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# Triglyceride Synthesis by DGAT1 Protects Adipocytes from Lipid-Induced ER Stress during Lipolysis

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## SUMMARY

Triglyceride (TG) storage in adipose tissue provides the major reservoir for metabolic energy in mammals. During lipolysis, fatty acids (FAs) are hydrolyzed from adipocyte TG stores and transported to other tissues for fuel. For unclear reasons, a large portion of hydrolyzed FAs in adipocytes is re-esterified to TGs in a “futile”, ATP-consuming, energy dissipating cycle. Here we show that FA re-esterification during adipocyte lipolysis is mediated by DGAT1, an ER-localized DGAT enzyme. Surprisingly, this re-esterification cycle does not preserve TG mass, but instead functions to protect the ER from lipotoxic stress and related consequences, such as adipose tissue inflammation. Our data reveal an important role for DGAT activity and TG synthesis generally in averting ER stress and lipotoxicity, with specifically DGAT1 performing this function during stimulated lipolysis in adipocytes.

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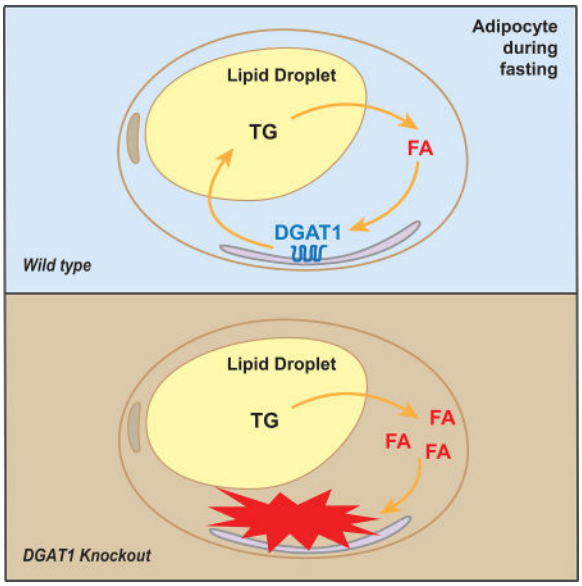
**AUTHOR CONTRIBUTIONS**

C.C., R.V.F., and T.C.W. planned the study and designed the experiments. C.C. performed most of the experiments. J.H. performed DGAT activity assays. G.D.R. and C.A.G. helped to maintain mouse colonies. C.C., R.V.F., and T.C.W. wrote the manuscript.

## In Brief




Chitraju *et al.* unravel a 60+-year old mystery of why a large portion of hydrolyzed FAs in adipocytes is re-esterified to TGs during lipolysis. They show show the ER enzyme DGAT1 mediates this FA re-esterification, not preserve TG mass, but instead to protect the ER from lipotoxicity.

## INTRODUCTION

Triglycerides (TGs) provide the major storage form of fatty acids (FAs), which in turn serve as reservoirs of metabolic fuel or membrane building blocks. During times of excess energy availability, more TG is synthesized, and in mammals, this TG is predominantly packaged in cellular lipid droplets (LDs), the organelle for neutral lipid storage (Farese and Walther, 2009; Fujimoto and Parton, 2011; Walther and Farese, 2012). TG storage in mammals occurs primarily in adipocytes in white adipose tissue (WAT). However, when adipose depots of TG are overwhelmed, for instance during obesity, other cells will synthesize and accumulate TG in LDs. This is often accompanied by tissue dysfunction due to cellular lipotoxicity (Unger and Zhou, 2001), which can lead to lipotoxicity-related diseases, such as type 2 diabetes, non-alcoholic fatty liver disease, and metabolic cardiomyopathy.

When energy supply is limited, lipolysis of adipocyte TGs is stimulated, releasing large amounts of hydrolyzed FAs that can be transported to other tissues for use as fuel (Duncan et al., 2007; Zechner et al., 2012). However, during lipolysis, up to two-thirds of hydrolyzed FAs from TG is re-esterified to TG in WAT (Edens et al., 1990; Leibel et al., 1985; Nye et al., 2008; Vaughan, 1962; Wood et al., 1960). Re-esterification of FAs to glycerol backbones could occur at any step along the glycerolipid-TG synthesis pathway. This re-esterification cycle is targeted by thiazolidinedione drugs, which promote glyceroneogenesis (Guan et al., 2002; Tordjman et al., 2003) to provide glycerol and glycerol-phosphate for TG synthesis during fasting, and which promote fat storage in humans. The reason for this “futile”, ATP-

consuming re-esterification cycle has remained a mystery for decades. For instance, this cycle of lipolysis and re-esterification might provide a mechanism to fine-tune the release rate of fatty acids, thereby preserving fat mass during fasting or other conditions associated with lipolysis, but evidence for this hypothesis is lacking.

The enzyme responsible for re-esterification of FAs to diacylglycerol to form TGs during adipocyte lipolysis is unknown. In mammals, TG synthesis is catalyzed by acyl CoA:diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 or DGAT2 (Liu et al., 2012; Yen et al., 2008). Both DGAT enzymes catalyze the same reaction, utilizing diacylglycerol and fatty acyl CoAs as substrates, but are evolutionarily unrelated (Liu et al., 2012; Yen et al., 2008). DGAT1 is localized exclusively to the ER (Stone et al., 2009; Wilfling et al., 2013) and has broader substrate specificity (with respect to acyl acceptors) than DGAT2 (Cases et al., 1998; Cases et al., 2001; Yen et al., 2005; Yen et al., 2008), and mice lacking DGAT1 are viable and metabolically healthy (Chen et al., 2002a; Smith et al., 2000; Yen et al., 2008). In contrast, DGAT2 localizes to both the ER and around LDs (Stone et al., 2009; Wilfling et al., 2013) and appears to be the major enzyme accounting for TG synthesis (Cases et al., 2001; Stone et al., 2004), as mice lacking the enzyme have >90% reduction in TGs and die shortly after birth (Stone et al., 2004). DGAT1 and DGAT2 have been functionally linked to storage of TGs in different types of LDs. TG synthesis mediated by DGAT1 in the ER leads exclusively to the formation of relatively small (400–800 nm diameter) LDs, called initial LDs (or iLDs; (Wilfling et al., 2013)). In contrast, when FA levels are high in cells, DGAT2 relocalizes from the ER around a subset of LDs (Kuerschner et al., 2008; Stone et al., 2009; Wilfling et al., 2013), where it acts in concert with other LD-localized enzymes of TG synthesis to locally synthesize TGs and generate expanding LDs (or eLDs, typically 1–2 μm in diameter). Which DGAT enzyme and related pathway of TG synthesis for LDs mediate the FA re-esterification cycle in adipocytes is unclear.

Here, using a combination of cell and murine models, we investigated how TG synthesis is mediated in adipocytes during lipolysis. We found that the re-esterification cycle is mediated exclusively by DGAT1, and that DGAT2 is inactive during these conditions. Surprisingly, we found that the function of DGAT1-mediated re-esterification during lipolysis is not to preserve TG mass, but instead DGAT1 activation serves to protect the adipocyte ER from lipotoxic stress and associated inflammatory consequences. Our findings, thus, uncover that TG synthesis is important to protect cells from lipid-induced ER stress.

## RESULTS

### DGAT1 and DGAT2 in murine WAT are reciprocally regulated in fasting and re-feeding

We investigated mRNA regulation of DGAT1 and DGAT2 in gonadal WAT, brown adipose tissue (BAT), and livers from mice in different nutritional states. In WAT, DGAT2 mRNA levels were decreased after 16 h fasting and increased after 4 hours of re-feeding (Figure 1A). These expression changes closely mirrored those of sterol regulatory element binding protein 1c (SREBP1c) and leptin (Figure 1A), two known master regulators of the WAT response to changes in food intake. Consistent with changes in mRNA levels, DGAT2 enzymatic activity in WAT was increased during re-feeding (Figure 1B). In contrast, DGAT2 mRNA levels in liver and BAT remained unregulated during fasting and re-feeding.

Unexpectedly, the level of DGAT1 mRNA was increased during 16 h fasting, a physiological condition associated with increased lipolysis and reduced TG synthesis (Figure 1A). Upon re-feeding, DGAT1 mRNA levels normalized to the levels observed during *ad libitum* feeding conditions. These changes in mRNA levels were reflected in increased enzymatic DGAT1 activity during fasting (Figure 1B). The up-regulation of DGAT1 was not only observed in WAT but also in liver, as reported (Villanueva et al., 2009), and to some extent in BAT.

## DGAT1 mediates the majority of TG re-esterification during stimulated lipolysis *in vitro*

Our data indicate that DGAT1-mediated TG synthesis has a more active role in WAT during fasting than during fed conditions, suggesting that DGAT1 mediates FA re-esterification during this condition. To test this hypothesis, we studied differentiated 3T3-L1 adipocytes and examined TG synthesis by DGAT1 or DGAT2 under different conditions. Under basal conditions, inhibiting either DGAT1 or DGAT2 only modestly reduced TG synthesis in intact cells (measured by [$^{14}$C]-oleic acid incorporation), but inhibiting both DGAT1 and DGAT2 nearly completely abolished TG synthesis (Figure 2A). Under these conditions, there was a concomitant increase in label in cellular 1,2-DAG (or a racemic mixture of *sn*-1,2 DAG and *sn*-2,3 DAG isomers) and free FAs. These findings suggest DGAT1 and DGAT2 activities compensate for each other to maintain TG synthesis in adipocytes under basal conditions.

We next examined TG synthesis during lipolysis (stimulated with 10 μM isoproterenol). Using labeled fatty acid as tracer, we found that a considerable amount of TG was synthesized at a 3-hour time point, consistent with previous reports on fatty acid re-esterification during lipolysis (Edens et al., 1990; Leibel et al., 1985; Nye et al., 2008; Vaughan, 1962). Notably, DGAT1 inhibition nearly abolished TG synthesis during stimulated lipolysis (Figure 2A), and free fatty acids and 1,2-diacylglycerol accumulated in cells. DGAT1 inhibition also resulted in accumulation of monoacylgycerol (Figure S2B). In contrast, DGAT2 inhibition alone had no effect on [$^{14}$C]-oleic acid incorporation into TG during stimulated lipolysis. Also, inhibiting DGAT2 in addition to DGAT1 did not change the findings for DGAT1 inhibition alone, indicating that DGAT2 is apparently not active during stimulated lipolysis.

To exclude the possibility that the reduced TG synthesis found with DGAT1 inhibition was due to increased dilution of the tracer ([$^{14}$C] oleic acid) from release of hydrolyzed FAs during lipolysis, we examined tracer incorporation into TG and other lipids for a range of tracer concentrations. We found that the incorporation of the tracer into PC and PE in DMSO-treated or DGAT1-inhibited cells was linearly increased and similar for each tracer specific activity (Figures S1A and S1B), arguing against a significant tracer dilution effect accounting for the findings in DGAT1-inhibited cells. The incorporation of tracer into TG also increased under the DGAT1-inhibition conditions, although at a slightly lower proportion than in the control cells, perhaps because TG synthesis was nearly absent.

Together, these data indicate that DGAT1 mediates most of TG synthesis under stimulated lipolysis conditions in cultured adipocytes. This conclusion predicts that DGAT1 inhibition during isoproterenol-stimulated lipolysis leads to increased FA release into the medium.

Consistent with this, we observed a roughly two-fold increase of FA release from 3T3-L1 adipocytes when DGAT1 was inhibited during lipolysis (Figure 2B). In contrast, no changes in FA release were found upon inhibition of DGAT2. Inhibition of both DGAT1 and DGAT2 resulted in similar effects as for DGAT1 inhibition alone.

The increase of released FAs from adipocytes with DGAT1 inhibition during lipolysis could result from either an increase in lipolysis activity or a decrease in re-esterification of FAs liberated from TG. To examine these possibilities, we stimulated lipolysis in adipocytes under conditions in which we inhibited the major adipocyte lipases. Consistent with previous reports, inhibition of adipose triglyceride lipase (ATGL), the rate-limiting enzyme of adipocyte TG hydrolysis, reduced FAs release from cells by 80% (Figure 2C). Inhibition of DGAT1, but not DGAT2, under these conditions greatly increased FA release into the medium. Blocking hormone-sensitive lipase (HSL) alone reduced FA release to a lesser degree than ATGL inhibition. Similar to ATGL inhibition, however, inhibition of DGAT1, but not DGAT2, resulted in increased FA release (Figure 2C). These data suggest that increased FAs release during DGAT1 inhibition is due to a block in DGAT1 mediated re-esterification, rather than changes in lipolysis activity *per se*. Consistent with this interpretation, we did not observe any changes in ATGL and HSL protein levels or HSL phosphorylation with DGAT1 inhibition (Figure S2A).

## DGAT1 forms initial LDs in cultured adipocytes during stimulated lipolysis

Under conditions of stimulated lipolysis, if FAs are not sequestered from cells by incubating with albumin in the medium, 3T3-L1 cells form larger numbers of new iLDs (Ariotti et al., 2012; Hashimoto et al., 2012; Marcinkiewicz et al., 2006; Paar et al., 2012). Inasmuch as we found DGAT1 is specifically active during lipolysis, we tested whether the new iLDs are formed by the DGAT1-coupled iLD pathway. Consistent with previous findings (Ariotti et al., 2012; Paar et al., 2012), we detected by confocal microscopy a multitude of relatively uniformly sized, small (median diameter ~ 0.75 μm) iLDs that were newly formed when lipolysis was triggered in 3T3-L1 adipocytes (Figure 3A). Treatment with Triacsin C, which blocks the activation of FAs with a coenzyme A moiety and hence the incorporation of fatty acids into glycerolipids, effectively blocked the formation of these newly formed iLDs, as reported (Paar et al., 2012). Testing the roles of the two different DGAT enzymes in this process, we found that inhibition of DGAT1, but not DGAT2, blocked the formation of newly formed iLDs (see Figure 3B, in particular LDs with < 1 μm diameter).

## Decreased re-esterification in cultured WAT from mice lacking DGAT1 in adipocytes

Our findings in cultured 3T3-L1 adipocytes show that DGAT1, but not DGAT2, re-esterifies FAs during stimulated lipolysis. To determine whether this was also the case in *in vivo*, we performed studies of mice lacking DGAT1 specifically in adipocytes. To avoid complications due to altered whole-body physiology and cross-talk between tissues observed in mice lacking DGAT1 in all tissues (Chen et al., 2002a; Smith et al., 2000; Yen et al., 2008), we generated mice lacking DGAT1 specifically in adipocytes (ADGAT1 KO) by crossing *Dgat1*$^{Flox/Flox}$ mice (Villanueva et al., 2009) with transgenic mice expressing Cre-recombinase under the adiponectin promoter (*Adipoq-Cre*; (Eguchi et al., 2011)). ADGAT1 KO mice lacked detectable DGAT1 protein and had markedly reduced DGAT activity in

adipose tissue (under conditions optimized to detect DGAT1 activity), but had normal DGAT1 expression in the liver and small intestine (Figures 4A–4B and S3A).

ADGAT1 KO mice fed a chow diet *ad libitum* had smaller adipose tissue depots (20% less weight than controls) (Figure S3B), and had similar levels of plasma glucose, TG, glycerol, and FAs under standard *ad libitum* fed conditions (Figure 4C). The lack of increase in plasma FAs in the fasted ADGAT1 KO mice was unexpected and could be due to increased oxidation of FAs in tissues, since these mice exhibit increased energy expenditure (C.C., T.W., and R.F., unpublished observations). ADGAT1 KO mice also showed no differences in liver TG levels after fasting (Figure S3C). To test the role of DGAT1 during lipolysis in adipocytes of adipose tissue, we measured glycerol release from isoproterenol-treated WAT explants of control or ADGAT1 KO fat pads. At baseline and during lipolysis, we found that ADGAT1 KO fat released more glycerol into the medium than fat pads isolated from control mice (Figure 4D). Increased release of glycerol and FAs were also observed in WAT explants treated with DGAT1 inhibitor (Figure 4E) consistent with a role for DGAT1 in re-esterification during lipolysis.

## DGAT1 mediated re-esterification does not preserve fat mass during fasting or calorie restriction

Identification of DGAT1 as the enzyme responsible for catalyzing fatty acid re-esterification during lipolysis in adipocytes, both in culture and in WAT, enabled us to next address the function of this process. One possibility is that DGAT1-mediated re-esterification during lipolysis prevents loss of fat mass when energy input is limited. To test this hypothesis, we submitted ADGAT1 knockout mice to nutrient deprivation caused either by fasting or calorie restriction. During conditions of a 16-h fast, both ADGAT1 and control mice lost similar amounts of body weight, lean mass, and fat mass (Figure 4F). Similarly, during calorie restriction for 20 days with a diet consisting of 40% of normal food intake (60% calorie restriction; (Zhao et al., 2010)), body weights and reductions in lean and fat masses were similar between genotypes (Figures 4G and 4H). These data suggest DGAT1-mediated re-esterification of FAs does not play a major role in preserving fat mass during conditions of acute or chronic energy deprivation.

## DGAT1 protects against the ER stress response during simulated lipolysis in 3T3-L1 adipocytes

ER function is closely monitored by a homeostatic system, termed the “unfolded protein response” (UPR), which is activated during the accumulation of misfolded proteins, an overload of the ER with secretory cargo, or during changes in the lipid composition of the ER (Volmer and Ron, 2015; Walter and Ron, 2011). DGAT1 has been functionally linked to preventing cellular lipotoxicity (Koliwad et al., 2010; Listenberger et al., 2003). We, therefore, hypothesized that DGAT1 activation during lipolysis protects the ER from lipotoxic stress due to the large increase in FAs. The UPR in mammals consists of three branches, employing distinct stress sensors (Ire1, Perk, and Atf6) and response programs (Walter and Ron, 2011).

We first tested for UPR activation in differentiated 3T3-L1 adipocytes during basal conditions. Although inhibiting DGAT1 or DGAT2 alone did not trigger the UPR, inhibiting them simultaneously resulted in strong induction (e.g., ~80-fold induction of XBP1s after 10 h of inhibition) of many UPR targets genes, including spliced XBP1, Bip, CHOP, and ATF3 (Figures 5A–5B and 5E). These changes were similar in magnitude to those found with treatment with thapsigargin, an agent that induces ER stress by blocking calcium uptake and thereby disrupting ER homeostasis. If the UPR is unresolved, this leads to cleavage and degradation of messages encoding some ER proteins, a process known as RIDD, or *R*egulated *I*re1 *D*ependent *D*ecay of mRNAs (Han et al., 2009; Hollien et al., 2009; Hollien and Weissman, 2006; Maurel et al., 2014). Indeed, we found decreased abundance of RIDD target mRNAs during prolonged inhibition of both DGAT1 and DGAT2 under basal conditions, findings that were also consistent with those after thapsigargin treatment. These data indicate that under basal conditions in differentiated adipocytes, either DGAT1 or DGAT2 activity is sufficient to protect the ER from lipotoxic stress, but in the absence of TG synthesis, the UPR and ER stress are markedly activated. The increase in ER stress caused by inhibition of both DGAT1 and DGAT2 in the basal conditions was partially blocked by pharmacologic inhibition of FA synthesis (data not shown), suggesting that excess FAs from either *de novo* synthesis or TG hydrolysis can lead to an ER stress response.

During lipolysis in adipocytes, our studies show that TG synthesis activity by DGAT2 appears to be inactivated (Figure 2A). We thus hypothesized that activation of DGAT1 functions during lipolysis primarily to re-esterify FAs in the ER and prevent disruption of ER function. Indeed, during stimulated lipolysis, inhibition of DGAT1 alone (or DGAT1 inhibition combined with DGAT2 inhibition) triggered strong induction of the UPR (16-fold induction of XBP1s after 3 h of DGAT1 inhibition) and reduced abundance of RIDD targets (Figures 5C–5D and 5E). These effects were dependent on the generation of non-esterified FAs, as increasing the extracellular concentration of albumin, which binds to and effectively buffers excess FAs, decreased the magnitude of UPR activation (Figure S4). However, we found no effect on ER function as measured by the amount of proteins secreted by differentiated adipocytes into the medium (Figure S5). As expected from the observation that DGAT2 was inactive during lipolysis, inhibition of DGAT2 did not result in induction of the UPR or RIDD under these conditions (Figures 5C–5D and 5E).

## Lack of DGAT1 in adipocytes increases the unfolded protein response in WAT during fasting or cold exposure

To test whether DGAT1 protects the ER from lipotoxic stress and UPR activation *in vivo*, we studied conventionally housed wild-type or ADGAT1 KO mice under two conditions that stimulate lipolysis in WAT: a 16-h fast or 6-h exposure to cold in the absence of food. Even at basal conditions, we found a small, but significant, activation of XBP1s and the ER chaperone BIP in WAT of ADGAT1 compared with controls (Figure 6A). After 16 hours of fasting, or after cold exposure, several UPR targets (e.g., XBP1s and Bip) were upregulated two- to threefold in WAT. In contrast, mRNA levels of Atf3, a transcription factor not directly involved in the UPR, were not altered in adipose tissue of ADGAT1 mice, compared with controls. Additionally, some UPR targets were not changed at the mRNA level under this experimental paradigm. In some instances, changes in mRNA levels of UPR genes

between ADGAT1 and control adipose tissue were mirrored by changes in protein expression levels. Specifically, WAT of 16 h fasted ADGAT1 mice showed increased levels of Bip and CHOP (an effector of the PERK branch of the UPR), compared with controls (Figures 6B and S6).

### Adipocyte-specific DGAT1 deficiency induces the inflammatory activation of adipose tissue macrophages during fasting or cold exposure

Prolonged ER stress in WAT is usually associated with increased inflammation (Hotamisligil, 2010; Kaser et al., 2008; Keestra-Gounder et al., 2016; Montane et al., 2014; Urano et al., 2000). Thus, we next examined the level of inflammation in WAT during fasting or in the cold. After a 16-h fast or a 6-h cold exposure in the absence of food, WAT from ADGAT1 mice showed higher mRNA levels of F4/80 and CD68, reflecting a larger number of activated macrophages in this tissue than in controls. We also observed greater expression of M1, proinflammatory markers, such as TNFα, IL1β, and MCP1 in WAT from fasted or cold exposed ADGAT1 mice than in controls, which was associated with modest increases in serum cytokines and inflammatory markers (Figures 6C and 6D) In contrast, transcripts reflecting the presence of anti-inflammatory M2 macrophages, such as IL4, IL10 and arginase1, were similar between the genotypes (Figure 6C).

### Inverse correlation of DGAT1 expression with ER stress genes in adipose tissue of human subjects

To test whether our findings in murine model systems are valid in humans, we differentiated human multipotent adipose-derived stem (hMADS) cells-3 to adipocytes and measured the ER stress response in basal conditions and with stimulated lipolysis under DGAT1- or DGAT2-inhibition conditions. As we found in murine cells, the mRNA levels of ER stress genes XBP1s and Bip were increased in cells treated with both DGAT inhibitors under basal conditions, whereas these markers were elevated with DGAT1 inhibition alone with stimulated lipolysis (Figure S7), validating this response for human adipocytes.

To further examine the relationship between DGAT1 expression and the expression of ER stress genes in human adipose tissue in pathophysiological conditions, we analyzed published mRNA expression profiles of human white adipose tissue obtained from 26 non-obese and 30 obese women who were fasted overnight (Arner et al., 2012). Expression levels of DGAT1 and DGAT2 and genes regulated by the unfolded protein response (GO: 0006986) were extracted from the dataset and correlated. We found that the abundance of numerous mRNAs encoded by UPR genes displayed a negative correlation with DGAT1 mRNA levels (Figure 7A). This type of relationship with UPR genes was not found for DGAT2. The mRNA levels for six of genes that correlate negatively with DGAT1 are shown for different individuals in Figure 7B. Taken together, these data corroborate the apparent relationship of DGAT1 to ER stress in human adipose tissue.

## DISCUSSION

During lipolysis, adipose tissue FAs are liberated from TG, but a large fraction of these FAs is re-esterified back to TG (Edens et al., 1990; Leibel et al., 1985; Nye et al., 2008; Vaughan,

1962). Neither the mechanism for TG synthesis nor the reasons for this FA re-esterification cycle have been clear. Here, we find that during lipolysis, DGAT1 is activated and mediates this re-esterification, whereas DGAT2 is inactivated. Surprisingly, we find that the function of DGAT1-mediated FA re-esterification is not to preserve TG mass, but instead protects the ER from lipotoxic stress and, as shown in mice lacking DGAT1 in adipocytes, associated adipose tissue inflammation.

More generally, our data indicate that DGAT-mediated TG synthesis is important to prevent adipocyte ER stress under both basal and lipolysis-stimulating conditions. Our findings are consistent with earlier reports that generally suggested DGAT1 function is important to avert cellular lipotoxicity. For example, murine fibroblasts lacking DGAT1 are sensitive to oleate-induced cell death (Listenberger et al., 2003). Additionally, several tissue-specific transgenic models of DGAT1 overexpression have reported beneficial effects that are consistent with a role of DGAT1-mediated ER protection (Chen et al., 2002b; Koliwad et al., 2010; Liu et al., 2009), and deletion of DGAT1 in heart increased lipotoxicity in a murine model (Liu et al., 2014). We now show a mechanism for this protection: the detoxification of lipid species by DGAT1 prevents buildup of toxic lipids in the ER that can lead to sustained UPR activation. Although this study focused on DGAT1, activity of either DGAT enzyme was sufficient to protect the adipocyte ER from lipotoxic stress under basal conditions. We note that our findings do not address the fed condition, where re-esterification has also been reported, and is under the influence of insulin and acylation stimulating protein (Van Harmelen et al., 1999).

The detoxifying function of DGAT1 is consistent with several features of the enzyme. First, DGAT1 localizes exclusively to the ER (Wilfling et al., 2013), where toxic lipids can accumulate. Second, DGAT1 has broad substrate specificity and can esterify monacylglycerols, retinol, and long chain alcohols in addition to DAG (Yen et al., 2005; Yen et al., 2008), and so therefore likely protects additionally against toxic accumulation of these lipids in the ER. Third, DGAT1 has high activity at high substrate concentrations (Cases et al., 2001). Finally, DGAT1 belongs to the membrane-bound *O*-acyltransferase (M-BOAT) gene family (Yen et al., 2008), in which several other members function to prevent toxic buildup of lipids in the ER. For example, acyl CoA:cholesterol acyltransferase (ACAT) enzymes, or the yeast orthologues Are1 and Are2, esterify sterols, a reaction that protects cells from accumulation these lipids. For ACAT enzymes, cellular function has been clearly linked to preventing ER stress due to excess sterols (Devries-Seimon et al., 2005). Maintenance of the ER lipid composition and fluidity is likely of crucial importance to cell function, so it is perhaps not surprising that enzymes, such as DGATs and ACATs, monitor and defend against changes to this composition.

We currently do not know how toxic lipids trigger the UPR when DGAT1 activity is absent in adipocytes. The possibility that alterations in the ER lipids result in the accumulation of unfolded proteins in the ER seems unlikely, as we did not find effects on protein secretion in the absence of DGAT1. Instead, our data support a model in which excess amounts of fatty acids liberated from lipolysis alter the ER membrane lipids, which in turn activate the UPR directly. This is consistent with other studies showing a direct activation of the UPR by altered membrane lipid changes (Promlek et al., 2011; Volmer et al., 2013). Which lipid

species mediate the activation of the UPR during lipolysis in the absence of DGAT activity is unknown. One possibility is changes in ER phospholipid composition (e.g., a higher degree of saturated fatty acid side chains), which trigger activation of the Ire1 and PERK UPR sensors (Robblee et al., 2016; Volmer and Ron, 2015; Volmer et al., 2013).

UPR induction is a homeostatic response to resolve cellular ER stress (Volmer and Ron, 2015; Walter and Ron, 2011). At least two lines of evidence suggest that this resolution is not achieved in the absence of DGAT1 during lipolysis. First, cells activate RIDD to remove messages encoding secreted proteins, which is a sign of unresolved ER stress *in vitro* (Han et al., 2009; Hollien et al., 2009; Maurel et al., 2014), and we found evidence of RIDD activation in our studies. Second, in mice lacking DGAT1 in adipocytes that were subjected to lipolysis-stimulating conditions, we found evidence of inflammation, a known consequence of prolonged ER stress (Hotamisligil, 2010; Kaser et al., 2008; Keestra-Gounder et al., 2016; Montane et al., 2014; Urano et al., 2000).

In contrast to DGAT1, we found that DGAT2 appears to be upregulated with re-feeding and inactivated during lipolysis. This is consistent with a primary function for DGAT2 in mediating TG storage (Stone et al., 2004). Under basal conditions in adipocytes, DGAT2 is active and can play a role in FA re-esterification, as suggested previously (Eichmann et al., 2012) and by our data. The inactivation of DGAT2 during lipolysis suggests that adipocytes coordinate the regulation of TG synthesis and lipolysis: when lipases, such as ATGL and HSL, are activated by β-adrenergic signaling during lipolysis (Zechner et al., 2012), DGAT2-mediated TG synthesis is inhibited. The inactivation of DGAT2 activity at LDs during stimulated lipolysis would serve to prevent re-esterification from occurring directly at the LD surface where lipolysis is taking place, allowing for FAs to be available for export from cells.

Although in the current study we focused on the role of DGATs in re-esterification, our data showing increased release of both glycerol and during induced lipolysis in the setting of DGAT1 inhibition suggest that lipolysis completely hydrolyzes TGs to their glycerol and FA components. This implies that glycerol 3-phosphate, from glycolysis or glyceroneogenesis, is the likely backbone of ER-based FA re-esterification, a hypothesis that can be tested in future studies.

How DGAT2 is inactivated in adipocytes during lipolysis is unknown. We found that DGAT2 mRNA levels were down-regulated in WAT during lipolytic conditions. Additionally, DGAT2 has multiple potential phosphorylation sites (Yen et al., 2008), suggesting post-translational regulation of its stability or activity. DGAT2 is a short-lived protein ($T_{1/2}$ of ~ 30 min) that is degraded by ER-associated protein degradation (Brandt et al., 2016; Choi et al., 2014). Therefore, fast turnover of DGAT2 could also enable efficient down-regulation of its activity.

Several findings, from this study and others, indicate that an ER protective role of DGAT1 activity is likely conserved in humans. In the current study, we found that DGAT1 inhibition resulted in increased UPR gene expression in cultured hMADS cells. Further, we found a negative correlation of DGAT1 mRNA levels with many UPR genes in adipose tissue of

fasted human subjects. In previous studies, we and others found that homozygous *DGAT1* loss-of-function mutations in humans leads to a severe congenital diarrheal disorder involving enterocyte dysfunction, which is triggered by a fat-containing diet (Gluchowski et al., 2017; Haas et al., 2012; Stephen et al., 2016). Similarly, DGAT1 inhibition caused dose-related diarrhea in clinical studies of humans (Denison et al., 2014; Meyers et al., 2015). Importantly, DGAT2 appears not to be expressed in human intestine (Haas et al., 2012), creating a situation similar to adipocyte lipolysis, with only DGAT1 active. Thus, DGAT1 might have a particularly important ER-protective role in the human intestine, and absence of this protective function might lead to ER toxicity and enterocyte dysfunction when cells are exposed to high lipid levels from the diet. Consistent with this model, mice express both DGAT enzymes in their intestine (Buhman et al., 2002), and DGAT1 knockout mice do not exhibit diarrhea or other overt intestinal consequences (Buhman et al., 2002; Smith et al., 2000).

In summary, our data provide new insight into the function of FA re-esterification in WAT during lipolysis. Periods of fasting in animals results in mobilization of large amounts of FA from WAT to provide fuel for other tissues. Re-esterification of a large portion of these FAs was thought to preserve fat mass. Instead, our findings indicate that DGAT1-mediated re-esterification plays a crucial role in protecting adipocytes from ER toxicity under such FA-liberating conditions. Protection of the ER by DGAT enzymes may be important in many tissues routinely challenged by huge fluctuations in fatty acid concentrations, such as adipocytes or intestine, allowing organisms to adapt to constantly changing energy conditions.

## STAR★METHODS

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## CONTACT FOR REAGENT AND RESOURCES SHARING

Further information and request for reagent and resources should be directed to Robert V. Farese, Jr. (robert@hsph.harvard.edu) or Tobias C. Walther (twalther@hsph.harvard.edu).

## EXPERIMENTAL PROCEDURES

### Animals and Dietary Intervention Studies

All animal experiments were performed under the guidelines established by Harvard Center for Comparative Medicine (HCCM). Mice were maintained in a barrier facility, at normal room temperatures, on a regular 12-h light and 12-h dark cycle and had *ad libitum* access to food and water unless otherwise stated. ADGAT1 KO mice were generated from DGAT1 floxed mice (Villanueva et al., 2009) that were crossed with adiponectin Cre mice (a gift from Dr. E. Rosen, (Eguchi et al., 2011)). The genetic background of mice in this study was C57BL/6J (Jackson Laboratory Bar Harbor, ME). Mice were fed on standard laboratory chow diet (less than 4.5% crude fat). Calorie restriction study was carried out as described (Zhao et al., 2010). Mice were individually housed for a week and fed chow diet *ad libitum*. During this week of acclimation, we monitored the daily food intake of each mouse. The following week onwards, mice were subjected to 60% calorie restriction such that each mouse was fed every day at 6 pm with an amount of food equal to 40% of their daily intake by the same mouse during the week of acclimation. Body weights were measured daily at 5:30 pm before feeding: lean and fat mass were determined every 5 days by DEXA.

### 3T3-L1 Cell Differentiation

3T3-L1 cells were cultured in high glucose DMEM containing 10% FBS and penicillin and streptomycin. Two days after full confluency cells were cultured for 3 days in differentiation medium containing 1μM dexamethasone, 100 μM isobutylmethylxantine and 5 μg/ml bovine insulin. Cells were then cultured in post differentiation medium containing 5 μg/ml bovine insulin for five to eight days before using them for metabolic labelling experiments. For confocal microscopy cells were cultured in gelatin coated glass bottom dishes.

### Culture and Differentiation of Human Multipotent Adipose-derived Stem Cells

hMADS-3 cells (a gift from Dominique Langin) were cultured and differentiated as described (Bezaire et al., 2009). Cells were cultured in proliferation medium (DMEM-low glucose containing 10% FBS, L-glutamine, HEPES Buffer, and 5 μg/ml of FGF2. Proliferation medium (+FGF2) was changed 3 days after cell plating, and then changed to proliferation medium without FGF2. 6 days after plating. Then cells were washed with 1× PBS for two times and differentiation medium (proliferation medium without serum/ Ham's F12 containing insulin, 5 μg/ml; transferrin, 10 μg/ml; T3, 0.2 nM; rosiglitazone, 0.1 μM; IBMX, 100 μM; dexamethasone, 1μM) was added. Cells were cultured for 3 days. Then cells were washed with 1× PBS for two times and differentiation medium without IBMX

and dexamethasone was added and incubated for 2–3 days followed by differentiation medium without rosiglitazone. At day 18 of culture, cells were utilized for experiments.

### [14C]- Oleic Acid Labelling of Lipids, Lipid Extraction and Thin Layer Chromatography

DGAT1 and DGAT2 specific activities in differentiated 3T3-L1 adipocytes were determined by [$^{14}$C]-oleic acid incorporation into TG in presence of inhibitors. Cells were pre-treated for 30 min with 5 μM inhibitors, cells were pulse labeled with [$^{14}$C]-oleic acid (50 μCi/μmol) for 3 h in presence of inhibitors. To determine activities during induced lipolysis, cells were treated with 10 μM isoproterenol. Cells were washed with phosphate buffer saline for 3 times. Lipids were extracted directly from 6-well cell-culture plates by adding hexane: isopropanol mixture (3:2) and gentle shaking for 10 min. The process is repeated second time for efficient extraction of all lipids. Lipids were dried under nitrogen stream and separated by TLC using hexane:diethyl ether:acetic acid (80:20:1) solvent system. TLC plate were exposed to phosphor imaging cassette overnight and revealed by Typhoon FLA 7000 phosphor imager. Lipids on TLC plate were stained with iodine vapors; bands were scraped and quantified by liquid scintillation counter. After lipid extraction from 6-well plates, 400 μl of lysis buffer (0.3 N NaOH and 0.1% SDS) was added to each well and kept for shaking for 3 h to lyse the cells for protein measurement by Bio-Rad *DC* Protein Assay kit.

### DGAT Activity Assay

DGAT1- and DGAT2-specific enzymatic activities were measured in membrane fractions isolated from WAT of wild-type and DGAT1 KO mice, respectively. Enzymatic activities were measured at $V_{max}$ substrate concentrations. Assay mixture contained 5–10 μg of membrane proteins, 100 μM of 1,2-dioleoyl-*sn*-glycerol, 25 μM of oleoyl-CoA, which contained [$^{14}$C] oleoyl-CoA as tracer, 25 mM $MgCl_2$ for the DGAT1 assay and 1 mM $MgCl_2$ for the DGAT2 assay in an assay in buffer containing 100 mM Tris-HCl (pH 7.4) and protease inhibitors. Reaction was carried out as described (Cases et al., 2001; Yen et al., 2005). After stopping the reaction, lipids were extracted, then separated by TLC using hexane:diethyl ether:acetic acid (80:20:1) solvent system. The TLC plates were exposed to phosphor imager screen and developed.

### *Ex Vivo* Lipolysis Assay

Mice which had *ad libitum* access to food and water were sacrificed and WAT was isolated and cut in to pieces in phosphate buffer saline (PBS). To measure basal lipolysis, small pieces (2–3 mm) of WAT were incubated in 96-well plate in serum-free medium containing 1% fatty acid–free BSA. To measure glycerol and fatty acid release during stimulated lipolysis, WAT explants were incubated in were incubated in 96-well plate in serum-free medium containing 1% fatty acid–free BSA and 10 μM isoproterenol. Glycerol release into medium was measured using free glycerol reagent kit from Sigma, fatty acid release was measured using NEFA kit from Wako diagnostics. according to manufacturer's protocol.

### Microscopy and Image Processing

Microscopy was performed on spinning disk confocal (Yokogawa CSU-X1) set up on a Nikon Eclipse Ti inverted microscope with a 100× ApoTIRF 1.4 NA objective (Nikon) in

line with 2× amplification. BODIPY 493/503 fluorophore was exited on 561-nm laser line, and fluorescence was detected by an iXon Ultra 897 EMCCD camera (Andor). Acquired images were processed using FIJI software (http://fiji.sc/Fiji). LDs were analyzed using IMARIS software (Bitplane).

### RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA from 3T3-L1 cells was isolated using the RNeasy Kit (Qiagen), according to the manufacturer's instructions. For isolating RNA from WAT, RNeasy Lipid Tissue (Qiagen) was used. Complementary DNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad), and qPCR was performed in triplicates using SYBR Green PCR Master Mix Kit (Applied Biosystems). Sequences of the qPCR primers used are listed in Table S1)

### Immunoblotting

Cells were lysed using RIPA lysis buffer. Tissues were lysed in buffer containing 250 mM sucrose, 100 mM Tris-HCl (pH 7.4), and protease inhibitors in a dounce homogenizer. Proteins were denatured in Laemmli buffer and were separated on 10% SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad). The membranes were blocked with blocking buffer for 2 h in TBST containing 5% BSA or 5% milk, and then incubated with primary antibodies overnight. The membranes were then washed with TBST for 10 min × 3 times, and incubated in mouse secondary antibodies (Santa Cruz Biotechnology) at 1:5000 dilutions in blocking buffer. Membranes was washed again with TBST for 10 min × 3 times and revealed using the Super Signal West Pico kit (Thermo Scientific). DGAT1 antibodies were a gift from Dr. Jin Ye from UT Southwestern Medical Center. CHOP and Bip antibodies were purchased from Cell Signaling Technology.

### DGAT inhibitors

DGAT2 and DGAT1 inhibitors were obtained from collaborators at Merck & Co., Inc. DGAT2 and DGAT1 inhibitors are well characterized and published. (Imbriglio et al., 2015; Liu et al., 2013). Inhibitors were dissolved in DMSO. For [14C]-oleic acid labelling studies, Cells were pre-treated for 30 min with 5 μM inhibitors, cells were pulse labeled with [14C]-oleic acid (50 μCi/μmol) for 3 h in presence of inhibitors.

### Statistical Analyses

Data are presented as means ± SD. Statistical significance was evaluated by unpaired two-tailed Student's t-test using Microsoft Excel software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- DGAT-mediated triglyceride synthesis prevents lipid-induced ER stress in adipocytes
- During lipolysis, DGAT1 is upregulated and mediates fatty acid re-esterification
- DGAT1 activity protects adipocytes from lipid-induced ER stress during lipolysis

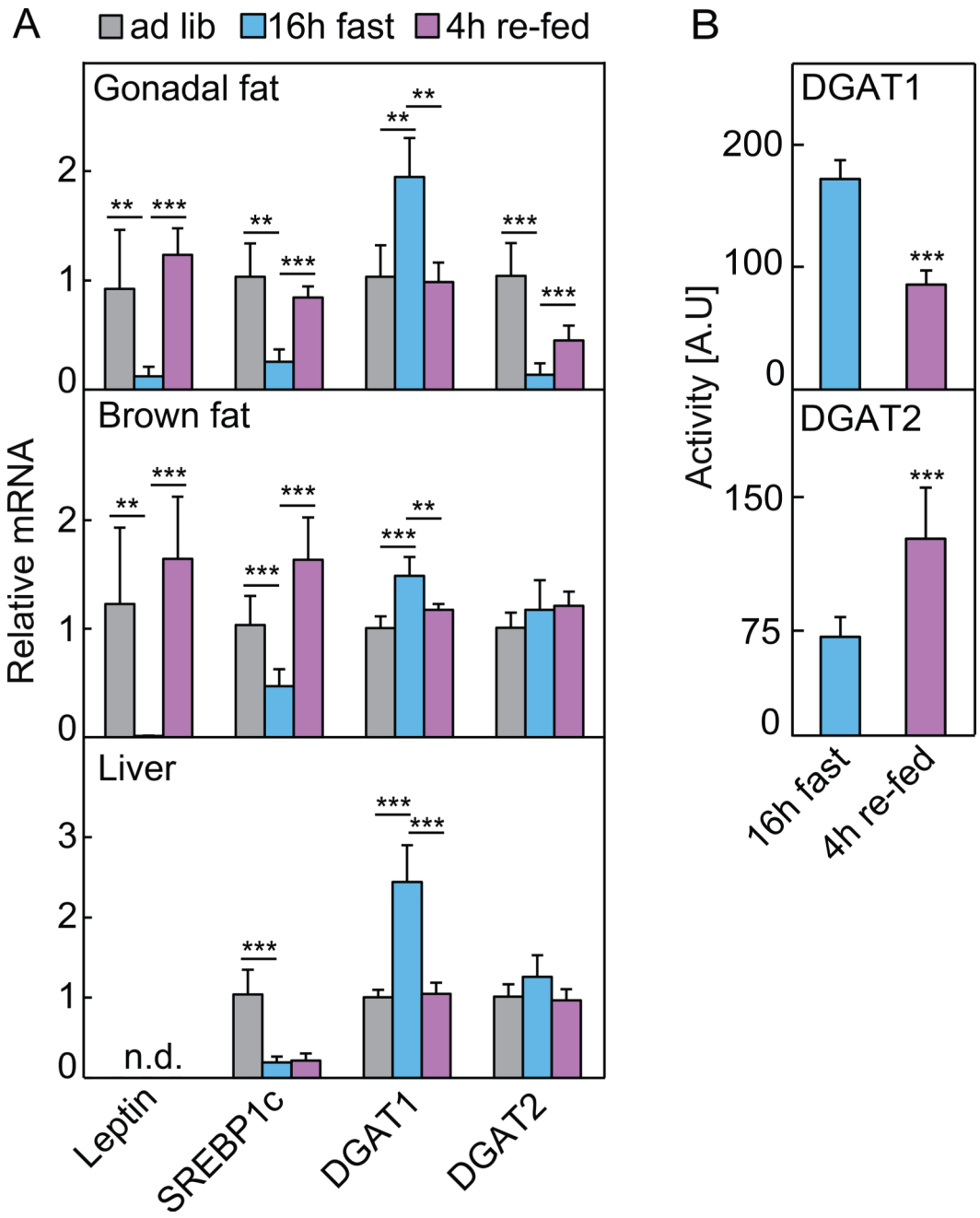


**Figure 1. DGAT1 and DGAT2 in murine WAT are reciprocally regulated in fasting and re-feeding**

(A) Relative mRNA levels in gonadal fat, brown fat and livers of *ad libitum* fed, fasted for 16-h, or after 16-h fasted and 4-h re-fed mice. Cyclophilin was used as reference gene. (B) DGAT1 and DGAT2 enzymatic activities were measured in microsomal fractions of WAT from wild-type mice and DGAT1 KO mice respectively. Data are presented as ± SD. (n=5 mice per group). Statistical significance was evaluated by unpaired two-tailed student's t test. **p<0.01; ***p<0.001; n.d., not detected.

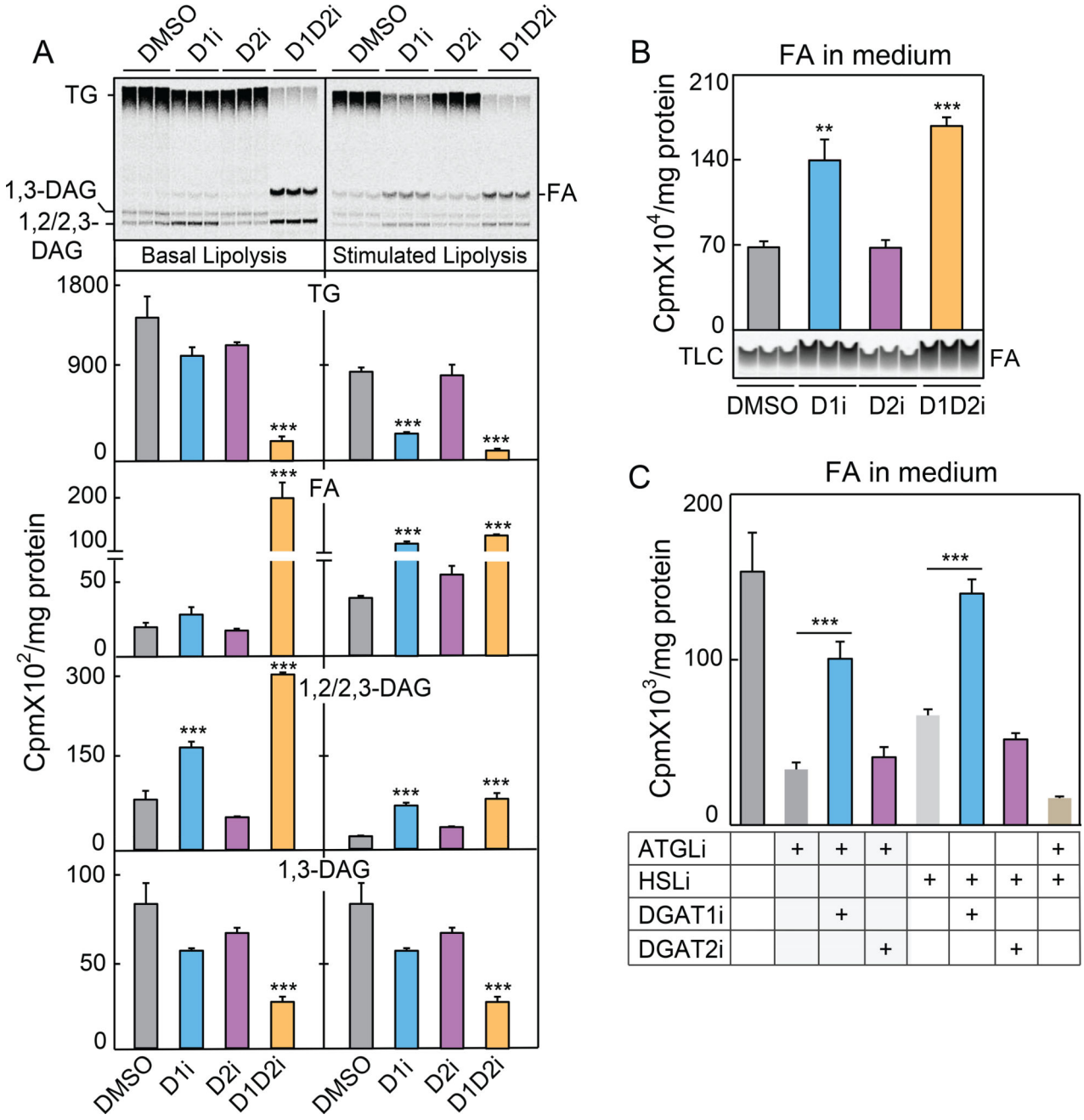


**Figure 2. DGAT1 mediates the majority of TG re-esterification during stimulated lipolysis *in vitro***

(A) Incorporation of [$^{14}$C] oleic acid into TG in differentiated 3T3-L1 adipocytes was measured during basal and 3-h stimulated lipolysis by inhibiting DGAT1 (D1i) or DGAT2 (D2i) or both (D1D2i). Lipids were extracted from cells and separated by TLC. Top: Autoradiographs of TLC plates. Lipids from TLC plates were scraped out and quantified by scintillation counter. Data shown represent two independent experiments. (B) FAs release into medium from differentiated 3T3-L1 adipocytes were measured by extracting lipids from the medium and separating them by TLC. FA region on TLC (shown in the graph) was scraped off and radioactivity was measured by liquid scintillation counter. (C) FAs release

into medium from differentiated 3T3-L1 adipocytes were measured by counting radioactivity in the medium (In figure 2B, we used a higher quantity of tracer. In subsequent experiments, we reduced tracer amount to label cells). Data are presented as ± SD. (n=3 biological replicates). Statistical significance shown is between DMSO control treatments and corresponding inhibitor treatments. Statistical significance was evaluated by unpaired two-tailed student's t test. **p<0.01; ***p<0.001. See also Figures S1 and S2.

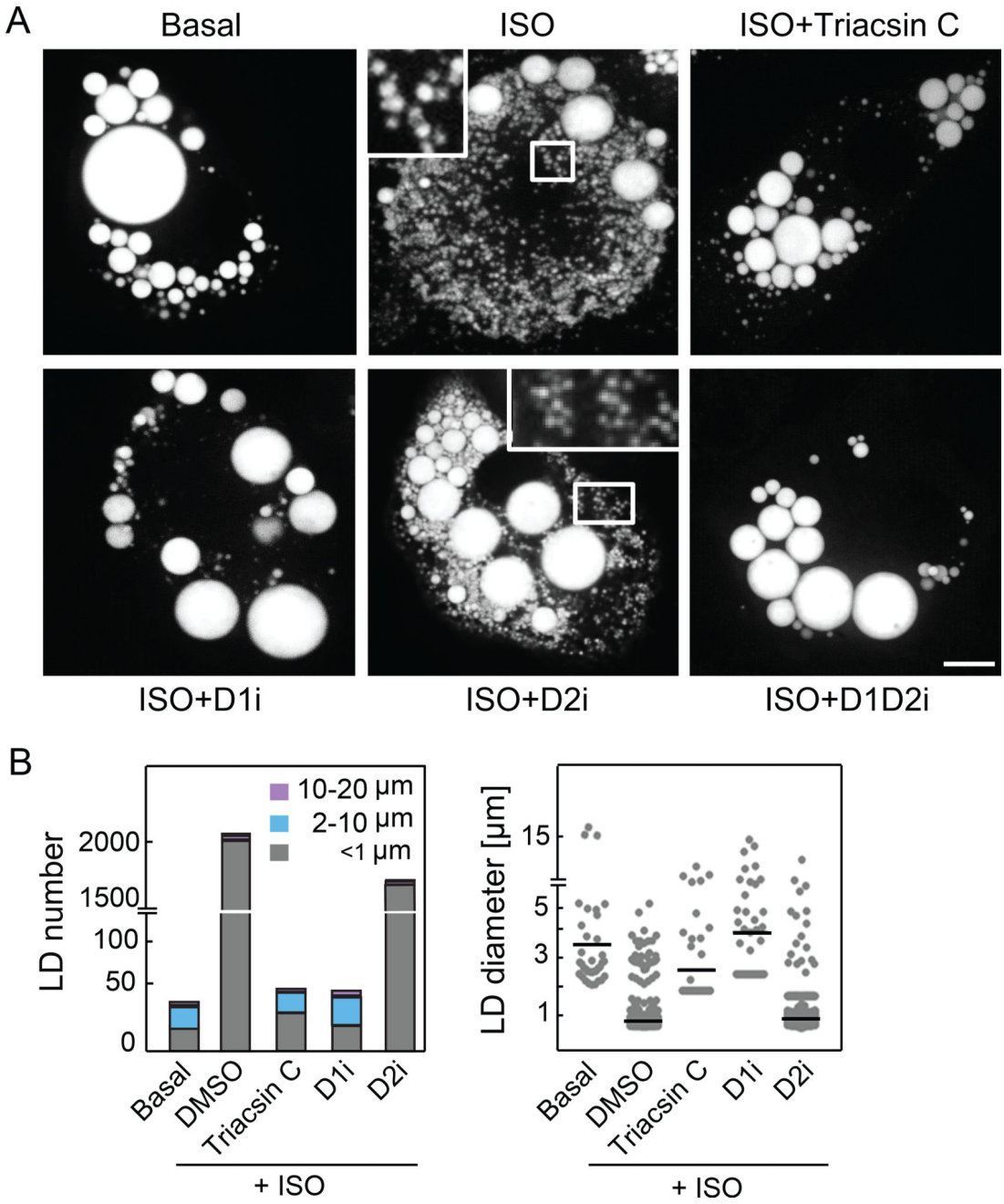


**Figure 3. DGAT1 forms iLDs during stimulated lipolysis**

(A) Confocal microscopic images of differentiated 3T3-L1 cells in basal and isoproterenol stimulated lipolysis (ISO). Lipolysis was induced by treating cells with 10 μM isoproterenol in the medium containing no serum and no BSA. LDs were stained with BODIPY 493/503. Scale bar, 10 μm. (B) LDs were quantified using IMARIS software. Representative results from four independent experiments are shown.

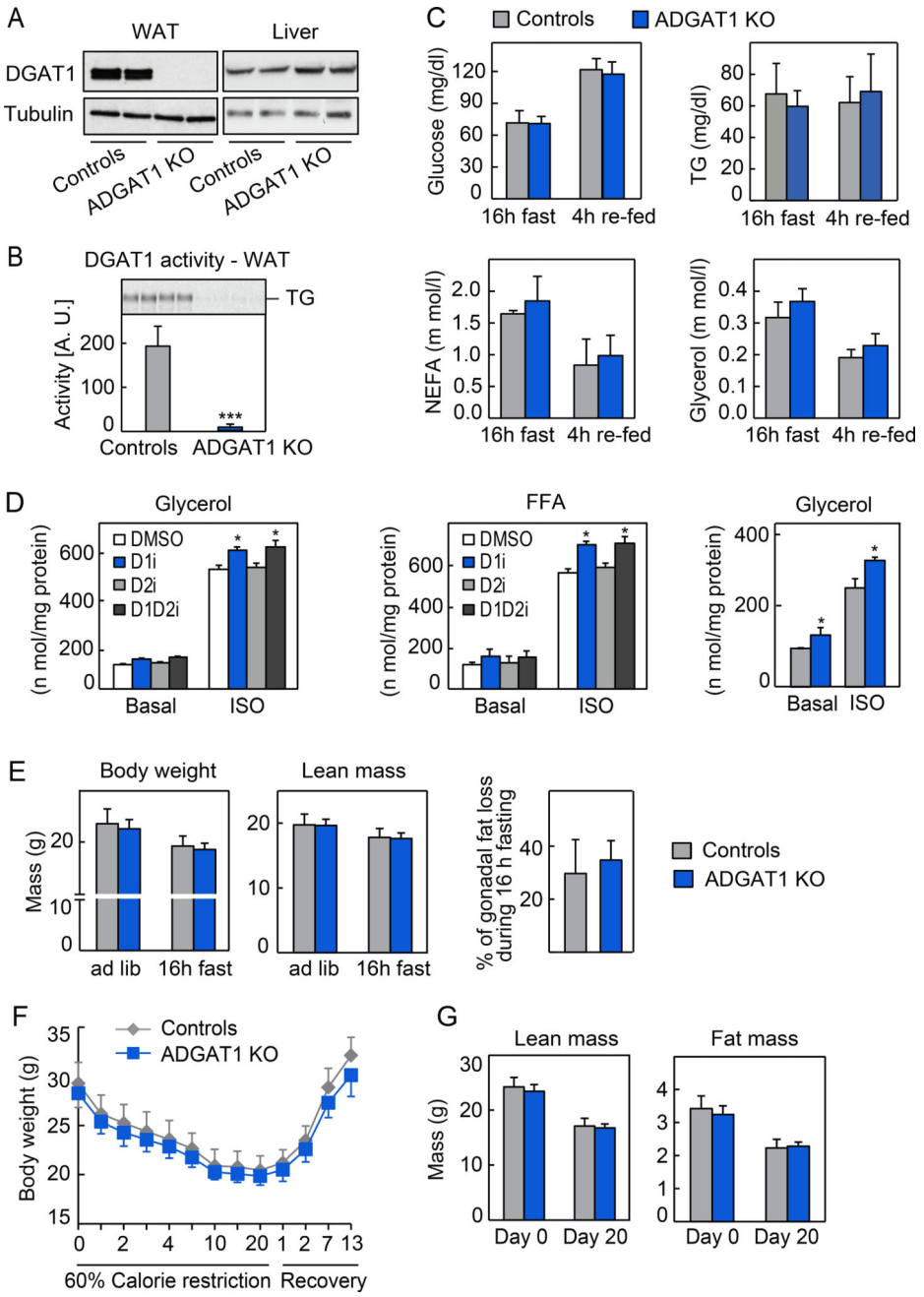


**Figure 4. DGAT1 mediated re-esterification does not prevent fat loss during fasting or calorie restriction in mice**

(A) Western blot analysis showing absence of DGAT1 protein in WAT lysates of ADGAT1 KO mice. (B) DGAT1 activity in WAT microsomal fractions of control and ADGAT1 KO mice. (C) Plasma glucose, glycerol, free FA and TG of 16-h fasted and 4-h re-fed mice (n= 5–6 mice per group). (D) Glycerol release from WAT explants under basal and 3-h stimulated (ISO) lipolysis conditions. (E) Glycerol and FA release from WAT explants treated with DGAT inhibitors under stimulated (ISO) lipolysis for 3-h. (F) Body weights and fat mass loss during 16-h fasting (n= 5–6 mice per group). (G and H) Body weights and change in lean and fat mass during 60% calorie restriction study for 20 days, followed by

recovery by *ad libitum* feeding. (n= 8–10 mice per group). Data are presented as ± SD. Statistical significance was evaluated by unpaired two-tailed student's t test. *p<0.05; ***p<0.001. See also Figure S3.

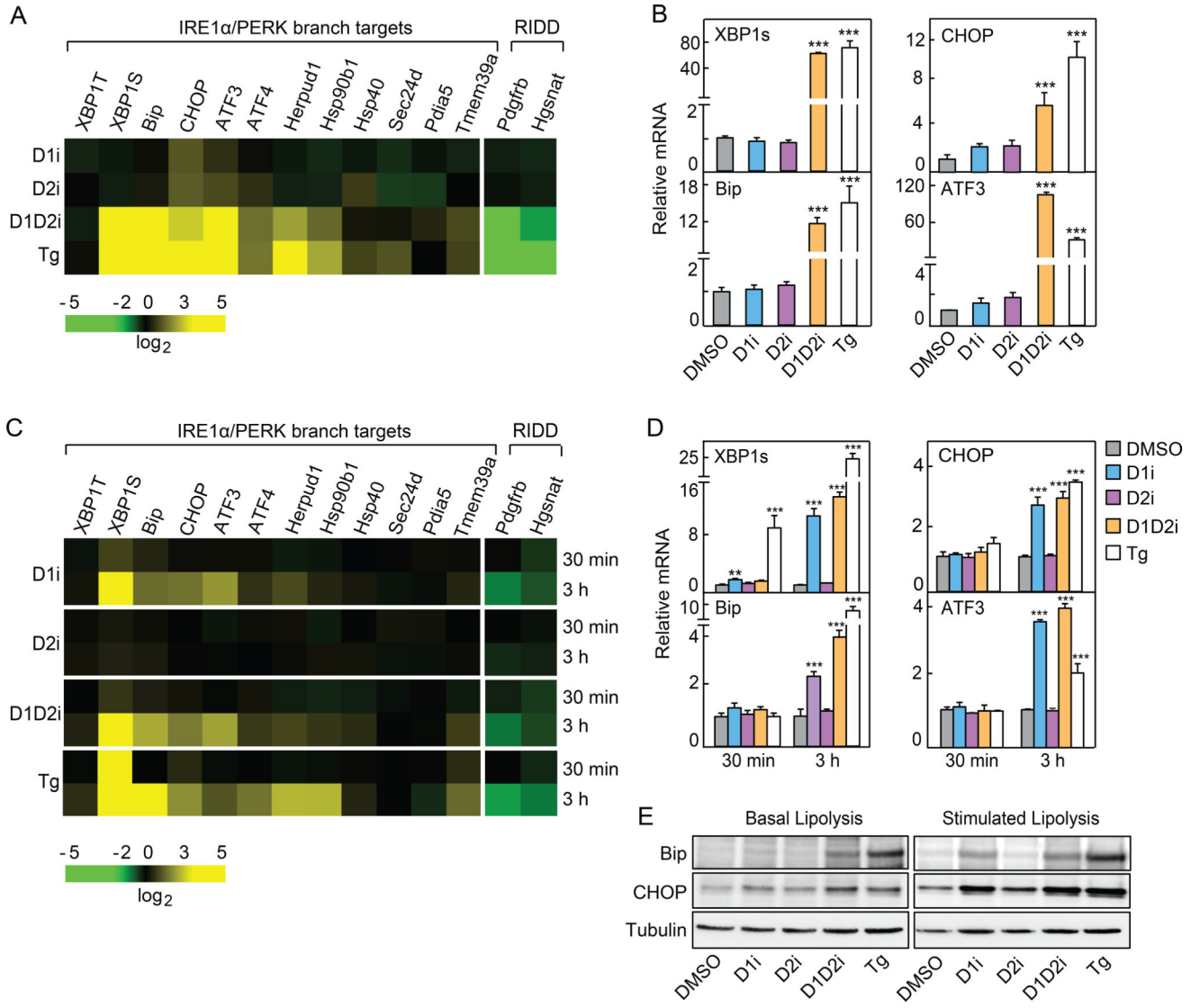


**Figure 5. DGAT1 inhibition during simulated lipolysis induces the ER stress response in 3T3-L1 adipocytes**

(A and B) Heat map and bar graphs showing relative mRNA levels of ER stress marker genes during basal lipolysis determined by RT-qPCR after 10-h treatment with DGAT1 (D1i), DGAT2 (D2i) or both (D1D2i) inhibitors. Thapsigargin (Tg) treated cells were used as positive controls. (C and D) Heat map and bar graphs showing relative mRNA levels of ER stress marker genes during induced lipolysis. Lipolysis was induced by treating cells with 10 μM isoproterenol in the medium containing no serum and no BSA. (E) Western blot analysis of Bip and CHOP during basal and induced lipolysis. Data are presented as ± SD (n=3 biological replicates). Statistical significance shown is between DMSO control treatments and corresponding inhibitor treatments. Statistical significance was evaluated by unpaired two-tailed student's t test. ***p<0.001. See also Figures S4 and S5.

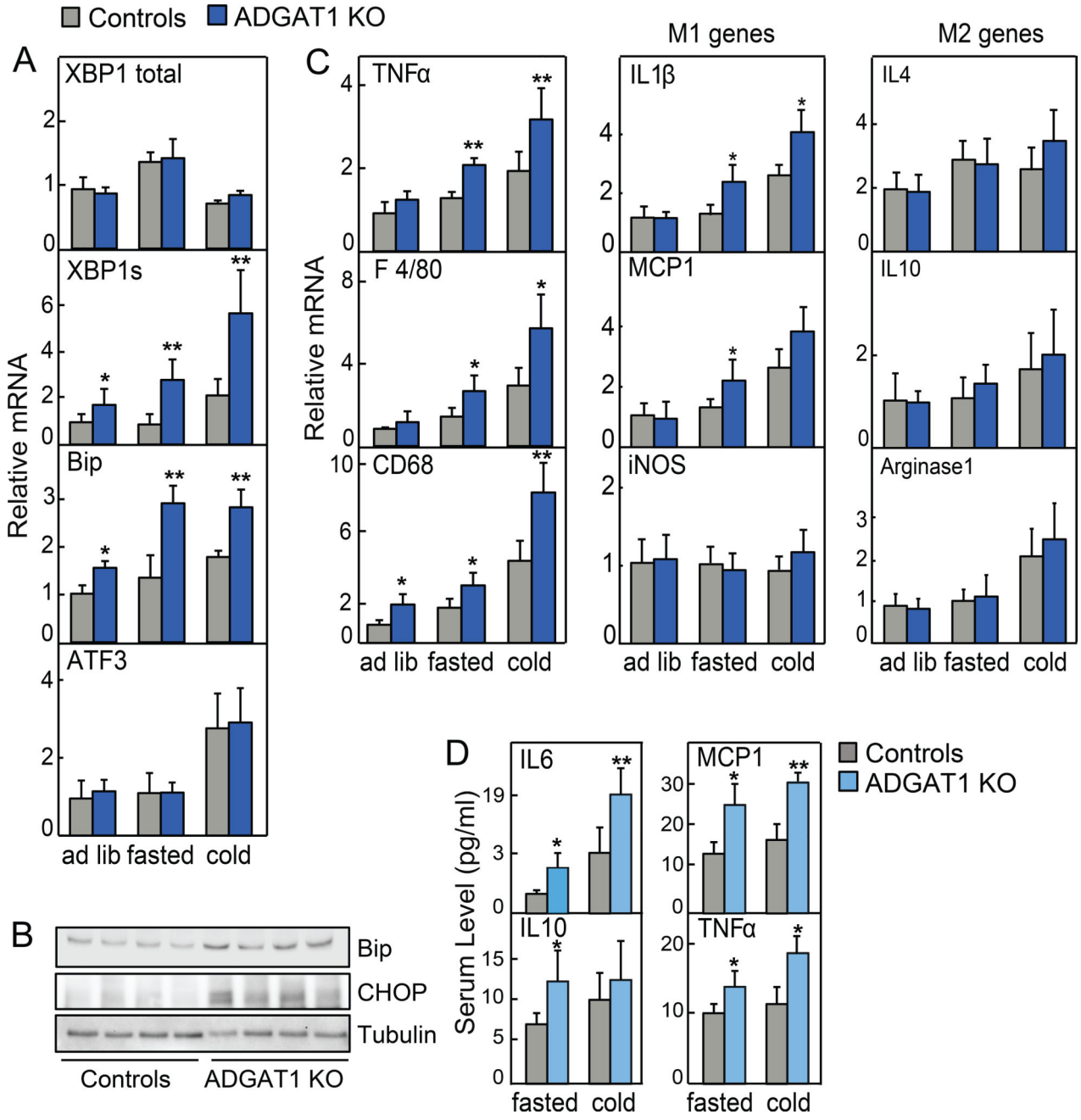


**Figure 6. Lack of DGAT1 in adipose tissue increases the unfolded protein response and associated inflammation during fasting or cold exposure**

(A) RT-qPCR analysis of ER stress marker genes in WAT of *ad libitum* fed, fasted for 16-h, or 6-h cold exposed (while fasting) ADGAT1 KO and control mice. (B) Western blot analysis of Bip and CHOP in WAT of 16-h fasted mice. (C). Relative mRNA levels of inflammatory genes in WAT of *ad libitum* fed, fasted for 16-h, or 6-h cold-exposed (while fasting) ADGAT1 KO and control mice determined by RT-qPCR. (D). Plasma inflammatory markers were estimated by ELISA. Data are presented as ± SD (n=5–6 mice per group). Statistical significance was evaluated by unpaired two-tailed student's t test. *p<0.05. **p<0.01. See also Figure S6.

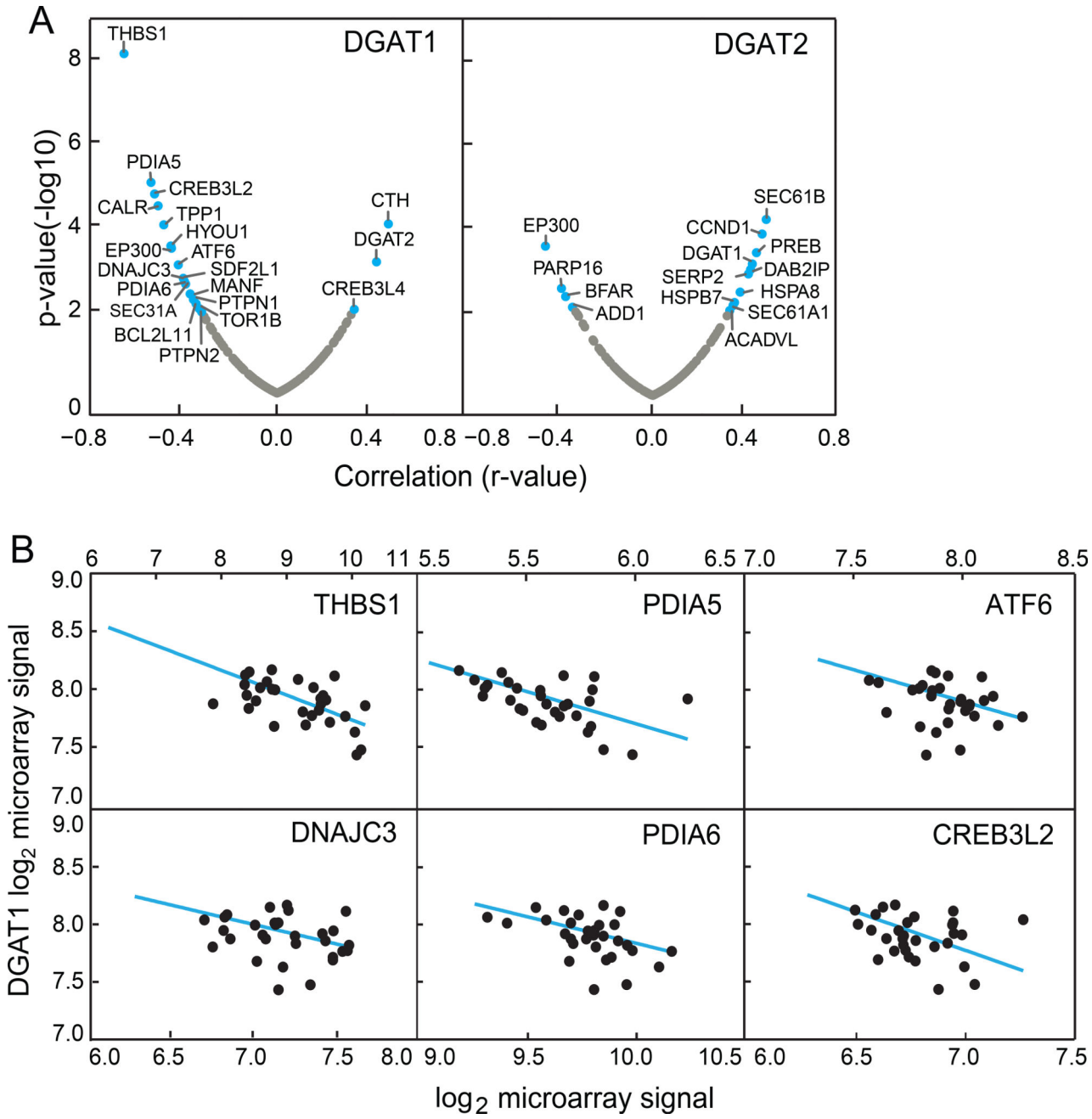


**Figure 7. DGAT1 expression is negatively correlated with ER stress genes in adipose tissue of human subjects**

(A) Correlation of DGAT1 or DGAT2 mRNA levels with genes regulated by the unfolded protein response (GO:0006986, n = 167). Results are based on previously published transcriptional profiles generated from human white adipose tissue derived from 26 non-obese and 30 obese women (Arner et al., 2012). Subjects were fasted overnight. Highlighted genes (blue circles) are significantly correlating ($p < 0.01$) with DGAT1 or DGAT2. (B) Correlation between DGAT1 and a subset of the significant genes from panel (A). Non-

obese (n=26) and obese (n=30) subjects are depicted as white and black circles, respectively. Data are expressed as $\log_2$ microarray signal.