Deacetylation of FoxO by Sirt1 Plays an Essential Role in Mediating Starvation-Induced Autophagy in Cardiac Myocytes

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Rationale: Autophagy, a bulk degradation process of cytosolic proteins and organelles, is protective during nutrient starvation in cardiomyocytes (CMs). However, the underlying signaling mechanism mediating autophagy is not well understood.

Objective: We investigated the role of FoxOs and its posttranslational modification in mediating starvation-induced autophagy.

Methods and Results: Glucose deprivation (GD) increased autophagic flux in cultured CMs, as evidenced by increased mRFP-GFP-LC3 puncta and decreases in p62, which was accompanied by upregulation of Sirt1 and FoxO1. Overexpression of either Sirt1 or FoxO1 was sufficient for inducing autophagic flux, whereas both Sirt1 and FoxO1 were required for GD-induced autophagy. GD increased deacetylation of FoxO1, and Sirt1 was required for GD-induced deacetylation of FoxO1. Overexpression of FoxO1(3A/LXXAA), which cannot interact with Sirt1, or p300, a histone acetylase, increased acetylation of FoxO1 and inhibited GD-induced autophagy. FoxO1 increased expression of Rab7, a small GTP-binding protein that mediates late autophagosome–lysosome fusion, which was both necessary and sufficient for mediating FoxO1-induced increases in autophagic flux. Although cardiac function was maintained in control mice after 48 hours of food starvation, it was significantly deteriorated in mice with cardiac-specific overexpression of FoxO1(3A/LXXAA), those with cardiac-specific homozygous deletion of FoxO1 (c-FoxO1<sup>−/−</sup>), and beclin1<sup>+/−</sup> mice, in which autophagy is significantly inhibited.

Conclusions: These results suggest that Sirt1-mediated deacetylation of FoxO1 and upregulation of Rab7 play an important role in mediating starvation-induced increases in autophagic flux, which in turn plays an essential role in maintaining left ventricular function during starvation. (*Circ Res. 2010;107:1470-1482.)*

Key Words: autophagy ■ starvation ■ FoxO ■ Sirt1 ■ Rab7 ■ deacetylation

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Macroutaphagy (termed hereafter as autophagy) is a dynamic process of intracellular bulk degradation in which cytosolic proteins and organelles are sequestered into double-membrane vesicles called autophagosomes to be fused with lysosomes for degradation. In the heart, autophagy maintains protein quality control, adapts to nutrient and oxygen deprivation during myocardial ischemia, and mediates cell death during reperfusion injury. Autophagy during nutrient deprivation is an adaptive mechanism that allows the cells to survive by degrading the intracellular protein and lipid cargo and recycling the amino and fatty acids to generate ATP. The nutrient status has a profound effect on cardiac contractility, and activation of autophagy during starvation is protective for the heart. The intracellular signaling mechanism by which nutrient starvation activates autophagy in cardiomyocytes (CMs) is not well understood, however.

The forkhead box, class O (FoxO) family of transcription factors are present as 4 distinct isoforms (FoxO1, FoxO3, FoxO4, and FoxO6) in mammals. FoxO proteins play an important role in several intracellular functions, such as metabolism, stress resistance, longevity, tumor suppression, and cell size regulation. The key to the myriad functions of FoxO proteins lies in the complex posttranslational modifications they undergo. They are phosphorylated in response to insulin and growth factors, dephosphorylated by protein phosphatases, ubiquitinylated in response to oxidative stress, acetylated by p300/CBP, and deacetylated by Sirt1. FoxO3 and FoxO1 regulate autophagy in skeletal and cardiac muscles by activating genes that are involved in autophagosome fusion.
formation. However, the specific role of FoxO posttranslational modification in mediating autophagy is not yet known.

Sirt1, the mammalian ortholog of yeast silent information regulator (Sir2), is a class III histone deacetylase. Sirt1 extends lifespan in lower organisms, whereas it suppresses aging-induced cardiomyopathy and protects against oxidative stress in the mouse heart. Sirt1 regulates autophagy by interacting with autophagy-related genes Atg5, Atg7, and Atg8 and deacetylating them. We have shown previously that endogenous Sirt1 positively regulates autophagy in CMs. Sirt1 deacetylates FoxO1, FoxO3, and FoxO4 and regulates FoxO-dependent gene transcription either positively or negatively. FoxO proteins bear a conserved coactivator-interacting LXXLL motif that is important for their interaction with Sirt1. However, whether or not the effect of Sirt1 on autophagy is mediated through FoxO proteins is unknown.

In this study, we used activation of autophagy by glucose deprivation (GD) in cultured CMs as a model and investigated the signaling mechanism mediating autophagy. Goals of this study were to (1) elucidate the role of FoxO1 in mediating starvation-induced increases in autophagy; (2) evaluate the role of FoxO1 deacetylation by Sirt1 in mediating starvation-induced autophagy; and (3) clarify the molecular mechanism by which FoxO1 can enhance autophagic flux.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Adeno- and Lentiviruses**

Adenoviruses harboring murine wild-type (WT) FoxO1 (Ad-FoxO1-WT), FoxO1 (3A/LXXAA) (Ad-3A/LXXAA), Sirt1 (Ad-Sirt1), short hairpin (sh)RNA Sirt1 (Ad-sh-Sirt1), GFP-LC3 (Ad-GFP-LC3), tandem fluorescent mRFP-GFP-LC3 (Ad-tf-LC3), mRFP (monomeric red fluorescent protein) short hairpin (sh)RNA Sirt1 (Ad-sh-Sirt1),10 shRNA Scramble (Ad-sh-Scr),10 GFP-LC3 (Ad-GFP-LC3),2 tandem fluorescent mRFP-GFP-LC3 (Ad-tf-LC3),15 tTA (Ad-tTA),16 and LacZ (Ad-LacZ)16 have been described. Adenovirus harboring inducible p300 (Ad-p300) was purchased from Cell Biolabs. The plasmid constructs of hemagglutinin (HA)-tagged human Rab7 (obtained from Missouri S&T cDNA Resource Center) and HA-tagged WT FoxO3a (from Dr Michael Greenberg) were used to generate adenoviruses Ad-HA-Rab7 (obtained from Missouri S&T cDNA Resource Center) and HA-tagged WT FoxO3a (from Dr Michael Greenberg). were used to generate adenoviruses Ad-HA-Rab7 (obtained from Missouri S&T cDNA Resource Center) and HA-tagged WT FoxO3a (from Dr Michael Greenberg). were used to generate adenoviruses Ad-HA-Rab7 (obtained from Missouri S&T cDNA Resource Center) and HA-tagged WT FoxO3a (from Dr Michael Greenberg). were used to generate adenoviruses Ad-HA-Rab7 (obtained from Missouri S&T cDNA Resource Center) and HA-tagged WT FoxO3a (from Dr Michael Greenberg).

**Transgenic Mice**

Transgenic mice with cardiac-specific overexpression of FoxO1 (3A/LXXAA) (Tg-FoxO1m) were generated on an FVB background with the α-myosin heavy chain (MHC) promoter (courtesy of Dr J Robbins, Children’s Hospital, Cincinnati, Ohio). FoxO1 homozygous floxed mice (fox) were bred with transgenic mice with cardiac-specific overexpression of Cre recombinase (mMHC-Cre-Tg) (courtesy of Dr M. D. Schneider, Imperial College, London, UK) to generate cardiac-specific FoxO1 homozygous knockout mice (c-FoxO1/−/−). Beclin1 heterozygous knockout mice (beclin1+/−) have been described.

**Results**

GD Increases Autophagic Flux in Cultured CMs

Cultured CMs were subjected to GD for 2 hours. As previously shown, GD induced a significant increase in the expression of LC3-II (Figure 1A and 1B). When CMs were transduced with Ad-GFP-LC3, GD significantly increased the number of CMs with numerous GFP-LC3 dots (Online Figure I, A). Expression of p62, a protein sequestered in autophagosomes for lysosomal degradation, was significantly reduced (Figure 1A and 1B). To separately evaluate the extent of autophagosome and autolysosome accumulation, we generated an adenovirus harboring tandem fluorescent mRFP-GFP-LC3 (Ad-tf-LC3).14,15 mRFP (monomeric red fluorescent protein) retains its fluorescence even in the acidic environment of lysosomes, where GFP loses its fluorescence. Thus, green LC3 puncta primarily indicate autophagosomes, whereas red LC3 puncta indicate both autophagosomes and autolysosomes. The red puncta that overlay with the green puncta indicate yellow in merged images are indicators of autophagosomes, whereas the free red puncta that do not overlay with the green puncta and appear red in merged images are indicative of autolysosomes.14,15 The numbers of green and red puncta were both significantly higher after GD (Figure 1C and 1D). Furthermore, yellow and red puncta were both significantly increased after GD in merged images, indicating increased autophagosomes and autolysosomes (Figure 1E). These results suggest that autophagic flux is enhanced in response to GD in CMs.

**FoxO1 Is Required for GD-Induced Autophagy**

FoxO1, a major isoform of the FoxO family in CMs, was significantly upregulated following GD (Figure 1A and 1B). Overexpression of FoxO1 by Ad-FoxO1-WT caused a significant increase in LC3-II and a decrease in p62, indicating increased autophagy (Figure 2A and 2B), whereas knockdown of FoxO1 by Ad-sh-FoxO1 caused significant accumu-
lation of both LC3-II and p62 indicating inhibition of autophagic flux in CMs (Figure 2A and 2B). To further evaluate the effect of FoxO1 on autophagic flux, CMs were transduced with Ad-tf-LC3. Expression of FoxO1 significantly increased both green and red puncta compared to that of LacZ (Figure 2C; Online Figure I, B). Merged images showed that the increase in red puncta was significantly greater than that in yellow puncta (Figure 2D). These results suggest that FoxO1 increases autolysosomes more than autophagosomes, and, thus, stimulates autophagic flux. In addition, there was a significant increase in the FoxO1-induced accumulation of LC3-II in the presence of lysosomal protease inhibitors, including phenyl methane sulfonyl fluoride and leupeptin, further supporting the idea that FoxO1 stimulates autophagic flux in CMs (Online Figure I, C and D). FoxO1 also increased the number of yellow puncta in merged images in the presence of bafilomycin A1, an inhibitor of autophagosome–lysosome fusion19 (Online Figure I, E through G), suggesting that FoxO1 increases autophagosome formation as well. In Ad-sh-FoxO1–transduced CMs, although the number of green and red puncta was robustly increased compared to Ad-FoxO1-WT (Online Figure I, B), most of the red puncta overlaid with the green ones (Figure 2C and 2D), suggesting that knockdown of FoxO1 inhibits autophagic flux. Similar to FoxO1, overexpression of FoxO3 also increased autophagic flux (Online Figure II).

To evaluate whether FoxO1 is required for GD-induced autophagy, CMs were transduced with Ad-sh-FoxO1 (Figure 2E). Knockdown of FoxO1 with Ad-sh-FoxO1 induced p62 accumulation in the presence and absence of GD, indicating that endogenous FoxO1 is required for GD-induced autophagy (Figure 2E and 2F). Analyses with Ad-tf-LC3 showed that on knockdown of FoxO1, even in the presence of GD, most of the red puncta overlaid with green puncta, indicating inhibition of autophagic flux (Figure 2G and 2H). These results suggest that FoxO1 is required for GD-induced autophagy.

Sirt1 Deacetylates FoxO1 During GD
Sirt1 is activated on starvation in cancer cell lines.20 We hypothesized that Sirt1 deacetylates FoxO1 during GD, thereby inducing autophagy in CMs. An acetylated form of FoxO1, evaluated with anti–acetylated FoxO1 antibody, was significantly decreased following GD (Figure 3A and 3B). Conversely, expression of Sirt1 was increased following GD (Figure 3A and 3B). Because the activity of Sirt1 is NAD⁺ dependent, we evaluated NAD⁺ content in starved CMs. The NAD⁺ content was significantly increased following GD (Figure 3C). GD-induced deacetylation of FoxO1 was abolished when Sirt1 was downregulated by Ad-sh-Sirt1 (Figure 3D and 3E). Taken together, these results suggest that GD upregulates and activates Sirt1, which in turn induces deacetylation of FoxO1.

Sirt1 Is Required for GD-Induced Autophagy
To evaluate the role of Sirt1 in mediating autophagy, CMs were transduced with Ad-Sirt1 and Ad-sh-Sirt1. Overexpre-
sion of Sirt1 increased LC3-II and decreased p62 accumulation, suggesting that Sirt1 stimulates autophagy (Figure 4A and 4B). Knockdown of Sirt1 inhibited autophagic flux, as evidenced by increased accumulation of both LC3-II and p62 at baseline, consistent with our previous results10 (Figure 4A and 4B). Analyses with tf-LC3 showed that the number of green and red puncta was higher in Ad-Sirt1–transduced CMs than in Ad-LacZ–transduced CMs (Figure 4C; Online Figure III). Furthermore, there were more red puncta than yellow puncta, indicating that Sirt1 increases autolysosome formation more strongly than autophagosome formation (Figure 4D). In Ad-Sirt1–transduced CMs, LC3-II accumulation was significantly enhanced in the presence of protease inhibitors, indicating that Sirt1 enhances autophagic flux in CMs (Online Figure III, A). Furthermore, there were more red puncta than yellow puncta, indicating that Sirt1 increases autolysosome formation more strongly than autophagosome formation (Figure 4D). In Ad-Sirt1–transduced CMs, LC3-II accumulation was significantly enhanced in the presence of protease inhibitors, indicating that Sirt1 enhances autophagic flux in CMs (Online Figure III, B and C). Ad-Sirt1 significantly enhanced accumulation of yellow puncta in the presence of Bafilomy-

Figure 2. FoxO1 is required for GD-induced autophagy in CMs. CMs were transduced with Ad-FoxO1-WT or Ad-LacZ, Ad-sh-FoxO1, or Ad-sh-Scr. A, Immunoblot analyses showing FoxO1, LC3, p62, and tubulin expressions. B, Densitometric analyses. C, Representative images of fluorescent LC3 puncta after Ad-tf-LC3 transduction. Insets in Ad-FoxO1-WT–transduced myocytes show higher magnification. D, Mean number of autophagosomes represented by yellow puncta in merged images and autolysosomes represented by red puncta in merged images per cell. E through H, CMs were transduced with Ad-sh-FoxO1 or Ad-sh-Scr and treated with glucose-free media. E, Immunoblots showing p62, FoxO1, and tubulin expressions. F, Densitometric analyses. G, Representative images of fluorescent LC3 puncta after Ad-tf-LC3 transduction. H, Mean number of autophagosomes represented by yellow puncta in merged images and autolysosomes represented by red puncta in merged images per cell. In D and H, graph with error bars is shown in Online Figure XI, B and C. Results represent means from at least 4 independent experiments. *P < 0.05, **P < 0.01. N.S. indicates not significant.
cin A1 (Online Figure I, E through G), suggesting that Sirt1 increases autophagosome formation as well. Although the number of green and red puncta were significantly greater in Ad-sh-Sirt1–transduced CMs than in Ad-LacZ or Ad-Sirt1–transduced CMs (Figure 4C; Online Figure III, A), most of the red puncta overlaid with green puncta in Ad-sh-Sirt1–transduced CMs (Figure 4D), suggesting that autophagic flux was inhibited in the absence of Sirt1.

To evaluate whether Sirt1 is required for GD-induced autophagy, Ad-sh-Sirt1–transduced myocytes were subjected to GD. Knockdown of Sirt1 inhibited GD-induced stimulation of autophagic flux, as evidenced by increased accumulation of p62 (Figure 4E and 4F). Furthermore, Ad-sh-Sirt1 inhibited GD-induced increases in autolysosome formation (Figure 4G and 4H), indicating that Sirt1 is required for GD induced increases in autophagic flux in CMs.

**Deacetylation of FoxO1 and the LXXLL Motif Are Required for GD-Induced Autophagy**

The LXXLL motif in FoxOs is important for interaction with Sirt1. Mutations in this motif are introduced in FoxO1(3SA), a mutant which cannot be phosphorylated by Akt and which is localized constitutively in the nucleus (FoxO1(3A/LXXAA)). Expression of FoxO1(3A/LXXAA) mutant abolishes interaction between Sirt1 and FoxO1 and Sirt1-induced stimulation of FoxO1-mediated transcription. FoxO1(3A/LXXAA) is a useful tool for investigating the role of Sirt1 in regulating the function of FoxO1. We confirmed that FoxO1(3A/LXXAA) is localized in the nucleus (Online Figure IV). We hypothesized that FoxO1-Sirt1 interaction is required for FoxO1 to induce autophagy in response to GD. We transduced CMs with Ad-3A/LXXAA or Ad-LacZ and subjected them to GD for 2 hours. In Ad-3A/LXXAA–transduced myocytes, acetylation of FoxO1 was increased at baseline and even after GD treatment (Figure 5A). In these myocytes, GD-induced stimulation of autophagy was inhibited, as indicated by reduced LC3-II and increased p62 accumulation (Figure 5A).

To show that deacetylation is required for autophagy, CMs were transduced with adenoviruses harboring inducible p300 (Ad-p300+Ad-tTA), a histone acetyl transferase. Overexpression of p300 significantly increased FoxO1 acetylation and inhibited autophagy, as indicated by decreased LC3-II and increased p62 accumulation, at baseline and on GD (Figure 5B and 5C). Increases in FoxO1 acetylation and suppression of autophagy were not observed when myocytes were transduced with Ad-p300 or Ad-tTA alone. Ad-3A/LXXAA and Ad-p300+Ad-tTA both increased accumulation of autophagosomes in Ad-tf-LC3–transduced myocytes, as indicated by increases in yellow puncta in merged images, but not autolysosomes, as indicated by significant decreases in red puncta (Figure 5D through 5F). These results suggest that deacetylation of FoxO1 by Sirt1, which is dependent on an intact LXXLL motif, is required for GD-induced autophagy.
FoxO1 and Sirt1 Act Synergistically to Induce Autophagy

Because deacetylation of FoxO1 mediated by Sirt1 is required for GD-induced autophagy, we hypothesized that FoxO1 is required for Sirt1-induced autophagy. To this end, the effect of FoxO1 knockdown on Sirt1-mediated autophagy was evaluated with tf-LC3 (Online Figure V, A through C). Although the number of autophagosomes was significantly greater, the number of autolysosomes was significantly smaller in CMs transduced with both Ad-Sirt1 and Ad-sh-FoxO1 than in those with Ad-Sirt1 alone (Online Figure V, C). These results suggest that FoxO1...
mediates Sirt1-induced increases in autophagic flux. Conversely, in CMs transduced with Ad-FoxO1 and Ad-sh-Sirt1, autophagosome formation was significantly increased, whereas autolysosome formation was robustly decreased compared to in those with Ad-FoxO1 alone (Online Figure V, D through F). These results suggest that Sirt1 also plays an important role in mediating FoxO1-induced increases in autophagic flux in CMs and that Sirt1 and FoxO1 act in concert to mediate autophagy in CMs (Online Figure VI).

**Figure 5. Interaction with Sirt1 and deacetylation of FoxO1 are required for autophagy in CMs.**

A, CMs were transduced with Ad-3A/LXXAA or Ad-LacZ and treated with glucose-free media. Immunoblots for acetylated FoxO1, total FoxO1, LC3, p62, and tubulin. B, CMs were transduced with Ad-p300 and Ad-tTA. Immunoblots for p300, acetylated FoxO1, total FoxO1, LC3, p62, and tubulin. C, Densitometric analyses. D, Representative images of fluorescent LC3 puncta after transduction with Ad-tf-LC3. E and F, Mean number of autophagosomes represented by yellow puncta in merged images and autolysosomes represented by red puncta in merged images per cell. Graph with error bars is shown in Online Figure XI, F G. Data represent means from at least 4 independent experiments. *P<0.05, **P<0.01. N.S. indicates not significant.

**FoxO1 Increases Expression of Rab7**

Because FoxO1 not only stimulates autophagosome formation but also prominently increases autolysosomes, we hypothesized that FoxO1 increases autophagic flux by increasing the expression of Rab7, a small GTPase protein which stimulates lysosomal biogenesis and maturation of autophagic vacuoles by promoting their fusion with endosomes and lysosomes. Both mRNA (Online Figure VII, A) and protein expression of Rab7 (Figure 6A) were significantly increased in Ad-FoxO1-WT–transduced myocytes, whereas they were
reduced significantly by knockdown of FoxO1 or transduction with Ad-3A/LXXAA (Figure 6A). Rab7 expression was also increased significantly on GD, but this increase was abolished when FoxO1 was knocked down (Figure 6B), indicating that the GD-induced increase in Rab7 is critically mediated through FoxO1. Rab7 is accumulated as puncta in the peri-nuclear region in response to GD (Online Figure VII, B). Rab7 costained predominantly with Cathepsin L, a lysosomal cysteine protease (Online Figure VII, B), but less so with GFP-LC3 (Online Figure VII, C). These results suggest that, in cultured CMs, Rab7 is localized to autolysosomes and late autophagic vacuoles, but not autophagosomes. Overexpression of Sirt1 upregulates Rab7, whereas knockdown of Sirt1 downregulates it (Online Figure VIII, D). Taken together, these results indicate that the Sirt1-FoxO1 pathway regulates expression of Rab7.

**FoxO1-Induced Autophagy Is Inhibited by Rab7 Knockdown**

To evaluate the role of Rab7 in mediating autophagic flux, we generated an adenovirus harboring HA-Rab7 (Ad-HA-Rab7). Overexpression of Rab7 in CMs increased LC3-II and decreased p62 (Figure 6C), indicating that Rab7 stimulates autophagy. To evaluate the extent of autophagosome and autolysosome formation, Rab7-overexpressing CMs were transduced with Ad-tf-LC3 (Online Figure VIII, A). Rab7 increased both yellow and red puncta, but it increased red puncta significantly more (Online Figure VIII, B and C), suggesting that Rab7 enhances autophagic flux in CMs.

To evaluate the role of endogenous Rab7 in mediating FoxO1-induced autophagy, we treated CMs with Lt-sh-Rab7. Knockdown of Rab7 was confirmed by immunoblots (Online Figure VIII, D). Expression of LC3-II was reduced and p62 was accumulated at baseline in Lt-sh-Rab7-transduced myocytes (Figure 6D). Furthermore, the FoxO1-induced increase in LC3-II expression and p62 degradation was significantly attenuated on knockdown of Rab7 (Figure 6D). These results suggest that stimulation of autophagic flux by FoxO1 is mediated through upregulation of Rab7.

**FoxO1 and Its Interaction With Sirt1 Are Required for Starvation-Induced Autophagy in Mouse Hearts**

We hypothesized that FoxO1 and its modulation by Sirt1 are required for starvation-induced autophagy in the heart in
vivo. We used transgenic mice with cardiac-specific overexpression of FoxO1(3A/LXXAA) (Tg-FoxO1m) and cardiac-specific FoxO1 homozygous knockout mice (c-FoxO1/H11002/H11002). These mice were subjected to starvation for 48 hours. LC3-II expression and p62 degradation were increased in control mice (nontransgenic mice and FoxO1flox/fox without Cre mice) after starvation, indicating increased autophagy (Figure 7). These changes were abolished in Tg-FoxO1m and c-FoxO1/H11002/H11002 mice, where LC3-II expression was reduced and p62 accumulation was increased at baseline and in response to starvation, indicating inhibition of autophagic flux (Figure 7). These results suggest that FoxO1 and its interaction with Sirt1 are required for starvation-induced autophagy in the heart in vivo.

To evaluate the role of FoxO1 in regulating cardiac function during starvation, echocardiographic analyses were conducted before and after starvation. In control mice, ejection fraction and fractional shortening were maintained after starvation (Figure 8A and 8B; Online Figure IX). However, in both Tg-FoxO1m and c-FoxO1/H11002/H11002 mice, cardiac function deteriorated significantly after starvation (Figure 8A and 8B; Online Table I). A decrease in cardiac function caused by starvation was also observed in beclin1/H11001/H11002 mice (Figure 8C and 8D), consistent with the notion that suppression of
autophagy may contribute to starvation-induced cardiac dysfunction. These results suggest that FoxO1 is required for maintaining cardiac function after starvation, possibly through stimulation of autophagy.

**Discussion**

We have demonstrated that (1) deacetylation of FoxO1 by Sirt1 is required for starvation induced autophagy in the heart; (2) Sirt1 and FoxO1 cooperatively mediate starvation-induced autophagy, (3) FoxO1 enhances autophagic flux by activating Rab7, and (4) FoxO1 is required to maintain cardiac function after starvation. Overall, deacetylation of FoxO1 and activation of autophagy form an essential adaptive mechanism for maintaining LV function under starvation conditions. Experiments with Ad-tf-LC3 allowed us to demonstrate that both GD and FoxO1 not only stimulate autophagosome formation but also enhance formation of autolysosomes, and, thus, they both stimulate autophagic flux. FoxO1 is upregulated by GD and upregulation of FoxO1 is necessary for stimulating starvation-induced autophagy. FoxO1 is a key molecule which is important for maintaining energy homeostasis and regulating metabolism in the liver, adipose tissue and skeletal muscle. During fasting and exercise, FoxO1 can activate gluconeogenic enzymes in the liver and turn on lipid metabolism to cope with conditions of nutrient and energy depletion. FoxO1-induced autophagy could be a part of such an adaptive mechanism, providing a source of energy by recycling amino and fatty acids and mediating cell survival during the energy crisis.

Multiple FoxO family transcription factors have many overlapping functions. Because FoxO3 also stimulates autophagy (Online Figure II), FoxO3 may be involved in GD-induced autophagy in CMs. However, because specific downregulation of FoxO1 potently suppresses GD-induced autophagy, FoxO1 may have nonoverlapping mechanisms to stimulate autophagy in CMs. Whether or not FoxO isoforms differentially contribute to starvation-induced autophagy remains to be clarified.

Different posttranslational modifications regulate the function of FoxO proteins. In the presence of insulin, FoxO is phosphorylated by Akt and translocates from the nucleus to the cytosol, and FoxO-mediated transcription is attenuated. In response to GD, FoxO proteins are dephosphorylated (Online Figure X) and localized in the nucleus (Online Figure IV), which in turn could stimulate transcription of autophagy genes. Besides phosphorylation, the acetylation status of FoxO proteins also affects their transcriptional activity. Here we show that FoxO1 is deacetylated on GD in a Sirt1-dependent manner. FoxO1(3A/LXXAA), which cannot interact with Sirt1, remains acetylated, indicating that interaction with Sirt1, remains acetylated, indicating that interaction with Sirt1 is required for FoxO1 to be deacetylated in the presence of GD. Importantly, FoxO1 cannot stimulate autophagy when it is acetylated in the presence of p300, a histone acetyltransferase known to acetylate Sirt1, and FoxO1(3A/LXXAA) inhibits GD-induced autophagy despite being localized in the nucleus, suggesting that interaction with Sirt1 and consequent deacetylation are required for FoxO1 to mediate GD-induced autophagy.
clear translocation of FoxO1 caused by dephosphorylation alone may not be sufficient for inducing autophagy in response to GD. Interestingly, nuclear localization of FoxO1 is enhanced in the presence of Sirt1 overexpression (Online Figure IV), raising the possibility that Sirt1-mediated deacetylation may also contribute to the nuclear localization of FoxO1 during GD. Taken together, our results suggest that Sirt1-mediated deacetylation of FoxO1 plays an important role in mediating GD-induced stimulation of autophagy in CMs. Our results do not exclude the involvement of phosphorylation-dependent regulation of FoxO1 in modulation of autophagy.

Although both sh-FoxO1 and FoxO1 (3A/LXXAA) inhibit autophagic flux, as evidenced by accumulation of p62 and decreases in autolysosomes, Ad-sh-FoxO1 increased LC3-II, whereas FoxO1 (3A/LXXAA) decreased LC3-I and LC3-II, suggesting that shRNA for FoxO1 inhibits autophagic flux primarily at autophagosome–lysosome fusion, whereas FoxO1 (3A/LXXAA) either inhibits expression of LC3 or interferes with autophagosome formation as well as autophagosome–lysosome fusion. It is possible that shRNA for FoxO1 induced incomplete knockdown of FoxO1 or that FoxO3 may compensate for downregulation of FoxO1. On the other hand, FoxO1 (3A/LXXAA), as a dominant negative (against FoxO1 and/or FoxO3), may be a stronger inhibitor of FoxO, so that not only genes involved in autophagosome–lysosome fusion but also those involved in autophagosome formation are affected.

Both expression of Sirt1 and the cellular amount of NAD⁺ are significantly increased during GD in CMs, which would
enhance the total activity of Sirt1, favoring deacetylation of FoxO1. The facts that upregulation of Sirt1 is sufficient to increase both autophagosomes and autolysosomes and stimulate autophagy, an effect very similar to that of FoxO1, and that stimulation of autophagy by Sirt1 is abolished in the absence of FoxO1 are consistent with the notion that Sirt1 is an upstream regulator of FoxO1 for induction of autophagy (Online Figure VI). Interestingly, however, FoxO1-induced autophagy also requires Sirt1. These results suggest that FoxO1 and Sirt1 in concert mediate autophagy under GD by making a functional complex in CMs.

Previous studies have shown that FoxO3 regulates autophagy in skeletal muscle cells by transcriptional activation of genes that are involved in autophagosome formation, including LC3, Gabarap1L, Atg12L, Atg4, Beclin1, Ulk2, Vps34, and Bnip3. It should be noted that the causative role of autophagy related molecules in mediating FoxO-mediated autophagy remains to be elucidated. Our results suggest that Rab7 plays an important role in mediating FoxO1-induced stimulation of autophagy. Because Rab7 plays an important role in the fusion of the matured autophagic vacuole with the lysosome, we speculate that Rab7 may primarily contribute to the late stages of autophagy and the overall increase in autophagic flux. Because FoxO1 upregulates other autophagy-related genes specifically involved in different steps of autophagy, and because autophagy is executed through multiple steps, including nucleation, autophagosome formation, autophagosome–lysosome fusion, and lysosomal degradation, other FoxO1 targets may also contribute to FoxO1-induced autophagy by modulating other processes of autophagy.

Starvation of newborn mice lacking atg5 increases perinatal death resulting from heart failure, suggesting that the heart function critically relies on autophagy during nutrient starvation. Our results show that downregulation of FoxO1 or expression of a FoxO1 mutant that cannot interact with Sirt1 not only inhibits autophagy but also results in LV dysfunction under starvation conditions. These results are consistent with the notion that the Sirt1-FoxO1 pathway is a compensatory signaling mechanism essential for maintaining LV function under starvation conditions. Because the suppression of autophagy in beclin1−/− mice also leads to LV dysfunction during food starvation, suppression of autophagy may in part mediate LV dysfunction in Tg-FoxO1m and c-FoxO1−/− mice under starvation conditions. When Sirt1 and FoxO proteins interact, transcription of genes mediating stress resistance, cell survival, and longevity is turned on, whereas transcription of those mediating apoptosis and cell death is turned off. We therefore speculate that FoxO1-induced transcription of autophagy genes is an important adaptive mechanism during starvation in the heart.

Both FoxOs and Sirt1 affect glucose metabolism, as well as insulin signaling. Thus, we should point out the possibility that manipulation of FoxOs and Sirt1 secondarily affects the extent of autophagy through changes in metabolism and insulin signaling in the heart and the myocytes therein.

In summary, Sirt1-mediated deacetylation of FoxO1 plays an essential role in mediating starvation-induced autophagy. Deacetylation of FoxO1 induces expression of genes involved in autophagy, including Rab7, which in turn stimulate autophagy. We propose that autophagy activated by the Sirt1-FoxO1 pathway is beneficial for the heart during nutrient and energy deficiency.

Acknowledgments

We thank Drs Tamotsu Yoshimori and Beth Levine for tf-LC3 and beclin1−/− mice, respectively. We thank Daniela Zablocki for critical reading of the manuscript.
Sources of Funding
This work was supported in part by US Public Health Service grants HL59139, HL67724, HL69020, HL91469, HL102738, and AG27211 and the Foundation of Leducq Transatlantic Network of Excellence.

Disclosures
None.

References

Novelty and Significance

What Is Known?

- The FoxO family transcription factors stimulate autophagy in cardiac myocytes.
- Autophagy is activated by nutrient deprivation.

What New Information Does This Article Contribute?

- In cardiac myocytes, deacetylation of FoxO1 by Sirt1, a histone deacetylase, is required for mediating starvation-induced autophagy.
- FoxO1 upregulates Rab7, which in turn mediates autophagosome–lysosome fusion, thereby enhancing autophagic flux.
- FoxO1-induced autophagy maintains cardiac function during starvation.

Activation of autophagy preserves ATP and increases survival during nutrient deprivation. The signaling mechanism by which nutrient deprivation activates autophagy in the heart is not well understood. Here, we demonstrate that in cardiac myocytes FoxO1 and its deacetylation by Sirt1 mediates autophagy in response to glucose deprivation. FoxO1 upregulates Rab7, a small GTP-binding protein, which in turn enhances fusion of autophagosomes and lysosomes and stimulates autophagic flux.

Deacetylation of FoxO1 and subsequent activation of autophagy are essential for the heart to maintain left ventricular contractility during nutrient starvation in adult mice. Our findings suggest a critical role of Sirt1-mediated deacetylation of FoxO1 in mediating starvation-induced autophagy and indicate that activation of autophagy through the Sirt1–FoxO1 pathway protects the heart during nutrient/energy starvation.
Deacetylation of FoxO by Sirt1 Plays an Essential Role in Mediating Starvation-Induced Autophagy in Cardiac Myocytes
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Circ Res. 2010;107:1470-1482; originally published online October 14, 2010;
doi: 10.1161/CIRCRESAHA.110.227371

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplement Material

Animal protocols
All animal protocols were approved by the review board of the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Antibodies
Antibodies used in the study include those against LC3 (MBL Intl. Corp., #PD014, 1:1000 dilution), p62 (ARP Inc., #03-GP62-C, 1:3000 dilution), FoxO1 (Cell Signaling Technology, #2880, #9454, Epitomics, #1874-1, 1:2000 dilution), Acetylated FoxO1 (Santacruz Biotechnology Inc., sc-49437, 1:500 dilution), Sirt1 (Upstate Biotech, #07-131, 1:2000 dilution), p300 (Millipore, 05-257, 1:1000 dilution), Rab7 (Sigma-Aldrich, #R4779, 1:2000 dilution), Cathepsin-L (Sigma-Aldrich, #C2970, 1:500 dilution), FoxO3 (Imgenex, #IMG-90489-2, 1:1000 dilution), phosphorylated FoxO1 (Cell Signaling Technology, #9461, 1:1000 dilution), α-sarcomeric actinin (Sigma-Aldrich, #A7811, 1:800 dilution) and α-Tubulin (Sigma-Aldrich, #T6199, 1:4000 dilution).

Primary Culture of Cardiac Myocytes
The method to isolate cardiac myocytes (CMs) from neonatal rats has been described previously. Following 24 hours of culture in gelatin coated culture dishes, the media was changed to serum-free Dulbecco's modified Eagle's medium (DMEM)/F-12 (Mediatech Inc.). For GD, myocytes were washed three times with Phosphate Buffered Saline (PBS) and incubated with glucose free medium containing glucose free-serum free DMEM (Gibco Inc. #11966) for 2 hours as described previously.1

Adenoviral and Lentiviral Transduction
Transductions with Ad-LacZ, Ad-FoxO1-WT, Ad-3A/LXXAA, Ad-Sirt1, Ad-p300, Ad-tTA, Ad-tf-LC3, Ad-GFP-LC3, Ad-HA-Rab7, and Ad-FoxO3-WT were carried out for 24 hours. Knockdown adenoviruses like Ad-sh-Scr, Ad-sh-FoxO1, Ad-sh-Sirt1, Lt-sh-Rab7 were transduced for 96 hours. Adenoviruses were transduced at 15 MOI.

Fluorescence Microscopy
The method to evaluate tandem fluorescent LC3 puncta using Ad-tf-LC3 has been described previously. Cardiac myocytes cultured on cover slips were transduced with Ad-tf-LC3 at 15 MOI. Twenty-four hours after adenovirus transduction, the cells were washed with PBS, fixed with 4% paraformaldehyde (PFA), mounted with a reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories Inc.), and viewed under a fluorescence microscope (Nikon Eclipse E800). The number of GFP and mRFP dots was determined by manual counting of fluorescent puncta from at least 4 different myocyte preparations with a 60X objective. At least 50 cells were scored in each experiment. The nuclear number was evaluated by counting the number of DAPI stained nuclei in the same field. The number of dots/cell was obtained by dividing the total number of dots by the number of nuclei in each microscopic field. For immunostaining, myocytes cultured on coverslips were fixed with 4% PFA, permeabilized in PBS containing 0.2% Triton-X, blocked with 3% BSA for 30 minutes followed by incubation with primary antibody (1:500 dilution). Alexa Fluor 488 Dye or Alexa Fluor 594 Dye conjugated secondary antibody (Invitrogen) was used for detecting indirect fluorescence.

NAD+ Content Measurement
NAD+ content as has been described previously, was measured using the Enzychrom NAD+/NADH Assay kit (ECND-100, BioAssay Systems) according to the manufacturer’s protocol. ³

Preparation of Cell Lysate and Immunoblot Analysis
The methods of preparation of cell lysates from in vitro and in vivo samples and immunoblot analyses have been described previously. ² For in vitro samples, protein lysates were prepared from myocytes cultured in 6 cm culture dishes using boiled (95°C for 2 minutes) 2X SDS sample buffer containing 4% SDS, 20% glycerol, 120mmol/L Tris-HCl (pH 6.8), 0.01% bromophenol blue, and 5% beta-mercaptoethanol. The protein samples were immediately boiled again at 95°C for 3 minutes. For detection of some proteins by immunoblot (Ac-FoxO1, Sirt1, p300, P-FoxO1), cultured CM samples were extracted on dry ice and ethanol instead of ice using 2X SDS sample buffer at room temperature. Heart tissue homogenates were prepared using RIPA buffer containing 50mmol/L Tris-HCl (pH 8.0), 150mmol/L NaCl, 0.1% SDS, 1% Igepal CA-630, and 0.5% Sodium deoxycholate with protease inhibitors (Sigma, P8340) at a 1:400 dilution. After determining the protein concentration by BCA Assay (Thermo Scientific), equal amounts of proteins were loaded on SDS-PAGE gels with 6X sample buffer containing 0.35mol/L Tris-HCl (pH 6.8), 10.28% SDS, 36% glycerol, 0.01% bromophenol blue, and 5% beta-mercaptoethanol.

Quantitative Reverse Transcription-PCR (qRT-PCR)
Total RNA was extracted from cultured CMs with TRIzol (Invitrogen). cDNA was synthesized with RETROscript kit (Ambion) following the manufacturer’s instructions. Real time-PCR was carried out as described previously.³ The following primer pairs were used –
Rab7:  Sense – TTACTTCGAGACCAGTGCCAAGGA
Antisense – TGTCCAGTTTGATGGGTTCAGGGA
GAPDH: Sense – GAGCTGAACGGGAAGCTCACT
Antisense – TTGTCATACCAGGAATGAGC

Chemical Inhibitors of Autophagy
To inhibit autophagic flux at the degradation step, cultured CMs were treated with lysosomal protease inhibitors (PI) - 500 μM Phenyl Methane Sulfonyl Fluoride (PMSF) and 50 μM leupeptin, and transduced with adenovirus for 24 hours. To inhibit autophagosome-lysosome fusion, CMs were treated with 50nmol/L Bafilomycin-A1 for 2 hours.

Echocardiography
Mice were anesthetized using 12 μL/g BW of 2.5% avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions) as previously described.³

Statistics
Data are expressed as mean ± SEM. Statistical analyses between groups of 2 were conducted with unpaired student’s t-test. Groups of 3 or more were analyzed with one-way ANOVA, following Newman-Keuls multiple comparison test. A value of p<0.05 was considered statistically significant.
References


Online Figure I-I:
A) CMs were transduced with Ad-LacZ and Ad-GFP-LC3 and treated with glucose free media. Number of cells expressing >5 GFP-LC3 vacuoles/cell were counted and are expressed as a percentage. B) CMs were transduced with Ad-tf-LC3, Ad-FoxO1-WT, Ad-LacZ and Ad-sh-FoxO1 or Ad-sh-Scr. Mean number of GFP and mRFP dots/cell is shown. C-D) To evaluate LC3 turnover, CMs were treated with lysosomal protease inhibitors (PI) - 500 μM Phenyl Methane Sulfonfonyl Fluoride and 50 μM leupeptin and transduced with adenovirus. C) Immunoblots showing LC3 and Tubulin expressions. D) Densitometric analyses. *p<0.05, **p<0.01.
Online Figure I-II:
E-G) CMs were transduced with Ad-tf-LC3, Ad-FoxO1-WT, Ad-LacZ and Ad-Sirt1 and treated with 50nM Bafilomycin A1 for 2 hours. E) Representative images of fluorescent LC3 puncta. F) Mean number of GFP and mRFP dots/cell. G) Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell.
Online Figure II:
CMs were transduced with Ad-LacZ or Ad-FoxO3-WT. A) Immunoblots showing expression levels of FoxO3, LC3 and Tubulin. B-D) CMs were transduced with Ad-tf-LC3 and Ad-FoxO3-WT or Ad-LacZ. B) Representative images of fluorescent puncta. C) Mean number of GFP and mRFP dots per cell. D) Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. Graph with error bars is shown in Online Figure XI-H. Results represent means from at least 4 independent experiments. * indicates p<0.05, ** indicates p<0.01.
**Online Figure III:**

A) CMs were transduced with Ad-tf-LC3, Ad-Sirt1 or Ad-LacZ, Ad-sh-Sirt1 or Ad-sh-Scr. Mean number of GFP and mRFP dots/cell is shown. B-C) CMs were treated with lysosomal protease inhibitors (PI) and transduced with Ad-LacZ and Ad-Sirt1. B) Immunoblot with LC3 (at two different exposures) and Tubulin expressions are shown. C) Densitometric analysis. Results represent means from at least 4 independent experiments. * indicates p<0.05, ** indicates p<0.01, N.S Not Significant.
Online Figure IV:
Representative images of FoxO1 sub-cellular localization. CMs were transduced with the indicated adenoviruses. For GD, Ad-LacZ transduced CMs were treated with glucose free-serum free media. The cells were immunostained with α-sarcomeric actinin (red) and FoxO1 (green) antibodies.
Online Figure V-I:
A-C) Cultured CMs were transduced with Ad-tf-LC3, Ad-Sirt1 or Ad-LacZ, Ad-sh-FoxO1 or Ad-sh-Scr. A) Representative images of fluorescent LC3 puncta. B) Mean number of GFP and mRFP dots per cell. C) Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. Graph with error bars is shown in Online Figure XI-I.
Online Figure V-II:
D-F) CMs were transduced with Ad-tf-LC3, Ad-FoxO1-WT or Ad-LacZ, Ad-sh-Sirt1 or Ad-sh-Scr. D) Representative images of fluorescent LC3 puncta are shown. E) Mean number of GFP and mRFP dots per cell. F) Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. Graph with error bars is shown in Online Figure XI-J. Data represent means from at least 4 independent experiments. * p<0.05, **p<0.01, N.S Not Significant.
Online Figure VI:
Schematic representation of the roles of FoxO1 and Sirt1 in regulating autophagy in CMs. Glucose deprivation (GD) activates Sirt1 which deacetylates and activates FoxO1. FoxO1 upregulates Rab7, a small GTP binding protein that mediates autophagosome-lysosome fusion and thereby enhances autophagic flux. Knockdown of Sirt1 (sh-Sirt1), FoxO1 (sh-FoxO1), Rab7 (sh-Rab7) or use of mutant FoxO1 with mutation in the Sirt1 interacting LXXLL motif and Akt phosphorylation sites (3A/LXXAA) inhibit autophagic flux.
Online Figure VII-I:

A) CMs were transduced with Ad-LacZ, Ad-FoxO1-WT, Ad-3A/LXXAA for 12 hours and Ad-sh-FoxO1 or Ad-sh-Scr for 96 hours. RNA was extracted and Rab7 expression was evaluated by quantitative real time polymerase chain reaction (qRT-PCR). Relative mRNA expression level is shown. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. B) CMs were treated with glucose free media for 2 hours and immunostained with Rab7 (red) and Cathepsin L (green) antibodies. DAPI (4',6-diamidino-2-phenylindole) stains the nuclei. Representative images of Rab7 and Cathepsin L localizations are shown. C) CMs were transduced with Ad-GFP-LC3, treated with serum-free glucose free media and immunostained for Rab7 antibody (red). Representative images of localization of Rab7 and GFP-LC3 are shown.
Online Figure VII-II:
D) CMs were transduced with Ad-LacZ, Ad-Sirt1, Ad-sh-Scr or Ad-sh-Sirt1. Immunoblots and densitometric analyses are shown. Results represent means from at least 4 independent experiments. * indicates p<0.05, ** indicates p<0.01.
Online Figure VIII:
A-C) CMs were transduced with Ad-tf-LC3, Ad-HA-Rab7 or Ad-LacZ for 24 hours. A) Representative images of fluorescent LC3 puncta. B) Mean number of GFP and mRFP dots per cell. C) Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. Graph with error bars is shown in Online Figure XI-K. D) CMs were transduced with Ad-sh-Scr or Lt-sh-Rab7. Immunoblot confirming knockdown of Rab7 is shown. Tubulin was used as the internal control. Data represent means from at least 4 independent experiments. * indicates p<0.05, ** indicates p<0.01.
Online Figure IX:
Representative images of echocardiographs of control, Tg-FoxO1m and c-FoxO1-/- mice measured at baseline and after 48 hours starvation.
Online Figure X:
CMs were treated with serum free-glucose free media for 2 hours. Immunoblots and densitometric analyses for phosphorylated FoxO1 (P-FoxO1) and FoxO1. Tubulin was used as the internal control.
Online Figure XI-I:
Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. A) corresponds to Fig.1E, B-C) correspond to Fig.2DH, D-E) correspond to Fig.4DH, F-G) correspond to Fig. 5EF. * indicates p<0.05, ** indicates p<0.01.
Online Figure XI-II
Mean number of autophagosomes represented by yellow dots in merged images and autolysosomes represented by red dots in merged images per cell. H) corresponds to Online Figure II-D, I-J) correspond to Online Figure V-EF, K) corresponds to Online Figure VIII-C. * indicates p<0.05, ** indicates p<0.01.
Online Table I:

Echocardiographic measurements of 5-6 month old NTg, Tg-FoxO1m and c-FoxO1-/- mice at baseline and after 48 hours of starvation.

<table>
<thead>
<tr>
<th>Abbreviations:</th>
<th>DSEP WT (mm)</th>
<th>LVEDD (mm)</th>
<th>DPW WT (mm)</th>
<th>SSEP WT (mm)</th>
<th>LVESD (mm)</th>
<th>SPW WT (mm)</th>
<th>EF (%)</th>
<th>%FS</th>
<th>HR (bpm)</th>
<th>BW (g)</th>
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<tr>
<td>NTg</td>
<td>1.11 ± 0.03</td>
<td>3.21 ± 0.10</td>
<td>0.84 ± 0.01</td>
<td>1.47 ± 0.09</td>
<td>2.18 ± 0.14</td>
<td>1.17 ± 0.09</td>
<td>74.17 ± 0.8</td>
<td>37.17 ± 1.80</td>
<td>440.22 ± 27.6</td>
<td>21.90 ± 0.74</td>
</tr>
<tr>
<td>Tg-FoxO1m</td>
<td>0.89 ± 0.03</td>
<td>3.01 ± 0.11</td>
<td>0.94 ± 0.02</td>
<td>1.26 ± 0.01</td>
<td>1.9 ± 0.11</td>
<td>1.10 ± 0.09</td>
<td>74.01 ± 0.01</td>
<td>36.9 ± 0.91</td>
<td>410.14 ± 37.28</td>
<td>19.60 ± 1.40</td>
</tr>
<tr>
<td>c-FoxO1-/-</td>
<td>0.96 ± 0.03</td>
<td>3.59 ± 0.16</td>
<td>0.98 ± 0.05</td>
<td>1.39 ± 0.07</td>
<td>2.30 ± 0.05</td>
<td>1.28 ± 0.08</td>
<td>72.31 ± 0.03</td>
<td>35.27 ± 2.37</td>
<td>455.01 ± 23.6</td>
<td>30.02 ± 1.63</td>
</tr>
<tr>
<td>beclin1 +/-</td>
<td>1.03 ± 0.04</td>
<td>3.12 ± 0.14</td>
<td>1.05 ± 0.07</td>
<td>1.36 ± 0.05</td>
<td>2.27 ± 0.11</td>
<td>1.15 ± 0.08</td>
<td>61.62 ± 0.02</td>
<td>27.41 ± 1.09</td>
<td>455.23 ± 12.27</td>
<td>23.50 ± 1.59</td>
</tr>
</tbody>
</table>

Baseline Starvation Baseline Starvation Baseline Starvation Baseline Starvation

| EF | 0.86 ± 0.02 | 1.01 ± 0.06 | 1.17 ± 0.03 | 0.74 ± 0.02 |
| HR | 0.94 ± 0.05 | 2.54 ± 0.16 | 0.94 ± 0.06 | 0.97 ± 0.0 |
| BW | 1.03 ± 0.04 | 0.83 ± 0.03 | 1.20 ± 0.03 | 1.20 ± 0.0 |
| FS | 1.0 ± 0.06 | 2.54 ± 0.16 | 0.94 ± 0.06 | 0.97 ± 0.0 |
| HR | 1.22 ± 0.06 | 0.94 ± 0.06 | 1.20 ± 0.03 | 1.20 ± 0.0 |
| BW | 3.12 ± 0.14 | 2.54 ± 0.16 | 0.94 ± 0.06 | 0.97 ± 0.0 |
| FS | 1.00 ± 0.05 | 2.54 ± 0.16 | 0.94 ± 0.06 | 0.97 ± 0.0 |
| HR | 1.00 ± 0.05 | 2.54 ± 0.16 | 0.94 ± 0.06 | 0.97 ± 0.0 |
| BW | 51.15 ± 0.02 | 51.15 ± 0.02 | 51.15 ± 0.02 | 51.15 ± 0.02 |
| FS | 21.38 ± 1.32 | 21.38 ± 1.32 | 21.38 ± 1.32 | 21.38 ± 1.32 |
| HR | 346.06 ± 40.32 | 346.06 ± 40.32 | 346.06 ± 40.32 | 346.06 ± 40.32 |
| BW | 20.86 ± 1.00 | 20.86 ± 1.00 | 20.86 ± 1.00 | 20.86 ± 1.00 |

| n  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  |

Online Table I: