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Skeletal Muscle-derived Myonectin Activates the Mammalian Target of Rapamycin (mTOR) Pathway to Suppress Autophagy in Liver*

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Background: Liver autophagy is dynamically regulated in fed and fasted states.
Results: Myonectin is secreted by skeletal muscle in response to nutrient availability, and it activates the mTOR signaling pathway to suppress autophagy in liver.
Conclusion: Myonectin is a novel regulator of liver autophagy.
Significance: Myonectin mediates muscle-liver cross-talk to control energy balance.

Cells turn on autophagy, an intracellular recycling pathway, when deprived of nutrients. How autophagy is regulated by hormonal signals in response to major changes in metabolic state is not well understood. Here, we provide evidence that myonectin (CTRP15), a skeletal muscle-derived myokine, is a novel regulator of cellular autophagy. Starvation activated liver autophagy, whereas nutrient supplementation following food deprivation suppressed it; the former and latter correlated with reduced and increased expression and circulating levels of myonectin, respectively, suggestive of a causal link. Indeed, recombinant myonectin administration suppressed starvation-induced autophagy in mouse liver and cultured hepatocytes, as indicated by the inhibition of LC3-dependent autophagosome formation, p62 degradation, and expression of critical autophagy-related genes. Reduction in protein degradation is mediated by the PI3K/Akt/mTOR signaling pathway; inhibition of this pathway abrogated the ability of myonectin to suppress autophagy in cultured hepatocytes. Together, our results reveal a novel skeletal muscle-liver axis controlling cellular autophagy, underscoring the importance of hormone-mediated tissue cross-talk in maintaining energy homeostasis.

Autophagy helps recycle intracellular organelles and proteins into their constituent building blocks, serving as potential fuel sources for maintaining cellular homeostasis in times of nutrient deprivation (1, 2). In fact, exercise, a form of nutrient deprivation, triggers autophagic turnover of aged organelles, such as mitochondria, to accommodate renewal of cellular content; this is thought to be the origin of some exercise-related health benefits (3).

In contrast, dysregulation of autophagy is linked to diseases like obesity and atherosclerosis (4–6), and the absence of autophagy prohibits survival. Global deletion of autophagy genes (*e.g. Atg5* (autophagy-related 5) and *Atg7*) in mice results in perinatal lethality (7, 8), which likely results from a failure in catabolic breakdown of liver protein and glycogen (9, 10). This process is needed to maintain energy homeostasis within the first few hours after birth, before neonates have acquired a full capacity for gluconeogenesis (11, 12). Thus, autophagy is essential to normal cellular and organismal functions.

One organ in which autophagy has particular importance is the liver. The liver responds dramatically to starvation; up to 40% of its cellular protein content can be degraded via autophagy during prolonged periods of nutrient depravation (13). Autophagy in liver is remarkably sensitive to hormonal regulation. Elevated levels of glucagon, a hormone produced in the pancreas, in the fasted state induce autophagy in liver (14). Conversely, the anabolic hormone insulin, secreted in response to food intake, and diet-derived amino acids suppress hepatocyte autophagy by activating the nutrient-sensing mTOR (mammalian target of rapamycin) signaling pathway (15). In diet-induced obese mice that are insulin-resistant, liver autophagy is dysregulated (16). Suppression of autophagy by insulin also extends to skeletal and cardiac muscles (17). Although insulin and glucagon play an important role in regulating cellular autophagy in the fed and fasted states, respectively, other hormonal signals may also affect this recycling process.

We recently described myonectin (also designated as CTRP15) as a nutritionally regulated hormone secreted by skeletal muscle (18). It belongs to the C1q/TNF-related protein family of secreted proteins known to regulate glucose and fatty acid metabolism (19–27). However, unlike many C1q/TNF-related proteins that are predominantly expressed by adipose tissue, myonectin is derived primarily from skeletal muscle. Myonectin expression and circulating levels in mice are highly induced by refeeding following an overnight (12-h) fast. Infusion of recombinant myonectin into mice lowers circulating levels of free fatty acids, in part by promoting cellular lipid uptake and up-regulating the expression of genes (*Cd36*, *Fabp*, and *Fatp*) involved in lipid uptake (18). Here, we established the functional significance of myonectin as a novel regulator of

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autophagy in liver, highlighting its role in mediating skeletal muscle-liver cross-talk to modulate metabolic processes critical to maintaining tissue homeostasis.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—Mouse monoclonal anti-FLAG M2 antibody and a rabbit polyclonal antibody recognizing LC3B (microtubule-associated protein-1 light chain 3 beta) were obtained from Sigma-Aldrich. Rabbit polyclonal anti-peptide antibody, which can specifically recognize mouse myonectin, has been described previously (18). Rabbit polyclonal antibodies recognizing p62, ULK1 (UNC-51-like kinase 1), phospho-ULK1 (Ser-757), Akt, phospho-Akt (Thr-308), mTOR, phospho-mTOR (Ser-2448), IRS1 (insulin receptor substrate 1), phospho-IRS-1 (Ser-612), FOXO3 (Forkhead box transcription factor O3), phospho-FOXO3 (Thr-24), and β-tubulin were obtained from Cell Signaling Technology (Danvers, MA). Rabbit monoclonal antibodies against Atg7 and Atg12 were obtained from Cell Signaling Technology. Akt inhibitor IV was obtained from Millipore (Billerica, MA). The mTOR inhibitor rapamycin and the PI3K inhibitor LY294002 were obtained from Cell Signaling Technology.

Animals—Eight-week-old male wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in polycarbonate cages on a 12-h light/dark photocycle and had *ad libitum* access to water and standard laboratory chow diet (LabDiet 5001, PMI Nutrition International, St. Louis, MO) throughout the study period. Animals were fasted for 24 h and injected via the tail vein with either 20% (w/v) glucose solution and saline (10 μl/g of body weight; control) or recombinant myonectin (1 μg/g of body weight). Serum and tissues were harvested in the *ad libitum* state at 10 min or 4 h post-injection as indicated. All animal protocols were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Protein Purification—Recombinant full-length mouse myonectin containing a C-terminal FLAG-tagged epitope was produced in mammalian cells as described previously (18). Briefly, HEK293 cells (GripTite™, Invitrogen) were cultured in DMEM (Invitrogen) containing 10% (v/v) FBS (Invitrogen) supplemented with antibiotics. Transfections were performed in HEK293 cells using the calcium phosphate method (28). At 48 h post-transfection, the medium was replaced with serum-free Opti-MEM I (Invitrogen) supplemented with vitamin C (0.1 mg/ml). Supernatants were collected three times every 48 h, pooled, and purified using an anti-FLAG affinity gel (Sigma-Aldrich) according to the manufacturer's protocol. Proteins were eluted with 150 μg/ml FLAG peptide (Sigma-Aldrich) and dialyzed against 20 mM HEPES buffer (pH 8.0) containing 135 mM NaCl in a 10-kDa cutoff Slide-A-Lyzer dialysis cassette (Pierce). Protein concentration was determined using Coomassie Plus protein assay reagent (Thermo Scientific), and samples were aliquoted and stored at −80 °C.

Isolation of Skeletal Muscle and Liver—Liver and muscle samples were immediately harvested from euthanized mice and snap-frozen in liquid nitrogen. Homogenized cell lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 10% glycerol) containing protease and phosphatase inhibitor mixtures (Sigma-Aldrich). Protein content was quantified using Coomassie Plus protein reagent.

Cell Culture—Mouse C2C12 myocytes and rat H4IIE hepatocytes (ATCC) were cultured in 24-well plates and differentiated as described previously (18). To induce autophagy, cells were placed in autophagy media (DMEM containing 0.1% BSA (Sigma-Aldrich) but lacking glucose, L-glutamine, and sodium pyruvate) for 3, 6, 12, or 24 h. To assess the effects of macronutrients on myonectin expression, myotubes were cultured in autophagy medium alone or supplemented with 5 mM glucose (Sigma) and/or a mixture of essential amino acids (Sigma M5550) for 6 h. For all other experiments on hepatocytes, control cells were incubated with DMEM containing 10% FBS. In all cell culture treatments, 5 μg/ml recombinant myonectin was used.

Serum and Blood Chemistry Analysis—Mouse serum was harvested by tail bleed. Samples were separated using Microvette® CB 300 (Sarstedt, Nümbrecht, Germany) and centrifuged at 10,000 × g for 5 min. Serum glucagon levels were quantified using a glucagon ELISA kit (Millipore).

Quantitative Real-time PCR Analysis—Total RNAs were isolated from tissues or cell lines using TRIzol® and reverse-transcribed using Superscript II RNase H reverse transcriptase (Invitrogen). Primer sequences are listed in supplemental Table 1. Quantitative real-time PCR analyses were performed on an Applied Biosystems Prism 7500 sequence detection system (Invitrogen). Samples were analyzed in 25-μl reactions with SYBR® Green PCR Master Mix (Applied Biosystems) following the manufacturer's directions. Data were normalized to β-actin and expressed as relative mRNA levels using the $\Delta\Delta C_t$ method (29).

Immunoblot Analysis—Serum samples were diluted 1:20 in SDS loading buffer (50 mM Tris-HCl (pH 7.4), 2% (w/v) SDS, 6% (w/v) glycerol, 1% (v/v) 2-mercaptoethanol, and 0.01% (w/v) bromphenol blue) and separated on NuPAGE 10% Bis-Tris[4] gels (Invitrogen). Each well was loaded with an equivalent of 1 μl of serum. For H4IIE hepatocytes and mouse liver lysates, 10 μg of protein were loaded and separated on NuPAGE 12% Bis-Tris gels (Invitrogen). Fractionated proteins were then transferred to Protran BA8 nitrocellulose membranes (Whatman), blocked in 2% nonfat milk for 1 h, and probed overnight with primary antibodies. Immunoblots were washed three times (10 min each) with PBS containing 0.1% Tween 20 and then incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Cell Signaling Technology) for 1 h. Blots were washed three times (10 min each) with PBS containing 0.1% Tween 20, developed in enhanced chemiluminescent reagent (Amersham Biosciences), and visualized with MultiImage III FluorChem® Q (Alpha Innotech, Santa Clara, CA). Signal intensity quantifications were performed using AlphaView software (Alpha Innotech).

Autophagy Assay—Autophagic degradation of long-lived proteins was assessed as described previously (30). Briefly, H4IIE hepatocytes (~80% confluence) were incubated for 18 h in complete medium (DMEM containing 5 mM glucose, penicillin/streptomycin, and 10% FBS) supplemented with 0.2 μCi/ml L-[^{14}C]valine (Moravek Biochemicals, Brea, CA). Cells

[4] The abbreviation used is: Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl) propane-1,3-diol.

were washed three times with PBS and placed in DMEM (lacking glucose, L-glutamine, and sodium pyruvate but containing 0.1% BSA and 0.5 mM cold valine) supplemented with 10% FBS; vehicle (20 mM HEPES (pH 8) containing 135 mM NaCl); 5 μg/ml myonectin; or myonectin plus LY294002 (50 μM), Akt inhibitor IV (10 μM), or rapamycin (10 ng/ml). Short-lived proteins were degraded within 1 h of incubation, so media were changed at 1 h. Cells were then incubated for an additional 4 h, after which the media were collected. Cells were washed three times with PBS, scraped, and collected. A matched volume containing 10% trichloroacetic acid and 1% phosphotungstic acid was added to cells, which were then centrifuged at 600 × *g* for 10 min at 4 °C. Cell media and the acid-soluble cell lysate fraction were combined and placed in a scintillation vial containing 2 ml of Soluene 350 (National Diagnostics). The acid-insoluble fraction was resuspended in PBS and placed in a separate scintillation vial containing 2 ml of Soluene 350. Percent protein degradation was calculated by dividing counts in the medium plus the acid-soluble fraction of the cell lysate by counts in the acid-insoluble fraction of the cell lysate.

Statistical Analysis—Comparisons were performed using two-tailed Student's *t* tests with 95% confidence intervals. Values were considered significant at $p < 0.05$. All data are presented as means ± S.E.

RESULTS

Myonectin Expression Is Repressed by Starvation and Induced by Nutrient Supplementation—We have previously shown that myonectin expression and circulating levels are highly up-regulated in mice by refeeding after a 12-h fast (18). However, effects on liver autophagy are observed only after a prolonged 24-h food deprivation (considered starvation in mice) (31). To evaluate changes in myonectin expression during starvation, mice were fed *ad libitum* or fasted for 24 h and injected with a bolus of glucose or saline. A striking increase in myonectin mRNA (~10-fold) and circulating protein levels (~3-fold) was observed when starved mice were given a bolus of glucose compared with a bolus of saline or mice fed *ad libitum* (Fig. 1, *A* and *B*). In accordance, myonectin mRNA expression was markedly reduced in differentiated C2C12 myotubes when cells were subjected to serum deprivation (Fig. 1*C*), mimicking starvation-induced autophagy (32). To evaluate which macronutrients could prevent the suppression of myonectin expression in nutrient-deprived cells, C2C12 myotubes were incubated in autophagic medium in the presence or absence of 5 mM glucose and/or a mixture of minimal essential amino acids. Glucose or amino acid supplementation was sufficient to inhibit the starvation-induced down-regulation of myonectin expression (Fig. 1*D*). The effects on myonectin expression were much greater if both glucose and essential amino acids were given to cells, reflecting the additive effects of both nutrients. These results indicate that myonectin expression is repressed by starvation but robustly induced by nutrient (glucose or amino acids) availability in cultured skeletal muscle cells and in mice.

Myonectin Suppresses the Expression of Autophagy Genes in H4IIE Hepatocytes and Mouse Liver—Expression of key autophagy genes in mouse liver is altered in response to nutrient deprivation. Atg7 is required for starvation-induced protein

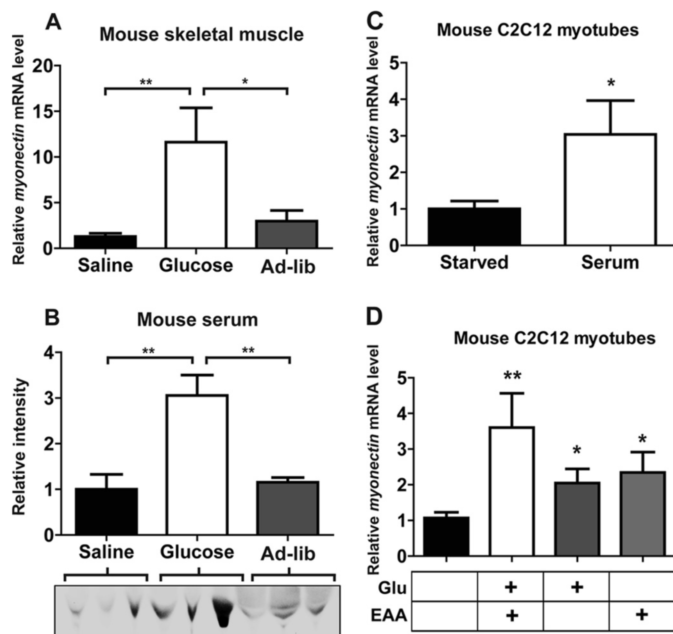

FIGURE 1. **Myonectin expression and circulating levels are reduced by starvation and increased by nutrient availability.** *A* and *B*, animals fed *ad libitum* (*Ad-lib*) or fasted for 24 h were injected via the tail vein with 20% (w/v) glucose (10 μl/g of body weight) or a matched volume of saline ($n = 6$). Skeletal muscle and sera were harvested 3 h later and subjected to quantitative real-time PCR analysis of myonectin expression (*A*) and immunoblot quantification of serum myonectin levels (*B*). *C*, quantitative real-time PCR analysis of myonectin expression in differentiated mouse C2C12 myotubes ($n = 6$) treated for 18 h in DMEM containing 5 mM glucose and 0.1% BSA with (*Serum*) or without (*Starved*) 10% FBS. *D*, quantitative real-time PCR analysis of myonectin expression in differentiated mouse C2C12 myotubes ($n = 6$) treated for 6 h in autophagy medium in the presence or absence of 5 mM glucose and/or a mixture of minimal essential amino acids (*EAA*). All quantitative real-time PCR data were normalized to β-actin values. *, $p < 0.05$; **, $p < 0.01$.

degradation in liver (8), whereas Atg12 is critical for the formation of autophagosome precursors (33). Time course treatments revealed a strong induction of *Atg7* (Fig. 2*A*) and *Atg12* (Fig. 2*B*) when H4IIE hepatocytes were serum-starved for 3 and 6 h. Addition of recombinant myonectin was sufficient to suppress the induction of *Atg* genes in response to serum deprivation (Fig. 2, *A* and *B*). Changes in the mRNA levels of *Atg7* and *Atg12* in response to serum starvation and myonectin treatment were confirmed at the protein levels (Fig. 2, *C* and *D*). Atg12 conjugates with Atg5, and the Atg12-Atg5 complex facilitates autophagosome formation (34, 35); however, we could not detect the covalently linked complex using the anti-Atg12 antibody. Western blot analysis of Atg12 reflects only the unconjugated form of the protein. To confirm our results *in vivo*, mice were fasted for 24 h and injected with a bolus of glucose, vehicle, or myonectin (1 μg/g of body weight). Recombinant protein administration resulted in a 4-fold increase in the circulating levels of myonectin 10 min after protein injection compared with saline injection (supplemental Fig. 1). As expected, starved mice injected with vehicle control showed a significant up-regulation of Atg7 and Atg12 levels in mouse liver, and this induction was suppressed when starved mice were given a bolus of glucose or injected with recombinant myonectin (Fig. 2, *E* and *F*). These results suggest that myonectin regulates the expression of key autophagy genes in cultured

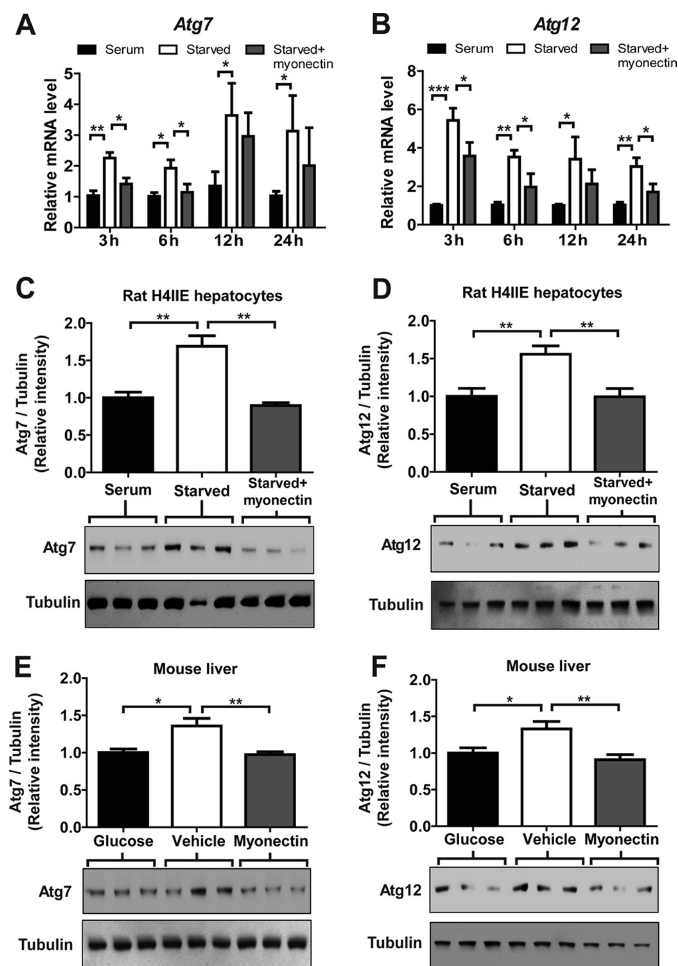

FIGURE 2. **Myonectin suppresses expression of starvation-induced autophagy genes.** *A* and *B*, quantitative real-time PCR analysis of *Atg7* (*A*) and *Atg12* (*B*) mRNAs in H4IIE hepatocytes ($n = 4$) cultured for 3, 6, 12, or 24 h in medium containing 10% FBS (*Serum*), HEPES buffer (*Starved*), or 5 μg/ml recombinant myonectin (*Starved + myonectin*). All quantitative PCR data were first normalized to β-actin values and then normalized to samples treated with serum. *C* and *D*, representative immunoblots and corresponding protein quantifications for Atg7 (*C*) and Atg12 (D) in H4IIE hepatocytes cultured for 6 h under the same conditions ($n = 6$). *E* and *F*, mice ($n = 6$) were fasted for 24 h and injected via the tail vein with 20% (w/v) glucose (10 μl/g of body weight), HEPES buffer (vehicle), or recombinant myonectin (1 μg/g of body weight). Four hours later, livers were harvested, and Atg7 (*E*) and Atg12 (*F*) levels were quantified by Western blot analysis. Immunoblots show three representative liver samples. Tubulin was used as a loading control for immunoblot analyses. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

hepatocytes and liver in response to nutrient availability. Furthermore, recombinant myonectin administration did not alter the circulating levels of glucagon, a hormone known to activate liver autophagy in the fasted state (14), at 10 min or 4 h post-injection (supplemental Fig. 2), suggesting that myonectin acts independently of glucagon.

Myonectin Inhibits LC3 Lipidation and Autophagosome-dependent p62 Degradation—Lipidation of LC3 is a critical initial step in autophagy, leading to its subsequent aggregation and autophagosome formation (31, 33). Autophagosomes contain p62/SQSTM1 protein complexes, which are eventually degraded by cellular autophagy (36, 37). Thus, the ratio of non-lipidated to lipidated LC3 and the extent of p62 degradation serve as markers for early and late stages of autophagy, respectively. H4IIE hepatocytes were deprived of serum for 2 h to activate autophagy, as indicated by a marked decrease in the proportion of non-lipidated (indicated by LC3-I) to lipidated (indicated by LC3-II) forms of LC3. Treatment of serum-starved cells with recombinant myonectin alone for 1 h was sufficient to reduce the lipidation of LC3 (Fig. 3*A*). Myonectin treatment increased the LC3-I/LC3-II ratio by ~50%, an effect comparable to cells cultured in the presence of serum (Fig. 3*B*). To confirm that the effects of myonectin on LC3 lipidation were indeed due to a shift in autophagic flux, the same experiments were repeated in the presence of chloroquine (38), a potent inhibitor of lysosomal degradation (Fig. 3, *A* and *C*). Treatment with chloroquine enhanced the extent of starvation-induced LC3 lipidation, as well as the ability of myonectin to abolish these effects (Fig. 3*C*).

To assess late-stage autophagosome-dependent protein degradation, H4IIE hepatocytes were cultured for 24 h in the presence of serum, serum-starved, or serum-starved in the presence of recombinant myonectin and probed for p62 levels. Serum starvation significantly reduced p62 levels relative to cells cultured in the presence of serum (Fig. 3*D*). Recombinant myonectin treatment essentially reversed this process in starved cells, as indicated by the complete inhibition of starvation-induced p62 degradation (Fig. 3*D*). Cells starved for a shorter period of time (3, 6, or 12 h) elicited only a modest reduction of p62 compared with serum- and myonectin-treated samples (data not shown). To ensure that the effects of myonectin on cellular autophagy are physiologically relevant *in vivo*, we subjected mice to a 24-h fast and then administered a bolus of glucose, vehicle, or recombinant myonectin. Consistent with our *in vitro* results, starved mice injected with vehicle control activated autophagy, as indicated by significantly greater hepatic LC3 lipidation and p62 degradation compared with starved mice injected with a bolus of glucose (Fig. 3, *E* and *F*). Importantly, myonectin administration largely suppressed starvation-induced LC3 lipidation and p62 degradation in liver to a similar extent as glucose administration (Fig. 3, *E* and *F*). These results indicate that myonectin inhibits early-stage autophagosome formation, as well as late-stage autophagy-dependent p62 degradation *in vitro* and *in vivo*.

Myonectin Activates the Akt/mTOR Pathway to Suppress Autophagy—Activation of the Akt/mTOR pathway is known to suppress autophagy (2). Thus, we next investigated if myonectin also acts on a similar signaling pathway to block autophagy. H4IIE hepatocytes were serum-starved for 1 h to reduce background signaling and then treated with vehicle control or recombinant myonectin for 5, 15, 30, or 60 min. Relative to vehicle-treated cells, myonectin dramatically induced phosphorylation of various components within the canonical Akt/mTOR pathway. Myonectin robustly induced the phosphorylation and activation of IRS1 (Ser-612), Akt (Thr-308), and mTOR (Ser-2448) in cultured H4IIE hepatocytes (Fig. 4*A*) and in mouse liver (Fig. 4*B*). Activation of Akt signaling elicits an mTOR-dependent suppression of cellular autophagy (39). Furthermore, mTOR-dependent phosphorylation of ULK1 at Ser-757 disrupts its association with AMP-activated protein kinase and abolishes AMP-activated protein kinase-mediated autophagosome formation (40, 41). Myonectin robustly induced ULK1 phosphorylation at Ser-757 in cultured hepato-

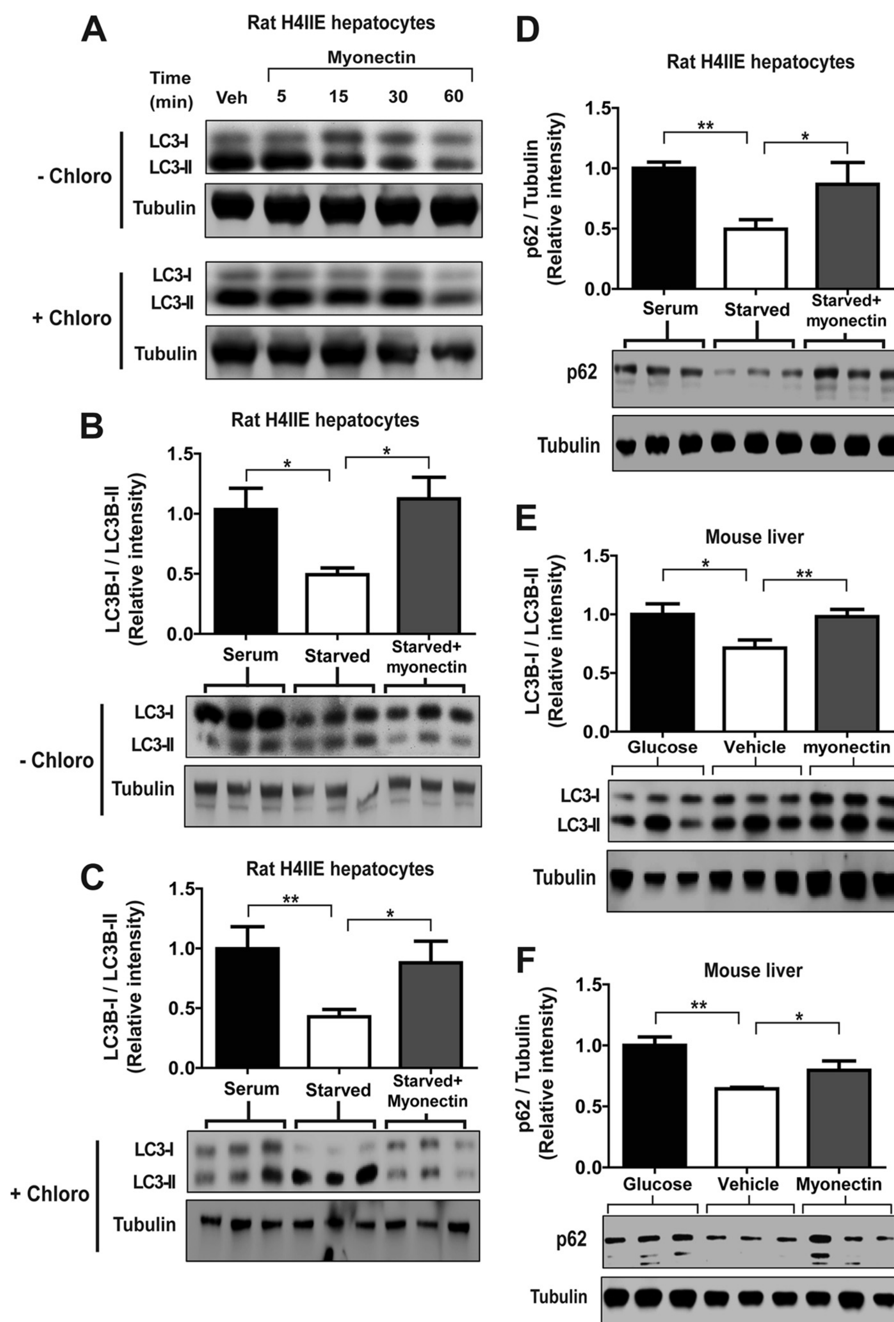

FIGURE 3. **Myonectin reduces autophagosome formation and autophagy-mediated p62 degradation.** *A*, representative immunoblots and quantifications of autophagy marker LC3 in H4IIE hepatocytes cultured for 2 h in starvation medium in the presence or absence of 10 μM chloroquine (*Chloro*) and then treated for 5, 15, 30, or 60 min with HEPES buffer (vehicle (*Veh*)) or 5 μg/ml myonectin. LC3-II denotes the lipidated form, observed in a slight size shift from the non-lipidated band, LC3-I. *B* and *C*, immunoblot and quantification ($n = 6$) of LC3 in H4IIE hepatocytes cultured for 1 h in medium containing 10% FBS (*Serum*), HEPES buffer (*Starved*), or 5 μg/ml myonectin in the absence (*B*) or presence (*C*) of 10 μM chloroquine. *D*, immunoblot and quantification ($n = 6$) of p62 in H4IIE hepatocytes cultured for 24 h in medium containing 10% FBS (*Serum*), HEPES buffer (*Starved*), or 5 μg/ml myonectin. *E* and *F*, mice ($n = 6$) were fasted for 24 h and injected via the tail vein with 20% (w/v) glucose (10 μl/g of body weight), HEPES buffer (*Vehicle*), or recombinant myonectin (1 μg/g of body weight). Four hours later, livers were harvested and subjected to immunoblot quantification of LC3 (*E*) and p62 (*F*). All blots show tubulin as the loading control. Each lane represents an independent cell or liver sample. *, $p < 0.05$; **, $p < 0.01$.

cytes and in mouse liver (Fig. 4). Activated Akt phosphorylates FOXO3 at Thr-24, resulting in its nuclear exclusion (42). Under conditions in which autophagic activity is high, FOXO3 binds to the promoters of relevant *Atg* genes (*e.g. Atg12*) to activate gene transcription; this process is disrupted when FOXO3 is phosphorylated by Akt (43). Myonectin induced FOXO3 phosphorylation at Thr-24 in cultured H4IIE hepatocytes and in mouse liver (Fig. 4). LC3B is a downstream transcriptional target of FOXO3 (44). Accordingly, myonectin treatment reduced the expression of *Lc3b* mRNA in H4IIE hepatocytes (supplemental Fig. 3).

Inhibition of mTOR Signaling Abrogates Myonectin Suppression of Autophagy—To confirm that myonectin indeed acts via the PI3K/Akt/mTOR pathway to inhibit autophagy, H4IIE hepatocytes were treated with recombinant myonectin in the presence or absence of LY294002, Akt inhibitor IV, or rapamy-

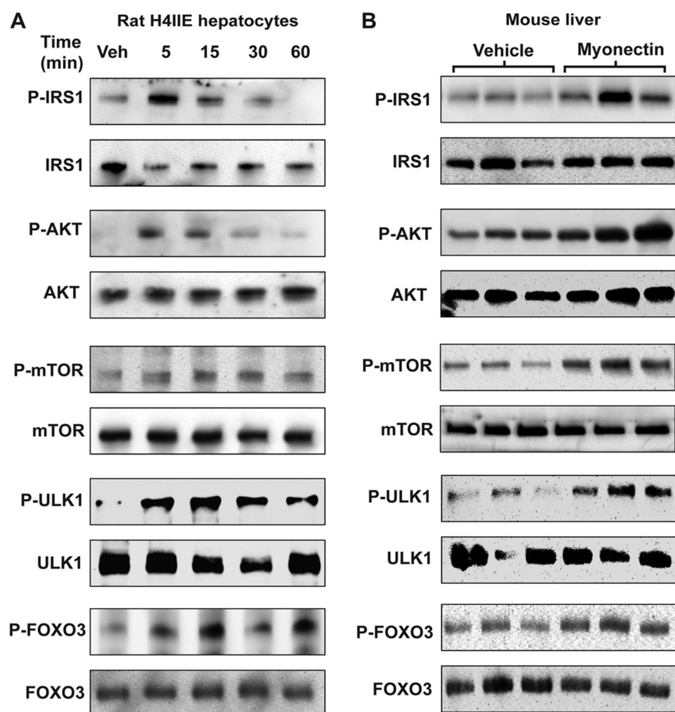

FIGURE 4. **Myonectin activates the Akt/mTOR pathway in H4IIE hepatocytes and mouse liver.** *A*, H4IIE hepatocytes were serum-starved for 2 h and then placed in DMEM containing 5 mM glucose and 5 μg/ml myonectin or HEPES buffer (vehicle (*Veh*)) for 5, 15, 30, or 60 min. Immunoblots were probed for phospho-IRS1 (Ser-612; *P-IRS1*), IRS, phospho-Akt (Thr-308; *P-AKT*), Akt, phospho-mTOR (Ser-2448; *P-mTOR*), mTOR, phospho-ULK1 (Ser-757; *P-ULK1*), ULK1, phospho-FOXO3 (Thr-24; *P-FOXO3*), and FOXO3. *B*, mice ($n = 6$) were fasted for 24 h and injected via the tail vein with HEPES buffer (*Vehicle*) or recombinant myonectin (1 μg/g of body weight). Livers were harvested 10 min after injection and subjected to immunoblotting for the same signaling molecules. Each lane represents an independent cell or liver sample.

cin, potent pharmacological inhibitors of PI3K, Akt, and mTOR function, respectively. Serum-starved cells treated with vehicle control or inhibitors activated autophagy, as indicated by increased LC3 lipidation, Atg7 and Atg12 expression, and p62 degradation. These processes were suppressed by myonectin treatment (Fig. 5). All three inhibitors partially or significantly abolished the ability of myonectin to inhibit starvation-induced LC3 lipidation and *Atg* gene expression and reduced its ability to prevent p62 degradation when simultaneously given to cells (Fig. 5).

LC3 lipidation and p62 degradation serve as useful and informative markers that represent the early and late stages of autophagy, respectively. However, we sought to functionally verify our results by using a biochemical assay to quantify the degradation of long-lived proteins in cells when autophagy is fully engaged (30). To do so, H4IIE hepatocytes were pulsed with ^{14}C-labeled valine to radiolabel intracellular proteins before starving cells. As expected, serum-starved cells had ~50% higher total protein degradation compared with cells cultured with serum (Fig. 6). Treatment with recombinant myonectin largely abolished starvation-induced protein degradation. However, LY294002, Akt inhibitor IV, and rapamycin treatment substantially inhibited the ability of myonectin to suppress starvation-induced cellular protein degradation (Fig. 6). Together, these results indicate that myonectin modulates cellular autophagy via the Akt/mTOR signaling pathway.

DISCUSSION

Here, we have described a novel function of myonectin as a nutrient-responsive regulator of liver autophagy. Prolonged periods of food deprivation turn on autophagy, the cell recycling pathway, by inducing the expression of autophagy-related genes and the formation of autophagosomes (1). This intracellular degradative pathway is highly regulated and sensitive to metabolic alterations. We provide evidence that skeletal muscle-derived myonectin, induced by food intake or the availability of nutrients (*e.g.* glucose and free fatty acids), conveys a hormonal signal to inhibit autophagy in hepatocytes; this is evidence of a novel skeletal muscle-liver axis in modulating tissue homeostasis.

The autophagy-related *Atg* genes, first characterized in budding yeast, are required for autophagosome formation (45). The Atg-regulated cellular recycling pathway is phylogenetically conserved in metazoans (46), playing an essential role in maintaining cellular homeostasis in response to prolonged nutrient deprivation (1). As expected, we observed a pronounced induction of several key *Atg* genes in serum-starved H4IIE hepatocytes and in the livers of starved mice; remarkably, myonectin administration suppressed starvation-induced *Atg* expression *in vitro* and *in vivo*.

Degradation of the cytoplasmic components of liver cells is metabolically regulated and tightly coupled to prolonged fasting or starvation (1). After a 24-h fast, hepatic glycogen stores are mostly depleted to buffer blood glucose. Autophagosome-mediated recycling of cytoplasmic contents provides the liver, a major gluconeogenic tissue, an additional fuel source to power *de novo* glucose synthesis for maintaining physiological blood glucose levels during starvation. When nutrients become available, autophagy will be promptly turned off to prevent excessive catabolic breakdown of cytoplasmic components. We have shown that nutrient-induced myonectin potently suppresses the LC3-dependent formation of autophagosomes and p62 degradation in cultured hepatocytes and in mouse liver. Importantly, myonectin inhibits autophagy to the same extent as conditions that maximally suppress autophagy (hepatocytes cultured in medium containing serum or starved mice given a bolus of glucose).

Activation of mTOR signaling, a nutrient-responsive anabolic pathway, potently inhibits autophagy (2, 15). We have shown that myonectin activates the Akt/mTOR pathway to inhibit autophagy. Consistently, pharmacological inhibition of PI3K, Akt, or mTOR function completely abolished the ability of myonectin to suppress autophagy in cultured hepatocytes. Because the receptor for myonectin is unknown, it is also unknown how skeletal muscle-derived myonectin conveys a hormonal signal to the liver to suppress autophagy through mTOR pathway activation. Identifying the receptor that mediates the cellular effects of myonectin will provide a better mechanistic understanding of its function and regulation.

The anabolic hormone insulin is a potent suppressor of autophagy in liver and skeletal muscles (17). The kinetics of insulin secretion is rapid; within 5–10 min after the ingestion

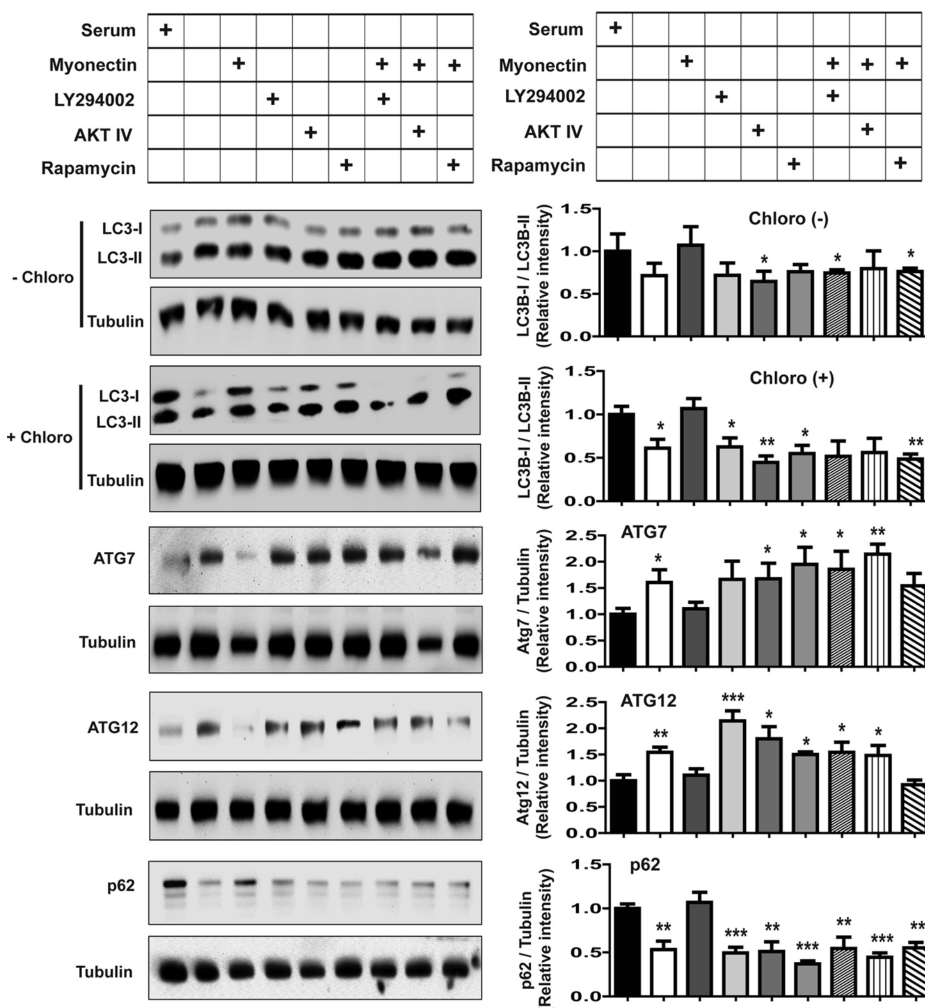

FIGURE 5. **Inhibition of PI3K/Akt/mTOR signaling prevents myonectin from suppressing autophagy-mediated LC3 lipidation, *Atg* expression, and p62 degradation.** Shown are representative immunoblots (*left*) and quantifications (*right*; $n = 5$) from Western blot analysis of H4IIE hepatocytes treated with or without 10% FBS (*Serum*) and recombinant myonectin (5 μg/ml) in the presence or absence of the signaling inhibitor LY294002 (50 μM), Akt inhibitor IV (10 μM), or rapamycin (10 ng/ml) for 1 h (assaying for LC3 lipidation), 6 h (assaying for Atg protein levels), or 24 h (assaying for p62 protein levels). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. All statistical comparisons were between the myonectin-treated group and other treatment conditions (inhibitors + myonectin). *Chloro*, chloroquine.

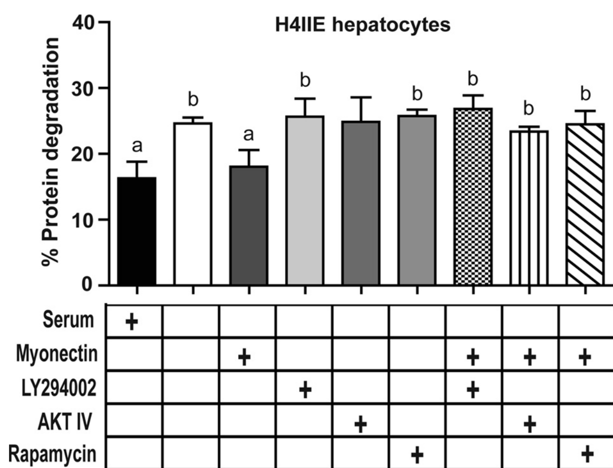

FIGURE 6. **Inhibition of Akt/mTOR signaling abolishes the ability of myonectin to suppress autophagy in hepatocytes.** The rate of degradation of [^{14}C]valine-labeled long-lived proteins was measured in H4IIE hepatocytes ($n = 5$) cultured in glucose-free medium (*Starved*) or glucose-free medium containing 10% FBS, myonectin (5 μg/ml), LY294002 (50 μM), Akt inhibitor IV (10 μM), rapamycin (10 ng/ml), or myonectin plus inhibitors. $a = p < 0.05$ compared with the starved vehicle control; $b = p < 0.05$ compared with the starved myonectin sample.

of food or a bolus of glucose, insulin is secreted from pancreatic β-cells to promote glucose uptake in skeletal muscle and adipose tissue (47), as well as to suppress hepatic glucose output in liver (48). Within 1 h, circulating levels of insulin revert to baseline levels. In contrast, the kinetics of myonectin expression and secretion in response to food intake are slower than the time scale of insulin secretion and clearance. Peak induction of myonectin expression and secretion occurs 3 h after food intake or glucose gavage. Thus, although the effect of insulin on liver autophagy is rapid and acute following food ingestion, myonectin acts in liver at a later postprandial time point to maintain liver autophagy in an "off" state.

Exercise induces autophagy in multiple tissues, leading to improved metabolic outcomes (3). We showed previously that myonectin mRNA and circulating levels are increased in mice that have access to a running wheel for 2 weeks (a model of chronic voluntary exercise) (18). The observed effect may result from a secondary response associated with increased bouts of feeding following voluntary exercise (49). Accordingly, the magnitude of myonectin induction resulting from refeeding following a fast is much greater compared with that induced

by chronic voluntary exercise. Further study is needed to determine whether acute muscle contraction or a single bout of exercise can regulate myonectin expression in skeletal muscle.

Collectively, this study provides evidence for direct endocrine communication between skeletal muscle and liver and highlights the complexity and importance of inter-tissue cross-talk in mediating integrated physiology. Elucidating hormone-mediated tissue cross-talk under different metabolic states, such as food deprivation and exercise, will undoubtedly provide insights into obesity and type 2 diabetes, two prevalent metabolic disorders characterized by failure to maintain systemic energy balance.

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