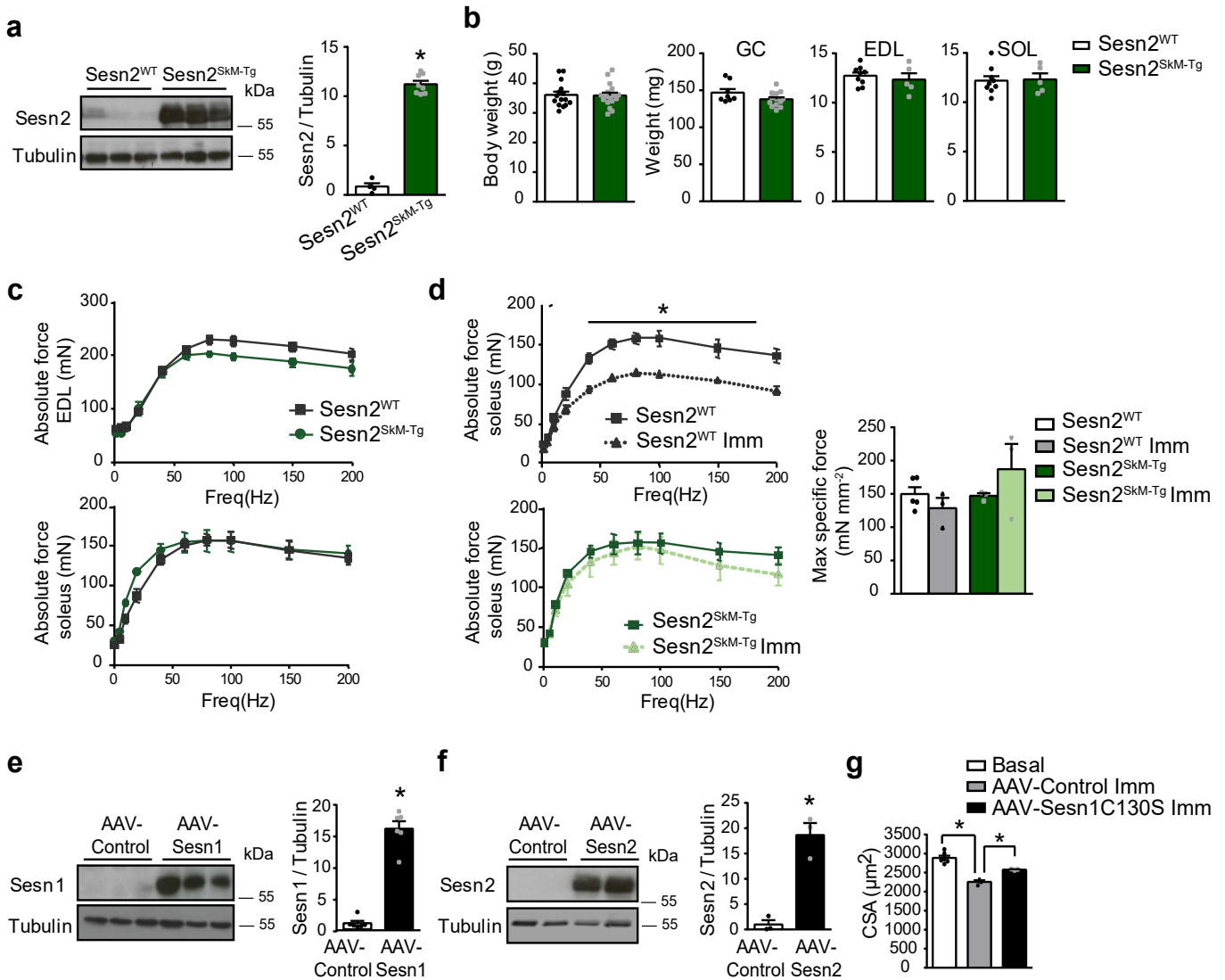


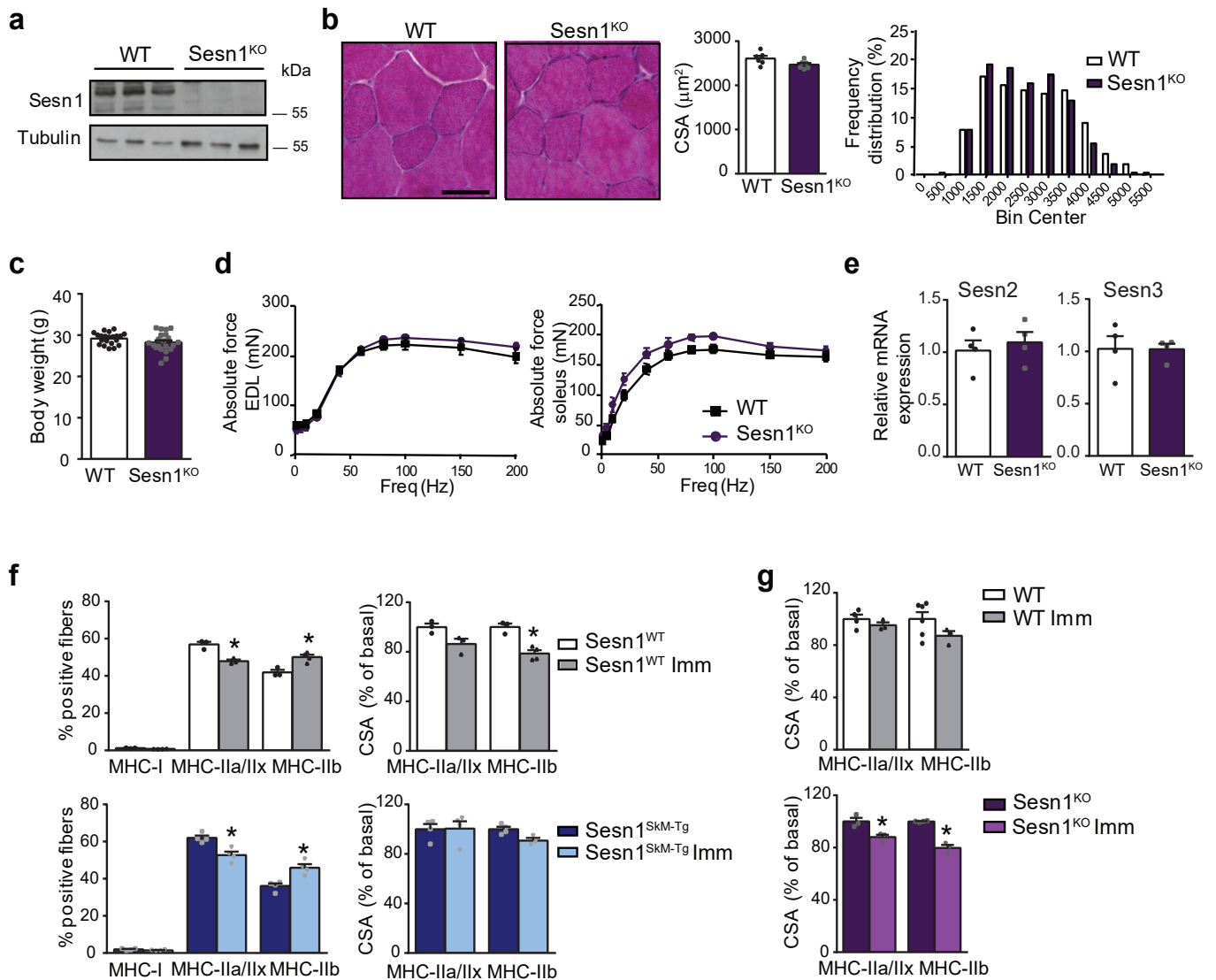
### Supplementary Figure 1. Functional characterization of muscles from Sesn1<sup>SKM-Tg</sup> mice

**a**, RNAseq-determined Sesn1-3 mRNA expression levels in skeletal muscle of young mice. **b**, qPCR (left) and Western blot analysis and its quantification (right) of human Sesn1 mRNA and protein expression respectively in skeletal muscle from Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> young mice (4 months old). **c**, qPCR analysis of Sesn2 and Sesn3 expression in TA muscles of Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> young mice. **d**, Body and muscle weight of young Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> mice. **e**, Force-frequency curves of EDL and soleus muscles from Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> mice in basal conditions. **f**, Quantification of Sirius Red staining of TA muscle sections from young Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> mice in basal state and 10 days post-immobilization. **g**, Force measurements in soleus muscles of Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> mice in basal conditions and after 10 days of limb immobilization. Charts show force-frequency curves (left) and maximum specific force (right). **h**, Myonuclei number in TA muscles from Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> mice in basal conditions and after 10 days of immobilization. **i**, Mean fiber CSA of TA muscles from Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> mice in basal conditions and at 10 days post-denervation. Results are expressed as percentage of CSA in basal conditions. **j**, Force measurements in EDL muscles of Sesn1<sup>WT</sup> and Sesn1<sup>SKM-Tg</sup> mice in basal conditions and after 10 days of denervation. All data are shown as mean with SEM. Comparisons by paired (i) or unpaired (others) Student t-test (\*p<0.05 and \*\*p<0.01). TA, tibialis anterior; SOL, soleus and GC, gastrocnemius. N=5-7 mice per genotype for **e**, n=3-4 mice per group for **f** and **h**, n=3-7 animals per group for **g** and n=4-6 mice for **i-j**. Source data are provided as a Source Data file.



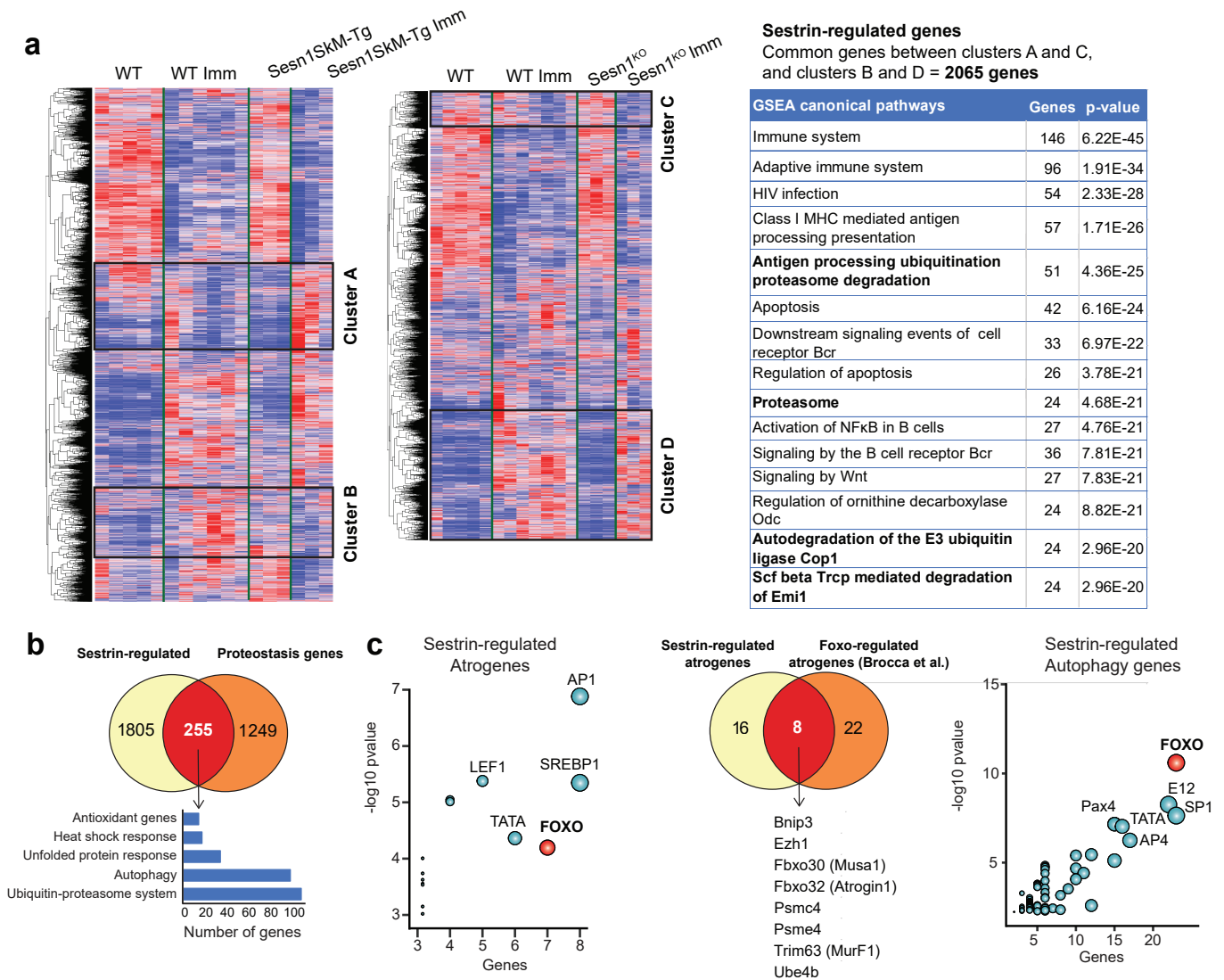
### Supplementary Figure 2. Functional characterization of muscles from Sesn2<sup>SkM-Tg</sup> mice

**a**, Western blot and quantification of Sesn2 protein levels in skeletal muscle from Sesn2<sup>WT</sup> and Sesn2<sup>SkM-Tg</sup> mice. **b**, Body and muscle weight of young Sesn2<sup>WT</sup> and Sesn2<sup>SkM-Tg</sup> mice (4 months old). **c**, Force-frequency curves of EDL and soleus muscles from Sesn2<sup>WT</sup> and Sesn2<sup>SkM-Tg</sup> mice in basal conditions. **d**, Force measurements in soleus muscles of Sesn2<sup>WT</sup> and Sesn2<sup>SkM-Tg</sup> mice in basal conditions and after 10 days of limb immobilization. Charts show force-frequency curves and maximum specific force. **e**, **f**, Western blot analysis and quantification of Sesn1 and Sesn2 expression in TA muscles transduced with AAV-Sesn1 (**e**) or AAV-Sesn2 (**f**). **g**, Mean myofiber CSA in TA muscles transduced with AAV-Sesn1C130S or AAV-Control and immobilized for 10 days. All data are shown as mean with SEM. Comparisons by unpaired Student t-test (\* $p < 0.05$ ). Sample numbers were  $n = 3-5$  mice per group for **c-d**,  $n = 3$  animals for **g**. Source data are provided as a Source Data file.



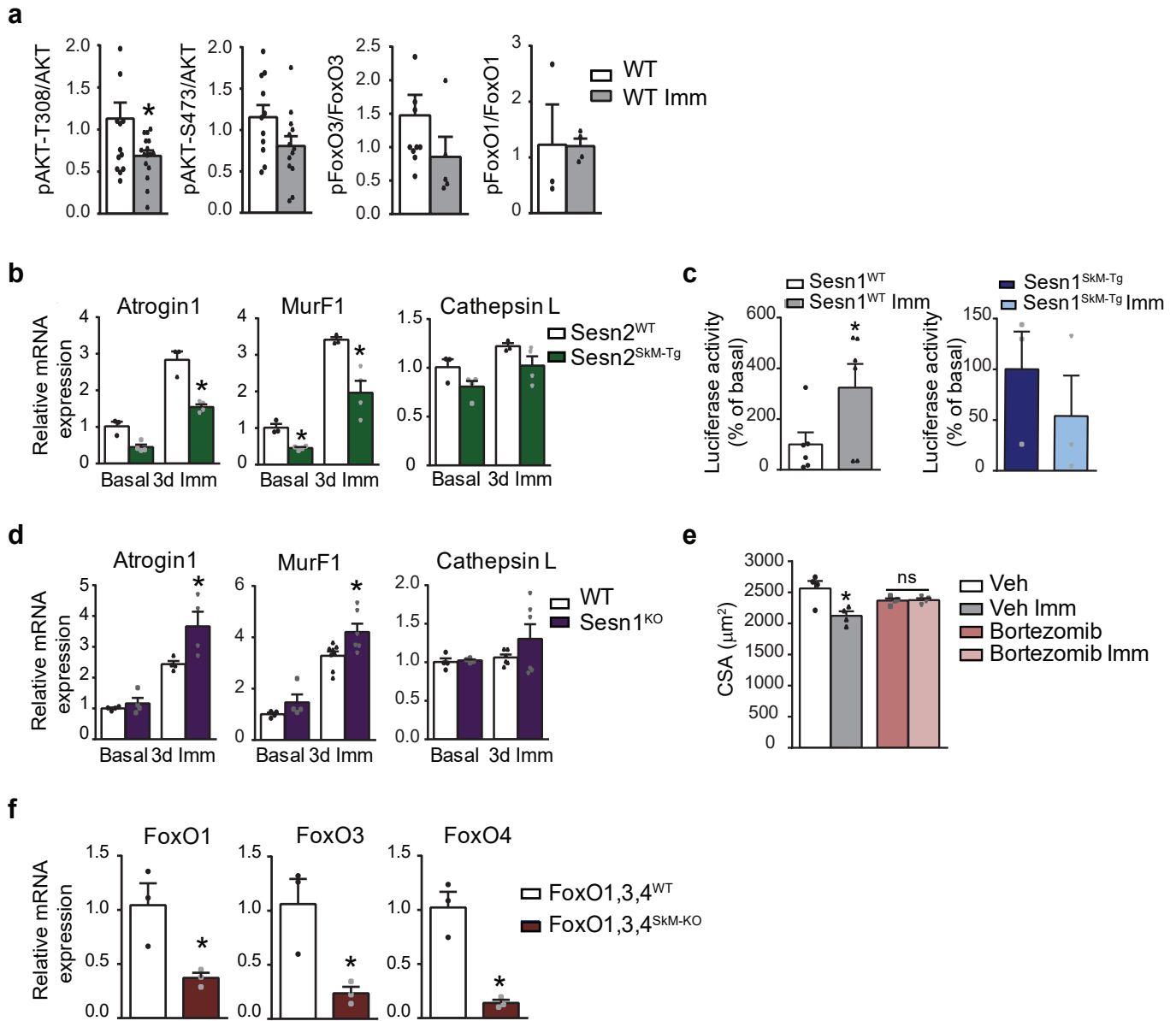
### Supplementary Figure 3. Functional characterization of muscles from Sesn1<sup>KO</sup> mice

**a**, Representative Western blot showing Sesn1 protein levels in WT and Sesn1<sup>KO</sup> mice. **b**, Representative H/E staining pictures of TA muscle sections from WT and Sesn1<sup>KO</sup> mice (left), quantification of mean myofiber CSA (center) and myofiber-size frequency distribution (right panel). Scale bar=50 μm. **c**, Body weight of young WT and Sesn1<sup>KO</sup> mice. **d**, Force-frequency curves of EDL and soleus muscles from WT and Sesn1<sup>KO</sup> mice in basal conditions. **e**, qPCR analysis of Sesn2 and Sesn3 mRNA expression in skeletal muscle from WT and Sesn1<sup>KO</sup> mice. **f**, Percentage of type I, IIA/IIX, and IIB fibers, based on myosin heavy chain (MHC) expression, in TA muscle sections from Sesn1<sup>WT</sup> (top) and Sesn1<sup>SkM-Tg</sup> mice (bottom) in basal conditions and after 10 days of immobilization (left). CSA of type IIA/IIX and IIB fibers in basal conditions and after 10 days of immobilization (right). **g**, Mean CSA of type IIA/IIX and IIB fibers in TA muscles from WT and Sesn1<sup>KO</sup> mice in basal conditions and after 10 days of immobilization. All data are shown as mean with SEM. Comparisons by unpaired Student t-test (\*p<0.05). Sample numbers were n=5-7 mice for **b-d** and n=3-5 mice per condition for **f-g**. Source data are provided as a Source Data file.



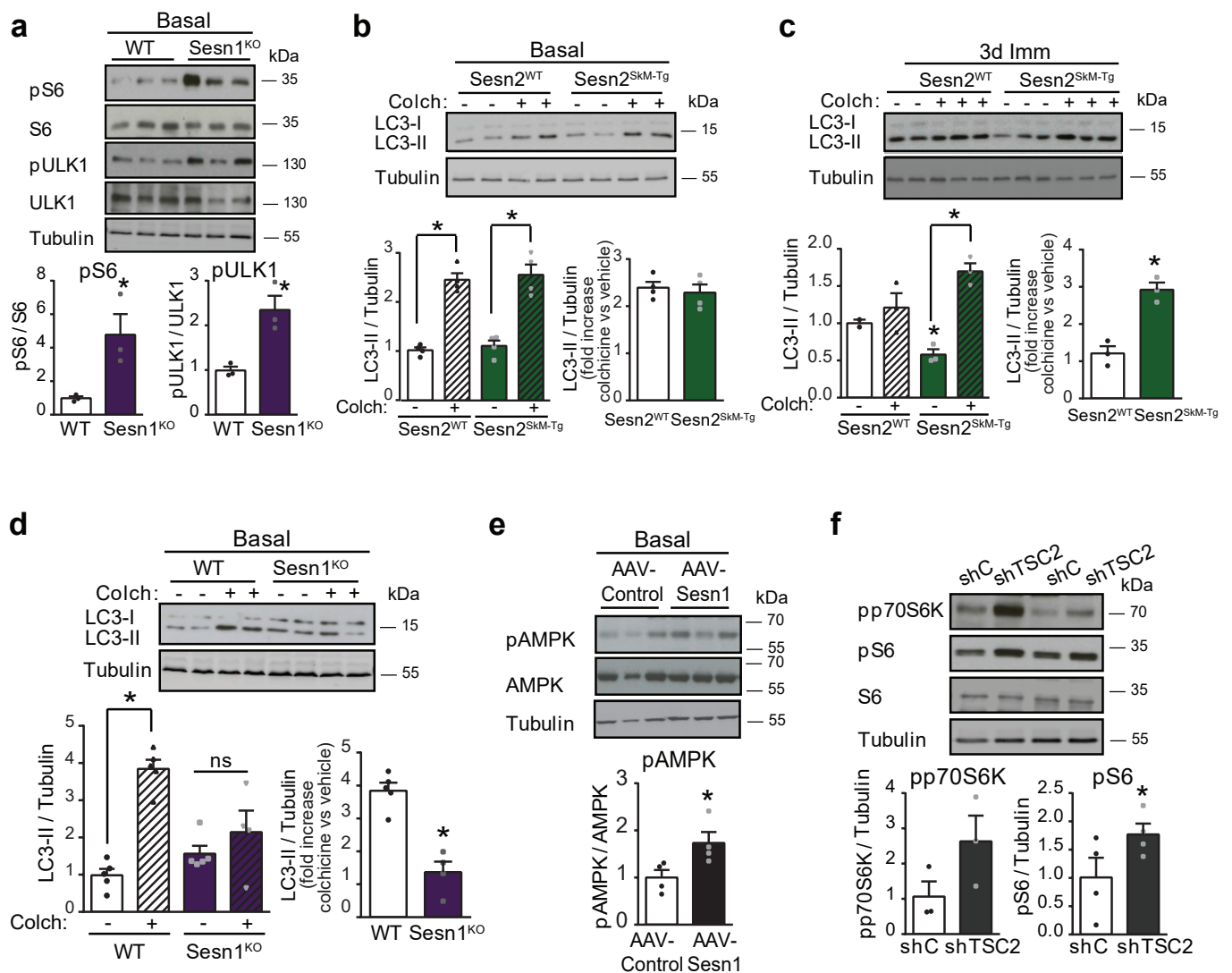
**Supplementary Figure 4. Sestrin regulates the expression of critical atrogenes in disused muscles**

**a**, Unsupervised hierarchical clustering heat map of normalized gene expression in 3-day immobilized TA muscles compared with basal conditions in the indicated mouse genotypes. Each column corresponds to an individual sample. Selected clusters are shown. Cluster A/B shows genes downregulated/upregulated in immobilized WT muscles compared to basal, which behave differently in Sesn-expressing muscles, and Cluster C/D to genes showing decreased/increased expression in Sesn1-deficient mice. Addition of the common genes between clusters A and C, and between cluster B and D defined the “sestrin-regulated gene set”. The table shows GSEA-identified canonical pathways enriched in the sestrin-regulated gene set. **b**, Venn diagram showing genes common to the sestrin-regulated gene set (defined in a) and a full set of genes implicated in proteostasis pathways<sup>32</sup>. The bar chart shows sestrin-regulated proteostasis genes subdivided into individual pathways. **c**, Bubble plot of enriched transcription factor binding sites (GSEA) among autophagy genes (right) and atrogenes (left) belonging to the sestrin-regulated gene set defined in a. Comparison of FoxO-regulated atrogenes from Brocca et al.<sup>24</sup> and the list of sestrin regulated atrogenes (middle).



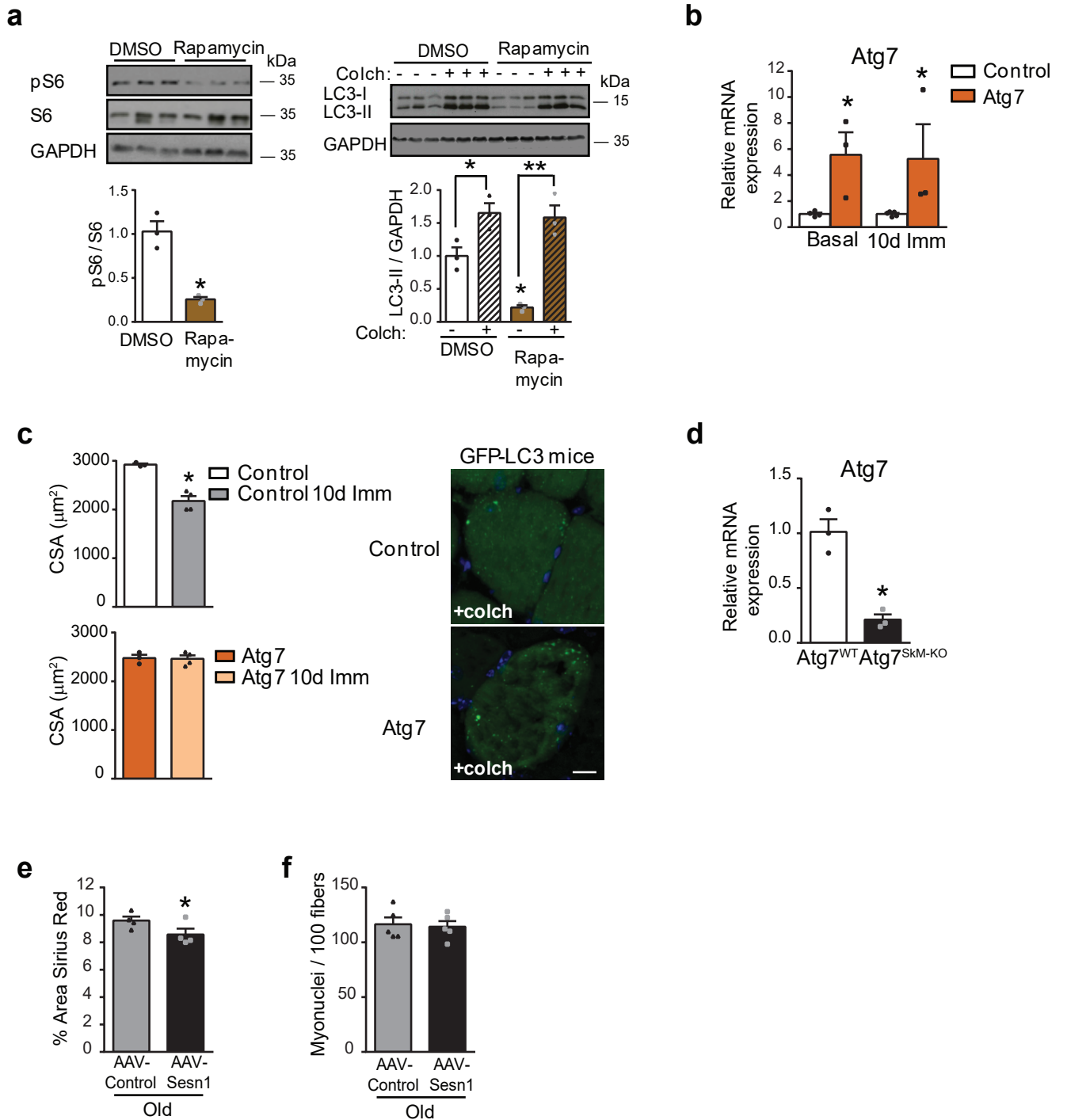
**Supplementary Figure 5. Sestrin regulates the expression of proteasome-related genes in atrophying muscles**

**a**, Quantification of the Western blot analyses of FoxO1, FoxO3 and AKT phosphorylation levels in muscles of WT mice in basal conditions and after 3 days of immobilization. **b**, Atrogin1, MurF1, and cathepsin L mRNA levels in muscles from *Sesn2*<sup>WT</sup> and *Sesn2*<sup>SKM-Tg</sup> mice in basal conditions and 3 days post-immobilization. **c**, Luciferase activity in TA muscle homogenates from *Sesn1*<sup>WT</sup> and *Sesn1*<sup>SKM-Tg</sup> mice electrotransferred with the FHRE-luciferase reporter plasmid and immobilized for 3 days. **d**, Atrogin1, MurF1, and cathepsin L mRNA levels in muscles from WT and *Sesn1*<sup>KO</sup> mice in basal conditions and 3 days post-immobilization. **e**, Mean fiber CSA of TA muscles from WT mice treated with vehicle or the proteasome inhibitor bortezomib in basal conditions and at 10 days post-immobilization. **f**, mRNA expression levels of FoxO1,3,4 in skeletal muscle from *FoxO1,3,4*<sup>WT</sup> and *FoxO1,3,4*<sup>SKM-KO</sup> mice. All data are shown as mean with SEM. Comparisons by paired (**c**) or unpaired (others) Student t-test (\**p*<0.05). Sample numbers were *n*=3-7 mice per group for **b** and **d**, *n*=3-6 mice for **c**, *n*=4 mice per condition for **e** and *n*=3 mice for **i**.



### Supplementary Figure 6. Sestrins inhibit mTORC1 and maintain autophagy in disused muscles

**a**, Western blot analysis of S6 and ULK1 phosphorylation in TA muscles from WT and Sesn1<sup>KO</sup> mice in basal conditions. **b** and **c**, Western blot analysis and quantification of LC3I and LC3II content in TA homogenates from Sesn2<sup>WT</sup> and Sesn2<sup>SKM-Tg</sup> mice in basal conditions (**b**) and after 3 days of immobilization (**c**). The right chart shows the fold increase in LC3II content in colchicine (Colch) treated versus vehicle-treated mice (autophagic flux). **d**, Western blot analysis of LC3I and LC3II in homogenates of WT and Sesn1<sup>KO</sup> TA muscles in basal conditions. **e**, Western blot analysis of AMPK phosphorylation in muscles transduced with AAV-Sesn1 or AAV-Control in basal conditions. **f**, Western blot analysis of mTORC1 signaling activation in TA muscles electrotransferred with control short hairpin (sh) and sh against TSC2 (shTSC2); blots were probed for phosphorylated p70S6K and S6 and total S6. All data are shown as mean with SEM. Comparisons by paired unpaired Student t-test (\* $p < 0.05$ ). For **b-d** values were normalized to control vehicle conditions. Sample numbers were  $n=3-4$  animals per condition for **a-c** and **f**,  $n=4$  mice for **e** and  $n=5$  mice for **d**.



### Supplementary Figure 7. Autophagy induction prevents disuse-induced muscle atrophy

**a**, Western blot analysis of mTORC1 inhibition and autophagy induction in WT mice treated with rapamycin or vehicle (DMSO); blots were probed for phosphorylated and total S6 and LC3. **b**, qPCR analysis of *Atg7* overexpression in WT TA muscles electrotransferred with a plasmid encoding *Atg7* and then immobilized for 10 days. **c**, Mean myofiber CSA of fibers from TA muscles of WT mice treated as in **b**. Right panels show representative confocal images of TA muscle from LC3-GFP mice electrotransferred with a plasmid encoding *Atg7* and treated with colchicine to assess autophagosome accumulation. Scale bar=10  $\mu\text{m}$ . **d**, mRNA expression levels of *Atg7* in skeletal muscle from *Atg7*<sup>WT</sup> and *Atg7*<sup>SKM-KO</sup> mice. **e**, Quantification of Sirius Red staining of TA muscle sections from 24 month-old WT mice transduced with AAV-Sesn1 for 28 days. **f**, Number of myonuclei in TA muscle of old mice transduced with AAV-Sesn1 for 28 days. All data are shown as mean with SEM. Comparisons by paired (**e**) or unpaired (others) Student t-test (\* $p < 0.05$ ). Sample numbers were  $n=3-4$  animals per condition for **a-d**,  $n=4$  mice for **e** and  $n=5$  mice for **f**.