Supplemental Information

Skeletal muscle myotubes of the severely obese exhibit altered ubiquitin-proteasome and autophagic/lysosomal proteolytic flux

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Method S1

Myotube Area Method

Myotube area was quantified by analyzing the amount of myosin heavy chain covering the culture area (immunofluorescence) and total number of nuclei counted after staining with 4',6-diamidino-2-phenylindole (DAPI). Myoblasts from lean and obese individuals (~40% confluency) were plated in six well plates, grown to confluency, differentiated for 6-7d, and cultured in starvation media (HBSS) for 24 h. Briefly, myotubes were washed with a cytoskeleton stabilizing buffer containing 80mM PIPES, 5mM EGTA, 1mM MgCl2, and 40g/L PEG 35,000 as described by Trendelenurg et al. (1). Cross-linking was performed with 4% paraformaldehyde, and myotubes were permeabilized with 0.1% Triton X-100. Non-specific binding was blocked with 10% rat serum. Myotubes were then incubated overnight at 4ºC with an antibody that detects all isoforms of myosin heavy chain (A4.1025, 1:100 dilution), which was developed by S. Schiaffino and obtained from Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa. Immunofluorescence was achieved using a fluorogenic secondary antibody (AlexaFluor 546, Invitrogen) and DAPI. Myotubes were then imaged at 10x magnification using a fluorescent microscope (Leica DMI 4000B). Antibody specificity was confirmed by the striated appearance of myotubes. Wells (n=2 per condition) were divided into 10 regions and a single image collected from each region (20 images/condition/individual). Images were then processed using open-access software from NCBI (Image J, rsbweb.nih.gov/ij/). Images were split from RGB format to individual components (TRITC and DAPI),

converted to threshold images, and analyzed for positive pixels (myosin heavy chain) or individual particles (nuclei count).

1. Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S, Glass DJ. Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *Am J Physiol Cell Physiol* 2009;296:C1258–70.

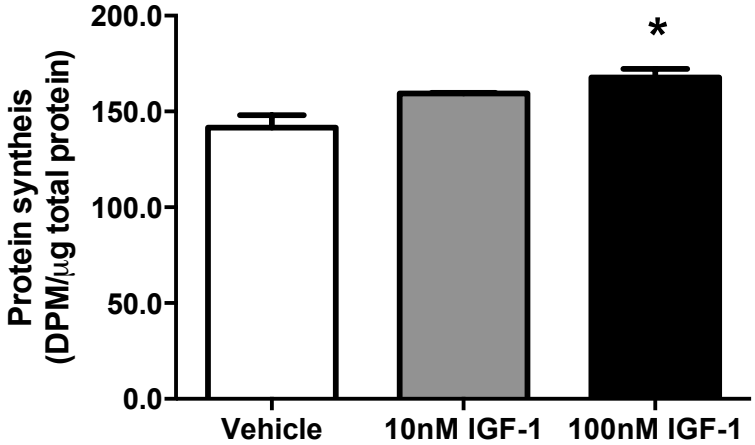

Figure S1. Primary human skeletal muscle myotubes were treated with vehicle, 10nM IGF-1, or 100nM IGF-1 for 24 h then radiolabeled with 5μCi/ml ^{3}H-tyrosine for 2 h, cellular proteins precipitated by 10% TCA, washed in 95% ethanol, and solubilized in 0.2N NaOH. TCA precipitable radioactivity was determined by scintillation counting and normalized to total protein (assessed by bicinchoninic acid assay). Mean ± SEM. n = 3 per condition. *Significantly different vs vehicle, $p<0.05$.

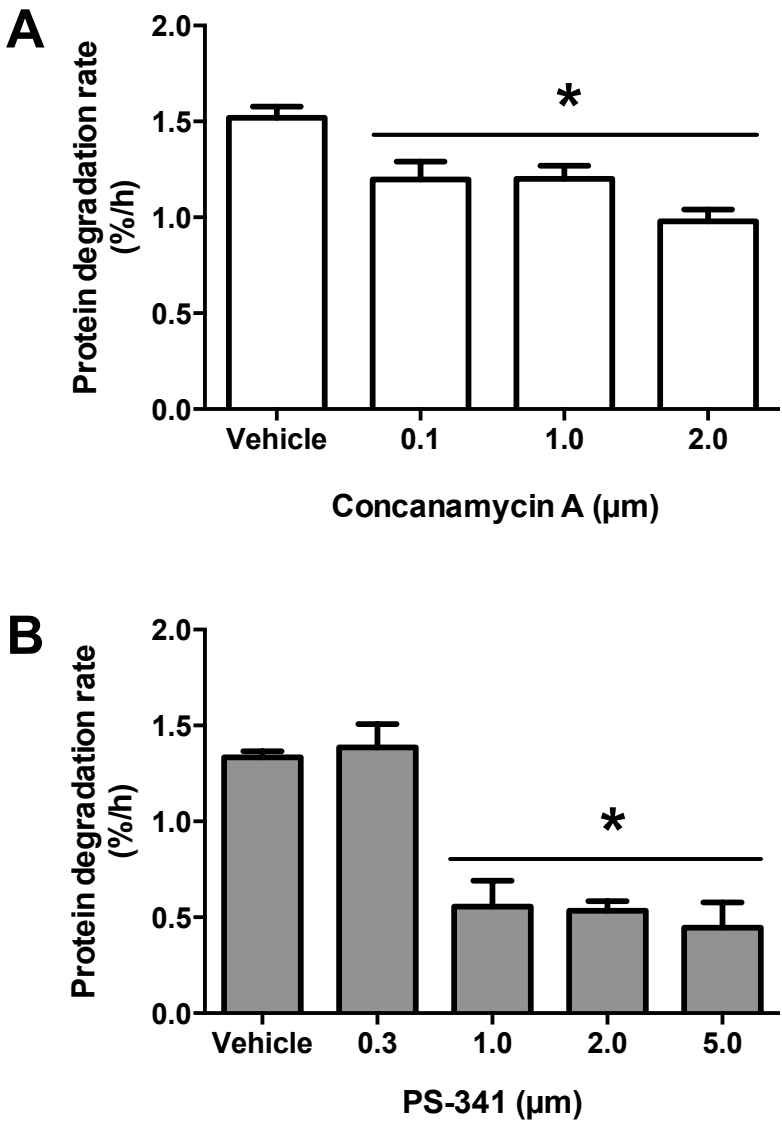

Figure S2. Pooled myotubes (three individuals per group) were radiolabeled with 5ìCi/ml 3H-tyrosine for 24 h, treated with the proteasome inhibitor PS-341 or inhibitor of lysosomal acidification, Concanamycin A, incubated in serum and amino acid free media, and protein degradation rate determine by ^{3}H-tyrosine release over 5 hours. *Significantly different vs vehicle, $p<0.05$.

```
run("Split Channels");

close();

close();

//run("Threshold...");

setAutoThreshold("Default");

setThreshold(7, 29);

run("Convert to Mask");

run("Measure");
```

Figure S3. Macro used in ImageJ64 (NIH) to convert fluorescent images to threshold (bianary) images for quantification of myotube area and nuclei number

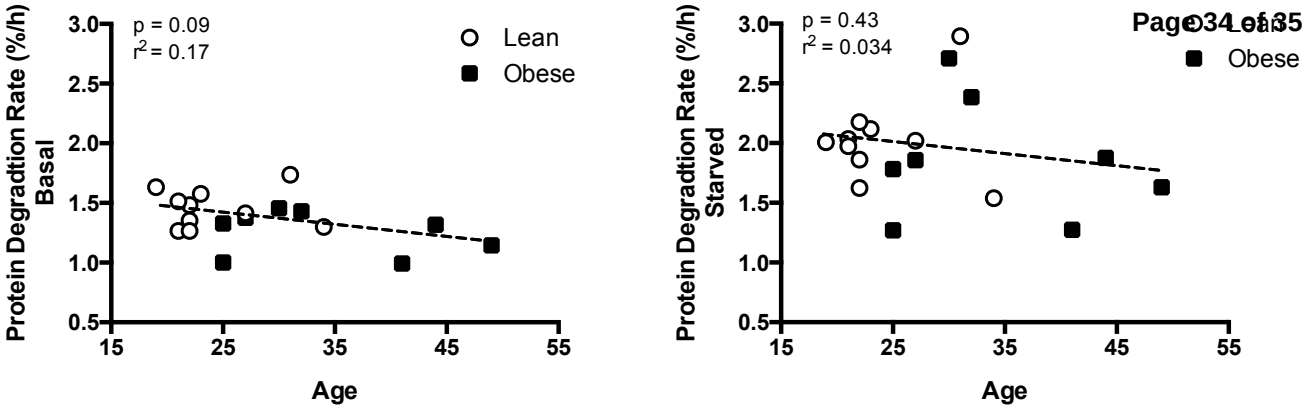

Figure S4. Scatter plot of protein degradation rate individual subject human skeletal muscle myotubes versus age of subject. Human skeletal muscle myotubes were radiolabeled with L-[3,5-3H]-tyrosine for 24h, chased with 2mM non-radioactive tyrosine for 2h, and then 3-4 media samples were taken over 5h. Protein degradation rate was calculated from the regression line of radiolabel release into the media over time as a % total radiolabel incorporated.

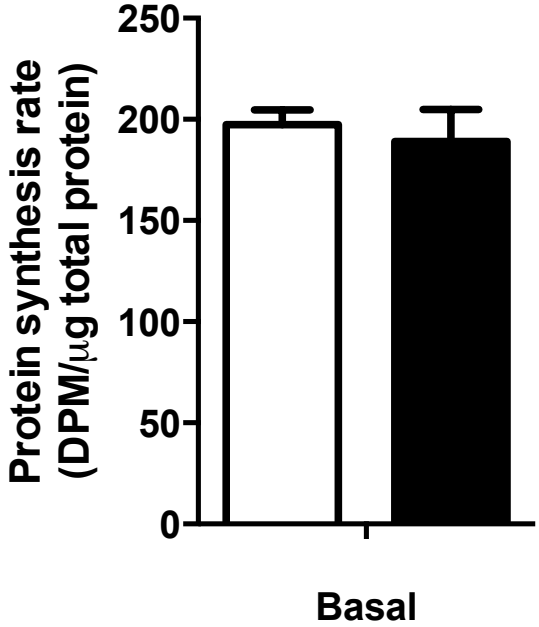

Figure S5. Primary human skeletal muscle myotubes cultured under basal conditions were radiolabeled with 5 μCi/ml ^{3}H-tyrosine for 2 h, cellular proteins precipitated by 10% TCA, washed in 95% ethanol, and solubilized in 0.2N NaOH. TCA precipitable radioactivity was determined by scintillation counting and normalized to total protein (assessed by bicinchoninic acid assay). Mean ± SEM. n = 3 per condition.