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# **Rescue of advanced Pompe disease in mice with hepatic expression of secretable acid $\alpha$ -glucosidase**

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**Short title: Rescue of advanced Pompe disease with secGAA**

## Abstract

Pompe disease is a neuromuscular disorder caused by disease-associated variants in the gene encoding for the lysosomal enzyme acid  $\alpha$ -glucosidase (GAA), which converts lysosomal glycogen to glucose. We previously reported full rescue of Pompe disease in symptomatic four-month-old  $Gaa^{-/-}$  mice by adeno-associated virus (AAV) vector-mediated liver gene transfer of an engineered secretable form of GAA (secGAA). Here, we showed that hepatic expression of secGAA rescues the phenotype of four-month-old  $Gaa^{-/-}$  mice at vector doses at which the native form of GAA have little to no therapeutic effect. Based on these results, we then treated severely affected nine-month-old  $Gaa^{-/-}$  mice with an AAV vector expressing secGAA and followed the animals for nine months thereafter. AAV-treated  $Gaa^{-/-}$  mice showed complete reversal of the Pompe phenotype, with rescue of glycogen accumulation in most tissues, including the central nervous system, and normalization of muscle strength. Transcriptomic profiling of skeletal muscle showed rescue of most altered pathways, including those involved in mitochondrial defects, a finding supported by structural and biochemical analyses, which also showed restoration of lysosomal function. Together, these results provide insight into the reversibility of advanced Pompe disease in the  $Gaa^{-/-}$  mouse model via liver gene transfer of secGAA.

## Introduction

Glycogen storage disease type II, also known as Pompe disease (PD), is an autosomal recessive disorder caused by mutations in the *GAA* gene which encodes for the lysosomal enzyme acid alpha-glucosidase (GAA) <sup>1</sup>. This enzyme converts glycogen into glucose within lysosomes, and its deficiency leads to the accumulation of the substrate in virtually all the tissues of the body <sup>2</sup>. Infantile-onset Pompe disease (IOPD) is the most severe form of PD characterized by cardiomegaly, respiratory failure, muscle weakness and cognitive impairment <sup>3</sup>. IOPD subjects show absent or barely detectable residual GAA activity and, in the absence of any treatment, die within the first years of life <sup>4</sup>. On the other hand, late-onset Pompe disease (LOPD) patients present a variable range of residual GAA activity and usually present a milder phenotype with progressive respiratory and muscle impairments with no signs of cardiac defects <sup>1,5</sup>.

Nowadays, the only approved treatment for PD is enzyme replacement therapy (ERT) based on high doses of recombinant human GAA (rhGAA) infused intravenously weekly in classic infantile patients and every two weeks in LOPD patients <sup>6</sup>. ERT relies on a cross-correction mechanism by which circulating rhGAA binds to the mannose-6-phosphate receptors (MPRs) on the cell surface and trafficked to the lysosome <sup>1</sup>. However, despite having a major impact on the course of the disease in both infantile and late onset Pompe patients, ERT presents important shortcomings due to the short half-life in plasma of rhGAA and its inability to cross the blood brain barrier (BBB), resulting in limited efficacy in skeletal muscle and brain <sup>3,7</sup>. Low expression of MPRs, and the autophagy block in skeletal muscle, are also among the factors limiting ERT efficacy <sup>1,8</sup>. Finally, rhGAA exhibits high immunogenicity, particularly in IOPD patients, with

consequent development of neutralizing antibodies that can reduce significantly the efficacy of ERT<sup>9</sup>.

Likely due to these limitations, emerging clinical data appear to indicate that ERT has more of a stabilizing effect on LOPD patients, who generally experience improvements of muscle and respiratory function only during the first months after initiation<sup>10,11</sup>. Similarly, early intervention with ERT appear to be crucial for a positive treatment outcome in IOPD patients<sup>12</sup>, possibly highlighting the inability of the current treatment to reverse PD.

Development of gene therapy approaches may be a promising alternative to ERT for the treatment of PD. Adeno-associated virus (AAV) vectors have been proven as a safe and promising platform for in vivo gene transfer in humans as demonstrated in numerous clinical trials<sup>13-20</sup>. In the context of Pompe disease, one reported trial was carried out in pediatric subjects by intra-diaphragmatic injection of an AAV1 encoding the human GAA transgene<sup>21</sup>. The procedure was demonstrated safe with evidence of increase in respiratory function<sup>21,22</sup>.

Several preclinical studies of gene therapy have been published (reviewed in<sup>23</sup>) using a well-established *Gaa* knockout mouse model (*Gaa*<sup>-/-</sup> hereafter)<sup>24, 25</sup>. Among these studies, we recently showed full correction of Pompe disease in symptomatic four-month-old *Gaa*<sup>-/-</sup> mice with AAV vector-mediated liver gene transfer of an engineered secretable GAA (secGAA) transgene. The high and stable circulating GAA plasma activity levels achieved with secGAA rescued muscle and central nervous system pathology without development of humoral immune responses to the engineered transgene<sup>26</sup>. However, questions about the approach remain, including the long-term efficacy of secGAA at limiting vector doses and its ability to

correct Pompe disease in severely affected mice. Published studies suggest that rescue of advanced PD in mice with gene transfer is challenging, as an only partial amelioration of the disease phenotype was observed even at relatively high vector doses<sup>27, 28</sup>. One possible explanation of these findings relies on the impairment of lysosomal function characteristic of PD. Lysosomes play a key role in regulating many cellular processes, and recent studies showed significant mitochondrial defects in Pompe skeletal muscle, which have been associated with autophagic block<sup>29-31</sup>, and may explain why advanced PD is poorly reversible.

Here, we first performed a study in four-month-old *Gaa*<sup>-/-</sup> mice to demonstrate that secGAA can drive enhanced therapeutic efficacy and survival compared to the native version of the transgene following long-term hepatic gene transfer with low doses of AAV8 vectors. Next, given the superior efficacy of secGAA compared to its native form, we tested the reversibility of PD phenotype by treating nine-month-old *Gaa*<sup>-/-</sup> mice with advanced disease by liver gene transfer with an AAV8-secGAA vector. AAV8-secGAA mediated gene transfer resulted in high and sustained circulating plasma activity of GAA, driving body-wide rescue of glycogen accumulation and normalization of muscle function. Further investigation of muscle ultrastructure by electron microscopy revealed that pathological features were partially corrected by AAV8-secGAA treatment. RNA sequencing revealed normalization of expression levels of majority of genes involved in bioenergetics homeostasis along with partial normalization of large part of transcriptomics alterations associated with PD. This correlated with functional, biochemical and structural findings, suggesting that liver gene therapy with secGAA could potentially represent an efficient therapeutic strategy for the treatment at advanced stages of Pompe disease.

## Results

### Liver expression of secretable GAA rescues PD at low vector doses

We previously showed that liver targeted expression of secGAA via AAV8 vectors drives superior therapeutic efficacy compared to the native version of the enzyme<sup>26</sup>. However, long-term follow-up (ten months post treatment) of animals injected with relatively high vector doses ( $2 \times 10^{12}$  vg/kg) showed no clear difference between treatment groups<sup>26</sup>. To more carefully compare the therapeutic efficacy of secGAA with that of the native form of the enzyme (GAAco), we injected four-month-old *Gaa*<sup>-/-</sup> mice at a dose of  $5 \times 10^{11}$  vg/kg and followed the animals for ten months (**Figure 1A**). At this limiting vector dose, GAAco failed to extend lifespan of animals, whereas secGAA-treated animals had identical survival to wild-type littermates (**Figure 1B**). Accordingly, secGAA expression in liver fully rescued cardiomegaly (**Figure 1C**) and muscle strength (**Figure 1D**). Conversely, liver expression of GAAco did not affect cardiomegaly (**Figure 1C**) and only partially rescued muscle strength (**Figure 1D**). We next looked at tissue GAA enzymatic activity and glycogen content. GAA activity (**Figure 1E**) and glycogen content (**Figure 1F**) in the diaphragm of animals treated with the secGAA or GAAco transgenes did not differ significantly. Similar results were observed in triceps (**Figures S1A and S1B**) and heart (**Figures S1C and S1D**). The lack of statistical significance between secGAA and GAAco cohorts was likely due to the low number of animals surviving to the end of the study in the GAAco cohort. A limited effect was observed in the brain of secGAA treated animals (**Figure S1E and S1F**), while no effect was observed in the spinal cord of animals from either secGAA or GAAco cohorts (**Figures S1G and S1H**). Vector genome copy number in liver was not different across cohorts of treated animals, confirming the equivalent efficiency of liver transduction of the vectors used in the study (**Figure S1I**). These findings confirm that, at

limiting vector doses, secGAA drives superior rescue of survival and muscle strength compared to GAAco in  $Gaa^{-/-}$  mice.

### **Hepatic expression of secGAA results in correction of muscle phenotype in $Gaa^{-/-}$ mice with advanced disease**

Previous reports of liver gene transfer with AAV vectors expressing native forms of GAA showed only marginal rescue of established PD phenotype in  $Gaa^{-/-}$  mice<sup>27, 28, 32</sup>. Based on the results obtained (**Figure 1**), we therefore wanted to test whether secGAA expression could provide therapeutic benefit in  $Gaa^{-/-}$  mice with advanced disease. We first measured the amount of glycogen in various tissues of naïve  $Gaa^{-/-}$  and  $Gaa^{+/+}$  mice at four, nine and fourteen months of age.  $Gaa^{-/-}$  mice showed greater glycogen accumulation compared to  $Gaa^{+/+}$  mice in all tissues (**Figures S2A-S2D**), with significantly higher amounts of glycogen measured at nine months in triceps, brain and diaphragm (**Figures S2A-S2C**). Lower glycogen content was observed in the tissues of fourteen-month-old  $Gaa^{-/-}$  mice (**Figures S2A-S2D**), consistent with previous findings in Pompe mice<sup>33</sup>. Survival of  $Gaa^{-/-}$  mice was less than 50% at fourteen months of age (**Figure S2E**). Grip-test measurement showed a significant decrease in  $Gaa^{-/-}$  mice (**Figure S2F**), with loss of muscle strength slightly more pronounced at nine and fourteen months (average -32%) compared to four months (average -26%) (**Figure S2F**).

Based on this natural history study, we intravenously injected nine-month-old  $Gaa^{-/-}$  mice with an AAV8-secGAA vector at a dose of  $2 \times 10^{12}$  vg/kg (**Figure 2A**). PBS-treated age-matched  $Gaa^{-/-}$  and  $Gaa^{+/+}$  male mice were used as control in all the experiments. Liver-mediated gene transfer of secGAA in  $Gaa^{-/-}$  mice resulted in high and stable enzyme activity in plasma throughout the nine months of follow-up (**Figure 2B**). This resulted in detection of



supraphysiologic enzyme activity in muscle (**Figure 2C**) and liver (**Figure S3A**). Muscle uptake of secGAA resulted in the complete clearance of glycogen in heart, diaphragm and triceps (**Figures 2D** and **S3B**).

In agreement with the correction of the disease phenotype at the biochemical level, we observed the normalization of cardiomegaly and body weight (**Figures 2E** and **S3C**) in AAV8-secGAA treated animals, to an extent undistinguishable from PBS-Gaa<sup>+/+</sup> mice. Mice were also subjected to grip-test to assess muscle strength before AAV vector injection and at four, six, and nine months thereafter (**Figure 2F**). Gaa<sup>-/-</sup> mice treated with the AAV8-secGAA vector showed a steady increase in muscle strength over time, achieving full recovery by the end of the study (**Figure 2F**). Improvement in muscle strength was accompanied by a normalization of the skeletal muscle weight (**Figure S3D**), whereas a significant loss of weight in triceps and gastrocnemii was detected in PBS-treated Gaa<sup>-/-</sup> mice (**Figure S3D**).

Plethysmography measurement of respiratory function<sup>34</sup> did not show any significant difference among treatment cohorts, due to the low number of mice in each group, although a tendency to improved tidal volume was noted in Gaa<sup>-/-</sup> mice treated with the AAV8-secGAA vector (**Figure 2G**). Altogether, these results highlight rescue of advanced muscle pathology at the biochemical and functional level in Gaa<sup>-/-</sup> mice upon AAV8-secGAA treatment.

### **SecGAA partially corrects glycogen accumulation in the central nervous system of 18-month-old Gaa<sup>-/-</sup> mice**

We next measured GAA enzymatic activity in the central nervous system (CNS) of mice, specifically, in brain and spinal cord (**Figure 3**). Although detectable, significant lower enzyme activity was found in Gaa<sup>-/-</sup> mice treated with the AAV8-secGAA vector compared to PBS-

treated  $Gaa^{+/+}$  mice (**Figure 3A**). The residual GAA activity detected was reflected in the partial correction of glycogen content in brain and spinal cord (about 30% lower than PBS- $Gaa^{-/-}$  cohort, **Figure 3B**). Western blot analysis of brain and spinal cord (**Figures 3C and 3D**) allowed to detect the precursor (110 kDa) and lysosomal (76 kDa) forms of human GAA in  $Gaa^{-/-}$  mice injected with AAV8-secGAA vector, documenting the presence of functionally active GAA in the lysosomes in the CNS. Finally, representative histological sections of the brain (**Figure 3E**) stained with periodic acid-Schiff (PAS) confirmed the partial reduction of glycogen content in the brain of AAV8-secGAA treated  $Gaa^{-/-}$  mice. Together, these results confirm that sustained levels of circulating GAA can at least partially ameliorate the CNS pathology in  $Gaa^{-/-}$  mice.

#### **Hepatic expression of secGAA restores muscle structure and lysosomal function in old $Gaa^{-/-}$ mice**

In order to assess reversal of muscle pathology at the structural level, we carried out hematoxylin and eosin staining and electron microscopy (EM) analysis on muscle sections. Histological sections revealed structural abnormalities in affected  $Gaa^{-/-}$  mice treated with PBS, whereas wild-type littermates ( $Gaa^{+/+}$ ) and secGAA-treated  $Gaa^{-/-}$  mice had similar muscle structure (**Figure 4A**). Quantification of p62, Lamp1, Beclin1 and LC3b, known markers of lysosomal homeostasis and autophagic buildup in Pompe disease <sup>35-37</sup>, showed significant phenotype reversal in secGAA-treated animals for most markers analyzed (**Figures 4B-4H**). High-resolution EM also revealed the presence of enlarged lysosomes throughout the muscle of  $Gaa^{-/-}$  mice (**Figure 4I**). Quantification of EM findings showed widespread presence of enlarged lysosomes in PBS-treated  $Gaa^{-/-}$  mice, which were undetectable in wild-type animals (**Figure**

**4I).** Significantly smaller lysosomes (~75% of reduction compared to PBS-treated  $Gaa^{-/-}$  mice) were observed in AAV8-secGAA vector-treated  $Gaa^{-/-}$  mice (**Figure 4J**).

Alterations of lysosomal function have been associated with mitochondrial defects in the skeletal muscle of  $Gaa^{-/-}$  mice<sup>30, 31</sup>. EM analysis of skeletal muscle showed the expected ultrastructure with mitochondria homogenously distributed along the muscle fibers in  $Gaa^{+/+}$  mice (**Figure 5A**). Muscle from PBS- $Gaa^{-/-}$  cohort exhibited areas of clustering of mitochondria and glycogen-filled lysosomes, which were less evident in  $Gaa^{-/-}$  mice treated with the AAV8-secGAA vector (**Figure 5A**). Western blot analysis was then performed to assess mitochondrial content using the three known mitochondrial markers: CoxIV (a subunit of complex IV), ATP-b (beta subunit of complex V), and Sdha (succinate dehydrogenase A, a subunit of complex II). This analysis showed that PBS-treated  $Gaa^{-/-}$  mice had a slight but not statistically significant increase in the amounts of CoxIV compared to  $Gaa^{-/-}$  mice treated with AAV8-secGAA and  $Gaa^{+/+}$  mice (**Figures 5B and 5C**). Significantly higher amounts of both ATP-b (**Figures 5D and 5E**) and Sdha (**Figures 5F and 5G**) were detected in  $Gaa^{-/-}$  mice compared to both AAV8-secGAA treated  $Gaa^{-/-}$  mice and wild-type littermates (PBS- $Gaa^{+/+}$ ) (**Figures 5E and 5G**).

These results demonstrate that hepatic expression of secGAA can normalize autophagy, lysosome size and mitochondrial content.

### **Liver targeted expression of secGAA largely normalizes the transcriptome in skeletal muscle**

In order to better understand the molecular signatures of diseased muscle in advanced Pompe disease, and analyze the effect of AAV8-secGAA at the molecular level, we performed transcriptome profiling by RNA sequencing using quadriceps of animals at the end of the study

(eighteen months of age, nine month post vector infusion). Principal Component Analysis (PCA) revealed that the global transcriptome of secGAA-treated animals was intermediate between wild-type littermates (PBS-Gaa<sup>+/+</sup>) and untreated Gaa<sup>-/-</sup> animals (PBS-Gaa<sup>-/-</sup>) (**Figure S4A**). Differential expression analysis was then performed to identify subsets of genes with significant expression changes across the experimental groups. Pairwise comparative analysis yielded in the highest number of Differentially Expressed Genes (DEGs) between PBS-treated Gaa<sup>+/+</sup> and PBS-treated Gaa<sup>-/-</sup> animals (2802 DEGs, **Figure S4B**). AAV8-secGAA treated Gaa<sup>-/-</sup> mice displayed a lower number of DEGs when compared to Gaa<sup>-/-</sup> and Gaa<sup>+/+</sup> animals, 434 and 371 respectively (**Figure S4B**). The decrease in DEGs in secGAA-Gaa<sup>-/-</sup> vs. PBS-Gaa<sup>+/+</sup> mice, as compared to PBS-Gaa<sup>-/-</sup> vs. PBS-Gaa<sup>+/+</sup>, is in agreement with the functional and biochemical data (**Figures 2, 4 and 5**), and are consistent with an phenotype of secGAA- Gaa<sup>-/-</sup> mice intermediate between PBS-Gaa<sup>+/+</sup> and PBS-Gaa<sup>-/-</sup>. Hierarchical clustering using the expression profile confirmed that muscle from AAV8-secGAA treated Gaa<sup>-/-</sup> animals was more similar to PBS-treated Gaa<sup>+/+</sup> animals than to PBS-treated affected Gaa<sup>-/-</sup> animals (**Figure S4C**). Functional enrichment analysis performed with Ingenuity Pathway Analysis (IPA) on the list of differentially expressed genes (2802 genes from the comparison PBS-Gaa<sup>-/-</sup> vs. PBS-Gaa<sup>+/+</sup>) identified 257 upstream transcription factors (TFs) including Nuclear Factor Kappa B (*NFκβ*), Signal Transducer And Activator Of Transcription 1 (*STAT1*) and RELA Proto-Oncogene (*RELA*) (**Figure S5A**). These TFs, which are mainly involved in inflammatory responses and in muscle tissue homeostasis pathways<sup>38-41</sup>, were found to be highly activated in Gaa<sup>-/-</sup> animals (**Figures S5A and S5B**).

After having identified genes with altered expression in the Gaa<sup>-/-</sup> background (relative to Gaa<sup>+/+</sup>), we focused on those whose expression had been restored to wild-type levels upon liver

targeted expression of secGAA. For this purpose, we grouped the complete collection of genes detected as differentially expressed in any of the three comparisons (**Figure S4B**) into six clusters, using K-Means. Each cluster contained around 450 genes with similar expression profiles. We grouped clusters with complementary profiles into three metaclusters that represented genes with different degrees of normalized expression towards  $Gaa^{+/+}$  (wild type) levels: full, partial or no rescue (**Figure 6A**). After AAV8-secGAA treatment, gene expression was either partially or fully normalized in approximately 75% of the identified DEGs, while the expression of 25% of the DEGs was not rescued. Functional enrichment analysis with IPA indicated that metaclusters containing genes with fully or partially rescued expression levels were associated to pathways involved in bioenergetics homeostasis such as Sirtuin signaling, mitochondrial dysfunction and Integrin signaling (**Figure 6B**). Pathways related to glucose metabolism (i.e. Glycolysis I, Gluconeogenesis I, Glycogen degradation II, Glycogen degradation III) were also associated to metaclusters with fully or partially rescued expression levels after hepatic expression of secGAA (**Figure S6**).

As defects in muscle regeneration have been recently documented in  $Gaa^{-/-}$  mice<sup>33, 42</sup>, we analyzed the expression pattern of several genes of interest. We observed partial normalization of the expression levels of genes involved in muscle regeneration and plasticity such as *Capn3*, *Myoz1*, and *Dysf*<sup>43-45</sup> (**Figure S7**). Interestingly, the expression level of the dystrophin gene (*Dmd*), a key gene for structure and function of muscle cells which mutations can result in Duchenne and Becker muscular dystrophy, was normalized<sup>46</sup> (**Figure S7**). Along the same line, we also evaluated fibrosis in muscle. At the histological level, Sirius red staining showed limited changes in signal intensity in the three cohorts (PBS- $Gaa^{+/+}$ , PBS- $Gaa^{-/-}$ , secGAA- $Gaa^{-/-}$ ) (**Figure S8**). Interestingly, at the transcriptomics levels, of approximately 75% of genes involved in

fibrosis and fibrogenesis, which were dysregulated in PBS-Gaa<sup>+/+</sup> mice, were fully or partially rescued upon AAV-secGAA treatment (**Figure S8**). Together, these results highlight a significant amelioration of Pompe disease at the transcriptional level.

### **AAV8-secGAA liver gene transfer partially re-establish mitochondria homeostasis in eighteen-month-old Gaa<sup>-/-</sup> mice**

Deeper analysis of mechanistic differences was performed using the transcriptome data from skeletal muscle of Gaa<sup>-/-</sup> compared to Gaa<sup>+/+</sup> mice to better evaluate the bioenergetics defects. Upstream Regulator Analysis (IPA) identified various TFs involved in oxidative stress which were associated to genes with altered expression in PBS-Gaa<sup>-/-</sup> mice relative to PBS-Gaa<sup>+/+</sup> mice, such as Hypoxia Inducible Factor 1 Alpha (*Hif1α*), Nuclear Factor Erythroid 2 Like 2 (*Nfe2L2*, also known as *Nrf2*) and mitochondrial homeostasis such as Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha (*PPARGC1α*), Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Beta (*PPARGC1β*) and Sirtuin1 (*Sirt1*) which were enriched in PBS-Gaa<sup>-/-</sup> mice (**Figure 7A**). Interestingly, *PPARGC1α* and *PPARGC1β*, central players of mitochondrial biogenesis<sup>47</sup>, were predicted as inhibited (negative z-score) in Gaa<sup>-/-</sup> mice (**Figure 7A**). This was consistent with the high mitochondrial content detected in skeletal muscle (**Figure 5**), linked to defective mitophagy and consequent inhibition of the mitochondrial biogenesis machinery as a compensatory mechanism<sup>48</sup>. Given that the oxidative stress related TFs (including *Nrf2* and *Hif1α*) were predicted to be more active in PBS-Gaa<sup>-/-</sup> than PBS-Gaa<sup>+/+</sup> (with positive z-score), we decided to assess global Reactive Oxygen Species (ROS) production in skeletal muscle. We observed slightly elevated amounts of ROS in PBS-Gaa<sup>-/-</sup> animals, which were normalized upon treatment with secGAA (**Figure 7B**).

To test whether the correction of mitochondrial content in skeletal muscle of AAV-treated *Gaa*<sup>-/-</sup> mice was accompanied by a recovery of mitochondrial function, we performed a Western blot analysis of key players in mitochondrial homeostasis in skeletal muscle. We looked at the “energy sensor” serine/threonine kinase AMP-activated protein kinase (*AMPK*) complex<sup>49</sup>, based on the fact that AMPK signaling was significantly enriched in *Gaa*<sup>-/-</sup> mice compared to *Gaa*<sup>+/+</sup> mice, and that genes regulated by AMPK signaling were rescued in AAV8-secGAA treated animals (**Figure 6B**). Interestingly, we observed increased levels of AMPK in PBS-treated *Gaa*<sup>-/-</sup> mice, which were normalized levels upon AAV8-secGAA treatment (**Figures 7C and 7D**). In summary, transcriptomic data and biochemical analysis suggest that long-term exposure to secGAA following hepatic gene transfer results in partial normalization of mitochondrial homeostasis in skeletal muscle of *Gaa*<sup>-/-</sup> mice with advanced disease.

## Discussion

Pompe disease is a progressive neuromuscular disease caused by accumulation of lysosomal glycogen, which results in tissue disruption, autophagy block and metabolic impairments <sup>1</sup>. Introduction of enzyme replacement therapy with rhGAA represented a major advance in the treatment of Pompe disease, being a lifesaving treatment for infantile patients <sup>3, 50, 51</sup> and providing benefit to the adult Pompe population <sup>11, 52, 53</sup>. In vivo gene delivery with AAV vectors is a promising alternative to ERT for Pompe disease, owing to the promising results in preclinical models of the disease <sup>26, 54, 55</sup> and the excellent safety and efficacy profile in human trials for a variety of diseases <sup>14, 16, 20, 56, 57</sup>. We previously demonstrated the full rescue of Pompe disease in four-month-old *Gaa*<sup>-/-</sup> mice using a secretable form of the GAA enzyme expressed in hepatocytes via AAV vectors <sup>26</sup>. Here, we further defined the therapeutic potential of this approach by comparing the long-term effect of low vector doses of AAV8 vectors expressing secretable GAA or its native counterpart in *Gaa*<sup>-/-</sup> mice treated at four months of age. Despite the small increase in GAA activity detectable in tissues of animals treated with secretable GAA, survival data clearly demonstrate that, at 5x10<sup>11</sup> vg/kg, native GAA is unable to provide any therapeutic benefit, while *Gaa*<sup>-/-</sup> mice treated with secretable GAA present a lifespan undistinguishable from that of wild-type littermates. These results are of fundamental importance in the prospective of translating these results to the clinic, as the use of transgenes with enhanced bioavailability, like secretable GAA, could potentially provide superior therapeutic benefit to patients, while reducing the AAV doses administered. Importantly, the use of transgenes with an improved therapeutic index in the context of hemophilia B gene therapy trials allowed to decrease the therapeutic vector dose thus reducing capsid immune mediated toxicities <sup>16</sup> and potentially lowering manufacturing requirements. Future side-by-side studies of gene transfer of



secretable GAA with the current standard of care for Pompe disease <sup>1</sup> will help highlight differences in pharmacokinetics and bioavailability between gene therapy and ERT.

An open question about ERT, which also concerns all novel therapies for Pompe disease, is whether providing a source of GAA enzyme could reverse an already established disease phenotype. Experience with ERT in IOPD patients shows clear beneficial effects including the resolution of hypertrophic cardiomyopathy, amelioration of muscle functions and, importantly, survival <sup>3, 50, 51</sup>. However, in this patient population, early intervention is crucial for a positive treatment outcome <sup>12</sup>, suggesting that the effects of glycogen accumulation are poorly reversible with ERT. Similarly, LOPD patients receiving ERT in the long-term tend to achieve, at best, stable muscle and respiratory functions <sup>11, 52, 53</sup>, possibly due to the fact that ERT is started when patients become symptomatic, thus with relatively advanced disease progression <sup>58</sup>. Presence of vacuolated fibers and autophagy impairment in muscle have also been demonstrated to hamper the trafficking of rhGAA in the context of ERT <sup>8, 29</sup>.

In the context of gene transfer, two studies attempted to correct Pompe disease in *Gaa*<sup>-/-</sup> mice with an advanced pathology. A first study described ten-month-old *Gaa*<sup>-/-</sup> mice treated with an AAV vector expressing the GAA transgene in the liver <sup>27</sup>. Despite the high vector dose used ( $1 \times 10^{12}$ /mouse), six months after treatment the animals showed only partial glycogen clearance in skeletal muscle and no improvements of muscle function <sup>27</sup>. In a second study, twelve-month-old *Gaa*<sup>-/-</sup> mice received an AAV vector expressing a GAA transgene carrying a heterologous signal peptide to enhance secretion from hepatocytes <sup>28</sup>. Also in this case, AAV-treated mice exhibited incomplete reduction of glycogen in muscle <sup>28</sup>. Additional published data in *Gaa*<sup>-/-</sup> mice with advanced phenotype show lack of correction of glycogen accumulation with muscle

directed expression of GAA<sup>59</sup>. Results presented here clearly show that hepatic expression of secretable GAA results in clearance of glycogen from multiple tissues, including a partial clearance in CNS, and restoration of muscle function to wild-type levels in Gaa<sup>-/-</sup> mice with advanced pathology. While this is somewhat in contrast with published findings<sup>27, 28, 59</sup>, the higher bioavailability of secretable GAA compared with the native form of the enzyme or to enzymes carrying heterologous signal peptides<sup>26</sup> could explain our findings. Alternatively, difference in time of follow up and age of inclusion of the animals in the study, as well as genetic background of the Gaa<sup>-/-</sup> colonies used in the study<sup>24, 25, 60</sup> could explain the observed differences across studies. The fact that animals presented a clear severe phenotype at the time of treatment, both in terms of glycogen accumulation and muscle strength, would argue that hepatic expression of secretable GAA can efficiently reverse an advanced disease phenotype in Gaa<sup>-/-</sup> mice. Transcriptomics analysis of genes involved in muscle regeneration and remodeling observed in our study further supports our findings, although similar studies in the context of gene therapy trials will be necessary to elucidate the effects of secretable GAA expression on human muscle plasticity. Morphological changes observed in skeletal muscle of Pompe patients followed by magnetic resonance imaging (MRI)<sup>61, 62</sup>, show gradual replacement of skeletal muscle mass with fat tissue, a hallmark of muscle degeneration<sup>63</sup>. This phenomenon has not been documented in Gaa<sup>-/-</sup> mice, which could indicate that the animal model fails to fully recapitulate the human condition when it comes to muscle pathophysiology. Results in our study show that, at 18 months of age, untreated Gaa<sup>-/-</sup> mice show a marked decrease of skeletal muscle mass, and secretable GAA treatment appears to restore both skeletal muscle and body weight to wild-type levels.

Another possible shortcoming of the current study is the small number of animals per cohort. While larger study could address this point, and help understand the reversibility of more variable endpoints of therapeutic efficacy like those reflecting respiratory function <sup>34, 64, 65</sup>, limited long-term survival of untreated *Gaa*<sup>-/-</sup> mice <sup>26</sup> limits the ability to conduct larger studies with aged Pompe mice. Nevertheless, statistical analysis and correlation of multiple endpoints at the biochemical, functional, and morphological level, and the changes in the transcriptional signature of skeletal muscle, support the robustness of the current findings on the reversibility of the advanced Pompe phenotype in mice.

Consistent with previous findings in Pompe disease <sup>26</sup> and other lysosomal storage disorders <sup>66</sup>, mice treated with secretable GAA showed a significant glycogen reduction in CNS, although a full evaluation of CNS rescue, previously reported by our lab <sup>26</sup>, was not presented in the current study. The mechanism underlying transport of GAA from the systematic circulation to the CNS is currently unknown and could involve transcytosis, transport via exosomes <sup>67</sup>, or lysosomal exocytosis at neuromuscular junctions <sup>68</sup>. Although encouraging, these results suggest that high plasma levels of GAA derived from the hepatic expression of secGAA are likely to be needed to fully correct the CNS manifestations characteristic of IOPD <sup>34, 69</sup>. Alternatively, direct targeting of neurons has also been explored <sup>70</sup>, although this strategy achieved full correction of CNS glycogen accumulation with little effect on peripheral tissues and muscle function. Notably, a GAA-expressing vector based on the recently developed AAV-PHP.B serotype <sup>71</sup>, delivered at high doses ( $5 \times 10^{12}$  vg/kg), completely cleared heart and brain glycogen in Pompe mice, while reducing glycogen content in quadriceps and gastrocnemius <sup>72</sup>.

Lysosomal defects have been linked to severe autophagy impairment in skeletal muscle of both Pompe patients and *Gaa*<sup>-/-</sup> mice<sup>29, 36</sup>. In addition, studies in primary cell cultures and muscle tissue from *Gaa*<sup>-/-</sup> mice, show secondary defects in mitochondria and mitophagy<sup>30</sup>, consistent with recent findings on the link between autophagy block and mitochondria impairment in juvenile *Gaa*<sup>-/-</sup> mice<sup>31</sup>. Clearance of defective mitochondria via mitophagy is essential to maintain a healthy mitochondrial network, whereas accumulation of dysfunctional mitochondria can have detrimental consequences in various diseases including LSDs<sup>73</sup>. Here we showed that liver expression of secretable GAA normalized both autophagy and mitophagy, as documented by electron microscopy and Western blot analyses, with normalization of lysosomal length and markers of autophagy such as p62, Lamp1 and LC3b, as well as normalization of mitochondria content and improvements in mitochondria metabolism in skeletal muscle as suggested by changes in AMPK levels. Impaired mitophagy at 18 months of age resulted in accumulation of mitochondria and modestly increased levels of ROS which were normalized by clearance of mitochondria. At the transcriptomics level, untreated *Gaa*<sup>-/-</sup> showed profound alterations of mitochondrial biogenesis and upregulation of inflammatory pathways in skeletal muscle, similar to findings in other lysosomal storage diseases<sup>74</sup>. Large transcriptional alterations were observed in the muscle of untreated Pompe mice at the end of the observational study, 18 months. Liver expression of secretable GAA resulted in either full or partial rescue of expression pattern of about two thirds of the genes studied. Signaling pathway analysis confirmed partial rescue of the alterations observed in Pompe mice. Those alterations were possibly related to the deep modifications of the muscle physiology and their partial rescue after gene transfer was likely expected. As Pompe patients have access to ERT, it is conceivable that the muscle of humans would present a somewhat less altered expression pattern. Work by Koeberl and colleagues

reported a short list of genes whose expression level were normalized after clenbuterol treatment in Pompe patients <sup>75</sup>. Interestingly, *NFE2* is one of the genes with normalized expression showing the importance of our findings on normalization of oxidative stress related transcriptomic changes and ROS levels <sup>75</sup>.

Characterization of satellite cells in muscle biopsies from both IOPD and LOPD patients suggest that satellite cells found in muscle are not altered in number but are less functional <sup>76</sup>. Two independent studies reported that *Gaa*<sup>-/-</sup> mouse has functionally normal satellite cells however they are not capable to repair disease damage probably due to a activation signal defect <sup>33, 42</sup>. Here, we observed partial rescue of genes related to muscle plasticity after gene transfer, although the activation state of satellite cells in old mice may be affected by age-related changes unrelated to Pompe disease <sup>77</sup>. Additional studies in Pompe mice and in PD patients will be required to further understand differences in plasticity of the muscle across species and in particular whether gene transfer with secretable GAA will result in rescue Pompe disease in older patients.

Further work is also needed to assess the therapeutic potential of our approach in IOPD patients. Because of the non-integrative nature of AAV vectors, treatment of newborn IOPD patients with liver-expressed secretable GAA might result in transient expression of the donated transgene. This would require the re-administration of the therapeutic vector<sup>78</sup> to counteract vector genome dilution and loss of transgene expression due to liver growth<sup>79</sup>.

In summary, this work confirms the superior therapeutic efficacy of secretable GAA compared to the native form of the enzyme. It also demonstrates that gene transfer with secretable GAA

drives efficient rescue of advanced Pompe phenotype in  $Gaa^{-/-}$  mice and in particular has a profound impact on the restoration of autophagy, mitophagy, and overall bioenergetic and inflammatory homeostasis of muscle. Future translation of the therapeutic modality based on secretable GAA to the clinic will help address similar mechanistic questions on the reversibility of the Pompe phenotype in humans.

## Materials and Methods

### GAA expression cassettes and AAV vectors

The GAA transgene expression cassettes containing the codon-optimized coding sequence encoding for the native and secretable forms of human GAA were previously described <sup>26</sup>.

AAV vectors used in this study were produced using an adenovirus-free transient transfection method as described earlier <sup>80</sup>. Titers of AAV vector stocks were determined using quantitative real-time PCR (qPCR) and confirmed by SDS-PAGE followed by SYPRO Ruby protein gel stain and band quantification using ImageJ software. All vector preparations used in the studies were quantified side-by-side at least 3 times before use.

### In vivo studies

Mouse studies were performed according to the French and European legislation on animal care and experimentation (2010/63/EU) and approved by the local institutional ethical board (protocol n° 2015-008).

The *Gaa*<sup>-/-</sup> mice was purchased from Jackson laboratory (B6;129-Gaatm1Rabn/J, stock number 004154, 6neo). This mouse model was originally generated by Nina Raben (Raben et al. 1998 J. Biol Chem. 273, 19086-19092). Wild-type mouse (*Gaa*<sup>+/+</sup>) are derived from the same colony and breed separately.

For the comparative analysis of native GAA (GAAco) with secGAA, AAV8-GAAco or AAV8-secGAA <sup>26</sup> were administered intravenously at four-month-old male mice. *Gaa*<sup>-/-</sup> mice were treated at a vector dose of 5x10<sup>11</sup> vg/kg and followed for 10 months (n=7 per treatment group). *Gaa*<sup>-/-</sup> and *Gaa*<sup>+/+</sup> mice were injected with PBS and used as controls (n=8 per group).

For the treatment of mice at advanced stages of Pompe disease, an AAV8-secGAA vector<sup>26</sup> was administered intravenously via the tail vein to nine-month-old male mice. *Gaa*<sup>-/-</sup> mice were treated at a vector dose of  $2 \times 10^{12}$  vg/kg and followed for 9 months (n=3/4 per treatment group). Age matched *Gaa*<sup>-/-</sup> and *Gaa*<sup>+/+</sup> mice were injected with PBS and used as controls. All of the animals were perfused at the time of sacrifice.

### **Vector genome copy number analysis**

Vector genome copy number was determined using qPCR as previously described<sup>26</sup>. The PCR primers used in the reaction were located on the human alpha-1 antitrypsin promoter sequence within the expression cassette. Forward primer, 5'-GGCGGGCGACTCAGATC-3'; reverse primer 5'-GGGAGGCTGCTGGTGAATATT-3'.

### **Measurement of GAA activity and glycogen content**

GAA activity was measured as previously described<sup>81</sup>. Briefly, 10  $\mu$ L of sample, either tissue homogenate or plasma, were incubated for 1 hour at 37°C with 20  $\mu$ L of 4-Methylumbelliferyl  $\alpha$ -D-glucopyranoside (3mM) diluted in acetate buffer solution, pH 4.65 (Sigma Aldrich). The reaction was stopped using the carbonate solution and the fluorescence ( $\lambda_{ex}$  360nm/ $\lambda_{em}$  449nm) was read with an EnSpire alpha plate reader (Perkin-Elmer, Waltham, MA). A standard curve was prepared using 4-Methylumbelliferone diluted in 0.5M of carbonate solution, pH 10.5.

Glycogen content was measured indirectly in tissue homogenates as the glucose released after total digestion with *Aspergillus Niger* amyloglucosidase (Sigma Aldrich, Saint Louis, MO). Samples were incubated for 5 min at 95°C and then cooled at 4°C; 25  $\mu$ L of amyloglucosidase diluted 1:50 in 0.1M potassium acetate pH 5.5 were then added to each sample. A control



reaction without amyloglucosidase was prepared for each sample. Both sample and control reactions were incubated at 37°C for 90 minutes. The reaction was stopped by incubating samples for 5 min at 95°C. The glucose released was determined using a glucose assay kit (Sigma Aldrich, Saint Louis, MO) and by measuring resulting absorbance at the EnSpire alpha plate reader (Perkin-Elmer, Waltham, MA) at 540 nm.

### **Western blot analyses**

Mouse tissues were mechanically homogenate in H<sub>2</sub>O using lysing matrix tubes (MP Biomedicals). Protein concentration was determined using the BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA).

SDS-page electrophoresis was performed in a 4-15% gradient polyacrylamide gel. After transfer, the membrane was blocked with Odyssey buffer (Li-Cor Biosciences, Lincoln, NE) and incubated with an anti-GAA antibody (rabbit monoclonal, Abcam, Cambridge, MA), anti-p62 (mouse monoclonal, Abcam, Cambridge, MA), anti-LAMP1 (rabbit polyclonal, Abcam, Cambridge, MA), anti-Beclin1 (rabbit polyclonal, Abcam, Cambridge, MA), anti-LC3B (rabbit polyclonal, Novusbio), anti-tubulin (mouse monoclonal, Sigma), anti-Actin (rabbit polyclonal, Sigma), anti-GAPDH (rabbit polyclonal, Thermo Fisher), anti-AMPK (rabbit polyclonal, Cell Signaling), anti-CoxIV (rabbit polyclonal, Abcam, Cambridge, MA), anti-ATP-B (mouse monoclonal, Abcam, Cambridge, MA), anti-SDHA (mouse monoclonal, Invitrogen) . The membrane was washed and incubated with the appropriate secondary antibody (Li-Cor Biosciences) conjugated with fluorescent probes with extended linear range, and visualized by

Odyssey imaging system (Li-Cor Biosciences), which allows to identify and exclude saturated signals during band quantification.

### **Histological evaluation**

For histology, immediately after euthanasia heart, triceps brachii, diaphragm, and brain were snap-frozen in isopentane (-160°C) previously chilled in liquid nitrogen. Serial 8 µm cross-sections were cut in a Leica CM3050 S cryostat (Leica Biosystems). To minimize sampling error, 3 sections of each specimen were obtained and stained with hematoxylin and eosin (H&E), Sirius red and periodic acid-Schiff (PAS) according to standard procedures.

### **Electron microscopy analyses**

Muscle samples were fixed with 2.5 % of glutaraldehyde (Sigma Aldrich) in 0.1M PHEM, pH 7.2. Post fixation was done with 1 % osmium tetroxide (Merck) and 1.5 % ferrocyanide (Sigma Aldrich) in 0.1M Hepes. After dehydration by a graded series of ethanol, the tissue samples were infiltrated with epoxy resin. Polymerized resin blocks were thin sectioned longitudinally using a Leica UC 7 microtome (Leica microsystems). The 70nm sections were collected on formvar coated slot grids (EMS) and were contrasted with 4% uranyl acetate and Reynolds lead citrate. Stained sections were observed with a Tecnai spirit FEI operated at 120 kV. Images were acquired with FEI Eagle digital camera. Quantification of lysosome length was performed using ImageJ software.

### **Functional assessment**

Respiratory function during quiet breathing was evaluated as already reported<sup>26, 34</sup>. Briefly, a flow-through (0.5 L/min) plethysmograph (EMKA technologies, Paris, France) was used to measure the breathing pattern in treated *Gaa*<sup>-/-</sup> mice and untreated *Gaa*<sup>-/-</sup> and wild type littermates. Before the assessment, the instrument was calibrated with known airflow and pressure signals for optimal data collection. Signals were analyzed by using the IOX2 software (EMKA technologies). Tidal volume was evaluated over a period of 5 minutes. Animals were allowed for acclimation into the plethysmograph chamber before testing. During both acclimation and data acquisition, mice were breathing normoxic air (21% O<sub>2</sub>, 79% N<sub>2</sub>).

Grip strength was measured as already reported by TREAT-NMD Network ([www.treat-nmd.org](http://www.treat-nmd.org)). The test was performed on AAV-treated *Gaa*<sup>-/-</sup> mice and untreated *Gaa*<sup>-/-</sup> and wild type littermates. Using a grip strength meter, (Columbus instruments, San Diego, CA) three independent measurements of the four limbs strength were calculated. Mean values of the grip strength were reported.

### **Electron paramagnetic resonance (EPR) spectroscopy**

Free radical production was measured by electron paramagnetic resonance (EPR) spectroscopy using 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine hydrochloride (CMH, Noxygen, Elzach, Germany) as a spin probe. In brief, muscles were frozen in liquid nitrogen and kept at -80°C until assay. Tissues were weighed and homogenized in Krebs-HEPES buffer (pH 7.4). Then, samples were incubated in Krebs-HEPES buffer containing 5 mM diethyldithiocarbamic acid silver salt (DETC), 25 mM deferoxamine (DF) and 50 mM of CMH in 24-well plates at 37°C. Blank wells without tissue were prepared in parallel. After a 60 minute incubation, samples were immediately frozen in liquid nitrogen to stop the reaction. Oxidized

CMH spectra were recorded with a Miniscope MS-200 (Magnettech, Berlin). Acquisition EPR parameters were: Bo-field 3325.96 G; microwave power 1 mW; modulation amplitude 5 G; sweep time, 60 s. Spectra intensity was expressed in arbitrary units, normalized per milligram of protein.

### **RNA Isolation and transcriptome profiling**

The RNAs were isolated from snap-frozen quadriceps tissues preserved at -80°C. Samples were initially homogenized in Trizol and total RNA were column purified by PureLink™ RNA Mini Kit (Invitrogen). NGS experiments were performed in the Genomics Unit of the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain). 200 ng of total RNA were used to generate barcoded RNA-seq libraries using the NEBNext Ultra RNA Library preparation kit (New England Biolabs). Briefly, poly A+ RNA was purified using poly-T oligo- attached magnetic beads followed by fragmentation and then first and second cDNA strand synthesis. Next, cDNA 3' ends were adenylated and the adapters were ligated followed by PCR library amplification. Finally, the size of the libraries was checked using the Agilent 2100 Bioanalyzer DNA 1000 chip and their concentration was determined using the Qubit® fluorometer (Life Technologies). Libraries were sequenced on a HiSeq2500 (Illumina) to generate 60 bases single reads. FastQ files for each sample were obtained using CASAVA v1.8 software (Illumina). Sequencing data discussed here have been deposited in NCBI's Gene Expression Omnibus and accessible through GEO Series accession number GSE150935.

### **Bioinformatics analysis of RNA sequencing data**

RNA-seq data analysis was performed by the Bioinformatics Unit of Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain). Sequencing reads were processed with a pipeline that used FastQC to evaluate their quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>, developed by Babraham Institute), and cutadapt to trim sequencing reads, eliminating Illumina adaptor remains, and to discard reads that were shorter than 30 bp. Resulting reads were mapped against mouse transcriptome GRCm38.91, and gene expression levels were estimated with RNA-Seq by Expectation Maximization (RSEM)<sup>82</sup>. Around 91% of the reads from any sample participated in at least one reported alignment. Expression count matrices were then processed with an analysis pipeline that used Bioconductor package linear models for microarray data (limma) for normalization (using trimmed mean of M-values normalization (TMM) method) and differential expression testing<sup>83</sup>. Changes in gene expression were considered significant if associated to Benjamini and Hochberg adjusted p-value < 0.05.

The expression profiles of 2,864 genes detected as differentially expressed in any of the three performed contrasts (Figure S4C) were clustered using kmeans with n=6. Clusters with complementary expression profiles were combined to define three metaclusters (Figure 7A). Each metacluster contained genes with expression levels that had been fully rescued (FR), partially rescued (PR) or not rescued (NR) in animals receiving the AAV8-secGAA vector.

Differentially expressed and metacluster gene collections were processed with Ingenuity Pathway Analysis (IPA, Qiagen) to identify associations with Canonical Pathways, Diseases and Biofunctions, and Upstream Transcriptional Regulators. Functional associations provided by IPA

are characterized by two parameters: p values to describe enrichment test significance and z-scores to indicate predicted activation state. Enrichments were considered significant if associated to p-value < 0.05. Calculation of z-score for a given pathway, or regulator, is based on the fold change value of associated genes and can only be applied, by definition, to pairwise contrast results. Positive and negative z-scores suggest higher activity in the first or the second condition being compared, respectively. Abs(z-score) values higher than 2 are considered relevant. Circular plots, representing the association between enriched transcriptional regulators and functional terms were produced with the GOplot package <sup>84</sup>.

### **Statistical analysis**

All the data showed in the present manuscript are reported as mean  $\pm$  standard deviation. The number of sampled units, n, upon which we reported statistic is the single mouse for the in vivo experiments (one mouse is n=1). GraphPad Prism 7 software (GraphPad Software) was used for statistical analyses. p-values<0.05 were considered significant. For all the data sets, data were analyzed by parametric tests, alpha=0.05, (one-way and two-way ANOVA with Tukey's post-hoc correction, and multiple t-tests with Sidak-Bonferroni post-hoc correction). The survival of mice was compared by Kaplan-Meier log-rank test. The statistical analysis performed for each data set is indicated in the figure legends.

### **Authors contribution**

F.P., P.C., O.B., G.R. and F.M. directed the study. U.C. and F.P. performed most of the experiments and data analysis. U.C, F.P. and F.M. wrote the manuscript. U.C. and M.J.G. analyzed the RNA sequencing data. F.P., P.C., U.C., G.R. and F.M. contributed to the

interpretation of results and provided critical insights into the significance of the work. P.S. contributed to the biochemical analyses. L.v.W. and N.D. performed the delivery of AAV vectors in mice and managed the harvesting of mouse samples. N.G. performed grip test and plethysmography experiments. B.G. performed histological staining of mouse muscle. U.C, M.M.N. and J.K.L. performed electron microscopy analysis on mice skeletal muscle. C.A. performed the ROS measurements. F.C., S.C and M.S.S. produced the AAV vectors.

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## **Conflict of interest statement**

F.P., P.C, G. R. and F.M. are inventors in patents describing the secGAA technology. F.M. is employee and equity holder of Spark Therapeutics, Inc. All other authors declare no conflict of interest.

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## Figure legends

### Figure 1. Liver expression of secretable GAA rescues Pompe disease at low vector doses.

(A-F) Four-month-old  $Gaa^{-/-}$  mice were injected with an AAV8-GAAco (GAAco- $Gaa^{-/-}$ ) or AAV8-secGAA (secGAA- $Gaa^{-/-}$ ) vector at a dose of  $5 \times 10^{11}$  vg/kg.  $Gaa^{+/+}$  (PBS- $Gaa^{+/+}$ ) and  $Gaa^{-/-}$  (PBS- $Gaa^{-/-}$ ) mice injected with PBS served as controls in the study. Animals were followed for ten months after treatment (n=7/8 per cohort). (A) Graphical representation of the study design (m.o., months old). (B) Survival curve. The numbers between brackets in the figure legend show the number of animals survived at the end of the study. (C) Hypertrophy of cardiac muscle reported as heart weight percentage of body mass. (D) Muscle strength tested by grip test at three, six and nine months post treatment presented as percentage of PBS- $Gaa^{+/+}$ . (E) GAA enzymatic activity and (F) Glycogen content in diaphragm. Statistical analysis: (B) Kaplan-Meier log-rank test. \*,  $p < 0.05$  (C, E, F) One-way ANOVA with Tukey's post hoc. ns, not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ . (D) Two-way ANOVA time per treatment. +,  $p < 0.05$ ; \*\* or ++ or ##,  $p < 0.01$ ; +++,  $p < 0.001$ ; ++++ or \*\*\*\*,  $p < 0.0001$ . In all graphs error bars represent the standard deviation of the mean.

### Figure 2. secGAA normalizes muscle phenotype in advanced stages of Pompe disease. (A-G)

Nine-month-old mice were injected with an AAV8-secGAA (secGAA- $Gaa^{-/-}$ ) vector at a dose of  $2 \times 10^{12}$  vg/kg.  $Gaa^{+/+}$  (PBS- $Gaa^{+/+}$ ) and  $Gaa^{-/-}$  (PBS- $Gaa^{-/-}$ ) mice injected with PBS served as controls in the study. Animals were followed for nine months after treatment (n=3/4 per cohort). (A) Graphical representation of the study design (m.o., months old). (B) GAA enzymatic activity measured in plasma at one, three, six and nine months post-injection. (C) GAA enzymatic

activity in heart, diaphragm, triceps muscles reported as percentage of PBS-Gaa<sup>+/+</sup> activity. **(D)** Glycogen content in heart, diaphragm, triceps muscles reported as percentage of PBS-Gaa<sup>-/-</sup> glycogen. **(E)** Hypertrophy of cardiac muscle reported as heart weight percentage of body mass. **(F)** Grip-test analysis reported as percentage of PBS-Gaa<sup>+/+</sup> muscle strength. **(G)** Tidal volume measured by whole-body plethysmography. Statistical analysis: **(B, E, G)** One-way ANOVA with Tukey's post hoc. \*\*, p<0.01. **(C, D)** Two-way ANOVA with Tukey's post hoc (treatment, tissue). \*, p<0.05; \*\*, p<0.01; \*\*\*\*, p<0.0001. **(F)** Two-way ANOVA with Tukey's post hoc (treatment, time). #, p<0.05; \*\* or ##, p<0.01; ###, p<0.001; \*\*\*\* or ####, p<0.0001. In all graphs error bars represent the standard deviation of the mean.

**Figure 3. Hepatic expression of secGAA partially normalizes glycogen content in central nervous system.** **(A-E)** Nine-month-old mice were injected with an AAV8-secGAA (secGAA-Gaa<sup>-/-</sup>) vector at a dose of 2x10<sup>12</sup> vg/kg. Gaa<sup>+/+</sup> (PBS-Gaa<sup>+/+</sup>) and Gaa<sup>-/-</sup> (PBS-Gaa<sup>-/-</sup>) mice injected with PBS served as controls in the study. Animals were followed for nine months after treatment (n=3/4 per cohort). **(A)** GAA enzymatic activity in brain and spinal cord reported as percentage of PBS-Gaa<sup>+/+</sup> activity. **(B)** Glycogen content in brain and spinal cord reported as percentage of PBS-Gaa<sup>-/-</sup> glycogen. **(C-D)** Western blot analysis of brain **(C)** and spinal cord **(D)** lysates nine months after treatment using anti-GAA monoclonal antibody. An anti-tubulin antibody was used as loading control. **(E)** Representative images of periodic acid-Schiff (PAS) staining of brain sections. Insets represent caudate putamen (striatum) region of the brain. Statistical analysis: **(A, B)** Two-way ANOVA with Tukey's post hoc (treatment, tissue). \*, p<0.05; \*\*\*, p<0.001; \*\*\*\*, p<0.0001. In all graphs error bars represent the standard deviation of the mean.



**Figure 4. AAV mediated secGAA expression restores histology and partially reverses autophagy block in muscle.** (A-E) Nine-month-old mice were injected with an AAV8-secGAA (secGAA-Gaa<sup>-/-</sup>) vector at a dose of 2x10<sup>12</sup> vg/kg. Gaa<sup>+/+</sup> (PBS-Gaa<sup>+/+</sup>) and Gaa<sup>-/-</sup> (PBS-Gaa<sup>-/-</sup>) mice injected with PBS served as controls in the study. Animals were followed for nine months after treatment (n=3/4 per cohort). (A) Representative images of hematoxylin and eosin (H&E) staining of diaphragm, heart and triceps. Scale bar represents 10µm. (B) Western blot analysis of triceps lysates using anti-p62/SQSTM1 monoclonal antibody. An anti-GAPDH antibody was used as loading control. (C) Western blot analysis of triceps lysates using anti-Lamp1 polyclonal and anti-Beclin1 polyclonal antibodies. An anti-GAPDH antibody was used as loading control. (D) Western blot analysis of triceps lysates using anti-LC3b monoclonal antibody. An anti-GAPDH antibody was used as loading control. (E) Quantification of p62 levels normalized to GAPDH levels. (F) Quantification of Lamp1 levels normalized to GAPDH levels. (G) Quantification of Beclin1 levels normalized to GAPDH levels. (H) Quantification of LC3b-I levels normalized to GAPDH levels. (I) Electron microscopy analysis of *tibialis anterior*. Arrows indicate enlarged lysosomes. Scale bars represent 2µm. (J) Quantification of lysosome length from the sections in electron microscopy. Statistical analysis: (E-H) One-way ANOVA with Tukey's post hoc. \*, p<0.05; \*\*, p<0.01. (J) Un-paired t-test. \*, p<0.0001. In all graphs error bars represent the standard deviation of the mean.

**Figure 5. AAV8-secGAA restores physiological mitochondrial content in skeletal muscle.** (A-G) Nine-month-old mice were injected with an AAV8-secGAA (secGAA-Gaa<sup>-/-</sup>) vector at a dose of 2x10<sup>12</sup> vg/kg. Gaa<sup>+/+</sup> (PBS-Gaa<sup>+/+</sup>) and Gaa<sup>-/-</sup> (PBS-Gaa<sup>-/-</sup>) mice injected with PBS served

as controls in the study. Animals were followed for nine months after treatment (n=3/4 per cohort). **(A)** Mitochondria analysis in *tibialis anterior* using electron microscopy. Arrows indicate regions with accumulated mitochondria. Squares indicate zoomed areas which are presented in the lower panels. Scale bars present 2 $\mu$ m. **(B)** Western blot analysis of triceps lysates using anti-CoxIV monoclonal antibody. An anti-GAPDH antibody was used as loading control. **(C)** Quantification of CoxIV levels from the corresponding western blots shown in **(B)**. **(D)** Western blot analysis of triceps using anti-ATPb monoclonal antibody. An anti-GAPDH antibody was used as loading control. **(E)** Quantification of ATPb levels from the corresponding western blots shown in **(D)**. **(F)** Western blot analysis of triceps using anti-SDHA monoclonal antibody. An anti-GAPDH antibody was used as loading control. **(G)** Quantification of SDHA levels from the corresponding western blots shown in **(F)**. Statistical analysis: **(C, E, G)** One-way ANOVA with Tukey's post hoc. \*\*\*, p<0.001; \*\*\*\*, p<0.0001. In all graphs error bars represent the standard deviation of the mean.

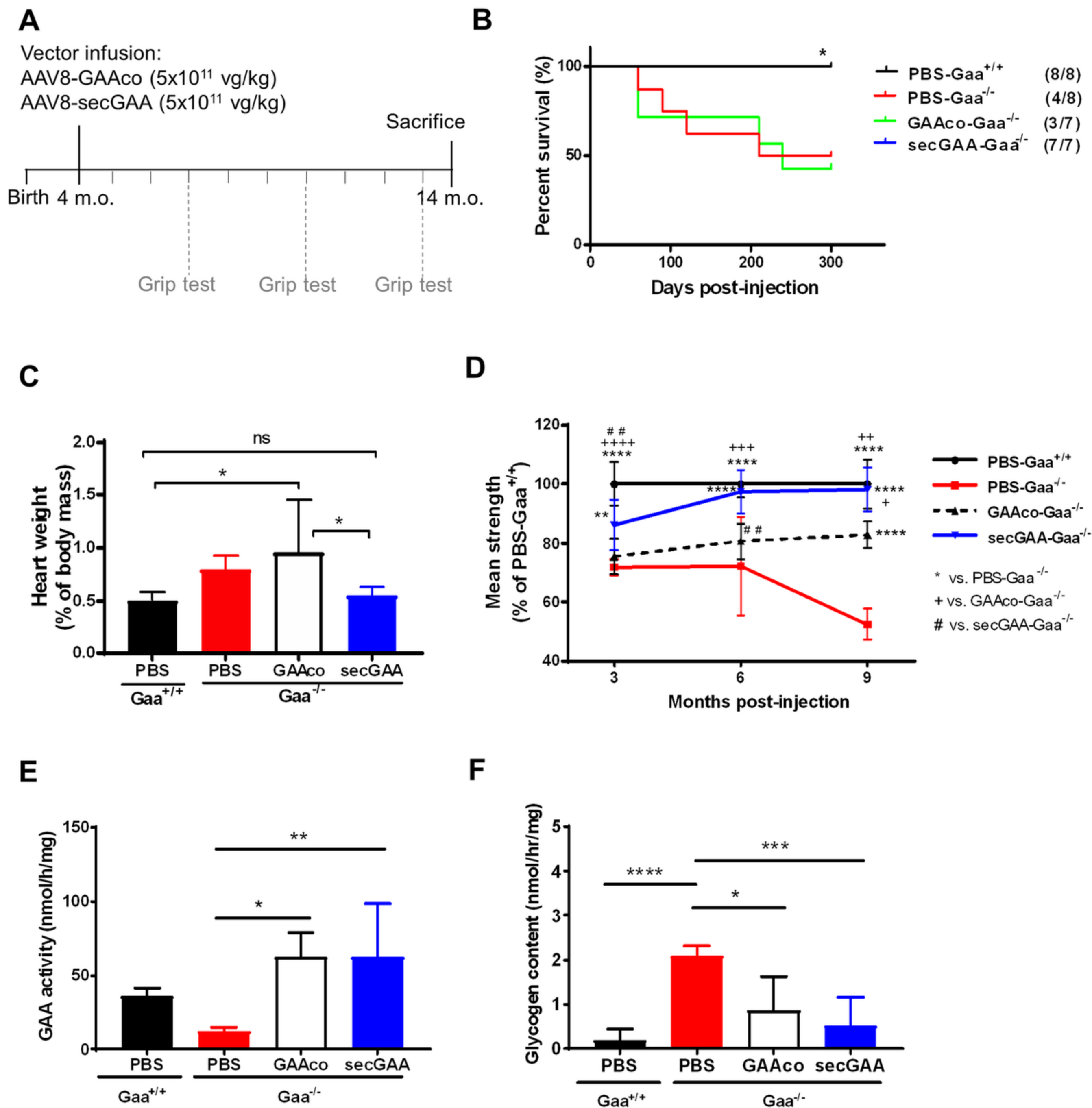
**Figure 6. AAV liver gene therapy largely normalizes transcriptome in muscle.** **(A-B)** Nine-month-old mice were injected with an AAV8-secGAA (secGAA-Gaa<sup>-/-</sup>) vector at a dose of 2x10<sup>12</sup> vg/kg. Gaa<sup>+/+</sup> (PBS-Gaa<sup>+/+</sup>) and Gaa<sup>-/-</sup> (PBS-Gaa<sup>-/-</sup>) mice injected with PBS served as controls in the study. Animals were followed for nine months after treatment (n=3/4 per cohort). **(A)** Gene expression in quadriceps muscle tissue from PBS-Gaa<sup>-/-</sup>, PBS-Gaa<sup>+/+</sup> and secGAA-Gaa<sup>-/-</sup> mice was analyzed by RNA sequencing. The expression profiles of 2,864 genes detected as differentially expressed in any of the three possible pairwise contrasts (Figure S4B) were assigned to clusters using kmeans. Three metaclusters, containing genes whose expression levels had been fully rescued (FR, 23.3%, 666 genes), partially rescued (PR, 51.3%, 1470 genes) or not

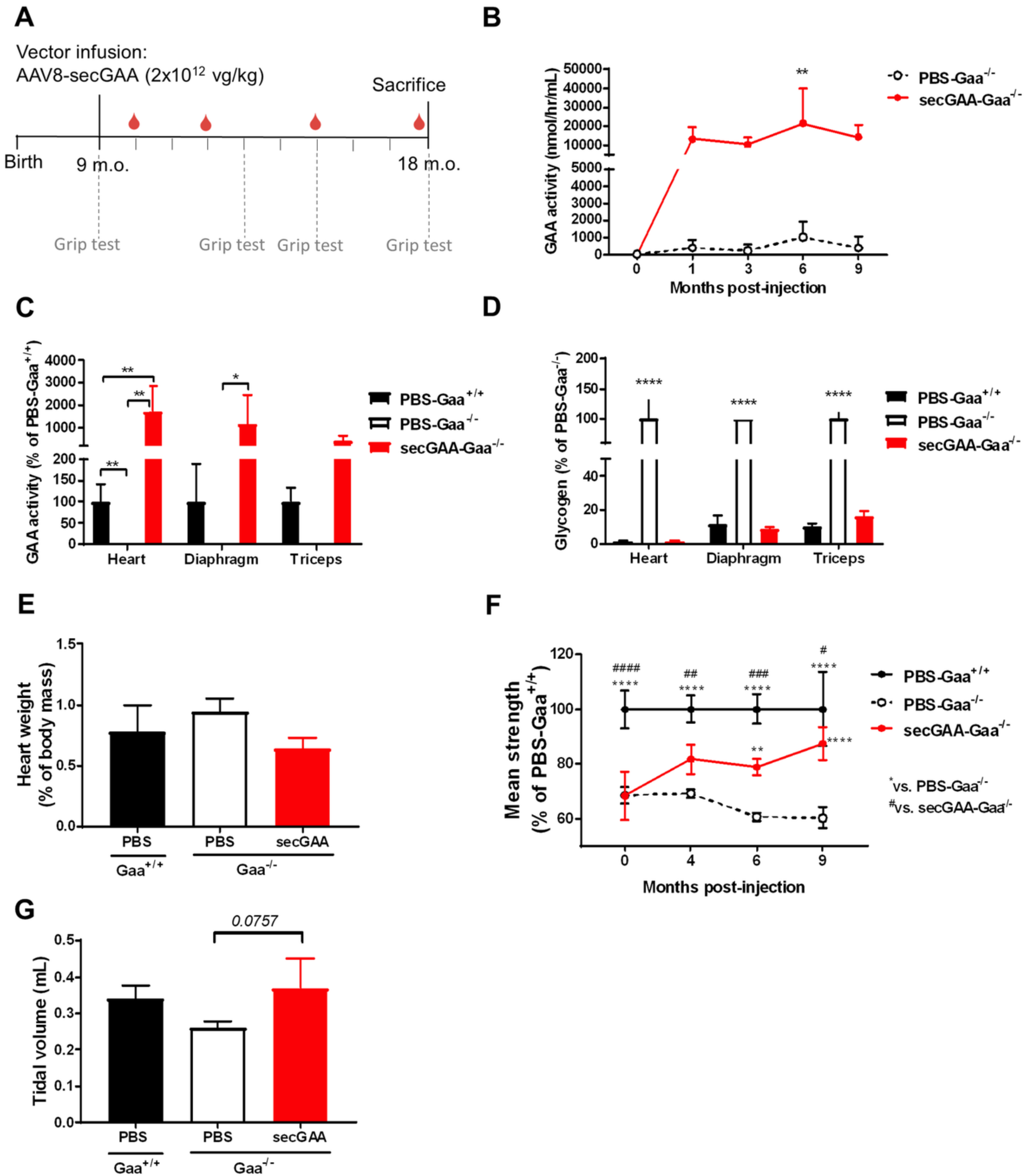
rescued (NR, 25.4%, 728 genes) by the expression of secGAA in  $GAA^{-/-}$  background, were defined by combining gene clusters with complementary expression profiles. Normalized expression counts, averaged for each condition and scaled for each gene, are represented on the Y-axis. Gray lines represent the expression profile of individual genes. Black lines represent average expression profiles for each of the combined clusters. **(B)** Significant associations in 9 of total 41 Canonical Pathways were found for the three metacluster gene lists (FR, PR, NR), after Ingenuity Pathway Analysis (IPA) enrichment analyses. Bar plots represent Fisher's test p values for nine of the pathways and the three metaclusters. Red lines indicate the significance threshold ( $p < 0.05$ ). Results for the remaining 33 pathways are presented in Supplemental figure 6.

**Figure 7. AAV8-secGAA administration partially normalize mitochondrial stress signaling in skeletal muscle.** **(A)** Significant associations to 257 upstream transcriptional regulators were found for the set of 2802 genes detected as differentially expressed in the comparison of PBS- $Gaa^{-/-}$  versus PBS- $Gaa^{+/+}$ , after IPA enrichment analyses. The circular plot represents a subset of 16 regulators involved in metabolic processes, and a selection of their connected functions, retrieved from IPA's knowledge base with the "grow" function. Circular crown sectors, representing transcriptional regulators, are colored according to activation z-score. Red and blue colors indicate a higher activation state in PBS- $Gaa^{-/-}$  or PBS- $Gaa^{+/+}$  mice, respectively. **(B-D)** Nine-month-old mice were injected with an AAV8-secGAA (secGAA- $Gaa^{-/-}$ ) vector at a dose of  $2 \times 10^{12}$  vg/kg.  $Gaa^{+/+}$  (PBS- $Gaa^{+/+}$ ) and  $Gaa^{-/-}$  (PBS- $Gaa^{-/-}$ ) mice injected with PBS served as controls in the study. Animals were followed for nine months after treatment (n=3/4 per cohort). **(B)** ROS production measured in snap-frozen tissues by EPR spectroscopy. **(C)** Western blot analysis of triceps lysates using monoclonal antibody anti-AMPK. An anti-GAPDH antibody

was used as loading control. **(D)** Quantification of AMPK levels normalized to GAPDH levels from the corresponding western blot shown in **(C)**. Statistical analysis: One-way ANOVA with Tukey's post hoc. \*\*,  $p < 0.01$ . In all graphs error bars represent the standard deviation of the mean.

**Figure 1**

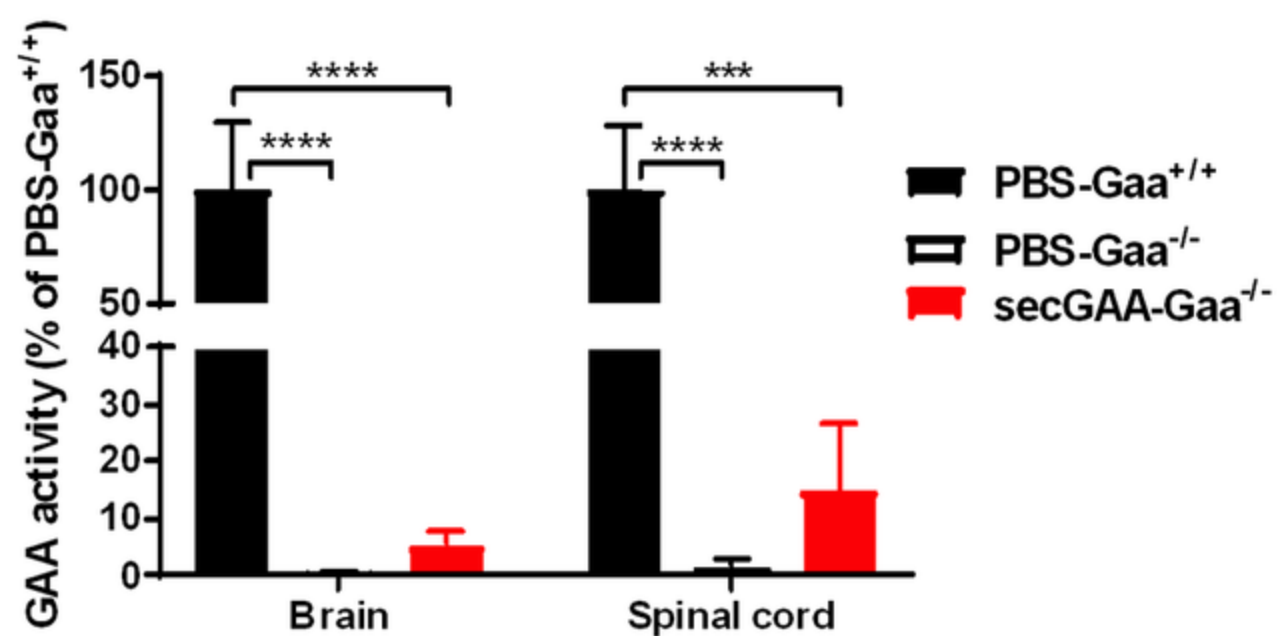


**Figure 2**

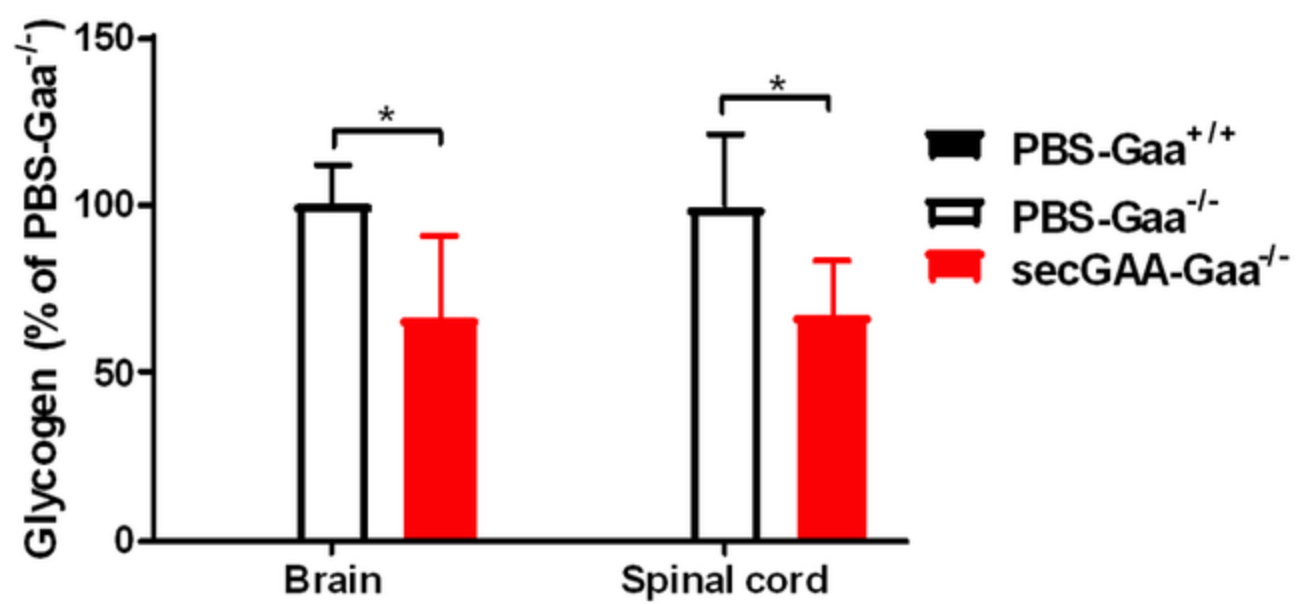


**Figure 3**

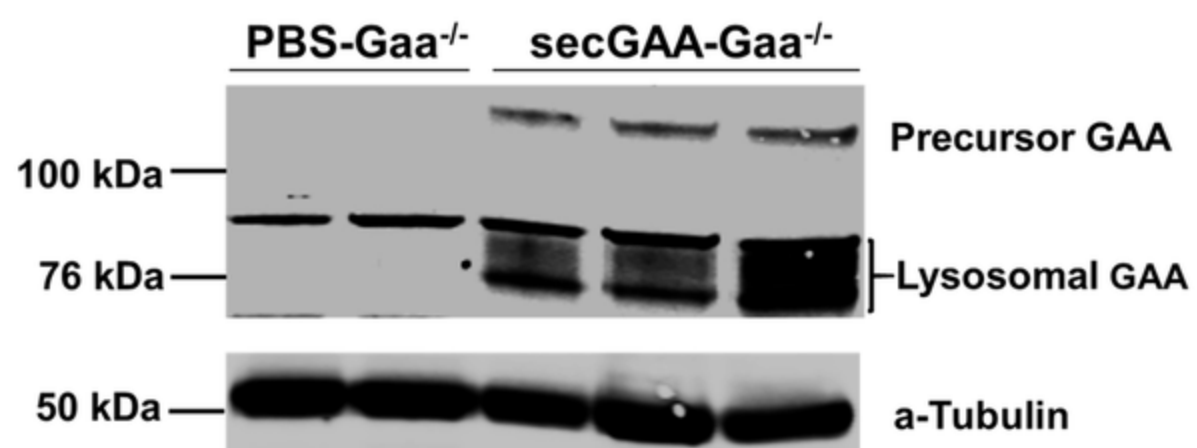
**A**



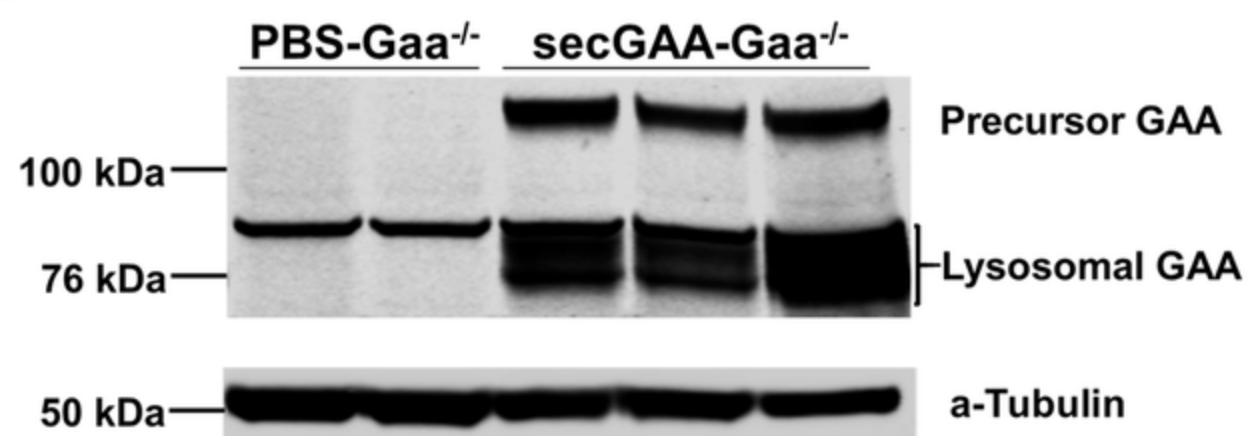
**B**



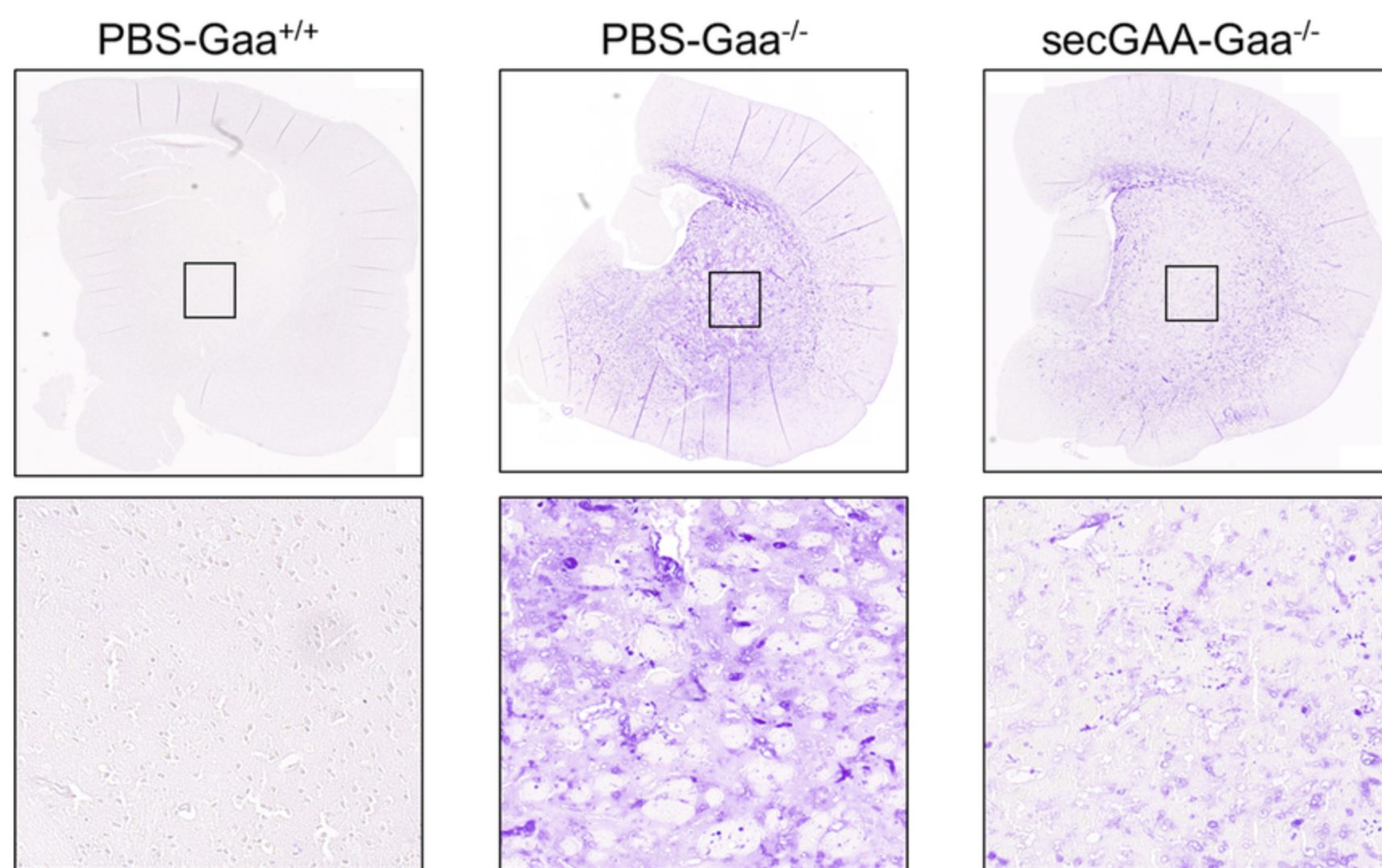
**C**



**D**



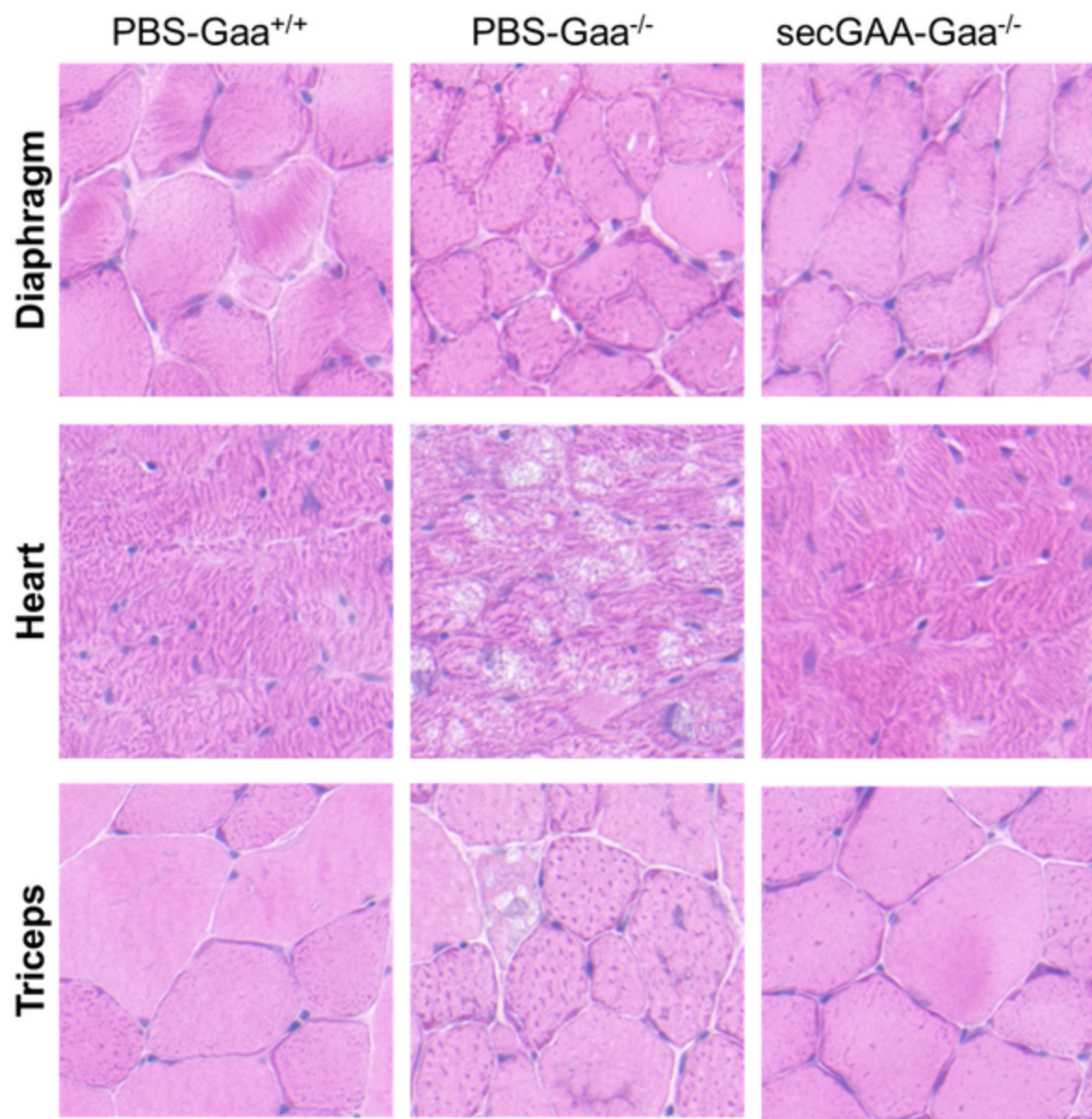
**E**



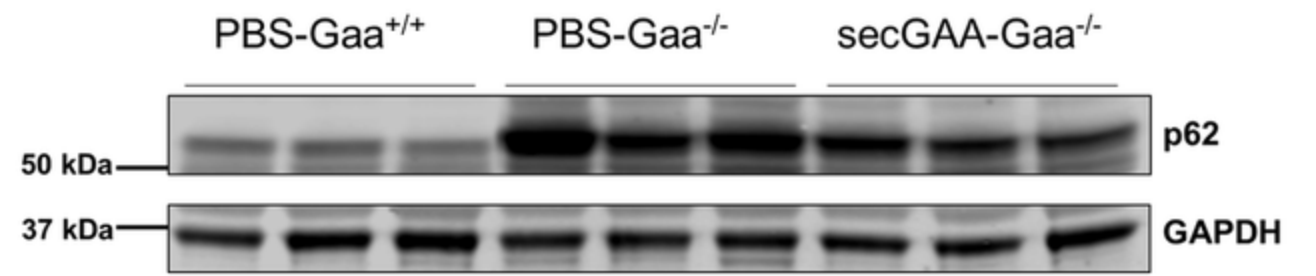


**Figure 4**

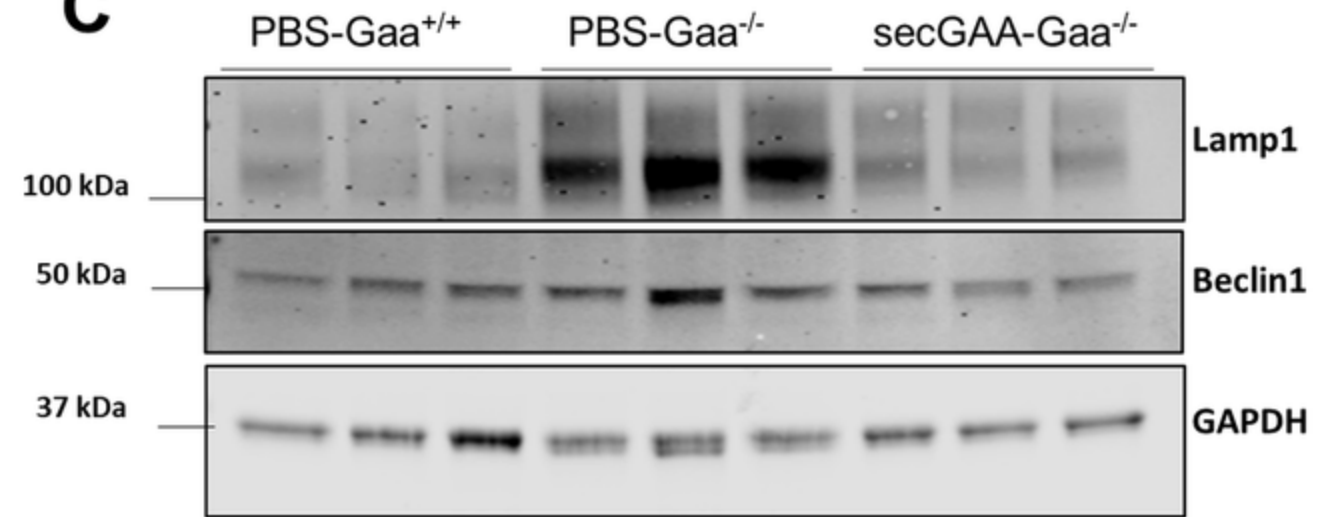
**A**



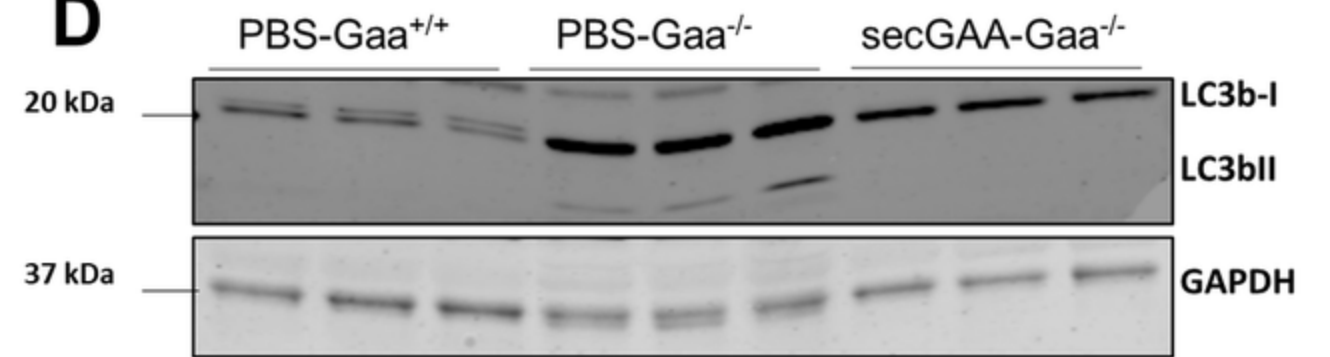
**B**



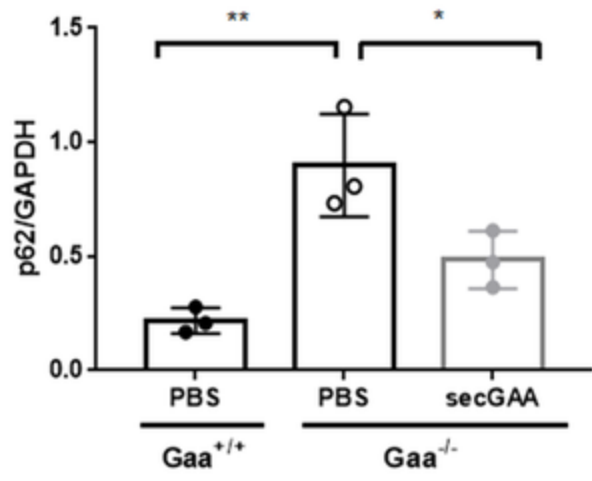
**C**



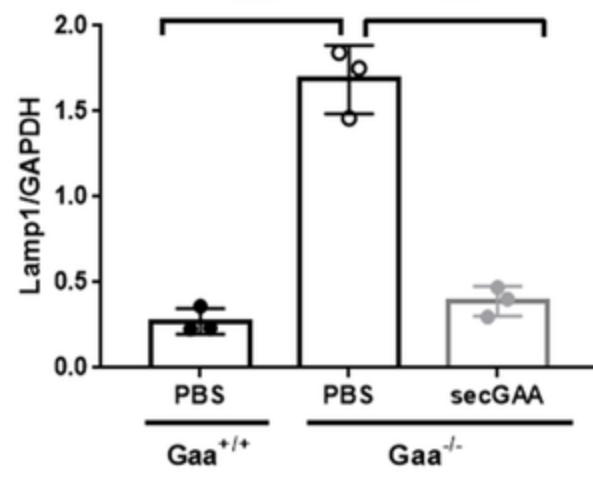
**D**



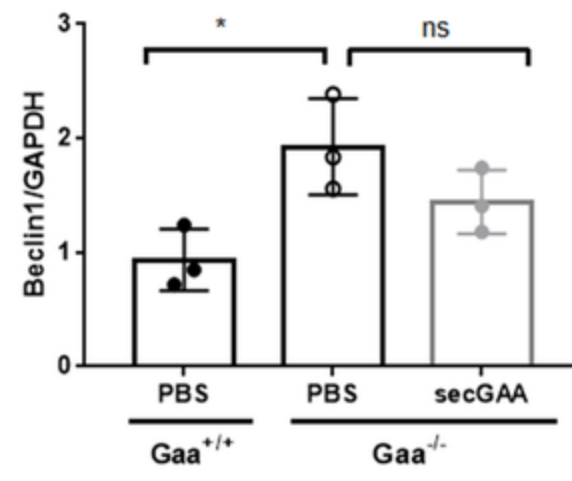
**E**



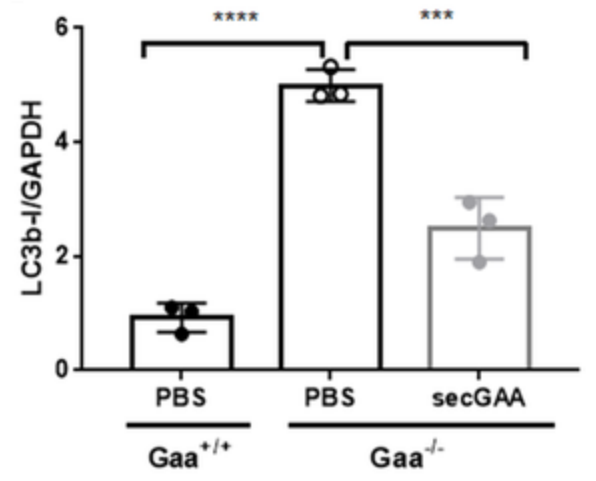
**F**



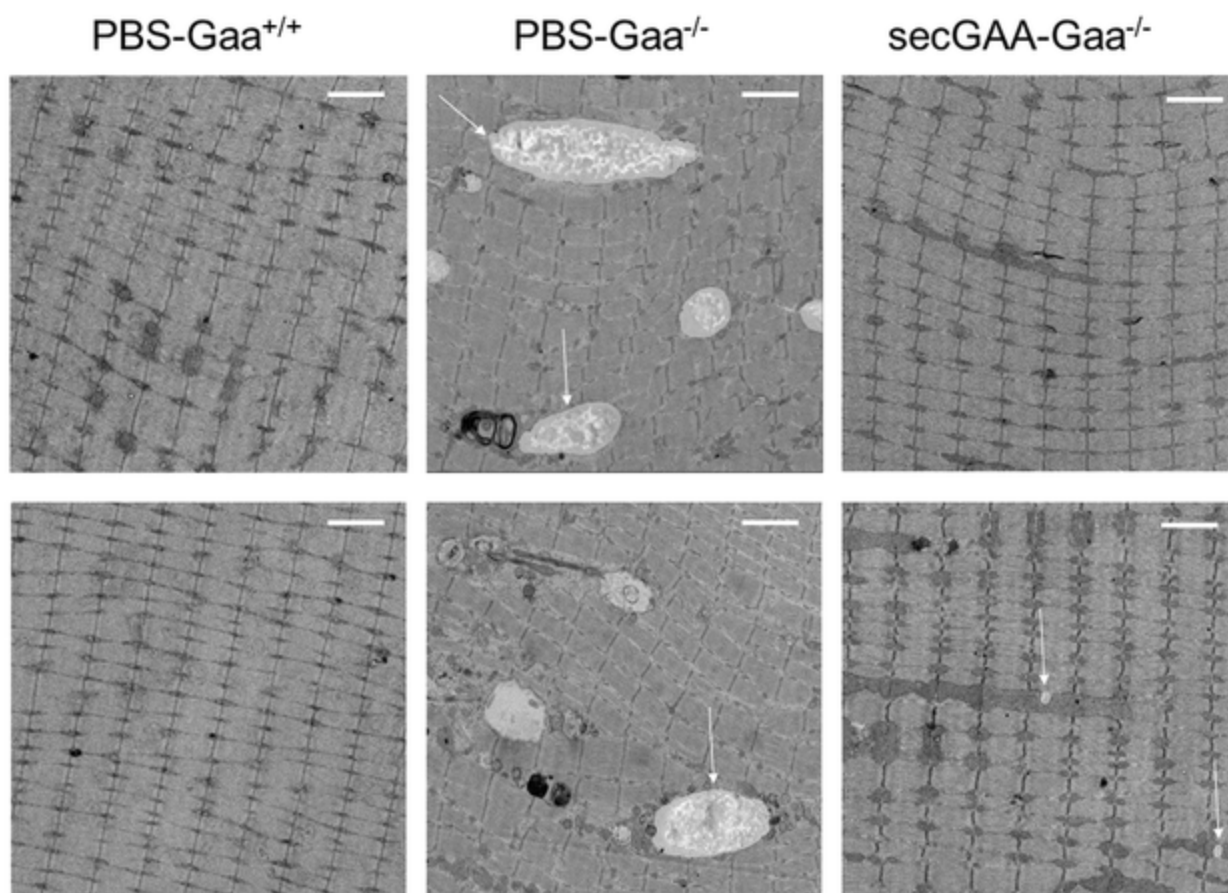
**G**



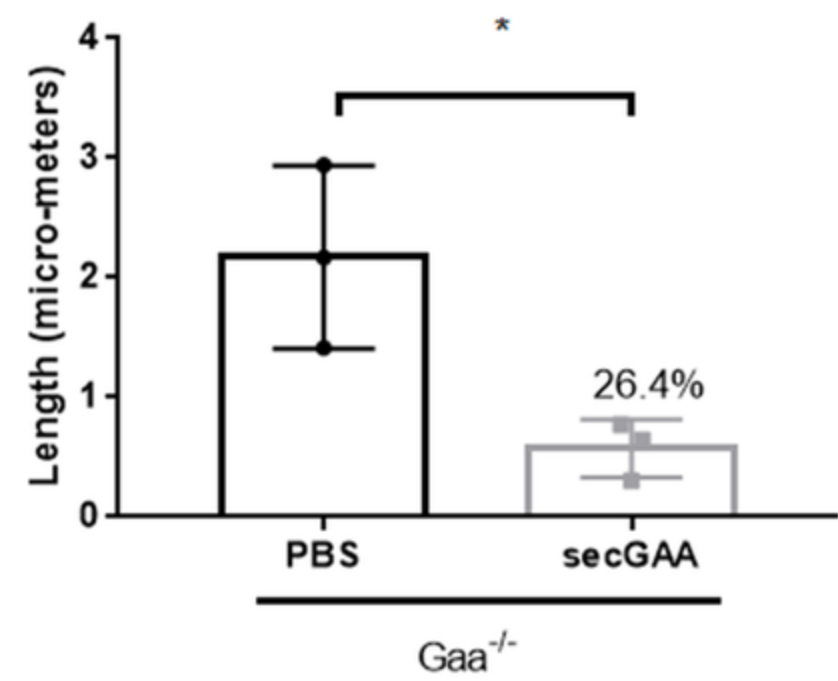
**H**



**I**



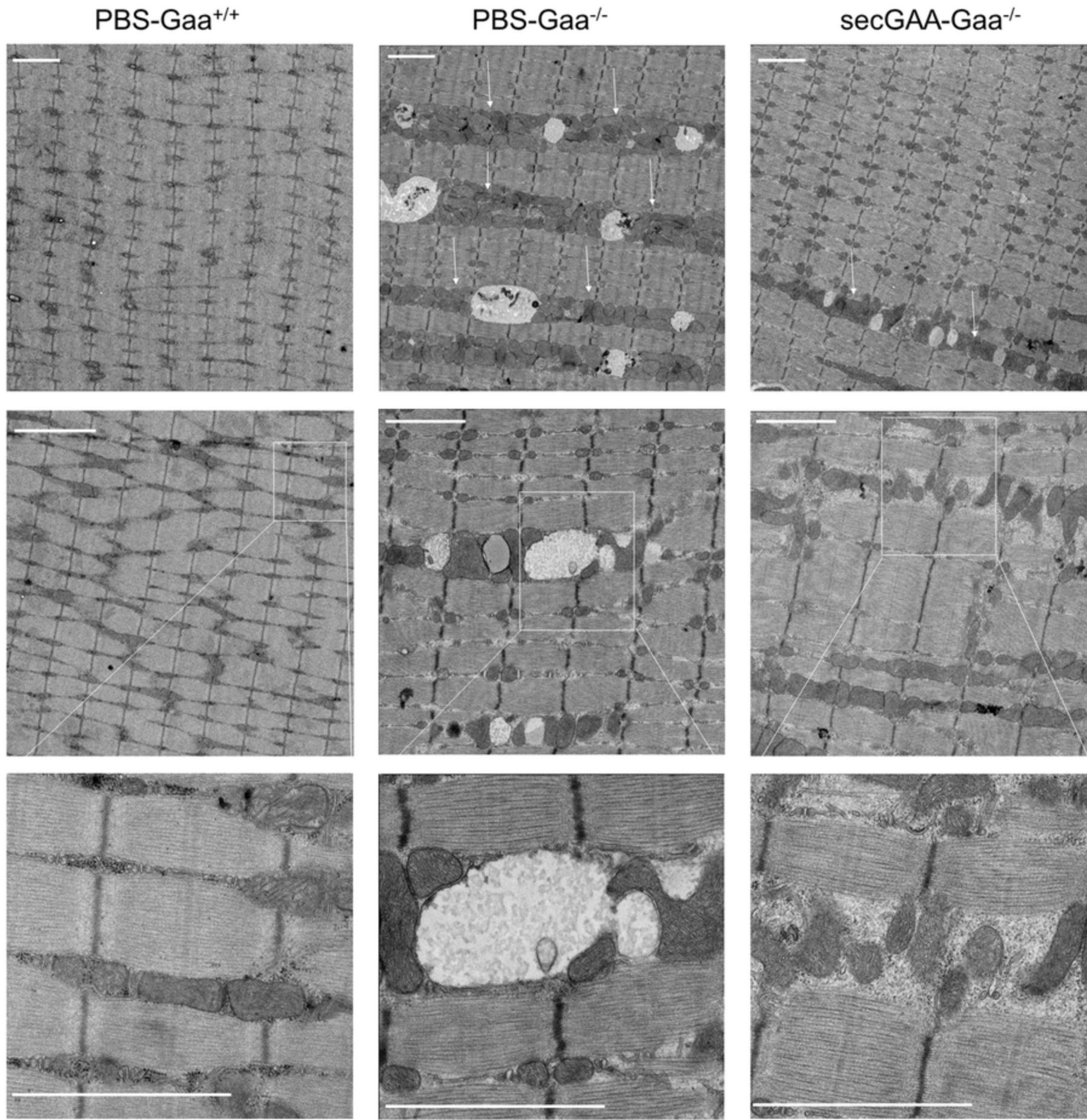
**J**



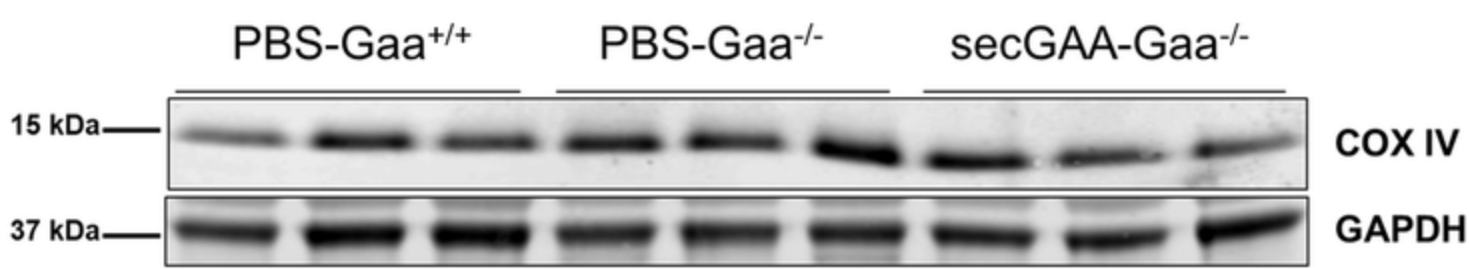


**Figure 5**

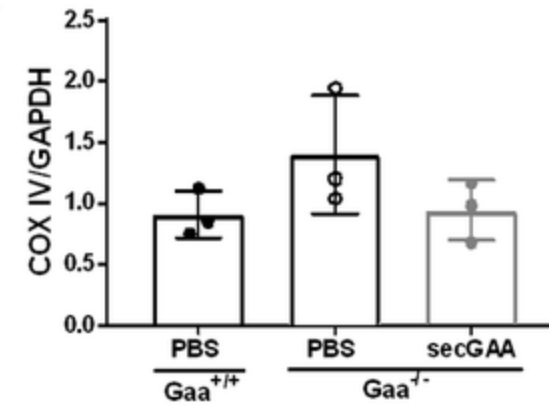
**A**



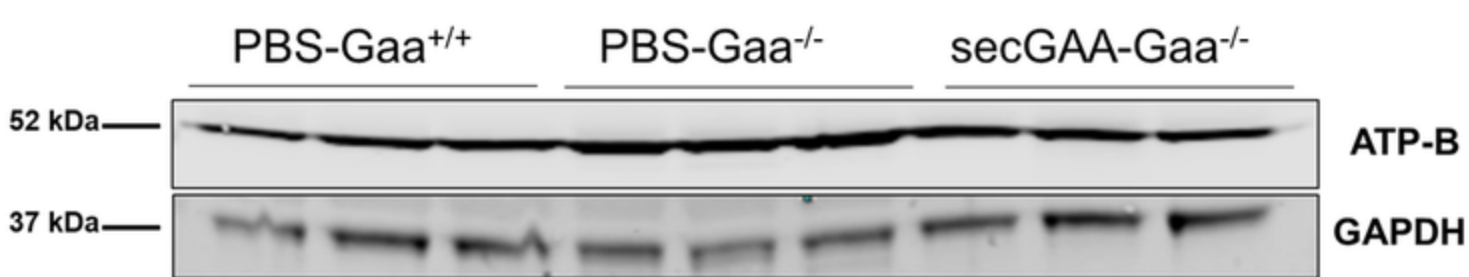
**B**



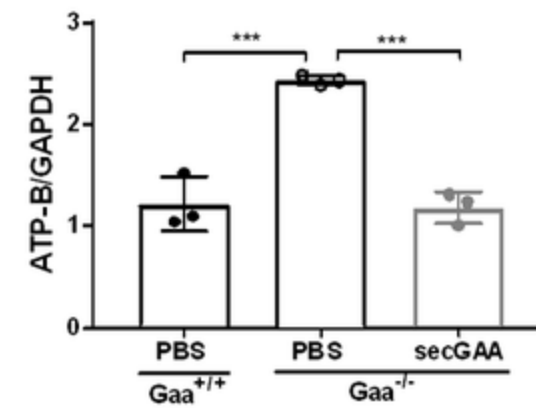
**C**



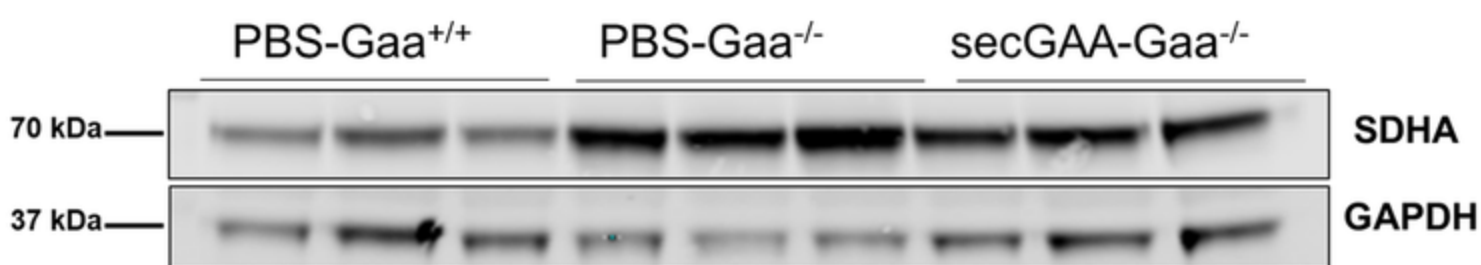
**D**



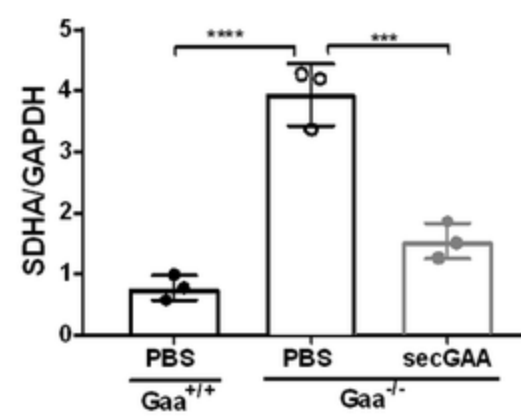
**E**



**F**



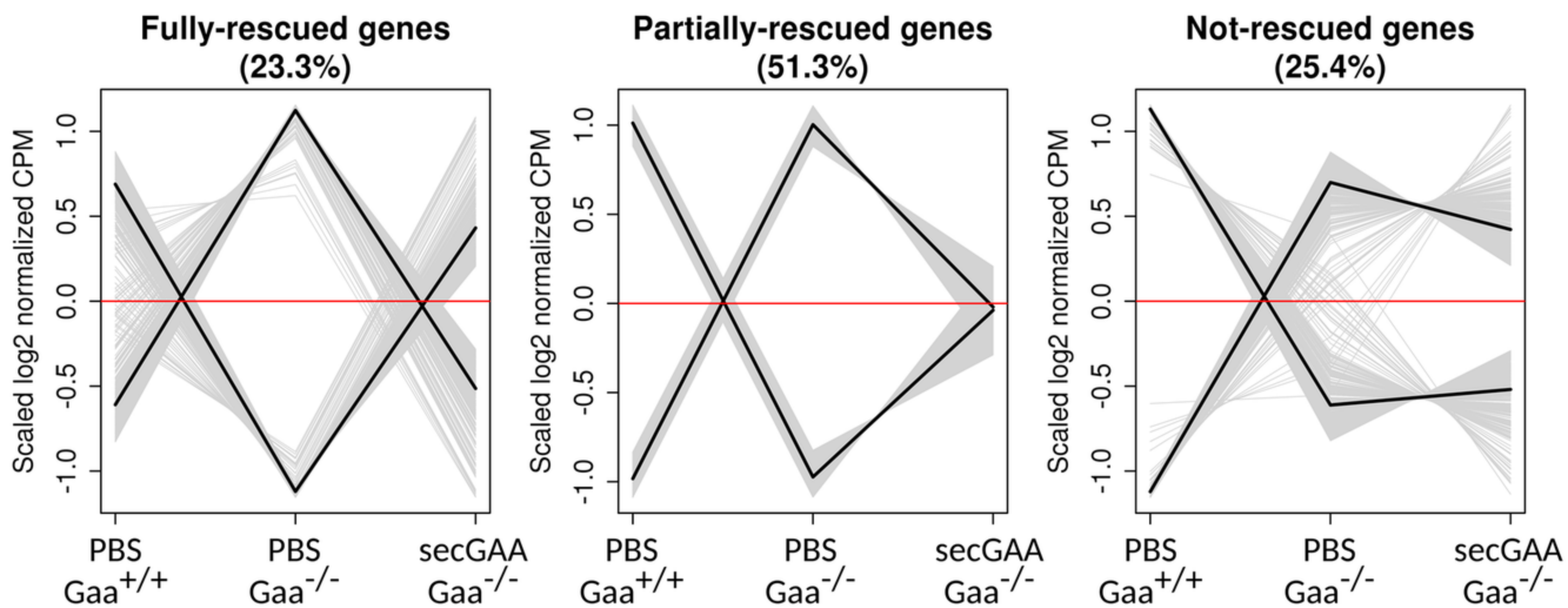
**G**



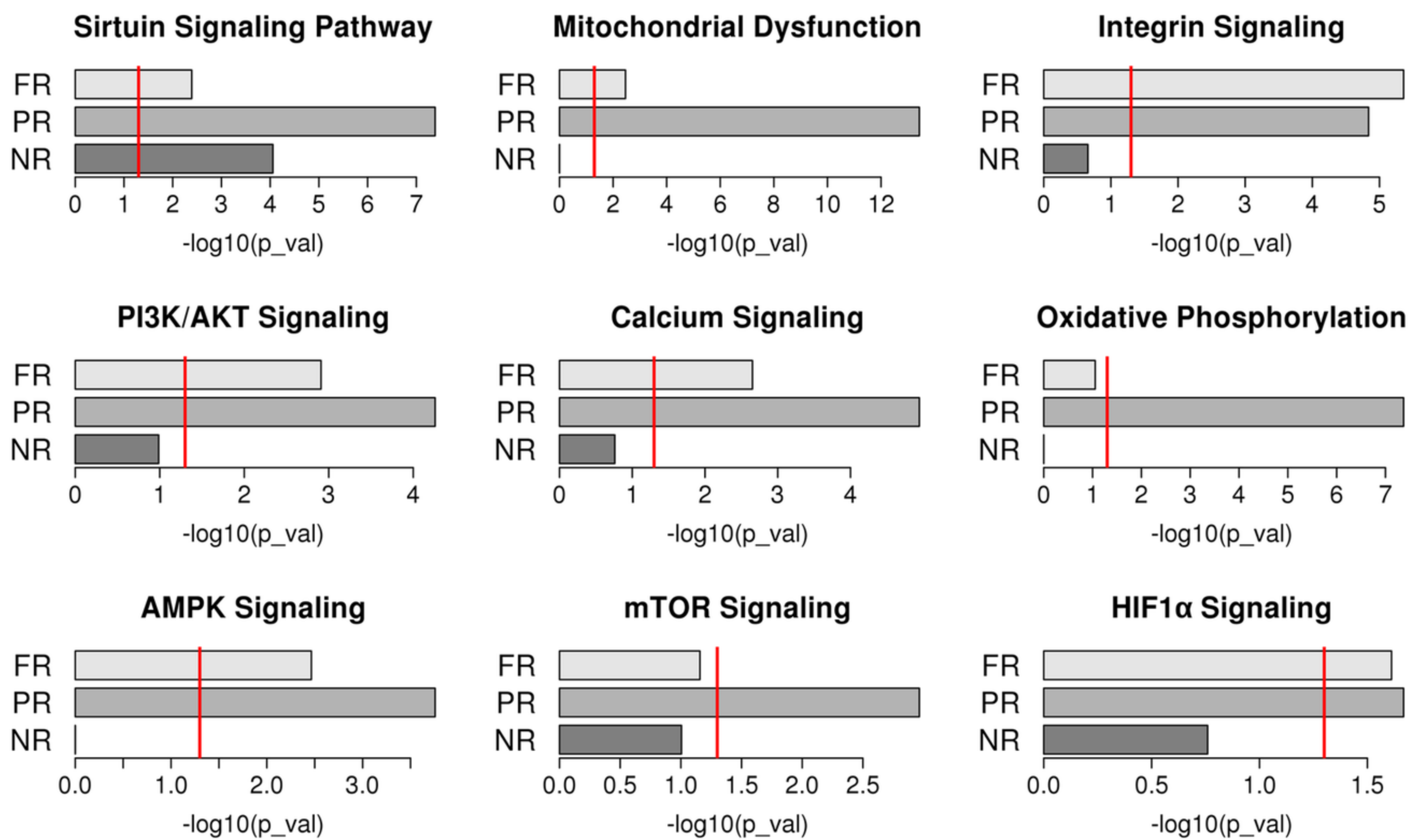


**Figure 6**

**A**

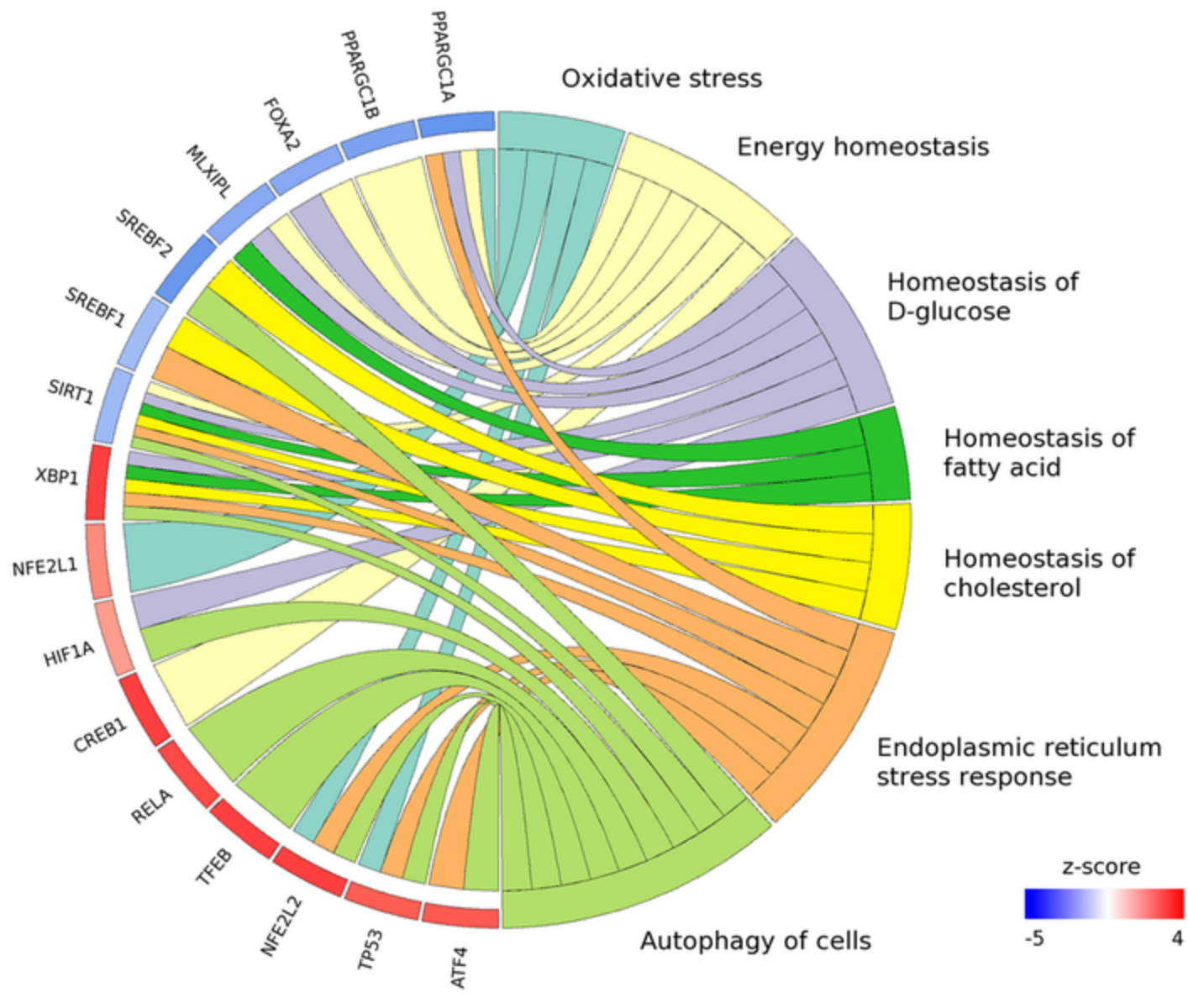


**B**

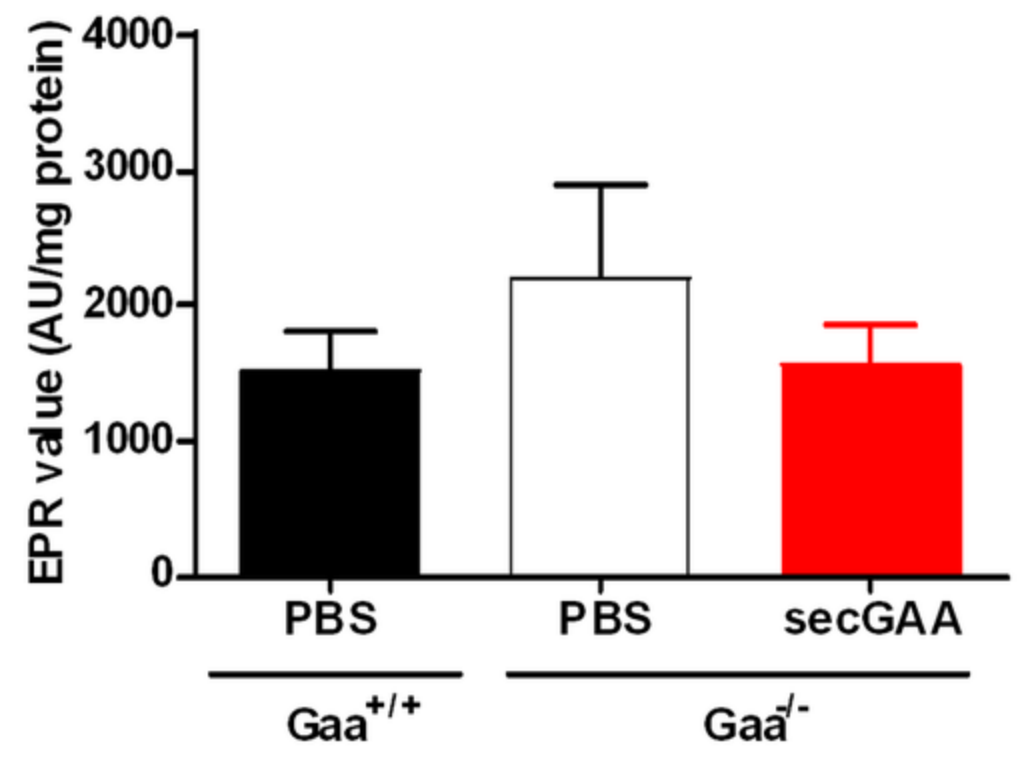


**Figure 7**

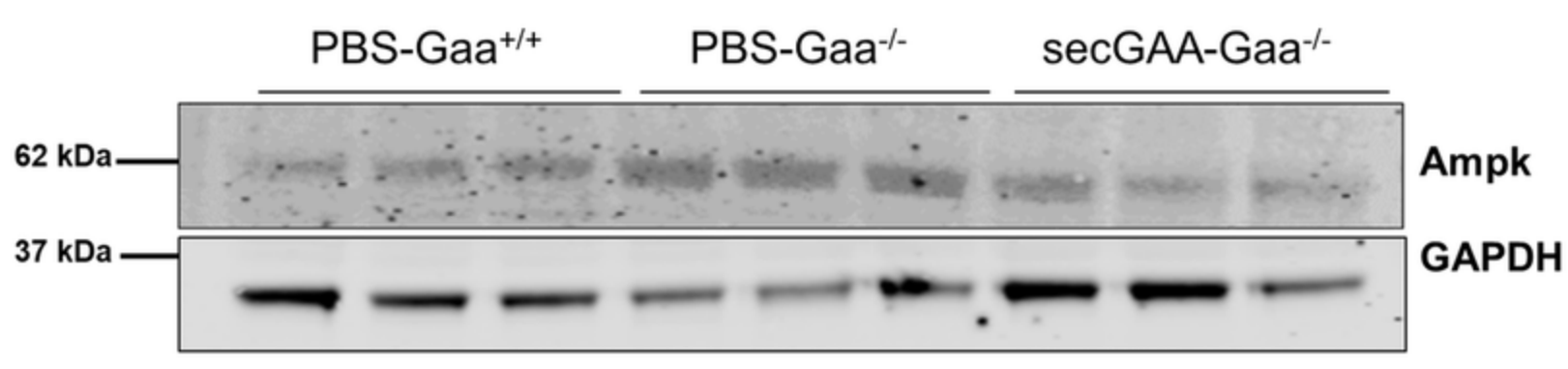
**A**



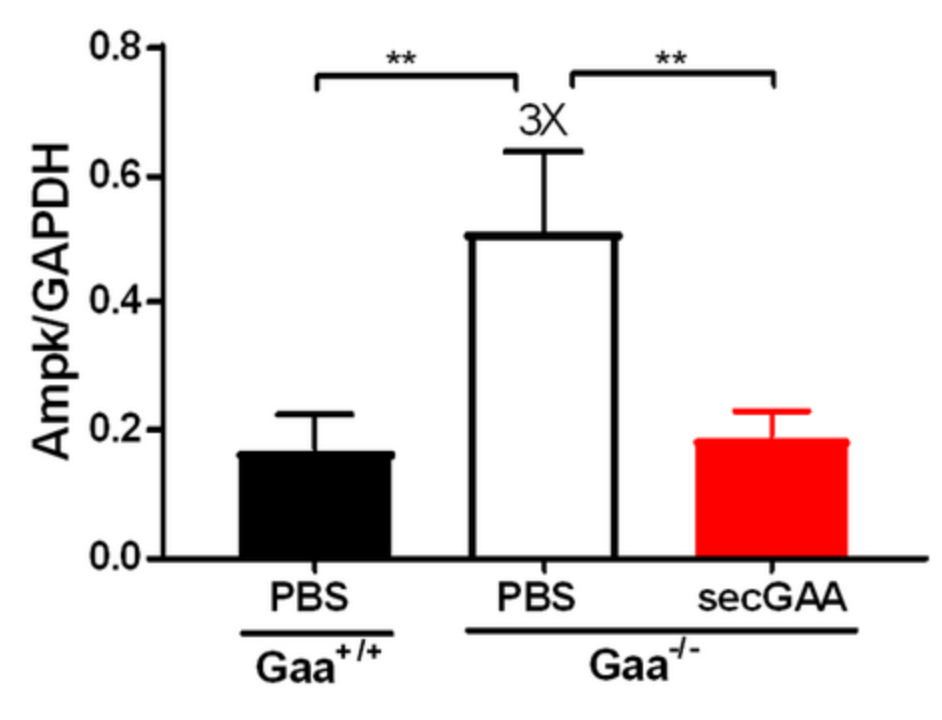
**B**

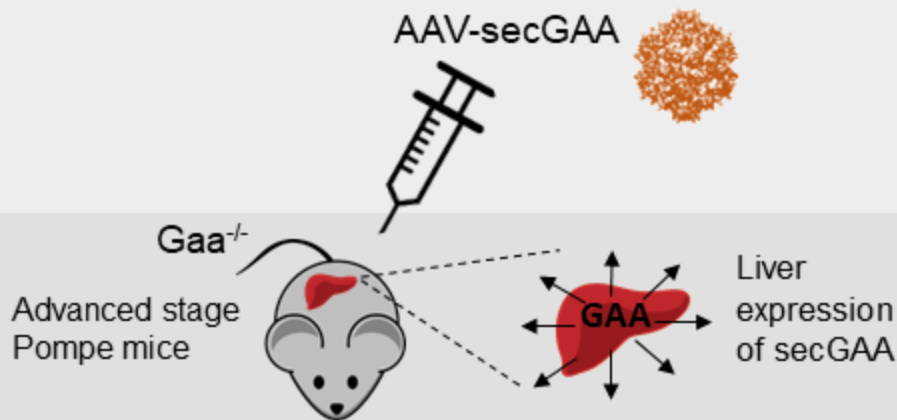


**C**



**D**





## Rescue of disease phenotype

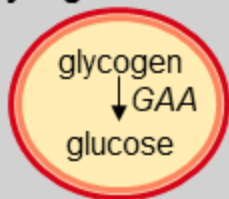
cardiomegaly



muscle strength

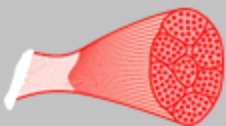


glycogen content



## Normalization of muscle

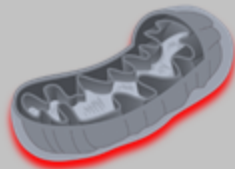
Ultrastructure



Autophagy



Mitophagy



Transcriptome

