CCAAT/enhancer binding protein beta protects muscle satellite cells from apoptosis after injury and in cancer cachexia

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CCAAT/enhancer binding protein beta (C/EBPβ), a transcription factor expressed in muscle satellite cells (SCs), inhibits the myogenic program and is downregulated early in differentiation. In a conditional null model in which C/EBPβ expression is knocked down in paired box protein 7+ (Pax7+) SCs, cardiotoxin (CTX) injury is poorly repaired, although muscle regeneration is efficient in control littermates. While myoblasts lacking C/EBPβ can differentiate efficiently in culture, after CTX injury poor regeneration was attributed to a smaller than normal Pax7+ population, which was not due to a failure of SCs to proliferate. Rather, the percentage of apoptotic SCs was increased in muscle lacking C/EBPβ. Given that an injury induced by BaCl₂ is repaired with greater efficiency than controls in the absence of C/EBPβ, we investigated the inflammatory response following BaCl₂ and CTX injury and found that the levels of interleukin-1β (IL-1β), a proinflammatory cytokine, were robustly elevated following CTX injury and could induce C/EBPβ expression in myoblasts. High levels of C/EBPβ expression in myoblasts correlated with resistance to apoptotic stimuli, while its loss increased sensitivity to thapsigargin-induced cell death. Using cancer cachexia as a model for chronic inflammation, we found that C/EBPβ expression was increased in SCs and myoblasts of tumor-bearing cachectic animals. Further, in cachectic conditional knockout animals lacking C/EBPβ in Pax7+ cells, the SC compartment was reduced because of increased apoptosis, and regeneration was impaired. Our findings indicate that the stimulation of C/EBPβ expression by IL-1β following muscle injury and in cancer cachexia acts to promote SC survival, and is therefore a protective mechanism for SCs and myoblasts in the face of inflammation.

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Efficient regeneration of skeletal muscle is dependent on tissue-resident stem cells termed satellite cells (SCs).¹ Quiescent and heterogeneous by nature, SCs are characterized by their histological localization between the muscle fiber sarcolemma and the basal lamina, as well as by expression of the paired box protein 7 (Pax7).²,³ Upon muscle injury, Pax7 expression declines and activated SCs progressively stimulate the expression of the myogenic basic helix-loop-helix regulatory factors that are required for the induction of myocyte-specific genes.⁴ CCAAT/enhancer binding proteins (C/EBPs) are a family of bZIP transcription factors involved in numerous biological processes.⁵–⁹ Cebpb null mice have defective liver regeneration,¹⁰ skin abnormalities,¹¹ impaired development of the mammary glands,¹² reduced adipose tissue,¹³ female sterility,¹⁴ and are immunodeficient.¹⁵–¹⁷ In healthy skeletal muscle, C/EBPβ expression is restricted to Pax7+ SCs. Highest in SCs, C/EBPβ levels decline early in differentiation and this downregulation is required for myogenesis to occur.¹⁸,¹⁹ Indeed, forcing C/EBPβ expression in myoblasts blocks myogenesis, and is accompanied by increased Pax7 and decreased myogenic differentiation marker 1 (MyoD), myogenin and myosin heavy chain expression. In addition, loss of C/EBPβ in SCs results in larger myotubes in culture, muscle hypertrophy in vivo and enhanced muscle regeneration following a single BaCl₂-induced injury.¹⁹

Normal skeletal muscle repair involves local inflammation that is required for efficient regeneration. M1-type macrophages are recruited early to the site of injury, produce interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNFα), and promote SC proliferation while inhibiting their differentiation.²⁰,²¹ Four days after injury, M2-type macrophages become the dominant subtype in the muscle and act to decrease local inflammation by deactivating M1 macrophages.²²,²³ The transient inflammatory response following acute muscle injury is accompanied by an increase in Pax7+ cells in the injured muscle that do not immediately contribute to repair.²⁴–²⁶ Resolution of inflammation, rather, promotes myogenesis.²⁷–³⁰ Although acute muscle injury is accompanied by transient inflammation, chronic inflammation and dysregulated cytokine production is a feature of cachexia, characterized by both adipose tissue and skeletal muscle atrophy, and occurs in many ailments including chronic obstructive pulmonary disease, AIDS, chronic kidney failure and sepsis.³¹–³⁴

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Abbreviations: C/EBPβ, CCAAT/enhancer binding protein beta; Pax7, paired box protein 7; IL-1β, interleukin-1 beta; SC, satellite cell; MyoD, myogenic differentiation factor 1; cKO, conditional knockout; CTX, cardiotoxin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; TPG, thapsigargin; PI, propidium iodine; LLC, Lewis lung carcinoma

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Relatively little is known about the effect of inflammation on muscle stem cell populations after acute injury, and even less under chronic conditions such as cachexia. Herein, we describe a protective mechanism where IL-1β production after acute muscle injury or in cachexia drives increased C/EBPβ expression in muscle SCs, rendering SCs more resistant to apoptosis. Loss of C/EBPβ expression triggers the loss of Pax7+ cells by apoptosis and impairs muscle regeneration.

Results

CTX injury increases apoptosis of Cebpb null SCs. C/EBPβ conditional knockout mice (cKO, Cebpbβ−/−Pax7+/−creER), where Cebpb is excised in Pax7+ cells, and non-Cre-expressing littermate controls (WT, Cebpbβ−/−Pax7−/+ ) were injured with cardiotoxin (CTX) and repair was assessed 7 days post-injury. An excision efficiency of approximately 75% was achieved in primary myoblasts isolated from cKO mice as compared with WT, and this correlated with a decrease in C/EBPβ protein expression in these same cells (Figures 1a and b). In sharp contrast to BaCl2-induced injury, where cKOs repaired the damage with greater efficiency than WT controls,19 after CTX injury, cKOs failed to appreciably repair muscle (Figure 1c). The number of fibers with centrally located nuclei, indicating regeneration, was decreased by approximately 33% in cKOs after CTX injury as compared with controls with an average cross-sectional area ~ 24% smaller than WT (Figures 1d and e). Given that SCs lacking C/EBPβ can differentiate efficiently in vitro,18 we reasoned that the CTX injury may be affecting the size of the SC population in cKO mice. Although uninjured WT and cKO muscle had equivalent percentages of Pax7+ cells (~2% of total nuclei), injury increased this value approximately twofold in WT mice (Figures 1f and g). However, the increase in the Pax7+ population was not observed in the cKOs after injury, but rather remained at uninjured levels. The smaller Pax7+ population in the cKOs was not because of a failure of these cells to proliferate, as the percentage of Pax7+ cells that were also Ki67+ was greater in cKO cultures 7 days after injury with BaCl2 as compared with WT muscle (Figures 1h and i). Rather, CTX injury provoked a ~ 2.5-fold increase in the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)/Pax7+ cells in cKOs after CTX injury when compared with WT mice, but not after BaCl2-induced injury, indicating that CTX injury promotes the apoptosis of SCs lacking C/EBPβ (Figures 1j and k). Interestingly, the remaining SCs in cKO muscle after injury with CTX were mostly C/EBPβ positive, suggesting that they are likely ‘recombination escapers’ (Figures 1l and m).

C/EBPβ is upregulated by IL-1β. Following injury to the skeletal muscle, recruited leukocytes express and secrete a constellation of cytokines that act in a paracrine manner in the injured micro-environment.21,35 Given the contrast in the regenerative response to CTX and BaCl2 in cKO animals, we hypothesized that the two injuries generate a different immune response. Among cytokines measured, IL-1β expression, which was not detected in uninjured muscle, was ~ 6-fold higher in CTX-injured muscles than after BaCl2 injury (Figure 2a). C/EBPβ levels after treatment with IL-1β were next assessed in primary myoblasts (Figure 2b). C/EBPβ expression was upregulated following exposure to IL-1β, and concomitant with the higher C/EBPβ, we observed an increase in Pax7 expression and reduced MyoD protein levels as previously reported.19

C/EBPβ protects myoblasts from apoptosis. Quiescent SCs are known to be resistant to apoptosis,36–38 although following activation, myoblasts become increasingly sensitive to apoptotic signals in a time frame concomitant with decreasing C/EBPβ levels.19,39 Indeed, C/EBPβ has previously been implicated in cell survival.40–42 Given the increase in apoptotic SCs lacking C/EBPβ after CTX injury, we tested if C/EBPβ could promote myoblast survival. C2C12 myoblasts were retrovirally transduced to express C/EBPβ or with empty virus (pLXSN) and pooled stable cell lines were treated with thapsigargin (TPG) to trigger apoptosis (Figures 3a and b). TPG promotes ER stress and results in activation of caspase-9 and/or caspase-12 by inhibiting the sarcoplasmic reticulum calcium ATPase SERCA.43–45 In the absence of treatment, approximately 7% of empty virus control and C/EBPβ-overexpressing myoblasts were dead by apoptosis, defined as being both propidium iodide-positive (PI+) and Annexin V-positive (Figure 3b). TPG treatment stimulated a twofold increase in dead cells in empty virus controls to approximately 20%, whereas ectopic expression of C/EBPβ protected against TPG-induced apoptosis, with an PI+/Annexin V+ population of ~ 12% that was not statistically different from controls. Analysis of the PI+, Annexin V+ and double-negative populations revealed that while TPG increased the Annexin V–/PI+ and Annexin V+/PI+ populations in both empty virus controls and C/EBPβ overexpressors, the only significant difference found between cell types was the percentage of dead cells (indicated by the asterisk) (Figure 3c).

Activity of the initiator caspase-9, which has been implicated in the apoptotic pathway downstream of TPG, was significantly reduced in C/EBPβ-overexpressing cells after TPG treatment, and was also reduced in vehicle-treated cells although not meeting statistical significance (Figure 3d). Caspase-12 activity, although trending toward an increase following TPG treatment in empty virus control cells, was highly variable and failed to change significantly in any of the conditions tested (Figure 3e). Consistent with the reduction in dead cells observed, caspase 3/7 activity was decreased 66% in TPG-treated C/EBPβ-overexpressing myoblasts as compared with empty virus controls (Figure 3f).

In primary myoblasts, treatment with TPG resulted in an almost twofold increase in dead cells in WT cultures to approximately 7.5% (Figure 3h). In primary myoblasts lacking C/EBPβ, the PI+/Annexin V+ population increased to ~ 20% following TPG treatment, 2.7-fold more dead cells than TPG-treated WT cultures (Figure 3h). Interestingly, the effect was not generalizable to other apoptotic stimuli. Although TNFα treatment could significantly increase the percentage of dead WT cells, this effect was not magnified with loss of C/EBPβ, suggesting that C/EBPβ’s anti-apoptotic effects may be limited to intrinsic ER stress-mediated pathways (Figure 3h). Following TPG treatment, cKO cultures had significantly more dead (Annexin V+/PI+), Annexin V+/PI− and...
Annexin V−/PI+ cells than WT controls, and significantly fewer Annexin V−/PI− cells (Figure 3i). In agreement with our gain-of-function data, loss of C/EBPβ expression resulted in increased activation of caspase-9 following TPG treatment, as compared with WT controls (Figure 3j). Further, although TPG treatment did not stimulate caspase-12 activation in WT cultures, caspase-12 activity was significantly increased in cKO cultures following TPG treatment (Figure 3k), suggesting that C/EBPβ may act to negatively regulate caspase-12 activity. In accordance with these results, caspase 3/7 activity was significantly increased by 50% in TPG-treated cKO cells as compared with WT (Figure 3l). Taken together, these data indicate that C/EBPβ regulates myoblast sensitivity to apoptosis.

Given that IL-1β can stimulate C/EBPβ expression, we asked whether treatment with IL-1β could protect myoblasts from TPG-induced apoptosis in a C/EBPβ-dependent manner. In WT primary myoblasts, treatment with IL-1β alone did not
increase the percentage of PI+/Annexin V+ cells as compared with vehicle-treated cultures (Figure 3m). Although treatment with TPG increased the percentage of WT cells dying, when IL-1β was added before TPG treatment to upregulate C/EBPβ, the percentage of PI+/Annexin V+ cells decreased significantly (Figure 3m). This protective effect was lost in cKO myoblasts, in which IL-1β failed to significantly reduce the population of dead cells following TPG treatment. Analysis of the Annexin V+ and PI+ double-negative populations revealed that the Annexin V+/PI− population was significantly larger in WT myoblasts treated with IL-1β and TPG as compared with cKO cells while the double-positive population was significantly smaller, consistent with higher levels of apoptosis in the cKO cultures that was not rescued by IL-1β treatment (Supplementary Figure S1).

Cancer cachexia increases C/EBPβ expression in SCs. High levels of proinflammatory cytokines including IL-1β are associated with muscle wasting and cachexia. To assess whether chronic inflammation could regulate C/EBPβ expression in myoblasts in vivo, cancer cachexia was induced in mice using the Lewis lung carcinoma (LLC) syngenic tumor graft model.46,47 LLC cells were transplanted into mice, which persist in vivo whether chronic inflammation could regulate C/EBPβ expression in myoblasts incubated with IL-1/β for 24 h in growth medium. The migration of molecular weight markers, in kDa, is shown. Cyclophilin B (CyPB) is a loading control.

To determine whether C/EBPβ could sensitize SCs to apoptosis in the context of cancer cachexia, we grafted the LLC tumor into cKO animals (Figure 5). Although there was no difference in body weight between sham-injected WT and cKO animals in the absence of tumor, both WT and cKO animals lost approximately 10% of their body weight following tumor graft, indicative of cachexia (Figure 5a). Tumor mass was equivalent in both genotypes, although more variable in the cKOs (Figure 5b). Cachexia was accompanied by a decrease in TA weight in both WT and cKOs, although no significant differences were noted between WT and cKO tumor-bearing animals (Figure 5c).

Further, cKO animals with cachexia had an ~2-fold increase in the percentage of TUNEL+/Pax7+ cells compared with WT animals bearing the LLC tumor (Figures 5f and g). TUNEL staining was undetectable in sham controls (Figure 4g). Further, the proportion of C/EBPβ+/Pax7+ cells was increased in the cachectic animals as compared with sham controls, suggesting that larger Pax7+ population was observed in cachectic animals was largely also C/EBPβ-expressing. (Figures 4k and l). C/EBPβ is required for SCs expansion in cancer cachexia. To determine whether loss of C/EBPβ could afford muscle wasting in cancer cachexia, the whole muscle from cachectic animals as compared with sham controls (Figure 4g). Further, immunohistochemical analysis of the SC population in cachectic mice revealed that the Pax7+ population increased more than twofold in the TA muscles of LLC-bearing animals as compared with sham controls, without differences in the total number of nuclei, indicating that SCs are present in greater numbers in cachectic mice (Figures 4i and j) consistent with previous observations.48 Further, the proportion of C/EBPβ+/Pax7+ cells was increased in the cachectic animals as compared with sham controls, suggesting that larger Pax7+ population was observed in cachectic animals was largely also C/EBPβ-expressing. (Figures 4k and l).
Figure 3  C/EBPβ promotes the survival of myoblasts. (a) C/EBPβ protein expression in proliferating C2C12 cells retrovirally transduced to express C/EBPβ or with empty virus (pLXSN). The migration of molecular weight markers, in kDa, is shown. Cyclophilin B (CyPB) is a loading control. (b) Percentage of dead cells determined by flow cytometry analysis of Annexin V and PI staining in vehicle and TPG-treated C2C12 stable cells. Means indicated with different letters are significantly different from one another, n = 4. Gating strategy and sample plots are provided in Supplementary Figure S2. (c) Percentage of cells found in the Annexin V+/PI+, Annexin V+/PI−, Annexin V−/PI+ and Annexin V−/PI− populations from cells treated as in (b). Two populations indicated with an asterisk are significantly different from one another. Gating strategy and sample plots are provided in Supplementary Figure S2. (d) Caspase-9 activation in vehicle and TPG-treated C2C12 stable cell lines treated as in (b), shown relative to vehicle-treated empty virus control cells. NS, nonsignificant, *P < 0.05, n = 3. (e) Caspase-12 activation in vehicle and TPG-treated C2C12 stable cell lines treated as in (b), shown relative to vehicle-treated empty virus control cells. n = 6. (f) Caspase-3/7 activity in TPG-treated C2C12-overexpressing C/EBPβ shown relative to C2C12 empty vector controls. *P < 0.05, n = 4. (g) Western analysis of C/EBPβ protein expression in primary myoblasts from WT and cKO mice treated with 4-OH tamoxifen for 48 h to induce excision. (h) Percentage of dead cells determined by flow cytometry in vehicle and TPG-treated WT and cKO myoblasts. Means indicated with different letters are significantly different from one another, n = 6. Gating strategy and sample plots are provided in Supplementary Figure S3. (i) Percentage of cells found in the Annexin V+/PI+, Annexin V−/PI−, Annexin V+/PI− and Annexin V−/PI− populations from cells treated as in (h). Matching populations in the cell lines indicated with an asterisk are significantly different from one another. Gating strategy and sample plots are provided in Supplementary Figure S3. (j) Caspase-9 activation in vehicle and TPG-treated primary myoblasts cell lines treated as in (h), shown relative to vehicle-treated WT cells. n = 3. (k) Caspase-12 activation in vehicle and TPG-treated primary myoblasts treated as in (h), shown relative to vehicle-treated WT control cells. **P < 0.01, n = 3. (l) Caspase-3/7 activity in TPG-treated cKO myoblasts relative to WT. *P < 0.05, **P < 0.01, n = 4. (m) Percentage of dead cells determined by flow cytometry in WT and cKO myoblasts pretreated with IL-1β and TPG for 24 hrs, as indicated. *P < 0.05, **P < 0.01, n = 3. Gating strategy and sample plots are provided in Supplementary Figure S4
regenerating fibers and not their size (Figure 6d). Taken together, these results suggest that loss of C/EBPβ can exacerbate the regeneration defect in cachetic mice.

**Discussion**

SCs and myotubes are known to be relatively resistant to apoptotic stimuli.\cite{36,49} Injury and subsequent activation of SCs increase their vulnerability to apoptosis. However, the mechanism by which SCs withstand apoptosis is poorly defined. Pax7 expression is considered protective from apoptosis, as deletion of Pax7 triggers cell cycle abnormalities characterized by an extended G2/M phase, and a progressive loss of muscle precursors to cell death.\cite{50,51} Recently, Brg1, a component of the Swi/Snf chromatin remodeling complex, was shown to be required for maintaining viability in myoblasts, and this through regulation of Pax7 expression.\cite{52} Interestingly, Pax7 is also a target of C/EBPβ in proliferating myoblasts and in differentiating cultures.\cite{19} Induction of C/EBPβ by IL-1β stimulates Pax7 expression and thus, C/EBPβ may act through Pax7 to protect muscle SCs from apoptosis. In addition, Myod1−/− myoblasts are resistant to apoptosis both in vitro and in vivo and C/EBPβ is a potent inhibitor of MyoD protein expression, making it straightforward to speculate that C/EBPβ could also modulate sensitivity to apoptosis through the control of MyoD expression.\cite{53} C/EBPβ can also directly inhibit caspase activity, and regulate p53 activity and expression, both of which could also regulate sensitivity to apoptotic signals.\cite{41,42} Our results suggest that C/EBPβ may also negatively regulate caspase-12.

In acute injury, when the inflammation is short-lived, regeneration is restrained and resolution of the inflammation would be expected to reduce C/EBPβ expression allowing for the initiation of myogenesis.\cite{27,30} The decrease in inflammation concomitant with a decrease C/EBPβ expression is indeed consistent with the known time frame of myoblast differentiation after injury.\cite{29} In chronic inflammation, the levels of proinflammatory cytokines remain high, leading to an expansion of the Pax7+ population, but a defect in muscle regeneration. It remains unknown whether the SC numbers and activity recover when the inflammatory milieu resolves, but given that tumor resection can improve cachexia, it is likely that the persistence of C/EBPβ in muscle SCs in the cachectic animal is transient.\cite{48} In our experiments, we can, however, detect an increase in C/EBPβ expression up to a week after removal from the cachetic milieu. As such, the induction of C/EBPβ in SCs may act as a sensor for inflammation providing both survival signals and concomitantly a blockade of regeneration.

Although a pro-survival role for C/EBPβ has been described in the development of cancer (hepatocellular carcinoma and melanomas), our results identify a function for C/EBPβ in an adult stem cell population. In *gallus gallus*, C/EBPβ (NF-M) promotes survival in hematopoietic progenitor cells, suggesting that our findings could extend to a broad range of stem cell populations as well as a diverse range of organisms.\cite{54}

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**Figure 4** Cancer cachexia increases C/EBPβ expression in SCs and inhibits myogenesis. (a) Average weight gain of female C57BL/6 sham and LLC-injected mice (8 weeks old) 4 weeks after grafting. ***P = 0.001, n = 15. (b) Average daily food consumption of sham and LLC tumor grafted animals. (c) Average TA mass in healthy and cachectic mice as in (a). *P < 0.05, n = 5. (d) H&E-stained TA cross-sections from sham and LLC-bearing mice 4 weeks after grafting. Mean fiber cross-sectional areas (XSA) are indicated. (e) Percentage of C/EBPβ+ cells (relative to total nuclei) in TA muscle isolated from mice as in (d). ***P = 0.001, n = 5. (f) C/EBPβ expression in primary myoblasts isolated from healthy and cachectic mice. Freshly isolated cells were plated at equal densities and expanded in culture for 3 days without passing before analysis. **P = 0.001, n = 6. (g) C/EBPβ expression in primary myoblasts isolated from healthy and LLC-bearing mice. Freshly isolated cells were plated at equal densities and expanded in culture for 3 days without passing before analysis. Actin is a loading control. (h) IL-1β expression in TA muscle of sham and LLC-bearing mice. The migration of molecular weight markers, in kDa, is shown. Tubulin is a loading control. (i) Representative images of immunohistochemistry for Pax7 expression in TA muscle isolated from mice as in (a). Scale bar = 50 μm. Arrowheads indicate positively stained cells. (j) Percentage of Pax7+ cells (relative to total nuclei) in TA muscle isolated from mice as in (a). ***P < 0.001, n = 6. (k) Representative images of Pax7 and C/EBPβ double staining on TA muscle sections from animals treated as in (a). Scale bar = 20 μm. Arrowheads indicate double-positive stained cells. (l) Percentage of C/EBPβ+/Pax7+ cells (relative to total Pax7+ cells) in TA muscle from healthy and cachectic mice from (k). n = 3

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**Table 1** C/EBPβ promotes survival of muscle satellite cells

| Condition          | C/EBPβ Expression | Pax7+ Population |
|--------------------|--------------------|------------------|
| Healthy            | +                  | +                |
| LLC                | +                  | −                |
| Cachetic           | +                  | −                |

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Injury as previously reported, the average regenerating fiber cross-sectional area after injury in tumor-bearing WT and cKO mice was reduced ~25% as compared with healthy controls, with no significant differences between genotypes (Figure 6c). However, the number of regenerating fibers was reduced by ~37% in cKO animals as compared with WT controls indicating that the repair defect is limited to the number of...
One of the limitations of murine cachexia models is the relatively short term in which muscle wasting is studied. Unlike human cachexia, the wasting in mice persists for only a few weeks before the cachexia is too profound for humane treatment. As such, it is impossible to know from our experiments, in which the animals were killed at approximately 10% weight loss with relatively mild cachexia, whether the induction of C/EBPβ expression by the cachectic environment persists as cachexia worsens or whether the cells eventually become desensitized to the effects of proinflammatory cytokines. In the case of cytokine resistance, we would expect loss of C/EBPβ expression in cachexia to trigger apoptosis of activated muscle precursors resulting in loss of the regenerative response and a more rapid loss of muscle mass. Indeed, an increase in SC apoptosis in a cachexia model with more severe weight loss has been observed.55 Moreover, muscle biopsies from patients suffering of gastrointestinal cancers had increased DNA fragmentation, typical of apoptosis, suggesting that loss of muscle cells contributes to wasting in humans.56 These findings would suggest that anti-inflammatory therapy could release the C/EBPβ-imposed blockade of muscle repair and therefore counteract the loss of muscle protein observed in cachexia.57

Figure 5 Smaller SC population in cancer cachexia with loss of C/EBPβ. (a) Average body weight of WT and cKO mice 4 weeks after sham or LLC tumor graft. Eight-week-old WT and cKO male mice in the C57BL/6 genetic background received IP tamoxifen injections 5 days before tumor graft. *P < 0.05, **P < 0.01, n = 5. (b) Tumor mass at necropsy in WT and cKO animals as in (a). (c) TA mass in WT and cKO sham or LLC-bearing mice. *P < 0.05 and ***P < 0.001, n = 5. (d) Representative images of TA cross-sections from WT and cKO sham and LLC-bearing mice. Scale bar = 50 μm. (e) Average TA fiber cross-sectional area (XSA) in WT and cKO sham or LLC-bearing mice. *P < 0.05, n = 4. (f) Representative images of immunostaining for Pax7 expression in WT and cKO sham and LLC-bearing mice. Scale bar = 20 μm. Arrowheads indicate positively stained cells. (g) Percentage of Pax7+ cells relative to total DAPI+ nuclei in WT and cKO sham and LLC-bearing mice. *P < 0.01, ***P < 0.001, n = 10. (h) Representative images of immunostaining for Pax7+ and TUNEL+ cells in TA muscle of WT and cKO sham and LLC-bearing mice. Scale bar = 20 μm. Arrowheads indicate double-positive cells. (i) Percentage of apoptotic Pax7+ cells (TUNEL+/Pax7+ cells relative to total Pax7+ cells) found in TA muscle of WT and cKO sham and LLC-bearing mice. *P < 0.05, n = 3

Figure 6 Inhibition of regeneration in cachetic mice lacking C/EBPβ in Pax7+ cells. (a) H&E-stained TA cross-sections from sham and LLC-grafted WT or cKO mice 7 days after BaCl2 injury. Scale bar = 20 μm. (b) Average TA mass in sham or LLC-grafted WT or cKO animals 7 days after BaCl2 injury. *P < 0.05, n = 5. (c) Average TA fiber cross-sectional area (XSA) in sham or LLC-grafted BaCl2-injured WT or cKO mice. *P < 0.05, n = 5. (d) Number of regenerating muscle fibers per mm2 7 days after BaCl2 injury to the TA in LLC-grafted WT and cKO mice. *P < 0.05, n = 5
Materials and Methods

Animal models. All animal handling procedures conformed to the guidelines established by the University of Ottawa Animal Care Service and the Canadian Council on Animal Care. Mice carrying a C/EBPβ-floxed allele18 and the mouse carrying the Pax7-CreERT2 allele19 were maintained in a mixed genetic background, to generate control (WT, Cebpbfl/flPax7−/−) and conditional null (cKO, Cebpbfl/flPax7creERT2) mice. Young WT and cKO littermates received daily intraperitoneal (iP) injections of tamoxifen (2 mg/g) for 5 days to excise Crepβ. To allow growth of LLC cells in transgenic animals, CEBPβfl/fl and Pax7CreERT2 mice were backcrossed to C57BL/6 (Jackson, Bar Harbor, ME, USA) mice for nine generations. For all LLC experiment, 6- to 8-week-old mice were used. For the induction of cancer cachexia, 5x10⁵ sub-confluent LLC cells washed in PBS were injected subcutaneously while PBS alone was used for sham animals.

Cell culture and differentiation. C2C12 cells (ATCC, Manassas, VA, USA) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Primary WT and cKO myoblasts cultures, obtained by enzymatic digestion and pre-plating as previously described,19 were maintained in DMEM supplemented with 20% FBS, 10% horse serum (HS), 10 ng/ml basic fibroblast growth factor and 2ng/ml hepatocyte growth factor (Peprotech, Rocky Hill, NJ, USA). For assessment of the manufacturer's instructions (caspase-3/7 (Promega, Madison, WI, USA), β-actin (Sigma) at 20 ng/ml for 24 h starting 6 h before the addition of 10-4 M or 50 μl of 1.2% BaCl2 (Sigma) both dissolved in PBS were injected into the left TA muscle using a 28 gauge syringe. PBS was injected in the right TA muscle for control. At necropsy, TA muscle was dissected and fixed in 10% formalin and paraffin embedded or flash frozen in melting isopentane.

Immunocytochemistry, immunohistochemistry and TUNEL. In situ TUNEL assays were performed according to the manufacturer's instructions (Roche, Laval, QC, Canada) and counter-stained with DAPI (0.5 μg/ml) for 5 min. For immunohistochemistry, muscle sections were air-dried 30 min at 65 °C and fixed 10 min in 4% paraformaldehyde. After washes, antigen retrieval was done for 20 min at 92 °C with citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) and sections were cooled to 20 °C. Sections were permeabilized 10 min in 0.5% Triton X-100, washed and blocked 1 h in 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA, USA). Antibodies used for detection were: mouse anti-Pax7 (DSHB, Santa Cruz Biotechnology, Dallas, TX, USA, SC-150), rabbit anti-IL-1β (Santa Cruz Biotechnology, mouse anti-Pax7 (DSHB), rabbit anti-IL-1β and rabbit anti-cyclophilin B from Abcam and mouse anti-p-Akt (Sigma).

Conflict of Interest

The authors declare no conflict of interest.

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