Impaired mitochondrial oxidative metabolism in skeletal progenitor cells leads to musculoskeletal disintegration

| Item Type       | Journal Article |
|-----------------|-----------------|
| Authors         | Lin, Chujiao;Yang, Qiyuan;Guo, Dongsheng;Xie, Jun;Yang, Yeon-Suk;Chaugule, Sachin;DeSouza, Ngoc;Oh, Won-Taek;Li, Rui;Chen, Zhihao;John, Aijaz A;Qiu, Qiang;Zhu, Lihua Julie;Greenblatt, Matthew B;Ghosh, Sankar;Li, Shaoguang;Gao, Guangping;Haynes, Cole M;Emerson, Charles P. Jr.;Shim, Jae-Hyuck |
| Citation        | Lin C, Yang Q, Guo D, Xie J, Yang YS, Chaugule S, DeSouza N, Oh WT, Li R, Chen Z, John AA, Qiu Q, Zhu L J, Greenblatt MB, Ghosh S, Li S, Gao G, Haynes C, Emerson CP, Shim JH. Impaired mitochondrial oxidative metabolism in skeletal progenitor cells leads to musculoskeletal disintegration. Nat Commun. 2022 Nov 11;13(1):6869. doi: 10.1038/s41467-022-34694-8. PMID: 36369293; PMCID: PMC9652319. |
| DOI             | 10.1038/s41467-022-34694-8 |
| Journal         | Nature communications |
| Rights          | Copyright © The Author[s] 2022. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/; Attribution 4.0 International |
| Download date   | 2025-03-01 01:38:08 |
| Item License         | http://creativecommons.org/licenses/by/4.0/ |
|---------------------|---------------------------------------------|
| Link to Item        | https://hdl.handle.net/20.500.14038/51491   |
Impaired mitochondrial oxidative metabolism in skeletal progenitor cells leads to musculoskeletal disintegration

Chujiao Lin1, Qiyuan Yang2,12, Dongsheng Guo3,12, Jun Xie4,5,6, Yeon-Suk Yang1, Sachin Chaugule1, Ngoc DeSouza1, Won-Taek Oh1, Rui Li2, Zhihao Chen1, Aijaz A. John1, Qiang Qiu1, Lihua Julie Zhu2, Matthew B. Greenblatt7,8, Sankar Ghosh9, Shaoguang Li1, Guangping Gao4,6,10, Cole Haynes2, Charles P. Emerson3,11 & Jae-Hyuck Shim1,4,10

Although skeletal progenitors provide a reservoir for bone-forming osteoblasts, the major energy source for their osteogenesis remains unclear. Here, we demonstrate a requirement for mitochondrial oxidative phosphorylation in the osteogenic commitment and differentiation of skeletal progenitors. Deletion of Evolutionarily Conserved Signaling Intermediate in Toll pathways (ECSIT) in skeletal progenitors hinders bone formation and regeneration, resulting in skeletal deformity, defects in the bone marrow niche and spontaneous fractures followed by persistent nonunion. Upon skeletal fracture, Ecsit-deficient skeletal progenitors migrate to adjacent skeletal muscle causing muscle atrophy. These phenotypes are intrinsic to ECSIT function in skeletal progenitors, as little skeletal abnormalities were observed in mice lacking Ecsit in committed osteoprogenitors or mature osteoblasts. Mechanistically, Ecsit deletion in skeletal progenitors impairs mitochondrial complex assembly and mitochondrial oxidative phosphorylation and elevates glycolysis. ECSIT-associated skeletal phenotypes were reversed by in vivo reconstitution with wild-type ECSIT expression, but not a mutant displaying defective mitochondrial localization. Collectively, these findings identify mitochondrial oxidative phosphorylation as the prominent energy-driving force for osteogenesis of skeletal progenitors, governing musculoskeletal integrity.

Mitochondrial diseases are associated with decreased energy production in organs with high energy requirements and are mainly due to defects in the oxidative phosphorylation (OXPHOS) machinery1–4. Releasing energy through OXPHOS requires the orchestrated action of five multi-heteromeric enzyme complexes (complexes I–V), located in the inner mitochondrial membrane. Mitochondrial complex 1 (CI) is the largest complex comprised of at least 45 different proteins and is the primary entry point that generates the proton motive force for

1Department of Medicine/Division of Rheumatology, UMass Chan Medical School, Worcester, MA, USA. 2Department of Molecular, Cell and Cancer Biology, UMass Chan Medical School, Worcester, MA, USA. 3Department of Neurology, UMass Chan Medical School, Worcester, MA, USA. 4Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA, USA. 5Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA, USA. 6Viral Vector Core, UMass Chan Medical School, Worcester, MA, USA. 7Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, Cornell University, New York, NY, USA. 8Research Divisions, Hospital for Special Surgery, New York, NY, USA. 9Department of Microbiology and Immunology, Columbia University Vagelos College of Physicians and Surgeons, New York, NY, USA. 10Li Weibo Institute for Rare Diseases Research, UMass Chan Medical School, Worcester, MA, USA. 11Wellstone Muscular Dystrophy Program, UMass Chan Medical School, Worcester, MA, USA. 12These authors contributed equally: Qiyuan Yang, Dongsheng Guo. e-mail: jaehyuck.shim@umassmed.edu
electrons into the electron transport chain driving ATP production$^8$, CI-deficiency is the most frequent cause of defects of the OXPHOS system that cause multisystem dysfunction$^{11}$, including skeletal disorders$^{12,13}$.

Emerging epidemiological evidence$^{16,17}$ and data from animal models$^{18,19}$ suggest an association of mitochondrial dysfunction and high oxidative stress with skeletal disorders, including a clinically significant decrease in bone strength, reduced bone mineral density, premature aging of bone or a high risk of fragility fractures. For example, Kearns Sayre Syndrome (KSS), a rare mitochondrial DNA deletion syndrome, shows clinical manifestations of muscle weakness and wasting, accompanied with multiple bone deformities and severe fractures, causing loss of the ability to walk and early lethality$^{20}$. In addition, mitochondrial respiratory chain deficiency is related to spontaneous bone fractures and myopathy$^{21}$. However, it is unclear how mitochondrial defects contribute to skeletal dysfunction associated with myopathy.

Glycolysis is the primary metabolic process that generates ATP for energy and intermediate metabolites, including active lipids, nucleotides, and amino acids, for biosynthesis$^{22,23}$. It serves as the major energy and carbon source for committed osteoblast-lineage cells during osteogenic differentiation$^{24,25}$. In particular, metabolic intermediates fuel the synthesis of amino acids to produce extracellular matrix proteins and hydroxyapatite in bone$^{26}$. In addition to glycolysis, glutamine metabolism also provides osteoblast progenitors with an additional energy source as well as a carbon and nitrogen source. Glutamine is converted to citrate through the tricarboxylic acid (TCA) cycle in the mitochondria, producing ATP through the OXPHOS pathway, while providing metabolic intermediates for the synthesis of amino acids, nucleotides, glutathione, and hexosamine$^{27}$. Recent studies demonstrated an in vivo role for glutamine and glucose metabolism in skeletal progenitors using conditional deletion of the enzyme Glut1-deciency by conditionally deleting Ecsit in multiple skeletal progenitors is dispensable for skeletal development$^{29}$. However, other energy metabolic pathways controlling osteogenesis of skeletal progenitors remain to be elucidated.

To investigate in vivo roles for mitochondrial oxidative metabolism in musculoskeletal system, we developed a mouse model for mitochondrial CI deficiency by conditionally deleting Ecsit in multiple skeletal and muscle cells. Herein, we highlight the importance of mitochondrial OXPHOS in determining the osteogenic commitment and differentiation of skeletal progenitors as a critical rheostat of skeletal and muscular development. Notably, restoration of impaired OXPHOS pathway using recombinant adeno-associated virus (rAAV) may represent an untapped therapeutic avenue for mitochondrial disorders in the musculoskeletal system.

**Results**

**Ecsit-deficiency in skeletal progenitors impairs early skeletal development**

The CI intermediate assembly (MCIA) complex, containing the core subunits (ECSIT, NDUFAF1, ACAD9, and TMEM126B) is required for the formation of mitochondrial complex I (CI)$^{30-31}$. Among these subunits, ECSIT is highly expressed in skeletal cells, including endosteal osteoblasts, osteocytes, periosteal cells, and osteoclasts (Fig. 1a), and its deficiency reduces the expression of other MCIA and CI components$^{32,33}$. Mitochondrial localization of ECSIT in human bone marrow-derived stromal cells (BMSCs) and mouse osteoblasts residing on the surface of trabecular and cortical bones was also confirmed by immunofluorescence and subcellular fractionation analyses (Fig. 1b, c).

The role of mitochondrial activity in bone formation was examined in vivo using mice with targeted deletion of Ecsit in skeletal progenitors (Ecsit$^{fl/fl}$). Deletion efficiency of Ecsit in these cells was confirmed by fluorescence microscopy and immunoblotting analyses (Supplemental Fig. 1a, Fig. 2c). Ecsit$^{fl/fl}$ mice developed limb deformities along with spontaneous fractures in long bones right after birth (Fig. 1d, right). At postnatal day 1 (P1), they displayed severe osteoporosis and delayed formation of primary and secondary ossification centers in long bones (Fig. 1d, e, Supplemental Fig. 1b, c). Severe hypomineralization and delayed formation of primary ossification centers was also evident in Ecsit$^{flox}$ embryos and neonates (Fig. 1f). Consistent with immunofluorescence analysis showing nearly complete absence of osteocalcin (OCN)-expressing mature osteoblasts in the femur of P21 Ecsit$^{fl/fl}$ mice (Fig. 1f, right), KO Prx1$^{+}$ skeletal progenitors, isolated from P0 Ecsit$^{fl/fl}$ hindlimbs as Prx1$^{CD45^{+} Tie2^{+} TER119^{+}}$ cells, failed to differentiate into mature osteoblasts (Fig. 1g). Of note, the bone marrow niche function of Ecsit$^{fl/fl}$ skeletal cells was also disrupted, as shown by a significant reduction in total bone marrow cell numbers and a low frequency of hematopoietic stem cells (HSCs), B cells, macrophages, granulocytes, and T cells (Fig. 1h, Supplemental Fig. 2). These results suggest that ECSIT is essential for osteogenic commitment of skeletal progenitors and for the formation of bone marrow HSC niche. Notably, GLUT1-mediated glucose metabolism mediates chondrogenesis, not osteogenesis of Prx1$^{+}$ skeletal progenitors while GLS-mediated glutamine metabolism in Prx1$^{+}$ skeletal progenitors is dispensable for skeletal development$^{35}$. Thus, ECSIT-mediated mitochondrial regulation, not GLUT1-mediated glucose metabolism or GLS-mediated glutamine metabolism, plays a critical role in the osteogenic activity of Prx1$^{+}$ lineage skeletal progenitors.

Prx1-lineage skeletal cells are major cell populations (Fig. 1i) that initiate callus formation and vascularization for successful bone fracture repair$^{36-39}$. Ecsit deletion in Prx1$^{+}$ skeletal progenitors resulted in nearly complete arrest of cortical bone and periosteal formation despite expansion of Ecsit-deficient periosteal cells (Fig. ij, Supplemental Fig. 1f). Accordingly, Ecsit$^{fl/fl}$ femurs show spontaneous fractures occurring after birth. The resulting fracture sites displayed persistent nonunion with a lack of callus mineralization and mature osteoblasts and the persistence of an unmineralized fibrous callus with cartilaginous elements (Fig. ij, k, Supplemental Fig. 1d). To define where ECSIT is required in the osteoblast maturation process, skeletal cell populations were isolated from P0 Ecsit$^{fl/fl}$ limbs and subjected to flow cytometry, demonstrating an increased frequency of skeletal stem cells (SSCs) and pre-bone, cartilage, stromal progenitors (pre-BCSPs) and a decreased frequency of BCSPs (Fig. il, m, Supplemental Fig. 3). Thus, Ecsit deletion in Prx1$^{+}$ skeletal progenitors impairs the differentiation of SSCs and pre-BCSPs to BCSPs, resulting in defective commitment and differentiation of skeletal progenitors to osteoblasts.

To test whether Ecsit deletion in skeletal progenitors is a primary cause of impaired fracturing healing in Ecsit$^{fl/fl}$ mice, Ecsit$^{fl/fl}$ mice were crossed with Prx1-Cre$^{fl/+}$-EGFP mice (Ecsit$^{fl/+}$Prx1-Cre$^{fl/+}$EGFP) to delete ECSIT expression in skeletal progenitors in an inducible manner. Fracture surgery was performed on the femurs of 6-week-old Ecsit$^{fl/+}$Prx1-Cre$^{fl/+}$-EGFP and Prx1-Cre$^{fl/+}$-ERT/GFP control mice three days after tamoxifen injection (Supplemental Fig. 4a). One month later, tamoxifen-induced expression of Cre recombinase and deletion of Ecsit were validated by fluorescence microscopy and RT-PCR, respectively (Supplemental Fig. 4b-4c). MicroCT analysis revealed that basal bone mass in control and Ecsit$^{fl/+}$Prx1-Cre$^{fl/+}$-ERT/GFP mice was comparable, as shown by equivalent trabecular bone mass and cortical bone thickness of non-fractured femurs (Supplemental Fig. 4d, e). Unlike Ecsit$^{fl/+}$ mice, tamoxifen-treated Ecsit$^{fl/+}$Prx1-Cre$^{fl/+}$-ERT/GFP mice displayed normal periosteal and callus formation and fracture unionization in the fractured sites (Supplemental Fig. 4b, f, g). These results demonstrate that inducible deletion of Ecsit in Prx1$^{+}$ skeletal
**Fig. 1** *Ecsit*^**−/−** mice show defects in skeletogenesis, fracture healing, and the bone marrow niche. a Immunostaining of ECSIT in 2-month-old wild-type femurs. Tb: trabecular bone, cb: cortical bone. b Immuno-fluorescence showing the localization of ECSIT in P21 mouse femurs. Arrows indicate co-localization of ECSIT and NDUFS3. c Immunoblot analysis showing mitochondrial fractionation of ECSIT in human BMSCs. d, e X-radiography (d, left) and microCT showing P21 whole body and hindlimbs (d, middle) and quantification of femoral bone mass (e, n = 5). Alizarin red/alcan blue staining of P1 neonates (d, right). Arrows indicate spontaneous fractures. Tra, BV/TV: Trabecular bone volume/total volume. f H&E staining of E16.5 and P0 femurs (left, middle). Immunofluorescence showing osteocalcin (OCN) expression in the femurs (right). Arrows indicate femoral trabecular bone. g Skeletal progenitors isolated by FACS from P0 *Ecsit*'**fl/fl**/Rosa26mTmG (cKO) and *Prx1*Rosa26mTmG (WT) limbs using GFP+CD45−Ter119−Tie2− markers. Osteogenic gene expression (n = 4) and alkaline phosphatase activity (ALP, n = 3) were assessed for osteogenic differentiation. h Flow cytometry analysis showing frequency of the indicated bone marrow cell populations at P10 (right, HSCs *Ecsit*'**fl/fl**, n = 7; *Ecsit*'**−/−**, n = 5); other subpopulations, n = 4–5) and total number of the isolated cells (left, *Ecsit*'**fl/fl**, n = 7; *Ecsit*'**−/−**, n = 6). Supplemental Fig. 2 demonstrates the gating strategy for this analysis. i GFP-expressing PRX1-lineage cells residing in the diaphyseal periosteum of P1 *PRX1-cre*Rosa26mTmG mice. m: muscle, bm: bone marrow. j P21 femoral cortical bones were stained for Von Kossa (top), H&E (bottom) and immuno-fluorescence for OCN (middle). k H&E staining of *Ecsit*'**−/−** femurs at the age of P0, P10, and P21. l, m Flow cytometry analysis showing frequency of SSCs, pre-BCSP, and BCSP in skeletal cells (l, n = 5). RT-PCR shows expression of Ecsit in pre-BCSP (m, n = 3). Supplemental Fig. 3 demonstrates the gating strategy for this analysis. A two-tailed unpaired Student’s t-test for comparing two groups (e, g, h, i, m; error bars, data represent mean ± SD). Data are representative of three independent experiments. Scale bars = a, 100 μm; b, 50 μm; d (left, middle), 2 mm; d (right), 1 mm; f, 60 μm; l, 75 μm; j, 40 μm; k, 300 μm.
progenitors in adult mice does not affect bone fracture healing and homeostasis. Thus, impaired fracture healing seen in EcsitPrx1 mice may result from pre-existing skeletal phenotypes and/or alteration of skeletal progenitor population during early skeletal development. However, it cannot be fully excluded that inadequately complete deletion of Ecsit in this system contributes to the lack of a phenotype observed. Taken together, ECSIT-mediated mitochondrial functions play critical roles in osteogenic commitment and differentiation of skeletal progenitors during early skeletal development.

ECSIT controls mitochondrial OXPHOS in skeletal progenitors

Despite the original identification of ECSIT as a key regulator of NF-κB signaling downstream of pro-inflammatory receptors, Toll-like receptor (TLR) and interleukin (IL)-1β signaling, transcriptome analysis showed an

Fig. 2 | Ecsit deficiency in skeletal progenitors impairs mitochondrial OXPHOS. a, b Gene ontology analysis showing biological processes with gene enrichment in cKO vs WT skeletal progenitors (a). Gene set enrichment analysis (GSEA) showing the enrichment of genes involved in OXPHOS, CI biogenesis, glycolysis, glutamine metabolism, and IKK-NFκB signaling (b). c Immunoblot analysis showing protein levels in cKO and WT skeletal progenitors. GAPDH was used for a loading control. d MitoTracker and TMRE staining (red) of GFP-expressing cKO and WT skeletal progenitors (green). DAPI was used for nuclear staining (left). The signal intensity of the stained cells was quantified using ImageJ software (right). (MitoTracker (WT), n = 6; MitoTracker (cKO), TMRE (WT, cKO), n = 5). e RT-PCR analysis showing the ratio of mitochondrial DNA (mtDNA, Ndufv1) to nuclear DNA (nDNA, 18s) in cKO and WT skeletal progenitors (n = 4). Intracellular ATP levels in cKO and WT skeletal progenitors were measured by colorimetric analysis (n = 9). g–i Seahorse assay showing a real time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR, l) of cKO and WT skeletal progenitors before and after the addition of mitochondrial inhibitors (n = 46). j) Diagram of constructs for ECSIT-full length and the mutant lacking mitochondrial localization sequence (MLS, 1-47 aa). A FLAG epitope tag was inserted into the C-terminus of ECSIT cDNA (top). Immunoblot analysis showing mitochondrial fractionation of FLAG-ECSIT proteins in cKO skeletal progenitors. (bottom). T total, S soluble, M mitochondrial fraction. k Immunoblot analysis showing expression of FLAG-ECSIT proteins in AAV-transduced cKO skeletal progenitors. l, m MitoTracker or TMRE staining of vector control or FLAG-ECSIT-expressing cKO and WT skeletal progenitors and quantification of deep red signal intensity (l). AAV-transduced cells were cultured under osteogenic conditions and ALP activity and osteogenic gene expression were assessed (m, n = 4). A two-tailed unpaired Student’s t-test for comparing two groups (d, e, f, g) or ordinary one-way ANOVA with Dunnett’s multiple comparisons test (l,m). (d–i, l–m; data represent mean ± SD). Data (e, f, g, m) are representative of three independent experiments. Scale bars = d (left), l, 10 μm.
enrichment of genes associated with metabolic pathways and mitochondrial function, not the NF-κB pathway in cKO Prx1- skeletal cells (Fig. 2a, b). Specifically, expression of the genes involved in mitochondrial CI biogenesis and OXPHOS was markedly downregulated in the absence of Ecsit while there was little to no altered expression in glucose and glutamine metabolism (Fig. 2b). This is consistent with previous reports demonstrating ECSIT as a key component of the MCIA complex that controls the stability of CI subunits23–33. To directly test the role of ECSIT in mitochondrial OXPHOS, the expression of mitochondrial CI, II, III, IV, and V subunit proteins in the OXPHOS pathway was examined in WT and cKO Prx1- skeletal progenitors, demonstrating that expression of core CI subunit proteins, including ND6, NDUFB8 and NDUFS3, was ablated in the absence of Ecsit while Ecsit deletion minimally affected the expression of proteins in other mitochondrial complexes (Fig. 2c, left). Similarly, Ecsit-deficiency significantly reduced expression of NDUFAF1, a key component of the MCIA complex23–33 (Fig. 2c, right), suggesting the importance of ECSIT in stabilizing the components of CI and MICA complexes in Prx1- skeletal progenitors. Accompanying our transcriptome analysis that show little to no enrichment of genes associated with mitochondrial biogenesis (Fig. 2b), mRNA and protein levels of Atfs14-16 and Hsp90α4-6 were largely intact in the absence of Ecsit (Fig. 2c, Supplemental Fig. 5a). Of note, expression of Lcs1 and Pink1, key regulators of mitophagy, was markedly increased in cKO Prx1- skeletal progenitors (Fig. 2c), suggesting enhanced mitophagy activity. These results suggest that Ecsit deletion in Prx1- skeletal progenitors destabilizes the components of the MCIA and CI complexes while enhancing mitophagy activity. Accordingly, cKO Prx1- skeletal progenitors displayed a significant decrease in mitochondrial numbers and transmembrane potential (Fig. 2d), in the ratio of mitochondrial to nuclear DNA levels (Fig. 2e), and in ATP production (Fig. 2f). Along with a decrease in basal and maximal mitochondrial oxygen consumption (Fig. 2g), cKO Prx1- skeletal progenitors failed to respond to mitochondrial respiratory modulators, demonstrating the nearly complete absence of oxygen consumption rates (OCR, Fig. 2h). Of note, the glycolytic proton efflux rate was markedly upregulated in these cells, as shown by greater extracellular acidification rate (ECAR, Fig. 2i). However, enhanced glycolysis was incapable of compensating for the reduced ATP production by the damaged mitochondrial OXPHOS. In contrast to the involvement of glucose metabolism in RUNX2-mediated osteogenesis45, expression and transcription activity of RUNX2 were largely intact in cKO Prx1- skeletal progenitors (Supplemental Fig. 5b, c), suggesting that ECSIT-mediated mitochondrial oxidative metabolism is dispensable for RUNX2-mediated osteogenesis.

Since ECSIT is localized on the membrane of mitochondria via an N-terminal mitochondrial localization sequence (MLS, 1–48 aa)32,46, to investigate mechanistic actions of ECSIT in regulating mitochondrial OXPHOS, cKO Prx1- skeletal progenitors were reconstituted with FLAG-tagged ECSIT full length (ECSIT-FL) or a MLS-deletion mutant (ECSIT-ΔMLS) that failed to localize to mitochondria via RAAV-mediated delivery (Fig. 2). Enforced expression of ECSIT-FL, not the ECSIT-ΔMLS mutant, reversed alterations in expression of the CI subunit NDUFS3, mitochondrial numbers and transmembrane activity, and osteogenic potential in cKO Prx1- skeletal progenitors (Fig. 2k–m). Thus, mitochondrial ECSIT regulates CI assembly and mitochondrial OXPHOS in Prx1- skeletal progenitors, which in turn determines the osteogenic commitment and differentiation of skeletal progenitors.

To confirm these findings in vivo, a bone-targeting RAAV was used to express FLAG-tagged ECSIT-FL and ECSIT-ΔMLS proteins in EcsitFlox mice (Fig. 3a). Given our previous report showing high transduction efficiency of the AAV9 serotype in osteoblast-lineage cells in vivo36, systemic delivery of AAV9 vector expressing mCherry to Prx1;Ro- sarΔneonate effective in transducing Prx1- osteoblast-lineage cells, including skeletal progenitors, osteoprogenitors, and mature osteoblasts, in both trabecular and cortical compartments of long bones in addition to lung, heart, liver, kidney, and skeletal muscle (Fig. 3b, c). AAV-mediated expression of FLAG-tagged ECSIT-FL and ECSIT-ΔMLS proteins in the femur and liver was confirmed (Fig. 3f, Suplemental Fig. 6). Remarkably, enforced expression of ECSIT-FL, but not a vector control or ECSIT-ΔMLS, markedly improved the survival rates of EcsitFlox mice (Fig. 3d). The osteogenic activity of EcsitFlox skeletal progenitors was recovered by ECSIT-FL expression (Fig. 3f–h), leading to recovery of bone formation and regeneration activity at multiple anatomic compartments. This led to an almost complete rescue of skeletal deformities in EcsitFlox forelimbs and hindlimbs (Fig. 3e) and abnormal bone marrow compartment (Fig. 3f, left) and impaired fracture healing and periosteal bone formation of EcsitFlox long bones (Fig. 3g, h). However, none of these phenotypes were improved by AAV-mediated expression of ECSIT-ΔMLS (Fig. 3d–h). As expression of ECSIT-FL, not ECSIT-ΔMLS, restored OCN+ mature osteoblasts and osteoblast-supporting CD31+EDMC+ type H endothelium48 in the fracture callus of EcsitFlox long bones, fracture healing progressed normally in these mice (Fig. 3g, h). Restoring ECSIT levels in Prx1- osteoblast-lineage cells, including skeletal progenitors, osteoprogenitors, and mature osteoblasts, reverses skeletal defects and retrieves bone regeneration during fracture in EcsitFlox mice. Thus, ECSIT-mediated regulation of mitochondrial OXPHOS in skeletal progenitors is crucial for bone accrual, fracture repair, and bone marrow niche maintenance during early skeletal development.

**ECSIT is dispensable of mitochondrial OXPHOS in committed skeletal cells**

Since ECSIT is highly expressed in osteix (OSX)+ committed osteoprogenitors and dentin matrix acidic phosphoprotein 1 (DMP1)+ mature osteoblasts and osteocytes (Figs. 1a and 4a), the role of ECSIT in these cells were examined using conditional deletion with OSX-cre (EcsitOsxFlox), Dmp1+ osteoblast-lineage cells, including skeletal progenitors, osteoprogenitors, and mature osteoblasts, which in turn determines the osteogenic differentiation and bone fracture healing were largely intact in the absence of Ecsit (Fig. 4f, Supplemental Fig. 7). In addition, osteogenic differentiation and bone fracture healing were largely intact in the absence of Ecsit (Fig. 4f, Supplemental Fig. 8). These results suggest that ECSIT function is dispensable for the development of committed osteoblast-lineage cells, including OSX+ osteoprogenitors and Dmp1+ mature osteoblasts and osteocytes.

To gain insights on this mechanism, mitochondrial OXPHOS was examined in EcsitFlox osteoprogenitors and EcsitFlox+ osteoblasts. Unlike EcsitFlox skeletal progenitors, expression of the CI protein NDUFS3, ND6, and NDUFB8, and the MCIA subunit NDUFAF1 (Fig. 4g), the ratio of mitochondrial DNA to nuclear DNA (Fig. 4h), mitochondrial numbers and transmembrane potential (Fig. 4i) were all intact in these cells, suggesting that ECSIT function is dispensable for mitochondrial OXPHOS in OSX+ committed osteoprogenitors and Dmp1+ mature osteoblasts and osteocytes. Notably, while a similar decrease in ECSIT expression was observed among EcsitFlox skeletal progenitors, EcsitFlox+ osteoprogenitors, and EcsitFlox+ osteoblasts, only EcsitFlox- cells showed a significant decrease in NDUFS3 expression (Supplemental Fig. 9). However, it cannot be fully excluded that inadequately deletion of Ecsit in osteoblasts may contribute to the lack of an observed phenotype. Thus, the role of ECSIT in controlling mitochondrial oxidative metabolism is context- and tissue-dependent. **Ecsit deficiency in skeletal progenitors induces muscle atrophy**

Accompanying the onset spontaneous fractures in EcsitFlox mice is an onset of myopathy and muscle atrophy. Specifically, the size and
weight of skeletal muscles, including tibialis anterior (TA) and gastrocnemius (GA) muscles, were dramatically decreased (Fig. 5a, b, Supplemental Fig. 10a). While muscle structure of EcsitPrx1 neonates is grossly normal after birth (Fig. 5c, Supplemental Fig. 10b), EcsitPrx1 mice gradually developed severe degeneration of skeletal muscle, as evident by significant decrease in mature myofibers, extracellular matrix in the interstitial space, and the expression of mature myoblast genes (Fig. 5d, e, Supplemental Fig. 10c). Despite a substantial decrease in

**Fig. 3 | AAV-mediated expression of ECSIT reverses Ecsit<sup>fl/fl</sup> skeletal phenotypes.** a Diagram summarizing the study and treatment methods. A single dose of 2 x 10<sup>11</sup> genome copies (GCs) of rAAV9 vectors carrying control vector or FLAG-ECSIT constructs was injected into P1 Ecsit<sup>fl/fl</sup> neonates via the facial vein and musculoskeletal phenotypes were assessed 21 days post-injection (created with biorender.com). b Single dose of 2 x 10<sup>11</sup> GCs of rAAV9 carrying mCherry was injected into P0 Prx1;Rosa26mTmG neonates via facial vein and mCherry expression was monitored by IVIS-100 optical imaging 21 days post-injection. c rAAV9 vector carrying mCherry was injected into Prx1;Rosa26mTmG neonates and 21 days later, mCherry expression was assessed by fluorescence microscopy of cryo-sectioned femurs. d Survival rate of AAV-treated Ecsit<sup>fl/fl</sup> mice up to 21 days post-injection (n = 6). e MicroCT analysis of the forelimbs (left) and hindlimbs (right) of P21 AAV-treated Ecsit<sup>fl/fl</sup> and Ecsit<sup>Prx1</sup> mice (n = 5–8). f Longitudinal sections of P21 AAV-treated Ecsit<sup>fl/fl</sup> and Ecsit<sup>Prx1</sup> femurs were stained for H&E (left) or immunostained for OCN (middle) or FLAG-ECSIT (right). Blue lines indicate the bone marrow area. g H&E staining (left) or immunofluorescence for CD31, EDMC, or OCN (middle, right) of diaphyseal cortical bones of P21 AAV-treated Ecsit<sup>fl/fl</sup> and Ecsit<sup>Prx1</sup> femurs. Blue lines indicate periosteum. h H&E staining (top) or immunofluorescence for CD31, EDMC (VEC, n = 7; ECSIT-FL, n = 8, ECSIT-MLS, n = 6), or OCN (n = 6, middle, bottom) of fracture sites of P21 AAV-treated Ecsit<sup>fl/fl</sup> and Ecsit<sup>Prx1</sup> femurs. Immunofluorescence intensity was quantified using ImageJ software. An ordinary one-way ANOVA with Dunnett’s multiple comparisons test (h; error bars, data represent mean ± SD). Data are representative of three independent experiments. Scale bars = c, 50 μm; e, 1 mm; f, h, 100 μm; g, 75 μm.

https://doi.org/10.1038/s41467-022-34694-8
total myocyte numbers in Ecsitflm mice, the frequency of skeletal muscle-resident satellite cells (β-integrin/CXCR4 Sca1CD45 CD11b TER119) and mesenchymal progenitors (Sca1 integrin α7 CD45 CD31) was dramatically increased in Ecsitflm muscle (Fig. 5f, Supplemental Fig. 1I), indicating a defect in myoblast differentiation, but not the maintenance of muscle-resident stem cells. Of note, this phenotype does not result from the intrinsic effects of Ecsit deficiency in muscle cells, as shown by intact expression of Ecsit mRNA and protein in Ecsitfli muscle (Fig. 5d, e). This is accompanied by fluorescence microscopy showing little to no Prx1-cre driven reporter activity in skeletal muscle cells of Prx1-cre;Rosa26tm1tm1 mice (Fig. 5g). To directly test this, mice with targeted deletion of Ecsit in myoblast precursors (Ecsitflm) were generated, demonstrating little to no gross phenotypes in the skeletal muscle, bone, and bone marrow (Fig. 5h). Likewise, the expression of mature myoblast genes in the skeletal muscle of these Ecsitflm mice was also normal (Fig. 5i). Additionally, primary human myoblast progenitors (17Ubi) with Ecsit knockdown were cultured under myogenic conditions, demonstrating no effect of Ecsit deficiency on myoblast differentiation (Fig. 5j). As seen in Oxs-cre committed osteoprogenitors and Dmp1+ mature osteoblasts (Fig. 4g, h), deletion of Ecsit in myoblast progenitors did not affect expression of CI protein NDUFS3, the ratio of mitochondrial DNA to nuclear DNA, mitochondrial number (Oxs-cre, n = 8; Ecsitflm, n = 7; Ecsitflm, n = 5) and cortical bone thickness (Oxs-cre, n = 8; Ecsitflm, n = 7; Ecsitflm, n = 6; Ecsitflm, n = 4, right). Oxs-cre limbs were used as controls for Ecsitflm mice. f MicroCT analysis showing sagittal and transverse views of fracture sites of 2-month-old Oxs-cre, Ecsitflm, and Ecsitflm femurs 10 weeks after the surgery, and the relative quantification of fracture union rates (n = 5). g-i Osteoprogenitors and osteoblasts were isolated from Oxs-cre and Ecsitflm calvaria and Ecsitflm and Ecsitflm long bones, respectively. Immunoblot analysis showing protein levels in the isolated cells. GAPDH was used as a loading control. A two-tailed unpaired Student's t-test for comparing two groups (b, c, d, e, f) was applied. Data are representative of three independent experiments. Scale bars = a, 75 μm; b, d, 100 μm; c, e (left, top) 2 mm; e (left, middle and bottom), 100 μm; f, 1 mm; i (left), 25 μm.
**Fig. 5** *Ecsit*<sup>Prx1<sup>−/−</sup> mice display skeletal muscle atrophy. **a** Graph shows the kinetics of fracture incidence and skeletal muscle weight of *Ecsit<sup>Prx1<sup>−/−</sup></sup>* and *Ecsit<sup>fl/fl</sup>* mice (n = 5, left). Representative macroscopic images of the gastrocnemius (GA) muscle at the age of P0 and P10 (right). **b** Quantification of skeletal muscle weight (left) and a ratio of skeletal muscle weight to body weight (right) are displayed (n = 5). **c** P0 (e) or P15 (d) *Ecsit<sup>Prx1<sup>−/−</sup></sup>* and *Ecsit<sup>fl/fl</sup>* tibialis anterior (TA) muscles were stained for H&E or immunostained for MF20, COL1α1, or ECSIT. **e** mRNA levels of myogenic genes in P10 *Ecsit<sup>Prx1<sup>−/−</sup></sup>* and *Ecsit<sup>fl/fl</sup>* TA muscles (n = 16). **f** Total cell number (n = 5) and flow cytometry analysis showing the frequency of muscle satellite cells (MuSCs) and skeletal muscle resident stem/progenitor cells (*Ecsit<sup>Prx1<sup>−/−</sup></sup>* n = 9, *Ecsit<sup>fl/fl</sup>* n = 5) in P10 *Ecsit<sup>Prx1<sup>−/−</sup></sup>* and *Ecsit<sup>fl/fl</sup>* GA muscles. **g** GFP-expressing Prx1<sup>+</sup> skeletal cells in P3 Prx1<sup>-creRosa2<sub>A</sub></sup> mice. Red: Prx1<sup>+</sup> bone marrow and muscle. **h**, **i** Tibialis anterior muscles or femurs of E21 *Ecsit<sup>Prx1<sup>−/−</sup></sup>* and *Ecsit<sup>fl/fl</sup>* embryos were stained for H&E, Von Kossa, or immunostained for MF20, ECSIT and NDUF3 (k, left) or the ratio of mtDNA (Ndufv1) to nDNA (18s, k, right) in shCtrl or shECSIT-expressing 17Ubic cells. Fluorescence microscopy shows MitoTracker- and TMRE-stained cells and relative quantification of deep red signal intensity (n = 6). **k** Protein levels of NDUFAF1, ECSIT and NDUF3 (k, left) or the ratio of mtDNA (Ndufv1) to nDNA (18s, k, right) in shCtrl or shECSIT-expressing 17Ubic cells. Fluorescence microscopy shows MitoTracker- and TMRE-stained cells and relative quantification of deep red signal intensity (n = 6). **l** Protein levels of NDUFAF1, ECSIT and NDUFS3 (k, left) or the ratio of mtDNA (Ndufv1) to nDNA (18s, k, right) in shCtrl or shECSIT-expressing 17Ubic cells. Fluorescence microscopy shows MitoTracker- and TMRE-stained cells and relative quantification of deep red signal intensity (n = 6). A two-tailed unpaired Student’s *t*-test for comparing two groups (b, e, f, i, j, k, l; error bars, data represent mean ± SD). Data are representative of three independent experiments. Scale bars = c, d, 60 μm; g, 75 μm; h, 30 μm; j, 100 μm; l, 10 μm.
skeletal muscle was monitored at fracture sites. Wild-type mice undergoing fracture did not experience muscle degeneration, showing normal callus formation at 7- and 14-days post-fracture, and restriction of wild-type Prx1-lineage skeletal cells to the callus and not adjacent skeletal muscle (Fig. 6a). On the other hand, EcsitPrx1 mice showed abnormal callus development and fibrotic tissue at fracture sites and failed to form periosteum, where EcsitPrx1 skeletal progenitors migrated into the skeletal muscle and induced degeneration of myofibers (Fig. 6b). In non-fractured long bone showing normal muscle structure, EcsitPrx1 skeletal progenitors were only detected inside the bone (Fig. 6c). Thus, impaired fracture healing and periosteal formation allow migration of EcsitPrx1 skeletal progenitors to adjacent skeletal muscle, resulting in muscle atrophy. To directly test this hypothesis, conditioned media (CM) harvested from WT or cKO Prx1+ skeletal progenitors were added to cultures of human myoblast progenitors (17Ubic). These studies demonstrated a significant reduction in myoblast differentiation in the presence of cKO-CM compared to WT-CM (Fig. 6d). Transcriptome and RT-PCR analyses of FACS-sorted WT and cKO Prx1+ skeletal progenitors and SSCs revealed elevated levels of the soluble myogenic suppressor TGF-β1 in cKO cells (Fig. 6e, f). Since TGF-β expression was unchanged in satellite cells (MuSCs) isolated from the skeletal muscle of EcsitPrx1 mice and Ecsit-deficient myoblasts...
progenitors (Fig. 6f, g), cKO Ptx1 MED skeletal progenitors and SSCs, not MuSCs and myoblast progenitors, are likely to be major contributors of TGF-β1 expression in skeletal muscle. Accordingly, the TGF-β1 inhibitor SB-431542 almost completely reversed the ability of cKO-CM to suppress myoblast differentiation (Fig. 6d). Since the TGF-β pathway plays a role in the pathogenesis of skeletal muscle myopathies and its inhibition is considered for the treatment of skeletal muscle fibrosis55,56, TGF-β1 is likely to be an important factor mediating the myoblast suppressive activity of Ecsit57,58 skeletal progenitors, though other pathways may contribute as well.

Intravenous administration of PI EcsitPRL neonates with bone-targeting rAAV9 expressing FLAG-tagged ECSI-FL, not ECSI-ΔMLS mutant, almost completely recovered degeneration of skeletal muscle (Fig. 6h). This was accompanied with little to no migration of EcsitPRL skeletal progenitors expressing ECSI-FL, not ECSI-ΔMLS mutant, to the skeletal muscle (Fig. 6i). Finally, enforced expression of ECSI-FL markedly decreased TGF-β1 transcription in FACS-sorted cKO Ptx1 MED skeletal progenitors while TGF-β expression was partially reversed by ECSI-ΔMLS mutant (Fig. 6j), suggesting the presence of additional functional domain(s) within Ecsit regulating TGF-β expression. Thus, impaired OXPHOS in skeletal progenitors is likely to be responsible for muscle atrophy seen in EcsitPRL mice. Taken together, Ecsit-mediated regulation of mitochondrial oxidative metabolism is a key determinant of the osteogenic commitment and differentiation of Ptx1 MED skeletal progenitors, governing musculoskeletal development and bone marrow niche functions.

Discussion

Our study provides in vivo evidence showing the importance of mitochondrial oxidative metabolism in determining osteogenic commitment and differentiation of skeletal progenitors during early skeletal development. Despite emerging evidence suggesting an association of mitochondrial dysfunction and high oxidative stress in skeletal cells with skeletal disorders17, only few genetic models with association of mitochondrial dysfunction and high oxidative stress in skeletal progenitors suggest the presence of any relevant phenotypes. Herein, we generated a mouse model with CI deficiency by conditionally deleting the key CI assembly factor Ecsit in various musculoskeletal cells. Deletion of Ecsit in Ptx1 MED skeletal progenitors disrupts mitochondrial OXPHOS for ATP production while enhancing glucose metabolism as a compensatory mechanism. Lack of OXPHOS activity ablates the osteogenic potential of skeletal progenitors, resulting in defects in skeletal development, bone marrow niche maintenance, and bone fracture repair. Surprisingly, breakdown of this regulatory circuit leads to the infiltration of Ptx1 MED skeletal progenitors into adjacent muscle, which induces myopathy mediated by TGF-β1 (Supplemental Fig. 12). This unexpected finding indicates that OXPHOS defects or TGF-β1 may be implicated in paired skeletal fragility/myopathy disorders, and there may be potential therapeutic targets in this context. Intriguingly, despite the requirement of mitochondrial localization of Ecsit in regulating mitochondrial oxidative metabolism in Ptx1 MED skeletal progenitors, our findings that cytosolic Ecsit (Ecsit-ΔMLS) can partially improve survival of EcsitPRL mice and TGF-β1 expression in EcsitPRL skeletal progenitors suggest the presence of additional functional domain(s) within Ecsit regulating survival and TGF-β1 expression. Further studies will be necessary to identify these functional domains in Ptx1 MED skeletal progenitors.

Glucose and glutamine metabolism and mitochondrial OXPHOS have been known as major pathways of energy metabolism regulating the production of ATP in osteoblast-lineage cells59. In particular, glucose and glutamine metabolism are prominent metabolic features of committed osteoblast-lineage cells60, and integral to collagen production and mineralizing activity by generating ATP and intermediate metabolites61. In contrast to our findings that Ecsit-mediated mitochondrial OXPHOS provides an energy source crucial to the osteogenic commitment and differentiation of Ptx1 MED skeletal progenitors during skeletal development, glutamine metabolism mainly contributed to biosynthesis and redox homeostasis, but not energy production, during bone remodeling. Of note, since amino-acid-transaminase-derived αKG is the critical downstream glutamine metabolite regulating proliferation of skeletal progenitors62, cell proliferation rates were largely not altered in EcsitPRL skeletal progenitors with normal glutamine metabolism. Intriguingly, despite extensive studies showing that glucose is a major nutrient for osteoblast development, mice lacking the glucose transporter Glut1 in Ptx1 MED skeletal progenitors displayed impaired cartilage development without any alteration in skeletal mineralization63. This suggests that glycolysis is critical for chondrogenesis, not osteogenesis, of Ptx1 MED skeletal progenitors during skeletal development. Notably, Ecsit deficiency in Ptx1 MED skeletal progenitors enhanced glucose metabolism as a compensatory mechanism while cartilage development occurred normally in EcsitPRL mice. Thus, our data implies that mitochondrial OXPHOS, not glucose or glutamine metabolism, is the major energy-driving force for osteogenesis in skeletal progenitors and that it is, therefore, critical for skeletal development and the maintenance of musculoskeletal integrity. We note that reduced mitochondrial mass in EcsitPRL skeletal progenitors also potentially results in a broader set of cellular abnormalities than just decreased ATP synthesis, as many TCA cycle metabolites, such as acetyl-CoA and α-ketoglutaric acid, have direct roles in lipid synthesis, protein acetylation, histone demethylation, and other processes64,65,66.

Since Ecsit function is dispensable for Osx MED committed osteoprogenitors and Dmp1 MED mature osteoblasts and terminally differentiated osteocytes, glycolysis may off this as glycolysis is the primary metabolic pathway for ATP production during the differentiation of committed osteoblast-lineage cells66. Further studies will be necessary to determine how Ecsit controls mitochondrial oxidative metabolism and osteogenic development in a context and tissue-dependent manner. Additional further studies are warranted to define the precise mechanisms by which mitochondrial OXPHOS impacts skeletal progenitors through other mitochondrial regulatory mechanisms, such as mitochondrial biogenesis, the degradation of damaged mitochondria via mitophagy, reactive oxygen species (ROS) generation, and mtDNA replication and/or transcription.

The unexpected finding that mitochondrial defects lead EcsitPRL MED skeletal progenitors to infiltrate into muscle and mediate pathogenic TGF-β1 secretion, provides a new potential explanation for a number of clinical disorders that pair prominent or primary skeletal phenotypes with myopathy. For instance, Kearns Sayre Syndrome displays linked skeletal and myopathy phenotypes due to mitochondrial dysfunction, raising the possibility that the myopathy may be in part secondary to pathogenic infiltration of skeletal progenitors into adjacent muscle67. rAAVs have been considered the most promising viral vector for gene therapy68, as they have a clinical track record of being well tolerated and have the potential to efficiently mediate genetic modifications that can persist for years after a single treatment. Remarkably, AAV-mediated expression of full length Ecsit almost completely restored impaired mitochondrial OXPHOS and osteogenic potentials of EcsitPRL MED progenitors, preventing musculoskeletal deformities of EcsitPRL MED mice. Thus, our findings provide proof-of-concept demonstration for gene therapy of developmental skeletal disorders. Collectively, this study reveals a previously unappreciated role of mitochondrial oxidative metabolism in determining osteogenesis of skeletal progenitors and demonstrates a new gene therapy approach for mitochondrial diseases in the musculoskeletal system.

Methods

Mice

Mice were housed in a constant environment under a standard mouse chow diet (up to 5 mice per cage); ambient temperature of 21 ± 2 °C,

Article

https://doi.org/10.1038/s41467-022-34694-8
circulating air, and constant humidity of 50 ± 10%, in a 12 h light, 12 h dark cycle. Mice were monitored every three days for the amount of their food and water intake and signs for distress. For signs of severe distress, including general malaise, severe cachexia, or more than 20% loss of body weight, humane euthanasia was performed in consultation with veterinary staff. Mice were euthanized in a carbon dioxide chamber, followed by cervical dislocation. Necropsies were euthanized by decapitation. Ecsftm mice were generated as previously described and maintained on a C57BL/6J background. Cre deleter mice (C57BL/6J) that express Cre recombinase under the control of the prx1 promoter (Prx1-cre), the osterix promoter (Osx-cre), and the dmpl promoter (Dmpl-cre) were purchased from The Jackson Laboratory. To label Cre-expressing cells, EcsftmPrx1-cre mice were further crossed with Rosa26tm1(CAG-EGFP)Cre reporter mice (C57BL/6J). Mouse genotypes were determined by PCR on tail genomic DNA and primer sequences are available upon request. Control littermates were used and analyzed in all experiments. A phenotypic summary of Ecsftm mice is described in Supplemental Table 1. All animals were used in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC).

Generation of rAAV vectors
DNA sequences for ECSIT-FL and ECSIT-ΔMSL were synthesized as gBlocks, cloned into the intronic region of the pAAVsc-CB6-Egfp plasmid at the restriction enzyme sites (PstI and BglII), and packaged into an AAV9 capsid. rAAV production was performed by transient transfection of HEK293 cells, purified by CsCl sedimentation, titered by droplet digital PCR (ddPCR) on a QX200 ddPCR system (Bio-Rad) using the Egfp prime/probe set as previously described. The droplet digital PCR (ddPCR) on a QX200 ddPCR system (Bio-Rad) was used for antigen retrieval and blocking, respectively. Sections were incubated with antibodies specific to ECSIT (A7804, ABclonal, 1:200) for 40 min at 37 °C, and a secondary antibody for 20 min at 37 °C. Subsequently, they were incubated in SA-HRP D for 16 min at 37 °C and then DAB + H2O2 substrate for 8 min, followed by hematoxylin and bluing reagent counterstain at 37 °C. Reaction buffer (pH 7.6 Tris buffer) was used as washing solution. Stained samples were visualized using an Aperio virtual microscope (Leica Microsystems, USA) and images of the sample were analyzed by the Aperio image scope program (ver. 12.3.2.5013, Leica Microsystems, USA).

MicroCT, radiography, and skeletal preparation
MicroCT was used for qualitative and quantitative assessment of trabecular and cortical bone microarchitecture and performed by an investigator blinded to the genotypes of the animals under analysis. Femurs excised from the indicated mice were scanned using a microCT 35 (Scanco Medical) with a spatial resolution of 7 μm. For cortical bone analysis of the distal femur, an upper 2.1 mm region beginning 280 μm proximal to the growth plate was contoured. For trabecular bone analysis of the femur and tibia, a midshaft region of 3500 μm thickness along the coronal plate from anterior to posterior. Decalcified femoral sections were stained with hematoxylin and eosin (H&E) or safranin O. Alternatively, intact hindlimbs containing bones and skeletal muscles were immediately fixed in ice-cold 4% paraformaldehyde solution for one day for histological analysis of skeletal muscles. Semi-decalcification was carried out for five days in 0.5 M EDTA pH 7.4 at 4 °C with constant shaking (age ≥1 week), and infiltration was followed with a mixture of 20% sucrose phosphate buffer for one day. All samples were embedded in a 50/50 mixture of 25% sucrose solution and OCT compound (Sakura) and cut into 10 μm thick cross sections using a cryostat (Leica Microsystems, USA).

Immunofluorescence staining and analysis were performed, as described previously. Briefly, after treatment with 0.2% Triton X-100 for 10 min, cryosections were blocked with 5% donkey serum at room temperature for 30 min and incubated overnight at 4 °C with primary antibody and then visualized with fluorescence-conjugated secondary antibody (1:400, Molecular Probes). Nuclei were counterstained with 4–6-diamidino-2-phenylindole (DAPI). An Olympus IX81 confocal microscope or Leica TCS SP5 II Zeiss LSM-880 confocal microscope or EVO epifluorescence microscope was used to image samples. Osteocalcin (OCN, sc-365797, Santa Cruz Biotechnology, 1:100), ECSIT (HPA042979, Sigma,1:100), ECSIT (gifted from Dr. Sankar Gosh, Columbia University), NDUFS3 (43–9200, Invitrogen,1:100), CD31/PECAM-1 (AF3628, R&D systems, 1:50), Endomucin (sc-63495, Santa Cruz Biotechnology, 1:100), COL1A1 (A1352, ABclonal, 1:100), MF20 (AB-2147781, DSHB, 1:100) and anti-FLAG-tag (MAI-142-A488, Sigma-Aldrich, 1:100) were used for primary antibodies.

For immunohistochemistry, paraffin sections were dewaxed and stained according to the manufacturer’s directions, using the Discovery XT automated IHC stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). CCl3 standard (pH 8.4 buffer contained Tris/Borate/EDTA) and inhibitor D (3% H2O2, Endogenous peroxidase) were used for antigen retrieval and blocking, respectively. Sections were incubated with antibodies specific to ECSIT (A7804, ABclonal,1:200) for 40 min at 37 °C, and a secondary antibody for 20 min at 37 °C. Subsequently, they were incubated in SA-HRP D for 16 min at 37 °C and then DAB + H2O2 substrate for 8 min, followed by hematoxylin and bluing reagent counterstain at 37 °C. Reaction buffer (pH 7.6 Tris buffer) was used as washing solution. Stained samples were visualized using an Aperio virtual microscope (Leica Microsystems, USA) and images of the sample were analyzed by the Aperio image scope program (ver. 12.3.2.5013, Leica Microsystems, USA).

Human myoblast and mouse osteoblast and osteoclast differentiation
Human primary myoblasts 17Ubic were kindly gifted from Dr. Charles P Emerson at UMass Chan Medical school. Informed consent was obtained from the patients who donated tissue for the production of cell lines. IRB protocols were approved by UMass Chan Medical School (H00006681-10 and H00006681-11) and by Kennedy Krieger Institute (B041008011). For human myoblast differentiation, human primary myoblasts 17Ubic were cultured in growth medium (20% FBS,0.5% calf serum) and maintained on a C57BL/6J background. Cre deleter mice (C57BL/6J) were purchased from The Jackson Laboratory. To label Cre-expressing cells, EcsftmPrx1-cre mice were further crossed with Rosa26tm1(CAG-EGFP)Cre reporter mice (C57BL/6J). Mouse genotypes were determined by PCR on tail genomic DNA and primer sequences are listed in Supplemental Table 2.

Histology, immunofluorescence, and immunohistochemistry
Histological analysis was performed on liver and hindlimbs, and skeletons and skeletal muscles were dissected from mice fixed in 4% PFA for one day, and decalcified by daily changes of 15% tetrasic acid EDTA for one to two weeks. Tissues were dehydrated by passage through an ethanol series, cleared twice in xylene, embedded in paraffin, and sectioned at 5 μm thickness along the coronal plate from anterior to posterior. Decalcified femoral sections were stained with hematoxylin and eosin (H&E) or safranin O. Alternatively, intact hindlimbs containing bones and skeletal muscles were immediately fixed in ice-cold 4% paraformaldehyde solution for one day for histological analysis of skeletal muscles. Semi-decalcification was carried out for five days in 0.5 M EDTA pH 7.4 at 4 °C with constant shaking (age ≥1 week), and infiltration was followed with a mixture of 20% sucrose phosphate buffer for one day. All samples were embedded in a 50/50 mixture of 25% sucrose solution and OCT compound (Sakura) and cut into 10 μm thick cross sections using a cryostat (Leica Microsystems, USA).
For mouse osteoblast differentiation, GFP-expressing PrxI skeletal cells were FACS-sorted from P1 Ecsitfl/fl;Prx1-Cre Rosa26loxM mice and Ecsitfl/fl;Prx1-Cre Rosa26loxM mice. Cells were cultured in cell culture medium with 20 ng/ml of M-CSF for 3 days, and then 10 ng/ml of RANKL were added to induce osteoclast differentiation. Twelve hours later, BMMs were treated with rAAV9 vectors expressing control vector or Cre recombinase, cultured in the medium containing 20 ng/ml of M-CSF for 3 days, and then 10 ng/ml of RANKL were added to induce osteoclast differentiation. Twelve hours later, the non-adherent cells were re-plated, cultured in the medium containing 20 ng/ml of M-CSF for 3 days, and then 10 ng/ml of RANKL were added to induce osteoclast differentiation. Twelve hours later, the non-adherent cells were re-plated, cultured in the medium containing 20 ng/ml of M-CSF for 3 days, and then 10 ng/ml of RANKL were added to induce osteoclast differentiation. Twelve hours later, the non-adherent cells were re-plated, cultured in the medium containing 20 ng/ml of M-CSF for 3 days, and then 10 ng/ml of RANKL were added to induce osteoclast differentiation.

Flow cytometry analysis
Bone marrow cells and splenocytes were isolated from p10 Ecsitfl/fl and Ecsitfl/fl mice. After incubation with red blood cells (RBC) lysis buffer containing NH4Cl, KHCO3, and EDTA), cells were passed through a 40 μm cell strainer, washed with FACS buffer (cold PBS (pH 7.2) containing 0.5% BSA (Fraction V) and 1 mM EDTA), and stained with mouse hematopoietic lineage antibody Cocktail (a mixture of antibodies against CD3, CD4, CD8, B20, Gr-1, CD11b and Ter119, 88-7772-72, Invitrogen, 1:100) and antibodies for ckit (12-1171-83, Invitrogen, 1:100) and Sca-1 (17-5981-82, Invitrogen, 1:100) for 30 min at 4 °C, washed with PBS, then 7-αmino-actinomycin D (7AAD, 00-6993-50, Invitrogen, 1:100) was added right before flow cytometry analysis to stain dead cells. Alternatively, cells were stained with Gr-1 (11-5931-85, Invitrogen, 1:100), B220 (48-0452-82, Invitrogen, 1:100), CD3ε (17-0031-82, Invitrogen, 1:100), CD11b (12-0112-82, Invitrogen, 1:100) to analyze immune cell populations. After washing three times, cells were re-suspended in cold PBS (pH 7.2) with 1 mM EDTA and analyzed with a LSRII (BD Biosciences) with the exclusion of 7AAD+ cells and doublets.

For flow cytometry analysis of muscle satellite cells, skeletal muscle tissues were dissected from P15 Ecsitfl/fl and Ecsitfl/fl hindlimbs and after 1 h enzymatic digestion at 37 °C, cells were stained with the following antibodies. CD45 (103103, BioLegend, 1:100), CD11b (101203, BioLegend, 1:100), Ter119 (116203, BioLegend, 1:100), Sca1 (108143, BioLegend, 1:100), integrin β-1 (102205, BioLegend, 1:100) and CXCR4 (146507, BioLegend, 1:100) for muscle-resident satellite cell staining while CD31 (102503, BioLegend, 1:100), CD45 (103103, BioLegend, 1:100), integrin-α7 (130-123-833, Miltenyi Biotec, 1:100), Sca1(108143, BioLegend, 1:100) for staining of skeletal muscle resident stem/progenitor cells. GHOST Dye (13–0865, Tion Biosiences, 1:100) was used to stain dead cells.

For flow cytometry analysis of skeletal stem cells, PI Ecsitfl/fl and Ecsitfl/fl limbs were dissociated by mechanical and enzymatic digestion (1 mg/ml of Collagenase D (108866601, Sigma-Aldrich), 1 mg/ml of Dispase II (494270801, Roche), 1 mg/ml of Hyaluronidase (H3506, Sigma-Aldrich) and 1000 unit/ml of DNase I (476728001, Roche) for 1 hour at 37 °C under gentle agitation. After blocking with purified rat anti-mouse CD16/CD32 (2342626, BioLegend, 1:100), PE-conjugated CD105 (120409, BioLegend, 1:100) for staining of skeletal muscle resident stem/progenitor cells. GHOST Dye (13–0865, Tion Biosiences, 1:100) was used to stain dead cells.

For flow cytometry analysis of muscle satellite cells, skeletal muscle tissues were dissected from P15 Ecsitfl/fl and Ecsitfl/fl hindlimbs and after 1 h enzymatic digestion at 37 °C, cells were stained with the following antibodies. CD45 (103103, BioLegend, 1:100), CD11b (101203, BioLegend, 1:100), Ter119 (116203, BioLegend, 1:100), Sca1 (108143, BioLegend, 1:100), integrin β-1 (102205, BioLegend, 1:100) and CXCR4 (146507, BioLegend, 1:100) for muscle-resident satellite cell staining while CD31 (102503, BioLegend, 1:100), CD45 (103103, BioLegend, 1:100), integrin-α7 (130-123-833, Miltenyi Biotec, 1:100), Sca1(108143, BioLegend, 1:100) for staining of skeletal muscle resident stem/progenitor cells. GHOST Dye (13–0865, Tion Biosiences, 1:100) was used to stain dead cells.
4/0 Nylon suture. Radiographs of the injured legs were performed to monitor fracture healing 2 weeks post-surgery. Ten weeks later, microCT and histology were performed for skeletal analyses. Alternatively, 6 week-old Prx1;Rosa26mTmG mice, while still under surgical plane of anesthesia, were placed into an Einhorn Device to create a closed femoral fracture, which was then confirmed by radiography. Nonunion fracture was defined as failure of bridging callus on anteroposterior and lateral tilted fracture cortical bones by coronal and sagittal reconstruction view of microCT and radiography. Fracture unionization was defined as osseous consolidation evident on reconstruction view of microCT and radiography.

**AAV-mediated expression of ECSIT proteins in mice**

A single dose of rAAV9 vectors carrying control vector or FLAG-ECSIT constructs (2 × 1011 GC, 50 μl) was randomly injected into P1 Ecsitfl/fl and Ecsitfl/fl neonates via the facial vein and 21 days later, skeletal and muscular phenotypes were assessed using X-ray, microCT, and histology. Alternatively, a single dose of rAAV9 vector carrying mCherry (2 × 1011 GC, 50 μl) was injected into P1 Prx1;Rosa26mTmG neonates via facial vein. Twenty-one days later, mCherry expression in individual tissues and the cryosectioned bone and its adjacent muscle were monitored by the IVIS-100 optical imaging and fluorescence microscopy, respectively.

**Mitochondrial fractionation**

Human bone marrow-derived stromal cells (BMSCs) were purchased from ScienCell Inc. (Cat #: 7500). Cells were resuspended in mitochondrial buffer (70 mM sucrose, 1 mM EGTA, 210 mM sorbitol, 10 mM MOPS (pH 7.4)) and then, the supernatant was collected after centrifugation for 10 min (450g, 4 °C). For total fraction, 54 ul of the supernatant was collected after centrifugation for 10 min (550 g, 4 °C). For cytosol fraction, 54 ul of the mitochondrial buffer was incubated on ice for 15 min, and centrifuged at 500 g for 10 min at 4 °C. The rest of supernatant was further centrifuged at 9500g for 10 min at 4 °C and then, the supernatant was collected for the cytosol fraction.

**Statistical analysis**

All data were presented as the mean ± SD. Sample sizes were calculated on the assumption that a 30% difference in the parameters measured would be considered biologically significant with an estimate of sigma of 10–20% of the expected mean. Alpha and Beta were set to the standard values of 0.05 and 0.8, respectively. No animals or samples were excluded from analysis, and animals were randomized to treatment versus control groups, where applicable. For relevant data analysis, where relevant, we first performed the Shapiro-Wilk normality test for checking normal distributions of the groups. If normally tests passed, two-tailed, unpaired Student’s t-test were used, but if normality tests failed, Mann-Whitney tests were used for the comparisons between two groups. For the comparisons of three or four groups, we used one-way ANOVA if normality tests passed, followed by Tukey’s multiple comparison test for all pairs of groups. If normality tests failed, Kruskal–Wallis test was performed and was followed by Dunn’s multiple comparison test. For the comparison of two proportions, we performed Fisher’s exact test. The GraphPad PRISM software (v.9.4.1, La Jolla, CA) and Microsoft Office Excel 2016 were used for statistical analysis.

**References**

1. Suomalainen, A. & Batterbsy, B. J. Mitochondrial diseases: the contribution of organelle stress responses to pathology. Nat. Rev. Mol. cell Biol. 19, 77–92 (2018).
2. Trifunovic, A. et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429, 417–423 (2004).
3. Kojot, G. C. et al. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science 309, 481–484 (2005).
4. Chinnery, P. & Turnbull, D. Mitochondrial DNA and disease. Lancet 354, 517–521 (1999).
5. Vercellino, I. & Sazanov, L.A. The assembly, regulation and function of the mitochondrial respiratory chain. Nat. Rev. Mol. Cell Biol. 1–21 (2021).
6. Guerrero-Castillo, S. et al. The assembly pathway of mitochondrial respiratory chain complex I. Cell Metab. 25, 128–139 (2017).
7. Hirst, J. Mitochondrial complex I. Annu. Rev. Biochem. 82, 551–575 (2013).
8. Jones, A. J., Blaza, J. N., Varghese, F. & Hirst, J. Respiratory complex I in Bos taurus and Paracoccus denitrificans pumps four protons across the membrane for every NADH oxidized. J. Biol. Chem. 292, 4987–4995 (2017).
9. Martin, M. A. et al. Leigh syndrome associated with mitochondrial complex I deficiency due to a novel mutation in the NDUF51 gene. Arch. Neurol. 62, 659–661 (2005).
10. Tripels, R., Van Den Heuvel, L., Tribjels, J. & Smeitink, J. Respiratory chain complex I deficiency. Am. J. Med. Genet. 106, 37–45 (2001).
11. Karamanlidis, G. et al. Mitochondrial complex I deficiency increases protein acetylation and accelerates heart failure. Cell Metab. 18, 239–250 (2013).
12. Kruse, S. E. et al. Mice with mitochondrial complex I deficiency develop a fat enchophalomyopathy. Cell Metab. 7, 312–320 (2008).
13. Koopman, W. J., Willems, P. H. & Smeitink, J. A. Monogenic mitochondrial disorders. N. Engl. J. Med. 366, 1132–1141 (2012).
14. Gorman, G. S. et al. Mitochondrial diseases. Nat. Rev. Dis. Prim. 2, 1–22 (2016).
15. Manolagas, S. C. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocr. Rev. 31, 266–300 (2010).
16. Gandhi, S. S., Murareksu, C., McCormick, E. M., Falk, M. J. & McCormack, S. E. Risk factors for poor bone health in primary mitochondrial disease. J. Inherit. Metab. Dis. 40, 673–683 (2017).
17. Yang, S., Feskanich, D., Williet, W. C., Ellisena, A. H. & Wu, T. Association between global biomarkers of oxidative stress and hip fracture in postmenopausal women: a prospective study. J. Bone Miner. Res. 29, 2577–2583 (2014).
18. Langdahl, H. J. et al. Mitochondrial point mutation m.3243a>g associates with lower bone mineral density, thinner cortices, and reduced bone strength: a case-control study. J. Bone Miner. Res. 32, 2041–2048 (2017).
19. Limnane, A., Ozawa, T., Maruzki, S. & Tankha, M. Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases. Lancet 333, 642–645 (1989).
20. Tzoufi, M. et al. A rare case report of simultaneous presentation of myopathy, Addison’s disease, primary hypoparathyroidism, and Fanconi syndrome in a child diagnosed with Kears–Sayre syndrome. Eur. J. Pediatrics 172, 557–561 (2013).
21. Cholley, F. et al. Mitochondrial respiratory chain deficiency revealed by hypothermia. *Neuropediatrics* **32**, 104–106 (2001).
22. Flanagan, B. & Nichols, G. Jr Metabolic studies of bone in vitro: v. glucose metabolism and collagen biosynthesis. *J. Biol. Chem.* **239**, 1261–1265 (1964).
23. Vander Helden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029–1033 (2009).
24. Lee, W.-C., Guntur, A. R., Long, F. & Rosen, C. J. Energy metabolism of the osteoblast: implications for osteoporosis. *Endocrin. Rev.* **38**, 255–266 (2017).
25. Guntur, A. R., Le, P. T., Farber, C. R. & Rosen, C. J. Bioenergetics during calvarial osteoblast differentiation reflect strain differences in bone mass. *Endocrinology* **155**, 1589–1595 (2014).
26. Esen, E. et al. WNT-LRP5 signaling induces Warburg effect through mTORC2 activation during osteoblast differentiation. *Cell Metab.* **17**, 745–755 (2013).
27. Stegen, S. et al. HIF-1α promotes glutamine-mediated redox homeostasis and glycogen-dependent bioenergetics to support postimplantation bone cell survival. *Cell Metab.* **23**, 265–279 (2016).
28. Lee, S.-Y., Abel, E. D. & Long, F. Glucose metabolism induced by Bmp signaling is essential for murine skeletal development. *Nat. Commun.* **9**, 1–11 (2018).
29. Wang, M. et al. Glutamine metabolism regulates proliferation and lineage allocation in skeletal stem cells. *Cell Metab.* **29**, 966–976.e964 (2019).
30. Heide, H. et al. Complexome profiling identifies TMEM126B as a component of the mitochondrial complex I assembly complex. *Cell Metab.* **16**, 538–549 (2012).
31. Formosa, L. E. et al. Dissecting the roles of mitochondrial complex I intermediate assembly complex factors in the biogenesis of complex I. *Cell Rep.* **31**, 107541 (2020).
32. Vogel, R. O. et al. Cytosolic signaling protein Ecsit also localizes to mitochondria where it interacts with chaperone NDUFA1 and functions in complex I assembly. *Genes Dev.* **21**, 615–624 (2007).
33. Nouw, J. et al. Acyl-CoA dehydrogenase 9 is required for the biogenesis of oxidative phosphorylation complex I. *Cell Metab.* **12**, 283–294 (2010).
34. de Lageneste, O. D. et al. Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin. *Nat. Commun.* **9**, 1–15 (2018).
35. Kawanami, A., Matsushita, T., Chan, Y. Y. & Murakami, S. Mice expressing GFP and CreER in osteochondro progenitor cells in the periosteum. *Biochemical biophysical Res. Commun.* **386**, 477–482 (2009).
36. Moore, E. R., Yang, Y. & Jacobs, C. R. Primary cilia are necessary for Prrx1-expressing cells to contribute to postnatal skeletogenesis. *J. cell Sci.* **131**, jcs217828 (2018).
37. Chan, C. F. et al. Identification and specification of the mouse skeletal stem cell. *Cell* **160**, 285–298 (2015).
38. Kopp, E. et al. Ecsit is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. *Genes Dev.* **13**, 2059–2071 (1999).
39. Wi, S. M. et al. TAK1-ECSIT-TRAF6 complex plays a key role in the TLR4 signal to activate NF-kappaB. *J. Biol. Chem.* **289**, 35205–35214 (2014).
40. Fiorese, C. J. et al. The transcription factor ATF5 mediates a mammalian mitochondrial UPR. *Curr. Biol.* **26**, 2037–2043 (2016).
41. Narugund, A. M., Fiorese, C. J., Pellegrino, M. W., Deng, P. & Haynes, C. M. Mitochondrial and nuclear accumulation of the transcription factor ATFS-1 promotes OXPHOS recovery during the UPRmt. *Mol. Cell* **58**, 123–133 (2015).
42. Kang, B. H. et al. Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. *Cell* **131**, 257–270 (2007).
43. Taipale, M., Jarosz, D. F. & Lindquist, S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat. Rev. Mol. Cell Biol.* **11**, 515–528 (2010).
44. Ashrafi, G. & Schwarz, T. The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death Differ.* **20**, 31–42 (2013).
45. Wei, J. et al. Glucose uptake and Runx2 synergize to orchestrate osteoblast differentiation and bone formation. *Cell* **161**, 1576–1591 (2015).
46. Carneiro, F. R. G., Lepelley, A., Seeley, J. J., Hayden, M. S. & Ghosh, S. An essential role for ecsit in mitochondrial complex I assembly and mitophagy in macrophages. *Cell Rep.* **22**, 2654–2666 (2018).
47. Yang, Y.-S. et al. Bone-targeting AAV-mediated silencing of Sbnuri-3 prevents bone loss in osteoporosis. *Nat. Commun.* **10**, 1–13 (2019).
48. Romeo, S. G. et al. Endothelial proteolytic activity and interaction with non-resorbing osteoclasts mediate bone elongation. *Nat. Cell Biol.* **21**, 430–441 (2019).
49. Liu, L., Cheung, T. H., Charville, G. W. & Rando, T. A. Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. *Nat. Protoc.* **10**, 1612–1624 (2015).
50. Joe, A. W. et al. Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nat. Cell Biol.* **12**, 153–163 (2010).
51. Jones, T. I. et al. Facioscapulohumeral muscular dystrophy family studies of DUX4 expression: evidence for disease modifiers and a quantitative model of pathogenesis. *Hum. Mol. Genet.* **21**, 4419–4430 (2012).
52. Girardi, F. et al. TGFβ signaling curbs cell fusion and muscle regeneration. *Nat. Commun.* **12**, 1–16 (2021).
53. Melendez, J. et al. TGFβ signalling acts as a molecular brake of myoblast fusion. *Nat. Commun.* **12**, 1–11 (2021).
54. Ismaael, A. et al. Role of transforming growth factor-beta in skeletal muscle fibrosis: a review. *Int. J. Mol. Sci.* **20**, 2446 (2019).
55. Borle, A. B., Nichols, N. & Nichols, G. Jr Metabolic studies of bone in vitro: I. Normal bone. *J. Biol. Chem.* **235**, 1206–1210 (1960).
56. Cohn, D. V. & Forscher, B. K. Aerobic metabolism of glucose by bone. *J. Biol. Chem.* **237**, 615–616 (1962).
57. Peck, W. A., Birge, S. J. & Fedak, S. A. Bone cells: biochemical and biological studies after enzymatic isolation. *Science* **146**, 1476–1477 (1964).
58. Lee, W.-C., Ji, X., Nissim, I. & Long, F. Malic enzyme couples mitochondrial TCA cycle metabolism to aerobic glycolysis in osteoblasts. *Cell Rep.* **32**, 108108 (2020).
59. Peck, W. A., Birge, S. J. Jr & Brandt, J. Collagen synthesis by isolated bone cells: stimulation by ascorbic acid in vitro. *Biochimica et Biophysica Acta (BBA)-Nucleic Acids Protein Synth.* **142**, 512–525 (1967).
60. Gerstenfeld, L. C., Chipman, S. D., Glowacki, J. & Lian, J. B. Expression of differentiated function by mineralizing cultures of chicken osteoblasts. *Developmental Biol.* **122**, 49–60 (1987).
61. Martinez-Reyes, I. & Chandel, N. S. Mitochondrial TCA cycle metabolites control physiology and disease. *Nat. Commun.* **11**, 1–11 (2020).
62. Martinez-Reyes, I. et al. TCA cycle and mitochondrial membrane potential are necessary for diverse biological functions. *Mol. Cell* **61**, 199–209 (2016).
63. Kuzmin, D. A. et al. The clinical landscape for AAV gene therapies. *Nat. Rev. Drug Discov.* **20**, 173–174 (2021).
64. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593–605 (2007).
65. Xie, J. et al. Short DNA hairpins compromise recombinant adeno-associated virus genome homogeneity. *Mol. Ther.* **25**, 1363–1374 (2017).
66. McLeod, M. J. Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. Teratology 22, 299–301 (1980).

67. Li, R., Hu, K., Liu, H., Green, M. R. & Zhu, L. J. OneStopRNAseq; a web application for comprehensive and efficient analyses of RNA-Seq data. Genes 11, 1165 (2020).

68. Andrews, S. & Bioinformatics. B. FastQC: a quality control tool for high throughput sequence data, citeulike-article-id: 11583827. arXiv Prepr. arXiv 0906, 2747 (2010).

69. Ewels, P., Magnusson, M., Lundin, S. & Käller, M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32, 3047–3048 (2016).

70. Hartley, S. W. & Mullikin, J. C. QoRTs: a comprehensive toolset for quality control and data processing of RNA-Seq experiments. BMC Bioinforma. 16, 1–7 (2015).

71. Harrow, J. et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 22, 1760–1774 (2012).

72. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930 (2014).

73. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 1–21 (2014).

74. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA 102, 15545–15550 (2005).

75. Bonnarens, F. & Einhorn, T. A. Production of a standard closed fracture in laboratory animal bone. J. Orthop. Res. 2, 97–101 (1984).

Acknowledgements

We would like to thank many individuals who provided reagents and the Flow Core for FACS sorting (S10 grant 151000D028576). This project was supported by a NIH NIAMS grant: R21AR077557 and AAVAA Therapeutics. M.B.G. holds a Career Award for Medical Scientists from the Burroughs Wellcome Fund, NIH grant: R01AR075585, and a Pershing Square Sohn Cancer Research Alliance award.

Author contributions

C.L. designed, executed, and interpreted the experiments. J.X. and G.G. generated the AAV vectors. Q.Y. and D.G. performed mitochondrial and skeletal muscle experiments, respectively. Y.Y., Z.C., and A.A.J. performed histology and microCT. S.C. N.D. and Q.Q. performed whole transcriptome analysis. S.G. generated Ecsit-floxed mice. M.G., S.L., C.H., and C.P.E. interpreted the experiments and helped draft the manuscript. J.H.S. supervised the research and prepared the manuscript.

Competing interests

G.G. and J.H.S. are co-founders of AAVAA Therapeutics and hold equity of this company. G.G. is a scientific co-founder of Voyager Therapeutics and Aspa Therapeutics Inc., and an inventor on patents with potential royalties licensed to other biopharmaceutical companies, in which they hold equity. Other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-34694-8.

Correspondence and requests for materials should be addressed to Jae-Hyuck Shim.

Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permissions information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022