Vitamin D repletion ameliorates adipose tissue browning and muscle wasting in infantile nephropathic cystinosis-associated cachexia

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Abstract

Background Ctns−/− mice, a mouse model of infantile nephropathic cystinosis, exhibit hypermetabolism with adipose tissue browning and profound muscle wasting. Ctns−/− mice are 25(OH)D3 and 1,25(OH)2D3 insufficient. We investigated whether vitamin D repletion could ameliorate adipose tissue browning and muscle wasting in Ctns−/− mice.

Methods Twelve-month-old Ctns−/− mice and wild-type controls were treated with 25(OH)D3 and 1,25(OH)2D3 (75 μg/kg/day and 60 ng/kg/day, respectively) or an ethylene glycol vehicle for 6 weeks. Serum chemistry and parameters of energy homeostasis were measured. We quantitated total fat mass and studied expression of molecules regulating adipose tissue browning, energy metabolism, and inflammation. We measured lean mass content, skeletal muscle fibre size, in vivo muscle function (grip strength and rotarod activity), and expression of molecules regulating muscle metabolism. We also analysed the transcriptome of skeletal muscle in Ctns−/− mice using RNAseq.

Results Supplementation of 25(OH)D3 and 1,25(OH)2D3 normalized serum concentration of 25(OH)D3 and 1,25(OH)2D3 in Ctns−/− mice, respectively. Repletion of vitamin D partially or fully normalized food intake, weight gain, gain of fat, and lean mass, improved energy homeostasis, and attenuated perturbations of uncoupling proteins and adenosine triphosphate content in adipose tissue and muscle in Ctns−/− mice. Vitamin D repletion attenuated elevated expression of beige adipose cell biomarkers (UCP-1, CD137, Tmem26, and Tbx1) as well as aberrant expression of molecules implicated in adipose tissue browning (Cox2, Pgf2α, and NF-κB pathway) in inguinal white adipose tissue in Ctns−/− mice. Vitamin D repletion normalized skeletal muscle fibre size and improved in vivo muscle function in Ctns−/− mice. This was accompanied by correcting the increased muscle catabolic signalling (increased protein contents of IL-1β, IL-6, and TNF-α as well as an increased gene expression of Murf-2, atrogin-1, and myostatin) and promoting the decreased muscle regeneration and myogenesis process (decreased gene expression of Ifg1, Pax7, and MyoD) in skeletal muscles of Ctns−/− mice. Muscle RNAseq analysis revealed aberrant gene expression profiles associated with reduced muscle and neuron regeneration, increased energy metabolism, and fibrosis in Ctns−/− mice. Importantly, repletion of 25(OH)D3 and 1,25(OH)2D3 normalized the top 20 differentially expressed genes in Ctns−/− mice.

Conclusions We report the novel findings that correction of 25(OH)D3 and 1,25(OH)2D3 insufficiency reverses cachexia and may improve quality of life by restoring muscle function in an animal model of infantile nephropathic cystinosis. Mechanistically, vitamin D repletion attenuates adipose tissue browning and muscle wasting in Ctns−/− mice via multiple cellular and molecular mechanisms.

Keywords Infantile nephropathic cystinosis; Vitamin D insufficiency; Cachexia; Adipose tissue browning; Muscle wasting
Introduction

Cystinosis is caused by mutations of the CTNS gene (17p13) and is inherited as an autosomal recessive disease.\(^1\)\(^2\) It is a multisystem genetic disorder characterized by the accumulation of cystine in different tissues and organs. Infantile nephropathic cystinosis (INC) is the most common and severe form of cystinosis.\(^3\) Patients suffering from INC exhibit signs and symptoms of renal Fanconi syndrome and chronic kidney disease in early childhood.\(^4\) Metabolic abnormalities are common complications in patients with INC, which are associated with poor quality of life and mortality and for which there is no current therapy.\(^3\)\(^4\) In order to elucidate disease mechanisms, we previously characterized the metabolic phenotype in Ctns\(^{-/-}\) mice, a mouse model of INC. We showed that Ctns\(^{-/-}\) mice exhibited cachexia characterized by hypermetabolism, profound adipose tissue browning, and muscle wasting.\(^5\) Serum concentrations of 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) were significantly lower in patients with INC relative to normal subjects.\(^6\)-\(^8\) In addition to its classic function of maintaining calcium and phosphate homeostasis, vitamin D plays a very extensive role as a cell differentiating and anti-proliferative factor with actions in a variety of tissues, including renal, cardiovascular, immune systems, adipose tissue, and muscles.\(^9\)\(^10\) Vitamin D insufficiency has been implicated in a wide range of metabolic disorders.\(^11\)\(^12\) Vitamin D plays a significant role in adipogenesis and inflammation.\(^13\) Furthermore, vitamin D has been proposed to modulate muscle growth and muscle function.\(^14\)\(^15\) Vitamin D deficiency is associated with decreased muscle size and strength, reduced physical function, and increased falls in the elderly.\(^16\)-\(^16\) These deficits in muscle function can be improved by vitamin D supplementation.\(^16\) Serum 25(OH)D\(_3\) may exert both paracrine and autocrine effects via \(\alpha\) hydroxylation and vitamin D receptor (VDR).\(^14\) Low serum 25(OH)D\(_3\) values are associated with muscle weakness and increased risk of falls in patients on dialysis, independent of 1,25(OH)\(_2\)D\(_3\) values.\(^16\) Concentration of serum 25(OH)D\(_3\) exhibited significant seasonal variation, and deficiency of 25(OH)D\(_3\) is common in patients with chronic kidney disease and is associated with poor outcomes such as progression to end-stage kidney disease, infections, fracture rates, hospitalizations, and all-cause mortality.\(^17\)\(^18\) Apart from the classical VDR signalling pathway, other cytosol receptors such as annexin II and membrane-associated rapid response steroid-binding proteins could induce rapid effects of vitamin D in muscle.\(^19\)\(^20\)

In this study, we investigated the effects of vitamin D repletion in a mouse model of INC-associated cachexia, with particular focus on adipose tissue browning and muscle wasting.

Materials and methods

Study design

C57BL/6 Ctns\(^{-/-}\) mice were kindly provided by Professor Corinne Antignac.\(^21\) Wild-type (WT) C57BL/6 control mice were acquired from Jackson Lab. Only male mice were used for this study. Mice were treated with 25(OH)D\(_3\) (Sigma, Catalogue 739650-1ML), 1,25(OH)\(_2\)D\(_3\) (Sigma, Catalogue 740578-1ML), or ethylene glycol as a vehicle control using subcutaneous osmotic Alzet 2006 pump. The study period was 6 weeks. Mice were fed with LabDiet 5015 Diet (3.83 kcal/g, 3.3 IU/g vitamin D\(_3\)). Ctns\(^{-/-}\) mice and WT mice were treated with 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) (75 \(\mu\)g/kg/day and 60 ng/kg/day, respectively) or vehicle. Two sets of experiments were performed. In the first set, all animals were fed ad libitum. In the second set, the food intake was all kept the same (pair feeding) (details were provided in the Results section). Whole-body fat and lean mass of mice were determined by quantitative magnetic resonance analysis (EchoMRI-100\(^\text{TM}\), Echo Medical System). A grip strength meter (Model 47106, UGO Basile) and AccuRotor Rota Rod (model RRF/SP, AccuScan Instrument) were used to quantify forelimb grip strength and motor coordination in mice, respectively. Oxygen consumption (VO\(_2\)) and energy expenditure were measured in mice using Oxymax calorimetry (Columbus Instrument).\(^5\) The study protocol was in compliance with institutional guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee at the University of California, San Diego.

Serum and blood chemistry

Serum concentration of bicarbonate, Ca, Pi, and blood urea nitrogen was assessed using VetScan VS2 Comprehensive Diagnostic Profile (Abaxis, Catalogue 500-0038). Concentrations of serum creatinine were analysed by liquid chromatography with tandem mass spectrometry method.\(^22\) Concentration of serum 25(OH)D\(_3\), 1,25(OH)\(_2\)D\(_3\) and parathyroid hormone were also analysed (Supporting Information, Table S1).

Protein assay for adipose and muscle tissue

Adipose and muscle tissue was prepared in a homogenizer tube (USA Scientific, Catalogue 1420-9600) containing ceramic beads (Omni International, Catalogue 19-646) using a Bead Mill Homogenizer (Omni International). Protein concentration of tissue homogenate was assayed using Pierce
BAC Protein Assay Kit (Thermo Scientific, Catalogue 23227). Uncoupling protein (UCP) protein content as well as adenosine triphosphate (ATP) concentration in adipose tissue and muscle homogenates were assayed. Protein concentration of CD137, Tmem26, TxnR1, Cox2, Pgf2α, NF-κB phosphorylated Ser337 p50 and total NF-κB p50, NF-κB phosphorylated Ser536 p65 and total NF-κB p65, and Ikkα phosphorylated Thr23, toll-like receptor 2 (Tlr2), myeloid differentiation primary response 88 (MyD88), and TNF receptor-associated factor 6 (Tra6) in adipose tissue homogenates and protein concentration of IL-1β, IL-6, TNF-α, and collagen content in muscle homogenates were assayed (Supporting Information, Table S2).

Muscle fibre size and muscle collagen content

Fibre cross-sectional area of soleus muscles was measured. Excised soleus muscles were snap frozen in isopentane cooled by liquid nitrogen and stored at −80 °C for subsequent analysis. Muscle cross sections (10 μm thick) were taken from muscle midbelly. Sections were first treated with 1% bovine serum albumin and normal goat and mouse serum as blocking agents. Sections were incubated overnight with a polyclonal anti-laminin antibody (Sigma, dilution 1:1000) and then with the secondary antibody, Alexa Fluor 594 goat anti-rabbit immunoglobulin G (Invitrogen, dilution 1:200). The laminin antibody was used to label the fibre perimeter and facilitate semiautomated fibre area quantification. Sections were imaged with a microscope (Leica CTR 6500, Buffalo Grove) fit with a fluorescent camera (Leica DFC365 FX) set for 594 emission fluorescence using a 10× objective. Fibre cross-sectional areas were measured using a custom-written macro in ImageJ (National Institutes of Health). Filtering criteria were applied to ensure measurement of actual muscle fibres. These criteria rejected regions with areas below 50 μm² and above 5000 μm² to eliminate neurovascular structures and ‘optically fused’ fibres, respectively. Excised soleus muscles were hydrolyzed, and hydroxyproline content was calculated using a colorimetric assay.

Quantitative real-time PCR

Total adipose and muscle RNA was isolated using TRIzol (Life Technology) and further purified with Direct-zol RNA MiniPre Kit (Zymo Research). cDNA was synthesized using SuperScript III Reverse Transcriptase and oligo (dT)12-18 primer (Invitrogen). The PCR reaction based on SYBR (KAPA Biosystems, SYBR FAST Universal qPCR kit, Catalogue KK4602) was performed with a 7300 Real-Time PCR System (ABI Applied Biosystems) using the reaction procedure as follows: 95 °C for 1 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Appropriate primers for target genes are listed (Supporting Information, Table S2). The comparative 2−ΔΔCt method was used to determine the relative quantity of each target gene. Final results were expressed in arbitrary units, with one unit being the mean mRNA level in WT control mice.

Muscle RNAseq analysis

Total gastrocnemius muscle RNA was isolated in the experimental mice (three mice in each group) using TRIzol (Life Technology) followed by RNeasy mini kit (Qiagen) for further purification. The extracted muscle RNA samples were analysed using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) with RNA integrity number >8.5 and rRNA 28S/18S >1.0. Samples were used to construct cDNA libraries (Illumina) and sequenced through an Illumina HiSeq2000 platform at BGI Hong Kong (www.bgi.com). Approximately 5.88 GB of raw reads was generated for each of the samples. The raw RNAseq data were filtered into clean reads, followed by mapping to the mouse reference genome using HISAT. On average, 93.45% reads were mapped, and the uniformity of the mapping results for each sample suggested that the samples were comparable. The gene expression level for each sample was analysed using RSEM quantification tool. Based on the gene expression level, differentially expressed genes (DEG) between experimental groups were identified using DESeq2 algorithms. Biological function analysis of the DGE was enriched by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway. To identify pathways related to phenotypic differences of the muscle between Ctns−/− mice and WT control mice, DEG between these two groups of mice were analysed using the Ingenuity Pathway Analysis software (Ingenuity Systems, http://www.ingenuity.com).

Results

Vitamin D supplementation normalizes serum vitamin D concentrations in Ctns−/− mice

Ctns−/− mice, at the age of 12 months, were both 25(OH)D₃ and 1,25(OH)₂D₃ insufficient (Table 1). We show that 25(OH)D₃ (75 μg/kg/day for 6 weeks) or 1,25(OH)₂D₃ (60 ng/kg/day for 6 weeks) alone did not normalize serum 25(OH)D₃ and 1,25(OH)₂D₃ concentrations in Ctns−/− mice (Supporting Information, Table S3). However, supplementation of 25(OH)D₃ and 1,25(OH)₂D₃ (75 μg/kg/day and 60 ng/kg/day, respectively, for 6 weeks) normalized serum vitamin D concentrations in Ctns−/− mice (Table 1). Vitamin D repletion normalized serum concentration of calcium and phosphorus in Ctns−/− mice. Vitamin D repletion in Ctns−/− mice significantly decrease but did not completely normalize serum parathyroid hormone levels.

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With equal food intake in all groups, supplementation of the other three groups of mice were given an equivalent food, and detailed daily intake was recorded. Subsequently, Ctns phenotype in Ctns Vitamin D repletion improves the cachexia phenotype in Ctns mice.

Twelve-month-old, male, WT, and Ctns mice were supplemented with 25(OH)D3 and 1,25(OH)2D3 (75 μg/kg/day and 60 ng/kg/day, respectively) or ethylene glycol as vehicle for 6 weeks. Four groups of mice were included: WT + vehicle, WT + 25(OH)D3 + 1,25(OH)2D3, Ctns + vehicle, and Ctns + 1,25(OH)2D3. Ctns + vehicle mice were fed ad libitum, while other groups of mice were fed the same food intake as that of Ctns + vehicle mice. Data are expressed as mean ± standard error of the mean.

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<th>Table 1 Serum and blood chemistry of mice</th>
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BUN, blood urea nitrogen; PTH, parathyroid hormone; WT, wild-type.

Vitamin D repletion improves the cachexia phenotype in Ctns mice

Cachexia is defined by weight loss in adults. However, it is characterized by lack of adequate weight gain in growing children and mice. Similar to our previous published studies, Ctns mice exhibited the cachexia phenotype consisting of anorexia, reduced weight gain, abnormal body mass (manifested as reduced gain of lean and fat mass), and hypermetabolism (manifested as elevated energy expenditure). We studied the impact of vitamin D repletion on the cachexia phenotype. First, we fed all four groups of mice ad libitum. The food intake of Ctns mice supplemented with 25(OH)D3 and 1,25(OH)2D3 was significantly increased compared with Ctns mice and was not different from WT controls with or without 25(OH)D3 and 1,25(OH)2D3. The weight gain in the Ctns mice with 25(OH)D3 and 1,25(OH)2D3 was also increased compared with Ctns mice control but still less than the WT mice with or without 25(OH)D3 and 1,25(OH)2D3 (Supporting Information, Figure S1). We then repeated the experiments using a pair-feeding strategy. Ctns mice treated with vehicle were given an ad libitum amount of food, and detailed daily intake was recorded. Subsequently, the other three groups of mice were given an equivalent amount of food as vehicle treated Ctns mice (Figure 1).

With equal food intake in all groups, supplementation of 25(OH)D3 and 1,25(OH)2D3 normalized weight gain as well as lean and fat mass gains in the Ctns mice. Muscle function (grip strength and rotator activity), muscle fibre size (cross-sectional area), and collagen content were decreased in Ctns mice and were normalized following 25(OH)D3 and 1,25(OH)2D3 supplementation. Light-phase and dark-phase volume of oxygen consumption (VO2) as well as respective energy expenditure were significantly elevated in Ctns mice relative to WT mice and were normalized with 25(OH)D3 and 1,25(OH)2D3. Taken together, these results show that vitamin D supplementation corrects the cachexia phenotype in Ctns mice.

Vitamin D repletion normalized uncoupling protein content and adenosine triphosphate in Ctns mice

We measured adipose and muscle protein contents of UCPs and ATP in Ctns mice. Protein content of UCPs in adipose tissue [inguinal white adipose tissue (WAT) and intercapsular brown adipose tissue] and gastrocnemius muscle was elevated in Ctns mice relative to control mice (Figure 2). In contrast, ATP content in adipose tissue and muscle was significantly decreased in Ctns mice relative to control mice. Replication of 25(OH)D3 and 1,25(OH)2D3 normalized UCPs and ATP content in adipose tissue and muscle in Ctns mice relative to control mice.

Vitamin D repletion attenuates adipose tissue browning in Ctns mice

The mRNA and protein content of beige adipose cell surface markers (CD137, Tmem26, and Tbx1) were elevated in inguinal WAT in Ctns mice compared with control mice (Figure 3), and this is consistent with increased UCP-1 in WAT shown in Figure 2, which is a marker for beige adipocyte, and usually undetected in WAT. Collectively, our results demonstrate the presence of beige adipocytes in Ctns mice. Replication of 25(OH)D3 and 1,25(OH)2D3 attenuated browning of beige adipocytes in Ctns mice. Protein content of UCP-1 and
mRNA and protein content of CD137, Tmem26, and Tbx1 were normalized in Ctns−/− mice treated with 25(OH)D₃ and 1,25(OH)₂D₃ relative to control mice (Figures 2 and 3). In addition, repletion of 25(OH)D₃ and 1,25(OH)₂D₃ ameliorated aberrant levels of key molecules implicated in adipose tissue browning in Ctns−/− mice. Inguinal WAT of Ctns−/− mice displayed higher inguinal WAT mRNA expression and protein content of Cox2 and Pgf2α than that of controls (Figure 4). Inflammation has been implicated in the pathogenesis of adipose tissue browning. One of the most important inflammatory pathways in mammalian cells is that of the NF-κB pathway, and therefore, we studied expression of transcription factors involved in pro-inflammatory pathways in Ctns−/− mice. Inguinal WAT NF-κB p50 (phosphorylated Ser337)/total NF-κB p50 ratio, NF-κB p65 (phosphorylated S536)/total NF-κB p65 ratio, protein content of Iκκ-α (phosphorylated Thr23), mRNA expression, and protein content of Tlr2, MyD88, and Traf6 were up-regulated in Ctns−/− mice relative to control mice. Repletion of 25(OH)D₃ and 1,25(OH)₂D₃ partially or fully normalized mRNA expression and protein content of the entire Tlr/NF-κB pathway in Ctns−/− mice relative to control mice (Figure 4).

**Vitamin D repletion attenuates aberrant muscle mass signalling pathway in Ctns−/− mice**

Muscle catabolic signalling (muscle lysate protein levels of inflammatory cytokines IL-1β, IL-6, and TNF-α and expression of muscle proteolytic genes, Murf-1, atrogin-1, and myostatin) was significantly increased in Ctns−/− mice than that in controls (Figure 5). In contrast, gene expression for myogenensis and skeletal muscle regeneration (Igf1, Pax7, and MyoD) was decreased in gastrocnemius muscle of Ctns−/− mice relative to controls. Repletion of 25(OH)D₃ and 1,25(OH)₂D₃ was able to increase skeletal muscle cell proliferation and muscle protein synthesis, and reduce muscle proteolysis in muscle of Ctns−/− mice. These results indicate that repletion of vitamin D can attenuate skeletal muscle atrophy in Ctns−/− mice.
Figure 2  Vitamin D repletion ameliorates uncoupling protein content and adenosine triphosphate in Ctns<sup>−/−</sup> mice. UCP and ATP content in inguinal white adipose tissue, brown adipose tissue, and gastrocnemius muscle were measured. Results are analysed and expressed as in Figure 1. BAT, brown adipose tissue; WAT, white adipose tissue; WT, wild-type.

normalized aberrant levels of key molecules implicated in muscle wasting pathways in Ctns<sup>−/−</sup> mice.

**Differentially expressed genes in muscle from Ctns<sup>−/−</sup> mice**

To characterize muscle transcriptome changes associated with vitamin D repletion in Ctns<sup>−/−</sup> mice, we performed RNAseq analysis on the gastrocnemius muscle. We identified ~17 000 genes in all experimental groups of mice. We compared DEG in muscle between Ctns<sup>−/−</sup> mice and WT mice. We identified 214 genes up-regulated and 76 genes down-regulated in Ctns<sup>−/−</sup> mice relative to WT mice (Figure 6). Completed information for those DEG were listed (Supporting Information, Table S4). These DEG are displayed in the heatmap by hierarchical clustering to illustrate the high degree of reproducibility in individual samples of the same experimental groups of mice. The DEG data were further analysed using the GO pathway through Kyoto Encyclopedia of Genes and Genomes. Detailed GO of biological process and molecular function in Ctns<sup>−/−</sup> mice vs. WT mice revealed significant differential expression of genes implicated in cellular processes, biological regulation and processes, and metabolic processes. Similarly, we also identified gastrocnemius muscle DEG in Ctns<sup>−/−</sup> + 25(OH)D<sub>3</sub> + 1,25(OH)<sub>2</sub>D<sub>3</sub> mouse vs. WT + 25(OH)D<sub>3</sub> + 1,25(OH)<sub>2</sub>D<sub>3</sub> mice as well as Ctns<sup>−/−</sup> + 25(OH)D<sub>3</sub> + 1,25(OH)<sub>2</sub>D<sub>3</sub> mice vs. Ctns<sup>−/−</sup> + vehicle mice. Results are shown (Figure 6). Completed information for those DEG were listed (Supporting Information, Tables S5 and S6).

**Canonical signalling pathways analysis in muscle from Ctns<sup>−/−</sup> mice**

We performed Ingenuity Pathway Analysis enrichment tests for DEG in muscle from Ctns<sup>−/−</sup> mice vs. WT mice. We particularly focused on pathways related to energy metabolism, skeletal and muscular system development and function, and organismal injury and abnormalities. Results are shown in Figure 7. Up-regulated genes include ANKR2D, CSRP3, CYFIP2, FHL1, LY6A, MUP1, MYL2, MYL3, PKD4, SELL, SLN,
SPP1, TNNC1, TNNI1, and TPM3, whereas down-regulated genes are ATF3, CIDEA, FOS, SNCG, S and TBC1D1 in Ctns\(^{-/-}\) mice relative to WT control mice. Functional significance of those DEG is listed (Table 2). Taken together, gene expression associated with regeneration of muscle and neurons is compromised in Ctns\(^{-/-}\) mice. Furthermore, expression of genes related to enhanced muscle thermogenesis and fibrosis is increased in Ctns\(^{-/-}\) mice. Importantly, repletion of 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) normalized the top 20 DEG as listed in Table 2 in Ctns\(^{-/-}\) mice.

**Discussion**

We previously described cachexia characterized by adipose tissue browning and muscle wasting in Ctns\(^{-/-}\) mice, an established mouse model of INC, but the aetiology was not clear. This study showed that Ctns\(^{-/-}\) mice, at 12 months of age, were both 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) insufficient. We show that vitamin D supplementation corrects the cachexia phenotype in Ctns\(^{-/-}\) mice. Importantly, vitamin D repletion attenuates adipose tissue browning and skeletal muscle wasting in Ctns\(^{-/-}\) mice. These findings are novel.

Muscle wasting is a life-threatening complication in patients with cystinosis. Muscle wasting is prevalent in patients with INC and may be associated with significant morbidity and mortality. In post-transplant cystinotic patients who did not receive long-term cystine-depleting therapy, cystinotic myopathy have been associated with swallowing myopathy and, as a result, aspiration pneumonia, which constitutes a severe and potentially lethal complication. Vitamin D insufficiency has been associated with muscle wasting and impaired muscle strength, and low vitamin D suppresses skeletal muscle tropism and contraction. Therefore, we studied the effect of vitamin D repletion on muscle fibre histomorphometry in Ctns\(^{-/-}\) mice. We showed that 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) repletion normalized mean soleus muscle cross-sectional area in Ctns\(^{-/-}\) mice (Figure 1).

Skeletal muscle fibrosis, a major pathological hallmark of myopathies, is evident in Ctns\(^{-/-}\) mice. Repletion of vitamin D normalized soleus collagen content in Ctns\(^{-/-}\) mice. Our results were consistent with a recent report in which vitamin D treatment significantly increased cross-sectional size of cultured mouse myoblast C2C12 cells. Vitamin D reduced the expression of collagen (collagen I, III, and other collagen informs) and key profibrotic factors (TGF-β1 and plasminogen activator inhibitor) in mesenchymal multipotent cells.

Adipose tissues and muscle are important in energy metabolism. UCP1 contributes to adaptive thermogenesis, while UCP2 and UCP3 are involved in the resting metabolic rate.
We, and others, have previously described the increased thermogenesis and up-regulation of UCPs in adipose tissues and muscle in rodent models of cachexia.\textsuperscript{35–39} Up-regulation of UCP expression promotes protein leak and reduces ATP production in exchange for the generation of heat.\textsuperscript{34,38} Protein contents of UCPs were increased in adipose tissues and muscle of Ctns\textsuperscript{-/-} mice compared with controls (Figure 2). In contrast, ATP contents in adipose tissues and muscle were decreased in Ctns\textsuperscript{-/-} mice relative to controls. Vitamin D insufficiency exacerbates adipose tissue and muscle metabolism. \textsuperscript{25} 25(OH)D\textsubscript{3} and 1,25(OH)\textsubscript{2}D\textsubscript{3} repletion attenuated perturbations of UCPs and ATP contents in adipose tissues and muscle in Ctns\textsuperscript{-/-} mice. 1,25(OH)\textsubscript{2}D\textsubscript{3} suppresses UCPs expression in primary brown adipocyte and suppresses differentiation and mitochondrial respiration of brown adipocyte\textsuperscript{39,40} 1,25(OH)\textsubscript{2}D\textsubscript{3}/VDR by binding to the promoter region of the UCP3 gene and modulating UCP3 gene transcription and subsequent energy metabolism in muscle.\textsuperscript{41}
Adipose tissue browning is an important cause of hypermetabolism in patients with chronic cachectic disorders. Recent studies revealed the detrimental effects of adipose tissue browning in the context of cachexia. Adipose tissue browning and its associated increases in energy expenditure precedes skeletal muscle atrophy and is important for the development and progression of cachexia. We demonstrated the presence of brown adipose tissue marker UCP1 protein as well as an increased mRNA expression and protein content of unique beige adipose cell markers (CD137, Tmem26, and Tbx1) in inguinal WAT of Ctns⁻/⁻ mice (Figures 2 and 3). Importantly, repletion of 25(OH)D₃ and 1,25(OH)₂D₃ attenuated expression of beige adipose cell markers in inguinal WAT of Ctns⁻/⁻ mice. Several mechanisms are important for biogenesis of WAT browning, including Cox2 signalling pathway and chronic inflammation. Activation of Cox2, a downstream effector of β-adrenergic signalling, is crucial for the induction of beige cells in WAT depots. Cox2 produces prostaglandins that enhance mitochondrial biogenesis and increase the uncoupling capacity when activated with adrenergics. We showed that 25(OH)D₃ and 1,25(OH)₂D₃ repletion normalized elevated inguinal WAT gene expression and protein content of Cox2 and Pgf2α in Ctns⁻/⁻ mice (Figure 4). In addition, increased expression of key molecules implicated in the pro-inflammatory pathways in inguinal WAT (NF-κB p50 and p65, Iκκ-α, Tlr2, MyD88, and Traf6) was normalized in Ctns⁻/⁻ mice treated with 25(OH)D₃ and 1,25(OH)₂D₃ relative to control mice. We investigated the effect of vitamin D repletion on muscle mass regulatory signalling pathways in Ctns⁻/⁻ mice. Repletion of 25(OH)D₃ and 1,25(OH)₂D₃ normalized molecular signatures of processes associated with muscle wasting, including increased expression of pro-inflammatory muscle cytokines (IL-1β, IL-6, and TNF-α) and muscle proteolysis (Murf-1, atrogin-1, and myostatin) as well as decreased gene expression associated with myogenesis and skeletal muscle regeneration (Igf-1, Pax7, and MyoD) in skeletal muscle of Ctns⁻/⁻ mice relative to controls (Figure 5). Elevation of pro-inflammatory cytokines has been implicated in many forms of muscle wasting including chronic kidney disease and age-related sarcopenia. We acknowledge that increased

**Figure 5** Vitamin D repletion corrects aberrant muscle mass signalling pathways in Ctns⁻/⁻ mice. Protein concentrations of inflammatory cytokine (IL-1β, IL-6, and TNF-α) and mRNA levels of key molecules associated with myogenesis (Murf-1, atrogin-1, and myostatin) and skeletal muscle regeneration (IGF-1, Pax7, and MyoD) in gastrocnemius muscle were shown. Results are analysed and expressed as in Figure 1. WT, wild-type.
pro-inflammatory cytokines may not be casually related to muscle wasting in Ctns−/− mice. Vitamin D administration has been associated with reduced concentration of serum TNF-α in renal patients. Epidemiological studies suggest an inverse association between serum 25(OH)D3 and inflammatory markers, including C-reactive protein and IL-6. Supplemental vitamin D decreased levels of inflammatory biomarkers. Vitamin D inhibited lipopolysaccharide-induced IL-6 and TNF-α production in a dose-dependent manner in monocytes by inhibiting the activation of NF-κB. Vitamin D also inhibits T-lymphocyte proliferation and the production of pro-inflammatory cytokines. Interestingly, T-cell cytokines modulate vitamin D metabolism in macrophages. IFN-γ, a Th1 cytokine, activates the macrophage CYP27B1, leading to the enhanced conversion of 25(OH)D3 to 1,25(OH)2D3. In contrast, the Th2 cytokine IL-4 induces catabolism of 25(OH)D3 to the inactive metabolite 24,25(OH)2D3, suggesting a potential mechanism by which vitamin D metabolism links the cell-mediated immune responses to the immune responses.

Vitamin D deficiency decreased muscle Pax7 expression while increased the expression of MuRF-1 and atrogin-1 in the gastrocnemius muscle of diabetic mice. Combined treatment of vitamin D and low-intensity aerobic exercise attenuated osteopenia and muscle atrophy by enhancing muscle anabolic markers (Pax7, MyoD, and myogenin) and decreasing expression of catabolic markers (atrogin-1, MuRF-1, and REDD1) in diabetic rats. 1,25(OH)2D3 promotes cultured skeletal muscle differentiation by promoting the expression of myogenic markers and myotube formation. Myostatin is a TGF-β family member that acts as a negative regulator of skeletal muscle mass. Follistatin, a myostatin-binding protein, inhibits myostatin activity and promotes muscle growth.
follistatin expression and decreasing the expression of myostatin in C2C12 skeletal muscle cells.\textsuperscript{58} Results of a recent investigation showed that skeletal muscle-derived satellite cells from C57BL/6J mice expressed VDR and its expression, and nuclear translocation was induced upon incubating the cells with 1,25(OH)\textsubscript{2}D\textsubscript{3}. Furthermore, incubation of 1,25(OH)\textsubscript{2}D\textsubscript{3} enhanced myogenic differentiation in satellite cells by promoting the expression of myogenic markers (MYH1, TNNI1, TNNI3, and MMP9), marker of myotube formation (BMP4), and marker of myogenic cell migration and engraftment (MMP9) and by modulating myogenic growth factors (IGF-1, IGF-2, FGF2, and FGF2). Additional pro-myogenic effects of vitamin D on satellite cells were further demonstrated by the increased expression of myogenic markers including MyoD, myogenin, and follistatin while inhibiting the expression of myostatin.\textsuperscript{59} Vitamin D deficiency has been associated with muscle wasting in diabetic mice.

We performed RNAseq analysis on muscle in Ctns\textsuperscript{−/−} mice. Our data demonstrate clear differentiation in muscle transcriptomics in Ctns\textsuperscript{−/−} mice relative to control WT mice (Figures 6 and 7 and Supporting Information, Table S3). We identified muscle DEG associated with reduced regeneration of muscle and neurons as well as elevated energy metabolism and fibrosis in Ctns\textsuperscript{−/−} mice (Table 2). Elevated muscle ANKRD2, CSRP3, MYL2, MYL3, PDK4, SELL, SLN, SPP1, TNNC1, TNNI1, and TPM3, and decreased expression of genes are ATF3, CIDEA, FOS, SNCG, and TBC1D1.
has been shown to promote cancer cachexia.\textsuperscript{75} Up-regulation of PDK4 expression is a biomarker for muscle energy deprivation.\textsuperscript{76} Increased expression of MYL3 has been used as a biomarker for muscle injury.\textsuperscript{77} In addition, increased expression of muscle FHL1 and TPM3 promotes muscle atrophy and muscle weakness.\textsuperscript{78,79} Importantly, repletion of 25(OH)D\textsubscript{3} and 1,25(OH)\textsubscript{2}D\textsubscript{3} normalized the top 20 DEG as listed in Table 2 in Ctns\textsuperscript{-/-} mice.

### Conclusion

We report the novel findings that correction of 25(OH)D\textsubscript{3} and 1,25(OH)\textsubscript{2}D\textsubscript{3} insufficiency reverses cachexia and may improve quality of life by restoring muscle function in an animal model of INC. Mechanistically, vitamin D repletion attenuates adipose tissue browning and muscle wasting in Ctns\textsuperscript{-/-} mice via multiple cellular and molecular mechanisms.

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### Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Immunoassay information for serum chemistry, adipose and muscle tissue homogenate assay analysis.

**Table S2.** PCR primer information.

**Table S3.** Serum and blood chemistry of mice. Twelve-month-old, male, wild type (WT) and Ctns/-/- mice were treated with 25(OH)D\textsubscript{3} (75 μg/kg per day) or 1,25(OH)\textsubscript{2}D\textsubscript{3} (60 ng/kg per day) versus ethylene glycol as vehicle for 6 weeks. Six groups
of mice were included: WT + Vehicle, WT + 25(OH)D3, WT + 1,25(OH)2D3, Ctns-/- + Vehicle, Ctns-/- + 25(OH)D3, Ctns-/- + 1,25(OH)2D3. All mice were fed ad libitum. Data are expressed as mean SEM. Ap < 0.05, significantly higher in Ctns-/- + Vehicle, Ctns-/- + 25(OH)D3 or Ctns-/- + 1,25(OH)2D3 versus WT + Vehicle, WT + 25(OH)D3 or WT + 1,25(OH)2D3, respectively. Bp < 0.05, significantly lower in Ctns-/- + Vehicle, Ctns-/- + 25(OH)D3 or Ctns-/- + 1,25(OH)2D3 versus WT + Vehicle, WT + 25(OH)D3 or WT + 1,25(OH)2D3, respectively. cp < 0.05, significantly difference between Ctns-/- + 25(OH)D3 versus Ctns-/- + Vehicle or Ctns-/- + 1,25(OH)2D3 versus Ctns-/- + Vehicle.

**Table S4.** List of differential expressed genes in gastrocnemius muscle from Ctns-/- + Vehicle versus WT + Vehicle mice

**Table S5.** List of differential expressed genes in gastrocnemius muscle from Ctns-/- + 25(OH)D3 + 1,25(OH)2D3 versus WT + 25(OH)D3 + 1,25(OH)2D3 mice

**Figure S1.** Ad libitum food intake and weight gain in Ctns-/- mice.

**Conflicts of interest**

None declared.

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