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ORIGINAL ARTICLE

p62-mediated autophagy affects nutrition-dependent insulin receptor substrate 1 dynamics in 3T3-L1 preadipocytes

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ABSTRACT

Aims/Introduction: Previous studies have shown that an organism's nutritional status changes the protein levels of insulin receptor substrate 1 (IRS-1) in a tissue-specific manner. Although the mechanisms underlying the regulation of IRS-1 in the nutrient-rich conditions associated with diabetes and insulin resistance have been well studied, those under nutrient-poor conditions remain unknown. The aim of the present study was to investigate how IRS-1 protein levels change depending on the nutritional status of 3T3-L1 preadipocytes.

Materials and Methods: 3T3-L1 preadipocytes were treated with glucose-, amino acid- and serum-free medium for starvation. IRS-1 protein levels were detected by western blot. Autophagy activity was observed by western blot and fluorescence microscopy. The effect of autophagy and p62, an adaptor for selective autophagy, on IRS-1 protein levels under starvation conditions was examined by western blot and immunocytochemistry.

Results: We showed that the levels of IRS-1, but not those of insulin receptor and protein kinase B, decreased when starvation activated autophagy. The inhibition of autophagy by chloroquine or autophagy-related 7 (*Atg7*) ribonucleic acid interference counteracted the starvation-induced decrease of IRS-1. Additionally, *Atg7* knockdown increased insulin-stimulated phosphorylation of protein kinase B under starvation conditions. Furthermore, p62 colocalized with IRS-1 under starvation conditions, and *p62* knockdown counteracted the starvation-induced degradation of IRS-1.

Conclusions: Autophagy through p62 plays an important role in regulating IRS-1 protein levels in response to nutritional deficiency. The present findings suggest that autophagy might function as energy depletion-sensing machinery that finely tunes insulin signal transduction.

INTRODUCTION

Insulin receptor substrate (IRS)-1 is one of the most widely distributed members of the IRS family, an important class of intracellular molecules that mediate insulin signaling[1–3]. IRS-1 is mainly localized in the skeletal muscle and adipose tissue, and it plays an important role in glucose metabolism. *Irs-1* knockout mice are insulin-resistant, showing that IRS-1 is essential for insulin signal transduction[4].

The nutritional status changes IRS-1 protein levels. In animal models, obesity is associated with decreased IRS-1 levels in the liver and skeletal muscle[5]. Additionally, a reduction in the protein levels of IRS-1 has been found in the skeletal muscle and adipocytes of insulin-resistant patients with obesity and type 2 diabetes[6–8]. Studies in animal models have shown that fasting decreases IRS-1 expression in the skeletal muscle, but not in the liver[9]. These reports suggest that IRS-1 levels are regulated in a tissue-specific manner under starvation conditions. The mechanisms underlying the change of IRS-1 expression in the nutrient-rich conditions associated with diabetes and insulin resistance have been well studied. Prolonged insulin exposure decreases IRS-1 through the ubiquitin-proteasome pathway[10–12]. Through a different pathway, tumor necrosis factor-α treatment reduces IRS-1 protein levels

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in adipocytes[13]. However, it is still not fully understood how IRS-1 protein levels are regulated in response to nutritional change. Additionally, little is known about the mechanisms that control the expression of IRS-1 in adipocytes under nutrient-poor conditions.

Macroautophagy (hereafter referred to as autophagy) and the ubiquitin-proteasome pathway play important roles in protein degradation in eukaryotes, and are activated by starvation[14]. In autophagy, double-membrane vesicles, called autophagosomes, deliver large cytoplasmic components to the lysosomes for degradation[15]. Autophagy degrades proteins and entire organelles, and thereby maintains intracellular homeostasis. In addition, autophagy supplies nutrient sources for cellular renovation during cell differentiation under starvation conditions[16]. Autophagy was initially considered a non-selective degradative pathway[17]. Recent studies, however, have shown that autophagy selectively degrades targeted proteins[18]. For example, selective autophagy is involved in the removal of aggregation-prone proteins, damaged organelles and microbes[19–21]. Although earlier studies reported that autophagy plays important roles in diabetes and insulin resistance[22–24], the role of autophagy in the alteration of insulin signaling is unclear.

p62 is one of the best-known autophagy substrates[25,26]. The domain structure of p62 includes a region interacting with the autophagosomal protein, microtubule-associated protein 1 light chain 3 (LC3), and a ubiquitin-associated domain. p62 recognizes ubiquitinated substrates and links them to LC3[27]. Furthermore, p62 directly interacts with IRS-1[28]. p62 has the SH2 domain at the amino terminus, which interacts with IRS-1 through its phosphotyrosine (YXXM) motifs at Tyr-608, Tyr-628 and/or Tyr-658 in a manner similar to the interaction between IRS-1 and p85 of phosphoinositol 3-kinase: this interaction plays an important role in insulin signaling[28].

In the present study, we hypothesized that autophagy is involved in the degradation of insulin-signaling molecules in 3T3-L1 preadipocytes in response to nutritional changes. Our results show that autophagy is involved in the degradation of IRS-1, but not of the insulin receptor (IR) or protein kinase B (Akt), under starvation conditions induced by lack of glucose, amino acids and serum, and that inhibition of autophagy increases insulin-stimulated phosphorylation of Akt. Furthermore, our data show that p62 is involved in the starvation-induced degradation of IRS-1. These findings indicate that p62 and autophagy play an important role in the changes in IRS-1 levels in response to nutritional deficiency.

METHODS

Materials

The antibodies against IR, IRS-1, Akt, β-actin and Akt (phosphorylated on serine 473) were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody against LC3 was purchased from Cell Signaling Technology and Thermo Fisher Scientific Inc. (Waltham, MA, USA). The antibody against p62/SQSTM1 (C terminus) was purchased from Progen (Heidelberg, Germany). The antibodies against Atg7 were purchased from Wako (Osaka, Japan). Chloroquine and digitonin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male C57BL/6J mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). All animals were maintained under a 12-h light–dark cycle, fed with a regular diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) for up to 12 weeks and provided with water *ad libitum*. For fasting, mice were fed with a 60% high-fat diet (D12492; Research Diets, Inc., New Brunswick, NJ, USA) for 2 weeks, followed by fasting for 48 h. Mice were then anesthetized and killed to allow for the isolation of epididymal fat tissue. The study protocol was reviewed and approved by the animal care and use committee of Kanazawa University.

Cell culture and treatments

The mouse fibroblast cell line 3T3-L1 was obtained from the National Institute of Biomedical Innovation, Health and Nutrition (Osaka, Japan). Cells were cultured in the growth medium (GM), consisting of Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.), 2 mmol/L L-glutamine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 100 units/mL penicillin and 0.1 mg/mL streptomycin (Wako Pure Chemical Industries, Ltd.). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For starvation, the cells were treated with the glucose-, amino acid- and serum-free starvation medium (SM), which was prepared by mixing 5.81 g of Nutrition-Free Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc., Kyoto, Japan), 1.85 g of $NaHCO_3$ and 5 mL of 0.1 mol/L sodium pyruvate in 500 mL Milli-Q water, and then sterilizing with a 0.22-μm filter (Millipore, Billerica, MA, USA). For pharmacological inhibition of autophagy, the cells were treated with chloroquine (50 μmol/L).

Short interfering ribonucleic acid transfection in 3T3-L1 cells

Mouse *Atg7* short interfering ribonucleic acid (siRNA; M-049953-02), *p62* siRNA (M-047628-01) and negative control siRNA (D-001206-13-05) were purchased from GE Healthcare Dharmacon (Lafayette, CO, USA), and transfected into 3T3-L1 preadipocytes using DharmaFECT-1 (GE Healthcare Dharmacon) following the manufacturer's instructions. Briefly, 3T3-L1 preadipocytes were grown in 12-well plates and transfected with 50 pmol of siRNAs and 3 μL of dDharmaFECT-1 per well. At 48 h after transfection, the medium was changed to GM or SM for 30 min. Subsequently, the cells were harvested for analysis.

Induction of adipocyte differentiation

Induction of adipocyte differentiation was carried out as previously described[29].

Western blotting

Western blotting was carried out as previously reported[30]. Densitometry analysis of the blotted membranes was carried out using ImageJ software (National Institutes of Health, Rockville, MD, USA).

Quantitative polymerase chain reaction

Total RNA isolation, complementary deoxyribonucleic acid synthesis and quantitative polymerase chain reaction analysis were performed as previously described[30]. Quantitative polymerase chain reaction was carried out using the Step One Plus Real-Time PCR System (Thermo Fisher Scientific Inc.) and the following TaqMan (Applied Biosystems, Carlsbad, CA, USA) probes: actin B, 4352341E_m1; IRS-1: Mn01278327_m1; autophagy related 7 (Atg7), Mn00512209_m1; and p62: Mm00448091_m1.

Autophagy flux imaging

Autophagy flux was determined by fluorescence microscopy (BZ-X700 microscope; Keyence, Osaka, Japan) using the Cyto-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA) in 3T3-L1 preadipocytes incubated in GM or SM for 60 min with or without chloroquine (50 μmol/L), according to the manufacturer's instructions. The nuclei were stained with 4′,6-diamidino-2-phenylindole.

Colocalization studies of IRS-1 and LC3

3T3-L1 preadipocytes grown on four-chamber slides were incubated in GM or SM for 60 min, with or without chloroquine (50 μmol/L), and then washed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde at room temperature for 15 min. After washing with PBS, the cells were permeabilized with 500 μL of a digitonin solution (100 μg/mL) at room temperature for 15 min. After washing with PBS, non-specific antibody binding sites were blocked with Protein Block Serum-Free Ready-to-Use (Agilent Technologies, Santa Clara, CA, USA) at room temperature for 10 min. The cells were then washed with 0.01% TWEEN 20 in PBS (PBS-T) and incubated at 4°C overnight with the anti-IRS-1 antibody (diluted 1:100 in PBS-T). After washing, the cells were incubated at room temperature for 60 min with goat anti-rabbit immunoglobulin G conjugated with Alexa Fluor 488 (Abcam, Cambridge, UK) diluted in PBS-T. Subsequently, the anti-LC3 antibody (Thermo Fisher Scientific Inc.) labeled with HiLyte Fluor 555 was applied to the cells at room temperature for 60 min. After washing, the cells were incubated in Fluoroshield Mounting Medium with 4′,6-diamidino-2-phenylindole (Abcam). Images were obtained by optical sectioning observation by structured illumination microscopy using a BZ-X700 microscope (Keyence).

Colocalization studies of IRS-1 and P62

3T3-L1 preadipocytes grown on four-chamber slides were incubated in GM or SM for 30 min, washed with PBS and fixed in 100% methanol at −20°C for 30 min. After fixation, the cells were washed with PBS-T for 15 min, and non-specific antibody binding sites were blocked with Protein Block Serum-Free Ready-to-Use at 4°C overnight. Cells were then incubated at 4°C overnight with the anti-IRS-1 and anti-p62 antibodies (both diluted 1:100 in PBS-T). After washing, the cells were incubated for 60 min with goat anti-rabbit immunoglobulin G conjugated with Alexa Fluor 488 and goat anti-guinea pig immunoglobulin G conjugated with Alexa Fluor 594 (Abcam) diluted in PBS-T. After washing, cells were incubated in Fluoroshield Mounting Medium with 4′,6-diamidino-2-phenylindole. Images were obtained by optical sectioning observation by structured illumination microscopy using a BZ-X700 microscope (Keyence).

Statistical analysis

Data from the experiments are shown as box plots with individual points[31], and graphics were prepared using R software (https://www.r-project.org). Significance was compared by unpaired two-tailed Student's *t*-test or one-way analysis of variance, and differences were considered statistically significant for $P < 0.05$.

RESULTS

IRS-1 is degraded under starvation conditions

We examined the effect of starvation on the levels of insulin-signaling molecules in 3T3-L1 preadipocytes incubated in GM or SM. SM decreased the levels of IRS-1, but not those of IR and Akt over time (Figure 1a,b). In contrast, SM temporally increased the expression of *Irs-1* (Figure 1c). These results show that, in 3T3-L1 preadipocytes, the levels of IRS-1 decrease under starvation because of protein degradation. In addition, we examined the effect of starvation conditions on the protein levels of IRS-1 in 3T3-L1 mature adipocytes and mouse epididymal adipose tissue. First, we assessed the protein levels of insulin-signaling molecules in 3T3-L1 adipocytes. In 3T3-L1 adipocytes, as well as in 3T3-L1 preadipocytes, SM time dependently decreased IRS-1, although it did not alter insulin receptors or Akt (Figure 1d). Next, to examine the effect of fasting on protein levels of IRS-1 in the epididymal adipose tissue of mice, we fed or fasted C57BL/6J mice for 48 h. Fasting decreased the protein levels of IRS-1, but not those of insulin receptors or Akt (Figure 1e). These findings suggest that the starvation-induced degradation of IRS-1 by autophagy might occur in 3T3-L1 adipocytes and mouse epididymal adipose tissue.

Starvation induces autophagy in 3T3-L1 preadipocytes

Next, we evaluated whether autophagy is activated by starvation. First, we examined autophagy flux by evaluating the immunofluorescence associated with autophagic vacuoles. When the cells were incubated in GM, they did not show specific fluorescence (Figure 2a). In contrast, after culture in SM for 60 min, 3T3-L1 preadipocytes showed significant green fluorescence, indicating the formation of pre-autophagosomes, autophagosomes and autolysosomes. Additionally, treatment

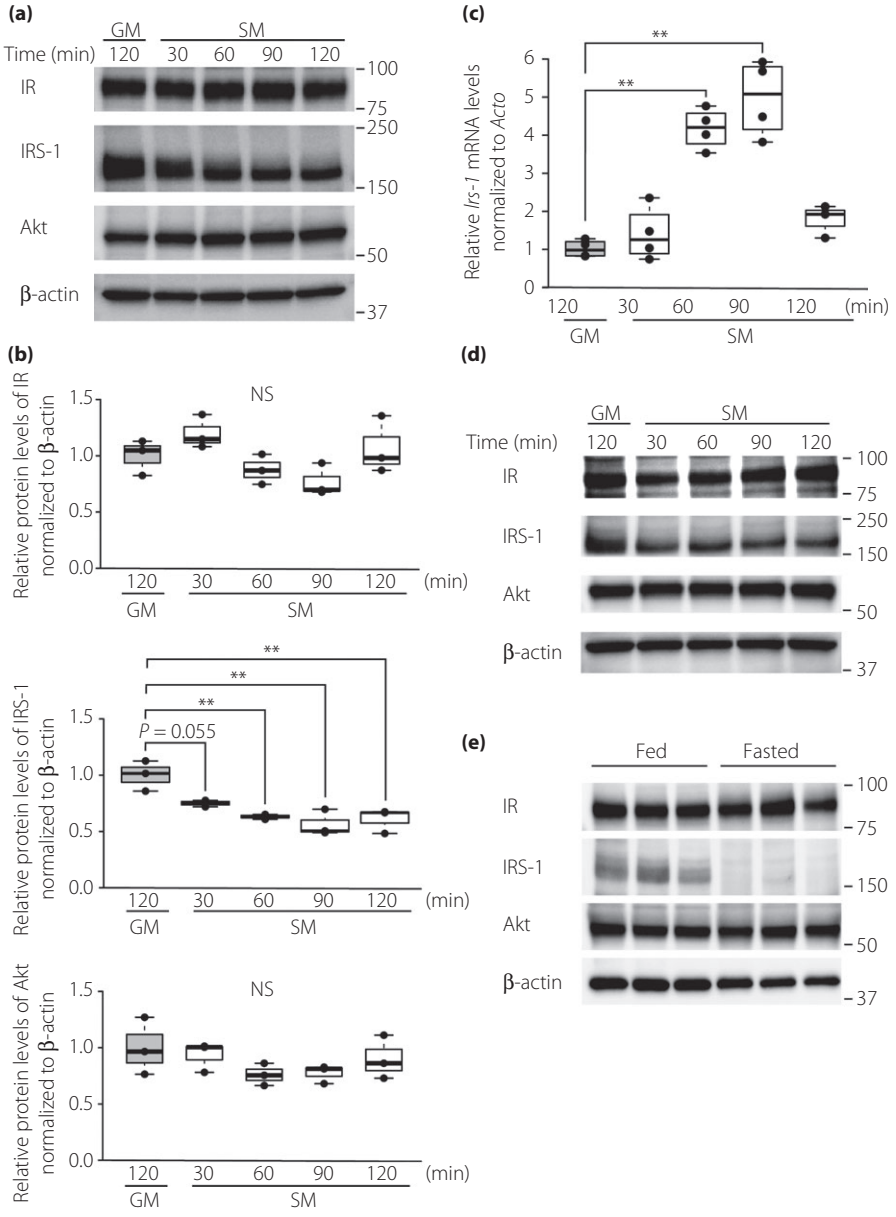

Figure 1 | Insulin receptor substrate 1 (IRS-1) is degraded under starvation conditions. (a) Representative western blot and (b) quantification of western blot images ($n = 3$) for insulin receptor (IR), IRS-1 and protein kinase B (Akt) in 3T3-L1 preadipocytes incubated in growth medium (GM) or starvation medium (SM) for the indicated times. (c) Quantitative polymerase chain reaction analysis of *Irs-1* expression in 3T3-L1 preadipocytes incubated in GM or SM for the indicated times ($n = 4$ per group). (d) Western blot images of the IR, IRS-1 and Akt in 3T3-L1 adipocytes treated with GM or SM for the indicated time. (e) Western blot images of IR, IRS-1 and Akt in the epididymal adipose tissue of male C57BL/6J mice that were fed or fasted for 48 h. In the box plots, the center lines represent the median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; and data points are plotted as circles. $^{**}P < 0.01$. mRNA, messenger ribonucleic acid; NS, not significant.

with chloroquine, an autophagy inhibitor, increased green fluorescence in cells that were incubated in SM. Furthermore, we tested the autophagy flux by analyzing LC3 turnover[32]. During autophagy, cytosolic LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II, and the two forms can be distinguished by western blot. We treated 3T3-L1 preadipocytes with GM or SM for 30 min in the presence or absence of 50 μmol/L chloroquine and assessed the difference in LC3-II protein levels. SM significantly increased LC3-II levels in the presence of chloroquine, indicating an increase of autophagy flux (Figure 2b,c). These findings show that autophagy is induced by starvation.

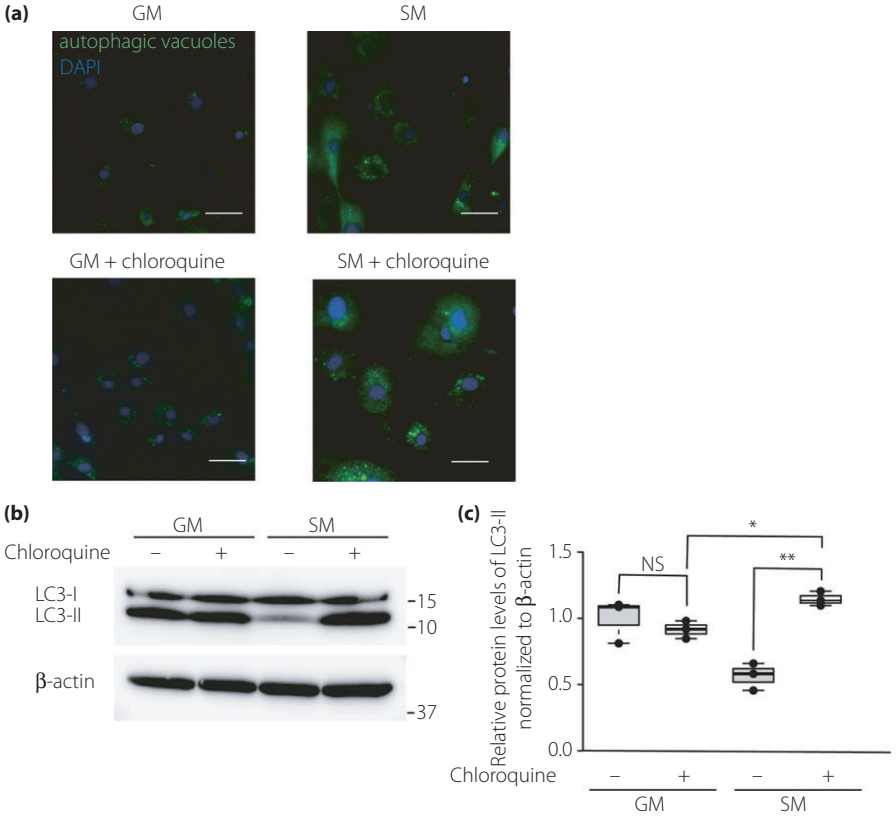

Figure 2 | Starvation induces autophagy in 3T3-L1 preadipocytes. (a) Immunofluorescence microscopy images of autophagic vacuoles (autophagic vacuoles, green) in 3T3-L1 preadipocytes incubated in growth medium (GM) or starvation medium (SM) for 60 min in the presence or absence of chloroquine. Scale bar, 50 μm. (b) Representative western blot and (c) quantification of western blot images ($n = 3$) of microtubule-associated protein 1 light chain 3 (LC3) in 3T3-L1 preadipocytes treated with GM or SM for 30 min in the presence or absence of chloroquine. In the box plots, the center lines represent the median; box limits indicate the 25th and 75th percentiles as determined with R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; and data points are plotted as circles. *$P < 0.05$; **$P < 0.01$. NS, not significant.

Autophagy activated by starvation decreases IRS-1 levels

We carried out experiments to elucidate the mechanism underlying the degradation of IRS-1 under starvation. To assess the involvement of autophagy in this process, we investigated the effect of the inhibition of autophagy on IRS-1 levels by treating the cells with chloroquine. The inhibition of autophagy counteracted the decrease in IRS-1 levels in cells incubated in SM (Figure 3a,b). Similarly, 10 μg/mL pepstatin A and 10 μg/mL E64-d, which are lysosomal inhibitors, counteracted the decrease in IRS-1 levels in cells incubated in SM (Figure S1). Furthermore, we genetically blocked autophagy through RNA interference against *Atg7* (Figure 3c). Knockdown of *Atg7* was associated with increased IRS-1 levels in cells incubated in SM (Figure 3d,e). Next, we assessed the localization of IRS-1 and LC3 in the cells by immunocytochemistry. 3T3-L1 preadipocytes treated with GM or SM for 30 min in the presence or absence of chloroquine were immunostained for IRS-1 and LC3 (Figure 3f). In cells incubated in SM and in the presence of chloroquine, some of the IRS-1-positive puncta colocalized with the LC3-positive puncta, suggesting that these two proteins form the same complexes. Next, we tested the effect of autophagy on insulin signaling. We treated control and *Atg7*-knockdown cells with SM for 30 min, followed by stimulation with or without 1 μg/mL insulin for 10 min. Knockdown of *Atg7* significantly increased insulin-stimulated Akt phosphorylation under starvation conditions (Figure 3g,h). In addition, we treated 3T3-L1 preadipocytes with SM in the presence or absence of chloroquine for 30 min, followed by stimulation with or without 1 μg/mL insulin for 10 min. Chloroquine also significantly increased insulin-stimulated Akt and extracellular signal-regulated kinase phosphorylation under starvation conditions (Figure S2). Because Akt and extracellular signal-regulated kinase is a downstream effector of IRS-1, these findings suggest that starvation-induced autophagy induces IRS-1 degradation, leading to decreased insulin-stimulated Akt phosphorylation.

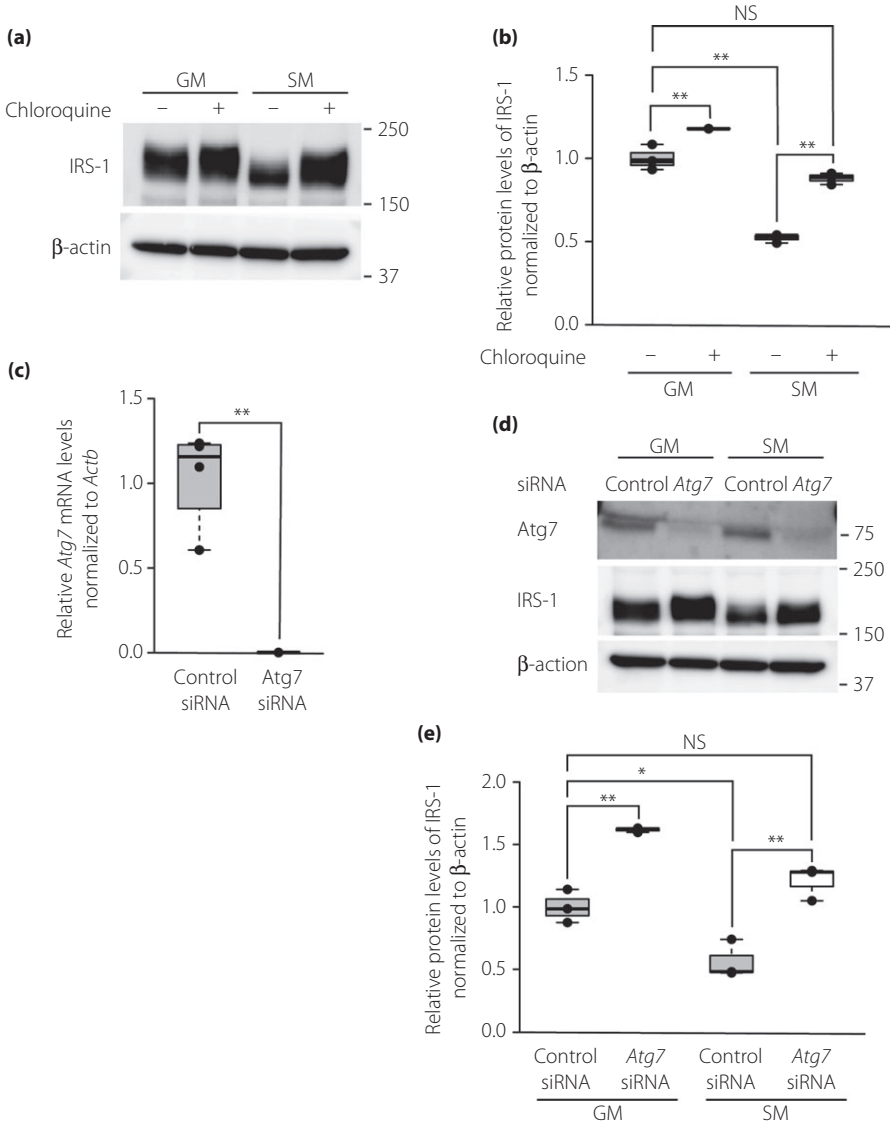

Figure 3 | Autophagy activated by starvation decreases insulin receptor substrate 1 (IRS-1) levels. (a) Representative western blot and (b) quantification of western blot images (n = 3) of IRS-1 in 3T3-L1 preadipocytes treated with growth medium (GM) or starvation medium (SM) for 30 min in the presence or absence of chloroquine. (c) Quantitative polymerase chain reaction analysis of autophagy-related 7 (*Atg7*) expression in 3T3-L1 preadipocytes in which *Atg7* was silenced (n = 4 per group). (d) Representative western blot of Atg7 and IRS-1, and (e) quantification of western blot images (n = 3) of IRS-1 in 3T3-L1 preadipocytes transfected with *Atg7* small interfering ribonucleic acid (siRNA) and incubated in GM or SM for 30 min. (f) Immunofluorescence microscopy images of IRS-1 (green) and microtubule-associated protein 1 light chain 3 (LC3; red) in 3T3-L1 preadipocytes treated with GM or SM for 30 min in the presence or absence of chloroquine. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). For each group, the squares indicate the region of interest, magnified in the right panels. White arrowheads in the region of interest indicate colocalization. Scale bar, 50 μm. (g) Representative western blot and (h) quantification of western blot images (n = 3 for each group) of insulin-stimulated phosphorylation of protein kinase B (pAkt) in 3T3-L1 preadipocytes transfected with the indicated siRNAs and treated with SM for 30 min. In the box plots, the center lines represent the median; box limits indicate the 25th and 75th percentiles as determined with R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; and data points are plotted as circles. *$P < 0.05$; **$P < 0.01$. NS, not significant.

P62 is associated with starvation-induced degradation of IRS-1

Because the autophagy adaptor protein, p62, plays an important role in insulin signaling by directly interacting with IRS-1[28], we examined whether p62 affects the starvation-induced degradation of this protein. First, we assessed the subcellular localization of IRS-1 and p62 in 3T3-L1 preadipocytes incubated in GM or SM by immunocytochemistry. Part of the IRS-1-positive puncta colocalized with the p62-positive puncta in

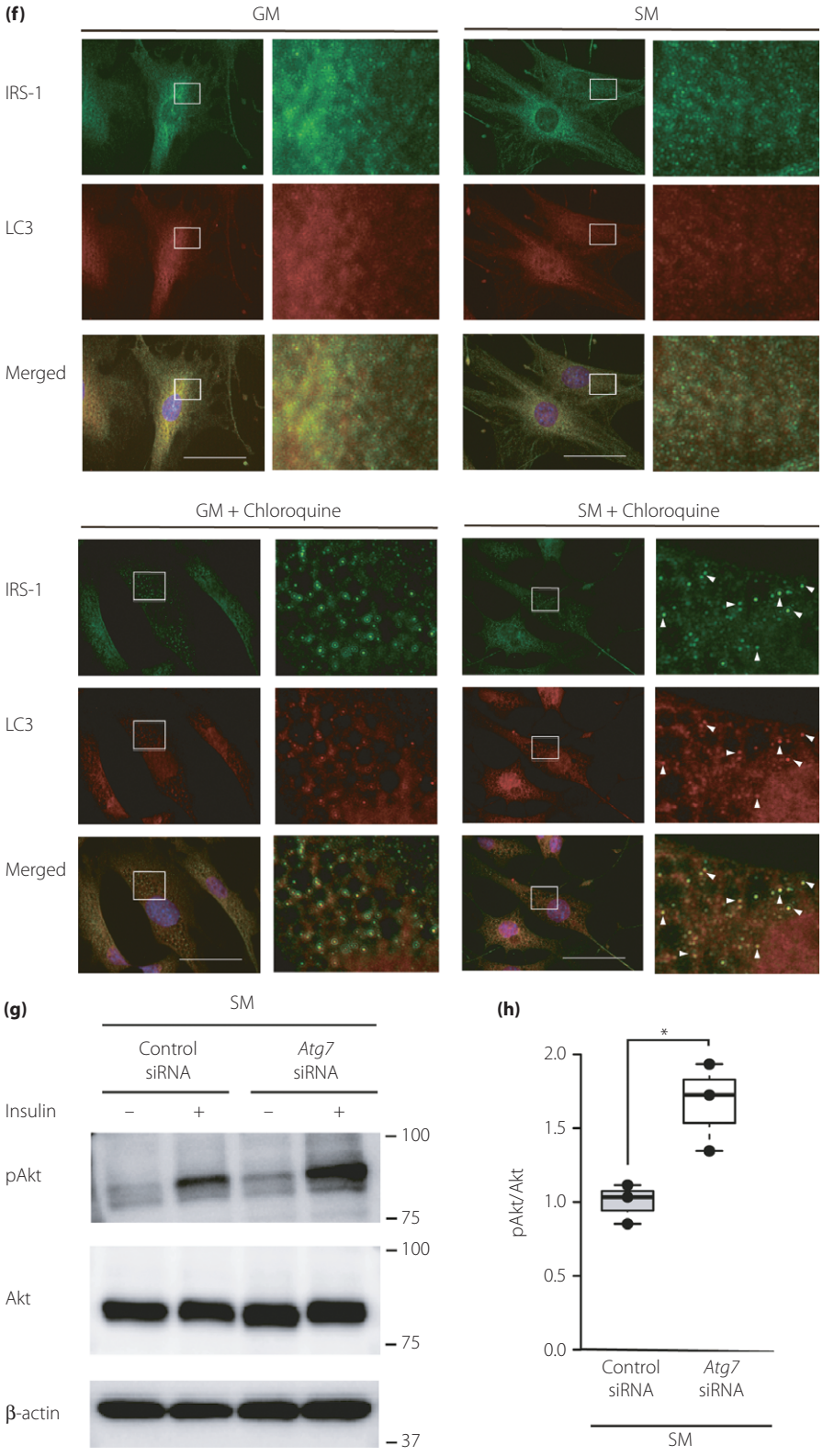

Figure 3 | (Continued)

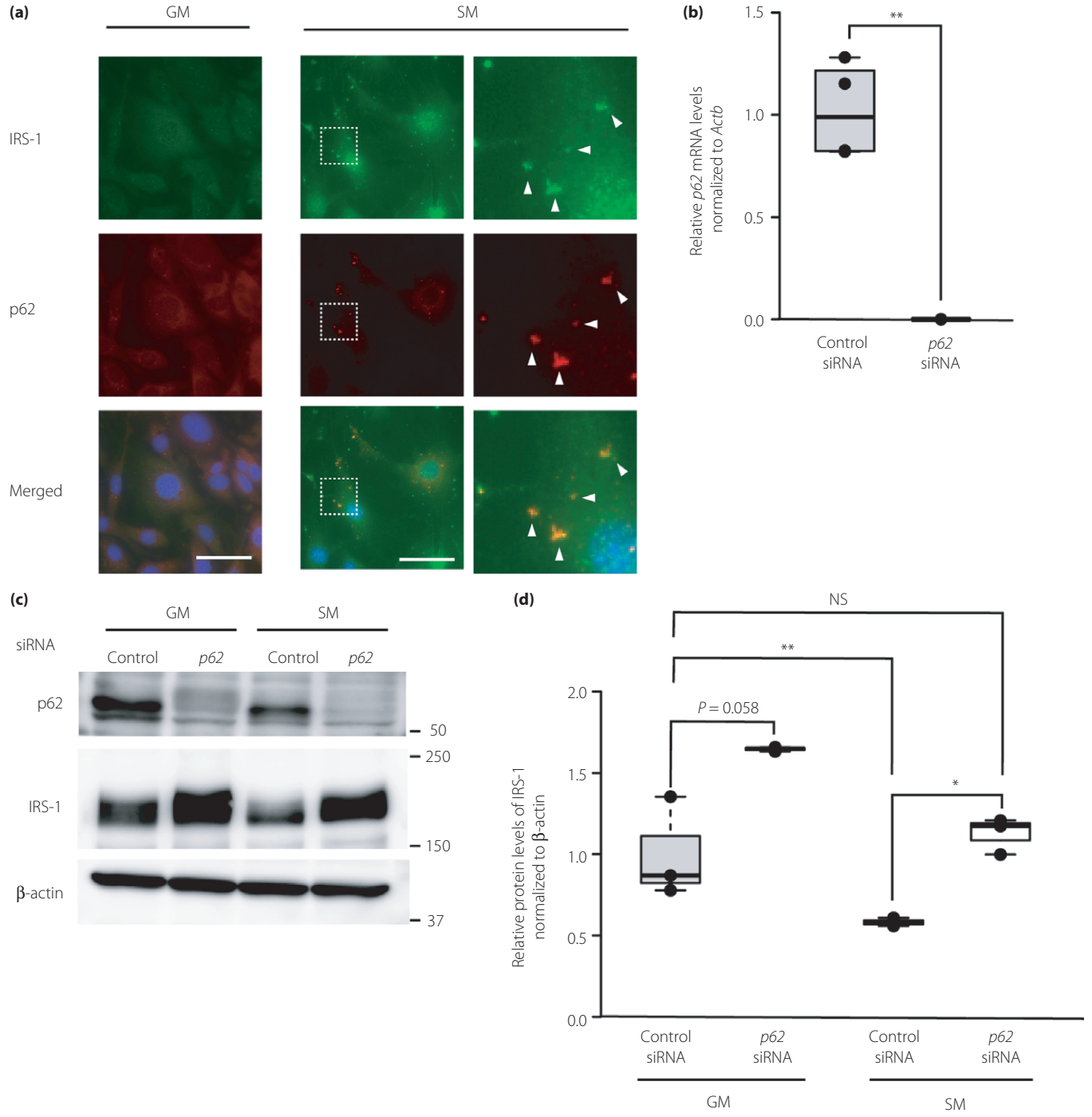

Figure 4 | p62 is associated with starvation-induced degradation of insulin receptor substrate 1 (IRS-1). (a) Immunofluorescence microscopy images of (IRS-1; green) and p62 (red) in 3T3-L1 preadipocytes incubated in growth medium (GM) or starvation medium (SM) for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). For each group, the squares indicate the region of interest, magnified in the right panels. White arrowheads in the region of interest indicate colocalization. Scale bar, 50 μm. (b) Quantitative polymerase chain reaction analysis of p62 expression in 3T3-L1 preadipocytes transfected with p62 small interfering ribonucleic acid (siRNA; $n = 4$ per group). (c) Representative western blot of p62 and IRS-1and (d) quantification of western blot images ($n = 3$ wells for each condition) of IRS-1 in 3T3-L1 preadipocytes transfected with p62 siRNA and incubated in GM or SM for 30 min. In the box plots, the center lines represent the median; box limits indicate the 25th and 75th percentiles as determined with R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; and data points are plotted as circles. *$P < 0.05$; **$P < 0.01$. NS, not significant.

the cytosol of cells incubated in SM (Figure 4a). Next, we silenced *p62* by RNA interference (Figure 4b,c). Knockdown of *p62* counteracted the starvation-induced decrease of IRS-1, which was consistent with the inhibition of autophagy (Figure 4c,d). These findings suggest that IRS-1 might be degraded through autophagy on p62 binding.

DISCUSSION

The present study provides evidence that p62 is involved in the degradation of IRS-1 through autophagy in 3T3-L1 preadipocytes under starvation conditions. We showed that starvation, induced by the absence of glucose, amino acids and serum, activated autophagy and was associated with lower IRS-1 protein levels, whereas inhibition of autophagy counteracted the starvation-induced degradation of IRS-1. Furthermore, p62 interacted with IRS-1 in starved 3T3-L1 preadipocytes, and knockdown of *p62* counteracted the starvation-induced decrease of IRS-1. These studies suggest that autophagy plays a major role in the regulation of insulin-signaling molecules in 3T3-L1 preadipocytes in response to nutritional changes.

Notably, pharmacological and genetic inhibition of autophagy also increased IRS-1 levels in cells incubated in GM (Figure 3a–e), indicating that autophagy constitutively degrades IRS-1 to maintain constant protein levels in growth conditions, and additionally induces IRS-1 degradation under starvation conditions. It is interesting to note that starvation temporally increased *Irs-1* messenger RNA levels (Figure 1c). Meng *et al.*[33] showed that the transcription factor activating enhancer-binding protein-2β, which increases during the maturation of adipocytes, negatively regulates IRS-1 gene expression. Starvation conditions might decrease activating enhancer-binding protein-2β, leading to induce *Irs-1* messenger RNA expression. These findings suggest that there exists a dynamic equilibrium between IRS-1 synthesis and degradation to fine-tune its protein levels in response to nutritional changes.

Autophagy was originally considered an unselective bulk degradation pathway. However, recent studies have shown that starvation induces selective autophagy. Onodera *et al.*[34] reported that cytosolic acetaldehyde dehydrogenase is selectively degraded by autophagy under nitrogen starvation in yeast. In addition, Kristensen *et al.*[35] analyzed global protein dynamics in human MCF7 breast carcinoma cells under amino acid starvation conditions by quantitative mass spectrometry, suggesting that starvation-induced degradation through autophagy is selective. In the present study, we found that the levels of IRS-1 decreased in 3T3-L1 preadipocytes under glucose-, amino acid- and serum-starvation conditions, although the levels of IR and Akt did not change (Figure 1a,b). Furthermore, we showed that p62, which is an adaptor for selective autophagy, mediated the degradation of IRS-1 by autophagy in starved preadipocytes (Figure 4). These results suggest the possibility that IRS-1, on interaction with p62, might be selectively degraded by autophagy.

Cellular models of insulin resistance and *in vivo* studies suggest an association between insulin resistance and changes in IRS-1 levels[7–9]. Decreased IRS-1 protein levels have been observed in animal models of insulin resistance[5,36] and *in vitro* models in which cells are treated with tumor necrosis factor-α or chronically stimulated with insulin[12,13,37]. We found that inhibition of autophagy counteracted the starvation-induced degradation of IRS-1, leading to increased insulin-stimulated phosphorylation of Akt (Figure 3g,h). These data suggest that the starvation-induced degradation of IRS-1 through autophagy might cause insulin resistance in response to nutritional deficiencies. Indeed, previous studies have shown that starvation causes insulin resistance in lean and obese participants with or without diabetes[38–41]. This is the physiological adaptation to fasting, leading to a shift in energy source from glucose to fat. The starvation-induced degradation of IRS-1 through autophagy might be associated with this adaptation.

Interestingly, Zhang *et al.*[42] found that inhibition of autophagy decreased insulin-stimulated phosphorylation of Akt under serum-starvation conditions in 3T3-L1 adipocytes. Our studies differ in that we used glucose-, amino acid- and serum-starvation conditions for a shorter time. These opposing observations raise the interesting notion that different starvation conditions can cause different insulin sensitivity.

We acknowledge there were several limitations to the present study. First, we assessed the protein levels of IR, IRS-1 and Akt under starvation conditions; however, it is still unknown whether starvation affects the levels of other insulin signaling molecules. We have just started a comprehensive analysis of the changes in such molecules under starvation conditions by proteomics. Additionally, the degradation of IRS-1 through autophagy under starvation conditions was only shown *in vitro*. Although Zhang *et al.*[24] have shown that an adipocyte-specific mouse knockout of *Atg7* decreases adipose mass and increases insulin sensitivity by increasing beige cells in white adipose tissue, it remains unclear how starvation conditions affect IRS-1 protein levels and insulin signaling in the mice. We have observed that IRS-1 levels decrease in starved 3T3-L1 mature adipocytes and epididymal adipose tissue of C57BL/6J mice fasted for 48 h (Figure 1d,e). However, a detailed *in vivo* analysis is required to understand the significance of the starvation-induced degradation of IRS-1 in the adipose tissue.

In conclusion, the present study highlights a novel mechanism for the nutrition-dependent dynamics of IRS-1. This new finding suggests the possibility that autophagy is involved in the degradation of proteins on their interaction with an autophagy adaptor, such as p62, and might therefore play an important role in the regulation of insulin signaling. The elucidation of this process might help to improve the current understanding of the dynamics of insulin signaling.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 | Pepstatin A and E64-d counteracted the decrease in insulin receptor substrate 1 (IRS-1) levels in 3T3-L1 preadipocytes under starvation conditions.

Figure S2 | Chloroquine increased insulin-stimulated protein kinase B (Akt) and extracellular signal-regulated kinase (Erk) phosphorylation under starvation conditions.