Regulation of Beclin 1 Protein Phosphorylation and Autophagy by Protein Phosphatase 2A (PP2A) and Death-associated Protein Kinase 3 (DAPK3)*

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Autophagy is an evolutionarily conserved intracellular degradation system that is involved in cell survival and activated in various diseases, including cancer. Beclin 1 is a central scaffold protein that assembles components for promoting or inhibiting autophagy. Association of Beclin 1 with its interacting proteins is regulated by the phosphorylation of Beclin 1 by various Ser/Thr kinases, but the Ser/Thr phosphatases that regulate these phosphorylation events remain unknown. Here we identify Ser-90 in Beclin 1 as a regulatory site whose phosphorylation is markedly enhanced in cells treated with okadaic acid, an inhibitor of protein phosphatase 2A (PP2A). Beclin 1 Ser-90 phosphorylation is induced in skeletal muscle tissues isolated from starved mice. The Beclin 1 S90A mutant blocked starvation-induced autophagy. We found association of PP2A B55α with Beclin 1, which dissociate by starvation. We also found that death-associated protein kinase 3 directly phosphorylates Beclin 1 Ser-90. We propose that physiological regulation of Beclin 1 Ser-90 phosphorylation by PP2A and death-associated protein kinase 3 controls autophagy.

Autophagy is an evolutionarily conserved intracellular degradation system by which cytoplasmic materials are delivered by autophagosomes to the lysosome, where the materials are degraded and recycled (1). Autophagy is considered to be a cell survival pathway that plays roles in development, cell death, and aging. In addition, autophagy has also been implicated in neurodegeneration, autophagy, and cancer. Multiple lines of evidence support the view that autophagy plays an essential role in the development of drug resistance, self-renewal, differentiation, and tumorigenic potential of cancer stem cells (2). Autophagy occurs at basal levels in cells, but it is also regulated developmentally and/or by environmental stimuli such as nutrient/energy availability, hypoxia, and reactive oxygen species.

Beclin 1, the mammalian ortholog of yeast Atg6, functions as a molecular scaffold that assembles an interactome comprising autophagy stimulators and suppressors that regulate the initiation of autophagosome formation (3). Beclin 1 is a haploinsufficient tumor suppressor that is often monoezonally deleted in human breast and ovarian cancers (4). Beclin 1 stimulates autophagy by functioning as part of a core complex that contains vacuolar sorting protein 34 (VPS34), a class III PI3K. The activity of the Beclin 1-VPS34 core complex is regulated by Beclin 1-binding proteins. Beclin 1 contains a BH3 (Bcl-2 homology 3) domain, and anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL, interact with Beclin 1 through the BH3 domain and thereby inhibit autophagy (5). By contrast, association with Atg14L through the coiled-coil domain of Beclin 1 promotes autophagy (6, 7). The interaction of Beclin 1 with its binding partners has been shown to be regulated by the phosphorylation of Beclin 1 by various Ser/Thr kinases. For instance, death-associated protein kinase 1 (DAPK1) phosphorylates Thr-119 of Beclin 1, which leads to the dissociation of Bcl-2 and Bcl-xL from Beclin 1 and promotes autophagy (8, 9). Conversely, Akt/PKB-mediated phosphorylation of Ser-234 and Ser-295 of Beclin 1 inhibits autophagy (10). Very recently, Wei et al. (11) reported that MAPKAPK2/3 (MK2/3) phosphorylates Ser-90 of Beclin 1 (11). However, the Ser/Thr phosphatases that regulate these phosphorylation events remain unknown.

Protein phosphatase 2A (PP2A)2 is an essential Ser/Thr phosphatase conserved from yeast to human that regulates a myriad of biological processes, including cell proliferation, apoptosis, development, and motility (12). Loss or inhibition of PP2A activity has been shown to play a critical role in tumorigenesis (13). Here we identify Ser-90 in Beclin 1 as a regulatory site whose phosphorylation is substantially enhanced in cells treated with okadaic acid, a PP2A inhibitor. We showed that Beclin 1 Ser-90 phosphorylation is physiologically regulated in mouse tissues. We also identified DAPK3 as a kinase that phosphorylates Ser-90 of Beclin 1.

Experimental Procedures

Animals—Male C57BL/6 mice purchased from Charles River Laboratories Japan were maintained in compliance with the guidelines of the Animal Care and Use Committee of Yamaguchi University. For the starvation/refeeding experiment, 2The abbreviations used are: PP2A, protein phosphatase 2A; NT, non-target; OA, okadaic acid; ROS, reactive oxygen species; EBSS, Earle’s balanced salt solution; KD, knockdown; DAPK, death-associated protein kinase; AMPK, AMP-activated protein kinase; MnTBAP, manganese (III) tetrakis (4-benzoic acid) porphyrin chloride.
mice were fasted for 48 h before refeeding. Mice were started to starve at the same time and were sacrificed at different time points. The experimental protocols were approved by the Yamaguchi University Animal Care and Use Committee.

Cell Cultures, Plasmids, Transfection, and Virus Production—HEK293T, HeLa, A549, and MCF7 cells were grown in DMEM supplemented with 10% FBS and 1× antibiotic/antimycotic solution (Life Technologies). Human Beclin 1 S90A, T119A, S234A, and S295A mutants were generated using the In-Fusion HD cloning kit (Takara), with pcDNA4 Beclin 1 (Addgene ID24388) serving as the template. Human Beclin 1 with a C-terminal FLAG tag was PCR-amplified and subcloned into pLV SIN-EF1α-IRE S-ZsGreen1 (Takara). Human B55α and B56α were PCR-amplified with human liver cDNA as a template and subcloned into pLV SIN-EF1α-FLAGx3-IRE S-ZsGreen1 (pLV FLAG). The FLAG-tagged constitutively active AMPK plasmid (1–312 of AMPKα1 T172D) was provided by Dr. Brautigan (University of Virginia), shRNA-expressing plasmids were generated as described previously (14). 19-mer shRNA sequences were as follows: non-target (NT), 5′-CACAAGTGGAGGACCCA-3′; PP2A, 5′-GATACAGGATCATCACA-3′; DAPK3 1, 5′-GGGACAGGATCTAGGAACA-3′; and DAPK3 2, 5′-GACGGACGTGTCCATCATC-3′. Cells were transfected with plasmids by using PEI Max (Polysciences Inc.) according to the instructions of the manufacturer.

To produce lentiviruses, 3 μg of pLV SIN, 2.3 μg of a packaging plasmid (psPAX2), and 1.3 μg of a coat protein plasmid expressing vesicular stomatitis virus G protein (pMD2.G) were transfected into Lentivirus 293T cells cultured in 60-mm dishes. Viral supernatants were collected after 48 h and, after filtering (0.22 μm), were added to 293T, A549, and MCF7 cells for 8 h.

Phosphospecific Antibody Generation—To generate a phospho-specific antibody against Ser(P)-90 Beclin 1, the phosphorylated peptide C+ARM Mp(S)TESAN was used to immunize rabbits. The antibody was purified from the antiserum by using phosphorylated peptide–conjugated SulfoLink coupling resin and then dialyzed against PBS, after which the antibody was further purified by passing it through nonphosphopeptide–conjugated SulfoLink coupling resin. The peptides and antibody were prepared by Operon Biotechnologies K.K. (Tokyo, Japan).

Immunoprecipitation and Immunoblotting—For immunoprecipitation, cells stably or transiently expressing FLAG-tagged proteins were lysed in 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 10 mM Na2VO4, 20 mM sodium pyrophosphate, and Roche complete protease inhibitor mixture. The supernatants were incubated with FLAG-M2 affinity gel (Sigma).

For immunoblotting, cells and tissue samples were lysed in a buffer containing 50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM Na2VO4, 20 mM sodium pyrophosphate, and Roche complete protease inhibitor mixture. For tissue samples, Multi Beads Shocker (Yasui Kikai) was used to homogenate tissues according to the instructions of the manufacturer. 20 μg of proteins was applied to SDS–PAGE. To detect Ser(P)-90 Beclin 1 and total Beclin 1, 40 μg of proteins was applied. Proteins were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Wako) or PVDF membrane (Bio-Rad). For detection of FLAG–Beclin 1 phosphorylation, Phos-tag biotin BTL-104 (Wako) was used according to the protocol of the manufacturer. For immunoblotting, membranes were blocked with 3% or 0.5% skim milk and treated with primary antibodies, and immunoreactive bands were visualized using an ECL Western blotting detection system (GE Healthcare) and LAS-3000 (Fujifilm). Band densities were quantified using ImageJ densitometry analysis software (National Institutes of Health). Antibodies used were as follows: anti-FLAG (Sigma, F7425), anti-LC3 (MBL, PM036), anti-DAPK3 (Gene Tex, GT102404), anti-valosin-containing protein (Gene Tex, GTHX13030), anti-Actin (Santa Cruz Biotechnology, sc-1615), anti–PP2A (Millipore, 07-324), anti–PP2A B55α (Abcam, ab185712), anti- Ser(P)-389 7056K (Cell Signaling Technology, 9234), anti–Beclin 1 (MBL, PD017), anti-Thr(P)-119 Beclin 1 (ABGent, RB34163), anti- Ser(P)-82 HSP27 (Cell Signaling Technology, 9709), MK2 (Cell Signaling Technology, 3042), anti- Ser(P)-792 raptor (Cell Signaling Technology, 2083), and anti-DAPK1 (Sigma, D1319). Anti–DAPK3 was provided by Dr. Tachibana (Osaka City University).

In Vitro Phosphorylation and Dephosphorylation of Beclin 1—For the phosphorylation assay, recombinant Beclin 1 (ProSpec Tech) was incubated with 0.1 μg of GST-tagged human active DAPK1 or DAPK3 (Signal Chem) in a kinase assay buffer (50 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM EGTA, 0.2 mM ATP, and 0.01% Brij-35) in a total volume of 40 μl for 60 min at 37 °C.

For the dephosphorylation assay, MCF7 cells stably expressing FLAG–Beclin 1 were treated with OA, and then Ser-90-phosphorylated FLAG–Beclin 1 was purified using FLAG M2 beads. Phosphorylated Beclin 1 was eluted from the beads by adding the FLAG peptide and then incubated with 0.4 μg of PP2A (Cayman Chemical) in a phosphatase assay buffer (40 mM Tris–HCl (pH 7.4), 34 mM MgCl2, 4 mM EDTA, 2 mM DTT, and 0.05 mg/ml BSA) in a total volume of 30 μl for 60 min at 37 °C.

Immunofluorescent Staining—MCF7 cells were grown on glass coverslips and subsequently fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized in 0.5% Triton X-100/PBS. Cells were blocked with 3% skim milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 1.76 mM KH2PO4, 10 mM Na2HPO4, and 0.05% Tween 20). After incubation with anti-LC3 antibody (MBL) overnight at 4 °C, Alexa Fluor 594–conjugated secondary antibodies (Invitrogen) were added, and cells were incubated for 1 h at room temperature. Fluorescence images were captured using a confocal laser-scanning microscope (LSM510, Zeiss, Tokyo, Japan).

Reactive Oxygen Species (ROS) Generation—1 × 104 HeLa cells were grown in 24-well plates for 2 days. Cells were pretreated with ROS scavengers, MnTBAP (AdipoGen) and EUK134 (Cayman), for 30 min and stimulated with OA for 6 h. ROS generation was detected using a total ROS detection kit for microscopy and flow cytometry (Enzo Life Sciences) according to the instructions of the manufacturer instruction.

Statistical Analysis—The results are expressed as mean ± S.E. Groups were compared using one-way analysis of variance, after which Student-Newman–Keuls test was used. For all analyses, p < 0.05 was considered statistically significant.
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**FIGURE 1.** Type 2A protein phosphatase inhibition induces Beclin 1 Ser-90 phosphorylation. A, a polyclonal population of MCF7 cells stably expressing FLAG-Beclin 1 WT was cultured with or without the type 1 protein phosphatase inhibitor tautomycin (TAU, 1 µM), the type 2A phosphatase inhibitor OA (100 nM), or the type 2B phosphatase inhibitor cyclosporin A (CSA, 10 µM) for 12 h. FLAG-Beclin 1 was immunoprecipitated (IP) using anti-FLAG M2 beads. Phosphorylation of FLAG-Beclin 1 was detected using Phos-tag Biotin HRP. Representative images from three independent experiments are shown. B, MCF7 cells stably expressing FLAG-Beclin 1 WT or S90A were treated with OA (100 nM) for 6 h. FLAG-Beclin 1 was immunoprecipitated. Immunoblotting was performed using an anti-Ser(P)-90 Beclin 1 antibody. Representative images from two independent experiments are shown. C, MCF7 cells stably expressing FLAG-Beclin 1 WT or S90A were treated with OA (100 nM) for increasing times, and the level of endogenous Beclin 1 Ser-90 phosphorylation was analyzed by immunoblotting. Actin was used as a loading control. Representative images from three independent experiments are shown.

**Results**

The Type2A Protein Phosphatase Inhibitor Enhances Ser-90 Phosphorylation of Beclin 1—MCF7 human breast carcinoma cells were used to stably express FLAG-Beclin 1. MCF7 cells were originally derived from a patient with 17q21 loss of heterozygosity and express very a low level of endogenous Beclin 1 (15). The function of Beclin 1 as an interactome scaffold is regulated by posttranslational modifications, including phosphorylation. To identify the Ser/Thr phosphatases that regulate Beclin 1 phosphorylation, we used the following inhibitors of Ser/Thr phosphatases: the type 1 protein phosphatase inhibitor tautomycin, the type 2A protein phosphatase inhibitor OA, and the type 2B protein phosphatase inhibitor cyclosporin A. The inhibitors were added to MCF7 cells that stably expressed WT FLAG-Beclin 1. In OA-treated cells but not in cells treated with tautomycin or cyclosporin A, phosphorylation of Beclin 1 was enhanced, as detected using Phos-tag Biotin HRP (Fig. 1A). To identify the OA-induced phosphorylation site of Beclin 1, we made these Ser/Thr-to-Ala substitutions in Beclin 1: S90A, T119A, S234A, and S295A. OA-induced phosphorylation of FLAG-Beclin 1 was drastically decreased in the S90A mutant but not in the T119A, S234A, or S295A mutants (Fig. 1B). We generated an antibody against Ser(P)-90 in Beclin 1 and demonstrated antibody specificity by comparing WT and S90A Beclin 1 staining in OA-treated cells (Fig. 1C). MCF7 cells stably expressing Beclin 1 WT or S90A were treated with OA, and Beclin 1 was immunoprecipitated using anti-FLAG M2 beads. Ser-90 phosphorylation was observed in Beclin 1 WT but not in Beclin 1 S90A. Furthermore, using this antibody, we characterized the time-dependent increase in endogenous Beclin 1 Ser-90 phosphorylation in OA-treated HeLa cells (Fig. 1D).

Beclin 1 Ser-90 Phosphorylation Is Induced by Starvation in Mouse Skeletal Muscle Tissues—To elucidate whether Beclin 1 Ser-90 phosphorylation is induced by starvation or growth factor stimulation, we performed in vivo starvation/refeeding experiments (Fig. 2A). Mouse tissues were collected at the following six time points (Fig. 2A, 1–6): before starvation, at 24 and 48 h of starvation, and 2, 4, and 8 h after refeeding. Immunoblotting for LC3 confirmed that starvation/refeeding up-regulated and down-regulated the autophagy pathway. In both gastrocnemius (white/fast muscle) and adductor muscle (red/slow muscle) tissues, Beclin 1 Ser-90 phosphorylation was induced by starvation, whereas refeeding dephosphorylated it (Fig. 2, B and C). In contrast, slight and no Ser-90 phosphorylation occurred in liver and heart tissues, respectively (Fig. 2, D and E). These data indicate that Beclin 1 Ser-90 phosphorylation is physiologically regulated in mouse tissues.

Beclin 1 Ser-90 Phosphorylation Promotes Autophagy—MCF7 cells expressing FLAG-Beclin 1 were treated with Earle’s balanced salt solution (EBSS) to examine whether starvation induces Ser-90 phosphorylation in cultured cells. Rapid Ser-90 phosphorylation was observed after EBSS treatment (Fig. 3A). Conversely, starvation did not affect the phosphorylation level of Thr-119, even though previous work reported starvation-induced Thr-119 phosphorylation (16). The specificity of the anti-Thr(P)-119 Beclin 1 antibody was confirmed using T119A Beclin 1 (Fig. 3B). Because Thr-119 phosphorylation can be detected even under nutrient-rich conditions, it might contribute to the regulation of basal autophagy. We further examined whether growth factor signaling suppresses Ser-90 phosphorylation levels. Cells were starved with FBS free medium for 24 h, and then insulin was added to the culture medium. Insulin stimulation rapidly triggered Ser-90 dephosphorylation (Fig. 3C). These data indicate that Beclin 1 Ser-90 phosphorylation is regulated by the nutrient status of cells.

We investigated whether Beclin 1 Ser-90 phosphorylation affected autophagy. In MCF7 cells stably expressing the Beclin 1 S90A dephospho-mimic mutant, autophagic activity was diminished. We observed that bafilomycin- and starvation-induced LC3-II accumulation was lower in cells expressing the S90A mutant than in cells expressing the WT protein (Fig. 3D). Moreover, in S90A-mutant cells, the number of LC3 puncta was decreased (Fig. 3E). These data indicate that Beclin 1 Ser-90 phosphorylation promotes autophagy.

PP2A Directly Dephosphorylates Beclin 1 Ser-90—OA blocks all type 2A protein phosphatases (PP2A, PP4, and PP6). However, among these, PP2A is the major type 2A phosphatase, and thus we inhibited PP2A expression in MCF7 FLAG-Beclin 1 cells by using shRNAs (Fig. 4A). PP2A knockdown enhanced
starvation-induced Ser-90 phosphorylation (Fig. 4B). The effect of PP2A KD on Ser-90 phosphorylation was confirmed by another shRNA for PP2A (data not shown). Consistent with this finding, the results of in vitro assays demonstrated that Ser-90 of FLAG-Beclin 1 was dephosphorylated by the recombinant human PP2A catalytic subunit (Fig. 4C). These data show that PP2A directly dephosphorylates Beclin 1 Ser-90.

PP2A forms heterotrimeric, each comprised of a catalytic subunit (C, or PP2Ac), a scaffolding subunit (A, or PP2A-A), and one regulatory B subunit from four different families of

FIGURE 2. Beclin 1 Ser-90 phosphorylation is induced by starvation in mouse skeletal muscle tissues. A, time course for mouse starvation/refeeding. Mice were euthanized before starvation (1), at 24 h of starvation (2), at 48 h of starvation (3), 2 h after refeeding (4), 4 h after refeeding (5), and 8 h after refeeding (6). B–E, gastrocnemius muscle (B), adductor muscle (C), liver (D), and heart (E) were isolated from mice at the indicated time points, and Beclin 1 Ser-90 phosphorylation was detected by immunoblotting. LC3 was used to monitor autophagy activity. Representative images from three independent experiments are shown.

FIGURE 3. Beclin 1 Ser-90 phosphorylation promotes autophagy. A, MCF7 cells stably expressing FLAG-Beclin 1 WT were treated with EBSS (starvation) for the indicated time periods. FLAG-Beclin 1 was immunoprecipitated (IP) using anti-FLAG M2 beads, and phosphorylation of Beclin 1 Ser-90 and Thr-119 was detected by immunoblotting. Representative images from three independent experiments are shown. B, FLAG-Beclin 1 was immunoprecipitated from MCF7 cells stably expressing FLAG-Beclin 1 WT or T119A. Immunoblotting was performed using an anti-Thr(P)-119 Beclin 1 antibody. Representative images from two independent experiments are shown. C, MCF7 cells stably expressing FLAG-Beclin 1 WT were starved for 24 h and stimulated with insulin for the indicated time periods. FLAG-Beclin 1 was immunoprecipitated, and phosphorylation of Beclin 1 Ser-90 was detected by immunoblotting. Representative images from three independent experiments are shown. D, MCF7 cells stably expressing FLAG-Beclin 1 WT or the S90A mutants were treated with bafilomycin A (100 nM) and/or EBSS (starvation) for 1 h. The levels of autophagy flux were quantified by LC3 immunoblotting. Actin was used as a loading control. Representative images from three independent experiments are shown. E, MCF7 cells stably expressing FLAG-Beclin 1 WT or the S90A mutants were fixed and stained with anti-LC3 antibodies, and images were captured using a confocal microscope. Representative images from three independent experiments are shown. The LC3 puncta in cells were counted, and 107–139 cells were analyzed per experiment.* p < 0.05 compared with Beclin 1 S90A.
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FIGURE 4. PP2A directly dephosphorylates Beclin 1 Ser-90. A and B, MCF7 cells stably expressing FLAG-Beclin 1 WT were expressing non-target shRNA (shNT) or shRNA targeting PP2A (shPP2A). A, the effect of PP2A knockdown was analyzed by immunoblotting. VCP, valosin-containing protein. B, cells were treated with EBSS for 30 min. FLAG-Beclin 1 was immunoprecipitated, and phosphorylation of Beclin 1 Ser-90 was detected by immunoblotting. Representative images from three and two independent experiments, respectively, are shown. C, in vitro dephosphorylation of FLAG-Beclin 1 by recombinant PP2A was performed as described under “Experimental Procedures,” and the level of Beclin 1 Ser-90 phosphorylation was analyzed by immunoblotting. Representative images from three independent experiments are shown. D, 293T cells were transiently expressing FLAG-B55α and FLAG-B56α. FLAG-B subunits were immunoprecipitated, and the association with endogenous Beclin 1 was analyzed. Representative images from three independent experiments are shown. WCL, whole cell lysate. E, 293T cells stably expressing FLAG-B55α were treated with EBSS (starvation) for the indicated time periods. FLAG-B55α was immunoprecipitated, and the association with endogenous Beclin 1 was analyzed. Representative images from three independent experiments are shown. F, 293T cells were transiently expressed FLAG-Beclin 1 WT or the S90A mutants. FLAG-Beclin 1 was immunoprecipitated, and the association with endogenous B55α was analyzed by immunoblotting. Representative images from three independent experiments are shown.

genes (17). Regulatory B subunits control PP2A specificity by targeting PP2Ac to substrates. To find out which B subunit associates with Beclin 1, FLAG tagged B55α and B56α were transiently expressed in 293T cells. Immunoprecipitation with FLAG M2 beads revealed that B55α, but not B56α, associates with Beclin 1 (Fig. 4D). Starvation induced dissociation of B55α from Beclin 1 (Fig. 4E). Moreover, B55α strongly associated with Beclin 1 WT compared with the S90A mutant (Fig. 4F). These data suggest that the PP2A-B55α complex regulates Beclin 1 Ser-90 dephosphorylation.

DAPK3 Directly Phosphorylates Beclin 1 Ser-90—Very recently, Wei et al. (11) reported that MAPKAPK2/3 (MK2/3) phosphorylates Ser-90 of Beclin 1. MK2/3 is directly regulated by p38 MAPK and involved in stress responses (18). ROS are known to activate p38 MAPK-MK2/3 signaling, and Wei et al. (11) showed that ROS generation induces Beclin 1 Ser-90 phosphorylation. Therefore, we examined whether ROS-p38 MAPK-MK2/3 signaling is involved in OA-induced Beclin 1 Ser-90 phosphorylation. HeLa cells were pretreated with two types of ROS scavengers, MnTBP and EUK134, for 30 min and stimulated with OA for 6 h. We observed OA-induced ROS generation in cells, and ROS scavengers effectively blocked it (Fig. 5A). However, ROS scavengers failed to block OA-induced Beclin 1 Ser-90 phosphorylation (Fig. 5B). HSP27 is known to be a MK2 substrate and is widely used as an indicator of MK2 activation (19). We observed an MK2 band shift (also an indicator of MK2 activation) and HSP27 phosphorylation in HeLa cells treated with anisomycin, a potent activator of p38 MAPK-MK2 signaling, even though anisomycin did not induce Ser-90 phosphorylation (Fig. 5C). On the other hand, OA only slightly activates MK2 and phosphorylates HSP27, but it markedly induced Beclin 1 phosphorylation. These data suggest that the other kinase(s) are involved in the phosphorylation of Beclin 1 Ser-90.

We sought to identify the kinase responsible for Beclin 1 Ser-90 phosphorylation induced by OA. Because starvation increases the level of Ser-90 phosphorylation, we first examined the involvement of AMPK. However, the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) did not induce Ser-90 phosphorylation (Fig. 5D). Moreover, expression of constitutively active AMPK failed to phosphorylate Ser-90 of Beclin 1 (Fig. 5E). The effectiveness of constitutively active AMPK was confirmed by the phosphorylation of the AMPK substrate raptor. Another molecule activated by starvation is ULK1, which is negatively regulated by mTORC1 (20). Thus, we tested whether Beclin 1 Ser-90 phosphorylation can be induced by activating ULK1 through rapamycin-dependent mTORC1 inhibition. However, rapamycin treatment did not increase Ser-90 phosphorylation (Fig. 5F). Here, the effectiveness of rapamycin was confirmed by the dephosphorylation of the mTORC1 substrate S6K. These data suggest that AMPK and ULK1 are not involved in Beclin 1 Ser-90 phosphorylation.

DAPK1 has been shown to phosphorylate Thr-119 of Beclin 1, which leads to the dissociation of the autophagy inhibitor Bcl-2 from Beclin 1 (8). DAPK1 is a member of the DAPK family that includes two other closely related kinases, DAPK2/DRP-1 and DAPK3/ZIPK (zipper-interacting protein kinase) (21). The Ser-90 residue of human Beclin 1 is evolutionally conserved among vertebrates, and the RMMS sequence in which Ser-90 phosphorylation occurs is identical to the DAPK phosphorylation motif RXYS/T (Fig. 6A). To investigate the involvement of the DAPK family in OA-induced Beclin 1 Ser-90 phosphorylation, we pretreated MCF7 FLAG-Beclin 1 cells with two types of DAPK inhibitors that inhibit both DAPK1 and DAPK3 but not DAPK2. Both Tc DAPK6 (Tocris) and DAPK inhibitor (Millipore) have an effect on OA-induced MK2 activation (Fig. 6B). These inhibitors partially suppressed OA-induced (Fig. 6C and D) and starvation-induced (Fig. 6E and data not shown) Ser-90 phosphorylation, suggesting the involvement of DAPK1 or DAPK3. To identify the specific DAPK family member responsible for Beclin 1 Ser-90 phosphorylation, we used shRNAs to suppress DAPK1 and DAPK3 expression in MCF7 FLAG-Beclin 1 cells (Fig. 6F). Starvation-induced Ser-90 phosphorylation was partially inhibited following DAPK3 knockdown (shDAPK3 #1) but not DAPK1 knockdown (Fig. 6G). To rule out the possibility of off-target effects and to reveal the role of
DAPK3 on endogenous Beclin 1 phosphorylation, we suppressed DAPK3 expression in A549 cells by another shRNA targeting DAPK3 (shDAPK3 #2, Fig. 6H). DAPK3 KD did not affect MK2 protein levels and activity (shown by a band shift). We revealed that OA-induced endogenous Beclin 1 phosphorylation was also partially inhibited by DAPK3 KD (Fig. 6f). Moreover, the results of in vitro kinase assays demonstrated that recombinant DAPK3 directly phosphorylated Beclin 1 Ser-90 (Fig. 6f). Although DAPK1 weakly phosphorylated Beclin 1 Ser-90 in vitro, our data indicate that DAPK3 is the kinase responsible for Beclin 1 Ser-90 phosphorylation.

Because we observed tissue specificity for the response to starvation/refeeding, we examined the protein levels of Beclin 1, DAPK3, MK2, PP2A, and B55α (Fig. 6k). We did not observe any relevance between these protein levels and tissue specificity. These results suggest that the differences in the levels of these proteins do not account for the differences of response to starvation/refeeding between mouse tissues.

### Discussion

Beclin 1 functions as a molecular scaffold that assembles an interactome comprising autophagy stimulators and suppressors (3). The association of Beclin 1 with its binding partners has been reported to be regulated by Beclin 1 phosphorylation (22). In this study, we discovered that Ser-90 of Beclin 1 is a target of PP2A and that this phosphorylation is physiologically regulated by nutrient status. We also revealed that DAPK3 directly phosphorylates this site. To our knowledge, this is the first report showing Beclin 1 regulation by a Ser/Thr phosphatase.

We identified Ser-90 in Beclin 1 as a regulatory site whose phosphorylation is markedly induced in cells treated with OA, an inhibitor of type 2A protein phosphatases. Under non-starved conditions, the B55α subunit, but not B56α, makes a complex with Beclin 1, suggesting constant dephosphorylation by the PP2A-B55α complex. Starvation induces dissociation of B55α from Beclin 1 and enables Ser-90 phosphorylation by kinases. The molecular mechanism underlying starvation-induced dissociation of B55α from Beclin 1 remains unknown. It has been reported that phosphorylation of B55α affects its holoenzyme assembly, suggesting that posttranscriptional modification of B55α may be involved in this (23).

The consecutive phosphorylation of Ser-93 after Ser-90 of Beclin 1 has been reported to be required for maximal autophagy. Fogel et al. (24) used Phos-tag gels, in which phosphorylated bands migrate as supershifted species (25), and showed that starvation induced supershifts in the Beclin 1 band. Kim et al. (26) reported Atg14L-dependent phosphorylation of Ser-93 and Ser-96 in Beclin 1 by AMPK, which induces...
autophagy. These data suggest that the consecutive phosphor-
ylation of Ser-93/Ser-96 after Ser-90 phosphorylation plays a
critical role in autophagy induction. The mechanism of
autophagy promotion by Beclin 1 Ser-90 phosphorylation has
not been fully understood, but Wei et al. (11) observed the
increased lipid kinase activity of the Beclin 1-VPS34 complex.
They also claimed that association with Bcl-2, a negative regu-
lator of Beclin 1, blocks MK2/3-dependent Beclin 1 phosphor-
ylation and starvation-induced autophagy.

In our study, ROS scavengers failed to block OA-induced
Ser-90 phosphorylation, and treatment with the MK2/3 activa-
tor anisomycin was not potent enough to induce Ser-90 phos-
phorylation. These data suggest that the other kinase(s) are
involved in this event. Supporting this idea, Wei et al. (11)
showed drastic but incomplete reduction of Ser-90 phosphor-
ylation in MK2/3 double KO cells. We found that DAPK3
directly phosphorylates Ser-90 of Beclin 1. In agreement with
our observation, shRNA-dependent inhibition of DAPK3 has
been reported to suppress starvation-induced autophagy (27).
Our result that DAPK3 KD only partially suppressed starva-
tion-induced Beclin 1 Ser-90 phosphorylation suggests the
involvement of other kinases, including MK2/3. The molecular
mechanism for DAPK3 activation has not been fully under-
stood. Although DAPK1 is suggested to be an upstream kinase
(28), our data indicated DAPK1-independent signaling for star-
vation-induced DAPK3 activation. Rho-associated protein
kinase is another possible upstream kinase for DAPK3 activa-
tion (29). However, we found that the Rho-associated protein

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**FIGURE 6.** DAPK3 directly phosphorylates Beclin 1 Ser-90. A, alignment of the conserved phosphorylation site in Beclin 1 (human Ser-90). B, HeLa cells were pretreated with or without DAPK inhibitor (10 μM) or Tc DAPK6 (10 μM) for 2 h before being treated with OA (100 nM) for 6 h. The level of MK2 activation (shown by a band shift) was analyzed by immunoblotting. Representative images from two independent experiments are shown. C-E, MCF7 cells stably expressing FLAG-Beclin 1 WT were pretreated with or without DAPK inhibitor (C and D) or Tc DAPK6 (D) for 2 h before being treated with OA (100 nM) for 6 h (C and D) or EBSS (Starvation) for 1 h (E), FLAG-Beclin 1 was immunoprecipitated (IP), and phosphorylation of Beclin 1 Ser-90 was detected by immunoblotting. Representative images from three independent experiments are shown. F-G, MCF7 cells stably expressing FLAG-Beclin 1 WT were expressing NT shRNA, shRNA targeting DAPK1, or DAPK3 (shDAPK3 #1). F, effects of DAPK1 and DAPK3 knockdown were analyzed by immunoblotting. G, cells were treated with EBSS (Starvation) for 1 h, FLAG-Beclin 1 was immunoprecipitated, and phosphorylation of Beclin 1 Ser-90 was detected by immunoblotting. Representative images and quantitative data from three independent experiments are shown. *, p < 0.05 versus shNT/starvation. H and I, AS49 cells were expressing NT shRNA or shRNA targeting DAPK3 (shDAPK3 #2). H, the effects of DAPK3 knockdown were analyzed by immunoblotting. VCP, valosin-containing protein. J, cells were treated with OA (100 nM) for 6 h, and phosphorylation of Beclin 1 Ser-90 was detected by immunoblotting. Representative images from three independent experiments are shown. J, in vitro phosphorylation of recombinant Beclin 1 by DAPK1 and DAPK3 was performed, and the level of Beclin 1 Ser-90 phosphorylation was evaluated by immunoblotting. Representative images from three independent experiments are shown. K, heart, liver, adductor muscle (AM), and gastrocnemius muscle (GM) were isolated from mice, and the expression levels of indicated proteins were detected by immunoblotting. Representative images from three independent experiments are shown.
kinase inhibitor Y27632 did not affect OA-induced Beclin 1 Ser-90 phosphorylation (data not shown). Therefore, the upstream kinase(s) for DAPK3 remain(s) unknown.

OA induced Ser-90 phosphorylation under non-starved conditions. On the other hand, PP2A KD did not induce Ser-90 phosphorylation under non-starved conditions even though it enhanced starvation-induced Ser-90 phosphorylation. This difference suggests that OA, but not PP2A KD, activates kinase(s) for Ser-90 phosphorylation. Although OA is a potent PP2A inhibitor, it produced oxidative stress in cells, suggesting activation of stress-responsive kinases such as p38 MAPK. However, ROS scavengers failed to block Ser-90 phosphorylation induced by OA. Because OA blocks all type2A protein phosphatases, PP2A, PP4, and PP6, it is possible that inhibition of PP4 and/or PP6 is involved in the activation of kinases, including DAPK3.

In this study, Beclin 1 Ser-90 was phosphorylated in skeletal muscle tissues obtained from starved mice. However, Ser-90 was phosphorylated only slightly in the liver and not phosphorylated in the heart. These data suggest that Beclin 1 Ser-90 phosphorylation plays a role in autophagy induction in skeletal muscle but is dispensable in the liver and heart. Our data showed that the differences in the levels of Beclin 1 Ser-90-related proteins do not account for the different responses to starvation/refeeding between mouse tissues. We speculate that Beclin 1 complex association is regulated in a tissue-specific manner, possibly involving another phosphosite on Beclin 1. The mechanism underlying such tissue-specific phosphorylation remains unclear. However, one possibility is the difference in the sustainability of autophagy. Autophagy activation in skeletal muscle tissues persists for up to 48 h but lasts only for a few hours in the liver (30). Another possibility is the use of divergent regulatory mechanisms in distinct tissues. Autophagy is differentially regulated by insulin and amino acids in a tissue-dependent manner. Although insulin regulates autophagy in skeletal muscle, amino acids regulate liver autophagy (31). We observed that insulin suppressed Ser-90 phosphorylation of Beclin 1 in cells, which suggests a role of insulin in controlling the levels of Ser-90 phosphorylation in vivo. Accumulating evidence has revealed that skeletal muscle is a crucial metabolic organ and that autophagy plays a central role in it (32). Our findings might provide an enhanced understanding for the mechanism through which metabolism and autophagy are linked.

Author Contributions—T. O. and N. F. designed the experiments. T. O. produced lentiviruses and stably expressing cells. N. F. performed the Western blotting analyses, immunoprecipitations, immunofluorescent staining, and in vitro kinase/phosphatase assays. T. O. and N. F. processed and analyzed the data and wrote and edited the manuscript. U. T. and K. S. provided suggestions and discussions. All authors read and approved the final manuscript.

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References
1. Mizushima, N. (2007) Autophagy: process and function. Genes Dev. 21, 2861–2873
2. Choi, K. S. (2012) Autophagy and cancer. Exp. Mol. Med. 44, 109–120
3. Kang, R., Zeh, H. J., Lotze, M. T., and Tang, D. (2011) The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 18, 571–580
4. Gong, C., Bavry, C., Tonelli, G., Yue, W., Deloméni, C., Nicolas, V., Zhu, Y., Domergue, V., Marin-Esteban, V., Tharinger, H., Delbos, L., Gary-Gouy, H., Morel, A. P., Ghavami, S., Song, E., Codogno, P., and Mehrpour, M. (2013) Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. Oncogene 32, 2261–2272
5. Pattengie, S., Tassa, A., Xu, Q., Garuti, R., Liang, X. H., Mizushima, N., Packing, M., Schneider, M. D., and Levine, B. (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939
6. Itakura, E., Kishi, C., Inoue, K., and Mizushima, N. (2008) Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol. Biol. Cell 19, 5360–5372
7. Sun, Q., Fan, W., Chen, K., Ding, X., Chen, S., and Zhong, Q. (2008) Identification of BARK as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. U.S.A. 105, 1921–1926
8. Zalckvar, E., Berissi, H., Eisenstein, M., and Kimchi, A. (2009) Phosphorylation of Beclin 1 by DAP-kine promotes autophagy by weakening its interactions with Bcl-2 and Bcl-XL. Autophagy 5, 720–722
9. Zalckvar, E., Berissi, H., Mizrachy, L., Idolchuk, Y., Koren, I., Eisenstein, M., Sabanay, H., Pinkas-Kramarski, R., and Kimchi, A. (2009) DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep. 10, 285–292
10. Wang, R. C., Wei, Y., An, Z., Zou, Z., Xiao, G., Bhagat, G., White, M., Reichelt, J., and Levine, B. (2012) Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. Science 338, 956–959
11. Wei, Y., An, Z., Zou, Z., Sumpter, R., Su, M., Zang, X., Sinha, S., Gaestel, M., and Levine, B. (2015) The stress-responsive kinases MAPKAPK2/ MAPKAPK3 activate starvation-induced autophagy through Beclin 1 phosphorylation. eLife 4, 10.7554/eLife.05289
12. Brautigan, D. L. (2013) Protein Ser/Thr phosphatases: the ugly ducklings of cell signalling. FEBS Lett. 580, 324–345
13. Eichhorn, P. J., Creyghton, M. P., and Bernards, R. (2009) Protein phosphatase 2A regulatory subunits and cancer. Biochim. Biophys. Acta 1795, 1–15
14. Yabe, R., Miura, A., Usui, T., Mudrak, I., Ogris, E., Ohama, T., and Sato, K. (2015) Protein phosphatase methyl-esterase PME-1 protects protein phosphatase 2A from ubiquitin/proteasome degradation. PLoS ONE 10, e0145226
15. Liang, X. H., Jackson, C., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402, 672–676
16. Gukar, A. U., Chu, K., Raj, L., Bouley, R., Lee, S. H., Kim, Y. B., Dunn, S. E., Mandinova, A., and Lee, S. W. (2013) Identification of ROCK1 kinase as a critical regulator of Beclin1-mediated autophagy during metabolic stress. Nat. Commun. 4, 2189
17. Jansens, V., and Goris, J. (2001) Protein phosphatase 2A: A highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem. J. 353, 417–439
18. Cargnello, M., and Roux, P. P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol. Mol. Biol. Rev. 75, 50–83
19. Stokoe, D., Campbell, D. G., Nikiel, R., Hidaka, H., Leevers, S. J., Marshall, C., and Cohen, P. (1992) MAPKAP kinase-2: A novel protein kinase activated by mitogen-activated protein kinase. EMBO J. 11, 3985–3994
20. Alers, S., Loffler, A. S., Wesselborg, S., and Stork, B. (2012) Role of AMPK-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. Mol. Cell Biol. 32, 2–11
21. Gozuacik, D., and Kimchi, A. (2006) DAPK protein family and cancer. Autophagy 2, 74–79
22. Abrahamsen, H., Stenmark, H., and Platta, H. W. (2012) Ubiquitination and phosphorylation of Beclin 1 and its binding partners: tuning class III phosphatidylinositol 3-kinase activity and tumor suppression. FEBS Lett. 586, 1584–1591
23. Schmitz, M. H., Held, M., Janssens, V., Hutchins, J. R., Hudecz, O., Ivanova, E., Goris, J., Trinkle-Mulcahy, L., Lamond, A. I., Poser, I., Hyman, A. A., Mechtler, K., Peters, J. M., and Gerlich, D. W. (2010) Live-cell imaging RNAi screen identifies PP2A-B55α and importin-β1 as key mitotic exit regulators in human cells. Nat. Cell Biol. 12, 886–893

24. Fogel, A. I., Dlouhy, B. J., Wang, C., Ryu, S. W., Neutzner, A., Hasson, S. A., Sideris, D. P., Abeliouicz, H., and Youle, R. J. (2013) Role of membrane association and Atg14-dependent phosphorylation in beclin-1-mediated autophagy. Mol. Cell Biol. 33, 3675–3688

25. Kinoshita, E., Takahashi, M., Takeda, H., Shiro, M., and Koike, T. (2004) Recognition of phosphate monoester dianion by an alkoxide-bridged dinuclear zinc(II) complex. Dalton Trans. 8, 1189–1193

26. Kim, J., Kim, Y. C., Fang, C., Russell, R. C., Kim, J. H., Fan, W., Liu, R., Zhong, Q., and Guan, K. L. (2013) Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. Cell 152, 290–303

27. Tang, H. W., Wang, Y. B., Wang, S. L., Wu, M. H., Lin, S. Y., and Chen, G. C. (2011) Atg1-mediated myosin II activation regulates autophagosome formation during starvation-induced autophagy. EMBO J. 30, 636–651

28. Shani, G., Marash, L., Gozuacik, D., Bialik, S., Teitelbaum, L., Shohat, G., and Kimchi, A. (2004) Death-associated protein kinase phosphorylates ZIP kinase, forming a unique kinase hierarchy to activate its cell death functions. Mol. Cell Biol. 24, 8611–8626

29. Graves, P. R., Winkfield, K. M., and Haystead, T. A. (2005) Regulation of zipper-interacting protein kinase activity in vitro and in vivo by multisite phosphorylation. J. Biol. Chem. 280, 9363–9374

30. Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., and Ohsumi, Y. (2004) In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol. Biol. Cell 15, 1101–1111

31. Naito, T., Kuma, A., and Mizushima, N. (2013) Differential contribution of insulin and amino acids to the mTORC1-autophagy pathway in the liver and muscle. J. Biol. Chem. 288, 21074–21081

32. Neel, B. A., Lin, Y., and Pessin, J. E. (2013) Skeletal muscle autophagy: a new metabolic regulator. Trends Endocrinol. Metab. 24, 635–643