BJ Signal

Biochem. J. (2012) **446**, 23–36 (Printed in Great Britain) doi:10.1042/BJ20112024

online data

## SUPPLEMENTARY ONLINE DATA

# Myostatin is a novel tumoral factor that induces cancer cachexia

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Supplementary Tables S1 and S2 are available at http://www.BiochemJ.org/bj/446/bj4460023add.htm

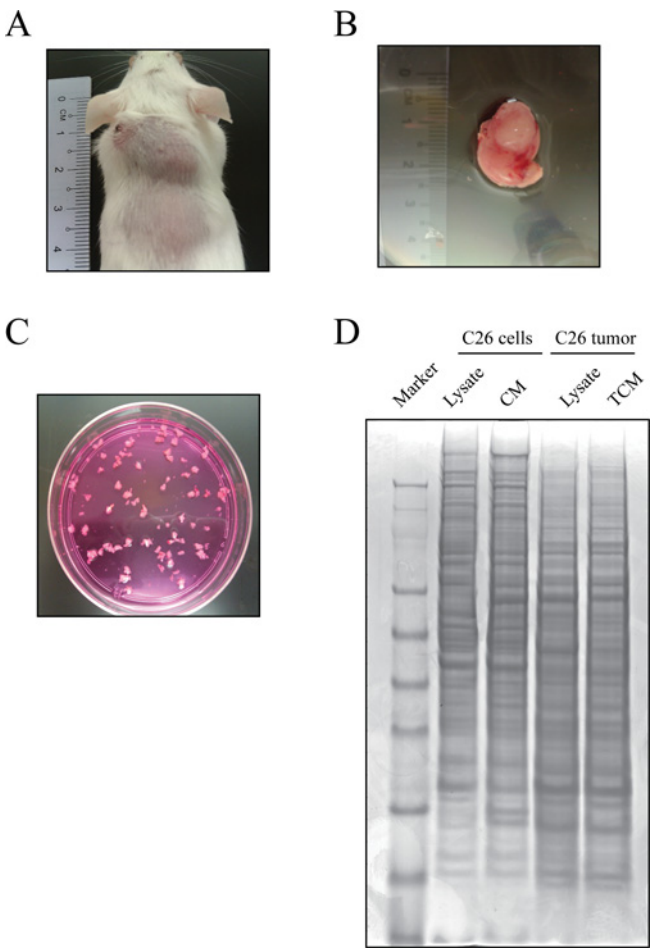


**Figure S1 TCM preparation**

(**A**) C26 cells ($0.5\times10^6$), in 100 $\mu$l of sterile PBS, were subcutaneously inoculated into the dorsal region of CD2F1 mice. The image demonstrates tumour development on day 10. (**B**) A photograph displaying the resected tumour collected from the CD2F1 mice. (**C**) The C26 tumour was diced and placed in serum-free DMEM for TCM collection. (**D**) A 4–12% NuPAGE gel stained with Coomassie Brilliant Blue displaying the proteins found in the lysate and CM from C26 cells and C26 tumours.

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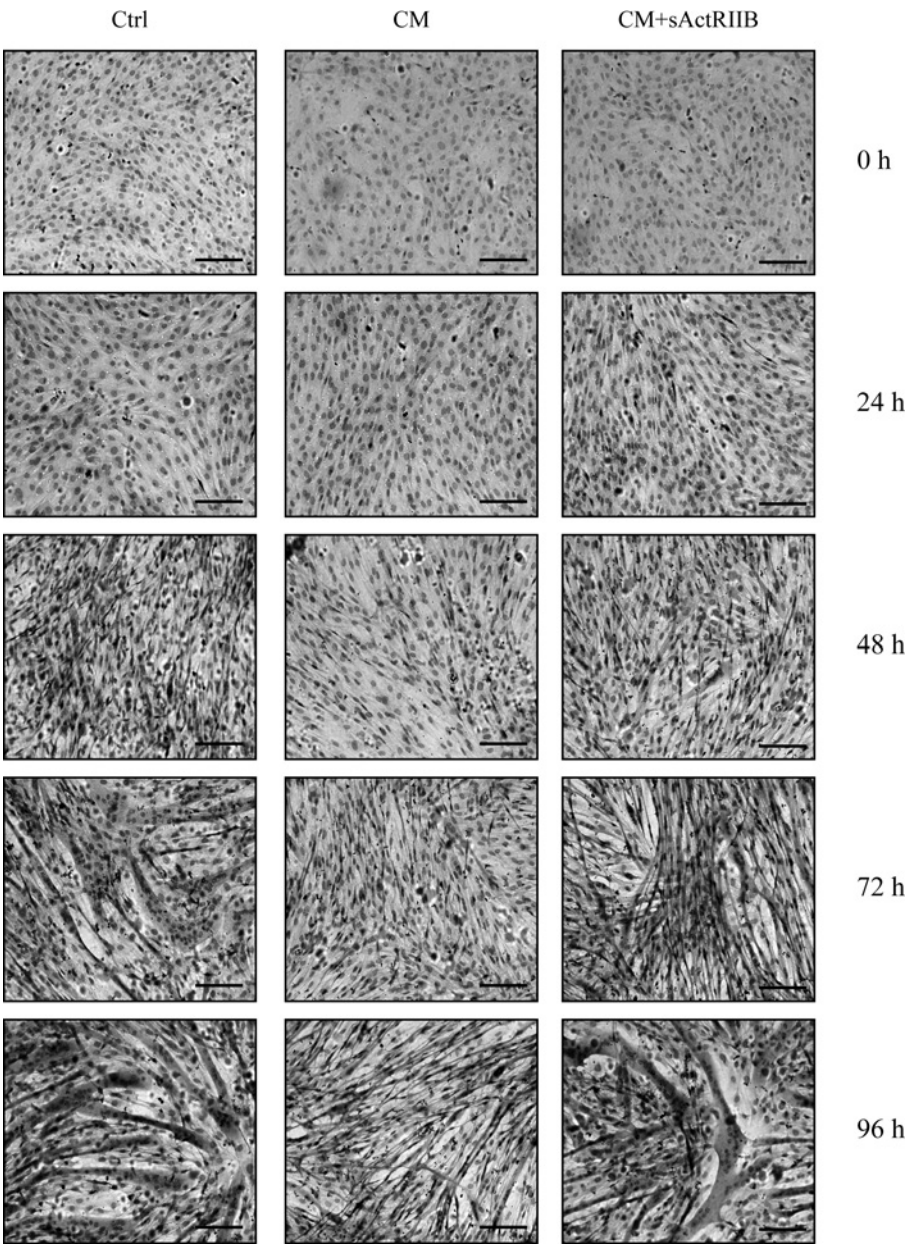


**Figure S2 ActRIIB partially alleviates C26 CM inhibition of C2C12 myoblast differentiation**

Haemotoxylin-and eosin-stained micrographs of C2C12 myoblasts differentiating in low-serum medium (Ctrl), C26 CM diluted 1:5 with low-serum medium and C26 CM diluted 1:5 with low-serum medium pre-incubated with ActRIIB (CM + sActRIIB). Scale bars represent 100 $\mu$m.

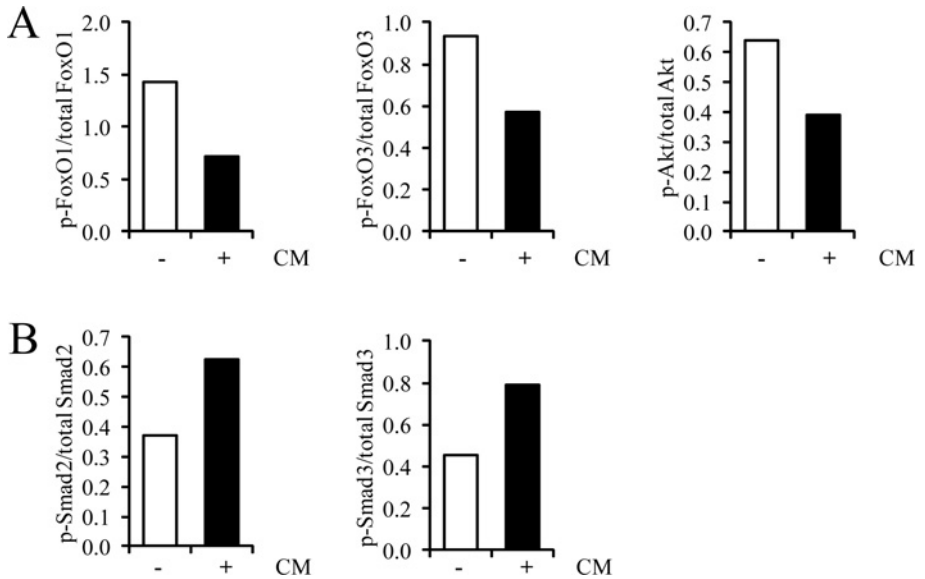


**Figure S3 Treatment of C2C12 myotubes with C26 CM activates signalling pathways involved in skeletal muscle wasting**

(**A**) Densitometric quantification of the relative levels of p-FoxO1, p-FoxO3 and p-Akt, expressed as a proportion of total FoxO1, FoxO3 and Akt respectively, corresponding to immunoblots in Figure 5(D) of the main text. (**B**) Densitometric quantification of the relative protein levels of p-Smad2 and p-Smad3, expressed as a proportion of total Smad2 and Smad3 respectively, corresponding to immunoblots in Figure 5(E) of the main text.

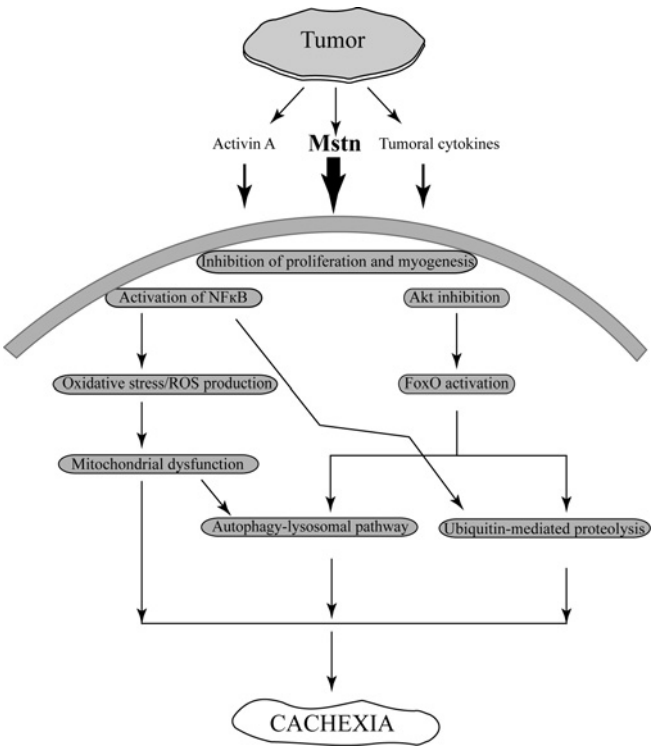


**Figure S4 Schematic diagram summarizing cancer-induced cachexia in skeletal muscle**

The C26 tumour produces several effectors, including Mstn, activin A and other tumoral cytokines, which impart deleterious consequences on skeletal muscle. Mstn family members present in the C26 CM may prevent the proliferation and myogenesis of myoblasts. Furthermore, components in C26 CM also induced the activity of NF-$\kappa$B and FoxO transcription factors, but inhibited Akt. Collectively, these signalling molecules are involved in regulating the activity of degradative pathways. Indeed, C2C12 muscle cell cultures exposed to C26 CM displayed the elevated activity of the ubiquitin–proteasome and the autophagy–lysosome pathway. Moreover, the C26 CM also increased oxidative stress and induced mitochondrial dysfunction in muscle cell cultures. Finally, selective inhibition of Mstn in the C26 CM demonstrated a greater rescue of the atrophy phenotype compared with selective activin A inhibition. Thus Mstn secretion by C26 may play a principal role in the pathogenesis of C26-induced cancer cachexia.

**Table S3 Values represent the relative gene expression changes observed upon treatment of differentiated C2C12 myotubes with different dilutions (1:1, 1:3, 1:5 and 1:10) of CM for 24 h**

Relative gene expression levels were measured by normalizing to *Gapdh*, *Actb* and *Hprt* using the $\Delta\Delta C_T$ method. Significance was calculated using Student's *t* test; $^*P < 0.01$. The experiment was repeated four times in duplicate.

| | | Relative expression compared with control | | | | |
|---|---|---|---|---|---|---|
| Function | Gene name | Control | CM (1:10 dilution) | CM (1:5 dilution) | CM (1:3 dilution) | CM (1:1 dilution) |
| E3 ligases | *Atrogin1* | 1 | 3.30* | 4.61* | 7.90* | 12.28* |
| | *Cblb* | 1 | 2.08* | 2.04* | 3.23* | 8.48* |
| | *March5* | 1 | 1.59 | 1.35 | 1.71 | 2.49* |
| | *Mul1* | 1 | 2.34* | 4.56* | 5.66* | 7.03* |
| | *MuRF1* | 1 | 2.39* | 3.96* | 3.48* | 5.51* |
| Mitochondria/oxidative stress | *Drp1* | 1 | 0.95 | 1.08 | 1.27 | 1.83 |
| | *Fis1* | 1 | 0.91 | 1.13 | 1.46 | 1.53 |
| | *Mfn1* | 1 | 0.72 | 1.16 | 1.49 | 1.05 |
| | *Mfn2* | 1 | 1.09 | 1.04 | 1.05 | 1.06 |
| | *Cat* | 1 | 0.78 | 1.03 | 1.18 | 1.02 |
| | *Bnip3* | 1 | 1.9 | 1.42 | 2.15* | 3.22* |
| | *Gsr* | 1 | 0.98 | 0.79 | 0.79* | 0.67* |
| | *Sod1* | 1 | 0.88 | 0.39* | 0.45* | 0.56* |
| Lysosomal/autophagy system | *Lc3* | 1 | 1.73* | 2.42* | 3.56* | 5.46* |
| | *Atg5* | 1 | 4.88* | 3.32* | 7.33* | 12.07* |
| | *Atg7* | 1 | 1.73* | 2.24* | 3.03* | 4.68** |
| | *Cath L* | 1 | 1.05 | 1.36 | 1.44 | 2.12* |
| Signalling | *Igf-1* | 1 | 0.87 | 0.55* | 0.43* | 0.56* |
| | *Igf-2* | 1 | 1.37 | 1.38 | 1.65 | 1.09 |
| | *Tgfb1* | 1 | 0.52* | 0.52* | 0.48* | 0.59* |
| | *Mstn* | 1 | 2.14* | 4.75* | 4.96* | 9.08* |
| Transcription factors | *Ppargc1a* | 1 | 1.01 | 0.85 | 0.75* | 0.65* |
| | *Ppargc1b* | 1 | 1.13 | 1.27 | 1.35 | 1.74 |
| | *FoxO1* | 1 | 0.91 | 0.99 | 2.02* | 2.27* |
| | *FoxO3* | 1 | 1.57 | 2.18* | 2.86* | 2.62* |
| | *Nrf1* | 1 | 1.12 | 0.99 | 1.07 | 1.79 |
| | *Tfam* | 1 | 1.31 | 0.77* | 0.32* | 0.50* |
| Structural proteins | *Myh1* | 1 | 1.85* | 1.93* | 1.99* | 2.38* |
| | *Myh2* | 1 | 1.63 | 1.17 | 1.42 | 2.13* |
| | *Myh3* | 1 | 1.14 | 1.12 | 0.84 | 1.6 |
| | *Myh4* | 1 | 2.22* | 1.82* | 2.60* | 3.11* |
| | *Myh7* | 1 | 1.94* | 1.97* | 2.13* | 2.69* |
| | *Myh8* | 1 | 2.16* | 2.66* | 2.93* | 3.65* |
| | *Myl1* | 1 | 1.2 | 1.62 | 1.52 | 2.29* |
| | *Myl2* | 1 | 0.86 | 0.78 | 0.79 | 1.4 |
| | *Myl3* | 1 | 1.4 | 1.39 | 1.33 | 1.59 |
| | *Mylpf* | 1 | 1.31 | 1.63 | 1.49 | 1.99* |
| Inflammatory cytokines | *Il1b* | 1 | 36.43* | 16.79* | 27.62* | 63.25* |
| | *Il2b* | 1 | 3.50* | 3.87* | 4.17* | 5.11* |
| | *Il4* | 1 | 4.45* | 2.96* | 4.31* | 7.52* |
| | *Il6* | 1 | 0.95 | 0.9 | 0.83 | 1.24 |
| | *Il10* | 1 | 9.16* | 5.69* | 9.94* | 12.11* |
| | *TNFa* | 1 | 3.36* | 2.18* | 3.64* | 3.22* |

**Table S4 Antibody list with dilutions**

Developmental Studies Hybridoma Bank.

| Antibody name | Catalogue number | Company Name | Antibody dilution with 5 % non-fat dried skimmed milk in 1× TBST |
|---|---|---|---|
| Atrogin-1 | Gift | Regeneron Pharmaceuticals | 1:500 |
| MuRF1 | Gift | Regeneron Pharmaceuticals | 1:200 |
| Myh | MF20a | DSHB | 1:5000 |
| Myl | T14 | DSHB | 1:5000 |
| $\alpha$-Tubulin | T9026 | Sigma | 1:10000 |
| Ubiquitin | sc-8017 | Santa Cruz Biotechnology | 1:10000 |
| FoxO1 | sc-11350 | Santa Cruz Biotechnology | 1:200 |
| p-FoxO1 | sc-16307 | Santa Cruz Biotechnology | 1:2000 |
| FoxO3 | sc-131351 | Santa Cruz Biotechnology | 1:200 |
| p-FoxO3 | sc-101681 | Santa Cruz Biotechnology | 1:100 |
| Smad2/3 | sc-6032 | Santa Cruz Biotechnology | 1:400 |
| p-Smad2/3 | sc-11769 | Santa Cruz Biotechnology | 1:400 |
| Mstn | MAB788 | R&D Systems | 0.5 $\mu$g/ml |
| Activin A | AF338 | R&D Systems | 0.5 $\mu$g/ml |
| p21 | 556430 | BD Pharmaceuticals | 1:400 |
| Cyclin D1 | sc-8396 | Santa Cruz Biotechnology | 1:400 |
| Cdk2 | sc-6248 | Santa Cruz Biotechnology | 1:400 |
| pRb | 554136 | BD Pharmaceuticals | 1:400 |
| LC3 | M152-3 | MBL International | 1:200 |
| MyoD | sc-304 | Santa Cruz Biotechnology | 1:400 |
| Myog | sc-576 | Santa Cruz Biotechnology | 1:400 |
| Akt | sc-8312 | Santa Cruz Biotechnology | 1:5000 |
| p-Akt | sc-7983 | Santa Cruz Biotechnology | 1:200 |
| p65 | 4764 | Cell Signaling Technology | 1:1000 |
| p-p65 | 3033 | Cell Signaling Technology | 1:1000 |
| Anti-rabbit HRP conjugate | 170-6515 | Bio-Rad Laboratories | 1:5000 |
| Anti-mouse HRP conjugate | 170-6516 | Bio-Rad Laboratories | 1:5000 |
| Anti-goat HRP conjugate | sc-2768 | Santa Cruz Biotechnology | 1:2000 |
| Anti-rat HRP conjugate | sc-2006 | Santa Cruz Biotechnology | 1:2000 |

Received 18 November 2011/12 March 2012; accepted 23 May 2012
Published as BJ Immediate Publication 23 May 2012, doi:10.1042/BJ20112024