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Growth arrest and DNA damage-inducible alpha regulates muscle repair and fat infiltration through ATP synthase F1 subunit alpha

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Abstract

Background Skeletal muscle fat infiltration is a common feature during ageing, obesity and several myopathies associated with muscular dysfunction and sarcopenia. However, the regulatory mechanisms of intramuscular adipogenesis and strategies to reduce fat infiltration in muscle remain unclear. Here, we identified the growth arrest and DNA damage-inducible alpha (GADD45A), a stress-inducible histone folding protein, as a critical regulator of intramuscular fat (IMAT) infiltration.

Methods To explore the role of GADD45A on IMAT infiltration and muscle regeneration, the gain or loss function of GADD45A in intramuscular preadipocytes was performed. The adipocyte-specific GADD45A knock-in (KI) mice and high IMAT-infiltrated muscle model by glycerol injection (50 μL of 50% *v*/v GLY) were generated. RNA-sequencing, histological changes, gene expression, lipid metabolism, mitochondrial function and the effect of dietary factor epigallocatechin-3-gallate (EGCG) treatment (100 mg/kg) on IMAT infiltration were studied.

Results The unbiased transcriptomics data analysis indicated that GADD45A expression positively correlates with IMAT infiltration and muscle metabolic disorders in humans (correlation: young vs. aged people, *Gadd45a* and *Cebpa*, $r^2 = 0.20, P < 0.05$) and animals (correlation: wild-type [WT] vs. *mdx* mice, *Gadd45a* and *Cebpa*, $r^2 = 0.38, P < 0.05$; NaCl vs. GLY mice, *Gadd45a* and *Adipoq/Fabp4*, $r^2 = 0.80/0.71$, both $P < 0.0001$). In vitro, GADD45A overexpression promotes intramuscular preadipocyte adipogenesis, upregulating the expression of adipogenic genes (*Ppara*: +47%, *Adipoq*: +28%, $P < 0.001$; *Cebpa*: +135%, *Fabp4*: +16%, $P < 0.01$; *Pparg*: +66%, *Leptin*: +77%, $P < 0.05$). GADD45A knockdown robustly decreased lipid accumulation (*Pparg*: −57%, *Adipoq*: −35%, $P < 0.001$; *Fabp4*: −37%, $P < 0.01$; *Leptin*: −28%, $P < 0.05$). GADD45A KI mice exhibit inhibited skeletal muscle regeneration (myofibres: −40%, $P < 0.01$) and enhanced IMAT infiltration (adipocytes: +20%, $P < 0.05$). These KI mice have impaired exercise endurance and mitochondrial function. Mechanistically, GADD45A affects ATP synthase F1 subunit alpha (ATP5A1) ubiquitination degradation (ubiquitinated ATP5A1, $P < 0.001$) by recruiting the E3 ubiquitin ligase TRIM25, which decreases ATP synthesis (ATP production: −23%, $P < 0.01$) and inactivates the cAMP/PKA/LKB1 signalling pathway (cAMP: −36%, $P < 0.01$; decreased phospho-PKA and phospho-LKB1 protein content, $P < 0.01$). The dietary factor EGCG can protect against muscle fat infiltration (triglyceride: −64%, $P < 0.05$) via downregulating GADD45A (decreased GADD45A protein content, $P < 0.001$).

Conclusions Our findings reveal a crucial role of GADD45A in regulating muscle repair and fat infiltration and suggest that inhibition of GADD45A by EGCG might be a potential strategy to combat fat infiltration and its associated muscle dysfunction.

Keywords GADD45A; EGCG; skeletal muscle; fat infiltration; intramuscular adipogenesis; metabolic disorder

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Introduction

Skeletal muscle plays a crucial role in locomotor function, respiration, and whole-body energy homeostasis.[1] Stress, ageing, obesity and muscle dysfunction are associated with sarcopenia and muscle fat infiltration, posing a major threat to muscle health in society.[2] In older adults, the combination of reduced muscle mass (sarcopenia) and increased intramuscular fat (IMAT or IMF) infiltration impairs muscle function, leading to a higher susceptibility for metabolic disorders, such as type 2 diabetes mellitus (T2DM) and insulin resistance.[3] Hence, effective and safe strategies for preventing IMAT infiltration and muscle loss are urgently required to maintain muscle function and extend life span.

IMAT infiltration is regulated by genetic, nutritional, and environmental factors, all of which determine the key signals that regulate skeletal muscle adipogenesis.[4] Available studies identified that ectopic IMAT formation results from adipogenic differentiation of several stem cells. Some transcription factors and signalling pathways have been shown to regulate IMAT infiltration, including CAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptor γ (PPARγ), fatty acid-binding proteins (FABPs), Wnt/β-catenin, Hedgehog, Notch and LKB1/AMPK.[4] These identified pathways mainly focus on the initial stages of adipogenesis, including the conversion of preadipocytes into adipocytes, the coactivators and co-inhibitors involved and their molecular interactions. However, the regulatory mechanisms of these pathways during intramuscular adipogenesis still deserve further investigation. Furthermore, it remains unclear whether other transcriptional factors or signals are involved in IMAT infiltration.

The growth arrest and DNA damage 45A (GADD45A) is a small, p53-controlled histone-fold protein induced by varieties of cellular stress.[5] Previous evidence has revealed the crucial role of GADD45A in DNA repair, cell cycle, apoptosis, DNA demethylation and energy metabolism.[6,7] *Gadd45a*$^{-/-}$ mice exhibit increased genome instability and an obvious metabolic phenotype.[6,8] The GADD45A/ING1 double-knockout mice display segmental progeria, lipodystrophy and several metabolic defects.[9] In human skeletal muscle biopsies, GADD45A was associated with ageing and T2DM.[10] In skeletal muscle, GADD45A may regulate mitochondrial biogenesis and myofibre size.[11,12] In brown and inguinal white adipocytes, GADD45A regulates preadipocyte differentiation and affects metabolism and thermogenesis.[6,13] These findings suggest that GADD45A might be a potential candidate to control muscle development and IMAT deposition. However, experimental evidence to show the exact role of GADD45A during fat infiltration and regeneration in skeletal muscle is still lacking. Furthermore, the regulatory mechanism and proteins interacting with GADD45A to control fat infiltration in skeletal muscle remain to be elucidated.

In this study, we leveraged transcriptomics data from human and animal skeletal muscle models with high IMF deposition and related them to ageing and metabolic disease. We identified GADD45A as a novel regulator of fat infiltration in skeletal muscle. Using functional genomic approaches both in vitro and in vivo, we found that GADD45A promotes intramuscular adipogenesis, impaired skeletal muscle regeneration and mitochondrial metabolism. We identified the molecular mechanism underlying GADD45A's function during IMAT infiltration. Finally, we revealed that EGCG could combat IMAT infiltration via downregulating GADD45A. Our findings demonstrate a novel role of GADD45A for muscle function and propose it as a potential target to combat muscle fat deposition and its related metabolic diseases.

Materials and methods

Animals

All procedures involving mice were approved by the Zhejiang University Animal Care and Use Committee. ROSA26-Gadd45a knockin (KI) mice were obtained from Cyagen Biosciences. The *Adipoq-Cre* (stock #010803), *Lkb1*$^{flox/flox}$ (stock #014143) and wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were on a C57BL/6J genetic background and housed inside the animal facility with free ad libitum access to water and standard rodent chow food. The age of the mice ranged between 8 and 10 weeks during the experiments. PCR genotyping was carried out as described by the supplier. Food intake was measured by weighing the total individual food consumption missing from the hopper once per week.

GLY injection model

Mice were first anaesthetised with an intraperitoneal injection of pentobarbital sodium, 0.02 mg per body weight (g). Then 50 μL of 50% *v*/*v* GLY was injected into the *tibialis anterior* (TA) muscle. Mice were allowed to recover for 14 dpi before the animal was euthanized, and muscles were collected for analysis. Supplementary methods and key reagent were shown in Table S1.

EGCG treatment in vivo

Mice were injected intraabdominally with 100-mg/kg EGCG (Sigma-Aldrich, catalogue PHR1333) for 14 consecutive days after the day of GLY injury. Control mice were treated with 0.9% saline solution. Mice were randomly allocated to each

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experimental group. TA muscles were harvested for analysis after 14 dpi.

Adipogenic differentiation

For adipogenic differentiation, intramuscular preadipocytes were stimulated with an induction medium (IM) containing DMEM, 10% fetal bovine serum (FBS), 2.85-mM insulin, 0.3-mM dexamethasone (DEXA) and 0.63-mM 3-isobutyl-methylxanthine (IBMX) for 4 days on confluence and then allowed to differentiate in differentiation medium (DM) containing DMEM, 10% FBS and 200-nM insulin for 2 days until all adipocytes matured. To avoid a cell density effect on adipogenic differentiation, cells were treated to differentiate when they reached 90% confluence.

Histological analysis

TA muscles from mice were fixed in 4% formalin for 24 h at room temperature (RT). Then, the tissues were embedded in paraffin and cut into 4-μm slices. For haematoxylin and eosin (H&E) staining, the sections were deparaffinized and rehydrated, and the nuclei were stained with haematoxylin for 15 min. Sections were then rinsed in running tap water and stained with eosin for 1 min, dehydrated, mounted and captured. Whole-slide digital images were collected at ×20 magnification with an Aperio Scan Scope slide scanner (Aperio, Vista, CA, USA). Scanned images of H&E staining were analysed by Photoshop CS6 to calculate the nuclei.

Statistical analysis

Data were presented as means ± standard error of the mean (SEM) from at least three independent experiments. GraphPad (Prism 8) was used for preparing the graphs and data analyses. Comparisons were made by two-tailed Student's *t*-tests. Differences between groups were considered significant at $P < 0.05$.

Results

GADD45A expression is positively associated with intramuscular fat infiltration

To identify potential regulators associated with skeletal muscle fat infiltration, we re-analysed publicly available microarray data derived from skeletal muscle samples obtained during ageing or metabolic disease. Notably, we found that GADD45A and several lipid metabolism and fat deposition-related genes, including *Fabp4*, *Cebpa* and *Cebpb*, were highly expressed in aged human muscle or patients suffering from metabolic syndrome (GSE136344) (*Figure* 1A), as well as in *mdx* mouse muscle (GSE162455) (*Figure* 1B). GADD45A expression was positively correlated with genes relating to adipogenic differentiation (*Fabp4*, *Cebpa* and *Cebpb*) whereas it was negatively correlated with genes relating to myogenic differentiation (*Myh4* and *Myh14*) (*Figure* 1B). Moreover, differentially expressed genes (DEGs) in the *longissimus dorsi* muscle (LDM) in pigs with high and low IMF deposition were selected for further analysis (*Figure* S1A–C).[14,15] Likewise, higher levels of *Gadd45a* were found in muscles of breeds with high IMF deposition (Jinhua and Lantang pigs) compared with lean breeds with low IMF deposition correspondingly (Landrace pig) (*Figure* S1A–C). GADD45A expression was positively correlated with IMF content. In addition, GADD45A was also identified as a potential gene regulating preadipocyte proliferation and differentiation in bulls having high IMF deposition (*Figure* S1D).[16] These results suggest that GADD45A expression is positively associated with fat infiltration and lipid metabolism in skeletal muscle.

To further confirm the participation of GADD45A in muscle fat infiltration, we used glycerol (GLY) injection to induce muscle injury as a fat infiltration model in which both body weight and tissue mass were not affected (*Figure* S2A–D). H&E staining showed disrupted myofibres and regenerated myofibres with a central nucleus surrounded by infiltrated cells in GLY-injected *tibialis anterior* (TA) at 14 dpi (*Figure* S2E). Immuno-staining with Perilipin-1 antibody further confirmed adipose accumulation in the GLY-injured TA (*Figure* 1C). To map transcriptional changes in skeletal muscle with IMF infiltration, we performed RNA-seq on a GLY-injected skeletal muscle at 14 dpi. DEGs based on *P*-values <0.05, and $|\log2(\text{fold change})| > 2$ were analysed. Among the DEGs, *Gadd45a* and other adipogenesis-related genes (*Pparg*, *Fabp4*, *Fasn* and *Adipoq*) were significantly increased in the GLY-injected muscle (*Figure* 1D). The correlation of *Gadd45a* expression with the fat deposition-related genes *Adipoq* and *Fabp4*, as well as our qPCR and western blotting results, further confirmed the results (*Figure* 1E,F). Overall, our results indicate that GADD45A is positively associated with skeletal muscle fat infiltration and might play an important role in regulating intramuscular adipogenesis.

GADD45A controls lipid accumulation and adipogenesis in intramuscular adipocytes

To determine the regulatory roles of GADD45A in regulating intramuscular adipocyte deposition, we isolated intramuscular preadipocytes from mice and examined GADD45A expression before and after adipogenic differentiation. Oil Red O showed that obvious lipid accumulation was observed in intramuscular adipocytes after adipogenic differentiation (*Figure* 2A). Consistently, the mRNA levels of adipogenesis-

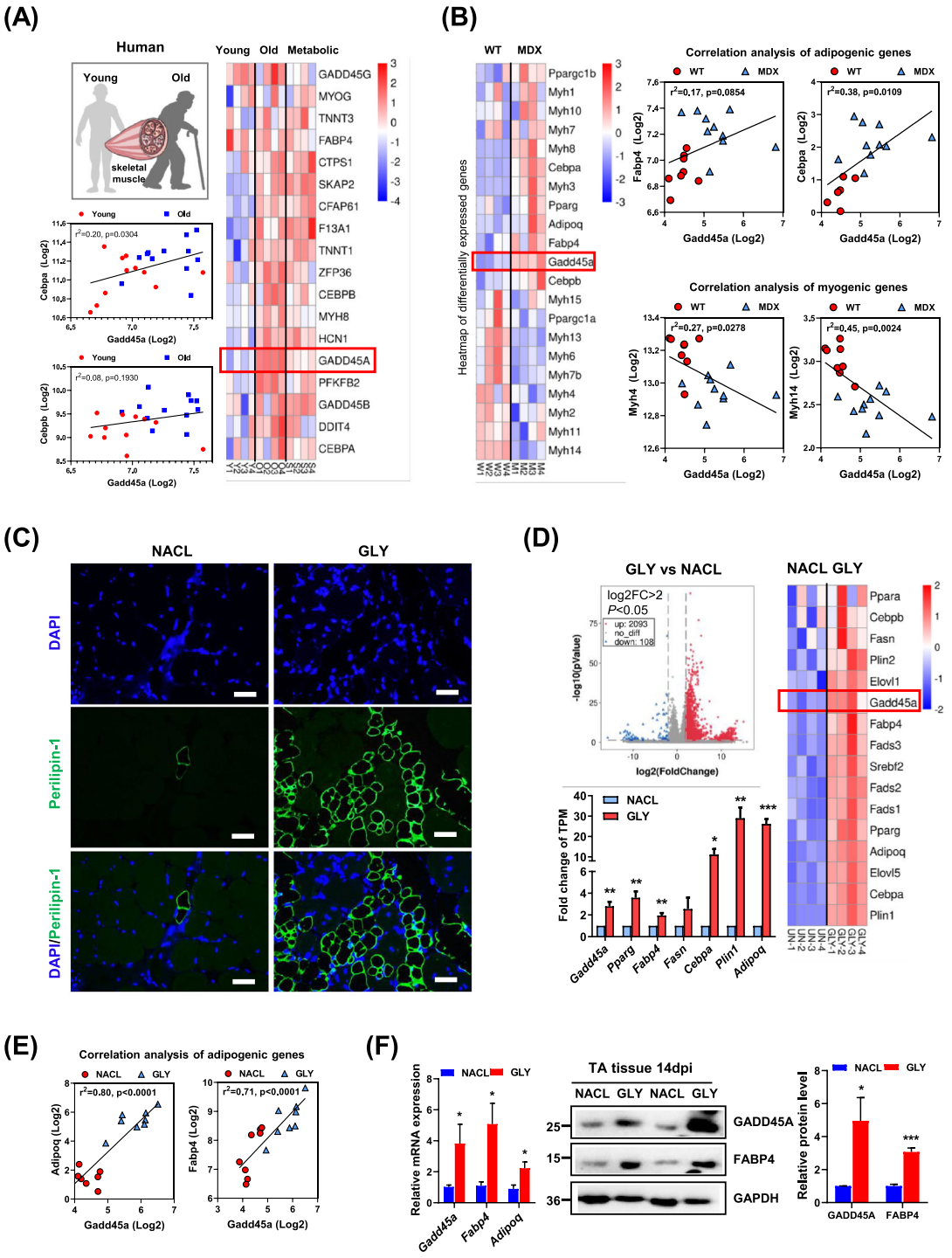

Figure 1 GADD45A expression is positively correlated with fat infiltration in skeletal muscle. (*A*) Schematic representation of skeletal muscles from young (21.8 ± 0.9 years) and aged (73.5 ± 0.2 years) people. Heatmap of differentially expressed genes in human ageing and muscle metabolism models. Correlation analyses of *Gadd45a* and *Cebpa* and *Cebpb* expression patterns in muscle models of human ageing. (*B*) Heatmap analysis of differentially expressed genes for the fat formation and muscle development in WT and *mdx* mice. Correlation analyses of *Gadd45a* and relative genes in WT and *mdx* mouse models. (*C*) Immunofluorescence for adipocytes (Perilipin-1, green) 14 dpi after NaCl or GLY injection. Nuclei were stained with DAPI (blue). Scale bars: 50 μm. (*D*) Scatterplot of gene expression changes induced by glycerol (GLY) that were measured by RNA sequencing. Shades of blue correspond to genes downregulated, and shades of red indicate upregulation in the GLY model. $N = 4$. $|\log2(\text{fold change})| > 2$, $P < 0.05$. Fold change of TPM and heatmap of expressions of selected specific adipogenic genes from the RNA-seq dataset in the GLY model. (*E*) Correlation analyses of gene expression levels between *Gadd45a*, *Adipoq* and *Fabp4*. $N = 8$. (*F*) qPCR and western blotting analysis in the GLY model. Error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test.

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related genes, including *Pparg* and *Adipoq*, were significantly upregulated (*Figure* 2B). Notably, the mRNA and the protein levels of GADD45A were dramatically increased (*Figure* 2B), suggesting that GADD45A is involved in intramuscular adipogenesis. Next, we did gain-of-function and loss-of-function experiments (*Figure* 2C–F). Compared with the control (Ctrl) cells, the adipocytes with GADD45A overexpression (OE, G45a-oe) accumulated higher levels of triglycerides (TGs) (*Figure* 2C) and significantly upregulated the mRNA levels of *Cebpa*, *Pparg*, *Lep, Adipoq*, *Fabp4* and the protein levels of Perilipin-1 (*Figure* 2D). In contrast, knockdown (KD, shG45a) of GADD45A robustly decreased the lipid accumulation and expression of these adipogenesis marker genes (*Figure* 2E,F). Our results demonstrate that GADD45A could enhance intramuscular adipocyte differentiation and lipid accumulation.

GADD45A promotes intramuscular fat infiltration and impairs skeletal muscle regeneration

To investigate the regulatory role of GADD45A in muscle fat infiltration, we generated an adipocyte-specific GADD45A KI mouse model using the Cre-loxP recombination system involving *Adipoq-Cre* and ROSA26-Gadd45a KI mice (*Figure* 3A). Previous studies verified the mature adipocyte-specific expression pattern of *Adipoq-Cre*.[17] Mice with the floxed *Gadd45a* alleles, which do not express Cre recombinase, were used as controls (WT). Genotyping confirmed the efficient overexpression of GADD45A in adipose tissues (*Figure* 3A). Western blotting and qPCR analysis confirmed that the protein and mRNA expression levels of GADD45A were significantly increased in adipose tissues (*Figure* S3A). Following GLY-induced muscle injury at 14 dpi (*Figure* 3A), compared with WT mice, *Adipoq-Cre/*ROSA26-Gadd45a KI mice displayed fewer myofibres with central nuclei and smaller myofibre cross-sectional area, suggesting impaired muscle regeneration (*Figure* 3B). Moreover, GADD45A KI mice exhibited higher IMAT infiltration with increasing Perilipin1-expressing adipocytes in TA muscle (*Figure* 3C). GADD45A KI significantly promoted TGs levels and the mRNA levels of mature adipocyte markers such as *Adipoq* and *Fabp4* (*Figure* 3D) and increased the protein levels of Perilipin-1 and slow skeletal myosin (*Figure* 3E). These results indicate that GADD45A overexpression promoted IMAT infiltration and impaired skeletal muscle regeneration. In addition, we found that the cell sizes of intramuscular adipocytes in KI mice are larger than that of the WT mice (*Figure* S3B,C), suggesting that GADD45A overexpression might promote intramuscular adipocyte hypertrophy. Furthermore, we isolated intramuscular preadipocytes from WT and KI mice and induced them to adipogenic differentiation for 12 days (*Figure* S3D). We found that GADD45A upregulated the expression of the mature adipocyte marker genes (*Adipoq* and *Fabp4*) (*Figure* S3E) and downregulated the expression of the lipolysis-related genes (*Atgl* and *Hsl*) (*Figure* S3E). These results collectively suggest that GADD45A might affect adipocyte differentiation and hypertrophy.

To further assess the effect of GADD45A on muscle function, we subjected mice to treadmill running and examined their endurance capacity. Total running time and distance until exhaustion were evaluated. We found that GLY injection induced a significant decrease in maximum running time and distance (*Figure* 3F). Additionally, the time to exhaustion and the running distance covered by GADD45A KI mice was significantly shorter than that of their control counterparts (*Figure* 3F). The exercise training data indicated that GADD45A overexpression could reduce the skeletal muscle response to exercise endurance. GADD45A decreases exercise endurance in skeletal muscles due to increased IMAT infiltration and impaired muscle regeneration.

GADD45A regulates intramuscular fat infiltration through downregulating the LKB1 pathway

We next investigated how GADD45A regulates muscle fat deposition and muscle regeneration. We measured the expression of genes involved in skeletal muscle development via knocking down or overexpressing GADD45A. Accordingly, we focused on serine/threonine kinase 11, commonly known as liver kinase b1 (LKB1), a tumour suppressor that regulates cellular energy metabolism and stem cell function.[18,19] Strikingly, we found that LKB1 and pLKB1 (C-1) protein levels were significantly decreased in GLY-induced TA muscle of the KI mice, compared with their WT littermates (*Figure* 4A). Because LKB1 is a crucial regulator of the mammalian target of rapamycin (mTOR) pathway,[20] our results suggest that GADD45A-mediated loss of LKB1 also may activate mTOR, which was confirmed by increased pS6 (ser240/244) level (*Figure* 4A). Similar results were obtained from inguinal white adipose tissue (iWAT) in GADD45A KI mice (*Figure* 4B). In contrast, GADD45A KD upregulated the expression of LKB1 and pLKB1 (C-1) and downregulated the expression of pS6 (ser240/244) in adipocytes after adipogenic differentiation (*Figure* 4B). Our results suggest that GADD45A might regulate IMF deposition and muscle regeneration by downregulating the LKB1 pathway.

To examine whether LKB1 deletion affects intramuscular adipogenesis in vivo, we used our previously generated adipocyte-specific LKB1 deletion model *Adipoq-Cre*/*Lkb1*$^{flox/flox}$ (LKB1-KO) mice.[18] After GLY injection for 14 dpi, we found that LKB1-KO mice exhibited significantly increased fat infiltration and decreased muscle regeneration in TA tissues (*Figure* 4C). Consistently, LKB1 deficiency triggered the protein level of Perilipin-1 and the content of TG (*Figure* 4D), as well as the expression of *Adipoq*, *Pparg* and *Fasn* (*Figure* 4E), and the protein levels of FABP4, slow skeletal

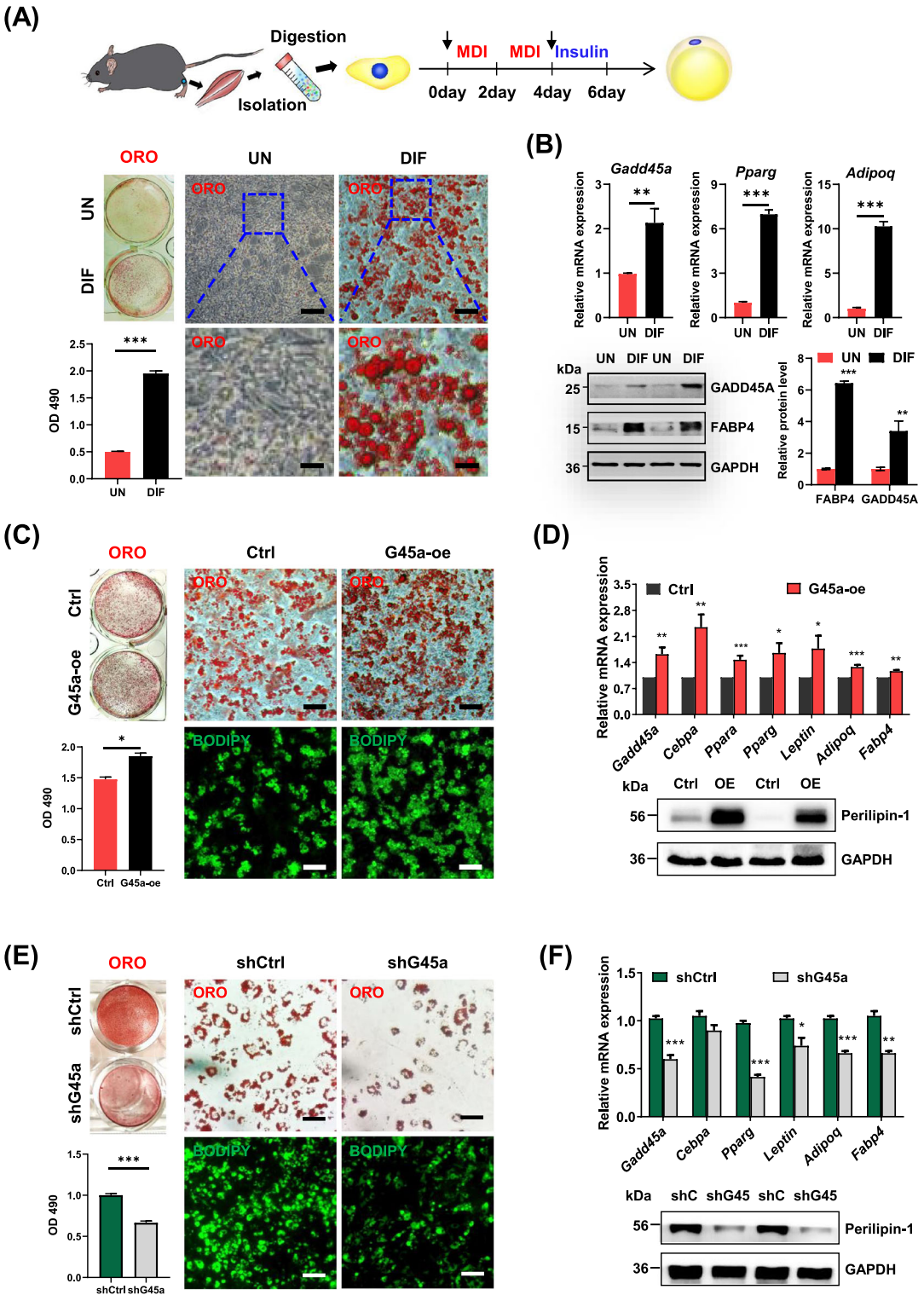

Figure 2 GADD45A promotes lipid accumulation and intramuscular adipocyte adipogenesis. (*A*) Schematic illustration of adipocyte differentiation. Oil red O staining of total lipids in differentiated intramuscular adipocytes. Intramuscular adipocytes were cultured in an adipogenic medium and collected on Day 6. (*B*) qPCR and western blotting of related genes. (*C*) Intramuscular adipocytes were infected with adenovirus expressing GADD45A and were allowed to differentiate. Oil red O and BODIPY staining of control and G45a-oe cells. (*D*) Relative mRNA and protein levels of *Gadd45a* and adipogenic related genes in control and G45a-oe cells after differentiation. (*E*) Lipid accumulation and gene expression (*F*) in differentiated intramuscular adipocytes with control and GADD45A-KD plasmids. Error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test. Scale bars: 200 and 100 μm.

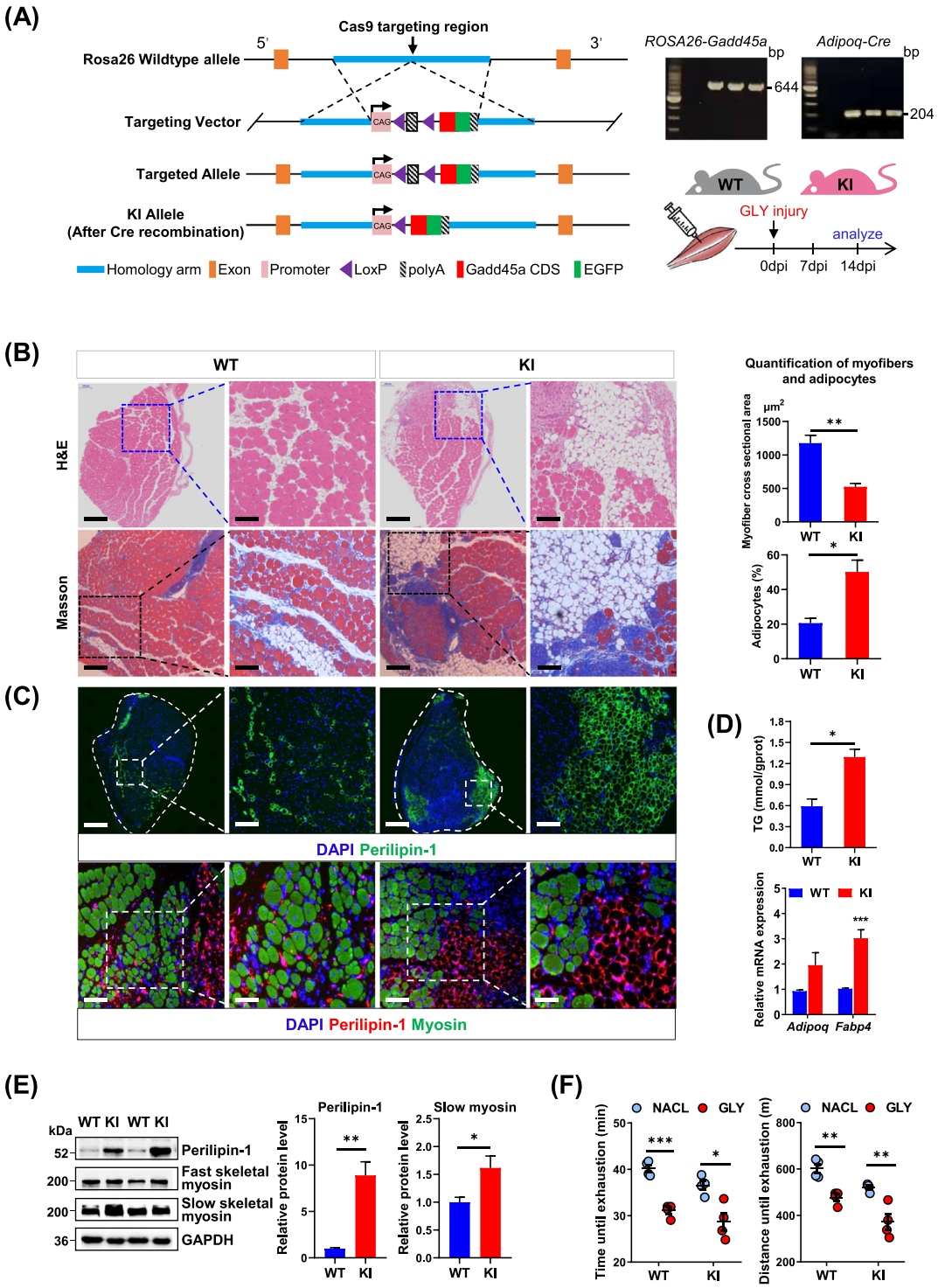

Figure 3 GADD45A overexpression promotes intramuscular adipogenesis at the cost of impaired muscle regeneration. (*A*) A targeted strategy for adipocyte-specific overexpression of GADD45A. Vertical lines represent exons, and triangles represent LoxP. Genotyping of ROSA26-Gadd45a KI mice, and schematic illustration of GLY injury 14 dpi in tibialis anterior (TA) tissues. (*B*) Haematoxylin and eosin and Masson trichrome staining of TAs injected with GLY from wild-type (WT) and GADD45A KI mice, scale bar: 200 μm. The average distribution of cross-sectional areas of myofibres in regenerated TAs. $N = 3$. Quantification of the percentage of adipocytes (%) in injured area 14 dpi after GLY injection. $N = 3$. (*C*) Immunofluorescence for adipocytes (Perilipin-1, green) and nuclei (DAPI, blue) at 14 dpi with GLY injection, as well as adipocytes (Perilipin-1, red), myofibres (fast skeletal myosin, green), and nuclei (DAPI, blue) of TAs. Scale bars: 200 and 50 μm. (*D*) TG content and qPCR from whole TAs with GLY injury. (*E*) Western blotting for Perilipin-1, fast skeletal myosin and slow skeletal myosin. (*F*) Running time and distance until exhaustion of WT and KI mice after GLY injection on 14 dpi. $N = 4$. Error bars represent SEM, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test.

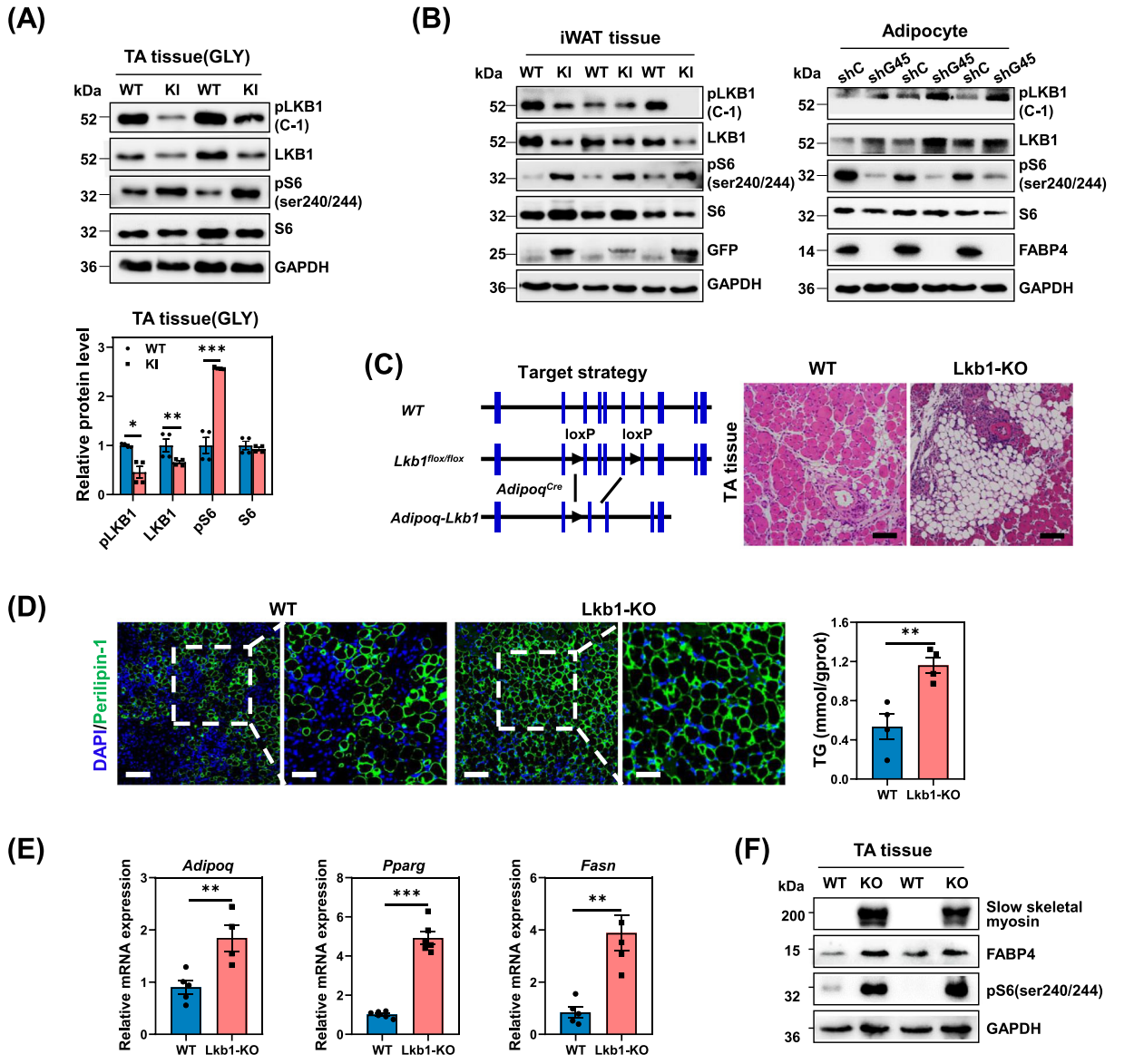

Figure 4 GADD45A regulates intramuscular fat infiltration by downregulating LKB1. (*A*) Protein levels of LKB1, pLKB1 (*C-1*), S6 and pS6 (ser240/244) in tibialis anterior (TA) muscles of wild-type (WT) and KI mice after injury with GLY. (*B*) Western blots in iWAT tissue of WT and GADD45A KI mice, as well as in 3T3-L1 cells with GADD45A KD after 6 days of adipogenic differentiation. (*C*) Target strategy for adipocyte-specific deletion of Lkb1. Vertical lines represent exons, and triangles represent LoxP. Haematoxylin and eosin staining of TAs injected with GLY from *Lkb1*$^{flox/flox}$ (WT) and *Adipoq-Cre: Lkb1*$^{flox/flox}$ (Lkb1-KO) mice, scale bar: 200 μm. (*D*) Immunofluorescence for Perilipin-1 (green) and DAPI (blue) at the indicated time points after injury with GLY. Scale bar: 200 and 50 μm. TG levels of TAs were measured. (*E*,*F*) qPCR (*E*) and western blotting (*F*) analysis in TA muscles after GLY injury in WT and Lkb1-KO mice. Scale bars: 100 μm. Error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test.

myosin and pS6 (ser240/244) (*Figure* 4F). To further investigate the function of LKB1 in intramuscular adipogenesis in vitro, we cultured intramuscular preadipocytes derived from the *Adipoq-Lkb1* mice. Compared with the WT cells, LKB1 KO robustly increased lipid accumulation in differentiated intramuscular fat cells (*Figure* S4A,B) and upregulated the expression of FABP4 consistently (*Figure* S4C). In contrast, adenovirus-mediated overexpression of LKB1 led to a remarkable decrease in lipid accumulation and related gene expression (*Figure* S4). Thus, LKB1 deficiency may facilitate intramuscular adipogenesis, and GADD45A may regulate IMAT infiltration and muscle regeneration via the LKB1 pathway.

GADD45A impairs mitochondrial function and regulates LKB1 via ATP/cAMP/PKA pathway

To understand the molecular mechanism through which GADD45A regulates LKB1 expression, we examined the mito-

chondrial function and cAMP/protein kinase A (PKA) pathway. We used an OXPHOS antibody cocktail to identify mitochondrial respiratory chain complex proteins. We found that the protein levels of complex CV (ATP5A), CIV (MTCO1) and CIII (UQCRC2) were increased in GADD45A KD adipocytes compared with the control cells (*Figure* 5A). Moreover, GADD45A KD increased the mitochondrial oxygen consumption rate (OCR) of adipocytes (*Figure* 5B). Adenosine triphosphate (ATP) production in mitochondria relies on the electron transport chain and oxidative phosphorylation.[21] Both basal respiration after oligomycin inhibition of ATP synthesis and maximal respiration after stimulation with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were significantly higher in GADD45A KD adipocytes than in control cells (*Figure* 5B). Likewise, GADD45A KD increased ATP content, but GADD45A OE had the opposite effect (*Figure* 5C). For adipocyte intracellular cyclic adenosine monophosphate (cAMP) accumulation, GADD45A KD had higher unstimulated cAMP levels compared with control cells, whereas GADD45A OE had lower cAMP levels (*Figure* 5D). These data collectively suggest that GADD45A impairs intracellular mitochondrial function and reduces ATP production and cAMP concentration. LKB1 is a vital downstream effector of the cAMP/PKA pathway. The highly conserved C-terminal residues of LKB1 are phosphorylated (Ser431) by PKA and then activate downstream signalling pathways, including the AMP-activated protein kinase (AMPK) and mTOR.[20,22] We hypothesize that GADD45A might impair mitochondrial function and LKB1 by inactivating the ATP/cAMP-PKA pathway (*Figure* 5E). Indeed, we found that protein levels of phosphorylated PKA (pPKA and Thr197) and phosphorylated LKB1 (pLKB1 and Ser431) were significantly increased in GADD45A-KD adipocytes (*Figure* 5F). In contrast, both proteins were decreased in GADD45A-OE adipocytes (*Figure* 5F). Our data suggest that GADD45A impairs mitochondrial function and LKB1 through the ATP/cAMP-PKA signalling pathway.

GADD45A impairs mitochondrial function and ATP production by interacting and downregulating ATP5A1

To explore how GADD45A affects mitochondrial function and ATP production, we analysed proteins interacting with GADD45A. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue revealed multiple proteins in the GADD45A pulldown sample, but they were absent in the control sample (*Figure* 6A). To discover specific proteins that interact with GADD45A, we performed mass spectrometry (MS) analyses and identified 868 different proteins in the GADD45A pulldown sample and 192 proteins in the control, respectively (Table S2). We found that GADD45A preferentially binds to ATP synthase F1 subunit alpha (ATP5F1A), also known as ATP5A1, a subunit of mitochondrial respiratory chain complex V (complex V), which controls ATP production from ADP in the presence of a proton gradient across the inner membrane (*Figure* 6A). Next, we used co-immunoprecipitation (Co-IP) to examine whether GADD45A interacts with ATP5A1. After overexpressing GADD45A in HEK293 cells, we found that ATP5A1 can be pulled down by Flag-GADD45A (*Figure* 6B). Likewise, endogenous interactions were also found in primary intramuscular adipocytes (*Figure* 6B). To determine how the binding between GADD45A and ATP5A1 affects mitochondrial function, we found that endogenous expression levels of ATP5A1 were markedly increased in GADD45A KD adipocytes, as shown by immunostaining and western blotting analyses (*Figure* S5A, B). In contrast, GADD45A OE decreased ATP5A1 protein levels (*Figure* S5A,C). Together, our results suggest that GADD45A interacts with ATP5A1 and downregulates its expression, which is responsible for impaired ATP synthesis and subsequently blocks the cAMP-PKA-LKB1 signal pathway.

GADD45A affects ATP5A1 ubiquitination degradation by recruiting the E3 ubiquitin ligase TRIM25

To better understand the molecular mechanism by which GADD45A decreases ATP5A1 levels, we first assessed the presence of endogenous ubiquitination, which is often coupled to the proteolysis of target proteins.[23] We found that ubiquitination was increased in the GADD45A-OE adipocytes, suggesting that some proteins have increased ubiquitination and some ubiquitinated proteins accumulated (*Figure* 6C). Next, to examine the ubiquitination status of ATP5A1, we immunoprecipitated endogenous ATP5A1 and then used anti-ubiquitin and anti-ATP5A1 antibodies for western blotting analysis. We found that intramitochondrial protein ATP5A1 with a higher molecular weight was significantly enriched in the immunoprecipitated fraction, which was probably due to the increased accessibility of ATP5A1 epitopes to the antibody on these isoforms (*Figure* 6C). Protein quantification results showed increased ubiquitination of ATP5A1 in GADD45A-OE adipocytes compared with control cells (*Figure* 6C). The results illustrate that ubiquitylation might represent a primary modality associated with the regulation of GADD45A-mediated ATP5A1 decrease. Finally, to further determine whether the ubiquitin-proteasome system (UPS) is involved in the increased ubiquitination of ATP5A1 in the presence of GADD45A, we treated control- or GADD45A-OE plasmid-transfected adipocytes with cycloheximide (CHX) for 16 h to block protein synthesis and then examined degradation of ATP5A1. In control-transfected cells, the ATP5A1 level was not significantly affected by treatment with CHX or the proteasome inhibitor MG132 within this time window, suggesting a relatively long half-life. This finding is consistent with an earlier study on ATP5A1 protein stability.[24] Intriguingly, the

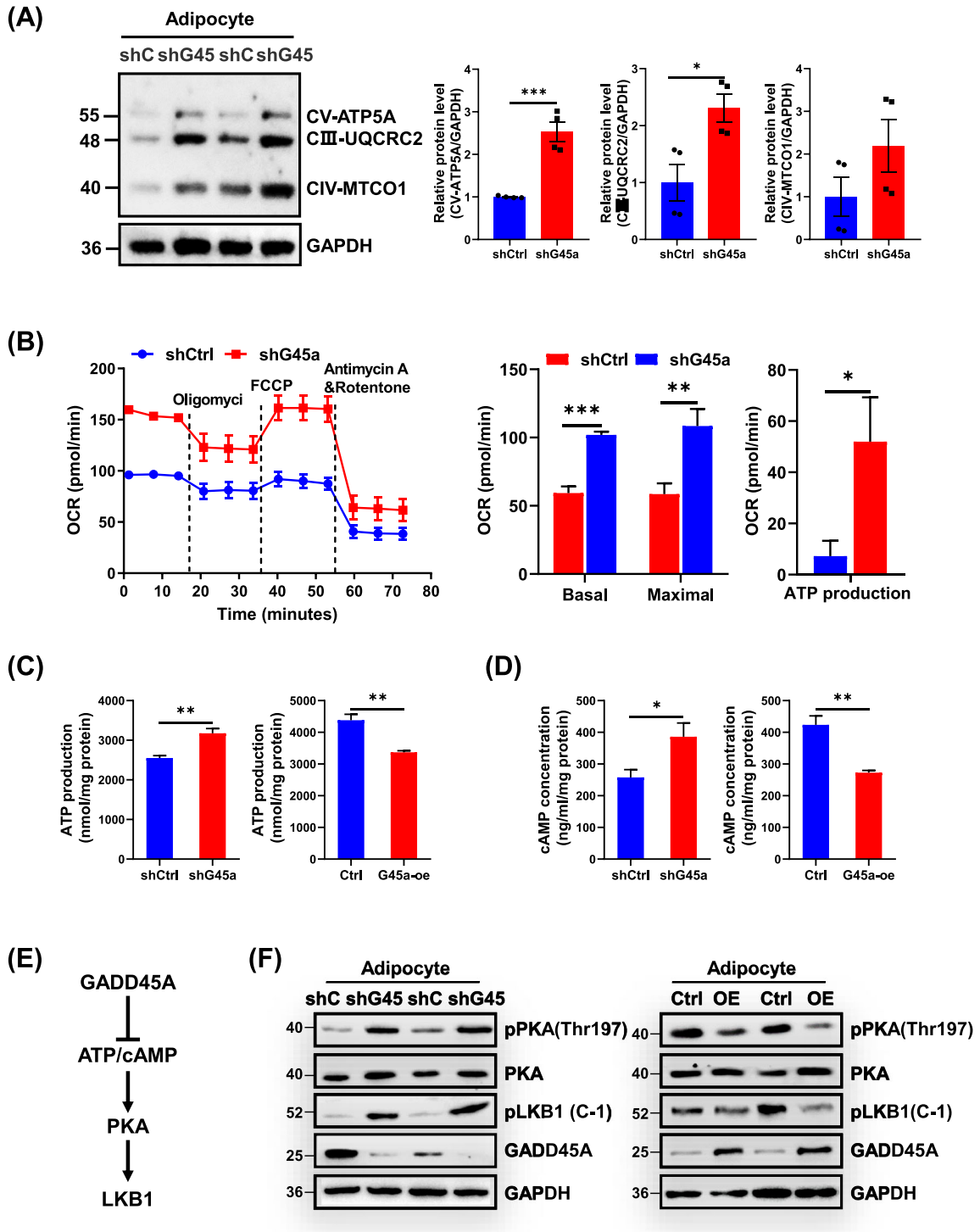

Figure 5 GADD45A impairs the mitochondrial function and downregulates LKB1 through ATP/cAMP-PKA pathway. (*A*) The protein levels of ETC (electron transport chain) complexes (ATP5A, ATP synthase, H + transporting, mitochondrial F1 complex, alpha 1; UQCRC2, ubiquinol-cytochrome c reductase core protein II; MTCO1, cytochrome c oxidase I) in 3T3-L1 cells with GADD45A KD. (*B*) Oxygen consumption rate (OCR) of 3T3-L1 cells with or without GADD45A KD, FCCP and antimycin A/rotenone to determine the uncoupled, maximal and nonmitochondrial-mediated OCR, respectively (n = 10). The average basal and maximal respiration rates and ATP production are shown on the right panel. (*C*) ATP production in GADD45A-KD and GADD45A-OE adipocytes. (*D*) Intracellular cAMP in 3T3-L1 cells transfected with GADD45A-KD and GADD45A-OE plasmids. (*E*) Schematic summary illustrating how GADD45A regulates the cAMP-PKA-LKB1 pathway. (*F*) Protein levels of GADD45A, pLKB1, LKB1 and pPKA with GADD45A KD or OE. Error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed Student's *t*-test.

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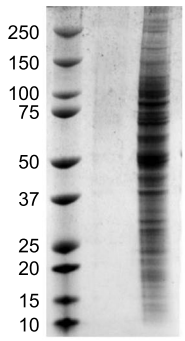
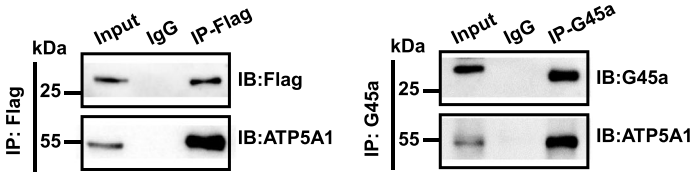
(A)

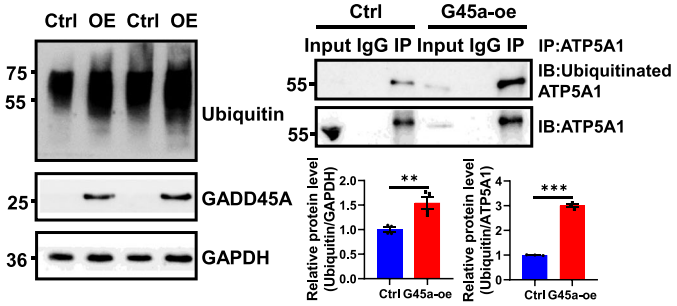


Accession	Gene name	Sum PEP Score	# Peptides	# PSMs	# Unique Peptides	MW [kDa]
P25705	ATP5F1A	22.98931853	8	8	8	59.714
P24522	GADD45A	18.62858967	3	4	3	18.324

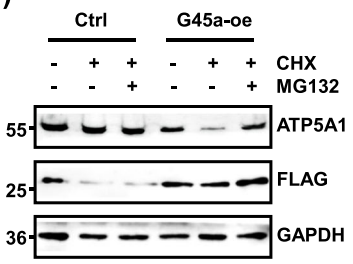
(B)



(C)

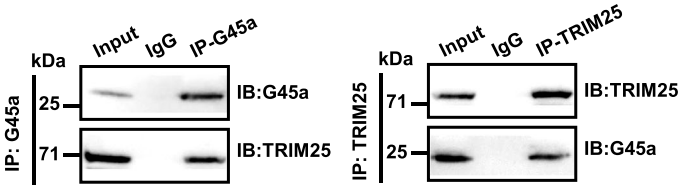


(D)



(E)

Gene name	TRIM25
Accession	Q14258
Sum PEP Score	1.60502328
# Peptides	1
# PSMs	1
# Unique Peptides	1



(F)

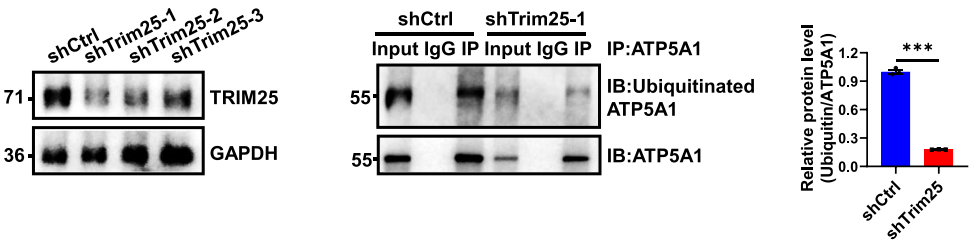


Figure 6 GADD45A compromises ATP production via interacting and decreasing ATP5A1 expression. (*A*) Sodium dodecyl-sulfate polyacrylamide gel electrophoresis and Coomassie brilliant blue staining of the GST-GADD45A pulldown potential binding proteins. Lane 1, marker; lane 2, GST-IgG protein (negative control); lane 3, GST-GADD45A protein with potential binding proteins. Proteins identified by mass spectrometry are indicated below. (*B*) GADD45A interacts with ATP5A1. HEK293 cells were transfected with pCDNA-FLAG-GADD45A, and the lysates were immunoprecipitated (IP) with FLAG antibody and blotted with FLAG and ATP5A1 antibodies. Endogenously expressed GADD45A binds to ATP5A1. Cell lysates from adipocytes were IP with GADD45A antibody and blotted with GADD45A and ATP5A1 antibodies. (*C*) Western blotting analysis of protein ubiquitination in adipocytes (3T3-L1 cell line). Immunoprecipitated ATP5A1 from HEK293 cells expressing GFP (control); GADD45A (OE) was analysed by western blot with anti-ubiquitin (top) and anti-ATP5A1 (bottom) antibodies. (*D*) ATP5A1 degradation in HEK293 cells expressing GFP (control) and GADD45A (OE) after treatment with CHX with or without MG132 for 16 h. (*E*) Potential interaction of GADD45A and TRIM25 proteins in mass spectrometry data. Co-IP analysis of GADD45A and TRIM25 interaction in vitro. HEK293 cells were co-transfected with pCDNA-FLAG-GADD45A and pCDNA-FLAG-TRIM25 plasmids. (*F*) Knockdown efficiency of TRIM25 shRNA detected by western blotting analysis. Immunoprecipitated ATP5A1 from 3T3-L1 cell line expressing shCtrl or shTrim25-1 was analysed by western blot with anti-ubiquitin (top) and anti-ATP5A1 (bottom) antibodies. Error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed Student's *t*-test.

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GADD45A-OE cells treated with CHX, ATP5A1, degraded faster, probably through the proteasome pathway, because parallel treatment with MG132 prevented its degradation (*Figure* 6D). Thus, ATP5A1 was more sensitive to MG132 treatment in GADD45A overexpressing cells and GADD45A increased the ubiquitination of ATP5A1 and its degradation through the proteasome pathway.

Because ubiquitination is usually catalysed by E3 ubiquitin ligase, we reasoned that GADD45A might act as an adaptor to recruit an E3 ubiquitin ligase to ATP5A1 for ubiquitination. To test this hypothesis, we further screened our mass spectrometry data and found a potential interaction between GADD45A and tripartite motif containing 25 (TRIM25), an E3 ubiquitin ligase (*Figure* 6E). The exogenous interaction of the two proteins was subsequently confirmed by Co-IP experiments (*Figure* 6E). Moreover, we knocked down TRIM25 using shRNA in the 3T3-L1 cell line (*Figure* 6F) and examined the total ubiquitination of ATP5A1. We found that TRIM25 deficiency decreased the total ubiquitination of ATP5A1 (*Figure* 6F). These results indicate that GADD45A might mediate the ubiquitination degradation of ATP5A1 by recruiting TRIM25.

Dietary factor EGCG protects against intramuscular fat infiltration via repressing GADD45A expression

Next, we determined whether downregulating GADD45A through dietary factors could alleviate IMAT infiltration. We examined the EGCG, a significant polyphenol having antioxidant, anticancer, antidiabetic, anti-inflammatory and anti-obesity functions,[25] which could inhibit the expression of GADD45A and that of adipogenic genes in intramuscular preadipocytes (*Figure* S6A–E). We further examined the effect of EGCG on IMAT infiltration in vivo. After EGCG treatment and GLY injury for 14 dpi, mice showed similar body weights but lower food intakes than the controls (*Figure* 7A). Masses of skeletal muscle (TA, EDL, SOL and GAS tissues), adipose tissue (BAT and iWAT tissue) and organs (heart, spleen and lung) were consistent (*Figure* 7B). Interestingly, the H&E and Masson trichrome staining results showed that TA muscle with EGCG + GLY treatment exhibited significantly decreased fat infiltration and fibrosis compared with the CON + GLY group (*Figure* 7C). The distribution of myofibre cross-sectional area showed increased proportions of myofibres with central nuclei in EGCG + GLY-treated mice compared with controls (*Figure* 7C). To better observe fat infiltration and muscle regeneration in the CON + GLY and EGCG + GLY groups, the presence of adipocytes and myofibres was confirmed by Perilipin-1 and myosin expression (*Figure* 7D). EGCG treatment increased the proportion of muscle myofibres in regenerating TA muscles (*Figure* 7D) and decreased fat infiltration in skeletal muscle following GLY injury (*Figure* 7E). Notably, GADD45A protein level was downregulated in the EGCG + GLY group, consistent with the expression of FABP4 (*Figure* 7F). Moreover, using the GADD45A KI mouse model, we found that GADD45A overexpression partially attenuates the effect of EGCG on GLY-induced intramuscular fat infiltration (*Figure* S7). Our results demonstrate that EGCG reduces fat infiltration and promotes muscle regeneration via suppressing GADD45A expression.

Discussion

Skeletal muscle fat infiltration and muscle wasting have adverse effects on human health.[3] Exploring the regulatory mechanism of IMAT infiltration and identifying novel regulators may release potential therapeutic molecular targets for the treatment of skeletal muscle fat infiltration associated with metabolic disorders or diseases. In this study, we identify GADD45A as a crucial regulator of intramuscular adipogenesis in vitro and in vivo. We found that GADD45A promoted intramuscular adipogenesis and inhibited muscle regeneration and mitochondrial function. Mechanistically, GADD45A regulates fat infiltration and mitochondrial function by binding to and promoting the degradation of mitochondrial complex protein ATP5A1, which leads to the reduction of ATP and the inactivation of the cAMP/PKA/LKB1 signalling pathway. In addition, we demonstrate that EGCG combats muscle fat infiltration via downregulating GADD45A. Our study presents a novel role of GADD45A in skeletal muscle fat infiltration and regeneration (*Figure* 8).

GADD45A is the first observed stress-inducible gene that can be activated by the p53 tumour suppressor. Previous studies showed that diverse stressors, including ageing, obesity and muscle disuse, cause muscle dysfunction.[11] Using unbiased transcriptome data analysis, we found that GADD45A and lipid metabolism-related genes (*Fabp4*, *Cebpa* and *Cebpb*) are highly expressed in skeletal muscle from aged humans and animals with high IMF deposition. We demonstrate that GADD45A expression is positively correlated with high IMAT infiltration and lipid metabolism in skeletal muscle. In primary isolated intramuscular preadipocytes, GADD45A gain or loss of function promotes or inhibits adipocyte differentiation, respectively. Intramuscular GLY injection is suitable for investigating the muscular pathologies and mechanisms regulating adipogenesis in skeletal muscle.[26,27] In vivo, we used an adipocyte-specific GADD45A KI mouse model combined with GLY injection and successfully simulated the onset of muscle fat infiltration. Our results show that GADD45A KI mice exhibited higher fat accumulation and collagen deposition in muscle tissues compared with control mice. By comparing the size of intramuscular adipocytes in the two mouse lines, we found that GADD45A might promote adipocyte hypertrophy. The KI mice had reduced muscle regeneration capacity, which led

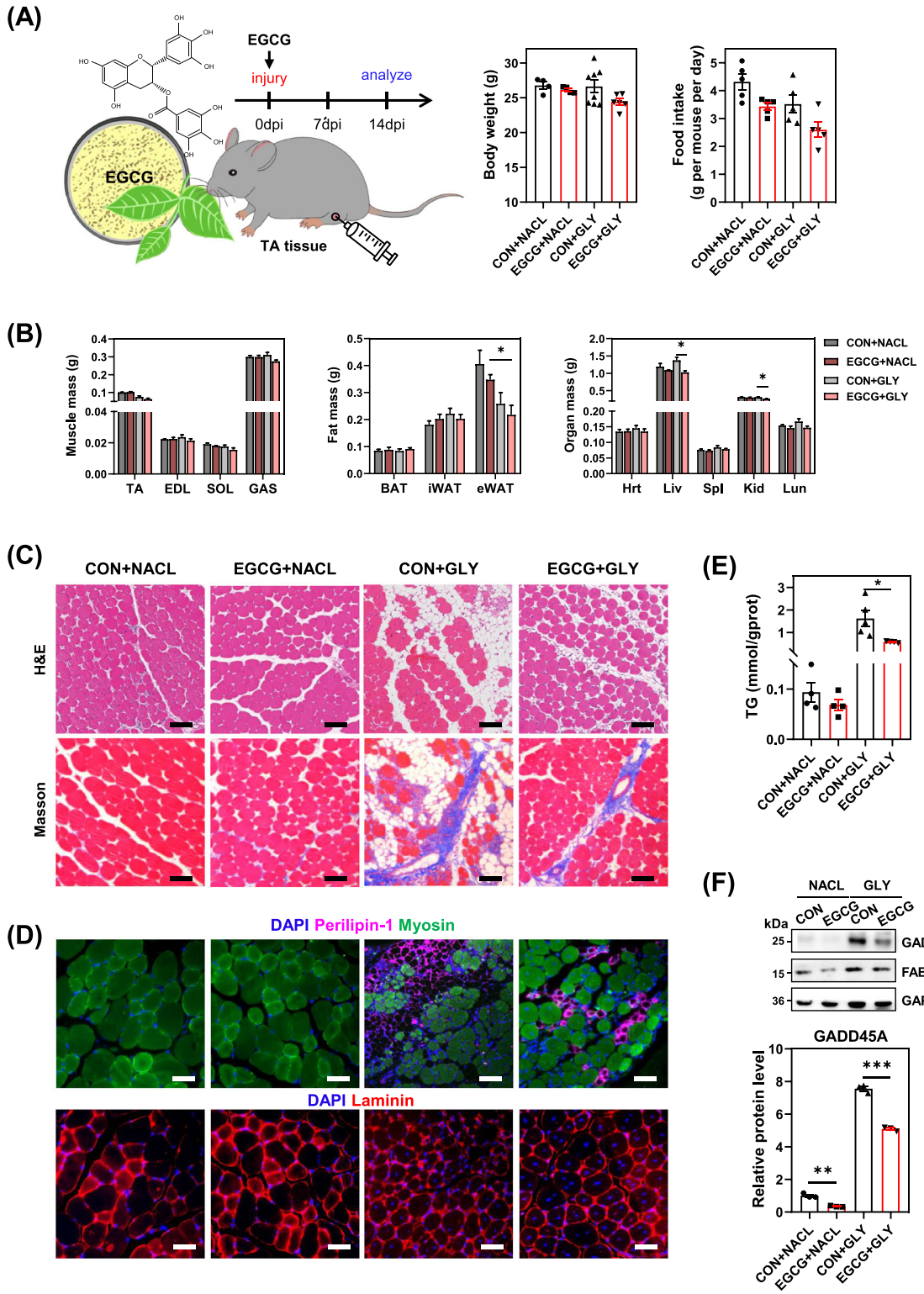

Figure 7 EGCG protects against intramuscular fat deposition via repressing GADD45A expression. (*A*) Schematic illustration of EGCG treatment (100 mg/kg) and GLY injury in vivo. Bodyweight and food intake of mice with EGCG treatment and GLY injury for 14 dpi. (*B*) Muscle mass, fat mass and masses of other organs. (*C*) Haematoxylin and eosin and Masson trichrome staining of TAs with EGCG treatment or GLY injection, scale bar: 200 μm. (*D*) Immunofluorescence for adipocytes (Perilipin-1, violet) and myofibres (fast skeletal myosin, green) and laminin (red) for the four groups. Scale bars: 50 μm. (*E*) TG levels were measured. (*F*) Western blot for GADD45A and FABP4 protein levels. Error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed Student's *t*-test.

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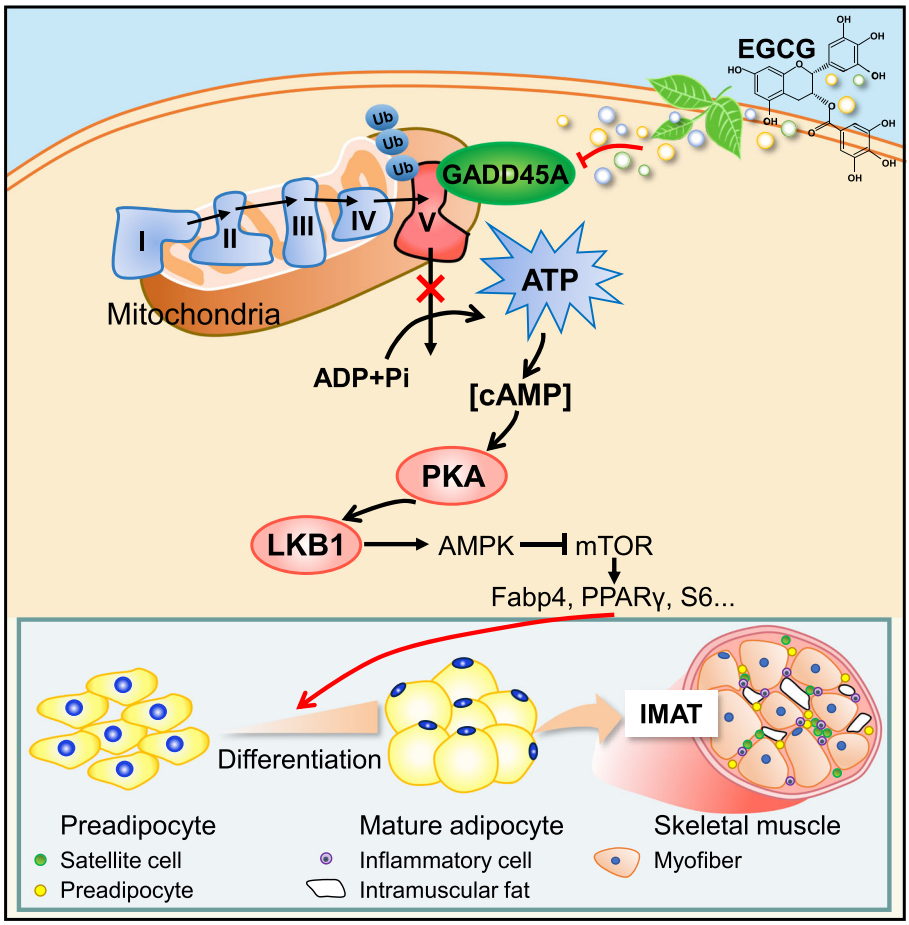

Figure 8 Scheme illustrating a working model of GADD45A in regulating fat infiltration in skeletal muscle. Supplementation with EGCG reduces the expression of GADD45A. GADD45A impairs mitochondrial function via preferentially binding to ATP5A1, leading to decreased ATP synthesis and intracellular cAMP concentration. As a result, the cAMP-PKA pathway gets inactivated, and the expression of LKB1 is downregulated. Furthermore, the inhibition of LKB1 activates mTOR signalling, thereby upregulating the expression of PPARγ and FABP4 and promoting fat accumulation in skeletal muscle.

to a decrease in exercise duration (shorter running time and distance). Importantly, our adipocyte-specific GADD45A KI mouse model will help to investigate the contribution of GADD45A gene modifiers.

At the mechanistic level, one important insight is that GADD45A preferentially binds to ATP5A1, a stable protein often used as a marker for mitochondrial complex V. Previous studies on GADD45A have focused on the role of GADD45A as a nuclear protein in the regulation of the cell cycle, DNA synthesis, muscle atrophy and epigenetic regulation. GADD45A can affect the activation of key transcription factors (*PPARG*, *C/EBPβ*, *ZFP423*, *OSTERIX* and *RUNX2*) and regulate stem cell lipogenesis and osteogenic differentiation through DNA demethylation.[9,13,28] In the current study, we showed that GADD45A preferentially interacts with and promotes the degradation of the mitochondrial protein ATP5A1 by using mass spectrometry and Co-IP experiments, which further demonstrates that GADD45A can cross the mitochondrial membrane and can function in the cytoplasm. The maintenance of mitochondrial homeostasis is crucial for muscle development.[29] Mitochondria produce energy in the form of ATP through a complex metabolic network.[24] Upregulation of ATP5A1 promotes ATP synthase activity, prevents mitochondrial superoxide production and oxidative stress and reduces cardiac hypertrophy and myocardial dysfunction in diabetic mice.[30] Few studies have reported that the function of ATP5A1 is to promote mitochondrial oxidation. In contrast, ATP5A1 deficiency leads to respiratory chain deficiency and mitochondrial disorders.[31] Mitochondrial dysfunction contributes to obesity or fatty acid-induced muscle atrophy.[29] Moreover, disruption of mitochondrial complex I (MCI) induces progressive Parkinson's disease and triggers motor deficits.[32] Intramitochondrial cAMP signalling also regulates the organization and activity

of complex V through mitochondrial proteases. cAMP-1 (Epac1) activation can disrupt mitochondrial function and intracellular lipid homeostasis, leading to cardiomyocyte death.[33] In this study, we observed that GADD45A binds to ATP5A, reduces ATP synthesis and cAMP release, and regulates mitochondrial metabolism in intramuscular adipocytes by inhibiting the cAMP/PKA/LKB1 signalling pathway. We show that GADD45A-mediated degradation of ATP5A1 causes mitochondrial dysfunction in adipocytes in skeletal muscle, leading to muscle metabolic disorders. Our study reveals the relevant function of GADD45A in regulating mitochondrial metabolism for the first time. It addresses the question that has long puzzled researchers: What is the role of GADD45A localized in the cytoplasm? Meanwhile, this finding confirms our previous study, that GADD45A deficiency promotes mitochondrial function and lipolysis with a distinct metabolic phenotype.[6]

We further explored the molecular mechanism by which GADD45A reduces the ATP5A1 level. Mitochondrial function plays an essential role in regulating skeletal muscle remodelling.[34] There is growing evidence that regulating ubiquitin-dependent degradation of mitochondrial proteins can regulate energy metabolism.[24,35] The UPS regulates many cellular functions by degrading key proteins.[24] Consistent with these recent studies, we found that GADD45A increased the ubiquitylation of ATP5A1 and degraded it via the ubiquitin–proteasome pathway. It will be interesting to determine how GADD45A increases ATP5A1 ubiquitination at the molecular level and which other mechanisms may lead to reduced ATP5A1 following GADD45A overexpression. We hypothesized that GADD45A might recruit the E3 ligase to ATP5A1 for ubiquitination. In our mass spectrometry data, we found a potential interaction between GADD45A and E3 ubiquitin-protein ligases, including TRIM25 and BRE1B, of which BRE1B mainly acts as a nuclear factor involved in histone ubiquitination.[36] Given that ATP5A1 is a mitochondrial protein, we focused on TRIM25 in this study. TRIM25, as a member of the E3 ubiquitination ligase family, has been reported to regulate the ubiquitination of various proteins.[37] Here, we showed that GADD45A might recruit TRIM25 and increase the total ubiquitination and degradation of ATP5A1. The biochemical link between GADD45A and ATP5A1 appears to be a critical pathogenic mechanism during the development of adipogenesis and skeletal muscle metabolic diseases.

Importantly, we reveal that GADD45A is negatively regulated by the dietary factor EGCG in GLY-induced muscle. Several nutritional approaches have been applied to skeletal muscle, including curcumin (CUR), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and resveratrol (RES), showing different effects on subcutaneous fat deposition and myogenic differentiation.[25,38] EGCG is an important polyphenol derived from green tea and exerts a wide range of health benefits.[25] EGCG has been reported to activate satellite cells and promote myogenic differentiation.[25] Short-term supplementation with EGCG can reduce postprandial plasma GLY and skeletal muscle interstitial lactate concentrations.[39] In addition, EGCG can regulate the expression of genes involved in lipid metabolism, such as Sirtuin 1 (*Sirt1*), *PPARγ* and PPARγ coactivator-1α (*PGC-1α*).[25] However, the molecular and cellular mechanisms linking metabolic reprogramming to its effects on IMAT infiltration and muscle regeneration remain poorly understood. In this context, we observed that supplementation with EGCG inhibits adipose infiltration and promotes muscle regeneration in vivo and in vitro. Mechanistically, our data explain the mechanism by which EGCG works and suggest that GADD45A-mediated signalling may become a new therapeutic tool for treating muscle diseases.

In conclusion, our results reveal the critical regulatory roles of GADD45A in skeletal muscle fat infiltration and regeneration. We demonstrate that EGCG can downregulate GADD45A to protect against IMAT infiltration. Our findings provide novel insights into the regulatory mechanism of muscle fat infiltration, which may become important for developing therapies to counteract fat infiltration and its related muscle diseases.

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Conflict of interest

The authors declare that they have no competing interests.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

References

1. Mathes S, Fahrner A, Ghoshdastider U, Rüdiger HA, Leunig M, Wolfrum C, et al. FGF-2-dependent signaling activated in aged human skeletal muscle promotes intramuscular adipogenesis. *Proc Natl Acad Sci U S A* 2021;**118**:e2021013118.
2. Rosa-Caldwell ME, Lim S, Haynie WA, Brown JL, Deaver JW, Morena da Silva F, et al. Female mice may have exacerbated catabolic signalling response compared to male mice during development and progression of disuse atrophy. *J Cachexia Sarcopenia Muscle* 2021;**12**:717–730.
3. Biltz NK, Collins KH, Shen KC, Schwartz K, Harris CA, Meyer GA. Infiltration of intramuscular adipose tissue impairs skeletal muscle contraction. *J Physiol* 2020;**598**: 2669–2683.
4. Du M, Yin J, Zhu MJ. Cellular signaling pathways regulating the initial stage of adipogenesis and marbling of skeletal muscle. *Meat Sci* 2010;**86**:103–109.
5. You WJ, Xu ZY, Shan TZ. Regulatory roles of GADD45 alpha in skeletal muscle and adipocyte. *Curr Protein Pept Sc* 2019;**20**: 918–925.
6. You W, Xu Z, Sun Y, Valencak TG, Wang Y, Shan T. GADD45alpha drives brown adipose tissue formation through upregulating PPARγ in mice. *Cell Death Dis* 2020; **11**:585.
7. Zhang X, Li Y, Ji J, Wang X, Zhang M, Li X, et al. Gadd45g initiates embryonic stem cell differentiation and inhibits breast cell carcinogenesis. *Cell Death Discov* 2021;**7**: 271.
8. Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, et al. Genomic instability in Gadd45a-deficient mice. *Nat Genet* 1999; **23**:176–184.
9. Schäfer A, Mekker B, Mallick M, Vastolo V, Karaulanov E, Sebastian D, et al. Impaired DNA demethylation of C/EBP sites causes premature aging. *Gene Dev* 2018;**32**: 742–762.
10. Su J, Ekman C, Oskolkov N, Lahti L, Ström K, Brazma A, et al. A novel atlas of gene expression in human skeletal muscle reveals molecular changes associated with aging. *Skelet Muscle* 2015;**5**:35.
11. Ebert SM, Dyle MC, Kunkel SD, Bullard SA, Bongers KS, Fox DK, et al. Stress-induced skeletal muscle Gadd45a expression reprograms myonuclei and causes muscle atrophy. *J Biol Chem* 2012;**287**: 27290–27301.
12. Ehmsen JT, Kawaguchi R, Kaval D, Johnson AE, Nachun D, Coppola G, et al. GADD45A is a protective modifier of neurogenic skeletal muscle atrophy. *JCI Insight* 2021;**6**: e149381.
13. Wang B, Fu X, Zhu MJ, Du M. Retinoic acid inhibits white adipogenesis by disrupting GADD45A-mediated Zfp423 DNA demethylation. *J Mol Cell Biol* 2017;**9**:338–349.
14. Wu T, Zhang Z, Yuan Z, Lo LJ, Chen J, Wang Y, et al. Distinctive genes determine different intramuscular fat and muscle fiber ratios of the longissimus dorsi muscles in Jinhua and landrace pigs. *PLoS One* 2013; **8**:e53181.
15. Yu K, Shu G, yuan F, Zhu X, Gao P, Wang S, et al. Fatty acid and transcriptome profiling of longissimus dorsi muscles between pig breeds differing in meat quality. *Int J Biol Sci* 2013;**9**:108–118.
16. Sadkowski T, Ciecierska A, Majewska A, Oprządek J, Dasiewicz K, Ollik M, et al. Transcriptional background of beef marbling—novel genes implicated in intramuscular fat deposition. *Meat Sci* 2014; **97**:32–41.
17. Lee KY, Russell SJ, Ussar S, Boucher J, Vernochet C, Mori MA, et al. Lessons on conditional gene targeting in mouse adipose tissue. *Diabetes* 2013;**62**:864–874.
18. Shan T, Xiong Y, Zhang P, Li Z, Jiang Q, Bi P, et al. Lkb1 controls brown adipose tissue growth and thermogenesis by regulating the intracellular localization of CRTC3. *Nat Commun* 2016;**7**:12205.
19. Shan T, Zhang P, Liang X, Bi P, Yue F, Kuang S. Lkb1 is indispensable for skeletal muscle development, regeneration, and satellite cell homeostasis. *Stem Cells* 2014;**32**: 2893–2907.
20. Lai LP, Lilley BN, Sanes JR, McMahon AP. Lkb1/Stk11 regulation of mTOR signaling controls the transition of chondrocyte fates and suppresses skeletal tumor formation. *Proc Natl Acad Sci U S A* 2013;**110**: 19450–19455.
21. Wang Y, Tang B, Long L, Luo P, Xiang W, Li X, et al. Improvement of obesity-associated disorders by a small-molecule drug targeting mitochondria of adipose tissue macrophages. *Nat Commun* 2021;**12**:102.
22. Ohtake Y, Sami A, Jiang X, Horiuchi M, Slattery K, Ma L, et al. Promoting axon regeneration in adult CNS by targeting liver kinase B1. *Mol Ther* 2019;**27**:102–117.
23. Heo JM, Rutter J. Ubiquitin-dependent mitochondrial protein degradation. *Int J Biochem Cell Biol* 2011;**43**:1422–1426.
24. Lavie J, de Belvalet H, Sonon S, Ion AM, Dumon E, Melser S, et al. Ubiquitin-dependent degradation of mitochondrial proteins regulates energy metabolism. *Cell Rep* 2018;**23**:2852–2863.
25. Wang L, Xu Z, Ling D, Li J, Wang Y, Shan T. The regulatory role of dietary factors in skeletal muscle development, regeneration and function. *Crit Rev Food Sci Nutr* 2022; **62**:764–782.
26. Mahdy MAA. Glycerol-induced injury as a new model of muscle regeneration. *Cell Tissue Res* 2018;**374**:233–241.
27. Xu Z, You W, Chen W, Zhou Y, Nong Q, Valencak TG, et al. Single-cell RNA sequencing and lipidomics reveal cell and lipid dynamics of fat infiltration in skeletal muscle. *J Cachexia Sarcopenia Muscle* 2021;**12**:109–129.
28. Zhang RP, Shao JZ, Xiang LX. GADD45A protein plays an essential role in active DNA demethylation during terminal osteogenic differentiation of adipose-derived mesenchymal stem cells. *J Biol Chem* 2011;**286**: 41083–41094.
29. Romanello V, Sandri M. Mitochondrial quality control and muscle mass maintenance. *Front Physiol* 2015;**6**:422.
30. Ni R, Zheng D, Xiong S, Hill DJ, Sun T, Gardiner RB, et al. Mitochondrial calpain-1 disrupts ATP synthase and induces superoxide generation in type 1 diabetic hearts: a novel mechanism contributing to diabetic cardiomyopathy. *Diabetes* 2016;**65**: 255–268.
31. Lieber DS, Calvo SE, Shanahan K, Slate NG, Liu S, Hershman SG, et al. Targeted exome sequencing of suspected mitochondrial disorders. *Neurology* 2013;**80**:1762–1770.
32. González-Rodríguez P, Zampese E, Stout KA, Guzman JN, Ilijic E, Yang B, et al. Disruption of mitochondrial complex I induces progressive parkinsonism. *Nature* 2021; **599**:650–656.
33. Laudette M, Sainte-Marie Y, Cousin G, Bergonnier D, Belhabib I, Brun S, et al. Cyclic AMP-binding protein Epac1 acts as a metabolic sensor to promote cardiomyocyte lipotoxicity. *Cell Death Dis* 2021;**12**: 824.
34. Romanello V, Sandri M. Mitochondrial biogenesis and fragmentation as regulators of muscle protein degradation. *Curr Hypertens Rep* 2010;**12**:433–439.
35. Segref A, Kevei E, Pokrzywa W, Schmeisser K, Mansfeld J, Livnat-Levanon N, et al. Pathogenesis of human mitochondrial diseases is modulated by reduced activity of the ubiquitin/proteasome system. *Cell Metab* 2014;**19**:642–652.
36. Kim J, An YK, Park S, Lee JS. Bre1 mediates the ubiquitination of histone H2B by regulating Lge1 stability. *FEBS Lett* 2018;**592**: 1565–1574.
37. Zhou S, Peng J, Xiao L, Zhou C, Fang Y, Ou Q, et al. TRIM25 regulates oxaliplatin resistance in colorectal cancer by promoting EZH2 stability. *Cell Death Dis* 2021;**12**:463.
38. Reggio A, Rosina M, Krahmer N, Palma A, Petrilli LL, Maiolatesi G, et al. Metabolic reprogramming of fibro/adipogenic progenitors facilitates muscle regeneration. *Life Sci Alliance* 2020;**3**:e202000646.
39. Most J, van Can JG, van Dijk JW, Goossens GH, Jocken J, Hospers JJ, et al. A 3-day EGCG-supplementation reduces interstitial lactate concentration in skeletal muscle of overweight subjects. *Sci Rep* 2015;**5**:17896.
40. von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2021. *J Cachexia Sarcopenia Muscle* 2021;**12**:2259–2261.