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C/EBPβ promotes the expression of atrophy-inducing factors by tumours and is a central regulator of cancer cachexia

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Abstract

Background CCAAT/enhancer-binding protein β (C/EBPβ) is a transcription factor whose high expression in human cancers is associated with tumour aggressiveness and poor outcomes. Most advanced cancer patients will develop cachexia, characterized by loss of skeletal muscle mass. In response to secreted factors from cachexia-inducing tumours, C/EBPβ is stimulated in muscle, leading to both myofibre atrophy and the inhibition of muscle regeneration. Involved in the regulation of immune responses, C/EBPβ induces the expression of many secreted factors, including cytokines. Because tumour-secreted factors drive cachexia and aggressive tumours have higher expression of C/EBPβ, we examined a potential role for C/EBPβ in the expression of tumour-derived cachexia-inducing factors.

Methods We used gain-of-function and loss-of-function approaches *in vitro* and *in vivo* to evaluate the role of tumour C/EBPβ expression on the secretion of cachexia-inducing factors.

Results We report that C/EBPβ overexpression up-regulates the expression of 260 secreted protein genes, resulting in a secretome that inhibits myogenic differentiation (−31%, $P < 0.05$) and myotube maturation [−38% (fusion index) and −25% (myotube diameter), $P < 0.05$]. We find that knockdown of C/EBPβ in cachexia-inducing Lewis lung carcinoma cells restores myogenic differentiation (+25%, $P < 0.0001$) and myotube diameter (+90%, $P < 0.0001$) in conditioned medium experiments and, *in vivo*, prevents muscle wasting (−51% for small myofibres vs. controls, $P < 0.01$; +140% for large myofibres, $P < 0.01$). Conversely, overexpression of C/EBPβ in non-cachectic tumours converts their secretome into a cachexia-inducing one, resulting in reduced myotube diameter (−41%, $P < 0.0001$, EL4 model) and inhibition of differentiation in culture (−26%, $P < 0.01$, EL4 model) and muscle wasting *in vivo* (+98% small fibres, $P < 0.001$; −76% large fibres, $P < 0.001$). Comparison of the differently expressed transcripts coding for secreted proteins in C/EBPβ-overexpressing myoblasts with the secretome from 27 different types of human cancers revealed ∼18% similarity between C/EBPβ-regulated secreted proteins and those secreted by highly cachectic tumours (brain, pancreatic, and stomach cancers). At the protein level, we identified 16 novel secreted factors that are present in human cancer secretomes and are up-regulated by C/EBPβ. Of these, we tested the effect of three factors (SERPINF1, TNFRSF11B, and CD93) on myotubes and found that all had atrophic potential (−33 to −36% for myotube diameter, $P < 0.01$).

Conclusions We find that C/EBPβ is necessary and sufficient to induce the secretion of cachexia-inducing factors by cancer cells and loss of C/EBPβ in tumours attenuates muscle atrophy in an animal model of cancer cachexia. Our findings establish C/EBPβ as a central regulator of cancer cachexia and an important therapeutic target.

Keywords CCAAT–enhancer-binding proteins; Muscular atrophy; Cachexia; Muscle, skeletal; Secreted proteins; Carcinoma, Lewis lung

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Introduction

CCAAT/enhancer-binding proteins (C/EBPs) are a family of six bZIP transcription factors that are involved in the regulation of numerous cellular activities including apoptosis, differentiation, proliferation, and immunity.[1–4] One family member, C/EBPβ, is expressed in most cancers.[5] While only rarely mutated, gain of copy number variants and increased *CEBPB* expression is observed in a subset of tumours including ~22% of stomach and ~30% of colon adenocarcinomas.[6] High C/EBPβ expression is associated with tumour aggressiveness and poor outcomes.[7–11] C/EBPβ drives the progression of non-alcoholic fatty liver to hepatocellular carcinoma,[12] glioblastoma growth *in vivo*,[13] tumour initiation, and malignancy in non-small cell carcinoma (lung cancer)[14] and skin cancers.[15]

Approximately 80% of advanced cancer patients will develop cachexia, a paraneoplastic syndrome characterized by systemic inflammation and skeletal muscle atrophy.[16–19] Cancer cachexia leads to significant morbidity and poor tolerance to chemotherapy and accounts for >20% of cancer mortalities,[17] and thus, therapeutic advances in this area are critical to improve both patient quality of life and treatment efficacy. The cause of cachexia is multifactorial and results from a complex interaction between the tumour and the host.[20] In particular, the pro-inflammatory cytokines TNF-α, IL-6, IL-1β, and IFN-γ are increased in cachexia and can induce muscle wasting. While multiple studies demonstrate that cytokines are key players in the pathogenesis of cancer cachexia,[21] the inhibition of any one single factor has failed to yield therapeutic benefits in all cancer patients.[21,22] Thus, the identification of a hierarchical transcription factor that is responsible for the cachexia-inducing secretome has tremendous therapeutic potential.

In cachexia, muscle injury is observed, accompanied by myofibre atrophy and inhibited muscle regenerative responses.[23–27] C/EBPβ is critical for skeletal muscle homeostasis.[4,28–30] In healthy muscle, C/EBPβ is expressed in muscle satellite cells where it promotes quiescence, stemness, and self-renewal while inhibiting myogenic differentiation.[29,31,32] Normally down-regulated after injury to allow for muscle repair, in the context of cachexia, C/EBPβ expression is induced in both muscle stem cells and myofibres.[28,33] *In vivo*, muscle regeneration is impaired in cachectic animals and is associated with an elevated number of muscle satellite cells that express higher levels of C/EBPβ when compared with controls.[33,34]

C/EBPβ, first identified as a regulator of *Il6*, has since been shown to stimulate acute-phase gene expression and that of cytokine genes *Tnf*, *Il8*, and *Csf3*.[35,36] C/EBPβ null mice are immunodeficient.[37,38] C/EBPβ expression in myoblasts results in a gene expression pattern that is heavily biased towards secreted proteins,[39] indicating that C/EBPβ is a major regulator of the myoblast secretome, including cytokines and chemokines. As treatment of myoblasts with IL-6, TNF-α, IFN-γ, and IL-1β blocks myogenic differentiation,[21] C/EBPβ expression and the resulting secretome are predicted to function in both an autocrine and paracrine fashion to inhibit myogenesis. Because tumour-secreted factors drive cachexia and aggressive tumours have higher expression of C/EBPβ, we investigated the role of C/EBPβ expression in tumours on the development of cachexia and the production of cachexia-inducing secreted factors in culture and *in vivo*.

Methods

Additional methods are available in the Supporting Information (Data S1). Primers are listed in Supplemental File S5.

Cell culture

Culture conditions are available in the Methods section in the Supporting Information. For the assessment of cell growth, cells were plated at equal numbers and allowed to grow for 24 h after which cells were counted using a haemocytometer or stained with crystal violet as previously described.[40]

For *in vitro* cachexia experiments, conditioned medium (CM) from cancer cell lines was prepared as previously described in Brown *et al.*[41] with the following modifications. Cancer cells (LLC, SKOV3, and EL4) were grown to ~80% confluency (or 5×10^5 cells/mL for EL4), after which fresh media were added. CM was collected after 2 days and diluted with fresh myoblast media (1:1) for a final concentration of 50% CM. The 1:1 conditioned media:fresh media were added to C2C12 for 2 days during proliferation (in GM) to measure effects on myogenic differentiation or after the formation of myotubes (4 days after differentiation, D4). For co-culture experiments, C2C12 cells were grown in wells and cancer cells in inserts using Corning™ Transwell™ dishes. For treatment with recombinant proteins, C2C12 myotubes were treated with SERPINF1 (0.5 μg/mL, 8295-SF, R&D Systems, ED_{50} is 0.1–0.6 μg/mL), TNFRSF11B (10 ng/mL, 6945-OS, R&D Systems, ED_{50} is 2–10 ng/mL), CD93 (6 μg/mL, 1696-CD, R&D Systems, ED_{50} is 6–30 μg/mL), or vehicle (0.1% BSA in PBS) for 2 days.

Retroviral infection

Phoenix Ampho packaging cells (ATCC) were used to generate replication-incompetent retroviruses as previously described.[29] Following retroviral infection, cells were maintained in media containing G418 (Wisent) for at least 7 days.

Immunofluorescence and myotube measurements

Cells were fixed in ice-cold methanol, incubated with primary antibody against myosin heavy chain (MyHC) (MF20, DSHB, 1:50) overnight at 4 °C, followed by incubation with secondary anti-mouse Cy3 antibody (anti-mouse Cy3, Jackson ImmunoResearch, 1:500) at room temperature. Nuclei were counterstained with DAPI. Random images were captured using an AxioObserver D1 microscope (Zeiss) with the ×10 objective. Myogenic differentiation was assessed using five random pictures per condition as the per cent differentiation (no. of nuclei in MyHC+ cells/total nuclei) and the fusion index (average number of nuclei/fibre). For assessment of myotube size, myotube diameter was measured as an average of three measurements (at the centre and at each of the two ends) for each myotube from five random pictures per condition using Fiji (ImageJ) and presented as the average of all measurements in μm as described.[42] The average per cent MyHC+ area was calculated for five random pictures from each condition.

Mice, animal care, and Lewis lung carcinoma tumour graft

Animal work was performed in accordance with the guidelines set out by the Canadian Council on Animal Care and was approved by the University of Ottawa Animal Care Committee. Animals were housed in a controlled facility (22°C with 30% relative humidity on a 12 h light/dark cycle) and provided with food and water ad libitum. Six-week-old C57BL/6 female mice (Charles River) were inoculated subcutaneously with 5×10^5 LLC cells (in 100 μL PBS),[33,34] 1×10^6 EL4 cells,[43] or PBS (sham controls). Three weeks after inoculation, tibialis anterior (TA) muscles were harvested and flash frozen for sectioning. Images for H&E-stained sections were taken using the EVOS M7000 imaging system (Invitrogen), and Fiji (ImageJ) was used to measure myofibre cross-sectional area from a total of 400 myofibres per section.

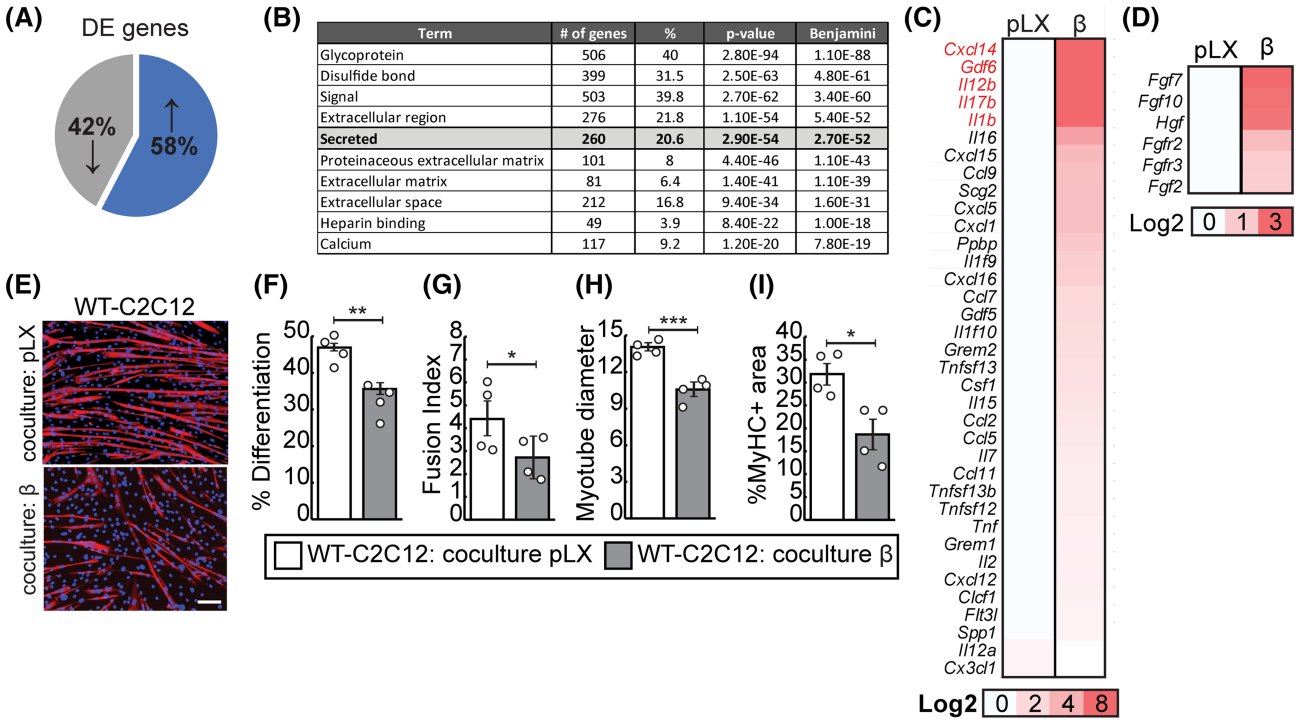
Results

C/EBPβ regulates the expression of secreted protein genes that inhibit myogenic differentiation

In cancer cachexia, misexpression of C/EBPβ in muscle satellite cells is central to their failure to differentiate.[33,34] To identify novel C/EBPβ target genes in muscle, we performed RNA-seq on proliferating C2C12 myoblasts retrovirally transduced to express C/EBPβ or with empty vector.[39] We identified 2210 protein-coding genes that were significantly differentially expressed (≥1.5-fold difference) in C/EBPβ-overexpressing cells as compared with controls, of which more than half (58%) were up-regulated (*Figure* 1A and *File* S1). Gene ontology analysis of the up-regulated genes revealed the GO term 'secreted' among the Top 5 terms (*Figure* 1B and *File* S1), representing 20% of the genes interrogated. Further analysis revealed that these secreted proteins are cytokines and chemokines (21%), collagens (24%), and growth factors (10%) with at least 9% associated with immune and inflammatory responses. Interestingly, of the known mouse cytokine genes (134 genes),[44] 66 (49%) were detected in C2C12 cells, and of these, 36 were up-regulated by ≥1.5-fold in C/EBPβ-overexpressing cells as compared with controls (*Figure* 1C). Of the 36 up-regulated cytokines in C/EBPβ-overexpressing myoblasts, more than 30% are known inhibitors of myogenesis, including *Tnf*,[21] *Il1b*,[21] *Cxcl14*,[45] *Cxcl12*,[46] and *Csf1*.[47] In addition to cytokine genes, growth factors and growth factor receptor genes (*Hgf*, *Fgf2*, *Fgf7*, *Fgf10*, *Fgfr2*, and *Fgfr3*) were also up-regulated by C/EBPβ overexpression (*Figure* 1D).

The up-regulation of secreted protein genes by C/EBPβ suggests that they contribute to the inhibition of myogenesis by C/EBPβ via autocrine and/or paracrine pathways. To test whether secreted proteins are responsible for the C/EBPβ-mediated inhibition of cell differentiation and fusion, we used an *in vitro* co-culture system that allows secreted factors to be shared between two cell types without permitting cell–cell contact. C2C12 cells were co-cultured with C2C12 myoblasts retrovirally transduced to express C/EBPβ (β) or with empty virus (pLX) for 2 days in growth medium before the induction of differentiation in low serum media. As compared with controls, C2C12 cells co-cultured with C/EBPβ-expressing cells had a 24% reduction in the per cent differentiation [no. of nuclei in myosin heavy chain-positive cells (MyHC+) cells/total nuclei] and a 38% reduction in the fusion index (no. of nuclei in myotubes/no. of myotubes) after 3 days of differentiation (*Figure* 1E–1G). Myotube size was similarly affected, with myotube diameter reduced by 25% (*Figure* 1H) and the area covered MyHC+ cells reduced by 41% (*Figure* 1I). Thus, C/EBPβ-regulated secreted proteins are central to the molecular mechanism by which C/EBPβ inhibits myogenic differentiation.

Interestingly, C/EBPβ is also up-regulated in a variety of cancer cells.[5] To expand our findings from myoblasts to cancer cells, we analysed RNA expression data sets from multiple human cancers. Using the CancerSEA database[48] that correlates gene expression with functional states in human cancers, we found that *CEBPB* expression significantly positively correlated with inflammation in at least five of the tested cancer types, including known cachectic ones such as colorec-



Term	# of genes	%	p-value	Benjamini
Glycoprotein	506	40	2.80E-94	1.10E-88
Disulfide bond	399	31.5	2.50E-63	4.80E-61
Signal	503	39.8	2.70E-62	3.40E-60
Extracellular region	276	21.8	1.10E-54	5.40E-52
Secreted	**260**	**20.6**	**2.90E-54**	**2.70E-52**
Proteinaceous extracellular matrix	101	8	4.40E-46	1.10E-43
Extracellular matrix	81	6.4	1.40E-41	1.10E-39
Extracellular space	212	16.8	9.40E-34	1.60E-31
Heparin binding	49	3.9	8.40E-22	1.00E-18
Calcium	117	9.2	1.20E-20	7.80E-19

Figure 1 C/EBPβ up-regulates gene coding for secreted proteins that inhibit myogenesis. RNA-seq was performed on proliferating C2C12 cells retrovirally transduced with empty vector (pLX) or to express C/EBPβ (β). (A) A pie chart showing the direction of change for genes that are significantly ($P_{adj} \leq 0.05$ and ≥1.5-fold change) differentially expressed (DE) in C/EBPβ-overexpressing myoblasts as compared with empty vector controls (pLX). (B) Top 10 terms produced by Gene Ontology analysis of the 1273 up-regulated genes in C/EBPβ-overexpressing myoblasts from (A). (C) Heat map of the expression of 35 cytokine genes that are up-regulated in C/EBPβ-overexpressing cells as compared with pLX. Genes highlighted in red are not detected in pLX conditions but induced in myoblast-overexpressing C/EBPβ. (D) Heat map for the expression of growth factors (*Hgf* and *Fgf*) and FGF receptor genes in control (pLX) and C/EBPβ-overexpressing cells. (E) MyHC (red) immunostaining of WT-C2C12 cells co-cultured with C2C12 cells retrovirally transduced with empty vector (pLX) or to express C/EBPβ (β) in growth medium for 2 days followed by differentiation medium for another 3 days. Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (F) Per cent differentiation (no. of nuclei in MyHC+ cells relative to the total nuclei) in cells cultured as in (E) ($n = 4$). (G) Fusion index (average no. of nuclei per MyHC+ cell) for cells cultured as in (E) ($n = 4$). (H) Average myotube diameter of cells from (E) in μm ($n = 4$). (I) Per cent myosin heavy chain-positive area for cultures from (E) ($n = 4$). Data information: for (F)–(I), data are presented as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Student's *t*-test).

tal cancer and lung cancers (LUAD and NSCLC)[17] (*Figure* S1A). Furthermore, in colorectal cancer (GSE166427[49]), C/EBPβ expression was increased in tumour cells as compared with healthy tissue (*Figure* S1B). In murine cancer cell lines used as models of cancer cachexia[50] [Lewis lung carcinoma (LLC) cells and colon carcinoma cell line (CT26)], higher C/EBPβ expression was observed than in the mouse T-cell lymphoblastoma cell line (EL4), which does not induce cachexia[43] (*Figure* S1C and S1D). Collectively, these data correlate C/EBPβ expression with inflammatory gene expression in cachectic tumours.

C/EBPβ is required for the secretion of cachexia-inducing factors by Lewis lung carcinoma cells

To evaluate a potential role for C/EBPβ in the expression of cachexia-inducing factors by tumours, we used a well-described *in vitro* cachexia model in which myotubes are incubated with 50% CM from LLC cells for 2 days. This treatment leads to a reduction in myotube size in response to cachexia-inducing factors in the CM produced by the cancer cells[28] (*Figure* 2A). To test if C/EBPβ expression is required for the secretion of cachexia-inducing factors by LLC cells, we retrovirally transduced LLC cells to express a shRNA against *Cebpb* (LLCshβ) or to express a shRNA against luciferase (LLC-shCtl) and confirmed the knockdown of C/EBPβ by western blot (*Figure* 2B). Conditioned medium from pooled stable LLC cells (shβ and shCtl) was added 1:1 with fresh media to myotubes for 2 days (*Figure* 2B). Immunostaining for MyHC was performed on myotubes at Day 4 (before treatment) and on Day 6 (2 days after treatment), and myotube maturation was evaluated by measuring the average myotube diameter, per cent area covered by MyHC+ cells, the fusion index, and the per cent differentiated as compared with unconditioned medium controls. As expected, incubation of C2C12 myotubes with 50% CM from LLC-shCtl cells resulted in a

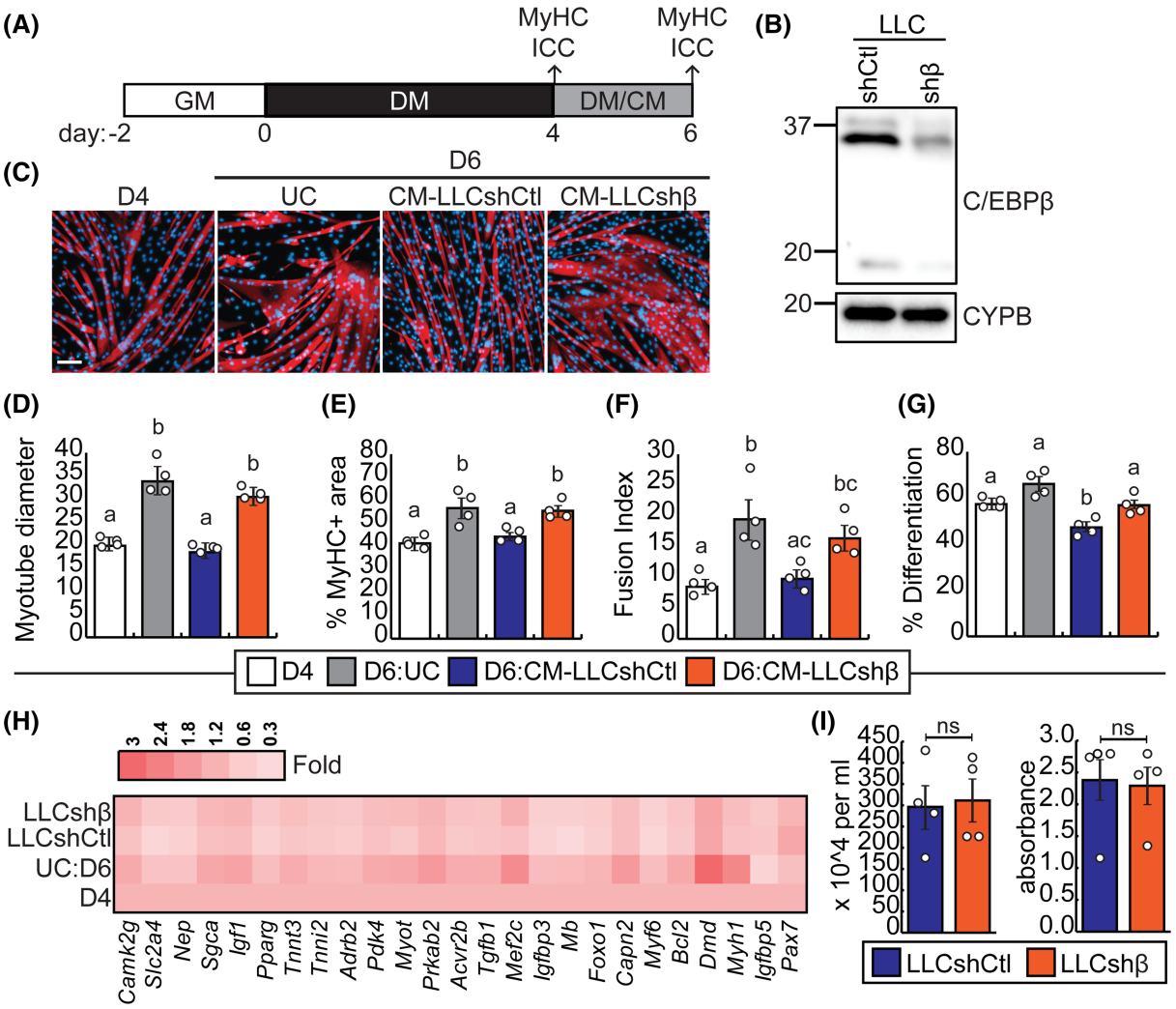


Figure 2 C/EBPβ is required for the expression of atrophy-inducing proteins by LLC cells. (A) Schematic representation of the experimental system. C2C12 myoblasts were grown to confluency in growth medium (GM) and differentiated in low serum medium [differentiation medium (DM)] for 4 days (D4). D4 myotubes were treated for two additional days with a mixture of conditioned medium (CM) and fresh DM at a ratio of 1:1 (50% CM final) from LLC-shCtrl or shβ cells. (B) C/EBPβ protein expression (isoforms LAP* at 37 kDa, LAP at 36 kDa, and LIP at 17 kDa) in Lewis lung carcinoma (LLC) cells retrovirally transduced with a control shRNA directed against luciferase (shCtl) or one directed against *Cebpb* (shβ). Cyclophilin B (CYPB) is used as a loading control. (C) Myosin heavy chain immunostaining (red) of myotubes on Day 4 of differentiation (D4), Day 6 of differentiation (D6) after treatment with unconditioned LLC medium (UC), or CM for 2 days (D6 LLC). Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (D) Average myotube diameter of cells treated as in (C) in μm (n = 4). (E) Per cent myosin heavy chain-stained area in cultures treated as in (C) (n = 4). (F) Fusion index (FI) for cells treated as in (C) (n = 4). (G) Per cent differentiation for cultures treated as in (C) (n = 4). (H) Heat map of differentially regulated genes in C2C12 myotubes treated with 50% CM from LLC-shCtl as compared with UC (D6) determined by RNA array for myogenesis and myopathy-related genes (RT2 Profiler Array, Qiagen). Relative gene expression values for myotubes treated with LLC-shβ CM are included for comparison. All data are presented as relative to D4 samples. The isolated mRNA was from three pooled trials. (I) Cell numbers (right) and crystal violet absorbance (left) of LLC-shCtl and LLC-shβ cell cultures after 48 h in growth medium (n = 4). Data information: for (D)–(G) and (I), data are presented as the mean ± SEM. Different letters above bars indicate statistically significant differences at a cut-off of $P < 0.05$ (one-way ANOVA).

55% reduction in myotube diameter as compared with the unconditioned controls (*Figure* 2C and 2D), whereas CM from LLC-shβ cells did not result in a significant reduction in myotube diameter (*Figure* 2C and 2D). Similarly, the area covered by MyHC+ cells was significantly reduced in myotubes treated with LLC-shCtl CM and unaffected by LLC-shβ CM (*Figure* 2E). Given that myotube size was observed to increase between D4 and D6 (*Figure* 2C–2E), we calculated the fusion index and the per cent differentiated to determine if the observed changes were due to impaired myotube maturation or atrophy. Incubation of C2C12 myotubes with LLC-shCtl CM reduced the fusion index by 45% as compared with the unconditioned D6 control, to a level comparable with D4 (*Figure* 2F). LLC-shβ CM did not significantly affect

the fusion index as compared with the unconditioned D6 controls (*Figure* 2F). Further, LLC-shCtl CM significantly reduced differentiation as compared with unconditioned controls, whereas LLC-shβ CM had no effect (*Figure* 2G), suggesting that tumour expression of C/EBPβ is necessary for LLC cells to produce cachexia-inducing factors that inhibit myogenic differentiation and negatively impact myotube size.

To quantify this effect, we performed an RT-qPCR array comparing the expression of 84 genes involved in myogenesis and myopathy in C2C12 cells at Day 4 (before treatment) and at Day 6 (2 days after treatment with 50% unconditioned medium, or 50% LLC-shCtl/LLC-shβ CM). Treatment of C2C12s with LLC-shCtl CM resulted in the down-regulation of 24 genes and the up-regulation of 2 genes as compared with untreated C2C12s at Day 6 using a 1.5-fold cut-off (*Figure* 2H and *File* S2). Among the down-regulated genes, we found well-known regulators of myogenic differentiation and myotube maturation including *Myh1*, *Mef2c*, *Myf6*, *Pparg*, *Igf1*, *Camk2g*, *Tnnt3*, *Tnni2*, and *Neb* (*Figure* 2H and *File* S2). Interestingly, cells incubated with 50% CM from LLC-shβ cells showed a partial rescue of the expression of 23 of these genes, with their expression returning almost to untreated levels (*Figure* 2H and *File* S2). These findings confirm a critical role for C/EBPβ in the production of a secretome that impairs myotube size.

Only two genes were found to be up-regulated in myotubes treated with LLC-shCtl CM, *Igfbp5* and *Pax7* (*Figure* 2H and *File* S2). The role of *Igfbp5* in myogenic differentiation is complicated and dependent on the ratio of IGF1 and IGFBP5 expression.[51,52] However, the overexpression of *Pax7* is known to be induced by exposure to CM from tumours or cancer patient serum,[53] consistent with our observations.

Because culture density can affect the concentration of secreted proteins in CM and C/EBPβ has been implicated in the regulation of proliferation, we assessed the growth of LLC-shCtl and LLC-shβ cells. Knockdown of C/EBPβ in LLC cells did not significantly affect cell proliferation as measured by cell counting and crystal violet staining (*Figure* 2I). Thus, the ability of C/EBPβ to promote myotube atrophy is not due to changes in culture density used to produce CM, but rather regulation of the LLC secretome itself.

Secreted factors from cancer cells not only cause muscle atrophy but can also inhibit satellite cell function,[21,33] and this can be tested using an *in vitro* cachexia model in which myoblasts are grown in a 1:1 mixture of CM from cancer cells to fresh medium prior to differentiation.[21,33] To assess the importance of C/EBPβ expression in tumour cells on the inhibition of myogenic differentiation, CM from LLC-shCtl or LLC-shβ cells was used to treat subconfluent C2C12 myoblasts for 2 days in growth medium (50% CM) after which differentiation was induced in fresh medium for 3 days (*Figure* 3A and 3B). The effect of CM on myoblast differentiation was evaluated using MyHC immunostaining and the calculation of the per cent differentiation and fusion index. As expected, myoblasts incubated with LLC-shCtl CM had a 25% reduction in differentiation and ~50% reduction in fusion as compared with unconditioned controls (*Figure* 3C and 3D). Myoblasts incubated with CM from LLC-shβ cells, by contrast, differentiated normally; however, the myotubes produced were smaller, reflected by a ~30% reduction in the fusion index (*Figure* 3C and 3D). While fusion was significantly inhibited by pretreatment with LLC-shβ CM, the cultures still produced larger myotubes that those exposed to LLC-shCtl CM, indicating a partial rescue of cell size. Furthermore, while the expression of myogenin, a differentiation marker, was reduced by ~60% in cells exposed to CM from LLC-shCtl cells as compared with untreated controls, incubation with LLC-shβ CM did not affect expression of this marker (*Figure* 3E and 3F). Taken together, these data suggest that C/EBPβ expression in LLC cells is required to produce cachexia-inducing factors that cause muscle atrophy and inhibit myogenic differentiation.

C/EBPβ converts the secretome of non-cachectic tumours to one that promotes wasting

To determine if C/EBPβ is sufficient to generate an atrophy-inducing secretome, we retrovirally transduced two cancer cell lines that are classified as non-cachectic,[33,43] the human ovarian adenocarcinoma cell line SKOV3 and the mouse T-cell lymphoblastoma cell line EL4, to overexpress C/EBPβ (SKOV3-β and EL4-β) or with empty virus (SKOV3-pLX and EL4-pLX), and overexpression of C/EBPβ was confirmed by western blot (*Figure* 4B and 4H). SKOV3-derived and EL4-derived CM was added to C2C12 myotubes in a 1:1 ratio with fresh medium (50% CM) in fresh media for 2 days (*Figure* 4A), and MyHC immunostaining was performed on myotubes at Day 4 (before treatment) and at Day 6 (2 days after treatment), and myotube maturation was evaluated as in *Figure* 1. While CM from SKOV3-pLX cells had no effect on C2C12 myotube size as compared with untreated controls, SKOV3-β CM resulted in a 32% reduction in myotube diameter (*Figure* 4C and 4D). The MyHC+ area was also significantly reduced following incubation with SKOV3-β CM (*Figure* 4E). Neither the fusion index nor the per cent differentiated were affected by exposure to the SKOV3-β CM as compared with SKOV3-pLX controls (*Figure* 4F and 4G); however, the differentiation index was significantly reduced as compared with D6 unconditioned medium controls (*Figure* 4G).

Similarly, CM from EL4-pLX cells had no significant effect on myotube diameter as compared with D6 unconditioned controls, while EL4-β CM reduced myotube diameter by 36% as compared with EL4-pLX, a reduction of 41% from untreated controls (*Figure* 4I and 4J). The area covered by MyHC + cells was also reduced in myotubes treated with CM from EL4-β as compared with unconditioned controls (*Figure* 4K).

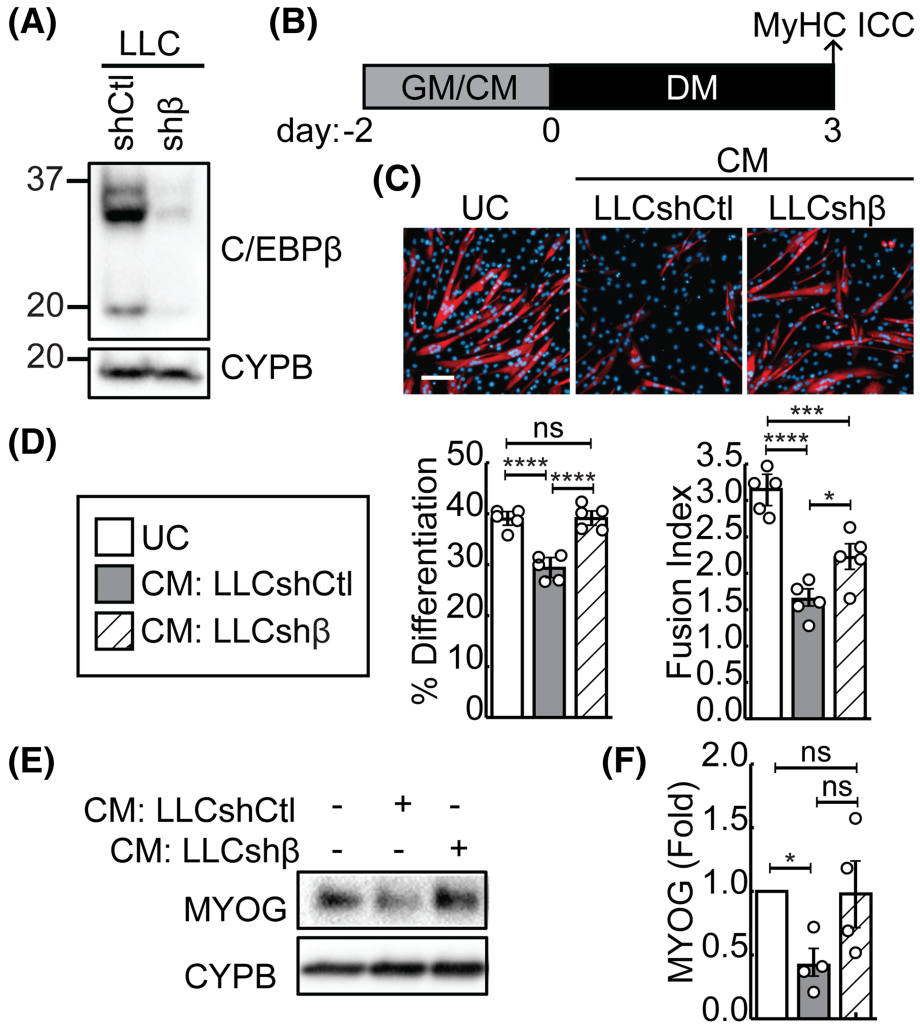


Figure 3 C/EBPβ is required for the expression of anti-myogenic proteins by LLC cells. (A) C/EBPβ protein expression in LLC-shCtl and LLC-shβ cells. Cyclophilin B (CYPB) is a loading control. (B) Schematic representation of the experimental system. C2C12 myoblasts were grown for 48 h in growth medium supplemented with 50% conditioned medium from LLC cells that were retrovirally transduced to express a shRNA directed against *Cebpb* (shβ) or against luciferase (ShCtl) and then differentiated in the absence of CM for 3 days (DM). Incubation with unconditioned LLC medium (UC) is shown as a control. (C) Immunostaining for myosin heavy chain (red) on Day 3 of differentiation of cells treated as in (B). Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (D) Per cent differentiation and fusion index (FI) for cells treated as in (B) and (C) (n = 5). (E) Myogenin protein expression on Day 1 of differentiation for C2C12 cells treated as in (B). Cyclophilin B (CYPB) is a loading control. (F) Quantification of myogenin protein expression from (E) (n = 4). Data information: for (D) and (F), bars are the mean ± SEM, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ (one-way ANOVA).

However, in contrast to the SKOV3 experiment, EL4-β CM significantly reduced both the fusion index (*Figure* 4L) and the differentiation index (*Figure* 4M) as compared with controls. These data indicate that C/EBPβ is sufficient to convert a cancer cell-derived secretome from neutral to one that negatively impacts myotube size.

To evaluate the effect of C/EBPβ expression in cancer cells on the differentiation of myoblasts, subconfluent C2C12 myoblasts were incubated with 50% CM from SKOV3-pLX cells, SKOV3-β cells, or unconditioned media for 2 days in growth medium then differentiated in fresh media for 3 days (*Figure* 5A and 5B). Following differentiation, cells were immunostained for MyHC, and the per cent differentiation and fusion index were assessed. Treatment with CM from SKOV3-pLX or SKOV3-β cells did not significantly impact the percentage of differentiated cells (*Figure* 5C and 5D). However, the fusion index was reduced in myoblasts that were treated with SKOV3-β CM as compared with unconditioned control, suggesting that C/EBPβ regulates secreted factors that influence myoblast fusion (*Figure* 5C and 5D). Consistent with our observations using SKOV3 cells, CM from EL4-pLX or EL4-β cells did not adversely affect differentiation (*Figure* 5E–5G). However, cell fusion was significantly reduced by pretreatment with EL4-β CM, like what was observed with SKOV3 CM

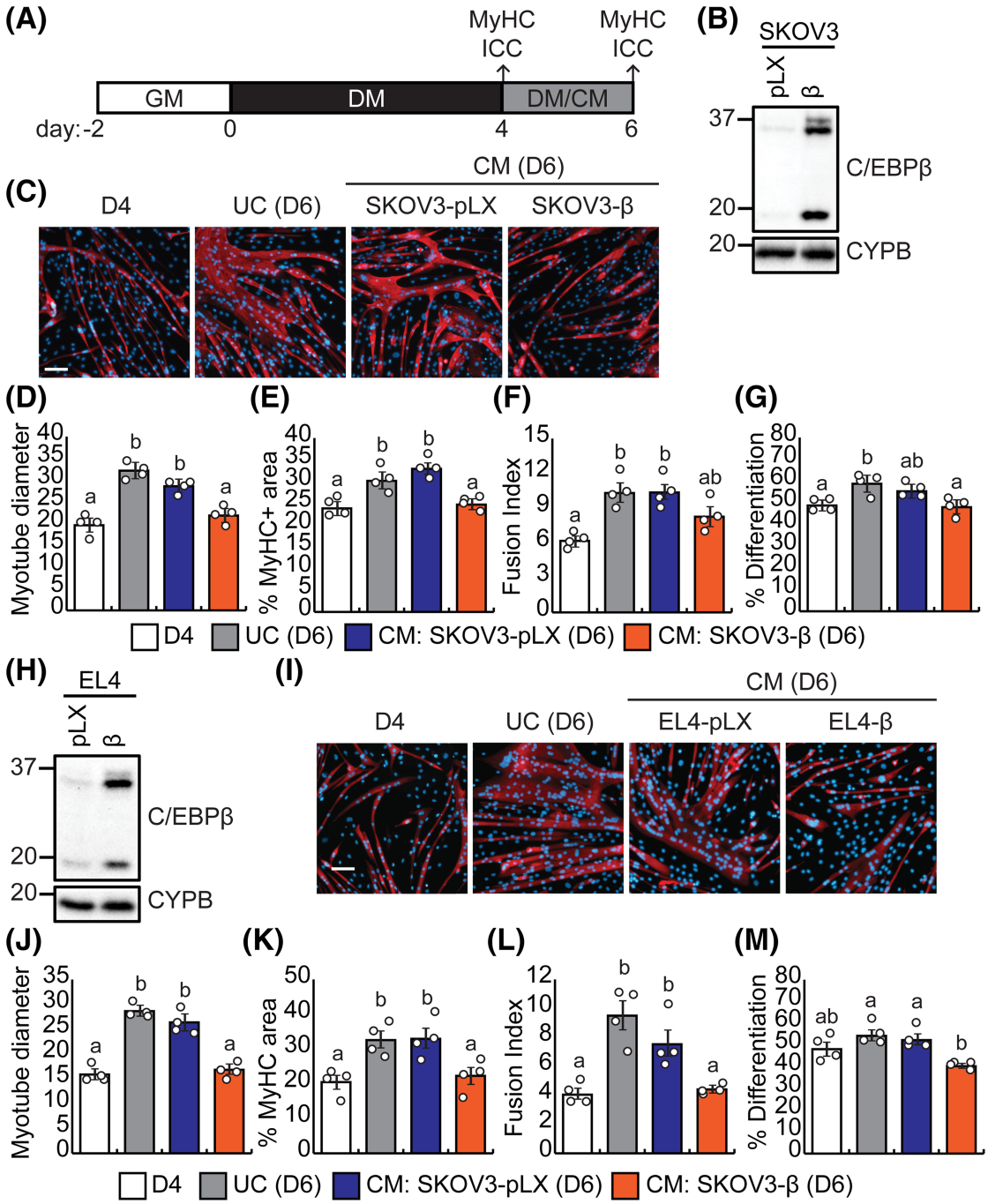


Figure 4 C/EBPβ expression converts non-atrophy-inducing secretomes to atrophy-inducing ones. (A) Schematic representation of the experimental system. C2C12 myoblasts were grown to confluency in growth medium (GM) and differentiated in low serum medium (DM) for 4 days (D4). Differentiated myotubes were then treated for 2 days with conditioned medium (CM) from tumour cells (SKOV3 or EL4) retrovirally transduced to express C/EBPβ or with empty vector in DM. (B) C/EBPβ protein expression in SKOV3 cells retrovirally transduced to express C/EBPβ (SKOV3-β) or with empty virus (SKOV3-pLX). Cyclophilin B (CYPB) is a loading control. (C) Myosin heavy chain immunostaining (red) of D4 myotubes and myotubes after SKOV3 CM treatment for 2 days [CM (D6)]. Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (D) Average myotube diameter of cells from (C) in μm (n = 4). (E) Per cent myosin heavy chain-positive area for cultures from (C) (n = 4). (F) Fusion index (FI) for cells from (C) (n = 4). (G) Per cent differentiation for cells from (C) (n = 4). (H) C/EBPβ protein expression in EL4 cells retrovirally transduced to express C/EBPβ (EL4-β) or with empty virus (EL4-pLX). Cyclophilin B (CYPB) is used as a loading control. (I) Myosin heavy chain immunostaining of D4 myotubes and myotubes after a 2 day treatment with EL4 CM [CM (D6)]. Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (J) Average myotube diameter of cells from (F) in μm (n = 4). (K) Per cent myosin heavy chain-positive area for cultures from (I) (n = 4). (L) Fusion index (FI) for cells from (I) (n = 4). (M) Per cent differentiation for cells from (I) (n = 4). Data information: for (D)–(G) and (J)–(M), bars are the mean ± SEM. Different letters above bars indicate statistically significant differences at a cut-off of $P < 0.05$ (one-way ANOVA).

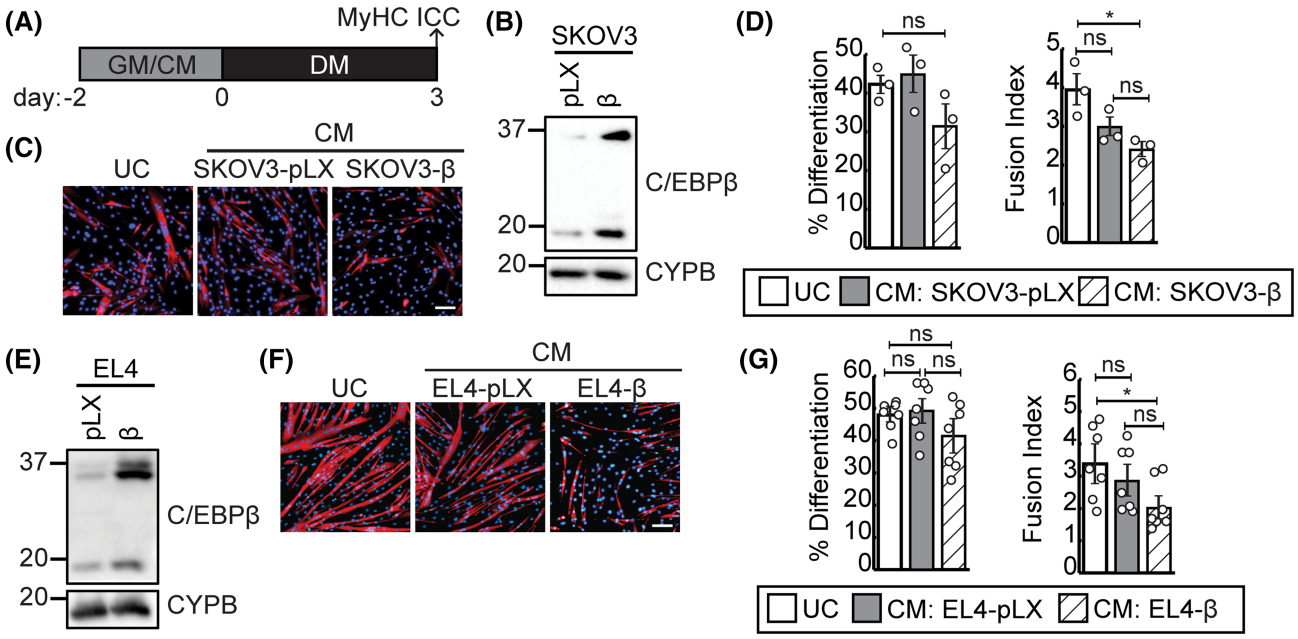


Figure 5 C/EBPβ converts the SKOV3 and EL4 secretomes from myogenesis-permissive to non-permissive. (A) Schematic representation of the experiment timeline. C2C12 myoblasts were cultured for 48 h in growth medium including 50% conditioned medium (1:1) from SKOV3 cells or EL4 cells that were retrovirally transduced to express C/EBPβ (SKOV3-β and EL4-β) or with empty vector (SKOV3-pLX and EL4-pLX) and then differentiated for 3 days in regular DM (D3). Unconditioned medium (UC) is a control. (B) C/EBPβ protein expression in SKOV3-pLX and SKOV3-β cells. Cyclophilin B (CYPB) is a loading control. (C) Myosin heavy chain immunostaining of D3 differentiated myoblasts from cells cultured as in (B) in the presence of conditioned medium from SKOV3-pLX or β cells. Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (D) Per cent differentiation and fusion index (n = 3) from cells cultured as in (C). (E) C/EBPβ protein expression in EL4-pLX and EL4-β cells. Cyclophilin B (CypB) is a loading control. (F) Myosin heavy chain immunostaining of Day 3 differentiated myoblasts after 48 h of proliferation in the presence of EL4 conditioned medium. Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (G) Percentage of differentiated cells and fusion index from cells cultured as in (F) (n = 7). Data information: for (D) and (G), bars are the mean ± SEM, *$P \leq 0.05$ and **$P \leq 0.01$. ns, not statistically significant (one-way ANOVA).

(*Figure* 5G). Taken together, these data suggest that C/EBPβ expression is sufficient to produce a secretome that inhibits myogenic fusion.

Loss of C/EBPβ in cancer cells attenuates muscle wasting in an in vivo model of cachexia

To understand the role of tumour C/EBPβ expression on the pathogenesis of cancer cachexia, mice were injected subcutaneously with 5 × 10^5 LLC cells that were retrovirally transduced with shRNA targeting *Cebpb* mRNA (LLC-shβ) or to express a shRNA against luciferase (LLC-shCtl) as control. Sham controls were injected with PBS. Tumours were allowed to grow and induce cachexia for 3 weeks, after which the tumours and TA muscles were harvested from sham and tumour-bearing mice. The knockdown of C/EBPβ in tumours was confirmed by western blotting (*Figure* 6A). As expected, we observed a significant reduction (−26%) in the myofibre cross-sectional area in TA sections from LLC-shCtl-bearing mice as compared with sham controls (*Figure* 6B and 6C). However, fibre cross-sectional area (XSA) from LLC-shβ-bearing mice was comparable with that of sham controls (*Figure* 6B and 6C), supporting a role for tumour C/EBPβ expression in the development of cancer cachexia. To further visualize the differences in fibre size between all the examined conditions, we analysed the distribution of myofibre XSAs for the TA muscle. We observed a trend towards increased smaller fibres (<1200 μm^2) and decreased larger fibres (>2500 μm^2) for muscles from LLC-shCtl-bearing mice as compared with sham controls (*Figure* 6D and 6E), whereas muscle fibres from LLC-shβ-bearing mice had a distribution of fibre sizes similar to sham controls (*Figure* 6D and 6E). When comparing the mass of dissected tumours from experimental animals, we observed a ~46% reduction in the weight of LLC-shβ tumours as compared with LLC-shCtl tumours and, while not statistically significant, could contribute to attenuation of cachexia observed (*Figure* 6F). To explore this possibility, we plotted the tumour mass against the average muscle fibre XSA for each mouse, revealing no strong correlation between tumour size and muscle wasting (*Figure* 6G). For example, both the smallest and largest LLC-shCtl tumours resulted in fibre XSA of ~1500 μm^2, and all LLC-shβ tumours, regardless of size, promoted larger myofibre XSAs (*Figure* 6G). Additionally, in a similar experiment using LLC cells transduced with empty vector (LLC-EV) or LLC-shβ, we observed a similar

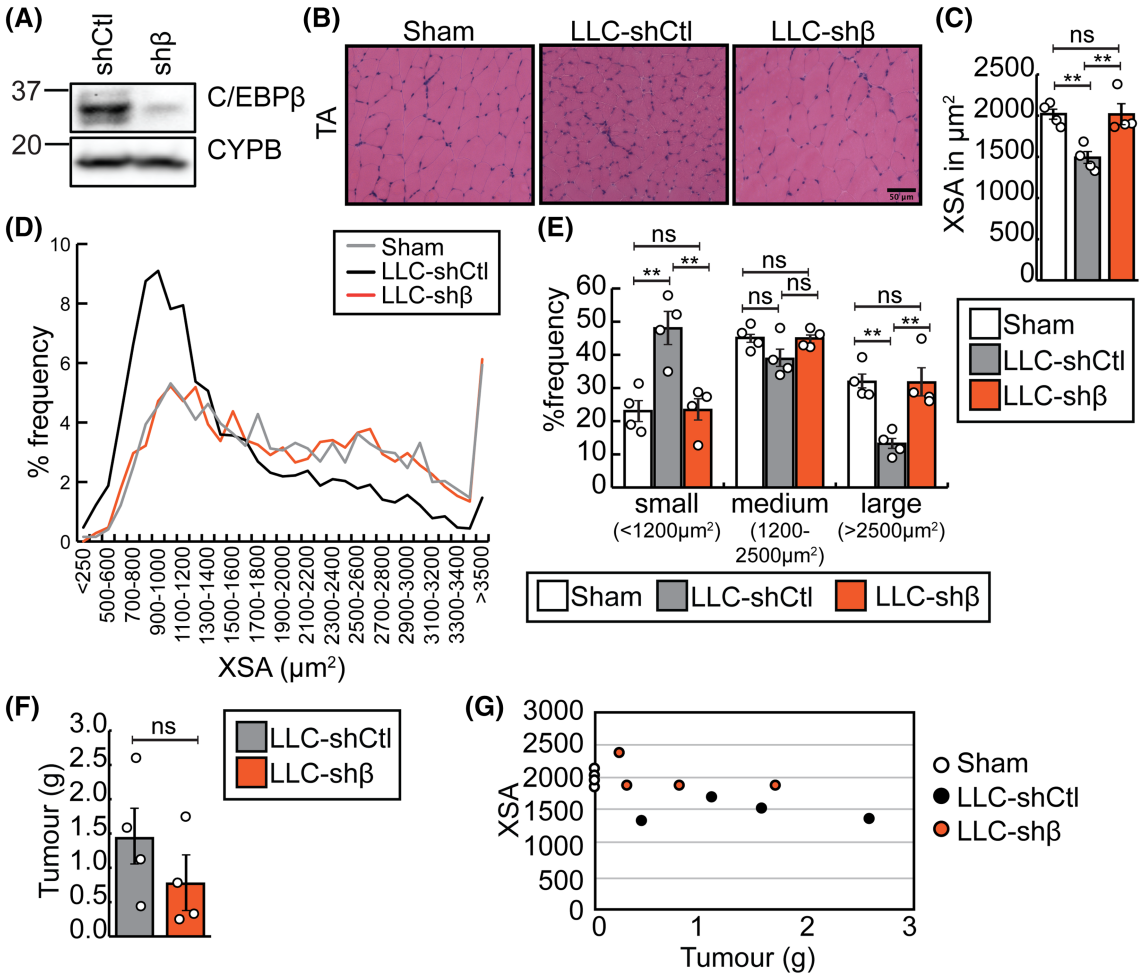


Figure 6 Loss of C/EBPβ expression in tumours prevents muscle atrophy in a mouse model of cachexia. Mice were inoculated subcutaneously into the right flank with 5 × 10^5 Lewis lung carcinoma (LLC) cells that were retrovirally transduced to express shRNA against *Cebpb* (LLC-shβ) or against luciferase as control (LLC-shCtl). Sham mice received PSB alone. Tumours were allowed to grow for 3 weeks to induce cachexia. (A) Representative western blot of C/EBPβ protein expression in LLC-shCtl and LLC-shβ tumours 3 weeks after inoculation. Cyclophilin B (CYPB) is a loading control. (B) H&E staining of cross sections of TA muscle from sham and tumour-bearing mice. Scale bar = 50 μm. (C) Average fibre cross-sectional area (XSA) in the TA muscle from (B) (n = 4). (D) Frequency distribution of myofibre XSA in the TA muscle. (E) Frequency of muscle cross-sectional areas grouped into small fibres (<1200 μm^2), medium-sized fibres (between 1200 and 2500 μm^2), and large fibres (>2500 μm^2) from (D). (F) LLC-shCtl and LLC-shβ tumour mass 3 weeks after inoculation (n = 4 per condition). (G) Average fibre cross-sectional area (XSA) from TA muscle plotted against tumour mass. Pearson r values are as follows: shCtrl r = −0.6259 and shβ r = −0.6525. Data information: for (C) and (E), bars represent the mean ± SEM, $^{**}P < 0.01$ (one-way ANOVA). For (F), bars represent the mean ± SEM. ns, not significant (two-tailed Student's t-test).

phenomenon, specifically that while LLC-EV tumours caused a reduction in XSA as compared with sham controls, LLC-shβ tumours did not induce cachexia (*Figure* S2). Even when tumour size was considered, LLC-EV tumours produced greater myofibre wasting than LLC-shβ tumours. Thus, the loss of C/EBPβ in LLC cells attenuates muscle atrophy *in vivo*.

Overexpression of C/EBPβ in non-cachectic tumours promotes muscle atrophy in vivo

Next, we tested if the overexpression of C/EBPβ in EL4 cells was sufficient to cause muscle wasting *in vivo*. Mice were injected subcutaneously with 1 × 10^6 EL4 cells retrovirally transduced to overexpress C/EBPβ (EL4-β) or with empty vector (EL4-pLX). Sham controls were injected with PBS. Tumours were allowed to grow for 3 weeks after which the tumour and TA muscles were harvested. The overexpression of C/EBPβ in the tumour was confirmed by western blotting (*Figure* 7A). As previously reported,[46] we did not observe a reduction in average TA myofibre XSA in EL4-pLX-bearing mice as compared with sham controls (*Figure* 7B–7E). However, C/EBPβ expression in EL4 tumours caused a significant reduction in average myofibre XSA as compared with sham and EL4-pLX-bearing mice, with a significant reduction in large-sized and medium-sized myofibres (>1200 μm^2) and a

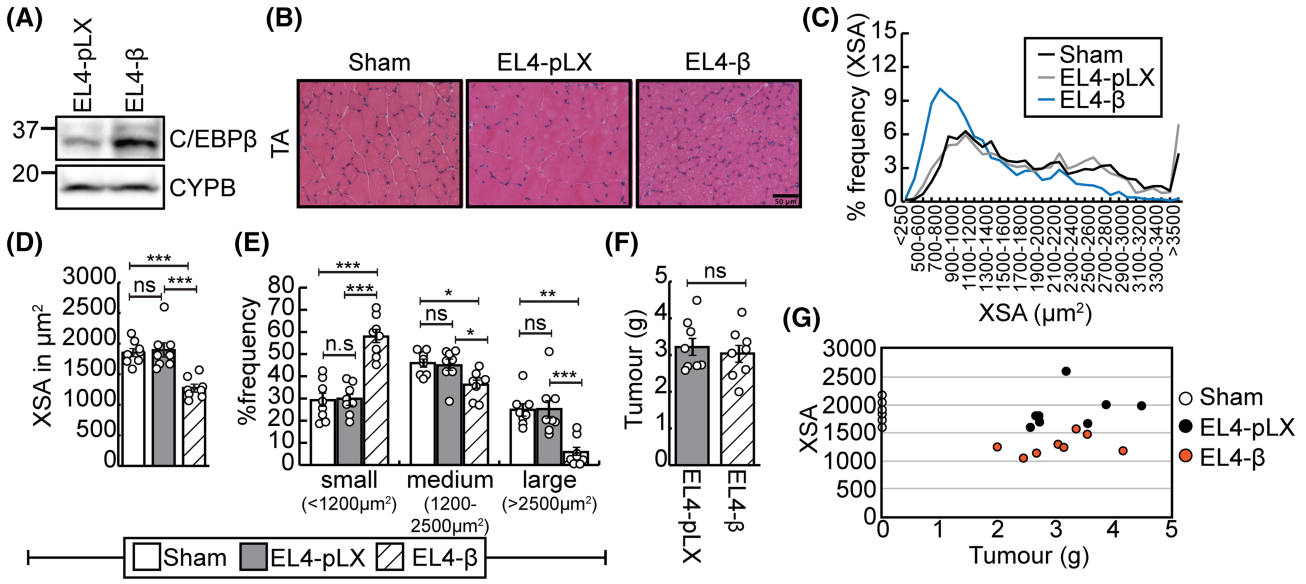


Figure 7 C/EBPβ overexpression converts non-cachectic tumours into atrophy-inducing ones *in vivo*. Mice were inoculated subcutaneously into the right flank with 1×10^6 lymphoblastoma (EL4) cells that were retrovirally transduced to express C/EBPβ (EL4-β) or with empty vector (EL4-pLX), and tumours were allowed to grow for 3 weeks. PBS alone was used for sham injections. (A) Representative western blot of C/EBPβ protein expression in EL4-pLX and EL4-β tumours 3 weeks after inoculation. Cyclophilin B (CYPB) is a loading control. (B) H&E staining of the TA muscle 3 weeks after inoculation with EL4 cells. Scale bar = 50 μm (C) Frequency distribution of myofibre XSA from (B). (D) Average TA fibre cross-sectional area (XSA) from (B) (n = 8). (E) Frequency of muscle cross-sectional areas grouped into small fibres (<1200 μm^2), medium-sized fibres (between 1200 and 2500 μm^2), and large fibres (>2500 μm^2) from (B). (F) EL4-pLX (n = 8) and EL4-β (n = 8) tumour mass. (G) Average fibre cross-sectional area (XSA) from TA muscle plotted against tumour mass. Pearson r values are as follows: EL4-pLX $r = -0.3057$ and EL4-β $r = -0.3698$. Data information: for (D)–(F), bars represent the mean ± SEM, $^*P < 0.05$. $^{**}P < 0.01$, and $^{***}P < 0.001$ (D, E: one-way ANOVA; F: two-tailed Student's t-test).

concomitant increase in small myofibres (<1200 μm^2) (*Figure* 7B–7E). C/EBPβ overexpression in EL4 cells did not result in an increase in tumour mass as compared with controls (*Figure* 7F), and no correlation between tumour mass and average myofibre XSA was observed (*Figure* 7G). Taken together, our data indicate that increased C/EBPβ expression in non-cachectic tumours (EL4) promotes muscle wasting.

C/EBPβ-regulated secretome shares similarity with the secretome of human cancers

Given that our data suggest that (i) C/EBPβ regulates secreted proteins that inhibit myogenesis and cause muscle wasting, (ii) about 80% of patients with advanced cancers are cachectic, and (iii) C/EBPβ is expressed in most cancer types, we sought to compare secreted proteins that are dysregulated in C/EBPβ-overexpressing myoblasts to those dysregulated in tumours. To do so, we used data that were generated by the Nielsen group[54] in which they compared the transcripts of secreted proteins from 27 different types of tumours obtained from The Cancer Genome Atlas to tumour equivalent healthy tissue profiles from the Genotype-Tissue Expression database.[54] Two lists of secreted proteins were used in their analysis, (i) proteins with a N-terminal signal peptide and annotated as 'secreted' in the UniProt database (1810 genes) and (ii) proteins detected experimentally in the secretome of any data set available in the Human Cancer Secretome Database (HCSD) (6543 genes, ~800 overlapping with the first list).[54] Thus, we compared differentially expressed genes from these human cancers to our list of differentially expressed genes from mouse C/EBPβ-overexpressing myoblasts. We only used genes from the two lists (secreted and HCSD) that are also expressed in mouse myoblasts. While only 45% of the 'secreted' list of genes were detected in mouse myoblasts, 82% of the HSCD list were detected. Thus, the HSCD list provides a more appropriate measure of the similarity between cancer secretome and C/EBPβ-regulated secreted proteins in mouse myoblasts. We performed the analysis using a cut-off of two-fold difference for significantly differentially expressed genes. Our analysis indicates that the similarity between C/EBPβ-regulated secreted proteins and secreted proteins from any cancer subtype[54] ranged from 10% to 19% using the HCSD lists and from 18% to 40% using the 'secreted' list (*Tables* S1 and S2). Differentially expressed secreted proteins from glioblastoma multiforme and brain lower grade glioma had the highest similarity to C/EBPβ-regulated secreted proteins (19%). Pancreatic adenocarcinoma (18% similarity) and stomach adenocarcinoma (17% similarity) ranked third and fourth, respectively (*Tables* S1 and S2). Interestingly, glioblastoma multiforme, pancreatic adenocarcinoma, and stomach adenocarcinoma are known to cause cancer cachexia,[55–57] and their

secretome likely plays a role in that process. Considering that most advanced cancers lead to cachexia, we generated lists of up-regulated and down-regulated secreted proteins in C/EBPβ-overexpressing myoblasts and in at least one type of cancer. This resulted in 314 up-regulated and 173 down-regulated genes (*File* S3). Gene ontology analysis of the up-regulated genes showed terms associated with proliferation, differentiation, migration, angiogenesis, and the inflammatory response (*File* S3). Gene ontology analysis of the down-regulated genes showed terms associated with muscle contraction, developmental and muscle proteins, migration, and angiogenesis (*File* S3).

C/EBPβ stimulates the expression of SERPINF1, TNFRSF11B, and CD93 to promote muscle atrophy

Given that C/EBPβ up-regulates several cytokines (*Figure* 1C), we assessed their protein expression in C/EBPβ-overexpressing C2C12 myoblasts and in LLC cells retrovirally transduced to express a shRNA against *Cebpb* (LLC-shβ). Lysates from C2C12-pLX, C2C12-β, LLC-shCtl, and LLC-shβ were incubated with membranes capturing antibodies against 111 mouse cytokines (Proteome Profiler Mouse XL Cytokine Array) (*Figure* 8A and 8B). C/EBPβ overexpression in C2C12s resulted in the up-regulation of 95 cytokines (51 proteins ≥1.5-fold) and the down-regulation of 16 cytokines (2 proteins ≥1.5-fold) out of the 111 tested factors (*Figure* 8C and *File* S4). Knockdown of C/EBPβ in LLC cells resulted in the down-regulation of 101 cytokines (31 proteins ≥1.5-fold) and the up-regulation of 10 cytokines (1 protein ≥1.5-fold) as compared with controls (*Figure* 8C and *File* S4). By comparing cytokines that are up-regulated in C2C12-β cells and down-regulated in LLC-shβ cells using a 1.5-fold cut-off, we narrowed the list to 16 candidate proteins (*Figure* 8D). Of these, the expression of 10 was up-regulated at the mRNA level in at least two tumour types from the 27 tumours that were analysed by the Nielsen group[54] (*Figure* 8E). Osteoprotegerin (TNFRSF11B) was up-regulated in 15 types of cancers followed by the matrix metallopeptidase 3 (MMP3), which was up-regulated in 10 tumours. WNT1-inducible signalling pathway protein 1 (WISP1), cellular communication network factor 4 (CCN4), and CD93 were up-regulated in eight tumours each (*Figure* 8E). To the best of our knowledge, a role for 15 of the 16 candidate cytokines identified has not been described in muscle atrophy (exception is *Serpinf1*).

We selected three secreted factors (TNFRSF11B, SERPINF1, and CD93) based on fold change in C/EBPβ-modified cells (*Figure* 8A–8D) and expression in human cachectic tumours to examine their role in muscle wasting, as a proof of concept (*Figure* 8E). TNFRSF11B (osteoprotegerin) is up-regulated in 15 tumours including brain, colon, pancreatic, and stomach cancers that are known as cachexia-inducing cancers.[55–57] SERPINF1 (pigment epithelium-derived factor member 1) and CD93 are up-regulated in six and eight tumours, respectively, including brain and pancreatic tumours.[55–57] To test the effect of SERPINF1, TNFRSF11B, and CD93 on myotubes, C2C12 myotubes were treated with recombinant SERPINF1, TNFRSF11B, and CD93 proteins, or vehicle for 2 days (*Figure* 8F). Untreated cells were kept as controls. MyHC immunostaining was performed on Day 4 (before treatment) and at Day 6 (2 days after treatment), and myotube size was evaluated by measuring the myotube diameter and the percentage of MyHC+ area. Myotubes treated with any recombinant protein (SERPINF1, TNFRSF11B, or CD93) showed ≥34% reduction in myotube diameter as compared with vehicle and untreated controls (*Figure* 8F and 8G). Moreover, the percentage of MyHC+ area was reduced by any of the recombinant proteins by at least 17% as compared with vehicle and untreated controls (*Figure* 8F and 8H). Thus, our data support the model that C/EBPβ is a central regulatory factor of a cachexia-inducing secretome in human cancers.

To determine if C/EBPβ regulates cytokine expression directly, we confirmed that overexpression of C/EBPβ resulted in its activation in EL4 cells (*Figure* S3A) and selected six putative C/EBPβ target genes: *Serpinf1*, *Tnfrsf11b*, *Cd93* (*Figure* 8), and (*Csf1*, *Spp1*, and *Grem1*) (*Figure* 1C). Using published ChIP-seq data for C/EBPβ (GSE36024), we identified C/EBPβ peaks that overlap with histone marks H3K27ac (GSE37525[58]) and H3K18ac (GSE25308[59]) in the regulatory regions of all six genes, suggesting that these regions are transcriptionally active (*Figure* S3B). Further, *Tnfrsf11b*, *Cd93*, *Csf1*, and *Grem1* expression was increased in EL4-β as compared with controls (*Figure* S3C). ChIP-qPCR revealed that in EL4-pLX cells, only the promoter region of *Cd93* and the 28 kb upstream region of *Csf1* were significantly enriched for C/EBPβ as compared with IgG (*Figure* S3D). However, in the EL4-β cells, the promoter regions of *Cd93*, *Tnfrsf11b*, *Grem1*, and *Csf1* and the upstream regions of *Csf1* (28 kb) and *Grem1* (7 kb) were significantly enriched for C/EBPβ as compared with IgG (*Figure* S3D). Thus, C/EBPβ overexpression in EL4 cells increases its occupancy at the regulatory regions of genes coding for secreted proteins, suggesting direct regulation of their expression by C/EBPβ. Taken together, our findings support a model placing C/EBPβ in a central role in the development of cachexia through regulation of tumour gene expression of secreted cachexia-inducing factors.

Discussion

In this study, we identified C/EBPβ as a critical regulator of cachexia-inducing factor expression by cancer cells, leading to both inhibition of myogenesis and reduction of muscle fibre size. C/EBPβ expression in tumours was found to be necessary and sufficient for the development of cachexia in tumour-bearing animals. Stimulation of C/EBPβ drives the ex-

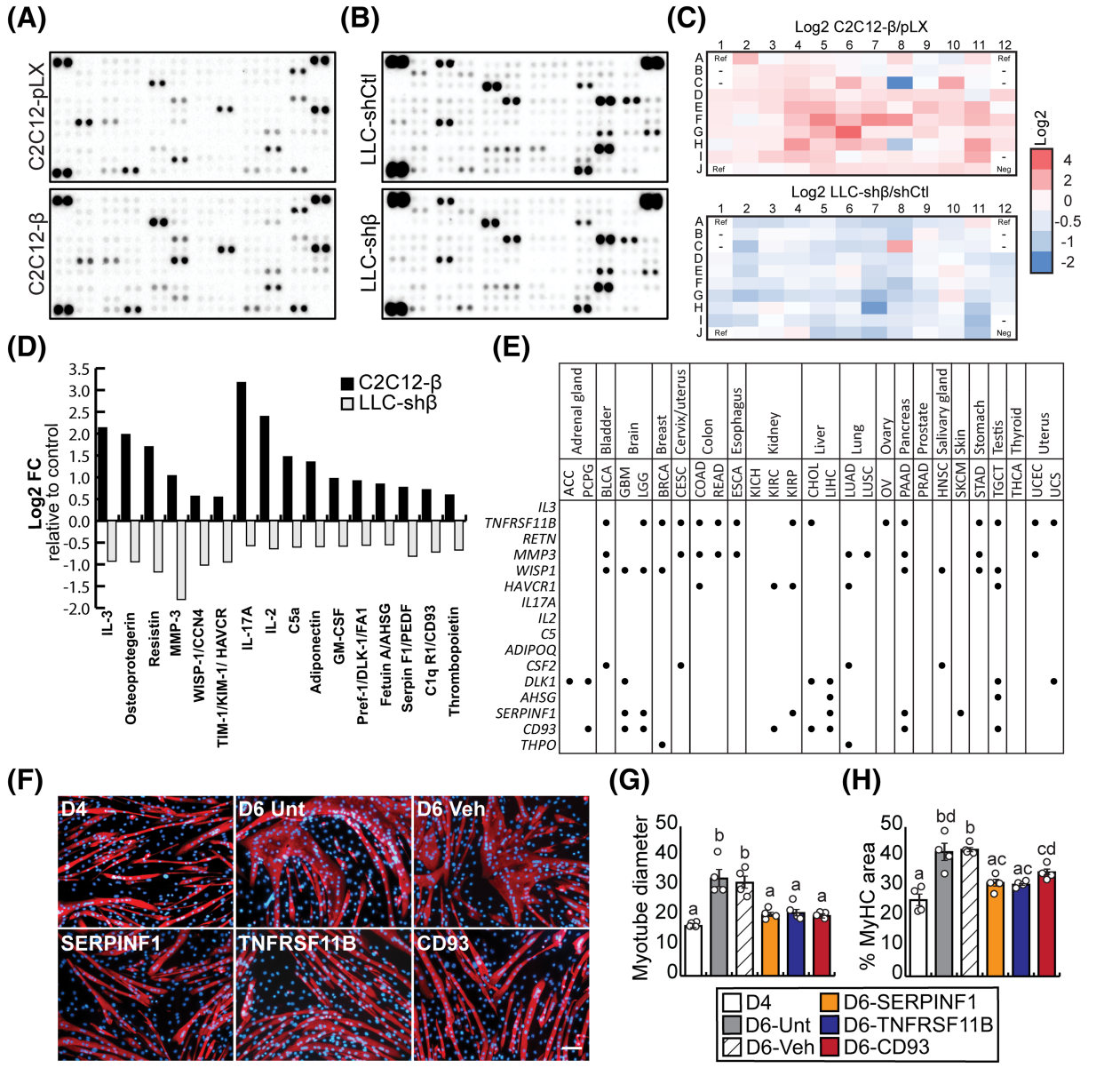


Figure 8 C/EBPβ regulates atrophy-inducing cytokines. (A) Lysates from proliferating C2C12 cells retrovirally transduced with empty vector (pLX) or to express C/EBPβ (β) were used to interrogate the expression of 111 mouse cytokines using the Proteome Profiler Mouse XL Cytokine Array (R&D, ARY028). (B) Lysates from LLC cells retrovirally transduced to express a shRNA directed against *Cebpb* (shβ) or against luciferase (ShCtl) were used to test the expression of 111 mouse cytokines. (C) Expression heat map for (A) and (B) presented as Log2 fold relative to respective controls (C2C12-pLX and LLC-shCtl). (D) Expression of 16 proteins from (C) that are up-regulated in C2C12-β as compared with C2C12-pLX and down-regulated in LLC-shβ as compared with LLC-shCtl by ≥1.5-fold change. (E) Tumours in which the mRNA expression of the 16 cytokines in (D) is up-regulated, generated using the 27 human cancer data sets from the Nielson's group.[54] (F) Myosin heavy chain immunostaining of C2C12 myoblasts differentiated for 4 days (D4) and then treated for 2 days (until D6) with recombinant SERPINF1 (0.5 μg/mL), TNFRSF11B (10 ng/mL), CD93 (6 μg/mL), vehicle, or left untreated (Unt). Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm. (G) Average myotube diameter of cells from (F) in μm (n = 4). (H) Per cent myosin heavy chain-positive area for cultures from (F) (n = 4). Data information: for (G) and (H), bars represent the mean ± SEM. Different letters above bars indicate statistically significant differences at a cut-off of $P < 0.05$ (one-way ANOVA).

pression of several secreted factors, including many cytokines, and we found that SERPINF1, TNFRSF11B, and CD93 can all negatively impact myotube size in culture. These findings are in line with the reported role for C/EBPβ in the immune response via the regulation of cytokines.[36,60]

While our work advances C/EBPβ as a central regulator of cachexia-inducing factor expression in tumours, certain limitations should be considered. The use of in culture models of cachexia is useful to determine the effect of secreted factors on myoblast differentiation and myotube size. As a

control, we opted to use unconditioned medium given that we primarily compared the secretomes of gain-of-function and loss-of-function models of the same cell line. However, we note that unconditioned medium cannot account for effects of nutrient depletion in cultures receiving CM. The corresponding *in vivo* experiments thus provide important support for the model that C/EBPβ expression in tumours underlies the cachexia-inducing secretome.

C/EBPβ knockout studies have demonstrated that this factor is indispensable for the development of immune cells and the immune response[37,61]; however, no specific report has investigated the association between C/EBPβ-regulated pro-inflammatory genes and the development of cancer cachexia. Factors that are derived from tumour cells and immune cells are well established as inducers of muscle atrophy, and yet therapies that target individual factors such as TNFα, IL-1α, or IL-6 have failed to demonstrate strong therapeutic benefit in clinical trials for the treatment of cachexia (reviewed in Prado and Qian.[22]). However, treatments that target multiple cytokines have proven more effective.[62,63] For example, thalidomide reduces cytokine expression[64] and, while its mechanism of action is not fully understood, can reduce body and muscle mass loss in patients with inoperable pancreatic cancer.[63] Interestingly, our findings demonstrate that C/EBPβ is a central regulator of cytokine production in the context of cachexia and thus an important therapeutic target. In support of this, ghrelin treatment prevents muscle atrophy in LLC-bearing mice via down-regulation of the p38/C/EBPβ/myostatin pro-inflammatory pathway supporting a role for C/EBPβ in the development of cachexia.[65] Our work focuses on a role for C/EBPβ expression in the tumour and suggests that therapies that target C/EBPβ expression or activity via post-translational modifications could be potent anti-cachexia agents, through modification of the tumour secretome and muscle protective effects.

Recently, proteomics was used to compare plasma proteins from tumour-bearing (LLC-bearing) and sham mice, finding 39 up-regulated proteins in plasma of cachectic mice.[66] Of these, the transcripts of 11 are also up-regulated in myoblast-overexpressing C/EBPβ (≥1.5-fold) as compared with control. Clinical studies, mainly in pancreatic and gastrointestinal cancer patients, report a correlation between secreted factors and the cachectic state.[67] Of the identified factors linked to cachexia, IL-10, VEGF, adiponectin, and resistin are all up-regulated in C/EBPβ-overexpressing myoblasts and down-regulated in LLC cells following C/EBPβ knockdown (*Figure* 8A–8E and *File* S4). The identification of the transcriptional targets of C/EBPβ (*File* S1) and their association with cachexia highlights the importance of targeting C/EBPβ as a therapeutic approach to treating cachexia.

Ethics statement

The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.[68]

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Conflict of interest

None declared.

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