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Muscle follistatin gene delivery increases muscle protein synthesis independent of periodical physical inactivity and fasting

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Abstract

Blocking of myostatin and activins effectively counteracts muscle atrophy. However, the potential interaction with physical inactivity and fasting in the regulation of muscle protein synthesis is poorly understood. We used blockade of myostatin and activins by recombinant adeno-associated virus (rAAV)-mediated follistatin (FS288) overexpression in mouse tibialis anterior muscle. To investigate the effects on muscle protein synthesis, muscles were collected 7 days after rAAV-injection in the nighttime or in the daytime representing high and low levels of activity and feeding, respectively, or after overnight fasting, refeeding, or ad libitum feeding. Muscle protein synthesis was increased by FS288 independent of the time of the day or the feeding status. However, the activation of mTORC1 signaling by FS288 was attenuated in the daytime and by overnight fasting. FS288 also increased the amount of mTOR colocalized with lysosomes, but did not alter their localization toward the sarcolemma. This study shows that FS288 gene delivery increases muscle protein synthesis largely independent of diurnal fluctuations in physical activity and food intake or feeding status, overriding the physiological signals. This is important for eg cachectic and sarcopenic patients with reduced physical activity and appetite. The FS288-induced increase in mTORC1 signaling and protein synthesis may be in part driven by increased amount of mTOR colocalized with lysosomes, but not by their localization toward sarcolemma.

KEYWORDS

activins, myostatin, mechanistic target of rapamycin protein, physical activity, fasting

Abbreviations: 4E-BP1, 4E-binding protein 1; (r)AAV, (recombinant) adeno-associated virus; ACVR2, activin receptor type II; AD, ad libitum feeding; CTRL, control; EDL, extensor Digitorum Longus muscle; EE, energy expenditure; ELISA, enzyme-linked immunosorbent assay; Exp, experiment; F, fasting; FS288, follistatin-288; GDF11, growth and differentiation factor 11; His, histidine; LAMP2, lysosome-associated membrane protein 2; LC3, microtubule-associated protein 1A/1B-light chain 3; MAP kinase, mitogen-activated protein kinase; mTOR(C1), mechanistic target of rapamycin (complex 1); p70 S6K, p70 ribosomal protein S6 kinase; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RER, respiratory exchange ratio; RF, refeeding; rpS6, ribosomal protein S6; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SUnSET, surface sensing of translation; TA, tibialis anterior; TGF-β, transforming growth factor-β; VCO_2, carbon dioxide production; VO_2, oxygen consumption.

1 | INTRODUCTION

Depletion of muscle tissue has detrimental effects on health and physical function, and it is associated with increased mortality and poor prognosis in different diseases,[1,2] while there is evidence in rodents that increasing muscle mass or prevention of muscle loss may improve survival in cancer.[3,4] Many diseases and aging are accompanied by physical inactivity and malnutrition that contribute to the decreased muscle mass and function[5] and may also diminish the effectiveness of therapies targeting skeletal muscles.[6] Muscle protein synthesis in the basal state and/or protein synthesis in response to nutrient stimulation are blunted in different models of disuse or inactivity-induced atrophies, such as immobilization, bed rest, and step reduction.[7-11] In contrast to the disuse atrophy that has largely been attributed to diminished muscle protein synthesis,[12] muscle atrophy induced by starvation and nutrient deprivation may arise from the combination of decreased protein synthesis and increased protein degradation.[13,14]

The mechanisms of the protein synthesis responses to activity and feeding may be associated with altered localization of the key regulatory protein mechanistic target of rapamycin (mTOR).[15-19] This is probably important, since the association of mTOR with the lysosome/late endosome appears to be required for maximal mTOR complex 1 (mTORC1) activation.[15,16,18,19] Based on the recent evidence, the activation of mTORC1 is additionally regulated via the cellular positioning of mTOR-lysosome complexes.[17,20,21] Both the colocalization and the translocation may be altered by nutrient availability and muscle activity.[15-19] This may also be relevant to several diseases accompanied by decreased food intake and physical inactivity as we have demonstrated decreased colocalization of mTOR with lysosomes in cachectic skeletal muscles of mice with experimental cancer.[3] However, it is still poorly understood if and how short-term fasting in vivo and the blocking of activin receptor ligands influence the colocalization and translocation of mTOR and lysosomes.

It is crucial to find the potential therapeutic targets to counteract negative protein balance in muscle and the resulting muscle wasting. Myostatin, activin A and B, and GDF11 belong to the transforming growth factor-β (TGF-β) superfamily of proteins, and they negatively regulate muscle mass through binding to activin receptors type 2A and 2B (ACVR2) on the cell membrane.[22] Blocking of ACVR2 ligands through various strategies increases muscle mass and decreases muscle wasting[3,4,22-25] In most cases, the blocking of ACVR2 ligands has increased muscle mass by strongly promoting protein synthesis rather than substantially affecting protein degradation pathways,[6,23-26] even though it has not restored the decreased physical activity and food intake in several rodent models of muscle wasting.[3,25,27] Furthermore, the blockade of ACVR2 ligands has successfully prevented disuse atrophy in mice.[6,28,29] These findings suggest the effectiveness of these treatment modalities in counteracting the negative effects of decreased physical activity and food intake on muscle mass. However, more direct studies investigating the underlying mechanisms required for enhanced muscle protein mass during these stress states are still warranted.

There are many different strategies to block or inhibit ACVR2 ligands, such as using systemic administration of a soluble ACVR2B trap.[3,4,22-24] Another often used and effective approach is the overexpression of follistatin, which is a secreted protein that binds myostatin, activin A and B, and GDF11 and thus effectively inhibits their biological function.[30] The 288 amino acid isoform of follistatin (FS288) contains heparan sulfate domains promoting its association with cell surfaces, which makes it an effective tool for local inhibition of myostatin, activin A and B, and GDF11.[31] Local adeno-associated virus (AAV)-mediated overexpression of FS288 has previously been shown to result in pronounced muscle hypertrophy and increased muscle protein synthesis and mTOR signaling.[32] However, it is not known if FS288 gene delivery influences mTOR localization and if it interacts with altered levels of food intake and/or physical activity in the regulation of muscle protein synthesis.

The purpose of this study was to investigate the local effects of muscle follistatin (FS288) overexpression using rAAV-mediated gene delivery on the regulation of muscle protein synthesis and mTOR signaling in response to i) diurnal fluctuations in physical activity and food intake and ii) food deprivation in mice.

2 | MATERIALS AND METHODS

2.1 | Cloning of DNA constructs and production of adeno-associated viral vectors

The FS288 + 6His-insert was amplified from pOTB7 AU24-F5 Follistatin plasmid, IMAGE clone #3688745 (Geneservice Ltd, Cambridge, UK) via PCR and cloned into pSupCAG vector for rAAV production. pSubCAG plasmid containing the gene of interest was then transfected to CHO-S cells and transgene expression was confirmed by Western blotting using anti-tetra-histidine antibody (1:5000, #34670, Qiagen, Hilden, Germany). To produce recombinant adeno-associated viral vectors (serotype 9), HEK293T cells were transfected with the pSubCAG-FS288 or pSubCAG-Scramble (rAAV9-CTRL) and helper plasmids. Media were collected after 2 and 3 days and the rAAV preparations were purified by ultracentrifugation using an iodixanol step gradient. The titers were determined by real-time qPCR. Finally, HEK293T cells were transduced with the AAVs encoding FS288 (rAAV9-FS288) or a non-gene coding control rAAV (rAAV9-CTRL), and the transgene expression was

confirmed by Western blotting using anti-tetra-histidine antibody (1:5000, #34670, Qiagen, Hilden, Germany). The rAAVs were manufactured by the AAV Gene Transfer and Cell Therapy Core Facility of Faculty of Medicine, University of Helsinki, Finland.

2.2 | Animals

Male mice from C57Bl/6Jrj background (Janvier, France) aged 10-12 weeks were used in all experiments. Mice were maintained in standard conditions (temperature 22°C, 12:12 hours light/dark cycle) with free access to food (R36; 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm, Sweden) and water. The treatment of the animals was in strict accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Directive 2010/63/EU). The protocols were approved by the National Animal Experiment Board, and all the experiments were carried out in accordance with the guidelines of that committee (permit numbers: ESAVI/10137/04.10.07/2014 and ESAVI/14457/2018).

2.3 | Experimental design

A schematic representation of the study design is presented in Figure 1. The experimental design includes a 3-week dose-response experiment and 1-week protein synthesis experiments. In all experiments, mice were anesthetized with an intraperitoneal injection of ketamine and xylazine (50-60 mg/kg and 7-8 mg/kg, respectively), and given an intramuscular injection of recombinant rAAV9 encoding FS288 (30 μL) into one tibialis anterior (TA) muscle and non-gene coding rAAV9-CTRL into the contralateral TA. The order of the experimental and control legs was randomized. Three different doses were tested in the dose-response experiment (experiment 1): 4×10^9, 1.6×10^{10}, and 6.4×10^{10} viral particles/leg, N = 4 mice/dose. Samples were collected 20 days after the rAAV injection. The lowest dose producing significant hypertrophy in TA and extensor digitorum longus (EDL) muscles without major effects on the mass of the other muscles was selected for the following experiments with rAAV9-FS288.

In the following short-term experiments, mice were injected intramuscularly with rAAV9-FS288 or rAAV9-CTRL (4×10^9 viral particles/leg) as above, 7 days before the collection of blood and muscle samples. In experiment 2, half of the mice (N = 8) were euthanized in the nighttime on average at 12 AM, and half of the mice (N = 8) were euthanized in the daytime on average at 3 PM. These time points were selected to represent periods of high physical activity and feeding (nighttime) in contrast to low physical activity and fasting (daytime), based on the light-dark cycle and our pilot measurements. Mice were housed individually in Promethion respirometry cages and respiratory gases and the amount and timing of food intake were monitored with Promethion High-Definition Multiplexed Respirometry System and MetaScreen software (Sable Systems, North Las Vegas, NV, USA) for 2 days before sample collection. In addition, physical activity was recorded with our validated force plate system described previously.[27,33]

In experiment 3, mice were either fed ad libitum overnight (AD, N = 8), fasted overnight (12 hours, F, N = 8), or fasted overnight (11 hours) and then, refed for an hour (RF, N = 8) before sample collection. The food was withdrawn an

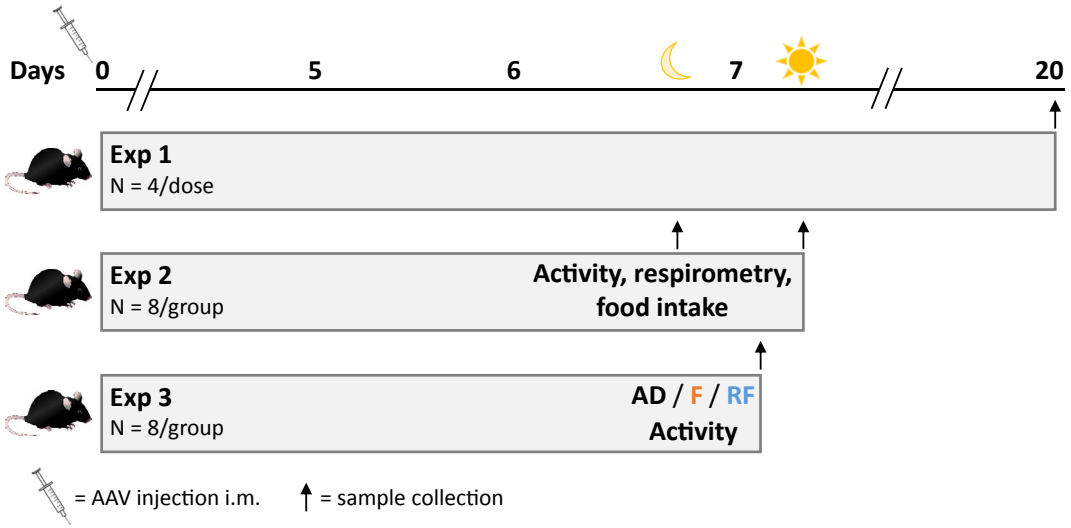

FIGURE 1 Schematic representation of the experimental design. Experiment (Exp) 1 is a dose-response experiment, based on which the rAAV9-FS288 dose was determined for the time of day (Exp 2) and the fasting (Exp 3) experiments. AD, food provided ad libitum; F, overnight fasting; RF, overnight fasting, and 1-hour refeeding

hour into the dark phase to avoid a more prolonged fasting by allowing feeding in the beginning of the dark phase and to better standardize the fasting time. Muscle and blood samples were collected in the morning, 7 days after the rAAV-injection. Physical activity was assessed by force plates as above.

2.4 | Sample collection

In all experiments, mice were euthanized by cardiac puncture followed by cervical dislocation under anesthesia (ketamine and xylazine, i.p.). Blood samples were collected in serum collection tubes, and centrifuged at 2000 *g* for 10 minutes after which the serum was separated and stored in −80°C. TA, EDL and gastrocnemius muscles were rapidly excised and weighed. The TA muscles were cross-sectionally dissected from the middle of the muscle belly, and the samples were either snap-frozen in liquid nitrogen for Western blot analyses (all experiments), or embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) and snap-frozen in isopentane cooled with liquid nitrogen for histological analyses. In experiments 1 and 3, the proximal parts of both TA muscles from each animal were used for histological analyses and the distal parts were used for Western blot analyses.

2.5 | Analysis of muscle protein synthesis

Muscle protein synthesis was analyzed in experiments 2 and 3 using the surface sensing of translation (SUnSET) method[34,35] as reported earlier by our group.[3,23,24] Briefly, 7 days after rAAV-injection, mice were anesthetized by i.p. administration of ketamine and xylazine, and subsequently injected i.p. with 0.040 μmol/g puromycin (Calbiochem, Darmstadt, Germany) dissolved in 200 μl of PBS. At 22 minutes after puromycin administration, mice were euthanized by cardiac puncture followed by cervical dislocation. Both TA muscles were isolated and weighed, and the samples were snap-frozen in liquid nitrogen exactly at 30 minutes after puromycin administration.

2.6 | Protein extraction and protein content

TA muscle samples were homogenized with Tissue Lyser II (2 × 2 minutes, 30 Hz, Qiagen, Hilden, Germany) in ice-cold HEPES-buffer with protease and phosphatase inhibitors [20 mM Hepes, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT, 1 mM Na_3VO_4, 100 mM β-glycerophosphate, 1% NP-40, and 3% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA)] followed by 30 minutes of agitation at +4°C. The samples were then centrifuged at 500 *g* for 5 minutes at +4°C for the analysis of the protein synthesis, and at 10 000 *g* for 10 minutes at +4°C for other analyses. Total protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Waltham, MA, USA) with an automated KoneLab device (Thermo Fisher Scientific, Vantaa, Finland).

2.7 | Western blotting

TA homogenates containing 30 μg of protein were solubilized in Laemmli sample buffer containing 5% β-mercaptoethanol and heated for 10 minutes at 95°C to denature proteins. Proteins were separated by SDS-PAGE in Criterion TGX Stain-Free gels (Bio-Rad Laboratories, Hercules, CA, USA) and then, transferred to PVDF membrane using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were then blocked and probed overnight with primary antibodies against phosphorylated ribosomal protein (rp) S6 at Ser240/244 (#2215), p70 S6 Kinase at Thr398 (#9234), 4E-BP1 at Thr37/46 (#2855), and SAPK/JNK at Thr183/Tyr185 (#4668), and total rpS6 (#2217), p70 S6K (#2708), SAPK/JNK (#9252, Cell Signaling Technology, Beverly, MA, USA), ubiquitin (sc-8017, Santa Cruz Biotechnology, Dallas, TX, USA), LC3 (L7543, Sigma-Aldrich, Darmstadt, Germany), and His-tag (anti-tetra-histidine, #34670, Qiagen, Hilden, Germany) at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Europe), the proteins were visualized by enhanced chemiluminescence (SuperSignal west femto maximum sensitivity substrate; Pierce Biotechnology, Waltham, MA, USA) with ChemiDoc MP device (Bio-Rad Laboratories, Hercules, CA, USA) and quantified (band intensity × volume) with Image Lab software (version 6.0; Bio-Rad Laboratories, Hercules, CA, USA). In the case of the analysis of puromycin-incorporated proteins, ubiquitinated proteins, and total protein loading (stain-free), the intensity of the whole lane was quantified. All Western blot results were normalized to the total protein loading analyzed from the stain-free image of the membrane.

2.8 | Analysis of respirometry and food intake data

In experiment 2, respirometry and food intake data acquired from Promethion respirometry system were analyzed with ExpeData software (version 1.9.14, Sable Systems, North Las Vegas, NV, USA). The total food intake was validated by weighing the food before and after the 2-day measurement. In experiment 3, food intake during the ad libitum feeding

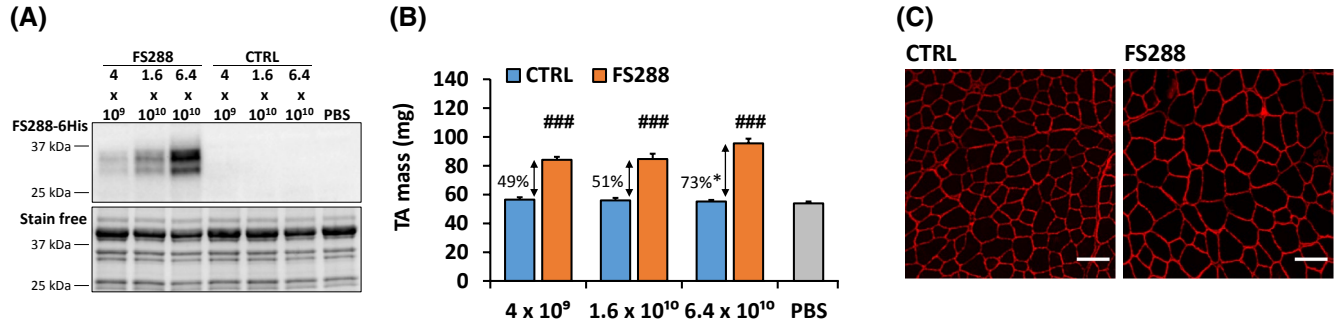

FIGURE 2 Intramuscular follistatin gene delivery induces local muscle hypertrophy. (A) Representative blot showing a dose-dependent expression of the FS288-6His protein in the TA muscles injected with rAAV9-FS288 and no FS288-6His protein when injected with rAAV-CTRL or PBS. (B) Tibialis anterior (TA) mass (N = 4/dose) and (C) representative dystrophin staining 20 days after rAAV-injection. Scale bar = 100 μm. ### = $P < .001$, compared with the respective CTRL. * = $P < .05$, compared with the other doses. CTRL, control; FS288, Follistatin

and the 1-hour refeeding period was analyzed by weighing the food.

2.9 | Serum insulin concentration

Insulin concentration was quantified from the serum samples by the enzyme-linked immunosorbent assay (ELISA) using the Ultra Sensitive Mouse Insulin ELISA Kit (#90080, Crystal Chem, Elk Grove Village, IL, USA) according to the manufacturer's instructions and an automated DS2 ELISA processing system (DYNEX Technologies, Chantilly, VA, USA).

2.10 | Muscle histology

For histological analyses, 10 μm of thick frozen sections were cut from OCT-embedded TA samples from experiments 1 and 3. The sections were air-dried, and fixed in −20°C acetone (dystrophin and mTOR-LAMP2 staining) or methanol (His-tag staining) for 10 minutes. To assess muscle fiber size, the sections from the dose-response experiment (experiment 1) were stained with anti-dystrophin antibody (1:500, ab15277, Abcam, Cambridge, UK) and Alexa Fluor 555 secondary antibody (1:200, A32732, Invitrogen, Waltham, MA, USA) to visualize the sarcolemma. To verify the expression of the His-tagged FS288 transgene, sections from the experiment 3 were stained with antibodies against His-tag (hexa histidine-tag, 1:100, ab9136, Abcam, Cambridge, UK) and dystrophin (1:500, ab15277, Abcam, Cambridge, UK) with Alexa Fluor 555 donkey anti-goat and Alexa Fluor 488 donkey anti-rabbit secondary antibodies (1:300, Invitrogen, Waltham, MA, USA), respectively. To analyze mTOR-LAMP2 colocalization, sections from the experiment 3 were stained with antibodies against LAMP2 (1:200, ab13524, Abcam, Cambridge, UK), mTOR (1:200, #2983, Cell Signaling Technology, Beverly, MA, USA), and dystrophin (1:200, NCL-DYS2, Novocastra, Newcastle upon Tyne, UK) and with Alexa Fluor 488 donkey anti-rat, Alexa Fluor 555 goat anti-rabbit, and Alexa Fluor 647 goat anti-mouse secondary antibodies (1:300, Invitrogen, Waltham, MA, USA), respectively. All stained sections were mounted with Mowiol.

2.11 | Image processing and analysis

The fluorescently labeled samples were scanned with a Zeiss LSM 700 confocal microscope and a Plan Apochromat 63x/1.4 NA Oil objective using ZEN black (2011 SP7). A stitched 4x4 tile scan (934 μm^2) consisting of an average of 241 cells was imaged for each subject. Data were deconvoluted with a theoretical PSF before achieving cell segmentation with the Trainable Weka Segmentation plugin[36] in Fiji.[37] Intracellular marker classification and distance calculations were then performed in Fiji and Matlab R2018b (MathWorks Inc), respectively. Colocalization between mTOR and LAMP2 was performed according to Costes et al through the Colocalization Threshold plugin in Fiji.[38] All the steps were performed blinded to the sample identification.

2.12 | Statistical analyses

The normality of the data was analyzed (Shapiro-Wilk test) and the equality of variances (Levene's test) was assessed. The main effects for FS288, time of day, and feeding status were analyzed using General Linear Model Analysis of Variance (GLM ANOVA) or Kruskal-Wallis test, when appropriate. Between group (time of day or feeding status) comparisons were conducted using Student's *t* test or nonparametric Mann-Whitney U test, and Holm-Bonferroni correction was applied when more than two groups were compared (feeding status). In case of the Western blot data, these

comparisons were always made within the genotype (CTRL or FS288, planned comparison). The differences between the CTRL and FS288 legs were analyzed within time of day or feeding status (planned comparison), unless otherwise stated, by nonparametric Mann-Whitney U test, and the result of the FS288 leg was expressed relative to the CTRL leg of the same animal. Statistical significance was set at $P < .05$. All results are presented as means ± SEM.

3 | RESULTS

3.1 | FS288 gene therapy results in substantial muscle hypertrophy

Western blot analysis showed a dose-dependent expression of the FS288-6His protein in the TA muscles injected with rAAV9-FS288 (Figure 2A). No expression was observed in the muscles injected with the non-gene coding control rAAV (rAAV9-CTRL, Figure 2A). All of the rAAV9-FS288 doses tested in Experiment 1 strongly increased TA and EDL masses (Figure 2B, Figure S1). The hypertrophy was explained by increased muscle fiber size as shown by the dystrophin staining (Figure 2C). The muscle masses of the control leg injected with rAAV9-CTRL were not affected and were also comparable to the muscle masses of the mice injected only with PBS into both TA muscles, showing that the hypertrophy effect of the intramuscular FS288 gene delivery was indeed local (Figure 2B, Figure S1). We selected the smallest dose for the following experiments, as the two larger doses caused some hypertrophy also in the gastrocnemius muscle of the treated leg (17.8% ± 4.3% and 25.2% ± 2.1%, respectively, Figure S1).

3.2 | Time of day influences physical activity, energy expenditure, feeding, and serum insulin

We next conducted an experiment, in which the effects of FS288 gene delivery were investigated at different times of day (Experiment 2, Figure 1). To evaluate, whether this was a suitable model to investigate the diurnal effects of different levels of activity and feeding on the local muscle responses to FS288 gene delivery, physical activity, respiratory gases, and food intake were analyzed. The mice in the night group were significantly more active and ate more during the 3-hour period preceding sample collection compared with the day group (Figure 3A,B). Oxygen consumption, carbon dioxide production, and energy expenditure followed a similar pattern with physical activity, and were also significantly higher in the nighttime (Figure 3C-E). In addition, the respiratory exchange ratio (RER) was significantly higher in the nighttime compared with the daytime (Figure 3F). In line with the

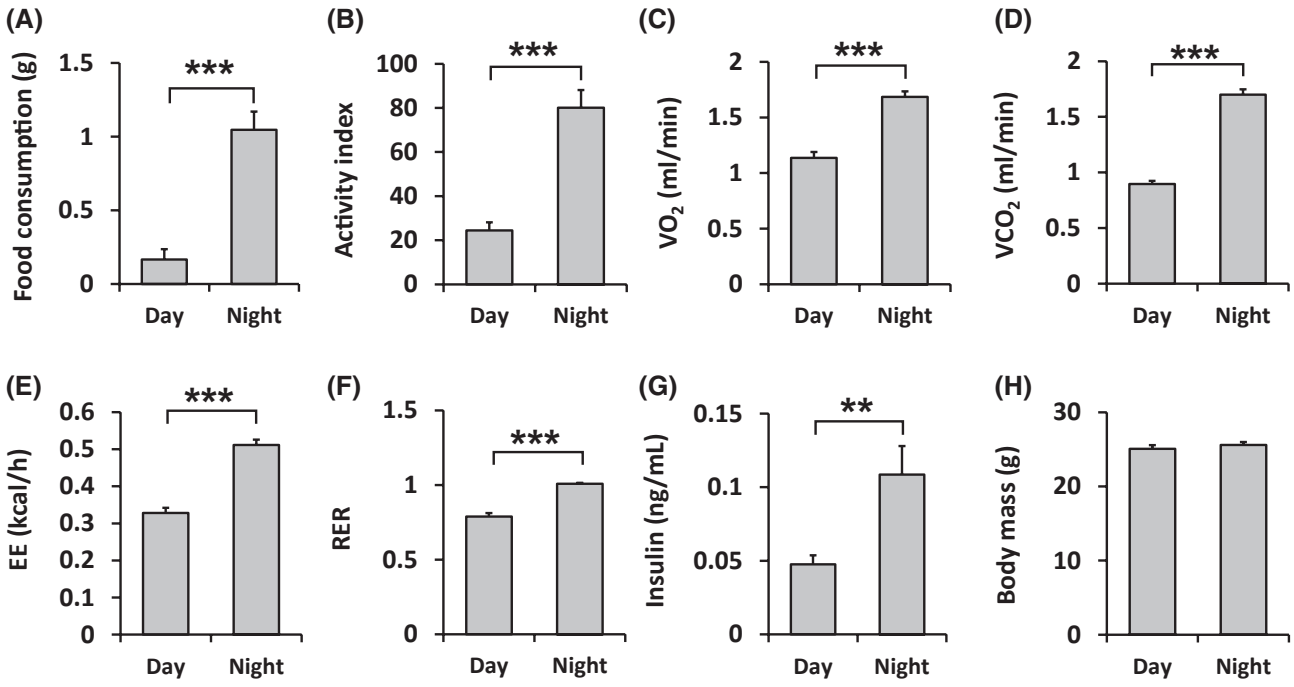

FIGURE 3 Food intake, serum insulin, physical activity, and respiratory gases are altered by the time of day. (A) Food consumption, (B) physical activity index, (C) oxygen consumption (VO_2), (D) carbon dioxide production (VCO_2), (E) energy expenditure (EE), (F) respiratory exchange ratio (RER), (G) serum insulin concentration, and (H) body mass in daytime and nighttime. Food consumption, physical activity, and respiratory parameters were analyzed from the last 3 hours preceding sample collection. **, and *** = $P < .01$, and .001, respectively [Student's *t* test (A, H), or Mann-Whitney U test (B-E, G)]. N = 8/group

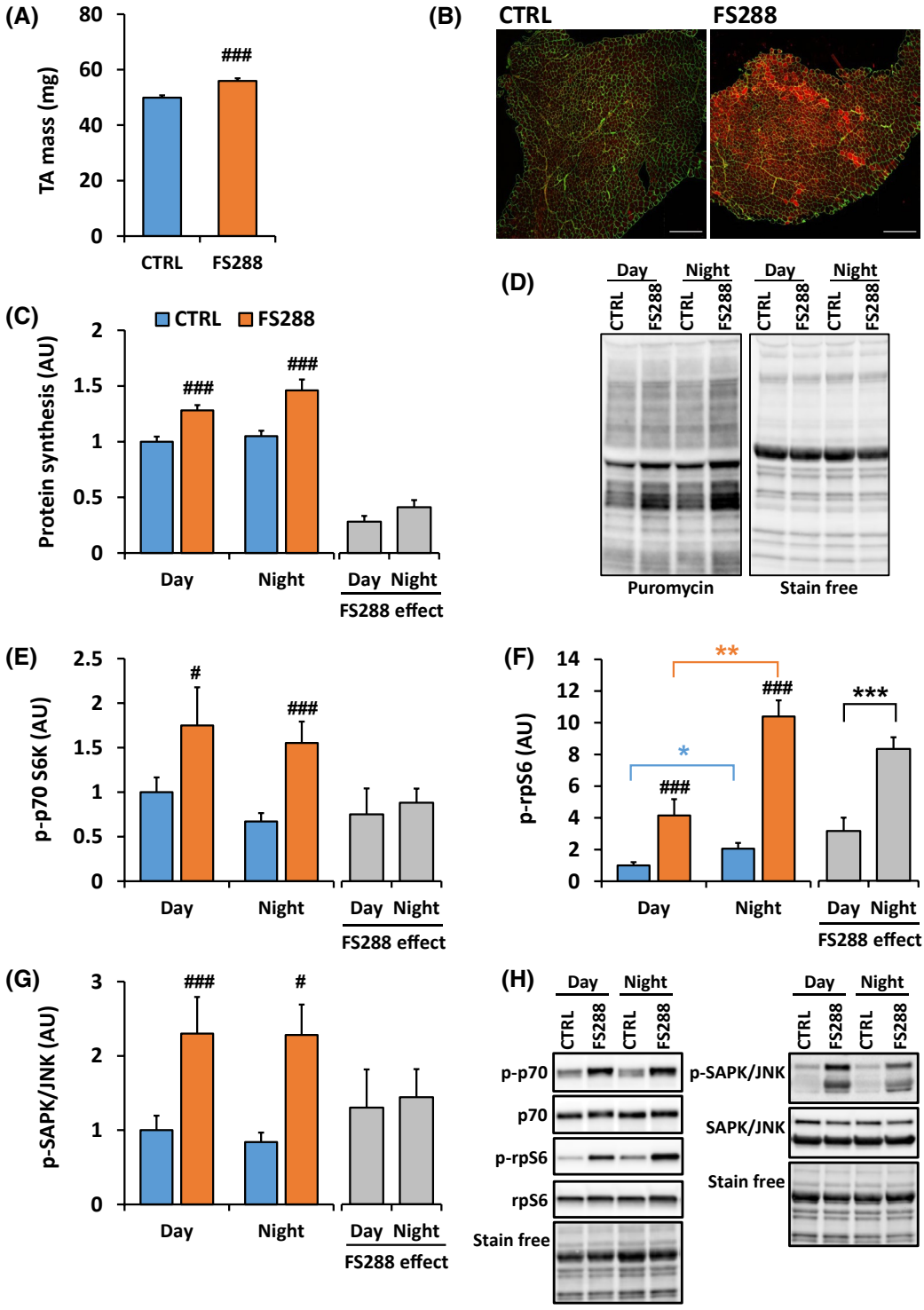

FIGURE 4 Effects of FS288 gene delivery on muscle protein synthesis and mTORC1 signaling on different times of the day. (A) Tibialis anterior (TA) mass at 7 days after rAAV injection (N = 16 per leg) and (B) representative image of anti-histidine staining showing FS288 expression. Scale bar = 500 µm. (C) TA protein synthesis analyzed with SUnSET and (D) the representative blots. Phosphorylation of (E) p70 S6 kinase (T389), (F) rpS6 (S240/244), and (G) SAPK/JNK (T183/Y185), and (H) the representative blots. All results are normalized to the stain free and to the mean of "Day CTRL." Time of day comparisons: *, **, and *** = $P < .05$, .01, and .001, respectively [Holm-Bonferroni corrected Student's *t* test (F)]. FS288 vs CTRL comparisons: #, and ### = $P < .05$, and .001, respectively [Student's *t* test (A), Mann-Whitney U test (C, E-H)]. N = 8/group

increased feeding, the serum insulin concentration was also significantly higher in the nighttime (Figure 3G). Body mass was not altered by the time of day (Figure 3H). These results demonstrate that the chosen model was suitable to compare the diurnal effects of different levels of activity and feeding on the local responses to FS288 gene delivery.

3.3 | FS288 gene delivery increases muscle protein synthesis and JNK phosphorylation independent of diurnal fluctuations in physical activity and feeding

After 7 days, the selected dose (4×10^9 vp/leg) already resulted in a small but significant increase in the TA mass compared with the control TA (Figure 4A). At this time point, the FS288 transgene expression was also verified with anti-histidine staining, showing wide-spread, mosaic expression pattern (Figure 4B). Muscle protein synthesis was markedly induced by FS288 gene delivery irrespective of time of day (Figure 4C,D). FS288 also increased the phosphorylation of p70 S6 kinase (p70 S6K) and both phosphorylated and total ribosomal protein S6 (rpS6) indicating increased mTORC1 signaling (Figure 4E, F, and H, Figure S2). Time of day did not influence muscle protein synthesis (Figure 4C,D). However, the phosphorylation of rpS6 was more pronounced in the nighttime compared with the daytime in both legs, while phosphorylated p70 S6K as well as total p70 S6K, and total rpS6 were similar between the day and the night (Figure 4E, F and H,

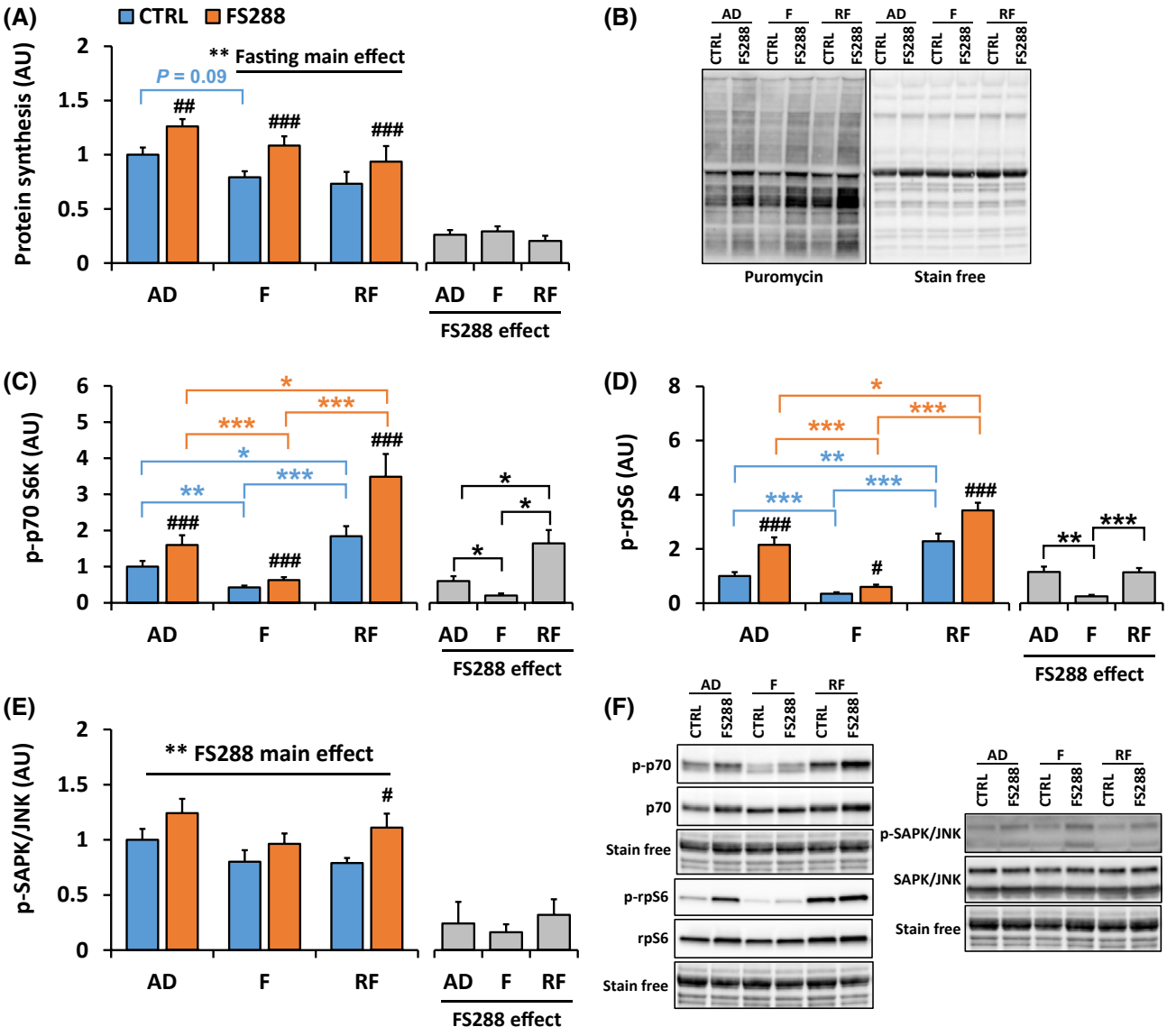

FIGURE 5 Effects of FS288 gene delivery on muscle protein synthesis and mTORC1 signaling after fasting and refeeding. (A) Tibialis anterior (TA) protein synthesis and (B) the representative blots. Phosphorylation of (C) p70 S6K (T389), (D) rpS6 (S240/244), and (E) SAPK/JNK (T183/Y185), and (F) the representative blots. All results are normalized to the stain free and mean of "AD CTRL." Feeding status comparisons: *, **, and *** = $P < .05$, .01, and .001, respectively [Holm-Bonferroni corrected Student's *t* test (A, C-D: FS288 effect, E-F), or Holm-Bonferroni corrected Mann-Whitney U test (C, D)]. FS288 vs CTRL comparisons: #, ##, and ### = $P < .05$, .01, and .001, respectively (Mann-Whitney U test). The main effects for fasting and FS288 were analyzed with GLM ANOVA. N = 7-8/group (A), N = 8/group (C-F). AD, food provided ad libitum; F, overnight fasting; RF, overnight fasting and 1-hour refeeding

Figure S2). In addition, FS288 increased the phosphorylation of rpS6 more strongly in the nighttime, without diurnal differences in p-p70 S6K response (Figure 4E, F and H,). FS288 gene delivery induced only a modest increase in the phosphorylation of 4E-binding protein 1 (4E-BP1), which is another downstream target of mTORC1 (FS288 main effect $P = .068$, Figure S2). In addition to mTORC1, phosphorylated mitogen-activated protein kinase SAPK/JNK has recently been identified to be a key driver for muscle hypertrophy through negative regulation of TGF-β/myostatin activity.[39] Interestingly, the FS288 gene delivery also markedly increased the phosphorylation of SAPK/JNK irrespective of time of day, without effects on total SAPK/JNK (Figure 4G and H, Figure S2).

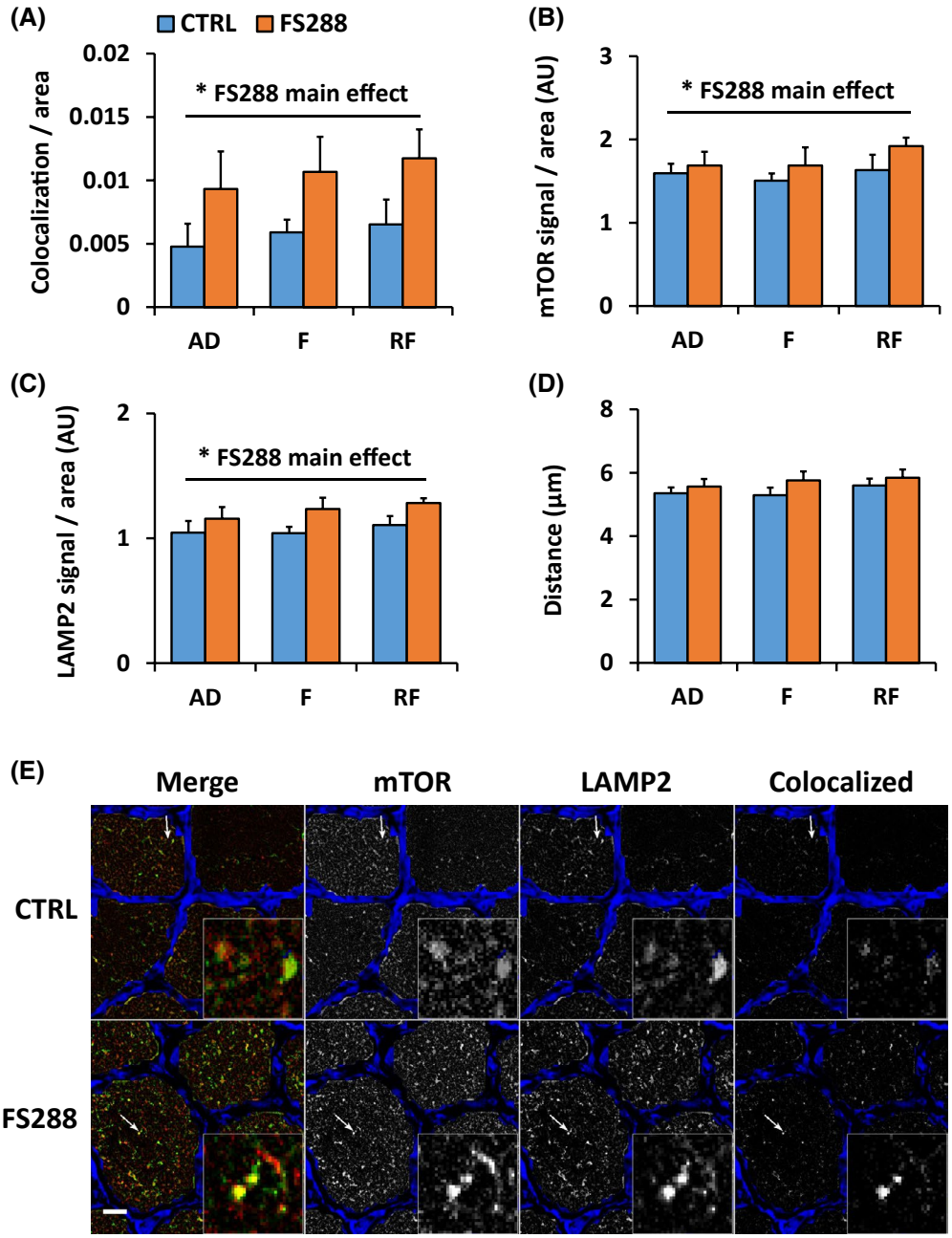

FIGURE 6 Effects of FS288 gene delivery and feeding status on mTOR and LAMP2 localization in TA muscle. (A) Quantification of the mTOR-LAMP2 colocalization per area, (B) the total mTOR signal per area, (C) the total LAMP2 signal per area, and (D) the distance of the colocalized mTOR-LAMP2 particles from the sarcolemma. (E) Representative image of the cytosolic mTOR (red), LAMP2 (green) and their colocalization (yellow) in the CTRL and FS288 muscles of AD. Dystrophin is represented in blue. Scale bar = 10 µm. * $P < .05$, the main effect for FS288 was determined by Kruskal-Wallis test (A, B), or GLM ANOVA (C). N = 7-8/group. AD, food provided ad libitum; F, overnight fasting; RF, overnight fasting and 1-hour refeeding

3.4 | FS288-induced mTORC1 signaling, but not protein synthesis, may be compromised by short-term fasting

In the experiment 3, mice were either fasted overnight, fasted overnight and then, refed for an hour, or fed ad libitum to more accurately elucidate the effect of feeding status on FS288 responses (Figure 1). Fasting caused a slight decrease in serum insulin concentration, whereas refeeding induced a threefold and a fourfold increase in serum insulin concentration when compared with ad libitum and fasted groups, respectively (Figure S3). In addition, the mice tended to be more active during fasting than during ad libitum feeding (Figure S3). As in the time of day experiment (experiment 2, Figure 4C), muscle protein synthesis was increased by the FS288 gene delivery irrespective of the feeding status (Figure 5A,B). However, the muscle protein synthesis was slightly lower in both CTRL and FS288 muscles of the fasted groups (F and RF pooled $P < .05$, main effect for fasting $P < .01$) compared with the ad libitum feeding (Figure 5A). Fasting also decreased the phosphorylated p70 S6K and rpS6 in both CTRL and FS288 legs, whereas an hour of refeeding was sufficient to reverse this effect of fasting on mTORC1 signaling (Figure 5C, D and F). The FS288 gene delivery increased the phosphorylation of p70 S6K and rpS6 in all groups, but the response to FS288 was significantly attenuated by fasting (Figure 5C, D, and F). The total levels of p70 S6K and S6 proteins were not significantly affected by the feeding status, but they were slightly increased by FS288 (Figure S4). As in the experiment 2 (Figure S2), the FS288 gene delivery induced only a modest increase in phosphorylated 4E-BP1, which reached statistical significance only in the AD group (Figure S4). The FS288 gene delivery increased the phosphorylation of SAPK/JNK without significant interaction with the feeding status (Figure 5E and F, Figure S4).

3.5 | Colocalization of mTOR with lysosomes is increased by FS288 irrespective of the feeding status

To analyze the potential mechanisms underlying the alterations in mTORC1 signaling, the colocalization of mTOR with a lysosomal marker LAMP2 was analyzed, as association of mTOR with the lysosome has been shown to be essential for mTORC1 activation.[15-19] FS288 significantly increased the colocalization of mTOR and LAMP2, while the feeding status did not have any significant effect on the mTOR-LAMP2 colocalization (Figure 6A,E). Overall, the proportion of the total mTOR colocalized with LAMP2 (or the proportion of the total LAMP2 colocalized with mTOR) was not altered by FS288 or the feeding status (Figure S5). Thus, the increased colocalization by FS288 may have been accompanied by an increased abundance of mTOR and LAMP2. Indeed, the signals of mTOR and LAMP2 were significantly increased by FS288 (Figure 6B, C, and E).

In addition to the association of mTOR with the lysosome, the translocation of the mTOR-lysosome complex to the cell periphery has been suggested to increase mTORC1 activation.[20] Thus, we analyzed the distance of the colocalized mTOR-LAMP2 particles from the sarcolemma as well as the colocalization of mTOR-LAMP2 positive particles with the sarcolemmal marker dystrophin. FS288 did not cause translocation of mTOR-LAMP2 toward sarcolemma (Figure 6D), or its colocalization with dystrophin (Figure S5). The distance of the colocalized mTOR-LAMP2 particles from the sarcolemma was also not altered by the feeding status (Figure 6D), but the fasted groups tended to exhibit a slight decrease (approximately 6%) in the colocalization of mTOR-LAMP2 with dystrophin (Main effect for fasting $P = .054$, Figure S5).

4 | DISCUSSION

In the present study, we show that the diurnal fluctuation in feeding and activity behavior, and fasting have little or no effect on the potency of the intramuscular FS288 gene delivery to increase muscle protein synthesis. In addition, FS288 gene delivery is able to increase the amount of mTOR colocalized with lysosomes, and this effect occurs irrespective of the feeding status. However, not all effects of feeding status on muscle protein synthesis and mTORC1 signaling responses to FS288 can be ruled out, as we show that fasting attenuates the phosphorylation of p70 S6K and rpS6 in both control and FS288-treated muscles.

As mice are nocturnal animals, they are more active and eat more during the dark phase.[40] This was corroborated by the results of the present study, as significantly higher levels of activity, oxygen consumption, carbon dioxide production, and food intake were recorded in the nighttime. Thus, this model was appropriate to study whether the FS288 gene delivery has differential effects on muscle protein synthesis and its regulation during the active and inactive times of the day. As shown earlier, muscle protein synthesis was significantly increased by the FS288 gene delivery,[32,41] but as a novel result, we show that this response occurs irrespective of the time of day. As blocking activin receptor ligands usually has only minor effects on the markers of protein degradation,[6,24] and because FS288 did not have any significant effects on the amount of ubiquitinated proteins and slightly decreased lipidated LC3 as a marker of autophagy (Figure S2), we propose that the constant expression of FS288 from the skeletal muscle also increases muscle growth irrespective of the time of day.

The ability of FS288 to induce anabolic effects on skeletal muscle tissue despite altered levels of physical activity and

food intake is consistent with the previous studies showing that blocking myostatin or activin receptor ligands protects from atrophy resulting from, for example, disuse,[6,28,29] and experimental cancer cachexia associated with decreased physical activity and food intake.[3,25,42] Similar pharmacological studies with blocking activin receptor ligands have not been conducted in humans, but muscle hypertrophy induced by resistance exercise with strong effects on muscle protein synthesis[43] has been found to be relatively little influenced by the time of day when training is performed.[44] These findings suggest that the ability of a strong anabolic stimulus, such as blocking activin receptor ligands or resistance training, to enhance muscle hypertrophy is relatively little influenced by the time of day.

The lack of an interaction between the effects of FS288 gene delivery and the diurnal variation in physical activity and feeding on muscle protein synthesis was perhaps surprising. It is well established that the combination of exercise and feeding stimulates mTORC1 signaling and consequently muscle protein synthesis and growth.[43,45] Moreover, muscle disuse, such as immobilization and bed rest, has been shown to cause muscle atrophy and decreased muscle protein synthesis in basal state and in response to nutrient stimulation.[8-11] Similarly, reduced muscle activity accomplished by the reduction of daily step count decreases muscle protein synthesis and muscle mass.[7,46] Indeed, muscle atrophy in response to inactivity has been attributed mostly to decreased muscle protein synthesis.[11,12] Moreover, nutrient deprivation decreases mTORC1 signaling activity resulting in decreased protein synthesis.[13,26,47] Thus, we hypothesized that muscle protein synthesis and mTORC1 signaling would be higher in the nighttime, which is characterized by higher levels of food intake and physical activity, and lower in the daytime, which is characterized by more passive behavior and diminished eating. In contrast to our hypothesis, muscle protein synthesis was similar between the two different times of day investigated in mice, which may also be a reason why FS288 gene delivery did not interact with the time of a day in the protein synthesis responses. It can be speculated that compared with the more severe models of disuse atrophy or starvation, mere diurnal fluctuation in physical activity and feeding may not be dramatic enough to cause substantial variation in muscle protein synthesis or in the effects of strong anabolic stimuli, such as the FS288 gene delivery. In addition to physical activity and feeding, other diurnally fluctuating factors and the circadian clock may also contribute to the results. All in all, these findings suggest that effective blocking of activin receptor signaling has such strong effects that it overrides the physiological regulatory mechanism of muscle protein synthesis.

As there was no interaction between the FS288 gene delivery and the diurnal variation in physical activity and feeding in muscle protein synthesis, we next sought to clarify whether FS288 interacts with altered levels of food intake in a more controlled setting. Therefore, similarly to the time of day experiment, 7 days after the FS288 gene delivery, mice were either fed ad libitum, fasted overnight, or fasted overnight and then, refed for an hour. The overnight (approximately 12 hours) fasting resulted in a mild decrease in muscle protein synthesis, the magnitude of the decrement being similar to previous studies reporting an approximately 20% decrease in muscle protein synthesis after a comparable fasting protocol in rats[48] and a 15%-19% nonsignificant decrease after a 24-hour starvation in mice.[49] However, the FS288 gene delivery again markedly increased muscle protein synthesis also in the fasted mice without any interaction with the feeding status, thus supporting the results of the time of day experiment. Previously, myostatin depletion did not have an influence on the muscle protein synthesis responses to overnight food deprivation, supporting the lack of interaction between myostatin signaling and food deprivation.[26] Moreover, myostatin deficiency does not protect mice from fasting-induced muscle atrophy or decrease in muscle protein synthesis.[26,49] However, we have demonstrated previously that the blocking of ACVR2 ligands by soluble ACVR2B is able to restore muscle mass and protein synthesis in mice receiving chemotherapy, which was associated with decreased food intake.[24] These findings suggest that the blocking of all activin receptor ligands instead of just myostatin might more effectively counteract the fasting-induced muscle atrophy, and future studies should, therefore, address this hypothesis in settings of strongly decreased food intake in the absence of other catabolic stimuli.

The increased muscle protein synthesis induced by the FS288 gene delivery was in both experiments associated with a robust increase in the markers of mTORC1 signaling, phosphorylated p70 S6K and rpS6, in all conditions investigated. This is consistent with a previous study showing that FS288 induces mTORC1 signaling, protein synthesis and muscle hypertrophy[32] similarly to other modalities used to block activin receptor ligands.[23] In that study, the effect of FS288 on muscle protein synthesis was attenuated by approximately 50% with rapamycin showing that the activation of mTORC1 signaling is in part responsible for the increased muscle protein synthesis and muscle hypertrophy response to FS288 overexpression.[32]

To elucidate the potential mechanisms via which FS288 modulates mTORC1 signaling and protein synthesis, the subcellular localization of mTOR was analyzed. We report here that FS288 overexpression caused a significant increase in the colocalization of mTOR with the lysosomal marker LAMP2, potentially indicating increased association of mTOR with lysosomes. This is consistent with the increased mTORC1 signaling and protein synthesis in response to FS288. As with muscle protein synthesis,

this occurred irrespective of the feeding status, which did not have any independent effect on the colocalization of mTOR with lysosomes. The present results expand our earlier study in which we showed that systemic blockade of activin receptor ligands with soluble ACVR2B was able to restore the colocalization of mTOR with the lysosomes in cachectic skeletal muscle,[3] whereas here we report for the first time the independent effect of blocking activin receptor ligands on the mTOR-lysosome colocalization. Thus, the blocking of activin receptor ligands may induce muscle protein synthesis and mTORC1 signaling at least in part via increasing the amount of mTOR colocalized with the lysosomes and this effect seems to be influenced relatively little by the feeding status. However, further mechanistic evidence with other feeding/starvation protocols is required to confirm this hypothesis.

The lack of an effect from feeding per se on mTOR localization was perhaps unexpected as nutrient availability has been shown to regulate mTOR-lysosome association.[15-17] Moreover, in tumor-bearing mice with strongly decreased muscle protein synthesis and markedly reduced food intake and physical activity, reduced colocalization of mTOR with lysosomes has been reported.[3] However, supporting the results of the present study, recent studies have suggested, that milder, more physiological nutrient deprivation alone would not cause the dissociation of mTOR from the lysosome.[17,20,21] In physiological nutrient deprivation, mTORC1 activity has been suggested to be regulated via the subcellular localization of the mTOR-lysosome complexes instead of altered levels of mTOR associated with lysosomes.[17,20] Specifically, it has been suggested that the translocation of the mTOR-lysosome complexes to the cell periphery, and thus closer to the upstream signals, substrates for protein synthesis and downstream targets, would be associated with increased mTORC1 activation.[17,20] Thus, we analyzed the distance of the mTOR-lysosome complexes from the sarcolemma as well as the colocalization of the mTOR-lysosome complexes with the sarcolemmal marker dystrophin. However, neither fasting nor refeeding caused any significant changes in the subcellular localization of mTOR-lysosome complexes, despite a slight tendency for fasting to decrease the colocalization with dystrophin. It is possible that a more severe or prolonged fasting protocol is required to observe substantial changes in mTOR-lysosome localization, and thus, future studies should aim to clarify the effects of fasting on mTOR-lysosome complex translocation further in physiological conditions.

Moreover, mTOR and lysosomes have been found to translocate toward the cell membrane in response to anabolic stimuli, namely, nutrients and resistance exercise, resulting in enhanced mTORC1 signaling.[20,21,50] However, we showed for the first time that blocking activin receptor ligands via FS288 gene delivery does not appear to cause translocation of mTOR-lysosome complexes toward the sarcolemma. Thus, the response to blocking activin receptor ligands may differ from that induced by nutrient stimulation or resistance exercise in a manner through which they regulate mTORC1 signaling,[17,21,50] but further studies are needed to thoroughly investigate this phenomenon. Taken together, the present and the previous studies suggest that there are multiple factors regulating mTORC1 activity, including its localization, and the importance of each regulatory factor may be dependent on the stimulus or the condition.

The decreased phosphorylation of mTORC1 signaling markers p70 S6K and rpS6 in fasted mice is in line with the previous studies.[51-53] Interestingly, we found that also the rpS6 phosphorylation response to FS288 gene delivery was reduced in the fasted state and during the day. Moreover, even though FS288 increased the phosphorylation of p70 S6K throughout the different feeding status, the response of phosphorylated p70 S6K was significantly blunted in fasted mice. The attenuated responses in both p-rpS6 and p-p70 S6K to FS288 gene delivery were restored, or even augmented in the case of p-p70 S6K, by refeeding. These effects might be due to additive effects of FS288 and nutrient availability and/or insulin stimulation, as higher insulin levels were observed in the nighttime compared with the daytime, while the insulin levels were decreased by fasting and robustly increased by refeeding, consistent with previous evidence.[40,51,54,55] Indeed, in a recent study, AAV-mediated FS288 overexpression was found to increase insulin stimulated but not basal phosphorylation of p70 S6K, which is upstream kinase for rpS6.[41] Thus, it is possible that even though the FS288 gene delivery increased muscle protein synthesis irrespective of the time of day or the feeding status, the other physiological effects of FS288 may in part be dependent on the time of day or the feeding status. As the time of day and the feeding status did not alter the protein synthesis responses to FS288, the relevance of these findings concerning mTORC1 signaling requires further investigation.

Furthermore, other FS288-induced factors, beyond or synergistic to mTORC1, potentially contributing to the FS288-induced muscle hypertrophy should not be disregarded. Indeed, the inhibition of mTOR via rapamycin administration did not fully abolish the FS288-induced increase in muscle protein synthesis in a previous study,[32] suggesting a role for FS288-induced effects on factors other than mTORC1 signaling in the regulation of muscle protein synthesis. For example, repression of an ankyrin repeat and SOCS box protein 2 (ASB2) was recently shown to be essential for FS288-induced hypertrophy.[56] Moreover, the MAP kinase JNK induced by anabolic stimuli, such as resistance exercise,[39,57] has been recently identified to be a key driver for muscle hypertrophy through negative regulation of TGF-β/myostatin activity.[39] Interestingly, the phosphorylation of JNK was increased by the overexpression

of FS288 in the present study suggesting another pathway worth investigating in the future. In addition to the mechanistic studies, future studies aiming for more therapeutic applications should focus also on the systemic effects, with a systemically delivered therapeutic agent.

In conclusion, we showed for the first time that the significant increase in muscle mass and muscle protein synthesis by follistatin gene delivery is largely independent of the diurnal fluctuations in physical activity and food intake, and the feeding status, suggesting that follistatin gene therapy may be an effective strategy to counteract muscle atrophy in conditions of mildly reduced activity and nutrient intake. In addition, our novel results demonstrate that the follistatin-induced increases in mTORC1 signaling and protein synthesis may potentially be driven by the increased amount of mTOR colocalized with the lysosomes, but not by the translocation of this complex toward the sarcolemma and that these effects are relatively little influenced by the feeding status. However, further mechanistic evidence is required to confirm the proposed mechanism. Finally, we also observed that the mTORC1 signaling response to follistatin gene delivery is attenuated by fasting and during the day, but the physiological relevance of this finding requires further investigation.

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CONFLICT OF INTEREST

Tuuli A. Nissinen, Jaakko Hentilä, Vasco Fachada, Juulia H. Lautaoja, Arja Pasternack, Olli Ritvos, Riikka Kivelä, and Juha J. Hulmi declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

TA Nissinen, JJ Hulmi, and R. Kivelä designed the study; TA Nissinen, J. Hentilä, and JH Lautaoja performed the research; TA Nissinen and V. Fachada analyzed the data; O. Ritvos, A. Pasternack, and R. Kivelä contributed new reagents or analytic tools; TA Nissinen and JJ Hulmi wrote the manuscript with the help of R. Kivelä. All authors have read and approved the final manuscript.

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SUPPORTING INFORMATION

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