca^{2+} is required for the reaction to occur. Waelsch and his co-workers named the enzyme responsible for catalysing this reaction transglutaminase Since then, several other proteins with similar enzymatic properties have been discovered, not only in mammals but also in other vertebrates as well as in invertebrates, plants, and microorganisms. The common feature of all these proteins is the ability to catalyse a reaction that not only ends with the incorporation of primary amines into glutamine residues of specific protein substrates, as first described for transglutaminase from guinea pig liver, but also forms an isopeptide bond between the lysine of one protein substrate and the glutamine of a second protein substrate [2]. This ε-(γ-glutamyl)-lysine isopeptide bond is thus responsible for the cross-linking of proteins, a reaction that can lead to significant biological effects in tissues, as will be discussed later. As shown in Figure 1, the reaction can also end with the deamination of the target glutamine, leading to the formation of glutamic acid and free ammonia. This outcome is favoured by slightly acidic conditions and when suitable primary amines are either absent or present in less than saturating amounts. Under these conditions, water plays the role of acyl acceptor normally performed by a primary amine or a lysine side chain of a protein substrate, and a glutamic acid is formed in the protein substrate. As we will discuss in more detail later, this reaction is one of the main causes of the symptoms associated with celiac disease.

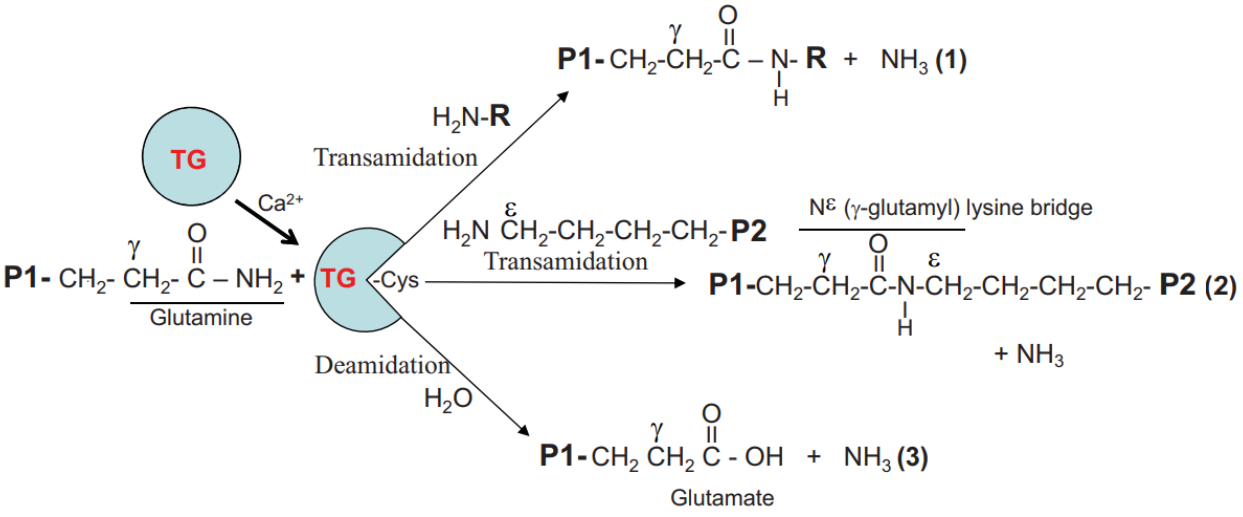


Figure 1. Illustration of the enzymatic reactions catalysed by transglutaminases. The reaction always starts with the formation of an intermediate between the enzyme and the protein substrate, with three different outcomes. If the molecule performing the nucleophilic attack is a primary amine, the reaction leads to the incorporation of the primary amine into the protein substrate (1). Otherwise, if the nucleophile is the side chain of a lysine residue of a second protein substrate, the reaction ends with the crosslinking of the two protein substrates (2). Ultimately, under slightly acidic conditions, a water molecule can act as the nucleophile, resulting in deamination of the protein substrate (3). Readapted from Eckert R. L., et al (2014) [3].

Although the main findings on transglutaminases (TGs) initially focused on their ability to catalyse crosslinks between different protein substrates and their involvement in the blood coagulation process, it was also soon pointed out that all these observations do not prove that cross-linking activity is the actual biological function of any transglutaminase.

1.2 The gene family of human transglutaminases

In mammals, nine members of the transglutaminase gene family have been identified, clustered on five different chromosomes and evolved in vertebrates by successive duplications: *TG1-7*, *F13* encoding coagulation Factor XIII, and *EBP42* encoding the inactive protein 4.2 (Figure 2) [4]. The transglutaminase proteins exhibit a high degree of structural homology, as their coding genes are the product of genomic duplication and rearrangement.

Transglutaminase 1 (TG1) is encoded in humans by the *TGM1* gene and is found in keratinocytes in the stratified squamous epithelia of the skin, upper digestive tract, and lower female genital tract. For this reason, TG1 is also referred to as keratinocyte transglutaminase [3]. TG1 is commonly described as a membrane-bound protein of approximately 90 kDa. In fact, TG1 exists in several different forms that are the result of proteolytic cleavages. A distinctive feature of TG1 is that the 92 kDa protein is the largest of the transglutaminase family. While it retains a high degree of sequence and structural homology in the central catalytic core of the enzyme, it has additional flanking stretches of residues at both the N- and C- termini. These additional residues are significant because they comprise a cluster of cysteines at the N- terminus that are responsible for the fatty acyl linkage to the plasma membrane [5]. Originally, TG1 was thought to be present in keratinocytes only in a membrane-bound form. However, it was later demonstrated that as a result of post-translational proteolysis, soluble forms of 10, 33 and 67 kDa are also present in the cytoplasm, which are differentially active and likely have different substrate specificity. In addition, a species with a higher molecular weight of 106 kDa was also identified [6]. TG1 crosslinking activity is critical for the formation of the cornified cell envelope (CE) of terminally differentiating epidermis, a 15 nm thick layer of crosslinked proteins on the inner

surface of keratinocytes that is crucial for the barrier function of the skin. Mutations of the *TGM1* gene lead to a skin disorder called autosomal recessive lamellar ichthyosis, a disease with several variants whose common phenotype is altered keratinization of the epidermis [7][8].

Another TG with crucial functions in skin and in terminal differentiation of keratinocytes is transglutaminase 3 (TG3), which was first discovered in hair fibers and hair follicle, but was later found in epidermis and brain, as well as in other tissues and organs such as skeletal muscle, testis, spleen, stomach, and small intestine [9]. Despite this wide distribution, the biological functions of TG3 have only been described in detail for the skin. Similar to TG1, the *TGM3* gene encodes an inactive precursor polypeptide that undergoes proteolysis, releasing two fragments of 30 and 47 kDa that remain associated to form the active 77 kDa protein [10][11]. Defects in TG3 activity have been associated with a condition termed "uncombable hair syndrome," a rare pathology characterized by visible changes in hair structure [12]. Current evidence suggests that TG3 does not play a vital role in global physiology and pathology, as both *Tgm3-/-* mice and humans with defects in TG3 function exhibit phenotypic changes mainly related to hair structure. However, TG3 is associated with gluten-sensitive autoimmune diseases, a property shared with two other members of the transglutaminase family, as will be discussed later. In particular, the production of autoantibodies to TG3 causes a condition called dermatitis herpetiformis (DH), commonly referred to as the skin manifestation of celiac disease. People with DH suffer from severe itching and blistering, symmetrical rash and may rarely experience gastrointestinal symptoms.

A much more tissue-specific expression is exhibited by the fourth member of the TG family, transglutaminase 4 (TG4), also known as prostate TG, as it is predominantly expressed in the prostate [13][14]. The exact functions of this protein in humans are unknown, but studies in rodents have shown that TG4 is necessary for physiological fertility [15]. In addition, TG4 has also been studied as a potential biomarker for prostate cancer aggressiveness [16].

Transglutaminase 5 (TG5) is another member of the TG family with a size of approximately 80 kDa that, like TG1 and TG3, is expressed in the skin, particularly in the upper layers of the epidermis, and in the hair follicle [17]. In addition, as previously discussed for other TGs, transglutaminase 5 undergoes proteolytic cleavage leading to the formation of much more active 53-kDa and 28-kDa fragments [18]. TG5 is one of the least described and understood members of the family, but mutations of the encoding gene have been associated with a disorder called acral peeling skin syndrome (APSS), characterized by painless superficial blistering, and peeling of the hands and feet [19].

Little is known about transglutaminase 6 and 7. Expression of the two proteins appears to be restricted to the testis, lung, and brain. Of particular importance is the localization of TG6 in the nervous system, as this protein has been associated with several neurodegenerative diseases, although these observations are still controversial [20]. However, the exact biological functions of TG6 and TG7 and their involvement in pathological processes are still unknown [21].

Factor XIIIa (FXIIIa), also called plasma TG, is an important player in the coagulation cascade because the crosslinking of fibrin catalysed by this protein stabilizes fibrin clots by making them stiffer and more compact and thus more resistant to fibrinolysis. As expected, FXIIIa is expressed in platelets and plasma, but also in many other cell types and tissues such as macrophages, astrocytes, chondrocytes, etc. The human *F13A1* gene encodes the FXIII zymogen, which must be proteolytically cleaved to release the active form of the enzyme (FXIIIa), a feature we now know to be quite common in the TG family. However, a distinctive feature of the inactive form of FXIII is that the zymogen is a heterotetramer consisting of two A and two B subunits. Both subunits weigh about 80 kDa, but only the A subunit contains the active site, while the B subunit serves as the carrier polypeptide [22]. Since the formation of fibrin clots is essential for hemostasis, FXIII deficiency results in a rare disorder characterized by bleeding manifestations and sometimes life-threatening central nervous system (CNS) hemorrhage. Although in the past FXIIIa was mainly associated with the study of the coagulation cascade, subsequent research has shown that this protein is indeed involved in several other areas and biological processes, such as healing, bone metabolism, pregnancy, immunity, adipogenesis, and blood and solid tumours [23].

Band 4.2 is the only member of the transglutaminase family that lacks the transamidation activity that defines this protein family. This peculiarity is due to an amino acid substitution in the active site of the protein. Although band 4.2 is mainly known as a membrane protein of erythrocytes, it is also found in platelets, lymphocytes, spinal cord tissue, and kidneys [24]. In red blood cells, band 4.2 plays a crucial role in the membrane cytoskeleton as a linker protein that maintains the stability and flexibility of red blood cells [25].

Gene	Protein	Chromosomal Location	Molecular Mass, kDa	Main Function	Tissue Distribution	Alternate Names
TGM1	TG1	14q11.2	90	Cell envelope formation during keratinocyte differentiation	Membrane-bound keratinocytes	TG_k, keratinocyte TG, particulate TG
TGM2	TG2	20q11-12	80	Apoptosis, cell adhesion, matrix stabilization, signal transduction	Many tissues: cytosolic, nuclear, membrane, and extracellular	Tissue TG, TG_c, liver TG, endothelial TG, erythrocyte TG, $Gh\alpha$
TGM3	TG3	20q11-12	77	Cell envelope formation during keratinocyte differentiation	Hair follicle, epidermis, brain	TG_E, callus TG, hair follicle TG, bovine snout TG
TGM4	TG4	3q21-22	77	Reproduction, especially in rodents as a result of semen coagulation	Prostate	TG_p, androgen-regulated major secretory protein, vesiculase, dorsal prostate protein 1
TGM5	TG5	15q15.2	81	Cell envelope formation in keratinocytes	Foreskin keratinocytes, epithelial barrier lining, skeletal muscular striatum	TG_x
TGM6	TG6	20q11	78	Not known	Testis and lung	TG_y
TGM7	TG7	15q15.2	81	Not known	Ubiquitous but predominately in testis and lung	TG_z
F13A1	FXIIIa	6q24-25	83	Blood clotting, wound healing, bone synthesis	Platelets, placenta, synovial fluid, chondrocytes, astrocytes, macrophages, osteoclasts and osteoblasts	Fibrin-stabilizing factor, fibrinoligase, plasma TG, Laki-Lorand factor
EPB42	Band4.2	15q15.2	72	Membrane integrity, cell attachment, signal transduction	Erythrocyte membranes, cone marrow, spleen	B4.2, ATP-binding erythrocyte membrane protein band 4.2

Figure 2. The human transglutaminase gene family. The table reports the nine genes of the transglutaminase gene family in humans, with the chromosomal location and the basic characteristics of the encoded proteins, like the molecular weight, the main functions described so far and the tissue distribution. Readapted from Eckert R. L., et al. (2014).

1.3 Transglutaminase 2

1.3.1 Introduction

Human transglutaminase 2 (hTG2) is a protein of 687 amino acids and a molecular weight of approximately 77 kDa, which in humans is encoded by the *TGM2* gene. This protein is widely expressed in various tissues in the human body and is also known as tissue transglutaminase (tTG), with high levels of expression in endothelial cells and smooth muscle cells [26]. As it will be discussed later, hTG2 exerts multiple enzymatic and non-enzymatic functions and is found in both the intracellular and extracellular environments. Given its presence in multiple cell types and subcellular compartments, and its controversial association with often conflicting mechanisms such as cell survival and cell death, TG2 is the best studied, but arguably the most complex, member of the transglutaminase family [27].

1.3.2 Transglutaminase 2 gene: *TGM2*

TGM2 is a large gene of 40 kb that comprises 13 exons and 12 introns and generates multiple isoforms by alternative splicing (Figure 3). Variant 1, which encodes canonical TG2 and is also referred to as isoform 1 or a, is a primary RNA transcript of 5144 bp that is eventually converted into a mature 2 kb mRNA. Eight other splice variants are known, all shorter than canonical TG2, whose different

tissue-specific expressions and biological functions are not well described, although some of them have been associated with neurodegenerative diseases and neoplasms [28].

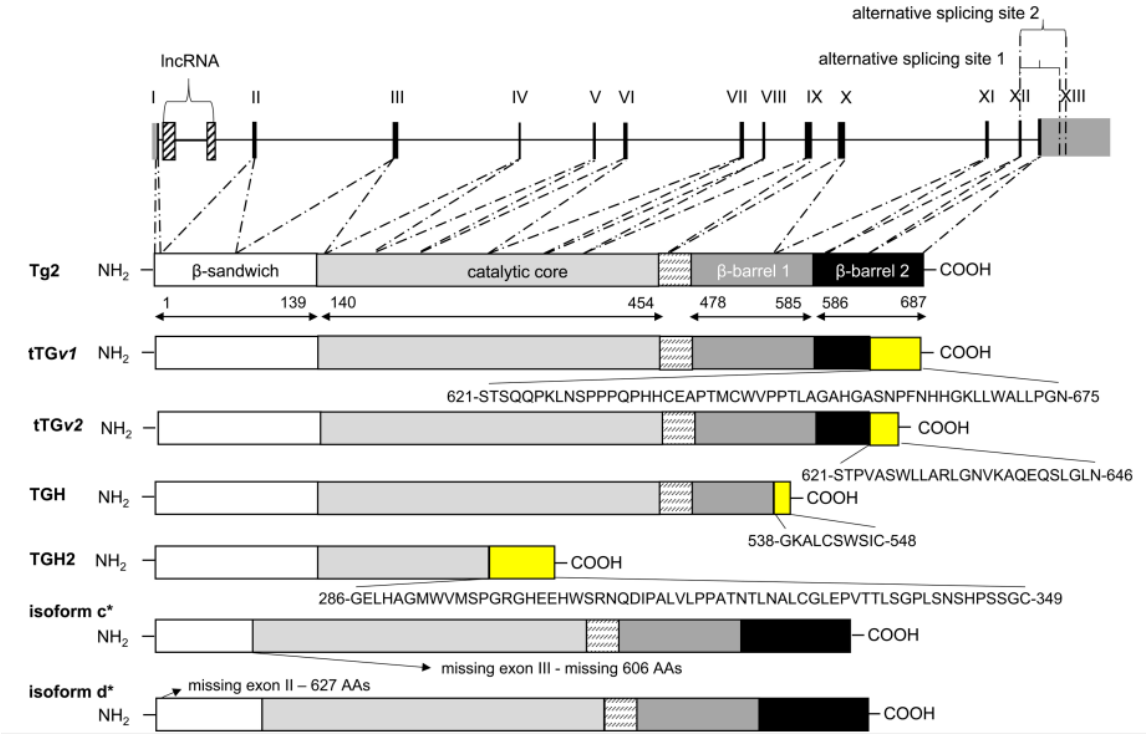


Figure 3. Structure of the *TGM2* gene. The 13 exons of the *TGM2* gene are indicated with roman numbers and the correspondence between exon structure and protein domains is highlighted by the dotted lines. Canonical TG2 and the other known variants are represented below the gene structure. The N terminal β-sandwich domain is depicted in white, the catalytic core domain in a light shade of grey and the two C terminal β-barrels domain are painted in a darker shade of grey and in black. The figure also reports the position of the 2 known alternative splicing sites, which produce the TG2 variants 1 and 2 (tTGv1 and tTGv2), and the altered isoforms TGH and TGH2, with the modified tails highlighted in yellow. Readapted from Bianchi et al. (2018).

1.3.3 *TGM2* gene expression and regulation

The *TGM2* promoter contains response elements for several crucial molecules, particularly for important agents involved in tissue injury or cell stress response [29]. A well- known inducer of TG2 expression is retinoic acid (RA), as the *TGM2* promoter contains two retinoic acid response elements, and indeed RA is frequently used as a TG2 inducer in various assays (Figure 4). During inflammation, the pro-inflammatory transcription factor NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) induces the expression of TG2, which in turn can activate NF-kB by cross-linking and inactivating its inhibitor IkBα, creating a positive loop that maintains the inflammatory response. Another inflammatory molecule involved in a positive loop with TG2 is transforming growth factor β (TGF-β), an anti-inflammatory molecule that is activated in the ECM by TG2 and in turn positively regulates *TG2M* expression. Several cell stressors have been associated with upregulation of *TGM2*,

such as hypoxia, oxidative stress, UV irradiation, and other inflammatory cytokines such as IL-6, TNF-α, and IFN-γ [30].

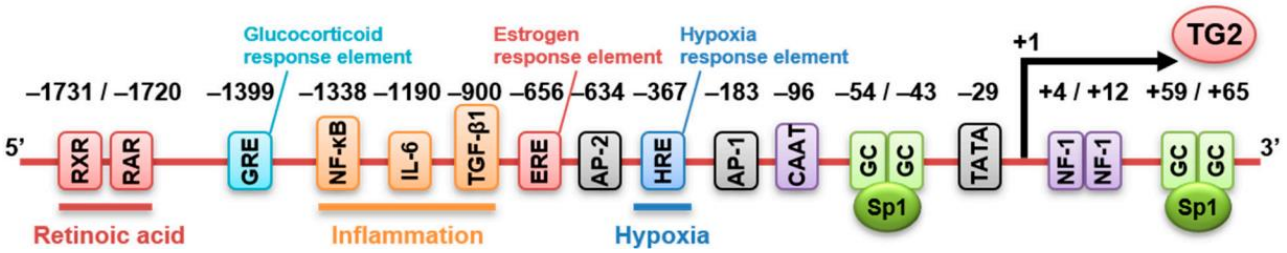


Figure 4. *TGM2* gene promoter region. Graphic illustration of the known regulatory regions of TG2 expression, with the positions of the transcription factors binding sites. Multiple factors bind to the *TGM2* promoters and promote TG2 expression, such as retinoic acid, inflammatory molecules (NF-kB, IL-6, TGF-β) and hormones. Readapted from Tatsukawa et al. (2021).

1.3.4 Transglutaminase 2 structure

The canonical variant encodes a protein consisting of 4 domains: an N-terminal β-sandwich, a central catalytic core responsible for crosslinking activity, and two C-terminal β-barrels.

The N-terminal domain extends from amino acid 1 to 139 and it is important for the cell adhesion functions of TG2 as it contains interaction sites for fibronectin and integrins. The catalytic domain, extending from residue 140 to 454, is the only one with α-helical structures and contains residues critical for enzymatic activity: the catalytic triad responsible for transamidation activity and most residues thought to be involved in calcium ion binding. Moreover, the catalytic core also contains the heparan-binding site, responsible for the interaction with syndecan-4 and cell adhesion to the extracellular matrix. The two C-terminal β-barrels comprise amino acids 479-585 and 586-687 and contain an important phospholipase C δ (PLC-δ)-binding site as well as a unique feature of TG2 as a member of the TG family: a nucleotide-binding site responsible for binding GTP/GDP (Figure 5) [31], [32].

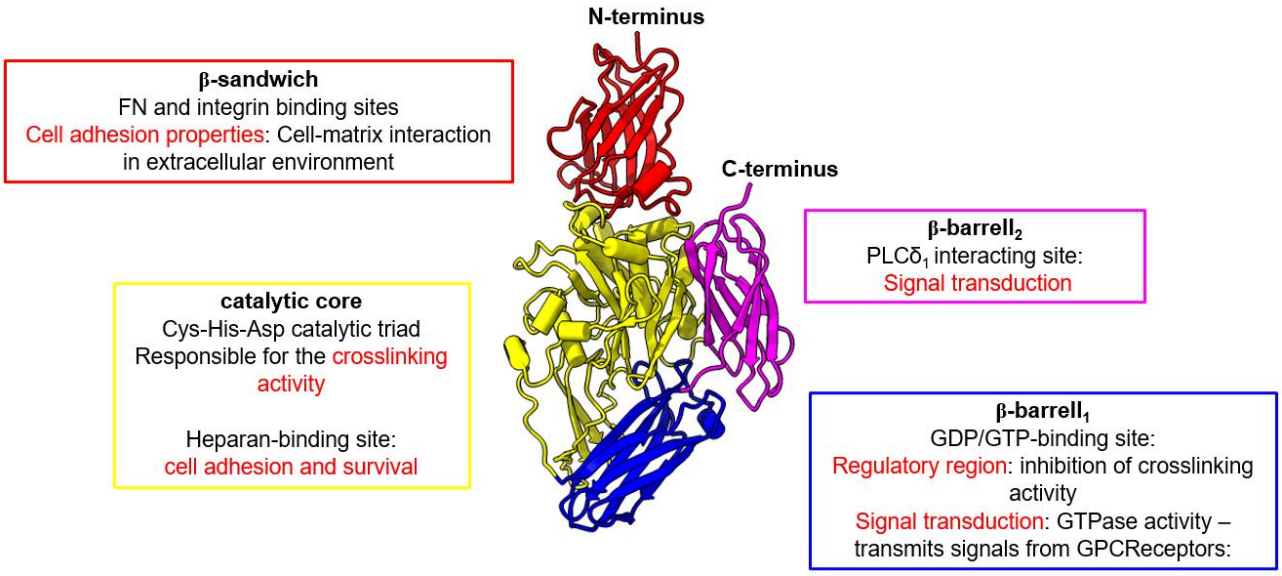


Figure 5. Structure of the transglutaminase 2 protein. The N terminal β-sandwich domain is painted in red, the catalytic core in yellow and the two C-terminal domains in blue and purple. Some essential interactions and functions are also reported for the four domains. The 3D model is rendered with ChimeraX.

1.3.5 TG2 transamidation activity

The structure of transglutaminase 2 is strictly associated to its enzymatic functions, which are not limited to the transamidation activity, as TG2 has been shown to possess also GTPase, PDI and kinase activities [33].

As previously described for the transglutaminase family of proteins, the transamidation activity of TG2 catalyses a reaction which can lead to the formation of an isopeptide bond between a lysine and a glutamine of two different protein substrates, thus resulting in their crosslinking, or to the incorporation of a small primary amine into a glutamine acceptor, as well as to the deamination of the target glutamine to form glutamic acid. Crucial for this enzymatic activity are three residues of the catalytic core, the so-called catalytic triad, namely cysteine 277, histidine 355 and aspartic acid 358, which are involved in a two-step reaction characterized by a ping-pong mechanism. First, the enzyme forms an acyl intermediate with the substrate through a chemical bond between the thiol group of Cys277 and the target glutamine in the protein substrate, releasing free ammonia. The formation of this intermediate is facilitated and stabilized by two critical tryptophan residues, Trp241 and Trp332. The outcome of the reaction depends on which molecule is responsible for the nucleophilic attack on the intermediate that releases the enzyme. If it is the ε-amino group of a lysine residue in the second protein substrate, the reaction ends with the crosslinking of the two proteins. If it is a primary amine, it is incorporated into the substrate. Finally, if a water molecule is the acceptor

nucleophile, hydrolysis occurs, and a glutamic acid is formed on the target protein. As with all catalytic triads of enzymes, the action of the nucleophile (Cys277 in the case of human TG2) is made possible by the close presence of a base and an acid (His335 and Asp358). Indeed, natural amino acids do not have strong nucleophilic properties and require an acid residue to align and polarize a basic residue, which is fundamental for the polarization and deprotonation of the nucleophile, increasing its reactivity. In addition, the basic amino acid supports the reaction by protonating the first product of the two-step reaction. In the case of transglutaminases, aspartic acid aligns and polarizes histidine, which in turn deprotonates the thiol group of the catalytic cysteine, making it a strong nucleophile, and protonates the -NH_2+ group, producing free ammonia (Figure 6). In addition, His335 and Asp358 are also essential for the second step of the reaction, as the histidine polarized by the aspartic acid can deprotonate an incoming amine, making it a strong enough nucleophile to attack the acyl-enzyme intermediate, resulting in the enzyme and the final product: a protein with an incorporated amine or two cross-linked proteins [32].

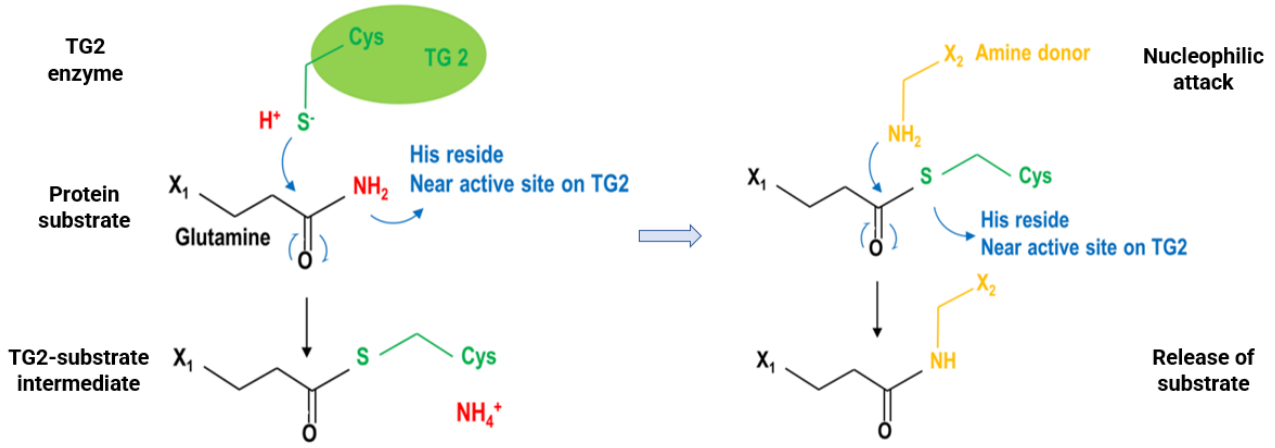


Figure 6. Transamidation reaction of TG2. The reaction starts with the formation of an intermediate between the enzyme and the protein substrate. Histidine 335 of the catalytic triad promotes the reaction by deprotonating the thiol group of cysteine 277, making it a stronger nucleophile, and protonating the terminal ammino group of the target glutamine, with the release of ammonia. Moreover, in the second step of the reaction His335 deprotonate an incoming amine, making it a stronger nucleophile, which then performs a nucleophilic attach on the intermediate, ultimately leading to the release of the final product: two cross-linked proteins, or a target protein modified by incorporation of an amine or by deamidation. Readapted from Lee et al. (2017) [34].

1.3.6 TG2 conformational switch

The classical view is that TG2 transamidation activity is calcium-dependent, as Ca^{2+} ions are necessary to keep the enzyme in an active open conformation, whereas at low Ca^{2+} concentrations and in the presence of GTP/GDP, the enzyme binds the nucleotide and undergoes a conformational switch, which brings the enzyme into a closed inactive form. In the closed conformation, the two C-

terminal β-barrels fold over the catalytic core and block substrate access into the pocket of the catalytic triad. The nucleotide binding site can accommodate GTP, GDP, and ATP, and the crystal structure of human TG2 bound to these molecules has been published (Figure 7) [35].

The nucleotide-binding site is located in a cleft between the catalytic core and the first C-terminal β-barrel. A peculiar feature of this interaction is that TG2 lacks critical serine/threonine residues normally present in other GTP-binding proteins that are responsible for binding to GTP by interacting with phosphate groups and coordinating Mg^{2+} ions. In TG2, interaction with GTP is instead mediated by positively charged arginine residues (Arg476, Arg478, and Arg580) which surround the negatively charged phosphate groups, and by hydrophobic residues (Phe174, Met483 and Val479) which stabilize the hydrophobic aromatic moieties of guanine. GTP-binding stabilizes the closed conformation of TG2 and favours the formation of precise disulfide bonds between cysteine residues, especially Cys230 and Cys370, which keep the enzyme in a compact structure. In fact, the redox potential is also critical for TG2 activity, as the different cysteine residues can form different combinations of disulfide bonds, which can potentially have dramatic effects on the protein conformation, and react with the Cys277 in the active site, inactivating the enzyme [36].

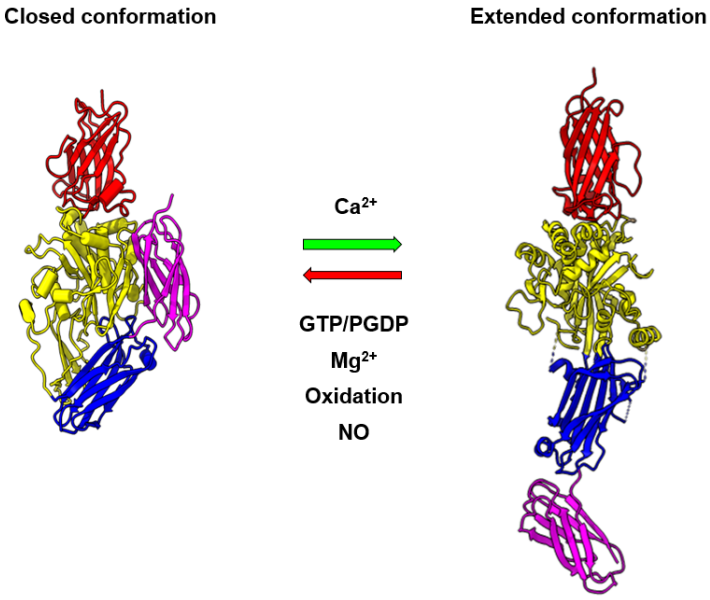


Figure 7. TG2 conformational switch. The binding of Ca^{2+} ions to TG2 favours the extended conformation of the protein, where the substrate can access the active site and TG2 can catalyse transamidation activity. Conversely, the binding of GTP, as well as the competitive binding of Mg^{2+} to the Ca^{2+}-binding sites and TG2 oxidation, lead to a conformational switch where the C terminal β-barrel 2 (in purple) folds over the catalytic domain (in yellow) and blocks access of the substrate to the active site. The 3D models are rendered with the ChimeraX software.

1.3.7 TG2: a Ca^{2+}-dependent enzyme

The exact mechanisms underlying Ca^{2+}-mediated activation of TG2 are not known. The protein is capable of binding up to six calcium ions, and for years the identification of binding sites was based on sequence comparisons with the resolved crystal structures of Ca^{2+}-bound FXIIIa and TG3, and on the study of negatively charged amino acids with high surface potential on TG2, since its Ca^{2+}-bound crystal structure was not available[37]–[39]. Five sites were initially identified as responsible for the binding to Ca^{2+}, which differed from the canonical Ca^{2+}-binding domains found in other proteins [40]. Mutations of these sites and the resulting loss of Ca^{2+} binding have profound effects on transamidation activity, the ability of TG2 to bind and hydrolyse GTP, and its affinity for antibodies that specifically recognize the fully open structure of the enzyme. A recent study describing a crystal structure of Ca^{2+}-bound hTG2 describes two binding sites: one represented by the side chains of Glu437, Tyr159, and Glu155, located in the catalytic domain, and another formed by Asn497, Phe537, Glu539, and Asn498 [41]. It has been demonstrated that the negatively charged amino acids Glu437 and Glu539 are crucial for Ca^{2+} binding and transamidation activity. While Glu437, located in the catalytic core, is part of one of the 5 previously hypothesized Ca^{2+}-binding sites, Glu539, located in the C-terminal β-barrel 1, represents a newly discovered interacting residue. A curious feature of the two residues is that they are both located in two loops in the hinge region of hTG2, where the conformational switch between the open and closed conformation occurs. Moreover, these two loops undergo conformational changes upon Ca^{2+} binding, as they are structurally different in the hTG2 crystal structure when bound to calcium or not. Since ion binding to these two residues is a prerequisite for the subsequent interaction of calcium with the other sites, it is plausible that these two residues cause a conformational change that promotes an open active conformation, even though the Ca^{2+}-bound crystal structure differs from the fully open conformation described for the inhibitor-bound hTG2, as it is a more compact GDP-bound structure (Figure 8) [35].

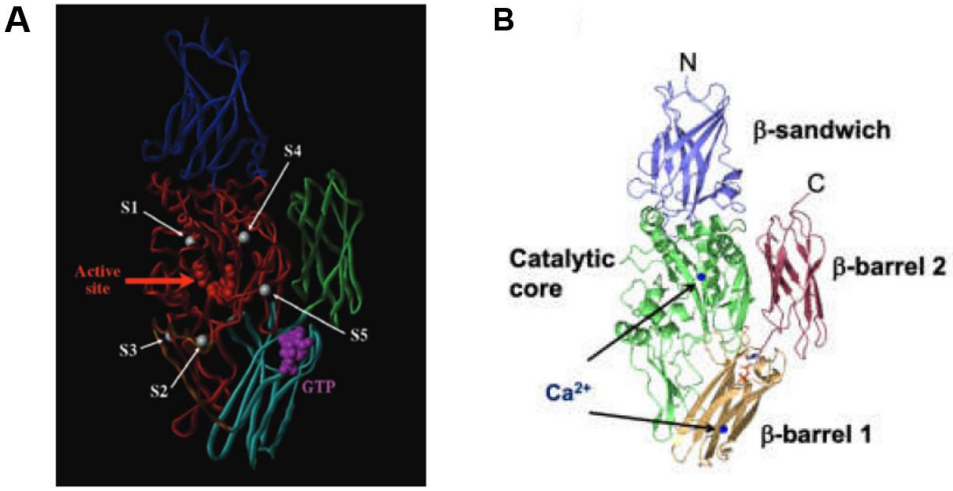


Figure 8. Transglutaminase 2 calcium-binding sites. A) 3D model of TG2 with highlighted the five calcium-binding sites identified by Kiraly and co-workers. The red spheres show the TG active site, while the purple ones highlight the bound GTP. B) 3D model of TG2 with indicated the two additional calcium-binding sites identified by Jeong and co-workers. Readapted from Kiraly et al. (2009) and Jeong et al. (2020).

Another interesting feature of Glu437 and Glu539 as Ca^{2+}-binding sites is that these amino acids can also be bound by magnesium ions, which compete with Ca^{2+} and inhibit transglutaminase activity. This competitive binding of Mg^{2+} is particularly important because GTP binding and hydrolysis by hTG2 requires Mg^{2+} ions, and mutations of Glu437 and Glu539 drastically reduce GTP binding and GTPase activity. Thus, Mg^{2+} is responsible for allosteric regulation of TG2 activity, and Mg^{2+} concentration plays an important role in determining whether TG2 is bound by Ca^{2+}, adopts an open conformation, and can catalyse crosslinking of available protein substrates, or remains in an inactive form. Because the cytosolic Ca^{2+} concentration is 10^4-fold lower than the Mg^{2+} concentration, magnesium ions play a crucial role in keeping intracellular TG2 inactive under physiological conditions, as we will discuss later [41].

1.3.8 GTPase activity

Transamidase activity is the hallmark enzymatic activity of TG2, being a member of the transglutaminase family, but this protein has been shown to catalyse other biochemical reactions. As anticipated, after binding to GTP in the closed conformation, hTG2 is able to hydrolyze GTP, an activity that is independent of the active site. Indeed, hTG2 is also considered to be a monomeric G protein, namely Gh, which has been shown to mediate signal transduction downstream of membrane receptors such as α1-adrenoreceptors, thromboxane A2, and oxytocin receptors and to activate phospholipase C δ (PLCδ) [42].

1.3.9 Other enzymatic functions

In addition to the transamidation activity and its role as a GTPase and G protein, which have been frequently discussed and represent the two main antagonistic enzymatic functions of hTG2, it has been reported that this enzyme can catalyse two different activities: phosphorylation and isomerization of protein substrates, thus being referred to capable of protein kinase and protein disulfide isomerase (PDI) activities [43], [44]. However, these putative hTG2-mediated enzymatic functions have not been thoroughly explored, and it might be premature to ascribe an actual role as protein kinase and PDI to hTG2, as the observed transfer of phosphoryl groups and renaturation of a protein substrate are biochemical processes that can be driven by a favourable chemical environment and are not actual demonstrations of enzymatic catalysis [42].

1.3.10 Regulation of TG2 activity

The transamidation activity of transglutaminases can have dramatic biological effects, as exemplified by the crosslinking of keratinocyte proteins to form the cornified cell envelop essential for the barrier function of the skin, and by the crosslinking of fibrin molecules to stabilize the clot in the coagulation process. Given the distribution of TG2 is several cell types and its localization both in the intracellular compartment (in the cytosol as well as in various organelles) and in the extracellular matrix (ECM), it is not surprising that the enzymatic activities of this enzyme can be regulated by different mechanisms. As mentioned above, the conformation of the enzyme is crucial for its transamidation activity, as the substrate must be able to enter a defined pocket in the catalytic core of hTG2 in order to react with the catalytic triad. The shape of this pocket and the chemical properties of the residues forming its surface determine the specificity of the enzyme, as TG2 is likely to interact with an incredible number of proteins, not all of which, however, represent good substrates for the post-translational modifications catalysed by TG2.

A restricted spectrum of substrates can also be viewed as an indirect regulatory mechanism, ensuring that TG2 does not indiscriminately crosslink all available glutamine and lysine residues, but only those that fit into and have access to the catalytic site. It should be kept in mind, however, that TG2 can indeed exert its transamidation activity on a wide variety of protein substrates, although the affinity for the different substrates and, consequently, the rate of the catalysed reaction are very different vary a lot. With all that said, the relevance of the conformation for the regulation of TG2 activity resides in the fact that accessibility to the substrate is only possible in the first place if the enzyme is in its open conformation. Indeed, as already discussed, in the closed conformation, the two C-terminal β-barrels are folded over the catalytic core and shield the active site. Crucial to whether

TG2 adopts the open and active or the closed and inactive conformation is the relative concentration of Ca^{2+}, GTP, and Mg^{2+}, although the exact nature of the active enzyme structure is still controversial. A cornerstone in this question seems to be that Ca^{2+} ions must bind an open conformation for catalysis to occur, since TG2 is still inactive in the open conformation in the absence of bound calcium [45]. As described earlier, competitive binding of magnesium ions to calcium binding sites negatively affects the transamidating activity of TG2, and GTP binding keeps the enzyme in its closed conformation. Other factors affect TG2 activity, such as the redox state, on which depends the correct formation of disulfide bonds between the numerous thiol groups of hTG2, and nitric oxide, since nitrosylation of TG2 has been shown to inhibit transamidation of TG2 [32].

1.3.11 Non-enzymatic functions

As a member of the transglutaminase family, the research on TG2 was initially very focused on the mechanisms underlying its enzymatic activity and its importance in biological events, as the functions of FXIII, the transglutaminase which initially draw more attention being involved in the vital process of the coagulation cascade, were mediated by its transamidation activity. However, it later became evident that the enzymatic activity of TG2 might be even less relevant than its non-enzymatic functions. Indeed, in physiological conditions, the intracellular compartment does not represent a favourable environment for TG2 transamidation activity, as the concentration of Ca^{2+} is low while the levels of inhibitory nucleotides such as GTP/GDP, as well as Mg^{2+} ions are high. Conversely, in the extracellular environment calcium ions abound while GTP does not, an ideal scenario for transamidase activity, which results in the crosslinking of ECM components. However, the extracellular enzymatic activity of TG2 has been detected in *in vitro* conditions of forced TG2 overexpression or of potential cell stress, where TG2 is known to be active. In fact, it has been suggested that TG2 is actually latent in the extracellular environment and might be activated under specific conditions, such as tissue injury, or that TG2 is initially active upon secretion in the ECM but then gets oxidized and inactivated. With all that said, transamidation activity of TG2 in the ECM does occur and it has a major importance for matrix deposition, crucial for matrix turnover after tissue injury and wound healing, fibrosis, and also for bone maturation[46].

The more TG2 is studied, the more it becomes clear that this protein can mediate numerous biological processes unrelated to its enzymatic activities. In the extracellular environment, the role of hTG2 as an adhesion molecule is well documented, as TG2 functions as an integrin co-receptor for fibronectin (FN), a function that does not require its enzymatic activity. In particular, TG2 can bind to integrin subunits β1, β3, and β5 and form ternary complexes with integrins and fibronectin. Indeed, FN has

only low affinity for cell surface integrins, whereas TG2, which in contrast binds both FN and integrins strongly, confers great stability to FN-integrin interactions and acts as a bridge between these molecules [47].

Moreover, TG2 has been shown to revert the loss of cell adhesion on FN when integrin binding to fibronectin is disrupted by blocking the interaction through small peptides containing the Arg-Gly-Asp sequence (RGD), which is found on many proteins of the ECM such as FN and is responsible for integrin binding [48]. Transglutaminase 2 binds fibronectin in the extracellular matrix with high affinity and mediates cell adhesion by binding the heparan sulfate chains of syndecan-4 [49]. This RGD-independent cell adhesion function of TG2 is thought to be essential under conditions where RGD peptides are released in the extracellular environment, such as matrix turnover when matrix metalloproteinases (MMPs) are secreted and digest the ECM, and tissue injury when TG2 is externalized and deposited in the ECM where it binds FN. Competitive binding of RGD peptides to cell surface integrins leads to loss of cell binding to the ECM and eventual cell death by a process called anoikis. Binding of the TG2-FN complex to syndecan-4 rescues cell adhesion to the ECM and triggers cell survival signaling. Specifically, upon binding of TG2 to its extracellular heparan sulfate chains, the cytosolic portion of syndecan-4 activates protein kinase C-α (PKC-α), which in turn interacts with integrins, particularly α5β1, and activates inside-out signaling. This eventually leads to activation of focal adhesion kinase (FAK) and mitogen-activated protein kinase ERK1/2. In addition, PKCα activates another member of the heparan sulfate proteoglycan family, sindecan-2, which, unlike syndecan-4, does not bind directly to TG2 but activates Rho-associated protein kinase (ROCK), mediating actin cytoskeleton reorganization and driving fibronectin fibril deposition in the ECM [50]. This fibronectin and syndecan-4 interaction with TG2 is then crucial in several biological processes, as matrix turnover and remodeling are fundamental in wound healing, bone remodeling, and angiogenesis, and TG2 can be considered to play an anti-apoptotic role in this process, as the cell signaling triggered by these cell surface interactions promotes the expression of pro-survival genes in the nucleus (Figure 9) [51].

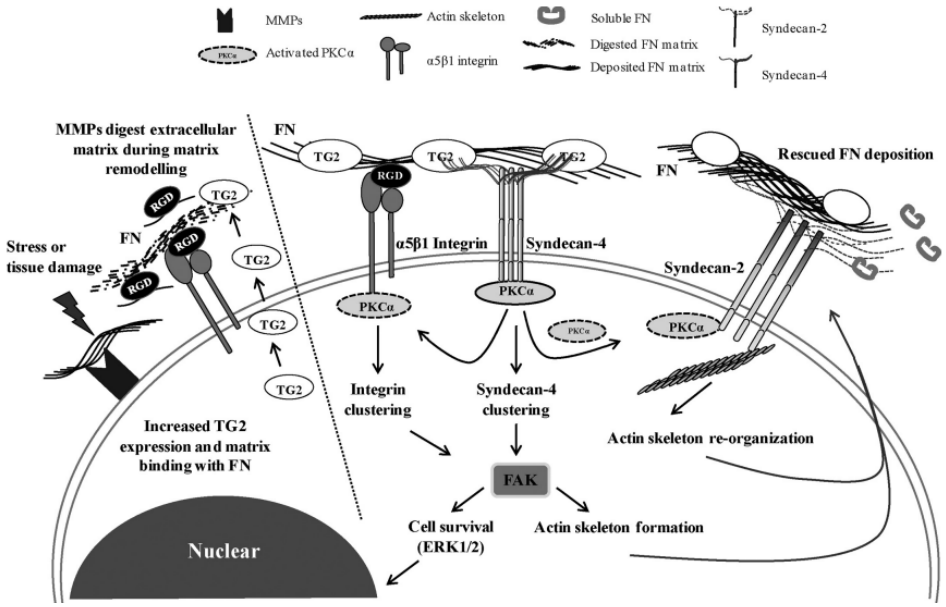


Figure 9. TG2 role in cell adhesion and matrix deposition. Graphic illustration of the mechanisms underlying the importance of TG2-FN complexes in the extracellular environment for RGD-independent cell adhesion, spreading and new fibronectin fibril formation. After stress of tissue damage, expression and externalization of TG2 is increased. In the ECM, matrix metalloproteinases (MMPs) degrade matrix protins for matrix turnover, resulting in the release of peptides containing the RGD motif, which compete and block the binding of cell surface integrins to ECM components, thus inducing loss of cell adhesion and anoikis. Cell adhesion is rescued by TG2, which interacts with matrix fibronectin and cell surface syndecan-4, acting as a bridge between the ECM and the cell. This interaction leads to activation of PKC-α, which in turn activates the inside-out signalling of α5β1 integrins. Ultimately, FAK activation leads to cell survival signals via ERK1/2 and focal adhesion formation. PKC-α also activates another HSPG, syndecan-2, which promotes actin cytoskeleton re-organization and fibronectin fibril deposition, leading to cell and matrix homeostasis. Readapted from Z. Wang et al. (2010).

Given its biological importance, the nature of TG2 interaction with heparan sulfate proteoglycans has been the subject of several studies by different groups. Heparan sulfate proteoglycans (HSPGs) are glycoproteins composed of a core protein with attached one or more sulfated glycosaminoglycan molecules, which are present both as secreted proteins in the extracellular matrix and as cell surface transmembrane receptors. As discussed, the biological importance of TG2 binding to syndecan-4 has been demonstrated, but the exact molecular basis of the interaction between TG2 and HSPGs in general is still debated, as three groups identified different TG2 heparan sulfate-binding sites (HBS), each team relying on different expermental approaches, which might partly explain the discrepancies [52]. Canonical heparan sulfate-binding motifs are eihter XBBXBX or XBBBXXBX, where B stands for a basic residue, whose side chin is exposed to the protein surface, and X is a neutral or hydrophobic residue, with a side chain facing the protein interior.

In particular Teesalu and colleagues identified 261-LRRWKNHGCQRVKY-274 as the aminoacidic stretch responsible for the interaction, while Lortat-Jacob and co-workers described two clusters of residues, 261-LRRWK-265 and 598-KQKRK-602, which in the closed conformation of the enzyme form a pocket lined by basic residues [53], [54]. Both teams used the surface plasmon resonance

technology to identify these regions, and an overlap between their data points out to a region comprising the 261-LRRWK-265 peptide as the best candidate as HBS. However, the group of Griffin and colleagues ruled out the 261-LRRWK-265 as a good candidate for the heparan sulfate binding, as they pointed out that this sequence is too short and is comprised in an α-helix, whereas a β-sheet is required for the basic residues of the XBBXBX consensus motif to be oriented in the same direction. Instead, they investigated two sequences, 590-KIRILGEPKQKRK-602 (HS1) and 200-NPKFLKNAGRDCSRRSS-216 (HS2) as potential binding sites. They identified HS2 peptide as the true HBS of hTG2, based on a molecular docking approach and mutagenesis studies, and also demonstrated how a synthetic mimicking peptide could bind syndecan-4 and mediate biological functions similar to the TG2-syndecan-4 interaction [55].

RGD-independent cell adhesion is not the only phenomenon in which the involvement of TG2 in a cell signaling cascade has been documented. We have previously discussed how after binding to GTP, this protein hydrolyses the nucleotide to GDP and acts as a G protein downstream of membrane G protein-coupled receptors (GPCRs) to activate PLC δ, a molecule central to many signaling pathways [56]–[58]. TG2 was first identified as an atypical high molecular weight G protein for the α1B-adrenergic receptor, as the α-subunit of heterotrimeric G proteins weighs between 30 and 35 kDa [59]. Later, TG2 was shown to mediate signaling through other GPCRs such as the α1D-adrenergic receptor, thromboxane A2 receptor, and oxytocin receptor by activating PLC-δ. In addition, it has been shown that the interaction between TG2 and GPCRs may also involve its transamidase activity, as TG2 has been shown to mediate angiotensin II AT2 receptor crosslinking and dimerization during the progression of Alzheimer disease (AD). The formation of AT2 oligomers catalysed by TG2 has been associated with disruption of Gαq/11 signaling, which has been linked to the neurodegeneration symptoms of AD [60]. In addition, TG2 has also been described as an extracellular GPCR ligand, particularly for the orphan receptor GPR56, and their interaction has been shown to inhibit tumour growth and metastatic spread in melanoma (Figure 10) [61].

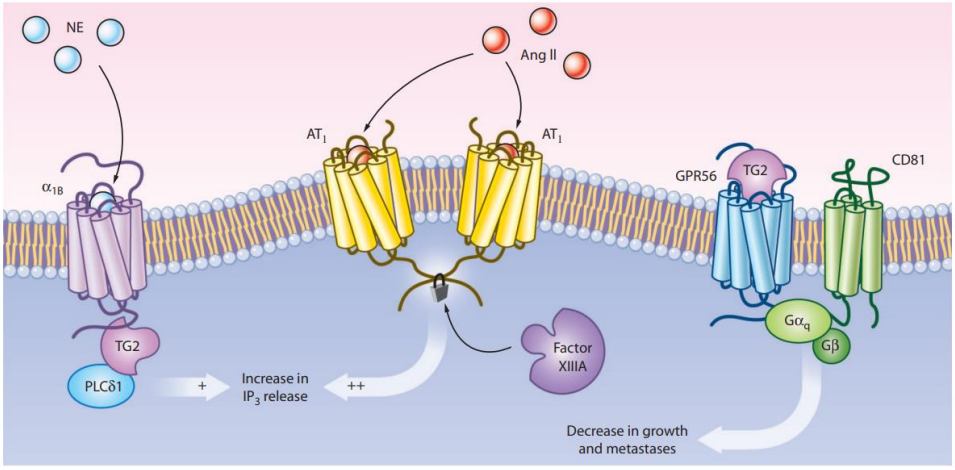


Figure 10. Transglutaminase and GPCR signaling. Graphic illustration of some of the described interactions and activities of TG2 with GPCRs. At the inner surface of the plasma membrane, intracellular TG2 can act as a G protein downstream α-adrenergic receptors to mediate the activation of PLC-δ. In the extracellular environment, TG2 has been identified as ligand of the orphan receptor GPR56, with inhibitory effects on growth and metastatic spread of melanoma cells. Moreover, TG2, but also Factor XIIIA, have been shown to mediate the oligomerization of angiotensin receptors (AT). Readapted from Iisma et al. (2006).

1.3.12 Cellular distribution

As mentioned above, TG2 can be found in the extracellular matrix, but the majority of TG2 is intracellular (~90% of total TG2), where it can localize at the inner surface of the plasma membrane, where it mediates cell signaling as a G protein, or interacts with a variety of proteins in the cytosol. In addition, TG2 is also found in several organelles such as the nucleus, mitochondria, and lysosomes.

Nuclear TG2 represents 5-7% of total cellular TG2, with variability depending on treatment, experimental conditions, and cell type. The exact mechanisms underlying translocation of TG2 to the nucleus are not clear. Importin-α3 has been identified as the molecule responsible for translocation of TG2, and two putative sites have been described as potential nuclear localization signals (NLSs), although only the ^{598}KQKRK^{602} residues appear to have the proper requirements to function as NLS. However, it has also been shown that TG2 might accumulate in the nucleus by interacting with other molecules that shuttle between the nucleus and the cytosol, such as NF-kB. In the nucleus, TG2 is thought to be involved in regulating chromatin structure, as it can both crosslink and phosphorylate histone proteins. In addition, TG2 has been reported to interact with retinoblastoma protein (Rb) in the nucleus, exerting both pro- and anti-apoptotic effects, and also with other known transcription factors such as hypoxia-inducible factor 1 (HIF1) and Sp1. TG2 is also involved in the DNA repair mechanism. Upon DNA double-strand breaks (DSBs), cytosolic TG2 is phosphorylated by specific DNA PKcs and translocated to the nucleus, where it aggregates at DSB sites, interacts with topoisomerase IIa, and mediates DSB repair via an unknown mechanism [62]. Although nuclear TG2

represents a relatively small fraction of the total cellular pool, it might be of great importance given its emerging role in various processes such as cell survival and cell death, but also in neurodegenerative diseases such as Alzheimer's disease and Huntington's chorea [63].

The role of TG2 in mitochondria is still debated as new functions continue to emerge. TG2 has been shown to be involved in energy metabolism, as several key enzymes of glycolysis and the Krebs cycle have been identified as TG2 substrates, as well as in calcium homeostasis and in the regulation of basic mitochondrial genes. However, it appears that activation of TG2 transamidating activity in mitochondria occurs only under stress or in "mitochondrial diseases," i.e., conditions characterized by abnormal calcium levels. A distinctive feature of mitochondrial TG2 is that its PDI activity in this compartment has been shown to be essential for the correct assembly of respiratory chain complexes and to catalyse important posttranslational modifications of key mitochondrial proteins (Figure 11) [30], [64].

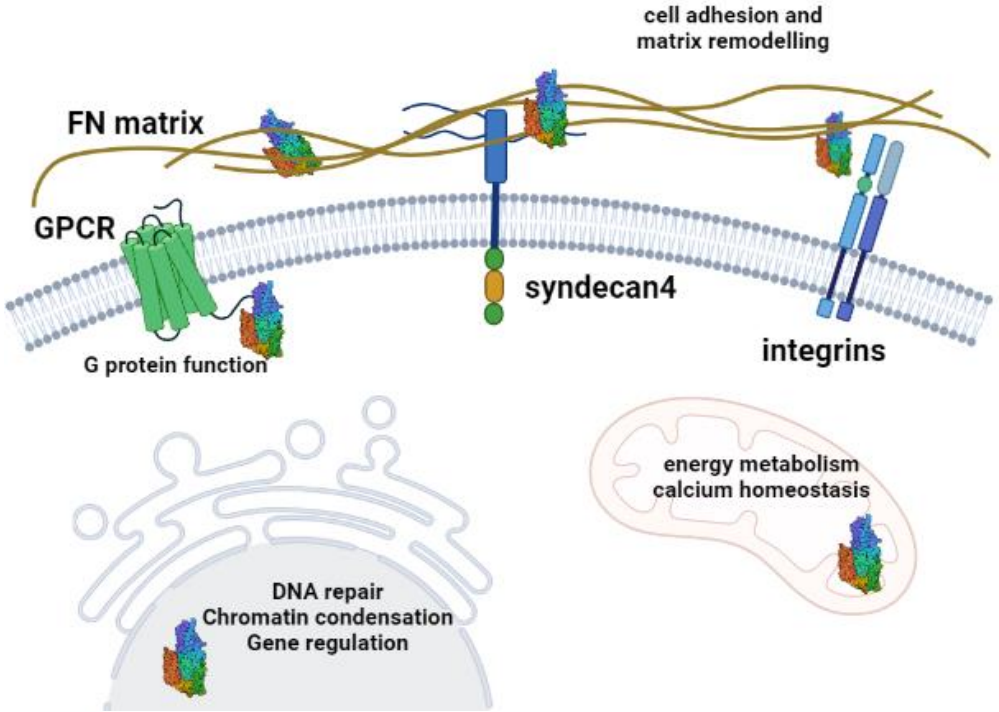


Figure 11. TG2 cell distribution. The majority of TG2 is present in the intracellular environment in the cytosol, but TG2 has also been found in several organelles. Indeed, TG2 can translocate in the nucleus, where it has been associated to DNA repair mechanisms, chromatin condensation and gene regulation by interacting with transcription factors and histone proteins. TG2 is also present in mitochondria, TG2 has been associated with energy metabolism and calcium homeostasis and in recycling exosomes. At the inner surface of the plasma membrane, TG2 interacts with several surface receptors, and can acts as a non-canonical G protein upon activation of GPCRs. In the extracellular environment, TG2 can interact with cell surface integrins, but also matrix fibronectin and cell surface syndecan-4, mediating cell adhesion, matrix deposition and remodelling.

TG2 was also found in recycling endosomes. The significance of this localization lies in the fact that TG2 has been suggested to be externalized into the ECM via an atypical pathway involving recycling endosomes. How TG2 is secreted is still controversial because TG2 lacks secretory signal sequences

and hydrophobic domains to cross the cell membrane. It has been reported that TG2 is not secreted via the classical endoplasmic reticulum/Golgi-dependent secretory pathway and is instead directed to perinuclear recycling endosomes that bring TG2 to the cell surface and release it into the extracellular environment after fusion with the plasma membrane. Moreover, treatments that impair endosome trafficking or endosome fusion with the plasma membrane impair TG2 externalization. An alternative mechanism of TG2 secretion that has been reported depends on the association of TG2 with heparan sulfate proteoglycans, particularly syndecans, cell surface receptors that interact with TG2 in the ECM and mediate cell adhesion and pro-survival signaling, as previously discussed. TG2 secretion depends on syndecan shedding, a process that occurs mainly under conditions that cause upregulation of TG2 and promote its overexpression and activation in the ECM, such as during tissue injury, wound healing, cancer migration, and bone development. Moreover, inhibition of syndecan release in tissue injury affects TG2 activity in the injured area, indicating that this process is critical for TG2 secretion in the ECM. The proposed mechanism is that intracellular TG2 binds syndecan in a closed form and that stressors such as tissue injury cause syndecan to be secreted and TG2 to be released into the extracellular environment. Due to high Ca^{2+} concentrations in the ECM, TG2 changes to the extended and active conformation, which has a low affinity for HSPs and promotes syndecan detachment and deposition in the matrix.

1.3.13 TG2 and wound healing

As mentioned above, TG2 can be considered as a stress response protein, as during cell stress TG2 activity is upregulated by increased TGM2 gene expression, increased secretion into the ECM, and activation of enzymatic activity. All these events occur during wound healing, which is a complex, multistep process that requires the concerted action of multiple cell types. When tissue injury occurs, an inflammatory response is initiated in the wound area, which must later be stopped to return to homeostasis. In addition, the ECM must be deposited and degraded in a tightly regulated manner to first repair the injured tissue and then remodel a provisional matrix into its physiological composition and structure, a process that is accompanied by revascularization of the injured area through angiogenesis [65].

In the initial phase of wound healing, TG2 has a pro-inflammatory function and is involved in the recruitment of leukocytes to the injured area. Indeed, tissue injury leads to local inflammation with increased blood vessel permeability, which allows leukocyte penetration into the injured area. Vasodilatation is driven by histamine released by mast cells and maintained by activation of phospholipase A2 enzymes (PLA2), which generate vasodilating eicosanoid molecules such as prostaglandins and leukotrienes. TG2 has been shown to mediate an essential post-translational

modification of PLA2 that drives its activation, as inhibition of TG2 modification of PLA2 has anti-inflammatory effects, whereas upregulation of TG2 activity enhances the eicosanoid cascade, suggesting that TG2 promotes vasodilation and leukocyte recruitment. Conversely, at later stages of the healing process, TG2 has anti-inflammatory functions by mediating activation of latent transforming growth factor β (TGF-β) and promoting phagocytosis of macrophages, which is essential for clearing tissue of debris and any pathogens. Activation of TGF-β promotes secretion of anti-inflammatory cytokines, which ensure that inflammation is terminated so that repair mechanisms and re-epithelialization can occur.

Upon matrix injury, fibronectin fibrils as well as other components of the ECM are degraded, releasing RDG-containing peptides. As mentioned earlier, RGD peptides saturate integrin receptors on the cell surface, leading to loss of cell adhesion and eventually anoikis. In this context, TG2 is critical for mediating RGD-independent cell adhesion interacting with syndecan-4, whose expression on the cell surface is upregulated during cell stress and tissue injury. Fibronectin-bound TG2 interacts strongly with syndecan-4 and promotes integrin clustering and outside-in pro-survival signaling, as well as the formation of new focal adhesions. TG2, as a cell adhesion molecule in the ECM, is important not only for cell attachment but also for spreading and migration, two fundamental processes in tissue repair, as both fibroblasts and endothelial cells must migrate to the injured area to deposit new matrix and form new blood vessels, respectively. Fibronectin fibril formation is also favoured by TG2, as RGD-independent cell adhesion mediated by TG2 promotes the deposition of new fibronectin fibrils, which are required for the formation of new ECM to heal the tissue injury.

1.3.14 TG2 and angiogenesis

A process that is fundamental to wound healing, but really to any biological event in which new tissue is formed, whether physiologically as in embryonic development or pathologically as in tumour growth, is angiogenesis. Defined as the formation of new blood vessels from pre-existing ones, angiogenesis is a hallmark of physiological and pathological tissue repair, expansion, and remodeling because the formation of new tissue requires the development of a new blood supply. Angiogenesis is a complex multistep process that depends on the activity of various growth factors, enzymes, cell types, and remodeling of the extracellular matrix. The first part, called vascular morphogenesis, begins with endothelial cells (ECs) sprouting and association into tubular structures in a concerted mechanism that involves EC differentiation, proliferation, and migration. Subsequently, mesenchymal cells recruited by the ECs migrate along the tubule surrounding it and differentiate into either perivascular stromal cells called pericytes or smooth muscle cells. Once the new vessel is

formed, migration and proliferation of ECs decrease dramatically, as they are normally quiescent cells under physiological conditions (Figure 12).

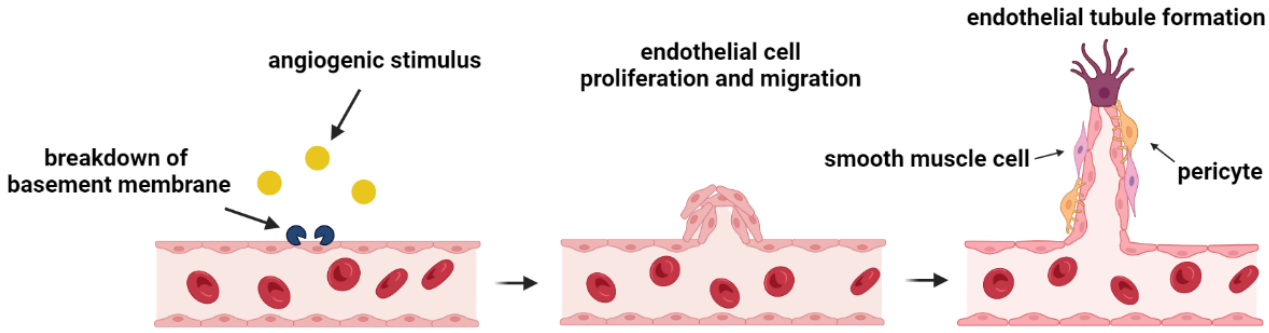


Figure 12 Angiogenic process. Graphic illustration showing the essential steps of the angiogenic process. An angiogenic stimulus triggers the breakdown of the endothelial basement membrane by matrix metalloproteinases and increased vascular permeability. Then, endothelial cells start to proliferate and to migrate forming an endothelial sprout. The endothelial tubule is then surrounded by mesenchymal cells, which differentiate in pericyte and smooth muscle cells.

During angiogenesis, the behavioural properties of endothelial cells change drastically, and these changes are highly dependent on a lot of modification of the surrounding matrix [66]. ECs express TG2 at high levels and the functions of TG2 in their behaviour and consequently on the angiogenic process have been the subject of extensive studies. However, there are several observations linking TG2 in ECs to both pro- and anti-angiogenic effects. Initial studies using an *in vivo* rat model showed that the addition of recombinant TG2 to a wounded area increased neovascularization in the skin, as the enzymatic activity of TG2 promotes fibrin matrix stabilization and scar formation and activates the anti-inflammatory TGF-β [67]. However, it was later demonstrated that TG2 crosslinking activity can actually inhibit endothelial cell tubule formation, as matrix deposition catalysed by TG2 impairs proliferation and migration of endothelial cells required for angiogenesis [68]. The antiangiogenic effect of TG2 enzymatic activity was also noted in other studies, in which the use of anti-TG2 antibodies from celiac patients that increased TG2 transamidation activity resulted in defects in the angiogenic process [69]–[73]. However, it was later reported that endothelial TG2 crosslinking activity is critical for angiogenesis and that extracellular TG2 transamidation promotes angiogenesis via deposition of matrix-bound vascular endothelial growth factor (VEGF) and activation of VEGF and VEGF receptor signaling [74]. In addition, because TGF-β1 signaling is also required for tubule formation, the TG2 transamidation activity of ECs has been reported to be essential for angiogenesis and to activate latent matrix-bound TGF-β1 [75]. Since reports from different groups provide a conflicting picture regarding the mechanisms of action of TG2 in the angiogenic process, it is still difficult to define the pro- or anti-angiogenic nature of its involvement. Because matrix deposition and remodeling are necessary for endothelial cell migration and proliferation, it seems clear that

physiological levels of TG2 are required for angiogenesis, because inhibition of TG2 leads to impaired VEGF and TGF-β1 signaling and impaired matrix remodeling. However, abnormal TG2 activity leads to increased ECM stiffness and altered matrix deposition, which impair blood vessel formation. Therefore, it stands to reason that alteration of TG2 activity by downregulation or upregulation would affect its physiological role in the angiogenic process (Figure 13). Nevertheless, the extent of its involvement is not yet clear, because TG2 knockout animals do not show phenotypic alterations in angiogenesis, as is the case in several other biological processes in which TG2 has been reported to be involved. Nevertheless, the data from TG2 knockout animals may not be as reliable as desired, because several compensatory mechanisms, dependent on other TG proteins, have been shown to occur in absence of TG2 [76].

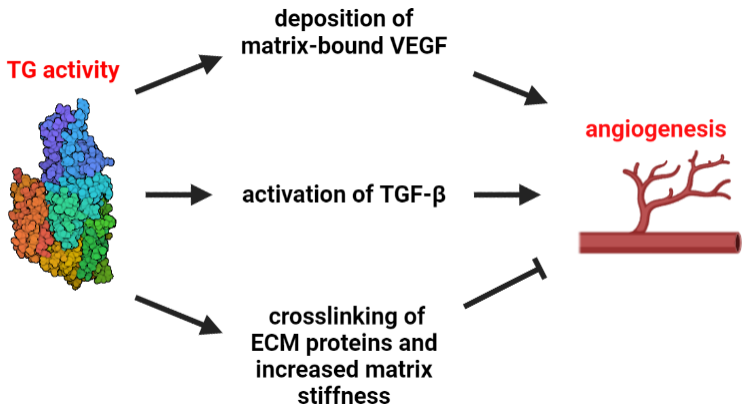


Figure 13. TG2 and angiogenesis. Graphic scheme of different ways TG2 has been shown to affect and be involved in the angiogenic process. Transglutaminase activity has been demonstrated to be critical for the deposition of matrix-bound VEGF, thus increasing VEGF signalling and promoting angiogenesis. Moreover, TG2 is critical for activation of matrix-bound latent TGF-β, which is essential for tubule formation. However, increased TG2 activity in the ECM has also been associated to excessive matrix stiffness, which impair tubule formation and ultimately angiogenesis.

1.3.15 TG2 and apoptosis

The term apoptosis was first introduced in the 1970s to refer to a morphologically peculiar form of cell death. Apoptosis is a physiological process that occurs during embryonic development and aging, as well as during tissue remodeling to maintain homeostasis. In addition, apoptosis is critical to the development of the immune and nervous systems, both of which share the common characteristic of an initial overproduction of cells. Cells that fail to establish functional synaptic connections or to exhibit proper antigen specificity are subjected to apoptosis, which thus represents a selection mechanism essential for the development of functional tissues [77]. Alterations in physiological apoptosis are involved in several pathologies, such as cancer, in which neoplastic cells generally escape apoptosis by various mechanisms, and neurodegenerative diseases, which are characterized by progressive abnormal nervous cell.

Cells undergoing apoptosis show peculiar morphological changes, as first the cells shrink, and chromatin condenses in the nucleus. Then there is a characteristic blebbing of the plasma membrane, which ends with fragmentation of the cells into apoptotic bodies that are phagocytosed by macrophages or parenchymal cells. Apoptotic cells are recognized by phagocytic cells through the exposure of typical surface markers: phosphatidylserine, normally directed inward in the phospholipid bilayer of the plasma membrane, is flipped and exposed on the surface.

Apoptosis is mediated by specific proteolytic enzymes called caspases (cysteine-aspartic proteases), which are activated through two different pathways: the extrinsic and the intrinsic pathway. The extrinsic pathway relies on signals activated by interactions at transmembrane receptors called death receptors, e.g., binding of Fas ligand to Fas receptor and binding of TNF-α to TNF receptor. The signaling cascade activates the death-inducing signaling complex (DISC), which activates the proenzyme caspase-8, an initiator cascade, which cleaves and activates execution caspase-3. Once caspases are activated, the cell is irreversibly committed to apoptosis, and caspase-3 activity leads to cell death via activation of endonucleases, which cause DNA degradation, and proteases, which break down cytoskeleton and nuclear proteins. The intrinsic pathway relies on stimuli that do not involve activation of surface receptors. The absence of pro-survival hormones, growth factors, and cytokines is considered a negative signal that activates the intrinsic pathway, while positive signals include cell irradiation as well as hypoxia, infection by certain pathogens, and oxidative stress.

Both positive and negative signals cause the cytosolic pro-apoptotic molecule Bax to translocate to the outer membrane of mitochondria, where it oligomerizes forming a pore in the membrane. This event allows the release of several pro-apoptotic molecules into the cytosol, including cytochrome c, Smac/ DIABLO, and HrtA2/Omi. These molecules ultimately lead to the activation of initiator caspase-9 via several mechanisms, which in turn activates caspase-2, triggering cell death (Figure 14) [78].

Similarly to angiogenesis, the involvement of TG2 in apoptosis has been extensively described but the overall role is not yet clear, as TG2 has been reported to both promote and inhibit apoptosis. Another shared feature with the involvement of TG2 in the angiogenic process is that TG2 knockout mice do not exhibit a phenotype associated with defective or abnormal apoptosis, calling into question the actual role of TG2 in this process, although, as mentioned earlier, compensatory mechanisms may occur through other TG isoforms. In the late 1980s, the induction of TG2 in cells undergoing apoptosis was reported, and it was first suggested that TG2 plays a protective role in tissues, as TG2-mediated crosslinking of cellular proteins in apoptotic cells prevents the release of pro-inflammatory harmful intracellular content into the extracellular milieu [79].

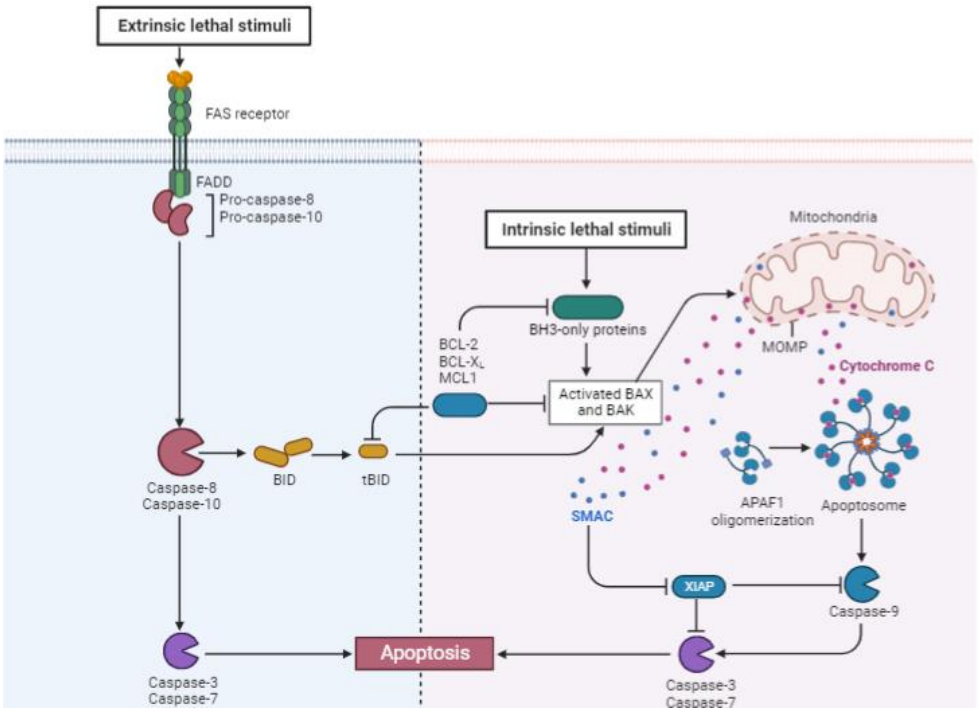


Figure 14. Extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is triggered by the binding of their ligands to so-called death receptors. Conversely, the intrinsic pathway is promoted by the absence of some pro-survival signals or the presence of some positive signals, such as cell irradiation, hypoxia, oxidative stress or infection by some pathogens. Ultimately, both pathways lead to the activation of effector caspases, such as caspase-3 and caspase-7, that promote apoptosis by activating proteases, which degrade cytoskeleton and nuclear proteins, and endonucleases, which digest cellular nucleic acids.

However, subsequent studies on TG2 in apoptosis showed that increased TG2 activity promotes apoptosis. For example, treatment of promonocytic U937 cells with the TG2 inducer RA was shown to drive the cells into apoptosis and decrease anti-apoptotic Bcl-2. Moreover, cell death was rescued by downregulation of TG2 expression in U937 cells subjected to apoptosis. Ca^{2+}-dependent cross-linking of intracellular proteins was the identified mechanism underlying the involvement of TG2 in apoptosis, as the ionophore molecule A23187, which increased intracellular Ca^{2+} levels, was able to elicit the same response as treatment with RA [80]. It was later demonstrated that TG2 might differentially modulate apoptosis in a stimuli-dependent fashion. Indeed, in human neuroblastoma cells exposed to three different apoptotic stimuli, increased caspase-3 activation, and apoptotic nuclear changes with concomitant increase in enzymatic transamidation activity were observed only in cells transfected with TG2 after osmotic stress or staurosporine treatment. Conversely, after heat shock, which did not lead to activation of TG2 activity, TG2 expression led to a decrease in caspase-3 activity. These observations suggested that TG2 has a pro-apoptotic function when apoptotic stress increases transamidation activity, otherwise TG2 acts as an anti-apoptotic molecule [81]. Moreover, it has been shown that the role of TG2 in apoptosis also depends on its localization. Indeed, in the nucleus, TG2 promotes apoptosis when catalytically active and inhibits it when inactive by interacting with Rb, whereas in the cytosol TG2 has anti-apoptotic effects [82]. Studies in human embryonic

kidney cells (HEK293) have shown that overexpression of TG2 has anti-apoptotic effects. In particular, TG2 increased the survival of cells treated with a calcium ionophore, which, increasing intracellular Ca^{2+} levels, triggers mitochondrial permeabilization and cytochrome c release, thus activating caspases. TG2 expression blocks the mitochondria-mediated apoptotic pathway by downregulating the expression of Bax, thereby reducing mitochondrial permeability. This leads to decreased mitochondrial release of cytochrome c and ultimately downregulation of caspase-3 and caspase-9 activation, thereby inhibiting apoptosis [83]. The anti-apoptotic functions of TG2 in renal cells have also been confirmed in renal cell carcinoma (RCC), where overexpression of TG2 leads to enhanced autophagy with protective anti-apoptotic effects [84]. Specifically, TG2 mediates the cross-linking of p53 in autophagosomes, leading to depletion of p53 and preventing p53-induced cell death signaling in renal cell carcinomas. In addition, combined inhibition of TG2 and treatment with an anticancer drug such as doxorubicin was shown to significantly reduce tumour growth in a xenograft model [85]. TG2 has also been reported to mediate apoptosis through caspase-independent mechanisms, for example, crosslinking and inactivation of transcription factors essential for cell survival [86]. Alterations of TG2 leading to apoptosis have also been described in various neurogenerative disorders, like Parkinson's, Huntington’s, and Alzheimer's disease, as TG2 has been shown to mediate the crosslinking of huntingtin as well as amyloid-β peptide and tau protein [29].

1.3.16 TG2 and bone development

Bone is a complex and dynamic tissue which need continuous remodelling through the coordinated activity of bone-forming osteoblasts and bone-resorbing osteoclasts. When the balance between these two activities is altered, deregulated bone remodelling causes several pathologies, such as rheumatoid arthritis and osteoporosis. The involvement of TG2 in bone development and remodeling has been the subject of several studies, as basic components of mineralized tissue such as fibronectin, collagen, and osteopontin have been identified as TG2 substrates. It has been reported that TG2 can actually assemble these proteins into polymeric forms involved in matrix stabilization. In addition, a crucial role of TG2 in the differentiation of osteoblasts and osteoclasts has also been reported. Indeed, it was demonstrated that during osteoblast differentiation, TG2 expression was upregulated together with bone morphogenetic protein 2 (BMP2), a known member of the TGF-β superfamily that is essential for bone cell differentiation and bone formation. Moreover, reducing TG2 expression led to a downregulation of BMP2, indicating a strong relationship between the two [87]. However, there is not much cumulative evidence for a key role of TG2 in bone formation and osteoblast differentiation and TG2 KO mice do not show clear alterations in bone development. However, as FXIIIa is another transglutaminase that has been shown to be involved in bone formation, it is possible that it may act

in concert with TGF-β to compensate for the loss of TG2. Conversely, as TG2 appears to be the only TG selectively expressed in osteoclasts, TG2 KO mice showed phenotypic alterations in osteoclast differentiation. In particular, ablation of TG2 was shown to lead to an increased number of osteoclasts on bone surfaces, resulting in increased bone resorption and decreased bone mass. Moreover, overexpression of TG2 suppresses osteoclastogenesis, with a negative regulation mechanism involving NF-kB and B-lymphocyte-induced maturation protein 1 (Blimp1), a suppressor of osteoclast differentiation [88]. These observations were supported by another animal model in which both TG2 and FXIIIa were ablated. The combined absence of both TGs resulted in dramatic bone loss due to increased osteoclastogenesis and bone resorption [89].

1.3.17 TG2 and cancer

As we have seen, TG2 is involved in several biological processes via different mechanisms that depend on its crosslinking activity but also on its scaffold and G-protein functions, and it is not surprising that alterations in TG2 expression and activity have been found in different types of cancers. As with most biological processes, the relationship between TG2 and malignant neoplasms has been extensively studied, and new data are continuously being generated, given the paramount importance of cancer research to modern medicine. However, conflicting observations have been made due to the inherent complexity and heterogeneity of both TG2 functions and cancer. TG2 is overexpressed in most cancers, whereas it is normally present at low levels in healthy tissues. In particular, several reports suggest that TG2 is initially expressed at low levels in primary tumours, but then becomes upregulated in metastatic cancer [90]–[92]. Indeed, TG2 is generally considered a negative prognostic marker for several types of cancer, as TG2 is associated with increased metastatic potential and drug resistance [93].

TG2 has been shown to increase the proliferative potential of tumour cells through interaction with TGF-β and MAPK signaling. In addition, TG2 negatively regulates Rb and p53 proteins, reducing pro-apoptotic and anti-proliferative signals and promoting tumour growth. The rapid metabolism of malignant proliferating cells leads to a hypoxic microenvironment, and angiogenesis is promoted to meet the increasing demand for blood supply. TG2 expression and activity is increased in response to cell stress and hypoxia by various mediators such as Hypoxia-Inducible Factor, which together with TG2 triggers an anti-apoptotic signaling in cancer cells [94]. HIF also induces the formation of new blood vessels to improve the supply of oxygen and nutrients to cancer cells, and as we have already seen, TG2 can modulate angiogenesis via both enzymatic and non-enzymatic functions [95]. Enhanced cell adhesion mediated by the interaction of TG2 with matrix fibronectin, syndecans, and integrin promotes cancer cell survival through MAPK signaling. Rescuing cancer cells from anoikis

might be particularly critical in a tumour microenvironment with increasing matrix turnover and highly migratory cells. However, TG2-mediated crosslinking of extracellular matrix proteins, such as fibronectin and collagen, increases the stiffness of the ECM and its resistance to the proteolytic cleavage by matrix metalloproteinases, limiting tumour mass expansion and basement membrane breakdown. This is particularly important because invading tumour cells must cross the basement membrane to metastasize. In the early stages of tumour progression, TG2 crosslinking activity plays a protective role in limiting tumour expansion, and several studies have shown that tumours with downregulation of TG2 have a higher invasive and metastatic potential. Thus, TG2 expression and activity can have completely different effects depending on the cancer type and the stage of tumour progression. For this reason, although TG2 has been addressed as a potential prognostic marker and a promising therapeutic target, its actual use in diagnostics and therapy is far from straightforward, as the role of its up- or downregulation in healthy and malignant tissues varies from case to case [91].

1.3.18 TG2 and fibrosis

Fibrosis is a scarring process characterized by abnormal accumulation of fibroblasts and extracellular matrix proteins, especially collagen I and III. It is a pathological event that can affect almost any organ and eventually leads to its failure by completely altering its functional architecture [96]. The involvement of TG2 in fibrosis has been established in several studies of renal, pulmonary, cardiac and hepatic fibrosis, and there are several mechanisms by which TG2 can promote this process [97]–[99]. Crosslinking of extracellular proteins such as collagen and fibronectin increases the stiffness of the ECM and makes it more resistant to proteolysis. In addition, TG2, as a scaffold protein that mediates cell adhesion, promotes the deposition of new fibrillar fibronectin (Figure 15). A key interaction in the fibrotic process is that between TG2 and TGF-β, which are involved in a positive activation loop, as mentioned earlier. TG2 activates latent TFG-β in the ECM, which promote epithelial-mesenchymal transition (EMT) and endothelial-mesenchymal transition (EndMT) and ultimately enhance abnormal matrix deposition. Inhibition of TG2 has been shown to ameliorate the fibrotic process both in vitro and in vivo. Indeed, both treatment with a selective TG2 enzymatic inhibitor and TG2 gene knockout have been demonstrated to reduce fibrosis by blocking the transition to the mesenchymal phenotype of epithelial and endothelial cells induced by TGF-β.

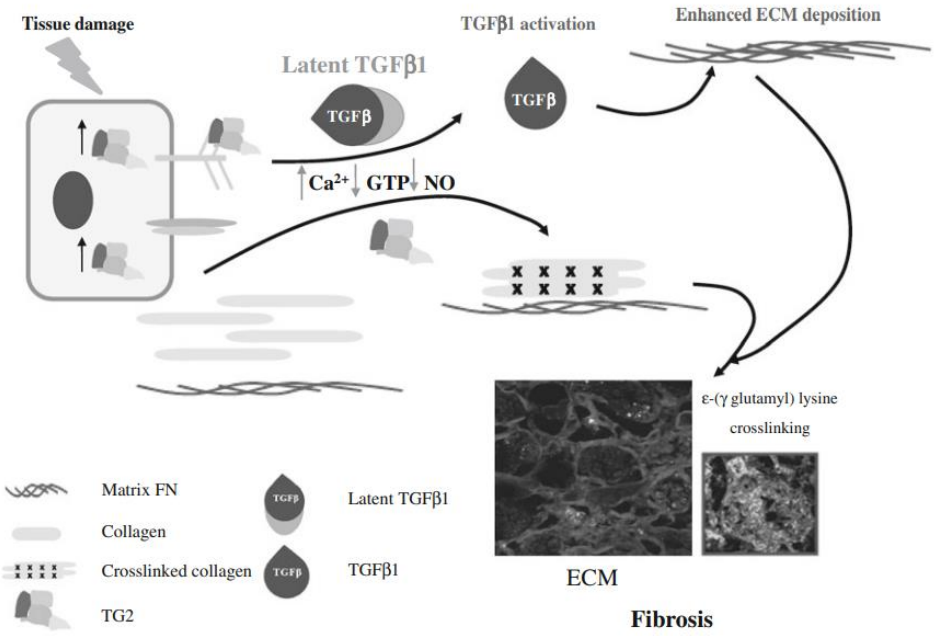


Figure 15. Transglutaminase 2 activity and fibrosis. After tissue damage, TG2 is upregulated and its deposition into the extracellular environment is increased. Here, TG2 can promote the deposition of new matrix fibronectin, mediate the crosslinking of ECM proteins and the activation of latent TGF-β. All these activities are crucial for wound healing and return to homeostasis, but a deregulation of these mechanisms can lead to fibrosis, with abnormal accumulation of scar tissue and loss of functional physiological tissue. Illustration from Wang Z. & Griffin M (2012) [100].

1.3.19 TG2 and celiac disease

Celiac disease (CD) is a chronic, immune-mediated disorder that presents a special challenge to modern medicine because of its unique features. Symptoms are triggered by the ingestion of dietary gluten, which is present in various grains, such as wheat, rye and barley, and nowadays there is no effective treatment other than a lifelong strict gluten-free diet. Moreover, the clinical manifestations of CD are broad, and some patients may even remain asymptomatic. This heterogeneity makes CD a largely unrecognized and undiagnosed disease worldwide. Celiac disease is best known for the gastrointestinal symptoms, namely diarrhoea and malabsorption with resultant anaemia, weight loss, and growth retardation, triggered by the ingestion of gluten, which is a complex mixture of gliadins and glutenins. These two classes of proteins are rich in prolines, which make them particularly resistant to proteolysis by digestive enzymes, and in specific sequences containing glutamine residues, which make them an ideal substrate for the enzymatic activity of TG2, particularly gliadins.

A specific genetic background is associated with the development of CD, in particular the major histocompatibility complex class II molecules HLA-DQ2 and HLA-DQ8. In fact, CD occurs almost exclusively in people with these HLA variants, but only a small proportion of individuals with this

genetic background develop CD, as other genetic and environmental factors are likely to play a role [101].

In the intestine, gluten peptides derived from incomplete digestion of gluten-containing foods penetrate the epithelial layer and reach the lamina propria, a thin connective tissue layer of the intestinal mucosa, where they trigger activation of both innate and adaptive immune responses in celiac patients, which are responsible for damage to the intestinal mucosa and lead to gastrointestinal symptoms and malabsorption. The immune response is triggered by TG2-mediated deamidation of target glutamines in gliadin peptides. The resulting glutamic acids in gliadin peptides are potent immunostimulatory molecules with increased affinity for the HLA-DQ2 and HLA-DQ8 complexes of antigen-presenting cells, including dendritic cells and B cells. Dendritic cells activate gliadin-specific CD4+ T cells, which trigger inflammation by secreting proinflammatory molecules such as interferon-γ and interleukin (IL)-21 and elicit a Th1 response with activation of intraepithelial lymphocytes that drive apoptosis of intestinal epithelial cells and tissue damage, leading to villous atrophy. B cells recognize both gliadin peptides and TG2-gliadin complexes via B cell receptors and present the processed antigen to CD4+ cells. This interaction activates both CD4+ T cells and antigen-presenting B cells, which differentiate into secreting plasma cells responsible for producing antibodies against gliadin peptides and autoantibodies against TG2 (Figure 16). TG2-specific immunoglobulin A (IgA) autoantibodies are found in the serum of celiac patients and are also bound to TG2 in the lamina propria, both under the basement membrane of the intestinal epithelia and around the capillaries of the mucosa [102]–[104]. Detection of anti-TG2 IgA in the serum of patients is the first screening step for the diagnosis of active celiac disease. The biological effects of these IgA autoantibodies have been the subject of several studies, which have reached quite contradictory results [105]–[107]. Anti-TG2 IgA molecules from celiac patients have been shown to affect the intestinal mucosa by altering its architecture and increasing its permeability to inflammatory molecules and leukocyte binding via mechanisms involving increased TG2 and RhoA activity. Anti-TG2 IgA antibodies also lead to upregulation of RhoB, altering endothelial cell behaviour. Indeed, several reports have linked anti-TG2-IgA to impairment of the angiogenic process because anti-TG2-IgA can alter the actin cytoskeleton of both endothelial and vascular mesenchymal cells, reducing cell adhesion and motility, which are essential for the formation of new blood vessels [70], [72], [73], [108].

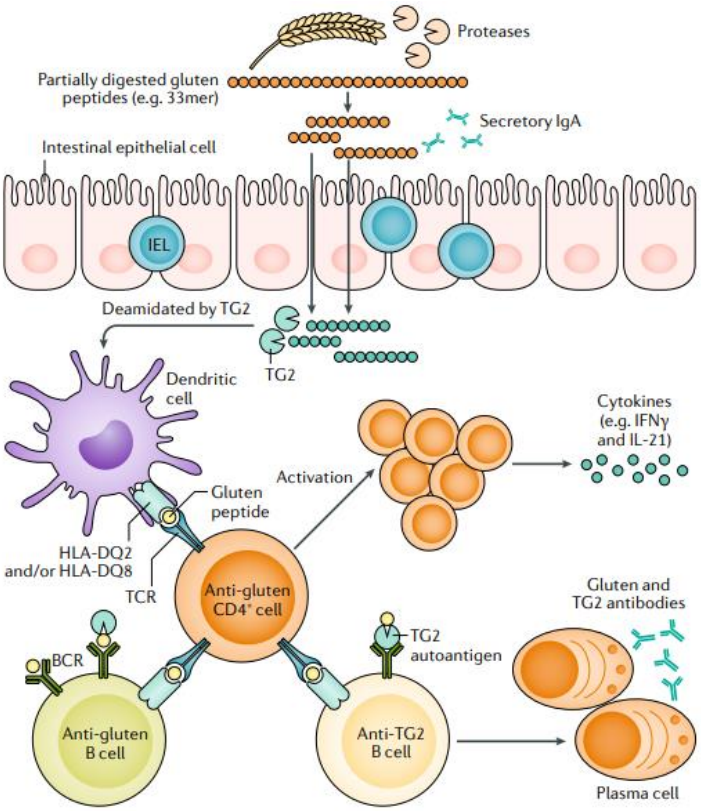


Figure 16. Celiac disease immune reaction. Food-derived gluten peptides are only partially digested due to their resistance to the proteolytic enzymes. Once in the intestinal lamina propria, gluten peptides are deamidated by TG2, resulting in a strongly immunogenic molecules with increased affinity for the HLA-DQ2 and HLA-DQ8 complexes of antigen-presenting cells, including dendritic cells and B cells. In celiac patients, intestinal TG2 is also recognized by anti-TG2 B cells. Antigen-presenting cells activate pro-inflammatory CD4+ T cells, which secrete pro-inflammatory cytokines, and promotes the maturation of plasma cells secreting autoantibodies. Readapted by Lindfors et al. (2019) [101].

Celiac disease is also characterized by extra-intestinal symptoms affecting various tissues and organs such as bone, liver, skin, and the nervous system. As mentioned earlier, these manifestations are also associated with the production of autoantibodies against other members of the transglutaminase family encoded by genes all located on chromosome 10q21. In particular, immunoglobulins against TG3, which is mainly found in the skin, are the typical marker for the dermal manifestation of CD, dermatitis herpetiformis. Moreover, immunoglobulins directed against TG6, also known as neuronal TG6, produce neuronal deposits in patients affected by gluten ataxia, a rare neurological disorder that results in decreased motor skills and loss of coordination [11], [109].

1.4 The zebrafish

1.4.1 Introduction on Danio rerio

The zebrafish (Danio rerio) is a small freshwater fish originally native to South Asia. It is a teleost fish of the Cyprinidae family with rapid embryonic development lasting few days and a life cycle of 3 to 4 years. In the wild, the zebrafish lives in water temperatures of 25 to 39 °C and shows no evident signs of heat stress, although the optimal temperature used in animal facilities is around 28 °C. Male and female adults can be distinguished by the fuller and rounder abdomen of gravid females, which upon breeding can lay hundreds of eggs weekly (Figure 17). Indeed, in research facilities the adults of domesticated strains of zebrafish breed year-round, whereas in the wild they generally breed during the monsoon season [110].

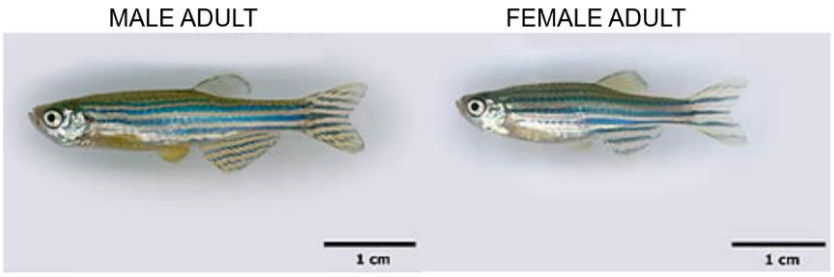


Figure 17. Image of a male and a female zebrafish adults. Readapted from the aquariumfishparadise website.

The zebrafish has become a well-established animal model in biology for several reasons, including ease of handling and maintenance, prolific breeding, external fertilization, high numerosity, rapid development and high genetic similarity to humans. The high numerosity of eggs makes it possible to work with a large number of embryos, characterized by rapid embryonic development, which can be easily followed thanks to the transparency of the embryos [111]. This feature sets the zebrafish apart from more complex animal models such as rodents, which being mammals share a higher similarity to human biology but also come with the drawback of internal embryonic development. In order to study a mouse embryo directly, the pregnant adult female must be sacrificed, making the study more complicated both from an ethical perspective and in terms of experimental simplicity, whereas zebrafish embryos can be followed in real time with microscopic analysis without the need to scarify any animals. Moreover, the high numerosity of individuals is an asset of other simple and established models, but the zebrafish extends this advantage to vertebrate biology, which makes it relatively similar to humans in many developmental processes and biological events. Indeed, this model has a high degree of genetic conservation with Homo sapiens, and compared to humans, the zebrafish genome has a similar number of genes, 26 thousands, distributed over a similar number of

chromosomes, 25 pairs, but for a total number of 1.4 billion bases, which is half the human amount [112].

For these reasons, the zebrafish was initially employed for developmental studies and forward genetics, for which it is particularly suited because it allows to mutagenize a huge number of individuals and screen a variety of phenotypes in a relatively short period of time. Later, with the advent of reverse genetics techniques, the zebrafish was also used to analyse the role of numerous genes, as its ease of handling and injection at the single cell stage made the use of antisense morpholino oligonucleotides (MO) a very effective technology for loss-of-function mutation studies. The MO technology, although still often used as a preliminary analysis technique, has been largely replaced with the introduction of directed gene editing techniques and the development of the CRISPR/Cas9 system because of its toxicity and off-target effects. In addition to its suitability for developmental biology and genetic studies, the zebrafish has also been studied for its regenerative capacity, and the ease of handling combined with the conservation of important signaling pathways and biological processes have made the zebrafish an excellent model for the study of numerous physiological and pathological processes [113].

1.4.2 Zebrafish as a model for the study of angiogenesis

The zebrafish is a highly versatile and robust vertebrate model system for studying vascular development and angiogenesis *in vivo*, as the mechanisms underlying these processes are similar to those found in mice and humans. This is particularly relevant as invertebrate organisms do not have a homologous structure for vertebrate blood vessels. Moreover, the external fertilization and optical transparency of zebrafish embryos and larvae allow easy tracking of blood vessel formation and circulatory defects in real time, which is generally a difficult task in other vertebrates due to the opacity or the internal development of the embryos. In addition, during the first days of zebrafish development, the embryos, being quite thin, can survive via passive diffusion of oxygen, which make them an extremely useful tool for studying phenotypes involving non-functional circulation. The rapid embryonic development of the zebrafish allows the pro- or antiangiogenic effects of a treatment or of genetic mutations to be observed in a much shorter period of time compared to mammalian animal models. In the zebrafish, the circulation begins at about 24-26 hours post-fertilization (hpf), with the formation of a simple circulatory loop and within 72 hours the basic architecture of the entire vasculature is already formed, with slight changes still occurring in the later stages of zebrafish development [114].

Two days after the fertilization, blood vessels called sub-intestinal veins (SIVs) begin to form, and within two days they grow into two vascular plexuses whose development can be easily followed, and observation of their morphological alterations has been used as a tool to study the effects of gene downregulation and drug treatment on the angiogenic process (Figure 18) [115]–[117]. Other structures have also been used to measure angiogenic effects, such as retinal blood vessels and stem microvasculature [118].

Figure 18. Vasculature of the zebrafish embryo. Graphic illustration of the basic vasculature loop of the zebrafish embryo at 1 day post-fertilization (dpf) on the left, and at 3 dpf on the right. DC=Duct of Cuvier; DA=dorsal aorta; AV=axial vein; H=heart; SIVs=sub-intestinal veins. Readapted from Serbedzija et al. (1999) [119]

There are several techniques that can be used to visualize the zebrafish vasculature. Some of them require the fixation of embryos or adult fish, while others can be performed with live animals. The first and most classical technique is non-vital staining, in which blood vessels are filled with a dye or resin by injection. Nowadays, these methods are often replaced by vital imaging techniques, but sometimes they still represent the best way to visualize the majority of the blood vessels, especially in adult fish whose thickness and opacity are incompatible with other methods. Confocal microangiography, in which fluorescent microspheres are injected into the bloodstream of a living animal, allowing very detailed visualization of all blood vessels reached by the active circulation, is very useful for embryos and young larvae, but the loss of transparency at later stages limits the use of this technique. In the early 2000s, genome editing enabled the development of transgenic zebrafish strains, expressing fluorescent proteins at the level of the vasculature, such as fli1a:EGFP and kdrl:EGFP. The introduction of these transgenic strains made it possible to study angiogenesis in live animals without the need for prior staining or injection, and also to perform real-time imaging of the animals, resulting in high-resolution time-lapse videos of the angiogenesis process [120].

1.4.3 Zebrafish as a model for the study of cancer

The zebrafish has proven to be an extremely versatile and suitable model for cancer research. Similarly to angiogenesis, the cancer process in zebrafish mimics very closely what happens in humans. Moreover, the possibility of working with an extremely high numerosity of small individuals in parallel, with a rapid development and ease of handling, makes zebrafish embryos an extremely useful tool for potential anti-cancer drug screening. Indeed, the small body size of the embryos, which can fit even into 384-well plate, combined with their permeability to small molecules diluted in fish water make them a very effective platform to screen thousands of compounds each week.

Two main methods have been employed to study cancer biology in zebrafish [115]. The genetic cancer model is based on genetic modifications of zebrafish genes (downregulation of an oncosuppressor or overexpression of an oncogene) that promote the growth of tumour masses. The main advantage of this method is that it better mimics the real dynamics of the tumour progression in humans, as the tumour forms spontaneously after the genetic modification and develops in a slow multistep process. The obvious drawback lies in the fact that the different genetic background and overall physiology of the fish might introduce discrepancies with the actual tumour biology found in humans [121].

The second method to investigate cancer in zebrafish is the transplantable model, which involves grafting tumour cells in the fish. The malignant cells can then grow, form a mass, and metastasize, and cancer progression can be easily followed by fluorescent labelling of the engrafted cells in the optically clear embryos and even in the transparent *casper* zebrafish strain. Transplantation usually involves the use of tumour cells from mice or humans, and it is then referred to as xenograft, but it can also consist of an allograft, which relies on zebrafish tumour cells. The main advantage of this technique over the genetic model is that the tumour mass emerges more rapidly after transplantation, allowing rapid which allows a quick analysis of tumour progression and mimicking highly proliferative primary tumours. A drawback of this technique is that xenografts in adult fish require immunosuppression to avoid rejection of the engrafted tumour. Xenografts are not rejected by zebrafish embryos as the immune system is not fully developed, but the adult model better mimics tumour biology in humans [122].

Interestingly, the zebrafish model has also been employed as a precision medicine tool. Thanks to the high degree of conservation of cancer-related genes as well as signaling pathways, receptors, and ligands involved in the proliferation, invasion, and differentiation of malignant cells, zebrafish avatars can be used as a preclinical screening platform to test the response to different treatments of a given

tumour. For example, tumour cells from patients can be engrafted in numerous zebrafish larvae and tested with different molecules to determine the best candidate for clinical treatment and whether a patient is likely to respond to a particular therapy [123].

1.4.4 Zebrafish as a model for the study of other pathologies

The more the physiology of the zebrafish is studied, the more this organism finds its application as a model for human pathologies. The transparency of embryos and larvae and the establishment of multiple transgenic strains make the zebrafish a very effective tool for monitoring angiogenesis, and the large number of individuals and similar genetic background provide an incredible platform for drug screening and cancer biology studies. The high similarity of tissues, developmental biology, and disease-associated proteins has allowed researchers to use zebrafish to study all sorts of pathologies, from blood disorder to heart failure and cardiovascular defects, from neurogenerative diseases to osteoporosis, from inflammation to type 2 diabetes. It has been reported that in the zebrafish we can find an orthologue for more than 80% of human disease-associated genes, and the strong genetic conservation combined with its versatility makes zebrafish a unique model for the study of most physio-pathological processes [124], [125].

1.4.5 Zebrafish transglutaminases

The family of the zebrafish transglutaminase genes was first studied by Deasey and coworkers, who identified 13 zebrafish TG genes (zTGs): five homologues of human *TGM1*, three homologues of human *TGM2* and three homologues of human *F13A1*. Each set of homologues likely originated from gene amplification following divergence from the common ancestors of the human orthologous gene. Conversely, no orthologues were described for *TGM3*, *TGM4*, *TGM5*, *TGM6*, and *TGM7*, but they identified two new TG isoforms similarly distant from all human TGs, which they named zTG-84 and zTG-91 [126]. The authors performed gene expression analysis of zTGs genes, which are characterized by a specific temporal expression pattern in larval and adult fish and described the involvement of zTGs proteins in zebrafish bone mineralization, as treatment with a TG inhibitor resulted in a decrease in vertebral mineralization. The TG2 orthologues were designated as zTG2b, zTG2c, and zTG2-12, and zTG2b was identified as the isoform with the most ubiquitous expression in tissues of all developmental stages, whereas expression of the other two was detected to a good extent only in the larval stage. The three zebrafish TG2 protein orthologues (zTGs2) were later defined as zTG2a, zTG2b, and zTG2like.

After this initial description of TGs in zebrafish and a later study on the role of transglutaminase proteins in nuclear localization of serotonin, in which the zebrafish was one of the models used to

study the effects of TGs inhibition by cystamine, only one recent publication provided new insights into zebrafish orthologues of TG2 [127]. The authors used employed zebrafish as a tool to study the role of TG2 in the Wnt/β-catenin signaling pathway and downregulated the 3 zTGs2 genes with the use of morpholino oligonucleotides. Visible morphological alterations, loss of pigmentation, and increased mortality were observed in embryos injected with the MO targeting zTG2b, while downregulation of the other two proteins had no effect on embryo viability and morphology, suggesting that zTG2b might be the most important TG2 orthologue in zebrafish embryonic development. Anti-zTG2b MO also caused suppression of the Wnt/β-catenin pathway, as evidenced by a decrease in fluorescence of transgenic Tg7xTCFX.lasiam:EGFP embryos, where Wnt signalling correlates with fluorescence signaling (Figure 19) [128].

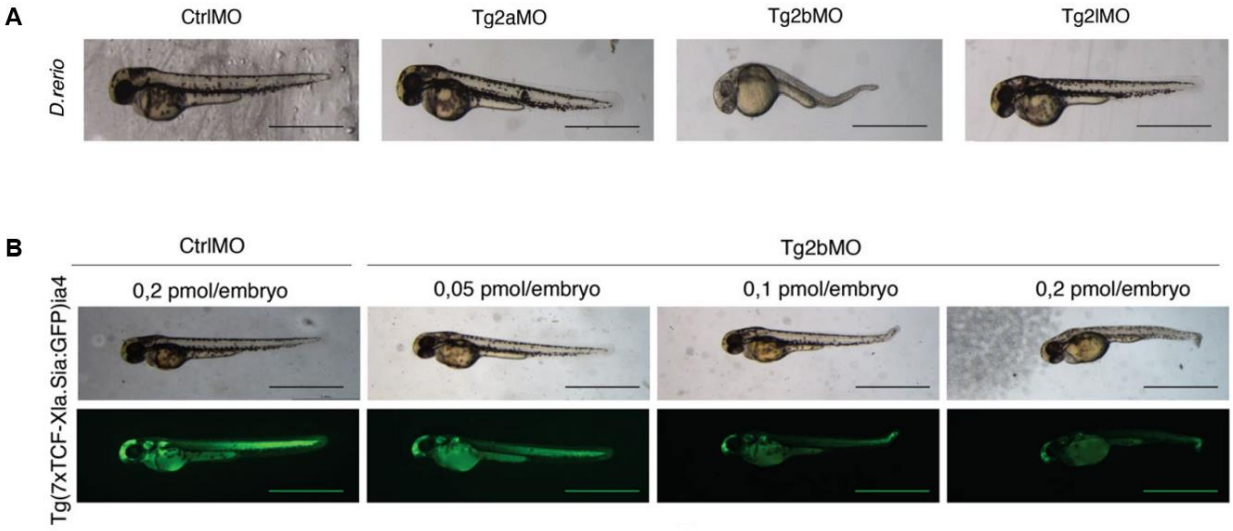


Figure 19. Knockdown of TG2b and Wnt signalling downregulation in zebrafish embryos. A) Transient knockdown of the three zebrafish TGs2 gene by injection of morpholino oligonucleotides (MO) resulted in visible alteration only in embryos treated with MO against zebrafish TG2b. A standard control MO was also used. Representative brightfield images of the embryos at 24 hours post fertilization (hpf) are reported. Bars = 1 mm. B) Zebrafish TGs2 gene knockdown by injection of MO in Tg(7xTCFX.lasiam:GFP)ia4 Wnt-dependent reporter embryos resulted in visible reduction of Wnt signalling fluorescence signal. Three increasing dosages of morpholinos (0.05, 0.1, and 0.2 pmol) were used. A standard control MO was also used. Representative brightfield and epifluorescence images of the embryos at 48 hpf are reported. Bars = 1 mm. Readapted from Rossin et al. (2021).

1.4.6 Transglutaminase 2 in medaka, another fish model

Because little is known about transglutaminase 2 in zebrafish, the study of TG2 in another fish model, the medaka, may provide some insights on this topic. The medaka fish (*Oryzias latipe*), a relatively recent animal model, shares many features and advantages of the zebrafish and has been the subject of a few studies on transglutaminases [129]–[132]. In medaka, one orthologue of human TG2 protein has been identified and called OlTGT. Moreover, this protein has been produced and purified as a recombinant 75 kDa protein from bacteria, and was shown to possess a transamidation activity insensitive to the regulatory action of GTP. Furthermore, immunohistochemical analysis showed that OlTGT is present only in the eye, spinal cord, gas gland, and brain, in contrast to the ubiquitous expression of the human orthologue [131]. In a recent study, genetic ablation of the *OlTGT* gene in medaka was shown to cause no morphological defects and no alteration in the propagation rate and lifespan of the animal. However, the mutants were characterized by movement retardation, when evaluated by a tank-diving test. Moreover, the mutants showed reduced expression of OlTGT in a region of the brain called periventricular layer of the optic tectum, where medaka TG2 was particularly expressed in wild type animals, suggesting that TG2 in this region might be important for motor functions [130].

2. AIMS OF THE PROJECT

Transglutaminase 2 is a multifunctional enzyme that has been demonstrated to be involved in several physiological and pathological processes. However, due to its diverse enzymatic and non-enzymatic functions and its widespread distribution in tissues, its actual role in biological events is often controversial. The aims of the project are

1. to clone and sequence the three zebrafish TGs2 (zTGs2), and describe their homology with the human orthologue,
2. to produce and purify the zTGs2 as recombinant proteins, in order to study their enzymatic properties and determine if the transamidation activity of hTG2 is conserved in the zebrafish orthologues,
3. to describe the functions of the zTGs2 both in the extracellular environment and in the intracellular compartment, in order to determine if the zebrafish orthologues play some of the important functional roles of hTG2 and might be involved in the same biological processes of the human enzyme.

Ultimately, this study aims to lay the foundations of the use of the zebrafish, a versatile and robust model of Vertebrate biology, as a valid tool to better characterize TG2 functions *in vivo*.

3. MATERIALS AND METHODS

Genetic analysis of TG2 orthologues in the zebrafish genome.

The human transglutaminase 2 and the Danio rerio orthologs has been aligned with MUSCLE. The resulting multiple alignment has been processed with Gblocks to remove the noninformative phylogenetically regions that were too divergent or corresponding to significant small insertions and deletions (indels). The output has been used to generate a phylogenetic tree with a Neighbor-joining (NJ) algorithm, with 100 bootstrap replicates, which allowed us to evaluate the solidity of the generated tree topology. The tree was reported as a cladogram, and the group of sequences corresponding to FXIIIa was used as outgroup for rooting.

Zebrafish embryos handling and maintenance

Fertilized zebrafish eggs were placed in E3 Medium (5mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) supplemented with methylene blue 0.5% and incubated at 28°C. 24 hours post-fertilization (hpf) the eggs were dechorionated with Pronase 1 mg/mL for 5 minutes at room temperature (RT). Following a wash in E3 Medium, the embryos were placed in E3 Medium supplemented with Phenylthiourea (PTU) 0.2 mM (final concentration) to prevent melanogenesis and thus the pigmentation of the larvae. Zebrafish embryos were handled according to standard rules and procedures for animal wellness (https://zfin.org).

RNA extraction from zebrafish embryos

Zebrafish embryos were euthanized with 0.2% Tricaine. Total RNA was extracted from experimental groups of 20-30 embryos with the PureLink™ RNA Mini Kit (Invitrogen™, cat#12183020) after embryos' lysis with TRIzol® reagent (Life technologies, cat#15596), following the manufacturer's instructions.

Gene expression study of the three zebrafish TG2 genes during the embryonic development

The expression of the three zebrafish TG2 2 (zTGs2) genes was studied by Quantitative Real-Time PCR (RT-qPCR) at 1, 3 and 5 days post-fertilization (dpf).

Primers pairs were designed using the open source Primer3 program (http://frodo.wi.mit.edu/) in order to map on different exons, to generate an amplicon of about 250-300 bp, and to have a melting temperature (tm) of about 60°C. Two housekeeping genes were selected as normalizers, LSM12 homolog b (lsm12b) and beta-actin (β-actin). The primer pair of lsm12b was previously validated by the work of Yu Hu and co-workers, whereas the primer pair of β-actin was previously validated by the work of Tang et co-workers [133], [134]. All the primers used for the gene expression analysis are listed in **Table 1**.

Table 1. List of primers used for the RT-qPCR experiments.

Primer name	**Sequence 5'-3'**
zTG2a Forward	CCAAAGCAGTGGGTCGAGAT
zTG2a Reverse	GTGCGTAAAACATCACCCGG
zTG2b Forward	GATGGGTCGTTCCGATCTCC
zTG2b Reverse	GCTGATCTTCTGGCCCACTT
zTG2like Forward	CACCTGCAGCCCTGAAAAAC
zTG2like Reverse	TCTGGTTGGGATGCCAAGAC
Beta actin Forward	CGAGCTGTCTTCCCATCCA
Beta actin Reverse	TCACCAACGTAGCTGTCTTTCTG
Lsm12b Forward	AGTTGTCCCAAGCCTATGCAATCAG
Lsm12b Reverse	CCACTCAGGAGGATAAAGACGAGTC
Hsp70 Forward	TCAAGCGCAACACAACCATC
Hsp70 Reverse	ATTTGCCCAGCAGGTTGTTG

RNA was extracted as previously described from 1, 3 and 5 days post fertilization (dpf) zebrafish embryos. After treatment with ezDNase (Invitrogen™, cat#11766051), 5 µg of total RNA was reverse transcribed to produce cDNA using SuperScript™ IV Reverse Transcriptase (Invitrogen™, cat#18090200). cDNA was diluted fivefold and used as a template to perform a quantitative Real Time PCR (qRT-PCR) with iQ™ SYBR® Green Supermix (Bio-Rad, cat#1708880) following the manufacturer's instructions in a CFC96 thermocycler (Bio-Rad). The relative amounts of the transcript were calculated with the comparative ΔΔCt method using β-actin and lsm12b as normalizers.

DNA sequencing

The three zTG2 coding sequences has been PCR-amplified with Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific, cat#F530S), purified with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, cat#NA1020) and sequenced with Sanger method. The complete list of the primers used for the sequencing is reported in **Table 2**.

Table 2 List of primers used for the sequencing of the three zTG2 coding sequences.

Primer name	**Sequence 5'-3'**
zTG2a inside sense	TTGGACTCTGTGTATCTGGCG
zTG2a inside anti	CATCCTGTCCTGAGTTATGGC
zTG2b inside sense	TGGTGTCCACTTGATGTG
zTG2b inside anti	GACGGATGAGGTTATCTTCTG
zTG2like inside sense	CTGCTGTTTAATCCCTGGTGC
zTG2like inside anti	GCTGTAACTCTTATCATGTGG
T7 promoter	TAATACGACTCACTATAGGG
T7 terminator	GCTAGTTATTGCTCAGCGG

Protein sequence analysis

The ExPASy Translate tool was used to obtain the protein sequences for the three zTG2. The percentage of similarity between three zebrafish TG2 and the human TG2 (accession #AAT79353) protein sequence was calculated with a pairwise sequence alignment performed with EMBOSS Needle online tool.

Molecular cloning of zebrafish transglutaminase 2 sequence into bacterial expression vector

The three zebrafish TG2 coding sequences have been cloned in the pET30a(+) bacterial expression vector, in order to produce the three TGs2 as hexahistidine-tagged (His6-tagged) recombinant proteins. Zebrafish TGs sequences were PCR-amplified with Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific, cat#F530S), using 2 dpf zebrafish embryos cDNA as template and zTGs2-specific primers, designed to insert restriction sites for KpnI and HindIII respectively at the 5' and 3' of the coding sequences (the primers' sequences are reported in **Table 3**). The PCR products

were gel purified with the GenElute™ Gel Extraction Kit (Sigma-Aldrich, cat#NA1010), following the manufacturer's instructions, and digested with KpnI and HindIII (New England BioLabs, cat#R3142 and cat#R3104) together with the recipient plasmid. The digested vector and DNA fragments were ligated with T4 DNA ligase (Thermo Scientific, cat#EL0011) and cloned into DH5α competent cells. Plasmid minipreps were obtained from overnight culture at 37°C in 2xTY medium with GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, cat#PLN70), and transformed into BL21(DE3) competent cells.

Table 3 List of primers used for the cloning of zTGs2 coding sequences in pET30a(+) bacterial expression vector.

Primer name	**Sequence 5'-3'**
zTG2a sense KpnI	AGATCTGGGTACCATGGAGAGAGTGGTGGAG
zTG2a anti HindIII	TCCTCGAGAAGCTTTTATTCATAGATAACCAG
zTG2b sense KpnI	AGATCTGGGTACCATGGCTCTGGACATCGGC
zTG2b anti HindIII	TCCTCGAGAAGCTTTCATTTCCCGATGATGAC
zTG2like sense KpnI	AGATCTGGGTACCATGGCCAGCTATAATGCC
zTG2like anti HindIII	TCCTCGAGAAGCTTCTAAATTTCTGGGACAGC

Recombinant protein production and purification

BL21(DE3) bacteria with the pET30a(+) plasmid vector bearing the zTG2 coding sequence were plated on a fresh 2xTY agar plate with 50 µg/mL Kanamycin (Kan) and left to grow overnight (O/N) at 30°C. For each zTG2, a single colony was inoculated in 5 mL of 2xTY broth with 50 µg/mL Kan and 1% (v/v) glucose. After 5 hours of growth at 37°C in an orbital shaker, the bacterial cultures were diluted 1:100 in 2xTY with Kan 50 µg/mL and left growing at 25°C in an orbital shaker. When the optical density at 600 nm (OD600nm) was about 0.6/0.8, the recombinant protein production was induced with 0.2 mM isopropyl-β-D-1-thiogalattopiranoside (IPTG) and was carried out for 16 hours at 25°C. The bacterial cultures were centrifuged at 4500 x g for 15 minutes at 10°C. Lysis of the bacterial pellet was performed in lysis buffer (20 mM Tris/HCl pH 8, 500 mM NaCl, 1% Triton X-100, 5 mM imidazole, 100 µg/mL lysozyme (Sigma-Aldrich, cat#34046) and 50 µg/mL DNase I (Sigma-Aldrich, D5025), supplemented with cOmplete™ Protease Inhibitor Cocktail Tablets (Roche, cat#04693116001). The bacterial lysate was sonicated three times at 30% amplitude for 30'' and then centrifuged at 11000 x g for 30 minutes at 10°C. The recombinant 6His-Tagged protein was column affinity-purified with HisPur™ Ni-NTA Resin (Thermo Scientific cat#88221) from the supernatant,

using a 20 mM Tris/HCl pH8, 500 mM NaCl, 10 mM imidazole solution as equilibration buffer, a 20 mM Tris/HCl pH8, 500 mM NaCl, 20 mM imidazole solution as wash buffer, and a 20 mM Tris/HCl pH 8, 500 mM NaCl, 300 mM imidazole solution as elution buffer. The purified proteins were dialysed for 16 hours at 8°C in Tris/Acetate-EDTA buffer supplemented with 5 mM dithiothreitol (DTT). Recombinant human TG2 (hTG2) was produced in the same way starting from BL21(DE3) competent cells, previously transformed with the expression vector pET28a(+) containing hTG2 coding sequence.

Protein quantification

Recombinant protein samples were denatured for 5 minutes with Laemmli buffer in boiling water and resolved on SDS-polyacrylamide gels. For quantification, the samples of unknown concentration were resolved together with a calibration curve of BSA (Sigma-Aldrich, cat#P0834). The polyacrylamide gels were coloured with Coomassie brilliant blue and bands volume was measured with Image Lab Software (Bio-Rad) after imaging with ChemiDoc™ MP Imaging System (Bio-Rad).

SDS-PAGE and Western blot of TGs2 recombinant proteins

10 nanograms of each recombinant affinity-purified protein were tested in western blot (WB) using an anti-hexahistidine Tag (6His-Tag) antibody. The samples were denatured for 5 minutes with loading buffer in boiling water and resolved on SDS-polyacrylamide gel. The proteins were transferred with Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) onto a nitrocellulose membrane. After a blocking step of 45 minutes with 5% skim milk in PBS at room temperature (RT), the membrane was incubated with the for 1 hour with HRP-conjugated anti-6His-Tag antibody diluted in the blocking buffer in volumetric ratio 1:5000. After three washes with PBS-Tween 20 0.1% and three with PBS, the membrane was developed with Pierce™ ECL Western Blotting Substrate (Thermo Scientific, cat#32209) and imaged with ChemiDoc™ MP Imaging System (Bio-Rad).

zTGs2 transamidation activity curve

ELISA wells were coated O/N at 4°C for 16 hours with 120 µL of 15 µg/mL N,N'-dimethyl casein (DMC) in PBS. After one wash with PBS, 100 µL of reaction mix (100 mM Tris-HCl, pH 8.0, 0.2 mM EZ-Link™ Pentylamine-Biotin from Thermo Scientific cat#21345, 10 mM DTT, 5 mM CaCl2) with increasing amounts of recombinant TG2 were added in each well and the plate was incubated

for 1 hour at 37°C. Alternately, 100 µL of reaction mix with 50 ng of recombinant TG2 and increasing concentrations of $CaCl_2$ were added in each well and the plate was incubated for 1 hour at 37°C. Following three washes with PBS-Tween 20 0.1% and three with PBS, the plate was incubated for 1 hour at 37°C with 100 µL per well of Pierce™ High Sensitivity Streptavidin-HRP (Thermo Scientific, cat#21134) diluted in a solution of 1% BSA in PBS in volumetric ratio 1:200. After washes, the colorimetric reaction was developed by adding 100 µL per well of 3,3',5,5-tetramethylbenzidine (TMB) and stopped after 5 minutes by adding 50 µL per well of 2.5 mM H_2SO_4. The absorbance was measured at 450 nm using a microplate reader (TECAN®).

In solution zTGs2 transamidation activity assay

In solution transamidation activity assay was performed by testing the incorporation of pentylamine-biotin catalysed by 100 ng of affinity-purified TG2 into 3 µg of DMC substrate in three conditions: 50 µL of reaction mix (100 mM Tris-HCl, pH 8.0, 0.2 mM EZ-Link™ Pentylamine-Biotin, 10 mM DTT, 5 mM CaCl2); 50 µL of reaction mix without 0.2 mM pentylamine-biotin; and 50 µL of reaction mix with 5 mM EDTA instead of $CaCl_2$, as a negative control. The reaction was allowed to proceed for 1 hour at 37°C. Then, the reaction mixture was denatured by heating for 5 minutes in boiling water in Laemmli buffer and resolved on SDS-polyacrylamide gel. The proteins were transferred with Trans-Blot SD Semi-Dry Transfer Cell onto a nitrocellulose membrane. After a blocking step of 45 minutes with 5% skim milk in PBS at RT, three washes with PBS-Tween 0.1% and three with PBS, the membrane was incubated for 1 hour at RT with HRP-conjugated streptavidin diluted in PBS-BSA 1% in volumetric ratio 1:200. After three washes with PBS-Tween 20 0.1% and three with PBS, the membrane was developed with Pierce™ ECL Western Blotting Substrate and imaged with ChemiDoc™ MP Imaging System.

zTGs2 transamidation activity GTP-dependent inhibition assay

ELISA wells were coated O/N at 4°C for 16 hours with 120 µL of 15µg/mL N,N'-dimethyl casein (DMC) in PBS. After one wash with PBS, 100 µL of reaction mix with 50 ng of recombinant TG2 was incubated for 1 hour at 37°C, in the presence or in the absence of 100 µM guanosine 5'-triphosphate (GTP). Following three washes with PBS-Tween 20 0.1% and three with PBS, the plate was incubated for 1 hour at 37°C with 100 µL per well of Pierce™ High Sensitivity Streptavidin-HRP diluted in a solution of 1% BSA in PBS in volumetric ratio 1:200. After washes, the colorimetric reaction was developed by adding 100 µL per well of TMB and stopped after 5 minutes by adding 50

µL per well of 2.5 mM H_2SO_4. The absorbance was measured at 450 nm using a microplate reader (TECAN®).

zTGs2 transamidation activity inhibition assay with hTG2- specific inhibitors

ELISA wells were coated O/N at 4°C for 16 hours with 120 µL of 15 µg/mL N,N'-dimethyl casein (DMC) in PBS. After one wash with PBS, 100 µL of reaction mix with 50 ng of recombinant TG2 was incubated for 1 hour at 37°C, in the presence or in the absence of hTG2-specific peptide inhibitors 1-155, R281 or ZDON (Sigma-Aldrich, cat#616467**)** at three different concentration (25 mM, 2.5 mM and 0.25 mM). Following three washes with PBS-Tween 20 0.1% and three with PBS, the plate was incubated for 1 hour at 37°C with 100 µL per well of Pierce™ High Sensitivity Streptavidin-HRP diluted in a solution of 1% BSA in PBS in volumetric ratio 1:200. After washes, the colorimetric reaction was developed by adding 100 µL per well of TMB and stopped after 5 minutes by adding 50 µL per well of 2.5 mM H_2SO_4. The absorbance was measured at 450 nm using a microplate reader (TECAN ®).

RGD-independent cell adhesion assay

Tissue culture plastic was coated overnight at 4 °C with 50 µL of human fibronectin (Sigma-Aldrich, cat# F0895) 5 µg/mL in 50 mM Tris-HCl, pH 7.4. After one wash with 100 µL of 50 mM Tris-HCl, pH 7.4, the fibronectin (FN) matrix was blocked with 100 µL of 3% (w/v) heat-inactivated BSA in PBS for 30 minutes at 37°. After one wash, hTG2 and zTGs2 were immobilized on FN matrix by incubating 100 µL of 5 µg/mL TGs2 in 2 mM EDTA in PBS, pH 7.4 at 37°C for 1 hour. Exponentially growing human foreskin dermal fibroblasts (HFDFs) were detached with 0.25% (w/v) trypsin in 5 mM EDTA, washed twice in serum free medium to remove the traces of serum proteins and incubated with or without 150 µg/ml G**RGD**TP synthetic peptide for 20 min at 37°C in a 5% CO_2, 95% (v/v) air atmosphere. Then, cells were seeded on matrices at a density of 3 x 10^5 cells/mL for a maximum of 20 minutes to minimize the secretion of any endogenous protein at 37°C in a 5% CO_2, 95% (v/v) air atmosphere. After 1 gentle wash with PBS to remove non-attached cells, the cells were fixed in 3.7% (w/v) paraformaldehyde in PBS for 15 minutes at room temperature, then washed twice with PBS and permeabilized in 0.1% (v/v) Triton X-100 in PBS for 15 min at room temperature. After two washed with PBS, the cells were stained first with May-Grunwald stain, then with Giemsa stain. Digital images of 3 non-overlapping fields covering the central portion of each well were captured using a video digital camera (Nikon E5400). At least 9 images of separate fields per sample were examined.

Actin cytoskeletal staining

Nunc™ Lab-Tek™ Chamber Slide System (Thermo Scientific, cat#177445) was coated overnight at 4°C with 150 µL of 5 µg/mL FN in 50 mM Tris-HCl, pH 7.4. After one wash with 200 µL of 50 mM Tris-HCl, pH 7.4, the FN matrix was blocked with 200 µL of PBS buffer supplemented with 3% (w/v) heat-inactivated BSA for 30 minutes at 37°. After one wash, hTG2 and zTGs2 were immobilized on FN matrix by incubating 200 µL of 5 µg/mL TGs2 in 2 mM EDTA in PBS, pH 7.4 at 37°C for 1 hour. Exponentially growing HFDFs were detached with 0.25% (w/v) trypsin in 5 mM EDTA, washed twice in serum free medium to remove the traces of serum proteins and incubated with or without 150 µg/ml GRGDTP synthetic peptide for 20 min at 37°C in a 5% CO_2, 95% (v/v) air atmosphere. Then, cells were seeded on matrices at a density of 6 x 10^4 cells per well for a maximum of 20 minutes to minimize the secretion of any endogenous protein at 37°C in a 5% CO_2, 95% (v/v) air atmosphere. After 1 gentle wash with PBS to remove non-attached cells, the cells were fixed in 3.7% (w/v) paraformaldehyde in PBS for 15 minutes at room temperature, then washed twice with PBS and permeabilized in 0.1% (v/v) Triton X-100 in PBS for 15 min at room temperature. Cells were then blocked in PBS buffer supplemented with 3% (w/v) heat-inactivated BSA and then incubated with FITC-labelled phalloidin (20 µg/ml) in blocking buffer. After three washes with PBS, Coverslips were mounted with Vectashield mountant containing propidium iodide (Vector Laboratories) and examined by epifluorescence microscopy.

Molecular cloning of zebrafish transglutaminase 2 sequence into eukaryotic expression vector

The hTG2 and zTGs2 coding sequences have been cloned in a modified version of pCDNA 3.1 HYGRO(+) eukaryotic expression vector, in order to express the TGs2 as SV5-tagged proteins in HEK293 cells. Zebrafish TGs cds were PCR-amplified with Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific, cat#F530S), using pET30a(+) plasmid vector bearing the TGs2 coding sequences as template and TGs2-specific primers, designed to insert restriction sites for XbaI or NheI and HindIII respectively at the 5' and 3' of the coding sequences, as well as the nucleotide sequence encoding the SV5 tag (the primers' sequences are reported in **Table 4**). The PCR products were gel purified with the GenElute™ Gel Extraction Kit, following the manufacturer's instructions, and digested with XbaI or NheI and HindIII (New England BioLabs, cat#R0145, cat#R3131 and cat#R3104) together with the recipient plasmid. The digested vector and DNA fragments were ligated with T4 DNA ligase and cloned into DH5α competent cells. Plasmid minipreps were obtained from overnight culture at 37°C in 2xTY medium with GenElute™ Plasmid Miniprep Kit.

Transient cell transfection and selection of stable TG2-expressing HEK293 cells

HEK293 cells were cultured in DMEM (Gibco™, cat#41966) supplemented with 10% (w/v) FBS and 1% (w/v) Penicillin-Streptomycin (Gibco™, cat#15140122). For transfection, HEK293 cells were seeded in 6-well plate and eukaryotic expression vector pCDNA 3.1 HYGRO(+) containing the sequence encoding hTG2 or zTGs2 fused to SV5 tag was transfected with Lipofectamine™ 2000 Transfection Reagent (cat#11668019), according to manufacturer's instructions. After 48h, the cells were either detached with 0.25% (w/v) trypsin in 5 mM EDTA for cell lysis or cultured with culture medium supplemented with 200 μg/mL Hygromycin B Gold (InvivoGen).

Table 4 List of primers used for the cloning of hTG2 and zTGs2 coding sequences in eukaryotic expression vector pCDNA 3.1 HYGRO(+).

Primer name	**Sequence 5'-3'**
hTG2-HYGRO-Sense_NheI	GCTGGCTAGCTGCCACCATGGCCGAGGAGCTGGTC
hTG2-HYGRO-anti_SV5	GATTGGTTTGCCACTAGTGGCGGGGCCAATGATGAC
zTG2a-HYGRO-Sense_Xba	AGCTGTCTAGATGCCACCATGGAGAGAGTGGTGG
zTG2a-HYGRO-anti_SV5	GATTGGTTTGCCACTAGTTTCATAGATAACCAGATTC
zTG2b-HYGRO-Sense_NheI	GCTGGCTAGCTGCCACCATGGCTCTGGACATCGGC
zTG2b-HYGRO-anti_SV5	GATTGGTTTGCCACTAGTTTTCCCGATGATGACGTTC
zTG2like-HYGRO-Sense_Xba	GCTGTCTAGATGCCACCATGGCCAGCTATAATGCC
zTG2like-HYGRO-anti_SV5	GATTGGTTTGCCACTAGTAATTTCTGGGACAGCATTTAC
SV5_HindIII_anti	AGCTAAGCTTTTAAGTACTATCCAGGCCCAGCAG TGGGTTTGGGATTGGTTTGCCACTAGT

HEK293 anti-SV5 tag Western Blot

HEK293 cells expressing hTG2, zTGs2, or the non-transfected negative control cells were lysed in lysis buffer (Tris-HCl 20 mM pH 8, NaCl 500 mM, 1% Triton X-100, supplemented with cOmplete™ Protease Inhibitor Cocktail Tablets), sonicated and centrifuged at 14000 x g for 15 minutes at 4°C. The clear protein lysates were collected, quantified with Bradford reagent (Supelco, cat#B6916), denatured for 5 minutes with loading buffer in boiling water and resolved on SDS-polyacrylamide gel. The proteins were transferred with Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) onto a nitrocellulose membrane. After a blocking step of 45 minutes with 5% skim milk in PBS at room temperature (RT), the membrane was incubated with the for 1 hour with anti-SV5 antibody (isolated

and produced by the laboratory), diluted in the blocking buffer in volumetric ratio 1:5000. After three washes with PBS-Tween 20 0.1% and three with PBS, the membrane was incubated with anti-mouse IgG antibody (Jackson) diluted in the blocking buffer in volumetric ratio 1:2500. After three washes with PBS-Tween 20 0.1% and three with PBS, the membrane was developed with Pierce™ ECL Western Blotting Substrate (Thermo Scientific, cat#32209) and imaged with ChemiDoc™ MP Imaging System (Bio-Rad).

HEK293 cell lysates transamidation activity assay

ELISA wells were coated O/N at 4°C for 16 hours with 120 µL of 15µg/mL N,N'-dimethyl casein (DMC) in PBS. After one wash with PBS, 100 µL of reaction mix (100 mM Tris-HCl, pH 8.0, 0.2 mM EZ-Link™ Pentylamine-Biotin from Thermo Scientific cat#21345, 10 mM DTT, 5 mM CaCl2) with HEK293 cell protein lysates were added in each well and the plate was incubated for 1 hour at 37°C. For protein lysates of HEK293 cells after transient transfection, 1 µg of lysate per well was used. For protein lysates of hygromycin-selected HEK293 stable clones expressing TGs2, the amount of samples used was normalized on the level of TG2 expression, measured by quantifying the relative protein band on the nitrocellulose membrane with Image Lab Software (Bio-Rad). Following three washes with PBS-Tween 20 0.1% and three with PBS, the plate was incubated for 1 hour at 37°C with 100 µL per well of Pierce™ High Sensitivity Streptavidin-HRP diluted in a solution of 1% BSA in PBS in volumetric ratio 1:200. After washes, the colorimetric reaction was developed by adding 100 µL per well of TMB and stopped after 5 minutes by adding 50 µL per well of 2.5 mM H_2SO_4. The absorbance was measured at 450 nm using a microplate reader (TECAN ®).

HEK293 cells flow cytometry analysis

HEK293 cells expressing TGs2 and wild type HEK293 were detached with 0.25% (w/v) trypsin in 5 mM EDTA, washed in PBS and fixed in 3.7% (w/v) paraformaldehyde in PBS for 15 minutes at room temperature, then washed twice with PBS and permeabilized in PBS supplemented with 0.1% (v/v) Triton X-100 and 2% (w/v) BSA for 30 min at room temperature. After two washes, cells were either stained with anti-SV5 tag antibody conjugated with phycoerythrin (α-SV5-PE) on ice for 30 minutes, washed and analysed with Attune NxT Flow Cytometer (Invitrogen).

Apoptosis assays

HEK293 cells expressing TGs2 and wild type HEK293 cells were detached with 0.25% (w/v) trypsin in 5 mM EDTA and seeded in 24-well plate in culture medium supplemented with 2% FBS. After 16 hours, cells were treated with 0.4 mM H_20_2 for 4 hours. Untreated cells were used as control. After treatment, the cells were detached with 0.25% (w/v) trypsin in 5 mM EDTA, washed in annexin binding buffer (HEPES 20 mM, 150 mM NaCl, 2.5 $CaCl_2$) and stained for 15 minutes with Annexin V-fluorescein isothiocyanate (FITC) (ImmunoTools, cat#31490013) and Propidium Iodide (PI) Ready-Flow™ Reagent (Invitrogen™, cat#R37169). Unstained wild type cells and wild type cells stained with Annexin V-FITC only and PI only were used as control samples for fluorescence compensation. Cells were analysed with Attune NxT Flow Cytometer (Invitrogen).

4. RESULTS AND DISCUSSION

In this thesis, we report the biochemical and functional characterization of the zebrafish orthologues of human TG2. After a phylogenetic and gene expression analysis, we cloned and analysed the sequences of the zebrafish TGs2, describing a high conservation of regions important for the hallmark transamidation activity of transglutaminases. We successfully produced and purified the zebrafish TGs2 as recombinant proteins and demonstrated that they can catalyse a Ca^{2+}-dependent transamidation activity, similarly to the human enzyme. However, we showed that this enzymatic activity is not inhibited by the presence of GTP in the zebrafish proteins but can be modulated by hTG2-specific inhibitors. Finally, we confirmed a high conservation of the functional properties of human TG2 in the zebrafish enzymes. Indeed, we demonstrated that, similarly to human TG2, these proteins can mediate an important cell adhesion function when bound to matrix fibronectin in the extracellular environment, and also play an anti-apoptotic role in the intracellular compartment.

4.1 Phylogenetic analysis and gene expression study of the zebrafish transglutaminase 2 genes

In 2012, a publication from Deasey and co-workers described for the first time the transglutaminase gene family in Danio rerio, and since then no additional evidence on the genomic organization of the TG gene family and on its genes have been provided [126]. For this reason, we performed a phylogenetic analysis on updated datasets. We confirmed that the TG gene family in the zebrafish genome comprises 13 genes, with five orthologues of *TGM1*, three for *F13A1* and three for *TGM2*, highlighted in the red box in Figure 20. Among the *TGM2* orthologue, we confirmed the presence of two genes with a common ancestor with the human TG2 gene, which according to the current nomenclature are named *tgm2a* and *tgm2b*, and one more distant predicted gene, called *tgm2l*.

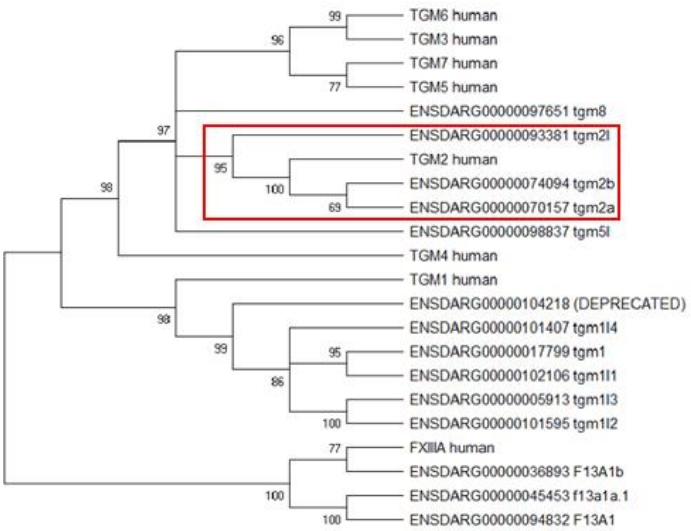


Figure 20. Phylogenetic analysis of the zebrafish transglutaminase gene family. Cladogram showing the evolutionary relationships between the human transglutaminase gene family and the zebrafish orthologues. The red box highlights the three TG2 orthologues present in the zebrafish genome: *tgm2a*, *tgm2b*, and *tgm2l*.

Confirmed the existence of three orthologues of the gene encoding the hTG2 protein, we then decided to investigate if these genes were expressed during the embryonic development. Published transcriptomic data of zebrafish embryonic development showed good expression levels of *tgm2a* e *tgm2b* in the first days of development, and very low levels of *tgm2l* gene expression [135]. We choose day 1, day 3 and day 5 post-fertilization as experimental points to measure the gene expression of the 3 zebrafish TGs2 orthologues, and we extracted total RNA from different pools of embryos at these three days of development. After DNase digestion and reverse transcription, the cDNA was used as template for RT-qPCR, and three sets of specific oligonucleotides were designed to amplify the three genes. According to our results, all the three genes are expressed throughout the embryonic development. In particular, we observed that *tgm2a* is the most expressed gene among the three, and its expression decreases during the first days of development. A different expression profile was detected for *tgm2b*, whose expression levels does not substantially change from 1 to 5 days post-fertilization (dpf). Compared to the other two genes, very low levels of expression were observed for *tgm2l*, which also shows a progressive decrease in gene expression along the first days of development (Figure 21).

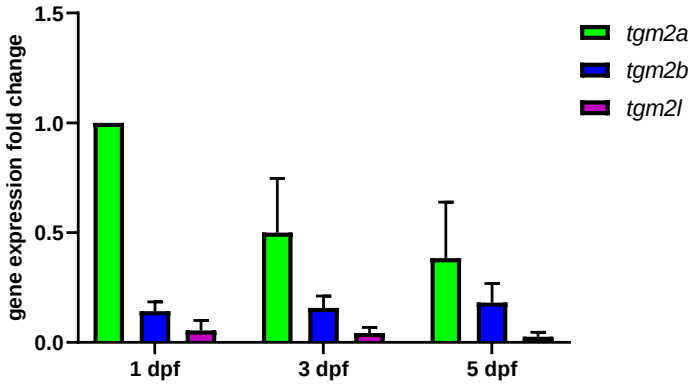


Figure 21. Gene expression analysis of the zebrafish transglutaminase 2 genes. RT-qPCR expression analysis of the zebrafish transglutaminase 2 genes. The plot shows the gene expression fold change of the 3 zebrafish TG2 orthologues at 1, 3 and 5 dpf. The expression data are normalized with the Ct of the zebrafish b-actin gene. The fold change has been calculated with ΔΔCt method and represented setting the value of *tgm2a* at 1dpf as 1. All conditions have been tested in triplicate and each plot represents the averaged result of five independent experiments (n=5).

According to these data, we confirmed that the zebrafish genome has three genes identifiable as orthologues of the human transglutaminase 2 gene, named *tgm2a*, *tgm2b* and *tgm2l*. Moreover, we demonstrated that all the three genes are expressed throughout the embryonic development.

4.2 Zebrafish TGs2 sequence homology

We then decided to better characterize the three transglutaminase 2 genes and the encoded proteins sequences. For this purpose, we cloned and sequenced the three TGs2 genes and compared the sequencing outputs with the sequences deposited into the NCBI database. Sequenced *tgm2a* showed seven mismatches with the NCBI entry, with two amino acid changes: an aspartic acid instead of a glutamic acid at position 147, and a threonine instead of an alanine at position 620. Sequenced zTG2b showed only one mismatch resulting in a glutamic acid instead of an aspartic acid at position 200, while zTG2like had 100% identity with the sequence deposited into the NCBI database (Table 5). Overall, we found only few nucleotide differences, which are likely due to allelic differences found among individual zebrafish organisms or strains.

	n° of nucleotide mismatches	Aminoacidic differences
tgm2a	7	**D147E; T620A**
tgm2b	1	**E200D**
tgm2l	0	/

Table 5. Nucleotide and amino acid differences between the sequencing outputs and the zebrafish TGs2 gene sequences deposited in the NCBI database.

Starting from the genes sequences, we calculated the predicted encoded proteins sequences for the three zebrafish TGs2 (zTGs2), named zTG2a, zTG2b, and zTG2like, and we aligned them with the human transglutaminases TG1-7 and FXIIIa (Table 6).

	hTG1	hTG2	hTG3	hTG4	hTG5	hTG6	hTG7	hFXIIIa
zTG2a	42.2	**64.6**	56.7	53.2	57.4	54.5	54.1	44.4
zTG2b	43.2	**67.9**	55.7a	51.3	55.1	55.4	55.2	47.5
zTG2like	44.2	**58.3**	52.9	49.6	53.3	54.6	54.9	49.1

Table 6. Similarity between the zTGs2 and the human transglutaminases. The percentage of similarity between three zebrafish TG2 and the human transglutaminases protein sequences was calculated with pairwise sequence alignments performed with EMBOSS Needle online tool.

We found that the zTG2b, encoded by the *tgm2b* gene, is the zebrafish TG2 with the higher degree of similarity with the human TG2 enzyme, with a score of 68% of homology. When compared with other members of the human TGs protein family, the homology ranges from 43% for hTG1 and 56 for hTG3. The same applies for the other two zebrafish TGs2, as zTG2a, encoded by the *tgm2a* gene, has a 65% of homology for hTG2, compared to 42% for hTG1, while the zTG2like, encoded by the *tgm2l* gene, shows a 58% of homology for hTG2 and indeed is the zebrafish isoform with the lowest similarity. However, zTG2like shows higher similarity for human TG2 compared to other TGs members, as the homology values goes from 44% of for hTG1 to 55 for hTG7. This analysis on the TGs protein sequences reflects the evolutionary relationships among their encoding genes shown above in the cladogram, as zTG2a and zTG2b proteins are more similar to hTG2 than to zTG2like (Table 7).

	zTG2a	zTG2b	zTG2like	hTG2
zTG2a	-	68.2	56.4	64.6
zTG2b	68.2	-	58.2	67.9
zTG2like	56.4	58.2	-	58.3

Table 7. Similarity among the three zTG2 protein sequences. The percentage of similarity was calculated with pairwise sequence alignments performed with EMBOSS Needle online tool.

Given the high similarity of the zTGs2 amino acid sequences compared to the human enzyme, we looked at the three-dimensional folded structure of the zebrafish TG2, to see if the overall conformation of the proteins is conserved (Figure 22).

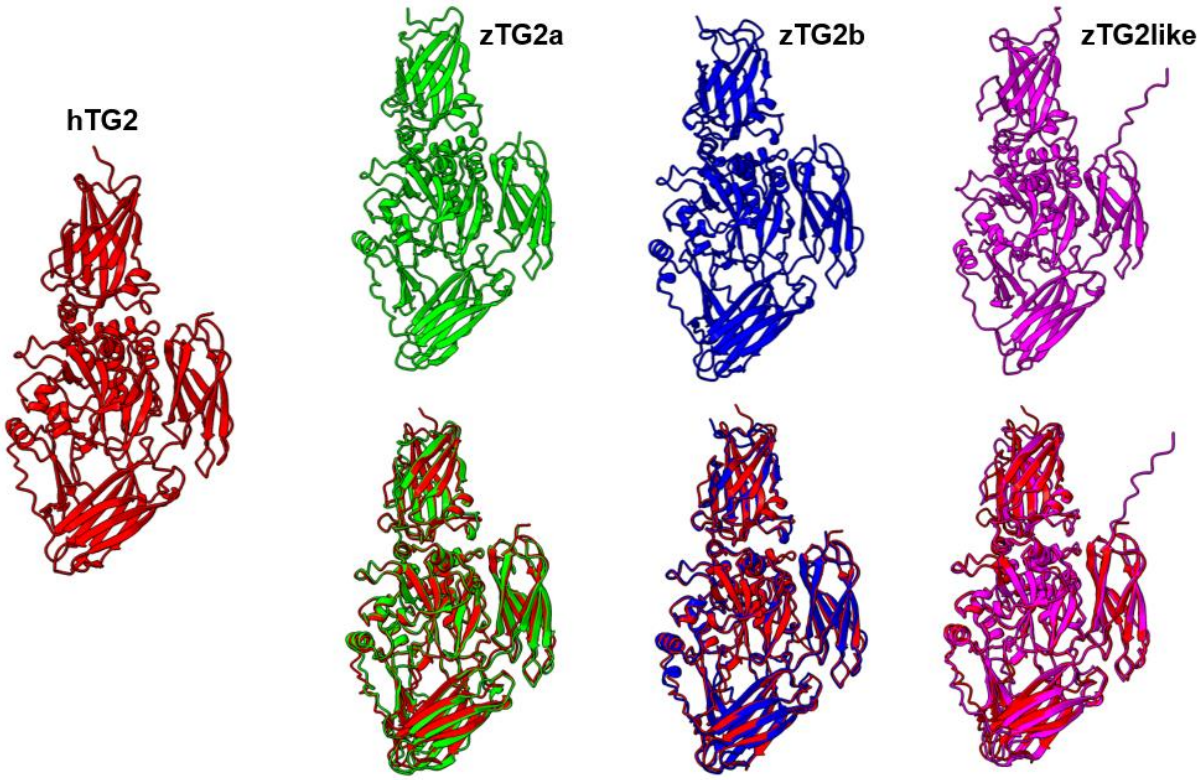


Figure 22. 3D models of the predicted structures of the zTGs2 proteins. The figure shows the predicted 3D models of the three zebrafish TGs2 proteins and the human orthologue for comparison (coloured in red, on the left). Moreover, 3D models representing the superpositions of the 3 zebrafish TGs2 structure with the human TG2 are also shown, below each of the three zTGs2. The overall structure is highly similar among the 4 proteins, with high conservation of both the secondary and tertiary structures. The three zebrafish TG2 present the same 4 domains as the human orthologue: a N terminal β-sandwich, a catalytic core and two C terminal β-barrels. The 3D models are predicted by the AlphaFold tools and rendered with the ChimeraX software.

We used the AlphaFold online tools to obtain the models of the 3D structures of the zebrafish TGs2, based on the protein sequences encoded by the three zTGs2 DNA sequences resulting from the sequencing. The zTGs2 3D models, together with the 3D structure of human TG2 in the closed conformation for comparison, were rendered and superimposed by UCSF ChimeraX [136]–[139].

The 3D models of the three zebrafish proteins show very high similarity of both secondary and tertiary structures with the human enzyme. Moreover, according to these models, the three zTGs2 appear to maintain the same structural organization in 4 domains: a N terminal β-sandwich, a catalytic core and two C terminal β-barrels.

These data point out that the three zebrafish proteins are indeed closer to human TG2 compared to the other members of the transglutaminase family of proteins in humans, with zTG2like being equally distant from the other two zebrafish isoforms and the human orthologue. Moreover, the high similarity is also conserved at the structural level, as all the three orthologues present a structure very close to the one reported for the human enzyme, with the typical subdivision in 4 domains.

4.3 Zebrafish TGs2 protein production and purification

Given the high sequence and structural homology between the zebrafish and human proteins, we decided to produce the three zTGs2 as recombinant proteins to test their enzymatic and non-enzymatic functions.

In order to produce and purify the three zebrafish TG2 as recombinant proteins, we designed three pairs of primers to PCR-amplify the three sequences and clone them in the bacterial expression vector pET30a+. We PCR-amplified the three zTGs2 coding sequence starting from cDNA deriving from total RNA extracted from a pool of thirty 3 dpf embryos and cloned them into the recipient plasmid. In this way, we obtained three different plasmids, each encoding one of the three zTG2 as recombinant protein fused with a N terminal 6HisTag (Figure 23a). For the production of zTGs2 proteins, the plasmids were then transformed in BL21(DE3) competent cells, an excellent expression system for heterologous proteins production. Indeed, previous work from the team led to an adapted protocol for the production and purification of human TG2 from BL21(DE3), with quite consistent levels of yields, purity, and functionality, and indeed, hTG2 produced with this system has been used as positive control sample for many of the experiments reported in this thesis. Given the high similarity of both sequence and predicted structure between the zebrafish and human TGs2, we decided to employ the same system as a starting point. However, in order to obtain sufficient amounts of recombinant zTGs2 we had to change several parameters in the production and purification protocol,

including duration and temperature of the bacterial growth, concentration of IPTG for the induction of heterologous protein synthesis, bacterial strain, and relative position of the 6HisTag (N terminal or C terminal). Figure 23b illustrates the optimization phase that led to the final protocol used for the production and purification from soluble protein lysates of BL21(DE3) bacteria of the zTGs2 as N-terminal 6His-tagged recombinant protein.

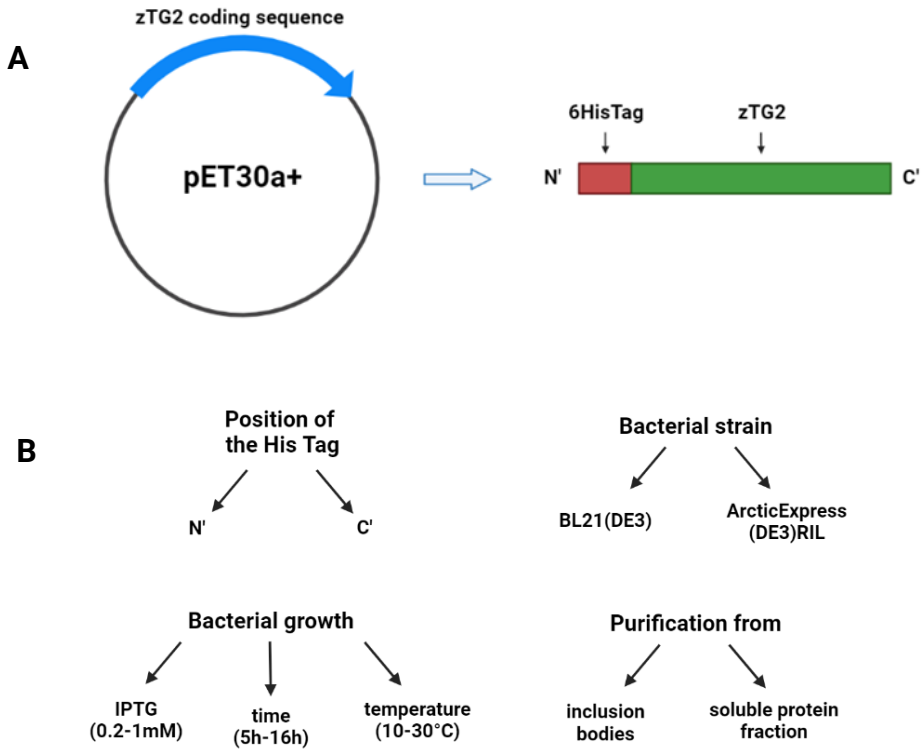


Figure 23. Optimization of the production and purification protocol. A) The zTGs2 coding sequences were cloned into the pET30a+ bacteria expression vector and produced as N terminal 6His tagged-recombinant proteins from BL21(DE3) bacteria. B) The zTGs2 production and purification protocol optimization involved changes to several parameters. We cloned the zTGs2 in the recipient vector with the 6HisTag at the N terminal or the at C terminal of the protein sequence. Moreover, we tried to produce and purified the zTGs2 from two different bacterial strains: BL21(DE3) and ArcticExpress (DE3)RIL. The recombinant protein production was induced with different concentrations of IPTG, and then the bacterial culture was grown at different temperatures and for different times. Finally, we tried to purify the protein from the soluble fraction of the bacterial lysates and also from the insoluble inclusion bodies.

Once produced and purified, we tested the three zTGs2 proteins both in SDS-PAGE and Western Blot (Figure 24), together with the human TG2 as a positive control. As shown, we observed the TGs2 protein bands at the expected molecular weight (75-80 kDa) in the SDS-PAGE (Fig. 24a) and with an anti-6His-Tag antibody in the Western Blot (Fig. 24b). The different heights of the protein bands in the Figure reflect the different sizes of the TGs2, as predicted by their aminoacidic sequences, confirming the purification of the three proteins, even though at suboptimal levels of purity. Indeed, the protein gel shows the presence of several bands in the lane of the purified proteins samples, corresponding either to undesired impurities either degradation products of the

transglutaminases. In particular, the Western blot assay shows the presence of several bands at lower molecular weights, which are detected by the anti-6His-Tag antibody and are likely degradation products of zTG2b cleaved from the C-terminus of the protein, as they retain the N-terminal tag. Moreover, compared to the yield normally associated to the production and purification process of the human TG2 in our laboratory, the amount of the final purified zTGs2 is very low, as the majority of the protein produced in bacteria accumulates as insoluble inclusion bodies. In particular, compared to an average of 2 mg of soluble recombinant hTG2 per litre of bacterial culture, which represents only 10% of the total hTG2 actually produced by the bacterial culture, we purified a maximum of 0,85 mg per litre for zTG2a and a minimum of 0.12 mg per litre for zTG2like, which highlights the peculiar insolubility of the recombinant zebrafish TGs2.

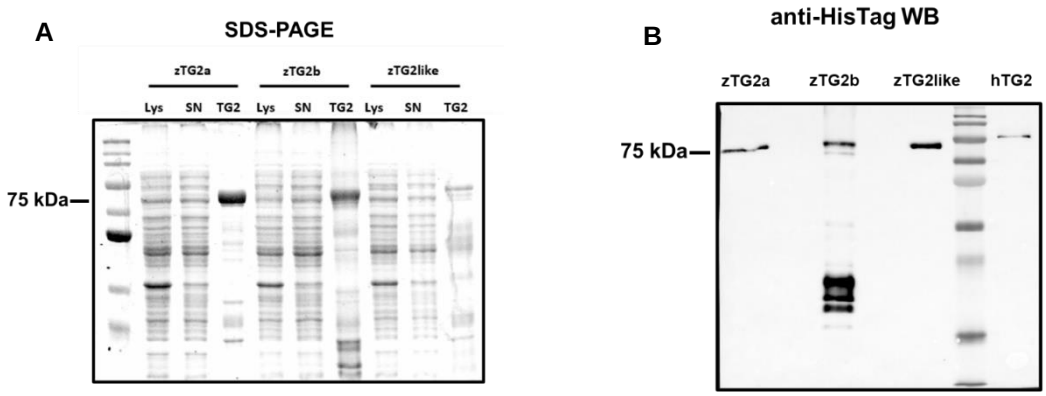


Figure 24. Zebrafish TGs2 production and affinity-purification. A) The three affinity-purified zTGs2 were analysed in SDS-PAGE together with the total lysate (Lys) and soluble protein fraction (SN). B) The purification of the three zTG2 was confirmed with a Western blot, using an anti 6His-Tag antibody. The 6His-tagged proteins were detected at the expected height (75-80 kDa). 6His-tagged affinity-purified human TG2 was used as positive control.

4.4 Enzymatic tests: Transamidation activity assays

At this point we had purified recombinant zTGs2, characterized by high sequence and structural homology to the human enzyme, so we decided to study if enzymatic and non-enzymatic properties of the human protein were also conserved in the zebrafish TGs2.

The hallmark property of any transglutaminase is the ability to catalyse a transamidation reaction, responsible for deamination, incorporation of amines, or crosslinking of target protein substrates. For this reason, the starting point of the biochemical and functional characterization of the zebrafish TG2 orthologues was investigating if the three recombinant proteins were able to perform this enzymatic function. Studies on the Medaka TG2 orthologue (OlTGT) showed high conservation of the active site at the sequence level and transamidation activity *in vitro* [131]. So, before testing the

transamidation activity of three zTGs2, we looked at the catalytic triad. In the human enzyme, the three residues of the triad are Cys277, His335 and Asp358. We performed a multiple sequence alignment with MUSCLE and found that the triad is conserved in all the three zebrafish proteins (Figure 25a).

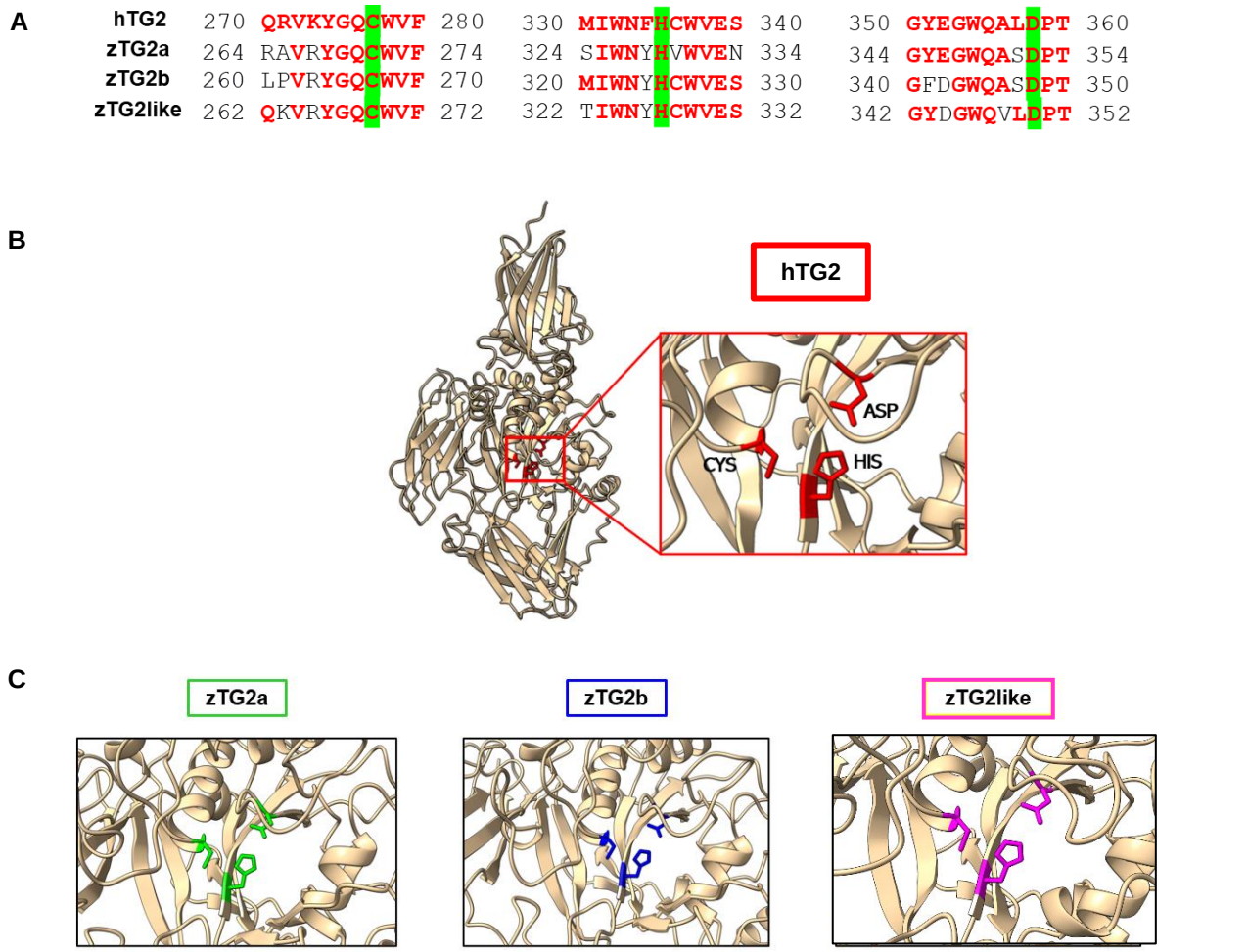


Figure 25. Transglutaminase catalytic site conservation. A) Multiple protein sequence alignment of the zTGs2 proteins and the hTG2. The three key residues involved in the transamidation reaction, the catalytic triad Cys-His-Asp, are conserved and highlighted in green. The hTG2 residues which are conserved in the zebrafish enzymes are in bold and highlighted in red. The protein sequence alignment was performed with MUSCLE. B) 3D of the human TG2, with the three residues of the catalytic triad highlighted in red, inside the catalytic core. A zoom on the catalytic triad is reported on the right. C) Zoom on the 3D structure of the catalytic triad residues of the three zTGs2 protein. The 3D models are rendered with ChimeraX. The catalytic triad residues are coloured in red for hTG2, in green for zTG2a, in blue for zTG2b and in purple for zTG2like.

Moreover, the relative position between the three residues in the sequence is conserved and the protein regions flanking these residues have a higher degree of homology compared with other regions of the zTG2 sequences. A high similarity of the catalytic core regions containing the triad is likely to be reflected in a conserved structural arrangment of the catalytic triad. The right conformation of the enzyme is crucial for the substrate accessibility to the active site and conservation of the three residues alone does not necessarily mean that zTGs2 can in fact catalyse transamidation

activity, nor that they might have similar substrate to the human enzymes. For this reasons, we looked at the arrangment of the catalytic triad in the predicted 3D structures of zTGs2 and the three amino acids are indeed close in the catalytic domain of the proteins, in a very similar fashon to the human enzyme, as we exptected given the high conservation of not only the key residues of the triad, but also the flanking regions in the sequence (Figure 25b-c).

Acording to these observations, we expected the three zTGs2 to be albe to catalyse transamidation acitivity in a similar manner to the human enzyme. So, we tested the transamidation activity of the three zTGs2, adding recombinant hTG2 as positive control, by measuring the incorporation of pentylamine-biotin into the substrate N,N-dimethyl-casein (DMC), both in solid phase and in solution. For the first assay, we measured the TG2-catalysed incorporation of pentylamine-biotin into DMC coated on the surface of ELISA wells, at increasing TGs2 concentrations demonstrating the functionality of the affinity-purified zTGs2 as catalytically active proteins (Figure 26a). Moreover, we tested the TG2-catalysed incorporation of pentylamine-biotin into DMC in solution, both in the presence and in the absence of Ca^{2+}. The reaction mixture was resolved by SDS-PAGE and blotted into a nitrocellulose membrane, and the biotinylated substrate detected with the used of HRP-conjugated streptavidin. Without free Ca^{2+} (assured also by the addition of 20mM EDTA) no transamidation activity was detected, as shown by the absence of biotinylated DMC bands in the Western Blot (Fig. 26b).

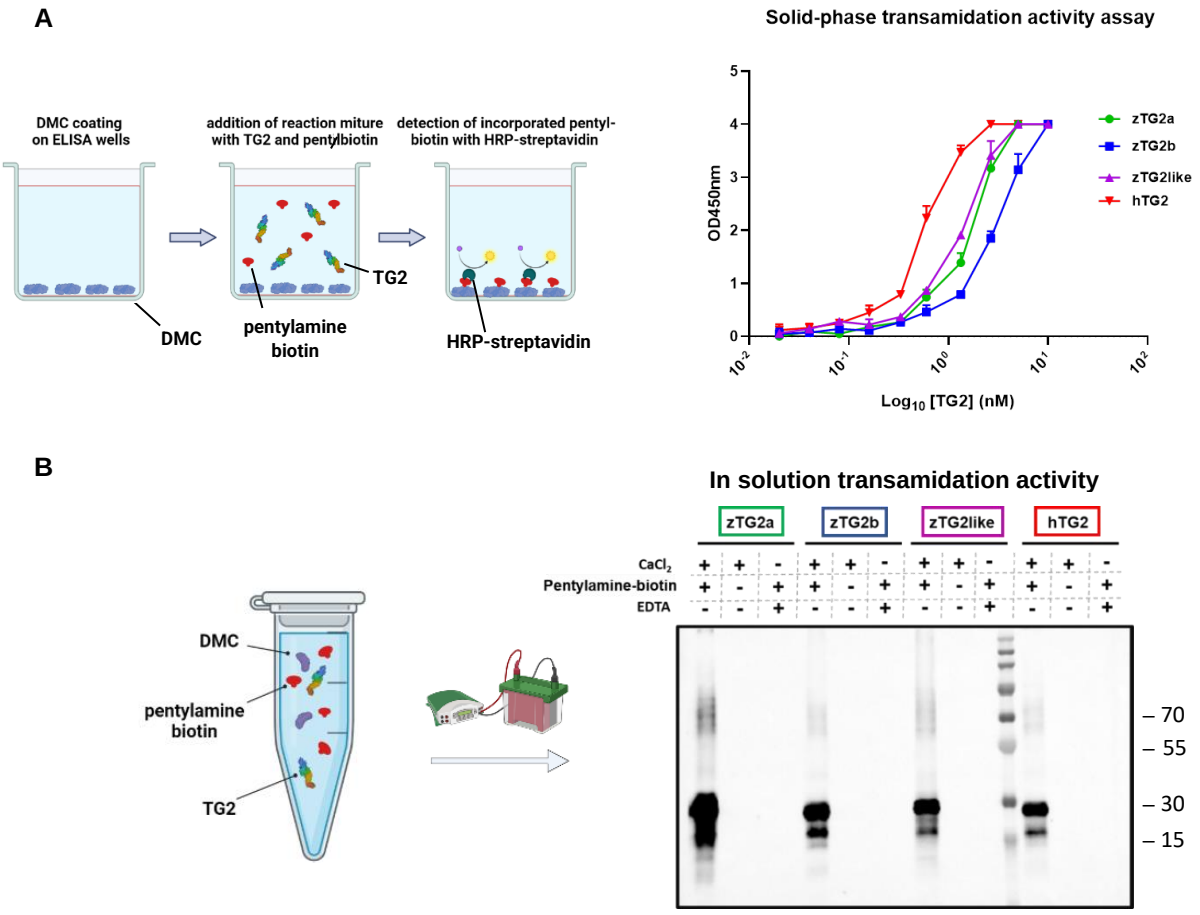


Figure 26 Transamidation activity assays. A) The three affinity-purified zTGs2 recombinant proteins were tested at increasing concentrations of proteins in ELISA by measuring the TG2-catalysed incorporation of pentylamine-biotin into a coated substrate, DMC (N,N-dimethylcaseine). 6His-tagged affinity-purified human TG2 was used as positive control. The 450nm absorbance values are plotted against the Log_{10} of the enzyme concentrations in the reaction. On the left, illustration of the basic steps of the experiment. All conditions have been tested in triplicate and each plot represents the averaged result of three independent experiments (n=3). B) In solution transamidation activity assay. The zTG2-catalysed incorporation of pentylamine-biotin into DMC was detected by HRP-conjugated streptavidin in Western Blot. 6His-tagged affinity-purified human TG2 was also tested as a positive control. In presence of 5 mM CaCl2 and 2 mM pentylamine-biotin, sharp bands of biotinylated DMC can be observed at the expected molecular weight (~25 kDa). Conversely, in absence of $CaCl_2$ and presence of 20 mM EDTA, no bands can be observed, demonstrating no enzymatic activity. The activity was also tested in absence of pentylamine biotin, as an additional negative control condition. The two assays demonstrate the functionality of the affinity-purified recombinant zTGs2, and showed that they possess a Ca2+-dependent transamidation activity similar to the human enzyme

4.5 Calcium and GTP-dependend activity assays

The dependence on calcium ions binding is one of the hallmarks of the transamidation activity of TG2, as high levels of Ca^{2+} promote the open and catalytically active conformation of the enzyme. Conversely, low levels of Ca^{2+}, the competitive binding of Mg^{2+} to the Ca^{2+}-binding sites and the presence of GTP/GDP, promote the conformational switch to the closed and inactive form. As we observed that the purified zTGs2 proteins showed transamidation activity in presence of Ca^{2+} in ELISA, we decided to better characterize their regulatory mechanisms. So, we also tested the zTGs2

at increasing concentrations of $CaCl_2$ in ELISA, observing a dose-dependent increase in transamidation activity, similarly to the human enzyme (Figure 27).

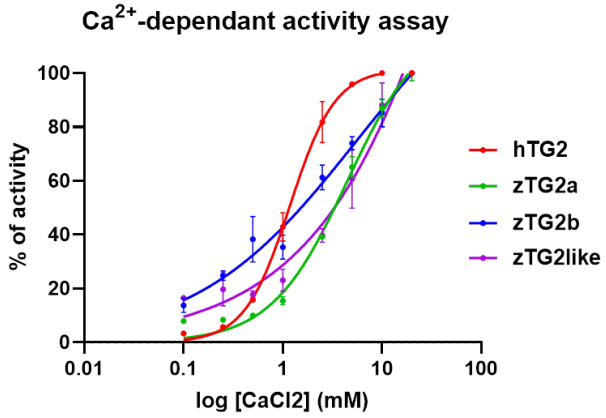


Figure 27. Calcium-dependent activity assay. A) The TGs2-catalysed incorporation of pentylamine-biotin into coated DMC was tested at increasing concentrations of $CaCl_2$, from 0.1 to 20 mM. Similarly to the human enzyme, the zTGs2 show a dose-dependent activity in response to Ca^{2+}. The data are expressed as percentage of activity with respect to the sample tested with the highest concentration of calcium. All conditions have been tested in triplicate and each plot represents the averaged result of three independent experiments (n=3).

Then, we also investigated if the zebrafish TG2 could be sensitive to the inhibitory effect of GTP. As already discussed, the human enzyme is able to exerts its transamidation reaction only when the catalytic triad is exposed and fully accessible to the substrates. This is made possible thanks to a well-described conformational switch, that moves the two N-terminal β-barrel domains away from the core domain. This conformational change is prevented when GTP is bound to a nucleotide binding pocket, located in a cleft between the catalytic core and the first β-barrel domain. GTP binding to this pocket stabilizes the closed conformation, where the substrates cannot access the active site and TG2 is unable to exert its transamidation activity. First, we looked at the protein sequences to investigate if the zTGs2 could have the putative regions responsible for the GTP binding described for the human enzyme. Sequence alignment shows no conservation of the putative key residues for the interaction, with the zTG2a being the most similar (Fig. 28b). So, we assumed that zTG2a could somehow bind and be inhibited by GTP, while we did not expect any inhibitory effect on the other two proteins. These assumptions were confirmed with an ELISA activity assay, which shows that zTG2a is indeed the only zebrafish TG2 whose activity is affected by the presence of GTP, albeit only to a small extent. (Fig. 28c).

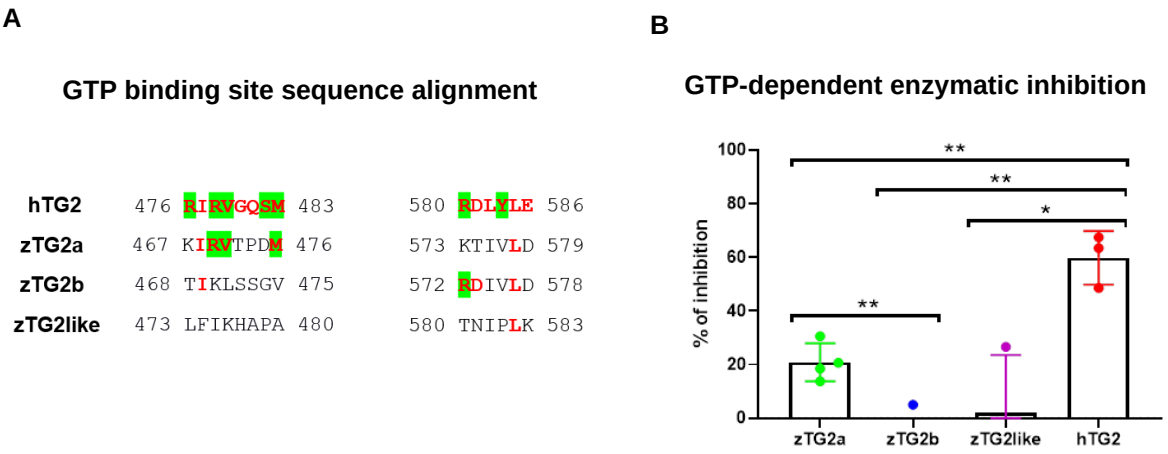


Figure 28. GTP inhibition activity assay. A) Multiple protein sequence alignment performed with MUSCLE of the putative GDP/GTP-binding site of the human and zebrafish TGs2. The key residues identified as the responsible for the interaction are highlighted in bold. Only zTG2a shows some degree of conservation of the key residues for the binding. (B) GTP-dependent enzymatic inhibition assay. The transamidation activity of the human and zebrafish recombinant TG2 proteins have been tested in the presence and absence of 100mM GTP. The decrease in the OD_{450nm} absorbance value is reported as percentage of enzymatic inhibition in the plot. In accordance with the sequence alignment, the only zTG2 whose transamidation activity is affected by the presence of GTP is the zTG2a isoform All conditions have been tested in triplicate and the plot represents the averaged result of three independent experiments (n=3).

Our observations on zebrafish TGs2 reflects what already reported in literature. In particular, according to Jang et al., hTG2 presents GTP binding cleft formed by Phe174, Ser482, Met483, Arg476, Arg478, Arg580 and Tyr583, which is well conserved in mammals, but not in birds, frogs and fishes. Indeed, our data are very similar to what has been described for the TG2 orthologue of medaka fish. OlTGT was demostrated to possess the Cys-Asp-His residues of the catalytic triad, with high homology in the flanking regions of the catalytic core, but to lack most of the putative residues responsible for the GTP binding. OlTGT transamidating acitive was indeed shown to be insensitive to the inhibitory action of GTP. This scenario is similar to what observed for zTG2b and zTG2like. Conversely, we reported that zTG2a shows slightly diminished activity in presence of GTP, and it might be reasonable to assume that zTG2a could bind GTP with low affinity. Indeed, even though only few residues are conserved, several mutations preserve the physicochemical properties of the substituted amino acid (two lysines instead of arginines and one aspartate instead of a glutamate. Structural studies should be performed to assess if GTP does not bind at all to the zTGs2s as they do not present a nucleotide binding cleft, and if the zTGs2 undergo a conformational switch like the human enzyme.

4.6 Zebrafish TGs2 inhibition assays

The high conservation of the catalytic core sequence and the similar Ca^{2+}-dependent transamidation activity suggest that the catalytic site of the zebrafish TGs2 might possess a similar structure to the one found in the human enzyme, and then accommodate similar protein substrates. High degree of homology in the catalytic site would also allow to modulate the activity of the three zebrafish enzymes with human TG2-specific enzymatic inhibitors. In order to confirm this assumption, we tested the sensitivity of the recombinant zTGs2 to three inhibitors: the commercial inhibitor from Zedira, ZDON, and two inhibitors developed by the group of Griffin and co-workers, R281 and 1-155 [140], [141].These three molecules have different applications, as ZDON and 1-155 are cell permeable molecules while R-281 is unable to cross the cell membrane. Moreover, these inhibitors are characterized by different values of IC50 for hTG2, which ranges from 6 nM for 1-155 to 150 nM of ZDON. We tested in ELISA the transamidation activity of the three zTGs2, in the absence (as a positive control condition) and in the presence of the inhibitors, used at three different concentrations: 250 nM, 2.5 μM and 25 μM (Figure 29). At 25 μM of inhibitors, the highest concentration used in the assay, we observed about 50% of inhibition of the 3 zTGs2 with the ZDON, while both R281 and 1-155 led to nearly complete inhibition of zTGs2 activity, with the only exception of zTG2a which is less sensitive to the inhibitory effect of 1-155 compared to the isoforms B and Like. Indeed, this effect is present at all the three concentrations tested, and it is particularly evident at 2.5 μM of 1-155, where zTG2b and zTG2like shows about 80% of inhibition while zTG2a activity is decreased only of 20%. At 2.5 μM, the inhibitory activity of ZDON is very low compared to the other two inhibitors, and at 250 nM, the lowest concentration tested, all the three inhibitors show very little inhibitory activity.

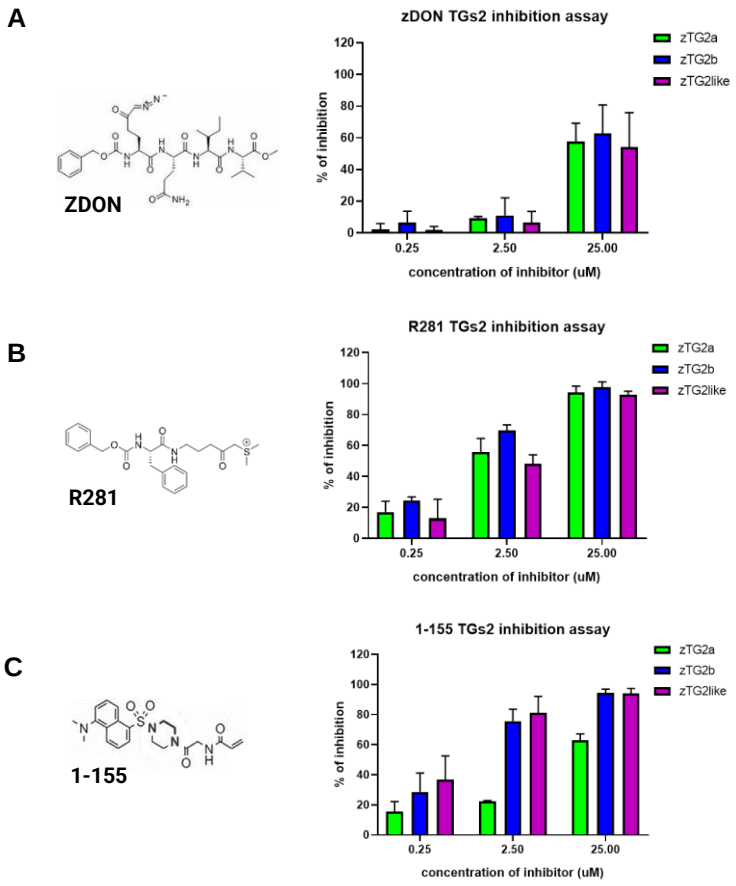


Figure 29. Solid phase zTGs2 enzymatic inhibition assays. The transamidation activity of the zTGs2 recombinant proteins were tested in ELISA as already described, with three different human TG2-specific inhibitors. The inhibitors were tested at 0.25, 2.5 and 25 μM. A) Enzymatic activity assay with the commercial hTG2-specific inhibitor ZDON (Zedira). B) Enzymatic activity assay with the hTG2-specific inhibitor R281, developed and provided by the group of Griffin M. C) Enzymatic activity assay with the hTG2-specific inhibitor 1-155, developed and provided by the group of Griffin M. The plots represent as percentage of inhibition the reduction in the measured signals ($O.D._{450\ nm}$ absorbance values) with respect to the control samples (no inhibitors). All conditions have been tested in triplicate and each plot represents the averaged result of three independent experiments (n=3).

We demonstrated that different hTG2-specific inhibitors can bind and successfully inhibit the activity of all the three zTG2 proteins, even though higher concentration of inhibitors are required to significantly affect the enzymatic activity of zTGs2 compared to what reported for the human enzyme. Indeed, the IC50 values of the inhibitors for the human enzyme are far lower (nanomolar range) than the concentrations at which we observed significant inhibition of zTGs2, which are 2.5 μM for R281 and 1-155, and 25 μM for ZDON. However, it is noteworthy that we reported catalytic inhibition with all the three inhibitors, as their different structures mimic the polypeptide substrates in different ways. These observations correlate with a high homology of the aminoacidic regions forming the catalytic site, as we can expect that the structure of the zTG2 catalytic core assumes a conformation highly similar to that of the hTG2, even though with some differences, which result in less effective inhibitory effects.

4.7 zTGs2 extracellular function: RGD-indipendent cell adhesion assay

The biological roles of transglutaminase 2 do not rely only on its enzymatic activities, but also on other non-enzymatic functions. Among those, TG2 plays an important role as a cell adhesion molecole in the extracellular environment, acting as a bridge between fibronectin and syndecan-4 with a RGD-indipendent mechanism, critical for the preventing loss of adhesion during matrix turnover and tissue injury, and promoting pro-survival signalling. In order to investigate if the zebrafish TG2 orthologues could mediate a similar cell adhesion role, we started with a protein sequence analysis of zTGs2, focusing at the regions responsible for the interaction with the other two macromolecules directly involved in the RGD-indipendent cell adhesion complex: fibronectin and syndecan-4. As already discussed above, TG2 interaction sites with heparan sulfate proteoglycans have been the subject of several studies by different groups, which identified different putative regions as responsible for syndecan binding.

We performed a multiple protein sequence alignment of the human and zebrafish TGs2 and looked at three distinct regions: the 590-KIRILGEPKQKRK-602 sequence, described as HS1 by Wang and co-workers and containing the 598-KQKRK-602 cluster proposed by Lortat-Jacob and co-workers; the 202-NPKFLKNAGRDCSRRSSPVYVGR-222 sequence, described as HS2 by Wang and co-workers; and 261-LRRWKN-266 sequence, proposed by both Teesalu and Lortat-Jacob (Figure 30).

Overall, we observed that zTG2b has the highest homology in the heparan-binding site, as HS1 is nearly completely conserved, while the other two regions show about 50% of homology with hTG2, even tough most of the basic amino acids identified as the key residues for the interaction are not present in zTG2b sequence. The other two zebrafish isoforms, zTG2a and zTG2like, feature only a low degree of conservation of the three regions, with zTG2like mantaining more of the key basic residues compared to zTG2a, despite being more evolutionarily distant to the human orthologue.

Based on these observations, it is diffucult to make solid assumptions on the likelihood of zTGs2 proteins being actually involved in high affinity interaction with syndecan molecules like the human orthologue. It is noteworthy that zebrafish has been demonstrated to possess orthologues of three of the four syndecans found in mammals: syndecan-2, 3 and 4. In particular, zebrafish syndecan-4 has been demostrated to possess several of the crucial roles played by the mammalian orthologue. Indeed, it has been reported to be able to mediate cell adhesion, bind phosphatidylinositol 4,5-bisphosphate and promote protein kinase C-α and RhoA signalling, driving focal adhesion formation and actin cytoskeleton reorganization. Binding of zTGs2 to heparan sulfate proteoglycans in zebrafihs would be a relevant biological event not only for the formation of the cell adhesion complex with fibronectin,

but also for the actual presence of zTGs2 in the extracellular environment as well. Indeed, as already discussed, human TG2 has been shown to associate with syndecan in the intracellular compartment and then be translocated to the extracellular environment with syndecan shedding.

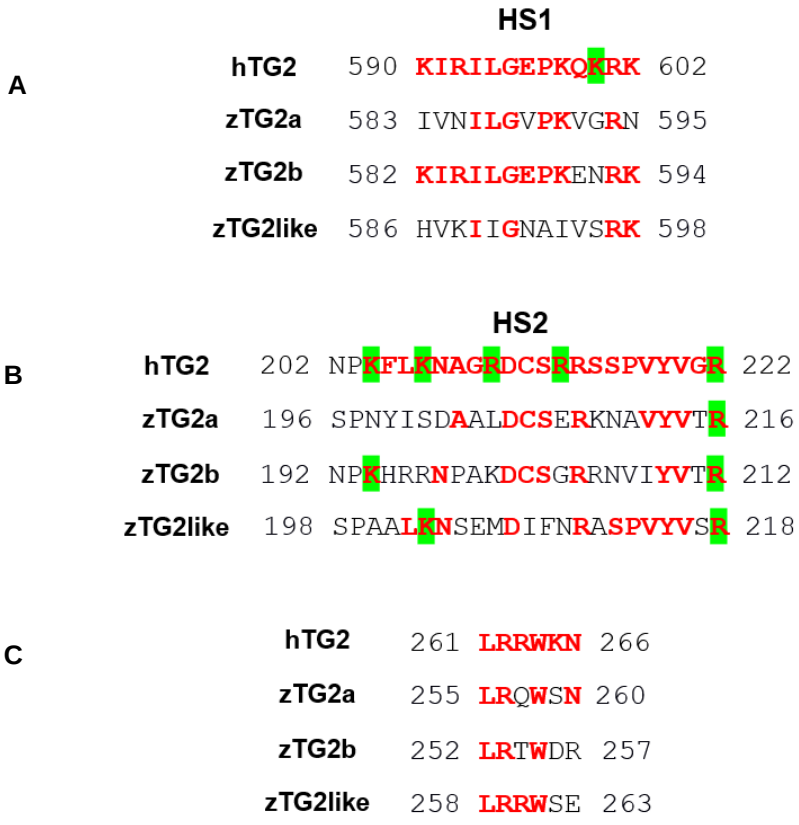


Figure 30. TG2 Heparan-binding sites protein homology. Multiple protein sequence alignment performed with MUSCLE of three putative heparan-binding sites. A) Protein sequence alignment of hTG2 and zTGs2 of the HS1 described by Wang et co-workers, comprising the KQKRQ peptide proposed by Lortat-Jacob and co-workers. B) Protein sequence alignment of hTG2 and zTGs2 of the HS2 described by Wang et co-workers as the true region responsible for the interaction. C) Protein sequence alignment of hTG2 and zTGs2 of the peptide identified as the best candidate HBS according to the publications of Teesalu and co-workers and Lortat-Jacob and co-workers. The basic amino acids described as the key residues for the binding are highlighted in green. The conserved residues are in bold and highlighted in red.

The moecular nature of the interaction between fibronectin and human TG2 relies on the interaction of a 15 kDa fragment, comprising three modules called I_7, I_8 and I_9, of the N terminal Gelatin-binding domain (GBD) of fibronectin, with specific residues of the N terminal portion of TG2. In particular, according to Cardoso and co-workers, the binding involves mainly amino acids from the N terminal β-sandwich domain: Lys30, Tyr58, Arg116, Glu120, Ser129, and His134 [142]. They demonstrated that TG2 binding is dramatically reduced upon mutation of Lys30, Arg116 and His134, but also of Asp198 of the catalytic core. For this reason, we investigated if these residues are conserved in zTGs2 with a multiple sequence alignment (Figure 31).

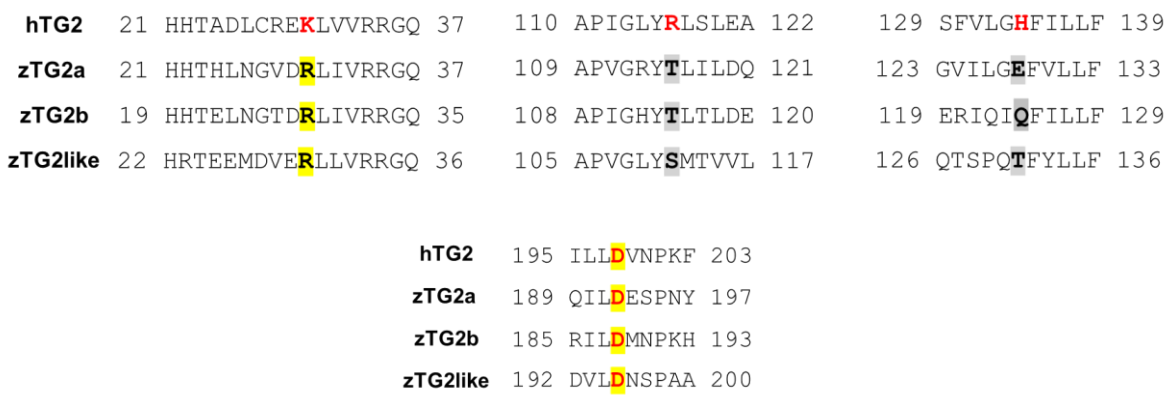


Figure 31. Fibronectin binding site sequence homology. Human and zebrafish TGs2 multiple protein sequence alignment performed with MUSCLE of the regions comprising the residues identified by Cardoso and co-workers (2017) as the key amino acids responsible for the binding to fibronectin. Lys30, Arg116, His134 and Asp198 are highlighted in red, as they are the critical residues for the binding. While Asp198 is conserved, the other residues are not found in the zebrafish sequences.

Looking at the multiple sequence alignment, we can observe a certain level of conservation of the regions analysed, even though the key residues Lys30, Arg116 and His134 are not conserved. However, Lys30 is replaced by another basic residue in the zebrafish orthologues, which also present the critical aspartic acid of the catalytic domain. According to the data reported by Cardoso and colleagues, the loss of Arg116 and His134 should lead to dramatic loss of binding affinity to fibronectin, so the likelihood of zTGs2 being able to bind fibronectin with high affinity as the human orthologue is debatable.

Considering the similarity of predicted structure and the conservation of some of the residues involved in the interaction with HSPGs and fibronectin, we decided to test the functionality of zebrafish TGs2 recombinant proteins as cell adhesion molecules. As discussed, TG2 plays an important function in the extracellular environment mediating RGD-independent cell adhesion and spreading, by forming complexes with matrix fibronectin and binding cell surface HSPGs, acting as a bridge between the cell and the ECM. For this reason, we tested the ability of zebrafish TGs2 to rescue the loss of cell adhesion and spreading caused by the use of RGD-containing peptides, that saturate cell surface integrins and impair cell-matrix interactions mediated by integrin binding to RGD-containing ECM proteins like fibronectin. In our experiment, Human Foreskin Dermal Fibroblasts (HFDFs), a primary cell line with very low endogenous levels of TG2 expression, were incubated with 150 µg/mL of GRGDTP synthetic peptide and seeded on wells coated with human fibronectin (hFN), and on wells coated with hFN complexed with zTGs2. Human FN complexed with hTG2 was used as a positive control condition, as we know that hTG2 binds FN and rescues the loss of cell adhesion caused by the RGD peptide (Figure 32).

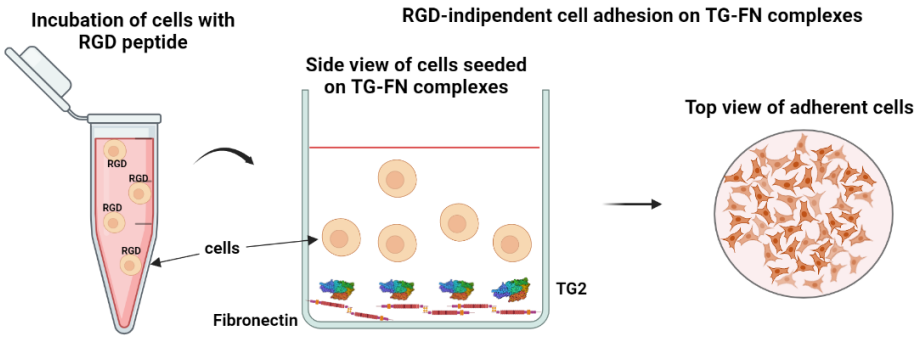


Figure 32. RGD-independent adhesion assay. Graphic illustration of the experiment. Fibroblasts were incubated for 20 minutes with or without GRGDTP synthetic peptide, and then seeded on wells coated with either fibronectin alone or fibronectin complexed with TG2 (human or zebrafish isoforms). After ~20 minutes, non-adherent cells were washed away and the number of cells attached and spread on the wells were counted, after fixing and staining.

With a preliminary experiment where we tested cell adhesion and spreading on FN complexes with all the three zTGs2, after incubation with either the GRGDTP synthetic peptide or a control peptide, we observed that cells seeded either on hTG2-FN or zTGs2-FN complexes showed a significant increase of both cell attachment and cell spreading, compared to cells seeded on only fibronectin, after incubation with the RGD-peptide (data not shown). In light of this preliminary observation, we assumed that zTGs2 could in fact form complexes with fibronectin in the ECM, mediating increased cell attachment and spreading, but we could not exclude that the observed rescue of cell adhesion was actually mediated simply by zTGs2 proteins interacting with cell surface receptors, without involvement of fibronectin. In order to investigate this alternative mechanism of cell adhesion, we repeated the assay testing HFDFs also on wells simply coated with TGs2, without hFN (Figure 33a). We did not repeat the assay testing zTG2like, as we decided to focus on the zTG2a and zTG2b proteins, which show significant discrepancies in their conservation of the putative key residues for the interaction with HSPGs, while being the two isoforms with the higher overall homology to hTG2. As represented in Figure 31a, the presence of human and zebrafish TGs2 alone was not sufficient to mediate cell adhesion. Moreover, we confirmed that both hTG2 and zTGs2 can form complexes with hFN and rescue the loss of both cell attachment (Fig. 33b) and spreading (Fig. 33c) induced by the inhibitory RGD peptide.

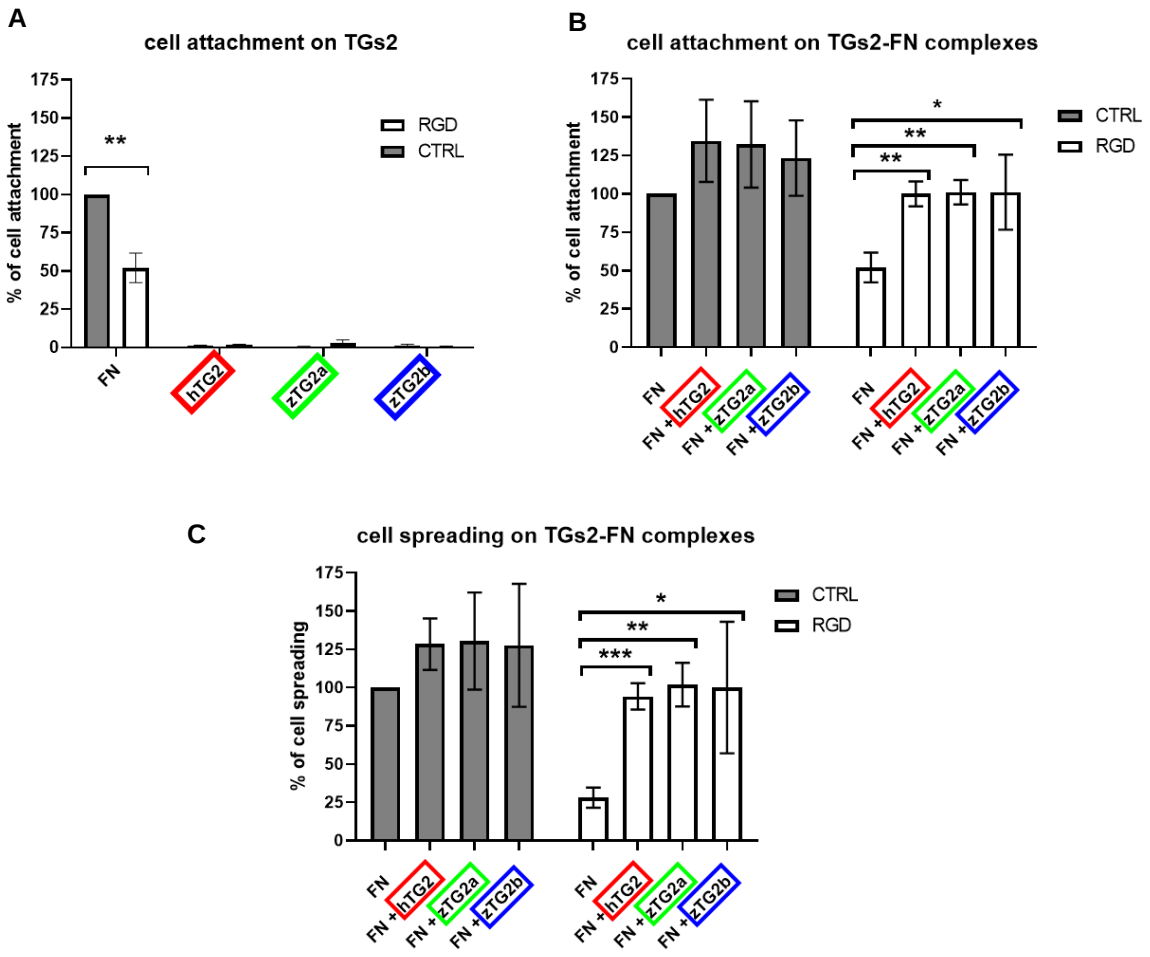


Figure 33. RGD-independent cell adhesion of HFDFs. Cell attachment (A, B) and cell spreading (C) on FN and TGs2 (A) or on FN and TGs2-FN (B, C) were assessed ~20 minutes after seeding cells pre-incubated with 150 µg/mL GRGDTP synthetic peptide (white bars) or serum-free medium (CTRL, grey bars). A) TGs2 by themselves cannot mediated cell adhesion, as HFDFs do not adhere on wells coated with human or zebrafish TGs2. No spread cells were present on TGs2-coated wells. B, C) Fibroblasts seeded on hTG2-hFN or zTGs2-hFN complexes show increased attachment (B) and spreading (C) compared to cells seeded on FN alone. Moreover, RGD-treated fibroblasts seeded on hTG2-FN and zTGs2-FN complexes show a significant rescue of the loss of attachment and spreading observed for the RGD-treated cells seeded on fibronectin alone, demonstrating the occurrence of a compensating mechanism mediated by the TGs2-FN complexes. All conditions have been tested at least in triplicate and the plots represent the averaged result of three independent experiments (n=3). Data are expressed as mean ± S.D. of the percentage of cell attachment or cell spreading, compared to the control values on FN (100%). Mean attached cells value on FN control was 96 ± 25; mean spread cells value on FN control was 52 ± 10. The average number of total cells analysed for the control sample was 543. *= $p<0.05$; **=$p<0.01$; ***=$p<0.001$.

In particular, incubation with RGD peptide led to a reduction of 50% in cell attachment and of 70% of cell spreading on fibronectin compared to the control condition, while seeding cells on fibronectin-TG2 complexes led to the complete rescue of both attachment and spreading.

Representative images from the experiment show how the RGD peptide promotes a significant reduction of both the number of cells attached to the wells and the number of cells undergoing spreading on fibronectin alone. The spreading cells are identifiable as the cytoplasm, detectable by the lighter staining compared to the darker nucleus, appear considerably larger than cells that are simply adhering to the matrix. Indeed, several degrees of the cell adhesion process are identifiable,

from very rounded cells, which have just formed attachment to the surface, to flatter and bigger cells, which are starting to spread and eventually very large cells whose focal adhesions promoted the reorganization of the actin cytoskeleton and show protrusive structures such as lamellipodia and filopodia (Figure 34).

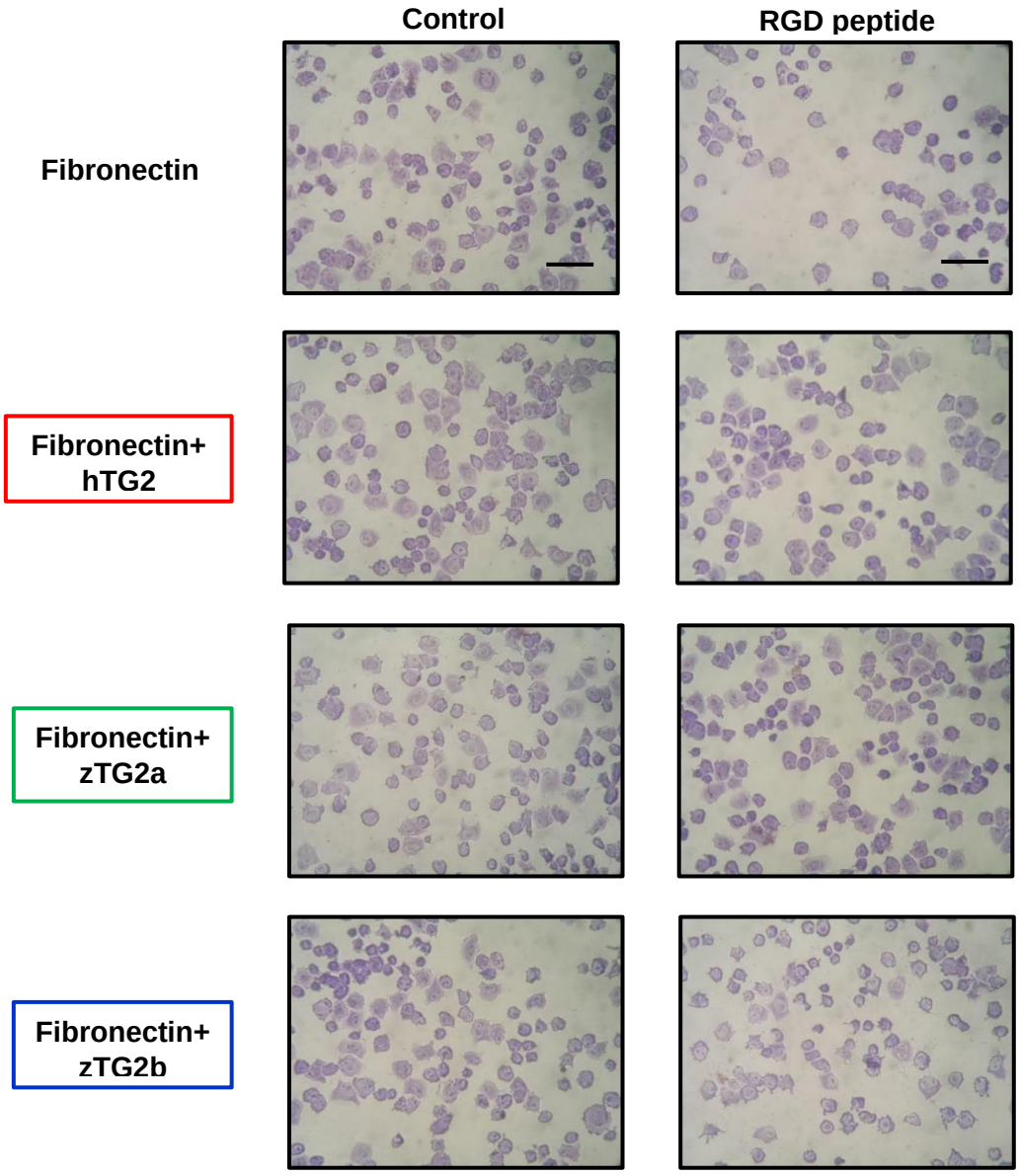


Figure 34. Representative images of RGD-independent cell adhesion assay. Human Foreskin Dermal Fibroblasts were fixed, permeabilized and stained with May-Grunwald staining for the cytoplasm and Giemsa staining for the nucleus. For each condition, at least three wells were analysed and three non-overlapping fields covering the central portion of each well were captured using a video digital camera (Nikon E5400). Incubation of the cells with the GRGDTP synthetic peptide led to a reduced fibroblast attachment and spreading on human fibronectin-coated wells (upper right panel) compared to the control fibroblasts (upper left panel). Fibroblasts seeded on hTG2-hFN or zTGs2-hFN complexes show increased attachment (higher cell density) and spreading (larger and brighter cells) compared to cells seeded on fibronectin alone. Moreover, RGD-treated fibroblasts seeded on hTG2-FN and zTGs2-FN complexes show a significant rescue of the loss of attachment and spreading observed for the RGD-treated cells seeded on fibronectin alone, demonstrating the occurrence of a compensating mechanism mediated by the TGs2-FN complexes. Scale bar = 50 μm.

In order to confirm that, as reported in literature for human TG2, also the zebrafish proteins are able to mediate cell adhesion by promoting the formation of focal adhesions and the reorganization of the actin cytoskeleton, we performed the RGD-independent cell adhesion assay and stained the cells with fluorescent phalloidin. Then, we observed the actin cytoskeleton of the seeded fibroblasts by epifluorescence microscopy (Figure 35). As expected, similarly to hTG2, fibronectin complexes with zTGs2 promotes cell attachment and spreading, which is evidenced at the intracellular level by the reorganization of the actin cytoskeleton into stress fibers.

According to this observation, zTGs2 in the extracellular environment mediate a similar cell adhesion function as the human orthologue, promoting the intracellular reorganization of the actin cytoskeleton, with the formation of focal adhesions and protrusive structures. Then, similarly for the human protein, the zebrafish TGs2 are likely to play essential role in biological events where matrix turnover and remodelling occur, like angiogenesis and bone remodelling, as well as during would healing after a tissue injury.

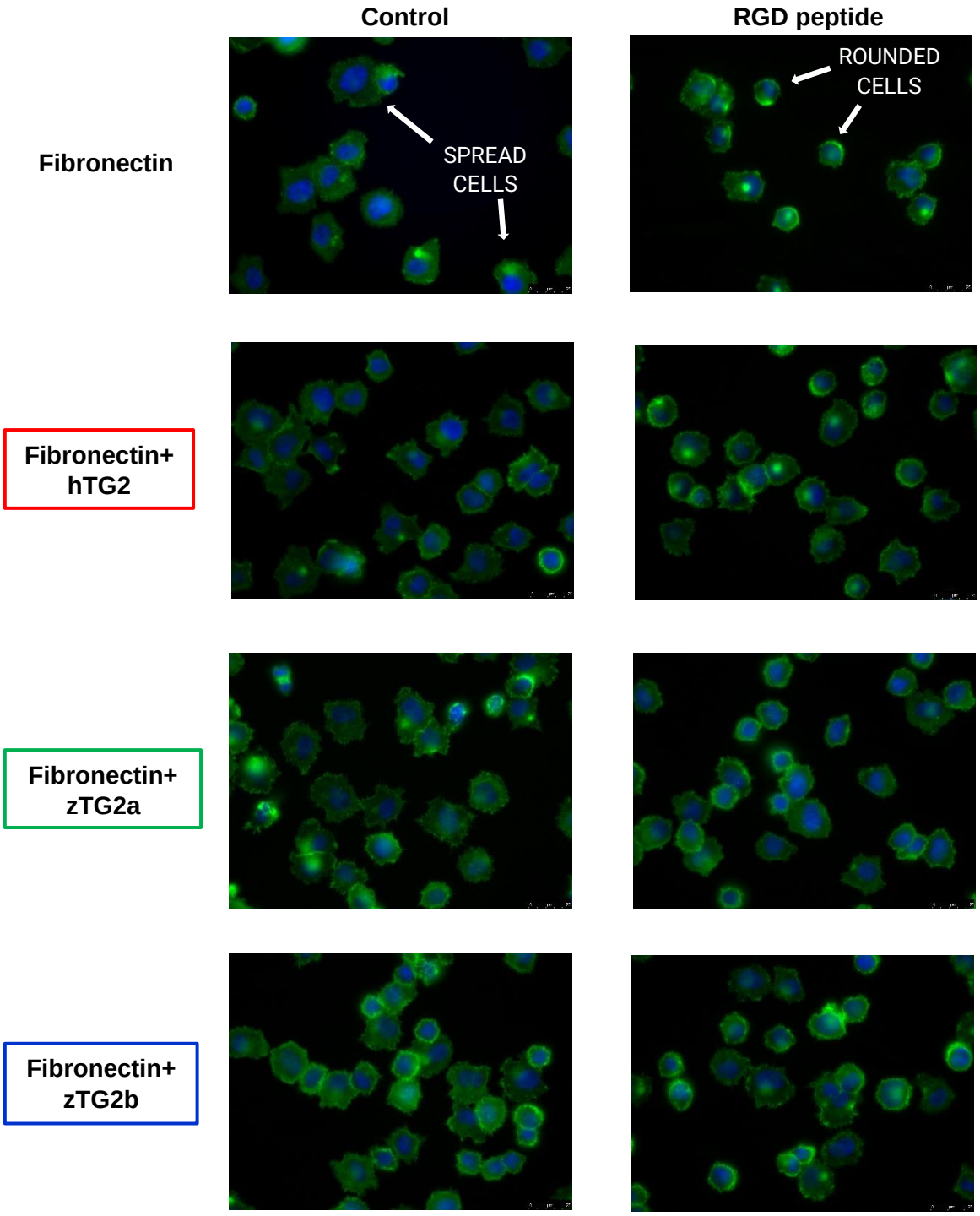


Figure 35. Epifluorescence microscopy of RGD-independent actin cytoskeleton organization in response to TGs-hFN. Human Foreskin Dermal Fibroblasts were seeded in 0.79-cm2 -wells of chamber slides previously coated with hFN and on hTG2-hFN or zTGs2-hFN, after incubation with GRGDTP synthetic peptide or serum-free medium. Adherent cells were fixed, permeabilized and actin stress fibers were visualized at 60X magnification using fluorescein isothiocyanate (FITC)-labelled phalloidin. At least 6 non-overlapping fields covering the central portion of each well were captured using an epifluorescence microscope. RGD-treated cells seeded on FN (upper right quadrant) show reduced attachment and rounded morphology compared to control cells (upper left pane). The rescue of cell attachment and spreading mediated by hTG2-hFN or zTGs2-hFN complexes is demonstrated by higher cell density and increased number of spread cells, complex morphology, and visible actin stress fibers.

4.8 zTGs2 cloning into eukaryotic expression plasmid

In light of the results of the RGD-independent assay, which demonstrate how zTGs2 protein can mediate homologous functions to human TG2 in the extracellular environment, we decided to investigate if these proteins could also mediate intracellular activities attributed to the human orthologue. For this reason, we decided to clone the zTGs2 and the hTG2 (as a control) coding sequences into a modified version of the eukaryotic expression vector pCDNA 3.1 HYGRO(+). We choose this vector as it allows to perform transient transfection of a gene of interest, which is expressed under the strong CMV promoter, and results in the constitutive high-level expression of a C-terminal SV5 tagged-protein. (Figure 36).

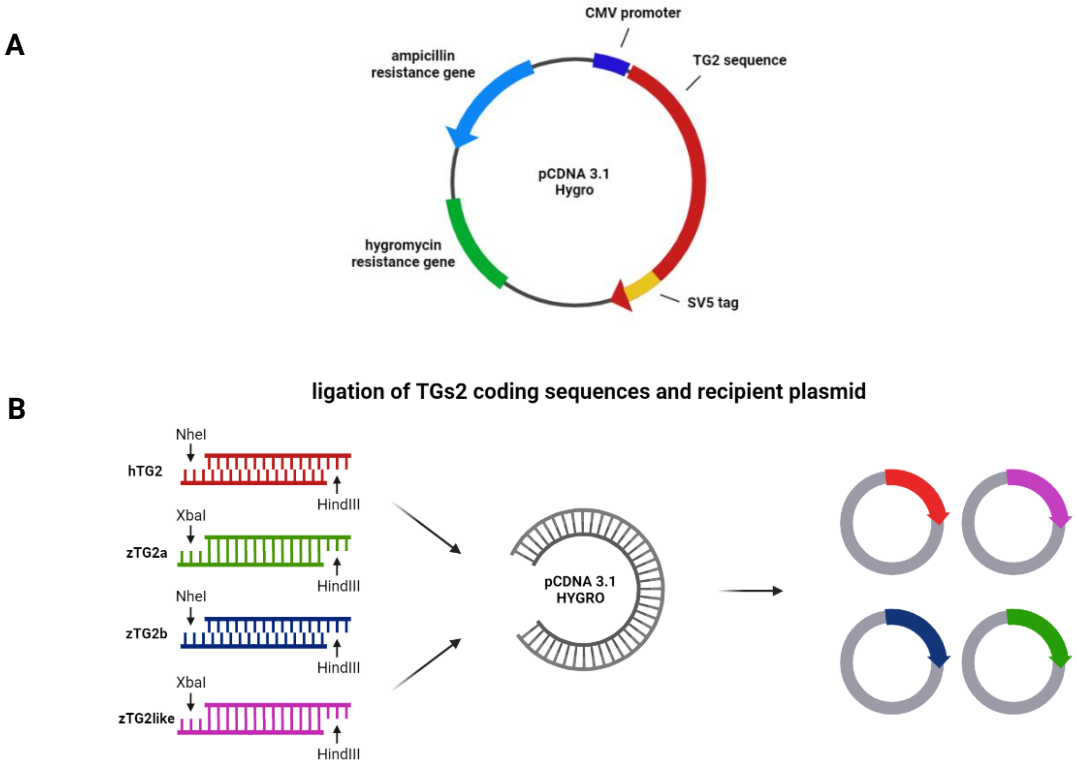


Figure 36. Zebrafish TG2 cloning into the eukaryotic expression vector. A) We cloned the zTGs2 and hTG2 as control in a modified version of the pCDNA 3.1 HYGRO (+) eukaryotic expression vector. The TGs2 sequences are expressed under the strong CMV promoter, and the resulting protein present a SV5 tag a C terminal SV5 tag. B) The PCR-amplified TGs2 and the recipient pCDNA 3.1 HYGRO (+) plasmid were digested and ligated, and the products were cloned into DH5α bacteria to produce the plasmid minipreps.

We successfully PCR-amplified the zTGs2 and hTG2 sequences with the two rounds of amplifications, digested and ligated the PCR products and the recipient vector and cloned the resulting products in DH5α competent cells (Figure 37a). The resulting colonies were screened for the presence of the correct DNA fragments and used for the production of plasmid minipreps of the four eukaryotic

expression vectors, each encoding one of the three zTGs2 or hTG2 as recombinant protein fused with C-terminal SV5 tag (Fig. 37b).

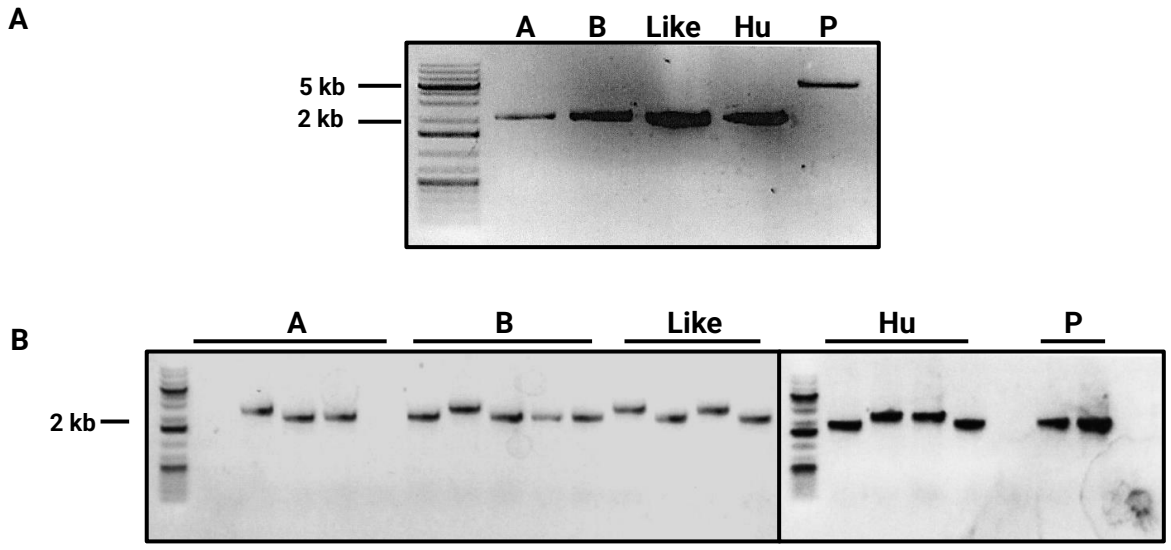


Figure 37. Zebrafish TGs2 and hTG2 cloning into eukaryotic expression vector. A) Agarose gel electrophoresis of the restriction enzymes-digested and gel-purified TGs2 coding sequence (A = zTG2a; B = zTG2b; Like = zTG2like; Hu = hTG2) and recipient plasmid pCDNA 3.1 HYGRO (P). The DNA fragments show the expected size: ~ 2 kb for TGs2 and ~ 5.3 kb for the recipient vector. B) Screening by agarose gel electrophoresis of the DH5α bacterial colonies resulting from the molecular cloning of zTGs2 and hTG2 into the modified pCDNA 3.1 HYGRO plasmid. For each TGs2, four of five colonies were tested for the presence of the expected molecular size of 2 kb. The gel shows how some colonies present the sequence of the TG2 as the PCR product has the correct molecular size, while others show a product with smaller size, attributable to the sequence of the gene harboured by the parental vector, also tested as a control (P).

4.9 zTGs2 transfection into HEK293 cells and isolation of stable clones

With the aim of investigating the intracellular functionality of zTGs2 proteins, we decided to express them in HEK293 cells, as this cell line has very low endogenous levels of TG2 expression and indeed has already been employed to study the effects of TG2 overexpression in biological processes, such as apoptosis.

The eukaryotic plasmid vectors harbouring the zTGs2 and hTG2 sequence were transiently transfected into HEK293 cells with lipofectamine 2000 and the expression of the proteins was assessed from cell lysates at day 3 post-transfection in western blot with the use of anti-SV5 tag antibody (Figure 38a). HEK293 cells transfected with a control GFP-expressing vector and cells treated with only lipofectamine without any plasmid were also used as negative controls. The presence of a band at the expected molecular weight confirms the expression of the four TGs2 in transfected HEK293. In order to assess if the proteins are expressed as functional transglutaminases in our cell model, we tested in ELISA the transamidation activity of soluble proteins lysates of the

transfected cells (Fig. 38b). The lysates of TGs2-expressing cells showed catalytic activity, demonstrating the expression of functional proteins in HEK293 transfected cells.

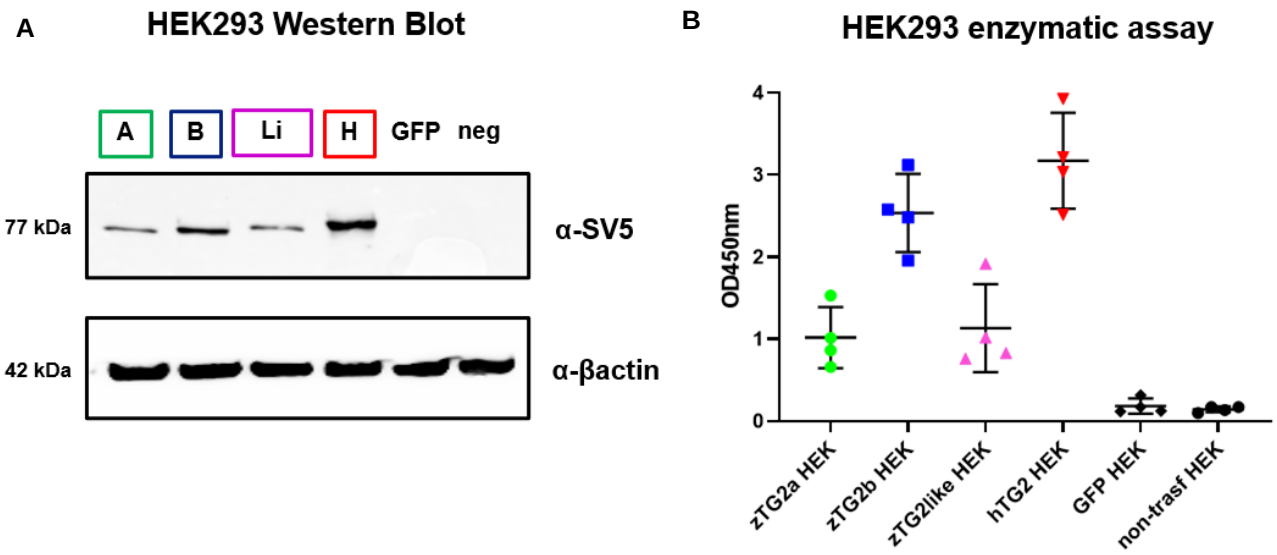


Figure 38. HEK293T cell transient transfection of zTGs2 proteins and enzymatic assay. A) HEK293 cells were transiently transfected with pcDNA 3.1 HYGRO (+) plasmid vectors containing the coding sequence of the three zTGs2 and the human TG2 as a control. Cells transfected with a control GPF-expressing plasmid vector and non-transfected cells served as additional negative controls. The expression of the SV5-tagged TGs2 was confirmed with α-SV5 tag antibody in Western Blot. In the figure, A=zTG2a; B=zTG2b; Like=zTG2like; hum=hTG2; GFP= GFP-expressing control vector; neg= non-transfected cells. B) The functionality of the expressed TGs was assessed in ELISA testing soluble protein lysates with an enzymatic activity assay, by measuring the TG2-catalysed incorporation of pentylamine-biotin into DMC, coated into a microtiter plate. 1 µg of protein lysate was used for each well. All conditions have been tested in triplicate and each plot represents the averaged result of four independent experiments (n=4).

As represented in Figure 38, the levels of TGs2 expression and the measured catalytic activity are quite different among the four TGs2, with zTG2b and hTG2 being more expressed than zTG2a and zTG2like. The different levels of expression are also reflected in the enzymatic activity assay, where the lysates of zTG2b- and hTG2-expressing cells show higher catalytic activity than those of cells expressing zTG2a and zTG2like. In order to obtain more homogenous expression of the proteins in the different samples, which we considered an essential prerequisite for the reproducibility and the reliability of any functional data on the zTGs2 role in the intracellular environment, we decided to select stable clones of HEK293 expressing the four different TGs2.

For the selection of stable TGs2-expressing polyclonal populations of HEK293 cells (HEK^{TG2}), we added Hygromycin 200 µg/mL at the culture media 3 days after transfection, as the transfected

pCDNA 3.1 HYGRO(+) plasmid vector harbours the hygromycin resistance gene. After some rounds of passaging and expansion, the polyclonal populations expressing the four different TGs2 were tested for confirming the expression of the heterologous protein. Western blot with α-SV5 tag antibody confirmed the expression of the four TGs2 and a solid-phase enzymatic activity assay demonstrated the functionality of the proteins in the cell lysates (Figure 39).

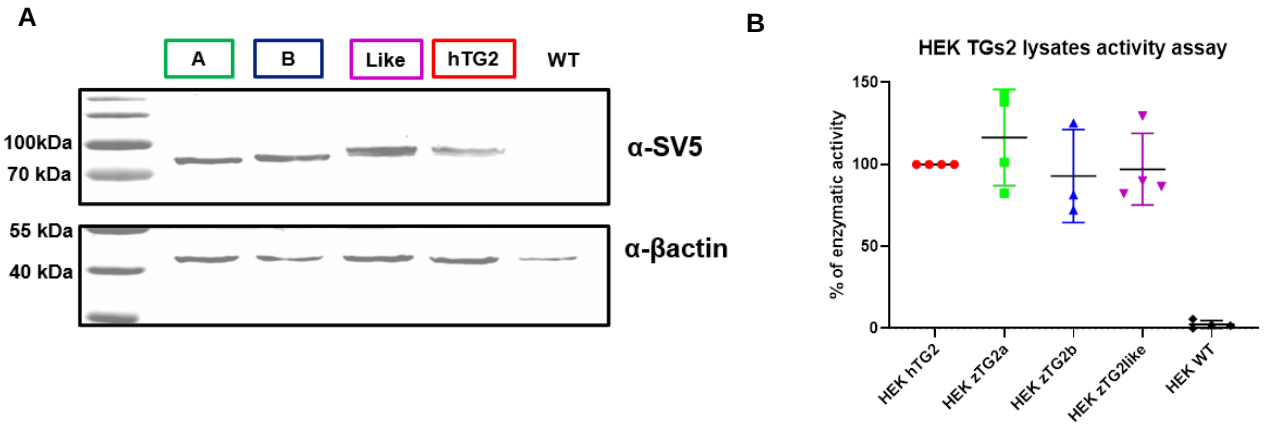


Figure 39. Stable TGs2-expressing HEK293 polyclonal populations. A) Stable HEK293 cells expressing TGs2 were selected adding 200 µg/mL hygromycin in the culture medium. The expression of the SV5-tagged TGs2 was confirmed with α-SV5 tag antibody in Western Blot. In the figure, A=zTG2a; B=zTG2b; Like=zTG2like; hum=hTG2. B) The functionality of the expressed TGs in the selected HEK^{TG2} polyclonal populations was assessed in ELISA, testing cell protein lysates with a solid phase enzymatic activity assay, by measuring the TG2-catalysed incorporation of pentylamine-biotin into DMC, coated into a microtiter plate. The amount of each protein lysate was normalized on the expression level of the TGs2, quantified from the SV5-bands in the Western blot. The data are expressed as percentage of enzymatic activity relative to the hTG2-expressing HEK293 cell lysate, used as positive control. All conditions have been tested in triplicate and each plot represents the averaged result of four independent experiments (n=4).

Morphological analysis by optical microscopy of the TGs2-expressing HEK293 cells showed some alterations compared to the wild type HEK293 cells (Figure 40).

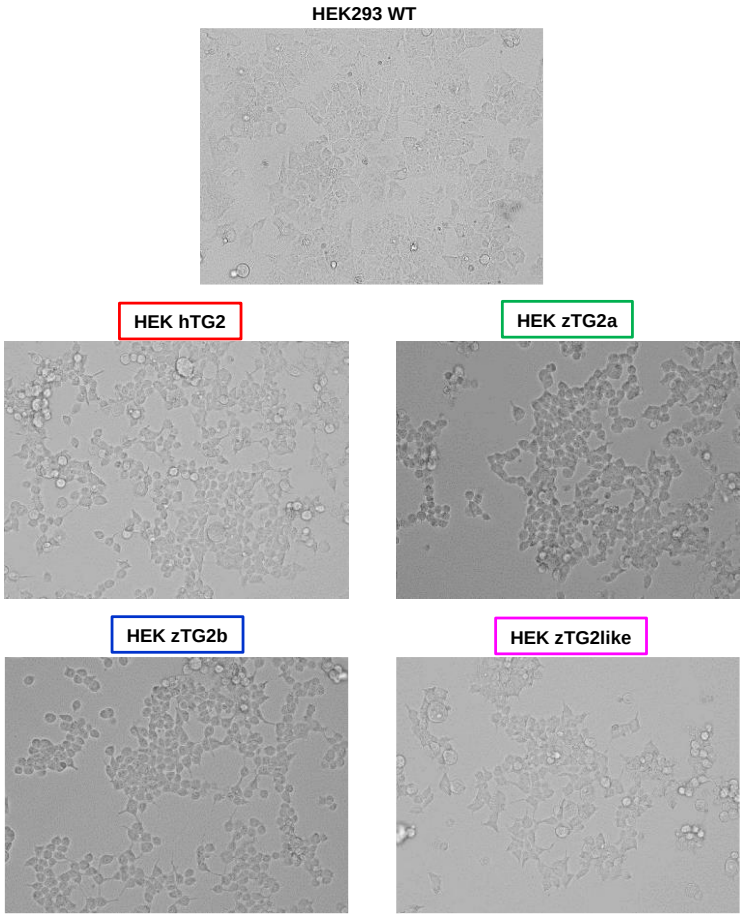
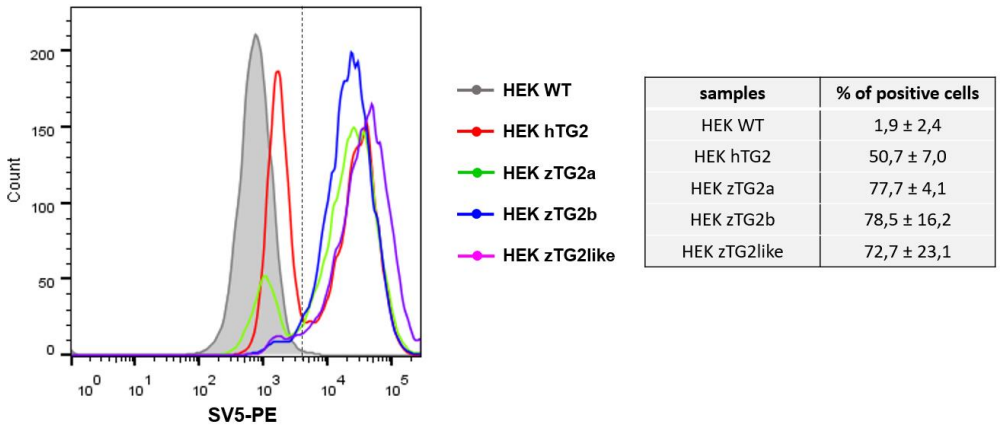


Figure 40. Stable HEK^{TG2} clones' morphology. Representative optical microscopy images at 20X magnification of the different morphology between the TGs2-expressing HEK293 cells compared to the wild type non-transfected cells.

At this point, we wanted to appreciate, for each of the four selected hygromycin-resistant HEK^{TG2} polyclonal populations, the percentage of cells actually expressing the heterologous TG2. So, we performed a flow cytometry analysis, staining the cells with fluorescent anti-SV5 tag antibody (α-SV5-PE) after fixing and permeabilization, to assess the expression of either hTG2 or zTGs2 at the single cell level (Figure 41). Wild type HEK293 cells were used as negative control for the analysis.



samples	% of positive cells
HEK WT	1,9 ± 2,4
HEK hTG2	50,7 ± 7,0
HEK zTG2a	77,7 ± 4,1
HEK zTG2b	78,5 ± 16,2
HEK zTG2like	72,7 ± 23,1

Figure 41. Flow cytometry analysis of TGs2 expression. Hygromycin-selected HEK293 cells expressing the human and zebrafish TGs2 were tested with an anti-SV5 tag antibody after fixing and permeabilization. Wild type HEK293 cells were also tested as negative control. The hygromycin-selected stable HEK293 polyclonal populations show a high percentage of cells expressing the SV5 tagged-TGs2. The histogram plot shows the results of one of three independent experiments, whose data are indicated in the table as mean ± S.D (n=3).

The flow cytometry assay showed that the hygromycin-resistant stable polyclonal HEK293 populations show a high percentage of cells expressing the SV5-tagged protein. In particular, the averaged percentage of cells positively stained by the α-SV5 antibody is 78% for HEK^{zTG2a}, 79% for HEK^{zTG2B}, 73% for $HEK^{zTG2like}$ and 51% for HEK^{hTG2}.

4.10 zTGs2 Intracellular functions: Apoptosis assay

As already discussed, TG2 has been reported to affect the apoptotic process in several ways. Both pro-apoptotic and anti-apoptotic roles has been proposed and demonstrated, involving different cell models, apoptotic stimuli, and intracellular mediators. We already demonstrated how zebrafish TGs2, like hTG2, can mediate cell adhesion, rescuing the loss of cell attachment, cell spreading, and cytoskeleton organization caused by the RGD peptide. The compensation mechanism involving FN-TG2 complexes promotes FAK and MAPK signalling, which lead to pro-survival stimuli for the attached cells, which otherwise would have undergone apoptosis, because of the lack of pro-survival signalling mediated by integrins activation. At this point, we wanted to investigate if the zebrafish proteins could also play a protective, anti-apoptotic role in the intracellular compartment of HEK293 cells.

For this reason, after an initial phase of protocol optimization, we exposed both HEK293 cells expressing hTG2 and zTGs2 and control wild type HEK293 cells at 0.4 mM hydrogen peroxide for 2 hours. Indeed, in the optimization phase, we exposed wild type cells at different concentration of H_2O_2 (0.2 and 0.4 mM) and evaluated the effects on cell death at different time point (after 1, 2 or 4 hours), and we found that exposure of 0.4 mM for 2 hours was the most suitable condition to appreciate the effects of the treatment. After treatment, we analysed by flow cytometry the percentage

of cells undergoing cell death by staining with Annexin V-FITC and propidium iodide (PI). We confirmed that the presence of hTG2 has a protective role in HEK293 cells against apoptotic stimuli, and we also observed that the zTGs2 expression produce similar protective effects, as the percentage of treated cells undergoing cell death is reduced compared to the wild type HEK293 cells (Figure 42).

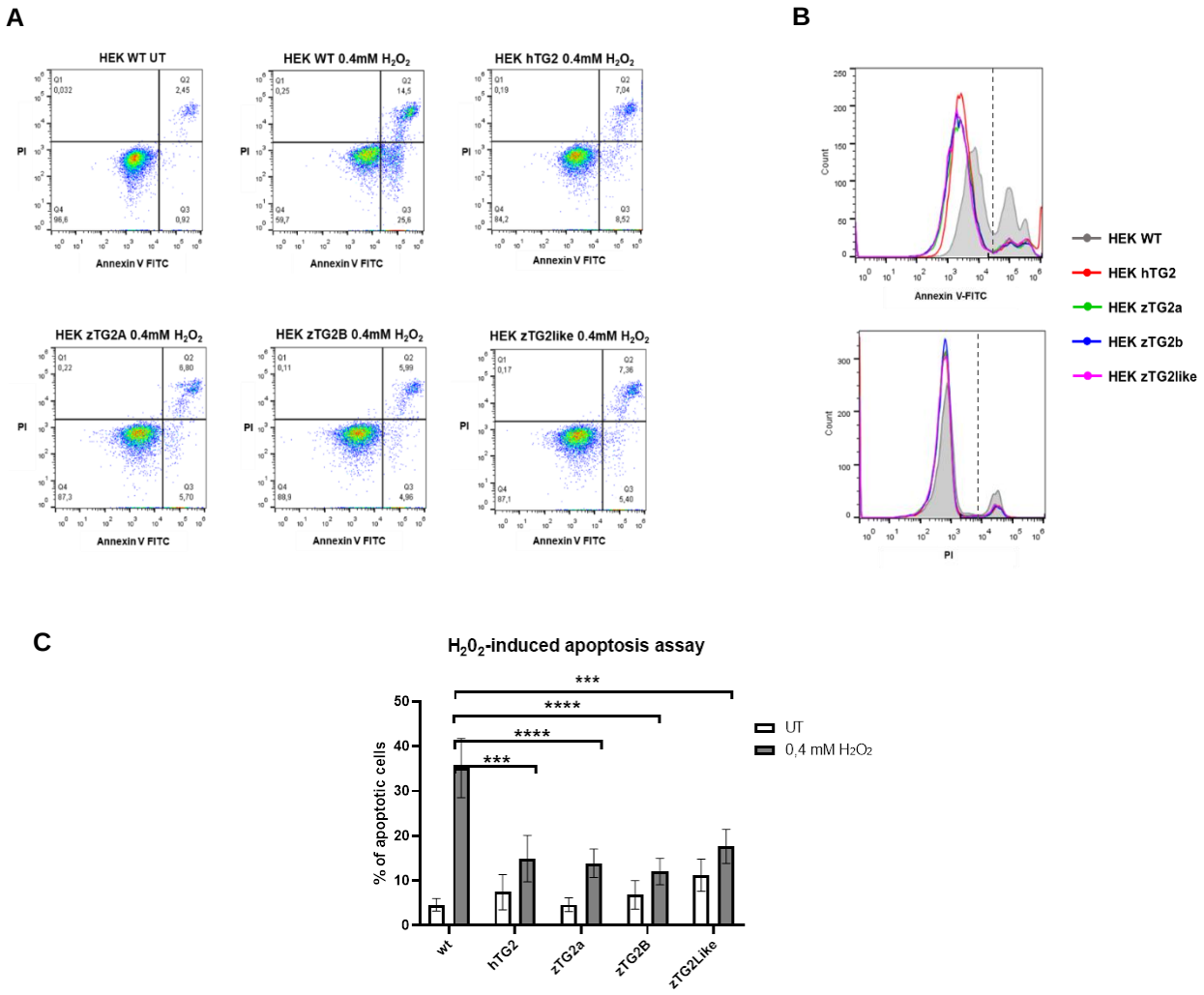


Figure 42. Hydrogen peroxide-induced apoptosis assay. A) HEK293 cells expressing hTG2 and zTGs2, and wild type HEK293 cells were treated with 0.4 mM H_2O_2 for 2 hours, then stained with Annexin V-FITC and propidium iodide (PI) and analysed by flow cytometry to assess apoptosis. The dot plot of the Annexin V-FITC – PI staining represents the results of one of five independent experiment (n=5). B) Histogram plots of Annexin V and PI staining of treated samples. The number of cells stained with Annexin V-FITC (upper panel) and PI (lower panel) is reduced in HEK293 cells expressing either hTG2 or zTGs2 compared to the wild type control cells, as shown by the lower peaks in the positive cells' region. The grey dashed line marks the gate between the negative cells, on the left, and the positive cells, on the right. The wild type sample is coloured with a grey shade. The plot shows the result of one representative experiment (n=5) C) Plot representing the percentage of apoptotic cells, resulting from the sum of the Annexin V positive cells and the PI positive cells. Treated HEK293 cells expressing hTG2 or zTGs2 show reduced number of apoptotic cells compared to the wild type control cells. The data are the averaged results of six independent experiments, shown as mean ± SD. ***=p<0.001; ****=p<0.0001.

Indeed, as showed in Fig. 42a, the number of cells positive for Annexin V staining and PI staining is higher in the wild type sample compared to the cells expressing TGs2. Figure 42b allows the better appreciation of the reduction in the percentage of cells undergoing cell death (sum of Annexin V-positive cells and double positive cells) in treated HEK293 cells expressing TGs2 compared to the control wild type treated cells. In particular, as showed in Fig. 42b, compared to 34% of cells undergoing cell death after H_2O_2 exposure in the wild type sample, we observed a 19% reduction of dying cells in HEK^{hTG2}, a 20% reduction in HEK^{zTG2a}, a 23% reduction HEK^{zTG2b}, and a 16% reduction in $HEK^{zTG2like}$ cells, that, however, show a higher percentage of dying cells in the untreated sample compared to the others.

Our observations demonstrated that the zebrafish TGs2 has the same anti-apoptotic effects reported for the human orthologue in HEK293 cells. In RCC, the anti-apoptotic activity of TG2 was shown to depend on TG2 interaction with p53, which determines degradation of p53 by routing this pro-apoptotic protein into the autophagosomes. In order to appreciate if, also in our case, the expression of TG2 and zTGs2 protein correlated with a downregulation of p53 as a potential mechanism underlying the observed effects on apoptosis, we tested the levels of p53 with a Western blot in wild type HEK293 and HEK^{TG2} cell lysates (Figure 43).

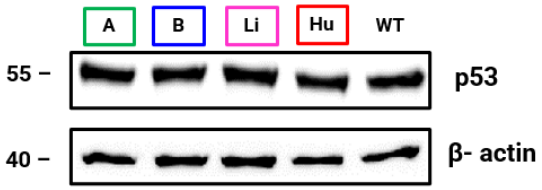


Figure 43. Correlation between TG2 and p53 expression. Western blot analysi of cell lysates of HEK293 wild type control cells (WT) and hygromycin-resistant HEK293 cells expressing hTG2 (Hu) and zTGs2 (A=zTG2a; B=zTG2b; Li=zTG2like). The levels of p53 do not show significant different between the WT and TG2-expressing cells. The levels of β-actin were tested for normalization.

The analysis demonstrated that in our HEK^{TG2} cells, the overexpression of hTG2 or zTGs2 does not impact the protein levels of p53, and we concluded that the observed anti-apoptotic effects must be caused by another mechanism.

5. CONCLUSIONS

In this study we laid the basis for the use of the zebrafish (*Danio rerio*) as a model organism to *in vivo* study TG2 and the biological processes in which it is involved.

Initially, we confirmed the presence in the Zebrafish genome of three orthologue genes of the human TG2, which we named zTG2a, zTG2b and zTG2like, and demonstrated with RT-qPCR that the three genes are expressed during the embryonic development of the zebrafish. We sequenced the three zTGs2 and showed that the encoded protein sequences are indeed more similar to TG2 than other members of the TG family, with zTG2like being the isoform with less homology of the three. We performed a multiple sequence alignment of zTGs2 and human TG2 (hTG2) and observed a high level of conservation in the protein region responsible for the transamidation activity.

In order to test the enzymatic properties of the zTGs2, we cloned the zTGs2 coding sequence into the bacterial expression vector pET30a+, and affinity-purified the zTGs2 as 6His-tagged recombinant protein produced in BL21(DE3) bacteria. We tested the zTGs2 in different enzymatic assays, demonstrating that they possess a transamidation activity, which, like the human enzyme, is Ca2+-dependent, but is not inhibited by the presence of GTP. This observation is supported by the low level of conservation of the putative GTP-binding residues in the zTGs2 protein sequence, with zTG2a being the only zebrafish isoform whose activity is somewhat affected by GTP. Moreover, we showed that the zTGs2 protein can be inhibited in vitro by three different hTG2-specific inhibitors, even though the inhibitory effects are significantly lower for zTGs2 compared to the human enzyme. The enzymatic assays demonstrate the biochemical similarity between the zebrafish and the human isoforms, but also highlight their differences.

In order to characterise also the non-enzymatic functions of zTGs2, we performed an RGD-independent adhesion assay with Human Foreskin Dermal Fibroblasts, and demonstrated that the zebrafish proteins, like the human orthologue, are able to form complexes with fibronectin molecules, which significantly rescue the loss of cell attachment and spreading caused by RGD-peptides. Moreover, we showed with epifluorescence microscopy that zTGs2-fibronectin complexes promote cell adhesion by mediating the reorganization of the actin cytoskeleton, with the formation of protrusive structures and focal adhesions. These data indicate that zTGs2 not only retain the catalytic function of protein crosslinking but can also mediate a very important cell adhesion function in the extracellular environment.

Then, in order to assess the functional properties of the zebrafish TGs2 in the intracellular compartment, we cloned the zTGs2 coding sequences into the pcDNA3.1 Hygro (+) eukaryotic expression vector and transfected HEK293 cells. We confirmed the expression of functional zTGs2

proteins after transient transfection, testing the transfected cells lysates with Western blot and enzymatic assays. With the aim of obtaining a stable over-expression of zTGs2, we selected the hygromycin-resistant HEK293 cells after transfection with the zTGs2-encoding plasmids. We confirmed the isolation of polyclonal populations of HEK293 with stable over-expression of zTGs2 (HEK^{zTGs2}) with Western blot and enzymatic activity assays on cell lysate, and with a flow cytometry analysis, which showed that the great majority of cells of the hygromycin-resistant clones overexpress the TG2. Confirmed their expression, we tested whether the zTGs2 have an anti-apoptotic function in HEK293 cells, as demonstrated for the human enzyme. We exposed wild type and zTGs2-expressing HEK293 cells at 0.4mM H_2O_2 for 2 hours and demonstrated with a flow cytometry assay that the expression of the zebrafish TGs2, like hTG2, have a protective role in HEK293. In particular, after the treatment, HEK^{zTGs2} show a significant lower percentage of cells undergoing cell death compared to wild type treated cells. Ultimately, we demonstrated that the anti-apoptotic mechanism observed for HEK^{zTGs2} does not rely on a TG2-dependent downregulation of p53, as previously reported in literature. Further studies are required to define the molecular mechanism underlying the anti-apoptotic function of the zebrafish transglutaminases. Moreover, downregulation of zTGs2 in HEK^{zTGs2} is required to definitively attribute the anti-apoptotic phenotype to the expression of the zebrafish proteins.

Finally, our results show that enzymatic properties and biological functions of the human transglutaminase are conserved in the three zebrafish orthologues. High functional conservation strongly supports the future use of the zebrafish as an animal model to *in vivo* study TG2 and its functions.

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