

AAV-mediated FGF21 gene therapy promotes health span extension by whole-body tissue-specific adaptations

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The decline of organ function during aging limits health span. Despite the potential of lifestyle interventions to improve health, sustained maintenance of health span is challenging, and no gerotherapeutic drugs have been approved. Here, we demonstrated that aged and geriatric male and female mice treated with muscle-directed adeno-associated viral (AAV) vector-mediated fibroblast growth factor 21 (FGF21) gene therapy extended health span and lifespan with sustained organ benefits. This treatment normalized body weight and adiposity, improved insulin sensitivity and glucose homeostasis, preserved hepatic detoxification capacity, counteracted age-related kidney disease, promoted cardiac health and muscular function, and enhanced cognition. Transcriptomic and histopathological analyses indicated improved whole-body energy homeostasis and cellular fitness, which were mediated by tissue-specific adaptations, including enhanced mitochondrial function, restored proteostasis, and reversion of inflammation, fibrosis, and amyloidosis. AAV-FGF21 treatment also activated AMPK signaling. These results highlight FGF21 gene therapy as a potential strategy to promote health span and delay age-related deterioration.

INTRODUCTION

Deterioration of physiological integrity and growing risk of disease during the aging process results from progressive impairment of cellular fitness and organ function.¹ Demographic projections indicate a dramatic expansion of the global aging population, with more than 2.1 billion individuals expected to be over 65 years old and 426 million over 80 by 2050 (www.who.int/z/news-room/fact-sheets/detail/ageing-and-health). Yet, while chronological lifespan continues to steadily increase, a comparable parallel prolongation of health span has not been achieved. Prevalence of age-associated disorders, such as type 2 diabetes (T2D), obesity, and cardiovascular or neurodegenerative diseases, is increasing with epidemic proportions (www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases).

The aging process is accompanied by progressive metabolic dysregulation characterized by weight gain, increased adiposity, insulin resistance, and impaired cellular metabolism and energy homeostasis.^{1–5} These metabolic shifts are recognized as central hallmarks of aging and key drivers of major age-related diseases, tissue functional decline, frailty, and diminished quality of life in the elderly.^{2,3} Conversely, metabolically healthy individuals, centenarians, and long-lived populations demonstrate remarkable resistance to age-related metabolic deterioration.³ They exhibit lower adiposity and improved insulin sensitivity, lipid metabolism, mitochondrial function, and protein homeostasis.^{6–9}

Lifestyle interventions, such as exercise, healthy diet, caloric, protein, or amino acid restriction, and intermittent fasting, can enhance metabolic health, reduce chronic diseases, and promote health span.¹⁰ These effects are mediated by a complex network of molecular pathways, in which AMPK, a master regulator of cellular metabolism,¹¹ is a critical regulatory hub.^{10,12} However, these approaches show variable efficacy and suffer from poor long-term adherence. Recently, metabolic-targeting drugs like metformin and rapamycin have been proposed as potential gerotherapeutics. Metformin, a first-line treatment for T2D, mimics exercise and dietary interventions, extending health span in animals primarily through AMPK-dependent pathways.¹³ Metformin also lowers the incidence of age-related diseases and reduces mortality in both diabetic and non-diabetic subjects.¹⁴

Similarly, the endocrine factor fibroblast growth factor 21 (FGF21) is a key metabolic regulator that coordinates inter-organ metabolism and energy homeostasis and has emerged as a promising therapeutic agent

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for chronic metabolic diseases.¹⁵ In this regard, one-time administration of adeno-associated viral (AAV) vector-mediated FGF21 gene therapy resulted in sustained reversal of metabolic dysfunction-associated steatohepatitis (MASH), T2D, and obesity in mice.^{16,17} This gene therapy approach is advancing toward clinical translation in humans (<https://kriyatherapeutics.com/pipeline/>). In this study, the therapeutic benefits of the muscle-directed AAV-FGF21 gene therapy in old and geriatric (13-, 19-, and 22-month-old) male and female mice were assessed. This single intervention extended both health span and lifespan through targeted tissue adaptations that delay age-related whole-body organ deterioration. This study establishes AAV-FGF21 gene therapy as a translatable strategy to promote healthy aging with a favorable long-term efficacy and safety profile.

RESULTS

Treatment of old mice with AAV-FGF21 increases health span and lifespan

Overweight and insulin-resistant 13-month-old chow-fed male mice (Figure 1A; Figure S1A) were treated intramuscularly (i.m.) with 3×10^{11} viral genomes (vg) of AAV vectors of serotype 1 encoding FGF21 (AAV-FGF21) to assess whether this gene therapy could reverse established metabolic alterations and to prevent age-related decline in whole-body cellular fitness. While control (AAV-Null-treated) mice steadily increased their body weight, AAV-FGF21-treated mice progressively lost weight and reached a mean body weight similar to that of young (2-month-old) mice throughout the follow-up period (Figure 1A). At 21 months of age, obese AAV-Null-treated mice began losing weight and gradually increased mortality (Figures 1A and 1B). These mice showed a median lifespan of 28.14 months, which was expanded to 33.92 months in the AAV-FGF21-treated group ($p = 0.0098$), representing a 20.54% increase in midlife survival (Figure 1B). Moreover, by ~34 months of age, 90% of AAV-Null mice had died (Figure 1B), whereas AAV-FGF21 treatment significantly extended longevity. Similarly, male mice treated with AAV-FGF21 at 22 months of age (Figure 1A) remained alive (4/4) at 26 months, while AAV-Null-treated mice showed increased mortality (8/31, ~25.8%) during this 5-months period (Figure 1B). This benefit resulted from sustained elevation of circulating FGF21 levels (Figure 1C), due to specific transduction and FGF21 overexpression in the AAV-FGF21-treated skeletal muscles (Figures S1B and S1C). As a result of the i.m. administration of AAV1 vectors, vector genome copy numbers and AAV-derived FGF21 expression were not detected in other tissues (Figures S1B and S1C), consistent with previous reports.^{16–19} The decreased body weight paralleled the reductions in the weight of the main white (WAT) and brown (BAT) adipose tissue depots, as well as the liver (Figures 1D and 1E; Figure S1D). These tissues also showed increased gene expression of the FGF21 co-receptor β -klotho (*Klb*) (Figures S1E and S1F). Although AAV-FGF21-treated mice were leaner, food intake remained unchanged (Figure S1G). Similar metabolic improvements were observed in both male and female geriatric mice, treated at 22 and 19 months old, respectively, with the same dose of AAV-FGF21 vectors (Figures 1A, 1C–1E; Figures S2A–S2F).

Moreover, AAV-FGF21 treatment decreased glycemia, normalized insulinemia, and increased insulin sensitivity (at both high and low insulin doses) and glucose tolerance, indicating improved peripheral tissue metabolism (Figures 1F–1I; Figure S1H). Consistent with insulin resistance, AAV-Null mice developed islet hyperplasia and increased β cell mass, both of which were normalized in AAV-FGF21-treated mice (Figure 1J; Figures S1I and S1J). AAV-FGF21-treated mice also exhibited reduced blood glucose levels in the pyruvate tolerance test (Figure 1K) reflecting decreased hepatic glucose production, which is typically elevated in insulin-resistant states. Similar insulin-sensitizing effects were observed in geriatric male and female mice treated with AAV-FGF21 vectors (Figures 1F and 1G; Figures S2G–S2I). Collectively, these results demonstrated that AAV-FGF21 treatment improved glucose homeostasis in aged mice. These whole-body benefits likely resulted from enhanced cellular fitness in key tissues.

Contribution of white and brown adipose tissues to extension of health span

The aging process exhibits organ-specific dynamics, with WAT showing the earliest onset and the most pronounced distress, particularly in metabolic and inflammation-related processes.^{20,21} Consistently, old AAV-Null-treated animals exhibited increased lipid deposition in visceral (epididymal WAT, eWAT) and subcutaneous WAT (inguinal WAT, iWAT) as well as in BAT (Figures 2A and 2B). Conversely, aged AAV-FGF21-treated mice showed white adipocyte size comparable to that of 2-month-old controls (Figures 2A and 2B). Moreover, reduced expression of the macrophage markers protein Cd68 antigen (*Cd68*) and adhesion G protein-coupled receptor E1 (*Adgre1*, also known as *F4/80*), along with the proinflammatory cytokine interleukin-1 β (*IL1 β*), was observed in eWAT and iWAT, indicating decreased age-associated low-grade inflammation (Figure 2C; Figure S3A). This paralleled the increased serum levels of adiponectin (Figure 2D), an anti-inflammatory and insulin-sensitizing adipokine, and a key biomarker for FGF21 biological activity.²² The reduction of inflammation in adipose tissue and increased adiponectin levels would contribute to prevent systemic low-grade inflammation and development of insulin resistance (Figures 1G and 1H; Figures S1H, S2H, and S2I).

The normalization of body weight and adiposity in FGF21-overexpressing mice resulted from higher energy expenditure during both light and dark cycles (Figure 2E; Figure S4A). In this regard, gene set enrichment analysis (GSEA) of adipose tissues from 21-month-old male mice revealed over-representation of oxidative phosphorylation (OXPHOS) and other key mitochondrial-related metabolic processes, not only in iWAT but also in eWAT of AAV-FGF21-treated mice (Figures S3B and S3C). Cluster mapping of the GSEA leading-edge genes revealed enrichment in these depots of most enzymes involved in pyruvate metabolism and tricarboxylic acid (TCA) cycle and of the majority of components of all electron transport chain (ETC) complexes (Figures 2F and 2G; Figures S3D–S3F). AAV-FGF21-treated female mice also showed upregulation of key markers of these metabolic pathways (Figures S4B–S4D). The TCA cycle is the central metabolic hub of

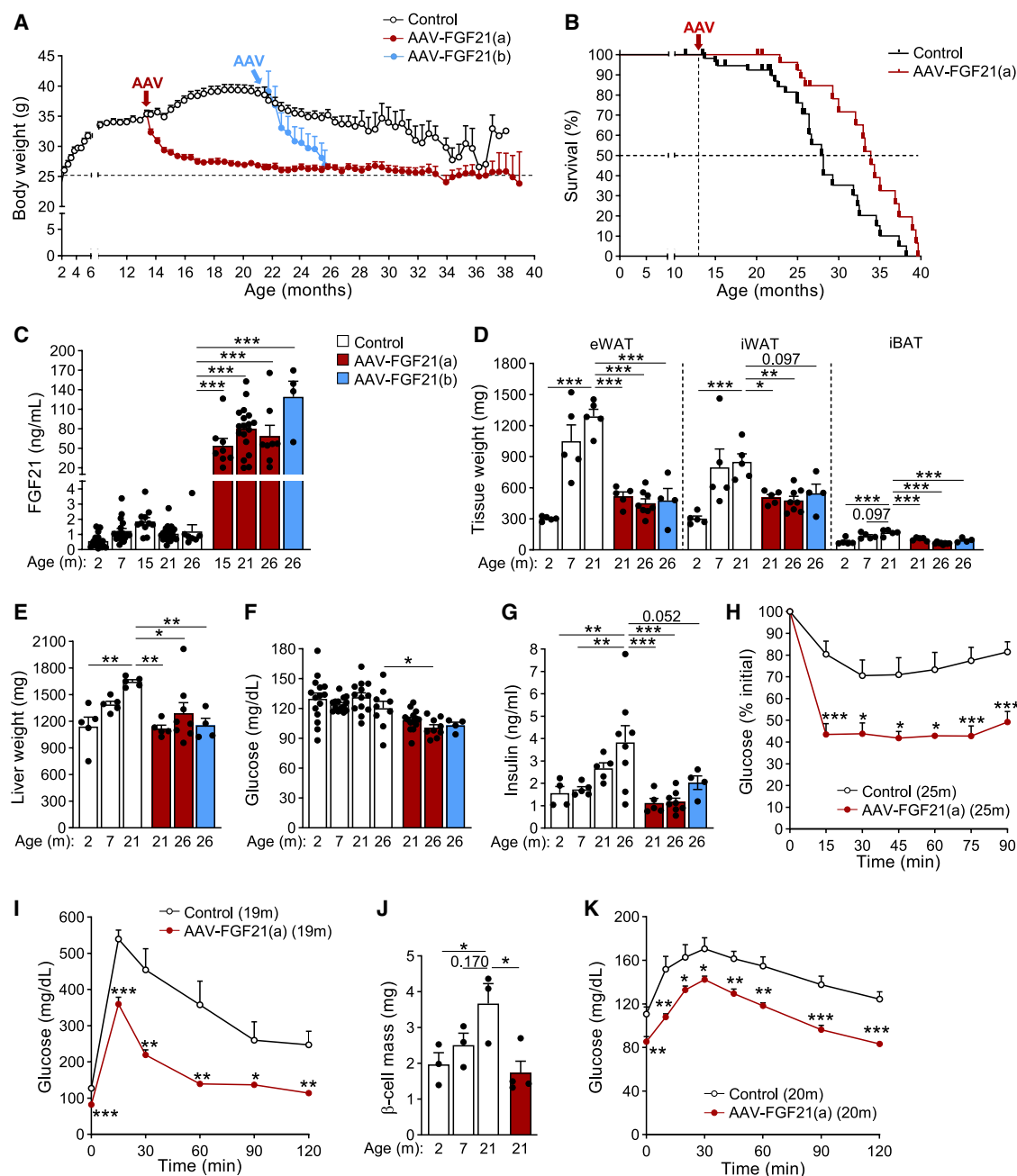
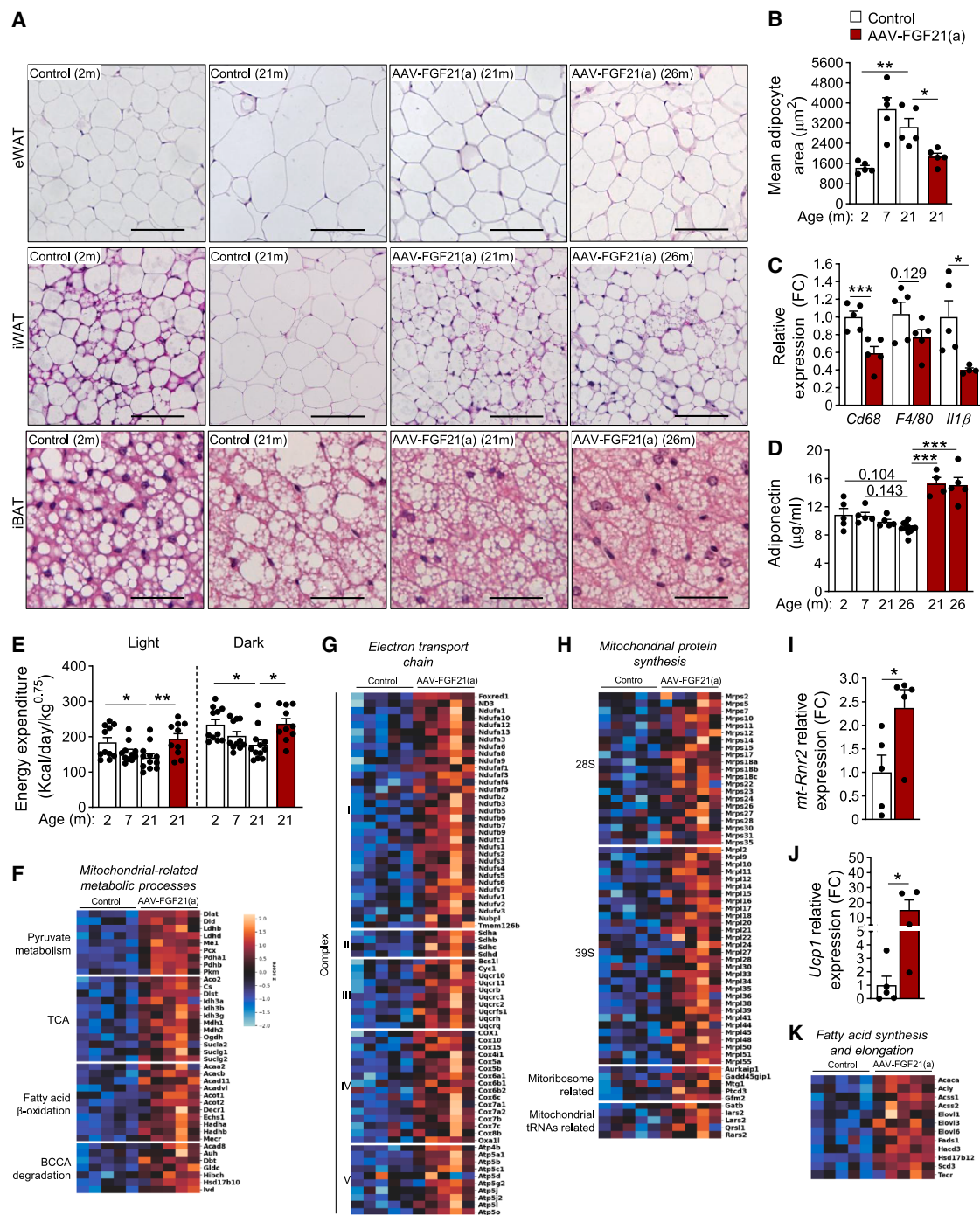


Figure 1. Treatment with AAV-FGF21 extends lifespan, counteracts body weight gain, and improves metabolism

(A) Body weight follow-up in mice treated with either AAV-Null (control, $n = 61$) or AAV-FGF21 vectors at 13 (AAV-FGF21 [a], $n = 32$) or 22 (AAV-FGF21 [b], $n = 4$) months of age. (B) Kaplan-Meier survival analysis ($n = 32$ –61/group). (C) Serum FGF21 levels ($n = 4$ –24/group). (D) Weight of the epididymal (eWAT) and inguinal (iWAT) white adipose tissue depots and the interscapular brown adipose tissue depot (iBAT) ($n = 4$ –8/group). (E) Weight of the liver ($n = 4$ –8/group). (F and G) Fed blood glucose (F, $n = 4$ –15/group) and insulin (G, $n = 4$ –8/group) levels. (H) Insulin sensitivity was determined after an intraperitoneal injection of insulin (0.375 units/kg body weight) at 25 months of age. Results were calculated as the percentage of initial blood glucose levels ($n = 3$ –6/group). (I) Glucose tolerance test was determined after an intraperitoneal injection of glucose (2 g/kg body weight) at 19 months of age ($n = 7$ /group). (J) Quantification of β cell mass ($n = 3$ –4/group). (K) Hepatic gluconeogenesis was assessed by measurement of blood glucose excursion after an intraperitoneal injection of pyruvate (1 g/kg body weight) at 20 months of age ($n = 6$ /group). Data are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (C–G and J) or Student's two-tailed t test (H, I, and K). m, months of age. Data were obtained in male mice.



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the cell producing NADH and FADH₂ to drive ETC and generate ATP through OXPHOS. Genes involved in fatty acid β -oxidation, which supplies substrates for both TCA cycle and ETC, were also enriched in iWAT (Figure 2F). Degradation of branched chain amino acids (BCAA; leucine, isoleucine, and valine) was also activated, likely to maintain intermediates for the TCA cycle (Figure 2F; Figures S3D and S3F). Upregulation of TCA cycle, OXPHOS, and amino acid metabolism was also observed in WAT of mice subjected to 17 lifespan-extending interventions.²³

A high number of genes involved in translation of mtDNA-encoded polypeptides, which are essential structural components of ETC complexes I, III, IV, and V, were also enriched in iWAT (Figure 2H; Figure S4E). These included structural components of the small and large mitoribosomal subunits (28S and 39S: *Mrps* and *Mrpl* genes), genes involved in assembly, stabilization, and recycling of mitoribosomes (*Ptcd3Mtg1*, *Aurkaip1*, *Gadd45gip1*, *Gfm2*), as well as enzymes mediating synthesis and modification of mitochondrial tRNAs (*Iars2*, *Lars2*, *Rars2*, *Gatb*, *Qrs1l*) (Figure 2H; Figure S4E). Mitochondrial protein synthesis is essential for adipose tissue function, and its impairment contributes to metabolic dysregulation during aging.^{24,25} In agreement with the globally enhanced expression of mitochondrial proteins, AAV-FGF21-treated mice exhibited increased mtDNA content in WAT (Figure 2I; Figure S3G).

The lack of enrichment for all these pathways in BAT (Figure S3H) likely stemmed from the tissue's pre-existing activation, a result of housing the mice at sub-thermoneutrality (22°C). Nevertheless, decreased lipid deposition in BAT of old AAV-FGF21-treated mice (Figure 2A) was indicative of enhancement of non-shivering thermogenesis (NST) in this depot. Accordingly, treatment with AAV-FGF21 vectors increased the expression of UCP1, the main molecular marker and effector of NST, in BAT (Figures S3I and S4F). Similarly, and in agreement with the induction of multilocular beige adipocytes in iWAT (Figure 2A), increased levels of UCP1 were observed in this tissue (Figure 2J; Figure S4G). Moreover, FGF21-overexpressing mice showed enrichment of genes related to fatty acid synthesis and elongation in iWAT (Figure 2K; Figure S4H). These processes sustain lipid reserves depleted by β -oxidation and optimize fatty acid composition for NST, which allows for a rapid substrate turnover to support high metabolic rates during NST.²⁶ Indeed, some of the upregulated genes (*Elovl3*, *Elovl6*) are key markers of NST (Figure 2K; Figure S4H). Collectively, these results indicated that NST was enhanced in iWAT and iBAT of aged mice treated with AAV-FGF21, contributing to the increased energy expenditure observed in these mice (Figure 2E; Figure S4A).

Development of fibrosis and dysregulated extracellular matrix (ECM) remodeling in WAT are also hallmarks of aging.²⁷ These processes are

regulated by TGF- β and Wnt signaling pathways,²⁸ which were downregulated in iWAT of AAV-FGF21-treated mice (Figures 3A and 3B). Consequently, these animals also showed decreased expression of genes encoding ECM components (such as collagens) and of genes involved in ECM assembly and integrity (*Fbn1*, *Ctsk*, *Mmp11*) (Figure 3C). Adipose fibrosis impairs metabolic homeostasis, and ECM remodeling can alter mitochondrial homeostasis via TGF- β signaling.^{29,30} Moreover, increased TGF- β signaling impairs browning of WAT.³¹ Therefore, downregulation of the TGF- β and Wnt signaling pathways in AAV-FGF21-treated mice would not only prevent the development of fibrosis but also contribute to maintaining mitochondrial function and induction of iWAT browning.

Development of fibrosis in WAT has been reported to induce inflammation in this tissue, which also regulates adipose tissue aging.^{32,33} Moreover, chronic low-grade inflammation, or "inflammaging," is a hallmark of aging and major contributor to age-related diseases.³⁴ In eWAT of AAV-FGF21-treated male mice, downregulation of the chemokine signaling pathway (Figure 3D) was consistent with the lack of low-grade inflammation in this depot (Figure 2C). Likewise, downregulation of immune response genes in WAT occurs across multiple health-span- and lifespan-extending interventions.²³ Moreover, the p53 signaling pathway was downregulated in eWAT of these mice (Figure 3E). Similarly, AAV-FGF21-treated female mice also showed reduced expression of markers of the chemokine and p53 signaling pathways in visceral WAT (Figures S4I and S4J). p53 downregulation in WAT has been reported to reduce senescence-associated changes, which decreases production of proinflammatory cytokines and macrophage infiltration, increases adiponectin levels, and improves insulin sensitivity.^{35,36} In contrast, upregulation of p53, induced by aging or by other stimuli, causes an inflammatory response in WAT that leads to insulin resistance.³⁵ In addition, p53 is involved in energy metabolism. Downregulation of p53 in adipocytes can increase expression of mitochondrial enzymes involved in TCA cycle, ETC, and β -oxidation.^{36–38} Similar observations were made in AAV-FGF21-treated mice (Figures S3D and S3E), suggesting that downregulation of p53 signaling also contributed to the improved energy metabolism observed in these mice. Additionally, activation of AMPK, a key mediator of FGF21 signaling,³⁹ was also observed in iWAT and iBAT of AAV-FGF21-treated mice (Figures 3F and 3G). Altogether, treatment with AAV-FGF21 vectors appeared to induce metabolic reprogramming in adipose tissue that enhanced mitochondrial function and mitigated aging-related signaling pathways, thereby improving cellular energetics and fitness.³⁹

AAV-FGF21 treatment improves detoxifying metabolism in the liver

Although body and liver weight, as well as hepatic triglyceride content, increased with aging (Figures 1A and 1E; Figure 4A),

depicts the normalized expression patterns of leading-edge genes involved in fatty acid synthesis and elongation in iWAT at 21 months of age. In (B)–(E), data are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (B–E) or Student's two-tailed t test (I and J). BCAA, branched chain amino acids; eWAT, epididymal white adipose tissue; iBAT, interscapular brown adipose tissue; iWAT, inguinal white adipose tissue; FC, fold change; m, months of age; TCA, tricarboxylic acid cycle.

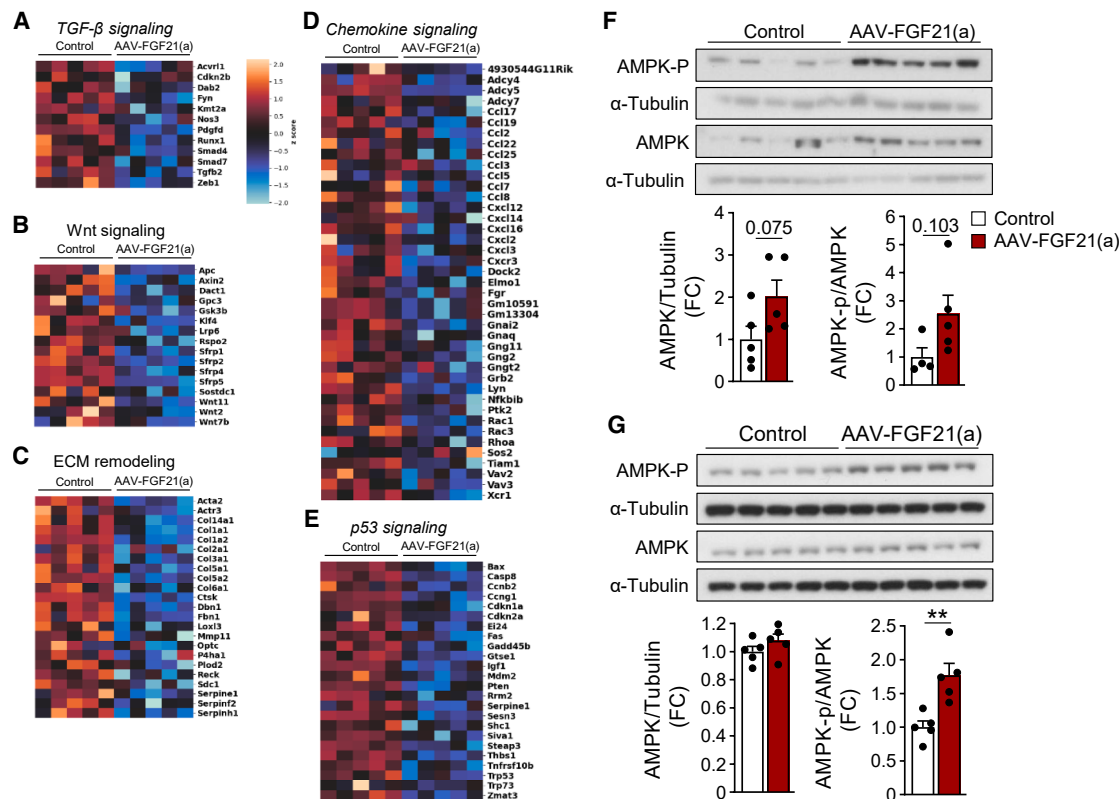


Figure 3. Effects of treatment with AAV-FGF21 in fibrosis- and inflammation-related processes in adipose tissue

Male mice were treated with either AAV-Null (control) or AAV-FGF21 vectors at 13 months of age (AAV-FGF21 (a)). (A–E) The heatmaps depict the normalized expression patterns of leading-edge genes involved in TGF- β (A) and Wnt (B) signaling, and ECM remodeling (C) in iWAT, and in chemokine (D) and p53 (E) signaling in eWAT at 21 months of age. (F and G) Western blot analysis of AMPK and AMPK-P content in iWAT (F) and iBAT (G) at 21 months of age. Representative immunoblots are shown (upper panels). Histograms depict the densitometric analyses of the immunoblots (lower panels). ($n = 5$ /group). In (F) and (G), data are expressed as the mean $\pm$ SEM. ** $p < 0.01$ by Student's two-tailed t test. ECM, extracellular matrix; FC, fold change.

the levels reached were much lower than those observed during obesogenic states, such as high-fat diet feeding or genetic obesity.^{16,17} In agreement with these findings, no steatosis was observed in liver sections of old control and AAV-FGF21-treated mice (Figure 4B). Consistent with the lack of hepatosteatosis, no striking differences in inflammation and collagen deposition were observed between groups at 21 months of age (Figures S5A and S5B). Nevertheless, at 26 months of age, histopathological analysis of the liver of control mice showed morphological alterations compatible with age-related amyloidosis, which were absent in AAV-FGF21-treated mice, indicating that this treatment prevented liver damage (Figures 4B and 4C).

The liver also plays a pivotal role in detoxification of drugs, environmental toxins, and endogenous molecules, which is crucial for maintaining cellular homeostasis. Hepatic detoxification occurs through two sequential phases: phase I introduces reactive or polar groups via oxidation, reduction, and hydrolysis, while phase II conjugates water-soluble molecules to these modified compounds, increasing their solubility for excretion. GSEA of the liver revealed that gene

sets related to detoxification were overrepresented in AAV-FGF21-treated mice (Figure S5C). Moreover, cluster map analysis of the leading-edge genes demonstrated that genes of both phases were enriched in these animals (Figures 4D and 4E). A significant number of genes encoding cytochrome P450 (CYP) enzymes, principal catalysts of phase I metabolism, were upregulated in AAV-FGF21-treated mice (Figure 4D). Several of them belong to the CYP2A, CYP2B, CYP2C, CYP3A, and CYP4A families, which metabolize lipids, steroid hormones, bile acids, and arachidonic acid (Figure 4D).^{40–43} Conversely, expression and activity of CYP enzymes and total CYP content decline during aging.⁴⁴ Flavin-containing monooxygenases (FMOs) are another class of key phase I enzymes involved in oxidative metabolism of xenobiotics and endogenous compounds. FGF21-overexpressing mice showed increased levels of FMO1–FMO4 (Figure 4D).

The balance between phase I and II is critical for effective detoxification. Treatment of aged mice with AAV-FGF21 vectors upregulated numerous phase II enzymes including glutathione S-transferases (GSTs), sulfotransferases (SULTs), and acyl-coenzyme A amino

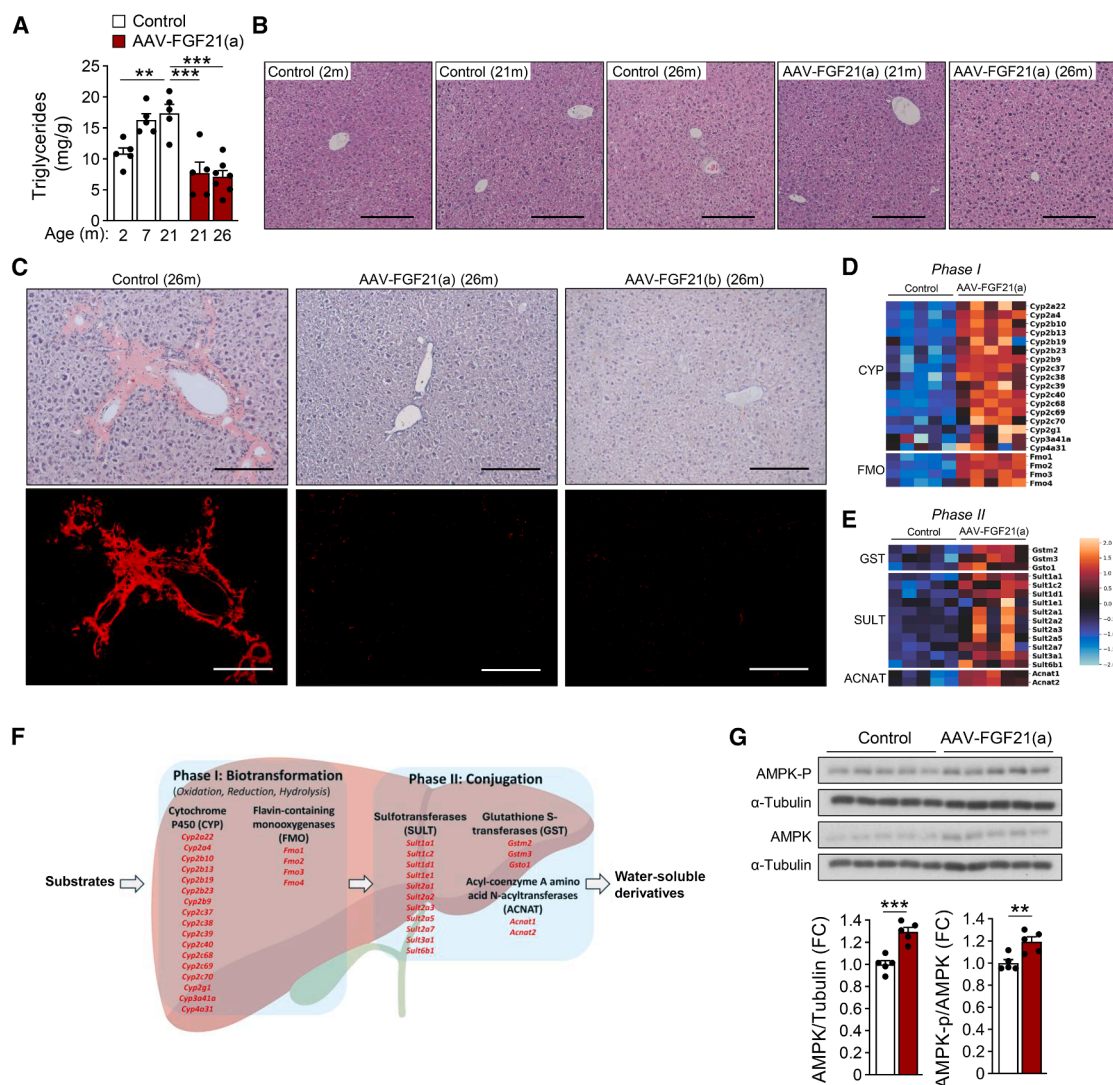
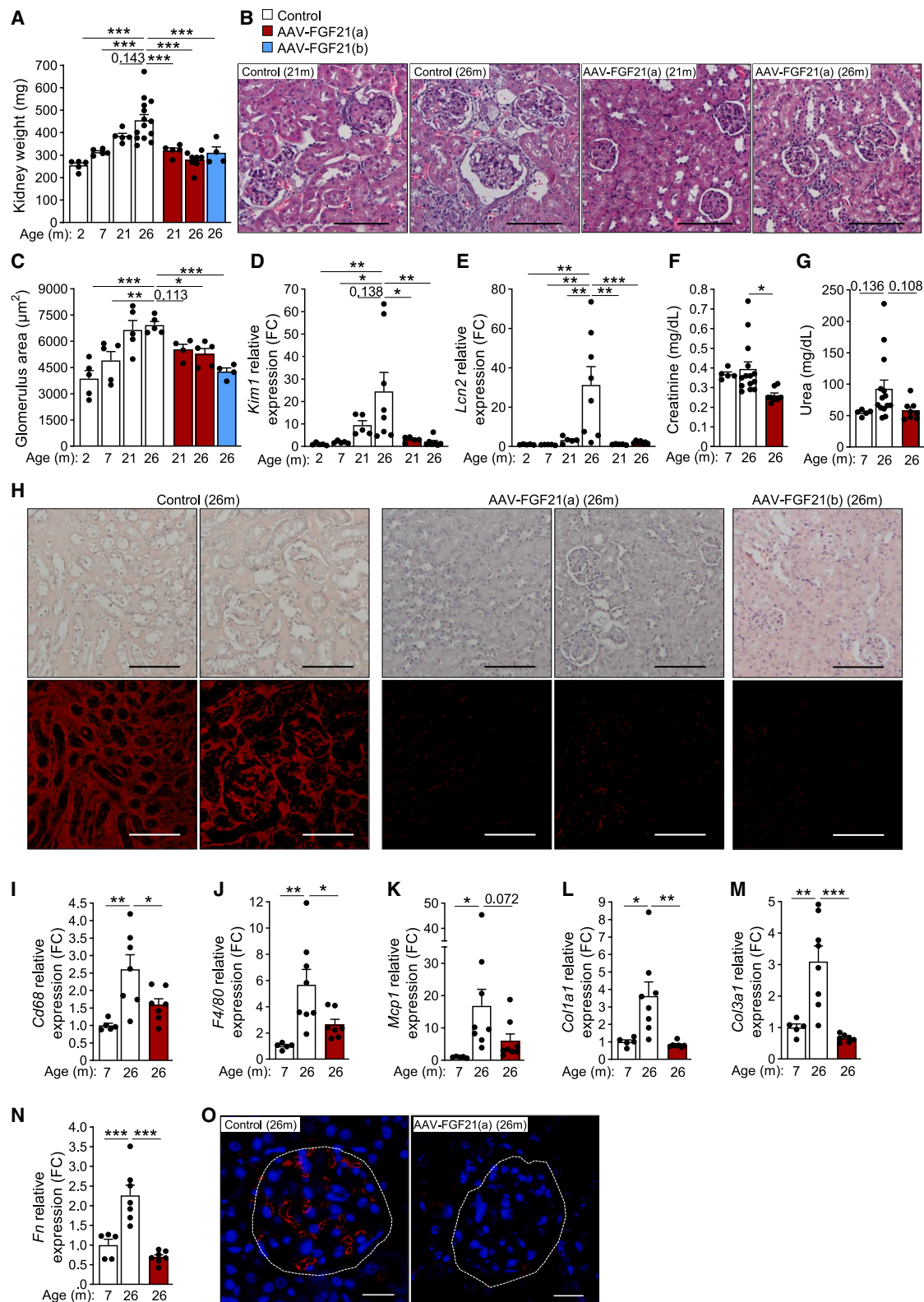


Figure 4. AAV-FGF21 gene therapy improves hepatic detoxification capacity

Male mice were treated with either AAV-Null (control) or AAV-FGF21 vectors at 13 (AAV-FGF21 (a)) or 22 (AAV-FGF21 (b)) months of age. (A) Liver triglyceride content ($n = 5$ –7/group). (B and C) Representative images of hematoxylin-eosin (B) and Congo red (C; bright field, upper panel, and fluorescence signal, lower panel) staining of liver sections. Scale bars, 200 μ m. (D and E) The heatmaps depict the normalized expression patterns of leading-edge genes of the phase I (D) and II (E) of liver detoxification at 21 months of age. (F) Scheme depicting the upregulated leading-edge genes involved in detoxifying metabolism in the liver of 21-month-old AAV-FGF21-treated mice (highlighted in red). (G) Western blot analysis of AMPK and AMPK-P content in the liver at 21 months of age. Representative immunoblots are shown. The histograms depict the densitometric analyses of the immunoblots ($n = 5$ /group). In (A) and (G), data are expressed as the mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (A) or Student's two-tailed t test (G). ACNAT, acyl-coenzyme A amino acid-N-acyltransferases; CYP, cytochrome P450; GST, glutathione S-transferases; FC, fold change; FMO, flavin-containing monooxygenases; m, months of age; SULT, sulfotransferases.

acid-N-acyltransferases (ACNATs) (Figure 4E). GST and SULT play critical roles in detoxification, oxidative stress regulation, and hormone metabolism with their expression declining during aging.^{45,46} ACNAT1 and ACNAT2, which were increased in AAV-FGF21-treated mice (Figure 4E), catalyze the conjugation of fatty acids to the amino acid taurine, forming N-acyl taurines. These bioactive lipids modulate lipid metabolism and improve glucose homeostasis^{47,48} and are also upregulated after caloric restriction.⁴⁹

Phase I and II leading-edge genes upregulated in the liver of aged mice treated with AAV-FGF21 are depicted in Figure 4F. Similar to the observations made in adipose tissue (Figures 3F and 3G), AAV-FGF21 treatment enhanced AMPK signaling in the liver (Figure 4G). Collectively, these results demonstrate that treatment with AAV-FGF21 counteracts hepatic age-related perturbations, thereby preserving liver detoxification capacity and function.



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AAV-FGF21 treatment reverses age-related kidney disease

The aging process is associated with a time-dependent decline in kidney function and is recognized as a major contributor to the increased incidence of acute kidney injury and chronic kidney disease.⁵⁰ As animals aged, progressive increases, particularly pronounced at 26 months, were observed in key markers of impaired kidney function and age-related kidney disease, including kidney weight, glomerulus area, expression of Kidney Injury Molecule 1 (*Kim1*) and Lipocalin 2 (*Lcn2*), and serum levels of creatinine and urea (Figures 5A–5G).^{51,52} In addition, 26-month-old control mice showed age-related amyloidosis in the kidney (Figure 5H). Treatment of old and geriatric male and female mice with AAV-FGF21 vectors normalized these parameters (Figures 5A–5H; Figures S6A–S6D). Moreover, AAV-FGF21-treated mice reversed the age-associated overexpression of inflammatory and fibrotic markers in the kidney, including *Cd68*, *F4/80*, *Mcp1*, collagen type I alpha 1 chain (*Col1a1*), collagen type III alpha 1 chain (*Col3a1*), and fibronectin (*Fn*) (Figures 5I–5N; Figures S6E–S6J). Consistent with this, reduced fibronectin immunostaining was observed in kidney sections of AAV-FGF21-treated mice (Figure 5O). All these findings confirmed the absence of age-related kidney damage following FGF21 gene therapy.

AAV-FGF21 treatment prevents heart aging

The aging process causes progressive structural and functional deterioration of the heart in elderly individuals and represents the dominant risk factor for cardiovascular diseases development—the leading cause of death globally.⁵³ Histopathological analysis of hematoxylin-eosin-stained myocardium sections revealed pathological alterations in 26-month-old mice receiving AAV-Null vectors (Figure 6A). These sections stained positive for Congo red, confirming development of heart amyloidosis (Figure 6B), a condition closely associated with the aging process.⁵⁴ Moreover, Picrosirius red staining demonstrated abundant collagen fibers in the heart of 26-month-old control animals (Figures 6C and 6D). In contrast, FGF21-overexpressing mice showed no signs of amyloidosis or fibrosis (Figures 6B–6D). In agreement, decreased expression levels of *Col1a1*, *Col3a1*, and *Fn* were observed in male and female mice treated with AAV-FGF21 (Figure 6E; Figure S7A). Accordingly, transcriptomic evaluation of the heart in these mice revealed downregulation of TGF- β , the master regulator of fibrosis, and several genes associated with the Wnt signaling pathway and ECM remodeling (Figures 6F and 6G). Chronic activation of the TGF- β and Wnt signaling pathways in the aging heart promotes pathological remodeling (including hypertrophy and ECM deposition), reduces elasticity, and impairs contractility, ultimately leading to heart failure.⁵⁵

Additionally, pathways related to enhanced protein synthesis capability were enriched in the hearts of AAV-FGF21-treated mice (Figure S7B). These mice showed upregulation of leading-edge genes encoding many structural proteins of both the small (40S) and large (60S) cytoplasmic ribosomal subunits (*Rps* and *Rpl* genes, respectively) and translation-associated factors (Figure 6H). The 40S subunit is critical for mRNA binding and translation initiation, whereas the 60S subunit is essential for peptide bond formation and elongation. Moreover, AAV-FGF21-treated mice showed overrepresentation of leading-edge genes involved in mitochondrial protein synthesis (*Mrps* and *Mrpl*) (Figure 6I). These findings suggested that AAV-FGF21 gene therapy not only enhanced cardiac proteostasis but also improved mitochondrial function through more efficient synthesis of ETC components encoded by mtDNA.

AAV1-FGF21 therapy improves muscular performance

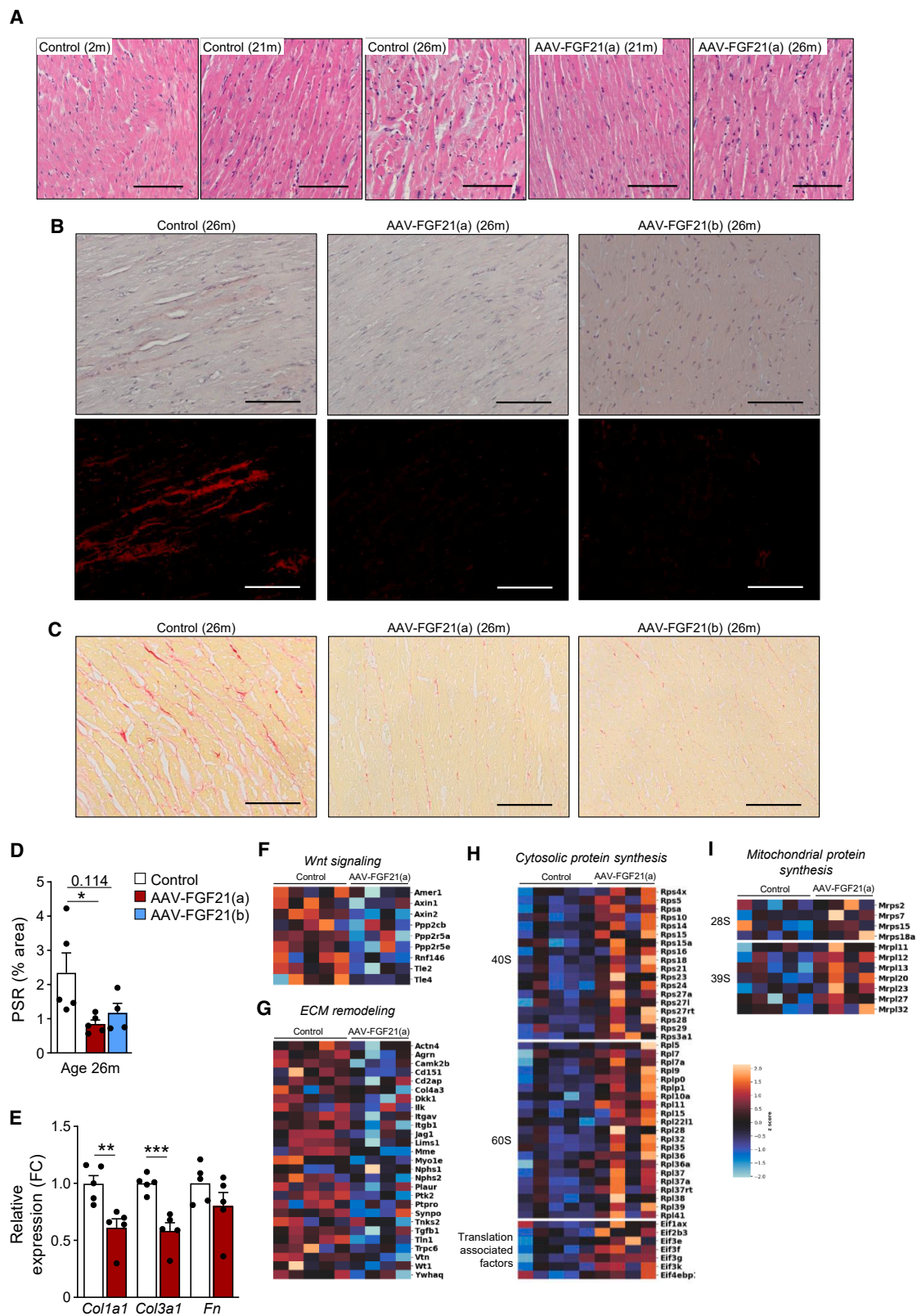
Muscle function and physical performance progressively declined during aging (Figures 7A–7D).⁵⁶ However, old mice treated with AAV-FGF21 vectors exhibited enhanced motor coordination, balance, locomotor activity, endurance, and muscle strength, performing similarly to their young counterparts (Figures 7A–7D; Figures S8A and S8B). These results demonstrated preservation of muscular performance at advanced ages.

No alterations in myofiber morphology and integrity or muscle weight were observed between 21-month-old AAV-FGF21- and AAV-Null-treated mice (Figure 7E; Figures S9A–S9D). Nevertheless, at 26 months of age, Picrosirius red staining of quadriceps sections evidenced development of fibrosis in control mice, a hallmark of aging muscle, which was absent in FGF21-overexpressing mice (Figures 7F and 7G). In agreement, decreased expression levels of *Col1a1*, *Col3a1*, and *Fn* were also observed in mice treated with AAV-FGF21 (Figure 7H; Figure S8C). Similar to the transcriptomic changes observed in cardiac muscle, AAV-FGF21-treated mice also showed downregulation of leading-edge genes involved in TGF- β signaling and ECM remodeling in quadriceps (Figures 7I and 7J). This would prevent fibrosis development, thereby preserving muscle structure and preventing functional decline.^{57,58}

Moreover, pathways involved in protein synthesis were overrepresented in quadriceps of AAV-FGF21-treated animals (Figure S9E). This gene therapy upregulated numerous *Rps* and *Rpl* genes as well as genes encoding translation-associated factors (Figure 7K). Expression of structural ribosomal constituents is also induced in skeletal muscle by other lifespan-promoting interventions.²³ Among

Figure 5. AAV-FGF21 treatment reverses age-related kidney disease

Male mice were treated with either AAV-Null (control) or AAV-FGF21 vectors at 13 (AAV-FGF21 (a)) or 22 (AAV-FGF21 (b)) months of age. (A) Kidney weight ($n = 5$ –14/group). (B) Representative images of hematoxylin-eosin staining of kidney sections. Scale bars, 100 μ m. (C) Glomerulus area ($n = 4$ –5/group). (D and E) Quantification by real-time quantitative PCR of the expression of the kidney damage gene markers Kidney Injury Molecule 1 (*Kim1*) (d) and Lipocalin 2 (*Lcn2*) (e) ($n = 5$ –14/group). (F and G) Serum creatinine (F) and urea (G) levels ($n = 5$ –14/group). (H) Representative images of Congo red staining of kidney sections (bright field, upper panels; fluorescence signal, lower panels). Scale bars, 100 μ m. (I and N) Quantification by real-time quantitative PCR of the expression of the inflammatory and fibrosis gene markers *Cd68* (I), *F4/80* (J), *Mcp1* (K), *Col1a1* (L), *Col3a1* (M), and fibronectin (*Fn*) (N) ($n = 5$ –8/group). (O) Representative images of kidney sections immunostained against fibronectin. Scale bars, 20 μ m. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test. FC, fold change; m, months of age.



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translation-associated factors, AAV-FGF21 treatment upregulated genes encoding the following: (1) critical ribosomal assembly and maturation factors, such as *Rps15*, *Rpl10A*, and *Fau*, a pivotal gene for 40S subunit maturation; (2) translation initiation regulators including *Eif4ebp1*, which controls cap-dependent translation, and *Eif3f* and *Eif3k*, which form the eIF3 complex critical for ribosome assembly; and (3) mRNA processing and translational regulation factors like *Rnps1*, an RNA-binding protein involved in splicing and surveillance, and *Srp14* and *Srp72*, signal recognition particle components important for targeting secretory and membrane proteins (Figure 7K). Collectively, these AAV-FGF21-mediated changes suggested maintenance of protein synthesis capacity, whose decline is a hallmark of aged muscle contributing to reduced function.^{59–61}

In addition to muscular performance, the effects of long-term increased FGF21 circulating levels on bone in aged mice were also assessed. At 26 months of age, mice treated at 13 months did not exhibit alterations in serum markers of bone formation, including procollagen type 1 N-terminal propeptide (PINP) and osteocalcin, nor in the bone resorption marker tartrate-resistant acid phosphatase 5b (TRAcP 5b), despite sustained elevation of circulating FGF21 for more than 1 year (Figures S9F–S9H). Moreover, circulating carboxy-terminal crosslinked telopeptide of type 1 collagen (CTX-1) was lower in these mice than in age-matched controls (Figure S9I), indicating reduced degradation of the type I collagen bone matrix. These data demonstrated that sustained elevated skeletal muscle-derived FGF21 circulating levels did not induce bone toxicity in aged mice. These findings are consistent with previous studies showing that long-term AAV-mediated increased FGF21 levels in obese, insulin-resistant mice did not induce bone alterations.^{16,62} Moreover, non-human primates treated for 1 year with an FGF21 mimetic did not exhibit adverse effects on bone mineral density or bone structure.⁶³

AAV-FGF21 treatment reverses age-associated cognitive decline

Impaired memory and learning are the most common consequences of the aging process in the central nervous system in the absence of neurodegenerative diseases.⁶⁴ By 27 months of age, male mice treated with FGF21-encoding vectors showed enhanced memory compared with age-matched controls, equivalent to that of 2-month-old animals in the novel object recognition test (Figures 8A and 8B). Moreover, evaluation of performance in the rotarod test during subsequent trials demonstrated that AAV-FGF21-treated mice exhibited improved motor learning ability to age-matched counterparts (Figure S10A). Treatment with AAV-FGF21

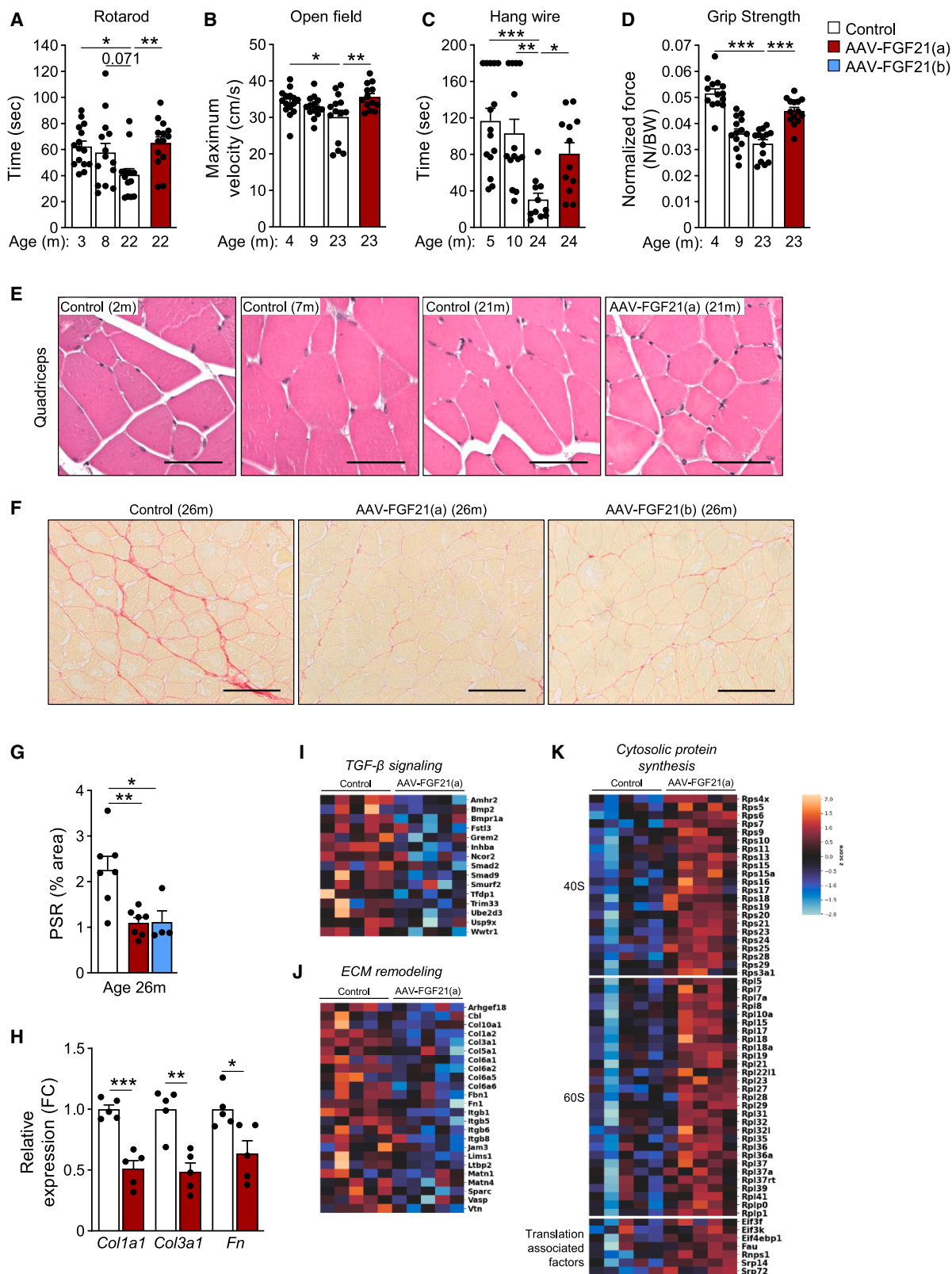
vectors also improved cognition in old female mice (Figures S10B and S10C).

Similar to observations in adipose tissue, skeletal muscle, and heart, GSEA of brain tissue from old mice treated with FGF21-encoding vectors revealed over-representation of processes related to mitochondrial energy metabolism (TCA cycle, OXPHOS, and ETC) as well as pathways associated with enhanced protein translation (Figure S10D). Specifically, AAV-FGF21-treated mice showed enrichment of leading-edge genes encoding key TCA cycle enzymes and components of the ETC complexes I–V (Figures 8C and 8D). The brain is a highly energy-demanding organ that relies on ATP production via the TCA cycle and OXPHOS, which is essential for synaptic function, neurotransmission, ion pump activity, and overall neuronal survival.^{65,66} Moreover, mitochondrial function and energy metabolism decline significantly with age, and their impairment is critical for the development of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.^{65,66} In 25-month-old AAV-FGF21-treated mice, measurement of key OXPHOS markers by qPCR further corroborated the transcriptomic findings and suggested efficient ATP production in cortex and hippocampus (Figure 8E), the main brain areas involved in cognitive function. Similarly, these mice also showed increased expression of key glycolysis-related genes in these regions (Figure 8F). The brain almost exclusively uses glucose as a substrate for energy metabolism, which is metabolized through glycolysis and TCA cycle.^{65,66} Nevertheless, in agreement with previous reports,^{67,68} treatment with AAV-FGF21 increased circulating levels of β -hydroxybutyrate (Figure S10E), which could also be utilized by the brain as an alternative energy substrate in addition to glucose.⁶⁹

AAV-FGF21-treated mice also showed upregulation of *Rps* and *Rpl* genes, as well as genes encoding translation initiation (EIFs), elongation factors (EEFs and TSFM), and additional translation-related factors (SRP68, PABPCL1, FAU) (Figure 8G). Upregulation of these leading-edge genes may also support cognitive function by maintaining synaptic plasticity, which depends heavily on rapid, on-demand synthesis of new proteins at the synapse.^{70,71} Mitochondrial perturbations and reduced ATP production also contribute to synaptic dysfunction and degeneration, which strongly correlate with cognitive deficits and memory loss.^{66,72,73} Notably, by 25 months of age, animals treated with AAV-FGF21 showed increased expression of genes encoding key synaptic proteins in cortex and hippocampus, including synaptophysin, and subunits of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type and N-methyl-D-aspartate (NMDA)-type ionotropic glutamate receptors

Figure 6. AAV-FGF21 treatment prevents heart aging

Male mice were treated with either AAV-Null (control) or AAV-FGF21 vectors at 13 (AAV-FGF21 (a)) or 22 (AAV-FGF21 (b)) months of age. (A–C) Representative images of hematoxylin-eosin (A), Congo red (B; bright field, upper panels; fluorescence signal, lower panels), and PicroSirius red (C) staining of heart sections. Scale bars, 100 μ m. (D) Histomorphometric quantitative assessment of heart fibrosis (PSR) ($n = 4$ –5/group). (E) Quantification by RT-qPCR of the expression of the fibrosis gene markers *Col1a1*, *Col3a1*, and *Fn* ($n = 5$ /group). (F–I) The heatmaps depict the normalized expression patterns of leading-edge genes involved in Wnt signaling (F), ECM remodeling (G), cytosolic (H), and mitochondrial protein synthesis (I) in heart at 21 months of age. Data in (D) and (E) are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (D) or Student's two-tailed t test (E).



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(Figure 8H). Overall, the coordinated upregulation of mitochondrial, metabolic, and protein synthesis-related genes would maintain the brain's energy-producing capacity and support brain health during aging, thereby preventing cognitive decline.

In aged mice treated with AAV-FGF21 vectors, FGF21 levels in the brain increased (Figure S10F), although they remained markedly lower than the circulating concentrations (Figure 1C). Since native FGF21 can cross the blood-brain barrier,⁷⁴ these direct central effects may contribute to the therapeutic outcomes. In addition, efficacy may reflect AAV-FGF21-driven amelioration of obesity, tissue inflammation, and insulin resistance, which are metabolic perturbations that negatively impact brain function.^{75–77}

DISCUSSION

This study demonstrated that a single administration of the muscle-directed AAV-FGF21 gene therapy led to sustained, long-term increases in circulating levels of FGF21 in aged mice, making the factor continuously available to all organs, including the brain.⁷⁴ The AAV-FGF21 treatment drove four interconnected adaptive programs that counteracted fundamental aging mechanisms. First, enhanced mitochondrial function was evident across most tissues, characterized by upregulation of TCA cycle enzymes, ETC components, and mitochondrial ribosomal proteins. This coordinated enhancement would address mitochondrial dysfunction, a primary hallmark of aging,^{1,78} and provide energetic capacity essential for tissue function. Energy-dependent organs including adipose tissue, brain, heart, and skeletal muscle showed particularly robust mitochondrial improvements correlating with functional preservation. Second, restored proteostasis was evident through widespread upregulation of cytoplasmic and mitochondrial ribosomal proteins and translation factors. Maintenance of protein synthesis capacity, which declines progressively with age,⁷⁹ would have contributed to preserved muscle function, cardiac health, and synaptic plasticity underlying cognitive improvements. Third, suppression of pro-fibrotic and proinflammatory pathways, such as TGF- β , Wnt, and p53 signaling, would have prevented pathological tissue remodeling, fibrosis, and chronic inflammation characterizing aging tissues.³⁴ Prevention of amyloidosis in liver, kidney, and heart demonstrated protection against age-related protein aggregation. Fourth, the coordinated upregulation of phase I and phase II detoxification enzymes likely maintained the capacity of the liver to efficiently metabolize and eliminate toxic compounds and metabolic by-products, thereby attenuating cellular stress and supporting the preservation of systemic metabolic homeostasis.

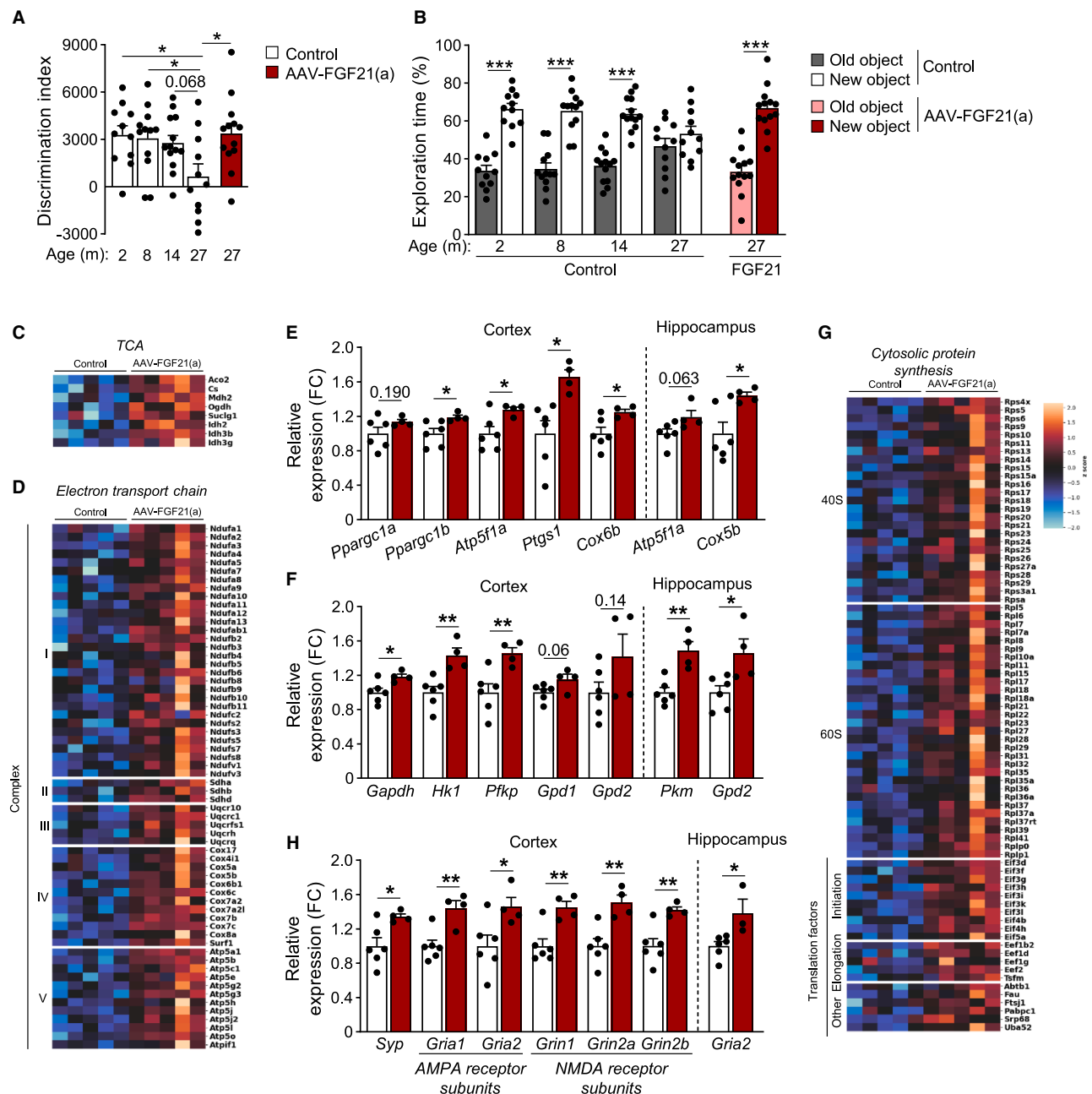
Enhanced hepatic detoxification capacity is a molecular signature of multiple lifespan-extending interventions.²³ Together, these four processes link molecular changes to functional health span improvements and extension of midlife survival.

Widespread expression of FGF21 receptors (FGFR1–4) and the co-receptor β -Klotho have been reported throughout tissues in the body⁸⁰ and likely enabled the observed multi-organ gerotherapeutic benefits. In this regard, all tissues examined showed detectable β -Klotho expression, with the highest levels in the liver and adipose depots, that were further induced in these tissues following AAV-FGF21 treatment. In tissues such as skeletal muscle, heart, kidney, and brain where bulk β -Klotho expression was comparatively low, global measurements may underestimate functionally relevant expression in specific cellular subpopulations.^{81–84} Nevertheless, the widespread phenotypic improvements observed after muscle-derived increased circulating FGF21 levels may also be mediated by indirect, systemic mechanisms. In particular, increased circulating adiponectin, a well-established downstream effector of FGF21,^{68,85} has been reported to exert protective effects in kidney, heart, and skeletal muscle through anti-inflammatory, anti-fibrotic, anti-oxidative, and metabolic pathways,^{68,86–89} thus likely contributing to the FGF21-derived therapeutic effects. Moreover, the improved systemic metabolic homeostasis induced by AAV-FGF21 gene therapy would reduce lipotoxic and oxidative stress in kidney, heart, and skeletal muscle, even in the absence of robust local β -Klotho signaling.

Additionally, AAV-FGF21 gene therapy in previous studies was able to counteract already established MASH, T2D, and obesity, major chronic age-associated metabolic diseases, and prevented development of hepatocellular carcinomas and obesity-associated cognitive decline and anxiety in obese mice,^{16,17} supporting its clinical translation to treat MASH (<https://kriyatherapeutics.com/pipeline/>). Unlike these earlier studies, which focused on genetically induced or diet-induced relevant mouse models of these severe metabolic diseases,^{16,17} the current study used naturally aged mice, which had already developed spontaneous multi-organ age-related deterioration. This shift from a pathological to a physiological context allowed to demonstrate, for the first time, that a single i.m. administration of AAV-FGF21 vectors not only improved metabolic parameters but extended health span and lifespan, producing robust and systemic geroprotective effects. In addition, integrated whole-organ transcriptomic and histopathological analyses revealed

Figure 7. AAV1-FGF21 therapy improves muscular performance in old male mice

Male mice were treated with either AAV-Null (control) or AAV-FGF21 vectors at 13 (AAV-FGF21 (a)) or 22 (AAV-FGF21 (b)) months of age. (A) Time before falling during the rotarod test ($n = 14$ –15/group). (B) Maximum velocity was measured in the open field test ($n = 13$ –15/group). (C) Hang time during the hang wire test ($n = 11$ –15/group). (D) Forelimb grip strength ($n = 14$ /group). (E) Representative images of hematoxylin-eosin staining of quadriceps sections. Scale bars, 100 μ m. (F) Representative images of PicroSirius red staining of quadriceps sections. Scale bars, 100 μ m. (G) Histomorphometric quantitative assessment of fibrosis in quadriceps (PSR) ($n = 4$ –7/group). (H) Quantification by real-time quantitative PCR of the expression of the fibrosis gene markers *Col1a1*, *Col3a1*, and *Fn* ($n = 5$ /group). (I–K) The heatmaps depict the normalized expression patterns of leading-edge genes involved in TGF- β signaling (I), ECM remodeling (J), and cytosolic protein synthesis (K) in quadriceps at 21 months of age. In (A)–(D) and (F), data are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (G) or Student's two-tailed t test (H). BW, body weight; m, months of age.



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tissue-specific adaptive responses that collectively preserved cellular fitness during natural aging. Whereas previous publications positioned AAV-FGF21 primarily as a promising treatment for metabolic disorders,^{16,17} the present work established its gerotherapeutic potential capable of modulating core biological hallmarks of aging, thereby enhancing its translational relevance for an expanding aging population.

While other previous studies have delineated the biological role of FGF21 in aging, their translational relevance remains intrinsically constrained as they used transgenic mouse models but not developed FGF21-based therapeutic strategies.^{90–94} In this regard, hepatocyte-specific FGF21 transgenic mice exhibited improved metabolic parameters and markedly extended lifespan, but in this model, FGF21 overexpression was already activated during embryogenesis,^{67,90,95} thereby precluding inference on the efficacy of therapeutic intervention in adult organisms. Moreover, these transgenic mice were dwarf and exhibited low bone mass, and as they aged, they failed to maintain reduced adiposity and showed no improvement in glucose tolerance or phosphorylated AMPK levels in liver and adipose tissue.^{90,96} More recently, a doxycycline-inducible adipocyte-FGF21 transgenic mouse model was developed to avoid overexpression from embryogenesis, and it demonstrated that FGF21 promotes longevity and confers metabolic benefits.⁹³ Nevertheless, in contrast to the present work, in this study using transgenic mice, FGF21 overexpression was induced in young ages (10–12 weeks of age), rather than in old animals. Additionally, the chronic high-fat-diet regimen limited the experimental context to diet-induced metabolic dysfunction instead of natural aging physiology.⁹³ Moreover, doxycycline-inducible systems cannot be used in gene therapy approaches to drive expression of therapeutic genes because of their immunogenicity.⁹⁷

On the other hand, protein restriction confirmed that FGF21 upregulation is required for metabolic and lifespan improvements, but the intervention was initiated in young male mice.⁹⁷ When 12-month-old male mice were subjected to an 8-week methionine-restricted diet, they also exhibited increased serum FGF21 levels and a reversal of age-associated changes in body weight, adiposity, physical activity, and glucose tolerance.⁹⁸ However, due to the short duration of the intervention, lifespan outcomes were not assessed, nor were potential therapeutic benefits on cognitive function evaluated in these animals.⁹⁸ Moreover, the deleterious consequences of long-term restriction of the essential amino acid methionine remain unexplored.

In addition, compared with the robust gerotherapeutic effects observed in the present study, previous AAV-FGF21-based strategies have produced partial and limited outcomes. Systemic administration of liver-targeted AAV8-FGF21 vectors to 18-month-old male mice reduced body weight in a 5-month follow-up, but this was

the only aging-related phenotype reported.⁶² In the same study, AAV8-FGF21 vectors were tested against surgically induced renal fibrosis and cardiac failure in mice.⁶² However, these experimentally induced models do not reflect the spontaneous age-associated organ decline of physiologically aged mice. Likewise, adipose-directed Rec2-AAV-FGF21 gene therapy in 17-month-old female mice modestly reduced body weight and slightly increased energy expenditure, without improving glucose tolerance, grip strength, rotarod performance, circulating adiponectin, liver weight, hepatic triglycerides, or β -Klotho expression in liver or adipose tissue.⁹⁹ This limited efficacy was consistent with the minimal serum FGF21 elevation achieved,⁹⁹ in contrast to the robust and sustained systemic FGF21 levels induced by i.m. AAV-FGF21 administration in the present study.

The absence of long-term adverse events in the present work, and in previous studies in mice and dogs,^{16,17} highlighted the high safety profile of the AAV-FGF21 gene therapy. In this regard, a large body of work in large animals and human patients has demonstrated that i.m. delivery of AAV vectors to genetically engineer skeletal muscle is safe and well tolerated.^{17,18,100–108} Moreover, durability of transgene expression in large animals and humans has been documented for 8 and 10 years, respectively, demonstrating that the capacity of AAV-engineered muscles to produce and secrete therapeutic proteins persisted as treated animal and human subjects grew older.^{18,106} Notably, in type 1 diabetic Beagle dogs treated i.m. with AAV vectors encoding insulin and glucokinase, using the same delivery route, AAV1 serotype, and CMV promoter as in the present study, transgene expression and therapeutic efficacy were sustained for a duration that essentially spanned the animals' lifetimes.^{18,100} This program is currently in the IND-enabling phase of development (<https://kriyatherapeutics.com/pipeline/>).

While AAV vectors are generally regarded as safe, systemic administration of high doses of these vectors may elicit adverse outcomes in clinical studies in patients, including hepatotoxicity, complement activation, severe immune-mediated responses, and, in certain contexts, life-threatening responses.^{109–112} Nevertheless, AAV vectors administered via the i.m. route exhibit restricted systemic biodistribution, especially the AAV1 serotype, which largely remains in transduced muscle fibers, as it lacks the capacity to cross endothelial barriers.^{16–18,100} Furthermore, in the AAV-FGF21 approach, genetic engineering of a limited subset of skeletal muscles in hindlimbs is sufficient to establish a therapeutic pump for FGF21 secretion into bloodstream, thereby reducing vector dose requirements and, consequently, the risk of detrimental host responses.^{16,17} Additionally, i.m. administration of AAV vectors for gene delivery is not substantially hindered by pre-existing neutralizing antibodies against the AAV capsid,^{107,108,113,114} a critical consideration given the

dehydrogenase; *Hk1*, hexokinase 1; *Pfkf*, platelet isoform of phosphofructokinase; *Gpd1*, glycerol-3-phosphate dehydrogenase 1; *Gpd2*, glycerol-3-phosphate dehydrogenase 2; *Pkm*, pyruvate kinase M; *Syp*, synaptophysin; *Gria1* and *Gria2*, GluR1 and GluR2 subunits of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type ionotropic glutamate receptor; *Grin1*, *Grin2a*, and *Grin2b*, NR1, N2A, and N2B subunits of the N-methyl-D-aspartate (NMDA)-type ionotropic glutamate receptor; FC, fold change; m, months of age.

high prevalence of anti-AAV immunity in the general population, which can otherwise limit the clinical applicability of AAV-based therapies.¹¹⁵

The geroprotective effects demonstrated in the current study, alongside a robust safety profile, support translation of AAV-FGF21 gene therapy as a therapeutic strategy to promote health span extension. Nonetheless, additional studies in large animals are required prior to human application. Prolonging health span encompasses profound individual benefits, including enhanced functional capacity, preserved autonomy, and reduced multimorbidity, and it alleviates societal burdens by lowering healthcare utilization and costs and productivity loss. This is the priority of the United Nations Decade of Healthy Aging (2021–2030), whose implementation is led by the World Health Organization, focused on advancing interventions designed to link increasing longevity with extended well-being (<https://www.decadeofhealthyageing.org/>). In this context, our AAV-FGF21-based gene therapy holds potential to be transformative for health span extension by targeting whole-body hallmarks of aging.

MATERIALS AND METHODS

Animals

Male and female C57BL/6J mice were kept in a specific pathogen-free facility (SER-CBATEG, UAB) and maintained under a light-dark cycle of 12 h at 22°C. Mice were fed *ad libitum* with a standard diet (2018S Teklad Global Diets, Envigo). For tissue sampling, mice were anesthetized with inhalational anesthetic isoflurane (IsoFlo, Abbott Laboratories) and decapitated. Tissues of interest were excised and kept at –80°C or in formalin until analysis. Animal care and experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (UAB).

Recombinant AAV vectors

An AAV expression cassette was obtained by cloning, between the ITRs of AAV2, the murine codon-optimized FGF21 coding sequence (*moFGF21*) under the control of the cytomegalovirus (CMV) promoter (Table S1). A non-coding cassette carrying the CMV promoter but no transgene was used to produce AAV-Null vectors. Single-stranded AAV1 vectors were produced by triple transfection in HEK293 cells and purified using a double CsCl gradient-based purification protocol that renders vector preps of high purity with negligible amounts of empty capsids.¹¹⁶ Viral genome titers were determined by PicoGreen (Quant-iT PicoGreen dsDNA assay kit, Invitrogen), using phage lambda DNA to generate the standard curve. The same batch of AAV-FGF21 vectors was used for all the mouse experimental work.

Intramuscular administration of AAV vectors

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Hindlimbs were shaved, and AAV vectors were i.m. administered in a volume of 180 μ L divided into six injection sites distributed between quadriceps, gastrocne-

mius, and tibialis cranialis of each hindlimb (one site per skeletal muscle).

Insulin tolerance test

Insulin (Humulin Regular; Eli Lilly) was injected intraperitoneally to fed mice at a dose of 0.375 or 0.75 IU/kg body weight. Glycemia was measured in tail vein blood samples at the indicated time points.

Glucose and pyruvate tolerance tests

Awake mice were fasted overnight (16 h) and administered with an intraperitoneal injection of glucose (2 g/kg body weight) or pyruvate (1 g/kg body weight). Glycemia was measured in tail vein blood samples at the indicated time points.

Indirect calorimetry

An indirect open circuit calorimeter (Oxylet, Panlab) was used to monitor O₂ consumption and CO₂ production. Mice were individualized and acclimated to the metabolic chambers for 24 h, and data were collected in each cage for 5 min, every 45 min, for 24 h. Data were taken during the light and dark cycles and adjusted by body weight.

Rotarod test

Mice were placed on a rotating rod (Panlab), spinning at 4 rpm. Lane width was 50 mm; rod diameter was 30 mm. Once stabilized, mice were subjected to an incrementally increasing speed from 4 to 40 rpm in 5 min. The first day of the experiment was used to train the animals in the use of the device. Each animal underwent 3 trials. The latency to fall from the rod was recorded. Then, animals underwent 1 day resting, and the third day, mice took 3 more trials on the rod. The average of 3 trials was analyzed.

Open field test

The open field test was performed between 9:00 a.m. and 1:00 p.m. Mice were placed in a corner of a square arena surrounded by high white walls (45 $\times$ 45 $\times$ 40 cm), and motor and exploratory activities were evaluated during the first 6 min using a video tracking system (SMART Junior, Panlab).

Hang wire test

The hang wire test was conducted using a 55-cm-wide, 2-mm-thick metallic wire, which was secured to two vertical stands. The wire was maintained 35 cm above a layer of bedding material to prevent injury to the animal when it falls down. Mice, handled by the tail, were allowed to grasp the middle of the wire with their forelimbs. The time until mice fell down was measured. Mice that reached the limit suspension time of 180 s, regardless of the trial number, were allowed to stop the experiment, while the others were directly retested for a maximum of three trials (a 30-s recovery period was used between trials). The average of 3 trials was analyzed.

Grip strength test

A grip strength test meter (Panlab) was used to assess forelimb grip strength. The grip strength meter was positioned horizontally, and

mice were held by the tail and lowered toward the apparatus. Animals were allowed to grasp the metal bar with their forepaws and were then pulled backward in the horizontal plane. The force applied to the bar just before it lost grip was recorded as the peak tension. The average of 3 trials was analyzed.

Novel object recognition test

The novel object recognition tests were conducted in the open field box.¹⁷ Open field test was used to acclimatize the mice to the box. The next day, to conduct the first trial, two identical objects (A and B) were placed in the upper right and upper left quadrants of the box, and then mice were placed backward to both objects. After 10 min of exploration, mice were removed from the box and allowed for 10 min break. In the second trial, one of the identical objects (A and B) was replaced with object C (new object). Mice were then put back into the box for a further 10 min of exploration. The amount of time animals spent exploring the novel object was recorded and evaluated using a video tracking system (SMART Junior; Panlab). Mice with exploration times for both objects shorter than 5 s, either during the first or the second trial, were excluded from analysis, as it cannot be confirmed that they spent enough time exploring to learn/discriminate the objects. The evaluation of novel object recognition test memory was expressed as a percentage of the discrimination ratio calculated according to the following formula: Discrimination ratio (%) = $(N - F)/(N + F) \times 100\%$, where N represents the time spent in exploring the new object, and F represents the time spent in exploring the same object.

Serum parameters and metabolite assays

Hepatic triglyceride content was determined by chloroform:methanol (2:1 vol/vol) extraction of total lipids.¹⁷ Triglycerides and β -hydroxybutyrate were quantified spectrophotometrically using an enzymatic assay (Horiba-ABX). Glycemia was determined using a Glucometer Elite (Bayer). The Mouse/Rat FGF-21 ELISA kit (MF2100, R&D Systems) was used to determine FGF21 levels in serum and brain. Brain samples (cerebellum) were homogenized with T-PER buffer (Thermo Fisher Scientific) using a sonicator (VC130PB, Sonics & Materials). Buffer was supplemented with protease inhibitors (Complete, Roche) and phosphatase inhibitors (orthovanadate). Once homogenized, samples were centrifuged at $10,000 \times g$ during 10 min at 4°C, and supernatant was kept at -80°C until analysis. Serum insulin, adiponectin, osteocalcin, CTX-1, TRAcP 5b, and PINP levels were determined using the Rat Insulin ELISA kit (90010, Crystal Chem), the Mouse Adiponectin ELISA kit (80569, Crystal Chem), the ELISA Kit for Osteocalcin (SEA471Mu, Cloud-Clone Corp.), the RatLaps (CTX-1) EIA kit (AC-06F1, Immunodiagnosics Systems Limited), the MouseTRAP (TRAcP 5b) ELISA kit (SB-TR103, Immunodiagnosics Systems Limited), and the Rat/Mouse PINP EIA kit (AC-33F1, Immunodiagnosics Systems Limited), respectively. Serum creatinine and urea levels were measured by spectrophotometry at the Servei de Bioquímica Clínica Veterinària (Veterinary School, UAB).

Immunohistochemistry

Tissues were fixed for 12–24 h in 10% formalin, embedded in paraffin, and sectioned. Sections were incubated overnight at 4°C with guinea pig anti-insulin (1:100; I-8510; Sigma-Aldrich) or rat anti-fibronectin (1:100; F3648; Sigma). Biotinylated rabbit anti-guinea pig coupled to peroxidase (1:300, P0141, Dako) or Donkey anti-rabbit 568 (a10042, Invitrogen) were used as secondary antibodies. The ABC peroxidase kit (Pierce) was used for immunodetection, and sections were counterstained in Mayer's hematoxylin. Hoechst (B2261; Sigma-Aldrich) was used for nuclear counterstaining of fluorescent specimens. PicroSirius red staining and Congo red staining were used to evaluate fibrosis and amyloidosis, respectively. Morphometric analysis of β cell mass, adipocyte size, and glomerulus area were performed as previously described.^{17,117} Tissue fibrosis was assessed by the percentage of the PSR-positive area quantified in 10 randomly selected $\times 100$ fields per tissue section.¹⁷ Images were obtained with a Nikon Eclipse 90i microscopy (Nikon).

Western blot analysis

For total protein extracts, tissues were homogenized in protein lysis buffer and centrifuged at $15,000 \times g$ during 15 min at 4°C. Proteins were separated by 12% SDS-PAGE and analyzed by immunoblotting with Anti-AMPK (2532, Cell Signaling), Antiphospho-AMPK-Thr172 (2531, Cell Signaling), and Anti- α -tubulin (ab4074, Abcam). Detection was performed using ECL Plus detection reagent (Amersham Biosciences).

RNA analysis

Total RNA was obtained from different tissues using isolation reagent (Tripure, Roche, for liver, kidney, heart, skeletal muscle, and brain, or QIAzol, Qiagen, for adipose depots) and an RNeasy Minikit (Qiagen) and treated with DNaseI (Qiagen). 1 μ g of RNA was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis kit (Roche). Real-time quantitative PCR was performed in a Lightcycler (Roche) using the Lightcycler 480 SyBr Green I Master Mix (Roche) and the following primers:

moFGF21: 5'CCTAACCAGGACGCCACAAG3', 5'GTTCCACCA TGCTCAGAGGG3'; *Cd68*: 5'GGGGCTCTTGGGAACACAC3', 5'CAAGCCCTCTTTAAGCCCCA3';

F4/80: 5'CTTTGGATGGGCTTCCAGTC3', 5'GCAAGGAGGAC AGAGTTTATC3';

Il1 β : 5'5'TGTAATGAAAGACGGCACACC3', 5'TCTTCTTTGGG TATTGCTTGG3'; *Mcp1*: 5'AACTGCATCTGCCCTAAGGTC3', 5' AAGTGCTTGAGGTGGTTGTG3';

Ucp1: 5'GGATTGGCCTCTACGACTCAG3', 5'TGTAGGCTGCCC AATGAACA3';

mt-Rnr2: 5'-CCGCAAGGGAAAGATGAAAGAC-3', 5' TCGTTTG GTTTCGGGGTTTC3'; *mt-Nd1*: 5' CTAGCAGAAACAAACCGGG

<i>C3'</i> , 5' CCGGCTGCGTATTCTACGTT3'; <i>Kim1</i> : 5' AAGCCGCGAG AAAAACCTAC3', 5' AACACGCTTAGAGATGCTG3';	<i>Grin1</i> : 5'TGACTACCCGAATGTCCATC3', 5'TTGTAGACGCGC ATCATCTC3';
<i>Lcn2</i> : 5'CAGAAAGGCAGCTTTACGATG3'; 5'CCTGGAGCTTGG AACAAATG3';	<i>Grin2a</i> : 5'TGTGAAGAAGTGCTGCAAGG3', 5'CGCCTATCATT CCATTCCAC3';
<i>Col1a1</i> : 5'GACTGGAAGAGCGGAGAGTA3', 5'CCTTGATGGCG TCCAGGTT3'; <i>Col3a1</i> : 5'AGGATCTGTCCTTTGCGATG3', 5'TC TCCAAATGGGATCTCTGG3';	<i>Grin2b</i> : 5'TTGGTGAGGTGGTCATGAAG3', 5'TGCGTGATACC ATGACACTG3';
<i>Fn</i> : 5'AAGATTGGCGACAAGTGGAG3', 5'TAGGTTTGCAGGT CCATTCC3';	<i>Klb</i> : 5'ACGAGGGCTGTTTTATGTTGG3', 5'TTCAAAGGGAAG CCGTTGTC3';
<i>Ppargc1a</i> : 5'TTTGGCCGACGACACGACTTTTC3', 5'TTGTGT GGGCGAGAGAAAAG3';	<i>Pcx</i> : 5'TTCCTGCAGGGCTACATTGG3', 5'GGCAGGTCTTTA GCACCTT3';
<i>Ppargc1b</i> : 5'AGAAGCGCTTTGAGGTGTTTC3', 5'GGTGATAAAA CCGTGCTTCTGG3'; <i>Atp5f1a</i> : 5'GGCCATTTTGTGCCAGTCG3', 5'CTGACCAATCGCGACGTAGA3';	<i>Pdha1</i> : 5'TGTGTGATGGTCAGGAAGCC3', 5'CCGAGTGAAGG TGAAGCCAT3';
<i>Ptgs1</i> : 5'TATCCAACCTCATCCCTTGAC3', 5'GAGTAGCGTCGTG GTATTCC3';	<i>Cs</i> : 5'CCCACAGAGGAACAGGTGTC3', 5'ACCACATGAGAAG GCAGAGC3';
<i>Cox6b</i> : 5'AGTCCCTCTGTCCCGTGTC3', 5'ATATGCTGAGGT CCCCCTTT3';	<i>Ndufa1</i> : 5'CAACGGGGGCAAGGAAAAAC3', 5'CCAGGCCCTTG GACACATAG3';
<i>Cox5b</i> : 5'CTCGTCAGCCTCAGCCAGT3', 5'TAGCAGCGAATGG AACAGAC3';	<i>Sdha</i> : 5'GTTGCAGCACAGGGAGGTAT3', 5'TCGGAGCCTTTCA CAGTGTC3';
<i>Gapdh</i> : 5'CCTTCCGTGTTCTTACCC3', 5'CAACCTGGTCCTCA GTGTAG3';	<i>Uqcqr</i> : 5'TTCAGCAAAGGCATCCCCAA3', 5'TAGACCACTACA AACGGCGG3';
<i>Hk1</i> : 5'GACCGTTCCTCAGACACCTG3', 5'CTCTGTGAGAGGG GGTGTA3';	<i>Cox5a</i> : 5'TTGCAGCATGTAGACGGTT3', 5'TGAGGTCTTGCT TTGTCCTT3';
<i>Pfkfb</i> : 5'TGTGTCTGAAGGAGCAATCG3', 5'GGCCAAAATCCTG TCAAATG3';	<i>Mrpl12</i> : 5'GGCAAAGCCTGTGGACAAAG3', 5'CTACCAGCTTC TTGGCCTGG3';
<i>Gpd1</i> : 5'AGACACCCAACTTTTCGCATC3', 5'TATTCTTCAAGGC CCCACAG3';	<i>Mrps35</i> : 5'CACCACAGACAGATGCCCTT3', 5'CATCTGCAGCA GGGTCTGAA3';
<i>Gpd2</i> : 5'TTGCTTGGGAGAAGATGAC3', 5'AGTTCCGCACTTC ATTCAGG3';	<i>Elovl3</i> : 5'AAACCGTGTGCTTTGCCATC3'; 5'CCGTGTCTCCCA GTTCAACA3';
<i>Pkm</i> : 5'GCTTTGCATCTGATCCCATT3', 5'AGTCCAGCCACAG GATGTTCT3';	<i>Cdkn1a</i> : 5'CAGACCAGCCTGACAGATTTCT3', 5'CTGACCCA CAGCAGAAGAGG3';
<i>Syp</i> : 5'ACATGGACGTGGTGAATCAG3', 5'AAGATGGCAAAGA CCCAGTG3';	<i>Cdkn2a</i> : 5'TCTTGGTGAAGTTCGTGCGA3', 5'TGCCCATCATC ATCCTGG3';
<i>Gria1</i> : 5'CCATGCTGGTTGCCTTAATC3', 5'CCGTATGGCTTC ATTGATGG3';	<i>Rplp0</i> : 5'TCCCACCTTGTCTCCAGTCT3', 5'ACTGGTCTAGG ACCCGAGAAG3'; <i>nqHK2</i> : 5'GCCAGCCTCTCCTGATTTTAGT GT3', 5'GGGAACACAAAAGACCTCTTCTGG3'.
<i>Gria2</i> : 5'AACGGCGTGTAATCCTTGAC3', 5'TTTCAGCAGGTC TCCATCAG3';	Data were normalized to <i>Rplp0</i> expression, except for mtDNA-en coded genes, which were normalized to <i>nqHK2</i> expression.

Transcriptomic analysis

Microarray plate processing was carried out at the High Technology Unit (UAT) of the Vall d'Hebron Research Institut (VHIR) (Barcelona, Spain). The Agilent 2100 Bioanalyzer was used to assess sample RNA integrity prior to the microarray protocol. The Affymetrix GeneTitan microarray platform and the Genechip Mouse Clariom S array plate were used in this study. This array plate analyzes gene expression patterns on a whole-genome scale on a single plate with probes covering many exons on the target genomes and, thus, permits an accurate expression summarization at gene level. The starting material was 200 ng of total RNA for each sample, obtained as previously described. The quality of isolated RNA was first measured by Bioanalyzer Assay (Agilent). Briefly, sense single-stranded DNA (ssDNA) suitable for labeling was generated from total RNA with the GeneChip WT Plus Reagent Kit from Affymetrix (Thermo Fisher-Affymetrix) according to the manufacturer's instructions. Sense ssDNA was fragmented, labeled, and hybridized to the arrays with the GeneChip WT Terminal Labeling and Hybridization Kit from the same manufacturer. Arrays plates were scanned to obtain.cel files, and quality control was performed with the Affymetrix Expression Control Software.

GSEA was performed to interpret transcriptomic data from a microarray analysis. This method uses gene sets, which are groups of genes that share common features based on existing biological knowledge from databases like the Molecular Signatures Database (MSigDB) (<https://www.gsea-msigdb.org/gsea/msigdb>). The analysis was conducted by first ordering genes in a ranked list based on their differential expression between two classes: AAV-FGF21 and AAV-Null. Next, an enrichment score was calculated for each gene set to determine if its members were overrepresented at the top or bottom of the ranked list. This score reflects the degree of enrichment for a particular gene set. Gene lists from several sources were used to gain biological insights, including Biological Process, KEGG Pathway, Reactome pathways, and Wikipathways. After GSEA, a leading-edge analysis was performed to identify the specific genes that contributed most to the enrichment of each category. These leading-edge genes and their corresponding categories were then clustered in a cluster map (Table S2). This process identified overlapping genes in enriched categories, which helped summarize the most relevant biological information and supported manual curation based on bibliographic research. GSEA and leading-edge identification were performed using the easy Visualization and Inference Toolbox for Transcriptome Analysis (eVITTA), and further data analysis and visualization were conducted in Python programming language.

Vector genome copy number

Tissue samples were digested overnight at 56°C in 300 µL of Tissue Lysis Solution supplemented with Proteinase K (0.2 mg/mL). Total DNA was isolated from supernatants using the MasterPure DNA purification kit. DNA was resuspended in Milli-Q water and quantified using a SynergyTM HTX Multi-ModeMicroplate reader. Vector

genome copy number in 40 ng of total DNA was determined by quantitative PCR and primers and probe specific for the murine codon-optimized FGF21 coding sequence (*moFGF21*): forward primer: 5'GAGCCTGCTGGAAGTGAAG3'; reverse primer: 5'TCAGGATCGAAGTGAGGAGAG3'; probe: 5'/56-FAM/CTTCACGC C/ZEN/CAGGATCTGGATCAC/3IABkFQ/3'. A reference standard curve was built from serial dilutions of the linearized AAV plasmid bearing the moFGF21 sequence spiked into 20 ng/µL of non-transduced mouse genomic DNA.

Statistical analysis and data processing

Sample size determination was based on previous experience with similar studies. Randomization was performed using the excel function Roundup() or by GraphPad QuickCalcs to allocate mice in each group. In addition, we tested that the mean body weight and the mean glycemia were statistically not different for each experimental group prior to assignment to treatment groups. Furthermore, each experimental group was caged separately to avoid any caging effects. All tests (ITT, GTT, open field, etc.) were performed by investigators blinded to the treatment. All results are expressed as mean ± SEM. The GraphPad Prism 7 software was used for statistical analyses. Data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, except for those parameters involving comparison of only two experimental groups, in which case Student's two-tailed *t* test was used. The Kaplan-Meier method was used to analyze survival, and the log rank test was used for comparisons. Differences were considered significant when *p* < 0.05.

DATA AND CODE AVAILABILITY

Array data have been deposited in the ArrayExpress database (<https://www.ebi.ac.uk/arrayexpress/>; accession code E-MTAB-15771). Other data supporting the findings of this study are available within the paper and its supplemental information.

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AUTHOR CONTRIBUTIONS

F.B. and V.J. conceived the project, designed and supervised experiments, and wrote the manuscript. V.J., V.S., and C.J. generated reagents and performed experiments. M.G. performed experiments and transcriptomic analyses. X.L. generated reagents. E.C., S. Muñoz, L.V., I.G., M.L.J., C.R., S. Marcó, M.M., A.R., I.E., J.R., and T.F. performed experiments. V.J., V.S., M.G., and F.B. analyzed the data, contributed to discussion, and reviewed/edited the manuscript.

DECLARATION OF INTERESTS

V.J., V.S., M.G., C.J., E.C., I.E., I.G., A.R., and F.B. are co-inventors on patent applications for the use of AAV vectors for the treatment of metabolic diseases. F.B. is a member of the Scientific Advisory Board of Kriya Therapeutics.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.ymthe.2026.05.025>.

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