Supplementary Files

Suppl. Tables

Suppl. Table 1. Glycogen levels in muscles from GAA -/- and autophagy-deficient GAA-/- strains

Genotype	Tissue	µg glucose/ hr/ mg protein	% glycogen/ hr/ wet weight	% Reduction
GAA-/-	gastroc/quads (n=39)	73.9 ± 23.7	5.2 ± 1.9	
	soleus (n=8)	149.7 ± 26.7		
MLCcre:Atg7F/F:GAA-/-	gastroc/quads (n=18)	29.9 ± 16.6 $p < 0.01$	2.3 ± 1.3 $p < 0.01$	57-60
	soleus (n=8)	188.4 ± 62.5		
HSAcre:Atg5F/F:GAA-/-	gastroc/quads (n=43)	46.5 ± 17.0 $p < 0.01$ *$p < 0.01$	3.7 ± 1.8 $p < 0.01$ *$p < 0.01$	29-37

* Comparison between the two muscle-specific autophagy-deficient strains.

Each value is the mean ± sd. from the indicated number of animals.

Glycogen levels for gastrocnemius/quadriceps are presented as percent glycogen/wet weight in addition to µg glucose/mg protein to compare these results with our previous data [4.0 ± 2.6% and 5.0 ± 2.8% for HSAcre:Atg5F/F:GAA-/- and GAA-/- respectively; (4)] which showed a slight but not statistically significant decrease in HSAcre:Atg5F/F:GAA-/- compared to GAA-/-; the difference reached statistical significance with the increased number of samples. The animals' ages ranged from 4 to 7 months. Since the glycogen levels in gastrocnemius/quadriceps muscles from 4-5 month-old mice were not significantly different from those in 6-7 month-old mice, the combined data are presented.

Suppl. Table 2. Functional measurements of EDL muscles from WT, GAA-/-, and muscle-specific autophagy-deficient mice

Genotype	Muscle CSA (mm^2)	Maximal Force (mN)	Specific Force (kN/m^2)
WT (n=5)	1.68 ± 0.17	373 ± 53	222 ± 18
HSAcre:Atg5F/F:WT (n=4)	1.37 ± 0.34 P=0.2	303 ± 90	220 ± 36
GAA-/- (n=5)	1.13 ± 0.20 P<0.01	221 ± 19	199 ± 27
HSAcre:Atg5F/F:GAA-/- (n=5)	1.0 ± 0.09 P<0.01	202 ± 24	201 ± 17.5
GAA-/- Treated (n=5)	1.2 ± 0.1 P<0.01	246 ± 37	202 ± 32
HSAcre:Atg5F/F:GAA-/- Treated (n=6)	1.1 ± 0.17 P<0.01	212 ± 31.6	196 ± 28.7

Each value is the mean ± sd. from the indicated number of animals.

P values were analyzed by comparing the indicated strains to the WT.

Suppl. Table 3. Isometric forces generated by single psoas fibers from WT, GAA-/-, and muscle-specific autophagy-deficient mice

Genotype	Fiber Diameter (μm)	Force (mg)	Force/Area (N/cm^2)
* WT	66 ± 3	40.8 ± 2.4	12.0 ± 0.8
GAA-/-	43 ± 2 P<0.001	8.9 ± 0.5 P<0.001	7.0 ± 0.4 P<0.001
MLCcre:Atg7F/F:WT	52 ± 2 P<0.01	19.4 ± 1.1 P<0.001	9.2 ± 0.6 P<0.05

Isometric forces generated by fully Ca^{2+} activated single psoas muscle fibers. Each value is the mean ± s.e.m from 9 single fibers of five WT mice, 16 single fibers of five GAA-/- mice, and 13 single fibers of four MLCcre:Atg7F/F:WTmice. Sarcomere length = 2.4 – 2.5 μm, temperature = 21 ^{o}C.

P values were analyzed for MLCcre:Atg7F/F:WT and GAA-/- compared to the WT.

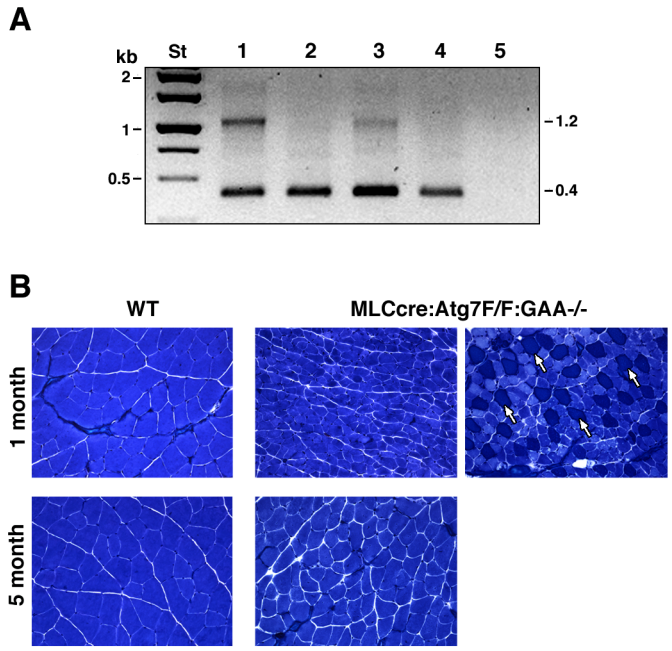
Suppl. Table 4. Muscle strength of WT and MLCcre:Atg7F/F:WT mice

Genotype	Grip Strength (KGF/kg)	
	Forelimb	**Hindlimb**
WT (n=3)	3.74 ± 0.21	6.75 ± 0.59
MLCcre:Atg7F/F:WT (n=4)	2.61 ± 0.38 P=0.004	5.37 ± 0.38 P=0.035

Each value is the mean ± sd. from the indicated number of animals. Five consecutive fore- and hindlimb strength measurements were recorded daily for each mouse over a 5-day-period. The maximum values for each day were used for the analysis. The data were normalized to body weight and expressed as KGF/kg. Six month-old male mice were used for the experiments.

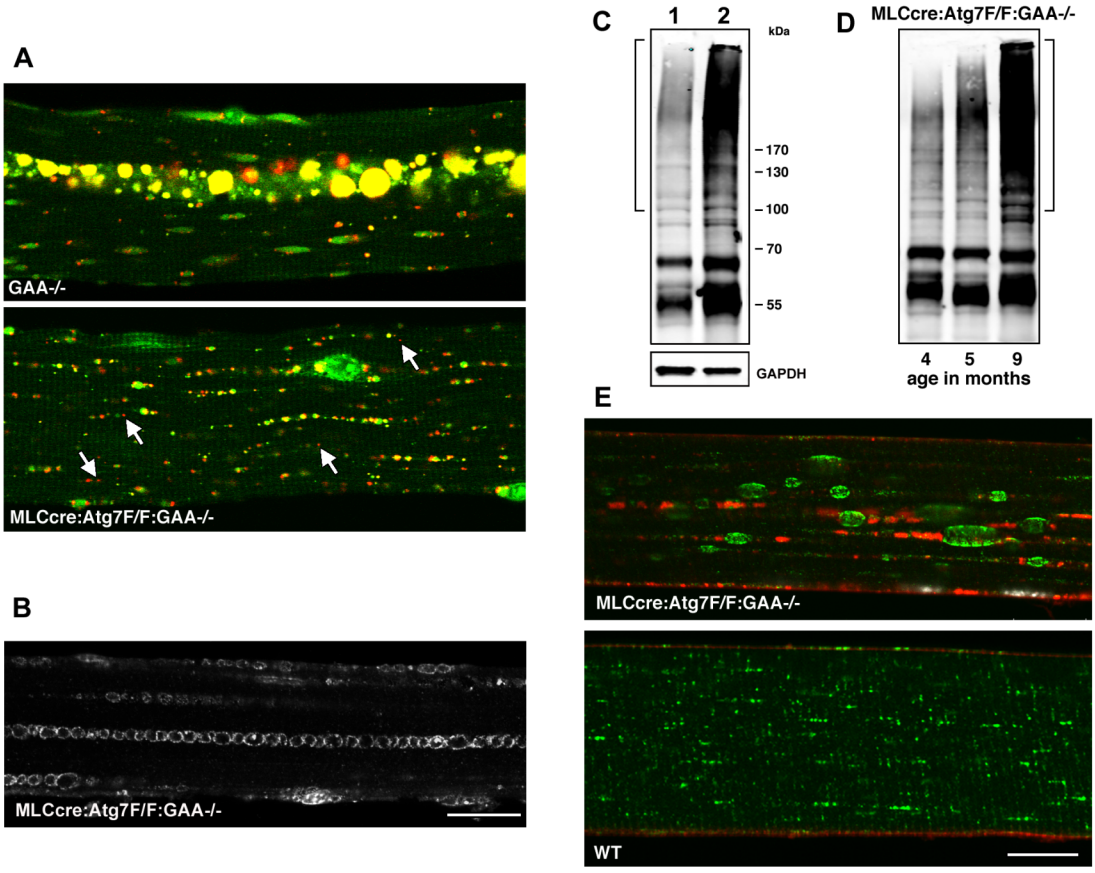
Suppl. Figure and Figure Legends

Suppl. Fig. 1.



Analysis of the suppression of autophagy in young and old MLCcre:Atg7F/F:GAA-/-mice (**A**) PCR analysis of genomic DNA from gastrocnemius and brain of 1 month-old (lanes 1-2) and 7 month-old (lanes 3-4) MLCcre:Atg7F/F:GAA-/-mice. A combination of three primers detects both excised (~1.2 kb) and non-excised/floxed (~0.4 kb) *Atg*7 alleles. Both alleles are detected in muscle from young (lane 1) and old (lane 3) mice, but only the non-excised/floxed allele is detected in brain (lanes 2 and 4). Lane 5: no DNA. The higher level of the Atg7 protein in young MLCcre:Atg7F/F:GAA-/- animals (shown in Fig. 1A) cannot be accounted for by less efficient Cre-mediated recombination since the relative amount of excised /non-excised *Atg*7 DNA is no less in young than in old mice. (**B**) Since the MLC1f promoter is activated in predominantly fast fibers, we analyzed fiber types in young and old WT and MLCcre:Atg7F/F:GAA-/- mice by metachromatic staining of gastrocnemius muscle. All fibers in old mice are type-II. A number of type-I fibers [top right panel, dark-rimmed cells (arrows)] is seen in some sections from young MLCcre:Atg7F/F:GAA-/-, but not WT animals. This shift toward type-I fibers may be partially responsible for the higher level of Atg7 in young MLCcre:Atg7F/F:GAA-/- mice.

Suppl. Fig. 2.



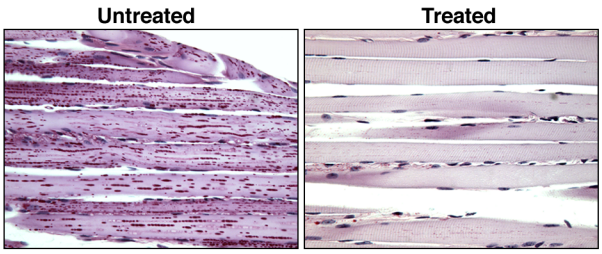
Characterization of MLCcre:Atg7F/F:GAA-/- mice

(A) Acidification of lysosomes in muscle from GAA-/- and MLCcre:Atg7F/F:GAA-/- mice. Acridine orange staining of live single fibers from 4 month-old GAA-/- and MLCcre:Atg7F/F:GAA-/- mice. The fibers were isolated from gastrocnemius (fast) muscle. Acridine orange enters and becomes protonated and sequestered inside lysosomes. In the acidic pH of the lysosomes, the dye emits orange light. Arrows point to acidic normal sized lysosomes, many of which can be seen in the MLCcre:Atg7F/F:GAA-/-.

(B) Distribution of lysosomes in fast fibers from MLCcre:Atg7F/F:GAA-/- mice. Single fiber from psoas (fast) muscle of a 4 month-old MLCcre:Atg7F/F:GAA-/-mouse. LAMP-1 staining shows clustering of late endosomes/lysosomes in the core of the fiber. This peculiar disposition of the lysosomes is never seen in autophagy-competent Pompe fast muscle fibers. Similar clustering of lysosomes in the core of fast fibers

was observed in another muscle-specific autophagy-deficient Pompe model: HSAcre:Atg5F/F:GAA-/-. Bar: 20 microns. **(C-E**) Accumulation of Ub-proteins in fast muscle of MLCcre:Atg7F/F:GAA-/- mice. (**C and D**) Western blotting of protein lysates from gastrocnemius (fast) muscles derived from WT and MLCcre:Atg7F/F:GAA-/- mice with anti-ubiquitin (FK2) antibody. (**C**) Muscle samples were taken from 9 month-old WT (lane 1) and MLCcre:Atg7F/F:GAA-/- (lane 2) mice. An age-dependent accumulation of Ub-proteins in MLCcre:Atg7F/F:GAA-/- mice is shown in **(D)**. High molecular mass Ub-proteins are marked by brackets. The data are representative of at least 3 independent experiments. (**E**) Distribution of Ub-proteins in fast (psoas) muscle of MLCcre:Atg7F/F:GAA-/- mice. Single fibers stained for ubiquitin (Ub) (red) and LAMP-1 (green) show the presence of Ub-positive structures which are dispersed throughout the fiber and located next to the lysosomes in the MLCcre:Atg7F/F:GAA-/-. These structures are not seen in the WT. Bar: 20 microns.

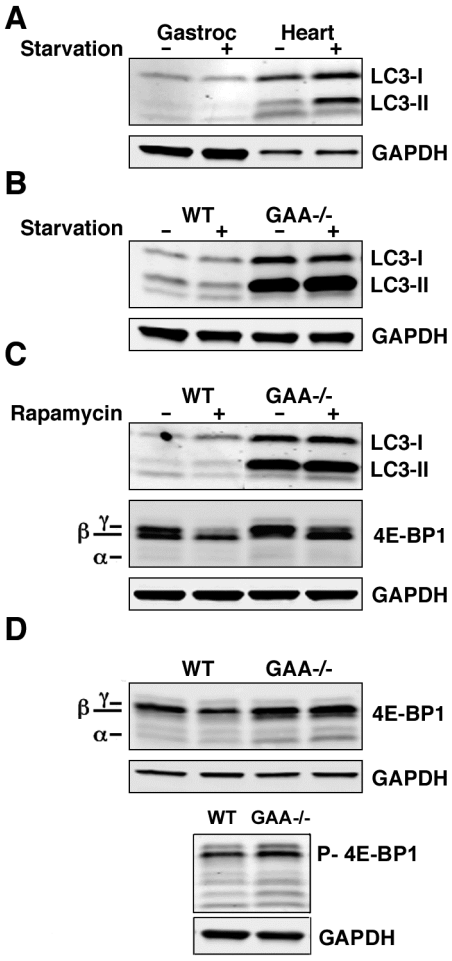
Suppl. Fig. 3.



Glycogen clearance in fast muscle of HSAcre:Atg5F/F:GAA-/- mice upon ERT

PAS-stained sections of gastrocnemius (fast) muscle from 4 month-old HSAcre:Atg5F/F:GAA-/- mice. Left: untreated mice; Right: ERT-treated. PAS-positive material (small purple dots), which represents glycogen, is seen in untreated but not in treated mice.

Suppl. Fig. 4.



The role of classical inducers of autophagy in skeletal muscle of WT and GAA-/- mice

Western blotting of protein lysates (gastrocnemius and cardiac muscle) with the indicated antibodies. (**A**) The level of LC3-II is increased upon starvation (48h) in cardiac, but not in gastrocnemius muscle from WT mice. (**B**) A slight increase in the LC3-II/LC3-I ratio is observed in muscle from GAA-/- mice. At least 5 mice were used for the starvation experiments for each group. (**C**) Rapamycin, an mTOR inhibitor, does not up-regulate autophagy in muscle from WT or GAA-/- mice as shown by the levels of LC3-II. The inhibition of mTOR by rapamycin is reflected by the decrease in the phosphorylation of a downstream target of mTOR - eukaryotic initiation factor 4E binding protein 1 (4E-BP1). The phosphorylation status of 4E-BP1 is characterized by the relative amount of the γ, β, and α forms (γ represents the hyper-phosphorylated form, whereas β and α represent intermediately and low-phosphorylated forms). A marked

decrease in the γ form is observed upon rapamycin treatment in both WT and GAA-/- mice compared to treatment with vehicle alone. (**D**) An increase in all three forms of 4E-BP1 is observed in muscle from GAA-/- mice.

Suppl. Videos

Suppl. Video 1. Suppression of autophagy in fast muscle of Pompe mice prevents the formation of autophagic buildup

Myofiber isolated from psoas (fast) muscle derived from a MLCcre:Atg7F/F:GAA-/- mouse. The fiber is stained for the lysosomal marker, LAMP-1 (green) and the autophagosomal marker, LC3 (red). Hoechst dye was used for staining of the nuclei (blue). Expanded lysosomes are clearly seen in the fiber from a MLCcre:Atg7F/F:GAA-/- mouse; autophagic buildup is not present in the core of the fiber.

Suppl. Video 2. Poor muscle response to ERT in GAA-/- mice

Myofiber isolated from psoas (fast) muscle derived from an ERT-treated GAA-/- mouse. The fiber is stained for the lysosomal marker, LAMP-1 (green) and the autophagosomal marker, LC3 (red). Hoechst dye was used for staining of the nuclei (blue). Both grossly expanded lysosomes and autophagic buildup in the core of the fiber persist upon therapy.