Supplemental Information:

Pan, et al., Prostate tumor-derived GDF11 accelerates androgen deprivation therapy-induced sarcopenia

Supplemental Methods

RNA isolation and quantitative RT-PCR. Tissues were harvested after euthanasia, snap frozen in liquid nitrogen, and stored at –80°C for mRNA expression analysis. Total RNA was extracted from pulverized QUA or tumor tissue using TRIzol (Invitrogen). Five micrograms of total RNA were reverse transcribed using pdN15 with the Superscript III reverse transcription kit (Invitrogen, Waltham, MA, USA) to generate single-strand cDNA. PCR was performed using SYBR® Green qPCR Master Mix (Molecular Probes, Eugene, OR, USA) in a StepOnePlus instrument (Applied Biosystems, Waltham, MA, USA). PCR primers, spanning at least one intron are listed in Supplemental Table S3. Standard curves were generated using linearized plasmids encoding the corresponding cDNAs to assure linearity of quantitation. The mean expression levels of the combination of GADPH and eEf2 (eukaryotic elongation factor 2) were used to normalize target gene expression for total cDNA input from tumor tissue. The mean expression levels of ribosomal protein L32 were used to normalize target gene expression for total cDNA input from QUA muscle tissue. RNA from tissues from at least three mice, each reverse transcribed three times, were analyzed, and qPCR was performed in triplicate for each sample.

Mouse assessments. Total body mass absent tumor was calculated using total body mass by weighing less the mass of the tumor, estimated as 1 mg per cubic millimeter tumor volume for correlative analysis in Supplemental Fig. S3.

Supplemental Discussion

TGFß-family proteins exist largely in a latent pro-domain or inhibitor-bound form and the active, soluble dimeric TGFß-family myokines represent a small proportion of the overall protein (1, 2). Therefore, measurement of functional ligands requires that soluble active TGFß-family myokines be examined using dimer-specific assays (3). Active myostatin c-terminal dimer was measured using a specific immunoblot assay, since available ELISA assays do not discriminate this form from latent pro-domain or inhibitor-bound myostatin c-terminal dimer (4). Dimer-specific ELISA assays used for each of the activins and GDF11, and the myostatin immunoblots, demonstrated distinct patterns of induction for each catabolic myokine in muscle after castration (Fig. 3). Measurement of myostatin and GDF11 has been problematic due to protein homology (5, 6). In muscle, serum, and tumor, the patterns of myostatin and GDF11 expression were distinct (Figs. 3 and 5). Similarly, the function-neutralizing anti-GDF11 monoclonal antibody employed did not cross-react with myostatin (Supplemental Fig. S8). The observed ADT-regulation of soluble active myostatin c-terminal dimer levels and soluble GDF11 are also distinct from the ADT-unregulated and quantitatively ~100-fold higher levels of total acid-solubilized myostatin (Supplemental Fig. S9). These data strongly suggest specific measurement of the castration-induced expression level changes of the active dimers of activins, myostatin, and GDF11, and specific neutralization of circulating GDF11.

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48   References

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53   inhibitory binding proteins of myostatin in normal serum. *J Biol Chem.*
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63   Cardiovascular Disease. *Cell Metab.* 2016;23(6):1207-15.

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66 **Supplemental Table S1. Analysis of tumor volume correlation with physiological assessments.**
67 Regression analysis for tumor-bearing cohort #1 of weekly measures of tumor volume versus initial pre-
68 castration tumor volume, and grip strength and total body mass versus tumor volume at the week of
69 assessment.
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A: Post-castration tumor volumes versus initial (pre-castration) tumor volume

Relevant to supplemental Fig. S3A

Week	n	slope (% change/1000 mm^3)	Pearson Correlation ('r')	p-Value
wk2	12	-13.4	0.451	0.141
wk4	12	-16.1	0.409	0.186
wk5	12	-21.3	0.476	0.117
wk7	12	-19.5	0.386	0.215
wk8	12	-26.4	0.489	0.106
wk9	12	-22.2	0.496	0.101
wk10	12	-29.9	0.626	0.029
wk14	12	-24.5	0.472	0.121
wk16	12	-27	0.578	0.049

B: Grip strength versus tumor volume at that same week

Relevant to supplemental Fig. S3B

Week	n	slope (N/ml)	Pearson Correlation ('r')	p-Value
wk2	12	-0.032	0.164	0.610
wk4	12	-0.033	0.167	0.603
wk5	12	-0.056	0.342	0.277
wk7	12	-0.043	0.210	0.513
wk8	12	-0.047	0.173	0.590
wk9	12	-0.035	0.237	0.459
wk10	12	-0.033	0.249	0.435
wk14	12	-0.008	0.095	0.769
wk16	12	-0.011	0.130	0.686

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73 **Supplemental Table S2. Analysis of tumor volume correlation with end-point measures.** Regression
74 analysis of final measures of individual skeletal muscle mass and body composition for tumor-bearing
75 cohort #1 versus initial pre-castration tumor volume.

A: Body composition versus tumor volume after 16 weeks castration

Relevant to supplemental Fig. S3D, S3E

	n	slope (% change/1000 mm^3)	Pearson Correlation ('r')	p-Value
%fat	12	-2.16	0.545	0.067
%fluid	12	0.52	0.065	0.841
%bone	12	0.04	0.277	0.383
%lean	12	1.7	0.599	0.040

B: Muscle mass versus tumor volume after 16 weeks castration

Relevant to supplemental Fig. S3F

	n	slope (% change/1000 mm^3)	Pearson Correlation ('r')	p-Value
TA	12	-2.7	-0.287	0.366
EDL	12	-1.3	-0.198	0.537
SOL	12	-1.6	-0.358	0.253
GAS	12	-2.3	-0.080	0.805
QUA	12	11	0.328	0.298
TRI	12	-0.8	-0.033	0.926

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78 **Supplemental Table S3. Oligonucleotide primers used in qRT-PCR analysis.** Forward and reverse
79 primers used to quantitate the indicated gene in RT-qPCR analyses.
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Gene	Forward primer sequence	Reverse primer sequence
B-Actin	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC
EF2	TGCCTTGCGTGTCACCGATGGA	TCAGGACGGGCTTGATGCGTTCAG
GADPH	CTTTGGCATTGTGGAAGGGC	CAGGGATGATGTTCTGGGCA
RpL32	CCTGGTCCACAATGTCAAGGA	TGGGATTGGTGACTCTGATGG
Atrogin-1	GTTCACAAAGGAAGTACGAAGG	AAGCTTTCAACAGACTGGACTTC
MuRF1	ACCTGCTGGTGGAAAACATC	AGGAGCAAGTAGGCACCTCA
Acvr1b	AAAGCCCTTCTACTGCCTGA	GATGATGCCGACCAGCTC
Acvr1c	GGCTGTGAAGCACGATTCTA	TCCAACTGAACACCTTCGAG
Acvr2a	CGTTCGCCGTCTTTCTTATC	GCCCTCACAGCAACAAAGT
Acvr2b	GTGGGAGCTCGTCTCTCG	GGTGTTTCAGCCAGTGATCC
Inhibin Ba	GGAGATAGAGGACGACATTGGC	ACGCTCCACTACTGACAGGTCA
Inhibin Bb	CTCCGAGATCATCAGCTTTGCAG	GGAGCAGTTTCAGGTACAGCCA
GDF-11	TTTCGCCAGCCACAGAGCAACT	CTCTAGGACTCGAAGCTCCATG
Myostatin	AACCTTCCCAGGACCAGGAGAA	GGCTTCAAAATCGACCGTGAGG

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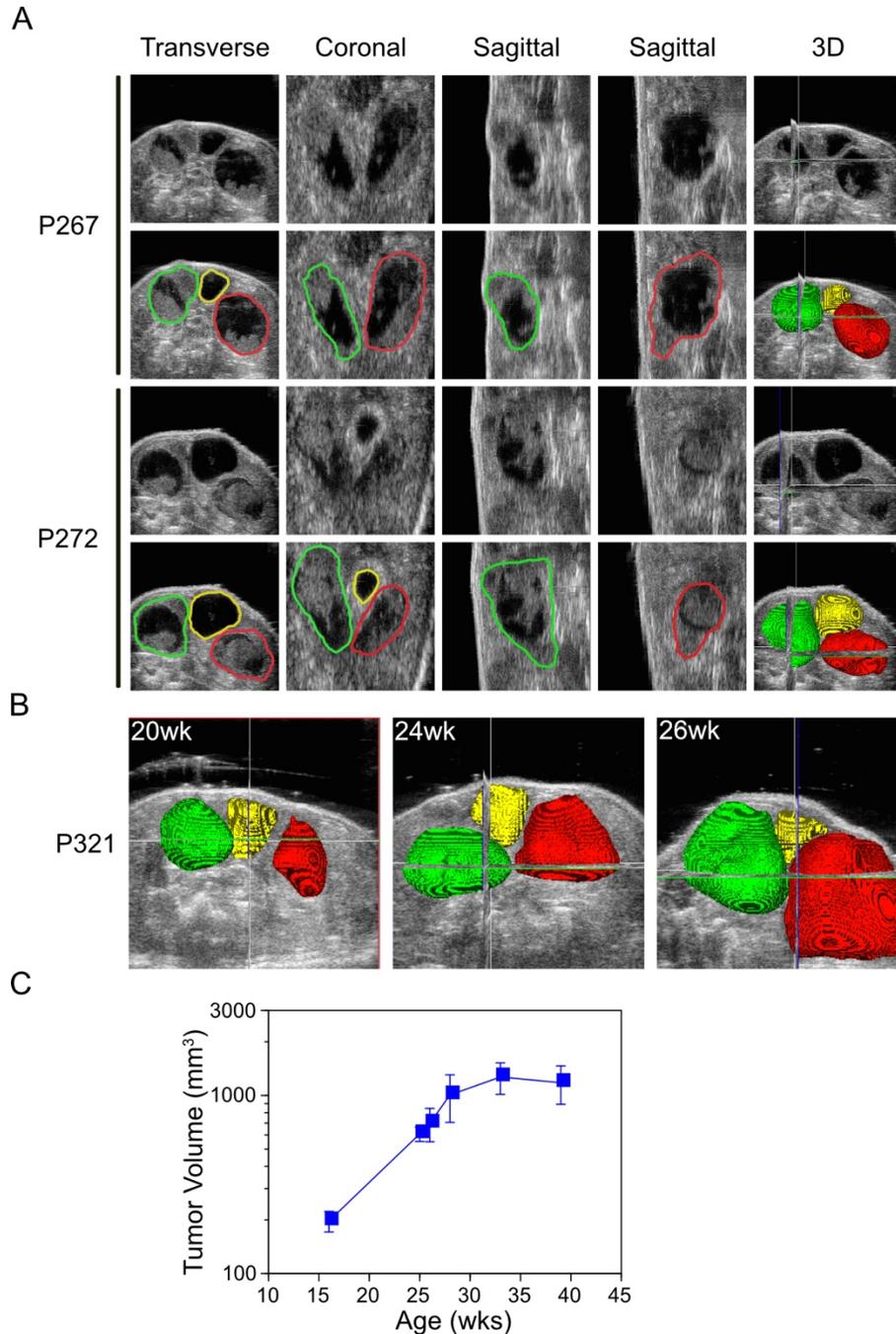
Supplemental Figure S1. High Frequency Ultrasound imaging (HFUS) with 3D reconstruction quantitated development of PTEN prostate KO tumor. (**A**) Representative HFUS image slices of the genitourinary region from 2 PB-Cre4:Pten^{fl/fl} mice. Orthogonal planes (transverse, coronal and sagittal at each tumor lobe). The 3D image is an integrated reconstruction using Amira visualization. Bladder (yellow), left (green) and right (red) prostate tumor lobes. (**B**) Panels show Amira 3D reconstructions of HFUS images from a single PB-Cre4:Pten^{fl/fl} mouse over time. Leftmost illustrates tumor when the mouse is 20 wk (tumor volume = 215 mm^3); Middle panel, at 24 wk (tumor volume = 387 mm^3); Right panel at 26 wk (tumor volume = 730 mm^3) Colors as in **A**. (**C**) Growth of tumor volume in mice prior to analysis in Figure 1 (n=13). The later slowing of the overall mean growth rate was due to removal from the analysis of mice bearing the fastest-growing tumors as they were entered into the experimental protocol.

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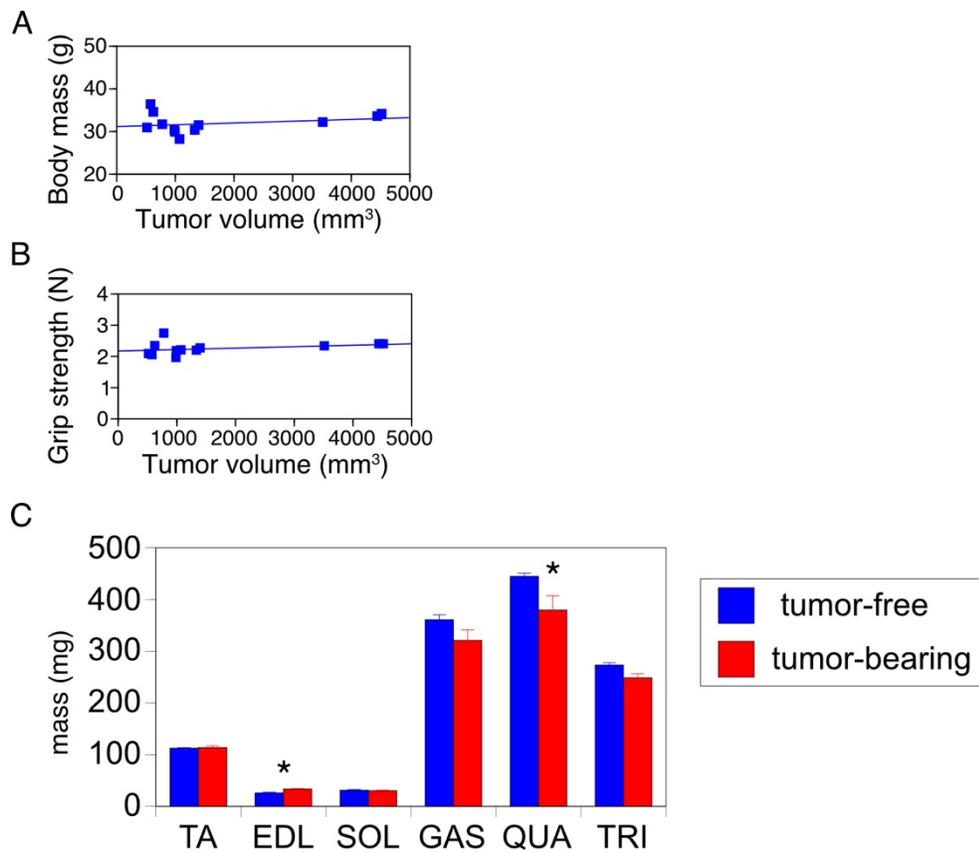
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96 **Supplemental Figure S2. Tumor burden did not influence total body mass, grip strength, or individual**
97 **skeletal muscle mass.** (**A**) Individual total body mass (BM), corrected for imputed tumor mass, was not
98 correlated with tumor volume (R^2=0.07, p=0.41). (**B**) Grip strength (GS) was not correlated with pre-castration
99 tumor volume (R^2=0.12, p=0.28). (**C**) Mass of TA, SOL, GAS, and TRI skeletal muscles from tumor-free mice
100 (blue) did not differ from muscles from tumor-bearing mice (red). EDL was increased in tumor bearing mice,
101 while QUA was decreased in tumor bearing mice (*p<0.05), determined using one-way ANOVA with Dunnett's
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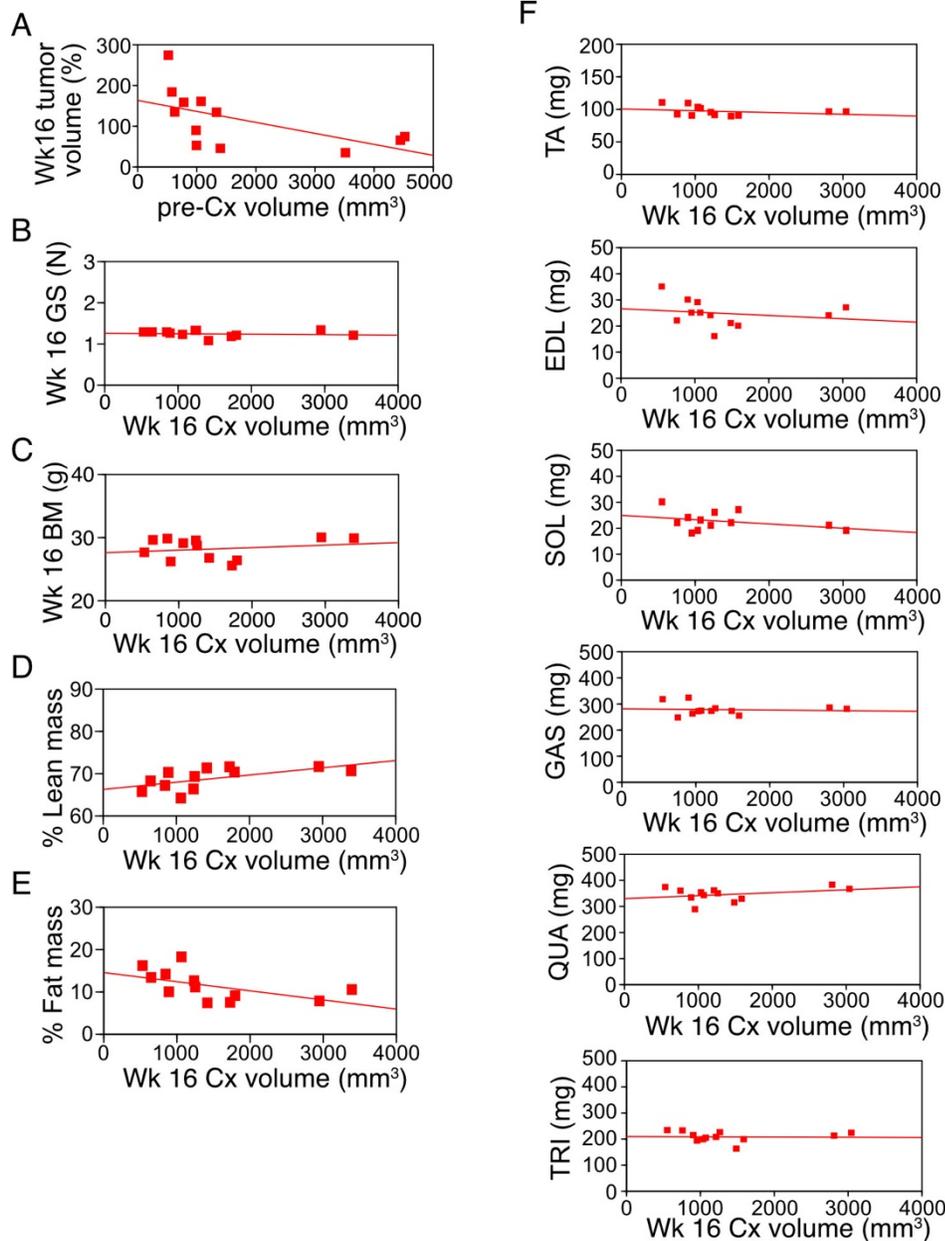
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108 **Supplemental Figure S3. Large tumors regressed more, but tumor burden did not alter grip strength,**
109 **body mass, body composition, or skeletal muscle mass 16 weeks after castration.** (**A**) Tumor regression
110 (tumor volume as a percentage of initial volume) after 16 weeks castration (Cx) was negatively correlated
111 (~ -25% / 1000 mm^3) with pre-Cx tumor volume (p=0.049). (**B**) Grip strength (GS), in Newtons (N), was not
112 correlated with tumor volume at any time up to 16 weeks after Cx. (**C**) Individual mean total body mass (BM),
113 corrected for imputed tumor mass, was not correlated (R^2=0.21, p=0.51) with tumor volume 16 weeks after Cx.
114 (**D**) Whole-body lean mass by qNMR (including tumor) increased 1.7% per 1000 mm^3 of increased tumor
115 volume (p= 0.040) 16 weeks after Cx. (**E**) Whole-body fat mass by qNMR was not correlated (p= 0.067) with
116 increased tumor volume 16 weeks after Cx. (**F**) Mass of individual skeletal muscles was not correlated with
117 increased tumor volumes 16 weeks after Cx. Line indicates linear regression, n=12 for each muscle. See
118 Supplemental Table S1 for intermediate time point (wk2-wk14) data, and Table S2 for end point data including
119 slopes, Pearson correlations, and p-values.
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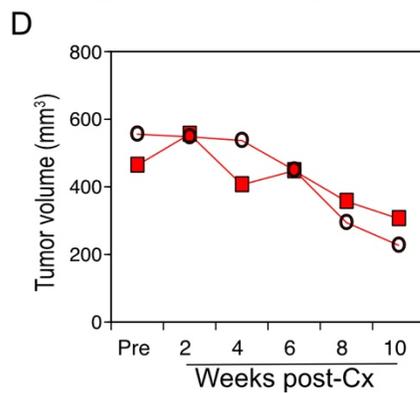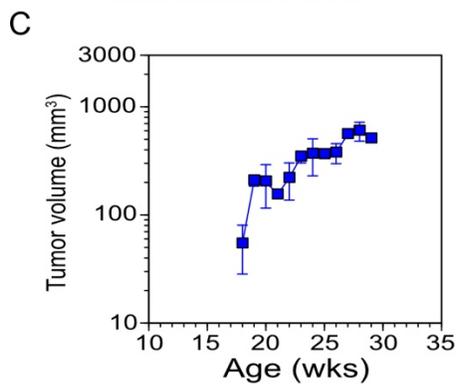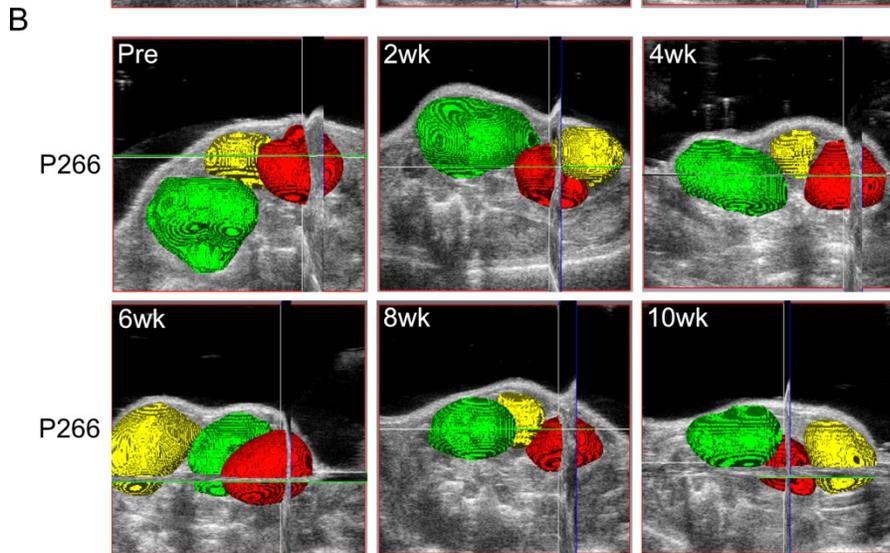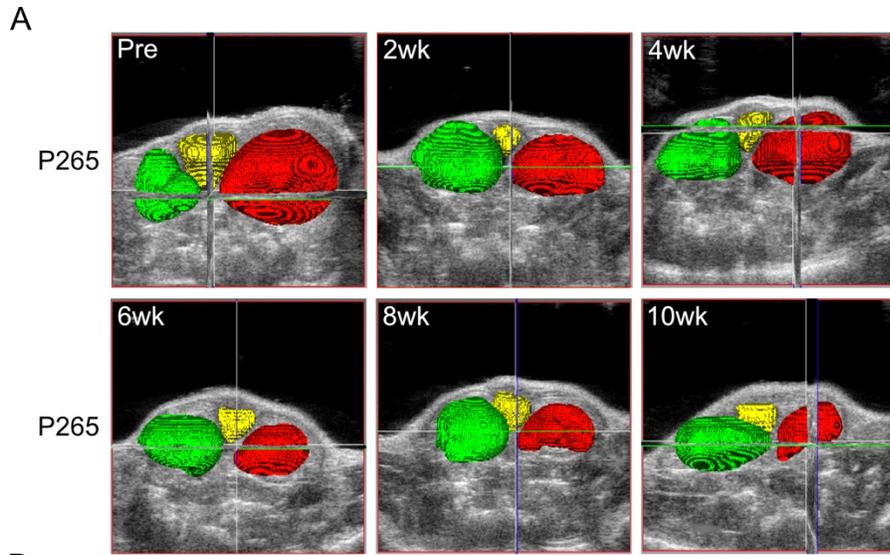
Supplemental Figure S4. High Frequency Ultrasound imaging with 3D reconstruction quantitated
regression of PTEN prostate KO tumor. (**A**) Panels show Amira 3D reconstructions of HFUS images of prostate tumor from a single PB-Cre4:Pten$^{fl/fl}$ mouse imaged prior to castration (pre) and every two weeks for 10 weeks after castration. Bladder (yellow), left (green) and right (red) prostate tumor lobes. (**B**) As in A. for a second tumor-bearing mouse. (**C**) Growth of tumor volume in mice prior to castration (n=12). (**D**) Quantitation of the tumor volumes of the mice depicted in each panel of **A**. (P265, open circles) and **B**. (P266, solid squares).

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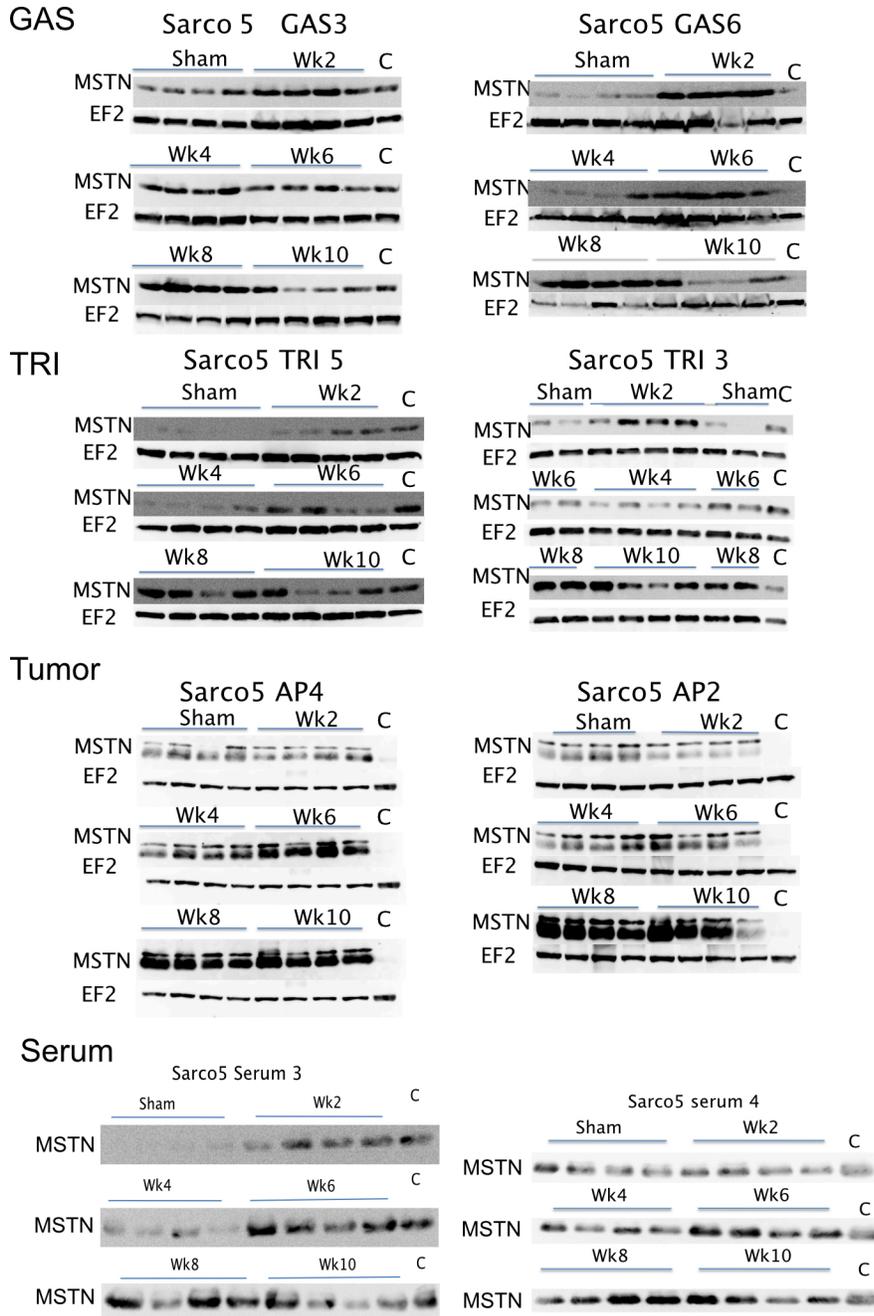
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132 **Supplemental Figure S5. Second and third replicates of immunoblots for myostatin quantitation in**
133 **Figures 3 and 5.** Images of the additional immunoblots of myostatin (MSTN), including eukaryotic elongation
134 factor 2 (EF2) expression in prostate tumor tissue from sets of 4 mice, castrated for the indicated times or sham-
135 castrated. Lanes of immunoblots marked "C" contain identical control sample for inter-blot comparison.
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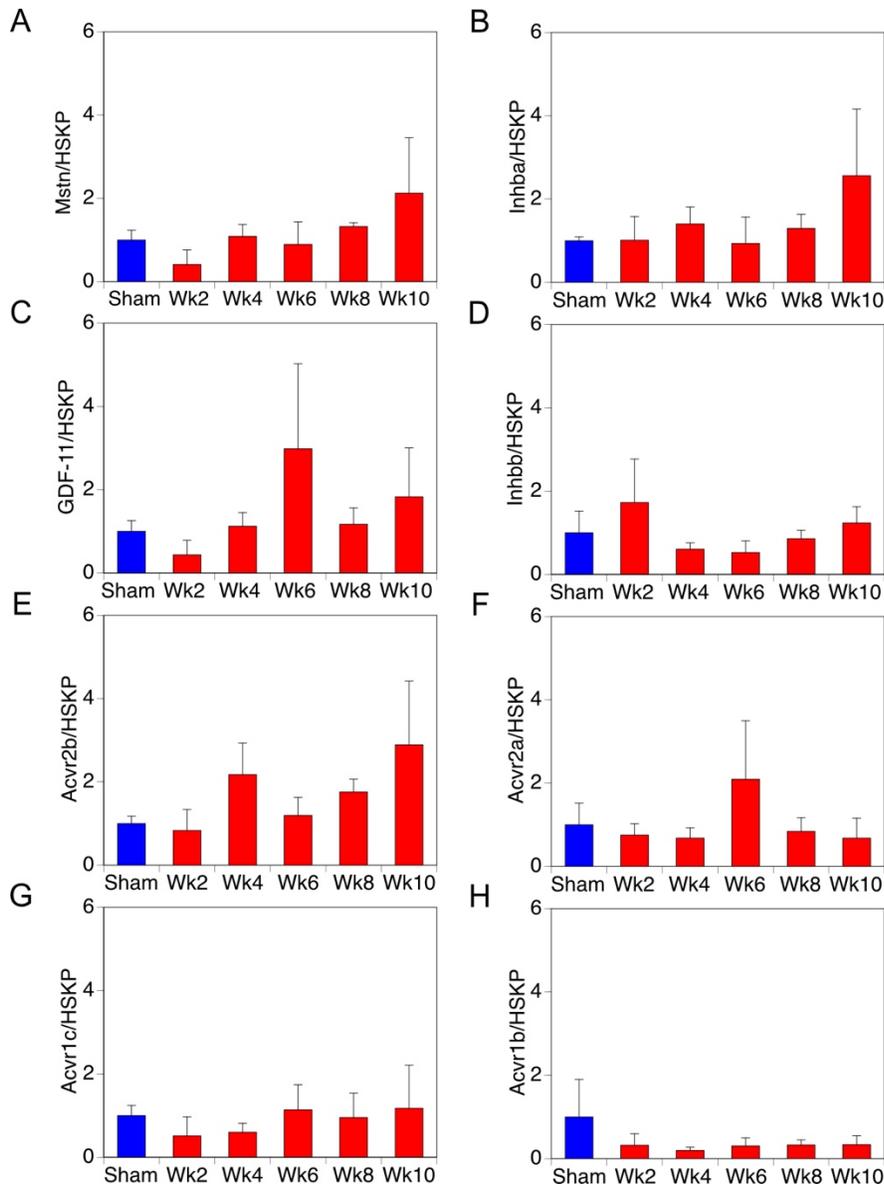
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143 **Supplemental Figure S6. Quantitation of TGFß-family myokine related mRNAs in muscle.** Expression of
144 the indicated ligand and receptor mRNAs (**A**-**H**) from QUA muscle tissue of PTEN prostate KO mice sacrificed
145 at the indicated number of weeks after castration. Expression of each gene was normalized to control (HSKP),
146 and subsequently to the sham castrate expression levels, from 4 mice at each time, measured 3 times.
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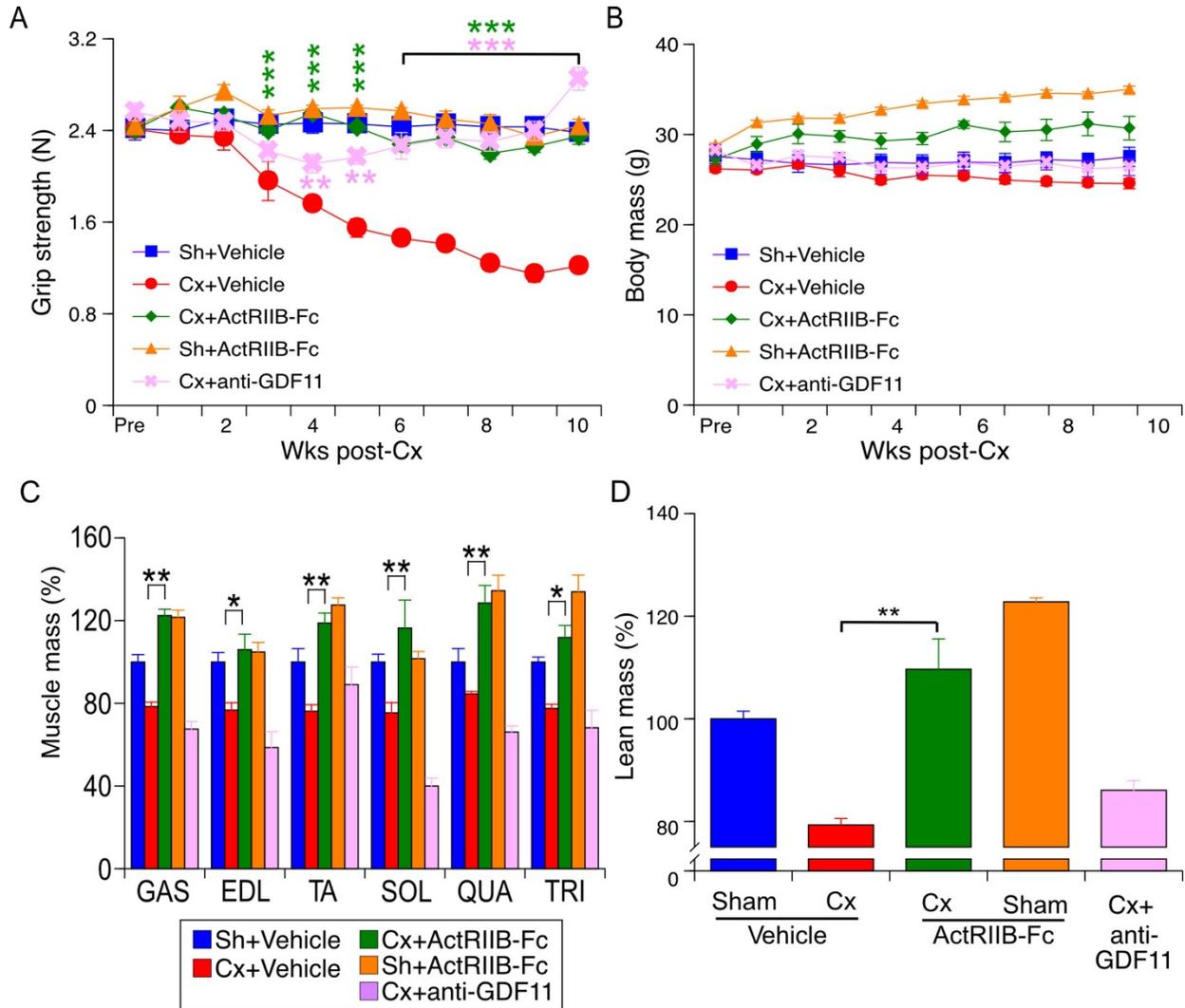
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151 **Supplemental Figure S7. Additional data for TGFß-family myokine ligand blockade treated cohorts of**
152 **Fig. 4, including Sham-castrated/ActRIIB-Fc treated cohort and Total body mass. (A)** Grip strength after
153 castration or sham-castration of mice treated with PBS, ActRIIB-Fc, or anti-GDF11 antibody. In addition to the
154 indicated comparisons (green and purple asterisks), mice castrated and vehicle treated were also different (p
155 < 0.001) from either sham castrated group from three weeks after castration. (**B**) Total body mass. (**C**)
156 Individual skeletal muscle mass after 10 weeks castration by dissection and weighing. (**D**) Lean body mass by
157 qNMR, as % of sham-castrated. Not indicated: Castration induced a significant reduction of each muscle mass
158 and lean body mass (p < 0.05, blue versus red columns). ActRIIB-Fc treated groups were not different from
159 each other. Mean (lines or columns), SEM (bars). n = 3-5/group, * p<0.05, **p<0.01, ***p<0.001 for ActRIIB-
160 Fc treated castrated (or anti-GDF11 treated castrated) versus vehicle treated castrated mice determined using
161 two-way ANOVA with Tukey's HSD test.
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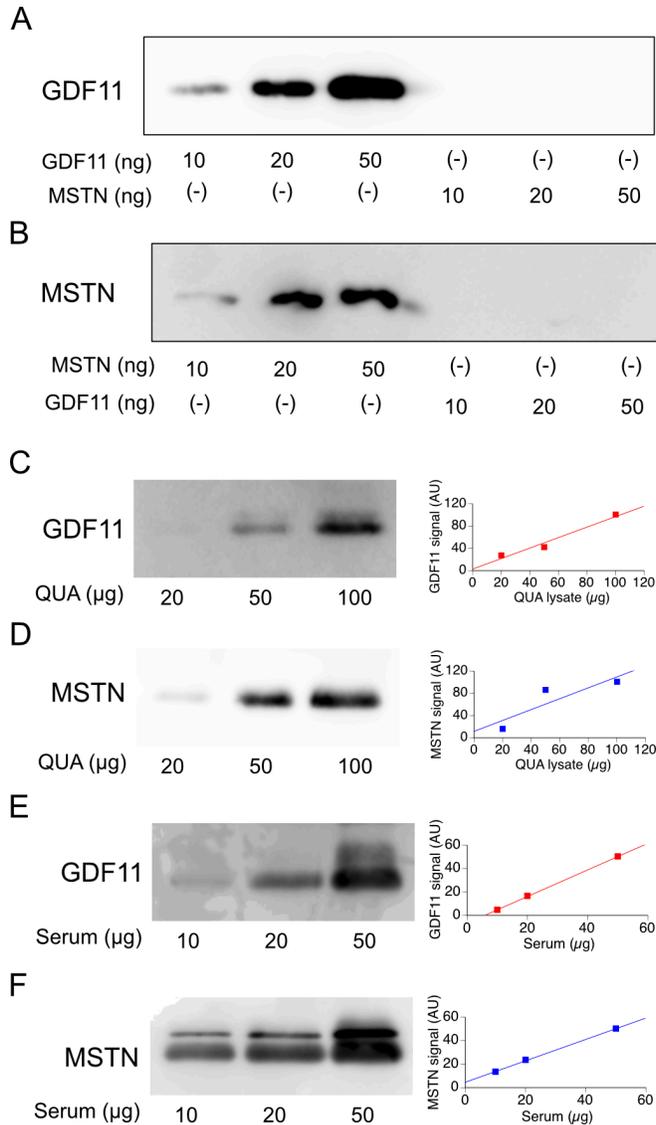
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166 **Supplemental Figure S8**. **Anti-GDF11 and anti-MSTN antibodies are sensitive and specific.** (**A**)
167 Immunoblot using anti-GDF11 (R&D #MAB19851) of an SDS-PAGE gel containing the indicated quantity of
168 purified GDF11 and MSTN. (**B**) Immunoblot using anti-MSTN antibody (R&D #788-G8) of an SDS-PAGE gel
169 containing the indicated quantity of purified MSTN and GDF11. (**C**) Immunoblot of GDF11 active dimer
170 expression in the indicated quantity of mouse quadriceps (QUA) muscle extract. Right, quantitation of GDF11
171 active dimer expression versus total protein, R^2 = 0.96. (**D**) Immunoblot of MSTN active dimer expression in
172 mouse quadriceps (QUA) muscle extract. Right, quantitation of MSTN active dimer expression versus total
173 protein, R^2 = 0.76. E, Immunoblot of GDF11 active dimer expression in the indicated quantity of serum. Right,
174 quantitation of GDF11 active dimer expression versus total protein, R^2 = 0.99. (**F**) Immunoblot of MSTN active
175 dimer expression in the indicated quantity of serum. Right, quantitation of MSTN active dimer expression versus
176 total protein, R^2 = 0.99.

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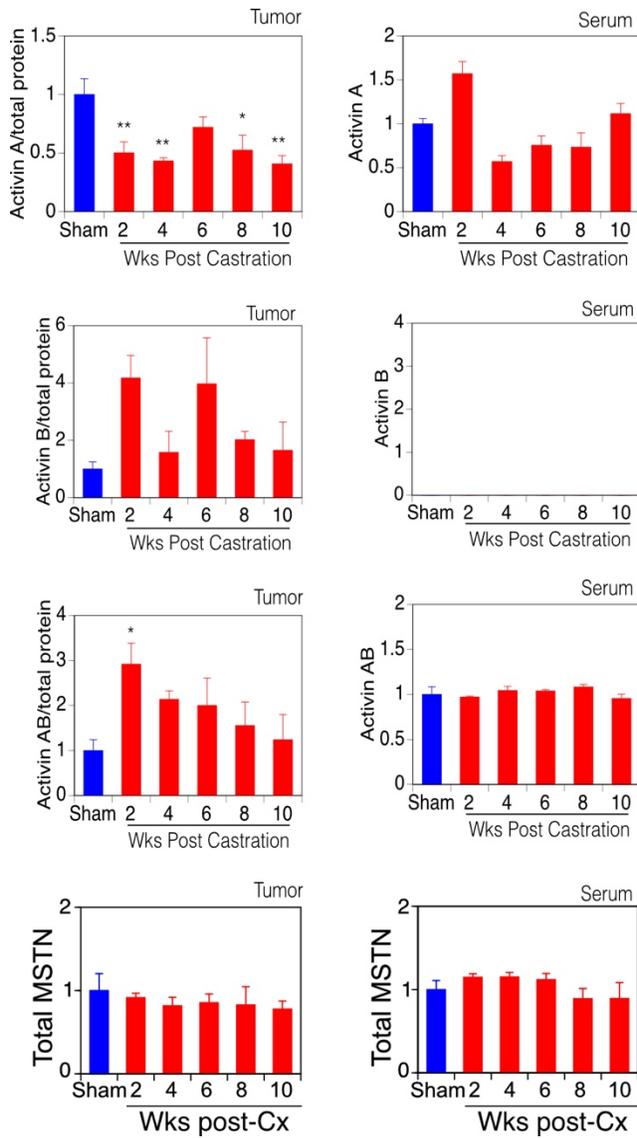
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182 **Supplemental Figure S9. Activins and total myostatin in tumor and serum after castration.** ELISA
183 determined levels of soluble, active activin A, Activin B, and Activin AB proteins, and total acid-solubilized
184 myostatin (MSTN) in tumor and serum at the indicated time after castration. Activin B was not detected in
185 serum. Mean protein levels after castration were normalized to sham-castrate group, from 4 mice at each time,
186 measured 3 times.
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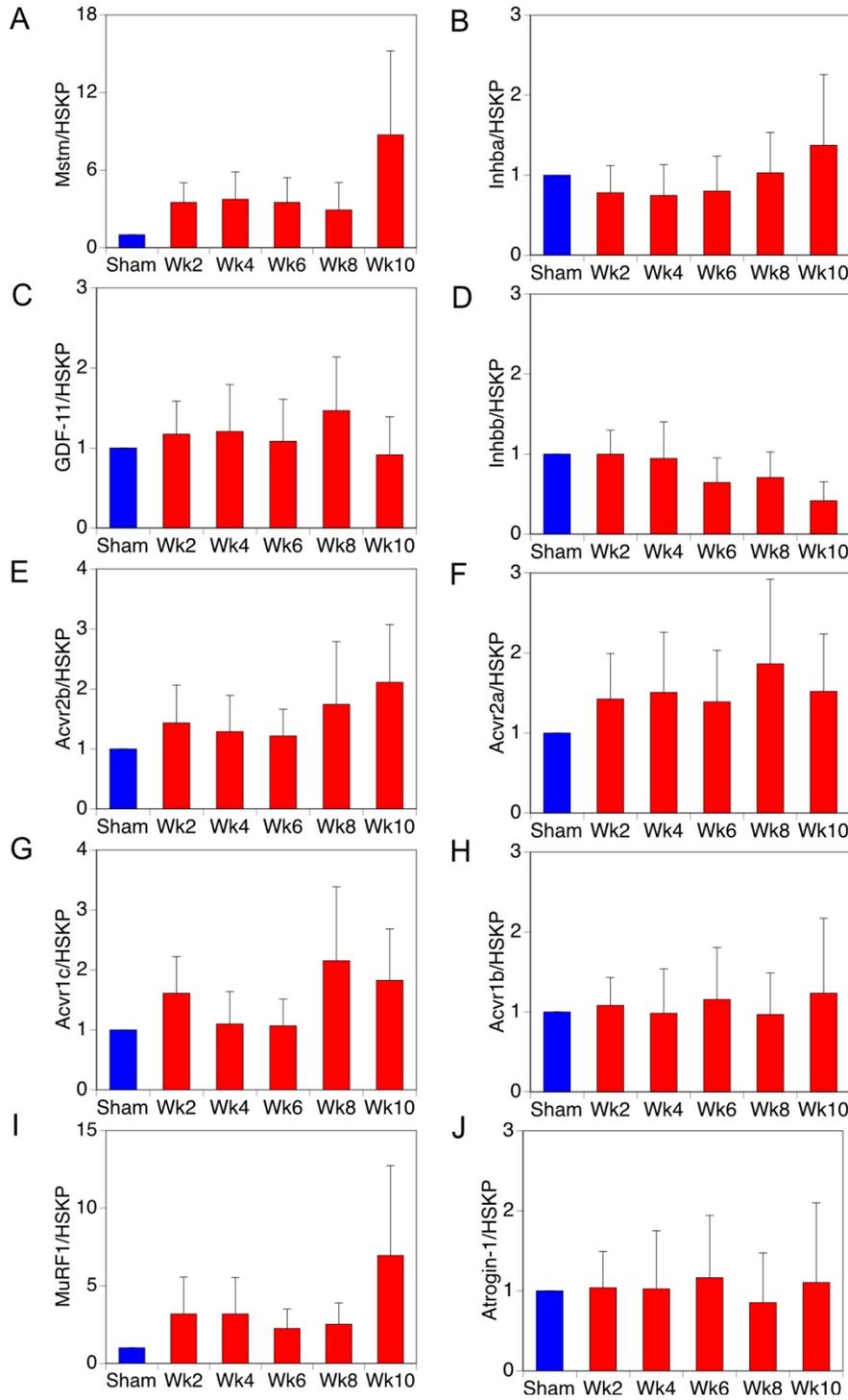
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196 **Supplemental Figure S10. Quantitation of catabolic TGFß-family myokine-related mRNAs in tumor.**
197 Expression of the indicated myokine associated mRNAs (**A-J**) from tumor tissue of PTEN prostate KO mice
198 sacrificed at the indicated number of weeks after castration. Expression of each gene was normalized to control
199 (HSKP) gene expression levels, and subsequently to the sham castrate expression levels, from 4 mice at each
200 time, measured 3 times.
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