Mechanisms involved in follistatin-induced increased insulin action in skeletal muscle

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Background. Skeletal muscle wasting is often associated with insulin resistance. A major regulator of muscle mass is the transforming growth factor β (TGF-β) superfamily, including activin A, which causes atrophy. TGF-β superfamily ligands also negatively regulate insulin-sensitive proteins, but whether this pathway contributes to insulin action remains to be determined.

Methods. To elucidate if TGF-β superfamily ligands regulate insulin action we used an adeno-associated virus gene editing approach to overexpress the activin A inhibitor, follistatin (Fst288) in mouse muscle of lean and diet-induced obese mice. We determined basal and insulin-stimulated 2 deoxy-glucose uptake using isotopic tracers in vivo. Furthermore, to evaluate whether circulating Fst and activin A concentrations are associated with obesity, insulin resistance, and weight loss in humans we analysed serum from morbidly obese subjects before, 1 week, and 1 year after Roux-en-Y gastric bypass (RYGB).

Results. Fst288 muscle overexpression markedly increased in vivo insulin-stimulated (but not basal) glucose uptake (+75%) and increased protein expression and intracellular insulin signalling of AKT, TBC1D4, PAK1, and p70S6K. Importantly, Fst288 completely normalized muscle glucose uptake in insulin-resistant diet-induced obese mice. RYGB surgery doubled circulating Fst and reduced Activin A (-24%) concentration 1 week after surgery before any significant weight loss in morbidly obese normoglycemic patients, while major weight loss after 1 year did not further change the concentrations.

Conclusions. We here present evidence that Fst is a potent regulator of insulin action in muscle and in addition to AKT and p70S6K, we identify TBC1D1, TBC1D4 and PAK1 as Fst targets. A possible role for Fst in regulating glycemic control is suggested because circulating Fst more than doubled post RYGB surgery, a treatment that markedly improved insulin sensitivity. These findings demonstrate the therapeutic potential of inhibiting TGF-β superfamily ligands to improve insulin action and Fst's relevance to insulin resistant conditions in mice and humans.

Introduction

Skeletal muscle is essential for maintaining an independent lifestyle and muscle strength is inversely associated with all-cause mortality [1]. Furthermore, skeletal muscle plays a critical role in glycemic control as it accounts up to 75% of insulin-stimulated glucose disposal in humans [2]. Several common conditions are associated with muscle wasting and/or insulin resistance, including cancer cachexia [3], [4], sarcopenia [5], [6], and type 2 diabetes [2], [7], [8]. However, there are currently no effective therapeutic strategies to improve muscle function in such conditions. Restoration of muscle mass, strength, and insulin action could be highly effective in improving these debilitating conditions.

A major regulator of muscle mass is the transforming growth factor β (TGF-β) superfamily, including activin A and myostatin, among which activin A is most relevant to humans [9]. Activin A and myostatin bind to the activin type II receptor (ActRII) causing atrophy. Clinical trials show that blockade of ActRII using an ActRII antibody (bimagrumab) effectively prevents muscle wasting in sarcopenia [10], with marked improvements in insulin sensitivity and HbA1c in insulin resistant individuals [11], suggesting that TGF-β signalling integrates muscle mass regulation and insulin action.

Follistatin (Fst) is an endogenously expressed inhibitor of activin A and myostatin. Fst binds to and thereby relieves activin A- or myostatin-induced Smad3-mediated suppression of AKT/mTOR signaling [12] and thereby promotes skeletal muscle hypertrophy [13]–[15]. Accordingly, Fst has emerged as a potential therapeutic to ameliorate the deleterious effects of muscle wasting [16]–[18] but it remains to be tested whether the action of Fst involves increased muscular insulin action.

Circumstantial evidence suggests that targeting Fst could indeed be a novel approach to improve insulin sensitivity in muscle. Recent proteomic profiling has shown that Fst regulates PAK1 [19] that is a downstream target of Rac1. Rac1 regulates insulin-stimulated translocation of the glucose transporter, GLUT4 [20], [21] and this pathway is compromised in muscles from obese and type 2 diabetic patients [22]. Furthermore, Fst increases signaling through the AKT/mTOR pathway in unstimulated mouse muscle [12], likely due to upregulation of Akt and mTOR protein expression [17]. Thus, Fst affects insulin regulated proteins but it is unknown if this translates into increased insulin-stimulated intracellular signaling and glucose uptake.

Inspired by these findings, we hypothesized that Fst could be a central regulator of muscular insulin sensitivity in addition to its known effects on muscle mass. We confirmed this hypothesis by showing that Fst overexpression markedly increased insulin sensitivity and completely normalized glucose uptake in insulin resistant mouse

muscle. Furthermore, Roux-en-Y gastric bypass, a treatment that rapidly and markedly improves insulin sensitivity and glycemic regulation, increased circulating Fst together with a decrease in the ActRII ligand activin A. These findings demonstrate the therapeutic potential of Fst and of inhibiting TGF-β superfamily ligands and highlight the relevance of this pathway to insulin resistance in humans.

Methods

Animals. Female C57BL/6J (Taconic, Lille Skensved, Denmark) mice age 20-22 weeks were maintained on a 12 h:12 h light–dark cycle, and group housed at 21-23°C with nesting materials. A 10-week diet intervention was started at 12 weeks of age and mice received either a standard rodent chow diet (Altromin no. 1324; Brogaarden, Horsholm, Denmark), or a 60E% high-fat diet (HFD) (no. D12492; Brogaarden, Denmark) and water ad libitum. Body weight was assessed biweekly. All experiments were approved by the Danish Animal Experimental Inspectorate.

Production of rAAV6 vectors. Production of recombinant adeno-associated viral vectors comprising serotype 6 capsids (rAAV6) and serotype 2 recombinant adeno-associated viral (rAAV6) plasmids encoding the short isoform of human follistatin, known as Fst288, and a non-coding control vector, designated MCS, has been described previously [12] and are herein referred to as rAAV6:Fst288 and rAAV6:MCS respectively. Co-transfection of HEK293T cells with the pDGM6 packaging and the above mentioned rAAV6 plasmid generated type-6 pseudotyped viral vectors that were harvested and purified as described previously [23]. In short, HEK293T cells were plated at a density of 3.2–3.8 × 10^6 cells onto a 15-cm culture dish 8–16 h before transfection with 10 µg of a vector-genome-containing plasmid and 20 µg of the packaging/helper plasmid pDGM6, by means of the calcium phosphate precipitate method to generate pseudotype 6 vectors. 72 h after transfection, the media and cells were collected and homogenized through a microfluidizer (Microfluidics) before 0.22 µm clarification (Millipore). The vector was purified from the clarified lysate by affinity chromatography over a HiTrap heparin column (GE Healthcare), and ultracentrifuged overnight before resuspension in sterile physiological Ringer's solution. The purified vector preparations were titered with a customized sequence-specific quantitative PCR-based reaction (Applied Biosystems).

Transfection of mouse muscles using rAAV6 vectors. Viral particles were diluted in Gelofusine (B. Braun, Germany) (Tibialis anterior: 30 µl, Gastrocnemius: 50 µl) to a dosage of 1×10^9 vector genomes for both muscles. For muscle specific delivery of rAAV6 vectors, 18-20-weeks old mice were placed under general anesthesia (2%

isofluorane in O_2) and mice were injected intramuscularly with rAAV6:Fst288 in the muscles of one leg and control rAAV6:MCS (empty vector) in the contralateral leg. Mice were terminated 2 weeks after rAAV6 administration.

Body composition analysis. For the HFD study, fat mass and lean body mass changes were determined by quantitative magnetic resonance using an Echo MRI scanner at week 9 into the diet intervention.

In vivo 2 deoxy-glucose experiments in vivo. To determine 2 deoxy-glucose (2DG) uptake in muscle, [^{3}H]2DG (Perkin Elmer) was injected retro-orbitally in a bolus of saline containing 66.7 μCi mL^{-1} [^{3}H]2DG (6 μL g^{-1} body weight) in chow and HFD fed mice. The injectate also contained 0.3 U kg^{-1} body weight insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) or a comparable volume of saline. Prior to stimulation, mice were fasted for 3-5 h from 07:00 h and anaesthetized (intraperitoneal injection of 7.5/9 mg (chow/HFD) pentobarbital sodium 100 g^{-1} body weight 15 minutes before the retro-orbital injection). Blood samples were collected from the tail vein immediately prior to insulin or saline injection and after 5 and 10 min and analyzed for glucose concentration using a glucometer (Bayer Contour; Bayer, Münchenbuchsee, Switzerland). After 10 min, tibialis anterior and gastrocnemius muscles were excised and quickly frozen in liquid nitrogen and stored at −80°C until processing. Once tissues were removed, blood was collected by punctuation of the heart, centrifuged and plasma frozen at -80°C. Plasma samples were analyzed for insulin concentration and specific [^{3}H]2DG tracer activity. Plasma insulin was analyzed in duplicate (Mouse Ultrasensitive Insulin ELISA, #80-INSTRU-E10, ALPCO Diagnostics, USA). Tissue specific 2DG uptake was analyzed as described [24], [25].

Human serum Fst and activin A analysis. Serum from 9 obese glucose-tolerant subjects (3 men, 6 women) age 40.1 ±2.8 years, body weight 116.9 ±4.9 kg) scheduled for laparoscopic RYGB at Hvidovre Hospital (Denmark) were examined after a mandatory diet-induced weight loss of minimum 8% (weight loss was 9.2 ± 1.2% pre-surgery) and at 1 week and 1 year post-surgery. Detailed description of the study design and subject characteristics have been reported elsewhere [26]. In the present study, we included patients that were followed for 1 week and 1 year post-surgery. Serum was stored at -80ºC and Fst and activin A analyzed in duplicates using DFN00 and DAC00B ELISA kits, respectively from R&D Systems performed according to manufacturer's instructions. Clinical experiments were approved by the Ethics Committee of Copenhagen and complied with the ethical guidelines of the Declaration of Helsinki II 2000. Written informed consent was obtained from all participants prior to entering the study. The clinical study is registered at www.ClinicalTrials.gov (NCT01202526).

Muscle tissue processing. Mouse muscles were pulverized in liquid nitrogen and homogenized 2 × 0.5 min at 30 Hz using a TissueLyser II bead mill (Qiagen, USA) in ice-cold homogenization buffer (10% glycerol, 1% NP-40, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 20 mM β-glycerophosphate, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 2 mM Na_3VO_4, 10 μg mL^{-1} leupeptin, 10 μg mL^{-1} aprotinin, 3 mM benzamidine). After rotation end-over-end for 30 min at 4°C, supernatants were collected by centrifugation (10,000 g) for 20 min at 4°C.

Immunoblotting. Lysate protein concentrations were measured using the bicinchoninic acid method with bovine serum albumin (BSA) as standard (Pierce). Total protein and phosphorylation levels of relevant proteins were determined by standard immunoblotting techniques loading equal amounts of protein. The primary antibodies used are presented in Table 1. Polyvinylidene difluoride membranes (Immobilon Transfer Membrane; Millipore) were blocked in Tris-buffered saline (TBS)-Tween 20 containing 3% milk or 5% BSA protein for 30–60 min at room temperature. Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Coomassie brilliant blue staining was used as a loading control [27]. Bands were visualized using the Bio-Rad ChemiDoc MP Imaging System and enhanced chemiluminescence (ECL+; Amersham Biosciences).

Antibody	**Dilution (Primary)**	**Cat number**	**Company**
Follistatin	1:100 (in 5% BSA)	#sc-365003	Santa Cruz
AKT2	1:1000 (in 3% milk)	#3063	Cell Signaling Technology
Phosphorylated (p)-AKT^{Ser473}	1:1000 (in 3% milk)	#9271	Cell Signaling Technology
p-AKT^{Thr308}	1:1000 (in 3% milk)	#9275	Cell Signaling Technology
P70S6K	1:1000 (in 3% milk)	#9202	Cell Signaling Technology
p-p70$S6K^{Thr389}$	1:1000 (in 3% milk)	#9205	Cell Signaling Technology
TBC1D4	1:1500 (in 5% BSA)	#ab189890	Abcam
p-TBC1$D4^{Thr642}$	1:1000 (in 5% BSA)	#D27E6	Cell Signaling Technology
TBC1D1	1:1000 (in 5% BSA)	#5929	Cell Signaling Technology
GLUT4	1:1000 (in 3% milk)	#PA1-1065	Thermo Fisher Scientific
PAK1	1:1000 (in 5% BSA)	#2602	Cell Signaling Technology
p-$PAK^{Thr423/402}$	1:1000 (in 5% BSA)	#2601	Cell Signaling Technology
Hexokinase II	1:1000 (in 3% milk)	#2867	Cell Signaling Technology

OXPHOS	1:1000 (in 3% milk)	MS604-300	Abcam
p-AMPK^{Thr172}	1:1000 (in 3% milk)	#2531	Cell Signaling Technology

Statistical analyses. Results are shown as mean ± standard error of the mean (SEM) with the individual values shown for figures with a paired design (rAAV6:MCS vs rAAV6:Fst288 treated legs within the same mouse). Statistical testing was performed using two-way (repeated measures when appropriate) ANOVA as applicable. Sidak post hoc test was performed when ANOVA revealed significant main effects or interactions for multiple comparisons. Statistical analyses were performed using GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA, USA). The significance level was set at $\alpha = 0.05$.

Results

Insulin-stimulated glucose uptake is improved by Fst288 overexpression. Mice were injected with rAAV6:Fst288 into the muscles of one leg while the contralateral leg was injected with an empty viral vector, rAAV6:MCS (Fig. 1A). Two weeks after the injection, Fst protein expression was markedly increased compared to the control muscle in tibialis anterior (TA) and gastrocnemius muscles injected with rAAV6:Fst288 (Fig. 1B). Fst288 delivery increased TA muscle mass by 33% on average compared to the control muscle (Fig. 1C). This was similar to previous reports [12]. Mice (one Fst288 treated leg and one control leg) were stimulated with an insulin dose of 0.3U kg^{-1} as described in [25], which lowered blood glucose concentration by 3 mM at the 10 minutes time point compared to saline injected mice (Fig. 1D). Fst288 overexpression resulted in almost a doubling of insulin-stimulated glucose uptake in TA compared to the control muscle (Fig. 1E). Similarly, in gastrocnemius muscle insulin-stimulated glucose uptake was 72% higher in rAAV6:Fst288 treated muscles compared to the contralateral rAAV6:MCS-treated control 2 weeks after rAAV administration (Fig. 1F). Basal glucose uptake was not affected by rAAV6:Fst288.

Fst288 overexpression augments insulin-stimulated AKT and PAK1 signaling in muscle. As insulin stimulated, but not basal, glucose uptake was markedly increased, we next sought to determine the molecular explanation for this and used western blotting techniques to analyze intracellular insulin signaling as illustrated in Fig. 2A. Overexpression of Fst288 significantly upregulated insulin-stimulated p-AKT^{Thr308} (+120%) in TA, but not in gastrocnemius muscles (Fig. 2B). This was also the case for the mTORC2 site, p-AKT^{Ser473} [28] (TA:+50%, Fig. 2C). The AKT signaling pathway bifurcates into TBC1D4, important for glucose uptake, and mTORC1, crucial

for muscle mass regulation. In line with increased p-AKT in TA, we observed a 68% increase in insulin-stimulated p-TBC1D4^{Thr642} compared to rAAV6:MCS (Fig. 2D). Insulin-stimulated TBC1D4^{Thr642} was also increased (+25%) in gastrocnemius with Fst288 overexpression even though p-AKT was not significantly increased. Fst288 overexpression resulted in a remarkable increase (+400%) in insulin-stimulated (but not basal) p-p70S6K^{Thr389}, an endogenous substrate of mTORC1 (Fig. 2E), suggesting that Fst increases anabolic insulin action in muscle. A lesser described, but likely equally important signalling pathway for insulin action, is the Rac1-mediated pathway. Rac1's downstream target, PAK1 has been identified by proteomic muscle profiling to be a putative target for Fst [19], [29], although actual activation through this pathway has not been investigated. Indeed, insulin-stimulated phosphorylation of PAK at its activation site Thr423, was upregulated by rAAV6:Fst288 (TA: +56%, gastrocnemius: +29%) compared to rAAV6:MCS control (Fig. 2F). Thus, Fst288 augments signaling in skeletal muscle to increase both glucose uptake and muscle mass in response to insulin. In contrast, no effect of Fst288 was observed on any of the analyzed signaling proteins during saline treatment, suggesting that the molecular modifications in this signaling pathway in response to Fst depend on the stimulation with insulin.

Fst288 overexpression increases muscle content of key insulin sensitive signaling proteins but does not affect proteins involved in glucose handling. We next sought to investigate whether the increase in insulin sensitivity could be due to an increased capacity of the muscles to enhance insulin-mediated molecular signals. We therefore analyzed the total content of proteins involved in insulin signal transduction and glucose handling. As expected, we observed no difference on protein expression between the 10 minutes saline or insulin-stimulated conditions (not shown), and thus they were pooled to increase statistical power and accuracy. In TA muscles, we found a marked upregulation of AKT2 by rAAV6:Fst288 (+90%, Fig. 3A) and TBC1D4 (+60%, Fig. 3B). In contrast, TBC1D1, a protein with 90% homology to TBC1D4 [30], was downregulated to half of the rAAV6:MCS treated control muscles (Fig. 3C). Similar to AKT2 and TBC1D4, PAK1 protein was doubled (Fig. 3D) and total p70S6K protein tripled (Fig. 3E). Because the levels of AKT2, TBC1D4, and PAK1 increased, the ratio of p-protein/total protein was similar for rAAV6:Fst288 and rAAV6:MCS administered muscles (Supplemental Fig. 3), suggesting that Fst288 overexpression increased protein expression and activity in the insulin signaling pathway to a similar extent. In contrast, the glucose handling machinery eg. GLUT4 (glucose transporter) and hexokinase (a limiting enzyme in glucose uptake [31]), was not regulated by Fst288 (Fig. 3F+G). We also analyzed complexes of the electron transport chain and found that all the investigated complexes were unaffected by Fst288, except for CIV which was mildly downregulated (-13%) (Fig. 3H). We found no effect in the phosphorylated levels of the cell stress sensor AMP-activated protein kinase (AMPK) (Supplemental Fig. 3), suggesting that Fst did not alter

intracellular metabolic stress. We observed similar regulation of proteins in gastrocnemius muscle (data not shown). These findings show that Fst alters the expression of key insulin sensitive proteins but does not regulate proteins directly involved in glucose handling or β-oxidation.

Fst288 overexpression restores insulin resistance in diet-induced obese mice. Since we observed that Fst markedly increased insulin-stimulated glucose uptake in skeletal muscle, we next asked if Fst288 could be relevant for restoring insulin resistant conditions. We fed mice a 60E% high-fat diet (HFD) for 10 weeks, an intervention known to induce obesity and muscular insulin resistance [32]. Mice were injected with rAAV6: Fst288 eight weeks into the diet intervention, at a time point when the HFD mice weighed significantly more than chow fed control mice (Fig. 4A), displayed markedly increased adiposity (Fig. 4B), and reduced relative lean body mass (Fig. 4C). Importantly, at this time of the HFD intervention, muscular insulin resistance has already set in [33], [34], thus allowing us to test the therapeutic potential for Fst in restoring insulin sensitivity in already insulin resistant muscle. That the mice were indeed insulin resistant on the experimental day was evidenced by a blunted blood glucose response to insulin (dose 0.3U kg^{-1}) in the HFD fed mice compared with chow (Fig. 4D) [35], [36]. We verified that TA muscle mass was increased by 33% after 2 weeks (Fig. 4E), and that Fst was expressed in the rAAV6:Fst288 treated muscles (Fig. 4F). The HFD intervention did not alter basal or insulin-induced intracellular signaling judged by p-AKT^{T308} and p-AKT^{S473} (data not shown) in agreement with some other reports [25], [37]. Also, basal muscle glucose uptake in HFD mice was unaffected by diet and Fst288 administration (Fig. 4G). HFD induced a 40% reduction in insulin-stimulated glucose uptake in TA and gastrocnemius muscles (Fig. 4H) when comparing with the rAAV6:MCS muscles from chow fed mice. Remarkably, Fst288 treatment completely restored insulin-stimulated glucose uptake (Fig. 4H,I,J) in the insulin resistant muscles of HFD fed mice. Having found that Fst288 overexpression increased glucose uptake in muscle and could restore insulin action, we next asked what the metabolic fate of the extra glucose taken up was. We therefore measured muscle glycogen. In both insulin sensitive chow fed and insulin resistant HFD fed mice, Fst288-treated TA muscles contained significantly more glycogen (+45%), compared to controls (Fig. 4K). These findings suggest that the larger amount of glucose that is taken up by the rAAV6:Fst288-administered muscles is, at least in part, incorporated into glycogen. Together, our findings suggest that Fst treatment could be a potent strategy to increase insulin sensitivity, also in insulin resistant muscles. A summary of our findings is graphically illustrated in Fig. 5.

Bariatric surgery increases Fst and reduces activin A in obese insulin resistant individuals. Having established that Fst regulate insulin action in skeletal muscle, we next investigated the relationship between insulin action and

TGF-β ligands in humans. We measured circulating Fst and activin A concentrations in a group of obese insulin resistant (but normoglycemic) individuals before, 1 week, and 1 year after treatment by Roux-en-Y gastric bypass surgery (RYGB). This procedure has consistently been shown to produce marked and rapid improvements in insulin resistance and glycemic control and those data for the currently studied subjects has been published previously [26]. One week following RYGB circulating Fst drastically increased (+108%, Fig. 6A) while circulating activin A was mildly reduced (-26%, Fig. 6B). At the same time hepatic insulin sensitivity double, while the peripheral insulin resistance did not differ from before surgery [26], [38]. Importantly, the ratio between Fst and activin A (which determines actual activin A signaling through the ActRII), was 217% increased 1 week post RYGB (Fig. 6C) where weight loss has not yet set in (Fig. 6D). These levels were largely maintained 1 year post surgery (Fst +111%, activin A +13%, ratio +168%) when peripheral insulin sensitivity was improved (+200%), and hepatic insulin sensitivity was further improved (+50 %) compared with 1 week after surgery. These findings indicate that in humans circulating Fst is markedly altered by metabolic status beyond the effect of body weight loss and could thus play an unrecognized critical role in maintenance of insulin sensitivity, glucose homeostasis and muscle mass.

Discussion

A major discovery in the current study was that Fst288 overexpression markedly augmented insulin action on glucose uptake and intracellular signaling and completely restored insulin resistance in diet-induced obese mouse muscles. To the best of our knowledge, we are the first to describe and mechanistically explain this insulin sensitizing effect of Fst in muscle. Our findings are supported by a recent study using a transgenic (TG) approach to overexpress Fst in the entire skeletal muscle mass [39]. In that study, Fst-TG mice displayed better glucose tolerance compared with control mice, however tissue-specific insulin action was not investigated. Other circumstantial evidence supports our results that Fst is a positive regulator of insulin sensitivity in muscle. For example, pharmacological inhibition or genetic deletion of the Fst targets, myostatin or activin A, protects against HFD-induced obesity [40]–[42]. Such findings support our results that Fst is a positive regulator of insulin sensitivity in muscle. Importantly, the role for this pathway in insulin action is also evident from clinical trials in humans. Here, the ActRII inhibitor bimagrumab markedly improved insulin sensitivity and HbA1c in insulin resistant individuals [11]. None of the above studies were able to ascribe the increased insulin sensitivity to a specific tissue, but our results suggest that likely, improved skeletal muscle glucose uptake underlies, at least some of, the beneficial effects of inhibiting ActRII or its ligands.

Intriguingly, these and our results appear to contradict a recent study showing that when Fst was overexpressed in the liver, insulin sensitivity and glucose tolerance was impaired in mice with genetically-induced liver insulin resistance [43]. However, in that study viral liver-expression of Fst markedly altered RNA expression profiles of the liver, probably affecting insulin sensitivity by confounding unknown factors. Furthermore, Fst was not overexpressed in skeletal muscle. Our data firmly show that Fst288 overexpression improves insulin action in a set-up where the effect can be isolated to muscular effects, as the Fst288 variant used in our muscle study typically remains localized in the immediate vicinity of the cell from which it is secreted [44]. This is in contrast to the longer circulating Fst315 isoform. Clearly, future work should investigate the tissue-specific metabolic effects of distinct Fst isoforms.

Another major finding of our study was the marked up-regulation of circulating Fst in obese insulin resistant humans following RYGB surgery, a treatment that markedly and rapidly improved insulin sensitivity [26], [38]. Furthermore, the TGF-β ligand, activin A was reduced following RYGB. As a result, the Fst/activin A ratio increased, suggesting a marked reduction of the ActRII ligand, activin A available to activate TGB-β signaling. These results may have broader implications also to adipose tissue function, as Fst can induce browning [45], which could promote increased energy expenditure and thereby protect from obesity. While our findings show that circulating Fst is markedly upregulated in humans by a treatment that improves glucose homeostasis, future studies should confirm inactivation of ActRII signaling in muscle biopsy samples.

In humans, the liver is a key contributor to circulating Fst, which is regulated by the glucagon-to-insulin ratio [46], further suggesting that circulating Fst is closely related to energy metabolism. However, contrasting with Fst´s proposed beneficial role in insulin sensitivity, plasma Fst is actually slightly elevated in patients with type 2 diabetes [47] and in nonalcoholic fatty liver disease [48]. Those studies did not analyze activin A. Thus the Fst/activin A ratio could not be determined, which would be the relevant read-out for actual activation/inactivation of ActRII. Thus, the exact cause(s) and consequence(s) of circulating Fst remain to be determined.

The molecular mechanism by which Fst regulates muscular insulin sensitivity was investigated in our study. Fst288 overexpression caused upregulation of key mediators of glucose uptake, including, AKT, TBC1D4, and PAK1 and enhanced their responsiveness to insulin. Furthermore, the insulin-responsiveness of p-p70S6K, a reporter of mTORC1 activity, was increased by 4-fold. Seminal studies have shown that phosphorylated AKT induces mTOR-mediated phosphorylation of p70S6K in skeletal muscle [49], resulting in muscle hypertrophy [50]. Although p70S6K seems to be largely dispensable for Fst-induced hypertrophy [12], [51], our study shows that Fst288 might also augments insulins action on anabolic signals that regulate muscle mass. This could be relevant as a blunted

protein anabolic response to insulin has been proposed in obesity [52] and T2D [7], although this was not directly tested in our study. Interestingly, despite an upregulated total amount of proteins in the insulin signaling pathways, we observed no effect of Fst288 on basal intracellular signaling or basal glucose uptake. This suggests that, while Fst increases the capacity for insulin signaling to be activated, insulin is needed to make use of this capacity. Being able to turn this pathway off in the presence of Fst is likely essential to maintain cellular homeostasis, as constitutive activation of Akt-mTOR would result in activation of negative feedback loops with proven adverse effects on muscle [53]. Whether Fst could improve skeletal muscle protein synthesis in situations of anabolic insulin resistance often found in ageing [6], would be important to determine and could have implications for the progression of sarcopenia in older subjects.

Interestingly, we observed that Fst288 regulated the Rab GAP proteins in opposite directions, TBC1D4 up and TBC1D1 down. These proteins regulate GLUT4 translocation and protein expression [54]. Downregulation of TBC1D1 would be expected to reduce insulin actin on glucose uptake, as TCB1D1 knock-out mouse muscles are insulin resistant, likely due to a 50% reduction in GLUT4 protein [55]. However on the contrary, reduced TBC1D1 protein in our study occurred with improved insulin sensitivity and normal GLUT4 protein expression. Interestingly, similar to the increased TBC1D4 phosphorylation observed in our study, some (but not all [56], [57]) studies find that greater post-exercise insulin-stimulated glucose uptake was accompanied by greater TBC1D4 (but not TBC1D1) phosphorylation [58], [59]. Thus, an outstanding question is whether Fst could increase post-exercise insulin sensitivity. Given that our findings show TBC1D4 phosphorylation as a target for Fst, it would be relevant for future studies to elucidate a potential role for Fst in post-exercise enhanced glucose uptake, which also impinges on TBC1D4 [58], [59].

That Fst markedly affects insulin action in muscle could have significant implications, as Fst treatment and ActRII inhibition are currently under investigation to treat muscle wasting conditions, including Becker muscular dystrophy [18] and sarcopenia [10]. While our study only investigated the effect of short-term Fst288 treatment, clinical trials have found no adverse effects of Fst or pharmacological ActRII inhibition months after gene transfer or treatment in humans [11], [60]. Results from our study suggest that this could be ascribed to the fact that Fst does not affect basal intracellular signaling, while enhancing insulin action. This underlines the therapeutic potential and safety for Fst and ActRII based therapies to improve both muscle mass and as reported in our study, to enhance muscular insulin sensitivity and glucose uptake.

Declaration of Interests: The authors declare no competing interests.

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Author contributions: X.H., L.L.V.M., E.A.R, and L.S. designed the study. X.H., L.L.V.M., E.D.G., and L.S., designed and conducted the mouse study, with experimental contribution from T.E.J, C.H.O, Z.L., J.R.K., J.D., and P.G. K.N.B-M and S.M. carried out the human experiments. X.H., L.L.V.M., E.A.R., and L.S. drafted the paper. All authors contributed to the final version of the manuscript.

Figure 1. Effect of Follistatin (Fst288) overexpression on muscle glucose uptake. A) Illustration of experimental set-up: recombinant adeno-associated virus encoding Fst288 (rAAV6:Fst288) was injected into tibialis anterior and gastrocnemius muscles of one leg, while the contralateral muscles were injected with rAAV6:MSC as control. **B)** Representative blots of Follistatin in muscle. **C)** Muscle mass of tibialis anterior (n=14). **D)** Blood glucose levels measured before (0 min) and 5 min and 10 min following retro-orbital insulin (0.3U kg^{-1} body weight) or saline injection 2 weeks after rAAV6:Fst288 administration in mice (n=6-8). Basal and insulin-stimulated 2-deoxy-glucose (2DG) uptake and fold-change (over basal) in **E)** tibialis anterior and **F)** gastrocnemius muscles from mice 2 weeks after intramuscular rAAV6:Fst288 administration (n=6-8). Effect of insulin-stimulation is indicated by †† $p < 0.01$; ††† $p < 0.001$. Statistically significant effect of Fst288 overexpression is indicated by */***$p < 0.05/0.001$. Values are shown as individual values or/and mean ± SEM.

Figure 2. Insulin-stimulated intracellular signaling in skeletal muscle following rAAV6:Fst288 administration. A) Graphic illustration of the signaling pathways analyzed. Basal and insulin-stimulated phosphorylated (p), **B)** p-AKT^{Thr308}, **C)** p-AKT^{Ser473}, **D)** p-TBC1D4^{Thr642}, **E)** p-p70S6K^{Thr389}, **F)** p-PAK1^{Thr423} in tibialis anterior and gastrocnemius muscles (n=5-8, one mouse sample excluded due to poor quality blot). **G)** Representative blots. Statistically significant effect of Fst288 overexpression on insulin signaling is indicated by *p < 0.05; ***p < 0.001. Effect of insulin-stimulation in Fst288 groups or main effect of insulin stimulation is indicated by††/††† p < 0.01/0.001. Values are shown as individual values and mean ± SEM.

Figure 3. Effect of Fst288 overexpression on proteins involved in insulin signaling and glucose handling. A) AKT2, **B)** TBC1D4, **C)** TBC1D1, **D)** PAK1, **E)** p70S6K, **F)** GLUT4, **G)** HKII, and **H)** complexes of the electron transport chain in tibialis anterior muscle following 2-week rAAV6:Fst288 overexpression in muscle (n=13-14, one mouse excluded due to poor blot). **I)** Representative blots. Statistically significant effect of Fst288 overexpression is indicated by *$p<0.05$; **p < 0.01; ***p < 0.001. Values are shown as individual values and mean ± SEM.

Figure 4. Effect of Fst288 overexpression in insulin-resistant obese mice. A) Body weight, **B)** Fat mass, and **C)** lean mass of mice fed a standard chow diet or 60E% high-fat diet (HFD) for 10 weeks (n = 14–16). **D)** Blood glucose in response to insulin (retro-orbitally injected, 0.3U kg^{-1} body weight) (n=8-9). **E)** Muscle mass of tibialis anterior muscle (n=14-16). **F)** Representative blots of Fst in tibialis anterior and gastrocnemius muscle 2 weeks following intramuscular rAAV6:Fst288 injection. **G)** Basal and **H)** insulin-stimulated 2 deoxy-glucose (2DG) uptake in muscles intramuscularly injected with rAAV6:Fst288 or rAAV6:MCS (control). Fold change of insulin-stimulated 2DG uptake in HFD over chow rAAV6:MCS in **I)** tibialis anterior muscle and **J)** Gastrocnemius. **K)** Glycogen content in tibialis anterior muscle (n=6-8). Statistically significant effect of Fst288 overexpression is indicated by */***p < 0.05/0.001. Statistical significance between HFD and chow control mice is indicated by ##/### p < 0.01/0.001. Values are shown as individual or/values and mean ± SEM.

Figure 5. A graphical illustration of our findings. Fst288 muscle overexpression markedly increased insulin-stimulated (but not basal) intracellular insulin signalling of AKT, TBC1D4, PAK1, and p70S6K. In lean insulin sensitive mice, muscle-directed Fst288 overexpression increased skeletal muscle insulin-stimulated glucose uptake. Fst288 completely normalized muscle glucose uptake in insulin-resistant obese mice.

Figure 6. Serum Fst and activin A concentrations in obese insulin resistant individuals before and following Roux-en-Y gastric bypass surgery (RYGB). Serum concentrations of **A)** Fst, **B)** activin A, **C)** ratio between Fst

and activin A before and 1 week and 1 year after RYGB, a procedure that markedly improves glycemic control. **D)** body weight. (n=9, however for 2 data points a value is missing, as indicated in the graph showing individual values). Statistically significant effect of RYGB is indicated by #/##/###$p < 0.05/0.001/0.0001$. Values are shown as individual values or/and mean ± SEM.

Supplemental Figure 3. The ratio of phosphorylated (p)-protein/total (t)-protein **A)** of AKT^{Thr308} and **B)** $\mathbf{AKT}^{Ser473}$, **C)** $TBC1D4^{Thr642}$, **D)** $p70S6K^{Thr389}$, and **E)** $PAK1^{Thr423}$ in tibialis anterior and gastrocnemius. **F)** Phosphorylated level of $AMPK^{Thr172}$ in tibialis anterior. Statistically significant effect of Fst288 overexpression is indicated by $*p < 0.05$. Main effect of insulin-stimulation is indicated by †† $p < 0.01$; ††† $p < 0.001$. Values are shown as mean with individual values. n=5-8, One or two samples excluded due to poor blot.

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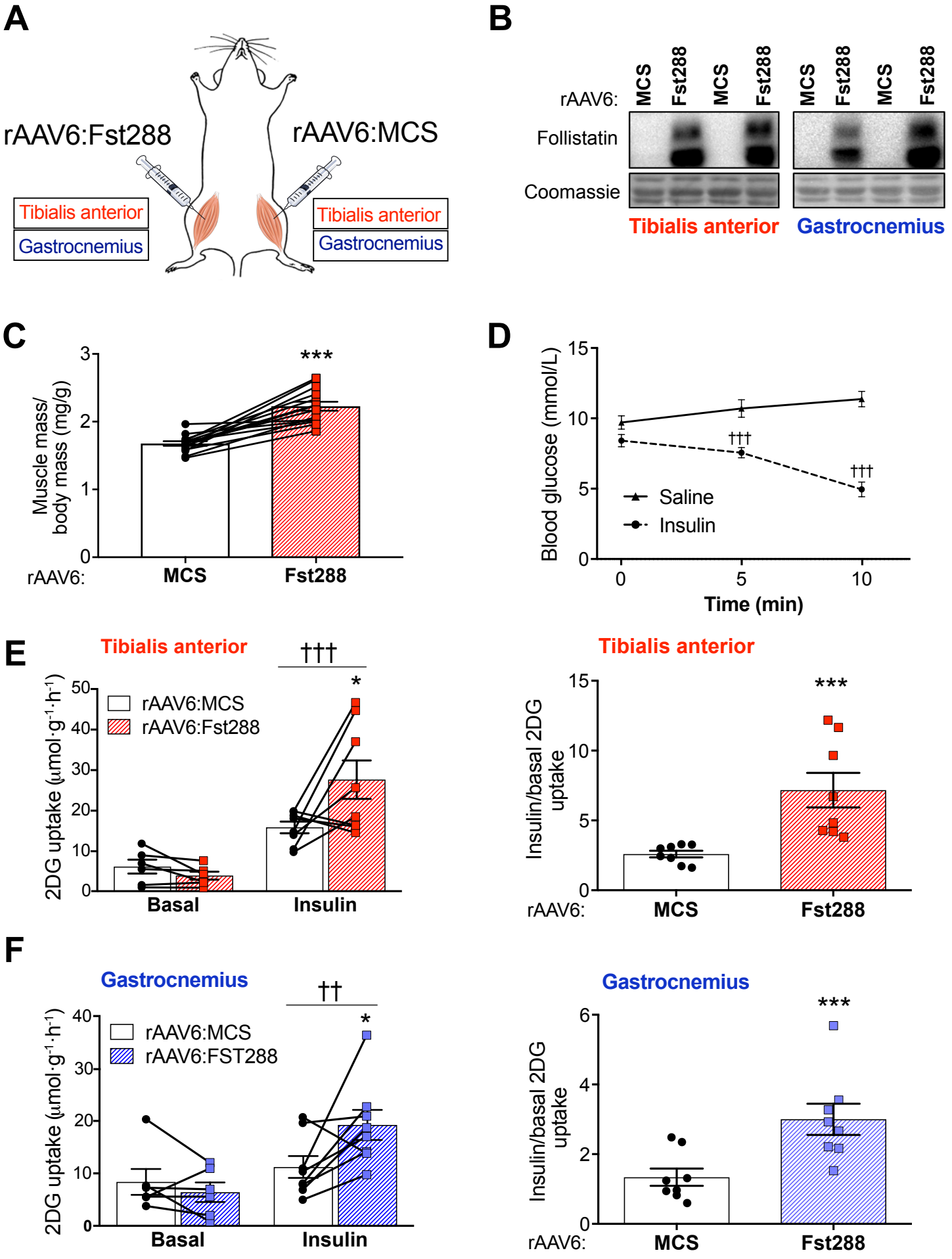
Figure 1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Gastrocnemius
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Gastrocnemius
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Fst288
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Gastrocnemius
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Muscle mass/ body mass (mg/g)
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Insulin
Tibialis anterior
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2DG uptake (µmol·g-1·h-1)
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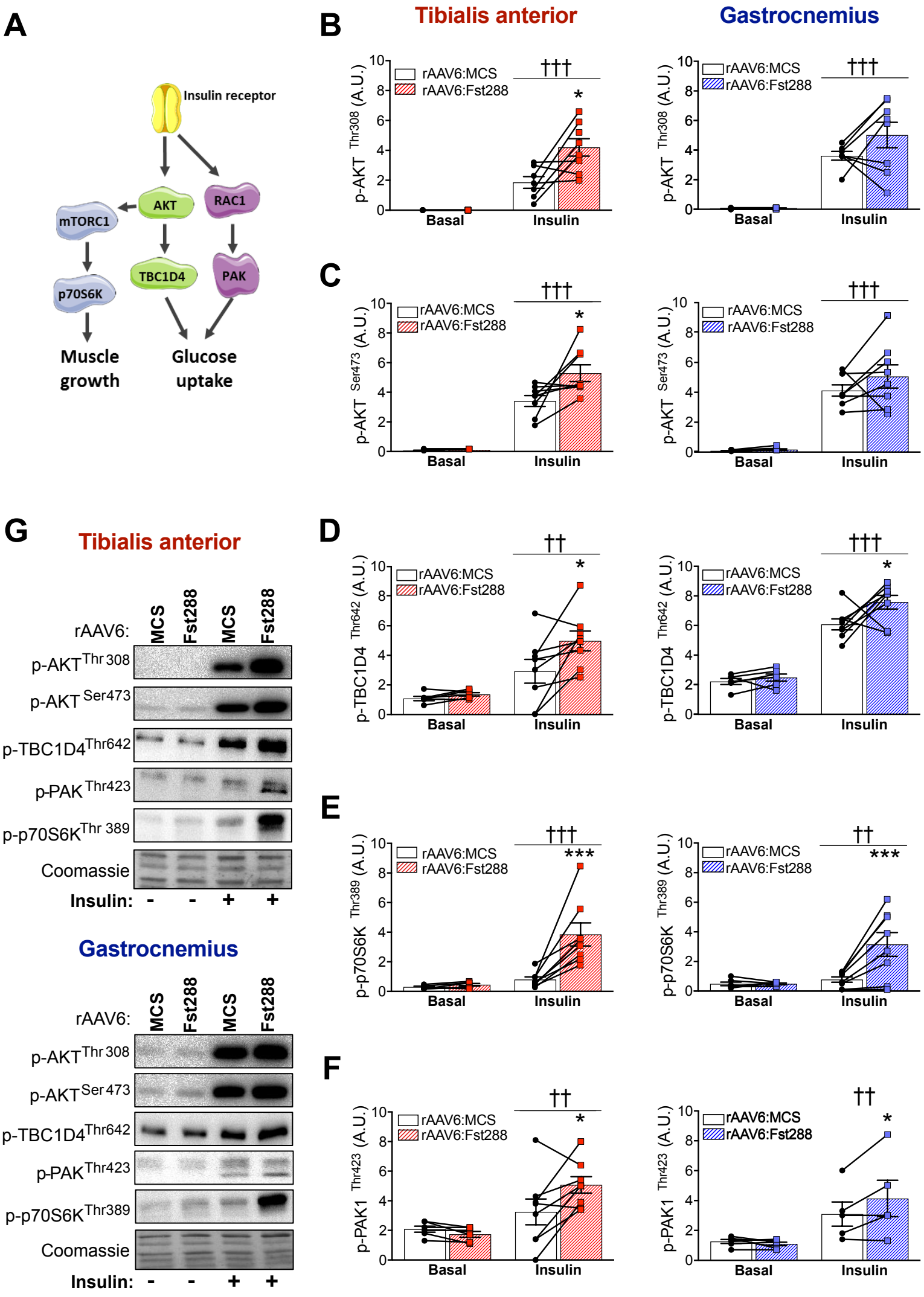
Fst288

Figure 2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p-AKT Thr308 (A.U.)
Basal
Insulin
C
p-AKT Ser473 (A.U.)
D
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F
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Figure 3

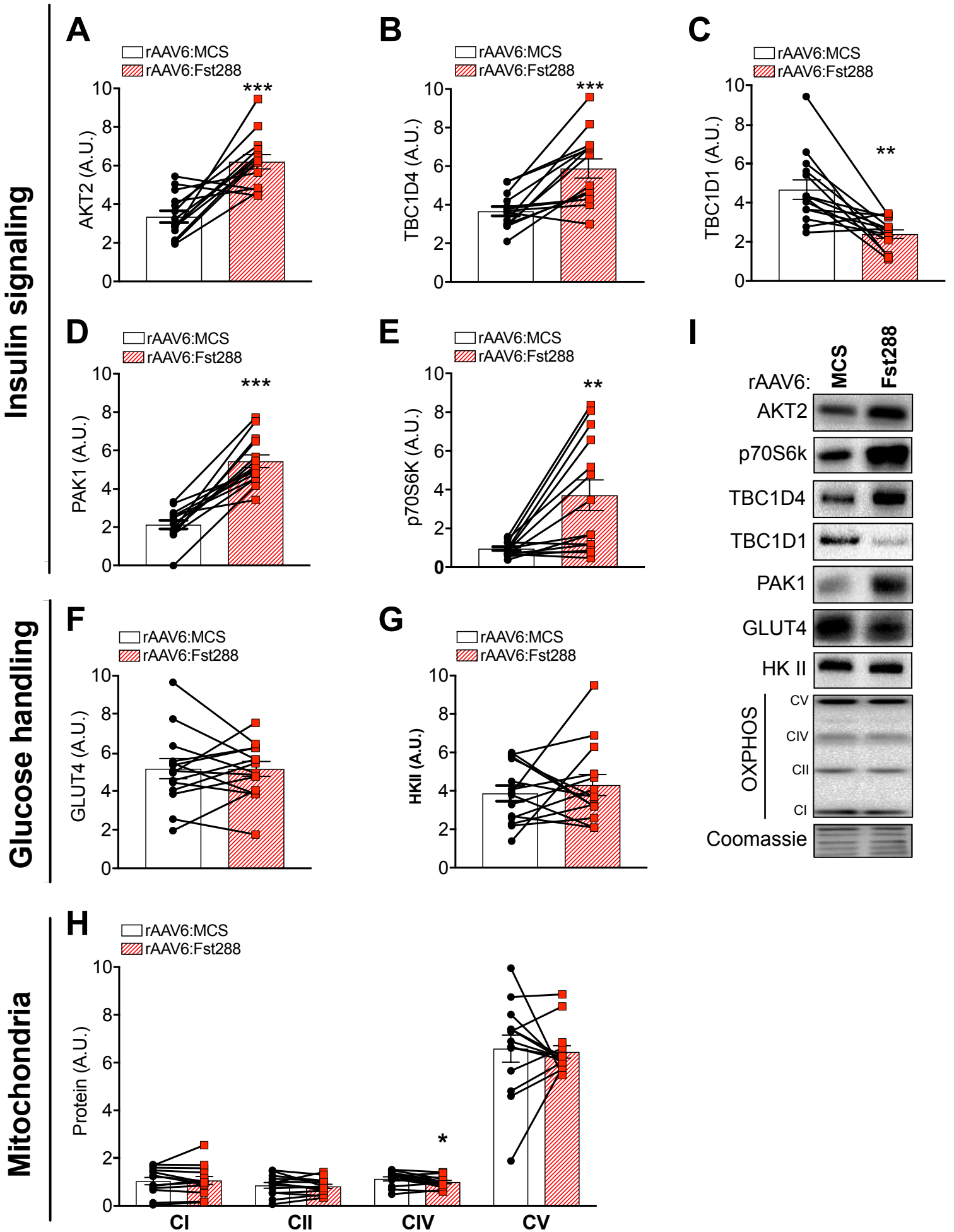
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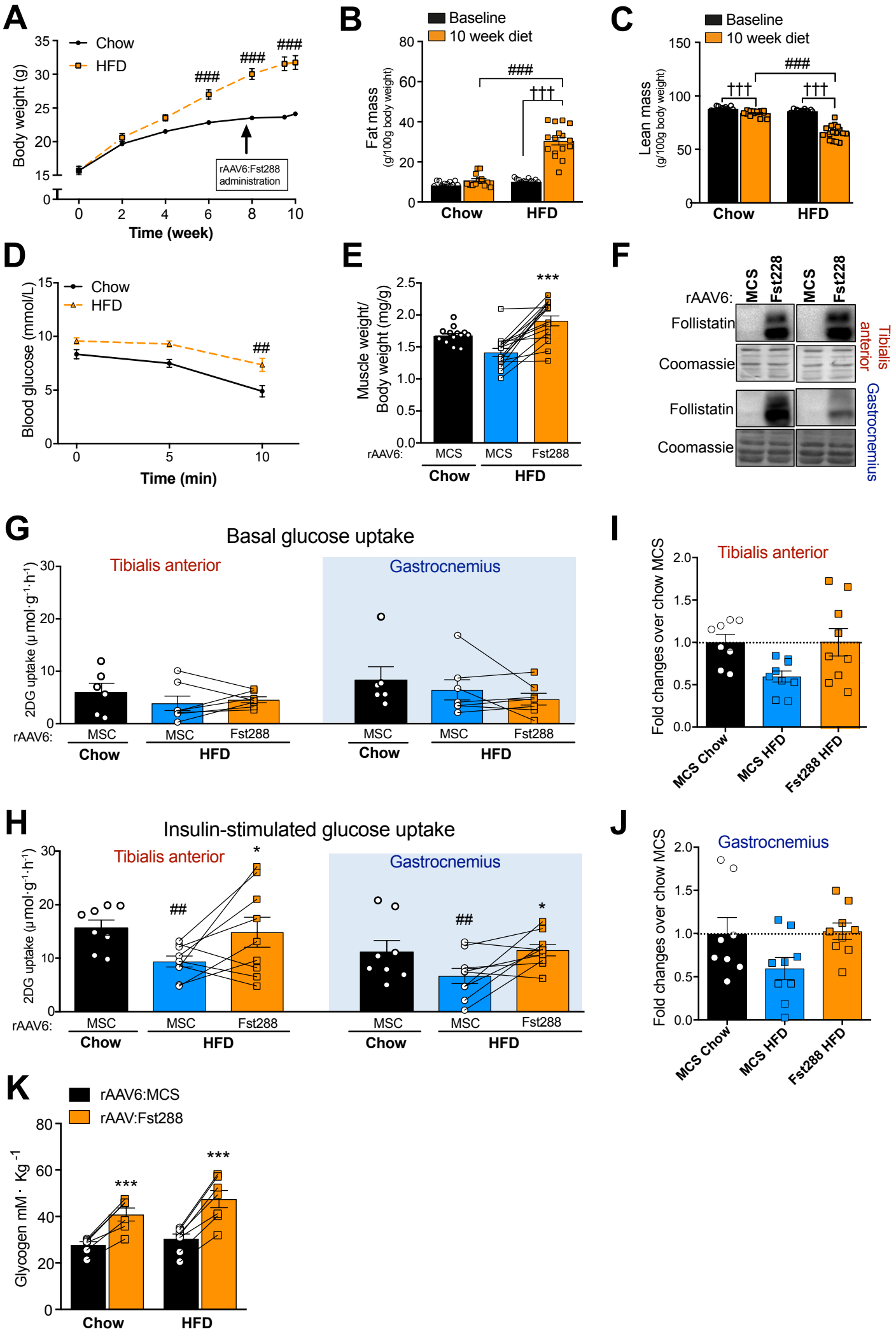
Figure 4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Chow
HFD
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Time (week)
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10 week diet
Fat mass (g/100g body weight)
Chow
HFD
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†††
C
Baseline
10 week diet
Lean mass (g/100g body weight)
Chow
HFD
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†††
D
Chow
HFD
Blood glucose (mmol/L)
Time (min)
##
E
Muscle weight/ Body weight (mg/g)
rAAV6:
MCS
MCS
Fst288
Chow
HFD
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F
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MCS
Fst228
MCS
Fst228
Follistatin
Coomassie
Follistatin
Coomassie
Tibialis anterior
Gastrocnemius
G
Basal glucose uptake
Tibialis anterior
Gastrocnemius
2DG uptake (μmol·g-1·h-1)
rAAV6:
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MSC
Fst288
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MSC
Fst288
Chow
HFD
Chow
HFD
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Tibialis anterior
Fold changes over chow MCS
MCS Chow
MCS HFD
Fst288 HFD
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Insulin-stimulated glucose uptake
Tibialis anterior
Gastrocnemius
2DG uptake (μmol·g-1·h-1)
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MSC
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MSC
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Gastrocnemius
Fold changes over chow MCS
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Chow
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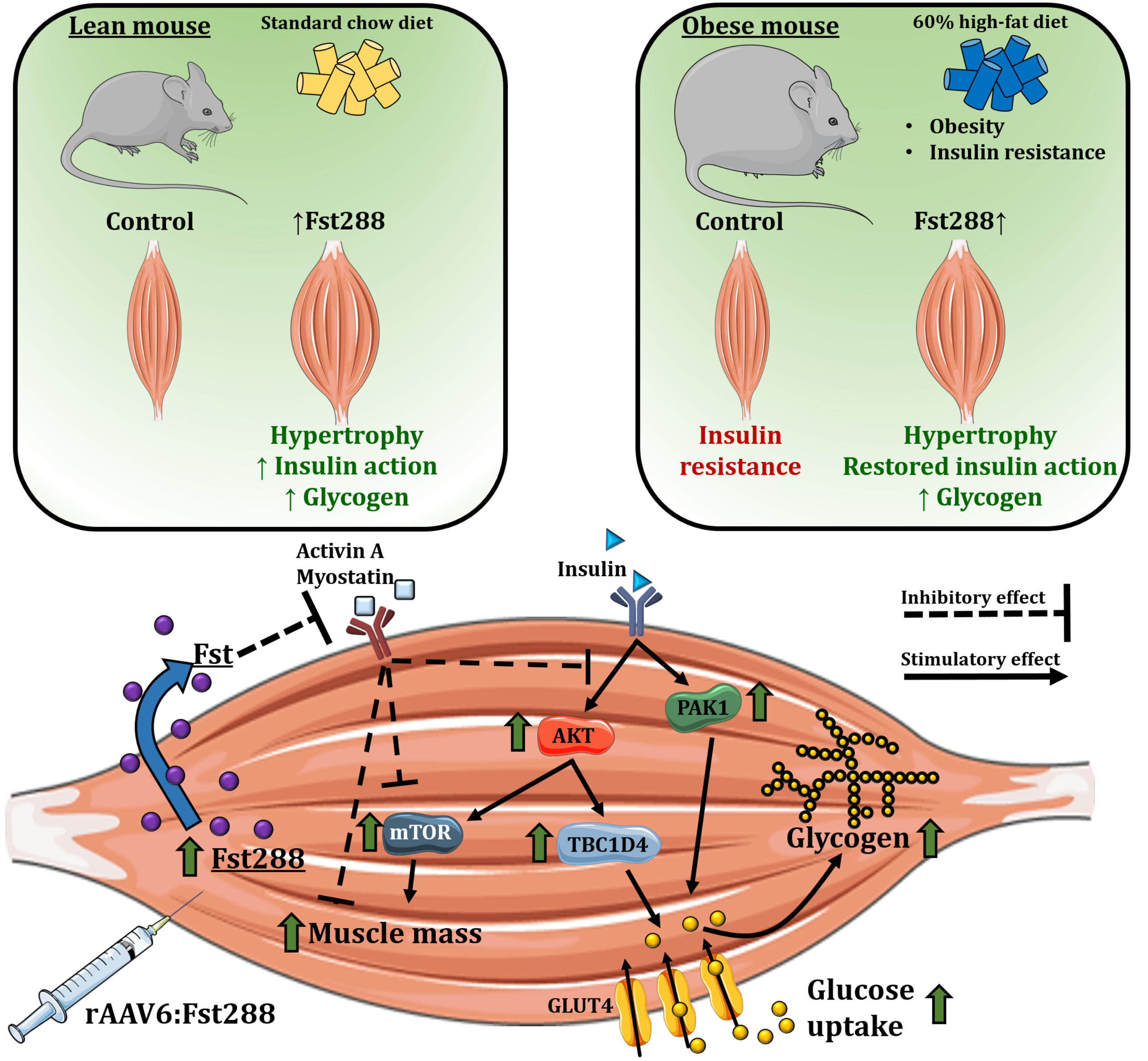
Lean mouse
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Hypertrophy
↑ Insulin action
↑ Glycogen
Obese mouse
60% high-fat diet
• Obesity
• Insulin resistance
Control
Fst288↑
Insulin resistance
Hypertrophy
Restored insulin action
↑ Glycogen
Activin A
Myostatin
Insulin
Inhibitory effect
Stimulatory effect
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Muscle mass
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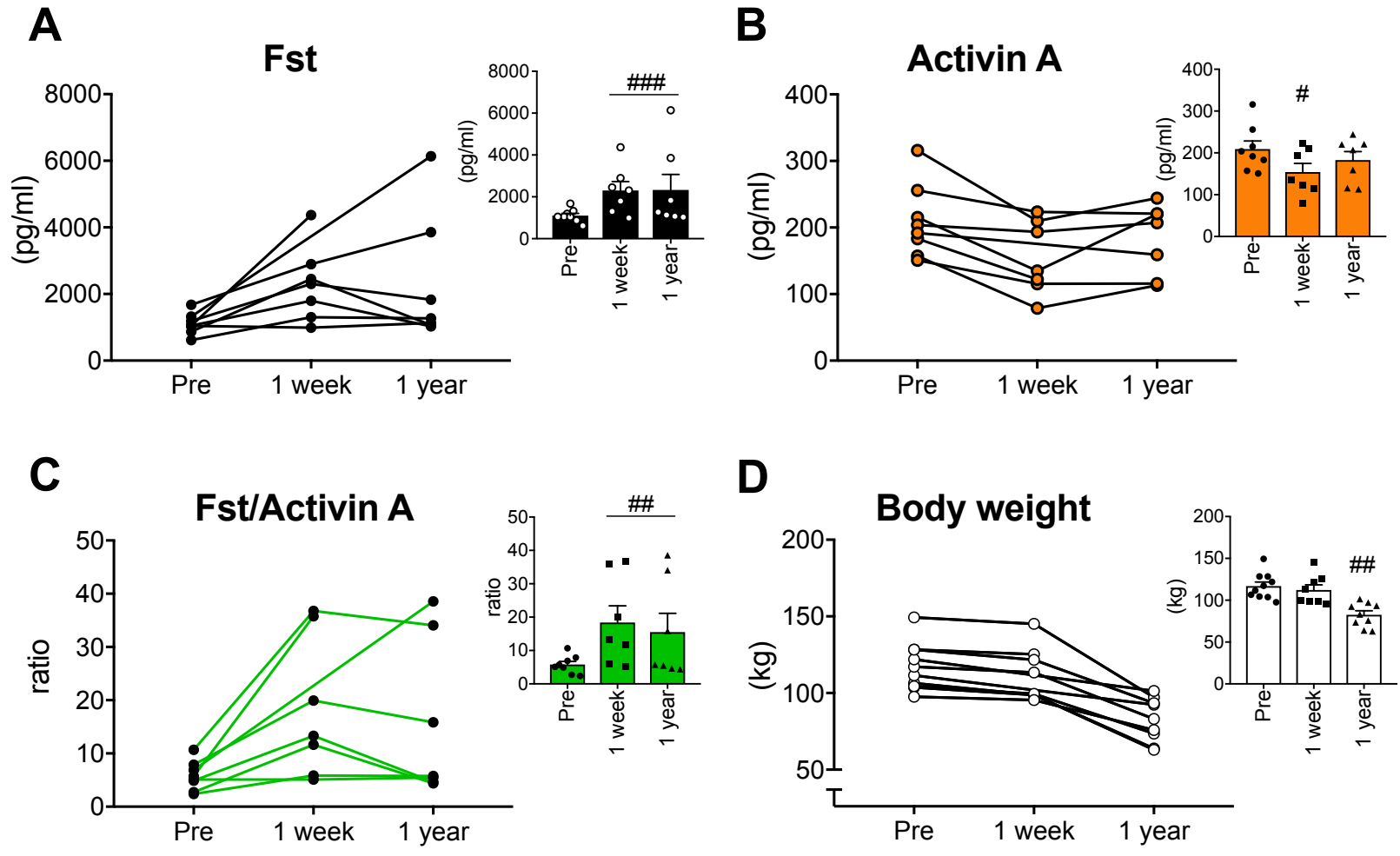
Glucose uptake
rAAV6:Fst288

Figure 6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1 year
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Activin A
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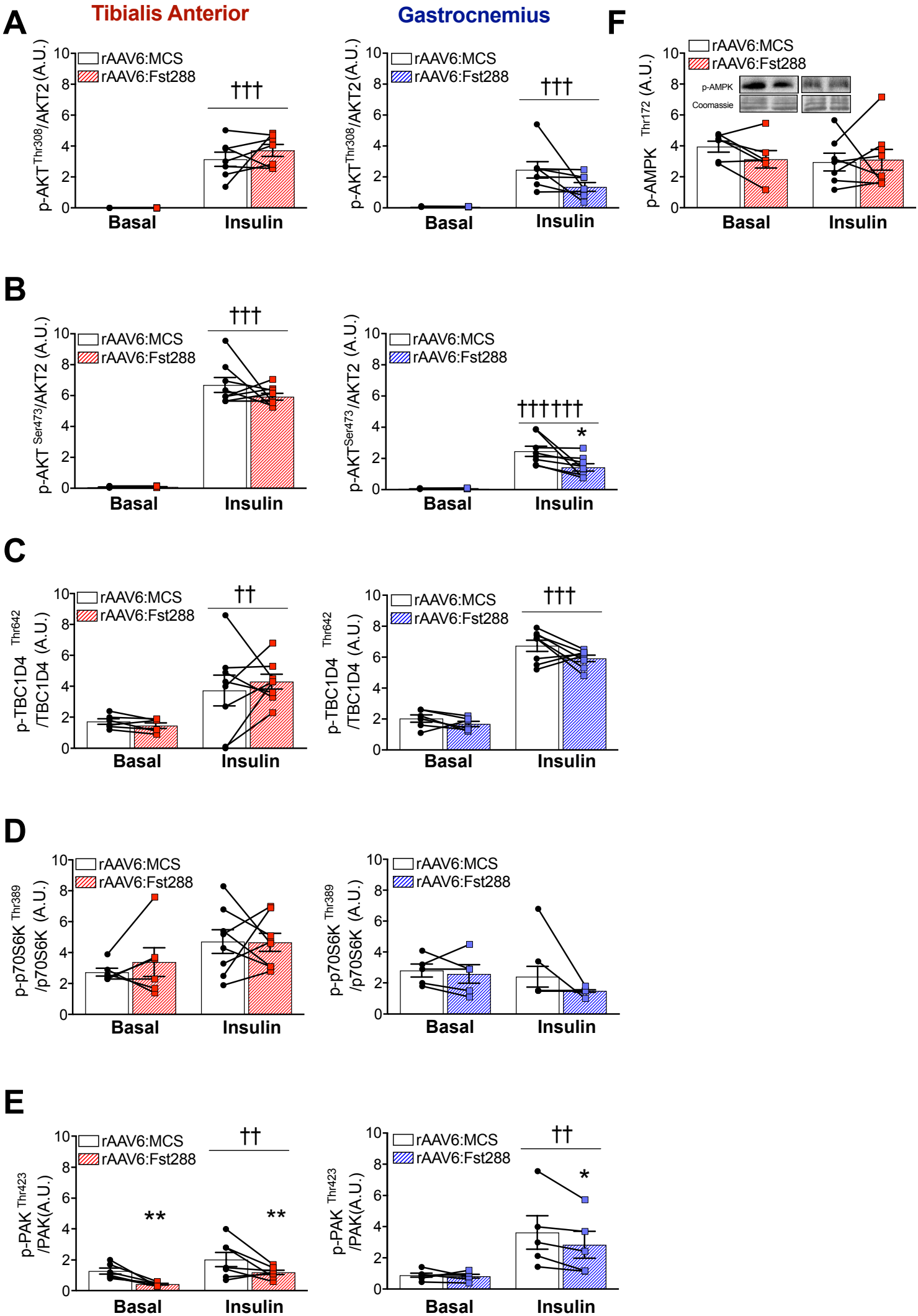
100
#
C
Fst/Activin A
ratio
50
40
30
20
10
##
D
Body weight
(kg)
200
150
100
50

Supplemental Figure 3