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p300 mediates muscle wasting in Lewis lung carcinoma

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Abstract

C/EBPβ is a key mediator of cancer-induced skeletal muscle wasting. However, the signaling mechanisms that activate C/EBPβ in the cancer milieu are poorly defined. Here we report cancer-induced muscle wasting requires the transcriptional co-factor p300 which is critical for the activation of C/EBPβ. Conditioned media from diverse types of tumor cells as well as recombinant HSP70 and HSP90 provoked rapid acetylation of C/EBPβ in myotubes, particularly at its Lys39 residue. Overexpression of C/EBPβ with mutated Lys39 impaired Lewis lung carcinoma (LLC)-induced activation of the C/EBPβ-dependent catabolic response, which included upregulation of E3 ligases UBR2 and atrogin1/MAFbx, increased LC3-II, and loss of muscle proteins both in myotubes and mouse muscle. Silencing p300 in myotubes or overexpressing a dominant negative p300 mutant lacking acetyltransferase activity in mouse muscle attenuated LLC tumor-induced muscle catabolism. Administration of pharmacological p300 inhibitor C646, but not PCAF/GCN5 inhibitor CPTH6, spared LLC tumor-bearing mice from muscle wasting. Furthermore, mice with muscle-specific p300 knockout were resistant to LLC tumor-induced muscle wasting. These data suggest that p300 is a key mediator of LLC tumor-induced muscle wasting whose acetyltransferase activity may be targeted for therapeutic benefit in this disease.

Precis:

Findings demonstrate that tumor-induced muscle wasting in mice is abrogated by knockout, mutation of Lys39 or Asp1399, and pharmacological inhibition of p300.

Keywords

Cancer Cachexia; HSP70; HSP90; Lys39 of C/EBPβ; C646

INTRODUCTION

Cancer has been increasingly recognized as a systemic disease that causes metabolic disorders in multiple organs that are not resided by cancer *per se*. Cachexia is a wasting

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

syndrome frequently seen in cancer patients and accounts for one third of cancer-associated deaths, which involves predominantly loss of skeletal muscle mass and function known as muscle wasting. The etiology of cancer-induced cachexia remains poorly defined and consequently there has been no FDA-approved medication for this lethal metabolic disorder[1, 2].

Cancer-induced muscle wasting is associated with accelerated muscle catabolism due to hyper-activation of the ubiquitin-proteasome pathway (UPP) leading to the degradation of myofibrillar proteins, as well as the autophagy-lysosomal pathway (ALP) leading to the degradation of cytoplasmic protein aggregates and organelles[3]. Although these proteolytic pathways are also activated in non-cancer-induced muscle atrophy including fasting, disuse and denervation, the signaling mechanism that mediates their activation in the cancer milieu appears distinct. For example, the Akt/FoxO pathway inversely regulates both UPP and ALP activation by fasting, disuse, or denervation[4–6], however, it appears not essential for muscle catabolism in animal cancer models[7, 8] or cancer patients[9, 10] due to activation of Akt that inhibits FoxOs[11]. In contrast, inflammation-activated signaling plays a key role in mediating the activation of UPP and ALP in response to cancer. In mouse models of cancer, TLR4 mediates muscle catabolism through a direct effect in muscle cells as well as an indirect effect by increasing circulating inflammatory cytokines, all of which activate such intracellular signaling molecules as p38 MAPK and NF-κB to promote muscle catabolism[12, 13]. Further, we recently found that diverse types of cachexia-inducing cancer cells release high levels of HSP70 and HSP90 via extracellular vesicles that activate TLR4-mediated muscle wasting[14]. We also observed that downstream of TLR4 and cytokine receptors, activation of transcription factor C/EBPβ by p38β MAPK is required for cancer-induced muscle catabolism through upregulation of E3 ligases atrogin1/MAFbx[8, 15] and UBR2[16], as well as key autophagy-related genes LC3b and Gabarapl1[17]. Thus, C/EBPβ plays a key role in mediating cancer-induced activation of UPP and ALP in cachectic muscle, which makes it a potential therapeutic target of cancer cachexia.

As a transcription factor involved in numerous cellular processes, the signaling mechanisms that mediate C/EBPβ activation is highly complex[18, 19]. The mechanisms by which C/EBPβ is activated in muscle cells in the cancer milieu have only been deciphered partially. C/EBPβ activity is regulated primarily by posttranscriptional modifications. The transactivation activity of C/EBPβ is normally auto-repressed in the absence of an activating signal, which is yet to be identified in cancer cachexia. Although we showed previously that p38β MAPK-mediated phosphorylation of C/EBPβ at Thr188 activates its DNA-binding activity in muscle cells[15], the signaling mechanism that removes the auto-repression is undefined. The regulation of C/EBPβ activity involves acetylation in addition to phosphorylation at various sites by transcriptional co-activators/acetyltransferases p300, GCN5 or PCAF[18, 19]. In cultured myotubes, dexamethasone induces p300-dependent acetylation of C/EBPβ, although the acetylation sites are unknown[20]. Intriguingly, different C/EBPβ-responsive promoters require different patterns of acetylated lysine in C/EBPβ for transcriptional activation[21]. In HEK293T cells, binding of p300 to and acetylation of the N-terminal transactivation domain (aa 22–104) of C/EBPβ removes the auto-repression, although the specific acetylation site(s) involved in this critical process remains unknown[22]. These data suggest that acetylation of the N-terminal transactivation domain of C/EBPβ by p300 plays a

primary role in the regulation of C/EBPβ activity. However, it is not clear whether and how p300-mediated acetylation of C/EBPβ releases it from auto-repressed state in muscle cells in response to a tumor burden.

In the present study, we tested the hypothesis that cancer activates C/EBPβ-mediated muscle catabolism through specific acetylation of its N-terminal transactivation domain. We demonstrate that multiple types of cachexia-inducing cancer as well as exogenous HSP70 and HSP90 induce C/EBPβ acetylation at its Lys39 residue within the N-terminal transactivation domain in myotubes. In addition, activation of muscle catabolism by Lewis lung carcinoma (LLC) in mice requires p300-mediated acetylation of Lys39 in C/EBPβ. Critically, we show that the acetyltransferase activity of p300 is essential for the development of muscle wasting in LLC tumor-bearing mice by genetic as well as pharmacological manipulations of p300. These data suggest that p300 is a key mediator and a promising therapeutic target of cancer-induced muscle wasting.

MATERIALS AND METHODS

Muscle and cancer cell cultures

Murine C2C12 myoblasts (American Type Culture Collection, ATCC) were grown in growth medium (10% fetal bovine serum in DMEM) at 37°C under 5% CO_2. Myoblast differentiation was induced at 85–90% confluence by changing to differentiation medium (4% heat-inactivated horse serum in DMEM) for 96 h with medium replacement in every 24 h interval to form myotubes. Conditioned medium from 48-hour cultures of cancer cells including Lewis lung carcinoma, C26 adenocarcinoma cells (both from National Cancer Institute, Frederick, MD in 2007), BxPC-3 and AGS (both from ATCC in 2011 and 2009, respectively) were collected and centrifuged (1000 × g, 5 min). The supernatant was used to treat myotubes (25% final volume in fresh medium) when indicated, and replaced every 24 h. Baculovirus expression system-derived human recombinant protein HSP70 (Sigma-Aldrich) and human HSP90α generated as previously described[14] were added to myotube culture medium (100 ng/ml each) when indicated. Pre-treatment with 10 μM C646 (Sigma, St. Louis, MO) or 10 μM CPTH6 (Glixx Laboratories, Hopkinton, MA) was performed 30 mins prior to the conditioned medium challenge when indicated. All cell lines were aliquoted upon receipt and kept in liquid nitrogen. Each frozen aliquot of cells were passaged for < 10 times. All cell lines were verified to be mycoplasma free using the Mycoplasma Detection Kit (Lonza Bioscience; in March 2017 and October 2018). Cell culture-based experiments were replicated independently for three times.

Animal use

Experimental protocols were pre-approved by the institutional Animal Welfare Committee at the University of Texas Health Science Center at Houston. For LLC-induced cancer cachexia model, 100 μl LLC cells (1×10^6), or an equal volume of vehicle (PBS) was injected subcutaneously into the flanks of 8-week-old male C57BL/6 mice (#000664, The Jackson Laboratories, Bar Harbor, ME). When indicated, C646 dissolved in DMSO and then diluted with equal volume of PBS, or the vehicle (50% DMSO in PBS), were administered 10 mg/kg/day from day 7 after cancer cell injection, when the tumor became palpable, through

a subcutaneously implanted Alzet® Osmotic Pump (Alzet, Cupertino, CA). Plasmids encoding dominant negative p300 mutant or C/EBPβ Lys39 mutants were transfected into TA muscle on day 7 and repeated on day 14 following cancer cell injection using a protocol adopted from Witczak et al.[23] and McMahon et al.[24]. Briefly, mice were anaesthetized prior to intramuscular injection of 0.4 U/μl of bovine hyaluronidase (H-4272; Sigma-Aldrich Products, Poole, UK) in 30 μl saline. After 2 h, the right TA muscle was injected with 30 μg K39A-encoding plasmids whereas the left TA was injected with the empty vector as a contralateral control. Ten square-wave electrical pulses (100 V/cm) were applied with an electrical pulse generator (Model 830, BTX) at a rate of 1 pulse/s to both muscles immediately after plasmid injection. Each pulse (20 ms in duration) was delivered through a pair of stainless steel needles that were 5 mm apart. Development of cachexia was monitored by body weight and grip strength test, and usually took place within 21 days after cancer cell injection. On day 21, mice were euthanized and muscle samples were collected immediately for analyses.

Generation of mice with muscle specific knockout of p300

Muscle-specific p300 knockout mice (p300 mKO) were created by crossbreeding p300-floxed mice[25] (#025168, The Jackson Laboratory, Bar Harbor, ME) in C57BL/6 background with muscle creatine kinase-Cre mice in the same background (#006475, The Jackson Laboratory, Bar Harbor, ME). Expected PCR genotyping band sizes for the p300-floxed transgene (400 bp) and MCK-Cre (450 bp) were assigned to the p300 mKO mice (Figure S1). Primer sets used in genotyping have the following sequences: for p300-floxed transgene-Forward 5'GTGAGTTGATGTCCCTGTCG3' and Reverse 5'CAGACACCCTCTTGCACTCA; for MCK-Cre: Forward 5'TAAGTCTGAACCCGGTCTGC3' and Reverse 5'GTGAAACAGCATTGCTGTCACTT.

Transfection of plasmids and siRNA in C2C12 myotubes

Plasmids encoding various proteins and siRNAs specific for p300 (SASI_Mm01_00159721), PCAF (SASI_Mm01_00043888 & SASI_Mm01_00043889, Sigma-Aldrich) or scrambled control siRNA (Ambion, Austin, TX) were transfected into C2C12 myoblasts using the jetPRIME reagent (Polyplus-transfection Inc., Illkirch, France) according to the manufacturer's protocol. In 24 h, myoblasts were changed into differentiating medium and experiments were started in another 96 h when myotubes were formed. Transfection of p300 siRNA caused an initial delay in differentiation during the first 24 h, however, at 96 h there was no difference in the degree of differentiation between control and p300 siRNA-transfected myotubes. Transfection of PCAF did not alter differentiation.

Western blotting

Cell lysate was prepared and Western blotting was performed as described previously[8]. The primary antibodies used for western blotting were as follows: anti-acetylated lysine (1:1000, 9814, Cell Signaling Technology), anti-p300 (1:500, sc584, Santa Cruz Biotechnology), anti-PCAF (1:1000, p3378, Cell Signaling Technology), anti-C/EBPβ (1:1000, MA1–827, ThermoFisher), anti-atrogin1/MAFbx (1:1000, AP2041, ECM Bioscience), anti-UBR2 (1:500, NBP1–45243, Novus Biologicals), anti-LC3 (1:2000, NB100–2220, Novus

Biologicals), anti-p62 (1:1000, H00008878-M01, Novus Biologicals), anti-p38 MAPK (1:1000, 9212, Cell Signaling), anti-pp38 MAPK (1:1000, 4511, Cell Signaling), anti-p65 (1:250, 8008, Santa Cruz), anti-pp65 (1:500, 166748, Santa Cruz) and anti-MHC (1:1000, MAB4470, R&D Systems); whereas the custom-antibody targeting acetylated Lys39 of C/EBPβ (1:2000) were generated by Pocono Rabbit Farm & Laboratory from rabbits using a peptide (CLAYGAK(Ac)AARAAPRA) synthesized by Novoprotein Scientific Inc (Summit, NJ 07901). Data were normalized to α-Tubulin (antibody was from Development Studies Hybridoma Bank at the University of Iowa, Iowa City, IA).

Fluorescence microscopy and histology study

C2C12 myotubes were stained with anti-MHC antibody (1:1000, MAB4470, R&D Systems) and anti-mouse Alexa Fluor® Plus 488 secondary antibody (1:200, A32723, ThermoFisher), and examined using a Zeiss Axioskop 40 microscope and a Zeiss Axiocam MRM camera system controlled by Axiovision Release 4.6 imaging software. Acquired images were edited using the Photoshop software. Myotube diameter was measured in MHC-stained myotubes as previously described[26]. Cross-sectional area of H&E stained muscle sections was quantified by using the ImageJ software (NIH). Five view-fields with ~100 myofibers per field in each section were measured.

Immunoprecipitation

Immunoprecipitation was carried out as previously described[8] using an anti-C/EBPβ antibody (2 μg per 1 mg total protein, MA1–827, ThermoFisher).

Statistical analyses

Statistical analyses were conducted using the SPSS 22.0 software package (IBM, Chicago, IL). A normality test was performed to examine data distributions. All data were expressed as means ± standard deviation (SD). Comparisons were made by one-way ANOVA followed by Tukey post-hoc test, Paired Student t-test or Chi-Square test as appropriate. Statistical significance was considered at $p < 0.05$.

RESULTS

Cancer cell-released cachexins cause C/EBPβ acetylation at Lys39

Given that murine Lewis lung carcinoma (LLC) and C26 adenocarcinoma trigger muscle catabolism through releasing HSP70 and HSP90 that activate Toll-like receptor 4 (TLR4) on muscle cells[14], we first examined if their conditioned media (denoted as LCM and CCM respectively) induce C/EBPβ acetylation in muscle cells. C/EBPβ was immunoprecipitated from the lysates of LCM/CCM-treated C2C12 myotubes followed by Western blotting using an antibody against acetylated lysine residues. As shown in Figure 1A, the total acetylation status of C/EBPβ was rapidly increased within 1 h and then gradually returned to basal levels around 8 h. To identify acetylated lysine residues that are relevant to C/EBPβ activity, we examined C/EBPβ acetylation on Lys39, which is within the N-terminal transactivation domain of C/EBPβ (aa 22–104) that is acetylated by p300 in HEK293T cells to remove auto-repression[22], by using a custom-made polyclonal antibody specific for C/EBPβ acetylated on Lys39. We observed that LCM increased Lys39 acetylation robustly with a

time course similar to the total acetylation of C/EBPβ (Figure 1B). In addition, treatment with conditioned media of human gastric adenocarcinoma AGS (ACM) and pancreatic adenocarcinoma BxPC-3 (BCM) resulted in similar increases in Lys39 acetylation and up-regulation of C/EBPβ-controlled E3 ligase UBR2[16]; thus indicating that C/EBPβ acetylation at Lys39 is induced by diverse types of cachexia-inducing cancer cells (Figure 1C). Given that the four types of cancer cells tested above all release high levels of HSP70 and HSP90 that are responsible for the activation of muscle catabolism in diverse tumor models[14], we treated myotubes with recombinant HSP70 and HSP90, which resulted in a similar increase in Lys39 acetylation and upregulation of the E3 ligase UBR2 (Figure 1C). Collectively, these data indicate that acetylation of C/EBPβ at Lys39 is a catabolic response induced by diverse types of cachexia-inducing cancer cells through releasing cachexins, primarily HSP70 and HSP90.

C/EBPβ acetylation at Lys39 is required for LLC-induced skeletal muscle catabolism

We then attempted to determine the significance of acetylation of C/EBPβ at Lys39 in the induction of atrophic signaling. Two Lys39 acetylation-defective mutants of C/EBPβ in which Lys39 was replaced with alanine (K39A) or arginine (K39R) and an acetylation-mimicking mutant in which Lys39 was replaced with glutamine (K39Q)[27] were over-expressed in C2C12 myotubes. Western blot analysis of the cell lysate using the custom-made antibody specific for C/EBPβ acetylated on Lys39 revealed that overexpressed K39A and K39R mutants abrogated the LCM-provoked Lys39 acetylation whereas the K39Q mutant mimicked Lys39 acetylation (Figure 2A). This result also demonstrates that the custom-made antibody is specific for Lys39-acetylated C/EBPβ. Consequently, C/EBPβ-dependent catabolic events including the up-regulations of UBR2[16] and atrogin1/MAFbx[8], increase in LC3-II and resultant loss of myofibrillar protein myosin heavy chain (MHC) in LCM-challenged myotubes were halted by K39A and K39R, but not by K39Q (Figure 2B & 2C). On the other hand, LCM-induced elevation of C/EBPβ phosphorylated at Thr188 (which regulates its DNA-binding activity[15]) was not altered by any of the mutants studied (Figure S2). This suggests that Thr188 phosphorylation is independent of Lys39 acetylation. Thus, Lys39 acetylation, not Thr188 phosphorylation, is likely the primary regulatory mechanism of C/EBPβ activity.

We then validated these findings *in vivo* by over-expressing K39A in the tibialis anterior (TA) muscle of LLC-bearing mice. Compared with the vector-transfected contralateral TA, forced expression of K39A attenuated the upregulations of atrogin1/MAFbx and UBR2 and the loss of MHC (Figure 2D). Furthermore, K39A ameliorated the loss of TA mass (Figure 2E) and the decrease in myofiber cross-sectional area under the tumor burden (Figure S3). These data suggest that C/EBPβ acetylation at Lys39 is required for cancer-induced skeletal muscle catabolism.

p300 mediates LLC-induced C/EBPβ acetylation at Lys39 and muscle catabolism

C/EBPβ can be acetylated by multiple acetyltransferases including p300[28], PCAF and GCN5[29]. To identify the acetyltransferase responsible for C/EBPβ acetylation at Lys39 in muscle cells in cancer milieu, p300 or PCAF were knocked down in C2C12 myotubes with specific siRNAs. LCM-induced acetylation of C/EBPβ at Lys39 was abrogated in p300-

deficient myotubes, but not in that deficient in PCAF (Figure 3A). Consequently, p300 silencing abolished LCM-induced catabolic responses including upregulations of UBR2 and atrogin1/MAFbx, increase in LC3-II (Figure 3B) and loss in MHC (Figure 3C) and myotube mass (Figure 3D). It is noteworthy that the protein level of p300 was not altered in LCM-treated myotubes (Figure 3A), suggesting the regulation of p300 activity by cancer primarily involves post-transcriptional mechanisms.

To verify whether the acetyltransferase activity of p300 is required for cancer-induced muscle catabolism *in vivo*, a dominant negative p300 mutant (DN-p300) that lacks acetyltransferase activity due to the conversion of aspartic acid 1399 to tyrosine[30] was transfected into the TA of LLC tumor-bearing mice. Expression of this mutant mitigated the elevations of UBR2, atrogin1/MAFbx and LC3-II, loss of MHC (Figure 4A) and TA mass (Figure 4B). In addition, DN-300 abrogated the loss of myofiber cross-sectional area (Figure S4).

Next, we investigated whether cancer-induced muscle wasting can be intervened by pharmacological inhibition of p300. C2C12 myotubes were pretreated with the specific p300 inhibitor C646[31] or a dual inhibitor of PCAF and GCN5, CPTH6[32]. Myotube catabolism provoked by LCM was impeded significantly by C646, but not by CPTH6 (Figure 5A & 5B). Furthermore, administration of C646 to LLC tumor-bearing mice hampered the catabolic responses (Figure 5C) and attenuated the loss of muscle mass (Figure 5D) as well as body weight (Figure 5E), without affecting tumor growth (Figure 5F). These data support that the acetyltransferase activity of p300 specifically mediates C/EBPβ acetylation at Lys39 and the ensuing muscle wasting in the tumor milieu. Therefore, pharmacological inhibition of p300 appears to be a promising strategy for combating cancer cachexia.

Mice with muscle-specific deletion of p300 are resistant to LLC-induced muscle wasting

Finally, to further verify the key role of p300 in mediating cancer-induced muscle wasting, we generated mice with skeletal muscle-specific knockout of p300 (p300 mKO) by crossing p300-floxed mice ($p300^{f/f}$)[25] to MCK-Cre mice (Figure S4), both were in the C57BL/6 background. Muscle-specific knockout of p300 was confirmed by Western blot analysis of p300 in various tissues (Figure 6A). There was no difference in tumor growth in p300 mKO mice compared to $p300^{f/f}$ mice (Figure 6B). LLC tumor-provoked increase in C/EBPβ acetylation on Lys39 was absent in the TA of p300 mKO mice, so as the C/EBPβ-dependent activation of the catabolic pathways (Figure 6C). Given that LLC activates TLR4 and provokes muscle catabolism through its release of HSP70 and HSP90[12, 14], the activities of TLR4 downstream effectors p38 MAPK and NF-κB were monitored. The increase in the active forms of p38 MAPK and NF-κB were not altered in LLC tumor-bearing p300 mKO mice (Figure S5). Consequently, p300 mKO mice were spared from muscle wasting as measured by body weight (Figure 6D), muscle strength (Figure 6E), muscle weight (Figure 6F), and muscle fiber cross sectional area (Figure 6G). In agreement with a previous report[33], p300 mKO mice did not manifest significant phenotypic differences as compared to $p300^{f/f}$ mice in terms of body weight (Figure 6D), muscle strength (Figure 6E), muscle weight (Figure 6F), and histology (Figure 6G). These data allow us to conclude that p300 is a critical mediator of LLC tumor-induced muscle wasting.

DISCUSSION

The present study demonstrates for the first time a key role of p300 in mediating cancer-induced muscle wasting by activating C/EBPβ through specific acetylation of its Lys39 residue. This study not only extends our scarce understanding of the etiology of cancer-induced muscle wasting through detailed analyses of the underlying signaling mechanism that activates muscle catabolic pathways, but also identifies a critical therapeutic target p300 for this metabolic disorder. Remarkably, from the therapeutic point of view, our findings that genetic or pharmacological inhibition of p300 is sufficient to abrogate muscle wasting in a widely studied mouse cancer model (LLC) may have significant translational values. Given the unsatisfactory results in intervening cancer cachexia with various therapeutic strategies in previous clinical trials[3435], our success in abrogating cancer-induced muscle wasting both *in vitro* and *in vivo* by targeting p300 suggests that improved understandings in pathogenic mechanisms can lead to the design of more specific and effective therapeutic strategies for the intervention of cancer cachexia.

Our data demonstrate that in response to a tumor burden, p300 activates C/EBPβ-mediated catabolic pathways in muscle cells through acetylating its Lys39 residue. In HEK293T cells, the auto-repressed state of C/EBPβ is released upon the binding of p300 to acetylate its N-terminal transactivation domain (aa 22–104)[22]. Our data suggest that p300-mediated acetylation of Lys39, which is within the N-terminal transactivation domain, serves the same purpose in muscle cells. In addition, our data indicate that in absence of Lys39 acetylation, LCM-induced C/EBPβ phosphorylation of Thr188 remains intact (Figure S1), which enables C/EBPβ to bind DNA[15]. Thus, Lys39 acetylation, but not Thr188 phosphorylation appears to play a primary role in the regulation of C/EBPβ activity by removing auto-repression. According to previous structural analyses by Lee et al., the N-terminal transactivation domain is mostly unstructured with pockets of hydrophobicity. Lys39 resides in a subdomain within this region (aa 22–40) that is populated by short hydrophobic helices[22]. It is likely that the Lys39 acetylation of C/EBPβ alters conformation of this domain to allow access of regulatory proteins or interaction with the target genes.

Exerting important roles in development, physiology, and disease via its network with many proteins, p300 serves not only as a histone acetyltransferase (HAT), but also as a factor acetyltransferase (FAT) for certain transcription factors[36, 37]. The effect of p300-mediated acetylation on muscle mass is highly complex. Previous studies reported that p300-mediated acetylation modulates FoxO1 and FoxO3 activity differentially in response to denervation, disuse, starvation, insulin or dexamethasone[38–41]; and HDAC1 promotes disuse-induced muscle atrophy by activating FoxO through deacetylation[42]. In contrast, the current study identifies p300 as a key mediator of cancer-induced muscle wasting by acetylating C/EBPβ at Lys39. Thus, the regulation of muscle mass by acetylation of transcription factors appears to be stimulus and substrate-dependent.

Our observations that conditioned media of diverse types of cancer cells as well as recombinant HSP70 and HSP90 similarly activate p300-mediated C/EBPβ acetylation at Lys39 in myotubes (Figure 1) suggest that this effect is mediated by HSP70 and HSP90 that are released by various cachectic cancer cells and cause muscle wasting by activating TLR4

as previously demonstrated[14]. Thus, p300-mediated C/EBPβ acetylation at Lys39 is likely important for muscle wasting induced by diverse types of cancer that release HSP70 and HSP90. We also observed that the abundance of p300 was not altered in response to cancer (Figure 3A), which implies that the regulation of p300 activity by cancer is primarily at the post-transcriptional level through an unknown mechanism downstream of TLR4. NF-κB and p38 MAPK are downstream effectors of TLR4 that mediate muscle wasting[12]. However, we observed that neither the activation of NF-κB nor p38 MAPK was attenuated in the muscle of tumor-bearing p300 mKO mice. This data suggests that p300 activation is downstream of or unrelated to NF-κB and p38 MAPK. Given that p300-mediated C/EBPβ acetylation on Lys39 takes place within 1 h of exposure to cancer cell-released cachexins (Figure 1), transcription factor NF-κB is unlikely to be involved in this event.

Acetylation of Gcn5-related *N*-acetyltransferases (GNATs) has been shown to promote gene transcription that governs diverse biological processes such as lipid metabolism and oxidative homeostasis[43]. In skeletal muscle, the mRNA levels of p300 and PCAF were elevated concomitantly during atrophy associated with denervation and nutrient deprivation[44] whereas forced expression of enzymatic-inactive GCN5 down-regulated a panel of atrophic genes in fasted animals[45]. However, our data that ablation of PCAF alone or in combination with GCN5 by either genetic or pharmacological approach failed to avert C/EBPβ-dependent catabolism suggest that these acetyltransferases are not essential for cancer-induced muscle wasting. In contrast, we demonstrated that mice with muscle-specific deletion of p300 are resistant to cancer-induced muscle wasting. Mice with muscle-specific knockout of p300 (p300 mKO) was generated by crossbreeding $p300^{f/f}$ mice to mice with the Cre transgene controlled by the *muscle creatine kinase* promoter. This approach allows p300 mKO mice to bypass the myogenic differentiation stage for which p300 is required[46], resulting in normal development of skeletal muscle. Concordantly, we observed a remarkable effectiveness of the specific p300 inhibitor C646 in blocking C/EBPβ acetylation at Lys39 and C/EBPβ-dependent muscle catabolism. These data support specific p300 inhibitors as potential therapeutic agents for cancer-induced muscle wasting.

Taken together, the current study reveals p300 as an indispensable mediator of LLC tumor-induced muscle wasting due to its activation of C/EBPβ by acetylating Lys39. Thus, specific inhibitors of p300 could be further tested for the intervention of cancer-associated muscle wasting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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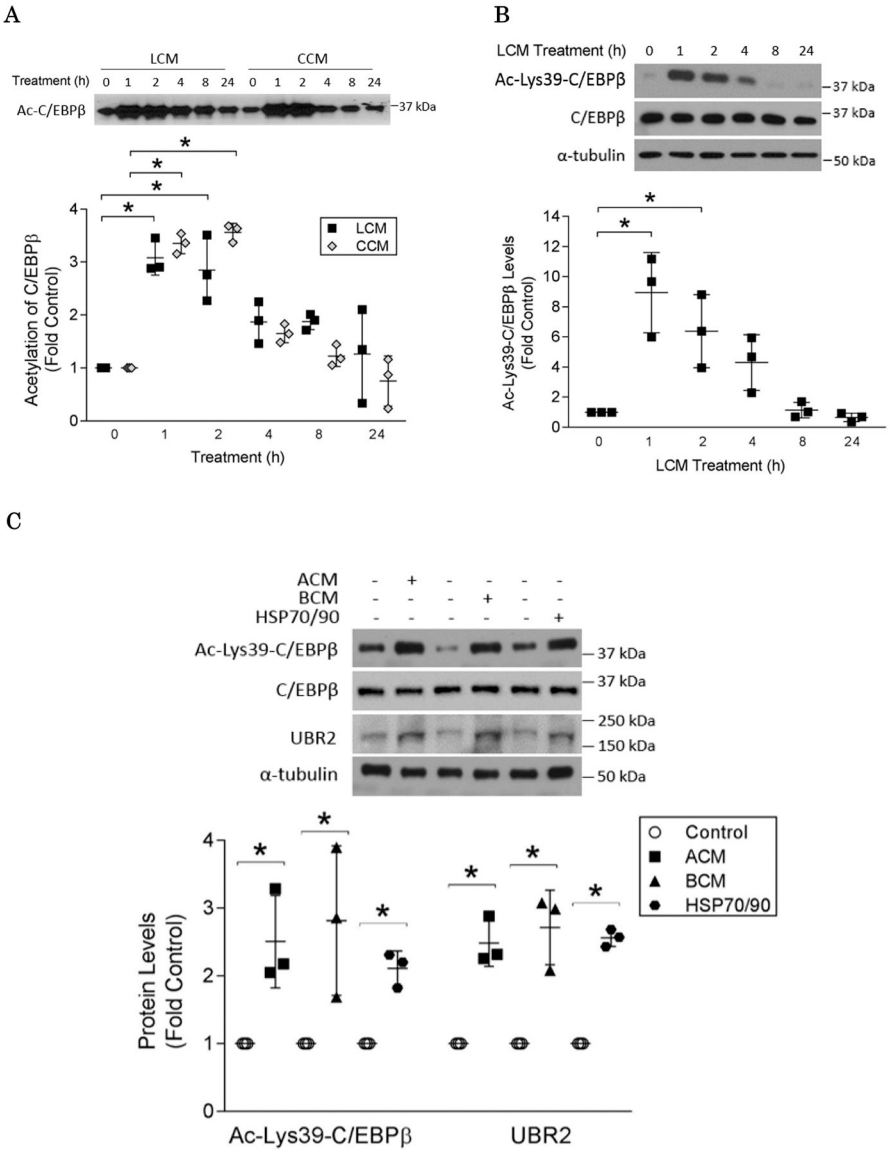

Figure 1. Cancer cell-released cachexins stimulate acetylation of C/EBPβ at Lys39 in myotubes.

C2C12 myotubes were incubated with diverse cancer cell-conditioned media or HSP70 and HSP90 as indicated in durations ranging from 0 to 24 hrs. (A) LLC and CCM induces acetylation of C/EBPβ. Cell lysates were subjected to immunoprecipitation with an anti-C/EBPβ antibody. The immunocomplexes were analyzed by Western blotting using an anti-acetyl lysine antibody. (B) LCM induces acetylation of C/EBPβ at Lys39. Western blotting was conducted to analyze lysate of LCM-treated myotubes using a custom-made antibody targeting acetylated Lys39 of C/EBPβ. (C) ACM and BCM as well as HSP70 and HSP90 induce acetylation of C/EBPβ at Lys39 (2 h) and up-regulation of UBR2 (8 h) in myotubes as analyzed by Western blotting. * $p<0.05$ signifies a statistically significant difference after One-way ANOVA analyses.

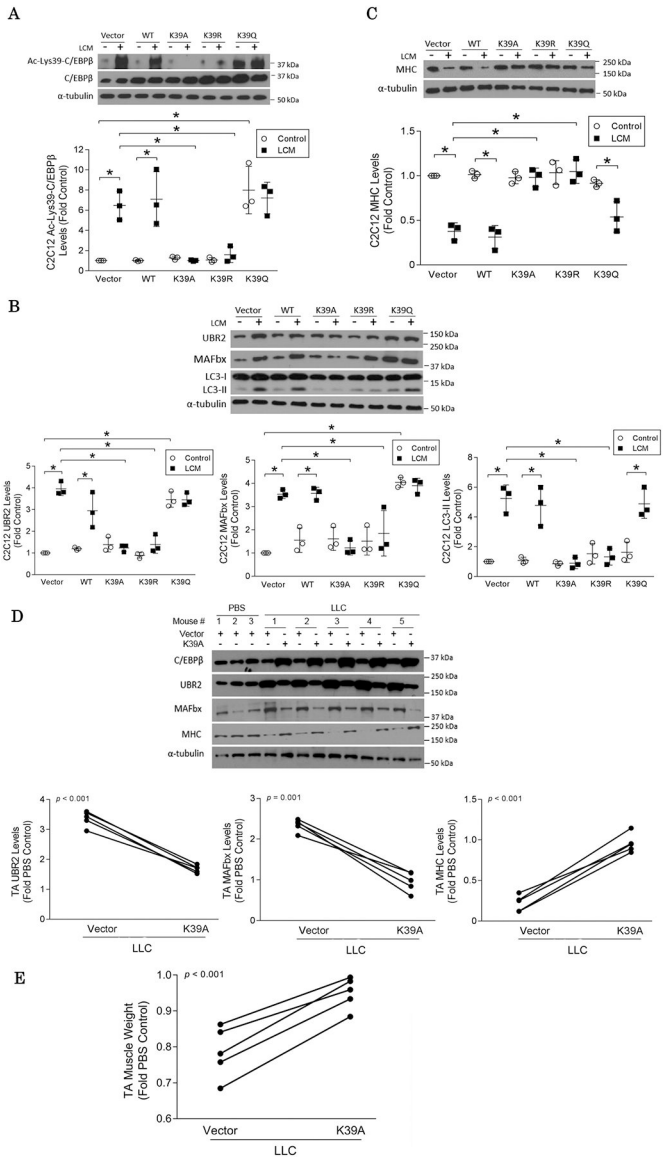

Figure 2. LLC-induced muscle wasting is dependent on Lys39 acetylation of C/EBPβ

(A) Over-expressed mutants of C/EBPβ alters acetylation of C/EBPβ at Lys39. C2C12 myotubes over-expressing Lys39 acetylation-defective C/EBPβ (K39A or K39R) or Lys39 acetylation-mimicking C/EBPβ (K39Q) were treated with LCM for 2 h. Cell lysates were analyzed by Western blotting using our custom-made antibody against C/EBPβ acetylated at Lys39. (B) LCM-induced catabolism in C2C12 myotubes requires C/EBPβ that is acetylated at Lys39. C2C12 myotubes over-expressing the indicated C/EBPβ mutants were treated with LCM for 8 h. Levels of catabolic markers UBR2, atrogin1/MAFbx and LC3-II were analyzed by Western blotting. (C) LCM-induced MHC loss requires C/EBPβ acetylated at Lys39. C2C12 myotubes over-expressing the indicated C/EBPβ mutants were treated with LCM for 72 h. Levels of MHC in cell lysates were analyzed by Western blotting. (D) C/EBPβ acetylation at Lys39 is critical to the activation of muscle catabolism in LLC tumor-bearing mice. The K39A mutant was over-expressed in TA of LLC tumor-bearing mice with

vector overexpressed in contralateral TA as control. After the mice had developed cachexia (day 21), markers of muscle catabolism and MHC in muscle lysate were analyzed by Western blotting. (E) C/EBPβ acetylation at Lys39 is critical to muscle mass loss in LLC tumor-bearing mice. TA was excised from the mice and weighed. * $p<0.05$ signifies a statistically significant difference after One-way ANOVA analyses or Paired t-test (A to E).

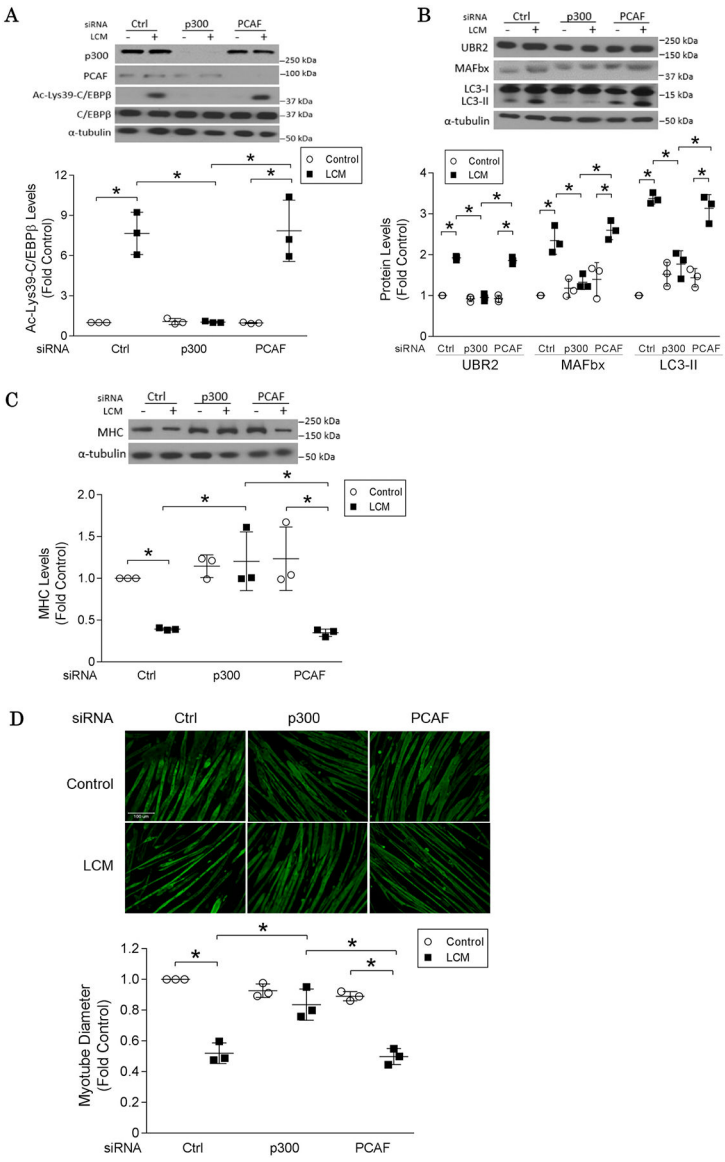

Figure 3. p300 mediates LLC-induced C/EBPβ acetylation at Lys39

(A) Cancer cell-induced Lys39 acetylation of C/EBPβ requires p300 specifically. Transcriptional repression of p300 or PCAF in myotubes was achieved by specific siRNA. After 2 h of LCM treatment, the expression of p300 and PCAF, as well as C/EBPβ acetylation at Lys39 in C2C12 myotubes were analyzed by Western blotting. (B) Cancer cell-induced activation of muscle catabolic pathways requires p300 specifically. p300 or PCAF in myotubes was knocked-down by specific siRNA. After 8 h of LCM treatment, the levels of UBR2, MAFbx and LC3-II were analyzed by Western blotting. (C) Cancer cell-induced MHC loss is dependent on p300. Myotubes in which p300 or PCAF was knocked-down by siRNA were treated with LCM for 72 h, and subjected to Western blot analysis of MHC. (D) Cancer cell-induced loss of myotube mass is dependent on p300. Myotubes treated as described in (C) were subjected to immunofluorescence staining using an anti-

MHC antibody and myotube diameter was measured. * $p<0.05$ signifies a statistically significant difference determined by One-way ANOVA analyses.

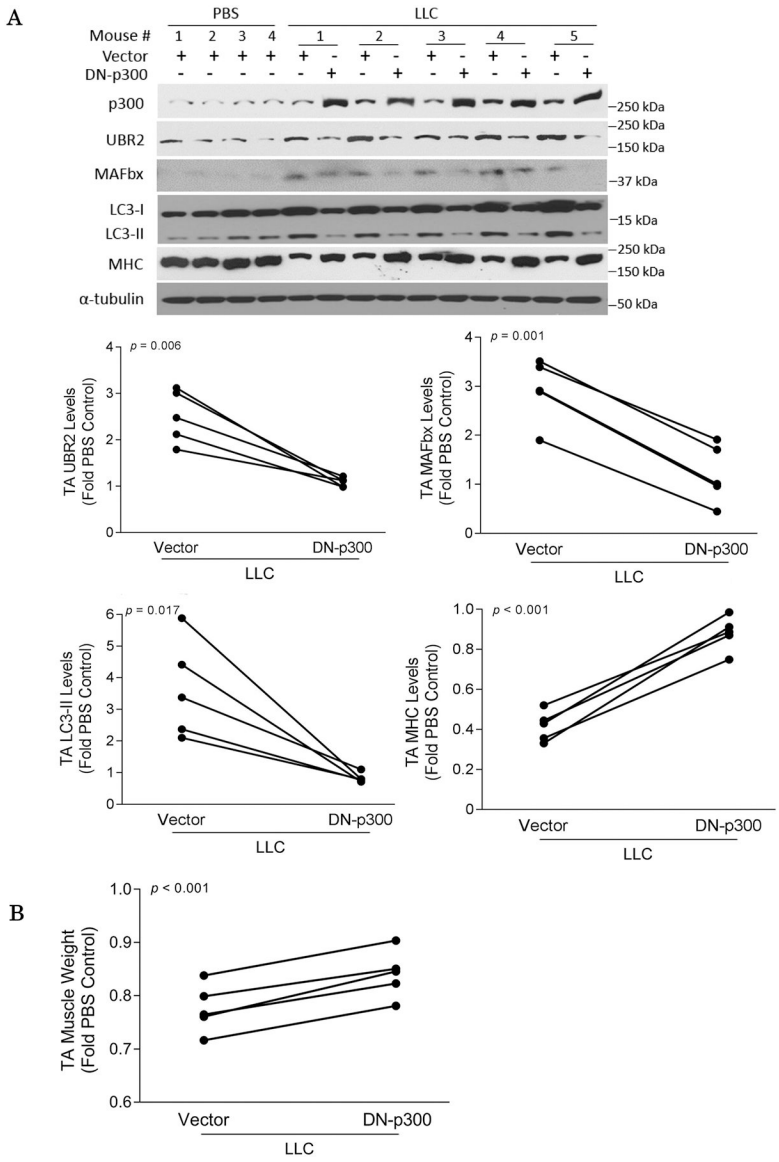

Figure 4. Acetyltransferase activity of p300 is essential to LLC-induced muscle wasting

(A) Cancer-induced activation of catabolic pathways in muscle requires p300 with intact acetyltransferase activity. A dominant negative p300 mutant (DN-p300) with defective acetyltransferase activity was over-expressed in the TA muscle of LLC tumor-bearing mice through plasmid transfection. Empty Vector was transfected into the contralateral TA as control. Muscle catabolism measured as UBR2, atrogin1/MAFbx, LC3-II and MHC, was analyzed by Western blotting. (B) Cancer-induced muscle loss is dependent on p300 with intact acetyltransferase activity. TA of mice described in (A) was excised and weighed. * p<0.05 signifies a statistically significant difference after One-way ANOVA analyses or Paired t-test (A and B).

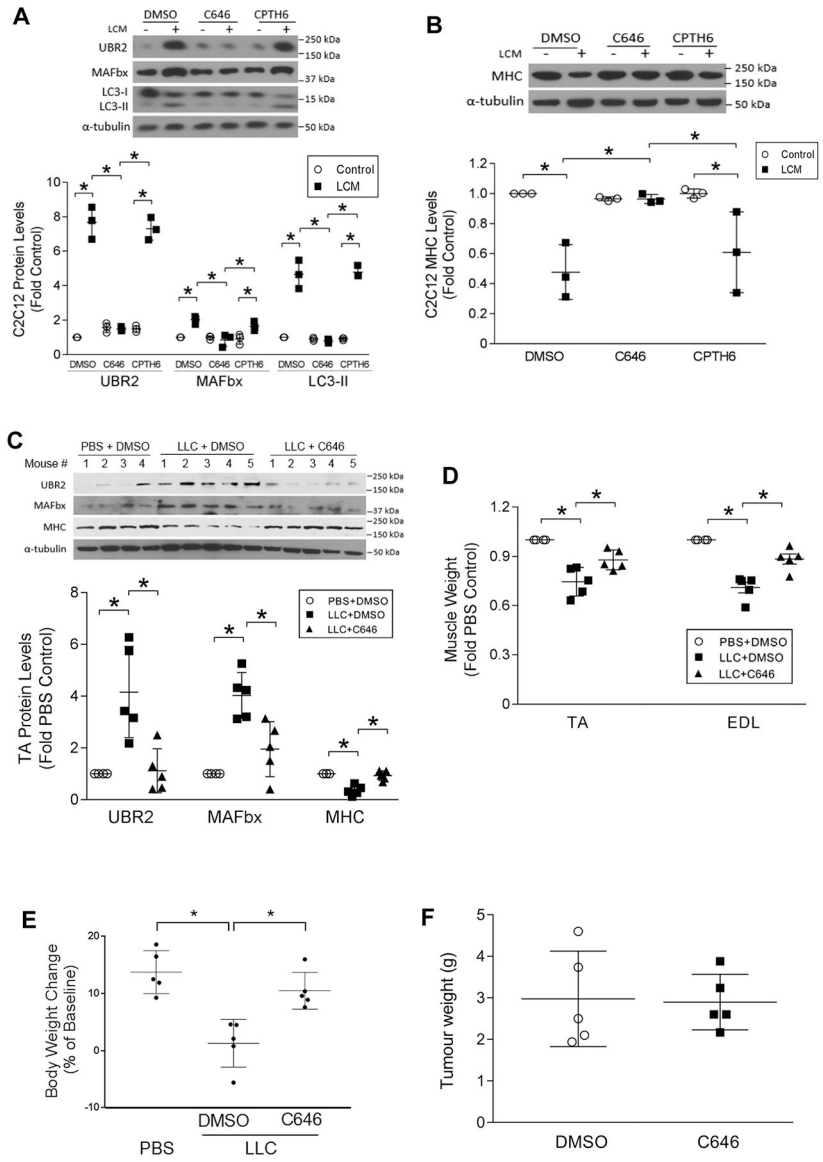

Figure 5. Pharmacological inhibition of p300 ameliorates LLC-induced muscle wasting

(A) C646, a specific pharmacological inhibitor of p300, abrogates the LCM-induced myotube catabolism. C2C12 myotubes were pre-treated (30 min) with pharmacological inhibitor of p300 (C646, 10 μM) or PCAF/GCN5 (CPTH6, 10 μM) prior to LCM exposure for 8 h. Markers of catabolic pathways were analyzed by Western blotting. (B) C646 abrogates the LCM-induced MHC loss. Myotubes were pre-treated with pharmacological inhibitor of p300 (C646) or PCAF/GCN5 (CPTH6) prior to LCM treatment for 72 h. The expression of MHC was analyzed by Western blotting. (C) C646 abrogates muscle catabolism in LLC tumor-bearing mice. Subcutaneous administration of C646 was initiated 7 days after the implantation of LLC. On day 21, mice were euthanized and analyzed for muscle catabolism markers by Western blotting. (D) C646 attenuates muscle mass loss in LLC tumor-bearing mice. (E) C646 attenuates body weight loss in LLC tumor-bearing mice.

(F) C646 does not alter tumor growth. * $p<0.05$ signifies a statistically significant difference after One-way ANOVA analyses.

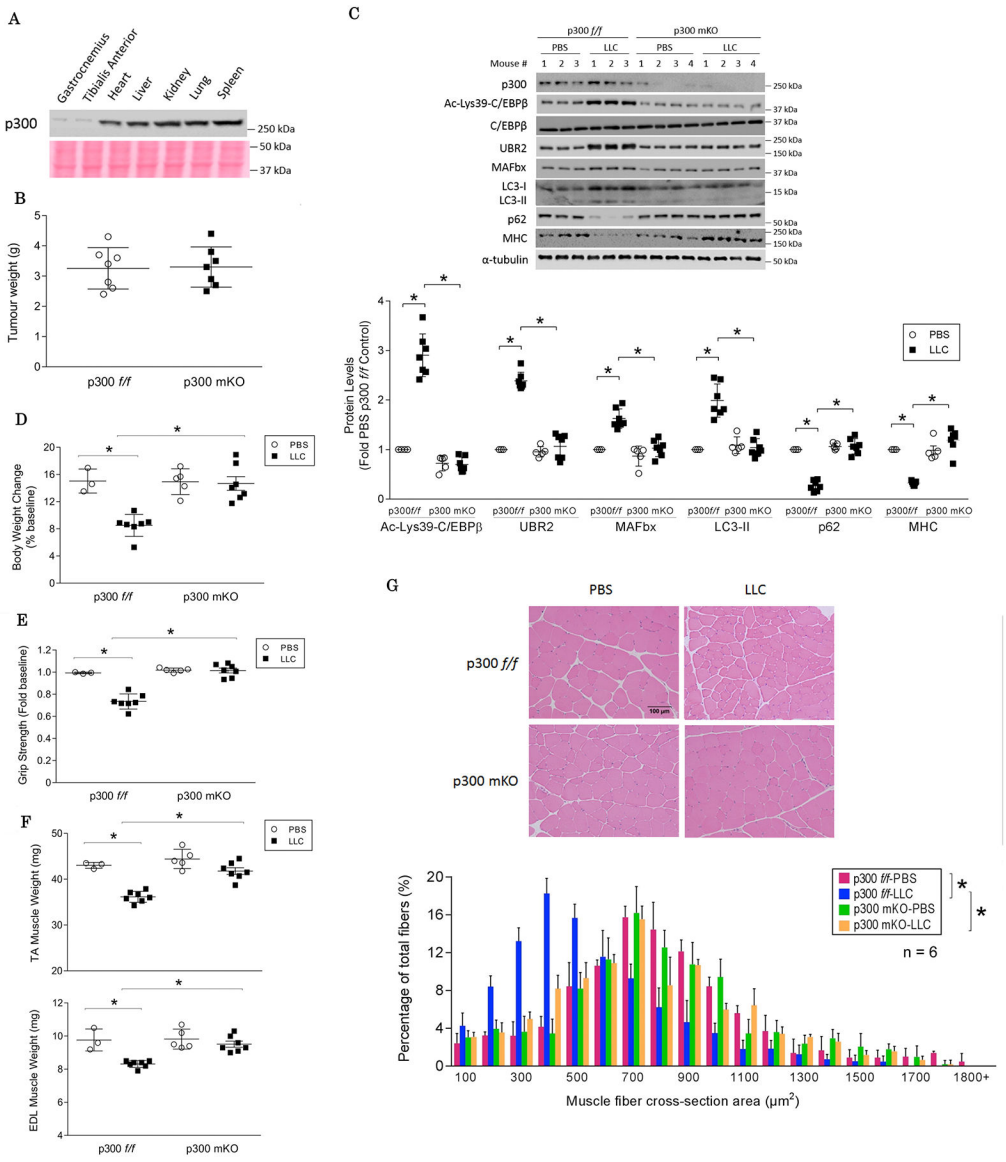

Figure 6. Mice with muscle-specific knockout of p300 are resistant to LLC-induced muscle wasting

(A) Muscle-specific knock out of p300 in mice (p300 mKO). p300 mKO mice were generated by crossing p300-floxed mice (p300 f/f) to MCK-Cre mice. The expression of p300 in multiple tissues of mKO mice were analyzed by Western blotting with Ponceau S staining of the membrane as loading control. (B) Muscle-specific knock out of p300 did not alter tumor growth (day 21). (C) Muscle catabolic response is abrogated in LLC tumor-bearing p300 mKO mice. p300 mKO and $p300^{f/f}$ mice were implanted with LLC cells or received PBS as control and euthanized on day 21. Lys39 acetylation of C/EBPβ, activation of muscle catabolic markers and the level of MHC in the TA lysates were analyzed by Western blotting. (D) Body weight loss is attenuated in LLC tumor-bearing p300 mKO mice. (E) Muscle strength was preserved in LLC tumor-bearing p300 mKO mice. (F) Muscle weight (TA and EDL) was preserved in LLC tumor-bearing p300 mKO mice. (G) Reduction of muscle fiber cross-sectional area was prevented in LLC tumor-bearing p300

mKO mice. * $p<0.05$ signifies a statistically significant difference after One-way ANOVA or Chi-square test.