Supplement

Materials & Methods

Construction of Fluorescent TG2 expression vectors

The mouse TG2 encoding IMAGE clone 3256943 (GenBank: BC016492) in the expression vector pCMV-SPORT6 was obtained from imaGenes (Berlin, Germany). In order to remove the stop codon, nt 1655-2101 were amplified (Taq PCR core kit, Qiagen) using primers 1 and 2 (Supplemental Table 1). The resulting PCR fragment (447 bp) was cloned into pGEM-Teasy (Promega) and verified by nucleotide sequence analysis using BigDye Terminator v3.1 (Applied Biosystems), and primers 3-4. This amplified TG2 PCR fragment was then used to replace the 3' end of TG2 in pCMV-Sport6 using the restriction enzymes NotI (New England Biolabs) and BglII (Roche). The complete TG2 open reading frame without stop-codon was subsequently amplified by touchdown PCR (Advantage GC-2 PCR kit, Clontech) starting at 70ºC, with 20 steps of 0,5ºC using primers 16 and 17. After 3'-T tailing, the PCR fragment was cloned into pGEM-Teasy and verified by nucleotide sequencing using primers 3-8 and 10-14. The TG2 open reading frame without stop-codon fragment was excised from pGEM-Teasy using restriction enzyme XmaI and EcoRI-HF (New England Biolabs) and cloned into either pEGFP-N1 or pmCherry-N1 (Clontech 632469 resp. 632523), generating vectors that express TG2 with a C-terminal fluorescent tag under control of a CMV promoter. Finally, the desired sequence was confirmed using primers 5-9, 11-15 and 18-19.

→ *Supplemental Table 1*

Western Blot

All TG2 fusion proteins were characterized in cell lysates and isolated microparticles (MPs), both obtained from HEK/293T cells. Cell lysate samples (40-50 µg), prepared as previously described, were boiled for 5 minutes and separated on a 7% SDS-polyacrylamide gel. The proteins were subsequently blotted on an Immobilon-P Transfer membrane (Milipore) in a tank system. After o/n blocking with a 1:1 solution Odyssey Blocking Buffer (LI-COR, 927-40000) and PBS, blots were probed with rabbit polyclonal TG2 Ab-4 (Neomarkers RB-060-P) at 1:1000. Donkey anti-rabbit InfraRed IRDye 680 was used as secondary antibody at 1:15.000. As a loading control, β-actin was detected using mouse monoclonal antibody A1978 (Sigma) at a 1:1000 dilution for and IRDye 800 CW donkey anti mouse (Westburg) at 1:15.000. Finally, antibodies against eGFP (ClonTech 632375, Living Colors GFP monoclonal) and mCherry (ClonTech 632393, Living Colors DsRed monoclonal) were used to confirm the appropriate size of fluorescently-tagged TG2. All antibodies were incubated for 1 hr at room temperature. Blot signal was measured using an Odyssey infrared detector, and protein molecular weight was estimated using Odyssey Prestained Molecular Weight marker (LI-COR, 928-40000).

Due to the limited amount of TG2 fusion proteins in the microparticles secreted by HEK293T cells, a more sensitive western blot approach was required. Culture supernatant was collected after a 48-hrs

incubation period in the presence of 10% FBS as previously described. Isolated fractions of microparticles were resuspended in a reducing sample buffer in a two-fold dilution, containing SDS and 6% β-mercapto-ethanol. Guinea pig liver TG2 (Sigma T5398) was used as positive control. All samples were boiled for 5 minutes, loaded onto a 4-15% gradient gel (Biorad, Criterion Precast gel, 345-0029) and blotted to PVDF membrane (Millipore, immobilon-FL, IPFL 00010). Membranes were incubated with 5% protifar (Nutricia, Protifar Plus) for 1 hour as to block non-specific staining, followed by incubation with a rabbit anti-TG2 (1:1000, Neomarkers, RB-060-P) and mouse anti-eGFP (1:20.000, ClonTech 632375, Living Colors GFP monoclonal). Respectively a secondary antibody goat anti-rabbit peroxidase (1:30.000, DakoCytomation, P0448) and goat anti-mouse peroxidase (1:30.000, DakoCytomation, P0447) was used for visualisation of the bands. Membranes were incubated with a five-fold diluted peroxidase substrate (Roche, Lumi-Light Western blotting substrate, 12.015.196.001) followed by analysis of luminescence using a LAS3000 luminescent image analyzer.

Results

Partial Degradation of TG2/mCherry

We tested the functional characteristics of TG2 tagged with either eGFP or mCherry, since the latter would provide a better signal-to-noise ratio in tissues with a high green autofluorescence level. Moreover, GFP was previously reported not be a substrate for transamidation, excluding the participation of our fusion protein as a substrate in transamidation. Expression of TG2 with or without fluorescent tags was not significantly different, as determined from quantification of western blots (Supplemental Table 1). Non-modified TG2 showed a strong band at the expected size of 80 kDa. However, when either eGFP or mCherry was tagged at the C-terminus of TG2, in addition to the expected band at 110 kDa, a second band at about 90 kDa was observed (Supplemental Fig. 1). The lower band was particularly apparent in the TG2/mCherry fusion product, where it constituted about half of the protein recognized by the TG2 antibody (Supplemental Table 2). In cells expressing TG2/eGFP on the other hand, the 90 kDa band contributed only about 10%. When western blots were probed with antibodies against eGFP or mCherry, both the 90 and 110 kDa bands were recognized (data not shown), excluding the possibility of cleavage of the complete fluorescent tag from the TG2 fusion protein. All subsequent imaging experiments below were carried out using TG2/eGFP. At the moment, this difference between these closely related fluorescing proteins cannot be explained. Therefore, all localization experiments were conducted using TG2/eGFP. Although about half of the TG2/mCherry protein was partially degraded, this did not lead to a diminished in vitro transamidation activity (Supplemental Table 2).

→ *Supplemental Fig. 1 & Supplemental Table 2*

→ *Supplemental Fig. 2*

Confirmation of presence of TG2 in microparticles

Our study on the secretion of TG2 via MPs was based on the fluorescent signal of TG2 fusion proteins in a flow cytometry setup (Fig. 5). We confirmed by western blotting that the eGFP signal of MPs from transfected cells indeed represented the corresponding TG2 protein. Control TG2 showed a distinct band at 80 kDa, control eGFP was observed at 30 kDa and at the dimeric weight of 60 kDa. The TG2 fusion proteins (wild-type TG2 and the mutants C277S and W241A) could be detected by antibodies against both TG2 and eGFP at the expected weight of 110 kDa (Supplemental Fig. 3). Thus the eGFP signal in these samples represents the full-length TG2 fusion protein.

→ *Supplemental Fig. 3*

→ *Supplemental Fig. 4*

Supplemental Figures

Supplemental Fig. 1: Example of western blot for determination of TG2 protein content in cell lysates. HEK/293T cells were transfected with full-length TG2, TG2 fused to eGFP or mCherry, or only these fluorescent proteins. Two different proteins were visualized simultaneously using 2 antibodies in the infrared spectrum: TG2 is shown in red, green represents respectively the loading control protein β-actin, mCherry and eGFP. Non-tagged TG2 shows a distinct band at 80 kDa; fluorescently-tagged TG2 appears as expected around 110 kDa, but has an additional band at ~90 kDa.

Supplemental Fig. 2: Subcellular localization of fluorescent TG2 on fibronectin. HEK/293T cells were transfected with TG2/eGFP and reseeded on a coating of fibronectin: TG2 appears distributed randomly throughout the cytosol, extracellular TG2/eGFP particles are absent.

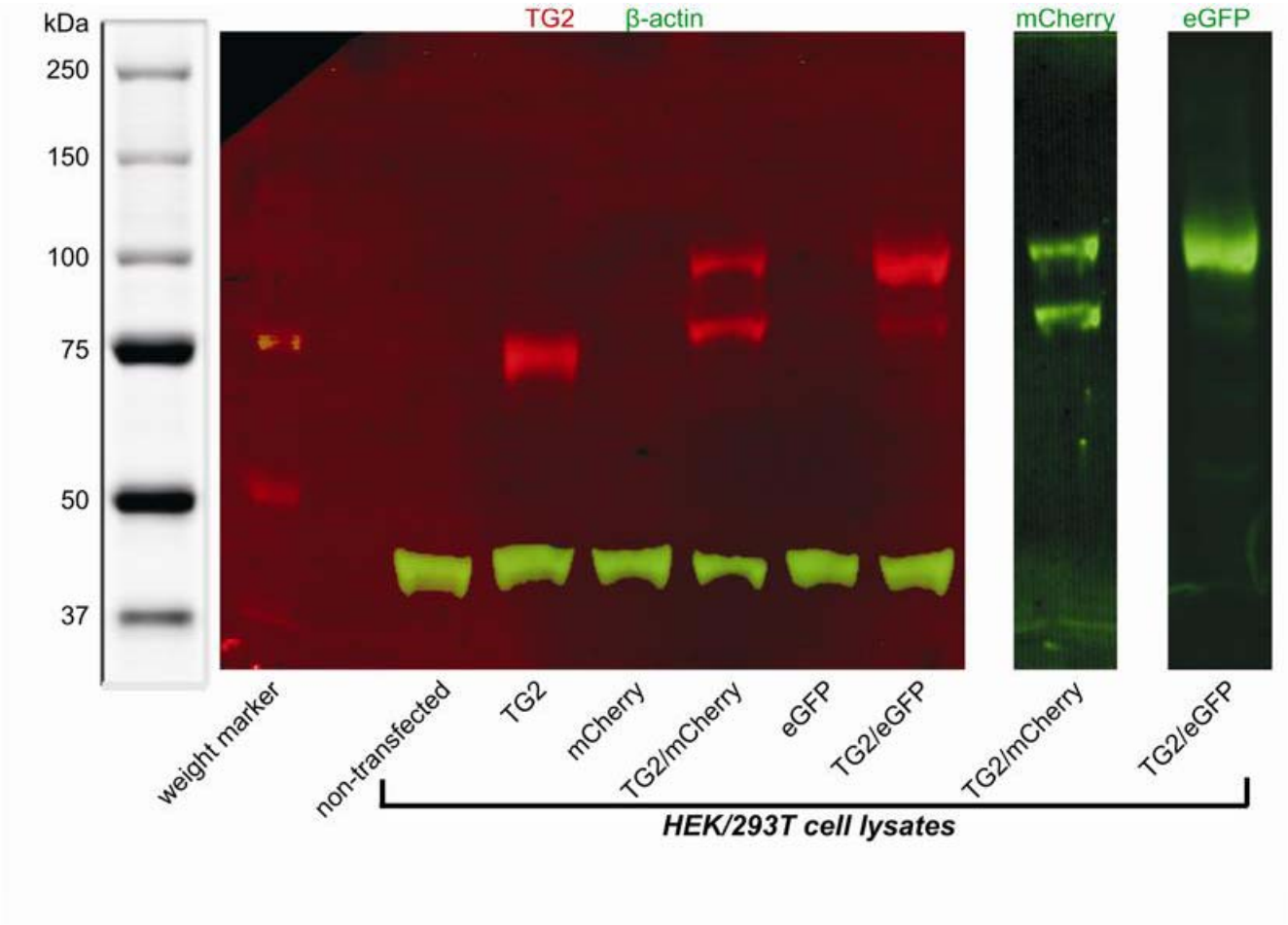
Supplemental Fig. 3: Example of western blot for characterization of TG2 fusion proteins in microparticles. HEK/293T cells were transfected with full-length TG2, TG2 fused to eGFP, or regular eGFP. The left panel shows the signal for a TG2 antibody, the right panel displays eGFP: this illustrates that the eGFP signal can be reliably used for flow cytometry measurements on TG2 fusion proteins (Fig. 5).

Supplemental Fig. 4: Flow cytometry measurements of FSC and SSC characteristics for beads of known sizes. Silica beads of 500, 1000 and 5000 nm were used, PMT settings are identical to those used for measurement of SMC-derived microparticles (Fig. 5). The gated region indicated by 'R1', including the majority of the 500 and 1000 nm beads, corresponds to the MP settings.

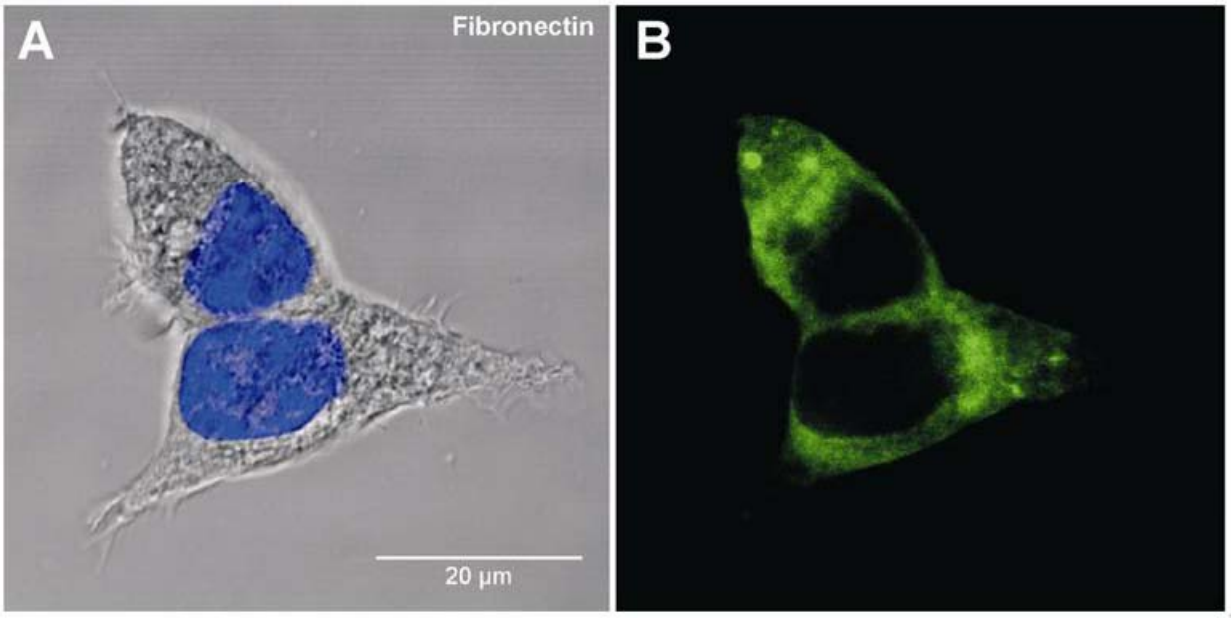
Supplemental Table Legends

Supplemental Table 1: Primers used for construction of fluorescent TG2.

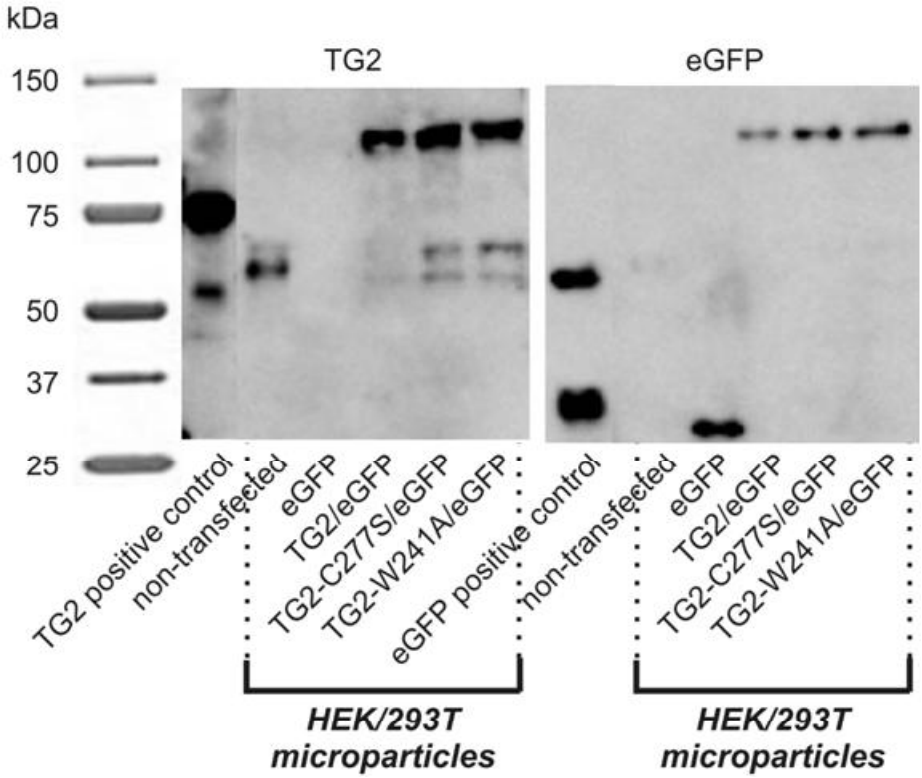
Supplemental Table 2: Quantification of TG2 western blot and transamidation activity assay. The protein bands were quantified by integrating pixel intensity after subtraction of the local background (see Supplemental Fig. 1). The amount of TG2 was then normalized to protein content as determined by Bradford assay, for fluorescent TG2 the sum of both bands was used. Transglutaminase activity was measured by incorporation of biotinylated cadaverine and is represented in units that were defined according to the activity of transglutaminase isolated from guinea pig liver.



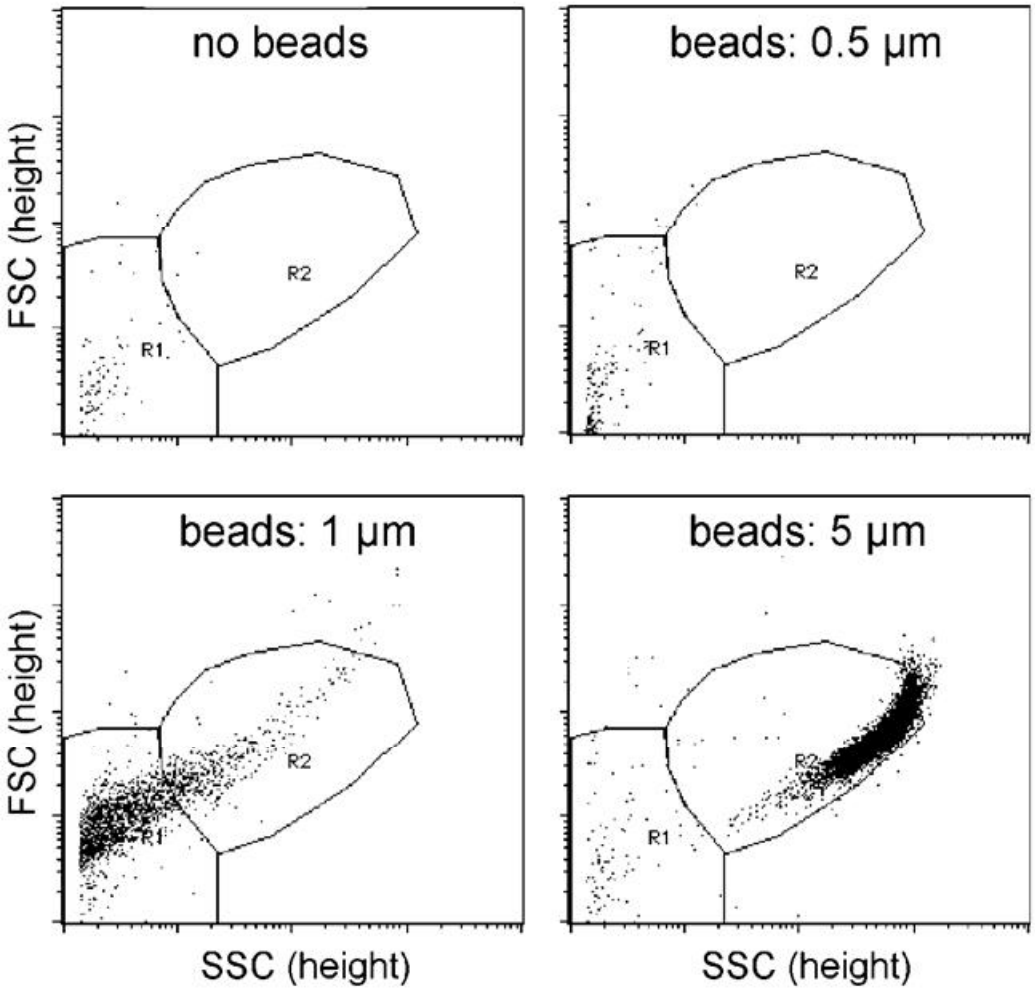
Supplemental figure 1



Supplemental figure 2



Supplemental figure 3



Supplemental figure 4

#	Primer Name	Sequence 5’ → 3’
1	Tgm2m-for	TCC CAC TTC GAA TCC TCT ACG A
2	Tgm2m-nostop-SmaI-MluI-rev	ACG CGT CCC GGG CCG GGC CGA TGA TAA CAT T
3	M13-rev	CAG GAA ACA GCT ATG AC
4	M13-fwd	CTG GCC GTC GTT TTA C
5	Tgm2-cDNA-Fwd1	AAC AGG ACA ATG TCC TCT
6	Tgm2-cDNA-Fwd2	CTG CAA TGA TGA CCA GG
7	Tgm2-cDNA-Fwd3	AGA AGA GCG AAG GGA CA
8	Tgm2-cDNA-Fwd4	AAC GAC ACC TCG GAG AC
9	Tgm2-cDNA-Fwd5	GGA GCA GAA GTC TGT GG
10	Tgm2-cDNA-Rev1	TTC TGA AGA GGC CCC AGG CA
11	Tgm2-cDNA-Rev2	GGA CCC GGA TCT TGA TT
12	Tgm2-cDNA-Rev3	GCC TTG GTG AAG ACT TC
13	Tgm2-cDNA-Rev4	TTG TTG CTC TCC AGC TCC CC
14	Tgm2-cDNA-Rev5	TAT CCA GGA TTC CAT CC
15	Tgm2-cDNA-Rev6	AAC GTG AGG CTG TCC AC
16	Tgm2/nostop-cDNA-For	GAA TTC GCG GCC GCT CTG AGC TGT CGC CGC
17	Tgm2/nostop-cDNA-Rev	CCC GGG TGG CCG GGC CGA TGA TAA CAT T
18	EGFP-N-Fwd	CGT GTA CGG TGG GAG GTC TA
19	EGFP-C-Rev	GTT GTG GTT TGT CCA AAC TCA

Sample *(n = 3)*	Western Blot 80 kDa *(intensity)*	90 kDa *(intensity)*	110 kDa *(intensity)*	Total *(intensity)*	Transglutaminase Activity *(µUnits/µgram)*
control	<1	-	-	<1	<10
TG2	35 ± 6.4	-	-	**35 ± 6.4**	**654 ± 212**
mCherry	<1	-	-	<1	<10
TG2/mCherry	-	16 ± 4	12 ± 4.4	**28 ± 8.4**	**602 ± 268**
eGFP	<1	-	-	<1	<10
TG2/eGFP	-	2.7 ± 1.1	24 ± 7.1	**26 ± 8.1**	**660 ± 292**