

Inhibition of *CERS1* in skeletal muscle exacerbates age-related muscle dysfunction

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Abstract

Age-related muscle wasting and dysfunction render the elderly population vulnerable and incapacitated, while underlying mechanisms are poorly understood. Here, we implicate the *CERS1* enzyme of the de novo sphingolipid synthesis pathway in the pathogenesis of age-related skeletal muscle impairment. In humans, *CERS1* abundance declines with aging in skeletal muscle cells and, correlates with biological pathways involved in muscle function and myogenesis. Furthermore, *CERS1* is upregulated during myogenic differentiation. Pharmacological or genetic inhibition of *CERS1* in aged mice blunts myogenesis and deteriorates aged skeletal muscle mass and function, which is associated with the occurrence of morphological features typical of inflammation and fibrosis. Ablation of the *CERS1* orthologue *lagr-1* in *C. elegans* similarly exacerbates the age-associated decline in muscle function and integrity. We discover genetic variants reducing *CERS1* expression in human skeletal muscle and Mendelian randomization analysis in the UK biobank cohort shows that these variants reduce muscle grip strength and overall health. In summary, our findings link age-related impairments in muscle function to a reduction in *CERS1*, thereby underlining the importance of the sphingolipid biosynthesis pathway in age-related muscle homeostasis.

eLife assessment

This **solid** study presents **valuable** insights into the role of Cers1 on skeletal muscle function during aging, although further substantiation would help to fully establish the experimental assertions. It examines an unexplored aspect of muscle biology that is a relevant opening to future studies in this area of research.

Introduction

We are currently experiencing the biggest demographic shift in human history with a prospective doubling of the 65+ year old population by the year 2050, thereby constituting almost a quarter of the world's population for the first time¹. While the increase in human lifespan could provide unprecedented opportunities for aged individuals, healthspan is not quite following this trend: One-fifth of an individual's life will be lived with morbidity and reduced mobility². Health burden escalates with age, which not only incapacitates the elderly but also puts tremendous stress on the healthcare system exemplified by the 20% increased healthcare spending due to the growing elderly population in the last 10 years alone³. Hence, understanding the underpinnings of aging is an important first step to inform development of biological tools to promote healthy aging.

Skeletal muscle is one of the organs most affected by aging. 3-8% muscle mass and strength are lost yearly after 30 years of age and 6-15% after 65 years of age during normal human aging⁴. Exacerbated age-related loss of muscle mass and strength has recently been recognized as the disease sarcopenia, which predicts and correlates with mortality⁵. The importance of sarcopenia is further highlighted by the high prevalence of sarcopenia in the common 60+ year old population (10-20%)⁶, especially considering that sarcopenia is one of the leading causes for frailty, loss of independence and reduced quality of life⁷. Several mechanisms have been implicated in the skeletal muscle aging process, including reduced mitochondrial bioenergetics coupled with increased levels of reactive oxygen species, dysfunctional proteostasis, reduced hormone levels and signaling, nutritional intake, increased, inflammation, loss of motor units, as well as reduced capacities for muscle regeneration and maturation/synthesis (for a review, see⁸). Recent evidence further suggests the involvement of sphingolipids in skeletal muscle homeostasis upon aging⁹.

Sphingolipids are bioactive lipids exerting pleiotropic cellular functions such as inflammation, proliferation, myelination, cell growth, and cell death¹⁰. Ceramides are the building blocks of most complex sphingolipids, which contain a sphingoid base (typically 18 carbon dihydrosphingosine or sphingosine) attached to a variable length fatty acyl side-chain. While sphingolipids have been shown to be involved in skeletal muscle bioenergetics^{11,12}, insulin sensitivity¹³ and diabetes¹⁴, the contribution of ceramides to skeletal muscle aging is not well understood. We recently reported that the first enzyme of the *de novo* sphingolipid biosynthesis pathway (SPT) is involved in skeletal muscle aging¹⁵. However, less is known about the involvement of ceramide synthases (Cers) in muscle aging, despite their prominent role in membrane homeostasis and cellular signaling. Six ceramide synthases (Cers1-6) catalyze ceramide synthesis in mammals resulting in a range of ceramides from C14:0 to C36:0¹¹. Depending on the enzyme, different length fatty acyl-coenzyme A (CoA) are transferred to the amine group of the sphingoid base¹⁶. For example, Cers1 only uses 18 carbon (C18) fatty acids to create C18 (d18:1/18:0) ceramides, whereas Cers2 predominantly synthesizes d18:1/24:0 (C24:0) and d18:1/24:1 (C24:1) ceramides and Cers5 uses C16 acyl-CoA substrates for acylation of the free primary amine group of sphingoid bases to form the corresponding C16:0 ceramides¹⁷. *Cers1* and *Cers5* are the two most expressed ceramide synthases in skeletal muscle¹⁸. Reducing *Cers1* function by genetic or pharmacological means has been shown to reduce adiposity and body weight upon exposing mice to a high fat diet^{11,19}. Moreover, BMI was found to correlate with C18:0 ceramide species, pointing towards *Cers1* as a desirable target for metabolic interventions²⁰. On the other hand, recent evidence demonstrates that skeletal muscle-specific deletion of *Cers1* in young mice reduces muscle fiber size and force, suggesting that *Cers1* is needed for proper skeletal muscle function in young mice²¹. However, *Cers1*' involvement during organismal aging, and particularly its mechanistic function and relevance in human skeletal muscle is not well characterized.

In the present study, we show that *CERS1* is involved in the age-related loss in muscle mass and function. Performing unbiased gene set enrichment analyses of *CERS1*-correlating transcripts in human skeletal muscle, we discover that *CERS1* levels correlate most strongly with muscle function and myogenesis. Our experiments validate the increase in *CERS1* abundance in mouse and human skeletal muscle cells during myogenic differentiation. Conversely, the selective pharmacological inhibitor of *CERS1*, P053¹¹, impairs myogenic maturation across several timepoints by blunting expression of myogenic regulatory factors and consequently, myosin heavy- and light chains. In aged mice, P053 treatment or AAV9-mediated silencing of *Cers1* in skeletal muscle attenuates C18:0/C18:1 ceramide and dihydroceramide species, and exacerbates the age-related decline in muscle fiber size while elevating inflammation and fibrosis. These morphological alterations coincide with reduced myogenesis and reduced muscle mass and function. Lenti- or adenovirus-mediated *CERS1* inhibition specifically in mouse or human muscle cells recapitulates maturation defects of muscle cells. Pharmacological or genetic inhibition of the *CERS1* orthologue *lagr-1* in *C. elegans* similarly impairs age-related muscle function and morphology suggesting conserved *CERS1* function across species. Finally, we identify single nucleotide variants in the genomic *CERS1* locus, which reduce *CERS1* levels in skeletal muscle and reduce muscle grip strength as well as overall health in humans.

Results

CERS1 expression correlates with muscle contraction and myogenesis

The sphingolipid synthesis pathway produces ceramides and other sphingolipids by using fatty acids and amino acids as substrates (Figure 1A). SPT converts L-serine and palmitoyl-CoA to 3-ketosphinganine, which is rapidly converted to sphinganine. Coupling of sphinganine to long-chain fatty acid is accomplished by one of 6 distinct mammalian ceramide synthases. Among these, *Cers1* expression in skeletal muscle tissue was shown to correlate negatively with aging²¹. To expand on this finding and assess whether the age-related decline in *CERS1* abundance is mediated specifically by skeletal muscle cells, we measured *CERS1* expression in primary muscle cells isolated from young and old human donors. We found reduced *CERS1* transcript levels in aged compared to young myoblasts which is in line with the previous report in whole muscle tissue²¹ (Figure 1B).

To study the role of *CERS1* in human skeletal muscle, we performed unbiased gene set enrichment analyses of *CERS1* correlating transcripts in human skeletal muscle biopsies. Molecular pathways involved in muscle contraction and myogenesis were among the strongest correlating pathways (Figure 1C). In line with this finding, we also observed significant positive correlations of *CERS1* expression with transcripts involved in myogenesis (*MYF5*, *MYH2*, *MYH7*, *MYL1*; Figure 1D) and muscle contraction (*TPM2*, *ACTA1*, *TMOD2*, *TNN1*; Figure 1A). Assaying the myogenic differentiation process in mouse and human muscle cells confirmed the upregulation of *Cers1*/*CERS1* at several timepoints once muscle differentiation was induced (Figure S1B and Figure 1E). *CERS1* has been shown to synthesize C18:0 and C18:1 ceramides^{17,22,23}. Measurement of ceramide levels during myogenic differentiation confirmed particularly the increase of C18:1 during mouse myoblast maturation (Figure S1C), whereas C14:0, C22:0, as well as C18:0 ceramide species were found to be induced during human myoblast differentiation suggesting some species-specific effects (Figure S1D).

Importantly, the *CERS1*-specific pharmacological inhibitor P053, which has been shown to reduce fat mass¹¹, inhibited mouse and human skeletal muscle cell differentiation as evidenced by downregulated expression of the early expressed myogenic transcription factors *Myog*/*MYOG* and

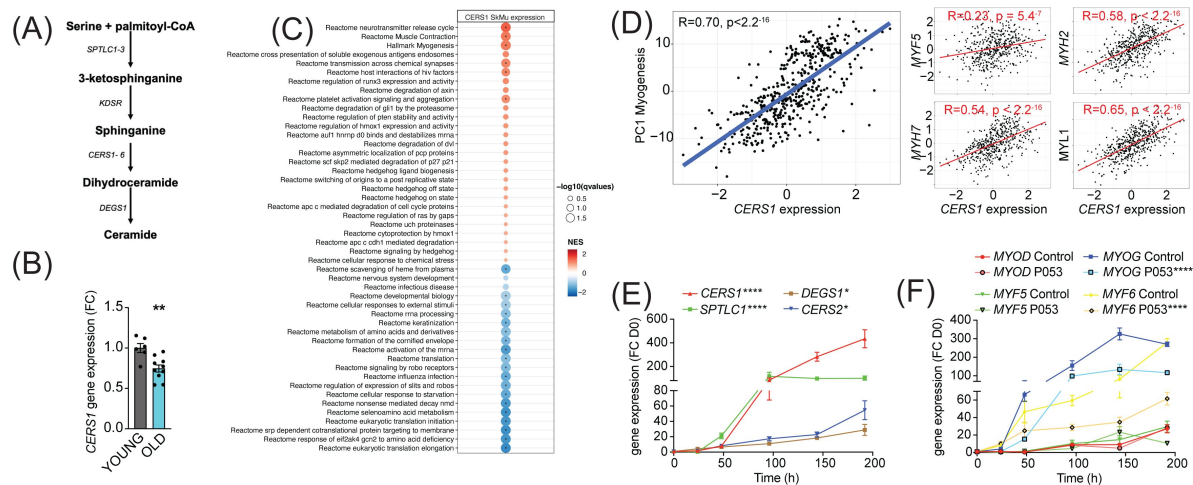


Figure 1.

CER1-correlated pathways in human skeletal muscle associate with muscle function and myogenesis. **(A)** Overview over the *de novo* sphingolipid biosynthesis pathway. **(B)** *CER1* expression in young (22 ± 3.61yo) and old (74 ± 8.4yo) human skeletal muscle cells (n=3-5 biological replicates). **(C)** Gene set enrichment analysis of *CER1* mRNA correlated transcripts in the human skeletal muscle from the Genotype-Tissue Expression (GTEx) dataset (n=469). Normalized effect size (NES). **(D)** Spearman correlation between skeletal muscle expression of *CER1* and the first principal component (PC1) of the myogenesis pathway (left) from **(C)** and expression of key genes involved in myogenesis (right). **(E-F)** Gene expression of enzymes involved in the sphingolipid *de novo* synthesis pathway **(E)** and myogenic regulatory factors **(F)** upon horse serum induced differentiation of primary human skeletal muscle cells (n=2-3 biological replicates). The pharmacological inhibitor P053 or DMSO was used at 1uM in **(E-F)**. Data: mean ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001.

Myf6/MYF6 (Figure S1E and Figure 1F) as well as of myosin light- and heavy chains (Figure S1F and Figure S1G). In aggregate, these findings suggest that *CERS1* is involved in skeletal muscle cell maturation.

Pharmacological inhibition of *Cers1* impairs skeletal muscle in aged mice

We next assessed the systemic effect of the pharmacological *Cers1* inhibitor P053 on skeletal muscle homeostasis in aged mice. As expected, systemic administration of P053 over 6 months (Figure 2A) reduced C18:0 and C18:1 ceramides in skeletal muscle tissue (Figure 2B), suggesting that P053 indeed targeted *Cers1* as previously shown¹¹. Interestingly, this was coupled with an increase in C24:0/C24:1 ceramides (Figure 2B) and C24:0/C24:1 dihydroceramides (Figure S2A), which are synthesized by *Cers2*^{17,24,25}. This might reflect a compensatory mechanism to maintain the flow of sphinganine and fatty acid substrates. An increase in C24:0/C24:1 ceramide and dihydroceramides upon *Cers1* inhibition is in line with a previous reports on *Cers1* inhibition^{11,21}. Laminin staining (Figure 2C) followed by minimum ferret diameter measurement (Figure S2B) and fiber size distribution (Figure 2D) showed that fiber size was reduced upon aging with a frequency shift towards smaller muscle fibers. Treatment with P053 exacerbated this age-related reduction in muscle fiber size. We next automated the pipeline to detect skeletal muscle fibers in mouse tissue sections by custom training and application of the recently described deep learning-based segmentation algorithm cellpose²⁶. Ground truth data, model training and validation, as well as the script to run on the data are publicly available on Zenodo (DOI: 10.5281/zenodo.7041137). Using our trained model in muscle sections stained with laminin and CD45, we were able to capture most muscle fibers (Average Precision: 0.97 ± 0.02, n=4, Table S1). Results showed an age-related decrease in cross sectional area, which worsened with P053 treatment (Figure 2E, top and Figure S2C) and is in agreement with our manual curation of fiber diameter (Figure 2C-D). We also observed an increase in CD45⁺ neighboring muscle fibers with age and P053 treatment (Figure 2E, bottom). The effect of age and P053 on muscle inflammation was confirmed by another CD45 staining with DAPI showing an increased proportion of CD45⁺ cells upon aging, which was exacerbated by P053 administration (Figure S2D). Analyses of other morphological markers of skeletal muscle aging, fibrosis and centralized nuclei, revealed that pharmacological inhibition of *Cers1* led to increased fibrosis (Figure 2F) and occurrence of centralized nuclei (Figure 2G). In line with our findings of differentiating skeletal muscle cell cultures (Figure 1F and Figure S1E-G), P053 treatment downregulated expression of myogenic regulatory factors and of myosin heavy chain transcripts in mice (Figure S2E). Of note, P053-mediated alterations in skeletal muscle morphology and expression of myogenic factors were correlated with impairments in skeletal muscle mass and function. Lean body mass was reduced upon aging and aged mice treated with P053 presented further reductions in muscle mass (Figure 2H), which is in line with reduced fiber diameters (Figure 2C-D) and cross section area (Figure S2C). Measurement of functional muscle parameters revealed that P053 administration worsened the effect of aging by reducing grip strength (Figure 2I) and treadmill running performance in aged mice (Figure S2F-G). Our observation of pharmacologic *Cers1* inhibition in aged mice is in line with a previous study where genetic knockout of *Cers1* in young mice impaired muscle contractility²¹.

Muscle-specific genetic inhibition of *CERS1* impairs aged muscle and blunts myogenesis

Systemic administration of P053 in aged mice might have exerted effects on other tissues that affect aged skeletal muscle. Moreover, pharmacological strategies might have undesired off target effects. Therefore, we investigated the effect of direct genetic inhibition of *Cers1* in aged mouse skeletal muscle. A single intramuscular injection of AAV9 with short hairpin RNA (shRNA) against mouse *Cers1* (Figure 3A) in 18-month-old mice reduced *Cers1* mRNA expression by ≈ 50% at 24

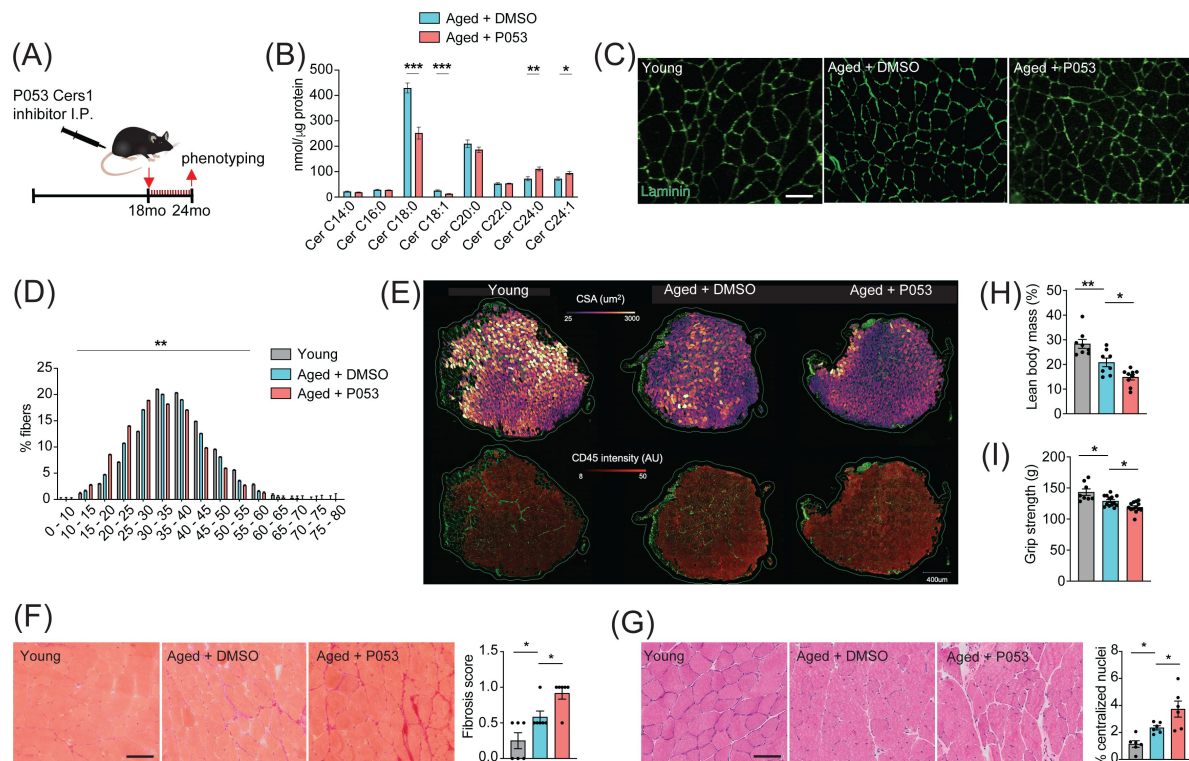


Figure 2.

P053 administration inhibiting *Cers1* in aged mice deteriorates skeletal muscle function and morphology. **(A)** Overview over the experimental pipeline that was used to administer P053 in aged C57BL/6j mice using intraperitoneal injections (I.P.) three times a week for 6 months. **(B)** Skeletal muscle ceramide levels in aged mice treated with DMSO or P053 (n=7-8 per group). **(C)** Representative images of laminin-stained tibialis anterior of young control or mice injected with DMSO or P053 (n=5-6 per group). Scale bar, 50 μ m. **(D)** Quantification of minimal Ferret diameter distribution in skeletal muscle cross sections from young control or mice injected with DMSO or P053 (n=5-6 per group). **(E)** Automated detection of laminin/CD45⁺ stained muscle fibers from young control or aged mice injected with DMSO or P053 showing cross sectional area (top) and inflammation-adjacent signal intensities (bottom). **(F)** Representative brightfield images and quantification of Sirius red stained muscle sections from young and aged mice treated with DMSO or P053 (n=6 per group). **(G)** Representative brightfield images and quantification of muscle cross sections from young and aged mice treated with DMSO or P053 stained with hematoxylin/eosin (n=6 per group). **(H-I)** Phenotyping measurements of young control and aged mice injected with DMSO or P053 showing **(H)** lean body mass and **(I)** grip strength (n=7-14 per group). Data: mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

months of age (**Figure S2H**) and hence, reduced C18:0 and C18:1 ceramide metabolites (**Figure 3B**). Similarly to our observation in P053-treated animals, genetic inhibition of *Cers1* in aged skeletal muscle tended to increase *Cers2*-derived very long chain ceramides (**Figure 3B**) and dihydroceramides (**Figure S2I**). Genetic silencing of *Cers1* reduced average skeletal muscle fiber size (**Figure 3C** and **Figure S2J**), shifting muscle fibers towards smaller fibers (**Figure 3D**). This was in line with our custom cellpose model for fiber detection in muscle cross sections showing that *Cers1*-deficient, aged muscles had smaller cross-sectional area (**Figure 3E**, top and **Figure S2K**), and showed more signs of inflammation (**Figure 3E**, bottom and **Figure S2L**). Furthermore, ablation of *Cers1* in aged muscle showed a trend towards increased fibrosis (**Figure 3F**) and more centralized nuclei (**Figure 3G**). We also found that genetic targeting of *Cers1* downregulated expression of myogenic regulatory factors and myosin heavy chains (**Figure S3A**). These findings were coupled with reduced muscle mass (**Figure 3H**), as well as reduced muscle grip strength (**Figure 3I**) and running performance in *Cers1*-deficient aged mice (**Figure S3B-C**). Reduced grip strength upon *Cers1* inhibition in aged mice is in line with reduced ex vivo contraction capacity of young muscles deficient of *Cers1*²¹. Overall, our results indicate that genetic, AAV9-mediated silencing of *Cers1* in skeletal muscle of aged mice deteriorates skeletal muscle morphology and function and therefore, recapitulate the effects observed by pharmacological inhibition of *Cers1*.

***Cers1* mediated deterioration of myogenesis is specific to skeletal muscle cells**

Several different cell types reside within human and mouse skeletal muscle tissue²⁷. To investigate whether the observed detrimental effects of genetic *Cers1* inhibition on muscle cell maturation are specific to skeletal muscle cells, we generated mouse and human skeletal muscle cells deficient of *Cers1/CERS1*. Lentivirus-mediated silencing of *Cers1* using shRNA in mouse C2C12 muscle progenitor cells reduced *Cers1* expression (**Figure S3D**) and with it, C18:0/C18:1 ceramide species (**Figure S3E**). This was coupled by an increase in potentially toxic very long chain ceramides (**Figure S3E**) and dihydroceramides (**Figure S3F**). Differentiation of these *Cers1* deficient myoblasts towards myotubes revealed diminished myogenesis evidenced by immunostaining (**Figure S3G**), which shows reduced myotube diameter, myotube area and number of multinucleated myotubes (**Figure S3H**). Expression profiling revealed reduced transcript expression of myogenic differentiation markers (**Figure S3I**), further suggesting that *Cers1* is indispensable for proper myogenic differentiation of mouse muscle cells. Notably, *Cers1* inhibition did not affect protein synthesis but upregulated the Myostatin pathway including the Foxo3/Smad3 transcription factors and its downstream E3 ubiquitin ligases, suggesting an increase in protein degradation (**Figure S4A-D**). We next silenced *CERS1* in isolated primary human myoblasts using adenovirus-mediated delivery of a shRNA construct targeting the human *CERS1* transcript (**Figure S4E**). As expected, reducing *CERS1* expression in human primary myoblasts reduced C18:0/C18:1 ceramides (**Figure 4A**). Similar to mouse tissue and cells, *CERS1* inhibition increased very long chain C24:0/C24:1 ceramides (**Figure 4A**) and C24:0/C24:1 dihydroceramides (**Figure S4F**) and impaired myogenic differentiation, as indicated by immunostaining (**Figure 4B**) of differentiating myoblasts showing smaller myotube diameter, myotube area and reduction in multinucleated myotubes (**Figure S4G-I**) and expression profiling (**Figure 4C**). Therefore, *CERS1* appears indispensable for intact myogenic maturation in mouse and human myoblasts.

Inhibition of the *CERS1* orthologue *lagr-1* in *C. elegans* deteriorates healthspan

Maintaining proper muscle function is vital to healthy aging and potent modulators of this process are typically conserved across species. Hence, the effect of inhibiting *Cers1* on skeletal muscle function and morphology upon *C. elegans* aging was evaluated. We used the RW1596 transgenic

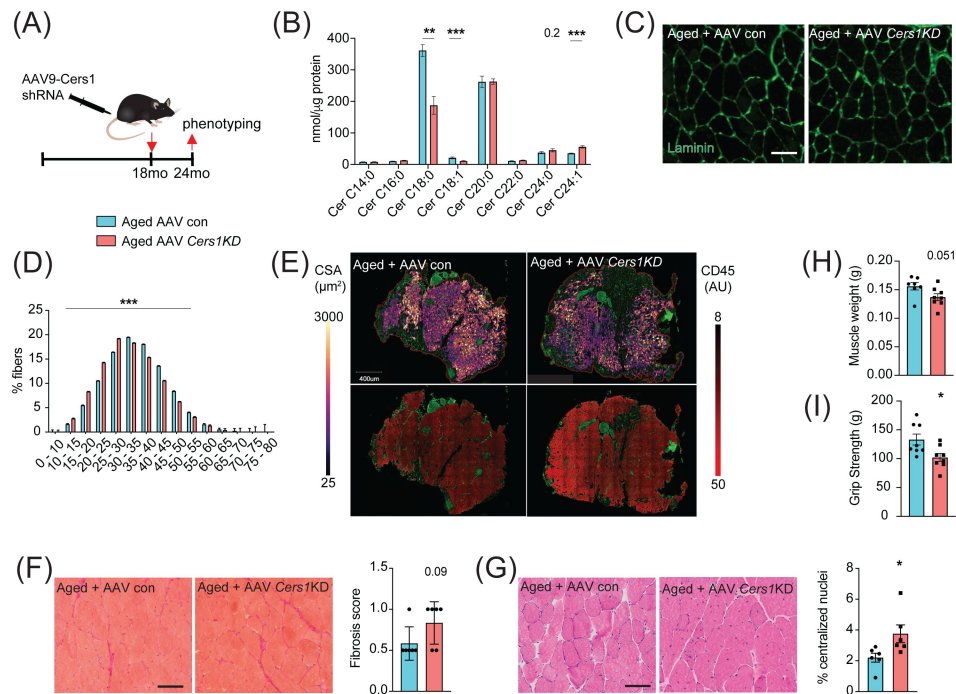


Figure 3.

Adeno-associated virus 9 (AAV9) mediated knockdown of *Cers1* expression in aged skeletal muscle reduces skeletal muscle function and morphology. **(A)** Schematic showing a single gastrocnemius intramuscular injection of adeno-associated virus particles containing short hairpin RNA against *Cers1* in aged C57BL/6J mice. **(B)** Skeletal muscle ceramide levels in aged mice intramuscularly injected with shRNA targeting *Cers1* (n=6-8). **(C)** Representative images of laminin-stained aged mice muscle cross sections intramuscularly injected with shRNA targeting *Cers1* (n=6 per group). Scale bar, 50μM. **(D)** Quantification of minimal Ferret diameter distribution in aged mice intramuscularly injected with shRNA targeting *Cers1* (n=6 per group). **(E)** Automated detection of laminin/CD45⁺ stained skeletal muscle fibers from aged mice intramuscularly injected with shRNA-*Cers1* showing cross sectional area (top) and inflammation-adjacent signal intensities (bottom). **(F)** Representative brightfield images and quantification of Sirius red stained skeletal muscle cross sections from mice intramuscularly injected with AAV9 particles containing scramble, or shRNA targeting *Cers1* (n=6 per group). **(G)** Representative brightfield images and quantification of hematoxylin/eosin-stained muscle cross sections from mice intramuscularly injected with AAV9 particles containing scramble, or shRNA targeting *Cers1* (n=6 per group). **(H-I)** Phenotyping measurements of aged mice intramuscularly injected with shRNA-*Cers1* or a scramble shRNA with **(H)** gastrocnemius muscle weight and **(I)** grip strength (n=6-8 per group). Data: mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

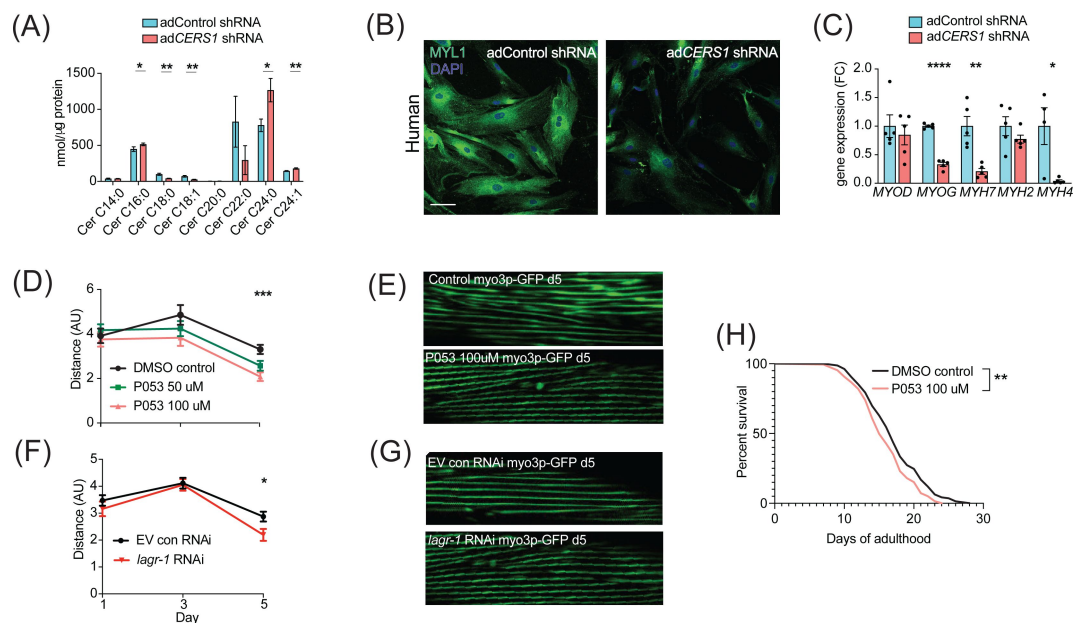


Figure 4.

Inhibition of *Cers1* in muscle cells impairs myogenic differentiation and *C. elegans* healthspan. **(A)** Primary human skeletal muscle cell ceramide levels infected with adenovirus containing silencing RNA targeting *CERS1* (n=5-6 per group). **(B)** Representative confocal immunocytochemistry images of differentiating human primary muscle cells deficient of *CERS1* (n=4 per group, left). Scale bar, 50µM. **(C)** Gene expression profiling of differentiating human primary muscle cells deficient of *CERS1* (n=4-5 per group, right). **(D)** Travelled distance in *C. elegans* treated with DMSO, 50µM P053 or 100µM P053 (n=40-57 per group). **(E)** Representative confocal muscle images of RW1596 *C. elegans* (myo3p-GFP) treated with DMSO or 100µM P053 (n=6 per group). **(F)** Travelled distance in *C. elegans* (myo3p-GFP) treated with EV control, or RNAi against *lagr-1* (n=46-56 per group). **(G)** Representative confocal muscle images of RW1596 *C. elegans* (myo3p-GFP) treated with control, or RNAi against *lagr-1* (n=6 per group). **(H)** Lifespan of *C. elegans* treated with control, or 100µM P053 (n=144-147 per group). Data: mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

worm strain expressing GFP under the control of the muscle-specific *myo3p* worm promoter, which allowed us to visualize muscle fibers as shown previously²⁸. Exposure of *C. elegans* to P053 mixed within the agar (50uM and 100uM) reduced muscle function at the onset of worm aging (day 5) when movement distance and speed are declining (**Figure 4D**, **Figure S5A-B**). In line with our observation of reduced muscle function when using P053 in mice, imaging muscle fibers of P053-treated worms showed deteriorated muscle morphology at day 5 as shown by the presence of rigged fibers compared to the smoother muscle fibers observed in the control worms that were exposed to DMSO vehicle (**Figure 4E**). We next evaluated the effect of genetic inhibition of the *CERS1* orthologue *lagr-1* on worm muscle function and morphology. Feeding RW1596 worms with an interference RNA (RNAi) targeting the *lagr-1* transcript reduced its expression by ~80% (**Figure S5C**), and reduced worm muscle function as measured by travelled distance and movement speed (**Figure 4F** and **Figure S5D**). Assessment of muscle fiber morphology revealed deteriorated muscle fibers in worms treated with *lagr-1* RNAi at day 5 (**Figure 4G**). Moreover, inhibition of *Cers1* using P053 reduced worm lifespan (**Figure 4H**). Taken together, these results suggest that pharmacological or genetic inhibition of the *Cers1* orthologue impairs certain hallmarks of healthy worm aging as suggested by reduced motility and muscle fiber morphology. This is most evident at the onset of worm aging, suggesting that observed effects in *Cers1* function might be conserved across species.

***CERS1* genetic variants reduce muscle *CERS1* expression and muscle function in humans**

We next sought to relate our functional mouse and worm findings of *Cers1*/*CERS1* inhibition impacting on muscle function to humans. To this end, we searched for both common (allele frequency > 1%) and rare genetic variants (allele frequency <1%) that alter *CERS1* levels either indirectly via regulatory elements, or directly by mutations in the *CERS1* coding region, respectively.

By exploring the Genotype-Tissue Expression (GTEx) dataset, we discovered three independent (linkage disequilibrium $r^2 < 0.1$; **Figure S5E**), common genetic variants that significantly alter *CERS1* expression in human skeletal muscle (**Figure S5F**). The C-allele at rs117558072 and the G-allele at rs1122821 reduce muscle *CERS1* expression, while the T-allele at rs71332140 increases muscle *CERS1* expression. We next evaluated how these skeletal muscle expression quantitative trait loci (eQTLs) might affect overall health, and muscle function by performing Mendelian randomization analysis using the UK biobank traits “overall health” (data-field 2178), “right grip strength” (data-field 47) and “left grip strength” (data-field 46). Combined results of the Mendelian randomization analysis showed that a decrease in muscular *CERS1* expression leads to impaired overall health rating and grip strength (**Figure 5A**). In particular, the *CERS1* muscle expression reducing alleles (rs117558072-C; rs1122821-G) decrease overall health, as well as right and left grip strength. Notably, we find a linear relationship between the eQTL-mediated effect sizes on muscle *CERS1* expression and the eQTL-mediated effect sizes on the respective phenotypes (**Figure 5B**). Importantly, some of the *CERS1* expression-reducing alleles are common in the human population, as indicated by their allele frequencies ranging from rs117558072-C(2%) to rs1122821-G(44%) and rs71332140-C(77%) and might therefore, contribute to skeletal muscle health in the broad population.

We next evaluated the effect of rare coding variants within the protein-coding exons of *CERS1* on the same phenotypes in the UK biobank cohort. Assessing the association of such coding variants with overall health rating, right grip strength and left grip strength, we found that the large majority of the significant (suggestive significant association, $p < 0.05$) variants in the *CERS1* coding region (9 out of 11) showed negative effect sizes on overall health rating, right grip strength and left grip strength (**Figure 5C**). Results from these rare variants support the genetic finding of our Mendelian randomization analysis of common variants and therefore, underline the notion that *CERS1* might affect overall health and muscle function in humans.

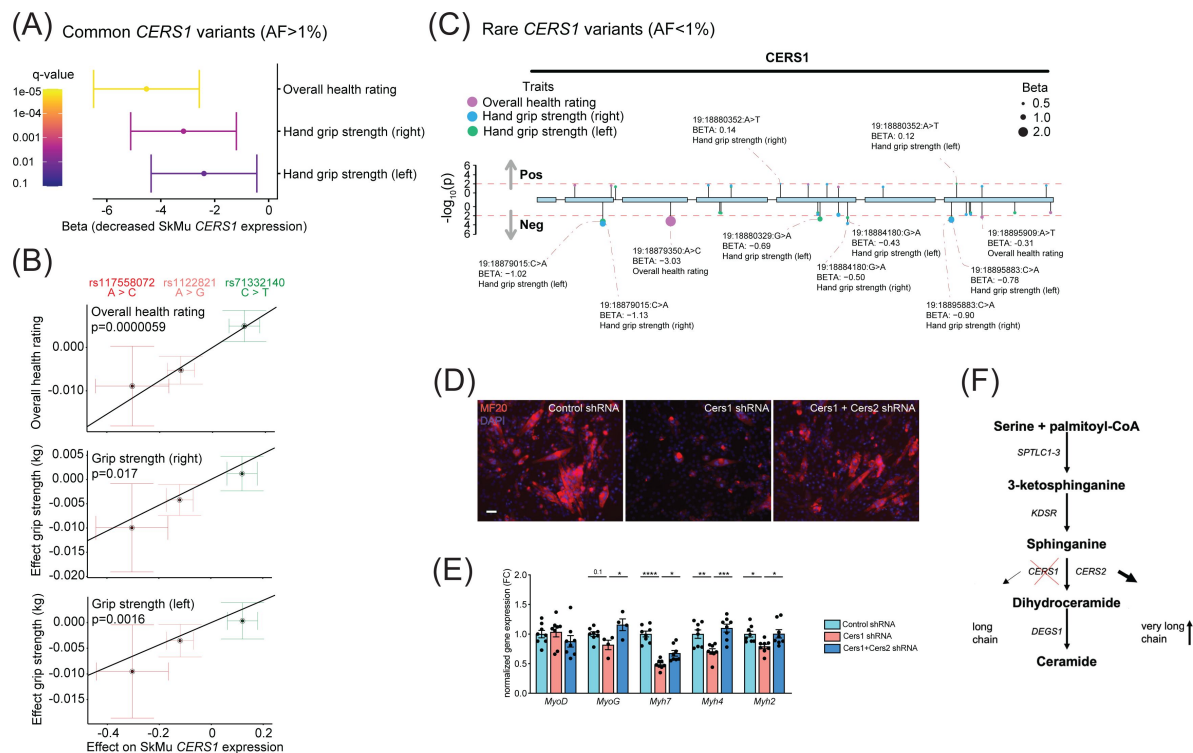


Figure 5.

Common and rare genetic variants in *CERS1* affect muscle function and health in humans. **(A)** Overall result of the Mendelian randomization analysis in the UK biobank cohort using skeletal muscle expression quantitative trait loci (*cis*-eQTL) of *CERS1*. **(B)** Scatter plot showing the effect of the three independent ($R^2 < 0.1$, see **Figure S5E**) *cis*-eQTLs rs117558072, rs1122821, rs71332140 on overall health, right grip strength, and left grip strength in the UK biobank. The slope of the regression line depicts the estimated causal effect with the inverse-variance weighted Mendelian randomization method. **(C)** Lollipop plot depicting rare variants in the coding region of *CERS1* and their effects on overall health, right grip strength, and left grip strength in the UK biobank. Colors indicate phenotypes, dot size indicates effect size, red dotted line indicates the suggested cut-off $p < 0.05$. **(D)** Representative immunocytochemistry images of differentiating mouse muscle cells treated with scramble control shRNA, or shRNA targeting *Cers1* alone, or shRNA against *Cers1* and *Cers2*. Scale bar, 50 μ m. **(E)** Gene expression profiling of differentiating mouse muscle cells treated with scramble control shRNA, or an shRNA targeting *Cers1* alone, or an shRNA against *Cers1* and *Cers2* ($n = 4-8$ per group). **(F)** Overview over the *de novo* sphingolipid biosynthesis pathway highlighting the hypothesis that *CERS1* inhibition leads to a compensatory upregulation of *CERS2*, which might inhibit muscle function in aging. Data: mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Dysfunctional muscle maturation and increased *Cers2* derived C24 ceramides upon *Cers1* inhibition, coupled with enhanced myogenic differentiation upon blockage of *Cers2*-derived C24 ceramides¹⁵ suggest that the observed inhibitory effect of *Cers1* knockdown on muscle cell maturation might be mediated by *Cers2*. Therefore, we tested whether *Cers1* knockdown was dependent on *Cers2*, and knocked down *Cers1* and *Cers2* simultaneously. Results showed that inhibition of *Cers1* together with *Cers2* alleviated the impaired differentiation of C2C12 myoblasts that is observed with *Cers1* knockdown alone (**Figure 5D-E**, **Figure S5G-I**). These findings suggest that very long chain ceramides might drive at least some of the negative muscular consequences observed upon reduced *Cers1* function.

In conclusion, our study reveals the necessity of intact *CERS1* expression for muscle cells to undergo muscle maturation, and that disruption of *CERS1* expression might contribute to muscle dysfunction across different species. Due to our consistent observation of increased very long chain C24:0/C24:1 ceramides and dihydroceramides upon *Cers1*/*CERS1* inhibition, we suggest a molecular network in which the compensatory upregulation of *CERS2* derived very long chain ceramide and dihydroceramide species in aging might accelerate age-related muscle wasting and dysfunction, hence contributing to sarcopenia (**Figure 5F**).

Discussion

In the present study, we sought to investigate the contribution of *CERS1* to the skeletal muscle aging process. Our main findings show that **I)***CERS1* expression correlates positively with skeletal myogenesis and function in human muscle biopsies; **II)**intact *CERS1* is necessary for proper muscle cell maturation; **III)***Cers1* is indispensable for intact organismal muscle aging, shown by deterioration of muscle mass, function and morphology upon genetic/pharmacologic inhibition of *Cers1*; and **IV)**common (5-70% allele frequency) and rare variants in humans affect muscle *CERS1* and hence, muscle function and perception of one's own health.

Our findings of reduced fiber size and muscle weight together with reduced muscle strength upon *Cers1* knockdown in aged mice are in line with previous findings by Tosetti et al²¹ who show that *Cers1* knockdown in young mice reduced muscle fiber size and contractility. Ceramide levels, and particularly *Cers1*-derived C18-ceramides were previously found elevated in skeletal muscle upon high-fat diet²⁹, streptozotocin-induced diabetes³⁰ and in obese, diabetic patients³¹. *Cers1* would therefore appear as an attractive target to improve metabolic outcomes due to its effect on C18 ceramide species. Indeed, the isoform-specific *Cers1* inhibitor P053 reduced C18 ceramide species (while increasing C24 ceramides) exclusively in skeletal muscle, which reduced fat mass in mice fed a high-fat diet¹¹. However, our study shows that the improvements in fat mass loss might come at the expense of muscle function/myogenesis, particularly upon aging. We show that inhibition of *Cers1* reduces myogenesis and exacerbates age-related muscle inflammation and fibrosis – key parameters associated with muscle aging. Furthermore, intact *Cers1* might not only be important for skeletal muscle homeostasis in aging; *Cers1* deficiency from birth was previously also reported to cause defects in foliation, progressive shrinkage, and neuronal apoptosis in the cerebellum, which translated to functional deficits including impaired exploration of novel objects, locomotion, and motor coordination²².

An interesting observation was that *Cers1* inhibition upregulated the levels of C24:0/C24:1 ceramides in all our *Cers1* targeting strategies and muscle models. This is consistent with previous literature^{11,21}. It suggests a possible compensatory upregulation of *Cers2* to make up for *Cers1* deficiency, or a re-routing of increased sphingoid base substrates towards very long chain (dh)ceramides by *Cers2* in the wake of reduced *Cers1* activity. Such a re-routing might reflect a cellular adaptation where a tradeoff between cell apoptosis mediated by sphingosine/sphinganine³²⁻³⁵ and reduced muscle maturation mediated by very long chain (dh)C24 ceramides, is tilted towards the latter. Of note, compensation effects in brain and liver

have also been reported in *Cers2* knockout mice^{36,37}, and upregulation of *Cers2* to compensate for *Cers1* deficiency in neurons reduced long chain C24:0/C24:1 ceramides and hence, ameliorated *Cers1* deficiency-mediated neurodegeneration³⁸. These studies combined with the dependency of the effects of *Cers1* inhibition on *Cers2* to reduce myogenic differentiation, therefore suggest that the long chain ceramides C24:0/C24:1 might exert detrimental effects at least in brain and skeletal muscle tissues. Confirmation of this hypothesis would open new avenues for targeted drug development, as *Cers1* targeting shows conflicting effects with regards to metabolic and muscle health.

We would like to point out that the pharmacologic inhibition of *Cers1* was superior to the genetic inhibition, as judged by the reduction in C18 ceramide levels. At the same time, mice treated with the pharmacologic inhibitor displayed more pronounced phenotypes. While it is intriguing to assume that changes in muscle phenotypes are correlated to the degree of ceramide inhibition, we cannot completely rule out multi-organ cross talk(s) of other organs that were affected by the systemic administration of the pharmacological inhibitor.

In summary, our study links *Cers1* with muscle cell maturation and muscle function in the context of aging. Disrupting *Cers1*, and hence the myogenic potential of aged skeletal muscle, exacerbates the age-related decline in skeletal muscle mass, function, and morphology. Given the highly beneficial effects of *Sptlc1* inactivation on healthy aging¹⁵, our current study further suggests that a significant part of the benefits of blocking the *de novo* ceramide biosynthesis pathway might come from inhibiting very long chain ceramides.

Methods

In vivo experiments

Animal experiments

Young (3mo) and aged (18-month-old) male C57BL/6J mice were obtained from Janvier Labs. Mice were randomized to the respective treatment groups according to their body weight. Animals were fed a standard chow diet. All animals were housed in micro-isolator cages in a room illuminated from 7:00AM to 7:00PM with ad libitum access to diet and water. The dose of P053 or DMSO was 5 mg/kg 3 times a week. Adeno-associated virus (AAV) 9 was bilaterally injected in gastrocnemius muscle at 18-month of age with 2×10^{11} viral particles (Vector Biolabs, United States) containing scramble short hairpin RNA (shRNA) or *Cers1*-targeting shRNA. 5-6 months after pharmacological or gene-modifying injections, phenotypic tests were performed on the animals. At sacrifice, muscles were removed for histochemical analyses or snap frozen in liquid N₂ for biochemical assays. Use of animals for all experimental studies were approved by animal licenses 2890.1 and 3341 in Canton of Vaud, Switzerland and were in compliance with the 1964 Declaration of Helsinki and its later amendments.

Endurance running test

After familiarizing with the treadmill, the exercise regimen started at a speed of 15 cm/s. Every 12 minutes, the speed was increased by 3 cm/s. Mice were considered to have reached their peak exercise capacity, and removed from the treadmill if they received 7 or more shocks (0.2 mA) per minute for two consecutive minutes. The distance traveled and time before exhaustion were registered as maximal running distance and time as shown previously³⁹.

Grip strength

Muscle strength was assessed by the grip strength test as previously described⁴⁰. Grip strength of each mouse was measured on a pulldown grid assembly connected to a grip strength meter (Columbus Instruments). The mouse was drawn along a straight line parallel to the grip, providing peak force. The experiment was repeated three times, and the highest value was included in the analysis.

Measurement of sphingolipids

Cell pellets (~1.0e6 cells) were extracted by the addition of 100 μ L of MeOH spiked with the stable isotope-labelled internal standards (Spa(d17:0), Cer(d18:1/16:0)-d9, Cer(d18:1/18:0)-d7, Cer(d18:1/24:0)-d7 and Cer(d18:1/24:1)-d7)). Sample homogenization was performed in the Cryolys Precellys Tissue Homogenizer (2 x 20 seconds at 10000 rpm, Bertin Technologies, Rockville, MD, US) with ceramic beads. The bead beater was air-cooled down at a flow rate of 110 L/min at 6 bar. Homogenized extracts were centrifuged for 15 minutes at 4000 g at 4°C and the resulting supernatants were collected for the LC-MS/MS analysis. Sphingolipids were quantified by LC-MS/MS analysis in positive ionization mode using a 6495 triple quadrupole system (QqQ) interfaced with 1290 UHPLC system (Agilent Technologies), adapted from Checa et.al.⁴¹. Briefly, the chromatographic separation was carried out in a Zorbax Eclipse plus C8 column (1.8 μ m, 100 mm $\times$ 2.1 mm I.D) (Agilent technologies). Mobile phase was composed of A = 5 mM ammonium formate and 0.2 % formic acid in water and B = 5 mM ammonium formate and 0.2% formic acid in MeOH at a flow rate of 400 μ L/min. Column temperature was 40 °C and sample injection volume 2 μ L. The linear gradient elution starting from 80% to 100% of B (in 8 min) was applied and held until 14 min. The column was then equilibrated to initial conditions. ESI source conditions were set as follows: dry gas temperature 230 °C, nebulizer 35 psi and flow 14 L/min, sheath gas temperature 400 °C and flow 12 L/min, nozzle voltage 500 V, and capillary voltage 4000 V. Dynamic Multiple Reaction Monitoring (dMRM) was used as acquisition mode with a total cycle time of 500 ms. Optimized collision energies for each metabolite were applied. Raw LC-MS/MS data was processed using the Agilent Quantitative analysis software (version B.07.00, MassHunter Agilent technologies). For absolute quantification, calibration curves and the stable isotope-labelled internal standards (IS) were used to determine the response factor. Linearity of the standard curves was evaluated for each metabolite using a 12-point range, in addition, peak area integration was manually curated and corrected when necessary. Sphingolipid concentrations were reported to protein concentrations measured in protein pellets (with BCA, Thermo Scientific) following the metabolite extraction.

Histology

Skeletal muscles were harvested from anesthetized mice, and immediately embedded in Thermo Scientific™ Shandon™ Cryomatrix™ and frozen in isopentane, cooled in liquid nitrogen, for 1 min before being transferred to dry ice and stored at -80 °C. 8 μ m cryosections were incubated in 4% PFA for 15 min, washed three times for 10 min with PBS, counterstained with DAPI, laminin (1:200, Sigma), CD45 (1:200, Life Technologies), coupled with Alexa-488 or Alexa-568 fluorochromes (Life Technology) and mounted with Dako Mounting Medium. Images was performed with VS120-S6-W slides scanner (Olympus) acquired using a 20x/0.75 air UPLS APO objective and an Olympus XM10 CCD camera. 3 channel images (DAPI, Laminin, and CD45-A568) were acquired defining a focus map based on the Laminin signal for fluorescence imaging whereas hematoxylin and eosin and Sirius Red were imaged with brightfield. Resulting VSI images could be opened in Fiji/ImageJ and QuPath using the BioFormats library. Minimum Feret diameter, and cross-sectional area were determined using the ImageJ software. For fiber size distribution, a minimum of 2,000 fibers were used for each condition and measurement. The minimum Feret diameter is defined as the

minimum distance between two parallel tangents at opposing borders of the muscle fiber. This measure has been found to be resistant to deviations away from the optimal cross-sectioning profile during the sectioning process.

Deep learning-based muscle segmentation and quantification

A subset of 4 muscle cross-section images was manually annotated in multiple regions to serve as ground-truth for cellpose training. In total, 15 areas were used for training and 4 for validation. Annotation and training was done through QuPath using the QuPath cellpose extension (<https://github.com/BIOP/qupath-extension-cellpose>). Original images were exported as “raw image plus labeled image” pairs and downsampled 4x in X and Y (Final pixel size for training: 1.2876 $\mu\text{m}/\text{px}$). The cellpose model was trained with the starting weights of cellpose’s ‘cyto2’ model, for 500 epochs. Predicted validation images were further run through a validation notebook derived from ZeroCostDL4Mic⁴² in order to assess the quality of the predictions (see **Table S1**). Segmentation of muscle fibers was performed in two steps. First, the whole cross-section was detected through a gaussian-blurred version of the Laminin signal ($\sigma=5 \mu\text{m}$), followed by an absolute threshold (threshold value: 5). The resulting region was then fed into the QuPath cellpose extension along with our custom model for individual fiber segmentation. Training images, training and validation scripts and Jupyter notebooks, as well as an example QuPath project are available on Zenodo (DOI: 10.5281/zenodo.7041137).

C. elegans motility assays

C. elegans N2 strains were cultured at 20°C on nematode growth medium agar plates seeded with *E. coli* strain OP50 unless stated otherwise. Synchronous animal populations were generated through hypochlorite treatment of gravid adults to obtain synchronized embryos. These embryos were allowed to develop into adulthood under appropriate conditions. The *Cers1* orthologue *lagr-1* was found on wormbase (<https://wormbase.org/>). For RNAi experiments, worms were exposed to *lagr-1* RNAi or an empty vector control plasmid using maternal treatment to ensure robust knock down. P053 was dissolved in DMSO, and used at a final concentration of 50 μM and 100 μM . Worms were exposed to P053 starting from L4 stage. P053 or DMSO was added to agar before preparing plates. To ensure permanent exposure to the compound and avoid bacterial contamination, plates were changed twice a week. *C. elegans* movement analysis was performed using the Movement Tracker software as done previously⁴⁰. Measurement of body thrashes was performed as previously described⁴³. Briefly, animals grown on plates containing either DMSO or P053 were placed in a 50 μl drop of M9 and the thrashes performed within a 90s interval were counted.

C. elegans muscle morphology

The transgenic RW1596 strain (Caenorhabditis Genetics Center, University of Minnesota) was used for muscle morphology. RW1596 expresses GFP under the control of the worm skeletal muscle promoter myo3 (myo3p-GFP). To perform muscle stainings, ~50 worms were washed in M9, immobilized with 7.5 mM tetramisole hydrochloride (Sigma-Aldrich) and immediately imaged afterwards. Confocal images were acquired with Zeiss LSM 700 Upright confocal microscope (Carl Zeiss AG) under non-saturating exposure conditions. Image processing was performed with Fiji.

C. elegans lifespan

20–25 worms were placed on nematode growth medium agar plates containing 100 μM P053 or DMSO as a control, along with 10 μM 5-FU, and seeded with HT115(DE3) bacteria. A minimum of 140 animals were tested per condition. Animals were assessed daily for touch-provoked movement. Worms that died due to internally hatched eggs, an extruded gonad, or desiccation resulting from crawling on the edge of the plates were excluded. Survival assays were repeated twice, and curves generated using the product-limit method of Kaplan and Meier. The log-rank (Mantel–Cox) test was used to evaluate differences between survivals and determine significance.

***In vitro* mouse and human studies**

Cell culture and cell transfection

The C2C12 mouse myoblast cell line was obtained from the American Type Culture Collection (CRL-1772TM). C2C12 cells or clones were cultured in growth medium consisting of Dulbecco's modified Eagle's medium (Gibco, 41966-029), 20 % Fetal Bovine Serum (Gibco, 10270-106) and 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, 15140-122). To induce differentiation, FBS was substituted with 2% horse serum (Gibco, 16050-122). Trypsin-EDTA 0.05% (GIBCO, 25300-062) was used to detach cells. Primary human skeletal muscle cells were obtained from Lonza (SkMC, #CC-2561) and the Hospices Civils de Lyon and were cultured in growth medium consisting of DMEM/F12 (Gibco, 10565018), 20 % Fetal Bovine Serum (Gibco, 10270-106) and 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, 15140-122). To induce differentiation, FBS was exchanged with 2 % horse serum (Euroclone) and kept in culture. All cells were maintained at 37 °C with 5% CO₂. Cell transfections were done using TransIT-X2 (Mirus) according to the manufacturer's protocol with a 3:1 ratio of transfection agent to DNA. C2C12 cells were grown confluent, and 1 μM P053 or DMSO was added, and cells were kept in growth medium for another 3 days to measure muscle cell differentiation. Protein synthesis was measured according to the instructions of the Click-iT HPG kit (C10428, Thermofisher Scientific). All cell lines were regularly tested for mycoplasma and inspected for fungi contamination.

RNA isolation and real-time qPCR

Tissues were homogenized with Trizol, whereas cells were homogenized, and RNA isolated using the RNeasy Mini kit (Qiagen, 74106). Reverse transcription was performed with the High-Capacity RNA-to-cDNA Kit (4387406 Thermofisher scientific) as shown previously⁴⁴. Gene expression was measured by quantitative reverse transcription PCR (qPCR) using LightCycler 480 SYBR Green I Master (50-720-3180 Roche). Quantitative polymerase chain reaction (PCR) results were calculated relative to the mean of the housekeeping gene *Gapdh*. The average of two technical replicates was used for each biological data point. Primer sets for qPCR analyses are shown in the **Table S2**.

Lentivirus production and transduction

Lentiviruses were produced by cotransfecting HEK293T cells with lenti plasmids expressing scramble shRNA or *Cers1* targeting shRNA (see plasmid list in **Table S3**), the packaging plasmid psPAX2 (addgene #12260) and the envelope plasmid pMD2G (addgene #12259), in a ratio of 4:3:1, respectively. Transfection medium was removed 24h after transfection and fresh medium was added to the plate. Virus containing medium was collected at 48h and concentrated using Lenti-XTM Concentrator (Takara). C2C12 mouse myoblasts were seeded 24h prior to infection and then transduced with virus-containing supernatant supplemented with 8μg/mL polybrene (Millipore) as shown previously⁴⁰. For the delivery of a shRNA construct targeting *CERS1* in human primary myoblasts, adenovirus containing Ad-U6-h-CERS1-shRNA or Ad-U6-scrmb-shRNA (Vector Biolabs) was infected using 1uL adenovirus per mL cell culture medium at a titer of 5×10¹⁰ PFU/mL.

Immunocytochemistry

C2C12 cells or Lonza SkMU cells cultured on a sterilized coverslip in 6-well plates (Greiner bio-one, CELLSTAR, 657160) were fixed in Fixx solution (Thermo Scientific, 9990244) for 15 min and permeabilized in 0.1% Triton X-100 (Amresco, 0694) solution for 15min at 20°C. Cells were blocked in 3% BSA for 1h at 20°C to avoid unspecific antibody binding and then incubated with primary antibody over night at 4°C with gentle shaking. MyHC was stained using the MF20 primary antibody (1:200, Invitrogen, 14-6503-82) for C2C12 cells and in Lonza muscle cells with a MYL1 antibody (1:140, Thermofisher, PA5-29635). Antibodies used in this study are shown in **Table S4**. The next day cells were incubated with secondary antibody (Thermo Fisher #A10037 for MF20 and #A-21206 for MYL1) for 1h at 20°C and nuclei were labeled with DAPI. The immunofluorescence

images were acquired using either fluorescence or confocal microscopy. The myofusion index was calculated as the ratio of nuclei within myotubes to total nuclei. Myotube diameter was measured for 8 myotubes per image using ImageJ. Myotube area was calculated as the total area covered by myotubes.

Human studies

Skeletal muscle gene expression in Genotype-Tissue Expression (GTEx) project

The Genotype-Tissue Expression (GTEx) project version 8 was accessed through accession number dbGaP phs000424.v8.p2 (approved request #10143-AgingX). Transcript expressions and covariates from male human muscle of 469 individuals were extracted from the GTEx Portal (<https://gtexportal.org>). To remove the known and hidden factors, the Probabilistic Estimation of Expression Residuals (PEER) was applied the expression residuals were used to further analysis. For gene set enrichment analysis, genes were ranked by Spearman correlation coefficients with the gene expression level of *CERS1* based on the Spearman correlation approach, and the *CERS1* enriched gene sets were calculated by GSEA using clusterProfiler R package (version 3.10.1). Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome and Hallmark gene sets were retrieved from the Molecular Signatures Database (MSigDB) using the msigdb R package (version 7.2.1).

Mendelian randomization

We used inverse variance-weighted Mendelian randomization to assess the causal effect of *CERS1* expression on muscle-related traits in humans. As exposures, we used the GTEx version 8 cis-eQTLs that were mapped by the GTEx consortium based on the normalized gene expressions in skeletal muscle from 706 deceased human individuals. These summary statistics are publicly available and can be accessed on the internet at the online GTEx webpage portal through https://storage.googleapis.com/gtex_analysis_v8/single_tissue_qtl_data/GTEx_Analysis_v8_eQTL.tar. As outcomes, we used 10648 publicly available summary statistics obtained from various sources through the “available_outcomes” function from the TwoSampleMR R package. Outcome IDs can be queried from the ieu gwas database at <https://gwas.mrcieu.ac.uk/>. We restricted the analysis to independent instrumental variables (genetic variants). We therefore followed an iterative pruning workflow whereby we included the variant with the most significant effect on *CERS1* expression, followed by pruning with a 0.05 squared correlation coefficient (r^2) threshold, then again selecting the most significant variant, and so on until no variants are left. This procedure retained three genetic variants: rs117558072 (A > C), rs1122821 (A > G), and rs71332140 (C > T). The causal effect of altered *CERS1* expression was inferred on the 10648 outcomes through inverse-variance weighted Mendelian randomization. The false discovery rate was controlled at the 5% significance level with the Benjamini-Hochberg procedure.

UK Biobank (UKBB) rare variant analysis

We performed genetic association analyses for hand grip strength and overall health in UK Biobank participants from 379,530 unrelated individuals. To run GWAS on the UK Biobank 450k Whole Exome Sequencing release (data field 23150) we filtered variants based on allele frequency and Hardy-Weinberg equilibrium. We then used Regenie v3.1.1 and accounted for sex, age, and body mass index. First, population structure estimation was performed using array sequencing to equally represent the whole genome. Second, GWAS burden tests were employed to measure SNP effects on the UKBB phenotypes overall health, right grip strength and left grip strength using the UKBB whole exon sequencing data set. The UKBB data was accessed under the application #48020.

Statistical analyses

For experimental conditions in which there were two independent factors and multiple comparisons, a factorial ANOVA with subsequent post hoc analysis was performed. Where appropriate, one-way ANOVA with post hoc analysis or t-tests was performed. All statistical analyses were performed using GraphPad Prism (9.4.0, San Diego, CA, USA). Results are reported as means $\pm$ standard error of the mean. Statistical significance was set to $P < 0.05$.

Acknowledgements

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Author contributions

The study was conceived and designed by MW, PPL and JA. Human and mouse bioinformatics analyses were performed by XL, GA and MW. Mouse phenotyping and injections were carried out by MW and PPL. *In vitro* work was performed by MW, BC, RM. HGA and JI performed sphingolipid measurements. Histological analyses and *ex vivo* measurements were performed by MW, BC, and RM. Worm experiments were performed by MW and ID. Genetic analyses were performed by LJEG, GA and MW. MW and JA wrote the manuscript, and all authors gave critical comments on it. JA supervised the work.

Conflict of interest

The other authors do not declare a conflict of interest. JA and PPL are inventors on a patent application (WO 2021/058497 A1) related to inhibition sphingolipid synthesis for the treatment of age-related diseases filed by the EPFL.

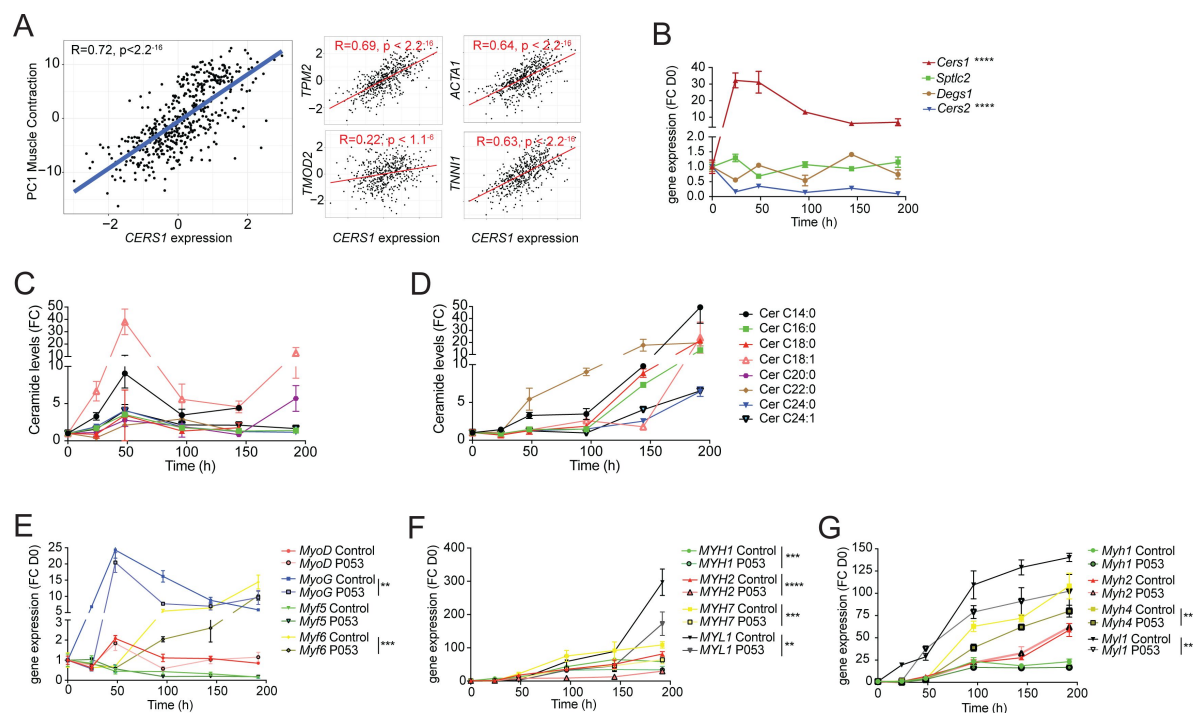


Figure S1.

Cers1 regulates muscle contraction in human muscle and myoblast maturation. **(A)** Spearman correlation between skeletal muscle expression of *CERS1* and the first principal component (PC1) of the muscle contraction pathway (left) and expression of key genes involved in muscle contraction (right). **(B)** Gene expression of enzymes involved in the sphingolipid *de novo* synthesis pathway upon horse serum induced differentiation of mouse C2C12 myoblasts (n=3-4). **(C-D)** Ceramide levels during myogenic differentiation of **(C)** mouse C2C12 myoblasts (n=3-4) and **(D)** human primary skeletal muscle cells (n=2-3). **(E)** Gene expression of myogenic regulatory factors upon horse serum induced differentiation of mouse C2C12 myoblasts (n=3-4). **(F-G)** Gene expression of myosin light- and heavy chains upon horse serum induced differentiation of isolated human primary myoblasts (n=2-3 biological replicates) **(F)** and mouse C2C12 (n=3-4) muscle cells **(G)** upon horse serum induced myoblast differentiation. The pharmacological inhibitor P053 or DMSO was used at 1uM in **(E-G)**. Data: mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

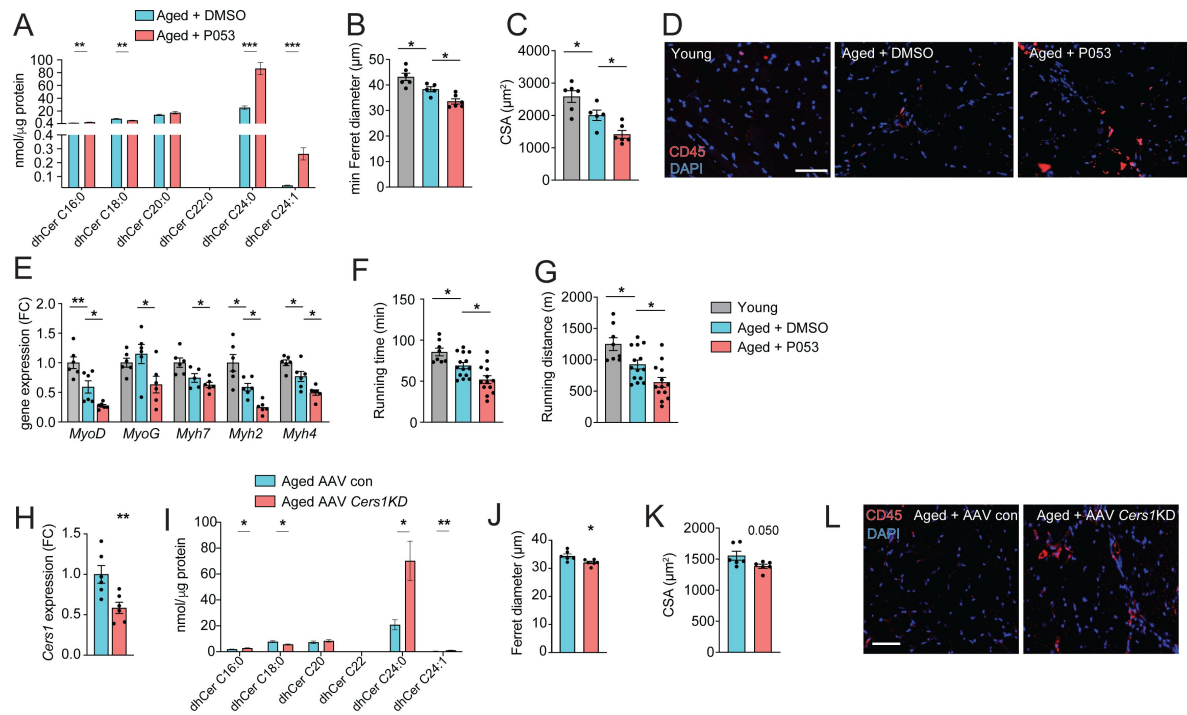


Figure S2.

Cers1 inhibition exacerbates mouse skeletal muscle aging. **(A)** Skeletal muscle dihydroceramide levels in aged mice treated with DMSO or P053 (n=7-8 per group). **(B)** Average minimal Ferret diameter in muscle of young and aged mice treated with DMSO or P053 (n=5-6 per group). **(C)** Quantification of muscle cross-sectional area in young and aged mice treated with DMSO or P053 (n=5-6 per group). **(D)** Representative immunohistochemistry images of muscle cross sections from young and aged mice treated with DMSO or P053 stained with DAPI and CD45 (n=5-6 per group). Scale bar, 50 μm. **(E)** Gene expression in young or aged muscle of mice treated with DMSO or P053 (n=5-6 per group). **(F-G)** Phenotyping of young control and aged mice injected with DMSO or P053 showing running time **(F)**, and running distance **(G)** (n=7-14 per group). **(H)** *Cers1* RNA expression in aged mouse muscle intramuscularly injected with scramble, or shRNA targeting *Cers1* (n=6 per group). **(I)** Dihydroceramide levels in gastrocnemius muscle injected with AAV9 particles containing scramble, or shRNA targeting *Cers1* (n=6-8 per group). **(J)** Average Ferret diameter in skeletal muscle of aged mice intramuscularly injected with scramble, or shRNA targeting *Cers1* (n=6 per group). **(K)** Quantification of cross-sectional area in aged mice treated with AAV9-shRNA *Cers1* (n=6 per group). **(L)** Representative immunohistochemistry images of muscle cross sections of CD45-stained muscle from mice intramuscularly injected with AAV9 particles containing scramble, or shRNA targeting *Cers1* (n=6 per group). Data: mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

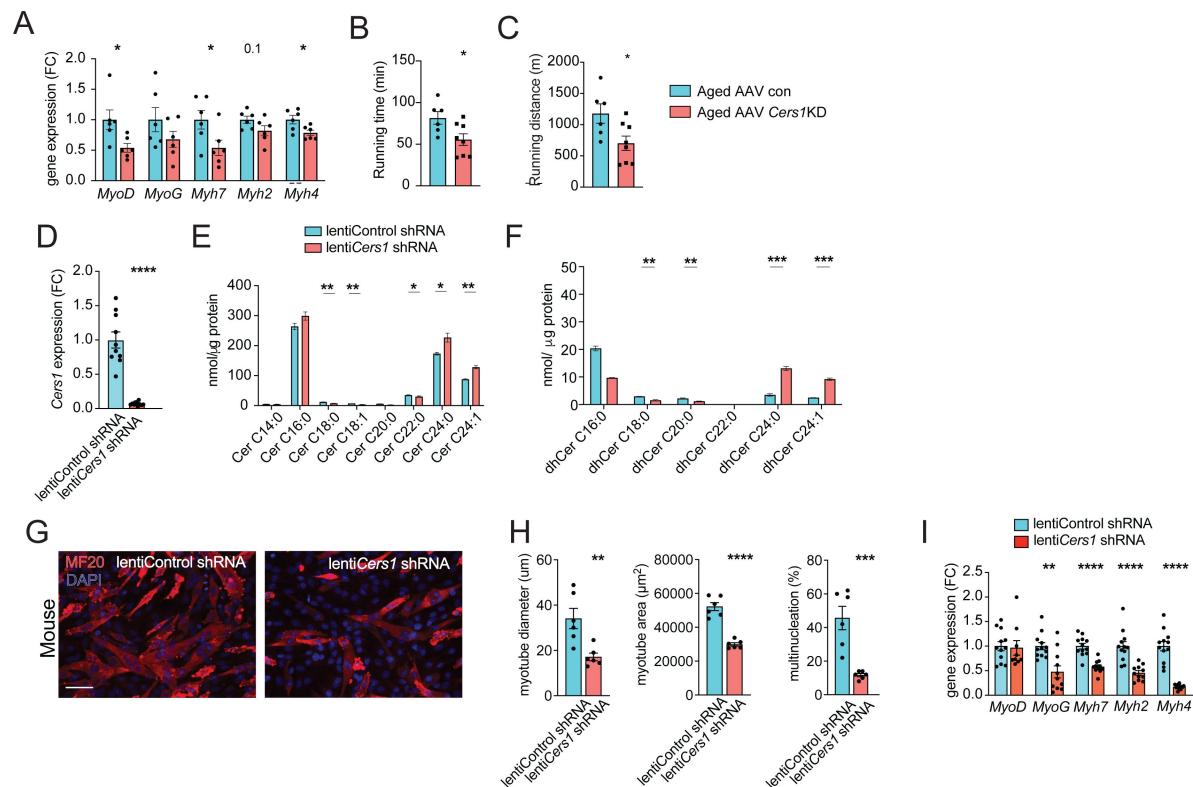


Figure S3.

Cers1 silencing deteriorates age-related running, ceramides and blunts myogenesis in myoblasts. **(A)** Muscle gene expression in of mice intramuscularly injected with AAV9 particles containing scramble, or shRNA targeting *Cers1* (n=6 per group). **(B-C)** Phenotyping measurements of aged mice intramuscularly injected with shRNA-*Cers1* shows running time **(B)** and running distance **(C)** (n=6-8 per group). **(D)** *Cers1* gene expression in C2C12 mouse muscle cells transduced with lentivirus containing scramble, or shRNA targeting *Cers1* (n=5-6 biological replicates per group). **(E)** C2C12 myoblast ceramide levels upon lentivirus mediated silencing of *Cers1* (n=3 per group). **(F)** Dihydroceramide levels in C2C12 cells transduced with lentivirus containing shRNA targeting *Cers1* (n=3 per group). **(G)** Representative immunocytochemistry images of differentiating C2C12 muscle cells deficient of *Cers1* (n=6 per group). Scale bar, 50μM. **(H)** Quantification of **(G)** showing myotube diameter (left), myotube area (middle) and % of multinucleated myotubes (right) in C2C12 mouse muscle cells transduced with lentivirus containing scramble, or shRNA targeting *Cers1* (n=6 per group). **(I)** Gene expression profiling of differentiating C2C12 muscle cells deficient of *Cers1* (n=8-12 per group). Data: mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

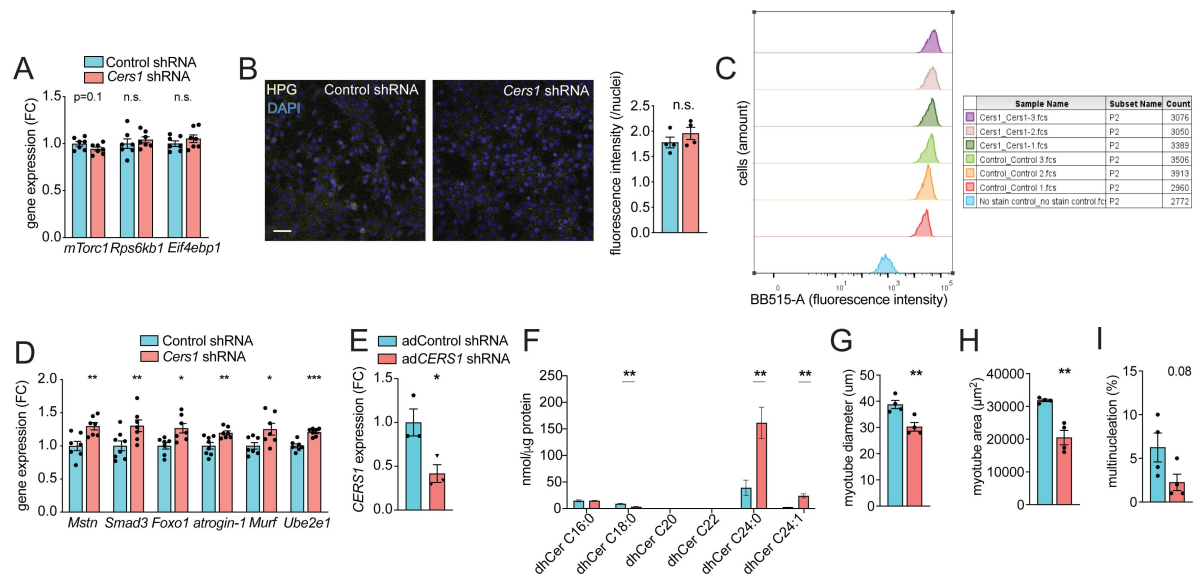


Figure S4.

Adenovirus mediated silencing of *CERS1* reduces ceramides and blunts myogenesis in human muscle cells. **(A)** Normalized gene expression of transcripts related to protein synthesis in scramble control and *Cers1* deficient differentiating myoblasts (n=7). **(B)** Representative confocal immunocytochemistry images of scramble control and *Cers1* deficient, differentiating myoblasts incubated with the Click-iT HPG dye to capture protein synthesis with quantification (n=4). Scale bar, 50µm. **(C)** Fluorescence-activated cell sorting of scramble control and *Cers1* deficient differentiating myoblasts incubated with the Click-iT HPG dye, to score for protein synthesis. **(D)** Normalized gene expression of transcripts related to protein degradation in scramble control and *Cers1* deficient, differentiating myoblasts (n=7 per group). **(E)** *CERS1* gene expression in isolated primary human muscle cells transduced with adenovirus containing scramble, or shRNA targeting *CERS1* (n=3 per group). **(F)** Dihydroceramide levels in differentiated primary human muscle cells transduced with adenovirus containing scramble, or shRNA targeting *CERS1* (n=5-6 per group). **(G-I)** Quantification of myotube diameter **(G)**, myotube area **(H)**, and % of multinucleated myotubes **(I)** in differentiated primary human muscle cells transduced with adenovirus containing scramble, or shRNA targeting *CERS1* (n=4 per group). Data: mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

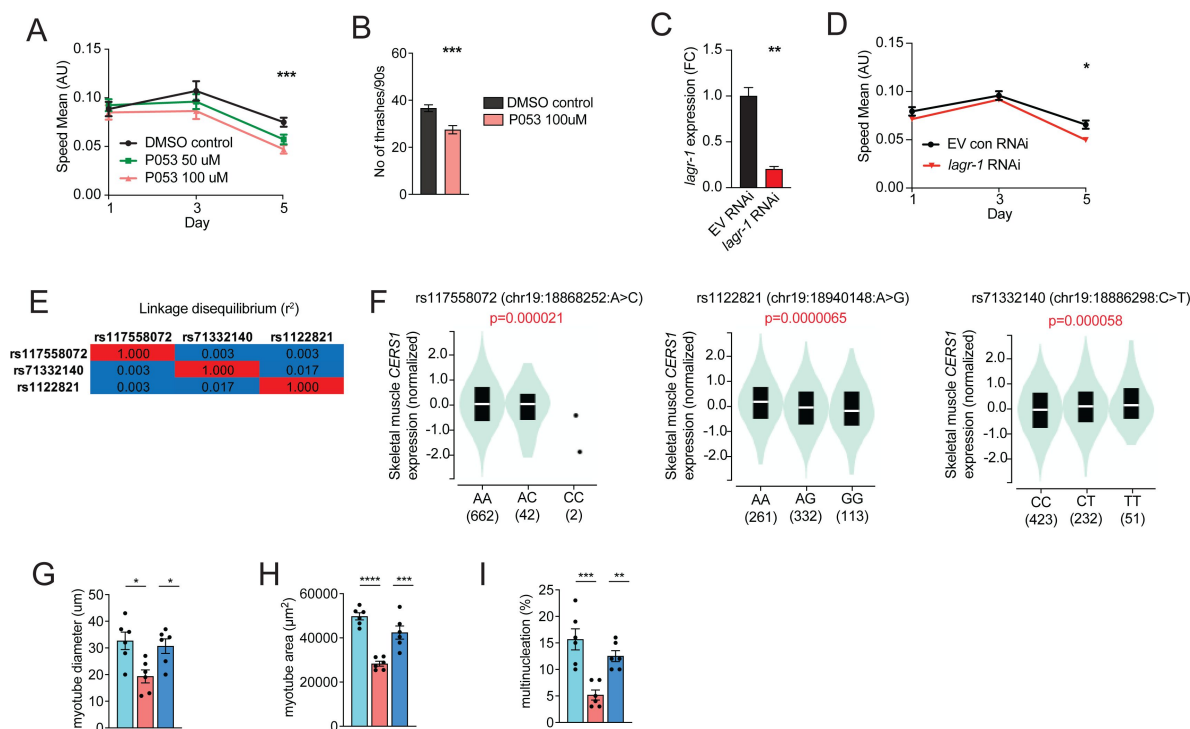


Figure S5.

CERS1 inhibition is associated with impaired health span. **(A)** Mean speed measured in *C. elegans* treated with DMSO, 50uM P053 or 100uM P053 (n=40-57 per group). **(B)** Number of thrashes recorded within 90 seconds in control or P053 treated *C. elegans* at day 5 of adulthood (n=33-40 per group). **(C)** Gene expression of the *Cers1* orthologue *lagr-1* in *C. elegans* treated with empty vector control, or RNAi targeting *lagr-1* (n=3 biological replicates per group). **(D)** Mean speed measured in *C. elegans* (myo3p-GFP) treated with EV control, or RNAi against the *Cers1* orthologue *lagr-1* (n=46-56 per group). **(E)** Linkage disequilibrium of the common *CERS1* expression quantitative trait loci (*cis*-eQTL) rs117558072, rs1122821, rs71332140 measured as r^2 in all populations (<https://ldlink.nci.nih.gov>). **(F)** Violin plots showing the allelic effect of rs117558072 (left), rs1122821 (middle), rs71332140 (right) on *CERS1* expression in human skeletal muscle in the Genotype-Tissue Expression (GTEx) dataset (n=706 human in total). **(G-I)** Quantification of **(G)** myotube diameter, **(H)** myotube area (middle) and **(I)** % of multinucleated myotubes in C2C12 mouse muscle cells treated with scramble control shRNA, shRNA targeting *Cers1*, or shRNA targeting *Cers1* and *Cers2* (n=6 per group). Data: mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Image	Prediction v. GT Intersect over Union	False positive	True positive	False negative	Precision
Image_LIS-123_region_0.tif	0.810790951	4	148	16	0.973684
Image_LIS-308_region_0.tif	0.747845652	4	68	27	0.944444
Image_LIS-140_region_0.tif	0.906795138	4	129	3	0.969925
Image_LIS-301_region_0.tif	0.881344966	2	258	9	0.992308

Table S1.

Cellpose training quality control.

Gene symbol (mouse)	Forward	Reverse
<i>Myod1</i>	AGCACTACAGTGGCGACTC	GTGGAGATGCGCTCCACT
<i>Pax7</i>	TCTCCAAGATTCTGTGCCGAT	CGGGGTCTCTCTTTATACTCC
<i>Myf5</i>	TGAGGGAAACAGGTGGAGAAC	TGGAGAGAGGGAAGCTGTGT
<i>Myf6</i>	AGATCGTCGGAAAGCAGC	CCTGGAATGATCCGAAACAC
<i>Myog</i>	TTGCTCAGCTCCCTCAACCAGGA	TGCAGATTGTGGGCGTCTGTAGG
<i>Myh4</i>	ACAAGCTGCGGGTGAAGAGC	CAGGACAGTGACAAAGAACG
<i>Myh1</i>	CCAAGTGCAGGAAAGTGACC	AGGAAGAGACTGACGAGCTC
<i>Myh2</i>	CAGAGGCAAGTAGTGGTGGA	CAAATTCTCTCTGAACAGGGCA
<i>Myh4</i>	ACACAGAGTCAGGCGAGTTT	CAGTGCCTTCTTGGCCTT
<i>Myh7</i>	GTGGCTCCGAGAAAGGAAG	GAGCCTTGGATTCTCAAACG
<i>Myl1</i>	AGAGGTAGAAGCGTTGCTGG	GGCCAGTCTTCCCAACATT
<i>Cers1</i>	CCACCACACATCTTTCGG	GCCTGACCTCCAGTCATAGA
<i>Sptlc2</i>	GCACTCGTCAGGAAATGGAAA	CTCCTAGAACCAGTGACGCA
<i>Degs1</i>	AATGGGTCTACACGGACCAG	GGACGAGAAGCATCATGGCTA
<i>Cers2</i>	GGCGCTAGAAGTGGGAAAC	TCGAATGACGAGAAAGAGCA
<i>mTorc1</i>	GGCATAACAGATCCTGACCCTGAT	CAGGGATGCCAAGACACAGTA
<i>Rps6kb1</i>	CGGGTACTTGGTAAAGGGGG	ATGTTCCGCTCTGCTTTCGT
<i>Eif4ebp1</i>	GGGGTCACTAGCCCTACCA	TCATTGCGTCTACGGCTG
<i>Mstn</i>	TCACGCTACCACGGAACAA	TGCCATCCGCTTGCAATTAGA
<i>Smad3</i>	CACTCCCAACAGCTCAG	CAACTGCCTGAACATCTCCTCT
<i>Foxo1</i>	AAGGATAAGGGCGACAGCAA	ATTTTCCGCTCTTGCTCTCC
<i>Atrogin-1</i>	TCTCTCTGGAAGGGCACTG	TGAGGGGAAAGTGAGACGGA
<i>Murf</i>	CACAGAGGGTAAAGAAGAACACC	TGGGGAGCCCTATGCTAGTC
<i>Ube2e1</i>	GTTGCTGCACCTCCGCTTC	GCGAACGGCCCTCTCTTT
<i>MYOD</i>	TCTCCTTGGTGTAGGCTCAG	CCTGACCTTGAACGTGAATC
<i>MYOG</i>	TTGCTCAGTCCCTCAACCAGGA	TGCAGATTGTGGGCGTCTGTAGG
<i>MYF5</i>	TGTGGCTCTCTCTCCGTATG	AATACAGACATGCAGGCTTCAC
<i>MYF6</i>	GTGGAGGAAGTGGTGAGAA	ACTTTTCCGCTCTGGGTTCT
<i>MYH1</i>	TGCTCCAAAGCCAAGGGAAA	CCCTCGAGAGCTGTGAAACT
<i>MYH2</i>	GTCTTGCTTTAAAAAGCTCCAAGA	TCAAAGGGCTATTCTGGGC
<i>MYH7</i>	TTGGCCCTTTCTCATCTGT	ATCAGGCACGAAGACATCCTT
<i>MYH4</i>	GCTGAAGAGGCTGAGGAACA	CCCGACTCTTCACTCTCAGC
<i>MYL1</i>	AACCACCACTCTCTTCCAA	AGGGTGGGTTAAAAAGAGAAGGA
<i>CERS1</i>	TCGTCTCCTCTACGCCTTC	GCGGAACCAAGAACACAGC
<i>SPTLC1</i>	AGTGGGTTCTGGTGGAGATG	TGGTAAGCAGGAGCCTCGTAA
<i>DEGS1</i>	AGCTAGTCTGCAAGCCACC	CTCTGGATACTTTGCCAGGAT
<i>CERS2</i>	GCTGGAGTCAGCCAAGATGT	AGGATCCAGAAGGGCAGGAT

Table S2.

List of mouse and human qPCR primers.

Plasmid	Reference
Lenti shRNA Cers1	Origene # 93898
psPAX2	Addgene # 12260
pMD2G	Addgene # 12259

Table S3.

List of plasmids.

Antibody	Supplier	Reference #
Myosin 4 Monoclonal (MF20)	ThermoFisher Scientific	# 14-6503-82
Anti-Myosin light chain 2	Abcam	# ab79935
Anti-Myosin light chain 1	ThermoFisher	# PA5-29635
Laminin	Sigma	#L9393
CD45 (1:200, eBioscience, eFluor450 conjugated)	eBioscience	# 48-0459-42
Donkey anti-Mouse IgG secondary antibody	ThermoFisher Scientific	# A10037
Donkey anti-Rabbit IgG secondary antibody	ThermoFisher Scientific	# A-21206

Table S4.

List of antibodies.

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Christopher Huang

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Reviewer #1 (Public Review):

Summary:

The authors identified that genetically and pharmacological inhibition of CERS1, an enzyme
implicated in ceramides biosynthesis worsen muscle fibrosis and inflammation during aging.

Strengths:

The study points out an interesting issue on excluding CERS1 inhibition as a therapeutic
strategy for sarcopenia. Overall, the article it's well written and clear.

<https://doi.org/10.7554/eLife.90522.2.sa1>

Reviewer #2 (Public Review):

Summary:

The manuscript by Wohlwend et al. investigates the implications of inhibiting ceramide
synthase Cers1 on skeletal muscle function during aging. The authors propose a role for Cers1
in muscle myogenesis and aging sarcopenia. Both pharmacological and AAV-driven genetic
inhibition of Cers1 in 18-month-old mice lead to reduced C18 ceramides in skeletal muscle,
exacerbating age-dependent features such as muscle atrophy, fibrosis, and center-nucleated
fibers. Similarly, inhibition of the Cers1 orthologue in *C. elegans* reduces motility and causes
alterations in muscle morphology.

Strengths:

The study is well-designed, carefully executed, and provides highly informative and novel findings that are relevant to the field.

<https://doi.org/10.7554/eLife.90522.2.sa0>

Reviewer #1 (Public Review):

Summary:

The authors identified that genetically and pharmacological inhibition of CERS1, an enzyme implicated in ceramides biosynthesis worsen muscle fibrosis and inflammation during aging.

Strengths:

The study points out an interesting issue on excluding CERS1 inhibition as a therapeutic strategy for sarcopenia. Overall, the article it's well written and clear.

Weaknesses:

Many of the experiments confirmed previous published data, which also show a decline of CERS1 in ageing and the generation and characterization of a muscle specific knockout mouse line. The mechanistic insights of how the increased amount of long ceramides (cer c24) and the decreased of shorter ones (cer c18) might influence muscle mass, force production, fibrosis and inflammation in aged mice have not been addressed.

We thank the reviewer for the assessment and would like to point out that Cers1 had not previously been studied in the context of aging. Moreover, our unbiased pathway analyses in human skeletal muscle implicate CERS1 for the first time with myogenic differentiation, which we validate in cell culture systems. To improve mechanistic insights, as suggested by Reviewer #1, we performed more experiments to gain insights how Cers1 derived c18, and Cers2 derived c24 ceramide species affect myogenesis. We recently showed that knocking out Cers2 reduces c24:0/c24:1 and promotes muscle cell maturation (PMID: 37118545, Fig. 6m-r and Supplementary Fig. 5e). This suggests that the very long chain ceramides c24 might indeed be driving the effect we see upon Cers1 inhibition because we observe an accumulation of c24 ceramides upon Cers1 (c18) inhibition (Fig 2B, Fig 3B, Fig 4A, Fig S3E), which is associated with impaired muscle maturation (Fig 4B-C, Fig S3G-I, Fig S4G-I). To study whether impaired muscle cell differentiation upon Cers1 inhibition is dependent on Cers2, we knocked-down Cers1 alone, or in combination with the knockdown of Cers2. Results show that reduced muscle cell maturation mediated by Cers1KD is rescued by the simultaneous knockdown of Cers2 as shown by gene expression analyses and immunohistochemical validation and quantification. Hence, we believe that reducing Cers1 function during aging might lead to an increase in sphingosine levels as has been shown previously (PMID: 31692231). Increased sphingosine triggers cell apoptosis due to its toxicity (PMID: 12531554). Therefore, channeling accumulating sphingosine towards C24 ceramides may avoid toxicity but, as we show in this manuscript, will reduce the myogenic potential in muscle. However, if also C24 production is blocked by Cers2 inhibition, sphingosine is forced towards the production of other, potentially less toxic or myogenesis-impairing ceramides. We added these new data to the revised manuscript as new Fig 5D-E and new Fig S5G-I.

Reviewer #2 (Public Review):

Summary:

The manuscript by Wohlwend et al. investigates the implications of inhibiting ceramide synthase Cers1 on skeletal muscle function during aging. The authors propose a role for Cers1 in muscle myogenesis and aging sarcopenia. Both pharmacological and AAV-driven genetic inhibition of Cers1 in 18month-old mice lead to reduced C18 ceramides in skeletal muscle, exacerbating age-dependent features such as muscle atrophy, fibrosis, and center-nucleated fibers. Similarly, inhibition of the Cers1 orthologue in C. elegans reduces motility and causes alterations in muscle morphology.

Strengths:

The study is well-designed, carefully executed, and provides highly informative and novel findings that are relevant to the field.

Weaknesses:

The following points should be addressed to support the conclusions of the manuscript.

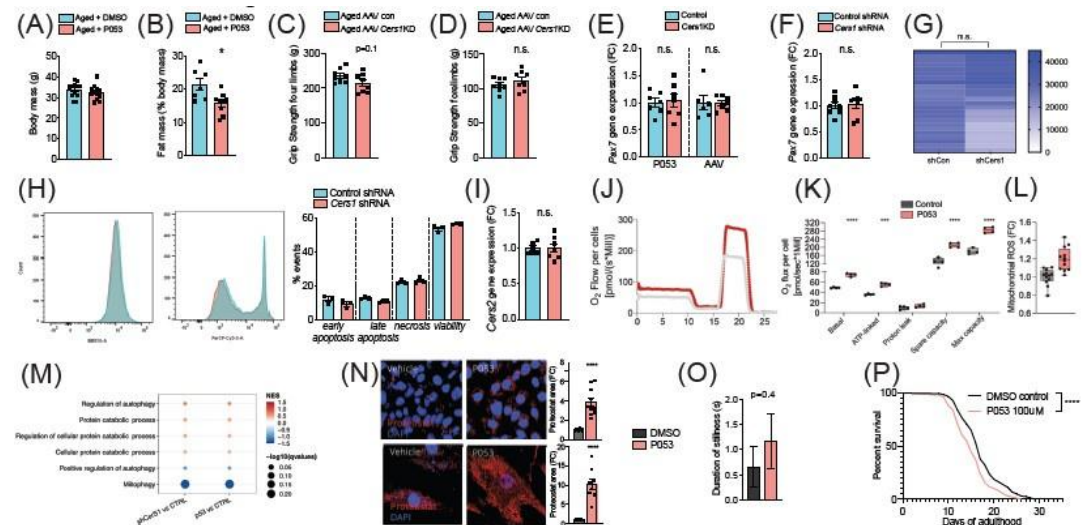
(1) It would be essential to investigate whether P053 treatment of young mice induces age-dependent features besides muscle loss, such as muscle fibrosis or regeneration. This would help determine whether the exacerbation of age-dependent features solely depends on Cers1 inhibition or is associated with other factors related to age-dependent decline in cell function. Additionally, considering the reported role of Cers1 in whole-body adiposity, it is necessary to present data on mice body weight and fat mass in P053treated aged-mice.

We thank the reviewer to suggest that we study Cers1 inhibition in young mice. In fact, a previous study shows that muscle-specific Cers1 knockout in young mice impairs muscle function (PMID: 31692231). Similar to our observation, these authors report reduced muscle fiber size and muscle force. Therefore, we do not believe that our observed effects of Cers1 inhibition in aged mice are specific to aging, although the phenotypic consequences are accentuated in aged mice. As requested by the reviewer, we attached the mice body weights and fat mass (Author response image 1A-B). The reduced fat mass upon P053 treatment is in line with previously reported reductions in fat mass in chow diet or high fat diet fed young mice upon Cers1 inhibition (PMID: 30605666, PMID: 30131496), again suggesting that the effect of Cers1 inhibition might not be specific to aging.

Author response image 1.

(A-B) Body mass (A) and Fat mass as % of body mass (B) were measured in 22mo C57BL/6J mice intraperitoneally injected with DMSO or P053 using EchoMRI (n=7-12 per group). (C-D) Grip strength measurements in all limbs (C) or only the forelimbs (D) in 24mo C57BL/6J mice intramuscularly injected with AAV9 particles containing scramble, or shRNA targeting Cers1 (n=8 per group). (E-F) Pax7 gene expression in P053 or AAV9 treated mice (n=6-7 per group) (E), or in mouse C2C12 muscle progenitor cells treated with 25nM scramble or Cers1 targeting shRNA (n=8 per group) (F). (G) Proliferation as measured by luciferase intensity in mouse C2C12 muscle muscle cells treated with 25nM scramble or Cers1 targeting shRNA (n=24 per group). Each column represents one biological replicate. (H) Overlayed FACS traces of Annexin-V (BB515, left) and Propidium Iodide (Cy5, right) of mouse C2C12 muscle myotubes treated with 25nM scramble or Cers1 targeting shRNA (n=3 per group). Quantification right: early apoptosis (Annexin+PI-), late apoptosis (Annexin+PI+), necrosis (Annexin-PI+), viability (Annexin-PI-). (I) Normalized Cers2 gene expression in mouse C2C12 muscle muscle cells treated with 25nM scramble or Cers1 targeting shRNA (n=6-7 per group). (J-K) Representative mitochondrial respiration traces of digitonin-permeabilized mouse C2C12 muscle muscle cells treated DMSO or P053 (J) with quantification of basal, ATP-linked, proton leak respiration as well as spare capacity and maximal capacity linked respiration (n=4 per group). (L) Reactive oxygen production in mitochondria of mouse C2C12 muscle muscle cells

treated DMSO or P053. (M) Enriched gene sets related to autophagy and mitophagy in 24mo C57BL/6J mouse muscles intramuscularly injected with AAV9 particles containing scramble, or shRNA targeting Cers1 (left), or intraperitoneally injected with DMSO or P053 (right). Color gradient indicates normalized effect size. Dot size indicates statistical significance (n=6-8 per group). (N) Representative confocal Proteostat® stainings with quantifications of DMSO and P053 treated mouse muscle cells expressing APPSWE (top) and human primary myoblasts isolated from patients with inclusion body myositis (bottom). (O) Stillness duration during a 90 seconds interval in adult day 5 C. elegans treated with DMSO or 100uM P053. (P) Lifespan of C. elegans treated with DMSO or P053. (n=144-147 per group, for method details see main manuscript page 10).



(2) As grip and exercise performance tests evaluate muscle function across several muscles, it is not evident how intramuscular AAV-mediated Cers1 inhibition solely in the gastrocnemius muscle can have a systemic effect or impact different muscles. This point requires clarification.

The grip strength measurements presented in the manuscript come from hindlimb grip strength, as pointed out in the Methods section. We measured grip strength in all four limbs, as well as only fore- (Author response image 1C-D). While forelimb strength did not change, only hindlimb grip strength was significantly different in AAV-Cers1KD compared to the scramble control AAV (Fig 3I), which is in line with the fact that we only injected the AAV in the hindlimbs. This is similar to the effect we observed with our previous data where we saw altered muscle function upon IM AAV delivery in the gastrocnemius (PMID: PMID: 34878822, PMID: 37118545). The gastrocnemius likely has the largest contribution to hindlimb grip strength given its size, and possibly even overall grip strength as suggested by a trend of reduced grip strength in all four limbs (Author response image 1C). We also suspect that the hindlimb muscles have the largest contribution to uphill running as we could also see an effect on running performance. While we carefully injected a minimal amount of AAV into gastrocnemius to avoid leakage, we cannot completely rule out that some AAV might have spread to other muscles. We added this information to the discussion of the manuscript as a potential limitation of the study.

(3) To further substantiate the role of Cers1 in myogenesis, it would be crucial to investigate the consequences of Cers1 inhibition under conditions of muscle damage, such as cardiotoxin treatment or eccentric exercise. While it would be interesting to study Cers1 in the context of muscle regeneration, and

possibly mouse models of muscular dystrophy, we think such work would go beyond the scope of the current manuscript.

(4) It would be informative to determine whether the muscle defects are primarily dependent on the reduction of C18-ceramides or the compensatory increase of C24-ceramides or C24-dihydroceramides.

To improve mechanistic insights, as suggested by Reviewer #2, we performed more experiments to gain insights how Cers1 derived c18, and Cers2 derived c24 ceramide species affect myogenesis. We recently showed that knocking out Cers2 reduces c24:0/c24:1 and promotes muscle cell maturation (PMID: 37118545, Fig. 6m-r and Supplementary Fig. 5e). This suggests that the very long chain ceramides c24 might indeed be driving the effect we see upon Cers1 inhibition because we observe an accumulation of c24 ceramides upon Cers1 (c18) inhibition (Fig 2B, Fig 3B, Fig 4A, Fig S3E), which is associated with impaired muscle maturation (Fig 4B-C, Fig S3G-I, Fig S4G-I). To study whether impaired muscle cell differentiation upon Cers1 inhibition is dependent on Cers2, we knocked-down Cers1 alone, or in combination with the knockdown of Cers2. Results show that reduced muscle cell maturation mediated by Cers1KD is rescued by the simultaneous knockdown of Cers2 as shown by gene expression analyses and immunohistochemical validation and quantification. We added these data to the manuscript as new Fig 5D-E, new Fig S5G-I. These data, together with our previous results showing that Dggs1 knockout reduces myogenesis (PMID: 37118545, Fig. 6s-x and Fig. 7) suggest that C24/dhC24 might contribute to the age-related impairments in myogenesis. We added the new results to the revised manuscript.

(5) Previous studies from the research group (PMID 37118545) have shown that inhibiting the de novo sphingolipid pathway by blocking SPLC1-3 with myriocin counteracts muscle loss and that C18-ceramides increase during aging. In light of the current findings, certain issues need clarification and discussion. For instance, how would myriocin treatment, which reduces Cers1 activity because of the upstream inhibition of the pathway, have a positive effect on muscle? Additionally, it is essential to explain the association between the reduction of Cers1 gene expression with aging (Fig. 1B) and the age-dependent increase in C18-ceramides (PMID 37118545).

Blocking the upstream enzyme of the ceramide pathway (SPT1) shuts down the entire pathway that is overactive in aging, and therefore seems beneficial for muscle aging. While most enzymes in the ceramide pathway that we studied so far (SPTLC1, CERS2) revealed muscle benefits in terms of myogenesis, inflammation (PMID: 35089797; PMID: 37118545) and muscle protein aggregation (PMID: 37196064), the CERS1 enzyme shows opposite effects. This is also visible in the direction of CERS1 expression compared to the other enzymes in one of our previous published studies (PMID: 37118545, Fig. 1e and Fig. 1f). In the current study, we show that Cers1 inhibition indeed exacerbates age-related myogenesis and inflammation as opposed to the inhibition of Sptlc1 or Cers2. As the reviewer points out, both C18- and C24-ceramides seem to accumulate upon muscle aging. We think this is due to an overall overactive ceramide biosynthesis pathway. Blocking C18-ceramides via Cers1 inhibition results in the accumulates C24-ceramides and worsens muscle phenotypes (see reply to question #4). On the other hand, blocking C24-ceramides via Cers2 inhibition improves muscle differentiation. These observations together with the finding that Cers1 mediated inhibition of muscle differentiation is dependent on proper Cers2 function (new Fig 5D-E, new Fig S5G-I) points towards C24-ceramides as the main culprit of reduced muscle differentiation. Hence, at least a significant part of the benefits of blocking SPTLC1 might have been related to reducing very long-chain ceramides. We believe that reduced Cers1 expression in skeletal muscle upon aging, observed by us and others (PMID: 31692231), might reflect a compensatory mechanism to make up for an overall overactive ceramide flux in aged muscles. Reducing Cers1 function during aging might lead to an increase in sphingosine

levels as has been shown previously (PMID: 31692231). Increased sphingosine triggers cell apoptosis due to its toxicity (PMID: 12531554). Therefore, channeling accumulating sphingosine towards C24 ceramides may avoid toxicity but, as we show in this manuscript, will reduce the myogenic potential in muscle. However, if also C24 production is blocked by Cers2 inhibition (new Fig 5E-D, new Fig S5G-I), sphingosine is forced towards the production of other, potentially less toxic, or myogenesis-impairing ceramides. These data are now added to the revised manuscript (see page 7). Details were added to the discussion of the manuscript (see page 8).

Addressing these points will strengthen the manuscript's conclusions and provide a more comprehensive understanding of the role of Cers1 in skeletal muscle function during aging.

Reviewer #1 (Recommendations For The Authors):

The authors identified that genetical and pharmacological inhibition of CERS1, an enzyme implicated in ceramides biosynthesis worsen muscle fibrosis and inflammation during aging.

Even though many of the experiments only confirmed previous published data (ref 21, 11,37,38), which also show a decline of CERS1 in ageing and the generation and characterization of a muscle specific knockout mouse line, the study points out an interesting issue on excluding CERS1 inhibition as a therapeutic strategy for sarcopenia and opens new questions on understanding how inhibition of SPTLC1 (upstream CERS1) have beneficial effects in healthy aging (ref 15 published by the same authors).

Overall, the article it's well written and clear. However, there is a major weakness. The mechanistic insights of how the increased amount of long ceramides (c24) and the decreased of shorter ones (cer c18) might influence muscle mass, force production, fibrosis and inflammation in aged mice have not been addressed. At the present stage the manuscript is descriptive and confirmatory of CERS1 mediated function in preserving muscle mass. The authors should consider the following points:

Comments:

(1) Muscle data

(a) The effect of CERS1 inhibition on myotube formation must be better characterized. Which step of myogenesis is affected? Is stem cell renewal or MyoD replication/differentiation, or myoblast fusion or an increased cell death the major culprit of the small myotubes? Minor point: Figure S1C: show C14:00 level at 200 h; text of Fig S2A and 1F: MRF4 and Myogenin are not an early gene in myogenesis please correct, Fig S2B and 2C: changes in transcript does not mean changes in protein or myotube differentiation and therefore, authors must test myotube formation and myosin expression.

Cers1 inhibition seems to affect differentiation and myoblast fusion. To test other suggested effects we performed more experiments as delineated. Inhibiting Cers1 systemically with the pharmacological inhibitor of Cers1 (P053) or with intramuscular delivery of AAV expressing a short hairpin RNA (shRNA) against Cers1 in mice did not affect Pax7 transcript levels (Author response image 1E). Moreover, we did also not observe an effect of shRNA targeting Cers1 on Pax7 levels in mouse C2C12 muscle progenitor cells (Author response image 1F). To characterize the effect of Cers1 inhibition on muscle progenitor proliferation/renewal, we used scramble shRNA, or shRNA targeting Cers1 in C2C12 muscle progenitors and measured proliferation using CellTiter-Glo (Promega). Results showed that Cers1KD had no significant effect on cell proliferation (Author response image 1G). Next, we assayed cell death in differentiating C2C12 myotubes deficient in Cers1 using FACS Analysis of Annexin V (left) and

propidium iodide (right). We found no difference in early apoptosis, late apoptosis, necrosis, or muscle cell viability, suggesting that cell death can be ruled out to explain smaller myotubes (Author response image 1H). These findings support the notion that the inhibitory effect of Cers1 knockdown on muscle maturation are primarily based on effects on myogenesis rather than on apoptosis. Our data in the manuscript also suggests that Cers1 inhibition affects myoblast fusion, as shown by reduced myonucleation upon Cers1KD (Fig S3H right, Fig S5I).

(b) The phenotype of CESR1 knockdown is milder than OP53 treated mice (Fig S5D and Figure 3F, 3H are not significant) despite similar changes of Cer18:0, Cer24:0, Cer 24:1 concentration in muscles . Why?

Increases in very long chain ceramides were in fact larger upon P053 administration compared to AAVmediated knockdown. For example, Cer24:0 levels increased by >50% upon P053 administration, compared to 20% by AAV injections. Moreover, dhC24:1 increased by 6.5-fold vs 2.5-fold upon P053 vs AAV treatment, respectively. These differences might not only explain the slightly attenuated phenotypes in the AA- treated mice but also underlines the notion that very long chain ceramides might cause muscle deterioration. We believe inhibiting the enzymatic activity of Cers1 (P053) as compared to degrading Cers1 transcripts is a more efficient strategy to reduce ceramide levels. However, we cannot completely rule out multi-organ, systemic effects of P053 treatment beyond its direct effect on muscle. We added these details in the discussion of the revised manuscript (see page 8 of the revised manuscript).

(c) The authors talk about a possible compensation of CERS2 isoform but they never showed mRNA expression levels or CERS2 protein levels aner treatment. Is CERS2 higher expressed when CERS1 is downregulated in skeletal muscle?

We appreciate the suggestion of the reviewer. We found no change in Cers2 mRNA levels upon Cers1 inhibition in mouse C2C12 myoblasts (Author response image 1I). We would like to point out that mRNA abundance might not be the optimal measurement for enzymes due to enzymatic activities. Therefore, we think metabolite levels are a better proxy of enzymatic activity. It should also be pointed out that “compensation” might not be an accurate description as sphingoid base substrate might simply be more available upon Cers1KD and hence, more substrate might be present for Cers2 to synthesize very long chain ceramides. This “re-routing” has been previously described in the literature and hypothesized to be related to avoid toxic (dh)sphingosine accumulation (PMID: 30131496). Therefore, we changed the wording in the revised manuscript to be more precise.

(d) Force measurement of AAV CERS1 downregulated muscles could be a plus for the study (assay function of contractility)

In the current study we measured grip strength in mice, which had previously been shown to be a good proxy of muscle strength and general health (PMID: 31631989). Indeed, our results of reduced muscle grip strength are in line with previous work that shows reduced contractility in muscles of Cers1 deficient mice (PMID: 31692231).

(e) How are degradation pathways affected by the downregulation of CERS1. Is autophagy/mitophagy affected? How is mTOR and protein synthesis affected? There is a recent paper that showed that CerS1 silencing leads to a reduction in C18:0-Cer content, with a subsequent increase in the activity of the insulin pathway, and an improvement in skeletal muscle glucose uptake. Could be possible that CERS1 downregulation increases mTOR signalling and decreases autophagy pathway? Autophagic flux using colchicine in vivo would be useful to answer this hypothesis

Cers1 in skeletal muscle has indeed been linked to metabolic homeostasis (see PMID: 30605666). In line with their finding in young mice we also find reduced fat mass upon P053 treatment in aged mice (Author response image 1A-B). We also looked into mitochondrial bioenergetics upon blocking Cers1 with P053 treatment using an O2k oxygraphy (Author response image 1J-L). Results show that Cers1 inhibition in mouse muscle cells increases mitochondrial respiration, similar to what has been shown before (PMID: 30131496). However, we also found that reactive oxygen species production in mouse muscle cells is increased upon P053 treatment, suggesting the presence of dysfunctional mitochondria upon inhibiting Cers1 with P053. We next looked into the mitophagy/autophagy degradation pathways suggested by the reviewer and do not find convincing evidence supporting that Cers1 has a major impact on autophagy or mitophagy derived gene sets in mice treated with shRNA against Cers1, or the Cers1 pharmacological inhibitor P053 (Author response image 1M).

We then assessed the effect of Cers1 inhibition on transcripts levels related to the mTORC1/protein synthesis, as suggested by the reviewer. Cers1 knockdown in differentiating mouse muscle cells showed only a weak trend to reduce mTORC1 and its downstream targets (new Fig S4A). In line with this, there was no notable difference in protein synthesis in differentiating, Cers1 deficient mouse C2C12 myoblasts as assessed by L-homopropargylglycine (HPG) amino acid labeling using confocal microscopy (new Fig S4B) or FACS analyses (new Fig S4C). However, Cers1KD increased transcripts related to the myostatin-Foxo1 axis as well as the ubiquitin proteasome system (e.g. atrogin-1, MuRF1) (new Fig S4D), suggesting Cers1 inhibition increases protein degradation. We added these details to the revised manuscript on page 7. We recently implicated the ceramide pathway in regulating muscle protein homeostasis (PMID: 37196064). Therefore, we assessed the effect of Cers1 inhibition with the P053 pharmacological inhibitor on protein folding in muscle cells using the Proteostat dye that intercalates into the cross-beta spine of quaternary protein structures typically found in misfolded and aggregated proteins. Interestingly, inhibiting Cers1 further increased misfolded proteins in C2C12 mouse myoblasts expressing the Swedish mutation in APP and human myoblasts isolated from patients with inclusion body myositis (Author response image 1N). These findings suggest that deficient Cers1 might upregulate protein degradation to compensate for the accumulation of misfolded and aggregating proteins, which might contribute to impaired muscle function observed upon Cers1 knockdown. Further studies are needed to disentangle the underlying mechanistics.

(f) The balances of ceramides have been found to play roles in mitophagy and fission with an impact on cell fate and metabolism. Did the authors check how are mitochondria morphology, mitophagy or how dynamics of mitochondria are altered in CERS1 knockdown muscles? (fission and fusion). There is growing evidence relating mitochondrial dysfunction to the contribution of the development of fibrosis and inflammation.

Previously, CERS1 has been studied in the context of metabolism and mitochondria (for reference, please see PMID: 26739815, PMID: 29415895, PMID: 30605666, PMID: 30131496). In summary, these studies demonstrate that C18 ceramide levels are inversely related to insulin sensitivity in muscle and mitochondria, and that Cers1 inhibition improves insulin-stimulated suppression of hepatic glucose production and reduced high-fat diet induced adiposity. Moreover, improved mitochondrial respiration, citrate synthase activity and increased energy expenditure were reported upon Cers1 inhibition. Lack of Cers1 specifically in skeletal muscle was also reported to improve systemic glucose homeostasis. While these studies agree on the effect of Cers1 inhibition on fat loss, results on glucose homeostasis and insulin sensitivity differ depending on whether a pharmacologic or a genetic approach was used to inhibit Cers1. The current manuscript describes the effect of CERS1 on muscle function and myogenesis because these were the most strongly correlated pathways with

CERS1 in human skeletal muscle (Fig 1C) and impact of Cers1 on these pathways is poorly studied, particularly in the context of aging. Therefore, we would like to refer to the mentioned studies investigating the effect of CERS1 on mitochondria and metabolism.

(2) C.elegans data:

(a) The authors checked maternal RNAi protocol to knockdown lagr-1 and showed alteration of muscle morphology at day 5. They also give pharmacological exposure of P053 drug at L4 stage. Furthermore, the authors also used a transgenic ortholog lagr-1 to perform the experiments. All of them were consistent showing a reduced movement. It would be important to show rescue of the muscle phenotype by overexpressing CERS1 ortholog in knockdown transgenic animals.

We used RNAi to knockdown the Cers1 orthologue, lagr-1, in C.elegans. Therefore, we do not have transgenic animals. Overexpressing lagr-1 in the RNAi treated animals would also not be possible as the RNA from the overexpression would just get degraded.

(b) The authors showed data about distance of C.elegans. It would be interesting to specify if body bends, reversals and stillness are affected in RNAi and transgenic Knockdown worms.

As suggested, we measured trashing and stillness as suggested by the reviewer and found reduced trashing (new Fig S5B) and a trend towards an increase in stillness (Author response image 1O) in P053 treated worms on day 5 of adulthood, which is the day we observed significant differences in muscle morphology and movement (Fig 4D-E, Fig S5A). These data are now included in the revised manuscript.

(c) Is there an effect on lifespan extension by knocking down CERS1?

We performed two independent lifespan experiments in C.elegans treated with the Cers1 inhibitor P053 and found reduced lifespan in both replicate experiments (for second replicate, see Author response image 1P). We added these data to the revised manuscript as new Fig 4H.

How do the authors explain the beneficial effect of sptlc1 inhibition on healthy aging muscle? Discuss more during the article if there is no possible explanation at the moment.

We believe that blocking the upstream enzyme of the ceramide pathway (SPT1) shuts down the entire pathway that is overactive in aging, and therefore is more beneficial for muscle aging. Our current work suggests that at least a significant part of Sptlc1-KD benefits might stem from blocking very long chain ceramides. While SPTLC1 and CERS2 revealed muscle benefits in terms of myogenesis, inflammation (PMID: 35089797; PMID: 37118545) and muscle protein aggregation (PMID: 37196064), the CERS1 enzyme shows opposite effects, which is also visible in Fig 1e and Fig 1f of PMID: 37118545. In the current study, we show that Cers1 inhibition indeed exacerbates aging defects in myogenesis and inflammation as opposed to the inhibition of Sptlc1 or Cers2. The fact that the effect of Cers1 on inhibiting muscle differentiation is dependent on the clearance of Cers2-derived C24-ceramides suggests that reducing very long chain ceramides might be crucial for healthy muscle aging. We added details to the discussion.