TPD52 expression increases neutral lipid storage within cultured cells

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Abstract

Tumor protein D52 (TPD52) is amplified/over-expressed in cancers of diverse cellular origins. Altered cellular metabolism (including lipogenesis) is a hallmark of cancer development, and protein-protein associations between TPD52 and known regulators of lipid storage, and differential TPD52 expression in obese versus non-obese adipose tissue, suggest that TPD52 may regulate cellular lipid metabolism. We found increased lipid droplet numbers in stably TPD52-expressing BALB/c 3T3 cell lines, compared with control and TPD52L1-expressing cell lines. TPD52-expressing 3T3 cells showed increased fatty acid storage in triglyceride (from both de novo synthesis and uptake), and formed greater numbers of lipid droplets upon oleic acid supplementation than control cells. TPD52 co-localised with Golgi but not ER markers, and also showed partial co-localisation with Adrp-coated lipid droplets, with a proportion of TPD52 being detected in the lipid droplet fraction. Direct interactions between ADRP and TPD52, but not TPD52L1, were demonstrated using the yeast two-hybrid system, with ADRP/TPD52 interactions confirmed using GST pull-down assays. Our findings uncover a novel, isoform-specific role for TPD52 in promoting intracellular lipid storage, which may be relevant to TPD52 overexpression in cancer.
Introduction

TPD52 is a candidate oncogene located at chromosome 8q21.13, which is frequently amplified or gained in human cancer (Byrne et al., 2012; Byrne et al., 2014; Shehata et al., 2008b). TPD52 overexpression has been reproducibly associated with poor outcomes in breast carcinoma (Byrne et al., 2014), and aggressive phenotypes in most cancers examined (Adler et al., 2006; Bismar et al., 2006; Byrne et al., 2014; Shehata et al., 2008b). TPD52 is the founding member of the TPD52-like protein family, where members share approximately 50% sequence identity. At the molecular level, TPD52-like proteins exhibit functional redundancy, in that heterologous partners identified through yeast two-hybrid screens using a single TPD52-like bait also interact with related TPD52-like proteins (Wilson et al., 2001; Proux-Gillardeaux et al., 2003; Shahheydari et al., 2014). However, stable expression of TPD52 or its parologue TPD52L1 in BALB/c 3T3 cells produced shared but also isoform-specific cellular effects (Lewis et al., 2007; Shehata et al., 2008a). Exogenous TPD52 but not TPD52L1 expression increased proliferation and anchorage independent growth of 3T3 cells, whereas both proteins produced similar morphological changes (Shehata et al., 2008a). Similarly, TPD52 but not TPD52L1 transcript levels were significantly higher in breast carcinoma samples, relative to normal breast tissue (Shehata et al., 2008a). These results suggest that isoform-specific functions for TPD52 not shared by TPD52L1 underpin the oncogenic effects of TPD52 overexpression.

A hallmark of cancer cells is deregulated cellular metabolism (Luo et al., 2009), with a number of studies focusing upon lipogenesis (Budhu et al., 2013; Kumar-Sinha et al., 2003; Wang et al., 2013). Actively proliferating cells require lipids to build new membranes, lipid cofactors and lipid-modified proteins (Brasaemle, 2007; Vander Heiden et al., 2009), yet the cytotoxicity of many lipid species requires their conversion to and storage as neutral lipids (e.g. triglycerides, TAG; cholesterol esters, CE) within lipid droplets (Listenberger et al., 2003). Lipid droplets are highly complex, dynamic organelles which actively participate in lipid metabolism and cellular signalling,
controlling intracellular lipid trafficking, and interacting with other organelles (Walther and Farese, 2012). Lipid droplets consist of a neutral lipid core surrounded by a phospholipid monolayer, and are coated by one or more members of the perilipin (PAT) family (perilipin/PLIN1, ADRP/PLIN2, TIP47/PLIN3, S3-12/PLIN4, and OXPAT/MLDP/PLIN5) (Brasaemle, 2007), and a diverse array of other proteins (Krahmer et al., 2009; Walther and Farese, 2012). It is commonly proposed that lipid droplets form within the endoplasmic reticulum (ER) and are transported from the ER to the Golgi apparatus, where more TAG is loaded and more proteins are attached (Fujimoto and Parton, 2011; Walther and Farese, 2012; Wilfling et al., 2014).

Lipid droplets are constitutively present in fat-storing cells, including adipocytes and steroidogenic cells. Although present in low numbers in most other cell types, increased numbers of lipid droplets can occur in cancer cells (Bozza and Viola, 2010). A lipogenic phenotype has been particularly associated with ERBB2-positive breast cancers. Increased FASN expression was noted in response to exogenous ERBB2 expression in breast cancer cells (Kumar-Sinha et al., 2003), and genes encoding other regulators of lipid metabolism may be co-amplified with ERBB2 at chromosome 17q (Kourtidis et al., 2010). TPD52 expression has been reproducibly associated with ERBB2 expression in human breast cancer cell lines and tissues, and in mammary tissues from Erbb2 transgenic mice (Byrne et al., 2014; Kourtidis et al., 2010; Roslan et al., 2014). On the other hand, knock-down of the C. elegans TPD52 orthologue F13E6.1 significantly reduced lipid storage in a genome-wide screening study (Ashrafi et al., 2003), and expression microarray analyses have identified increased TPD52 levels in mouse and human adipose tissue from obese versus lean subjects (Clement et al., 2004; Keller et al., 2008; Nadler et al., 2000). TPD52 was also identified as a perilipin binding partner in a yeast two-hybrid screen (Yamaguchi et al., 2006), and TPD52 co-immunoprecipitated with TIP47 and other proteins (Zhang et al., 2007). These studies suggest the possible involvement of TPD52 in regulating lipid metabolism.
Here, we show for the first time that TPD52 but not TPD52L1 expression increases lipid droplet numbers in cultured cells, and that TPD52 expression also promotes fatty acid storage in TAG. We also demonstrate that TPD52 co-localised with both Golgi markers and Adrp-coated lipid droplets, with further evidence supporting a direct interaction between TPD52 and ADRP.

Results

Stable TPD52 expression increased cellular lipid droplet numbers in 3T3 fibroblast and MDA-MB-231 breast cancer cells

We have previously reported the effects of TPD52 and TPD52L1 expression in 3T3 fibroblast cells (Shehata et al., 2008a), whose sequences are aligned in Fig. 1A. In the present study, we assessed lipid droplets in BALB/c 3T3 parental cells and those stably transfected with PG307 vector only (vector-2 and/or vector-3), PG307-TPD52 (D52-1-12, D52-2-1, and D52-2-7), or PG307-TPD52L1 (D52L1-4 and D52-L1-6), using BODIPY 493/503 staining. Compared with parent and vector (vector-3) cells, TPD52-expressing cell lines (D52-2-1, D52-2-7) showed strikingly increased cytosolic lipid droplet numbers (Fig. 1B, data not shown). TPD52L1-expressing 3T3 cell lines showed similar lipid droplet staining as parental and vector-3 control cell lines (Fig. 1B). Quantification of lipid droplets confirmed a significant increase in mean lipid droplet numbers per cell in two TPD52-expressing 3T3 cell lines, but not in two TPD52L1-expressing 3T3 cell lines, compared to vector control cells (Fig. 1C). This corresponded to an approximate 10- and 4-fold increase in mean lipid droplet numbers per cell in D52-2-7 cells and D52-2-1 cells, respectively, relative to vector-3 control cells (Fig. 1C). There was also a smaller but significant increase in the mean lipid droplet area (μm²) in D52-2-7 cells compared with vector-3 control cells (Fig. 1D). Levels of TPD52 or TPD52L1 in respective cell lines were validated using Western blot analyses (Fig. 1E). Electron microscopy analyses of vector-3 and D52-2-7 cells further confirmed lipid droplet accumulation in D52-2-7 cells (supplementary material, Fig. S1A,B). Numerous whorled,
laminated structures were also noted in D52-2-7 cells (supplementary material, Fig. S1B), which resembled phospholipid inclusions (O’Farrell et al., 2001).

To determine whether these phenotypes were confined to 3T3 cells, we examined a second stably TPD52-expressing cell line, derived from the breast cancer cell line MDA-MB-231 with low endogenous TPD52 expression levels (Roslan et al., 2014). Again, increased lipid droplets were detected in TPD52-transfected MDA-MB-231 cells (TPD52-H1D2) compared to vector control cells (supplementary material, Fig. S1C).

**TPD52-expressing 3T3 cells form more lipid droplets following oleic acid supplementation**

We next examined how 3T3 cell lines would respond to exogenous fatty acid supplementation. Following 400 μM oleic acid (OA) treatment for 24 hours, lipid droplet numbers per cell were significantly increased in all 3T3 cell lines examined, relative to parallel cultures harvested prior to OA supplementation (Fig. 2A,B). However, significantly more lipid droplets per cell were measured in TPD52-expressing cells, compared with parent and vector control cells (Fig. 2A,B). Quantitation of lipid droplets showed a significant increase (~2-fold) in the mean lipid droplet number per cell in all 3 TPD52-expressing cell lines post OA treatment, compared with OA-treated vector control cells (Fig. 2B). All 3 TPD52-expressing 3T3 cells showed very similar mean lipid droplet numbers per cell after OA treatment, despite different basal numbers of lipid droplets per cell (Fig. 2B). Individually, there were ~18- and ~14-fold increases in mean lipid droplet numbers per cell in parent and vector control cells, respectively, after OA treatment, but only approximately 4-fold increases in D52-1-12 and D52-2-1 cells, and a 2-fold increase in D52-2-7 cells (Fig. 2C). Increased lipid droplet numbers after 24 hours OA treatment were associated with significantly increased lipid droplet areas in each cell line (Fig. 2D), with similar relative fold changes in lipid droplet areas measured post OA treatment in all 3T3 cell lines analysed (Fig. 2E). However,
significantly larger lipid droplets were measured in 2/3 TPD52-expressing cell lines (D52-1-12 and D52-2-7) post OA treatment, compared with OA-treated vector control cells (Fig. 2D).

**TPD52 expression alters fatty acid metabolism in 3T3 fibroblast cells**

To explore the mechanisms by which TPD52 increases cellular lipid storage, we compared fatty acid metabolism in 3T3 cell lines. Consistent with significant increases in lipid droplet numbers, the levels of cellular TAG, the main component of lipid droplets, were also significantly elevated in TPD52-expressing cells, most notably in D52-2-7 cells, where TAG levels were approximately 4 times higher than those of vector controls (Fig. 3A). Cells were treated with [3H]acetic acid to trace fatty acid synthesis, and with [1-14C]oleate that can be taken up by cells. The rates of *de novo* fatty acid incorporation into TAG (*de novo* lipogenesis) were significantly increased in D52-1-12 (~2-fold) and D52-2-7 (~1.5-fold) cells compared with parent and vector cells (Fig. 3B). Similarly, the incorporation rates of media-supplied [1-14C]oleate into TAG were also significantly increased in D52-1-12 (~2-fold) and D52-2-7 (~1.5-fold) cells (Fig. 3C). In contrast, rates of [1-14C]oleate uptake differed significantly between vector control and parent 3T3 cells, but not between vector control and D52-2-7 cells (Fig. 3D), despite the latter consistently demonstrating the highest number of lipid droplets per cell (Fig. 1B,1C, Fig. 2A,2B).

To examine the molecular mechanisms contributing to increased fatty acid incorporation into TAG observed in TPD52-expressing cells, we examined the levels of key enzymes in 3T3 cell lines cultured without OA supplementation, using Western blot analyses. There were no significant differences in the levels of fatty acid synthase (Fasn) (Fig. 3E), acetyl-CoA carboxylase (Acc) (data not shown), stearoyl-CoA desaturase 1 (Scd1) (Fig. 3E), or diacylglycerol acyltransferase 2 (DGAT2) (data not shown), according to TPD52 expression status. Similarly, there were no obvious differences in Adrp levels, the major PAT protein that regulates lipid droplets in non-
adipocytes (Brasaemle et al., 1997) (Fig. 3E), and no perilipin was detected as a marker of adipocyte differentiation (data not shown).

Excess free saturated fatty acids such as palmitic acid are toxic to cells, whereas unsaturated fatty acids could rescue palmitate-induced apoptosis by channelling palmitate into triglyceride pools and away from pathways leading to apoptosis (Listenberger et al., 2003). We investigated whether TPD52 expression would provide protective effects to cells upon palmitic acid treatment. MTT assays performed over 4 days post 250 μM palmitic acid treatment showed significantly increased cell survival in D52-1-12 and D52-2-7 cells, compared with that of parent and vector cells (Fig. 3F).

Co-localisation of TPD52 with a Golgi but not an ER marker in TPD52-expressing cells

Studies have indicated that lipid droplet formation is initiated in the ER (Martin and Parton, 2006). Indirect immunofluorescence analyses of TPD52 show granular cytoplasmic staining with a perinuclear accentuation in MCF-7 breast cancer (Balleine et al., 2000) and TPD52-expressing 3T3 cells (Chen et al., 2013). As TPD52 further accumulated at the perinuclear region of TPD52-expressing 3T3 cells upon OA treatment (supplementary material, Fig. S2B), we examined whether TPD52 could be associated with the ER using protein disulfide isomerase (Pdi) (Ohsaki et al., 2008). While Pdi staining was detected throughout the cytoplasm in both D52-2-7 and vector control cells, with or without 24 hours of 400 μM OA treatment (supplementary material, Fig. S2), Pdi did not obviously co-localise with TPD52 in D52-2-7 cells (supplementary material, Fig. S2B).

The Golgi apparatus also plays an important role in lipid droplet growth (Hesse et al., 2013; Kalantari et al., 2010), so we compared TPD52 staining with that of the Golgi matrix protein Gm130 (Golgin 95, also GOLGA2) (Marra et al., 2001). Although there were no obvious differences in Gm130 staining between D52-2-7 (Fig. 4A) and vector control cells (supplementary
material, Fig. S3A,B), perinuclear TPD52 staining co-localised with Gm130 in D52-2-7 cells (Fig. 4A). Following 6 hours of 400 µM OA treatment, this co-localisation became more prominent in some cells (Fig. 4B, cells 1 and 2). Quantification of the extent of co-localisation between Gm130 and TPD52 (Fig. 4) using Pearson’s correlation and Manders’ overlap coefficients showed that this significantly increased from median values of 0.31 and 0.42 (no OA, n=28) to 0.38 and 0.48 (6 hr OA, n=23) (Pearson’s correlation coefficients, $P<0.0001$; Manders’ overlap coefficients, $P<0.0001$; Mann Whitney $u$ test, data not shown). Co-localisation of TPD52 and Gm130 was further confirmed in D52-1-12 and D52-2-1 cells with and without OA treatment (supplementary material, Fig. S4).

**Co-localisation of TPD52 with Arl1 on Golgi in TPD52-expressing cells**

The GTPase ARL1 (ADP-ribosylation factor-like 1) coordinates Golgin recruitment from the cytosol to Golgi membranes in a GTP-dependent manner (Gillingham and Munro, 2003), which also requires ARFRP1 (ADP-ribosylation factor-related protein 1) (Zahn et al., 2006). It has been proposed that the ARFRP1-ARL1-Golgin-Rab cascade participates in the control of lipid droplet formation (Hesse et al., 2013; Hommel et al., 2010). We investigated whether Arl1 co-localised with Gm130 as well as TPD52. As expected (Lu et al., 2004), Arl1 showed incomplete peri-nuclear co-localisation with Gm130 in D52-2-7 cells with or without OA treatment (Fig. 5A), and in vector control cells (supplementary material, Fig. S3). Arl1 also co-localised with TPD52 in untreated D52-2-7 cells, with more prominent co-localisation detected after OA treatment (Fig. 5B), and comparable results were obtained in D52-1-12 and D52-2-1 cells (data not shown). As for Gm130, quantification of the extent of co-localisation between Arl1 and TPD52 (Fig. 5B) using Pearson’s correlation and Manders’ overlap coefficients showed that this significantly increased from median values of 0.31 and 0.59 (no OA, n=21) to 0.41 and 0.66 (24 hr OA, n=21) (Pearson’s correlation coefficients, $P=0.0022$; Manders’ overlap coefficients, $P=0.0003$; Mann Whitney $u$ test, data not shown). As the fixation and permeabilisation method required for Arl1 detection did not allow for
BODIPY 493/503 co-staining, we used Adrp as a surrogate for lipid droplet staining (Imamura et al., 2002), having also confirmed the co-incidence of Adrp and BODIPY 493/503 staining in TPD52-expressing 3T3 cells (data not shown). Western blot analyses showed no differences in Arl1 or Arfrp1 levels between TPD52-expressing and control cells (supplementary material, Fig. S3C).

Limited co-localisation of TPD52 and Adrp in TPD52-expressing 3T3 cells

ADRP is the predominant PAT protein that coats small lipid droplets in non-adipocytes (Bickel et al., 2009). We therefore compared the sub-cellular distribution of Adrp and TPD52 in 3T3 cell lines. Adrp showed clustered ring structures in D52-2-7 cells, which were largely excluded from the Golgi/TPD52 perinuclear region (Figs. 5,6A,6B cell 1). However, a proportion of TPD52 was detected on Adrp-stained ring structures resembling lipid droplets in both untreated (Figs. 5B,6A) and OA-supplemented D52-2-7 cells, with the latter showing larger grape-like Adrp-stained clusters (Figs. 5B,6B cell 2), than those detected in vector control cells (supplementary material, Fig. S3A,B and data not shown). Quantification of the extent of co-localisation between Adrp and TPD52 (Fig. 6) using Pearson’s correlation and Manders’ overlap coefficients showed that this significantly increased from median values of 0.15 and 0.17 (no OA, n=25) to 0.29 and 0.33 (6 hr OA, n=19) (Pearson’s correlation coefficients, P<0.0001; Overlap coefficients, P<0.0001; Mann Whitney u test, data not shown).

Altered lipid droplet distribution in TPD52-expressing versus vector control 3T3 cells

We also noted differences in the distribution of Adrp-stained lipid droplets between TPD52-expressing and vector control cells. Adrp-stained lipid droplets formed clusters towards the periphery of D52-2-7 cells, which became more prominent upon OA treatment (Figs. 6,7A), whereas lipid droplets accumulated closer to the nuclei of vector-2 cells (Fig. 7A, supplementary material, Fig. S3A,B). To further address this, we quantified the distance between the centre of lipid
droplets to the centre of the respective cell nucleus, with more than 1,900 lipid droplets quantified for each setting. In vector control cells, 96.6% Adrp-stained bodies were distributed less than 100 pixels from the centre of each nucleus (Fig. 7B). In contrast, 83.7% objects in D52-2-7 cells were distributed less than 100 pixels from the centre of each nucleus, with OA treatment having no significant effect on either distribution (Fig. 7B). Statistical analyses confirmed significant differences in lipid droplet distributions between D52-2-7 and vector cells with and without OA treatment (Fig. 7C).

**Direct interactions between TPD52, but not TPD52L1, and ADRP/TIP47**

Based on the co-localisation of TPD52 and Adrp, and possible roles for TPD52 in lipid droplet formation/trafficking, we investigated whether TPD52 (Fig. 1A) directly interacts with Adrp. We first employed the yeast two-hybrid system which has previously identified TPD52 binding partners (Boutros et al., 2003; Byrne et al., 1998; Proux-Gillardueaux et al., 2003; Shahheydari et al., 2014). Co-transfection of 1-415 aa ADRP or full-length TIP47 bait and TPD52 prey constructs produced detectable Hf7c yeast growth on triple drop-out media after 5 days and 3 days, respectively (Fig. 8A). However, co-transfection of TIP47 or ADRP bait with TPD52L1 prey (Fig. 1A) resulted in no detectable yeast growth after 9 days (Fig. 8A).

To further confirm interactions between TPD52 and ADRP, we performed GST pull-down assays. Since the molecular weight of Adrp and GST-tagged TPD52/Tpd52 are both approximately 50 kDa, GST proteins were cross-linked to GSH beads. Adrp was recovered by GST-tagged mouse (Tpd52) or human TPD52, but not by the GST tag alone, from D52-1-12 and D52-2-7 3T3 cell lysates (Fig. 8B). Interactions between GST-tagged Tpd52/TPD52 and Rab5 were used as positive controls (Shahheydari et al., 2014) (Fig. 8B).
To examine whether TPD52 also localises in lipid droplet fractions, we isolated lipid droplet fractions from D52-2-7 cells treated with 400 μM OA for 24 hours using sucrose density centrifugation. As previously reported (Brasaemle and Wolins, 2006), lipid droplets floated to the top of the gradient and were enriched in Adrp (Fig. 8C). A small proportion of both TPD52 and Arl1 were detected in this fraction, as was Rab5 (Brasaemle et al., 2004; Cho et al., 2007; Liu et al., 2007; Sato et al., 2006) (Fig. 8C). However, we did not detect Snap23 or Vamp4 within this lipid droplet fraction (Fig. 8C), even after longer exposures (data not shown). This contrasts with the findings of Boström et al. (2007), who reported that Snap23 and Vamp4, as well as NSF, α-Snap and Syntaxin-5, were associated with lipid droplets in ADRP-transfected NIH-3T3 cells. We confirmed no obvious changes in the protein levels of Nsf1, Snap 23, Syntaxin-5 and -6, and Vamp 4, according to TPD52 expression status in 3T3 cells (Fig. 8D).

**Discussion**

Despite increasing recognition of TPD52 overexpression in various cancer types (Tennstedt et al., 2014; Byrne et al., 2014), little is known about the molecular or cellular functions of TPD52. This study has provided experimental evidence that TPD52 regulates cellular lipogenesis and lipid storage, which supports previous results of high-throughput studies, where for example F13E6.1 (TPD52) knock-down in *C. elegans* significantly reduced lipid storage (Ashrafi et al., 2003), and increased *Tpd52* or *TPD52* levels were identified in mouse and human adipose tissue from obese versus lean subjects (Clement et al., 2004; Keller et al., 2008; Nadler et al., 2000). Our findings are also consistent with TPD52 being androgen-inducible in prostate cancer (Rubin et al., 2004; Wang et al., 2004), with androgen representing a potent regulator of lipogenesis and lipid metabolism in this disease (Massie et al., 2011; Swinnen and Verhoeven, 1998).

Our results suggest that TPD52 may enhance lipid storage through more than one mechanism. Increased numbers of lipid droplets were consistently noted in TPD52-expressing 3T3 fibroblastic
cell lines, and MDA-MB-231 breast cancer cells with exogenously increased TPD52 levels, relative to vector controls. Following OA supplementation, all 3 TPD52-expressing 3T3 cell lines also showed significantly increased lipid droplet numbers compared with both untreated cells, and OA-treated vector control cells, without corresponding differences in fatty acid uptake rates which could underpin these phenotypes. This suggested that TPD52 may contribute to lipid droplet formation within the ER, which is supported by previous reports of TPD52 being an ER-associated protein (Hoja et al., 2000). However, TPD52 showed no detectable co-localisation with Pdi in TPD52-expressing 3T3 cells, but instead showed perinuclear co-localisation with the Golgi markers Gm130 and Arl, in the presence and absence of OA supplementation. TPD52, Gm130 and Arl1 detection became more prominent within this perinuclear compartment following OA treatment, as did the degree of co-localisation between TPD52 and Gm130 or Arl1 in some cells. This suggests that rather than influencing lipid droplet formation within the ER, TPD52 may contribute to lipid loading within the Golgi, leading to growth of lipid droplets within this compartment, and facilitating their detection by light microscopy. Expansion of the Golgi compartment and increased co-localisation between TPD52 and Gm130 and Arl1 in OA-supplemented 3T3 cell lines indicates that these cells increased their reliance upon Golgi function in response to increased exogenous lipid loading. In support of a Golgi-located function for TPD52 in regulating lipid droplets, TPD52 has been reported to interact with members of two protein families involved in the ARFRP1-ARL1-Golgin-Rab cascade (Hesse et al., 2013), namely Rab and Golgin proteins (Messenger et al., 2014; Shahheydari et al., 2014).

In addition to localising to the Golgi apparatus in TPD52-expressing 3T3 cell lines, TPD52 showed limited, more peripheral co-localisation with the lipid droplet protein Adrp/Plin2. A small proportion of TPD52 was detected within the lipid droplet fraction of D52-2-7 cells, which is supported by the Drosophila orthologue CG5174 having been previously detected within the lipid droplet proteome (Cermelli et al., 2006). TPD52 also bound both ADRP and TIP47 in the yeast two
hybrid system, and TPD52/ADRP interactions were validated using GST pull-downs. TPD52 was previously identified as a binding partner of perilipin residues 1-240 in a yeast two-hybrid screen (Yamaguchi et al., 2006), and as a TIP47 co-immunoprecipitating protein (Zhang et al., 2007). This indicates that TPD52 may directly co-operate with PAT proteins, to promote either lipid droplet trafficking and/or lipid storage at the lipid droplet surface.

Our results identified increased lipid droplet numbers in TPD52- but not TPD52L1-expressing 3T3 cell lines, and while we detected interactions between TPD52 and ADRP or TIP47, similar interactions were not detected for TPD52L1. At present, it is not known why TPD52 but not TPD52L1 may bind PAT proteins. TPD52-like genes commonly include alternatively-spliced exons encoding a 14-3-3 binding site (Boutros et al., 2003), where the relevant exon is more commonly included in TPD52L1 transcripts (Nourse et al., 1998), but less frequently included in TPD52 transcripts (Boutros et al., 2003; Nourse et al., 1998; Wang et al., 2004). A 14-3-3 binding site was present in the TPD52L1 isoform expressed in 3T3 cells and employed in interaction testing, but was absent from the TPD52 isoform employed in these and other functional analyses (Boutros et al., 2003; Shehata et al., 2008a). It is possible that the presence of a 14-3-3 binding site in TPD52-like proteins acts as a molecular switch, allowing interactions with 14-3-3 proteins and preventing interactions with PAT proteins. In support of this hypothesis, another TPD52-like protein TPD52L2 has been reproducibly identified within lipid droplet fractions (Hodges and Wu, 2010) from OA-treated A431 cells stably expressing stomatin (Umlauf et al., 2004), Hep39 cells (Sato et al., 2006), U937 cells (Wan et al., 2007), differentiated 3T3-L1 cells (Cho et al., 2007), and 3T3-L1 cells cultured to stimulate lipolysis (Brasaemle et al., 2004). Increased TPD52L2 levels were also reported in response to furan fatty acid treatment of Caco-2 cells (Lengler et al., 2012), and in the first reported knock-out model for any Tpd52-like gene, smaller body length and absent or minimal hepatic lipidosis were reported in Tpd52/2/- females (Adissu et al., 2014). Like TPD52, TPD52L2 isoforms are predicted to lack 14-3-3 binding sites in most tissues (Boutros et al., 2003).
In addition to increased lipid droplet numbers that were measured in all 3 TPD52-expressing 3T3 cell lines, 2/3 of these cell lines (D52-1-12 and D52-2-7, the latter harbouring the highest TPD52 levels and lipid droplet numbers) also showed significantly increased fatty acid incorporation into TAG from both de novo fatty acid synthesis, and fatty acid uptake. Significantly larger lipid droplets were also noted in D52-1-12 and D52-2-7 cells when supplemented with OA, and in D52-2-7 cells in the absence of OA treatment. This indicates that in addition to mechanisms regulating lipid droplet numbers, TPD52 can either directly or indirectly alter other aspects of lipid metabolism within cells, although TPD52 expression may not regulate fatty acid uptake rates. The functional significance of this phenotype was demonstrated by D52-1-12 and D52-2-7 cells also showing improved cell survival in response to palmitic acid supplementation.

Lipids play diverse roles in building cellular structures, forming membrane microdomains for functional scaffolding of protein complexes, serving as fat storage depots, and acting as signalling molecules (Baumann et al., 2013; Currie et al., 2013; Nomura and Cravatt, 2013; Zadra et al., 2013). All of these processes are important in cancer, and as such, altered lipid metabolism is now an established hallmark of cancer (Baumann et al., 2013; Currie et al., 2013; Nomura and Cravatt, 2013; Zadra et al., 2013). Both breast and prostate cancers show elevated levels of intracellular lipid (Fisher et al., 1977; Swinnen and Verhoeven, 1998), and are characterised by frequent TPD52 overexpression (Roslan et al., 2014; Rubin et al., 2004; Shehata et al., 2008a; Wang et al., 2004), as well as TPD52 amplification in clinically important disease subsets (Cornen et al., 2014; Guedj et al., 2012; Liu et al., 2013). As androgen upregulates lipogenesis in prostate cancer cells (Swinnen and Verhoeven, 1998; Massie et al., 2011), our results suggest that androgen-induced TPD52 upregulation may contribute to this important process. ERBB2-amplified breast cancers also show increased lipogenesis through ERBB2 both directly and indirectly regulating FASN function (Kumar-Sinha et al., 2003; Menendez and Lupu, 2007), and the co-amplification of other
chromosome 17q genes encoding regulators of lipid metabolism (Kourtidis et al., 2010). As TPD52 is co-expressed with ERBB2 in human breast cancer cell lines and tissues (Byrne et al., 2014; Kourtidis et al., 2010; Roslan et al., 2014), and is a known survival factor in ERBB2-amplified breast cancer cell lines (Kourtidis et al., 2010; Roslan et al, 2014), elevated TPD52 expression may similarly advantage ERBB2-amplified breast cancer cells by increasing both lipogenesis and lipid storage capacity. The isoform-specificity of these functions may partly explain why TPD52 but not TPD52L1 is targeted by gene amplification in cancer.

In summary, this study has identified a new, isoform-specific role for TPD52 as a promoter of both lipid storage and lipogenesis, which may contribute to an improved understanding of lipid droplet function under both physiological and pathological conditions. Given previous reports of increased TPD52 or Tpd52 transcript levels in adipose tissue from obese versus non-obese subjects (Clement et al., 2004; Keller et al., 2008; Nadler et al., 2000), inhibition of TPD52 function may have future relevance in the management of obesity. As TPD52 amplification and overexpression is also frequent in lipogenic cancers such as breast and prostate cancer (Roslan et al., 2014; Rubin et al., 2004; Shehata et al., 2008a; Wang et al., 2004), our results may also partially explain TPD52 targeting in these diseases, and could similarly be applied to target lipogenic cancers in future.
Materials and Methods

Cell lines and cell culture

Human breast carcinoma cells (MDA-MB-231) were cultured in GIBCO® RPMI 1640 (Life technologies, VIC, AU) medium supplemented with 10% FBS (Life technologies, VIC, AU), 6 mM L-glutamine (Life technologies, VIC, AU) in a humidified atmosphere containing 5% CO₂ at 37°C. Cell line identities were confirmed through short tandem repeat profiling by CellBank Australia (Westmead, NSW, AU). Vector-, TPD52- and TPD52L1-transfected BALB/c 3T3 cell lines have been previously reported (Shehata et al., 2008a).

Derivation of stably TPD52-expressing MDA-MB-231 cell lines

The PG307-TPD52 plasmid was constructed by subcloning a SalI-BamHI TPD52 fragment (GenBank NM_005079.3, encoding TPD52 isoform shown in Fig. 1A), representing 91 bp 5’UTR, 555 bp coding sequence and 2,599 bp 3’UTR, into the PG307 expression vector (Boutros et al., 2003). After verification of constructs using Sanger sequencing, the PG307-TPD52 construct or PG307 vector alone were stably transfected into MDA-MB-231 cells, as described previously (Boutros et al., 2003).

Indirect immunofluorescence analyses

Cells were plated onto glass coverslips in 6-well plates, and cultured overnight to reach 70-80% confluence the next day. For lipid droplet quantification, untreated cells or cells supplemented with 400 μM OA (Sigma-Aldrich, NSW, AU) complexed to 10% fatty acid-free BSA (OA/ FA-free BSA, Sigma-Aldrich) (Listenberger and Brown, 2007) for 24 hours were fixed in 3% formaldehyde in PBS for 30 minutes at RT, then stained with 1 μg/ml BODIPY 493/503 (Life technologies, VIC, AU) for 30 minutes at RT. DNA was counterstained using 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Sigma-Aldrich, NSW, AU).
For co-staining of lipid droplets and proteins, untreated cells or cells supplemented with 400 μM OA/FA-free BSA for 6 hours were fixed with 3% formaldehyde and 0.025% glutaraldehyde in PBS for 10 minutes at RT, treated with 50 mM ammonium chloride in PBS for 10 minutes, followed by permeabilization using 0.1% Triton X-100 in PBS (Ohsaki et al., 2005). Cells were stained with affinity-purified rabbit polyclonal TPD52 antisera (1:100) (Balleine et al., 2000) and GM130 mouse monoclonal antibody (1:1,000, BD Transduction Laboratories, San Jose, CA), followed by incubation in 3% BSA/PBS containing 1 μg/ml BODIPY 493/503 solution, Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA), and Alexa Fluor®633 anti-mouse IgG (Life technologies, VIC, AU) secondary antibodies.

For other indirect immunofluorescence analyses, cells treated with or without 400 μM OA were fixed in 3% formaldehyde in PBS for 15 minutes at RT, permeabilised with cold acetone: methanol (v/v, 1:1) for 15 minutes at -20°C, and then incubated with affinity-purified rabbit polyclonal TPD52 antisera and GM130 antibody as described above, or TPD52 mouse monoclonal antibody (1:10) (Tiacci et al., 2005), PDI (C81H6) rabbit monoclonal antibody (1:100, Cell Signaling Technology, Danvers, MA), ARL1 (EPR10595) rabbit monoclonal antibody (1:100, Abcam, VIC, AU), and Adipophilin/ADRP guinea pig polyclonal antibody (1:200, Progen Biotechnik, Heidelberg, Germany). This method was not compatible with BODIPY 493/503 staining, but allowed Adrp detection at lipid droplets. Alexa Fluor®488 anti-mouse IgG (Life technologies, VIC, AU), Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA), and Alexa Fluor®633 anti-guinea pig IgG (Life technologies, VIC, AU) were utilised as secondary antibodies. All samples were viewed with a Leica LCS SP5 II confocal microscope (NSW, AU) using a 63× objective lens.

Quantitative analysis of co-localisation between TPD52 and Gm130, Arl1 or Adrp with or without OA supplementation was performed using Image-Pro Analyzer 7.0 (MediaCybernetics, Rockville,
Scatterplots displaying intensity ranges of red and green pixels were generated from 19-28 representative single focal plane images for each case without pre-processing (TPD52, Gm130, Arl1), or after background subtraction to obtain lipid droplet structures (Adrp), with same parameters applied to all Adrp images. Pearson’s correlation coefficients and Manders’ overlap coefficients were calculated for each scatterplot (Zinchuk and Zinchuk, 2008).

**Electron microscopy**

Vector-3 or D52-2-7 cells were fixed in 2% buffered glutaraldehyde (ProSciTech) for 2 hours, washed in 0.1 M MOPS buffer, scraped from the culture flask, encapsulated in 10% BSA (Sigma-Aldrich, NSW, AU), then cross-linked with glutaraldehyde to form a pellet. Small blocks were post-fixed in 2% buffered osmium tetroxide for 3 hours followed by dehydration in a graded ethanol series. After infiltration in epoxy resin/acetone, cells were embedded in epoxy resin (TAAB TLV) and polymerized for 10 hours at 60°C. Semi-thin (500 nm) sections were cut on glass knives using a Leica Ultracut UC6 ultramicrotome (Leica Microsystems, Vienna) and stained with methylene blue for light microscopic evaluation. Selected areas were trimmed and ultrathin (70 nm) sections were cut with a diamond knife (Diatome, Switzerland), and collected onto 300 mesh thin bar copper grids (ProSciTech). Grids were stained with 2% uranyl acetate in 50% ethanol, followed by Reynold’s lead citrate, and examined using a Philips CM120 BioTWIN transmission electron microscope (FEI, The Netherlands) at 100 kV. Images were collected using an SIS Morada digital camera.

**Cellular lipid droplet quantification**

All image data intended for quantitative comparison were acquired using the same sub-saturating settings on a single focal plane. Numbers of lipid droplets per cell and diameters of each lipid droplet were quantified using Image-Pro Plus Version 5.1 software (MediaCybernetics, Rockville, MD). Lipid droplet areas were calculated from measured diameters. Based on expected lipid droplet
sizes in non-adipocytes ranging from 0.2-1 μm (Suzuki et al., 2011), objects with diameters smaller than 0.3 μm were filtered to reduce noise from potential non-specific staining. The intensity range was set according to the BODIPY 493/503 fluorescence intensity detected in one TPD52-expressing cell line, D52-2-7, and the same parameters were then used to quantify lipid droplets in all 3T3 cell lines. Images of at least 10 panels per cell line were quantified in each of 3 independent experiments.

The dispersal of cellular lipid droplets was investigated using ADRP and DAPI images. Measurements were carried out using Metamorph software Version 7.7 (Molecular Devices, Sunnyvale, CA). After thresholding and background subtraction, the locations of the centre of each cell nucleus and lipid droplet were used to calculate the distance between the centre of each lipid droplet and that of the respective cell nucleus, based on Pythagoras’s theorem.

Quantification of cellular TAG content
Cellular total lipid was extracted using the Folch method (Folch et al., 1957) and TAG concentrations were measured using a GPO-PAP kit (Roche Diagnostics) according to the manufacturer’s instructions, and normalised according to cellular protein levels.

Western blot analyses
Cells were lysed in 3% SDS lysis buffer as described previously (Boutros et al., 2003). Between 20-30 μg total protein extracts were resolved by SDS-PAGE on 12.5% mini polyacrylamide gels or NuPAGE® Novex 4–12% Bis-Tris mini gels (Life technologies, VIC, AU).

Affinity-purified rabbit polyclonal TPD52 (1:100) and TPD52L1 (1:100) antisera were raised in-house (Balleine et al., 2000; Boutros et al., 2003). Fatty Acid Synthase (FASN) mouse monoclonal antibody (A-5, 1:200) was purchased from Santa Cruz Biotechnology (Dallas, TX). SCD1 (M38,
(1:1,000) rabbit polyclonal and Syntaxin 6 (C34B2, 1:1,000) rabbit monoclonal antibodies were purchased from Cell Signaling Technology. ADRP (1:2,000) guinea pig polyclonal antisera were obtained from Progen Biotechnik. ARL1 (EPR10595, 1:2,000) and ARFRP1 (EPR3899, 1:1,000) rabbit monoclonal antibodies, mouse monoclonal anti-NSF (NSF-1, 1:2,000) and rabbit polyclonal anti-SNAP23 (1:500) antibodies were purchased from Abcam. Syntaxin 5 (1:1,000) and VAMP4 (1:1,000) rabbit polyclonal antibodies were purchased from Synaptic Systems (Goettingen, Germany). RAB5C rabbit polyclonal antibody (1:500) was purchased from Sigma-Aldrich (NSW, AU) and has been previously shown by our laboratory to cross-react with other RAB5 isoforms (Shahheydari et al., 2014). Mouse monoclonal α-tubulin antibody (DM1A) (1:5,000; Sigma-Aldrich, NSW, AU) and mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (6C5) (1:10,000; Life technologies, VIC, AU) were used as loading controls.

Assessment of lipid metabolism

Cells were incubated for 4 hours in GIBCO® DMEM media (Life technologies, VIC, AU) containing 2% FA-free BSA, [³H]acetic acid (1 µC/ml; Perkin Elmer, VIC, AU), [1-¹⁴C]OA (0.5 µC/ml; Perkin Elmer, VIC, AU), and 0.5 mM unlabelled oleate (Sigma-Aldrich, NSW, AU) to determine exogenous fatty acid oxidation and esterification into TAG, and TAG synthesis from de novo lipogenesis. Fatty acid oxidation was determined by measuring ¹⁴CO₂ in the culture media and acid soluble metabolite (ASM) production. To assess fatty acid incorporation from de novo lipogenesis or exogenous oleate into TAG, cellular lipids were extracted (Folch et al., 1957). After centrifugation of chloroform/methanol extracts (v/v, 2:1) for 10 minutes at 1000 g, the lower phase was collected and evaporated under a stream of nitrogen. Lipids were separated using thin layer chromatography by loading the dissolved pellet and an internal TAG standard on silica plates (Merck Millipore, VIC, AU), and then immersing plates in heptane/isopropyl ether/acetic acid (60:40:3). Plates were subsequently sprayed with chlorofluorescein dye (0.02% w/v in ethanol) and lipids were visualised under long-wave UV light. TAG bands were scraped and radioactivity was
counted. The rate of TAG synthesis from de novo lipogenesis was calculated as [3H] counts (dpm/min) in the TAG fraction per mg of protein of cell lysates, and the rate of TAG synthesis from exogenously supplied oleate was calculated as [14C] counts (pmol/min) in the TAG fraction per mg protein of cell lysates. The fatty acid uptake rate was calculated as the sum of [14C] counts (pmol/min) funnelled into oxidation (14CO₂ + ASM) and [14C] counts (pmol/min) in the TAG fraction, per mg of protein of cell lysates.

**Palmitic acid treatment and cell proliferation (MTT) assays**

Parental, vector control or TPD52-expressing 3T3 cell lines were plated in triplicate in 96-well plates (3x10³ cells/well). After 24 hours, cells were untreated or treated with 250 μM palmitic acid (Sigma-Aldrich, NSW, AU) in FA-free BSA. MTT assays were performed at indicated time points as described previously (Roslan et al., 2014).

**Plasmid constructs**

Plasmids encoding TPD52 bait protein, and TPD52, TPD52L1, RAB5C, GOLGA5 prey proteins have been previously described (Byrne et al., 1998; Shahheydari et al., 2014). Bait constructs encoding human TIP47 (Genbank NM_005817.4) and ADRP (Genbank NM_001122.3) were generated by subcloning respective EcoRI-XhoI fragments from pVP16-TIP47 and pVP16-ADRP, which were kind gifts from Professor Paul D. Bieniasz (The Rockefeller University) (Eastman et al., 2009), into the EcoRI and SalI sites of pAS2.1. Sanger sequencing confirmed that pAS2.1-TIP47 construct encodes full-length TIP47 (434 aa), whereas pAS2.1-ADRP encodes ADRP residues 1-415 (of 437 residues) due to a C>T substitution at nt 1456 (NM_001122.3) that produced a stop codon.

**Yeast two-hybrid system**

For direct interaction testing, *Saccharomyces cerevisiae* strain Hf7c cells were transformed with paired bait (pAS2.1) and prey (pACT2) constructs, and cultured at 30°C on solid synthetic drop-out
(SD) media lacking Leu and Trp (SD/-Leu-Trp) (Byrne et al., 1998). Bait-prey interactions were scored according to the time (in days) until visible colony growth on solid SD media lacking His, Leu, and Trp (SD/-His-Leu-Trp) (Boutros et al., 2003; Shahheydari et al., 2014). Bait or prey constructs paired with the opposing empty vector represented negative controls, while the interaction between TPD52 bait and TPD52L1 prey was used as a positive control.

**Pull-down assays**

Pull-down assays employed glutathione S-transferase (GST) and 6His-tagged recombinant mouse Tpd52 proteins expressed from pGEX3X (Byrne et al., 1998; Sathasivam et al., 2001), and GST-tagged recombinant human TPD52 expressed from pGEX6P-1 (Chen et al., 2013; Shahheydari et al., 2014). Purification of mouse or human TPD52 fusion proteins and respective GST controls was performed as previously described (Chen et al., 2013; Shahheydari et al., 2014). Purified proteins were assessed by SDS-PAGE. Vector and TPD52-expressing 3T3 cells were lysed in lysis buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris•HCl pH 7.5, 0.1% deoxycholate, 1% Triton X-100) (Lopez-Verges et al., 2006). One-hundred and fifty μg of recombinant Tpd52/TPD52 and respective GST proteins were cross-linked to GSH-agarose (Glutathione Sepharose 4B, GE Healthcare) as described (Shahheydari et al., 2014), and incubated with 3T3 cell lysates. Matrices were washed extensively before bound proteins were eluted into SDS sample buffer and subjected to Western blot analyses.

**Isolation of lipid droplets by cellular fractionation**

Cellular lipid droplets were isolated essentially as previously described (Brasaemle and Wolins, 2006). Briefly, D52-2-7 cells were treated with 400 μM OA/FA-free BSA for 24 hours and then lysed in hypotonic lysis medium (HLM) (40 mM Tris-HCl pH 7.4, 2 mM EDTA, 20 mM sodium fluoride, and EDTA-free Protease Inhibitor Cocktail Tablets (Roche Applied Science, NSW, AU)). After incubation on ice for 10 minutes, cell lysates were briefly sonicated and subjected to
centrifugation at 1,000 g for 10 minutes. The resulting supernatant with the floating fat layer was collected as a non-nuclear fraction into an ultracentrifuge tube and gently mixed with ice-cold HLM containing 60% sucrose (final 20% sucrose), then overlaid sequentially with 15% sucrose/HLM, 5% sucrose/HLM and HLM only to form discontinuous gradients. Gradients were centrifuged at 28,000 g in a Beckman SW41Ti rotor for 45 minutes at 4°C, and the lipid droplet fraction was collected from the top layer. Proteins from the lipid droplet fraction were concentrated by precipitation with 20% trichloroacetic acid (TCA, Sigma-Aldrich, NSW, AU) at -20°C overnight, and washed 3 times with 100% acetone. After air drying, the pellet was dissolved in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and EDTA-free Protease Inhibitor Cocktail Tablets). Protein concentrations were measured using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, VIC, AU), and 15 μg protein was subjected to Western blot analyses.

Statistical analysis

The SPSS for Windows package (version 21; IBM) was used for most graph generation and statistical analyses. Values from lipid quantitation analyses were expressed as means ± s.e.m. of 3 independent experiments, and comparisons between groups were made using a two-tailed, unequal variance Student’s t test. The Mann Whitney u test (GraphPad Prism 4, GraphPad Software, La Jolla, CA) was utilised to compare the dispersal of cellular lipid droplets, and Pearson’s correlation and Manders’ overlap coefficients for quantitative analysis of immunofluorescent staining.

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\textbf{Competing interests}

The authors declare no competing interests.

\textbf{Author contributions}

AK, NR, and YC performed experiments, analysed data and wrote the manuscript.

SF, AJH, and DW performed experiments, and analysed data.

LC, ADF, RKB, GEG, and BKS provided advice regarding experimental design and/or reagents.

JAB conceived of the study, and wrote the manuscript.

All authors read and approved the final manuscript.

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References


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Fig. 1. Increased lipid storage in TPD52- but not TPD52L1-expressing 3T3 cells. (A) Alignment of TPD52 (Uniprot identifier P55327-2) and TPD52L1 (Uniprot identifier Q16890-1) sequences, shown using the one-letter code, produced by the EMBOSS Needle algorithm. Numbers to the left and right of sequences refer to amino acid positions. Vertical lines and colons indicate identical and conserved residues, respectively, whereas hyphens represent gaps inserted to produce the
alignment. PEST domains (red text) (Byrne et al., 1996), coiled-coil domains (underlined text) (Byrne et al., 1998), the TPD52 Ser\textsuperscript{136} phosphorylation site (bold text) (Thomas et al., 2010), and the TPD52L1 14-3-3 consensus binding site (bold text) (Boutros et al., 2003) are indicated by arrows. A schematic diagram of the exon/ domain composition of TPD52 isoforms has also been reported by Byrne et al. (2014). (B) Immunofluorescence analyses of control (3T3-parent, vector-3), TPD52-expressing (D52-2-1, D52-2-7), and TPD52L1-expressing (D52L1-4, D52L1-6) 3T3 cells, stained with BODIPY 493/503 (green) for lipid droplets and DAPI (blue) for nuclei. Images are representative of those obtained in 3 independent experiments. Scale bar = 10 μm. (C) Quantitation of lipid droplet numbers per cell using Image-Pro Plus Version 5.1 software. Images of at least 10 panels per cell line in each of 3 independent experiments were quantified, and data represent means ± s.e.m. \( P \) values, Student’s t-test. (D) Quantitation of lipid droplet area (μm\textsuperscript{2}) as described in (C). \( P \) value, Student’s t-test. (E) Western blot analyses of 3T3 cells with antisera to the proteins shown (left). Gapdh served as a loading control. MW, molecular weight (right). Results are representative of 3 independent experiments.
Fig. 2. TPD52-expressing cells store more lipids upon OA supplementation. (A) Indirect immunofluorescence analyses of control (3T3-parent, Vector-3), and TPD52-expressing (D52-1-12, D52-2-1, D52-2-7) 3T3 cells with (right panels, 24 hr) or without (left panels, No) 400 μM OA/FA-free BSA. An enlarged image of a D52-2-7 cell post-supplementation (white boxed region) is shown. Lipid droplets were stained with BODIPY 493/503 (green) and nuclei were stained with
DAPI (blue). Images shown are representative of those obtained in 3 independent experiments. Scale bar = 20 μm. (B) Quantitation of lipid droplet numbers per cell using Image-Pro Plus 5.1 software as described in Fig. 1 (C). Data are presented as means ± s.e.m. from 3 independent experiments. Lipid droplet numbers were significantly increased in all cell lines post-OA supplementation compared with no treatment (3T3-parent: \( P = 5 \times 10^{-5} \); Vector-3: \( P = 2 \times 10^{-5} \); D52-1-12: \( P = 1 \times 10^{-5} \); D52-2-1: \( P = 3 \times 10^{-6} \); D52-2-7: \( P = 0.002 \); Student’s t-test). Comparisons between vector-3 and TPD52-expressing cells post-OA treatment are also shown. \( P \) values, Student’s t-test.

(C) Relative fold changes in mean lipid droplet numbers per cell (OA-treated/untreated) for each cell type. \( P \) values, Student’s t-test. (D) Quantitation of lipid droplet area (μm²) as described in Fig. 1C. Lipid droplet areas were significantly increased in all cell lines post-OA supplementation compared with no treatment (3T3-parent: \( P = 0.05 \); Vector-3: \( P = 1 \times 10^{-4} \); D52-1-12: \( P = 2 \times 10^{-6} \); D52-2-1: \( P = 0.004 \); D52-2-7: \( P = 4 \times 10^{-4} \); Student’s t-test). Comparisons between vector-3 and TPD52-expressing cells post-OA treatment are also shown. NS= not statistically significant, \( P > 0.05 \). (E) Relative fold changes in mean lipid droplet area.
Fig. 3. Altered fatty acid metabolism in TPD52-expressing 3T3 cells. (A) Triglyceride levels measured in parent, vector, and TPD52-expressing 3T3 cells. Incorporation rates of (B) newly-synthesised free fatty acid (from $[^3]$Hacetate) into TAG, as a measure of de novo lipogenesis, and (C) $[1^{-14}]$oleate into TAG, and (D) $[1^{-14}]$oleate uptake rates were measured in 3T3-parent, vector control, and TPD52-expressing 3T3 cells following 4 hours incubation with $[^3]$Hacetic acid (1
µC/ml), [1-14C]OA (0.5 µC/ml), and 0.5 mM unlabelled oleate. (A-D) Three independent experiments were performed and differences between vector controls and TPD52-expressing cells were examined by Student’s t-test. NS= not statistically significant, \( P > 0.05 \). The [1-14C]oleate uptake rate (D) was also significantly increased in parent versus vector control cell lines (\( P = 0.002 \), Student’s t-test). (E) Western blot analyses of 3T3 cells cultured without OA supplementation, with antisera to proteins shown (left). Gapdh served as a loading control. MW, molecular weight (right). Results are representative of at least 3 independent experiments. (F) MTT assays of parent, vector controls, and TPD52-expressing 3T3 cells treated with 250 µM palmitic acid for 4 days. Results are presented as percentages of MTT absorbance at indicated time points relative to that at day 0. Data are presented as means ± s.e.m. from 3 independent experiments. Significant differences (Student’s t-test) were detected between D52-1-12 and vector controls (Day 1: \( P = 0.03 \); Day 2: \( P = 0.005 \); Day 3: \( P = 0.01 \); Day 4: \( P = 0.004 \)), and D52-2-7 and vector controls (Day 1: \( P = 0.01 \); Day 2: \( P = 0.003 \); Day 3: \( P = 0.001 \); Day 4: \( P = 0.004 \)).
Fig. 4. Co-localisation of TPD52 and the Golgi marker Gm130 in (A) untreated and (B) OA-treated D52-2-7 cells. Cells were untreated (A) or supplemented with 400 μM OA/FA-free BSA for 6 hours (B), fixed and subjected to indirect immunofluorescence analyses. Cells were co-labelled with TPD52 (red), and Gm130 (green). Enlarged images of white boxed regions are shown below with co-localisation shown in yellow. Images shown are representative of those obtained in at least 3 independent experiments. Scale bar = 25 μm.
Fig. 5. Co-localisation of (A) Gm130 or (B) TPD52 with Arl1 and Adrp in untreated and OA-treated D52-2-7 cells. Cells were untreated or supplemented with 400 μM OA/FA-free BSA for 24 hours, fixed, and subjected to indirect immunofluorescence analyses. Cells were co-labelled with either (A) Gm130 (green) or (B) TPD52 (green), Arl1 (red), and Adrp (pseudo-coloured, red), with co-localisation shown in yellow. Images shown are representative of those obtained in 3 independent experiments. Scale bar = 10 μm.
**Fig. 6.** Co-localisation of Adrp and TPD52 in (A) untreated and (B) OA-treated D52-2-7 cells. Cells were prepared as described in Fig. 4. Cells were co-labelled with TPD52 (green) and Adrp (pseudo-coloured, red). Enlarged images of white boxed regions are shown below, with co-localisation shown in yellow. Images shown are representative of those obtained in 3 independent experiments. Scale bar = 10 μm.
Fig. 7. Altered distributions of lipid droplets in untreated and OA-treated D52-2-7 cells versus vector control cells. (A) Representative images of Vector-2 and D52-2-7 cells with or without 400 μM OA treatment. Adrp (pseudo-coloured, green), DAPI (blue). Scale bars = 5 μm. (B) Quantification of distances (pixels) between the centre of each lipid droplet object to the centre of the respective cell nucleus. At least 20 cells per cell type per treatment were assessed using images randomly chosen from 3 experiments, and frequencies (Y axis) of distances (X axis) were plotted on histograms. (C) Statistical analyses confirmed significant differences in lipid droplet distributions between D52-2-7 and vector cells with or without OA treatment (Mann Whitney u test). Boxes extend from the 25th percentile to the 75th percentile, with horizontal lines indicating median values. The whiskers extend above and below the box showing the highest and lowest values. Graphs were generated using GraphPad Prism 4.
Fig. 8. (A, B) Direct interactions between TPD52 and ADRP/ TIP47. (A) Analyses of direct interactions between TPD52 or TPD52L1 and ADRP or TIP47, along with known positive and negative controls using the yeast two-hybrid system. (+), growth on solid SD/-His-Leu-Trp media at 30°C after 5-6 days; (++) after 3-4 days; (+++), after 1-2 days; (-), no visible growth after at least 7 days; ND, not determined. Results were obtained from 3 independent experiments where all
interactions were tested in triplicate. (B) GST pull-down assays using GST-tagged full-length mouse Tpd52 or human TPD52 or GST tag, and D52-1-12 (left) or D52-2-7 (right) 3T3 cell lysates. Upper and middle panels show results of Western blot analyses using Adrp antibody and Rab5 antisera, respectively. The lower panel shows Ponceau S staining to reveal GST-Tpd52/TPD52 or GST tag before cross-linking (arrows). At least 3 independent experiments were performed. (C) Western blot analyses (proteins detected at left, molecular weights (MW) at right) using lipid droplet (LD) fractions from D52-2-7 cells treated with 400 μM OA/ FA-free BSA for 24 hours. Proteins detected in the LD fraction were compared with those detected in total protein from identically treated D52-2-7 cells. Gapdh served as a negative control. Results are representative of 3 independent experiments. (D) Detection of SNARE protein levels according to TPD52 expression status in 3T3 cell lines. Total proteins were extracted from 3T3 cells and subjected to Western blot analyses. Antisera to the proteins examined are shown at left, and molecular weights (MW) are shown at right. α-Tubulin served as a loading control. Results are representative of 3 independent experiments.