FoxO Transcription Factors Are Critical Regulators of Diabetes-Related Muscle Atrophy

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Abstract

Insulin deficiency and uncontrolled diabetes lead to a catabolic state with decreased muscle strength, contributing to disease-related morbidity. FoxO transcription factors are suppressed by insulin and thus are key mediators of insulin action. To study their role in diabetic muscle wasting, we created mice with muscle-specific triple knockout of FoxO1/3/4 and induced diabetes in these M-FoxO-TKO mice with streptozotocin (STZ). Muscle mass and myofiber area were decreased 20-30% in STZ-Diabetes mice due to increased ubiquitin-proteasome degradation and autophagy alterations, characterized by increased LC3 containing vesicles, and elevated levels of p-ULK1 and LC3-II. Both the muscle loss and markers of increased degradation/autophagy were completely prevented in STZ-FoxO-TKO mice. Transcriptomic analyses revealed FoxO-dependent increases in ubiquitin-mediated proteolysis pathways in STZ-Diabetes, including regulation of Fbxo32 (Atrogin1), Trim63 (MuRF1), Bnip3L, and Gabarapl. These same genes were increased 1.4- to 3.3-fold in muscle from type 1 diabetics after short-term insulin deprivation. Thus, FoxO-regulated genes play a rate-limiting role in increased protein degradation and muscle atrophy in insulin-deficient diabetes.
**Introduction**

Uncontrolled diabetes causes decreased muscle strength and aerobic capacity, both of which contribute to disability and mortality in patients with the disease. Likewise older individuals with type 2 diabetes lose muscle strength faster than those without diabetes, and this loss of muscle strength is highly correlated with death rates in these populations (1, 2). Patients with newly diagnosed or poorly-controlled type 1 diabetes also exhibit loss of lean mass (3, 4). Additionally, physical fitness in patients with type 1 diabetes is directly correlated with hemoglobin A1C levels, indicating that poor control of glycemia is associated with poor fitness in type 1 diabetes (5). Uncontrolled diabetes is accompanied by numerous metabolic abnormalities, including insufficient insulin levels, hyperglycemia, lipid abnormalities, hyperglucagonemia and glucocorticoid elevations, each of which may contribute to abnormalities in muscle function. However, the exact molecular mechanisms that account for muscle loss with loss of insulin action in diabetes are still not understood.

Insulin action induces profound effects on fuel metabolism and protein turnover. Insulin and amino acids coordinate protein synthesis and degradation in muscle (6). In patients with type 1 diabetes, insulin deprivation leads to a state of high protein turnover with a net loss of muscle mass. This is due to increased rates of protein degradation, which exceed the increased synthesis rate at the organismal level (7, 8). Conversely, insulin treatment suppresses protein degradation in muscle. Insulin also stimulates muscle protein synthesis, but only when co-infused with amino acids (9), mimicking the conditions of the post-prandial state. Thus, insulin’s role on protein degradation is more important than that on synthesis in muscle. The effect of insulin to suppress protein degradation is demonstrated *in vitro* and in rodent models (10, 11). Defining the rate-limiting steps in the pathways by which insulin regulates protein degradation
could provide an important target in treatment of diabetes that would help limit disability related to muscle atrophy.

Insulin acts via its tyrosine kinase receptor to mediate metabolic changes and cellular growth. Insulin receptor (IR) and the closely related IGF-1 receptor (IGF1R) have overlapping roles in mediating muscle growth and glucose homeostasis (12). IR and IGF1R act via the PI3-Kinase/Akt and the MAPK/ERK pathways to influence a broad range of cellular functions, including glucose uptake, growth, proliferation and protein turnover. Part of insulin’s action in muscle is to modulate transcription (13, 14). Activation of Akt in response to insulin or IGF-1 induces phosphorylation of FoxO transcription factors, which suppresses their transcriptional activity. Forkhead box-O (FoxO) proteins are metabolic and stress-responsive transcription factors that are ubiquitously expressed and conserved in the animal kingdom (15). Insulin’s ability to suppress FoxO1 mediated transcription is central to insulin action in the liver, and deletion of FoxO1 in liver can rescue many of the gene expression changes observed in mice with a liver-specific IR knockout (16, 17).

FoxOs control a broad range of atrophy-related genes in muscle, including Fbxo32 (Atrogin1), Trim63 (MuRF1), and autophagy genes (18-21). Indeed, deletion of FoxOs in muscle prevents muscle atrophy in response to starvation and denervation (22). Likewise, we have found that deletion of the three FoxO isoforms in muscle (FoxO1, FoxO3, and FoxO4) is able to rescue the profound muscle atrophy from muscle-specific deletion of IR and IGF1R, demonstrating the critical role of FoxOs in muscle protein degradation and proteostasis (23), but the degree to which FoxOs control muscle proteostasis in response to insulin-deficient diabetes, where numerous metabolic abnormalities are present (including hyperglycemia, lipid abnormalities, glucocorticoid elevations, and ketosis) has not been determined.
In the current study, we have explored the role of FoxO transcription factors in the muscle atrophy of insulin-deficient diabetes in both mice and humans. We show that muscle atrophy in STZ-Diabetes mice is prevented in STZ-FoxO-TKO. This occurs without any effect on hyperglycemia, hypoinsulinemia, or suppression of muscle protein synthesis by STZ treatment. Muscle mass is maintained in STZ-FoxO-TKO mice due to suppression of autophagy-lysosome and ubiquitin-proteasome degradation, without changes in myocyte number or oxidative/glycolytic distribution. Transcriptomic analysis reveals that induction of transcripts involved in protein degradation pathways in STZ-Diabetes mice is prevented in muscle from STZ-FoxO-TKO mice. Finally, we show that transcripts of the ubiquitin-proteasome and autophagy-lysosome systems are increased in muscle biopsies from people with type 1 diabetes that were deprived of insulin for as little as 8 hours. Thus, we demonstrate that muscle atrophy in response to insulin-deficient diabetes is mediated by FoxO-driven protein degradation and blocking this pathway can provide protection from this complication of diabetes.
Materials and Methods

Animal Care and Use. Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at both the Joslin Diabetes Center and at the University of Iowa. Male mice were used for studies unless indicated. M-FoxO-TKO mice were generated using ACTA1-Cre (Jackson Laboratory, stock number 006149) and FoxO1/3/4 triple floxed mice, provided by Dr. Domenico Accili, as previously described (23). Littermate controls were used for all experiments as the mice are on a mixed background containing C57Blk6, C57Blk6J, and 129 strains. Body composition was performed for total lean and fat mass by Dual Energy X-ray Absorptiometry (DEXA) in the Joslin DRC.

Diets and treatments. Animals were maintained on a standard chow diet (Lab Diet 9F, 5020). Fed mice were allowed ad libitum access to food and sacrificed at 9:00 am. For streptozotocin (STZ) treatments, mice were fasted overnight, then injected intraperitoneally with a single high-dose of STZ (150 mg/kg, Sigma S0130) dissolved in 100 mM citrate buffer (pH 4.5) or injected with Citrate buffer alone as a control. Female mice were given a 150 mg/kg dose followed by a 75 mg/kg dose of STZ on day 3, since female mice are less responsive to STZ (24). Mice were monitored for hyperglycemia on days 3, 7, 12, and 15, and only mice in which random blood glucose remained above 300 mg/dl for the following 9-12 days were used for experiments. For colchicine treatments, mice were injected intraperitoneally with colchicine (0.4 mg/kg/day, Sigma C9754) or saline daily for 2 days as previously described (23, 25). All mice were sacrificed between 12 and 15 days after STZ injection.

Proteolysis assay. Proteolysis was measured as previously described (11, 23), with the following modifications. Soleus and EDL muscles were isolated from 8 week-old mice and pre-incubated
in 1 ml of KRB buffer (in mM: 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 24.6
NaHCO₃, and 5 glucose) for 30 minutes with constant bubbling of 95% O₂/5% CO₂ prior to
transferring to fresh 1 ml of KRB containing 0.5 mM cycloheximide to inhibit protein synthesis
for 2 hours. Incubation buffer was collected and tyrosine concentration was determined in 0.5 ml
of incubation buffer as previously described (26), and was normalized to muscle weight.

**Protein Synthesis Assay.** Muscle protein synthesis was measured using the SUnSET method to
determine puromycin incorporation into newly synthesized proteins in Quadriceps tissue as
previously described (27). Briefly, mice were injected intraperitoneally with puromycin at a
dose of 0.04 µmol /g body weight, anesthetized 20 minutes later and sacrificed 30 minutes after
puromycin injection. Western blot analysis of puromycin-incorporated proteins was performed
using antibodies in Supplemental Table S3. Fed and 24-hour fasted mice were injected with or
without puromycin to validate the method.

**Proteasome Activity Assays.** Proteasome activity was determined from muscle homogenates
using substrates for trypsin-like activity of the 26S proteasome as previously described (23, 28).
Briefly, frozen powdered muscle was homogenized in 50 mM Tris-HCl, 5 mM MgCl₂, 250 mM
sucrose, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA, pH 7.5, then centrifuged (600 x g for 20 min)
at 4°C to pellet contractile proteins and membranes. The supernatant was removed and
centrifuged (16,000 x g for 10 min at 4°C) to yield the final supernatant for proteasome activity.
Twenty micrograms of protein from the proteasome supernatant was mixed with Boc-Leu-Ser-
Thr-Arg-7-amido-4-methylcoumarin (LSTR) (Sigma B4636, 300 µM final concentration) in
assay buffer (50 mM Tris-HCl pH7.5, 5 mM MgCl₂, 40 mM KCl, 2 mM ATP, 1 mM DTT, 0.5
mg/ml BSA) in a 96-well black plate and fluorescence was monitored (360 nm excitation, 460
nm emission) every 3 minutes for 1.5-hours at 37°C. Enzyme activity (Vmax) was determined
as the change in fluorescence during the linear phase of the reaction without MG132 minus the activity with 200 μM MG132, each converted to nmol/min/mg using a standard curve of 7-amido-4-methylcoumarin (Sigma A9891).

**Physiologic Measurements.** Whole blood glucose levels were measured using a glucose meter. Serum insulin levels were measured using an insulin ELISA according to the manufacturer’s protocol (Crystal Chem). Muscle tissue fluid amino acid levels were measured in mixed quadriceps and gastrocnemius muscle at the Mayo Clinic Metabolomics Resource Core using time-of-flight mass spectrometry as previously described (12). Please note that muscle tissue fluid amino acid levels from the Control group and M-FoxO-TKO group were incorporated in the previous publication (23), and are also presented as important control groups in the current study. Muscle grip strength was measured as previously described (23).

**Histology.** Frozen cross sections of tibialis anterior (TA) muscle were stained for succinate dehydrogenase (SDH) by immersing slides in staining buffer containing PBS with 0.5 M disodium succinate, 20 mM MgCl₂, and 0.5 mg/ml of nitro blue tetrazolium for 15 minutes at 37°C and quantified as previously described (12). For cross-sectional area, TA sections were immunofluorescently stained for laminin. For LC3 vesicle density, TA sections were co-stained with LC3A and Myosin IIa and quantified as previously described (23). See supplemental Table S3 for antibodies used.

**Western Analysis.** Muscle tissue was homogenized in RIPA buffer (Millipore) with protease and phosphatase A and B inhibitors (Bimake). Lysates were subjected to SDS-PAGE and blotted using antibodies as detailed in Supplemental Table S3.

**Transcriptomic Analysis and Quantitative RT-PCR.** For RNA-Seq experiments, RNA was extracted from a mixture of powdered quadriceps and gastrocnemius muscles using Trizol
reagent (Invitrogen). Each sample was verified for quality, then enriched for polyadenylated-RNAs used for library construction. All samples were run once for counts of reads, then twice for verification after adjusting to obtain greater than one million reads per sample. Each sample was aligned and annotated using STAR (29) to the mouse genome. In the alignment, non-canonical and unannotated splice junctions were removed. Mapped reads were counted for gene features using featureCounts (30). In this process, overlap features and multi-mapping reads were not counted. One sample was removed as it had almost empty reads. Filtering was performed and we only kept genes having counts per million (cpm) greater than 1 in at least 2 samples.

STZ-Diabetes vs. Controls and STZ-FoxO-TKO vs. M-FoxO-TKO were compared using linear modeling in the limma package (31). KEGG pathway analysis was performed using the sigPathway package (32). Heat maps were normalized by row (i.e. by gene) to compute z-scores, and were plotted with the gplots package. Volcano plots were plotted with the ggplot2 package (33). All statistical analysis was done in the R software.

For RT-PCR, RNA was reverse transcribed into cDNA (Applied Biosystems) according to the manufacturer’s protocol. RT-PCR was carried out using Sybr green (Bio-Rad or Bimake) with primers as detailed in Supplemental Table S1 (mouse) and S2 (human), and normalized to TBP, except for proteasome subunits which were normalized to GAPDH as TBP was different between groups for that experimental run.

Muscle cDNA from insulin treated/deprived Patients with Type 1 Diabetes. Muscle biopsy cDNA was obtained in collaboration with Dr. K. Sreekumaran Nair. The details of the study were previously published (34, 35). Briefly, participants with type 1 diabetes underwent two studies: one with insulin treatment and one with insulin deprivation for an average of 8.6 hours, separated by 1–2 weeks. Informed written consent was obtained after a detailed review of the
protocol, which had been approved by the Institutional Review Board of the Mayo Clinic and Foundation. Participants taking long-acting insulin were instructed to discontinue the long-acting for 3 days prior to the study day, and volunteers on insulin pumps were instructed to continue using ultra rapid-acting insulin until admission to the Clinical Research Unit (CRU). Volunteers were admitted to the CRU at 17:00 h on the evening before each study day and given a standard dinner at 18:00 h, after which subjects remained fasting until the completion of the study the next day. On the insulin-treated study day, regular human insulin was infused into a forearm vein to maintain blood glucose between 4.44 and 5.56 mmol/L overnight until 12:00 h the next day. The dose of insulin was adjusted based on plasma glucose levels every 30–60 minutes. On the insulin-deprived study day, the insulin infusion was discontinued for 8.6±0.6 hours. Vastus lateralis muscle samples were obtained at the end of the study (8.6 ±0.6 h after insulin withdrawal or similar time point on the insulin-treated day) under local anesthesia (lidocaine, 2%), with a percutaneous needle as described previously (34). RNA was isolated and cDNA was synthesized as mentioned above.

Statistical Analyses. All data are presented as mean ± standard error of the mean (SEM). Two-way ANOVA was performed for comparison of STZ treated groups to determine significance. For RT-PCR analyses of muscle cDNA from insulin treated/deprived patients with type 1 diabetes, paired t-tests were done since each patient contributed to both the insulin treated and the insulin deprived groups.
Results

*Deletion of FoxO isoforms in muscle prevents loss of muscle mass in insulin-deficient diabetes.*

We have previously shown that skeletal muscle expresses FoxO1, 3, and 4 and that deletion of all three isoforms of FoxOs in muscle can prevent atrophy from muscle-specific deletion of IR and IGF1R (23). To determine the impact of FoxO deletion on muscle proteostasis in the context of insulin-deficient diabetes, we utilized muscle-specific FoxO1, FoxO3, and FoxO4 triple knockout mice (M-FoxO-TKO) (23). These mice displayed a >95% depletion of FoxO proteins in quadriceps muscle (Figure 1A), without changes in FoxO protein expression in heart or liver from M-FoxO-TKO mice (Figure S1A-S1B).

Insulin-deficiency is the primary defect in type 1 diabetes, but is also accompanied by multiple abnormalities in circulating hormones and metabolites that could affect muscle including increases in glucagon, inflammatory cytokines, glucocorticoids as well as alterations of amino acids and lipid metabolites in muscle. To determine if muscle atrophy in response to insulin-deficient diabetes is mediated by FoxOs, we injected control and M-FoxO-TKO mice with streptozotocin (STZ) [single dose of 150 mg/kg] to induce diabetes and quantified muscle loss 12-14 days later. STZ-Diabetes induced marked hyperglycemia and insulin deficiency in both control and M-FoxO-TKO mice relative to vehicle-injected littermate mice within 14 days (Figure 1B-1C). During the 12-14 days after STZ treatment, body weight decreased in the STZ-Diabetes group by 3.75g (-15%), while Control and M-FoxO-TKO mice each gained 2.3g (+9%); STZ-FoxO-TKO mice also lost weight, but this was attenuated to 1.45g (-6%) (Figure 1D and S1C). Cardiac and adipose tissues atrophied to similar degrees in diabetic control and diabetic M-FoxO-TKO mice, while liver weights tended to increase (Figure S1D-S1E). However, STZ-Diabetes induced a dramatic 20-25% decrease in muscle size, and deletion of
FoxOs in STZ-FoxO-TKO mice prevented loss of muscle mass in response to diabetes (Figure 1E), accounting for the significant preservation of body weight even with uncontrolled diabetes.

FoxOs are critical regulators of cell cycle control and modulate muscle differentiation depending on the stage of myogenesis (36). Cell culture models have demonstrated that repression of FoxO4 increased muscle progenitor cell proliferation (37), but FoxO1 has been implicated in both the suppression of and activation of myogenic differentiation by influencing expression of components of the mTOR pathway and myostatin (38-40). To determine if our muscle-specific deletion of FoxO isoforms or insulin-deficient diabetes impacts expression of these genes and myocyte numbers in M-FoxO-TKO mice, we determined mRNA expression of myogenic factors in quadriceps (Quad) and counted total oxidative and glycolytic fibers in TA muscles. STZ-Diabetes tended to increase myogenin and IGF-2 (Figure S1F). In M-FoxO-TKO and STZ-FoxO-TKO Quad, MyoD and myogenin were significantly increased and IGF-2 tended to increase, while IGF-1, myoD, and myostatin remained unchanged (Figure S1F). However, quantitation of total oxidative and glycolytic fibers in TA cross-sections revealed no changes in total fiber numbers or fiber types in STZ-Diabetes, M-FoxO-TKO, or STZ-FoxO-TKO mice compared to controls (Figure 1F-1G). Thus deletion of FoxO isoforms in skeletal muscle prevents diabetes-induced muscle loss, but does not change myofiber number or oxidative type.

*Deletion of FoxOs prevents muscle atrophy in response to insulin-deficient diabetes.*

STZ-Diabetes mice showed a 24% decrease in the average myofiber cross sectional area (CSA) of TA muscle compared to controls, and this was completely prevented in STZ-FoxO-TKO (Figure 2A-2B), thus confirming that the maintenance of muscle mass in STZ-FoxO-TKO mice was due to prevention of myofiber atrophy. STZ-Diabetes mice showed an increased
percentage of fibers less than 1000 \( \mu m^2 \) compared to controls, and decreased the percentage of fibers greater than 2250 \( \mu m^2 \) (Figure 2C). This shift toward smaller fibers in STZ-Diabetes was reversed in STZ-FoxO-TKO mice. Finally, muscle grip strength was improved in M-FoxO-TKO and STZ-FoxO-TKO relative to controls and STZ-Diabetes mice (Figure 2D). These data indicate that insulin-deficient diabetes induces significant muscle atrophy, which is dependent on FoxO transcription factors.

*Induction of autophagy-lysosomal markers is prevented in STZ-FoxO-TKO muscle.*

FoxOs are critical regulators of protein degradation pathways in skeletal muscle (18-22). During states of energy deprivation, such as starvation or uncontrolled diabetes, bulk autophagy (macroautophagy) is activated and can be monitored by accumulation of the autophagy protein LC3 into puncta (or vesicles) (41). We found that in both type IIa oxidative fibers and non-type IIa fibers from TA muscle, LC3 vesicles per area increased more than 2-fold in STZ-Diabetes compared to controls (Figure 3A-3B). This was due primarily to an increase in LC3 vesicles coupled with a small decrease in myofiber cross-sectional area in STZ-Diabetes [11% and 15% in type IIa and non-IIa, respectively] (Figure S2A-S2B). The number of vesicles per muscle fiber also increased in M-FoxO-TKO, but this was not significant since the cross sectional area of the fibers also increased [27% and 44% in type IIa and non-IIa, respectively] (Figure S2A-S2B). Importantly, when compared to M-FoxO-TKO, STZ-FoxO-TKO mice showed no increase in LC3 vesicles per area (Figure 3B).

Phosphorylation of Ulk1 at serine 555, a marker of autophagy induction, tended to increase in STZ-Diabetes Quad compared to controls (Figure 3C-3D). Total Ulk1 levels also tended to increase in STZ-Diabetes compared to controls. By contrast, M-FoxO-TKO mice
showed decreased Ulk1 total protein levels (p<0.05 for genotype main effect by ANOVA) and prevented the increase in STZ-FoxO-TKO (Figure 3C). Levels of LC3-II were induced in STZ-Diabetes and p62/SQSTM also tended to increase, but not in STZ-FoxO-TKO muscle (Figure 3E-3F). To determine the impact on autophagy flux, STZ-Diabetes and STZ-FoxO-TKO mice were treated with or without colchicine for 2 days prior to sacrifice. Autophagy markers p-UlkS555, p62, and LC3II tended to increase in colchicine-treated STZ-Diabetes quadriceps muscle compared to saline treated STZ-Diabetes (Figure S2C-S2D). However, these same autophagy markers were significantly reduced in quad from saline-treated STZ-FoxO-TKO muscle and remained low in STZ-FoxO-TKO Colchicine muscle. In contrast, LC3-II increased equally in the heart of STZ-Diabetes and STZ-FoxO-TKO mice compared to their respective control, whereas no changes were observed between any of the groups in the liver (Figure S2E-S2F). Taken together, these data indicate that STZ-Diabetes causes an increase in autophagy markers in muscle relative to non-diabetic mice, perhaps due to increased flux or decreased degradation of autophagosomes. However, the increase in autophagy markers is not seen when FoxO transcription factors are absent.

Total ubiquitination levels, which play a role in both proteasomal degradation and autophagy, tended to increase with STZ-Diabetes, but was unchanged in STZ-FoxO-TKO muscle (Figure 3G). However, proteasome activity was unchanged and a proteasomal subunit was increased in M-FoxO-TKO and STZ-FoxO-TKO relative to controls (Figure S3S-S3B). This may not be surprising since the primary regulator of ubiquitin-mediated proteolysis is ubiquitination of the target and not proteasome activity. To determine how these alterations of protein degradation pathways influence total proteolysis, we measured tyrosine release from EDL and soleus muscles incubated ex vivo as previously described (23). Interestingly, we did
not observe significant increases in proteolysis of STZ-Diabetes muscle compared to control in this *ex vivo* experiment with EDL and soleus (Figure S3C-S3D), which display more mild or no atrophy in response to STZ treatment (Figure 1E). However, in soleus muscle, deletion of FoxOs did suppress proteolysis (Figure S3D) indicating that the regulation of autophagy and ubiquitin proteasome pathways by FoxOs controls muscle protein degradation.

*FoxO deletion does not change a majority of amino acid levels in muscle in response to STZ-Diabetes.*

Previous reports indicate that marked alterations in plasma (35) and muscle (23) amino acid levels occur in mice with insulin-deficient diabetes or mice with knockout of IR/IGF1R in muscle. To determine the role of increased protein degradation and muscle atrophy in STZ-Diabetes and the contribution of FoxO proteins to these changes, we measured amino acid (AA) levels in muscle lysates from control and M-FoxO-TKO mice rendered diabetic with STZ. Total AA levels in muscle were unchanged between all four groups (Figure 4A). Branch-chain amino acids (BCAA) increased in muscle from STZ-Diabetes mice (Figure 4B), mimicking changes in plasma of humans with type 1 diabetes during insulin withdrawal (35). Interestingly, FoxO deletion did not prevent this rise in BCAA, indicating that the suppression of protein degradation pathways by FoxO deletion in muscle does not contribute to the changing tissue BCAA levels. Histidine levels, on the other hand, decreased in muscle tissue from STZ diabetic mice, and this change was not dependent on FoxO signaling, while levels of other aromatic amino acids were unchanged by diabetes or FoxO deletion (Figure 4C). By contrast, methionine levels in muscle were increased in FoxO triple knockout, and were not further affected by STZ-diabetes (Figure 4D). Aspartate levels also increased with STZ-Diabetes, and this change was prevented by FoxO
deletion (Figure 4D). Aspartate has many fates including as a degradation product in the urea cycle. Citrulline combines with aspartate to initiate the urea cycle, and citrulline levels increased in STZ-FoxO-TKO muscle, perhaps indicated an increased capacity for urea cycle flux, but arginine and ornithine levels were unchanged (Figure 4D-4E). Thus, STZ-diabetes induces a rise in BCAA and decreases histidine levels in muscle, but these changes are not mediated by FoxO signaling. However, FoxO deletion in muscle does raise methionine and citrulline levels and prevents the increase in aspartate with diabetes, indicating a role for FoxOs in muscle amino acid metabolism.

Increases in ubiquitin-proteasome pathway transcripts in STZ Diabetic muscle depend on FoxOs, but abnormalities in fatty acid metabolism transcripts are FoxO-independent.

To determine the roles of FoxOs in transcriptional control of muscle protein turnover and metabolism, we performed RNA-Seq transcriptomic analyses on control and M-FoxO-TKO mice rendered diabetic with STZ. Using a false discovery rate of <0.1, a total of 1340 transcripts differed between STZ-Diabetes and Control, with 550 increased and 790 decreased (Figure 5A). In contrast, only 193 of these transcripts remained different between STZ-FoxO-TKO and M-FoxO-TKO with 78 increased and 115 decreased indicating that >85% of the transcriptional changes in STZ diabetic muscle are reversed by the deletion of FoxOs (Figure 5A). Volcano plots of these comparisons confirm that far more transcripts were altered in STZ-Diabetes vs. Control compared to STZ-FoxO-TKO vs. M-FoxO-TKO (Figure S5). These data indicate that FoxO transcription factors mediate most of the transcriptional changes in muscle due to diabetes.

Among the top five most significantly regulated KEGG pathways between STZ-Diabetes and Controls were pathways related to Type 2 diabetes and the “Ubiquitin Mediated Proteolysis”
pathway (Figure 5B). By contrast, in STZ-FoxO-TKO compared to M-FoxO-TKO, the Ubiquitin Mediated Proteolysis pathway was no longer significant indicating that regulation of these pathways in diabetes requires FoxOs. A heat map of all 125 genes within the Ubiquitin-Mediated Proteolysis pathway demonstrated a large cluster of genes that increased in STZ-Diabetes compared to controls, and this regulation was lost when FoxOs were deleted (Figure S6). We confirmed mRNA levels of 3 genes within this cluster, including a ubiquitin activating enzyme (Ube4a), an E2-ubiquitin conjugating enzyme (Ubeq2q), and an E3-ubiquitin ligase (Itch). The results show that Ube4a, Ube2q2, and Itch tended to increase with STZ-Diabetes, but were significantly reduced in STZ-FoxO-TKO mice (Figure 5C). Proteasomal subunit mRNAs were mildly altered by STZ-Diabetes and FoxO deletion. Psme4 significantly decreased in M-FoxO-TKO regardless of STZ treatment, while STZ-Diabetes tended to increase Psmb3 and Psmd8, with no effect of FoxO deletion (Figure 5D).

To determine the pathways that are diabetes-dependent but FoxO-independent, we compared STZ-FoxO-TKO to M-FoxO-TKO and found that fatty acid metabolism pathways ranked at the top (Figure 5E). Indeed, a heat map of the transcripts in the top 3 pathways from Figure 5E reveals a subset of genes which are highly induced in both STZ-Diabetes compared to Control and STZ-FoxO-TKO compared to M-FoxO-TKO (Figure S7). Fatty acid transport genes CD36 and Slc27a1 increased in muscle in STZ-Diabetes, and this increase was not blocked by loss of FoxOs in STZ-FoxO-TKO muscle, although baseline levels of Slc27a1 were decreased in M-FoxO-TKO (Figure 5F). Likewise, STZ-Diabetes increased Acsl1 and Acot2 (acyl-CoA modifying enzymes) expression independent of FoxO deletion (Figure 5G). This transcriptomic analysis reveals that insulin-deficient diabetes induces proteolysis pathways in a
FoxO-dependent manner, including the ubiquitin-proteasome system, but also increases fatty acid metabolism genes which are largely independent of FoxO regulation.

*Induction of ubiquitin-proteasome and autophagy-lysosomal mRNAs in muscle is dependent on FoxOs.*

Figure 6A shows a heat map of 49 atrogenes that are commonly regulated in all forms of muscle atrophy (42). These cluster into three main groups. Cluster 1 contained transcripts that increased with STZ-Diabetes and were rescued by FoxO deletion, i.e. are FoxO-dependent. These include many ubiquitin-proteasome genes, such as Fbxo32 (aka Atrogin-1/MAFbx), Ubc, Uba52, and proteasomal subunits. Cluster 2 was composed of transcripts that were down-regulated by STZ-Diabetes and not rescued by FoxO deletion. These included many metabolic genes, such as oxidative phosphorylation subunits, Mdh1, Dlat (a component of pyruvate dehydrogenase complex), and Ckmt2 (the mitochondrial creatine kinase). The third cluster contained genes that showed little regulation in response to either STZ-diabetes or FoxO deletion.

We confirmed mRNA levels of several genes in Cluster 1 along with other FoxO-target and autophagy genes in Quad muscle from a separate cohort of control and M-FoxO-TKO mice rendered diabetic with STZ. In agreement with the RNA-Seq data, mRNA levels of FoxO target genes Gadd45a, p27 Kip1, and Eif4ebp1 were significantly increased by STZ-Diabetes, but not in STZ-FoxO-TKO muscle (Figure 6B). Gadd45a is of particular interest since it can directly mediate skeletal muscle atrophy (43). 4EBP1 (the enzyme product of the Eif4ebp1 gene) is a negative regulator of protein synthesis that is inactivated upon phosphorylation by mTORC1, and we observed increase protein levels with a decrease in the p-4EBP/4EBP1 ratio in Quad
from STZ-Diabetes, which was reversed in STZ-FoxO-TKO mice (Figure S4A-S4B). Diabetes is known to decrease protein synthetic rates in muscle (44). To determine whether this regulation of 4EBP1 occurred simultaneously with alterations of protein synthetic rates in our mouse models, we determined protein synthesis by puromycin incorporation into muscle protein (27). Surprisingly, these data reveal that protein synthesis in Quad is decreased equally in STZ-Diabetes and STZ-FoxO-TKO groups compared to non-diabetic controls (Figure S4C-S4D). These data indicate that while FoxOs can control mRNA levels of 4EBP proteins, loss of FoxOs is not sufficient to prevent the decrease in muscle protein synthesis due to diabetes.

Muscle-specific E3-ubiquitin ligases Fbxo32 and Trim63 (also known as MuRF1) were up-regulated in STZ-Diabetes, but were decreased in M-FoxO-TKO and remained suppressed in STZ-FoxO-TKO (Figure 6C). However, other E3-ubiquitin ligases implicated in muscle atrophy, Fbxo21 and Fbxo30 (aka SMART and MUSA1) (22, 45), tended to increase in M-FoxO-TKO, and were not modified by STZ treatment (Figure 6C). Some autophagy genes, such as LC3A and LC3B, did not change significantly at the mRNA level with STZ-Diabetes. However, total mRNA levels of Bnip3, Bnip3L, Gabarapl, and Ulk1 were induced in STZ-Diabetes compared to controls, and this was blocked by FoxO deletion (Figure 6D). These changes in ubiquitin-proteasome and autophagy-lysosome genes did show some specificity based on muscle type. EDL, a glycolytic muscle, showed diabetes-induced increases in both autophagy genes and E3 ubiquitin ligases that were FoxO-dependent (Figure S4E), similar to Quad. However, oxidative soleus muscle showed very little change in autophagy genes, other than an upregulation of Lamp2 and decreases in Ulk1 expression that were independent of FoxOs (Figure S4F). E3-ligases increased in soleus with STZ-Diabetes in a FoxO regulated fashion.
Together these results show that FoxO transcription factors control the induction of a broad range of atrophy-related protein degradation genes in response to uncontrolled diabetes.

*Transcripts of FoxO-dependent ubiquitin-proteasomal and autophagy-lysosomal genes increase in muscle from patients with type 1 diabetes after insulin withdrawal.*

To determine if these FoxO-dependent atrophy and autophagy genes that increase in STZ-Diabetes mice were also increased in humans with type 1 diabetes, we obtained cDNA from muscle biopsies of patients with type 1 diabetes both before and after 8 hours of insulin deprivation (34). Following insulin deprivation, p27 Kip1 and three E3-Ubiquitin ligases FBXO30, FBXO32, and TRIM63 significantly increased by 2- to 3-fold compared to samples obtained from the same patients while insulin-treated (Figure 7A-7B). In addition, two out of 7 autophagy-related mRNAs, BNIP3L and GABARAPL, were increased significantly in the insulin-deprived group (Figure 7C), and mRNA levels of other atrophy and autophagy genes also tended to increase after insulin deprivation. Thus, FoxO-driven muscle atrophy-related pathways and protein degradation pathways are transcriptionally up-regulated in mice with STZ-diabetes and in humans with type 1 diabetes in as little as 8 hours after insulin withdrawal.
Discussion

While much attention has focused on the role of diabetes in risk of cardiovascular disease, blindness and renal failure, diabetes affects virtually all tissues of the body, often in more subtle, but very important ways. For example, patients with type 1 and type 2 diabetes have decreased muscle size and strength, and this contributes to poor physical fitness and increased risks of disability (3-5). Perhaps more striking is the strong association of decreased muscle strength with mortality. In the Health ABC study, individuals age 70-80 showed a near 20-30% mortality rate over 6 years if they were in the bottom quartile of muscle strength, and patients with uncontrolled diabetes, not only had decreased initial strength, but actually lost strength faster than non-diabetic individuals (1, 2, 46). Furthermore, patients with diabetes have decreased strength after major surgery, such as coronary artery bypass grafting (47). This would lead to delayed recovery, increased risk of post-surgical complications, increased falls, and disability, thus supporting the critical need to identify the factors that contribute to muscle loss in diabetes. Identifying the intracellular targets that are perturbed in diabetes and that lead to decreases in muscle fitness is of critical importance, as it is not clear that these effects on protein synthesis and degradation are simply linked to uncontrolled hyperglycemia.

In the present study, we have investigated the role of FoxO transcription factors in diabetic muscle disease by creating muscle-specific FoxO triple knockout mice and rendering them diabetic with STZ. Previous studies showed that overexpression of FoxO1 was sufficient to induce muscle atrophy (21, 48) and we now demonstrate that deletion of FoxOs specifically in muscle prevented diabetes-induced muscle atrophy without affecting glucose homeostasis. STZ-Diabetes induced a broad upregulation of both ubiquitin-proteasome and autophagy lysosome pathways, and this was blocked by FoxO deletion. A similar upregulation of these protein
degradation pathways occurs in muscle biopsies from people with type 1 diabetes after only 8 hours of insulin withdrawal, indicating the exquisite control of insulin over this process in humans. Thus, insulin deficiency induces muscle protein degradation via a FoxO-dependent pathway, providing new insight and potential for therapeutic investigations to prevent diabetes-induced muscle disease.

FoxOs target several genes that regulate muscle atrophy, and our study demonstrates that many of these genes are also upregulated in uncontrolled diabetes. The E3-ubiquitin ligases Fbxo32 and Trim63 are FoxO target genes known to modulate muscle atrophy in response to starvation, immobilization, and denervation (18-21, 49). Interestingly, of the proteolysis genes tested in humans with type 1 diabetes, FBXO32 and TRIM63 showed the largest induction after insulin withdrawal. Gadd45a, another known FoxO target, can promote muscle atrophy in response to various forms of stress by activating MEKK4 (43, 50). Diabetes also leads to a decrease in protein synthetic rates in muscle from rodents (44). The induction of Eif4ebp1, a negative regulator of protein synthesis, in our STZ-Diabetic mice was prevented in STZ-FoxO-TKO allowing FoxOs to regulate aspects of both synthesis and degradation of proteins. However, our direct measurements of puromycin incorporation into muscle protein revealed that loss of FoxOs does not prevent the decrease in protein synthetic rates in diabetes. This further strengthens our conclusions that FoxOs regulate protein degradation pathways to induce muscle atrophy in response to diabetes. Indeed, autophagy markers of LC3-II and p-UlkS555 accumulate in STZ-Diabetic muscle along with LC3-positive vesicles, indicating an alteration of autophagy, but not necessarily increased autophagy flux. Importantly, inhibition of autophagy in muscle can also lead to atrophy (51). Likely a combination of enhanced autophagy with impaired lysosomal degradation occurs with diabetes, but these alterations are prevented in STZ-FoxO-TKO,
indicating the necessity of FoxOs for this regulation. We provide evidence that FoxOs control of autophagy by transcriptional mechanisms since a number of autophagy-lysosomal genes are upregulated in a FoxO-dependent manner in mice (Bnip3, Bnip3L, Gabarapl, and Ulk1). But these genes were induced to a lesser degree in humans after insulin withdrawal. Whether this indicates that the two proteolytic pathways (ubiquitin-proteasome vs. autophagy-lysosome) contribute differentially to diabetes-induced muscle atrophy in humans and mice or whether this is simply the difference in duration of insulin deficiency in mice (which is chronic) and humans (which was only 8 hours) is unknown. Further studies on the time course of induction of ubiquitin-proteasome vs. autophagy intermediates may clarify if the response to insulin withdrawal involves acute upregulation of E3-ubiquitin ligase followed by a broad activation of autophagy pathways after chronic insulin deprivation.

The diabetes-induced upregulation in proteolysis pathways did not lead to a broad increase in tissue amino acids. Rather, selective amino acid pools were affected, such as increases in BCAAs and decreased histidine that were independent of FoxOs. Furthermore, STZ-Diabetes increased aspartate that was normalized with FoxO deletion, which could signify that FoxOs control synthesis or degradation of aspartate through regulation of enzymes in the urea cycle. Citrulline levels were also increased in STZ FoxO TKO muscle which may indicate an increased capacity for urea cycle flux, but these static metabolite measurements are unable to quantify flux. Aspartate can also be converted to other amino acids and even TCA cycle intermediates, and the enzymes that control these processes may be regulated by FoxOs. These data indicate that diabetes and FoxOs influence amino acid metabolism by mechanisms independent of proteostasis.
Transcriptomic analysis revealed that FoxOs are necessary for a majority of the transcriptional changes in response to STZ-Diabetes. Indeed, of the 1340 significantly changed genes in muscle from STZ-Diabetes mice, only 193 genes (14%) remained significantly changed in STZ-FoxO-TKO. However, FoxOs do not mediate all aspects of muscle gene regulation in response to insulin-deficient diabetes. For example, proteolysis pathways were highly upregulated in muscle from STZ-Diabetes mice, and these were prevented by deletion of FoxOs, whereas upregulation of fatty-acid import and biosynthesis genes which occurs in STZ-Diabetes was not mediated by FoxOs. Interestingly, of the 49 atrophy-related genes presented in the heat map, 19 were downregulated with STZ-diabetes, and 10 of the 19 genes are involved in metabolic pathways that were not rescued by FoxO deletion. Thus, while FoxOs do regulate some metabolism-related genes in muscle, such as the upregulation of PDK4 in the fasted state (52), FoxOs do not regulate genes related to fatty acid import and biosynthesis. FoxOs do have an important role in insulin-mediated metabolic regulation in the liver, controlling gluconeogenesis by gene regulation of G6Pase and PEPCK (17), and recent studies suggest that FoxOs may control both lipid and glucose metabolism in the liver (16, 53). Lastly, FoxOs have been implicated in mitochondrial dynamics related to muscle atrophy (54). How this might interact with the changes in protein degradation and autophagy remains to be determined.

Uncontrolled type 1 diabetes is caused by inadequate insulin levels. However, other hormonal and metabolite abnormalities also occur with insulin deficiency in uncontrolled diabetes including hyperglycemia, elevated cortisol levels and increased inflammation. The degree to which each of these contributes to diabetes-induced muscle atrophy and whether they all impinge on FoxO transcription factors is unknown. Previous reports have suggested that insulin signaling predominates over IGF-1 to control muscle size (23, 55). Other studies suggest
that glucocorticoid signaling contributes significantly to muscle atrophy in the context of diabetes (56), and glucocorticoid receptors act in a synergistic way with FoxO1 to induce the ubiquitin ligase Trim 63 (MuRF1) (57). The increase in inflammation and cytokine signaling during diabetes may also activate NF-kappaB signaling to promote muscle protein degradation (58). While these changes in circulating hormones, cytokines and metabolites could contribute to diabetes-induced atrophy, the fact that deletion of insulin and IGF-1 receptors induces marked muscle atrophy that is entirely reversed by triple FoxO deletion (6, 23) strongly supports the current data and points to a model where decreased insulin action in diabetes activates FoxO transcription leading to muscle atrophy. Indeed, regardless of the upstream cause, our study demonstrates that in the context of uncontrolled diabetes, FoxOs are necessary for the atrophic response.

In summary, insulin deficiency and uncontrolled diabetes lead to muscle atrophy and decreased muscle strength, which can contribute to decreased health and disability. Our study demonstrates that, in muscle, FoxO transcription factors mediate the majority of transcriptional changes in response to STZ-diabetes. FoxOs control the increases in proteolytic pathways that occur upon insulin deficiency, and these changes occur within 8 hours after insulin deprivation in humans with type 1 diabetes. Thus, FoxOs are critical regulators of diabetes-induced muscle atrophy and represent potential therapeutic targets to prevent muscle loss in patients with diabetes.
Figure Legends

**Figure 1. FoxO deletion in muscle prevents muscle loss after STZ-Diabetes without altering oxidative fiber type or number.** Western blot analysis (A) of FoxO isoforms 1, 3, and 4 in quadriceps. Blood glucose (B) and serum insulin (C) levels in control and M-FoxO-TKO mice 12-14 days after high-dose STZ treatment to induce insulin-deficient diabetes (n=10-12 for glucose and 5-7 for insulin levels). Total change (Δ) in body weight 12-14 days after STZ (D) and dissected muscle weights (E) from Control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO mice (n=10-12). Succinate dehydrogenase staining (F) and quantification (G) of total numbers of purple oxidative and gray glycolytic fibers from whole tibialis anterior (TA) muscle. (bar=500 μm) (*-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01 STZ-Diabetes vs. STZ-FoxO-TKO, #-p<0.05, ##-p<0.01 as indicated, Two-way ANOVA). Quad – quadriceps, EDL – extensor digitorum longus, Gastroc – gastrocnemius. Blots are from parallel samples run on separate gels.

**Figure 2. Deletion of FoxOs prevents muscle atrophy in response to insulin-deficient diabetes.** Immunofluorescent staining of laminin in TA muscle cross-sections (A) from Control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO mice (bar=100 μm) (n=5-6). Quantitation of cross-sectional area (B) and myofiber distribution (C) from images in Panel A. Grip strength (D) measured 12-15 days after STZ injection (n=8-11). (*-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01 STZ-Diabetes vs. STZ-FoxO-TKO, ◊-p<0.05 genotype main effect, Two-way ANOVA).

**Figure 3. Induction of autophagy-lysosomal markers is prevented in STZ-FoxO-TKO muscle.** Immunofluorescent staining of LC3A and Myosin IIa in TA muscle cross-sections (A)
from Control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO mice (bar=50 µm) (n=5-6).

Quantitation of LC3A-positive vesicles per cross-sectional area (B) in Type IIA positive and Non-IIA fibers. Western blot analysis (C) and Densitometric quantification of p-Ulk1S555 (D), p62 (E), LC3-II (F), and ubiquitin (G) levels in Control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO mice (n=5-6, other than ubiquitin where n=3 per group). (*-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01 STZ-Diabetes vs. STZ-FoxO-TKO, Two-way ANOVA). Blots are from parallel samples run on separate gels.

**Figure 4. FoxO Deletion does not prevent changes in most muscle-tissue amino acid levels in response to STZ-Diabetes.** Total tissue levels of 20 protein-forming amino acids (AA) in hind limb muscle from control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO mice (A). Fold change of Branch chain AA (BCAA) (B), Aromatic (C), other essential AA (D), and urea cycle metabolites (E) relative to controls represented by gold line (n=4-6 per group). (*-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01 STZ-Diabetes vs. STZ-FoxO-TKO, ##-p<0.01 as indicated, Two-way ANOVA). Note that Control and M-FoxO-TKO groups contain data from our prior publication (25), and are presented as important controls in the current study.

**Figure 5. Increases in ubiquitin-proteasome pathway transcripts in STZ-Diabetic muscle depend on FoxOs, but abnormalities in fatty acid metabolism transcripts are FoxO-independent.** Venn diagram of genes differentially regulated in STZ-Diabetes vs. Control (gray circle) and STZ-FoxO-TKO vs. M-FoxO-TKO (red circle) by RNA-Seq analysis of mixed quadriceps and gastrocnemius (A). KEGG pathway analysis (B) of RNA-Seq data from STZ-Diabetes vs. Control from panel A. Quantitative RT-PCR was performed to confirm mRNA levels of a ubiquitin activator (Ube4a), modifier (Ube2q2), and ligase (Itch) from the “Ubiquitin Mediated Proteolysis” pathway in quadriceps (C). Quantitative RT-PCR of was performed for
proteasomal subunit genes (D). KEGG pathway analysis (E) of RNA-Seq data from STZ-FoxO-TKO vs. M-FoxO-TKO from panel A. Quantitative RT-PCR of fatty acid import genes (E), and acyl-CoA modifying enzymes (F) in quadriceps from control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO mice. (n=4-6 per group. *-p<0.05, **-p<0.01 vs. Control, †-p<0.05, ††-p<0.01 STZ-Diabetes vs. STZ-FoxO-TKO, #-p<0.05, ##-p<0.01 as indicated, ^-p<0.05, ^^^-p<0.01 STZ main effect, Two-way ANOVA)

**Figure 6. Induction of ubiquitin-proteasome and autophagy-lysosomal mRNAs in muscle is dependent on FoxOs.** A heat map of transcripts of 49 atrophy-related genes from RNA-Seq analysis of mixed quadriceps and gastrocnemius (A). Quantitative RT-PCR of FoxO target genes (B), E3-ubiquitin ligases (C), and autophagy genes (D) from control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO quadriceps muscle. (n=4-6 per group. *-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01 STZ-Diabetes vs. STZ-FoxO-TKO, ##-p<0.01 as indicated, Two-way ANOVA)

**Figure 7. Transcripts of FoxO-dependent ubiquitin-proteasomal and autophagy-lysosomal genes increase in muscle from patients with type 1 diabetes after insulin withdrawal.**

Quantitative RT-PCR of FoxO target genes (A), E3-ubiquitin ligases (B), and autophagy genes (C) from vastus lateralis muscle biopsies from patients with type 1 diabetes either treated normally with insulin or deprived of insulin for 8 hours as previously reported (34). (n=9 per group. *-p<0.05, **-p<0.01 vs. insulin treated, paired t-test)
Author Contributions

B.T.O. designed the study, researched data, and wrote the manuscript. C.M.P., G.B., P.A.SB., and K.P. researched data, helped design experiments, and helped write the manuscript. M.T.K., M.L., and K.K. researched data and helped design experiments. J.M.D. and H.P. performed bioinformatic analyses. K.S.N. provided reagents, helped design experiments, and helped write the manuscript. C.R.K. designed the study, and helped write the manuscript.

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Figure 1

A. Insulin (ng/ml)

B. Glucose (mg/dL)

C. Insulin (ng/ml)

D. Δ Body Weight (g)

E. Muscle Weight (mg)

F. Control

STZ-Diabetes

M-FoxO-TKO

STZ-FoxO-TKO

G. Total Fiber Number

Oxidative Glycolytic
Figure 3

A. LC3A Myosin IIa DAPI

Control STZ-Diabetes
M-FoxO-TKO STZ-FoxO-TKO

B.

Vesicles (10^4/mm^2)

Control STZ M-FoxO TKO STZ M-FoxO-TKO

Densitometry (Fold Change)

p-Ulk1 S555

p=0.07

p=0.08

p=0.099

C.

STZ Diabetes

Control M-FoxO-TKO

p-ULK1S555

ULK1

p62

β-actin

LC3-II

β-actin

Ubiquitin

GAPDH

D.

Densitometry (Fold Change)

p-Ulk1 S555

p=0.099

E.

p62/Actin

F.

LC3-II/Actin

G.

Ubiquitin/GAPDH

p=0.093
**Figure 5**

A. 

**STZ-Diabetes vs. Control**

| Rank | Pathway Analysis of STZ-Diabetes vs. Control | q-Value |
|------|---------------------------------------------|---------|
| 1    | Renin Angiotensin System                     | <0.0001 |
| 2    | Systemic Lupus Erythematosus                | 0.004   |
| 3    | Type II Diabetes Mellitus                   | 0.004   |
| 4    | Ubiquitin Mediated Proteolysis              | 0.005   |
| 5    | Antigen Processing and Presentation         | 0.005   |

| Rank | Pathway Analysis of STZ-FoxO-TKO vs. M-FoxO-TKO | q-Value |
|------|-----------------------------------------------|---------|
| 1    | Biosynthesis of Unsaturated Fatty Acids       | <0.0001 |
| 2    | Fatty Acid Metabolism                         | 0.011   |
| 3    | PPAR Signaling Pathway                        | 0.017   |
| 4    | Limonene and Pinene Degrad.                   | 0.017   |
| 5    | TGF beta Signaling Pathway                    | 0.017   |

B. **Ubiquitin activator, conjugator, and ligase**

C. **Proteasomal Subunits**

D. **Acyl-CoA Modifying Enzymes**

E. 

F. **Fatty Acid Import**

G. **FDR<0.1 Proteasomal Subunits**

H. **Ubiquitin activator, conjugator, and ligase**

I. **Proteasomal Subunits**

J. **Acyl-CoA Modifying Enzymes**

K. **Fatty Acid Import**
Figure 6

A. Transcripts of Atrophy Genes

Cluster 2

Not Regulated

Cluster 1

B. FoxO Targets

C. E3-Ubiquitin Ligases

D. Autophagy and Lysosomal Genes
Figure 7

A. FoxO Targets

B. E3-Ubiquitin Ligases

C. Autophagy Genes

[Graphs showing mRNA expression levels for FoxO targets, E3-ubiquitin ligases, and autophagy genes under Insulin Treated and Insulin Deprived conditions.]

- **: Significant difference
- ***: Highly significant difference
Figure S1. Muscle-specific deletion of FoxOs mitigates weight loss, but does not prevent loss of cardiac/adipose tissue mass or alter myogenic factors in muscle in response to insulin-deficient diabetes. Western blot analysis for FoxO isoform expression in heart (A) and liver (B) from control and M-FoxO-TKO mice rendered diabetic with STZ. Body weight at sacrifice (C) in control, STZ-Diabetes, M-FoxO-TKO and STZ-FoxO-TKO mice (n=10-12). Heart weight (D), adipose and liver weights (E) after 12-15 days of diabetes after STZ treatment (n=10-12). mRNA levels of myogenic factors in quadriceps muscle (F) (n=4-6). (*-p<0.05, **-p<0.01 vs. control, †-p<0.05, STZ-Diabetes vs. STZ-FoxO-TKO, #-p<0.05, ##-p<0.01 as indicated, ◊-p<0.05 genotype main effect, ▲-p<0.05 STZ main effect, Two-way ANOVA). sWAT – subcutaneous white adipose tissue (WAT), eWAT – epididymal WAT.
Figure S2. FoxO Deletion prevents upregulation of autophagy markers in skeletal muscle. Quantification of total LC3A vesicles per fiber (A) and fiber cross-sectional area (B) from TA images in Figure 3A (n=5-6). Western blot analysis (C) and densitometry (D) of autophagy markers in Quad from STZ-Diabetes and STZ-FoxO-TKO mice treated with Saline or Colchicine for 2 days prior to sacrifice (n=7 per group). Western blot analysis and densitometric ratio of LC3-II/LC3-I in heart (E) and liver (F) from control, STZ-Diabetes, M-FoxO-TKO and STZ-FoxO-TKO mice. (*-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01, STZ-Diabetes vs. STZ-FoxO-TKO, ^^-p<0.01 STZ main effect, Two-way ANOVA)
Figure S3. Muscle Specific FoxO Deletion reduces proteolysis in Soleus muscle, but proteasome activity is unchanged and a 19S proteasome subunit is increased in Quad. Proteasome activity (A) and western of a 19S proteasomal subunit (B) in Quadriceps muscle. Proteolysis measured by tyrosine release in ex vivo EDL (C) and Soleus (D) muscle (n=4-5). (*-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01, STZ-Diabetes vs. STZ-FoxO-TKO, Two-way ANOVA)
Figure S4. Despite regulation of 4EBP1, FoxO Deletion does not restore the suppressed protein synthesis in STZ-Diabetes, and upregulation of autophagy markers by STZ-Diabetes is muscle-type specific. Western blot (A) and densitometry (B) of phospho- and total 4EBP1 protein in Quad. Protein synthesis by SUnSET method (C) and densitometry (D) in Quad (n=2-6 per group, Fed and Fasted mice treated +/- puromycin were used as positive and negative controls; ND=not diabetic and therefore excluded). qRT-PCR of autophagy/lysosomal genes and E3-ubiquitin ligases in EDL (E) and Soleus (F) from control, STZ-Diabetes, M-FoxO-TKO, and STZ-FoxO-TKO mice. (n=5-6 per group. *-p<0.05, **-p<0.01 vs. control, †-p<0.05, ††-p<0.01, STZ-Diabetes vs. STZ-FoxO-TKO, ^-p<0.05, ^^-p<0.01 STZ main effect, Two-way ANOVA)
Figure S5. A majority of the changes in transcript levels in response to STZ-Diabetes are normalized by deletion of FoxOs in muscle. Volcano plots of transcripts comparing STZ-Diabetes to Controls (A) and STZ-FoxO-TKO to M-FoxO-TKO (B) from quadriceps and gastrocnemius muscles determined by RNA-Seq (n=4-6 per group). Red shaded boxes indicate transcripts that are increased or decreased by more than 2 fold with a p-value less than 0.001. Red numbers indicate the total number of downregulated transcripts while green numbers indicate upregulated transcripts.
Figure S6. A subset of transcripts in the ubiquitin-proteasome pathway is increased by diabetes in a FoxO-dependent manner. Heat map of all 125 genes from the “Ubiquitin mediated proteolysis” KEGG pathway with cluster analysis based on Z-score.
Figure S7. A subset of transcripts in the Biosynthesis of Unsaturated Fatty Acids, Fatty Acid Metabolism and PPAR Signaling pathways is increased by diabetes independent of FoxOs. Heat map of all genes from the “Biosynthesis of Unsaturated Fatty Acids”, “Fatty Acid Metabolism”, and “PPAR Signaling” KEGG pathways from Figure 5E with cluster analysis based on Z-score.
### Supplemental Table S1. Primers for QPCR of Mouse genes.

| Common name | Gene name | 5' primer | 3' primer |
|-------------|-----------|-----------|-----------|
| **Mouse QPCR Primers** | | | |
| FoxO1       | Foxo1     | TGCTGTGAAGGGACAGATTG | GAGTGGATGTTGAAGAGCGT |
| FoxO3       | Foxo3     | ACAACCGGTCTACTTTGTCCAGA | TCTTGCCCGTGCTCTACTTCT |
| FoxO4       | Foxo4     | GTTGCCCTACTTCAAGGACAGA | AGCTTGTGCTCTGACTCAT |
| IGF-1       | Igf1      | GACCGAGGGGCTTTTACTTC | GGGGCACAGTACATCTCCA |
| IGF-2       | Igf2      | GAGCCGCGCTTCTACTTT | ACGTCCCTCTGAGACTTTG |
| MyoD        | Myod1     | AGCCTACAGTGCGACTCATG | TCCACTCATGCGAAGGACTG |
| Myogenin    | Myog      | TTGCTCAGCTCTCCACACAGA | AGATGTGAGGGCTTGGTCA |
| Myostatin   | Mstn      | TGGCTCAAACAGCTGGAACACA | TGGGTGCTGCTGCTACCTG |
| Psme4       | Psme4     | AATTTCCTCCAAAGAGGTCGA | CAGATCAAGGAAACTGGG |
| Ube4a       | Ube4a     | GTTTCGACGCAATCCCAAGAAG | CAGGCTCTCTGACACACTATTAC |
| Ubeq2q      | Ubeq2q   | GAATGCTCTGTTGAACACAGA | GCTGAGAGAGAGGAGAAGA |
| Itch        | Itch      | CCA CCC ACC CGA CAA AGA CC | CTA GGG CCC GAG CCT CCA GA |
| Psmb3       | Psmb3     | GAGCAATAGAAGGGCCTAGGA | GGGCAACCTCTGATGAGA |
| Psmd8       | Psmd8     | GGTGATCAGAGGACTGTTT | CCTGAGGCAAGACACTTCCC |
| Psmd11      | Psmd11    | CTAGAATGGAAGACACACCAAC | CAAATGCTGTGCGTAAAGAG |
| CD36        | Cd36      | CCTGAAATGTCAGAGGAAA | GGCACATGATTATGGACA |
| FATP1       | Sla27a1   | AGCCGAACACGAATACAGAAC | TTTGCTGATGCTGTTGG |
| Acs1        | Acs1      | ACCATCAGTGTTCAACGCTGA | CGCTACACGCTTGGTAT |
| Aco1        | Aco1      | CCCCTGTGACTATCTCTGAGAA | CAAACACTACTACCAACTGT |
| Aco2        | Aco2      | ATGCTTGCCCTGCTTCTTTTG | GAGCGGGGAGGTACAAAC |
| p27 Kip1    | Cdkn1b    | GGGGAACCGGTCTGAAACATTT | AGTGCAGGGAGGAGAAG |
| Gadd45a     | Gadd45a   | GGATCCTCTTCCATTGTGATGAA | TGCTACTGGAAGACAGCC |
| 4E-BP       | Eif4ebp1  | CCT CCT TGTTCCCTGTGCTTA | GCC TAA GGA AAG ATG GGT GT |
| Cathepsin   | Ctsl      | TATCCCTCAGGAAGGAAAGCCT | TCCCTCACTGCAATCCCACAA |
| LC3A        | Map1lc3a  | TCTGGCTCCAGACCCATGTTA | GGTGACGAGCCAGAAGAAG |
| LC3B        | Map1lc3b  | CACTGCTCTGCTTTGTAGGTGG | TCGTGGCTCCTTTATTAGTCATC |
| Lamp2a      | Lamp2     | ACAACCTGACTCTCTGCTGAGGA | AGTGGAGGTGATGGTGGTGG |
| Gabarap1    | Gabarap1  | GTCATGCGGAGAGGCTCTCAA | GAGGAGATGGGTTGGAGCAAA |
| Bnip3       | Bnip3     | CCCAGACACACAGAATACCAACA | GGTGCGACTTTGAACATTCCATCC |
| Bnip3L      | Bnip3l    | CACAAAAAGAGATGGGCAGATCA | TGGACACCTGATCCACATC |
| SMART       | Fbxo21    | TCA ATA ACC TCA AGG CTT GC | GTT TGG CAC ACA AGC TCC A |
| MUSA1       | Fbxo30    | TCG TGG AAT GGT AAT CTT GC | CCT CCC GAT TCT CA TCA CG |
| Atrogin-1   | Fbxo32    | CTGCTGCTGGTGCTCTCAATACA | CGTCATCAGCTCTGCTGAT |
| MuRF-1      | Trim63    | ATGAACTGATCATGGAGGCAGGCA | TTGACACAGAGGAGAAGGAGG |
| Ulk1        | Ulk1      | TGCATGGGAGGAGGCTCTCAA | AGCAGGGGCTTTGTGATACCTCG |
| TBP         | Tbp       | ACCCTTCCACAAATGACTCTATG | TGACTGCGCAATCCGGT |
| GAPDH       | Gapdh     | TGCTGCTGCGACTTACTGCTGCT | TCTGGTGCTCACACCATCACA |

Page 49 of 51 Diabetes
### Supplemental Table S2. Primers for QPCR of Human genes.

| Common name | Gene name | 5’ primer                        | 3’ primer                        |
|-------------|-----------|----------------------------------|----------------------------------|
| p27 Kip1    | CDKN1B    | TTCATCAAGCAGTGATGTATCTG          | AAGAAGCTGGCCTCAGAAG             |
| Gadd45b     | GADD45B   | GGATGAGCGTGAAGTGATT              | GTGTACGAGTCGGCCAAGTT            |
| 4E-BP       | EIF4EBP1  | AGTTCCGACACTCCATCAGG             | CGGGGACTACAGCAGCAGC             |
| Cathepsin L | CTSL      | AAAGGCAGCAAGGATGAGT             | GCCGCTGACTGTTGAG                |
| LC3B        | MAP1LC3B  | TTGTGGGATCCAGACAGGAG            | CGGAGAGACCTTCAAGCAG             |
| LC3C        | MAP1LC3C  | CCCAAGCGTCAGACCCCTTC            | GGGAACCTTTGCCCCGGATT            |
| Lamp2       | LAMP2     | CAATGCATAAGACCGCAG              | GTGTAGCAGCTGTTGGGT              |
| Gabarapl    | GABARAPL1 | TGGCAAGCATAGTACGGTGCA            | TCAGAAAAAGGAGGAGGAGAAA          |
| SMART       | FBXO21    | TGCCTTTGGGGATGTAGTGAG           | ATAACGGCTTGGTAGGAGGAC           |
| MUSA1       | FBXO30    | TCCACAAATGGGAAATGCTG            | GTCCAAGCCAGCTAGGG              |
| Atrogin-1   | FBXO32    | ATGCCACCTCAGGGGATGTGA           | TTCTCAACTGCCATTCTGGA            |
| MuRF-1      | TRIM63    | CTTCCTTGCTCCTGCACT              | ATCGTCAGGGAGTCTAGGG             |
| Bnip3       | BNIP3     | CTGTGGGTCCACAGCTATTT            | CATGCAAGGGAGGAGGCTGT            |
| Bnip3L      | BNIP3L    | CGCCTTTTCTTTCAAAGCCT            | GATGCAAGGATGCAAACCAAGAG         |
| TBP         | TBP       | GCGATAAGGCGATCCTGGGAG          | AACAACAGCTGACCCCTTA            |
### Supplemental Table S3. Antibodies used.

| Protein                     | Vendor                      | Catalog number | Dilution |
|-----------------------------|-----------------------------|----------------|----------|
| **Immunofluorescence Antibodies** |                             |                |          |
| LC3A                        | Cell Signaling              | 4599           | 1:200    |
| Laminin                     | Sigma                       | L9393          | 1:200    |
| Myosin IIa                   | DSHB University of Iowa     | SC-71          | 1:200    |
| Alexa-Fluor-594 Goat anti mouse | Life Technologies       | #A-11032       | 1:500    |
| Alexa-Fluor-488 Goat anti rabbit | Life Technologies   | #A-11008       | 1:500    |
| **Western Blot Antibodies** |                             |                |          |
| FoxO1                        | Cell Signaling              | 2880           | 1:1000   |
| FoxO3                        | Cell Signaling              | 12829          | 1:1000   |
| FoxO4                        | Abcam                       | 128908         | 1:1000   |
| β-Actin                      | Cell Signaling              | 4970           | 1:1000   |
| GAPDH                        | Cell Signaling              | 5174           | 1:1000   |
| LC3A/B                       | Cell Signaling              | 12741          | 1:1000   |
| p62/SQSTM1                   | Cell Signaling              | 5114           | 1:500    |
| p-ULKS555                    | Cell Signaling              | 5869           | 1:1000   |
| ULK1                         | Cell Signaling              | 8054           | 1:500    |
| Ubiquitin                    | Cell Signaling              | 3933           | 1:1000   |
| 19S Proteasome Subunit       | Enzo Life Sciences          | BML-PW8870     | 1:1000   |
| p-4EBP1                      | Cell Signaling              | 9451           | 1:1000   |
| 4EBP1                        | Cell Signaling              | 9452           | 1:1000   |
| Anti-Puromycin Sera (non-concentrated) | DSHB University of Iowa | PMY-2A4-S     | 1:20     |
| Secondary for Puromycin      | Jackson ImmunoResearch      | 115-035-208    | 1:10,000 |
| Rabbit secondary antibody    | Thermo Fisher               | SA5-35571      | 1:10,000 |
| Mouse secondary antibody     | Thermo Fisher               | SA5-35521      | 1:10,000 |