Transglutaminase 2—a novel inhibitor of adipogenesis

VD Myneni1, G Melino2 and MT Kaartinen*1,3

Differentiation of preadipocytes to lipid storing adipocytes involves extracellular signaling pathways, matrix remodeling and cytoskeletal changes. A number of factors have been implicated in maintaining the preadipocyte state and preventing their differentiation to adipocytes. We have previously reported that a multifunctional and protein crosslinking enzyme, transglutaminase 2 (TG2) is present in white adipose tissue. In this study, we have investigated TG2 function during adipocyte differentiation. We show that TG2 deficient mouse embryonic fibroblasts (Tgm2−/− MEFs) display increased and accelerated lipid accumulation due to increased expression of major adipogenic transcription factors, PPARγ and C/EBPα. Examination of Pref-1/Dlk1, an early negative regulator of adipogenesis, showed that the Pref-1/Dlk1 protein was completely absent in Tgm2−/− MEFs during early differentiation. Similarly, Tgm2−/− MEFs displayed defective canonical Wnt/β-catenin signaling with reduced β-catenin nuclear translocation. TG2 deficiency also resulted in reduced ROCK kinase activity, actin stress fiber formation and increased Akt phosphorylation in MEFs, but did not alter fibronectin matrix levels or solubility. TG2 protein levels were unaltered during adipogenic differentiation, and was found predominantly in the extracellular compartment of MEFs and mouse WAT. Addition of exogenous TG2 to Tgm2+/+ and Tgm2−/− MEFs significantly inhibited lipid accumulation, reduced expression of PPARγ and C/EBPα, promoted the nuclear accumulation of β-catenin, and recovered Pref-1/Dlk1 protein levels. Our study identifies TG2 as a novel negative regulator of adipogenesis.

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The prevalence of obesity is steadily increasing globally and is recognized as a major risk factor for diabetes, heart disease and certain cancers.1–3 During excess energy intake adipose tissue expands to store extra lipids. This expansion initially occurs via an increase in the size of existing adipocytes (hypertrophy) which is followed by an increase in adipocyte number via proliferation of preadipocytes (hyperplasia) and their differentiation into mature adipocytes (adipogenesis).3 Impaired adipogenesis and adipose tissue function are associated with the development of metabolic complications in obesity, such as the development of type 2 diabetes.4,5

Adipogenesis involves conversion of spindle-shaped preadipocytes to round lipid filled adipocytes, this morphological change requires conversion of filamentous actin to cortical actin6,7 which is associated with remodeling of extracellular matrix (ECM) fibronectin (FN) matrix to laminin-rich matrix. Adipogenesis is regulated by various factors that can either promote or inhibit adipogenesis. Many of these factors regulate ECM components and cytoskeletal tension, some of the factors or proteins which maintain preadipocyte state and act as inhibitors during early phase of adipogenesis include Wnt/β-catenin signaling, Pref-1/Dlk1, RhoA and ROCK kinases. These factors are amongst those which determine whether preadipocytes will be in quiescence, or undergo proliferation and differentiate to adipocytes.8–9

In our previous work we have identified two members of transglutaminase (TG) enzyme family, Factor XIII-A (FXIII-A) and transglutaminase 2 (TG2), in white adipose tissue (WAT).10 TGs are enzymes with ability to form isopeptide bonds between glutamine residue of one protein to a lysine residue of another protein by transamidation reaction.11–13 TGs can also have functions that do not involve their transamidase activity.11 In our recent work, we have shown that FXIII-A is responsible for the transamidase/crosslinking activity during adipocyte differentiation. In 3T3-L1 adipocyte and mouse embryonic fibroblasts (MEFs) cultures, FXIII-A crosslinking activity increased plasma FN assembly into preadipocyte matrix which promoted preadipocyte proliferation. Inhibition of TG activity of FXIII-A in these cultures resulted in increased adipocyte differentiation.10 The role of TG2 in adipogenesis remained unaddressed.

TG2 is the most ubiquitous of the TG family members and expressed in many tissues such as bone, cartilage, kidney, colon, liver, heart, lung, spleen, blood and nervous tissue.11,13–16 TG2 is expressed by many cell types such as osteoblasts,17 chondrocytes,18,19 mesenchymal stem cells
(MSCs), neuronal and glial cells, phagocytes, monocytes, neutrophils and T-cells and pancreatic β-cells. TG2 has been implicated in various biological functions including cell differentiation and maturation, cell morphology and adhesion, ECM stabilization, cell death, inflammation, cell migration and wound healing. TG2 is present in both extracellular and intracellular compartments of the cell. In the extracellular compartment, TG2 can be found on the cell surface and in the ECM. In the intracellular compartment, TG2 is mostly cytosolic but also found on the plasma membrane, in the nuclear membrane and in mitochondria. Dysregulation of TG2 function(s) has been implicated in pathogenesis of celiac disease, diabetes, neurodegenerative disorders such as Huntington’s, Alzheimer’s and Parkinson’s disease, as well as inflammatory disorders and cancer.

In this study, we have used MEFs from TG2 wild-type (Tgm2+/+) and TG2 deficient mice (Tgm2−/−) to address the potential role of TG2 during adipocyte differentiation. We report that TG2 deficiency results in accelerated and increased adipogenesis in MEFs due to increased expression of adipogenic transcription factors PPARγ and C/EBPα. We further examined the role of TG2 in several anti-adipogenic pathways and demonstrate that TG2 regulates adipogenesis via multiple factors – these include Pref-1/Dlk1 expression and modulation of Wnt/β-catenin signaling, ROCK kinase activity and Akt signaling.

Results

Tgm2−/− MEFs show increased and accelerated adipocyte differentiation. Our previous work identified two TG enzymes, FXIII-A and TG2, in mouse WAT and in the 3T3-L1 preadipocyte cell line, and identified FXIII-A as a regulator of preadipocyte proliferation. In this study, we investigated the role of TG2 in adipogenesis by using TG2 deficient and wild-type MEFs as a model, and examined Tgm2+/+ and Tgm2−/− MEFs capacity to differentiate into adipocytes under adipogenic conditions. Oil Red O staining for lipid and quantification on day 8 of adipogenesis, shows a 1.5-fold increase in adipose conversion in Tgm2−/− MEFs compared with Tgm2+/+ cells (Figures 1a and b). Increased adipogenesis was associated with an increase in mRNA expression levels of main adipogenesis transcription factors, Pparγ and Cebpα; Tgm2−/− MEFs showing a 1.8-fold and 1.5-fold increase, respectively, compared with Tgm2+/+ MEFs on day 8 (Figure 1c). The increase in the transcription factor mRNA in Tgm2−/− MEFs was also associated with

![Figure 1](image-url)
an increase in PPARγ protein levels and increased production of its downstream target GLUT4 (Figure 1d).

Time course analysis of lipid droplet accumulation in cells during early differentiation on days 0, 3, and 5, show that Tgm2−/− MEFS accumulate lipids earlier on day 3 compared with Tgm2+/+ MEFS that show lipids on days 4–5 (Figure 2a). Accelerated adipogenesis was associated with an increase in mRNA expression levels of Pparγ and Cebpα. Tgm2−/− MEFS showed a 5-fold and 4-fold increase, respectively compared with Tgm2+/+ MEFS on day 3 (Figure 2b). Increase in mRNA expression was also accompanied by significantly increased PPARG and C/EBPα positive nuclei in Tgm2−/− MEFS compared with Tgm2+/+ MEFS on day 3 indicative of their increased nuclear translocation and thus activation (Figures 2c and d). Western blot analysis of PPARG and C/EBPα showed that both are upregulated in Tgm2−/− MEFS compared with Tgm2+/+ MEFS. PPARγ was detected in Tgm2−/− MEFS but not Tgm2+/+ MEFS on day 3, and PPARG was detected in Tgm2+/+ MEFS by day 4 supporting the accelerated adipogenesis seen in Tgm2−/− MEFS (Figure 2e). These results indicate that TG2 is a negative regulator of adipogenesis.

**TG2 is critical for Pref-1 protein expression.** Because of the accelerated adipogenesis in Tgm2−/− MEFS, we examined Pref-1/Dlk-1 expression levels in these cells. Pref-1 inhibits adipogenesis during the early phase of differentiation, and Pref-1 downregulation coincides with upregulation of C/EBPα and PPARG31,32 Examination of Pref-1 protein levels in total cell lysate on days 3 and 4 show increased PPARG and C/EBPα protein levels in Tgm2−/− MEFS; actin used as loading control.

**TG2 is required for β-catenin nuclear translocation.** Because of the links of TG2 to canonical Wnt/β-catenin signaling30 and its inhibitory role in adipogenesis, and PPARG and C/EBPα expression,31,32 we examined canonical Wnt/β-catenin pathway to see if it is affected in Tgm2−/− MEFS. Examination of β-catenin nuclear translocation—a hallmark...
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Exogenous TG2 inhibits adipogenesis and increases PPARγ expression. Nuclear translocation of PPARγ and PPARγ target gene expression are increased in TG2−/− MEFs compared with TG2+/+ MEFs. The effect of TG2 deficiency on PPARγ expression was increased in TG2−/− MEFs compared with TG2+/+ MEFs. The addition of TG2 enzyme (ExoTG2) caused a significant decrease in PPARγ expression. (Figure 5e).

Exogenous TG2 inhibits adipogenesis and increases PPARγ expression. Nuclear translocation of PPARγ and PPARγ target gene expression are increased in TG2−/− MEFs compared with TG2+/+ MEFs. The addition of TG2 enzyme (ExoTG2) caused a significant decrease in PPARγ expression. (Figure 5e).

Figure 3: Western blot analysis of Pref-1 protein levels in TG2+/+ and TG2−/− MEFs. Pref-1 protein expression was significantly reduced in TG2−/− MEFs compared with TG2+/+ MEFs. (Figure 3, Supplementary Figure S2A). The addition of TG2 enzyme (ExoTG2) partially recovered the Pref-1 protein levels in TG2−/− MEFs (Figure 6g; Supplementary Figure S2C). However, in TG2−/− MEFs, the addition of TG2 enzyme (ExoTG2) caused a significant decrease in Pref-1 protein expression. (Figure 6g).

Figure 4: Pref-1 and PPARγ expression are increased in TG2−/− MEFs compared with TG2+/+ MEFs. Western blot analysis of Pref-1 and PPARγ expression in TG2+/+ and TG2−/− MEFs. Pref-1 protein expression was significantly reduced in TG2−/− MEFs compared with TG2+/+ MEFs. (Figure 3, Supplementary Figure S2A). The addition of TG2 enzyme (ExoTG2) partially recovered the Pref-1 protein levels in TG2−/− MEFs (Figure 6g; Supplementary Figure S2C). However, in TG2−/− MEFs, the addition of TG2 enzyme (ExoTG2) caused a significant decrease in Pref-1 protein expression. (Figure 6g).

Figure 5: TG2 activity is increased in adipose tissue of TG2−/− mice. Immunofluorescence staining of mouse epididymal WAT shows that TG2 is found in the extracellular space and increased cell surface expression of TG2 in TG2−/− mice compared with TG2+/+ mice. (Figure 5d).

Figure 6: TG2 inhibits adipogenesis. Exogenous TG2 inhibits adipogenesis and increases PPARγ expression. Nuclear translocation of PPARγ and PPARγ target gene expression are increased in TG2−/− MEFs compared with TG2+/+ MEFs. The addition of TG2 enzyme (ExoTG2) caused a significant decrease in PPARγ expression. (Figure 5e).

Figure 7: ROCK enhances Akt signaling, which has a crucial promoting role in adipogenesis. Exogenous TG2 inhibits adipogenesis and increases PPARγ expression. Nuclear translocation of PPARγ and PPARγ target gene expression are increased in TG2−/− MEFs compared with TG2+/+ MEFs. The addition of TG2 enzyme (ExoTG2) caused a significant decrease in PPARγ expression. (Figure 5e).
cytoskeleton is not mediated by FN matrix levels as $Tgm2^{-/-}$ MEFs assembled normal FN matrix and showed no changes in the amounts of FN in DOC-soluble or DOC-insoluble fractions (Supplementary Figure S3). mRNA expression of total and cellular FN (EDA-FN or EDB-FN) were also not altered (Supplementary Figure S4). This data suggests that TG2 modulation of actin cytoskeleton and Akt signaling also contributes to increased adipogenesis.

$Tgm2^{-/-}$ adipose tissue display increased adipocyte number. To see how an increase in adipogenesis in $Tgm2^{-/-}$ MEFs in vitro translates to adipose tissue in mice, epididymal fat pad of $Tgm2^{-/-}$ and $Tgm2^{+/+}$ mice were used to assess adipocyte size and number in vivo. Figures 8a and b show that the adipocyte size is significantly reduced in $Tgm2^{-/-}$ mice compared with $Tgm2^{+/+}$ mice. However, adipocyte number is significantly increased in $Tgm2^{-/-}$ mice compared with $Tgm2^{+/+}$ mice (Figure 8c). The increase in adipocyte number in $Tgm2^{-/-}$ mice suggest increased proliferation of precursor cells and/or preadipocytes (hyperplasia), and their differentiation into mature adipocytes (adipogenesis).

Discussion

Our previous work identified two members of TG family, FXIII-A and TG2, in WAT and demonstrated that FXIII-A can regulate adipocyte proliferation via promoting plasma FN matrix assembly which inhibits adipogenesis.10 In this study, we have examined the role of TG2 in adipogenesis and report for the first time that TG2 also acts as an inhibitor of adipocyte differentiation. We show that $Tgm2^{-/-}$ MEFs display accelerated and enhanced adipogenesis which is associated with downregulation of multiple anti-adipogenic signaling pathways that jointly lead to increased expression and activation of master transcription factors of adipogenesis, $Ppar\gamma$ and $Cebp\alpha$, and increased lipid accumulation in the cells. The pathways identified in this study are; regulation of Pref-1 protein levels, $\beta$-catenin signaling and modulation of ROCK-mediated cytoskeletal tension and Akt signaling.

The identification of TG2 in as an anti-adipogenic factor is not entirely unexpected as TG2 has been implicated in regulation of number of pathways in multiple location in cells.11 Our report for the first time show that in MEFs $Tgm2$ deficiency dramatically reduces Pref-1 protein expression. Pref-1 is an major inhibitor of adipocyte differentiation and
whose expression is highest during early differentiation and then gradually disappears as the cells differentiate into adipocytes. Pref-1 protein mediates its effects on adipocyte differentiation by directly binding to FN, which activates integrin signaling to engage the MAPK/ERK pathway. This induces Sox9 expression which inhibits adipocyte differentiation. Pref-1 knockout mice have increased adipose tissue mass, pre- and postnatal growth retardation and skeletal abnormalities and conversely, Pref-1 over-expressing mice have reduced adipose tissue mass, impaired glucose tolerance and reduced insulin sensitivity. Other effects of Pref-1 protein are also mediated by Sox9 which promotes chondrogenic commitment of MSCs, but inhibits chondrocyte maturation and osteoblast differentiation. Linked with Pref-1 function in chondrocyte maturation, TG2 has been shown to regulate the transition into the prehypertrophic stage during chondrocyte maturation. Premature, forced expression of TG2 accelerated progression toward prehypertrophy and it was shown that extracellular TG2 can increase Sox9 expression. Based on our work, it is highly possible that the effects of TG2 on chondrocytes may also be mediated via Pref-1. TG2 is also expressed by osteoblasts where it is located on the cell surface. TG2 knockout mice do not show any chondrogenic or osteogenic abnormalities during development or postnatally, which is likely due to compensatory function from upregulation of FXIII-A and TGFβ1. It is possible that TG2, jointly with other TG enzymes and Pref-1 may act as an upstream regulator of mesenchymal stem cell differentiation into the different lineages, particularly into chondrocytes, osteoblasts and adipocytes. In addition to Pref-1 we reported here that TG2 regulates β-catenin nuclear translocation in preadipocytes. Canonical Wnt signaling is a crucial pathway that regulates lineage determination of MSCs. In preadipocytes, Wnt signaling...
maintains preadipocytes in undifferentiated state by inhibiting PPARγ and C/EBPa.32 During early phase of adipogenesis PPARγ suppresses Wnt signaling by increasing β-catenin degradation and PPARγ upregulation coincides with decreased total and nuclear β-catenin levels, suggesting a reciprocal relation between Wnt and PPARγ.47–49 Here we report that Tgm2−/− cells display increase in Ppary and Cebpα mRNA expression and reduced β-catenin nuclear

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Figure 6 Exogenous, extracellular TG2 inhibits adipogenesis and activates β-catenin signaling and recovers Pref-1 protein levels. (a, b) Tgm2+/+ and Tgm2−/− MEF cultures were treated with increasing concentrations (0.5–5 μg/ml) of exogenous TG2 (ExoTG2) from days 0 to 8. Graphs show quantification of Oil Red O staining on day 8. Exogenous TG2 was able to reduce lipid accumulation in a significant manner in both Tgm2+/+ and Tgm2−/− MEFs. Results are mean values ± SEM (n = 3). **P < 0.001. *P < 0.05; **P < 0.01. (c) mRNA expression of Ppary and Cebpα in Tgm2+/+ and Tgm2−/− MEFs on days 0 and 1 with or without ExoTG2 (5 μg/ml); DM-differentiation medium. A reduced expression was observed with ExoTG2. (d) Western blot analysis of total β-catenin levels in total cell lysate of Tgm2+/+ and Tgm2−/− MEFs on day 1 with or without ExoTG2 (5 μg/ml) show no difference; actin used as a loading control. (e, f) Western blot analysis and quantification of β-catenin levels in cytosolic (c) and nuclear (N) fractions of Tgm2+/+ and Tgm2−/− MEFs on day 1 with or without ExoTG2 (5 μg/ml). Normalization was done to loading controls α-tubulin and histone H3. Tgm2−/− MEFs show significantly increased levels of β-catenin in the nucleus. Error bars ± SEM (n = 3), *P < 0.05; **P < 0.01. (g) mRNA expression of Pref-1 in Tgm2+/+ and Tgm2−/− MEFs on days 0 and 1 with or without ExoTG2 (5 μg/ml). ExoTG2 treatment recovered Pref-1 protein levels in Tgm2−/− MEFs.

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Figure 7  Tgm2−/− MEFs display reduced ROCK kinase activity, actin fibers and increased Akt phosphorylation. (a) Microplate ROCK kinase activity of Tgm2+/+ and Tgm2−/− MEFs in total cell lysate during differentiation show moderate but significant decrease in Tgm2−/− cells on days 1 and 2. Error bars ± SEM (n = 3), *P < 0.05. (b) Immunofluorescence staining of Tgm2+/+ and Tgm2−/− MEFs for F-actin on days 0 and 3. A moderate decrease in actin stress fibers is observed. Nuclei are stained with DAPI (blue). Scale bar 200 µm. (c, d) Western blot analysis and quantification of pAkt (Ser473) and total Akt in MEF cell lysates from day 0–3. An increase in Akt phosphorylation is seen on day 3 in Tgm2−/− MEFs compared with Tgm2+/+ MEFs. Results are mean values ± SEM (n = 3), *P < 0.05.

Figure 8  Increased adipocyte number in Tgm2−/− mouse WAT. (a) H&E stained sections of epididymal fat pads from Tgm2−/− and Tgm2+/+ mice at 24 weeks of age. (b) Average adipocyte area shows a significant decrease in the adipocyte area in Tgm2−/− compared with Tgm2+/+ mice. (c) Average adipocyte number was significantly increased in Tgm2−/− mice compared with Tgm2+/+. Results are mean values ± SEM (n = 3), *P < 0.05. Scale bar equals 100 µm.
accumulation during the early phase of adipogenesis. When exogenous TG2 was added, a significant decrease in lipid accumulation was seen and this was associated with an increase in nuclear accumulation of β-catenin as well as decreased Pparg and Cebpα mRNA expression. Interestingly, Pref-1 was shown to be a Wnt target gene and was, in fact, shown to be downregulated by TCF/β-catenin complex in fetal lung epithelial cells and MEFs. Furthermore, Pref-1 can act as a noncanonical Notch ligand and inhibit Notch signaling and, in turn, Wnt/β-catenin signaling is negatively regulated by Notch. This crosstalk between Notch and Wnt signaling may be one of the regulatory mechanisms for Pref-1 production. We are currently exploring the mechanisms how TG2 affects the Pref-1 protein regulation.

It is well documented that canonical Wnt signaling inhibits adipogenesis and promotes osteogenesis in MSCs. Canonical Wnt signaling can also inhibit adipogenesis in lineage committed preadipocytes. Furthermore, canonical Wnt receptor LRPs show increased adipogenesis. In smooth muscle cells, extracellular TG2 regulates canonical Wnt signaling and β-catenin nuclear translocation which promotes calcification of the cell culture system. This effect in smooth muscle cells is mediated by extracellular TG2 binding to LRPs on the smooth muscle cell surface and this reported interaction does not require transmembrane activity. In this study, we show that extracellular TG2 activates canonical Wnt signaling contributing to the inhibitory effect on adipogenesis. While we did not address the exact mechanism how exogenous extracellular TG2 here regulates β-catenin nuclear translocation, it is plausible that the mechanism is the same as in smooth muscle cells. It is also possible that the exogenous TG2 promotes β-catenin release from the plasma membrane E-cadherin to the cytosol and from there to nucleus. This concept is supported by the observation that exogenous TG2 addition caused an increase in cytosolic and nuclear pools of β-catenin without affecting the total β-catenin levels in protein extracts.

Consistent with previous work on the role of TG2 in maintaining cytoskeletal tension, we also report here that Tgm2-deficient MEFs have decreased ROCK kinase activity and decreased actin stress fibers, which also contributes to increased adipogenesis. Adipogenesis is characterized by change in cell shape, from spindle-shaped preadipocytes to round adipocytes and this transition is partly determined by the cytoskeletal tension. During adipogenesis filamentous actin from stress fibers is rearranged to cortical pattern and down regulation of ROCK kinase disrupts actin stress fibers. Inhibition of ROCK kinase was shown to promote adipogenesis and Akt signaling and conversely activating ROCK kinase inhibits adipogenesis. TG2 was reported to activate ROCK via two pathways - retinoic acid-induced TG2 enzymatic activity was reported to activate ROCK kinase by intracellular TG2 and cell surface TG2 was reported to amplify integrin mediated signaling to activate ROCK kinase in a non-enzymatic manner.

ECM quantity and quality is a major regulator of cytoskeleton and it is known that cell surface TG2 cooperates with α5β1 integrins to enhance FN-integrin binding which is required for FN assembly. Furthermore, TG2 has been suggested to stabilize ECM in a number of studies and factors such as TGFβ—an inhibitor of adipogenesis—was shown to increase cell surface expression of TG2 and increase FN assembly. FN matrix itself is a major inhibitor of adipogenesis and must be decreased for the preadipocytes to allow differentiation toward mature adipocytes. However, in our study, we show that the absence of TG2 does not affect FN matrix levels or solubility in preadipocytes which strongly suggests that TG2 is not involved in FN matrix assembly and that cytoskeletal alterations in Tgm2−/− MEFs are not mediated by ECM itself, but likely via cell surface TG2 and the manner cells adhere to ECM. Indeed, Tgm2−/− deficient fibroblasts have been demonstrated to have an adhesion defect. Moreover, the data strongly suggest that TG2 is not involved in MEF matrix assembly and does not appear to participate in MEF extracellular transamidation/crosslinking events. This is also supported by the fact that Tgm2−/− and Tgm2+/+ MEFs had similar levels of TGF activity—this activity likely deriving from FXIII-A. Interestingly, both TG2 and FXIII-A act as negative regulators of adipogenesis and thus they may have a complementary effect on adipogenesis.

Increased fat mass in obesity is associated with an increase in adipocyte cell size and/or adipocyte number which are reactions to expand adipose tissue upon need to increase energy storage. Defects in this expansion are linked to obesity-linked comorbidities such as development of type 2 diabetes. In this work, we have identified a new factor, TG2 that maintains preadipocyte state and thus acts as a negative regulator of adipogenesis. It is thus likely that regulation of TG2 in preadipocytes is tightly controlled to balance proliferation and differentiation. Further understanding of TG2 and its role and regulation in metabolic disorders would aid the development of new therapies to maintain healthy energy metabolism.

Materials and Methods

Animals. Tgm2+/+ mice were described before. Wild-type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Mice were kept under a normal diurnal cycle in a temperature-controlled room and fed with standard chow. Animal procedures (WAT extraction and MEF isolation) and study protocols were approved by the McGill University Animal Care Committee.

Antibodies and proteins. Antibodies against rabbit anti-Akt (pan), rabbit anti-phospho-Akt (Ser473) (D9E), rabbit anti-PPARγ, rabbit anti-histone, rabbit anti-Pref-1 were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). Rabbit anti-actin, mouse anti-tubulin antibodies were obtained from Sigma-Aldrich (St Louis, MO, USA). Rabbit anti-human recombinant MYPT1 (654–880) and rabbit anti-phospho-MYPT1 (Thr696) were from EMD Millipore (Billerica, MA, USA). Rabbit anti-β-catenin purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal TG2 Ab-3 antibody (Clones CUB 7402+TG100) was from Fisher Scientific (Fremont, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgM was purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Inc. Alexa Fluor 488 and 596, Fluor 568-phalloidin and Bodipy 493/503 were from Life Technologies (Grand Island, NY, USA).

Reagents. Dulbecco’s modified Eagle’s medium (DMEM) and 0.2 mg/ml EDTA from ATCC (Cedarlane, ON, Canada). Fetal bovine serum (FBS) and penicillin-streptomycin were from Gibco (Burlington, ON, Canada). Oil Red O, IGERAL, CA 630, dexamethasone, insulin, 3-isobutyl-1-methylxanthine (IBMX), 3,3’,5,5’-Tetramethylbenzidine (TMB) were from Sigma-Aldrich. Tretinoinzone were purchased from Santa Cruz Biotechnology. Sulfo-NHS-LC-biotin and...
5-(biotinamido)pentylamine were used from Pierce (Rockford, IL, USA). ECL kit was from Zymed Scientifique (Montreal, QC, Canada). All other reagents unless otherwise specified were purchased from Sigma-Aldrich or Fisher Scientific.

**MEF cell culture and differentiation.** Mouse embryonic fibroblasts (MEFs) were prepared from 13.5 days Tgm2+/+ and Tgm2−/− mouse embryos. MEFs isolation, culture and staining with Oil Red O was done according to previously published protocol. MEFs were differentiated into adipocytes with 10% FBS, 1 mM dexamethasone, 0.5 mM isobutyl-1-methylxanthine, 1 μg/ml insulin, and 10μM tiglatezone for 2 days. On day 2, media were replaced with maintenance medium which includes 10% FBS and 1 μg/ml insulin and 10μM tiglatezone. On day 4, maintenance media was replaced with medium containing 10% FBS and cells were cultured in this until the end of the experiment, that is, day 8. Intraglucercell triadicer was stained with Oel Red O and quantified; cells were counter stained with haematoxylin and photographed with a light microscope.

**Whole-mount staining, immunofluorescence and histology.** For whole-mount staining, mouse WAT from Tgm2+/- mice was fixed in 10% neutral-buffered formalin. Fixed tissue was cut with a scalpel to 5 mm x 5 mm sections, and blocked with 3% BSA, 0.3% Triton X-100, in PBS for 12-24 hr at 4 °C. Tissue pieces were incubated with primary antibodies overnight at 4 °C which was followed by incubation with Alexa Fluor-conjugated secondary antibodies for 1 hr at room temperature. Nuclei were stained with DAPI. Antibody omission and isotype specific incubation with Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature or with 1% formaldehyde for 15 min at room temperature were used to check specific binding. Antibodies used as controls. For histology, mouse epididymal fat pad was fixed in 10% neutral-buffered formalin. Tissues were cut with a scalpel to 5 mm × 5 mm sections, and fixed in 10% neutral-buffered formalin. Fixed tissue was cut with a scalpel to 5 mm × 5 mm sections, and fixed with 10% neutral-buffered formalin for 30 min at room temperature or with 1% formaldehyde for 15 min at room temperature for detection of cell surface protein staining. Staining was performed as previously described. Quantification was done with Image J (v1.34i, NIH, Bethesda, MD, USA).

**Protein extraction and western blotting.** Total cell lysate was prepared with lysis buffer containing 20 mM Tris-Cl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% DOC, 0.5% Triton X-100, 1 mM PMSE and 1 mM orthovanadate and protease inhibitor cocktail (Sigma). Cells were lysated in ice on 30 min with occasional vortexing and then centrifuged for 15 min at 10,000 x g at 4 °C. Nuclear and cytosolic fractions were prepared as described previously.63 Deoxoycholylate (DOC)-soluble and DOC-insoluble FN matrix extracts were prepared as described previously.64 Western blotting and quantification of bands with Image J (v1.34i, NIH) was done as previously described.10

**Cell surface biotinylation.** Cell surface biotinylation was done for Tgm2+/+ MEFs as previously described.10

**RT-PCR and Real-time PCR.** mRNA was isolated using Trizol method. RNA was treated with DNase (New England Biolabs, Ipswich, MA, USA), and PCR was performed with SuperScriptII One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada). PCR products were analyzed by 1% agarose gel electrophoresis. Primers used were previously described Pref-1, EDA, EDB, Fn, Gapdh, Pparα, Pparγ, Pref-1, and Gapdh. Real-time PCR was performed on an ABIHT7900 RT-PCR machine using the comparative C_T method in triplicate using the TaqMan Universal Master Mix II. Expression levels of Pref-1, EDA, EDB, Fn, Gapdh, Pparα, Pparγ, Pref-1, and Gapdh were determined and normalized to Rn18S (Mm 00184322_m1, Cebpb (Mm 514283_s1) and normalized to Rn18S (Mm 03928990_g1).
25. Belkin AM. Extracellular TG2: emerging functions and regulation. FEBS J 2011; 278: 4704–4716.
26. Klock C, Diraimondo TR, Khosla C. Role of transglutaminase 2 in celiac disease pathogenesis. Semin Immunopathol 2012; 34: 513–522.
27. Hudak CS, Sul HS. Pref-1 a gatekeeper of adipogenesis. Front Endocrinol (Lausanne) 2013; 4: 79.
28. Kim KA, Kim JS, Wang Y, Sul HS. Pref-1 (preadipocyte factor 1) activates the MEX/extracellular signal-regulated kinase pathway to inhibit adipocyte differentiation. Mol Cell Biol 2007; 27: 2234–2308.
29. Wang Y, Kim KA, Kim JS, Sul HS. Pref-1, a preadipocyte secreted factor that inhibits adipogenesis. J Biol Chem 2006; 281: 2953–2966.
30. Faverman L, Mikhaylova L, Malmquist J, Nurminskaya M. Extracellular transglutaminase 2 activates beta-catenin signaling in calcifying vascular smooth muscle cells. FEBS Lett 2008; 582: 1552–1557.
31. Christodoulides C, Lagathu C, Sethi JK, Vidal-Puig A. Adipogenesis and WNT signalling.
32. Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL et al. Cell surface transglutaminase promotes RhoA activation via integrin clustering and suppression of the Src-p190RhoGAP signaling pathway. Mol Cell Biol 2006; 26: 1608–1618.
33. Novoguchi M, Hoshida K, Fujikura J, Fujimoto M, Ikawa K, Tomita T et al. Genetic and pharmacological inhibition of Rho-associated kinase II enhances adipogenesis. J Biol Chem 2007; 282: 29574–29583.
34. Amano M, Nakayama M, Kikuchi KRho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity/Cytokeleton (Hoboken) 2010; 67: 545–554.
35. Aubin D, Gagnon A, Sorsisky A. Phosphorytosis 3-kinase is required for human adipocyte differentiation in culture. Int J Obes (Lond) 2005; 29: 1006–1009.
36. Kim JE, Chung J. Regulation of peroxisome proliferator-activated receptor-gamma activity by mammalian target of rapamycin and amino acids in adipogenesis. Diabetes 2004; 53: 769–776.
37. Smas CM, Sul HS. Pref-1 a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 1993; 73: 725–734.
38. Wang Y, Zhao L, Smas C, Sul HS. Pref-1 interacts with fibronectin to inhibit adipocyte differentiation. Mol Cell Biol 2010; 30: 3490–3492.
39. Mochida VS, Smas CM, Lee K, Villena JA, Kim KH, Yun EJ et al. Mice lacking paternal expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol Cell Biol 2002; 22: 5855–5862.
40. Lee K, Villena JA, Moon YS, Kim KH, Lee S, Kang C et al. Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor 1 (Pref-1). J Clin Invest 2003; 111: 453–463.
41. Villena JA, Choi CS, Wang Y, Kim S, Hwang YJ, Kim YB et al. Resistance to high-fat diet-induced obesity but exacerbated insulin resistance in mice overexpressing preadipocyte factor 1 (Pref-1): a new model of partial lipodystrophy. Diabetes 2008; 57: 3288–3286.
42. Wang Y, Sul HS. Pref-1 regulates mesenchymal cell commitment and differentiation through Sirpα. J Cell Metab 2009; 9: 287–302.
43. De Laurenzi V, Melino G. Gene disruption of tissue transglutaminase. Mol Cell Biol 2001; 21: 148–155.
44. Tarantino U, Oliva F, Taurisano G, Orlandi A, Pietroni V, Candi E et al. FXIIIa and TGF-beta over-expression produces normal muscle-skeletal phenotype in TG2-/ mice. Amino Acids 2009; 36: 679–684.
45. Molds M, Zuo Y, Morrison RF, Silva D, Park BH, Liu J et al. Peroxisome proliferator-activated receptor gamma suppresses Wnt/beta-catenin signaling during adipogenesis. Biochem J 2003; 378: 607–613.
46. Ginun GD, Smith WM, Droi S, Sarraf P, Mueller E, Eng C et al. APC-dependent suppression of colon carcinogenesis by PPARgamma. Proc Natl Acad Sci USA 2002; 99: 13771–13776.
47. Liu J, Wang H, Zuo Y, Farmer SR. Functional interaction between peroxisome proliferator-activated receptor gamma and beta-catenin. Mol Cell Biol 2006; 26: 5873–5877.
48. Weng T, Gao L, Bhaskaran M, Guo Y, Gou D, Narayanaperumal J et al. Pleiotrophin regulates lung epithelial cell proliferation and differentiation during fetal lung development via beta-catenin and Dkk1. J Biol Chem 2008; 284: 28021–28032.
49. Paul C, Sarder C, Fabbrizzo E. The Wnt-target gene Dkk-1 is regulated by the Prmt5-associated factor Copr during adipogenic conversion. Bio Cell 2015; 4: 312–316.
50. Galceran J, Suttmann C, Hsu SC, Fobert S, Grosschedl R. LEF-1-mediated regulation of Delta-like1 links Wnt and Notch signaling in somitogenesis. Genes Dev 2004; 18: 2718–2723.
51. Andersen P, Uosaki H, Shene J, Ltv, Kwok C. Non-canonical Notch signaling: emerging role and mechanism. Trends Cell Biol 2012; 22: 257–265.
52. Takada I, Kouzmenko AP, Wnt Kato S, and PPARgamma signaling in osteoblastogenesis and adipogenesis. Nat Rev Rheumatol 2009; 5: 442–447.
53. Kawai M, Mushiake S, Bassho K, Murakami M, Namba N, Kokubu C et al. Wnt/Lrp/beta-catenin suppression enhances adipogenesis by inhibiting mutual activation of PPARgamma and c-EBPalpha. Biochem Biophys Res Commun 2007; 363: 278–282.
54. McBeath R, Prime DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 2004; 4: 483–495.
55. Lee DH, Shl i, Jeoung NH, Kim MS, Zabolotny JM, Lee SW et al. Targeted disruption of ROCK1 causes insulin resistance in vivo. J Biol Chem 2009; 284: 11776–11780.
56. Singh US, Kumar MT, Kao YL, Baker KM. Role of transglutaminase II in retinoic acid-induced activation of Rho-associated kinase-2. EMBO J 2001; 20: 2413–2423.
57. Chiquet M, Gelman L, Lutz R, Maier S. From mechanotransduction to extracellular matrix gene expression in fibroblasts. Biochim Biophys Acta 2009; 1792: 911–920.
58. Akimov SS, Belkin AM. Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin: a role in TGFBeta-dependent matrix deposition. J Cell Sci 2001; 114: 2989–3000.
59. Telci D, Wang Z, Li X, Verdeno EA, Humphries MJ, Baccarin M et al. Fibronectin-tissue transglutaminase matrix rescues RGD-impaired cell adhesion through syndecan-4 and bet1 integrin co-signalizing. J Biol Chem 2008; 283: 20937–20947.
60. Wang Z, Collignon RA, Gross SR, Danen EH, Orend G, Telci D et al. RGD-independent cell adhesion via a tissue transglutaminase-fibronectin matrix promotes fibronectin fibril deposition and requires syndecan-4 alpha3beta1 integrin co-signalizing. J Biol Chem 2009; 284: 4022–4029.
61. Rosner M, Hengstschläger M. Cytosplasic and nuclear distribution of the protein complexes mTORC1 and mTORC2: rapamycin triggers dephosphorylation and delocalization of the mTORC2 components raptor and sin1. Hum Mol Genet 2006; 17: 2948–2954.
62. Palmon R, Yamada KM. Non-radioactive quantification of fibronectin matrix assembly. In: Bonifacino JS et al. (ed). Current Protocols in Cell Biology, Chapter 10, Unit 10. John Wiley & Sons: Hoboken, NJ, USA, 2004, p3.
63. Han J, Farmer SR, Kirkland JL, Corkey BE, Yoon R, Prakashalava T et al. Octanoate attenuates adipogenic conversion in 3T3-L1 preadipocytes. J Nutr 2002; 132: 904–910.
64. Han F, Adams CS, Tao Z, Williams CJ, Zaka R, Tuan RS et al. Transforming growth factor-beta1 (TGF-beta1) regulates ATDC5 chondrogenic differentiation and fibronectin isoform expression. J Cell Biochem 2005; 95: 750–762.
65. Tanabe Y, Koga M, Saito M, Matsunaga Y, Nakayama K. Inhibition of adipocyte differentiation by mechanical stretching through ERK-mediated downregulation of PPARgamma2. J Cell Sci 2004; 117: 3605–3614.

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