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Cellular Functions of Tissue Transglutaminase

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Abstract

Transglutaminase 2 (TG2 or tissue transglutaminase) is a highly complex multifunctional protein that acts as transglutaminase, GTPase/ATPase, protein disulfide isomerase, and protein kinase. Moreover, TG2 has many well-documented nonenzymatic functions that are based on its noncovalent interactions with multiple cellular proteins. A vast array of biochemical activities of TG2 accounts for its involvement in a variety of cellular processes, including adhesion, migration, growth, survival, apoptosis, differentiation, and extracellular matrix organization. In turn, the impact of TG2 on these processes implicates this protein in various physiological responses and pathological states, contributing to wound healing, inflammation, autoimmunity, neurodegeneration, vascular remodeling, tumor growth and metastasis, and tissue fibrosis. TG2 is ubiquitously expressed and is particularly abundant in endothelial cells, fibroblasts, osteoblasts, monocytes/macrophages, and smooth muscle cells. The protein is localized in multiple cellular compartments, including the nucleus, cytosol, mitochondria, endolysosomes, plasma membrane, and cell surface and extracellular matrix, where Ca^{2+}, nucleotides, nitric oxide, reactive oxygen species, membrane lipids, and distinct protein–protein interactions in the local microenvironment jointly regulate its activities. In this review, we discuss the complex biochemical activities and molecular interactions of TG2 in the context of diverse subcellular compartments and evaluate its wide ranging and cell type-specific biological functions and their regulation.

Keywords

Transglutaminase; Protein cross-linking; Transamidation; GTPase; Cell signaling; Stem cells; Therapeutic target

1. Introduction

Transglutaminase 2 (TG2), also known as tissue transglutaminase (TG), is an 80-kDa protein that consists of four domains (Gentile et al., 1991; Liu et al., 2002). TG2 is the only ubiquitously expressed member of the TG family of enzymes that all catalyze Ca^{2+}-dependent protein deamidation, transamidation, and cross-linking (Iismaa et al., 2009; Lorand and Graham, 2003). Since the discovery of TG2 in 1957, a large number of its enzymatic substrates have been identified in intracellular compartments, including the cytosol, nucleus, and mitochondria, and extracellularly, on the cell surface and in the extracellular matrix (ECM) (Csosz et al., 2008; Facchiano and Facchiano, 2009).

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Availability of the protein's crystal structure (Han et al., 2010; Liu et al., 2002; Pinkas et al., 2007) facilitated our understanding how the transamidating activity of TG2 is regulated in cells by reversible conformational changes of the protein. These include Ca^{2+}-dependent activation, which shifts TG2 to the "open" (extended) conformation, thereby unmasking the enzyme's active center, and inhibition by GTP, GDP, and ATP, which constrains it in the "closed" (compact) conformation (Begg et al., 2006a,b; Casadio et al., 1999; Di Venere et al., 2000; Kiraly et al., 2011; Liu et al., 2002; Monsonego et al., 1998; Pinkas et al., 2007; Zhang et al., 1998). Although recent studies suggested that transamidating activity of TG2 inside and outside the cells is tightly controlled and might be suppressed *in vivo* in the absence of mechanical or chemical stresses (Siegel et al., 2008), it is likely that precise regulation of the enzyme's activity involves other important mechanisms, including the binding of Ca^{2+} ions to noncanonical sites (Kiraly et al., 2009), reversible reduction/oxidation via a formation of intramolecular disulfide bonds (Stamnaes et al., 2010), and NO-mediated nitrosylation (Lai et al., 2001). The fact that sphingophospholipids were shown to sensitize TG2 to Ca^{2+} regulation (Lai et al., 1997) suggests that other lipids that bind to TG2, such as cholesterol and phosphoinositides (Harsfalvi et al., 1987; Zemskov et al., 2011a), small molecules, or as-yet-unidentified TG2-interacting proteins, may also modulate its transamidating activity (Singh et al., 2001). Finally, generation of alternative spliced isoforms (Antonyak et al., 2006; Festoff et al., 2002; Fraij et al., 1992; Lai et al., 2007; Tee et al., 2010) and limited proteolysis of the molecule (Fraij, 2011) was reported to influence the transamidating activity of TG2.

Besides its classical transamidating/protein cross-linking activity, TG2 possesses several other enzymatic functions (Iismaa et al., 2009, Lorand and Graham, 2003; Mehta et al., 2010; Park et al., 2010). Its GTPase activity allows intracellular TG2 to link transmembrane α_{1B}/α_{1D} adrenergic, thromboxane A2, and oxytocin receptors to cytoplasmic signaling targets such as phospholipase C (PLC)δ1, increasing inositol-1,4,5-trisphosphate levels upon stimulation of these receptors with appropriate agonists (Baek et al., 1993, 1996; Im and Graham, 1990; Im et al., 1990; Nakaoka et al., 1994; Park et al., 1998; Vezza et al., 1999). Biochemical studies revealed that the transamidating and GTPase activities of this protein are mutually exclusive: Ca^{2+}-bound TG2 has no GTPase activity, whereas GTP-bound TG2 does not exhibit TG activity (Feng et al., 1999a,b). The protein can also hydrolyze ATP (Iismaa et al., 1997), an activity which is believed to facilitate the promineralization capacity of TG2 in osteoblasts (Nakano et al., 2010).

Moreover, TG2 was found to display protein disulfide isomerase (PDI) activity *in vitro* (Hasegawa et al., 2003) and *in vivo* (Malorni et al., 2009; Mastroberardino et al., 2006). More recently, and even more surprisingly, TG2 was reported to phosphorylate insulin-like growth factor-binding protein-3 (IGFBP-3) on the cell surface, and p53 tumor suppressor protein, histones and retinoblastoma protein (Rb) in the nucleus, suggesting that it has an intrinsic serine/threonine protein kinase activity (Mishra and Murphy, 2004, 2006a,b; Mishra et al., 2006, 2007).

Finally, the vast array of TG2 functional activities in the cell is not limited to its enzymatic functions. TG2 was found engaged in the formation of noncovalent complexes with various cytoplasmic, cell surface, ECM, nuclear, and mitochondrial proteins (Iismaa et al., 2009; Lorand and Graham, 2003; Park et al., 2010). This emerging adapter/scaffolding function of TG2, which is independent of its enzymatic activities, appears to regulate cell adhesion, ECM remodeling, survival, growth, migration, and differentiation due to modulation of several signaling pathways (Belkin, 2011; Wang and Griffin, 2011).

An emerging theme in the field suggests that precise tuning of the numerous TG2 activities is defined by the microenvironment and localized protein–protein interactions within various

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cellular compartments (Park et al., 2010). Importantly, recent studies began to unravel the complex mechanisms of TG2 turnover, intracellular trafficking, and targeting to specific cellular compartments (Antonyak et al., 2011; Cho et al., 2011; Jeong et al., 2009; Luciani et al., 2009; Peng et al., 1999; Scarpellini et al., 2009; Zemskov et al., 2007, 2011a). In this review, we focus on the emerging mechanisms of spatial compartment-dependent regulation of TG2 activities in various cell types and their role in key cellular processes. We abstain from in-depth discussion of various mechanistic aspects of transamidating and GTPase functions of TG2, as excellent reviews on these topics are published elsewhere (Bergamini, 2007; Facchiano and Facchiano, 2009; Kiraly et al., 2011; Mhaouty-Kodja, 2004, Siegel and Khosla, 2007). Likewise, we do not extensively discuss the involvement of TG2 in human disease states, as recent comprehensive reviews in this field either elaborate on the numerous pathophysiological aspects of TG2 function (Iismaa et al., 2009) or focus on its role in inflammation (Elli et al., 2009; Iismaa et al., 2009; Kim, 2006), wound healing and tissue fibrosis (Collighan and Griffin, 2009; Verderio et al., 2004), autoimmunity (Briani et al., 2008; Sollid, 2000), cardiovascular diseases (Bakker et al., 2008; Sane et al., 2007), cancer (Chhabra et al., 2009; Mehta et al., 2010), and neurodegeneration (Bailey et al., 2005; Jeitner et al., 2009; Malorni et al., 2008; Mastroberardino and Piacentini, 2010).

2. Enzymatic and Nonenzymatic Activities of TG2

2.1. TG2 as transglutaminase

TG2 was the first identified member of the TG family of Ca^{2+}-dependent enzymes that is now known to contain eight enzymatically active and one inactive member in humans (Facchiano and Facchiano, 2009; Lorand and Graham, 2003). It shares the same overall four-domain tertiary structure and several conserved secondary structure elements with other mammalian TGs (Grenard et al., 2001; Liu et al., 2002; Lorand and Graham, 2003; Nemes et al., 2005). Unlike closely related TG1, TG3, and Factor XIIIA (FXIIIA) TGs, TG2 does not require proteolysis for activation. In humans, it is encoded by a single *TGM2* gene located on chromosome 20q11–12. TG2 has a highly conserved catalytic triad of Cys277-His335-Asp358, which is shared by all other enzymatically active TGs as well as cysteine proteases that belong to the papain-like superfamily (Lorand and Graham, 2003). While these residues form the enzyme's active site within a substrate binding channel of the second (catalytic) domain, the adjacent Trp241 and Trp332 residues are involved in stabilization of the transition state (Iismaa et al., 2003; Liu et al., 2002). Like other TGs, TG2 catalyzes covalent cross-linking, transamidation, and deamidation of proteins (Fig. 1.1). More than one hundred of its enzymatic substrates have been identified in a variety of cellular compartments (Esposito and Caputo, 2005; Facchiano and Facchiano, 2009). Therefore, this enzymatic activity enables TG2 to generate an immense array of posttranslational modifications in target proteins.

Despite sharing the same enzymatic reaction of forming acyl-enzyme intermediates with other TGs, both donor- and acceptor-group specificity for TG2 distinguish it from homologous TGs such as FXIIIA (Gorman and Folk, 1984, Hettasch et al., 1997; Khew et al., 2010), and TG1 and TG3 (Lorand and Graham, 2003). Although the distinction between reactive and nonreactive glutamines and lysines is dictated primarily by secondary and/or tertiary structural elements in the TG2 substrate proteins, the enzyme also displays preference at the level of primary sequence, mostly around reactive glutamine residues (Aeschlimann et al., 1992; Coussons et al., 1992). Using phage display combinatorial and bioinformatics approaches, the consensus sequences p-Q-X-(P,T,S)-I (Keresztessy et al., 2006) and Q-X-P-Φ-D-(P), Q-X-P-Φ, and Q-X-X-Φ-D-P (Sugimura et al., 2006) were defined as preferred for TG2-specific transamidation (where P and I stand for polar and aliphatic, and X and Φ stand for nonconserved and hydrophobic amino acids, respectively). Further developing these findings, a highly specific peptide for TG2-mediated

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transamidation, HQSYVDPWMLDH, was isolated from phage display libraries (Hitomi et al., 2009) and was subsequently shown to enable the detection of active TG2 *in situ* (Itoh et al., 2011). No such information is available with regard to consensus sequences containing the TG2-reactive lysines.

2.1.1. Transamidating enzymatic function of TG2—The transamidating function of TG2, which allows it to posttranslationally modify substrates by *de novo* formation of covalent bonds, is the best characterized enzymatic function of the protein. The substrates in this reaction can be broadly divided into two groups: (1) proteins and (2) numerous small molecules containing primary amino groups (Fig. 1.1). In turn, the protein substrates of TG2 can be subdivided into the subgroups containing (1) reactive glutamines and acting as acyl donors and (2) reactive lysines and acting as acyl acceptors (Lorand and Graham, 2003).

2.1.1.1. Protein-to-protein cross-linking by TG2: Among the numerous enzymatic reactions catalyzed by TG2, protein cross-linking is the most studied (Fig. 1.1(1)). First, TG2 is known to cross-link itself via its reactive lysine residues to various glutamine-containing substrates including the ubiquitous ECM protein fibronectin and fibrinogen (Barsigian et al., 1991) and gluten peptides (Fig. 1.2(1); Fleckenstein et al., 2004). Second, the simultaneous presence of both reactive glutamines and lysines enables TG2 to generate intramolecular isopeptide cross-links, which can profoundly affect protein conformation, interactions and stability, and the capacity to undergo polymerization (Fig. 1.2(2)). For example, TG2-induced intramolecular cross-linking of HIV-1 aspartyl protease was shown to increase its catalytic activity (Lentini et al., 2010). Likewise, TG2-driven intramolecular cross-linking of α-synuclein (Nemes et al., 2009) and β-amyloid Aβ peptide (Schmid et al., 2011) was shown to decrease their solubility and promote their aggregation and amyloid formation, the crucial aspects of neurodegeneration in conformational diseases. The third most common form of protein cross-linking mediated by TG2 involves the generation of intermolecular isopeptide bonds that leads to the formation of covalently linked dimers, oligomers, and polymers of various substrate proteins (Fig. 1.2(3)). TG2-induced formation of protein heterodimers and heteropolymers is typical for highly abundant and ubiquitous ECM proteins, such as fibrinogen and fibronectin on the surface of hepatocytes (Barsigian et al., 1988) or the laminin–nidogen complex of basement membranes (Aeschlimann and Paulsson, 1991). In these and other cases (Aeschlimann et al., 1996; Beninati et al., 1994), TG2 enables the generation of highly stable covalent protein heterocomplexes in the ECM.

The formation of homopolymers by TG2-driven cross-linking was demonstrated early during studies of the protein (Fesus et al., 1981; Lorand et al., 1976); later it was shown that TG2 could form homopolymers of more than hundred substrates inside (in the cytoplasm and the nucleus) and outside the cell (on its surface and in the ECM, Lorand and Graham, 2003; Park et al., 2010). It ought to be noted that TG2 contributes in two distinct ways to the cross-linking and generation of homopolymers of substrate proteins.

First, it is involved in the direct *de novo* polymerization of monomeric substrate proteins, which otherwise do not undergo this process in the absence of the enzyme (Fig. 1.2(3a)). This mechanism applies to most intracellular targets of TG2 cross-linking. An underlying principle of such reactions is that they proceed in a single phase where TG2 controls the rate of polymer formation. Their common outcome is that they alter the conformation, stability, and biological functions of TG2-polymerized proteins. A prominent example of this mechanism is cytoplasmic IκBα which can be polymerized by TG2 and then degraded following the enzyme-induced cross-linking in the cytoplasm (Fig. 1.3(1)). These TG2-mediated cross-linking events cause IκBα depletion without affecting its phosphorylation, thereby leading to a noncanonical activation of the NFκB pathway that can contribute to cancer progression and inflammation (Kim, 2006; Mehta et al., 2010). Direct TG2-mediated

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cross-linking also causes polymerization of SP1 transcription factor in the hepatocyte nuclei, thus decreasing its functional activity and downregulating transcription of SP1-dependent genes. TG2-driven SP1 cross-linking is implicated in the pathogenesis of alcoholic steatohepatitis, which is accompanied by increased TG2 expression and nuclear localization, as well as cross-linking of SP1 (Tatsukawa et al., 2009).

Second, TG2 is also implicated in the stabilization of preexisting protein assemblies via generation of isopeptide covalent bonds linking the adjacent monomers in the polymeric substrate (dubbed "enzymatic spotwelding," Lorand and Graham, 2003; Fig. 1.2(3b)). This type of TG2-mediated modification is particularly common for ECM protein polymers, such as fibronectin, collagen, fibrinogen, and others that undergo polymerization in the absence of TG2 (Collighan and Griffin, 2009). In this case, the TG2-dependent cross-linking takes place in a two-phase system that includes a preformed polymer scaffold.

The overall consequences of such TG2-driven cross-linking of protein assemblies are twofold. The generation of covalent cross-links in the ECM polymers by TG2 increases their mechanical stability and stiffness (Mangala et al., 2005; Santhanam et al., 2010; Spurlin et al., 2009) and also protects them from proteolytic degradation (Fisher et al., 2009; Jones et al., 2006), thus affecting both the biomechanical properties of tissues and the rate of ECM turnover (Larreta-Garde and Berry, 2002). Further, the covalent isopeptide bonds often alter the monomer's conformation within the polymer and unmask cryptic binding sites for other ECM components (van den Brule et al., 1998) and cell surface receptors such as integrins (Belkin et al., 2005; Khew et al., 2008; Nishimichi et al., 2009, 2011). Thus, this type of TG2-elicited modification of matrix polymers regulates ECM structure and also promotes cell–ECM adhesion and adhesion-dependent biological responses (Belkin et al., 2005; Chau et al., 2005; Forsprecher et al., 2011; Spurlin et al., 2009). The latter phenomenon gained much attention as an underlying principle in biotechnological applications of TG2-modified matrices for cell cultures (Collighan and Griffin, 2009).

2.1.1.2. Protein-to-small molecule cross-linking by TG2: Incorporation of amine-containing compounds, both mono- and polyamines, into substrate proteins was utilized early in the studies of TG2 transamidating activity *in vitro* and *in situ* (Fig. 1.1(2); Griffin et al., 2002; Iismaa et al., 2009; Kiraly et al., 2011; Lorand and Graham, 2003). Specifically, [^{14}C]-labeled putrescine was used to measure the transamidating activity of TG2, but the method proved tedious because it required extensive proteolysis and HPLC analysis (Folk, 1980). In addition, artificial polyamine substrates of TG2, including photoactivatable amine-containing compounds (Gorman and Folk, 1980), 5′-biotinamido-pentylamine (Slaughter et al., 1992), monodansyl-cadaverine (Lorand et al., 1986), biotin-cadaverine (Kunioka and Ando, 1996), and fluorescein-cadaverine (Griffin et al., 2002) were widely used to demonstrate this TG2 activity *in situ*.

Because mono- and polyamines, such as serotonin, histamine, dopamine, norepinephrine, putrescine, spermine, and spermidine, are abundant *in vivo*, protein-incorporated monoamines were detected a long time ago in various tissues and organs (Wajda et al., 1961). They were considered to be natural inhibitors of the cross-linking activity of TGs rather than functional modifiers of protein substrates. Their lack of recognized functionality resulted from the inability to identify specific target proteins. This view radically changed over the course of past decade, when convincing evidence of the functional alteration of substrate proteins by TG2-mediated monoaminylation was provided (Fig. 1.2(4)). The breakthrough came in 2003 when TG-mediated serotonylation of the small GTPases RhoA and Rab4A was found to be required for cytoskeletal rearrangement and exocytosis of platelet α-granules, respectively, and therefore, for platelet activation, adhesion, and aggregation (Walther et al., 2003). Although these modifications of RhoA and Rab4A could

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be replicated *in vitro* with TG2, the TG specificity of this reaction *in vivo* was not ascribed. TG2 and FXIIIA are, by far, the most abundant TGs in platelets (Lorand and Graham, 2003). Thus, the use of corresponding knockout mice should help clarify which TG is the physiological mediator of this reaction. Further, serotonylation of Rab3A and Rab27A in pancreatic β cells was found to be involved in the release of insulin after glucose stimulation (Paulmann et al., 2009). Again, the issue of TG specificity was not reported, although the findings that *TGM2* gene missense mutations were found in patients with early onset type 2 diabetes (Porzio et al., 2007), TG2 was the only TG significantly expressed in these cells, and its genetic deletion in mice impaired glucose-stimulated secretion (Bernassola et al., 2002) makes TG2 the most likely candidate. In vascular smooth muscle, TG2-mediated serotonylation of RhoA led to its transient activation and subsequent proteasomal degradation and depletion, causing increased Akt1 activation and inhibition of contractility (Guilluy et al., 2007). The TG2-mediated serotonylation of RhoA was also implicated in pulmonary artery remodeling and hypertension (Guilluy et al., 2009). Moreover, TG2-mediated serotonylation of α-actin and other key components of the contractile apparatus of vascular smooth muscle cells was shown to increase arterial isometric contraction (Watts et al., 2009). Mechanistically, similar TG2-mediated modifications of smooth muscle proteins with norepinephrine were shown to be important for vascular contraction (Johnson et al., 2010). Finally, another small GTPase, Rac1, was found to undergo TG2-mediated serotonylation and activation in cortical neurons, thereby suggesting a role for such modification in neuronal signaling (Dai et al., 2008). Importantly, in all the above mentioned cases, TG2-mediated incorporation of primary amines into various substrate proteins significantly altered their activity, leading to diverse biological effects in multiple tissues (Fig. 1.3(2); Walther et al., 2011). Therefore, over the past decade, TG2-induced monoaminylation of intracellular targets gained prominent significance in various pathophysiological processes and now represents a rapidly growing area of research.

2.1.2. Deamidating enzymatic function of TG2—Deamidation is a variant of the TG-mediated transamidation reaction in which water serves as nucleophile in the absence of amine cosubstrates (Facchiano and Facchiano, 2009; Lorand and Graham, 2003). The net result is a deamidation of glutamine residues to glutamic acid residues (Fig. 1.1(3)). This alteration of protein structure, apparently, may affect the conformation, activity, and interactions of target proteins (Fig. 1.2(5)). The deamidation reaction, which is favored with poor substrates, low TG concentrations, and low pH (Stamnaes et al., 2008), has a certain degree of substrate specificity. For example, TG2 caused deamidation of Gln66 and transamidation of Gln31 to Lys162 in Hsp20 protein (Boros et al., 2006). The deamidating function of TG2 received much attention when it was reported to catalyze deamidation of peptides derived from the wheat protein gliadin, causing them to become dominant epitopes for activating T cells associated with the pathogenesis of celiac disease (Shan et al., 2002; Sollid and Jabri, 2005). Other examples of TG2-induced protein deamidation that may have biomedical relevance were reported more recently. Glutamine residues in the N-terminal arms of βB_2 and βB_3 crystallins were shown to undergo rapid deamidation in the presence of TG2, causing a disruption of the β crystalline complex (Boros et al., 2008). This TG2-induced deamidation of crystallins is thought to contribute to age-dependent lens opacification in humans. Moreover, deamidation of Gln15 in β-amyloid peptide was shown to reduce its solubility (Schmid et al., 2011), whereas deamidation of Gln6 in substance P increased agonist potency toward its receptor (Fornelli et al., 2011), thus expanding the potential pathophysiological role of these TG2-dependent reactions (Fig. 1.3(3)).

2.1.3. Regulation of transamidating activity of TG2—A large body of work established a requirement for Ca^{2+} in the activation of TG2 transamidating activity (Kiraly et al., 2011). Ca^{2+} binding elicits a large conformational change in the TG2 molecule by

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moving the β-barrel domains 3 and 4 apart from the catalytic domain 2, thus opening up an access to its active center (Casadio et al., 1999; Liu et al., 2002; Mariani et al., 2000; Pinkas et al., 2007). A prevailing view in the field, however, ascribes that the transamidating activity of TG2 is latent in the absence of stresses, particularly inside cells (Kiraly et al., 2011; Lorand and Graham, 2003; Siegel et al., 2008). Measurement of the [Ca^{2+}] required for half-maximal transamidation of TG2 activation yielded the activation constants in the range of 3–100μM depending on the source of enzyme and substrate (Kiraly et al., 2011), thereby making the ~100nM free cytoplasmic [Ca^{2+}] generally nonpermissive for TG2 activation. In addition, the free cytoplasmic [GTP] in the range of ~100μM significantly exceeds the concentration needed to inhibit the enzyme, thus keeping it in the "closed" GTP-bound conformation (Mariani et al., 2000). Moreover, while high [Ca^{2+}] and low [GTP] outside the cells appear permissive for TG2 activation, the highly oxidative state in the extracellular space was reported to keep TG2 in the inactive state in the absence of mechanical and/or chemical stresses (Siegel et al., 2008), due to the formation of inhibitory disulfide bond between the residues Cys370 and Cys371 (Stamnaes et al., 2010). Intriguingly, the inactive disulfide-bonded conformation of extracellular TG2 was recently shown to be reversed by thioredoxin-mediated reduction, thus revealing the first plausible physiological mechanism for transient activation of the enzyme outside the cell (Jin et al., 2011). Likewise, S-nitrosylation of the cysteine residues by nitric oxide both *in vitro* (Lai et al., 2001) and in the vasculature (Santhanam et al., 2010) was shown to inhibit transamidating activity of TG2. Another posttranslational modification of TG2, acetylation, was also reported to suppress this TG2 activity *in vitro* (Lai et al., 2010). Finally, the apparently normal phenotype of *TGM2*–/– mice does not suggest a major role for TG2 transamidating activity *in vivo* (De Laurenzi and Melino, 2001; Nanda et al., 2001).

In contrast, numerous other studies challenge the view that TG2-mediated protein transamidation is entirely shut down in cells in the absence of stressors. The detectable levels of TG-generated isopeptide cross-links in cells (Fesus and Tarcsa, 1989), and in tissues and body fluids (Harsfalvi et al., 1992; Nemes et al., 2001), suggest the importance of transamidating reactions *in vivo*. Also, several studies showed that local Ca^{2+} levels can reach the range 0.5–1μM upon mobilization by toxins in neuroblastoma cells (Zhang et al., 1998), and upon treatments of human epithelial breast cancer cells with epidermal growth factor (EGF; Dadabay and Pike, 1987), platelets with thrombin (Lorand et al., 1987), and various cells with peroxide to generate reactive oxygen species (ROS, Shin et al., 2004). Even more strikingly, Ca^{2+} levels can reach 8–10μM in the submembrane regions (and possibly even higher levels attained within the nucleus) of pancreatic β cells in response to glucose stimulation (Walther et al., 2011). Thus, [Ca^{2+}] can act as a classical intracellular messenger that triggers TG2 activation inside the cell in response to outside cues. Inside the cell, the transamidating activity of TG2 can be sensitized to even lower concentrations of Ca^{2+} by a number of mechanisms, including expression of alternatively spliced isoforms, limited proteolysis of the molecule, and some still poorly characterized molecular interactions of TG2 with lipids and proteins in different cellular compartments. Extracellularly, transamidating activity of TG2 is suppressed by NO-mediated nitrosylation (Santhanam et al., 2010; Telci et al., 2009) and regulated by reversible redox-dependent formation of the inhibitory intramolecular disulfide bond (Jin et al., 2011; Stamnaes et al., 2010). On the cell surface and in the ECM, the direct binding of TG2 to heparan sulfate proteoglycans such as syndecan-4 was shown to increase transamidating activity of the enzyme (Scarpellini et al., 2009). It remains to be tested whether and how other principal cell surface/ECM-binding partners of TG2, such as integrins and fibronectin, or biomechanical forces (tension, shear stress), impact the TG2-mediated protein cross-linking activity outside the cell.

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Even more generally, an interaction of TG2 with some effector protein (s) may shift and stabilize the enzyme into the "open" conformation, thereby decreasing or even eliminating the need for Ca^{2+} activation. The existence of such still unidentified TG2-binding partners was postulated in the case of cell responses to retinoids (Singh and Cerione, 1996; Singh et al., 2001) and EGF (Antonyak et al., 2009), the stimuli that both trigger relocation of cytoplasmic TG2 to the inner leaflet of the plasma membrane and evoke a drastic upregulation of its transamidating activity.

The interaction of TG2 with membrane lipids (Fesus et al., 1983; Harsfalvi et al., 1987; Zemskov et al., 2011a) might be an additional important factor in helping the TG2 enzyme to overcome the Ca^{2+} activation barrier for transamidation. Notably, a sphingophospholipid sphingosine–phosphorylcholine markedly increased the transamidation activity of TG2 (Lai et al., 1997). The biological significance of this observation remains unclear due to the extremely scarce amount of this lipid in biological membranes. Nonetheless, an attractive hypothesis suggests that endomembrane-sequestered intracellular TG2 may undergo a conformational change upon membrane lipid binding, which allows transamidation to proceed due to locally increased concentrations of Ca^{2+} ions (Nemes et al., 2009).

Several alternatively spliced TG2 variants were shown to be coexpressed with the main canonical isoform, which contains 687 amino acids in humans. In erythroleukemia cells, Fraij and Gonzales (1996) detected the shortest currently known alternatively spliced isoform which consists of only 349 amino acids, 286 of which correspond to the N-terminal TG2 sequence. This group also described an alternatively spliced TG2 form (tTG-H), which contains 548 amino acids, 538 of which are identical to the canonical TG2 isoform (Fraij et al., 1992). Later, Antonyak and coauthors (2006) found that this latter truncated TG2 form has reduced transamidating activity upon expression in NIH3T3 fibroblasts but sensitizes these cells to apoptosis. Another alternatively spliced TG2 form (s-TGN) consisting of the same 622 amino acids of the canonical TG2 with additional divergent 30 amino acids at its C-terminus was found in tumor necrosis factor- (TNFα-) and interleukin-1β-treated astrocytes (Monsonego et al., 1997). This form appeared to be upregulated upon rat spinal cord injury (Festoff et al., 2002). Finally, tTG_{V1} and tTG_{V2} variants were identified in vascular smooth muscle cells, endothelial cells, and leukocytes and were found to be identical to TG2 in their initial 622 amino acids, but had divergent C-termini of 52 and 23 amino acids, respectively (Lai et al., 2007). With the clear exception of the shortest isoform which has to be inactive because it is missing a part of the catalytic triad (Fraij and Gonzales, 1996), all other currently described TG2 isoforms should retain transamidating activity. Given that their truncated or divergent C-termini lack either some parts or the entire GTP-binding pocket, their transamidating activity is not expected to be repressed even by high intracellular GTP levels, making them more sensitive to Ca^{2+} activation and catalytically active under physiological conditions.

By the same token, limited proteolysis of TG2 which cleaves the molecule within the β-barrel domains 3 and 4 is expected to relieve the inhibition of transamidation by opening the catalytic center of the enzyme. In agreement, bacterial expression of C-terminally truncated constructs TG2[1–464] and TG2[480] revealed their increased cross-linking activity (Fraij, 2011). The mechanism of TG2 activation by limited proteolysis might be applicable in the case of response to tissue injury.

2.2. TG2 as atypical GTPase and ATPase

Although the ability of TG2 to bind and hydrolyze GTP was discovered in 1987 (Achyuthan and Greenberg, 1987), a link between this activity and the function of G protein coupled receptors (GPCRs) was not established until 1994, when it was discovered that the GTP-binding protein termed Ghα (based on its atypically high ~75kDa molecular weight vs. 40–

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45kDa for the canonical α subunits of heterotrimeric G proteins), coisolated with the α_{1B} adrenergic receptor, was identical to TG2 (Nakaoka et al., 1994). By analogy, TG2/Ghα was also shown to mediate signaling by the α_{1D} adrenergic, thromboxane A2, oxytocin, and follicle stimulating hormone receptors, but not other GPCRs, by linking them to activation of PLCδ1, thereby increasing inositol-1,4,5-trisphosphate (IP_3) levels upon stimulation of these receptors with agonists (Fig. 1.4; Baek et al., 1993, 1996; Feng et al., 1996; Im and Graham, 1990; Im et al., 1990; Lorand and Graham, 2003; Mhaouty-Kodja, 2004; Park et al., 1998; Vezza et al., 1999). The GTPase activity and the associated signaling capacity of TG2/Ghα were found to be independent of its transamidating (protein cross-linking) activity (Chen et al., 1996). Moreover, given the high intracellular GTP levels under normal physiological conditions, the activity of TG2/Ghα as a GPCR-linked GTPase should be turned on inside the cell.

Several other findings allowed further characterization of the intracellular signaling pathways mediated by TG2/Ghα. The second subunit of Gh protein, Ghβ, was identified as the Ca^{2+}-binding protein calreticulin, which regulates the functions of TG2/Ghα by suppressing both its GTP binding/hydrolytic and transamidating activities, thus maintaining the molecule in the inactive conformation for signaling (Fig. 1.4; Feng et al., 1999b). Interaction of TG2/Ghα with α_1 adrenergic receptors in response to epinephrine switches off its transamidating activity and dissociates GTP-bound TG2/Ghα from Ghβ. This activation stimulates PLCδ1 due to the direct binding of TG2/Ghα thereby resulting in phosphoinositide hydrolysis and an increase in intracellular [Ca^{2+}] (Feng et al., 1996). TG2/Ghα binds and hydrolyzes GTP with an affinity and catalytic rate similar to those of canonical α subunits of heterotrimeric and monomeric G proteins. However, TG2/Ghα does not contain the four consensus GTP-binding motifs common to the classical G proteins. Using photoaffinity labeling and site-directed mutagenesis, Begg and colleagues (2006a,b) demonstrated that GTP binds mainly to residues from the first and last strands of its β-barrel 1 (amino acids 476–482 and 580–583) and to two core domain residues (Lys173 and Phe174) located on a loop protruding in the direction of β-barrel 1. Importantly, the allosteric regulation of transamidating activity of TG2/Ghα by GTP was demonstrated by mutating the critical Arg580 residue to Ala and revealed not only reduced GTP-binding affinity by ~100-fold but also uncoupled GTP-dependent inhibition of transamidation, resulting in dysregulated intracellular cross-linking (Begg et al., 2006a,b).

The activation/deactivation GTPase cycle of TG2/Ghα functions similarly to that of other heterotrimeric G proteins (Lorand and Graham, 2003; Mhaouty-Kodja, 2004). Upon agonist binding to GPCR, the receptor induces exchange of GDP to GTP and dissociation of TG2/Ghα-GTP from Ghβ. Deactivation occurs when TG2/Ghα hydrolyzes GTP to GDP, by virtue of its intrinsic GTPase activity, and reassociates with free Ghβ (Fig. 1.4). Two regions of TG2/Ghα, R564-D581 and Q633-E646, appear to be involved in its interaction with α_1 adrenergic receptors and activation of the GTPase function (Feng et al., 1999a). Unlike the Gβγ complex of heterotrimeric G proteins, Ghβ is not involved in the interaction of Gh with α_1 adrenergic receptors.

Notably, the specificity of TG2/Ghα function in GPCR signaling relates not only to the repertoire of receptors but also to the identity of downstream effectors. PLCδ1 is a key effector molecule for α_1 adrenergic receptor coupling with TG2/Ghα both *in vitro* and *in vivo* (Baek et al., 2001; Das et al., 1993; Feng et al., 1996). While Gqα protein activates PLCβ enzymes, TG2/Ghα interacts with PLCδ1. The Val665-Lys672 region in the C-terminal domain of TG2/Ghα is involved in effector binding and activation (Hwang et al., 1995). In turn, PLCδ1 activation increases phosphoinositide hydrolysis and raises intracellular [Ca^{2+}] (Feng et al., 1996; Kang et al., 2002). Unusually, PLCδ1 acts as both a guanine nucleotide exchange factor (GEF) and a GTP hydrolysis inhibitory factor (GDI) for

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TG2/Ghα (Baek et al., 2001), thus amplifying this signaling cascade. It remains mostly unknown how this pathway integrates negative signals. In Sertoli cells, nonmuscle myosin IIA links the follicle stimulating hormone receptor to the inactive GDP-bound TG2/Ghα, whereas agonist-induced indirect receptor-mediated phosphorylation of myosin molecules was shown to release the activated GTP-bound protein, thereby inducing the downstream activation of PLCδ1 and triggering Ca^{2+} influx into these cells (Lin et al., 2010).

TG2/Ghα also regulates other signaling pathways through its GTPase activity. It was reported to participate in the adrenergic activation of extracellular signal-regulated kinases (ERK) and their regulatory kinases (MEK) in cardiomyocytes (Lee et al., 2003). In fibroblasts and endothelial cells, overexpression of wild type or a transamidation-inactive mutant of TG2/Ghα inhibited adenylyl cyclase activity, while its downregulation led to the opposite effect (Gentile et al., 1997). TG2/Ghα also directly turns on the large conductance of Ca^{2+}-activated K^+ channels in vascular smooth muscle cells (Lee et al., 1997). In addition, GTP-bound TG2/Ghα binds to the cytoplasmic tail of α5 integrin, and this interaction inhibits vascular smooth muscle cell migration (Kang et al., 2004). On the contrary, TG2/Ghα was reported to promote cell migration in fibroblasts through its GTP-binding activity (Stephens et al., 2004). Finally, its GTPase activity was also found to regulate cell-cycle progression in fibrosarcoma cells (Mian et al., 1995) and to mediate cell proliferation induced by α_1 adrenergic receptors in hepatocytes (Wu et al., 2000) and visceral smooth muscle cells (Dupuis et al., 2004).

Despite all the cited progress in understanding the GTPase function of TG2/Ghα, the pathophysiological role(s) of the associated intracellular signaling remains poorly understood. For instance, its cardiac-specific overexpression failed to alter activation of PLCδ1 in the resting state and in response to agonists, suggesting that TG2/Ghα acts as TG rather than GTPase in the heart (Small et al., 1999). Nonetheless, intrinsic GTPase activity of the enzyme was found to be markedly decreased in the ischemic heart, suggesting that its downregulation is involved in cardiac failure in humans (Hwang et al., 1995). It may also be involved in the liver regeneration program due to its involvement in the α_1 adrenergic receptor signaling pathway (Sarang et al., 2005; Wu et al., 2000). Overall, the GTPase signaling by TG2/Ghα might be generally prosurvival and cytoprotective, as mutants defective in GTP-binding appeared to induce cell death in NIH3T3 and Hela cells independently of their transamidating activity (Datta et al., 2007). Likewise, the GTPase function and/or conformational state as well as intracellular localization of the protein were important in protecting HEK293 and mouse striatal cells from death due to oxygen glucose deprivation (Colak et al., 2011; Gundemir and Johnson, 2009). Apparently, further analysis is needed to assess the role of GTPase activity and the associated signaling function of TG2/Ghα in cells and *in vivo*.

In addition to GTP, TG2 was found to bind and hydrolyze ATP. Preliminary mapping of the ATP-binding site assigned it to the amino acids 145–185 of the core domain (Iismaa et al., 1997; Lai et al., 1996, 1998; Singh et al., 1995). Unlike GTP hydrolysis, the ATPase activity of TG2 was found to be surprisingly resistant to Ca^{2+} (Nakano et al., 2007). This finding suggested a novel role for the ATPase activity of extracellular TG2, which was further elevated as a result of limited proteolysis by membrane-type matrix metalloproteinase-1 (MT1-MMP), in the process of ATP-dependent mineralization of osteoblasts (Nakano et al., 2010). On the contrary, GTP-bound extracellular TG2 was implicated in hypertrophic differentiation and calcification of chondrocytes (Johnson and Terkeltaub, 2005), acting through $\alpha5\beta1$ integrin engagement and downstream signaling (Tanaka et al., 2007).

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2.3. Protein disulfide isomerase activity of TG2

PDI is an enzyme in the ER and on the surface of eukaryotic cells that catalyzes the formation, breakup, and exchange of disulfide bonds via cysteine residues in proteins (Gruber et al., 2006). A surprising finding by Chandrashekar and coauthors (1998) revealed that PDI-related protein in filarial parasite possesses transamidating activity. Later, several PDIs and related thioredoxins were found to display transamidating activity that depended on the same conserved, adjoining Cys, His, and Asp residues that are required by all TGs to catalyze the incorporation of primary amines into proteins (Blasko et al., 2003). This suggests that all these enzymes share some overlapping functions in cell and tissue homeostasis. The relatively low but detectable disulfide isomerase activity of TG2 with RNase A as its *in vitro* substrate was found to be independent of Ca^{2+} and GTP (Hasegawa et al., 2003). This enzymatic activity of TG2, which required free sulfhydryl groups of the protein for catalysis, was influenced by oxidants/antioxidants and strongly amplified by oxidized glutathione, but inhibited by its reduced form. An important *in vivo* role for the PDI function of TG2 was suggested based on the analysis of *TGM2*–/– mice which display abnormalities in the mitochondrial respiratory chain and ATP production (Bernassola et al., 2002). The underlying molecular mechanism may depend on defective disulfide bond formation in the ATP synthase complex and other key components of the respiratory chain (Battaglia et al., 2007; Mastroberardino et al., 2006), including mitochondrial ADP/ATP transporter adenine nucleotide translocator 1 (ANT1), which was incorrectly assembled and dysfunctional in the absence of PDI activity of mitochondrial TG2 (Malorni et al., 2009). It remains unclear whether other cellular and physiological functions of TG2 depend on its PDI activity in other compartments.

2.4. Protein kinase activity of TG2

Another unexpected enzymatic function of TG2 was described in 2004, when a novel intrinsic kinase activity of the protein was found to result in phosphorylation of IGFBP-3 on the surface of breast cancer cells. This activity was reproduced with purified TG2 protein (Mishra and Murphy, 2004). Further analysis determined that TG2 phosphorylates Ser and Thr, but not Tyr residues in IGFBP-3. The K_m and V_{max} for TG2-induced IGFBP-3 phosphorylation were in the physiological range and similar to that described for other kinases. Whereas Ca^{2+} activates the transamidating function of TG2, it was found to inhibit its protein kinase activity, as TG2-cross-linked IGFBP-3 polymers in the presence of Ca^{2+} appeared only weakly phosphorylated compared with the monomeric IGFBP-3 substrate. Interestingly, cystamine, an inhibitor of the TG2 transamidating function, was also found to block its protein kinase activity (Mishra and Murphy, 2004).

Later, the p53 oncoprotein was reported to serve as a substrate for the protein kinase activity of TG2 in the nucleus. TG2-induced phosphorylation of its residues Ser15 and Ser20 interfered with Mdm2 binding, suggesting that this TG2-dependent mechanism could facilitate apoptosis (Mishra and Murphy, 2006a). Additional nuclear substrates of TG2 protein kinase activity include histones H1 and H3, suggesting the ability of TG2 to regulate chromatin structure and function (Mishra et al., 2006). Likewise, in the nucleus TG2 was shown to phosphorylate Rb at Ser780, thus blocking its interaction with the E2F1 transcription factor (Mishra et al., 2007). Notably, TG2 itself appeared phosphorylated by protein kinase A (PKA), and this modification reduced the transamidating but increased the kinase activity of the protein (Mishra and Murphy, 2006b). Experiments with fibroblasts from *TGM2*–/– mice strongly suggested that PKA-induced phosphorylation of Rb is mediated, at least in part, by the kinase activity TG2, potentially explaining the antiapoptotic effects of TG2 in the nucleus (Mishra et al., 2007). Intriguingly, the PKA-dependent phosphorylation of TG2 at Ser216 residue was determined to generate the binding site for the 14-3-3 scaffolding protein *in vitro* and *in vivo*, thus providing additional avenues for the

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cross talk of TG2 with several signaling pathways (Mishra and Murphy, 2006b). Despite these initial striking observations on the protein kinase activity of TG2, the significance of such modifications in cell processes and tissue/organ homeostasis still awaits confirmation.

2.5. Nonenzymatic functions of TG2: A novel signaling/adapter protein

Over the past two decades, it has become increasingly clear that, in addition to enzymatic transamidating/protein cross-linking, GTPase, disulfide isomerase, and protein kinase activities, TG2 has other functions that are separate and independent from its enzymatic properties, but are rather dependent on direct noncovalent interactions of this protein with a number of binding partners localized in various cell compartments (Belkin, 2011; Lorand and Graham, 2003; Park et al., 2010; Zemskov et al., 2006). For instance, an interaction with nuclear protein α3-importin was suggested to be important for targeting TG2 to the nucleoplasm (Peng et al., 1999). Other TG2-binding proteins, such as PLCδ1 (Hwang et al., 1995; Kang et al., 2002), PKA anchor protein 13 (AKAP13, Lewis et al., 2005), 14-3-3 proteins (Mishra and Murphy, 2006b), Bcr (Yi et al., 2009), and Rac1 (Kim et al., 2010), are localized in the cytoplasm. Additional TG2 interactors include highly abundant ECM proteins such as fibronectin (Turner and Lorand, 1989) or minor ECM components, such as angiocidin (L'Heureux et al., 2010) and endostatin (Faye et al., 2010). On the cell surface, TG2 was found to directly bind matrix metalloproteinase-2 (MMP2; Belkin et al., 2004) and interact with extracellular domains of several transmembrane receptors, including several integrins (Akimov et al., 2000; Zemskov et al., 2006), an atypical orphan GPCR, GPR56 (Xu et al., 2006), syndecan-4 (Telci et al., 2008), platelet-derived growth factor receptor (PDGFR; Zemskov et al., 2009), low density lipoprotein receptor-related proteins 1 (LRP1; Zemskov et al., 2007) and 5/6 (Faverman et al., 2008). Finally, milk fat globulin EGF factor 8 (MFG-E8), a protein involved in bridging the apoptotic target cells to macrophage β3 integrins, was found to interact directly with TG2 on their surface (Toth et al., 2009a). In some cases, proteins that bind non-covalently to TG2 also serve as enzymatic substrates for transamidation/cross-linking (e.g., Bcr, Rac1, fibronectin, angiocidin); in other cases, proteins that bind noncovalently to TG2 are not enzymatically modified (e.g., integrins, PDGFR, MMP2). Thus, in addition to enzymatic functions, the wide variety of noncovalent interactions of TG2 implicates it in a plethora of adapter/signaling functions both inside and outside of cells, enabling it to impinge on a number of signaling pathways. In subsequent parts of this review, we discuss both enzymatic and nonenzymatic activities of TG2 with regard to particular cellular functions in individual cellular compartments.

3. Regulation of TG2 Expression and Localization

TG2 expression varies greatly in different types of cells, ranging from high constitutive levels in endothelium to low or undetectable levels in many other cell types (Iismaa et al., 2009; Lorand and Graham, 2003; Thomazy and Fesus, 1989). Remarkably, the expression of this protein is regulated on many levels and can be strikingly and acutely induced in response to a number of unrelated stressors, including injury, inflammation, and neoplastic transformation. Oxidants, hypoxia, oncogenes, cytokines, and growth factors all potently regulate TG2 in different cell types (Ientile et al., 2007). In agreement, a number of transcription factor-binding sites have been identified in the promoter region of the *TGM2* gene (Lu et al., 1995; Nagy et al., 1996; Ritter and Davies, 1998).

3.1. Epigenetic regulation

The role of promoter methylation/demethylation in the expression of the human *TGM2* gene was discovered by Lu and Davies (1997), who showed that the proximal promoter of the gene includes two GC-rich regions and that their hypomethylation correlated with basal levels of TG2 expression in normal endothelial and transformed erythroleukemia cells.

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Hypermethylation in promyelocytic leukemia cells and normal lymphocytes and monocytes led to a lack of constitutive TG2 expression. Moreover, *in vitro* demethylation of the promoter increased, while increased methylation reduced TG2 levels, thus suggesting that tissue-specific and transformation-induced alterations of DNA methylation regulate the rate of the *TGM2* gene transcription. Later, Cacciamani and coworkers (2002) mapped the 5-methylcytosine residues in the promoter and confirmed the essential role of this modification in maintaining the repressed state of the *TGM2* gene in various cell types.

Since TG2 expression is regulated by retinoids (Mehta et al., 1985), which are known to induce differentiation of myeloid cells, epigenetic changes in the regulatory regions of the *TGM2* gene were studied in relation to retinoid-induced maturation of these cells (Balint et al., 2005). The induction of the intermediary state of myeloid differentiation was found to correlate with increased methylation of Arg3 in histone H4 and decreased methylation of Lys4 in histone H3. These modifications occur before transcription and appear to prime the chromatin for subsequent hormone-regulated transcription of the *TGM2* gene. The authors concluded that histone H4 methylation alters the state of chromatin on the *TGM2* promoter, acting as a regulator of transcriptional responsiveness and signal integration mechanism during cell differentiation and the maintenance of epigenetic memory.

TG2 expression was also found to be coactivated during inflammation with that of metastatic tumor antigen 1 (MTA1). While studying the impact of MTA1 status on global gene expression in bacterial lipopolysaccharide (LPS)-stimulated mammalian cells, Ghanta and colleagues (2011) discovered that MTA1 depletion impairs the basal and LPS-induced expression of TG2 in multiple experimental systems. TG2 was identified as a chromatin target of MTA1 and of NFκB signaling in the LPS-stimulated cells. In addition, LPS-mediated stimulation of TG2 expression was accompanied by enhanced recruitment of MTA1, p65RelA, and RNA polymerase II to the NFκB consensus sites in the *TGM2* promoter. These findings revealed an obligatory coregulatory role of MTA1 in the induction of TG2 expression and of the MTA1-TG2 pathway, at least in part, in the inflammation-driven NFκB signaling in macrophages.

A novel mechanism of epigenetic repression of *TGM2* gene expression was identified in neuroblastoma and breast carcinoma cells, where, respectively, N-myc and c-myc acted as transrepressors by recruiting histone deacetylase protein to an SP1-binding site in the core promoter region (Fig. 1.5; Liu et al., 2007). Finally, aberrant hypermethylation of the *TGM2* gene promoter leading to its epigenetic silencing was detected in gliomas (Dyer et al., 2011). Despite these initial findings, much work is needed to fully characterize the role of chromatin structure in the regulation of *TGM2* gene expression *in vivo*.

3.2. Transcriptional regulation

Retinoids were historically the first factors found to markedly induce the acute upregulation of *TGM2* gene transcription in macrophages (Chiocca et al., 1989; Murtaugh et al., 1983, 1986) and other cells (Piacentini et al., 1992a,b; Vollberg et al., 1992). Accordingly, ~1.7kb upstream of the transcription start site, the *TGM2* promoter was found to contain a versatile tripartite retinoid response element which is activated by either retinoic acid receptor-retinoid X receptor (RAR/RXR) heterodimers or RXR homodimers (Fig. 1.5; Nagy et al., 1996). In addition, retinoid-dependent trans-activation of *TGM2* gene expression included the direct interaction of the SP1 transcription factor with the RAR/RXR complex within the GC-rich region of its promoter (Shimada et al., 2001). Unlike other inducers of TG2 expression, retinoids also amplify the transamidating activity of TG2 by sensitizing it to Ca^{2+} thus partially overcoming the requirement for Ca^{2+} activation and shift the cytoplasmic pool of TG2 to the plasma membrane (Singh and Cerione, 1996). While many stages in the pathway of retinoid-induced TG2 upregulation remain unknown, it has been shown to

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depend on PI3K activity in fibroblasts (Antonyak et al., 2002) and in differentiating neuroblastoma cells (Pan et al., 2005), to involve transamidation and activation of RhoA and downstream targets (Singh et al., 2001), and to include the nonenzymatic activation of the Rac1 and ERK1/2, JNK, and p38γ MAPK pathways (Singh et al., 2003).

Activation of the NFκB signaling pathway was reported to acutely induce TG2 mRNA expression in hepatocytes in response to chemical injury (Mirza et al., 1997), as well as interleukin-6 and TNFα (Kuncio et al., 1998). The same pathway was shown to drive the upregulation of TG2 expression at the transcriptional level in various other cells due to the binding of the p65RelA/p50 complex to a cognate response element in the *TGM2* promoter located ~1.35kb upstream of the transcription start site (Fig. 1.5; Ientile et al., 2007). A large and growing body of work indicates that excessive activation of the NFκB pathway might be particularly important for inducing increased levels of TG2 expression during inflammatory responses and in many types of tumor cells (Mehta et al., 2010). A principal regulator of NFκB-mediated TG2 expression, MTA1, which is also a master chromatin modifier, was recently shown to control both basal and LPS-induced levels of TG2 as an obligatory coactivator of TG2 expression and modifier of the NFκB signaling in macrophages (Ghanta et al., 2011).

The transforming growth factor (TGFβ) pathway is another important signaling cascade that has been shown to alter the transcription of the *TGM2* gene (George et al., 1990). TGFβ induces cell type-specific activation or deactivation of the *TGM2* promoter via a TGFβ response element located 868bp upstream of the transcription start site (Fig. 1.5; Ritter and Davies, 1998). In addition, bone morphogenetic proteins 2 and 4 have also been shown to regulate TG2 expression by acting on the TGFβ response element in the promoter. As TGFβ1 increases the TG2 levels in fibroblasts and many other cells, but downregulates them in epithelium, its impact on the *TGM2* promoter can be stimulatory or inhibitory depending on the cell type. TGFβ2 upregulates *TGM2* gene expression in optic nerve astrocytes and subconjunctival fibrobalsts. This upregulation involves the PI3K signaling pathway and, specifically, Akt1, while other upstream mediators were not identified (Fuchshofer et al., 2005; Jung et al., 2007). Meanwhile, the latest study by Tovar-Vidales and coworkers (2011) revealed that, in trabecular meshwork cells, TGFβ2 acts through the canonical Smad3-mediated signaling pathway to induce TG2 expression, while its action does not involve connective tissue growth factor (CTGF) as a downstream intermediate. The TGFβ-induced upregulation of *TGM2* gene expression in mesenchymal cells is likely to be involved in the regulation of ECM turnover during the normal wound healing response and pathologic tissue fibrosis (Collighan and Griffin, 2009; Telci and Griffin, 2006; Verderio et al., 2004).

Although interferon (IFN)-stimulated response elements in the *TGM2* promoter are not characterized, IFNα2b was shown to modestly increase the transcription of the gene in a squamous carcinoma cell line (Giandomenico et al., 1997) and in lung cancer cells (Esposito et al., 2003), likely acting through the JAK-STAT (Janus kinase—signal transducer and activation of transcription) pathway and IRF-1 (IFN regulatory factor 1) transcription factor.

Hox proteins are a family of homeodomain-containing transcription factors involved in pattern formation during embryonic development and regulation of hematopoiesis (van Oostveen et al., 1999). A sustained expression of Hox A7 in acute myeloid leukemia cells impaired their adhesion and migration on fibronectin during early differentiation, partly due to blockage of transcriptional induction of TG2 expression (Leroy et al., 2004). No details regarding this regulation were reported.

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Interleukin-1, interleukin-8, and growth-related oncogene α chemokines are elevated in osteoarthritic chondrocytes where they increase TG2 expression and activity via the p38MAPK pathway (Johnson et al., 2001; Merz et al., 2003).

The adaptive response to hypoxia is achieved by transcriptional changes of multiple genes mediated by hypoxia inducible factor 1 (HIF1), a heterodimeric transcription factor consisting of inducible HIF1α and constitutively expressed HIF1β subunits (Pouyssegur et al., 2006). Recent studies revealed that TG2 serves as transcriptional target of HIF1 during the survival of neurons exposed to oxygen and glucose deprivation (Filiano et al., 2008) and in hypoxic tumor cells (Jang et al., 2010). The response is due to the presence of six putative hypoxia response elements in the promoter of the *TGM2* gene (Fig. 1.5). In neurons, TG2 protected against hypoxia, likely as a result of its direct interaction with HIF1β and the subsequent attenuation of HIF1 signaling, whereas in tumor cells, it suppressed apoptosis by cross-linking and subsequent inactivating caspase-3 and promoted survival by activating the NFκB pathway.

The EGF/EGF receptor (EGFR) pathway, which is often hyperactivated in human malignancies, upregulated TG2 expression in cervical and breast epithelial cancer cells. The induction of TG2 was found to be essential for EGF-mediated cell migration, invasion (Fig. 1.5; Antonyak et al., 2009), and anchorage-independent growth (Li et al., 2010). This EGF signaling effect was mediated by Ras- and Cdc42-induced activation of PI3K and NFκB, and required Src activity and the formation of ternary cytoplasmic complexes between Src and keratin-19, mediated by TG2. Much like with retinoids, the EGF signaling through Ras and JNK was required for targeting TG2 to the leading edges of the cells and activating transamidation. Similar EGF/EGFR-dependent mechanism and JNK/ERK signaling pathways were implicated in the upregulation of TG2 in acquired tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance and invasiveness in lung cancer cells (Li et al., 2011). The functionally related PDGF/PDGFR signaling pathway was found to elevate TG2 mRNA and protein levels in vascular smooth muscle cells in culture and *in vivo* in response to blood vessel injury (Zemskov et al., 2011b). Nothing, however, is currently known about the signaling intermediates involved in this regulation. In addition, TG2 mRNA, TG2 protein levels, and its transamidating activity were shown to be upregulated by insulin-like growth factor (IGF) and estradiol in astrocytes (Campisi et al., 2008), dexamethasone in normal and transformed fibroblasts (Johnson et al., 1998), and endothelin-1 in cardiomyocytes (Li et al., 2009). The molecular mechanisms of TG2 modulation in all these cases remain to be defined. Finally, some of the pathways regulating TG2 expression operate in a cell type-specific manner. For example, oncogenic H-Ras increased the TG2 levels in the cells of epithelial origin (Antonyak et al., 2009; Li et al., 2010) but decreased them in fibroblasts acting via the JNK, p38γMAPK, and PI3K pathways (Akimov and Belkin, 2003).

3.3. Alternative splicing

Several alternatively spliced forms of TG2, all with truncated and some with unique short sequences at their C-termini, were described in astrocytes, neurons, lymphocytes, endothelial, and vascular smooth muscle cells (Section 2.1.3; Antonyak et al., 2006; Festoff et al., 2002; Fraij and Gonzales, 1996; Fraij et al., 1992; Lai et al., 2007; Monsonego et al., 1997). While some of these were shown to display altered transamidating and GTPase activities that impact cellular functions, it remains unknown how the splicing events leading to the generation of alternative TG2 transcripts are regulated.

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3.4. Degradation: Ubiquitination and SUMOylation

Currently, surprisingly little is known about TG2 turnover and its regulation in cells. One report revealed that, in lung carcinoma cells, TG2 is ubiquitinated and targeted to the proteasome for degradation, whereas these processes were attenuated by retinoic acid and IFNα2b (Fig. 1.5; Esposito et al., 2003). The identity of the ubiquitin-conjugating enzyme remains to be determined. A posttranslational modification of proteins, known as SUMOylation (SUMO—small ubiquitin-like modifier), represents a key cellular mechanism for the regulation of protein stability (Meulmeester and Melchior, 2008). Remarkably, human bronchial epithelial cells expressing functionally deficient cystic fibrosis transmembrane conductance regulator (CFTR) were found to upregulate TG2, leading to increased cross-linking and sequestration of its enzymatic substrate, anti-inflammatory peroxisome proliferator-activated receptor-γ (PPAR-γ) and thus indicating a central role of TG2 in mediating the intrinsic inflammation in cystic fibrosis (Maiuri et al., 2008). In these cells, oxidative stress increased the activity of the SUMO ligase, known as protein inhibitor of activated STAT-y, (PIASy) and its ability to interact with TG2 and mediate TG2 SUMOylation. This response reduced the ubiquitination of TG2, thus increasing its stability and transamidating activity in the cytoplasm (Fig. 1.5; Luciani et al., 2009). Three SUMO1 modification motifs Φ_KXE were tentatively identified in the TG2 sequence at positions 323–329, 361–366, and 466–470 but were not experimentally confirmed. Significantly, elevated ROS levels and SUMOylation of TG2 were demonstrated in the lung tissues of mice expressing the mutant ΔPhe508-CFTR, suggesting that the control of TG2 turnover may serve as a central link between oxidative stress and inflammation in cystic fibrosis. It will be important to determine whether, in addition to transcriptional effects, dysregulation of cytoplasmic TG2 turnover by ubiquitination and SUMOylation is involved in other pathological states, such as neurodegeneration and cancer, which are accompanied by increased expression levels of this protein.

4. TG2 in Diverse Cellular Compartments

Although it was initially identified and studied as a typical cytoplasmic protein, TG2 was later described to localize in other compartments, including the nucleus, mitochondria, endolysosomes, and in the extracellular space (Fig. 1.6; Gundemir and Johnson, 2009; Lorand and Graham, 2003; Malorni et al., 2008; Park et al., 2010; Zemskov et al., 2006). In this section, we overview and discuss compartment-specific enzymatic and nonenzymatic functions of TG2.

4.1. Cytoplasmic TG2

In most cells, cytoplasmic TG2 comprises the largest part of its cellular pool (Chowdhury et al., 1997; Lesort et al., 1998; Park et al., 2010). Whereas, in theory, GTPase activity should represent its main enzymatic function in the cytoplasmic environment of submicromolar [Ca^{2+}], TG2 also clearly displays TG properties by engaging in enzymatic cross-linking, transamidation, and deamidation of cytosolic substrates (Kiraly et al., 2011). Moreover, the majority of identified TG2 substrates are cytoplasmic proteins (Facchiano and Facchiano, 2009). The induction of TG function of cytoplasmic TG2 is likely triggered by a variety of factors, including excitoxins, ROS, growth factors, and chemokines, which all may drive a release of Ca^{2+} from intracellular stores and increase in local [Ca^{2+}], and by other small molecules and interacting proteins that can alter the TG2 conformation (see also Section 2.1.3). A role for binding partners in the regulation of TG2 enzymatic activities was suggested early when Singh and Cerione (1996) revealed that most TG2 is kept inactive as a GTPase in the cytoplasm of Hela cells as a part of a multiprotein (~600kDa) cytosolic complex, while retinoic acid shifts it to the ~150kDa plasma membrane-associated complex and induces the GTPase activity of the protein. A similar shift in TG2 localization from

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mainly cytosolic to membrane-associated was also observed in the case of EGF induction (Antonyak et al., 2009). Nonetheless, despite a lack of knowledge regarding TG2-binding cytoplasmic proteins that regulate its activities, there is a growing consensus that TG2 in the cytoplasm can be readily activated as a TG, whereas TG2 in the membrane-bound pool acts primarily as a GTPase (Fig. 1.6; Park et al., 2001). It remains unknown whether the conformational change of TG2, which accompanies its shuttling between these compartments, is regulated by its interaction with membrane lipids (Sections 4.2.2 and 4.2.3.1) and/or by yet uncharacterized posttranslational modifications of the protein.

When the cellular degradation machinery is impaired or overwhelmed, it causes a local accumulation of misfolded proteins in aggresomes, the inclusion bodies formed around the microtubule organizing center in eukaryotic cells (Caccamo et al., 2011). Aggresome formation is a general protective response to a high load of abnormal or damaged proteins within the cytosol that have failed to be eliminated by the ubiquitin proteasome system for protein degradation. Notably, TG2 overexpression was reported to drive the formation of α synuclein-containing perinuclear aggregates in a heterologous cell system and both proteins were localized in Lewy bodies in the neurons from Parkinson disease patients (Junn et al., 2003). Mallory bodies, a type of keratin-containing aggresome present in hepatocytes that are a hallmark of several chronic liver diseases, were determined to include TG2 (Riley et al., 2002). Their formation in a mouse model of response to chemical liver injury was reported to depend on TG2 (Strnad et al., 2007). As a principal link between oxidative stress and inflammation (Caccamo et al., 2011; Ientile et al., 2007), TG2 was also found to induce the formation of PPARγ aggregates in the perinuclear aggresomes typical for CFTR-defective bronchial epithelial cells. The oxidation-induced protein cross-linking function of TG2 appeared essential for this process (Maiuri et al., 2008). In summary, Ca^{2+}-mediated cross-linking of unrelated cytoplasmic protein substrates in several cell types by TG2 is involved in their sequestration in aggresomes. It is likely that this process plays a key role in the general pathophysiological response to accumulation of misfolded proteins.

While both the TG and GTPase enzymatic activities of cytoplasmic TG2 are well established (Iismaa et al., 2009; Lorand and Graham, 2003), gathering evidence points to additional nonenzymatic adapter/scaffolding functions of this protein in the cytoplasm (Fig. 1.6; Park et al., 2010). For example, cytoplasmic TG2 might be involved in the regulation of small GTPases. TG2 regulates Rho family GTPases through several distinct and unrelated mechanisms. These include enzymatic TG2-mediated serotonylation of RhoA and Rac1 in the cytoplasm (Walther et al., 2011) and nonenzymatic RhoA activation by surface TG2-mediated integrin clustering (Janiak et al., 2006). Recent work, however, reported that, in basophilic leukemia cells, cytoplasmic TG2 interacts with and activates Rac1 in a nonenzymatic manner (Kim et al., 2010). A likely mechanism for such activation was revealed when it was shown that TG2 directly interacts with Bcr, one of the GTPase-activating proteins for Rac1, *in vitro* and in cells. TG2 binding to the Rac-binding pocket blocks the GTPase activity of Bcr, thereby increasing Rac1 activation (Yi et al., 2009). Notably, TG2 in the extended rather than compact conformation preferentially binds to Bcr. This suggests that Ca^{2+} or other ligands that induce such conformational shift promote the interaction of TG2 with Bcr and the resulting upregulation of Rac1 activity. Other cytoplasmic signaling proteins, such as PKA-associated protein AKAP13 (Lewis et al., 2005) and 14-3-3, which binds to the PKA-generated pSer212 and pSer216 residues of TG2 (Mishra and Murphy, 2006b), were found to interact with TG2 *in vitro* and in the cytoplasm. These findings indicate the existence of PKA/AKAP13/TG2/14-3-3 cytoplasmic complexes that may potentially impinge on a number of signaling pathways. The mechanistic details, regulation, and signaling consequences of such interactions remain to be explored. Contradictory findings were reported when cytoplasmic TG2 was described to inhibit

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adenylyl cyclase activity in fibroblasts (Gentile et al., 1997) but found to activate it in neuroblastoma cells (Tucholski and Johnson, 2003).

Finally, some principal biological effects of cytoplasmic TG2, such as its impact on neuronal death upon oxygen–glucose deprivation, may depend on the protein conformation, rather than the balance between its TG and GTPase activities, as structural transitions of TG2 endow it with the ability to interact with conformation-specific binding partners (Colak et al., 2011; Pinkas et al., 2007). In the case of TG2, the protein conformation may define its key cellular functions, as it was shown for proapoptotic effect of GTP-binding-defective forms of TG2 in normal and transformed fibroblasts (Datta et al., 2007).

4.2. Extracellular TG2

Initial studies in the early 1990s revealed the presence of TG2 outside the cell, both on the cell surface in a close association with the plasma membrane and in the ECM (Fig. 1.6; Aeschlimann et al., 1993; Gentile et al., 1992; Upchurch et al., 1991). Several enzymatic substrates of TG2 were identified among ECM proteins, including fibrin(ogen), fibronectin, collagen, vitronectin, and osteopontin (Aeschlimann and Thomazy, 2000). In addition, extracellular TG2 was found to possess protein kinase activity (Mishra and Murphy, 2004) and, likely, PDI and GTPase/ATPase functions (Fig. 1.6; Hasegawa et al., 2003; Johnson and Terkeltaub, 2005; Nakano et al., 2010). Several subsequent findings established that, in addition to the cross-linking of various ECM substrates and other enzymatic functions, extracellular TG2 noncovalently interacts with several transmembrane receptors and ECM proteins, exhibiting an important nonenzymatic adapter/scaffolding function outside the cell.

4.2.1. Cell-surface TG2—Several types of TG2-containing protein complexes were identified on the surface of various cells.

4.2.1.1. Integrin–TG2–fibronectin complexes: TG2 has long been known to noncovalently interact with the ubiquitous and abundant ECM protein, fibronectin, *in vitro* (Turner and Lorand, 1989). More recent studies showed the ability of cell-surface TG2 to bind soluble fibronectin and to promote its deposition into the ECM (Akimov and Belkin, 2001b; Martinez et al., 1994). The ability of TG2 to promote cell–ECM adhesion, cell migration, and the assembly of fibronectin fibrillar matrices depends on this interaction (Akimov et al., 2000; Belkin, 2011; Hang et al., 2005; Zemskov et al., 2006). TG2 binds with high affinity to the region of fibronectin that consists of modules $I_6II_{1,2}I_{7–9}$, a part of fibronectin molecule that does not contain any known integrin-binding sites (Radek et al., 1993; Turner and Lorand, 1989).

Cell-surface TG2 collaborates with integrins in cell adhesion through a direct noncovalent interaction with the extracellular domains of structurally related β1, β3, and β5 integrin subunits and the formation of stable ternary complexes with both integrins and fibronectin (Fig. 1.7; Akimov and Belkin, 2001a,b; Akimov et al., 2000). The relatively weak affinity of integrin–fibronectin binding and the stable noncovalent association of TG2 with both these proteins suggest that cell-surface TG2 enhances the interaction of cells with fibronectin by acting as a bridge between integrins and this ECM protein (Akimov et al., 2000). In various cells, a sizeable integrin fraction (~40% of β1 integrins in macrophages) is associated with TG2 (Akimov et al., 2000; Janiak et al., 2006). Moreover, TG2 was reported to control integrin levels on the surface of cancer cells (Mangala et al., 2007; Satpathy et al., 2007) and macrophages (Toth et al., 2009a,b), however, the molecular mechanisms of such regulation remain unclear.

The functional collaboration between integrins and TG2 in cell adhesion is also reflected in the alteration of the state of integrins by cell-surface tTG even in the absence of fibronectin

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(Janiak et al., 2006). While no TG2-mediated changes in ligand-binding affinity of integrins were detected, TG2 was found to induce integrin clustering. In TG2-expressing fibroblasts, a significant integrin fraction was found within large protein complexes that were identified both biochemically and by immunofluorescence. The molecular mechanisms of integrin clustering by surface TG2 are currently unknown. Both the ability of TG2 to oligomerize (Liu et al., 2002; Janiak et al., 2006) and, potentially, interact with other integrin-binding proteins, including caveolin-1 and tetraspanins, within these complexes, may promote integrin aggregation. The observed codistribution of TG2 and β1 integrins in lipid rafts and caveolae (Zemskov et al., 2007) likely enhances the linkage of cell–ECM adhesions to these cholesterol-enriched membrane microdomains, affecting membrane protein trafficking and compartmentalization of cell signaling.

Importantly, the association of TG2 with integrins on the cell surface, and TG2-mediated integrin clustering, potentiates the outside-in signaling triggered by these transmembrane adhesion receptors (Belkin, 2011; Zemskov et al., 2006). The formation of stable complexes between β1 integrins and TG2 modulates the activities of focal adhesion kinase (FAK), src, and p190RhoGAP and upregulates the activation levels of RhoA GTPase and its downstream signaling target, ROCK. Therefore, these complexes contribute to increased formation of focal adhesions, stress fibers, and elevated actomyosin contractility in the cells expressing TG2 (Janiak et al., 2006). Additional targets of β3 integrin-mediated signaling, such as RhoG and Rac1, are upregulated by TG2 in macrophages (Toth et al., 2009a,b). It is likely that the activation of many other integrin-dependent signaling pathways is potentiated by TG2, suggesting that it serves as a general amplifier of the outside-in integrin signaling (Fig. 1.7). Accordingly, a significant impact of cell-surface TG2 on integrin-mediated adhesion, spreading, migration, survival, differentiation, fibronectin matrix assembly, and ECM contraction was described for a wide range of normal and transformed cells (Sections 5.1–5.4; Akimov and Belkin, 2001a,b; Akimov et al., 2000; Janiak et al., 2006; Mangala et al., 2007; Satpathy et al., 2007; Song et al., 2007; Stephens et al., 2004; Toth et al., 2009a,b).

4.2.1.2. TG2-syndecan-4 complexes: While early work indicated an interaction between TG2 and heparin *in vitro*, two latest studies with fibroblasts revealed that the heparan sulfate proteoglycan, syndecan-4, was another important binding partner for extracellular TG2 (Fig. 1.7; Scarpellini et al., 2009; Telci et al., 2008). A putative conserved heparan sulfate binding site ^{261}LRRWK^{265} was tentatively identified in several mammalian TG2s but appeared to be missing in other TGs (Verderio et al., 2009). Unlike syndecans-1, -2, and -3, syndecan-4 has been previously shown to accumulate in focal adhesions where it interacts via heparan sulfate chains with the Hep-2 region of fibronectin and collaborates with integrins in cell adhesion to fibronectin and in the adhesion-dependent, RhoA-mediated development of focal adhesions, stress fibers, and actomyosin contractility (Xian et al., 2010).

The high-affinity interaction of extracellular TG2 with syndecan-4 maintains the activation of PKCα, which, in turn, directly binds to the β1 integrin cytoplasmic tails. These interactions are important for controlling both integrin levels and their distribution throughout the cell surface, as well as integrin signaling to FAK and ERK1/2 (Parsons et al., 2002; Scarpellini et al., 2009; Telci et al., 2008; Wang et al., 2010, 2011). Recently, it has been shown that the ability of activated PKCα to maintain the RGD-independent adhesion of fibroblasts and osteoblasts through interaction of fibronectin–TG2 heterocomplexes in the ECM with cell-surface syndecan-4 is mediated by syndecan-2 (Wang et al., 2010, 2011). This receptor does not bind TG2 but rather acts as a downstream signaling effector in modulating the cytoskeletal organization through the ROCK pathway. These data also imply a major role for fibronectin/TG2/syndecan-4 complexes as a parallel adhesive/signaling platform that cells may utilize in the case of integrin function deficiency (Verderio and Scarpellini, 2010). In addition, the integrin- and syndecan-4-based adhesion systems are

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likely to physically interact, since these two receptors bind to separate and nonadjacent regions of fibronectin and functionally collaborate by jointly regulating p190RhoGAP activity and localization during cell adhesion to this ECM protein (Bass et al., 2008; Telci and Griffin, 2006). Therefore, an emerging model indicates the existence of quaternary adhesion/signaling complexes comprising integrins, syndecan-4, their joint ECM ligand fibronectin, and TG2, with the latter protein orchestrating the formation of such complexes due to its high affinity for all the other components (Fig. 1.7).

The interaction of integrin-bound TG2 on the cell surface and/or fibronectin-bound TG2 in the ECM with syndecan-4 might be required in response to extensive tissue damage and ECM degradation, which interferes with integrin-mediated adhesion and the associated outside-in signaling. Thus, increased TG2 expression during wound healing and tissue repair is likely to enhance the adhesive/signaling function of cell-surface TG2 and compensate for deficiency in the integrin-dependent adhesion and assembly of fibronectin matrices (Telci and Griffin, 2006; Verderio et al., 2003; Wang and Griffin, 2011). In turn, this should lead to clustering of its binding partners on the cell surface and enhanced adhesion, preventing deadhesion-mediated apoptosis (anoikis) and inducing prosurvival signaling, ultimately facilitating cell survival.

4.2.1.3. Interaction of TG2 with growth factor receptors: An important paradigm entails both the physical association and functional collaboration between integrins and receptor tyrosine kinases in the regulation of cell responses to both the ECM and soluble growth factors (Fig. 1.7). Various studies have shown that engagement of β1 and αvβ3 integrins with ECM ligands transiently activates EGF, PDGF, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) receptor tyrosine kinases even in the absence of their soluble ligands and promotes and sustains growth factor-initiated signaling by these receptors (Yamada and Even-Ram, 2002). Despite the significance of this synergistic signaling, the molecular mechanisms underlying the cross talk between the two receptor systems remain largely unknown. A physical interaction between these two types of signaling receptors was proposed to be enhanced by their cosequestering in cholesterol-enriched membrane microdomains (Baron et al., 2003). Since integrins and growth factor receptors share many downstream signaling targets, integrin–ECM interaction may also increase availability of signal relay enzymes and adapter proteins to growth factor receptors by promoting their recruitment from cytosol to the plasma membrane (DeMali et al., 1999).

A novel mechanistic insight into the cross talk between integrin and PDGFR signaling pathways was provided when TG2 was found to interact with PDGFR both *in vitro* and on the surface of fibroblasts and to mediate its physical association with integrins (Fig. 1.7). In fibroblasts and in vascular smooth muscle cells, TG2 enhances the PDGFR–integrin association by bridging these receptors on the cell surface (Zemskov et al., 2009, 2011b). The interaction between TG2 and PDGFR also reduces cellular levels of the receptor by accelerating its turnover. Moreover, the association of PDGFR with TG2 causes receptor clustering, increases PDGF binding, promotes both adhesion-mediated and growth factor-induced PDGFR activation, and upregulates the downstream signaling mediated by this receptor (Zemskov et al., 2009). Importantly, cell-surface TG2 appears to be required for efficient PDGF-dependent proliferation and migration of fibroblasts and smooth muscle cells. Likewise, TG2 localized on the cell surface was found to amplify PDGF-induced survival and dedifferentiation (phenotypic modulation) of vascular smooth muscle both in culture and *in vivo* (Zemskov et al., 2011b). These findings revealed a novel function of cell-surface TG2 in the regulation of the joint PDGFR/integrin signaling and PDGFR-dependent cell responses by coupling the adhesion-mediated and growth factor-dependent signaling pathways. They also suggest that this activity of TG2 might be involved in the proinflammatory function of this protein in normal wound healing and tissue fibrosis,

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vascular restenosis in response to vessel wall injury, and tumor metastasis, all diverse pathophysiological processes that often involve overactivation or dysregulation of PDGF/PDGFR-mediated signaling (Heldin and Westermark, 1999).

Moreover, the interaction of extracellular TG2 with a wide range of growth factor receptors might be a general phenomenon, as TG2 was also found to bind VEGFR on the surface of endothelial cells and modulate VEGF-induced signaling in this cell type (Dardik and Inbal, 2006). Unlike in the case of PDGFR binding, TG2 not only interacts with VEGFR but also generates covalently cross-linked VEGFR complexes that shuttle to the nucleus in response to VEGF. Future work should help to determine the molecular motifs involved in the association of cell-surface TG2 with growth factor receptors and address whether TG2 interacts with structurally related receptor tyrosine kinases, including EGFR and FGFR, and impacts their joint signaling with integrins.

4.2.1.4. Interaction of TG2 with LDLR family members: Recent work revealed the ability of cell-surface TG2 to interact with several structurally related members of low density lipoprotein receptor (LDLR) family, including LRP1, LRP5, LRP6, and VLDLR (Fig. 1.7; Faverman et al., 2008; Zemskov et al., 2007). TG2 directly binds via its catalytic domain to the α chain of the major endocytic receptor LRP1 both *in vitro* and on the cell surface (Zemskov et al., 2007). Somewhat surprisingly, the receptor-associated protein (RAP), which blocks the interaction of LRP1 with its numerous ligands, did not interfere with TG2 binding, thus indicating that TG2 interacts with the LRP1 site(s) distinct from the ligand-binding site(s). Biochemical cell fractionation also established that TG2 shifts a significant part of cell-surface LRP1 to the cell–matrix adhesive protein fraction enriched in β1 integrin and fibronectin. Notably, LRP1 deficiency or functional blockade prevented TG2 internalization and increased its surface levels, confirming a crucial role for this receptor in TG2 endocytosis from the cell surface.

Extracellular TG2 is also capable of binding to and signaling through LRP5 and LRP6 receptors (Fig. 1.7; Faverman et al., 2008). In a search for TG2-binding partners on the surface of vascular smooth muscle cells, the transmembrane receptors LRP5/6 were identified as its major interactors. The binding of TG2 to these receptors triggered the activation of the β-catenin pathway by driving nuclear translocation of β-catenin, inducing Tcf/Lef transcription factors, and decreasing p21 expression. In turn, TG2-mediated activation of the β-catenin pathway, which is inherently silent in vascular smooth muscle cells, was shown to promote calcification of these cells in culture. Additional *in vivo* studies should help assess the contribution of extracellular TG2 to pathologic calcification in the vessel wall.

4.2.1.5. TG2–GPR56 interaction: GPR56, an atypical GPCR that is downregulated in highly metastatic melanoma cells, was found to interact with the TG2 localized on the surface of surrounding cells in the tumor stroma (Fig. 1.7; Xu et al., 2006). Thus, cell-surface TG2 was proposed as a novel GPR56 ligand that may cooperate in the growth inhibitory and tumor suppressive roles of this orphan receptor. The precise mechanism involved in this activity remains unknown.

4.2.1.6. TG2–MMP2 interaction: TG2 was also shown to directly interact *in vitro* and to form complexes on the cell surface with secreted MMP2 (Belkin et al., 2004). MMP2, acting in concert with its proteolytic transmembrane activator, MT1-MMP (Belkin et al., 2001), cleaves cell-surface TG2, facilitating the effect initiated by MT1-MMP. In turn, TG2 is preferentially associated with the activation intermediate of MMP2 on the cell surfaces. This interaction regulates the rate of MMP2 maturation and protects TG2 against proteolysis by MMP2. Cell culture, *in vitro* experiments, and *in silico* modeling indicated that the

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MMP2 catalytic domain directly associates with the core enzymatic domain 2 of TG2, whereas a follow-up cleavage of this domain by MMP2 eliminates both the adhesive and enzymatic (transamidating) activities of TG2.

4.2.1.7. TG2 binding to MFG-E8: Recently, the mechanistic basis for functional collaboration between TG2 and β3 integrin in macrophages was revealed when TG2 was found to strongly interact with MFG-E8, also known as lactadherin, the protein involved in bridging the β3 integrin to apoptotic cells (Toth et al., 2009a,b). This suggests that the TG2-mediated stabilization of the b3 integrin/MFG-E8 complexes on macrophage surfaces is involved in phagocytic uptake of apoptotic cells.

4.2.2. TG2 in endolysosomal vesicles—Since the microenvironment inside endocytic vesicles is comparable to the one in the extracellular space rather than the cytoplasm, the activities of TG2 inside these vesicles are likely very similar to those of TG2 localized on the cell surface and in the ECM. TG2 is found to be associated with or inside various cytoplasmic vesicles, including early, late, recycling endosomes, and lysosomes (Fig. 1.6; Zemskov et al., 2007, 2011a). Uptake experiments with labeled surface TG2 using antibody Fab fragments in fibroblasts showed that the protein undergoes efficient dynamin-dependent internalization through clathrin- and caveolin-dependent mechanisms and is delivered to early, then late endosomes, and finally to lysosomes for degradation. Although the rate of TG2 endocytosis in fibroblasts was found to be similar to that of integrins, it remains unknown whether TG2 is cointernalized as a part of its cell-surface complexes with integrins and fibronectin. Given that the contents of endocytic vesicles undergo gradual acidification on the way from the cell surface to lysosomes, this pH shift is likely to impact the transamidating and GTPase activities of TG2, although experiments to test this notion appear to be technically challenging.

Although internalized TG2 is not recycled back to the cell surface, it was detected in association with and inside perinuclear recycling endosomes (Fig. 1.8(1); Zemskov et al., 2007, 2011a). The targeting of TG2 to these vesicles appears both to precede unconventional secretion of cytoplasmic TG2 and to be required for the process. The mechanism for recruitment of cytoplasmic TG2 to the recycling endosomes is not well understood but is known to involve the interaction of the phospholipid-binding site of the protein with endomembrane phospholipids such as phosphatidyl inositol (3)-phosphate [PI(3)P]. Inside the recycling endosomes, TG2 interacts with β1 integrins undergoing the recycling process. Likely, the TG2-β1 integrin complexes are initially formed inside these transport vesicles and subsequently delivered onto the cell surface. Other binding partners of TG2 on the membranes and inside the lumen of endosomal vesicles remain to be described.

4.2.3. Regulation of TG2 on the cell surface—The levels and functions of cell-surface TG2 are regulated on several levels, including externalization of cytoplasmic protein, internalization from the cell surface, proteolytic degradation, and translocation of the surface-associated protein to the ECM.

4.2.3.1. Unconventional secretion of TG2: TG2 is constitutively externalized from undamaged cells and various cell types including fibroblasts, osteoblasts, monocytes/macrophages, endothelial, and smooth muscle cells all contain it on their surface and in the ECM (Belkin, 2011; Wang and Griffin, 2011; Zemskov et al., 2006). There are no classical secretory signal sequences and hydrophobic or transmembrane domains in TG2 (Gentile et al., 1991), the protein is not localized in the ER/Golgi compartments, and little is known about the factors that control its secretion (Belkin, 2011, Lorand and Graham, 2003). While many growth factors and cytokines regulate TG2 cellular levels, biosynthesis, and degradation, they all concurrently modulate the levels of TG2 outside the cell, suggesting a

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general pathway(s) for the trafficking of this protein to the cell surface. Meanwhile, a significant portion of the protein is present in the so-called particulate fraction, indicating its association with membranes in various cell types (Lorand and Graham, 2003). This association may depend on stable TG2 interactions with transmembrane proteins, such as integrins or adrenergic receptors. Otherwise, despite the absence of posttranslational modifications of TG2 that might mediate association with the lipid bilayer, the *in vitro* identified lipid binding of TG2 (Harsfalvi et al., 1987; Lai et al., 1997) may target this protein to the intracellular membranes. While some reports proposed that fibronectin and heparan sulfate proteoglycans, two extracellular binding partners of TG2, and its own transamidating activity, may affect its export (Balklava et al., 2002; Cho et al., 2011; Gaudry et al., 1999; Scarpellini et al., 2009), they are more likely to impact the retention of TG2 on the cell surface rather than its intracellular trafficking *en route* to the surface. Although the available data suggested that TG2 is secreted by unconventional mechanisms, the pathway(s) of its externalization and mechanisms(s) of its translocation across lipid bilayers remained largely unknown.

Recent studies began to delineate the secretion pathway of cytoplasmic TG2 by focusing on its intracellular trafficking routes (Fig. 1.8(1); Zemskov et al., 2011a). In fibroblasts, recycling endosomes appear to be essential for TG2 externalization. Instead of being directed to the classical ER/Golgi-dependent secretion pathway, *de novo* synthesized cytoplasmic TG2 is targeted to and delivered inside perinuclear recycling endosomes prior to exportation. Functional ablation of recycling endosomes, blocking endo-some fusion with the plasma membrane, or downregulation of Rab11 GTPase that controls outbound trafficking of perinuclear recycling endosomes were all found to abrogate TG2 secretion. The initial recruitment of cytoplasmic TG2 to the recycling endosomes and its subsequent externalization depend on its binding to phosphoinositides on endosomal membranes (Fig. 1.8(1)). The interaction of TG2 with intracellular transport vesicles likely represents a two-step process with its initial tethering to endosomal phosphoinositides and subsequent tight binding to yet-unidentified endosomal membrane protein(s). It will be important to identify this TG2 “receptor” on the recycling endosomes. While the role of endosomal budding, fusion, and fission in the process of TG2 secretion is unknown, the protein is also often found inside multivesicular bodies. These findings begin to unravel an unconventional mechanism of TG2 secretion that utilizes the long loop of endosomal recycling pathway and indicate involvement of endosomal trafficking in nonclassical protein secretion. Unlike most routes of unconventional secretion, including the ones for FGF2 in fibroblasts or IL-1β in macrophages (Nickel and Rabouille, 2009), the default TG2 export pathway is likely to be common for many cell types that express this protein (Zemskov et al., 2011a). While TG2 exportation operates via a constitutive secretion route, it is likely modulated by a wide range of factors, including intracellular [Ca^{2+}] and regulatory proteins that control endosomal recycling pathways.

The emerging relationship of the TG2 trafficking pathway to the general recycling routes of transmembrane receptors has important functional implications. Several features of TG2 secretion, including its dependence on Rab11A/B function and VAMP3- and SNAP23-mediated endosome-to-plasma membrane fusion coincide with those governing integrin recycling (Caswell et al., 2009; Skalski et al., 2010), arguing that TG2 is likely exported inside the same vesicles that contain integrins undergoing the recycling process. While earlier studies indicated that TG2 binds β1 integrins within 30–60 min after the onset of biosynthesis (Akimov et al., 2000), given the lack of TG2 in the ER/Golgi, it remained unclear where these complexes were formed inside the cell. The targeting of cytoplasmic TG2 to the perinuclear recycling endosomal compartment may provide a plausible explanation for these earlier findings. β1 and β3 integrins are internalized and recycled back to the surface utilizing the long and the short endosomal recycling routes, respectively

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(Caswell et al., 2009). The localization of TG2 inside the recycling endosomes should facilitate its interaction with internalized integrins undergoing the recycling process inside these vesicles and lead to externalization of the newly formed integrin–TG2 complexes via the recycling routes. Likely, targeted delivery of these adhesive/signaling complexes to lamellipodia strengthens cell–matrix adhesion at the leading edge of migrating cell and contributes to the directionality of cell migration.

A distinct mechanism of TG2 secretion, which relies on transferring cell surface rather than cytoplasmic protein to neighboring cells using microvesicles derived from the plasma membrane, was recently described in breast carcinoma and glioma tumor cells (Antonyak et al., 2011). Importantly, this microvesicle-dependent mechanism allows the transfer of cancer cell-derived TG2 to normal recipient cells thereby causing their transformation by endowing them with the capacity for anchorage-independent growth and increased survival. In addition, TG2-generated cross-linked multimers of fibronectin appear to be present in the microvesicles as and required for the induction of integrin-dependent mitogenic signaling and transformation of the recipient fibroblasts. Although the mechanistic details and regulation of microvesicle-dependent secretion and transfer of TG2 to neighboring cells remain largely unknown, this process might be highly important for cell transformation and cancer progression *in vivo* (Antonyak et al., 2011). In addition, a novel microparticle-dependent process of TG2 secretion was recently described in normal smooth muscle cells (van den Akker et al., 2011). This process required the transamidating function of the protein. Because the origin and molecular components of the microparticles produced by smooth muscle cells remain to be defined, the extent of mechanistic similarity between these mechanisms of TG2 secretion in the transformed and normal cells is not clear.

4.2.3.2. Internalization of TG2 from the cell surface: A novel mechanism of cell-surface TG2 regulation was reported to operate via internalization and subsequent lysosomal degradation of the protein (Fig. 1.8(2); Zemskov et al., 2007). In fibroblasts, the constitutive endocytosis of cell-surface TG2 depends on plasma membrane cholesterol and requires the activity of dynamin-2 GTPase. Internalization of TG2 from the surface involves clathrin-coated pits and lipid rafts or caveolae. It proceeds through early and late endosomes and results in lysosomal accumulation and proteolysis of TG2. No recycling of the internalized TG2 occurs in fibroblastic cells. Endocytosis of TG2 in fibroblasts is rather efficient: the half-life of the protein on the surface is ~20min. Both soluble fibronectin and PDGF promote its endocytosis from the cell surface. On the contrary, fibronectin in the ECM anchors TG2 on the plasma membrane and prevents its internalization. Given that all cell-surface TG2 is bound to integrins, it appears plausible that these two proteins are internalized as a complex, however, experimental evidence for this is still lacking.

TG2 was found to interact with the major endocytic receptor, LRP1, both *in vitro* and on the cell surface, and internalization of TG2 from the surface requires the LRP1 function (Fig. 1.8(2)). It remains to be determined whether the direct interaction between TG2 and LRP1 triggers its endocytosis, or whether extracellular fibronectin facilitates this process by bridging TG2 to LRP1 on the cell surface. Notably, LRP1 deficiency or blockade of endolysosomal function both upregulate TG2 on the cell surface, thus leading to increased adhesion to the ECM. These findings reveal a novel pathway of TG2 internalization and degradation that might be crucial for regulation of the adhesive/signaling and transamidating capacities of cell-surface TG2. They also add to the emerging theme in the field that highlights a close functional relationship between cell–ECM adhesion and endocytosis. Future work will define the contribution of this endocytic mechanism to the regulation of the adhesive and signaling functions of cell-surface TG2 under pathophysiological conditions that include impairment of LRP1-mediated endocytosis and/or lysosomal function.

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4.2.3.3. Pericellular proteolysis controls the fate of extracellular TG2: Unlike its binding partners, integrins, which are extremely resistant to proteolysis, cell-surface TG2 is highly sensitive to proteolytic degradation (Belkin, 2011; Zemskov et al., 2006). Until recently, membrane-type (MT)-MMPs were thought to be primarily involved in the ECM degradation (Kessenbrock et al., 2010). However, recent findings showed that, in addition to the matrix breakdown, MT-MMPs are engaged in the proteolysis of TG2 as a principal adhesion receptor on tumor cell surfaces (Belkin et al., 2001). MT1-MMP overexpression in glioma and fibrosarcoma cells led to proteolytic degradation of TG2 at the leading edge of motile cancer cells. Likewise, structurally related MT1-MMP, MT2-MMP, and MT3-MMP efficiently degraded purified TG2 *in vitro*. Notably, the degradation of TG2 by MT1-MMP specifically suppressed cell adhesion and migration on fibronectin. However, fibronectin *in vitro* and in the ECM of cultured cells protected its surface receptor, TG2, from proteolysis by MT1-MMP, thus supporting cell adhesion and locomotion. These data suggest a novel regulatory function of membrane-anchored MMPs in cancer cell adhesion and locomotion (Zemskov et al., 2006).

MT1-MMP, a prototypic member of the MT-MMP subfamily, is an invasion-promoting protease and proteolytic activator of soluble metalloproteinase MMP2 (Kessenbrock et al., 2010). MMP2, functioning in concert with MT1-MMP, cleaves cell-surface-associated TG2, thereby further promoting the effect initiated by its proteolytic activator (Belkin et al., 2004). These findings illuminate the coordinated interplay involving the MT1-MMP/MMP2 protease tandem in the regulation of surface TG2 levels and functions. They also explain the underlying biochemical mechanisms of extensive TG2 proteolysis at the normal tissue/tumor boundary (Iismaa et al., 2009) and suggest that neoplasms, which express functionally active MT1-MMP and activate soluble MMP2, contribute to TG2 degradation on the surface of neighboring host cells.

The pathophysiological role of pericellular proteolysis of surface TG2 extends beyond its involvement in cancer cell invasiveness. Thrombospondin is a multifunctional ECM protein that is involved in cell responses to injury and angiogenesis, as well as the assembly and stabilization of collagen fibrils in the ECM (Adams and Lawler, 2004). Dermal fibroblasts from thrombospondin-2-null mice display an attachment defect that results from increased MMP2 levels in their conditioned media (Agah et al., 2005). A search for molecular mechanisms responsible for this defect identified surface TG2 as a key proteolytic target of MMP2 in thrombospondin-2-null fibroblasts. Notably, the thrombospondin-2-null mice have reduced TG2 levels and activity in the skin. Hence, thrombospondin-2 prevents the MMP2-induced degradation of TG2 in dermal fibroblasts, thus altering their adhesion and collagen fibril assembly capabilities. As in the case of cancer cells, the ECM composition and organization appears to control the TG2 levels and functions on the fibroblast surfaces by modulating its pericellular proteolysis. This ECM-mediated regulation of surface TG2 proteolysis may serve as a general mechanism that includes various cell types, matrices, and pericellular proteases.

Finally, although an extensive degradation of surface TG2 by MT1-MMP and related proteases abolishes its many functions, a limited proteolysis may induce some of its functions. For example, proteolytic removal of the C-terminal β-barrel domains 3 and 4 relieves the autoinhibition of the transamidating activity of TG2 (Fraij, 2011). Likewise, on osteoblast cell surfaces, a limited MT1-MMP-mediated TG2 cleavage generates the 56kDa N-terminal fragment containing the ATP-binding site, thus increasing the ATPase activity of the protein, which might be important for the mineralization process (Nakano et al., 2010).

4.2.3.4. Translocation of cell-surface TG2 to the ECM: It has been known for a long time that, in addition to its localization on the plasma membrane, the protein is also present in the

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ECM away from the cell surface (Aeschlimann and Thomazy, 2000; Upchurch et al., 1991). At the moment, the mechanism(s) of TG2 translocation from the cell surface to the ECM remains unknown. However, recent reports indicate that TG2 nitrosylation increases relative surface levels of the protein while reducing its deposition into the ECM (Telci et al., 2009). Likewise, treatment of cells with reducing agents decreases the levels of surface TG2 and integrin–TG2 complexes, suggesting that the noncovalent integrin–TG2 interaction is further stabilized by the formation of intermolecular disulfide bonds (Belkin, 2011). Therefore, the oxidation state of TG2, which might be regulated by nitric oxide, ROS, and disulfide modification/exchange (Lai et al., 2001; Santhanam et al., 2010; Stamnaes et al., 2010; Telci et al., 2009), appears crucial for the retention of TG2 on the cell surface and its translocation to the ECM. In addition, ternary integrin–TG2–fibronectin complexes might be mechanically disrupted during cell movement and contraction. Given that mechanical stretching alters the conformations of both integrin and fibronectin (Leiss et al., 2008), an excessive tension applied to the cytoskeleton–ECM scaffold may disrupt the integrin–TG2 complexes on the plasma membrane. These hypothetical mechanisms should be tested in the future studies.

4.2.4. TG2 in the ECM—TG2 localized in the ECM is engaged in enzymatic and nonenzymatic adapter/scaffolding activities. It plays a significant role in cell adhesion, migration, and ECM organization and turnover, contributing to normal wound healing, tissue regeneration, inflammation, and fibrosis.

4.2.4.1. TG2 as transglutaminase in the ECM: A number of TG2 cross-linking substrates were identified in the ECM, and the formation of covalent highly stable heteropolymers and homopolymers of ECM proteins was described for various types of cells and matrices (Aeschlimann and Thomazy, 2000; Wang and Griffin, 2011; Zemskov et al., 2006). There are at least four major consequences of such TG2-driven modification of ECM proteins. First, it increases ECM stability and reduces the rate of matrix turnover, which might have important biological effects (Lorand and Graham, 2003), such as TG2-mediated inhibition of tumor angiogenesis by the surrounding stroma (Jones et al., 2006), facilitation of experimental diabetic nephropathy (Huang et al., 2009), and potentially other fibrotic diseases. Second, this activity of TG2 increases the rigidity of cross-linked fibronectin (Nelea et al., 2008) and collagen fibrils (Spurlin et al., 2009) compared to native uncross-linked polymers of these ECM proteins. This, in turn, was shown to promote adhesion of fibroblasts and osteoblasts to less pliable matrices such as cross-linked collagen I, osteopontin, and bone sialoprotein (Chau et al., 2005; Forsprecher et al., 2009), thereby enhancing cell survival, growth, migration, and differentiation. Third, endothelial cell adhesion to the TG2-cross-linked compared to noncross-linked oligomers of fibrinogen αC domains amplified integrin clustering and focal adhesion formation, thereby elevating the outside-in integrin signaling to FAK and ERK1/2 (Belkin et al., 2005). This mechanism based on stimulation of integrin clustering is likely applicable to other cell types and TG2-cross-linked integrin ligands in the ECM. Fourth, the TG2-induced polymerization may lead to the exposure of cryptic functional sites in the ECM proteins. Accordingly, TG2-mediated cross-linking of osteopontin was reported to create a *de novo* binding site for neutrophil integrin α9β1 and to promote the chemotactic migratory activity of neutrophils *in vivo* (Nishimichi et al., 2009, 2011). Again, the TG2-induced modifications of other ECM ligands may unmask cryptic binding sites for cell-surface adhesion receptors or other ECM proteins. Combined, these examples underscore a wide range of functional effects of TG2-generated cross-linking of the ECM structural components.

An additional important function of TG2-induced protein cross-linking outside the cell involves the structural and functional modification of essential soluble growth factors (Lorand and Graham, 2003; Ientile et al., 2007; Wang et al., 2011). Midkine is a heparin-

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binding cytokine associated predominantly with the external surface of neural cell membranes. It promotes neurite sprouting in nerve cells and serves as a developmental morphogen in the brain (Mahoney et al., 1996). Interestingly, TG2-mediated cross-linking of midkine, which appeared to be stimulated by heparin, was shown to greatly enhance its functional activity and promote neurite outgrowth (Kojima et al., 1997; Mahoney et al., 1996).

TGFβ, a key regulator of ECM remodeling, is involved in wound healing, autoimmunity, inflammation, and pathological fibrosis (Worthington et al., 2011). The regulation of TGFβ biological activity includes the ECM storage and maturation of latent TGFβ precursor, which consists of the mature TGFβ homodimer associated noncovalently with the homodimeric propeptide, latency-associated peptide (LAP). The mature inactive LAP-TGFβ is stored in the ECM complexed with latent TGFβ-binding protein (LBTP). The process of latent TGFβ activation in the ECM is highly complex and implicates integrins, proteases, and other factors, including oxidative and mechanical stresses. It also involves TG2 as the principal enzyme that covalently cross-links LBTP to major ECM proteins such as fibronectin, thus controlling the rate of TGFβ maturation (Kojima et al., 1993; Lorand and Graham, 2003; Verderio et al., 1999). In agreement, upregulation of extracellular TG2 increases the levels of active TGFβ both in cell-culture models and *in vivo* in various pathological states (Wang and Griffin, 2011). In a positive feedback loop, TGFβ upregulates TG2 expression and function in the ECM, which appears to be important for many pathophysiological processes including epithelial–mesenchymal transition (EMT) and cancer progression (Kumar et al., 2010).

4.2.4.2. Cross-linking-independent functions of TG2 in the ECM: In addition to its key role in protein cross-linking, TG2 has important nonenzymatic adapter/scaffolding functions in the ECM. TG2 interacts noncovalently with the β1/β3/β5 integrin subunits and fibronectin. The formation of these stable TG2-containing ternary complexes was found to have a major role not only in the process of integrin-mediated cell adhesion to fibronectin (Akimov et al., 2000) but also in the assembly of fibronectin fibrils (Akimov and Belkin, 2001b). This latter activity was promoted by integrin-associated cell-surface TG2 but did not require its transamidating activity. Functionally, it was implicated in the TGFβ-dependent enhancement of fibronectin matrix deposition. Importantly, the enzymatically independent assembly of fibronectin fibrils, which is stimulated by TG2, precedes TG2-mediated cross-linking of these matrices (Fig. 1.2(3b); Zemskov et al., 2006).

Angiocidin, an antitumor ECM protein and integrin ligand produced by endothelial and tumor cells, was reported to inhibit angiogenesis and to interact with both collagen and the collagen-binding α2β1 integrin. More recently, angiocidin was found to colocalize with TG2 in the ECM of endothelial cells and to interact noncovalently with TG2 via its C-terminal integrin- and collagen-binding domain (L'Heureux et al., 2010). Intriguingly, the angiocidin–TG2 interaction was found to prevent the deposition of fibronectin in the ECM of tumor and endothelial cells, suggesting that angiocidin-mediated disruption of the TG2–fibronectin interaction is involved in its tumor suppressive activity. Angiocidin also serves as an enzymatic substrate of TG2 in the ECM, and TG2-generated angiocidin polymers appeared to inhibit endothelial cell migration and the ECM deposition/localization of fibronectin into tumor matrices more potently than monomeric angiocidin (L'Heureux et al., 2010). Hence, as in the case of fibronectin, angiocidin appears to act as a noncovalent binding partner and transamidating substrate of TG2.

The C-terminal fragment of the α1 chain of collagen XVIII, endostatin, binds to α5β1 and αvβ3 integrins, glypicans 1 and 4, and VEGFR2. It is a potent antiangiogenic protein localized on the surface of endothelial cells (Faye et al., 2010). It suppresses the integrin-

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mediated activation of FAK/c-Raf/MEK1/2-ERK1/2 signaling pathway and prevents binding of VEGF165 to endothelial cells, thereby inhibiting the VEGF-mediated activation of VEGFR. Endostatin was reported to bind TG2 with high affinity *in vitro* via its C-terminal integrin-binding domain and it colocalizes with TG2 in the ECM. This novel noncovalent interaction was suggested to play a role in the regulation of angiogenesis and tumor growth.

Unlike the integrin–TG2–fibronectin adhesion complexes in which TG2 and integrins can simultaneously bind to separate nonoverlapping sites on fibronectin, TG2 and integrins were reported to interact with the same sites in angiocidin and endostatin. Thus, on the surface of endothelial cells, TG2 may bridge angiocidin and endostatin to integrin receptors and promote their clustering, contributing to the antiangiogenic properties of these proteins. Additional analysis of these interactions and their role in cell functions is needed to prove this contention. Other interacting partners of TG2 might be identified on the cell surface and in the ECM, further expanding the complex adhesive/signaling function of TG2 in the extracellular space and helping to define its role in a wide range of pathophysio-logical processes.

4.2.4.3. TG2 in extracellular microvesicles: TG2 was identified in cancer cell-derived microvesicles, a special type of secreted vesicles derived from the plasma membrane (Antonyak et al., 2011). TG2 appears to both non-covalently bind fibronectin and generate covalently cross-linked fibronectin dimers on the surface of these vesicles. These microvesicles were shown to transfer TG2 and other proteins from the surface of donor cells to the surface of recipient cells. Also, in the case of cancer-derived microvesicles, TG2 and fibronectin were implicated in the transformation of the recipient normal fibroblasts. Evidently, similar TG2-containing microparticles were also described in normal smooth muscle cells (van den Akker et al., 2011). The activities and biological functions of microvesicular TG2 remain to be characterized.

4.3. Nuclear TG2

The presence of TG2 in the nucleus was reported three decades ago when elevation of TG2-mediated transamidation was detected in liver nuclei after partial hepatoectomy (Haddox and Russel, 1981). The TG and GTP-binding activities of nuclear TG2 were independently confirmed later (Singh et al., 1995). Lesort and colleagues (1998) identified TG2 in the nuclei of neuroblastoma cells and copurified the protein with chromatin from the nuclear fraction. Nuclear TG2 was demonstrated in a number of cell types and shown to represent ~5–7% of the total TG2 cellular pool.

4.3.1. Recruitment of cytoplasmic TG2 to the nucleus—Several inducers and stressors were shown to drive the nuclear translocation of TG2, including an increase in intracellular [Ca^{2+}] (Lesort et al., 1998), glutamate stimulation of astroglial cells (Campisi et al., 2003), retinoid induction during the differentiation of neutrophils (Balajthy et al., 2006), VEGF stimulation of endothelial cells (Dardik and Inbal, 2006), and hypoxia accompanied by protection against oxygen–glucose-deprivation-induced cell death in neuroblastoma cells (Filiano et al., 2008). A putative bipartite ^{259}DILRR$^{263/597}$PKQKRK^{602} nuclear localization signal (NLS) was identified in TG2 based on homology with influenza virus NS1 protein (Peng et al., 1999), however, its functionality remains questionable (McConoughey et al., 2010). It is likely that other TG2 motifs are involved in targeting this protein to the nucleus. Importin-α3, a nuclear transporter protein, was found to interact with TG2 both *in vitro* and in cells, suggesting that it might recruit the cytoplasmic TG2 to the nuclear compartment (Peng et al., 1999). Nonetheless, the mechanisms of TG2 recruitment into the nucleus

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remain unclear. It is also unknown whether exportin proteins are involved in the relocation of nuclear TG2 back to the cytoplasm.

4.3.2. Transamidating function of nuclear TG2—Of the numerous identified substrates of TG2 transamidating activity, ~15% represent nuclear proteins (Facchiano and Facchiano, 2009). Core histones H2A, H2B, H3, and H4 were found to serve as glutaminyl substrates of TG2 *in vitro*, and their cross-linking *in vivo* was suggested to contribute to apoptosis-induced condensation of chromatin (Ballestar et al., 1996, 2001). Other nuclear proteins, including lamins A and C (Glass et al., 1985), Rb protein (Oliverio et al., 1997), huntingtin (Kahlem et al., 1998; Karpuj et al., 1999), SP1 transcription factor (Han and Park, 2000; Tatsukawa et al., 2009), importin β1 subunit (Orru et al., 2003), and ataxin-1 (D'Souza et al., 2006), were all identified as TG2 substrates *in vitro* and/or *in situ*. In most cases, the pathophysiological significance of these modifications remains unclear.

However, in erythroleukemia cells, TG2-mediated cross-linking of nuclear Rb protected it from caspase-dependent degradation during retinoid-induced apoptosis (Boehm et al., 2002). Contrary to these findings, Milakovic and colleagues (2004) found that the noncovalent interaction of TG2 with Rb in the nucleus protected HEK293 cells from thapsigargin-induced apoptosis, whereas the transamidating function of TG2 appeared proapoptotic. Likely, the prosurvival effects of nuclear TG2 depend on the cell type and cell death inducer (Tucholski and Johnson, 2002).

Mounting evidence suggests a major role for nuclear TG2 in neurodegenerative disorders. Accordingly, TG2 was found to cross-link huntingtin *in vitro* and in the nuclear inclusions of Huntington disease patients and to colocalize with nuclear aggregates of huntingtin (Karpuj et al., 1999; Zainelli et al., 2003). McConoughey and colleagues (2010) demonstrated that TG2-mediated transamidation has a pivotal role in the pathogenesis of Huntington's disease. Importantly, TG2-induced transamidation was implicated in the broad transcriptional dysregulation in the mouse model of this disease, including the repression of nuclear-encoded genes that control mitochondrial metabolic functions, such as cytochrome *c* and PPAR-γ coactivator 1α. The proposed TG2-dependent mechanism of gene suppression was suggested to include the noncovalent interaction of TG2 with histone H3 and, potentially, its subsequent TG2-induced polyamination in the nucleus, leading to the profound epigenetic changes of chromatin that are characteristic of this disease (McConoughey et al., 2010).

Perhaps the best understood example of transamidation-dependent regulation of gene expression comes from studies on the impact of nuclear TG2 on SP1-mediated gene transcription (Fig. 1.9(1); Han and Park, 2000; Shimada et al., 2001; Tatsukawa and Kojima, 2010; Tatsukawa et al., 2009). A novel apoptotic mechanism involved in alcoholic liver injury was found to be mediated by nuclear TG2 via enzymatic cross-linking of SP1 *in vitro* and *in vivo* (Tatsukawa et al., 2009). The SP1 transcription factor appears to be cross-linked, oligomerized, and inactivated by the nuclear TG2, thereby decreasing expression of essential growth factor receptors such as c-Met, which, in turn, results in caspase-induced cell death. A similar mechanism was shown to operate in the case of free fatty acid-induced TG2 overexpression in hepatocytes and in nonalcoholic steatohepatitis.

4.3.3. Transamidation-independent activities of nuclear TG2—Besides transamidation/protein cross-linking, TG2 has other enzymatic and nonenzymatic functions in the nucleus. Although the phosphorylation of histones H1 and H3 by TG2 may be involved in regulation of chromatin structure, there is currently no experimental evidence in support of this hypothetical mechanism (Mishra et al., 2006). Likewise, while TG2-induced p53 and Rb phosphorylation in the nucleus may alter the balance between pro- and

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antiapoptotic TG2 functions in this compartment, further mechanistic analysis is needed to develop this concept (Mishra and Murphy, 2006a; Mishra et al., 2007).

Meanwhile, the ability of nuclear TG2 to regulate transcription factors via its nonenzymatic adapter/scaffolding function is gathering more evidence. The TG2-mediated downregulation of MMP9 gene transcription in cardiomyoblasts was suggested to be mediated by direct noncovalent binding of TG2 to c-Jun, thereby inhibiting its dimer formation with c-Fos and blocking the binding of the Jun-Fos complex to the AP1 site in the MMP9 gene promoter (Fig. 1.9(2); Ahn et al., 2008). This mechanism might be involved in the regulation of ECM turnover. A somewhat similar role of nuclear TG2 as an indirect transcriptional modulator was described in cortical neurons. In this case, its interaction with HIF1β prevents HIF1β from heterodimerizing with HIF1α to generate the functional HIF1 transcription factor (Fig. 1.9(3); Filiano et al., 2008). Notably, this interaction attenuated transcription of Bnip3 and other genes containing the hypoxic response element (HRE) in their promoters, thereby attenuating neuronal cell death in ischemia and underlying a potential prosurvival effect of nuclear TG2 in stroke.

4.4. Mitochondrial TG2

The initial evidence that TG2 acts as an important regulator of energy metabolism and mitochondrial functions came from observations with *TGM2*–/– mice whose heart appeared more sensitive to ischemia/reperfusion injury (Sarang et al., 2009; Szondy et al., 2006). Moreover, the phenotype of these mice resembled that of maturity-onset diabetes of the young (Bernassola et al., 2002), implying a broad TG2 involvement in mitochondrial functions outside cardiac muscle. Overexpression of TG2 in neural cells resulted in a much more rapid execution of the death program and accompanied by clustering of mitochondria, reduced cristae, and an extremely electron-dense matrix (Piacentini et al., 2002). The deletion of *TGM2* in mice caused significant dysregulation of the respiratory complexes I and II, reduction of ATP production, increased ATP/ADP carrier activity and mitochondrial membrane potential, and impairment of ATP synthase reverse activity and Bax recruitment (Malorni et al., 2009). Although a precise role of TG2 in the regulation of mitochondrial respiratory chain remains unclear, a significant progress during the past decade has shed light on the modulation of mitochondrial protein activities via both noncovalent interactions with TG2 and covalent TG2-mediated modifications.

4.4.1. Mitochondrial localization and targeting of TG2—While there is no classical N-terminal mitochondrial targeting signal in TG2, the protein is associated with mitochondria in various cell types; in neuroblastoma cells, it constitutes up to 50% of the total TG2 cellular pool (Piacentini et al., 2002; Rodolfo et al., 2004). Biochemical fractionation and electron microscopy revealed that the majority of mitochondrial TG2 is associated with the outer mitochondrial membrane and the inner membrane space, whereas 5–10% of the protein pool is present on the inner mitochondrial membrane and in the mitochondrial matrix (Park et al., 2010; Rodolfo et al., 2004). Analysis of the TG2 primary sequence showed a presence of an eight amino acid sequence ^{204}LKNAGRDC^{211} that shares 70% identity with the BH3 domain of Bcl-2 family proteins, suggesting that TG2 represents a novel BH3-only protein that regulates apoptosis. Significantly, mutation of the highly conserved Leu204 residue attenuated TG2-mediated staurosporin-induced neuroblastoma cell death, confirming previous results that showed that TG2-induced hyperpolarization of mitochondrial membrane sensitizes cells to the intrinsic pathway of programmed cell death (Piacentini et al., 2002; Rodolfo et al., 2004). Also, TG2-BH3 peptides delivered inside the cell as well as TG2 itself interacted with the proapoptotic protein Bax, but not with antiapoptotic Bcl-2. Cell death induction increased the TG2–Bax interaction and Bax served as one of the major substrates of TG2-mediated cross-linking in

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the mitochondria. This interaction may play a role in targeting cytoplasmic TG2 to this compartment, however, experimental evidence for such involvement is yet to be obtained. Other factors, such as phospholipids, may also be involved in the recruitment of TG2 to mitochondria. For example, cardiolipin, which is exclusively enriched in the inner mitochondrial membrane, was found to strongly bind TG2 *in vitro* (Zemskov et al., 2011a).

4.4.2. Protein disulfide isomerase TG2 activity as novel regulator of mitochondrial functions—Recent work revealed that TG2 plays a major role in mitochondrial physiology and energy metabolism acting as a PDI (Malorni et al., 2009; Mastroberardino et al., 2006; Sarang et al., 2009). Specifically, the genetic deletion of TG2 led to defective disulfide bond formation in NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), cytochrome *c* oxidase (complex IV), and ATP synthase (complex V). In addition, the PDI activity of TG2 might control the respiratory chain by modulating the formation of prohibitin complexes (Mastroberardino et al., 2006). Another principal target of the PDI activity of TG2 in mitochondria is the bifunctional ANT1, a protein involved in ADP/ATP exchange that serves as a core component of the permeability transition pore complex in the IMM (Malorni et al., 2009). While oligomerization of ANT1 is essential for its activity, *TGM2*–/– mice displayed increased thiol-dependent ANT1 oligomer formation and an elevated ADP/ATP exchange activity of ANT1 in heart mitochondria. Thus, by acting as a PDI, TG2 reduced the level of oligomerized ANT1 and inhibited its transporter activity by sequestering ANT1 monomers and preventing oligomer formation by its direct binding to ANT1. Further, both in steady state and during cell death, TG2 was required for the Bax/ANT1 colocalization and interaction in mitochondria. Together, these findings demonstrated for the first time the importance of TG2-PDI enzymatic activity *in vivo* and indicated the existence of a novel pathway that directly links it with the regulation of mitochondrial pathophysiology.

4.4.3. Transamidating function of mitochondrial TG2—Several studies identified the mitochondrial substrates of transamidating activity of TG2 *in situ* (Park et al., 2010; Sarang et al., 2009). While no such substrates were detected in the mitochondria in untreated cultured neural cells, a number of substrates were identified upon induction of the intrinsic apoptosis pathway with staurosporin. The proapoptotic protein and TG2-binding partner Bax appeared to serve as the major target of TG2-induced cross-linking during apoptosis (Rodolfo et al., 2004). Prohibitin is a membrane-bound chaperone essential for the correct folding of the respiratory chain components, Hsp70/Hsp90. Organizing protein Hsp60 cooperates with prohibitin and forms a membrane-tethered import motor complex involved in the unfolding of preprotein domains, while the ATP synthase β chain is a key component of complex V of the respiratory chain. Upon triggering mitochondrial-dependent apoptosis in neural cells, all these proteins were detected as prominent transamidation/cross-linking substrates of TG2 (Battaglia et al., 2007; Orru et al., 2003). A similar reaction occurred with the TG2-binding partner ANT1 *in vitro* and in cells where TG2 cross-linked it into oligomers detectable upon induction of cell death (Malorni et al., 2009).

While very few of any such TG2-mediated modifications take place in unaffected healthy tissues, they are likely to be involved in the pathogenesis of “mitochondrial diseases,” including cardiovascular ischemia/reperfusion injury and neurodegenerative disorders such as Huntington's disease. In keeping with this, a decrease in mitochondrial aconitase activity in parallel with the formation of high molecular weight aconitase aggregates was found in the regions of Huntington disease brain with elevated TG2 cross-linking activity (Kim et al., 2005). Likewise, TG2-catalyzed covalent cross-linking of mitochondrial matrix α-ketoglutarate dehydrogenase with polyglutamine containing polypeptides that are generated in CAG/Q(n) expansion diseases may cause enzyme inactivation and disruption of cerebral energy metabolism (Cooper et al., 2002).

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5. Roles of TG2 in Cellular Processes

Given the ubiquitous expression and vast array of enzymatic and nonenzymatic activities of TG2, it is not surprising that this protein appears intimately involved in the regulation of numerous cell functions, including cell adhesion, migration, survival and death, ECM assembly and turnover, cell growth and differentiation, exocytosis, and autophagy. In this section, we discuss the contribution of TG2 to specific cellular processes.

5.1. Cell adhesion and migration

The initial work that suggested an involvement of TG2 in cell–ECM adhesion and migration revealed a striking effect of its overexpression on fibroblast spreading and their resistance to detachment by trypsin (Gentile et al., 1992). Subsequently, a number of studies demonstrated a prominent role of extracellular TG2 in cell adhesion and migration (Belkin, 2011; Wang and Griffin, 2011). Importantly, in most cases, TG2 on the cell surface and in the ECM functions as a proadhesive and promigratory protein. Both the nonenzymatic adapter/scaffolding and enzymatic cross-linking properties of TG2 contribute to these effects (Fig. 1.5; Section 4.2). The proadhesive function of TG2 is based primarily on its ability to non-covalently bind to and collaborate with two types of transmembrane cell–ECM adhesion receptors: β1/β3/β5 integrins and syndecan-4. Both these receptors, as well as TG2 itself, interact with fibronectin (Belkin, 2011; Wang and Griffin, 2011; Zemskov et al., 2006). Besides strengthening the cell–ECM adhesion, surface-bound TG2 promotes receptor clustering and amplifies integrin and syndecan-4 outside-in signaling, increasing activation of multiple downstream targets, including FAK, RhoA, and PKCα. In turn, their increased activation further contributes to the TG2-mediated enhancement of cell adhesion and migration.

The transamidating activity of TG2 plays a major role in cell–ECM adhesion in at least three significant ways. The TG2-mediated cross-linking of ECM proteins (i) increases the rigidity of adhesive substrates (Forsprecher et al., 2009; Nelea et al., 2008; Spurlin et al., 2009), (ii) leads to the formation of highly ordered and stable ECM polymers promoting integrin clustering on the cell surface and amplifying integrin-dependent outside-in signaling (Belkin et al., 2005), and (iii) unmasks cryptic cell-binding sites in the ECM proteins (Nishimichi et al., 2009, 2011). Combined, these effects increase cell attachment to the ECM and promote outside-in signaling.

The interaction between integrin-bound TG2 and fibronectin on the cell surface and TG2-mediated ECM cross-linking are likely to be involved in various pathophysiological mechanisms. For instance, TGFβ-mediated upregulation of surface TG2 contributed to the enhancement of adhesion and migration of retinal epithelial cells on fibronectin (Priglinger et al., 2004). Hence, this TG2 function is implicated and might be targeted in some cases of proliferative vitreo-retinopathy, a protracted wound healing response in the eye and common consequence of surgical treatment of retinal detachment. In addition, the formation of ternary integrin–TG2–fibronectin complexes is thought to facilitate the anchoring of ovarian cancer cells to the mesothelial lining of the peritoneal cavity and promote a subsequent metastasis during the progression of this type of cancer (Satpathy et al., 2007). Notably, the interaction of integrin-bound TG2 with fibronectin on the surface of activated astrocytes was recently implicated in the recruitment of the cells to multiple sclerosis lesions and, consequently, the progression of multiple sclerosis (van Strien et al., 2011). Thus, targeting the TG2–fibronectin interaction might be a new promising venue for developing novel therapeutics that block the cell–ECM adhesion of tumor cells in ovarian cancer and activated astrocytes in multiple sclerosis. Rational design and generation of potent and specific inhibitors of the TG2–fibronectin complex formation are needed to delineate the role of this

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TG2-mediated mechanism in cell adhesion and migration *in vivo* and its contribution to the pathogenesis of metastatic cell spread, cardiovascular diseases, and autoimmune disorders.

In contrast, boosting the formation of integrin–TG2–fibronectin adhesive/signaling complexes on the cell surface might have important benefits for certain therapeutic applications. Transplantation therapy with autologous mesenchymal stem cells (MSCs) for repair of myocardial injury has inherent limitations due to poor viability of these cells after the implantation. Cell–ECM adhesion is a prerequisite for cell survival and also a key factor for MSCs differentiation. As a novel prosurvival improvement strategy, genetically engineered MSCs that overexpress TG2 were used to enhance cell adhesion and survival after the implantation (Song et al., 2007). The MSCs overexpressing TG2 showed significant retention in the infarcted rat myocardium and developed into cardiac myocyte-like cells as judged by the expression of cardiac-specific proteins. Transplantation of these cells into the ischemic or infarcted rat myocardium further restored cardiac function as compared with MSC transplantation alone, suggesting that TG2 is important for the integrin-mediated adhesion and prosurvival signaling of MSCs in the implanted tissues. Finally, intrinsic inhibition of this TG2-based adhesion mechanism might contribute to the pathogenesis of celiac disease, as IgA class autoantibodies to TG2 were reported to decrease motility of endothelial and vascular smooth muscle cells in culture and to disturb angiogenesis *in vivo* (Myrsky et al., 2008).

Syndecan-4/TG2/fibronectin complexes functionally cooperate with integrin-dependent cell adhesion and likely compensate for its deficiency during extensive tissue damage and generation of ECM degradation products that compete with intact ECM proteins in integrin-mediated cell adhesion (Fig. 1.5; Verderio et al., 2009; Wang and Griffin, 2011). Notably, the interaction of TG2 with heparan sulfate chains does not alter its transamidating activity; rather, it enhances its stability against thermal unfolding or proteolytic degradation (Signorini et al., 1988). The similar wound healing deficiencies observed in the *TGM2*–/– and *syndecan-4*–/– mice further indicated the interdependent functions of these proteins in tissue repair processes and fibrotic diseases such as renal scarring (Wang and Griffin, 2011). By bridging fibronectin in the ECM and syndecan-4 receptors on the cell surface, TG2 stabilizes cell–matrix adhesion in an integrin-independent manner and prevents anoikis in the case of perturbed integrin–ECM interactions. Recent findings indicate a novel role for these interactions in cell adhesion *in vivo*. Autoantibodies against TG2 perturbed the attachment of epithelial cells to TG2–fibronectin heterocomplexes by interfering with heparan sulfate binding (Teesalu et al., 2011), thus potentially broadening the involvement of TG2 in the pathogenesis of celiac disease (Schuppan et al., 2009).

A number of studies over the past decade demonstrated a prominent role for TG2 in cell migration (Belkin, 2011; Lorand and Graham, 2003; Wang and Griffin, 2011; Zemskov et al., 2006). As in the case of cell–ECM adhesion, the effects of TG2 on cell migration depend on a several complementary mechanisms. In most cases, the promigratory function of cell-surface TG2 paralleled its positive impact on cell adhesion. This correlation has been reported in fibrosarcoma and glioma cells (Belkin et al., 2001), monocyte-derived macrophages (Akimov and Belkin, 2001a), retinal epithelial cells (Priglinger et al., 2004), epithelial breast and ovarian cancer cells (Mangala et al., 2007; Satpathy et al., 2007), and MSCs (Song et al., 2007). Importantly, the ability of cell-surface TG2 to upregulate cancer cell motility also translated into a proinvasive function of this protein in breast and ovarian cancer cells (Mangala et al., 2007; Satpathy et al., 2007). The highly invasive phenotype of epidermoid cancer A431 cells depended on elevated TG2 and fibronectin levels, an enhanced β1 integrin–fibronectin interaction, and increased MMP9 secretion mediated by the upregulation of TG2. In all the above studies, the stimulatory effect of TG2 on cell locomotion depended on the integrin-coreceptor function of this protein on the cell surface

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and its capacity to interact with fibronectin in the ECM (Zemskov et al., 2006). In turn, these interactions stimulated a number of promigratory signaling pathways, including the activation of FAK, ERK1/2, RhoA, and Akt1 (Verma and Mehta, 2007; Zemskov et al., 2006).

An opposite scenario was reported by Balklava and colleagues (2002) who observed increased attachment and decreased migratory capacity of fibroblasts upon overexpression of TG2. Yet, it is known that the interaction between adhesion receptors and ECM ligands controls cell migration speed and directs the complex nonlinear relationship between the adhesion strength and the rate of cell migration (Palecek et al., 1997). Hence, a likely explanation for this seeming contradiction is that the levels of exogenous TG2 utilized in that study exceeded a threshold point where a further increase in adhesion strength counteracted cell locomotion by interfering with the efficient cell detachment from substrate needed for maintaining maximal cell migration speed.

Another nonenzymatic property of cell-surface TG2 that enhances chemotactic cell migration is its ability to activate growth factor signaling. TG2 interacts with growth factor receptors such as PDGFR and mediates their association with integrins to enhance the efficiency of PDGF-induced signal transduction (Zemskov et al., 2009, 2011b). This mechanism was recently described in both fibroblasts and vascular smooth muscle cells and shown to markedly increase PDGF-mediated cell migration and sensitize these cells to the action of this growth factor.

Extracellular TG2 may also contribute to cell migration through transamidation-dependent mechanisms. For instance, colon carcinoma and normal endothelial cells displayed increased adhesion and migration on polymeric TG2-cross-linked osteopontin compared with the monomeric protein (Higashikawa et al., 2007). As in the case of cell adhesion, TG2-mediated covalent cross-linking of proteins in the ECM stimulates cell migration by increasing ECM rigidity, facilitating integrin clustering, and increasing exposure of integrin-binding sites in the ECM proteins. The latter mechanism is especially important for neutrophil migration, as $\alpha 9\beta 1$ integrin on neutrophils does not bind to the cryptic SVVYGLR motif in monomeric osteopontin but interacts with this site upon its unmasking in TG2-cross-linked osteopontin polymers (Nishimichi et al., 2009, 2011).

Covalent cross-linking by extracellular TG2 also alters the properties of ephrins, membrane-associated proteins that bind transmembrane tyrosine kinase Eph receptors on neighboring cells and impact cell adhesion and migration via bidirectional signaling (Cowan and Henkemeyer, 2002). In addition, some A-type ephrins, including A1 and A5, are released from the cell surface by pericellular proteolysis and induce Eph receptor activation. Ephrins A1 and A5 were shown to serve as substrates for TG2 cross-linking, which mediated the formation of their oligomers (Alford et al., 2007). The TG2-cross-linked ephrins stimulated EphA2 kinase activity and promoted migration and invasion of Hela cells to a greater extent than monomeric ephrins. Hence, TG2-mediated oligomerization of soluble ephrins may represent a novel forward signaling mechanism through Eph receptors that extends the impact of A-type ephrins beyond cell–cell contact-mediated signaling and contributes to cell–ECM adhesion and migration.

It is well known that IGF-binding protein-1, IGFBP-1, the main secretory protein of decidua, binds IGFs and regulates their bioactivities. IGFBP-1 was recently found to undergo TG2-mediated polymerization on the surface of trophoblast cells, leading to its deactivation, the disinhibition of IGF, and, ultimately, enhanced IGF-dependent trophoblast cell migration (Shibuya et al., 2011). These findings suggested that progesterone might facilitate TG2-induced polymerization of decidua-secreted IGFBP-1 and increase IGF

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actions at the feto-maternal interface, thereby stimulating trophoblast invasion of the maternal uterus.

In addition to the TG2 present on the cell surface and in the ECM, cytoplasmic TG2 was also shown to indirectly contribute to the regulation of cell migration by a combination of nonenzymatic and enzymatic transamidation mechanisms. The transamidating activity of cytoplasmic TG2 was implicated in EGF/EGFR-induced migration and invasion of Hela cells; however, the identity of enzymatic TG2 targets was not reported (Antonyak et al., 2009). The accumulation of cytoplasmic TG2 at the leading edges of EGF-treated cancer cells was required for the enhancement of cell migration and found to depend on a novel interaction of TG2 with Hsp70 chaperone that altered the ATPase hydrolytic activity of Hsp70 (Boroughs et al., 2011). Similarly, EGF-induced upregulation of TG2 in TRAIL-resistant lung cancer cells elevated the levels of MMP9 expression, secretion, and activity, which led to a prominent enhancement of cell migration and invasiveness. The TG2-dependent mechanisms in this regulation remain to be defined (Li et al., 2011).

Further, Satpathy and colleagues (2009) reported that TG2-mediated transamidation controls MMP2 gene expression in ovarian cancer cells. The proposed TG2-induced mechanism suggests that TG2 interacts with and transamidates protein phosphatase 2A-α (PP2A-α), which leads to its degradation, thus raising the phosphorylation levels of cAMP-response element-binding protein (CREB transcription factor) at Ser133 and elevating CREB-mediated transactivation of MMP2 gene transcription. As in the case of TG2-induced upregulation of MMP9, this mechanism of TG2-mediated MMP2 induction might promote cancer cell invasiveness and metastasis.

Finally, cytoplasmic TG2 may indirectly impact cell migration through its transamidation-dependent serotonylation of the small regulatory RhoA and Rac1 GTPases (Section 2.1.1.2; Dai et al., 2008; Guilluy et al., 2007, 2009) and its transamidation-independent interaction with Rac1 (Section 2.5; Kim et al., 2010). Additional cytoplasmic targets of TG2-dependent transamidation are likely to be involved in the regulation of cell migration (Liu et al., 2011). Together, these examples illustrate the complex multifaceted roles of enzymatic and nonenzymatic TG2 activities in cell adhesion and migration.

5.2. Cell growth and proliferation

Accumulating data suggest a direct involvement of intracellular TG2 in the regulation of the cell cycle. Overexpression of TG2 or its transamidation-deficient mutant in malignant hamster fibrosarcoma cells resulted in impairment of the cell cycle. In these cells, TG2 affected the progression through the cell cycle from S phase to G_2/M, an effect that was suggested to depend on the GTPase activity of TG2 (Mian et al., 1995). In a subsequent study, downregulation of TG2 expression in endothelial cells led to cell-cycle arrest coupled to the elevated expression of cyclin E and decreased expression of cyclin B, proteins known to play essential roles in cell-cycle progression through G_1 to S and from G_2 to M phase, respectively (Nadalutti et al., 2011).

In breast and pancreatic cancer cells, TG2 was demonstrated to strongly amplify cell growth. This regulation involved overactivation of the NFκB and Akt1 pathways. In the latter pathway, TG2 was found to downregulate the tumor suppressor phosphatase PTEN, causing an increased activation of FAK and Akt1 (Herman et al., 2006; Mann et al., 2006; Verma et al., 2006, 2008a,b).

An emerging theme suggests an involvement of TG2 in the response of cells to soluble growth factors. Cell-surface TG2 was found to amplify the activation of PDGFR signaling in response to soluble PDGF in fibroblasts and smooth muscle cells and to promote their

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PDGF-induced proliferation (Zemskov et al., 2009, 2011b). The transamidating activity of TG2 was dispensable for this effect. Cytoplasmic TG2 was also found to be required for EGF/EGFR-induced anchorage-independent growth of breast cancer cells (Li et al., 2010). While the combined actions of Ras and Cdc42, leading to the activation of PI3K and NFκB, were involved in upregulation of TG2 in these cells, it was transamidation-dependent association of TG2 with the intermediate filament protein keratin-19 and activation of src kinase activity in ternary complexes that were implicated in the potentiation of cancer cell growth.

Last, the transamidating activity of TG2 was required for the proliferation of pulmonary artery smooth muscle cells induced by serotonin (Liu et al., 2011). The TG2-mediated serotonylation of fibronectin was suggested to be critical for this effect. In addition, TG2 was shown to mediate serotonylation of several cytoplasmic proteins integral for cytoskeletal functions and contractility, including smooth muscle α-actin, β-actin, γ-actin, myosin heavy chain, and filamin. Modifications of these proteins were also proposed to contribute to TG2-mediated enhancement of proliferation of the aortic smooth muscle cells (Watts et al., 2009).

5.3. Cell survival and apoptosis

It is well established that because of cell-cycle checkpoint signaling, blocking cell growth can lead to cell survival and permanent arrest or to cell death (Pietenpol and Stewart, 2002). Hence, it is not surprising that, in the past decade, numerous studies investigated the putative role of TG2 in cell survival and apoptosis (Fesus and Szondy, 2005; Mehta et al., 2006; Verma and Mehta, 2007). Apoptosis is a process of fundamental biological importance playing a critical role in normal tissue homeostasis as well as in disease. The genes that regulate both the initiation and execution of apoptosis are subject of intense scrutiny. Two decades ago, TG2 was identified among the genes whose expression most closely relates to the final execution of the apoptotic process (Fesus, 1992). Today, the dual role of TG2 acting either as a facilitator or attenuator of the apoptotic process is widely acknowledged (Fesus and Szondy, 2005). Several excellent reviews discuss the complex and important role of TG2 in programmed cell death (Caccamo et al., 2011; Chhabra et al., 2009; Fesus and Szondy, 2005; Iismaa et al., 2009). The current general concept implies that TG2 sensitizes cells to apoptosis when its transamidating activity is turned on; in contrast, it is protective when its transamidating activity is dormant (Antonyak et al., 2001; Milakovic et al., 2004; Tucholski and Johnson, 2002).

Activation of intracellular TG2, which is mostly quiescent except during extreme stress conditions, may depend on the level of calcium influx. When various stimuli increase cytosolic [Ca^{2+}] above a certain threshold, the transamidating activity of TG2 is no longer inhibited by GTP and it facilitates cell death processes. Multiple studies on the oxidative stress-induced cell death have shown that high levels of ROS trigger Ca^{2+} influx resulting in TG2 activation and, subsequently, in cell death (Caccamo et al., 2011; Iismaa et al., 2009). However, in many cell types, TG2 exhibits antiapoptotic prosurvival effects, which can be further amplified by specific inhibition of the TG2 transamidating activity.

Drug resistance in various cancers is often associated with high levels of TG2 (Verma and Mehta, 2007). TG2 expression in cancer cells leads to the constitutive activation of FAK and its downstream PI3K/Akt1 prosurvival pathway. Importantly, the inhibition of endogenous TG2 by siRNA resulted in the reversal of drug resistance and the invasive phenotype. Conversely, TG2 overexpression promoted cell survival, motility, and invasiveness of cancer cells. Increased Akt1 activity was suggested to mediate these effects (Verma and Mehta, 2007). In addition, TG2 mediated constitutive activation of NFκB in cancer cells (Mann et al., 2006) and this mechanism determined the resistance of epithelial ovarian cells

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to cisplatin-induced apoptosis (Cao et al., 2008). In HEK293 cells, TG2 exhibited antiapoptotic activity through the depletion of Bax, the suppression of caspase-3 and -9, and inhibition of cytochrome *c* release into the cytosol and mitochondria membrane depolarization in response to Ca^{2+} overload (Cho et al., 2010). A similar mechanism involving TG2-mediated inhibition of cross-linked caspase-3 was proposed to mediate the prosurvival effects of TG2 in hypoxic cancer cells (Jang et al., 2010). Likewise, TG2 depletion in endothelial cells resulted in cell-cycle arrest and apoptosis (Nadalutti et al., 2011), underscoring the significance of TG2 in endothelial cell-cycle progression and survival.

In addition, the subcellular localization and conformation of TG2 in neural cells were shown to define cell responses to apoptotic stimuli. Intriguingly, in the case of oxygen–glucose deprivation, the nuclear localization of the GTPase-deficient R580A mutant of TG2 was sufficient to counteract its prodeath role in the cytoplasm (Colak et al., 2011, Gundemir and Johnson, 2009). Thus, the prodeath effects of TG2 in hypoxic striatal cells appeared independent of transamidating activity but defined by the cytoplasmic localization of TG2 and its conformation. These data suggest that the adapter/scaffolding of cytoplasmic TG2 regulates these processes. In contrast, in the mouse model of Huntington disease, nuclear catalytically active TG2 was shown to regulate a large number of genes related to programed cell death, and retention of this enzyme in the cytoplasm resulted in reduced cytochrome *c* levels (McConoughey et al., 2010). In this study, the TG2-mediated modification of histone H3 was suggested to be the underlying proapoptotic mechanism of global epigenetic regulation by nuclear TG2. Therefore, the complex balance between the prosurvival and proapoptotic activities of TG2 appears to depend on its localization and conformation, as well as cell and stressor types.

5.4. Cell differentiation and phenotype modulation

Despite the normal development of *TGM2–/–* mice, studies with cultured cells imply an important role for TG2 in the differentiation and control of the phenotypic stability in various cell types. Compensation by other TGs for the loss of TG2 has been proposed to rescue the phenotype of *TGM2–/–* mice. Here, we summarize the available data for TG2-dependent cell differentiation and phenotypic modulation (Table 1.1).

5.4.1. Neurons—The first studies implicating TG2 in neuronal differentiation date back almost three decades when Maccioni and Seeds (1986) reported a 10-fold increase in TG activity associated with neurite outgrowth during morphological differentiation of neuroblastoma cells, indicating a prominent role for TG2 in the extent of microtubule assembly. Similarly, TG2 was necessary and sufficient for the neuronal differentiation of neuroblastoma cells: its overexpression in these cells caused spontaneous neurite outgrowth. TG2 was predominantly localized at the tips of the neurites, as well as in the perinuclear area, suggesting a role in stabilizing extended structural projections (Tucholski et al., 2001). In agreement with TG2 acting as a positive regulator of neuronal differentiation, its inhibitors prevented neurite outgrowth and neuronal marker expression in neuroblastoma cells induced to differentiate by retinoic acid (Singh et al., 2003). Finally, overexpression of catalytically active TG2 isoforms in neuroblastoma cell lines induced neurite outgrowth (Tee et al., 2010). The molecular mechanisms by which the transamidating activity of TG2 induces neuronal differentiation have yet to be resolved. TG2-mediated transamidation of RhoA was required for activation of ERK1/2 and p38γMAPK indicating a likely role for these pathways in neuronal differentiation. Yet, further studies revealed that RhoA transamidation was dispensable for retinoid-induced differentiation of neuroblastoma cells (Singh et al., 2003), and MAPK activation and neurite outgrowth were regulated by the PI3K-Rac1 pathway in transamidation-independent manner (Pan et al., 2005). It is possible

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that the TG2-dependent activation of JNK signaling in these cells may have a role in differentiation but this requires further investigation (Singh et al., 2003). Last, Tucholski and Johnson (2003) proposed a regulation of neuronal differentiation by TG2 via CREB phosphorylation and activation. They observed enhanced cAMP production and increased adenylyl cyclase activity in differentiating neuroblastoma cells overexpressing catalytically active TG2, but not its inactive mutant C277S. The fact that adenylyl cyclase levels remained unaltered suggested a TG2-dependent change in its conformation. Interestingly, this type of regulation appears specific for neuronal cells, since TG2 inhibited adenylyl cyclase activity in human fibroblasts and endothelial cells (Gentile et al., 1997) and decreased cAMP levels in mesenchymal cells undergoing chondrogenic differentiation (Nurminsky et al., 2011). This shows yet another example of cell type-specific biological activities of TG2.

5.4.2. Oligodendrocytes—A role for TG2 in the differentiation of glial cells is emerging. An increase in TG activity was seen in some regions of the developing brain including the cerebellar cortex, principally owing to the increasing preponderance of glial cell activity (Hand et al., 1993). In cell culture, KCC009, a pharmacologic inhibitor of TG2-mediated transamidation, attenuated the differentiation of myelin-producing oligodendrocytes from oligodendrocyte precursor cells (van Strien et al., 2011). An associated decrease in RhoA activity suggested a role for this small GTPase in TG2-dependent glial cell differentiation, but the precise mechanisms of this regulation remain to be defined. Further, genetic ablation of TG2 resulted in delayed remyelination *in vivo*. In addition to the delayed differentiation of *TG2–/–* oligodendrocytes, this phenotype may also depend on an attenuated TG2-dependent function in astrocytes—the cells that secrete regulatory proteins to promote the myelinating activity of oligodendrocytes. Astrocyte cell migration is required for proper remyelination (Campisi et al., 1992) and appears to be regulated by TG2-induced transamidation as revealed by their reduced motility in the presence of KCC009 (van Strien et al., 2011).

5.4.3. Dendritic cells—Accumulating evidence indicates a significant role for TG2 in cell-mediated immunity that does not involve antibodies/complement but is based on the activation of macrophages, natural killer (NK) cells, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to antigen. High TG2 levels were reported in various cell lineages that originated from a common bone marrow progenitor including monocytes, resident dendritic cells, and several macrophage subsets (Fogg et al., 2006). TG2 was required for dendritic cell maturation from monocytes stimulated by bacterial LPS. The TG2-specific inhibitor KCC009 attenuated the development of dendritic cells and their production of cytokines, and genetic ablation of TG2 resulted in conferred resistance to LPS-induced septic shock (Matic et al., 2010). Of note, TG2 was dispensable for dendritic cells differentiation induced by GM-CSF and IL-4, suggesting the involvement of specific LPS receptors in the regulation of the TG2 functions. Future studies should clarify the TG2-mediated mechanism(s) that regulate dendritic cell functions in response to bacterial compounds. Once activated, dendritic cells migrate to the lymph nodes where they interact with T cells and B cells to initiate and shape the adaptive immune response. Therefore, TG2 may regulate immune response via its role in dendritic cell differentiation.

5.4.4. Neutrophils—TG2 may also support inflammatory responses via its direct involvement in the differentiation of neutrophil granulocytes, as it appears essential for differentiation of these cells. Genetic ablation of TG2 in mouse neutrophils results in diminished superoxide anion production and impaired extravasation, indicating delayed differentiation (Balajthy et al., 2006). Similarly, TG2 silencing in a human promyelocytic

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leukemia cell line delayed its differentiation into mature neutrophils and downregulation of genes related to the innate immune system (Csomos et al., 2010). Microarray analysis showed that TG2 is required for retinoid-induced changes in the expression of a large number of genes. Although the scale of changes in gene expression suggested TG2 action at the genomic level, and partial TG2 translocation into the nucleus was observed in differentiating neutrophils (Balajthy et al., 2006), the precise mechanisms of this regulation are yet unknown.

5.4.5. Osteochondrogenic cells—TG2 and FXIIIA have been implicated in the regulation of bone formation (Aeschlimann et al., 1993, Nurminskaya and Linsenmayer, 1996; Thomazy and Davies, 1999). Recently, the critical role of TGs in bone calcification was reported *in vivo* using zebrafish model (Deasey et al., 2011). In this study, the TG2 inhibitor KCC009 reduced average vertebrae mineralization in growing fish by ~30%. It had no effect on the overall growth or vertebrae number. Pharmacological inhibition of total TG activity in the developing zebrafish allowed to overcome the compensation effect observed in mice lacking either *TGM2* or *FXIIIA* alone (Nurminskaya and Kaartinen, 2006; Tarantino et al., 2009), which display no skeletal phenotype (de Laurenzi and Melino, 2001; Nanda et al., 2001). The individual contribution of each enzyme in the regulation of skeletal formation *in vivo* remains to be determined. In cell cultures, TG2 was shown to regulate the differentiation of both chondrocytes and osteoblasts (Nurminskaya and Kaartinen, 2006).

5.4.5.1. Chondrocytes: A proper chondrogenic differentiation program is essential for the osteochondral ossification process by which long bones are formed. Chondrogenic differentiation is initiated by the condensation of mesenchymal cells followed by a sequential series of maturation stages, including a proliferation stage, a prehypertrophic stage, and terminal maturation (defined as chondrocyte hypertrophy). TG2 expression correlated with the transition into the prehypertrophic stage *in vivo* and in an *in vitro* model of spontaneous chondrogenesis of mesenchymal limb bud stem cells (Nurminsky et al., 2011). Forced premature TG2 expression resulted in accelerated progression toward prehypertrophy associated with disrupted deposition of the cartilaginous ECM (Nurminsky et al., 2011). Precautious hypertrophy was not induced. The cells arrested in the prehypertrophic stage and, as a result, bone formation were disrupted. Hence, TG2 regulates early stages of chondrogenic differentiation in the embryonic growth plate. The TG2-induced inhibition of the PKA signaling has been implicated as one of the major mechanisms underlying this regulation.

In contrast, in inflamed joints TG2 may contribute to cartilage destruction by inducing abnormal hypertrophy of articular chondrocytes in which differentiation seizes at the resting stage preceding the prehypertrophic transition. In cell-culture studies, GTP-bound extracellular TG2 was found to promote and be required for the hypertrophic differentiation of articular chondrocytes induced by retinoic acid and the chemokine CXCL1 (Merz et al., 2003). These effects of TG2 were independent from its transamidation activity and ability to bind fibronectin (Johnson et al., 2003). Integrin α5β1 mediated TG2-induced hypertrophy in articular chondrocytes using a mechanism that involved activation of Rac1 and p38MAPK (Johnson and Terkeltaub, 2005; Tanaka et al., 2007). Moreover, the GTP-binding and GTPase activity of extracellular TG2 were proposed to mediate these processes. In these cells, calgranulin S100A11 also mediated the TG2-induced hypertrophy in a manner dependent on the transamidating activity of TG2. The covalently bonded S100A11 homodimer acquired the capacity to induce chondrocyte hypertrophy and ECM catabolism, thereby coupling inflammation with chondrocyte activation to promote osteoarthritis progression (Cecil and Terkeltaub, 2008). The precise molecular mechanisms of this regulation remain unknown.

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In conclusion, TG2 regulated transition into the prehypertrophic stage in normal chondrogenic differentiation. However, in the context of osteoarthritic inflammatory cytokines, TG2 accelerated terminal differentiation in the articular chondrocytes leading to matrix calcification is the diseased joints. Thus, while targeting TG2 may be beneficial for inflamed joints, it could also affect normal homeostasis of the cartilaginous tissues. Further advances in the understanding of the downstream mediators of the TG2-dependent chondrogenic differentiation may resolve this dilemma.

5.4.5.2. Osteoblasts: In addition to regulating endochondral ossification through regulation of chondrogenic differentiation, TG2 is expressed in primary osteoblasts and is implicated in the direct regulation of osteoblast differentiation (Heath et al., 2001). In cell culture, TG2 accelerated the differentiation of primary osteoblasts leading to increased matrix calcification. This reaction likely resulted from TG2-induced inhibition of PKA signaling (Nurminskaya et al., 2003). Similarly, improved differentiation of human osteoblasts was reported on TG2-treated collagen type I scaffolds (Chau et al., 2005), although the molecular mechanism of this regulation remains unclear. Hedgehog proteins are well known as important regulators of osteoblast maturation. Recently, TG2-induced oligomerization of hedgehog proteins was implicated as a putative mechanism in the regulation of bone formation. Inhibitors that block TG activity strongly decreased the amounts of chondrocyte-secreted hedgehog protein oligomers (Dierker et al., 2009).

In addition, a truncated 56kDa form of TG2 (generated by MT1-MMP proteolysis), acting as an ATPase in a Ca^{2+}-rich environment, promoted matrix mineralization in preosteoblasts (Nakano et al., 2010). Inhibition of endogenous TG activity in preosteoblast cultures with cystamine resulted in complete abrogation of mineralization, attributable to reduced ECM accumulation and an arrested state of osteoblast differentiation (Al-Jallad et al., 2006); however, recent evidence indicated that FXIIIA rather than TG2 acted as the major regulator of ECM deposition (Al-Jallad et al., 2011). Finally, TG2-induced osteoblast-like transformation of phenotypically plastic cells, such as vascular smooth muscle cells (Faverman et al., 2008), suggested that TG2 may be critical for vascular calcification (Johnson et al., 2008a).

5.4.6. Vascular smooth muscle cells—TG2 was shown to regulate the phenotypic stability of vascular smooth muscle cells. When grown on TG2-treated collagen matrices, vascular smooth muscle cells stabilized their contractile phenotype (Spurlin et al., 2009), showing that TG2-induced ECM modifications support their differentiated state. Similarly, norepinephrine-induced contractility of these cells depended on TG2-mediated transamidation of cytoplasmic targets (Johnson et al., 2010). In contrast, in cells exposed to stress or growth factors, TG2 acts as a negative regulator of the contractile phenotype and promotes dedifferentiation. For example, TG2 induced an osteoblast-like transformation of vascular smooth muscle cells leading to vascular calcification (Faverman et al., 2008; Johnson et al., 2008a). The LRP5/6-β-catenin signaling pathway was implicated as a mediator of these TG2 effects in vascular smooth muscle cells (Faverman et al., 2008). In parallel, TG2 amplified the dedifferentiation of aortic smooth muscle cells by PDGF due to TG2-induced amplification of PDGF/PDGFR signaling in conjunction with increasing their survival, proliferation, and migration (Zemskov et al., 2011b). Thus, on the surface of vascular smooth muscle cells, TG2 acts as a negative regulator of their phenotypic stability. Accumulation of TG2 in blood vessels may underlie the phenotypic transformation of these cells in vascular diseases and the loss of blood vessel compliance (Bakker et al., 2008; Sane et al., 2007).

5.4.7. Epithelial cells—Like its effects on vascular smooth muscle cells, TG2 destabilizes mammary epithelial cells and confers stem cell-like properties to both untransformed and

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transformed cells (Kumar et al., 2011). Sustained TG2 expression induced an EMT that contributed to the progression of metastatic cancers. This EMT promoted the detachment of cancer cells from the primary tumor and facilitated migration via a loss of cell polarity and adhesion. In untransformed breast mammary epithelial cells, TG2 overexpression resulted in their transition to mesenchymal cells as defined by the upregulation of mesenchymal markers, such as fibronectin, vimentin, and N-cadherin, and transcriptional repressors Snail1, Zeb1, Zeb2, and Twist1 (Kumar et al., 2010). *In vivo*, these changes might result from TG2 acting downstream of TGFβ during EMT. Similarly, elevated TG2 induced the mesenchymal phenotype in epithelial ovarian cancer cells, characterized by a cadherin switch and invasive behavior. These changes were mediated at the transcriptional level by altering the levels and functions of several transcriptional repressors, including Zeb1, possibly via the activation of the NFκB complex (Shao et al., 2009).

5.4.8. Stem cells—Increased TG2 promotes differentiation of stem cells toward certain lineages (Nurminsky et al., 2011; Song et al., 2007). For example, the bone marrow-derived MSCs overexpressing TG2 displayed enhanced progression into cardiomyocyte-like cells on three-dimensional cardiogel. Transplantation of these cells into the ischemic rat myocardium restored normalized systolic and diastolic cardiac function and further restored the cardiac function of the infarcted myocardium as compared with MSC transplantation alone (Song et al., 2007). A similar effect of TG2 on the accelerated differentiation of stem cells was reported using mesenchymal limb bud cells undergoing spontaneous chondrogenic differentiation in high-density cultures (Nurminsky et al., 2011). In contrast, in differentiated epithelial cells, elevated TG2 levels may drive an induction of a stem cell-like phenotype as shown for mammary epithelium (Kumar et al., 2011). Thus, temporal and tissue-specific effects of TG2 on the stem cell phenotypes and differentiation appear commonly recognized. Additional exciting studies addressing the role of this protein in stem cell differentiation are expected.

5.5. ECM organization and turnover

Since matrix organization profoundly impacts multiple aspects of cell behavior, modification of the ECM by TG2 appears important. Several reviews have presented in-depth description of this TG2 function (Belkin, 2011; Collighan and Griffin, 2009; Telci and Griffin, 2006; Wang and Griffin, 2011; Zemskov et al., 2006). The cross-linking activity of extracellular TG2 increases the mechanical ECM stability due to “spotwelding” of preexisting polymers and formation of matrix protein homo- and heteropolymers (Fig. 1.2(3a,b); Section 4.2.4). In addition, TG2-mediated cross-linking reduces ECM turnover by raising its resistance to proteolysis, acting essentially as “reverse proteinase” (Larreta-Garde and Berry, 2002). This TG2 activity also reinforces cell–ECM interactions indirectly by increasing the rigidity of adhesive matrices, clustering the integrin–ECM attachment sites, and exposure of the cryptic interaction sites in ECM proteins (Belkin, 2011). Separately, high affinity noncovalent interactions of cell-surface TG2 with integrins, syndecan-4, and fibronectin were shown to promote the assembly of ECM fibrils in a transamidation-independent manner (Belkin, 2011; Wang and Griffin, 2011). Moreover, TG2 in the ECM is able to modulate the maturation and activities of MMP2, TGFβ, and other non-structural components that impact ECM composition, structure, and properties (Collighan and Griffin, 2009). Notably, transamidation-dependent activation of NFκB and TGFβ signaling pathways was shown to amplify not only the deposition but also the synthesis of fibronectin and collagen, indicating that the intracellular pool of TG2 may collaborate with extracellular TG2 in the regulation of ECM organization (Telci et al., 2009). Together, these TG2 activities in the ECM were reported to alter the ECM structure and accelerate wound healing (Telci and Griffin, 2006), promote fibrosis and scarring (Johnson et al., 2007), but inhibit tumor cell invasion into the TG2-modified matrices (Mangala et al., 2005) and suppress

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angiogenesis (Jones et al., 2006), thereby suggesting major implications for various pathophysiological states.

5.6. Exocytosis

An unexpected involvement of cytoplasmic TG in exocytosis of platelet α-granules was discovered when Walther and coauthors (2003) reported that TG-mediated serotonylation of the small regulatory GTPases RhoA and Rab4A, which renders them constitutively activated, induced vesicle release and subsequent platelet aggregation. Later, a modulation of insulin secretion by pancreatic β cells was found to be regulated by TG-driven serotonylation of Rab3A and Rab27A GTPases, as inhibition of this process was shown to block hormone release (Paulmann et al., 2009). These important findings open a new avenue of research indicating that TG2-driven monoaminylation of multiple regulatory GTPases is involved in several aspects of intracellular vesicular trafficking and vesicle-based secretion processes in various cell types (Walther et al., 2011).

5.7. Autophagy

Autophagy is a complex catabolic process involving the degradation of the cell's own components through autophagosomes and lysosomal machinery (Mizushima et al., 2008). This cytoprotective mechanism for degradation of misfolded polyubiquitinated proteins and damaged organelles through lysosomal self-digestion is important for maintenance of cell homeostasis and is dysregulated in many disease states. In addition to its impact on protein aggregation, stress-induced accumulation of cytoplasmic TG2 and activation of its protein-cross-linking function were found to regulate autophagy. Specifically, protein kinase C (PKC)δ-mediated TG2 induction in pancreatic carcinoma cells was shown to inhibit autophagy as a result of blocking beclin 1 function (Akar et al., 2007; Ozpolat et al., 2007). A mechanistically similar TG2-dependent mechanism of autophagy inhibition was reported to operate via covalent cross-linking of beclin 1, an essential regulator of autophagy. The TG2-induced cross-linking of beclin 1 led to sequestration of its interactome in aggresomes in CFTR-deficient epithelial cells under conditions of oxidative stress (Luciani et al., 2010). These findings were also confirmed and developed with cells from *TGM2*–/– mice when D'Eletto and colleagues (2009) determined that cytoplasmic TG2 potently inhibits the initial stage of autophagosome formation but is required for their subsequent maturation into autophagolysosomes. The TG2-mediated depletion of functionally active beclin 1 and its interactome was identified as a novel pathway involved in the inhibition of autophagy. This pathway emerged as the major cause of aggresome formation and lung inflammation in cystic fibrosis (Luciani et al., 2010). It will be important to define whether this mechanism is utilized by other cells such as neurons which undergo apoptosis under conditions of neurodegeneration due to formation of insoluble protein aggregates, a process accompanied by accumulation of TG2 and activation of its transamidating function.

6. Cell Type-Specific Functions of TG2

6.1. Endothelial cells

Although the reported data appear controversial, growing evidence implies an important role for TG2 in the functioning of the endothelial layer and in angiogenesis. Jones and colleagues (2006) reported transamidation-mediated suppression of angiogenesis in endothelial cultures by exogenous TG2. They identified TG2-induced covalent ECM stabilization as a major negative regulator of angiogenesis. Further support of this idea was provided by Dardik and Inbal (2006) who reported that inhibition of TG2-mediated cross-linking resulted in blockage of the association of TG2 with VEGFR, inhibition of the nuclear translocation of the complex, and the attenuation of VEGF-induced signaling and endothelial cell migration. On the contrary, blocking cell-surface TG2 on these cells with IgA from celiac disease

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patients inhibited endothelial cell sprouting (Myrsky et al., 2008), suggesting that TG2 acts as a positive regulator of angiogenesis. This discrepancy may result from the fact that in endothelium, as in other cell types, TG2 is present both intra- and extracellularly. Its localization outside the cell impacts adhesion and ECM stability, while inside the cell, TG2 controls growth and survival through its regulation of cell-cycle progression (Nadalutti et al., 2011).

6.2. Fibroblasts

The key TG2 functions in fibroblasts relate to its ability to regulate cell adhesion, migration, and ECM organization. Extracellular TG2 increases ECM stability, deposition, and accumulation by cross-linking numerous ECM proteins (Belkin, 2011; Collighan and Griffin, 2009; Lorand and Graham, 2003). In addition, TG2 present outside the cells regulates ECM indirectly by increasing the release of active TGFβ from its matrix stores (Nunes et al., 1997). In cultured fibroblasts and in animal models of kidney scarring, TG2 overexpression increased the levels of collagens I, III, and IV, as well as fibronectin synthesis and accumulation in the ECM in a transamidation-dependent manner (Johnson et al., 2007; Telci et al., 2009). This type of regulation is thought to be mediated by activated TGFβ and NFκB signaling (Telci et al., 2009). In parallel, TG2-dependent cross-linking of collagen fibrils was shown to enhance ECM contraction, the function of fibroblasts and myofibroblasts related to scar formation during wound healing *in vivo* (Stephens et al., 2004). TG2-mediated cross-linking of fibronectin follows its deposition into ECM during its assembly (Akimov and Belkin, 2001b; Verderio et al., 1998). A transamidation-independent function of cell-surface TG2 was reported as being central for α5β1 and αvβ3 integrin-mediated assembly of fibronectin fibrils in fibroblasts (Akimov and Belkin, 2001b). Also, the ability of surface TG2 to regulate the levels and activities of MMP2 (Belkin et al., 2004; Satpathy et al., 2009; Stephens et al., 2004) and MMP9 (Ahn et al., 2008; Li et al., 2011) is likely to be important for controlling the rate of ECM turnover. Notably, a destabilization of ECM due to excessive MMP2-dependent extracellular TG2 degradation was found to cause major matrix abnormalities in thrombospondin-null mice (Agah et al., 2005).

6.3. Macrophages

Macrophages perform the functions of recognition, binding, and internalization of apoptotic cells. Despite the upregulation of TG2 levels during monocyte differentiation into macrophages (Murtaugh et al., 1983; Akimov and Belkin, 2001a), the process is independent of TG2. Nonetheless, the *TGM2*–/– mice develop inflammation/autoimmunity and display elevated susceptibility to inflammatory pathologies due to the impaired ability of macrophages to engulf dying cells (Sarang et al., 2009; Szondy et al., 2003). The process of apoptotic cell removal includes the elaborate molecular machinery of both dying cells and phagocytes. Studies in wild type versus *TGM2*–/– mice revealed that phagocytosis of apoptotic cells by macrophages is TG2-dependent, whereas their recognition and binding are not (Falasca et al., 2005). Animal studies also showed that cell-surface TG2 enhances phagocytosis of apoptotic neutrophils by macrophages in a manner dependent on TGFβ activation, but not on the transamidating activity of TG2. This function of TG2 in macrophages was suggested to play a role in limiting peritoneal acute gout-like inflammation (Rose et al., 2006). Moreover, the exchange of purine nucleotides on extracellular TG2 and/or its GTPase activity was proposed to regulate its activity in inflammation. The primary role of TG2 in apoptotic cell clearance by macrophages was also shown to be involved in limiting the progression of atherosclerosis in *LDLR*–/– mice (Boisvert et al., 2006). Significant recent advances started to unveil the TG2-dependent mechanism in phagocytosis by revealing a principal role of the β3 integrin-coreceptor function of TG2 and its complex formation with MFG-E8 on macrophage surfaces in the

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regulation of downstream signaling to Rac1 and RhoG during engulfment of apoptotic cells (Toth et al., 2009a,b).

7. TG2 as a Novel Therapeutic Target

Although this review does not specifically address the emerging TG2-mediated pathophysiological mechanisms in neurodegenerative disorders, cancer, and autoimmune/inflammatory diseases, we briefly discuss the developing approaches of targeting this protein and its individual functions. Beneficial effects of inhibiting its transamidating/protein cross-linking activity were observed in *in vivo* models of neurodegeneration and fibrosis following delivery of the competitive inhibitor cystamine and, more recently, designed inhibitors, such as thiomidaziolium or norleucine derivatives, which irreversibly bind the active site cysteine (Caccamo et al., 2010). Targeting of TG2 with specific antibodies has also been shown to be a promising tool for celiac disease treatment. Based upon their mechanisms of inhibition, TG2 inhibitors are divided into three classes: competitive amine inhibitors (putrescine, cystamine, spermidine, histamine, and cadaverine analogs), reversible inhibitors (GTP, GDP, Zn^{2+}, and thieno[2,3-*d*]pyrimidin-4-one acylhydrazide family), and irreversible inhibitors (peptidomimetic inhibitors, iodoacetamide, and 3-halo-4,5-dihydroisoxazoles) (Wilhelmus et al., 2008). Among the competitive amine inhibitors, cystamine is probably the most extensively studied and most frequently used inhibitor in animal models, despite its low specificity toward TG2, its inhibition of thiol-dependent protease caspase-3, and its induction of antioxidant glutathione inside cells (Lesort et al., 2003). Nonetheless, the improved motor function and increased survival of cystamine-treated compared to untreated mice with Huntington's disease (Dedeoglu et al., 2002; Karpuj et al., 2002) suggested that inhibition of the transamidating activity of TG2 might also be a promising therapeutic target for other protein aggregation diseases including Alzheimer's and Parkinson's disease. While information is still limited, several irreversible inhibitors of TG2-mediated transamidation already showed a promise as therapeutic agents in human diseases. A newer class of selective and irreversible peptidomimetic TG2 inhibitors, such as KCC009, was evaluated for treatment of gliomas and reported to enhance apoptosis of glioblastomas *in vivo* in a murine orthotopic brain tumor model (Yuan et al., 2007). The potential use of KCC009 as a therapeutic agent in humans is supported by the fact that it is well tolerated at pharmacologically effective doses in rodents and that it has a short serum half-life, indicating a fast distribution into organs and tissues (Choi et al., 2005). Yet, further studies on its long-term use in humans and optimized design of additional TG2-specific inhibitors are required for their successful application in various diseases involving the TG2-mediated dysfunctions.

Further, in some diseases such as cancers, accumulating data suggest that the transamidating activity of TG2 is not involved in promoting EMT, chemoresistance, or metastasis. Therefore, alternate approaches to down-regulate TG2 expression in tumor cells hold greater promise in reversing chemoresistance and inhibiting metastasis (Mehta, 2009; Mehta et al., 2010). In this regard, application of siRNA oligonucleotides for TG2 may provide a novel approach for treating drug-resistant and metastatic tumors, which together account for >90% of cancer-related deaths. In addition, the upcoming design of small molecule inhibitors for intervention therapy may prove beneficial for inhibiting specific TG2 functions mediated by distinct parts of the protein, including its binding to fibronectin via the identified site within its N-terminal β-sandwich domain (Hang et al., 2005). Using the *in silico* docking approach, the novel small molecule inhibitor ITP-79 was recently selected and shown to interfere with the TG2–fibronectin interaction, suggesting its future application for blocking ovarian carcinoma cell adhesion and tumor metastasis (Khanna et al., 2011). Nonetheless, the rational design of even more potent and specific inhibitors based on deciphering the

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structure of the TG2–fibronectin complex is likely needed to meet the important threshold of targeting this interaction for future therapeutic use.

8. The Use of TG2 in Bioengineering Applications

TG2 is an emerging enzyme in bioengineering that has many potential uses including cross-linking natural polymers in order to enhance their mechanical properties and stability, obtaining *in situ* gelling hydrogels, and incorporating bioactive ligands or peptides into the scaffolds to direct cell differentiation and proliferation. Overexpression of TG2 in various cell lines grown on the polymers poly(DL lactide *co*-glycolide) (PLG), poly(ε-caprolactone) (PCL), and poly(L lactide) (PLA) showed that, with increased TG2 expression, endothelial-like cells displayed improved attachment and spreading on all these polymers, an effect shared by fibroblasts on PLA, and osteoblasts on PLG (Verderio et al., 2001). Nonetheless, because genetic cell alteration is undesirable in bioengineering, exogenous TG2 is being extensively tested as an ECM modifier to enhance cell proliferation and guide cell differentiation.

Collagens are the most abundant proteins in mammals, and their polymers are widely used in bioengineering. However, their *in vivo* applications are limited due to poor mechanical properties. Early studies showed that TG2 was able to enzymatically incorporate putrescine into nonhelical domains of collagen I and cross-link aminopeptides of collagen III (Bowness et al., 1987), heteropolymers of collagens V and XI (Kleman et al., 1995), and purified collagen XI (Shanmugasundaram et al., 2011). TG2-induced cross-linking of collagens resulted in increased denaturation temperature and enhanced resistance of these matrices to proteolysis (Jones et al., 2006; Orban et al., 2004). Further, TG2-cross-linked collagen scaffolds improved cell attachment, spreading and enhanced proliferation of dermal fibroblasts, osteoblasts, and bone marrow-derived MSCs (Chau et al., 2005; Shanmugasundaram et al., 2011).

Significantly, cell differentiation was also accelerated on the TG2-treated collagen matrices. Osteoblasts displayed an increased propensity to differentiate when plated on the TG2-cross-linked compared to untreated collagen I (Chau et al., 2005). Similarly, human MSCs differentiated more efficiently to the chondrogenic lineage when plated on TG2-treated scaffolds of collagen XI compared to untreated scaffolds (Shanmugasundaram et al., 2011). Several TG2-dependent mechanisms may account for these effects, including the determination of stem cell lineage specification by ECM rigidity and elasticity (Engler et al., 2006), exposure of the cell–ECM interaction sites (Collighan and Griffin, 2009), and direct interactions of the scaffold-autocross-linked TG2 with the cell surface (Shanmugasundaram et al., 2011). A new direction in bioengineering employs collagen-mimetic dendrimers mimicking the native collagen fibrillar architecture (Kinberger et al., 2002). The TG2-induced cross-linking of modified dendrimers supplemented with the cell-binding sequence GFQGER, and the substrate sequences EDGFFKI and APQQEA increased their melting temperature and enhanced adhesion of human hepatocarcinoma cells to these matrices (Khew et al., 2008). These effects were mediated by optimization of the triple helical conformation and increased integrin clustering. Thus, TG2-treated collagen-mimetic dendrimers are showing great promise as alternatives to collagen-based matrices (Collighan and Griffin, 2009).

TG2-mediated cross-linking of biologically active molecules to various scaffolds was shown to be an effective methodology to locally accommodate high morphogen concentrations, provide their sustained presence, and enhance cell invasion and directed differentiation. Local bone regeneration was shown with a matrix-bound engineered active fragment of human parathyroid hormone (PTH1–34), linked to a TG substrate for binding to fibrin as a

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delivery and cell-invasion matrix with an intervening plasmin-sensitive link. Notably, the PTH-fibrin matrix supported dose-dependent bone formation *in vivo*, with evidence of both osteoconductive and osteoinductive bone-healing mechanisms (Arrighi et al., 2009). Thus, the TG2-modified PTH-derivatized matrices may have potential utility in humans as replacement for bone grafts or to repair bone defects.

TG2 was also used for production of injectable hydrogels in controlled release systems for drug delivery and tissue engineering and as surgical sealants and adhesives. The formation of hydrogels under physiological conditions relies on enzymatic cross-linking to form polymer networks. Poly-ethyl-glycol (PEG) polymers modified with lysine and glutamine substrate peptides form hydrogels in the presence of TG2 under physiological conditions (Hu and Messersmith, 2005). The modified PEG polymers can be mixed with therapeutic agents or cells for targeted delivery and applied as surgical sealants and medical adhesives onto the tissue surface to be sealed. Several different synthetic and biopolymers are being investigated for use in TG2-mediated hydrogel polymerization after introduction into the body (Collighan and Griffin, 2009).

Last, TG2 on its own was tested as a biological glue, for the repair of articular cartilage. TG2 treatment increased the adhesive strength between two pieces of cartilage by ~40%, an effect that was greater than that achieved with a commercial tissue sealant (Jurgensen et al., 1997). In conclusion, TG2 as well as bacterial TGs are being widely tested in biomedical engineering, in addition to novel potential applications of TGs in the areas of material science, textiles, leather processing, and food industry.

9. Conclusions and Perspectives

Although TG2 was the first discovered member of TG family, its key pathophysiological roles still remain debatable despite impressive progress in our understanding of this protein (Iismaa et al., 2009). Several distinctive features, including its ubiquitous and regulated expression, its localization in multiple cellular compartments, and its multiple enzymatic and nonenzymatic activities, underscore its enormous complexity and set this fascinating protein apart from other TGs. Moreover, the intricate compartment-dependent regulation of its transamidating activity and the noncovalent interactions unique for this TG profoundly impact multiple cell functions and, therefore, are likely to contribute to a number of pathological states. Several lines of future research will likely be central for the elucidation of pathophysiological functions of this protein. Delineation of the pathways and mechanisms of intracellular TG2 trafficking and its targeting to various cellular compartments should be pivotal for manipulating its extracellular secretion as well as nuclear and mitochondrial recruitment, thus paving the way to a better understanding the compartment-specific functions of TG2. Identification of the key docking interactions and specific targeting sequences/sites in this protein that mediate its membrane association and its delivery outside the cell, into the nucleus, and into mitochondria are likely to aid in this arduous task. Further, generation of conformation-specific antibodies to TG2 and “clickable” inhibitors of TG2-induced transamidation (Dafik and Khosla, 2011) will facilitate the visualization of active TG2 in live cells. In turn, this should help to localize and inhibit this activity in celiac disease and other pathologies involving TG2-induced transamidation, such as cystic fibrosis and Huntington's disease. A better understanding of the physiological roles of the additional enzymatic functions of TG2 is also likely to involve the generation of new molecular tools, such as antibodies and specific probes that detect and block active GTP-bound TG2/Ghα, as well as the PDI and the protein kinase activities of TG2. In addition, rational design and generation of peptide and small molecule inhibitors of its noncovalent complexes with fibronectin might evolve as a novel approach for blocking the crucial cell adhesion and survival mechanisms of metastatic cancer cells or ECM accumulation pathways in fibrotic

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diseases. Similarly, deciphering the structure of TG2 complexes with transmembrane receptors, including integrins, LRP1/5/6, and PDGFR, might lead to approaches allowing their specific disruption in order to interfere with proinflammatory signaling on the surface of vascular smooth muscle cells and block a progression of major cardiovascular diseases. Finally, an advanced understanding of the compartment-specific molecular functions of TG2 and their regulation will likely help to elucidate the multifaceted roles of this intriguing protein in human pathologies.

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Abbreviations

AKAP13	protein kinase A anchor protein 13
ANT1	adenine nucleotide translocator 1
CFTR	cystic fibrosis transmembrane conductance regulator
CREB cAMP	response element-binding protein
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FXIIIA	Factor XIIIA
GPCR	G protein coupled receptor
HIF1	hypoxia inducible factor 1
HRE	hypoxic response element
IFN	interferon
IGF	insulin-like growth factor
IGFBP	insuline-like growth factor-binding protein
LAP	latency-associated peptide
LBTP	latent TGFβ-binding protein
LDLR	low density lipoprotein receptor
LPS	lipopolysaccharide
LRP	low density lipoprotein receptor-related protein
MEK	mitogen-activated protein kinase kinase

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MFG-E8	milk fat globulin EGF factor 8
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
MTA1	metastatic tumor antigen 1
MT-MMP	membrane-type matrix metalloproteinase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDI	protein disulfide isomerase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptor
Rb	retinoblastoma protein
ROCK	Rho kinase
ROS	reactive oxygen species
SUMO	small ubiquitin-like modifier
TG	transglutaminase
TG2	transglutaminase 2
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VLDLR	very low density lipoprotein receptor

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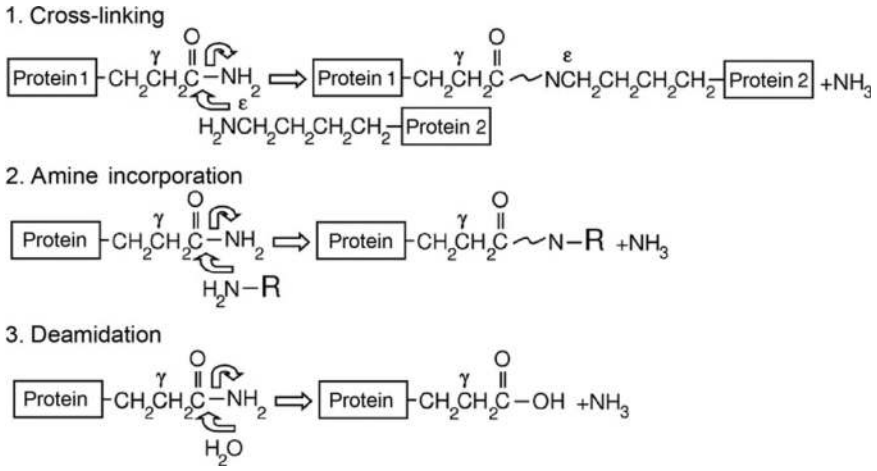

Figure 1.1.

TG2 acting as transglutaminase catalyzes several types of posttranslational modifications of proteins. (1) *Protein cross-linking*. TG2-mediated transamidation reactions proceed via formation of a $N^{\varepsilon}(\gamma$-glutamyl)lysine isopeptide bond between the acceptor Gln residue of the protein 1 and deprotonated Lys donor residue of the protein 2. TG2 displays specificities toward both their Gln and Lys substrates. (2) *Protein aminylation*. TG2-mediated transamidation reactions occur via incorporation of an amine (H_2NR) into the Gln residue of the acceptor protein. Diamines and polyamines may act as a tether in a bis-glutaminyl adduct between two protein molecules. (3) *Deamidation of proteins*. TG2-mediated hydrolysis reactions in the absence of amine cosubstrates convert the Gln residues of the reactive protein into the Glu residues. Electron movements are shown by curved arrows. The *de novo* formed covalent bonds are shown by curved lines.

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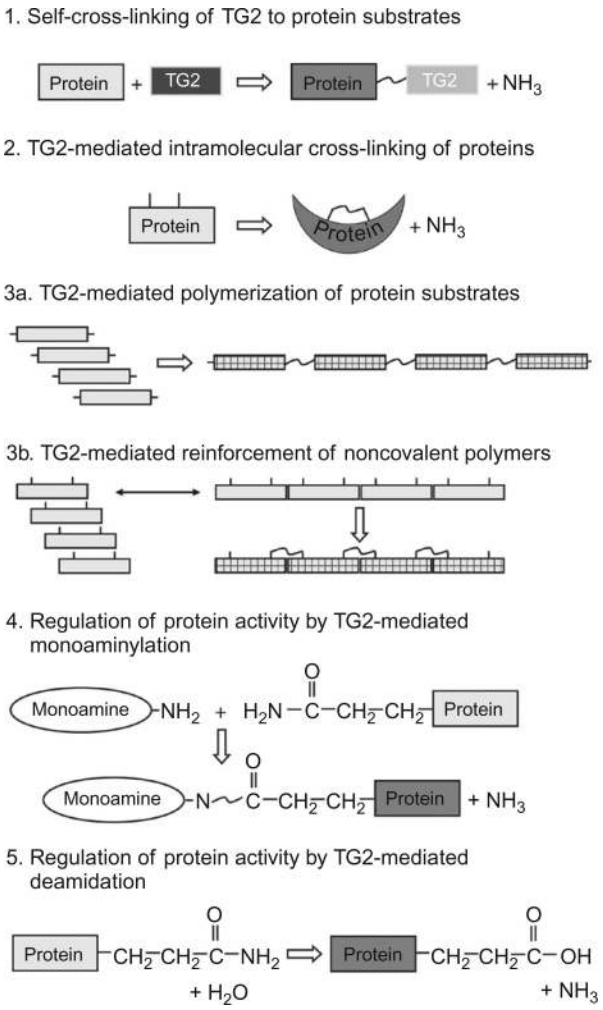

Figure 1.2.

Biological consequences of transglutaminase activity of TG2 on protein substrates. (1) Self-cross-linking of TG2 to protein substrates. TG2 incorporates itself into covalent complexes with protein substrates. (2) TG2 catalyzes the formation of intramolecular isopeptide cross-links between the selected Gln and Lys residues of protein substrates. In (1, 2), TG2 alters the conformation, stability, and functions of protein substrates. (3a) TG2-catalyzed *de novo* polymerization of protein substrates involves the formation of covalent isopeptide bonds between the protein monomers. (3b) Reinforcement of preexisting noncovalent protein polymers by TG2-mediated covalent cross-linking of protein monomers (enzymatic spotwelding). In (3a, 3b) TG2 modifies the properties of covalently cross-linked protein polymers compared with those of protein monomers (3a) or noncovalent polymers (3b). (4) TG2-mediated monoaminylation of protein substrates. (5) TG2-mediated protein deamidation. In (4, 5) TG2-induced protein modifications alter the activities of protein substrates. Altered biological activities of TG2-modified protein monomers are reflected by darker shades (1, 2, 4, 5); altered biological activities of TG2-modified protein polymers are shown as grid patterns (3a, 3b). The *de novo* formed covalent bonds are shown by curved lines.

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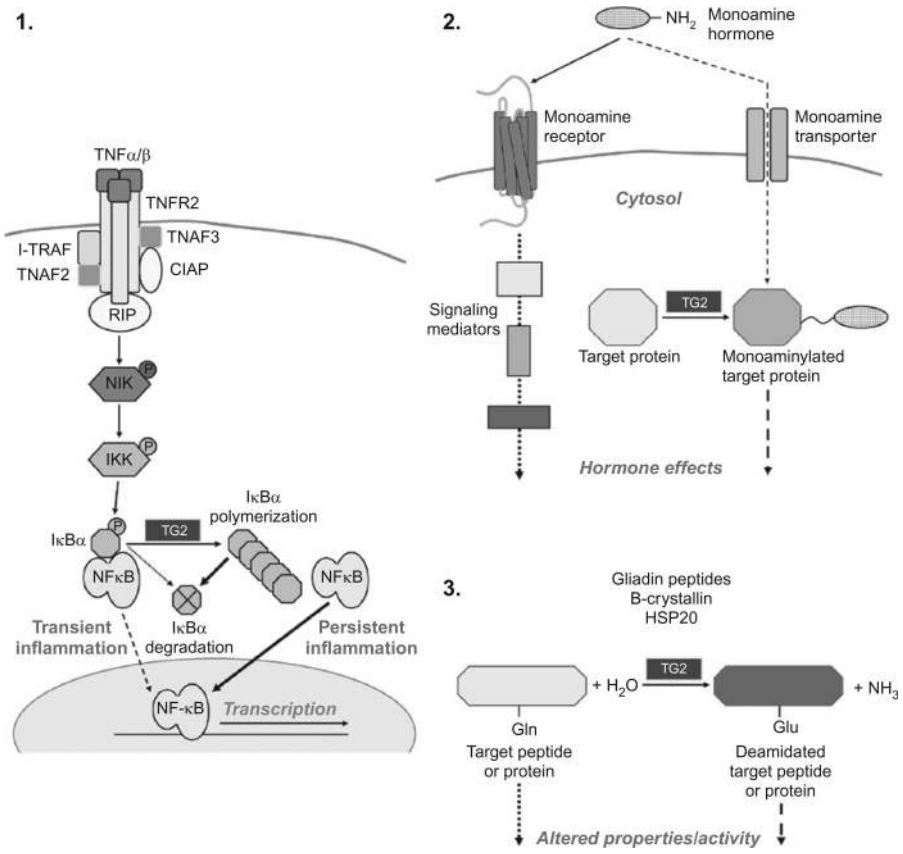

Figure 1.3.

Regulation of biological activities of protein substrates by TG2-mediated modifications and their pathophysiological implications. (1) TG2-mediated covalent cross-linking of IκBα leads to proteasomal degradation of the IκBα polymers and depletion of the active monomeric IκBα, causing a constitutive activation of NFκB. This TG2-related mechanism has important consequences for chronic inflammation and cancer. (2) Monoamine hormones (serotonine, norepinephrine, dopamine, etc.) delivered into the cell via monoamine transporters are covalently linked by TG2 to cytoplasmic target proteins, such as small regulatory GTPases Rho1, Rac1, Rab3A, Rab4a, Rab27A, or cytoskeletal components such as α-actin. These TG2-induced posttranslational modifications alter the biological activities of target proteins. The diverse biological effects of these TG2-driven modifications have important implications for diabetes and arterial hypertension. (3) TG2-mediated deamidation is described for several protein substrates such as gliadin peptides, B-crystallins, and Hsp20. These TG2-catalyzed protein modifications appear important for pathogenesis of celiac disease and cataract formation.

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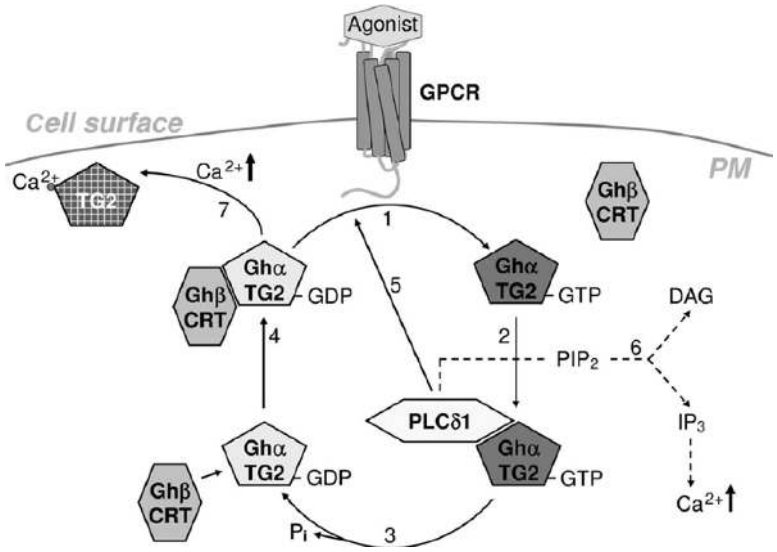

Figure 1.4.

GTPase activity of TG2/Ghα: the signaling cascade and regulation. GDP-bound heterodimer TG2/Ghα-calreticulin/Ghβ is inactive. (1) Agonist stimulation of transmembrane GPCRs induces exchange of GDP to GTP and dissociation of GTP-bound TG2/Ghα from calreticulin/Ghβ. (2) GTP-bound TG2/Ghα activates PLCδ1. (3, 4) Signal termination occurs with GTP hydrolysis (3) and reassociation of GDP-bound TG2/Ghα with free calreticulin/Ghβ (4). (5) PLCδ1 promotes coupling efficiency of this signaling system through its GEF function and stabilization of GTP-bound TG2/Ghα. (6) PLCδ1 catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), causing an increase in intracellular [Ca^{2+}]. (7) The switch of GTPase activity of TG2/Ghα to transglutaminase activity of TG2 in cells is triggered by elevation of intracellular [Ca^{2+}] and decrease of guanine nucleotides.

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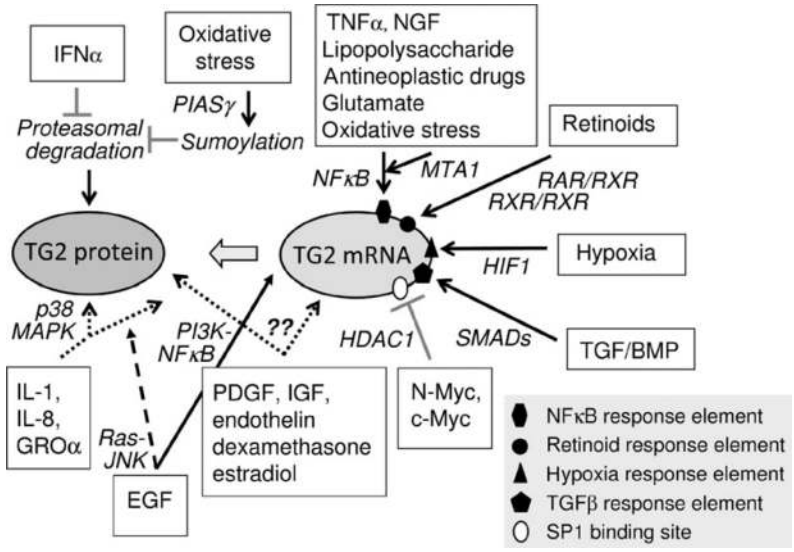

Figure 1.5.

Regulation of TG2 expression. A number of stressors, hormones, growth factors, cytokines, chemokines, and oncogenes impact TG2 mRNA expression levels through transcriptional regulation via several regulatory elements in the promoter region of the gene, or posttranslationally modulate TG2 protein levels by modulating the rate of its proteasomal degradation. Solid lines represent the established transcriptional or posttranslational regulatory cascades while dotted lines reflect currently undetermined pathways. Dashed line depicts the EGF-mediated effect of shifting cytoplasmic TG2 to the inner side of plasma membrane at the leading edge.

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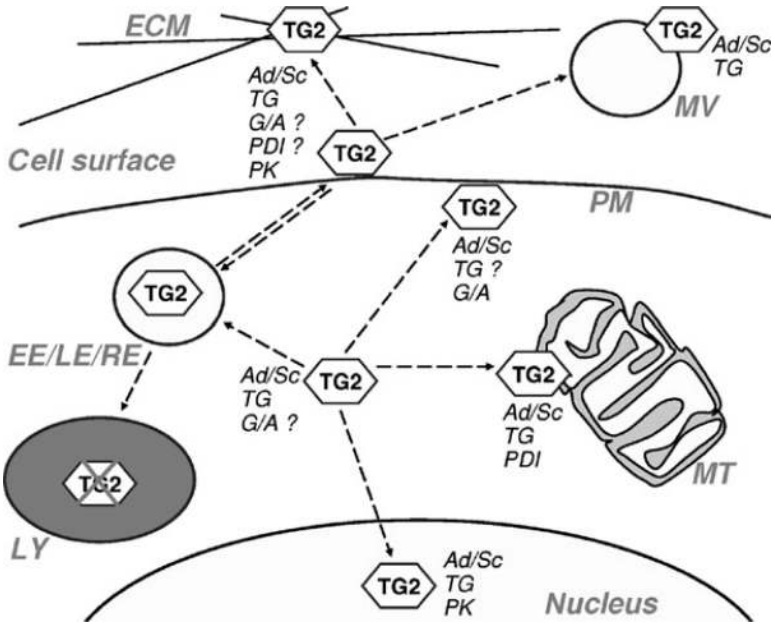

Figure 1.6.

Enzymatic and nonenzymatic activities of TG2 in diverse cellular compartments. The adapter/scaffolding nonenzymatic function of TG2 (*Ad/Sc*) and its transglutaminase (*TG*), GTPase/ATPase (*G/A*), protein disulfide isomerase (*PDI*), and protein kinase (*PK*) enzymatic activities are shown for the protein localized in the cytoplasm, underneath the plasma membrane (*PM*), in the nucleus, in mitochondria (*MT*), in early/late/recycling endosomes (*E/L/RE*) and lysosomes (*LY*), and on the cell surface, in the ECM, and in extracellular microvesicles (*MV*).

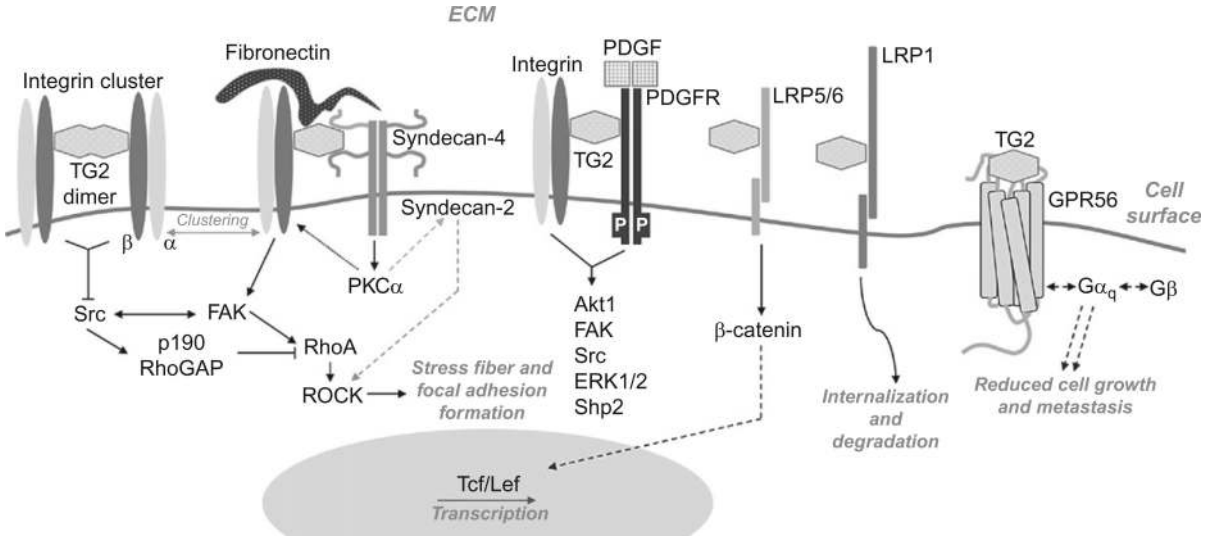

Figure 1.7.

The TG2-containing adhesive/signaling complexes on the cell surface. Solid black lines indicate TG2-mediated activation of cytoplasmic targets by transmembrane signaling receptors. Dotted black line marks binding of activated PKCα to the integrin cytoplasmic tails that causes their redistribution on the cell surface. Dashed gray lines outline the activation of syndecan-2 by intracellular PKCα and syndecan-2-mediated activation of ROCK that induces stress fiber and focal adhesion formation. Dashed black line marks the nuclear translocation of β-catenin that leads to its complex formation with Tcf/Lef and activation of gene transcription. Curved black line indicates the principal pathway of surface TG2 internalization. Dashed double black line depicts the unknown pathway of GPR56-induced $G\alpha_q$ activation that inhibits tumor cell growth and metastasis.

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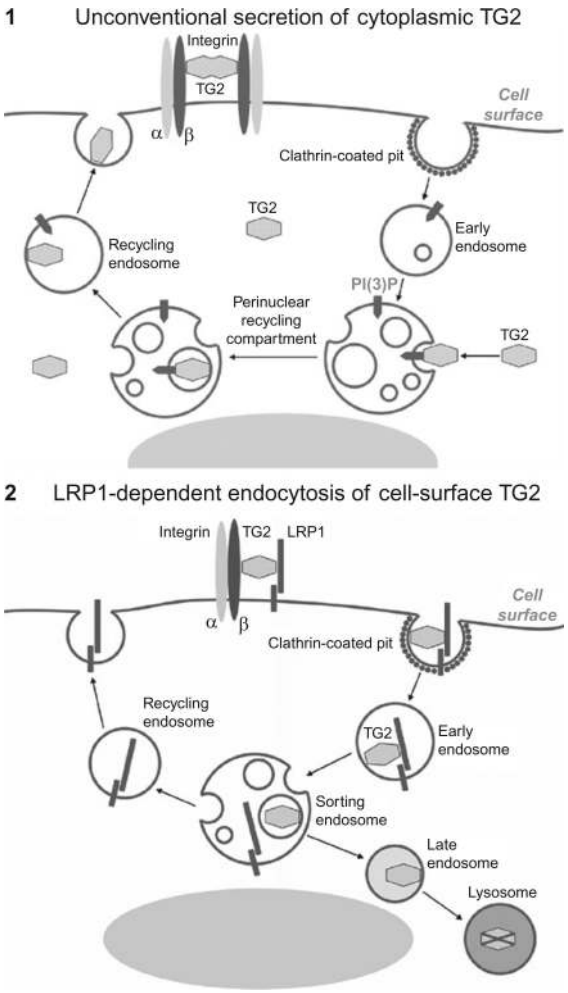

Figure 1.8.

Dynamic regulation of cell-surface TG2 levels and functions. (1) *TG2 externalization*. The unconventional pathway of cytoplasmic TG2 secretion involves phospholipid-dependent delivery into recycling endosomes. Solid lines mark the major endosomal recycling pathway that operates via the perinuclear recycling endosomal compartment. Dashed line indicates the PI(3)P-dependent recruitment of cytoplasmic TG2 (hexagons) to the membranes of the perinuclear recycling compartment. (2) *Endocytosis of TG2*. The constitutive LRP1-dependent internalization and lysosomal degradation of cell-surface TG2. Solid lines mark the major endosomal recycling and lysosomal degradative pathways.

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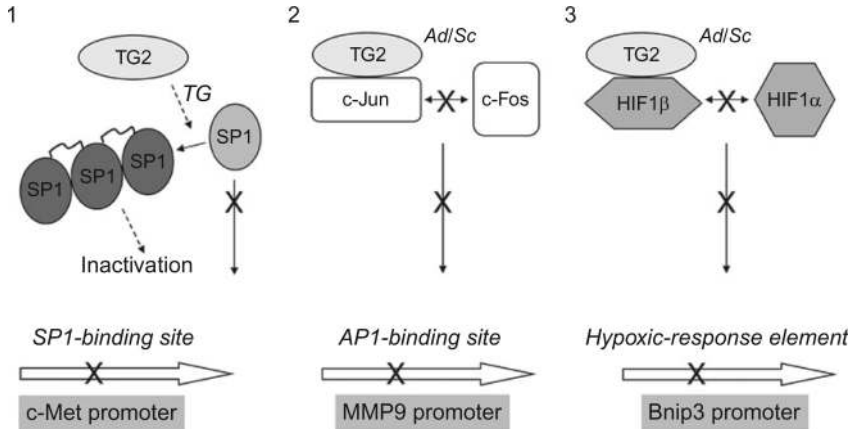

Figure 1.9.

TG2 as a novel transcriptional coregulator in the nucleus. (1) TG2-dependent enzymatic cross-linking and polymerization of the SP1 transcription factor in the nucleus causes its inactivation and inhibits SP1-mediated transcription of the prosurvival c-Met gene in hepatocytes. This transamidation-dependent mechanism mediated by nuclear TG2 is involved in liver steatohepatitis. (2) TG2 binds noncovalently to c-Jun in the nucleus and prevents c-Jun/c-Fos dimerization, thereby decreasing AP1-dependent transcription of the MMP9 gene in cardiomyoblasts. This nonenzymatic mechanism mediated by nuclear TG2 is thought to be involved in ECM remodeling. (3) TG2 interacts noncovalently with HIF1β in the nucleus and prevents its dimerization with HIF1α, thus inhibiting HIF1 binding to the HRE in the promoter region of Bnip3 gene and decreasing its transcription in neuronal cells. This nonenzymatic nuclear TG2-driven mechanism is implicated in the prosurvival effect of TG2 in stroke.

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Table 1.1

The role of TG2 in the regulation of cell differentiation and phenotype stability

Cell differentiation process	TG2 effects	Proposed mechanism	References
Neuronal differentiation of neuroblastoma cells	+	↑Adenylyl cyclase, ↑CREB	Tucholski et al. (2001)
	–/+	Balance of TG and GTPase activities? ↑JNK	Tucholski and Johnson (2003), Singh et al. (2003), Tee et al. (2010)
Myelinating oligodendrocytes	+	TG activity	van Strien et al. (2011)
Dentritic cell maturation	+	TG activity	Matic et al. (2010)
Neutrophil granulocyte differentiation	+	TG activity in the nucleus?	Balajthy et al. (2006), Csomos et al. (2010)
Early chondrogenesis	+	cAMP/PKA signaling	Nurminsky et al. (2011)
Chondrocyte hypertrophic differentiation	+	Surface GTP-bound TG2 acting via α5β1 integrin	Jonhson et al. (2005), Tanaka et al. (2007)
	+	TG activity in the ECM	Cecil and Terkeltaub (2008)
	+	Surface TG2 mobilization via FXIIIA binding to α1β1 integrin	Johnson et al. (2008b)
Osteoblasts	+	cAMP/PKA signaling	Nurminskaya et al. (2003)
	+	?	Chau et al. (2005)
	+	Hedgehog signaling	Dierker et al. (2009)
	+	ATPase activity	Nakano et al. (2010)
Vascular smooth muscle cells—phenotypic stability	+	TG2 in the ECM	Spurlin et al. (2009)
	–	Surface TG2 via LRP5/6 and ↑β-catenin signaling	Faverman et al. (2008)
	–	Surface TG2 via ↑PDGF/PDGFR signaling	Zemskov et al. (2011b)
Ovarian epithelial cancer cells	–	Promotes EMT via ↑NFκB activity and Zeb1 induction	Shao et al. (2009)
Breast epithelial cancer cells	–	Promotes EMT via induction of Zeb1/Zeb2, Snail1, Twist1	Kumar et al. (2010)
Mesenchymal stem cells	–	Enhanced differentiation into cardiomyocyte-like cells	Song et al. (2007)
	–	Accelerated chondrogenic differentiation (spontaneous or on TG2-modified collagen XI)	Nurminsky et al. (2011), Shanmugasundaram et al. (2011)