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The diamond anniversary of tissue transglutaminase: a protein of many talents

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Abstract

Tissue transglutaminase (tTG) is capable of binding and hydrolyzing GTP, as well as catalyzing an enzymatic transamidation reaction that crosslinks primary amines to glutamine residues. tTG adopts two vastly different conformations, depending on whether it is functioning as a GTP-binding protein or a crosslinking enzyme. It has been shown to have important roles in several different aspects of cancer progression, making it an attractive target for therapeutic intervention. Here, we highlight many of the major findings involving tTG since its discovery 60 years ago, and describe recent drug discovery efforts that target specific activities or conformations of this unique protein.

Keywords

Tissue transglutaminase; cancer; type 2 transglutaminase; inhibitors; transamidation; GTP-binding

Introduction

Tissue transglutaminase (tTG, EC 2.3.2.13), also referred to as type 2 transglutaminase, is one of nine members of the transglutaminase family of enzymes [1]. These proteins are best known for their ability to catalyze transamidations, or crosslinking reactions, in which an amide (primarily from a glutamine residue) reacts with a primary amine (often from a lysine residue) to form a new amide bond. The discovery of tTG is credited to Heinrich Waelsch, who in 1957 published an article showing that incubating guinea pig liver extracts with radiolabeled amines (ethanolamine, cadaverine or lysine) resulted in the Ca²⁺-dependent incorporation of the radiolabeled amines into lysate proteins [2]. This protein was later identified as tTG.

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Teaser: The GTP-binding and protein-crosslinking enzyme tissue transglutaminase plays crucial parts in the most malignant cancers. Targeting unique aspects of this protein holds great therapeutic potential.

In the 60 years since its discovery, tTG has been extensively studied [3]. It is a widely expressed protein and its expression and/or enzymatic activity is increased in several diseases, including Celiac disease, neurodegenerative diseases (e.g., Alzheimer's or Parkinson's disease) and highly aggressive cancers. Several key findings in tTG research are listed in Figure 1 [2,4–33]. Here, to commemorate the diamond anniversary of its discovery, we will highlight the groundbreaking work linking tTG to cancer progression, the potential of using tTG as a therapeutic target and a description of the future avenues of research still surrounding this important enzyme.

tTG structure and function

tTG is capable of protein crosslinking and GTP-binding activity [9]. The crosslinking reaction occurs through a ping-pong mechanism that involves the binding of a glutamine residue in a substrate protein to Cys277 in tTG (Figure 2a). Cys277 is essential for enzymatic activity and, together with His335 and Asp358, comprises the catalytic triad [21,28,34]. Iismaa and colleagues identified another highly conserved residue: Trp241 (Figure 2b), which was important for stabilizing the transition state of the reactive glutamine residue in the substrate protein, leading to two potential reaction mechanisms (Figure 2c) [1]. Either reaction pathway begins with deprotonation of Cys277 by His335, and subsequent stabilization of the His335 cation by Asp358. Trp241 then either forms a π -cation interaction with the substrate glutamine, to stabilize a more electrophilic transition state before nucleophilic attack (Figure 2c, left pathway), or forms a H-bond to the glutamate after it has been attacked by Cys277 (Figure 2c, right pathway). Transamidation then proceeds as expected: a primary amine attacks the thioester carbon (lower right), using Cys277 as a leaving group. This releases the transamidated product and regenerates the catalytic site. Mutation of any of the key residues that comprise the catalytic triad, as well as Trp241, has been shown to inhibit the crosslinking activity of tTG [1]. tTG can also hydrolyze the amide to a carboxylic acid or, to facilitate transesterification, amidation of esters, or ester hydrolysis reactions, but these activities are far less comprehensively studied [35].

In some early studies, it was determined that the crosslinking and GTPase activities of tTG were regulated by specific co-factors. The addition of excess Ca^{2+} to tTG promotes its crosslinking activity and inhibits GTP-binding activity, whereas the addition of GDP or GTP to tTG inhibits its crosslinking activity. In 2000, Di Venere *et al.* conducted a study where tTG, treated with either Ca^{2+} or GTP, was analyzed by circular dichroism (CD), intrinsic tryptophan fluorescence and binding strength to the fluorescent probe 1-anilino-8-naphthalenesulfonic acid (ANS) [36]. When excited at any of three different wavelengths, the fluorescence emission spectra for the Ca^{2+} - and GTP-bound forms of tTG were nearly identical. However, following that excitation, the emission from Ca^{2+} -bound tTG lasted significantly longer than that from GTP-bound tTG. This suggested that Ca^{2+} - and GTP-bound tTG adopted different conformations. Similarly, ANS bound more tightly to Ca^{2+} -bound tTG than to GTP-bound tTG (suggesting that the former had a greater solvent-accessible hydrophobic surface area), whereas CD measurements showed a destructuring of the aromatic region of the spectrum for Ca^{2+} -bound tTG, suggesting greater mobility for aromatic residues. The authors concluded that switching from the GTP-bound state to the

crosslinking active state of tTG was accompanied by what they characterized as only ‘a slight widening of the protein molecule’ [36].

The crystal structure of the guanine-nucleotide-bound conformation of tTG was described 2 years later by Liu and co-workers (PDB ID 1KV3). The structure revealed that the guanine-nucleotide-bound form of tTG adopted a compact conformation consisting of four domains: an amino-terminal β -sandwich domain, the primarily helical catalytic domain and two C-terminal β -barrel domains. The two β -barrel domains folded over onto the catalytic domain (Figure 3a) [21]. The structure also showed that the guanine nucleotide binding pocket was solvent-accessible (Figure 3b), and that Cys277, one of the key residues responsible for catalyzing the protein crosslinking activity of tTG, was located within a deep groove, access to which was occluded by the C-terminal β -barrels (Figure 3d). The authors hypothesized that GDP/GTP binding stabilized this conformation of tTG, and that, upon binding Ca^{2+} , the β -barrels would slightly move away from the active site, allowing substrates access to the catalytic site.

Subsequently, Pinkas *et al.* reported the structure of the crosslinking active form of tTG: PDB ID 2Q3Z [28]. The conformation of the crosslinking active form of tTG was dramatically different from that of the nucleotide-bound protein. The C-terminal β -barrel domains swung completely away from the crosslinking active site, such that they were nearly linear with the N-terminal β -sandwich and catalytic domains (Figure 3a). This crosslinking active state of tTG was unable to bind GTP/GDP, because the nucleotide-binding domain was largely disrupted in that conformation (Figure 3c). However, the crosslinking active site was available to bind substrates (Figure 3e). These large conformational changes in tTG have since been confirmed in solution using small-angle X-ray scattering (SAXS) [37]. The crosslinking active state, as represented by the more extended form of tTG, is now commonly referred to as the ‘open-state’ conformation, whereas the compact GTPase active state has come to be known as the ‘closed-state’ conformation.

Several other structural and mechanistic features of tTG are less well understood. One is the exact role of Ca^{2+} in stabilizing the tTG open-state, and activating crosslinking. To date, no crystal structure of tTG bound to Ca^{2+} has been described. Site-directed mutagenesis studies performed by Datta and colleagues, as well as Kiraly and colleagues, identified several Ca^{2+} -binding sites on tTG, that when mutated decreased the ability of tTG to bind Ca^{2+} and inhibited crosslinking activity [38,39]. TGM3 and FXIIIa have been crystallized in the presence of Ca^{2+} (PDB IDs 1NUG and 1GGU, respectively), and the two Ca^{2+} -binding sites identified in these enzymes correspond to sites predicted in the mutational studies performed on tTG [40,41]. However, Ca^{2+} -bound TGM3 and FXIIIa adopt conformations that resemble the closed-state of tTG, suggesting that not all Ca^{2+} -binding sites in tTG are important for inducing conformational changes.

Another important issue concerns how tTG conformation is regulated in biological systems. tTG is widely expressed, and has been detected in the cytosol, nucleus, cell membrane and even in the extracellular space [3,11,13,14,16,30]. Ca^{2+} concentrations outside the cell are significantly higher than nucleotide concentrations, suggesting that extracellular tTG

probably exists in the open-state. Inside cells, nucleotide concentrations exceed the levels of Ca^{2+} . Thus, it is expected that intracellular tTG should be predominantly in the closed-state [42,43]. However, it is worth mentioning that assorted tTG substrates, such as the fluorescent monodansylcadaverine (MDC), have been used to label cellular proteins, suggesting that at least some of the tTG expressed in cells is in the open-state and able to crosslink substrates [44,45].

Finally, the GTP-binding and hydrolytic activities of tTG are still poorly understood. In 1990, Im and Graham isolated an ~74 kDa GTP-binding protein that they reported was able to couple to the α_1 -adrenergic receptor and named $G_{\alpha h}$ [46]. A later report would demonstrate that $G_{\alpha h}$ and tTG were the same protein [9]. However, tTG has very little in common with classical GTP-binding proteins (i.e., G proteins). For example, most G proteins have highly conserved domains called switch regions which move upon binding GTP. These movements allow the G proteins to bind and activate effectors. The hydrolysis of GTP to GDP causes the switch regions to return to their original, inactive positions. No such switch regions have been identified in tTG. In fact, the binding of either GDP or GTP to tTG can cause it to adopt the closed-state. However, GTP has been shown to bind more tightly to tTG compared with GDP, and thus GTP enhances the conversion of tTG to the closed-state more potently than does GDP [9]. This is in good agreement with a study by Jang and colleagues, which reported that the crystal structure of GTP-bound tTG (PDB ID 4PYG) was nearly identical to the GDP-bound form of tTG reported by Liu and colleagues (Figure 3f,g) [47]. Han *et al.* showed that tTG in the closed-state could also bind ATP (PDB ID 3LY6) [48]. To date, there is no substantial evidence that the signaling activity of tTG is differently effected by its association with GDP, GTP or ATP.

Roles in cancer

Since the early 2000s, tTG has been recognized as a major player in cancer. Expression of tTG at the RNA transcript and protein levels correlates with grade and aggressiveness across several types of cancer, with the lower grade or less aggressive tumors generally exhibiting lower tTG expression, and the higher grade, more aggressive tumors typically showing high tTG levels (Figure 4a–c) [49–53]. However, it is worth emphasizing that increasing tTG expression and/or activation in cells has not been shown to induce cellular transformation or directly cause cancer. Rather, it appears that tTG functions downstream of several known oncogenes, where it has been shown to enhance various aspects of cancer progression, including promoting cell growth and survival, and increasing cell migration and invasive activity (Figure 5) [54,55].

Signaling: upregulation of tTG

The activation of several signaling pathways has been shown to influence the rates of tTG transcription. In 1995, the Davies laboratory published two of the earliest studies on this topic. In the first study, a 1.74 kb fragment of the promoter region of *TGM2*, the gene encoding tTG, was isolated and analyzed for regulatory domains [56]. Four binding domains for specificity protein (SP)1, a transcription factor involved in promoting cell differentiation and cell cycle regulation, were identified [57]. SP1 has been shown to be activated in

response to K-Ras, one of the most commonly mutated genes in cancer, raising the possibility that activation of K-Ras could induce tTG expression [58]. Putative binding sites for several other proteins known to influence transcription were also identified in this region of the *TGM2* promoter, including AP1 and AP2, glucocorticoids, neurofibromin (NF)1 and the inflammatory cytokine interleukin (IL)-6. However, only deletion of the SP1- and NF1-binding sites substantially diminished the transcriptional activity of this promoter. In the second study, a larger promoter region of tTG was isolated and found to contain retinoic acid (RA) response elements (RAREs) [10]. Consistent with this finding, treatment of a variety of cell types with RA strongly induces tTG expression [59–61].

Several other regulatory domains within the tTG promoter have since been described. Mirza *et al.* demonstrated that a transcription factor that functions downstream of many small GTPases and Wnt proteins, namely nuclear factor (NF)- κ B, bound to the tTG promoter and enhanced tTG expression in rats following liver injury [62–64]. A year later, Ritter and Davies discovered a response element for transforming growth factor β -1 (TGF- β), while Kuncio *et al.* identified a binding site for the inflammatory cytokine tumor necrosis factor (TNF)- α [65,66]. More recently, Jang and co-workers demonstrated that tTG expression is induced under hypoxic conditions. They also showed that the binding of hypoxia-inducible factor (HIF)-1 to the promoter mediated this outcome [67]. As shown in Figure 5, several of these inducers of tTG expression are also frequently implicated in oncogenesis.

Signaling: regulation by tTG

tTG has been shown to play a part in the activation of several common oncogenic signaling pathways, with one example being Sonic Hedgehog (Shh). Shh signaling is important for the growth and patterning of stem cells, and mutations in components of the Shh signaling pathway have been observed in aggressive cancers [54,68,69]. Dierker *et al.* found that tTG could crosslink Shh, forming oligomers of the ligand with elevated biological activity, compared with the monomeric Shh [68].

Similarly, tTG can mediate β -catenin signaling in response to platelet-derived growth factor (PDGF) stimulation [70]. Upon activation, β -catenin translocates to the nucleus where it activates the Tcf/LEF transcription factors, as part of the canonical Wnt pathway. Signaling by Wnt and β -catenin is involved in development and cancer progression [69]. Beazley *et al.* [70] showed the treatment of A10 smooth muscle cells with PDGF induced the translocation of β -catenin to the nucleus and stimulated cell growth, outcomes that could be inhibited when cells were treated with the specific tTG inhibitor ERW-1069. Interestingly, β -catenin-mediated signaling and cell growth could be induced by treatment of the same cells with recombinantly expressed tTG, suggesting that extracellularly expressed tTG is sufficient to induce these effects.

Another example of the oncogenic signaling of tTG involves NF- κ B. As already mentioned, NF- κ B can induce tTG expression [62]. However, tTG has also been shown to activate NF- κ B. Mann *et al.* found that, in breast, skin and pancreatic cancer cell lines that expressed varying amounts of tTG, the extent of NF- κ B activity detected in the cells correlated with the levels of tTG expression. Using tTG inhibitors and siRNAs targeting tTG, the authors discovered that the extent of NF- κ B activity observed in each cell line was dependent upon

tTG crosslinking activity. The mechanism responsible for this effect involved I κ B α , a protein that binds to NF- κ B in the cytosol and prevents it from translocating to the nucleus, where it regulates transcriptional events [26]. Normally, NF- κ B becomes activated when I κ B α is phosphorylated, causing it to dissociate from NF- κ B. Mann *et al.* showed that tTG crosslinks I κ B α and functionally inactivates its regulatory actions, resulting in constitutive NF- κ B activity.

tTG can also influence signaling events by modifying the small GTPase RhoA. Singh *et al.* showed that tTG transamidates RhoA, causing it to become constitutively active [19]. This then promoted binding of RhoA to ROCK-2, and subsequent activation of the ERK1/2, JNK1 and p38 γ MAP kinases [20]. ERK is involved in the activation of transcription factors such as c-myc, as well proteins involved in cell cycle progression and ATP synthesis. Similarly, JNK promotes the activation of the transcription factor c-Jun, which was recently demonstrated to upregulate expression of the metabolic enzyme glutaminase C, the activity of which is vital for the progression of numerous cancers [71].

A more definite link between tTG and cancer metabolism was provided in a study by Kumar *et al.*, who demonstrated that mammary epithelial cells stably expressing excess tTG exhibited enhanced glucose uptake and lactate production, each being a common phenotype in cancer cells [72]. This occurred as a result of NF- κ B activation, as observed by Mann *et al.*, leading to enhanced expression of HIF-1 α , and subsequent expression of the metabolic enzymes GLUT-1, HK2 and LDHA. These same proteins lost expression when either tTG or the p65 subunit of NF- κ B were knocked down with targeted shRNA.

Further, tTG plays a part in regulating the activity of growth factor receptors. Zhang *et al.* demonstrated that closed-state tTG binds to and sequesters c-Cbl, an E3-ubiquitin-ligase that catalyzes the ubiquitylation of the epidermal growth factor receptor (EGFR) and targets it for degradation in the lysosome. Sequestration of c-Cbl by tTG prevented the ubiquitination and downregulation of the EGFR in glioblastoma cells. The result was a build-up of EGFRs – an outcome that was important for the growth of brain tumor cells [53].

Promotion of cell survival

tTG plays an important part in promoting cell survival. Ironically, early on tTG was thought to only cause cell death. In 1987, Fesus, Thomazy and Falus showed that the induction of apoptosis in rat thymocytes was accompanied by a large increase in tTG expression and catalytic activation. This led the authors to hypothesize that tTG had a causative role in promoting apoptosis, a topic that received substantial attention for about a decade [6]. Today, we know that tTG can promote either apoptosis or cell survival in a stimulus-dependent manner. One of the earliest examples of this came when Tucholski and Johnson showed that SH-SY5Y neuroblastoma cells ectopically expressing tTG became more sensitive to osmotic-stress-induced apoptosis, but less sensitive to heat-shock-induced apoptosis, compared with control cells [73].

One potential mechanism that can account for the pro-survival function of tTG is its ability to modify the tumor suppressor retinoblastoma protein (pRB). Active pRB prevents progression through the G₁/S checkpoint of the cell cycle, and phosphorylation of pRB

inactivates the protein and allows cell cycle progression. pRB has also been strongly implicated in apoptotic processes [74]. Activation of pRB in rapidly dividing cancer cells often induces a strong apoptotic response, via interaction with E2F1 to enhance expression of caspase 7 and p73, or by binding monomeric Bax, causing the release of Ca^{2+} from mitochondria (an important step in promoting apoptosis) [75]. In 1997, Oliverio *et al.* found that pRB was a transamidation substrate for tTG. The authors showed that the crosslinking of pRB by tTG formed a pRB oligomer, and hypothesized that this larger species was important for apoptotic signaling [76]. However, it was subsequently shown that the modification of pRB by tTG was in fact antiapoptotic. When tTG crosslinked pRB, it inactivated its tumor suppressor activity, and thus promoted cell survival [74].

A second mechanism involves tTG helping cancer cells to adapt to metabolic stresses. Cancer cells tend to generate a large amount of lactate, and as a result acidify the tumor microenvironment (TME). In 2015, tTG was demonstrated to be a key modulator of this acidity [60]. As part of its crosslinking reaction, tTG produces ammonia, which helps to minimize the acidification of cell culture medium for T98G or U-87 MG brain cancer, or MCF7 breast cancer cells. In either case, small-molecule inhibition of tTG resulted in acidification of the culture medium. Moreover, acidification of the cell culture medium with hydrochloric acid resulted in the upregulation of tTG, strongly suggesting an important role for tTG in directly responding to this common metabolic stress.

Chemoresistance

A common approach in cancer chemotherapy is to use cytotoxic agents (such as doxorubicin or gemcitabine) to induce apoptosis in cancer cells. However, resistance to such therapies is a major hurdle confronted by oncologists, and tTG plays an important part here as well. tTG expression levels are often elevated in drug-resistant cancer cells. For example, Kim *et al.* showed that doxorubicin-resistant breast cancer cells express high levels of tTG, whereas their doxorubicin-sensitive counterparts express little or no detectable tTG. Inhibition of tTG activity using the small-molecule inhibitor cystamine rendered the cells sensitive to doxorubicin [77].

Further, tTG impacts gemcitabine resistance in pancreatic cancer. Verma *et al.* demonstrated that tTG expression correlated closely with gemcitabine resistance in Panc-28, MiaPaCa-2 and BxPC-3 pancreatic cancer cells. BxPC3 cells, with nearly undetectable levels of tTG, were very sensitive to gemcitabine (IC_{50} 0.01 μM), whereas the moderately tTG-expressing MiaPaCa-2 cells were less sensitive to the chemotherapeutic (IC_{50} 0.1 μM). Panc-28 cells, which had the highest levels of tTG expression, were shown to be the least sensitive to gemcitabine (IC_{50} 1 μM). Ectopic expression of tTG in BxPC-3 cells caused the cells to become highly resistant to gemcitabine, strongly suggesting that tTG plays an important part in promoting chemoresistance [27].

Cao *et al.* examined the role of tTG in mediating cisplatin resistance in ovarian cancer. Similar to the above studies, the authors found that tTG expression levels correlated with cisplatin resistance [29]. They showed that knocking-down the expression of tTG in the drug-resistant SKOV3 ovarian cancer cell line sensitized the cells to cisplatin. Moreover, they found that treating the cells with KCC009, a potent tTG inhibitor, could mimic this

effect. Interestingly, they then went on to transfect tTG knockdown SKOV3 cells with constitutively active forms of AKT or NF- κ B, and discovered that activated NF- κ B could rescue the cells from cisplatin-induced toxicity. Thus, it appears that tTG activates NF- κ B to promote drug resistance.

Migration, invasion and the epithelial-to-mesenchymal transition

Another well-established role for tTG involves its effects on cell adhesion and migration, both of which are necessary for promoting cancer cell invasion and metastasis. In 1992, Gentile *et al.* noticed that Balb-C 3T3 fibroblasts overexpressing tTG exhibited a highly flattened morphology, indicating enhanced cell attachment [78]. A report by Akimov *et al.* in 2000 demonstrated that, in W-38 fibroblasts and HEL erythroblasts, membrane-associated tTG tightly bound to the extracellular matrix protein fibronectin (Fn), leading to enhanced cell attachment and spreading on Fn-coated surfaces [15]. A related study by Balklava and co-workers suggested that, in Swiss 3T3 fibroblasts, Fn surface adhesion was enhanced by the ectopic expression of either wild-type or a crosslinking-deficient form of tTG, suggesting that the ability of tTG to promote cell attachment occurs independently of its crosslinking activity [79].

The role of tTG in the related process of cell migration is somewhat less well understood. Akimov and Belkin demonstrated that monocytic cells required tTG expression to migrate on a Fn-coated surface [17]. However, in the study by Balklava and colleagues, Swiss 3T3 fibroblast migration slowed when tTG was ectopically expressed in the cells [79]. Moreover, a study by Belkin *et al.* showed that matrix metalloproteinases degraded membrane-associated tTG, releasing cells from the Fn surface [16].

More recently, Antonyak *et al.* found that EGF-stimulated HeLa cervical carcinoma cell migration depended upon the activation of tTG, and its recruitment to the leading edges of migrating cells. This localization of tTG required EGF stimulation, because treatment of the same cells with RA activated tTG to a similar extent as EGF, but did not cause its accumulation at leading edges [59]. A follow-up study showed that this localization was dependent upon the ability of tTG to bind to the chaperone heat shock protein 70 (HSP70), which localizes to the leading edge of migratory cells [80]. Inhibition of either tTG crosslinking activity or the chaperone function of HSP70 blocked EGF-stimulated HeLa cell migration. Combined, these studies suggest a mechanism by which cells use tTG to adhere at the leading edge, anchoring the front of the cell in place while matrix metalloproteases degrade tTG at the rear, allowing the cells to detach. In this way, tTG effectively promotes directional migration.

An interesting report by Hwang and co-workers directly examined the tTG-dependent invasive potential of ovarian cancer cells [81]. Here, the authors demonstrated that ectopically expressing tTG into SKOV3 ovarian cancer cells dramatically increased their invasive potential, as measured by the movement of cells through Matrigel[®]. They complemented this finding by knocking-down tTG expression in HeyA8 ovarian cancer cells using siRNA, and showing that their ability to invade through Matrigel[®] was lost.

An important mechanism by which tTG influences cancer cell motility is through its role in the epithelial-to-mesenchymal transition (EMT). This process involves the conversion of epithelial-like cells (largely immobile cells with strong cell–cell adhesive properties) into mesenchymal-like cells, which are highly migratory. In 2009, Shao *et al.* showed that ectopic expression of tTG in ovarian cancer cells was sufficient to induce EMT [82]. In 2010, Kumar and co-workers showed an identical effect in mammary epithelial cells [32].

EMT is often induced by TGF- β , and a 2012 study by Cao and colleagues showed that TGF- β induced expression of tTG in ovarian cancer cells [31]. tTG expression was essential for EMT to occur in these cells, as read out using morphological changes and the expression of EMT markers such as Zeb1. Importantly, only the Fn-binding domain of tTG, residues 1–140, was required for EMT. However, in another study by Kumar and colleagues, it was shown that expression of wild-type tTG, or the crosslinking-defective C277S mutant, in mammary epithelial cells was able to induce EMT, whereas a GTP-binding defective mutant, tTG (R580A), was ineffective [83]. Thus, it is still unclear how tTG promotes EMT.

The tumor microenvironment

We have thus far focused primarily on the intracellular functions of tTG, but the enzyme has also been found to have important roles in the TME (i.e., the space immediately surrounding a tumor). One exciting function for extracellular tTG is in the context of microvesicles (MVs), a type of nonclassical secretory vesicle released from the surfaces of a variety of cancer cell types [30]. MVs from highly aggressive cancer cells can be transferred to other cell types that reside in the TME. The transfer of MVs from cancer cells to their target cells can promote cell growth and survival, as well as increase invasive activity and metastasis. Antonyak *et al.* found that tTG and Fn are major components of MVs isolated from aggressive MDA-MB-231 breast cancer and U87-MG glioma cells [30]. The tTG associated with these vesicles could crosslink Fn located along the surface of the MVs, creating a unique oligomeric form of Fn that enabled enhanced docking of the MVs to the recipient cell. This crosslinking event enhanced MV-initiated transformation of NIH-3T3 cells.

In a related report, Feng *et al.* recently showed that tTG can crosslink a 165 amino acid form of vascular endothelial growth factor (VEGF)-A (VEGF₁₆₅) in MVs to form an oligomeric, ~90 kDa form of VEGF (VEGF_{90K}). This oligomeric form of VEGF was capable of potentially activating VEGF receptors expressed on endothelial cells and promoting tumor angiogenesis [84]. Moreover, this effect was insensitive to antibody therapies that target VEGF (e.g., bevacizumab), thus offering a new way in which tTG can contribute to drug resistance. tTG can also influence the TME independently of MVs, because it has been shown that tTG can be released from cells as a soluble protein. For example, Lee *et al.* demonstrated that pancreatic cancers secrete tTG into their microenvironment, where it enhances tumor growth [85]. They first demonstrated that xenografts of tTG knockout AsPC1 or Panc1 cells had reduced growth compared with xenografts of wild-type cells. They then determined that tTG was being secreted by the cells, and was crosslinking collagen. This in turn stiffened the tumor stroma, and activated the YAP/TAZ pathway – activation of this pathway being a known promoter of tumor growth. Similar findings were reported by Yakubov and colleagues, who demonstrated that extracellular tTG was able to

promote metastasis in ovarian cancer [86]. They found that tTG, either naturally secreted from cells or added directly to culture medium, was able to upregulate NF- κ B through a noncanonical pathway involving phosphorylation of p65 and p100, although these events occurred only upon the addition of crosslinking competent tTG. Unfortunately, the authors were unable to determine the exact binding partner of tTG that initiated signaling. However, the effects also occurred *in vivo*, with injections of tTG able to stimulate EMT and invasion in ovarian cancer xenograft models.

Although tTG generally enhances the growth of tumors when regulated by cancer cells, some studies have suggested a contrary role. In 2006, Xu and co-workers investigated GPR56, a G-protein-coupled receptor often downregulated in metastatic cancers. They found that expression levels of GPR56 did not predict melanoma growth rate in cell culture but that overexpression of the receptor in MC-1 cells caused fewer metastases to form following their injection into the tail-vein [87]. Interestingly, it was shown that tTG in the TME bound the N terminus of GPR56, although it was not demonstrated that this binding interaction led to activation of the receptor or otherwise inhibited tumor metastasis. These results are similar to those from Jones *et al.*, who found that recombinantly expressed tTG inhibited angiogenesis during *in vitro* tube formation assays. This activity appeared to be due to protein crosslinking. They further showed that injection of tTG into CT26 colon cancer xenografts reduced tumor growth, and that tTG knockout mice implanted with B16-F1 mouse melanoma cells grew more rapidly than they did in wild-type mice, with the overall survival time being reduced by 4–8 days [88].

tTG in cancer stem cells

One recently appreciated role for tTG pertains to its actions in cancer stem cells (CSCs). These cells, also referred to as tumor-initiating cells, have recently begun attracting a good deal of attention, because they are thought to give rise to the entire tumor and promote tumor heterogeneity. CSCs are resistant to chemo- and radio-therapies, and are considered the principle drivers of tumor recurrence [33,61]. Recent work has shed light on an important role for tTG in the development and survival of CSCs. For example, in the context of brain cancer, two different classes of stem cells exist: highly aggressive mesenchymal stem cells (MES-GSCs) and less aggressive pro-neural stem cells (PN-GSCs). Fu *et al.* showed in 2013 that MES-GSCs have higher levels of tTG expression compared with PN-GSCs. Consistent with this finding, MES-GSCs were uniquely sensitive to tTG inhibition, which caused them to lose their stem cell phenotype [33]. Sullivan *et al.* recently expanded upon this finding, showing that treatment of MES-GSCs with tTG inhibitors and the DNA alkylating agent temozolomide was highly synergistic in causing cell death [61].

Fisher and co-workers demonstrated a role for tTG in epidermal CSCs. They found that tTG interacted with $\alpha 6/\beta 4$ integrins to activate a pathway that resulted in nuclear accumulation of YAP1. In the nucleus, YAP1 bound to and stabilized Np63 α against proteosomal degradation, leading to enhanced spheroid formation (a common phenotype of CSCs) and migration [89]. Supporting these findings, Kerr *et al.* recently showed that chemical inhibitors of tTG were detrimental to the survival of epidermal CSCs [90]. Further, while studying the role of tTG in EMT, Cao and co-workers also demonstrated that shRNA-

mediated knockdown of tTG in primary ovarian cancer cells blocked spheroid formation and reduced the number of cells expressing the CD44+/CD117+ CSC markers [31]. Stimulation of tTG expression by TGF- β had the opposite effect.

Inhibitors of tTG

The important roles for tTG in cancer progression make it an especially attractive target for drug discovery. Moreover, tTG knockout mice are phenotypically normal, suggesting that inhibiting tTG function would probably not have detrimental effects on healthy cells [18]. However, there are several issues to consider when designing inhibitors against tTG. Some members of the transglutaminase family are structurally similar. For example, tTG and TGM5 share ~40% sequence identity. Another issue to consider is that tTG has a weak consensus sequence for substrates, despite being able to discriminate between glutamine or lysine residues on substrates [91]. Examination of the assorted glutamine-crosslinking substrates in the transdab wiki (a repository of information pertaining to transglutaminase family substrates and interaction partners, located at <http://genomics.dote.hu/wiki/>) reveals no clear or conserved consensus sequence [92]. A similar lack of consensus sequence was determined for lysine-bearing substrates, with Murthy *et al.* concluding that the overall secondary structure of a substrate, rather than its specific sequence, was most likely to be responsible for tTG binding [91].

Pseudo-substrate inhibitors

Despite the moderate homology between tTG and each of its family members, and the potential promiscuity of tTG for substrates, some success has been achieved in developing tTG-specific inhibitors. Most of the tTG inhibitors that have been reported target its crosslinking activity, and generally fall into one of three categories: pseudo-substrates, peptidomimetics or novel scaffolds. The pseudo-substrates were among the first tTG inhibitors to be identified. Compounds such as MDC and cystamine (Figure 6a) contain a primary amine at the end of a long carbon chain, and act as amine-donor substrates for tTG. Thus, although they do not strictly inhibit tTG crosslinking activity, they prevent biologically relevant crosslinking events from occurring. Of these tTG inhibitors, MDC is generally considered to be the most potent [93]. However, these inhibitors tend not to be highly selective. For instance, MDC has known inhibitory activity against 17-ketosteroid reductase and phosphatidylcholine synthetase, as well as other transglutaminase family members [93]. Still, the pseudo-substrate inhibitors of tTG have been proven to be useful for studying tTG-crosslinking-mediated events in cell culture systems [20,30,59–61,77]. Unfortunately, they have not been successful in the clinic, with MDC being poorly tolerated in mice [93].

Peptidomimetic inhibitors

The best developed class of tTG inhibitors are the peptidomimetic inhibitors (Figure 6b). The first identified role for tTG in disease progression was in Celiac disease [11]. In 1997, Dieterich and colleagues reported that tTG was the major autoantigen in Celiac disease, a condition in which consumption of gliadin leads the immune system to attack the small intestine [11]. They also showed that gliadin was a direct substrate of tTG, and this led to the

development of gliadin-mimetic peptides as inhibitors of tTG. Most of these compounds are irreversible inhibitors, designed with cysteine-reactive functional groups at the glutamine position of the peptidomimetic that form a covalent bond to Cys277 and cannot be removed via reaction with primary amines or water.

Peptidomimetic inhibitors of tTG have been used with success to inhibit the growth of cancer cells. One widely used example is KCC009 (Figure 7b), which was identified in 2005 during a screening of compounds containing 3-halo-4,5-dihydroisoxazole rings. This ring system forms the reactive group in the naturally occurring glutamine analog acivicin [94]. KCC009 has a very low potency for tTG *in vitro* (reported K_i of 420 μ M) but was shown to be efficacious in enhancing the sensitivity of HCT-116 colon cancer cells to epothilone at much lower concentrations (100 μ M or less) while having no direct cytotoxic effect on those cells. In mice, an oral dose of 80 mg/kg KCC009/bodyweight resulted in 4.7 μ M compound being detected in the blood 1 h after treatment, with these doses substantially inhibiting the specific activity of tTG from the small intestines of the mice, despite being two orders of magnitude lower than the required dose to inhibit tTG crosslinking activity *in vitro*. The drug alone was unable to substantially reduce the growth of xenografted DBT glioblastoma cells, but co-dosing with BCNU (a DNA alkylating agent) was effective. A follow-up study showed that KCC009 functions primarily by blocking the interaction of tTG with Fn in the extracellular matrix, suggesting that the drug is not cell permeable [95].

Negligible cell permeability is common among peptidomimetics targeting tTG. A rare exception is Z-Don (Figure 6b), a member of a family of related inhibitors marketed by Zedira (Darmstadt, Germany), utilizing a reactive 6-diazo-5-oxo-L-norleucine functionality to bind Cys277. Compared with KCC009, Z-Don is substantially more potent (IC_{50} 20 nM), cell permeable to some extent (GI_{50} 40 μ M) and has been shown to inhibit the growth of several classical cell lines and CSCs that express tTG [60,61,96]. A related compound: ZED-1227 (Figure 6b), is a cell-impermeable inhibitor that was reported to have entered clinical trials for Celiac disease in 2015 as a first-in-class compound, and a recent press release claims that it was well tolerated in healthy male or female volunteers, and has been advanced to Phase IIa trials to test efficacy in Celiac patients (see web poster at http://www.zedira.com/resources/content/pdf/zedira_poster_oslo_2011.pdf and press release at http://zedira.com/News/Press-release-Dr-Falk-Pharma-and-Zedira-announce-completion-of-phase-1b-clinical-trial-of-ZED1227-for-the-treatment-of-celiac-disease-and-move-on-to-proof-of-concept-study_97).

Compound NC9 (Figure 6b), first described in 2008, is an example of a peptidomimetic utilizing a Michael-acceptor functionality to react with Cys277 [97]. NC9 was based on an earlier series of peptidomimetics, but includes a PEGylated tail with a fluorescent dansyl group. Although these modifications cost the molecule significant potency (K_i values ranging from 150 nM without the PEG tail to 29 μ M with the tail), the inhibitor can block the tTG-mediated mineralization of collagen in the extracellular matrix, and the fluorescent group gives it labeling capabilities similar to MDC. Moreover, the molecule is at least moderately cell permeable, as demonstrated in a recent report by Kerr *et al.* examining drug binding to an intracellular tTG chimera tagged with the FRET pair mCerulean and eYFP [90].

A final class of peptidomimetic inhibitors was recently described in a report by Badarau *et al.* These authors originally made use of dimethylsulfonium or imidazolium ions to irreversibly bind to tTG while ensuring substantial water solubility for the molecules. This prevented cell permeability and ensured that only extracellular tTG would be targeted. In their 2015 report, the authors optimized these molecules for tTG selectivity and potency, and also generated molecules that were cell permeable [98]. Using molecular mechanics simulations to determine the optimal conformation of their scaffold, they replaced the central residue of their peptidomimetic with a piperazine ring, resulting in compounds such as **1a** (Figure 6b). Further optimizations yielded compound **3h** (Figure 6b), which has an identical warhead and dansyl fluorescent label to NC9 but has a reported IC₅₀ value of 6 nM, compared with a K_i of 29 μ M for NC9. However, this newer class of compounds has not yet been exhaustively utilized.

Novel scaffolds

The third class of tTG inhibitors are novel scaffolds that do not serve as alternate catalytic substrates. This class has attracted significant attention in recent years. Several such compounds are currently marketed by Zedira, and are based around the same imidazolium functionality as the early Badarau peptidomimetics. These compounds (Figure 7) were originally reported by Freund and co-workers in 1994 and, like the peptidomimetics, are irreversible inhibitors of tTG [99]. The most potent of these compounds, T101, is cell impermeable and has been used to block tTG from crosslinking Fn in MVs, whereas the less potent D003 (also referred to as NTU283) has been shown to reduce tTG crosslinking inside cells, and thus appears to be cell permeable [30,45].

Other novel scaffold inhibitors are dominated by reversible inhibitors of tTG. One of the better studied classes of compounds are a series of nitrocinnamoyl inhibitors identified by the Keillor laboratory [100]. Although lead molecule CP4d (Figure 7) had comparable potency to many of the peptidomimetic scaffolds (K_i of 1 μ M), it also contains a Michael-acceptor functionality rendering it vulnerable to nucleophilic attack by common biomolecules like glutathione (GSH). Recently, Apperley *et al.* attempted to remedy this problem. Alkylation at the Michael-acceptor site led to a complete loss of potency against tTG, but replacement of the alkene with an alkyne yielded compounds **22b** (which was ten-times more potent than CP4d) and **27d** (which, although ten-times less potent than CP4d, was far less prone to react with GSH than the other compounds studied). Although CP4d has been shown to inhibit tTG-related phenotypes in epidermal cancer stem cells, no cellular data have yet been reported for **22b** or **27d** [90].

Several other alternate scaffolds that target tTG have been discovered using HTS efforts. Case and Stein led an effort in which 110 000 drug-like molecules were screened for their ability to inhibit tTG crosslinking activity, leading to the identification of LDN-27219 (Figure 7), a reversible inhibitor that exhibited slow binding kinetics when competing versus certain substrates, suggesting that it could bind effectively to multiple conformations of tTG [101]. Similarly, Kim and co-workers screened 1000 diverse molecules against several cancer cell lines, resulting in a series of thieno[3, 4-*b*]pyrazine compounds [102,103]. Two such compounds: GK-428 and GK-921 (Figure 7), were shown to inhibit the growth of renal

cell carcinoma in culture, as well as in xenograft models. The authors claimed to be particularly interested in administration of the compounds via an inhalation vector, but unfortunately have not yet demonstrated that ability. An earlier 2008 study screening known pharmaceutical agents in the LOPAC and Prestwick libraries found that the JAK inhibitor ZM39923, its metabolite ZM449829, the EGFR inhibitor tyrphostin 47 and several quinones, such as vitamin K3, all exhibited low- to sub-micromolar activity against tTG-mediated crosslinking [104]. These inhibitors were all found to be irreversible if dosed simultaneously with calcium, and many have a Michael-addition functionality. Further, they showed little selectivity for tTG versus FXIIIa. However, they are all known pharmaceutical agents, and were generally able to delay death in a tTG-mediated *Drosophila* model of Machado–Joseph disease, suggesting that they could indeed be able to target tTG in biological systems. A more recent screening of a ChemDiv library via an AlphaLISA assay (designed to measure tTG–Fn binding, rather than a crosslinking reaction) generated a number of pyrimidine-based compounds that inhibited tTG binding to Fn, with the most potent compound: TG53, showing good efficacy in preventing the migration, invasion or proliferation of SKOV3 ovarian cancer cells, while uniquely exhibiting an inability to inhibit tTG-mediated crosslinking in those cells [105]. Although many of these nonpeptidic scaffolds have not been subject to any further study or validation, they do present interesting opportunities for further tTG drug design efforts.

Imaging applications

Although most small-molecule development pertaining to tTG has focused on inhibiting its crosslinking activities for therapeutic applications, some molecules have also been developed for imaging purposes. Several of the molecules already described can serve such purposes: MDC, NC9 and compound **3h** have all been used to label tTG substrates fluorescently [44,97,98]. Another example of a fluorescent tTG imaging molecule is FITC-labeled T26 peptide. The T26 peptide tightly and specifically binds to tTG, and can be crosslinked to amine-donor proteins in the extracellular matrix. Lee *et al.* used a FITC-labeled derivative in their study on pancreatic cancer to demonstrate that tTG had crosslinking activity in the TME of their xenograft models [85].

The activity of tTG has been monitored via other techniques as well. In 2013, Hingorani and co-workers utilized chemical exchange saturation transfer (CEST) technology to develop a tTG magnetic resonance imaging (MRI) agent. CEST agents generate contrast by saturating an exchangeable proton on a MRI agent, using the resulting proton exchange to decrease the MRI water signal [106]. The authors designed a thulium-based probe conjugated to a cadaverine tail, and demonstrated that they could monitor the tTG-mediated addition of the probe to albumin via NMR, raising the possibility that such imaging agents might be of eventual clinical value. A similar, more-recent report by van der Wildt *et al.* demonstrated the ability to monitor tTG activity via positron emission tomography (PET) [107]. Here, the authors modified a series of known irreversible peptidomimetic inhibitors of tTG with the goal of being able to monitor its activity via PET. The inhibitors, each bearing an acrylamide reactive group, were synthesized to include an ^{11}C radiolabel in that functional group. The authors saw little accumulation of the molecules following IP injection into rats, suggesting that they were not binding to the generally inactive tTG in healthy animals. However, they

observed a strong signal in MD-AMB-231 breast cancer xenografts, which have a large amount of tTG in the active state. Thus, the molecules were able to distinguish between active and inactive tTG. The authors later developed an ^{18}F -labeled inhibitor based upon Z-Don with similar results [108].

An intriguing opportunity: the tTG open-state

Given the evidence that tTG plays an important part in cancer, the reasonable question might then be asked: why is tTG not being targeted in the clinic? What is holding back research on this enzyme? Primarily, we view this as a tipping point problem: clinical trials are the most expensive part of any drug discovery project, and it is difficult to support a trial on even the best tTG inhibitors in cancer patients. Because many cancer patients enrolling in clinical trials have already been failed by multiple standard therapies, they typically have advanced diseases and require treatments in which investigators have substantial confidence – tTG inhibitors simply have not yet reached that level. However, a potential new strategy could be on the horizon. This has to do with a recently appreciated biological activity of tTG that occurs independently of its catalytic properties. Several different mutants of tTG have been generated that alter its catalysis. For example, the tTG R580L mutant is defective for binding guanine nucleotides, whereas the C277S mutant lacks protein crosslinking activity [109]. Many of these tTG mutants stably adopt either the closed- or the open-state conformation. Moreover, those tTG mutants that constitutively adopt the open-state are highly cytotoxic. In 2007, Datta *et al.* reported that the ectopic expression of the GTP-binding-defective tTG mutants R478L, R580L and R580K in NIH 3T3 fibroblasts or SK-BR-3 breast cancer cells substantially increased their sensitivity to serum-starvation-induced cell death. The cell-death-promoting effects of these mutants still occurred under conditions where their crosslinking activity was also inhibited [109].

The results of the Datta study suggest that it is the ability of tTG to constitutively adopt the open-state, rather than its transamidation activity, which is responsible for cell death. Other studies have further supported this idea [3,37,53]. These findings potentially relate to tTG-short, an isoform of tTG truncated at the 3' end by 139 residues [8]. This mutant only weakly binds GTP, and exhibits minimal crosslinking activity, but it is highly toxic when expressed in cells [110]. Importantly, tTG-short has been shown to adopt a conformation similar to the tTG-wild-type open-state while in solution, which probably explains its cytotoxicity [37].

Despite the innate cytotoxicity of the open-state of tTG, there has been no direct effort to generate small molecules that stabilize the open-state of tTG. Several of the peptidomimetic inhibitors have been shown to stabilize the open-state of tTG as indicated by X-ray crystallography (e.g., Z-Don in PDB ID 3S3J, or other peptides in PDB IDs 3S3P, 3S3S and 2Q3Z), but stabilization of the open-state for such drugs was a consequence of their mode of action, and not necessarily the intention behind their design [28]. Similarly, Kerr *et al.* showed that several peptidomimetic inhibitors, such as NC9, cause tTG to adopt the open-state in cells, based on a readout involving a tTG chimera labeled with the FRET pair mCerulean and eYFP [90]. However, as discussed above, the peptidomimetic inhibitors of tTG generally show minimal cell permeability. Kerr *et al.* studied only a single nonpeptidic

drug, CP4d, and it was found to have a minimal ability to stabilize the cytotoxic open-state of tTG. Although the mechanism through which the open-state conformation kills cells is still unclear, it does offer an exciting avenue for drug discovery, because molecules designed to stabilize the open-state of tTG would be expected to elicit a cytotoxic response in cells overexpressing tTG (such as the most aggressive cancers), independent of any ability to inhibit crosslinking or GTP-binding activity.

Concluding remarks and future research directions

Several questions remain pertaining to the structure, function and therapeutic targeting of tTG. For some of these there is no answer in sight but for others, we suspect, they might be addressed in the next few years. For example, the mechanism by which Ca^{2+} ions activate tTG is still largely unknown, and no available crystal structure shows where Ca^{2+} ions bind the enzyme. Similarly, the structures of several other transglutaminase family members have yet to be determined, limiting the ability to rationally design transglutaminase-selective inhibitors. Further, no structure has yet been solved of tTG complexed to any binding partner, which might be especially valuable in determining how tTG participates in signaling events. Crystallization studies on tTG have historically been difficult, but the release of the GTP-bound structure in 2015 shows that efforts to improve crystallization conditions and determine novel structures are still progressing [47]. Such studies could be very helpful in further explaining the specificity of certain inhibitors, or investigating novel binding sites, and thus aiding future drug design.

In terms of drug discovery, tTG remains an open target, with no inhibitor yet available in the clinic, and the most promising inhibitor to date (the peptidomimetic ZED-1227) being cell impermeable. Indeed, the overwhelming trend in tTG drug discovery in the past decade has been toward peptidomimetic inhibitors, and we predict that, as these studies continue, peptidomimetics will continue to improve in potency and, particularly, cell permeability. However, the recent emphasis on HTS efforts has prioritized novel scaffolds, and these compounds could become more prominent in the coming years. Perhaps most importantly, however, is the increased appreciation of the role tTG has in cells via direct binding interactions, independent of its catalytic activities. The cytotoxicity of the constitutively maintained open-state conformation of tTG is particularly exciting in this regard, because the associated findings could drive the development of nonpeptidomimetic inhibitors capable of stabilizing open-state tTG, while having superior cell permeability.

When first discovered, tTG seemed to be nothing more than a curious enzyme that incorporated primary amines into other proteins. In the 60 years since, tTG has been extensively implicated in several different aspects of cancer progression, including EMT, cell migration and drug resistance. It has been shown to have two vastly different conformational states, with each having distinct and important functional consequences. Drug discovery efforts have moved toward more-potent and specific inhibitors with ever improving pharmacokinetic properties. However, few tTG drug candidates are in clinical trials, and none is available for use by physicians. Recent findings suggest that maintaining tTG in its open-state conformation is highly toxic, a finding that provides a novel and promising avenue of research for drug discovery. Hopefully the 60 years following this

diamond anniversary will result in a better understanding of the parts played by this fascinating enzyme, and development of novel tTG inhibitors of direct clinical value.

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Highlights

- Tissue transglutaminase plays a key part in the progression of cancer and survival
- It adopts two conformations, one of which is cytotoxic
- Drug discovery efforts have yet to develop a clinically relevant compound

Figure 1.

A timeline of major discoveries and accomplishments related to tTG. Events are classified as related to overall structure and mechanism (solid black outline), general biological function (dashed black outline) or disease states (dotted black outline).

Figure 2.

Catalysis of the tTG transamidation reaction. **(a)** The catalytic crosslinking site of tTG, from PDB ID 2Q3Z. The catalytic triad is shown in orange, whereas the transition state stabilizing Trp241 is shown in yellow. A loop formed by residues 329–334 is hidden for clarity. **(b)** The four catalytic residues are conserved between the eight catalytically active transglutaminase family members (alignment on the right, family members other than tTG are designated by gene symbol). Band 4.2 (*EPB42*), which has an alanine residue at position 277, is deficient in crosslinking activity. **(c)** Two proposed mechanisms for transamidation catalysis by tTG. These differ primarily by whether Trp241 serves to stabilize a cysteine–glutamine adduct via

H-bonding (right path), or if it turns glutamine into a better electrophile via a cation–pi interaction (left path, cation–pi interaction shown as red double-dotted line).

Figure 3.

Structure and conformations of tTG. (a) The tertiary structure of tTG. tTG adopts a 'closed' crosslinking inactive state (left, PDB ID 1KV3) and an 'open' crosslinking capable state (right, PDB ID 2Q3Z). The conformation that tTG adopts is controlled by the addition of either calcium or guanine nucleotides. The different domains in tTG are indicated by color: the N-terminal sandwich is red, the crosslinking catalytic domain is blue and the subsequent two β sheets are green and cyan. Panels b–e use the same coloring. (b) The nucleotide binding site of closed-state tTG (1KV3). The binding site is comprised nearly equally by residues from the crosslinking catalytic domain and the first β -sheet domain. GDP is shown

in orange. **(c)** The nucleotide binding site of open-state tTG (2Q3Z). The β sheets move away from the catalytic domain, and insufficient residues remain to provide a binding pocket for the nucleotide. GDP, in orange, is overlayed from 1KV3. **(d)** The crosslinking substrate-binding site of closed-state tTG (1KV3). A substrate-mimetic inhibitor from 2Q3Z was overlaid onto 1KV3. Although the binding site exists in the crosslinking catalytic domain, it is occupied by residues from the β sheets. **(e)** The crosslinking substrate-binding site of open-state tTG (2Q3Z). A substrate-mimetic inhibitor (orange) fits into a groove in the crosslinking catalytic site. **(f)** Overlay of GDP and GTP bound tTG structures (1KV3 and 4PYG, respectively). The GDP-bound structure is colored gray, and GDP is shown as gray spheres. The GTP-bound structure is colored by B-factor, where shades of blue are stable, immobile residues and shades of orange are flexible, poorly defined residues. The regions of the protein that have poor overlap between the GDP- and GTP-bound structures, indicated by red arrows, are naturally highly mobile in the crystal structures. **(g)** Zoomed in view of the lower indicated loop in (f).

Figure 4.

tTG is highly expressed in aggressive cancers. Mean survival time in cancers (top [50]) correlates to mRNA expression of the TGM2 gene (middle, images are representative sections of The Cancer Genome Atlas data [52] prepared with the UCSF Cancer browser [51]) and to tTG protein expression (as determined by staining of tissue slices with the tTG-specific antibody CAB002598 at <http://www.proteinatlas.org>). mRNA and protein expression for tumor samples or average healthy tissue are shown in similar color schemes, with red representing high expression, white moderate expression, blue low expression and gray no detectable expression.

Figure 5.

tTG regulates many signaling events that promote cancer cell phenotypes. The signaling pathways most often mutated in pancreatic cancers are shown in blue, whereas the phenotypes shared by most cancers are shown in purple. Most of the signaling pathways increase tTG expression, whereas positive feedback mechanisms allow tTG to enhance signaling in several such pathways. Of the phenotypes identified as hallmarks of cancer, tTG has demonstrated roles in about half of them.

Figure 6.

Inhibitors of tTG. The most common inhibitors of tTG are: **(a)** pseudo-substrate inhibitors, such as MDC and cystamine, which replace the amine-bearing substrate in tTG catalyzed crosslinking reactions; and **(b)** peptidomimetic inhibitors bearing 'warhead' functionality that form covalent bonds to the catalytic Cys277 and irreversibly inhibit tTG crosslinking. For peptidomimetic compounds, the warhead functionalities are shown in red. K_i or IC₅₀ values against tTG crosslinking *in vitro* are presented for each compound in (b) only because pseudo-substrates do not inhibit crosslinking, whereas all compounds have a GI₅₀ value against cell culture systems reported. References are provided to manuscripts providing the

related data. ^aData taken from http://www.zedira.com/resources/content/pdf/zedira_poster_oslo_2011.pdf.

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Figure 7.

Novel scaffolds of tTG inhibitors. A wide variety of inhibitors of tTG have been reported that are neither pseudo-substrates or peptidomimetics. Some are irreversible, but most are reversible inhibitors. IC₅₀ values for tTG *in vitro* are reported, as are cell growth GI₅₀ values where appropriate, along with the related references.