PHD1 controls muscle mTORC1 in a hydroxylation-independent manner by stabilizing leucyl tRNA synthetase

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mTORC1 is an important regulator of muscle mass but how it is modulated by oxygen and nutrients is not completely understood. We show that loss of the prolyl hydroxylase domain isoform 1 oxygen sensor in mice (PHD1KO) reduces muscle mass. PHD1KO muscles show impaired mTORC1 activation in response to leucine whereas mTORC1 activation by growth factors or eccentric contractions was preserved. The ability of PHD1 to promote mTORC1 activity is independent of its hydroxylation activity but is caused by decreased protein content of the leucyl tRNA synthetase (LRS) leucine sensor. Mechanistically, PHD1 interacts with and stabilizes LRS. This interaction is promoted during oxygen and amino acid depletion and protects LRS from degradation. Finally, elderly subjects have lower PHD1 levels and LRS activity in muscle from aged versus young human subjects. In conclusion, PHD1 ensures an optimal mTORC1 response to leucine after episodes of metabolic scarcity.

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Skeletal muscle is essential to life as it provides mechanical power for movement and at the same time plays a crucial role in whole body metabolism. From the age of 50, skeletal muscle mass is lost at a rate of 1–2% per year, resulting in diminished functional strength that correlates with lower overall quality of life and increased mortality. Muscle is also critical component of diseases such as chronic obstructive pulmonary disorder, diabetes, cancer, anaemia, and sepsis. The clinical and financial burden of loss of muscle mass to society is enormous, and it is therefore vital to develop strategies to prevent loss of muscle mass, or to maintain and even increase muscle mass. To do so, we need to improve our understanding of the underlying molecular mechanisms that control skeletal muscle mass.

Skeletal muscle mass is defined by a fine balance between protein synthesis and breakdown, processes which are governed by mechanistic target of rapamycin complex 1 (mTORC1), a master regulator of cellular metabolism. Growth factors, energetic stress, and contractions control mTORC1 directly or via its upstream inhibitor tuberous sclerosis complex 1/2 (TSC1/2). Amino acids regulate mTORC1 via alternative pathways involving Rag GTPases, which recruit mTORC1 to the lysosomal membrane. How TSC2-dependent signals regulate mTORC1 in skeletal muscle has been intensely studied, but how fluctuations of amino acids, and in particular the essential amino acid leucine, regulate mTORC1, and thus skeletal muscle mass in vivo is much less well understood.

Over the last years, intense research efforts have led to the discovery of leucine sensors, such as SESTRINs (SESNs) and leucyl-tRNA synthetase (LRS), which transmit intracellular leucine availability toward mTORC1. Growth factors, energetic stress, and contractions control mTORC1 directly or via its upstream inhibitor tuberous sclerosis complex 1/2 (TSC1/2). Amino acids regulate mTORC1 via alternative pathways involving Rag GTPases, which recruit mTORC1 to the lysosomal membrane. How TSC2-dependent signals regulate mTORC1 in skeletal muscle has been intensely studied, but how fluctuations of amino acids, and in particular the essential amino acid leucine, regulate mTORC1, and thus skeletal muscle mass in vivo is much less well understood.

De novo protein synthesis is a highly energy consuming process, while the energy cost of protein breakdown has been estimated to be much lower to even neglectable. Protein synthesis and mTORC1 activity therefore need to be tightly controlled upon metabolic stress such as hypoxia and nutrient deprivation, to ensure cell survival. Dependent on the specific context, hypoxia either strongly inhibits or promotes mTORC1 activity. Yet, the cellular machinery that ensures cell survival and orchestrates a fast and efficient cellular recovery upon restoration of oxygen and nutrient supply needs to be maintained. An excellent set of candidates to orchestrate such adaptive responses are proxyl-hydroxylase domain proteins 1–3 (PHD1–3). PHDs belong to a family of enzymes which use oxygen, Fe2+, ascorbic acid, and the TCA intermediate α-ketoglutarate (αKG) to hydroxylate proline residues in their target substrate proteins, the best characterized ones being the hypoxia-inducible factors 1–3α (HIF1–3α). During hypoxia, HIF1α inhibits mTORC1 by activating the transcription of its downstream target regulating in development and DNA damage response 1 (REDD1) which has been shown to activate TSC2 and eIF2α. On the other hand, HIF2α increases mTORC1 activity under low amino acid availability by increasing the expression of the LAT1 amino acid carrier.

Besides controlling HIF stability, PHDs can also change the activity and stability of other proteins, either or not in a hydroxylation-dependent fashion. But whether PHDs can directly control protein synthesis, is not known.

Results
Phd1-deficient mice have lower muscle mass
To study the role of PHD1 in mTORC1 activation and muscle mass control in vivo we used PHD1 KO mice (50% Swiss/50% 129S1). Both males (Fig. 1a) and females (Supplementary Fig. 1a) showed lower mass of both m. gastrocnemius (GAS), m. tibialis anterior (TA) and m. extensor digitorum longus (EDL). Magnetic resonance imaging (MRI) analysis confirmed that PHD1 KO mice have lower lean mass when compared with the corresponding controls (Fig. 1b). The reduction in lean mass resulted into lower body weight in males but not in females (Fig. 1d and Supplementary Fig. 1d), where the reduction in lean mass was completely compensated by an increase in fat mass (Supplementary Fig. 1c). This data confirms previous work reporting increased white adipose tissue mass in PHD1 KO mice. Analysis of fiber area in TA showed that lower muscle weight was accompanied with decreased fiber cross-sectional area (Fig. 1e). Differences in fiber cross-sectional area were not secondary to a shift in muscle fiber type composition (Supplementary Fig. 1e). We also did not find evidence for overt myopathy, indicated by the absence of centrally nucleated fibers (Supplementary Fig. 1f). Absolute force–frequency analysis of ex vivo contracted soleus showed reduced force production in PHD1 KO compared to WT mice (Fig. 1f). Relative force–frequency, which is corrected for muscle surface area, was unaffected (Supplementary Fig. 1h), further confirming that the lower force production in these muscles was caused by lower fiber area and likely not by defective intrinsic mechanical capacities.

Since muscle fiber size is determined by the balance between protein synthesis and protein breakdown, we first monitored the status of the ubiquitin-proteasome and autophagy-lysosome systems, the two main contributors to muscle protein breakdown. RT-qPCR analysis failed to show increased expression of the ubiquitin-proteasome related genes Atrogin-1, Murf1, Itch, Smart, Musa1, and Pbx031 (Fig. 1g). mRNA levels of autophagy related genes such as P62, LC3b, GabarapL, Bnip3, and CathL were also not affected by loss of Phd1 (Supplementary Fig. 1g). Accordingly, expression of microtubule-associated protein 1 light chain 3 (LC3-I) and lipidated LC3 (LC3-II) was not different between PHD1 KO compared to WT muscles which were harvested after 4 h of food withdrawal (Fig. 1h), neither did we find differences in P62 protein content, a marker for autophagy impairment (Fig. 1h). This data indicates that loss of Phd1 does not substantially promote muscle protein breakdown and prompted us to evaluate whether PHD1 controls muscle protein synthesis.
Fig. 1 Phd1-deficient mice have lower muscle mass. Bar graphs showing muscle weight (a), lean mass (b), fat mass (c), and body weight (d) of WT (white bars) and PHD1KO (red bars) male mice. e Quantification (left panel) and representative pictures (right panel) of fiber cross-sectional area distribution in WT (black line) and PHD1KO (red line) TA muscle. f Force–frequency curve in ex vivo stimulated soleus from WT (black line) and PHD1KO (red line) male mice. g mRNA expression levels of genes involved in ubiquitin-proteasome mediated protein degradation in TA muscle from WT (white bars) and PHD1KO (red bars) female mice. h Representative pictures and quantification of western blot analysis of LC3B and P62 protein levels in TA muscle from WT (white bars) and PHD1KO (red bars) female mice. Statistics: two-way ANOVA test, with a Holm-Sidak post hoc test (e, f) or unpaired t test (a, b, c, d, g, h) (*p < 0.05; **p < 0.01; ***p < 0.001; ns not significant). Each dot represents a single mouse (a, b, c, d, h). Bar graphs and line graphs represent mean ± SEM (error bars). Data is represented as fold change to WT (g, h). EDL m. extensor digitorum longus; GAS m. gastrocnemius; TA m. tibialis anterior. See also Supplementary Fig. 1. Source data are provided as a Source Data file.
PHD1 is required for leucine mediated mTORC1 activation. 

mTORC1 represents a main regulatory hub in the control of muscle protein synthesis in response to many anabolic signals, such as growth factors, eccentric contractions, and/or amino acids\textsuperscript{11,38}. To study whether loss \textit{Phd1} affects amino acid mediated activation of mTORC1 in muscle, we administered L-leucine (leucine), the most potent amino acid stimulator of mTORC1 and required for activation of muscle protein synthesis in vivo\textsuperscript{39}, to PHD1\textsuperscript{KO} and WT animals via oral gavage and subsequently analyzed mTORC1 activity. In WT TA muscle, leucine administration activated mTORC1, as judged by the increased phosphorylation states of its substrates S6 kinase 1 (p-S6K1), S6 ribosomal protein (p-RPS6), and the 4E-binding protein 1 (p-4E-BP1) (Fig. 2a and Supplementary Fig. 2a). In contrast, leucine-mediated phosphorylation of these mTORC1 substrates was abrogated in PHD1\textsuperscript{KO} muscle (Fig. 2a and Supplementary Fig. 2a). Immunofluorescent quantification of p-RPS6 levels in TA muscle confirmed these observations (Fig. 2b, c). Inhibition of leucine mediated mTORC1 activation upon deletion of \textit{Phd1} was observed in both females (Fig. 2a) as well as males (Supplementary Fig. 2a), so both genders were used for subsequent experiments. Moreover, inhibition of mTORC1 activity upon loss of \textit{Phd1} is fiber type independent, because we observed impaired mTORC1 activation in soleus, which is predominantly composed of a slower fibers, as well as EDL, which contains more fast glycolytic fibers (Supplementary Fig. 2b, c). To confirm that impaired mTORC1 activation resulted into impaired protein synthesis, we used SunSET analysis and measured puromycin incorporation into muscle protein after leucine injection. This data showed 50% lower protein synthesis in PHD1\textsuperscript{KO} muscle when compared to WT (Fig. 2d).

Growth factors such as insulin but also eccentric contractions are also potent regulators of mTORC1 in the muscle\textsuperscript{38,40}, albeit via different and independent upstream mechanisms\textsuperscript{41}. To explore whether PHD1 also controls insulin mediated activation of mTORC1, we injected mice with insulin (0.2 IU/g) and assessed mTORC1 activation. We first confirmed that insulin effectively activated the insulin signaling cascade by assessing the phosphorylation of AKT at Ser\textsuperscript{473} (p-AKT) (Supplementary Fig. 2d). In addition, insulin equally increased p-S6K1 in WT and PHD1\textsuperscript{KO} animals, indicating that PHD1 controls leucine, but not insulin, mediated activation of mTORC1 (Supplementary Fig. 2d). We subsequently isolated EDL muscle from PHD1\textsuperscript{KO} and WT mice and subjected them to an ex vivo eccentric contraction protocol which is known to activate mTORC1 directly\textsuperscript{42} or via the inactivation TSC2\textsuperscript{11}. Eccentric contractions effectively activated the stress-responsive C-Jun N-terminal kinase (JNK), a key regulator of adaptive remodeling after resistance training\textsuperscript{43} in both WT and PHD1\textsuperscript{KO} mice (Supplementary Fig. 2e). Moreover, eccentric contractions induced a similar activation of mTORC1 downstream signaling in both WT and PHD1\textsuperscript{KO} mice (Supplementary Fig. 2e). Thus, PHD1 controls leucine mediated, but not insulin nor contraction mediated activation of mTORC1 in the muscle.

Growth factors as well as eccentric contractions activate mTORC1 mainly through the inhibition of its inhibitory TSC complex\textsuperscript{11,44}, the latter being a direct target of HIF-dependent inhibition of protein synthesis via REDD1\textsuperscript{15}. Moreover, lack of oxygen availability leads to an AMPK dependent phosphorylation of the TSC complex as well as RAPTOR\textsuperscript{45}, leading to a general inhibition of protein synthesis. We did not find increased AMPK activation (Fig. 2e) nor phosphorylation of TSC2 at Ser\textsuperscript{1387} (Fig. 2f) in PHD1\textsuperscript{KO} muscle. We also did not pick up increased expression of the HIF target \textit{Redd1} (Fig. 2g). Our data suggest that PHD1 controls mTORC1 via selectively altering its response to leucine. We thus decided to further explore the role of PHD1 in leucine metabolism.

PHD1 controls muscle mass in a cell-autonomous fashion. To study whether the blunted leucine mediated mTORC1 activation upon \textit{Phd1} deletion was driven by muscle-intrinsic factors, we decided to cross \textit{Phd1}\textsuperscript{KO} mice with human skeletal muscle α-actin (HSA)-Cre-ERT\textsuperscript{2} mice\textsuperscript{46}, which upon tamoxifen treatment results into the generation of muscle specific \textit{Phd1} knockout mice (PHD1\textsuperscript{mKO}) (Fig. 3a). To evaluate the efficiency of our tamoxifen regimen, we also crossed HSA-Cre-ERT\textsuperscript{2} mice with Rosa\textsuperscript{TmG} mice\textsuperscript{47}, a double fluorescent Cre reporter line that expresses membrane-targeted Tomato (mT) prior to Cre-mediated excision and GFP (mG) after excision, and confirmed efficient recombinant which was restricted to skeletal muscles (Supplementary Fig. 3a). Indeed, one week after the last tamoxifen injection, \textit{Phd1} mRNA levels in muscle of PHD1\textsuperscript{mKO} mice were around 90% lower when compared to littermate controls (Fig. 3b).

We next confirmed that loss of \textit{Phd1} in muscle suffices to blunt the activation of mTORC1 downstream targets following leucine but not insulin stimulation (Fig. 3c and Supplementary Fig. 3b). Moreover, leucine-induced protein synthesis assessed via puromycin incorporation was reduced as well (Supplementary Fig. 3c). To further confirm the cell autonomous role of PHD1 in the control of leucine mediated mTORC1 activation, we isolated muscle stem cells from WT and PHD1\textsuperscript{KO} mice and differentiated them to myotubes. Myotubes were starved of amino acids and stimulated with leucine. In WT myotubes, leucine dose dependently activated mTORC1, and maximal activation was reached at 5 mM (Fig. 3d). Loss of \textit{Phd1} reduced the mTORC1 response at all leucine concentrations tested (Fig. 3d). Off note, we also measured lower mTORC1 activity under full medium conditions (containing 0.8 mM leucine), but consistent with our in vivo data, the response to insulin was preserved (Supplementary Fig. 3d).

PHD1 controls mTORC1 in a hydroxylation-independent manner. PHDs can control the stability of proteins by hydroxylation proline residues which targets them for proteasomal degradation\textsuperscript{48,49}. To evaluate whether PHD1 controls mTORC1 activity via hydroxylation dependent or independent mechanisms, we transduced primary satellite cells from PHD1\textsuperscript{KO} mice with retroviruses to reintroduce either the full length PHD1 (PHD1\textsuperscript{WT}) or a catalytically inactive PHD1 mutant (PHD1\textsuperscript{MUT})\textsuperscript{50}. Introduction of PHD1\textsuperscript{WT} as well as PHD1\textsuperscript{MUT} in PHD1\textsuperscript{KO} myotubes restored leucine-dependent mTORC1 activation (Fig. 3e), showing that PHD1 controls leucine-mediated mTORC1 activation in a hydroxylation-independent manner. Based on these observations, we decided to evaluate how PHD1 controls leucine mediated mTORC1 activation and hypothesized that this could occur through regulating leucine uptake or through interacting with leucine sensing mechanisms\textsuperscript{10,11}.

Loss of \textit{Phd1} does not impair leucine uptake. To act on mTORC1 and induce growth, large neutral amino acids such as leucine, enter the cell via coupled amino acid transport\textsuperscript{51}. Since leucine stimulated mTORC1 activation was blunted in PHD1\textsuperscript{KO} muscle, we investigated whether leucine uptake was impaired in PHD1\textsuperscript{KO} mice. First, we evaluated expression levels of main muscle amino acid transporters \textit{Snat}, \textit{Pat1}, and \textit{Lat1}, but these were unaffected in PHD1\textsuperscript{KO} mice (Fig. 3f). Second, oral administration of leucine did not alter blood leucine levels (Fig. 3g). And lastly, we measured leucine uptake into the muscle using 1.5 μCi L-[\textsuperscript{14}C(U)-leucine tracer labeling. Uptake of \textsuperscript{14}C-leucine by GAS and TA muscle was identical between WT and PHD1\textsuperscript{KO} mice (Fig. 3h). This data shows that leucine transport is not impaired in PHD1\textsuperscript{KO} mice, and that reduced leucine mediated activation of mTORC1 is likely due to defects in the intracellular leucine-mTORC1 activation cascade.
**Fig. 2** PHD1 is required for leucine-mediated mTORC1 activation in vivo. 

**a** Representative pictures (left panel) and quantification (right panels) of western blot analysis of S6K1, RPS6, and 4E-BP1 phosphorylation in TA muscles from WT (white bars) and PHD1KO (red bars) female mice 30 min after saline or leucine gavage. Representative pictures **b** and quantification **c** of p-RPS6 immunofluorescence analysis in TA muscle of WT (white bars) and PHD1KO (red bars) mice 30 min after saline (saline) or leucine (leucine) gavage. Intensity measurements are provided in arbitrary units (AU). **d** Representative pictures and quantification of western blot analysis of puromycin incorporation in TA muscle from WT (n = 4) and PHD1KO male mice (n = 4) 30 min after leucine gavage. Rapamycin (rapam) was used as a negative control. Representative pictures (top panel) and quantification (bottom panel) of western blot analysis of AMPK phosphorylation **e** and TSC2 phosphorylation **f** in TA muscle from WT (white bars) and PHD1KO (red bars) mice 30 min after leucine gavage. **g** Redd1 mRNA expression levels in TA muscle from WT (white bars) and PHD1KO (red bars) female mice.

Statistics: two-way ANOVA test with a Holm–Sidak post hoc test (a, c, e–g) (*p < 0.05; **p < 0.01; ***p < 0.001; ns not significant). Each dot represents a single mouse. Bar graphs represent mean ± SEM (error bars). Data are represented as fold change to WT saline (a, c, e–g) or fold change to WT (g). TA m. tibialis anterior. See also Supplementary Fig. 2. Source data are provided as a Source Data file.
PHD1 controls intracellular leucine sensing through LRS. Upon entering the cell, leucine is "sensed" by SESNs and LRS which transmit intracellular leucine availability towards mTORC1. To investigate whether PHD1 controls leucine mediated mTORC1 activation via affecting LRS or SESN1–2, we assessed protein levels of LRS and SESN1–2 in muscle. Interestingly, LRS protein levels were lower both in PHD1KO as well as PHD1mKO muscle when compared to WT animals (Fig. 4a and Supplementary Fig. 4a). In contrast, SESN1 nor SESN2 protein levels were affected by loss of Phd1 (Fig. 4b–d and Supplementary Fig. 4b–d).

To further investigate the role of LRS and SESNs, we evaluated whether Sesn knockdown or LRS overexpression could rescue leucine-dependent mTORC1 activation in Phd1-deficient myotubes. Silencing Sesn1 nor Sesn2 changed the responsiveness to leucine in PHD1KO myotubes (Supplementary Fig. 4e–h).
Conversely, lentiviral overexpression of LRS in PHD1KO myotubes restored LRS protein content and increased mTORC1 activation upon leucine stimulation (Fig. 4e). This data indicates that LRS, but not SESN1, is involved in PHD1-mediated mTORC1 activation in muscle cells. We therefore decided to focus on LRS and study its role in leucine-dependent mTORC1 activation in muscle.

LRS belongs to a family of proteins known as aminoacyl tRNA synthetases whose canonical function is to ensure that the genetic code is accurately deciphered by attaching the correct amino acid to the equivalent tRNA. However, LRS also serves as a leucine sensor for mTORC1 by functioning as a GTP activating protein for RagD, thereby promoting lysosomal translocation of mTORC1. GTP-bound RagD subsequently promotes the LRS-dependent uncoupling of RagA at K142 (KLeu142) upon leucine stimulation. Whether LRS levels are controlled by metabolism is, however, not clear. In fact, the leucine sensing ability of LRS in muscle is not described. To confirm that LRS is required for leucine-mediated mTORC1 activation in myotubes, we inhibited the interaction of LRS with RagD by using BC-LI-018652 and confirmed reduced leucine mediated activation of mTORC1 (Supplementary Fig. 4i). This data shows that LRS is involved in leucine-dependent mTORC1 activation in muscle cells.

To confirm that PHD1 reduces LRS downstream signaling to mTORC1, we stimulated WT and PHD1KO myotubes with leucine and evaluated RagA KLeu142 levels. In Phd1-deficient myotubes, leucine failed to increase RagA KLeu142 (Fig. 4e). Moreover, we observed reduced RagA KLeu142 levels in vivo (Fig. 4f). We also monitored the lysosomal localization of mTOR by performing immunofluorescent stainings for mTOR and the lysosomal marker LAMP2 under starved and stimulated conditions. Whereas we saw a clear increase in LAMP2/mTOR colocalization in WT myotubes upon (amino acid) stimulation, increased colocalization was not observed in PHD1KO myotubes (Fig. 4g, h). The data shows that loss of Phd1 reduces LRS protein content, leading to impaired mTORC1 translocation to the lysosomes and impaired RagA KLeu142.

PHD1 interacts with LRS and controls LRS stability. Although PHDs are mainly known for their hydroxylation dependent functions, it has been shown that they interact with other proteins to modulate their stability and/or activity via hydroxylation-independent mechanisms. To study whether PHD1 and LRS interact, we overexpressed both PHD1-flag and LRS-myc in HEK 293T cells and found that LRS and PHD1 communoprecipitated (Fig. 5a). The ability to interact with LRS was independent of PHD1’s enzymatic activity, since PHD1MUT also interacted with LRS (Fig. 5a). Furthermore, we performed an in vitro hydroxylation assay using recombinant PHD1 and LRS using HIF1α as a positive control, but did not detect any hydroxylated prolines on LRS, whereas HIF1α was clearly hydroxylated under the same experimental conditions (Fig. 5b and Supplementary Fig. 5a).

Interestingly though, whereas the interaction between PHD1 and LRS was weak under normal culture conditions, it was further promoted when the enzymatic activity of PHD1 was inhibited upon treatment with the hypoxia mimetic, dimethyl 2-oxoglutarate (DMOG), or during amino acid starvation (Fig. 5c). Based on these observations, we wondered whether the interaction between PHD1 and LRS improves LRS stability and whether LRS stability is enhanced when the interaction is promoted, such as during nutrient and oxygen deprivation. We first performed a cycloheximide chase assay for LRS in WT vs. PHD1KO myotubes, and noticed that upon translation elongation inhibition, LRS levels dropped faster when Phd1 is absent, indicating that loss of Phd1 reduces LRS protein stability (Fig. 5d). Second, we found that LRS protein levels remain remarkably stable (or even increase) during short-term amino acid starvation as well as hypoxia in WT myotubes, but rapidly go down in PHD1KO myotubes (Fig. 5e, f and Supplementary Fig. 5b, c) showing that PHD1 is required for the maintenance of LRS stability during oxygen and amino acid deprivation. Altogether, we show that upon metabolic stress, including amino acid starvation and hypoxia when PHD1 enzymatic activity is inhibited, PHD1 interacts with LRS and protects it from degradation in a manner which does not require its enzymatic activity. The protection of LRS during low oxygen and amino acid levels ensures a rapid and efficient activation of mTORC1 as soon as nutrient levels are restored.

PHD1 levels and LRS activity decline during aging. Aging is associated with a loss of muscle mass. Moreover, the ability of older muscle to efficiently activate protein synthesis in response to amino acids, a condition termed anabolic resistance, is impaired. Several molecular mechanisms underlying anabolic resistance have been proposed, including reduced amino acid delivery and uptake into the muscle, but it is not known whether leucine sensing mechanisms are affected. Thus, to explore the relevance of our findings in a human setting, we compared PHD1 protein content in muscle samples from a small cohort where samples were obtained from healthy old (n = 8, 4 women and 4 men, 72.6 ± 2.3 y (mean ± SEM)) vs. young (n = 8, 4 women and 4 men, 26.1 ± 1.1 y) volunteers after an overnight fast. In this cohort, old people showed reduced activation of muscle protein synthesis in response to milk protein (containing a high dose of leucine) ingestion when compared to young subjects. We found that older muscle on average showed an almost 50% decrease in PHD1 levels (Fig. 6a). Lower PHD1 content was confirmed using immunofluorescent stainings (Fig. 6b, c).
Importantly, in other cell types PHD1 localizes mainly in the nucleus, but several groups have also reported cytosolic localization and function of PHD1. We saw clear cytosolic localization of PHD1 in muscle fibers whereas nuclear staining was detected, but to a much lower extent. LRS levels were also lower but this failed to reach statistical significance because variability between subjects was larger (Fig. 6a). Notwithstanding, we found a clear reduction in LRS induced leucylation of RagA (RagA K142) (Fig. 6d), and RagA K142 levels correlated with PHD1 levels (r = 0.56; p = 0.02). Thus, in aged humans, impaired activation of muscle protein synthesis in response to leucine coincides with reduced PHD1 content and LRS activity.
Discussion

Protein synthesis is a highly energy consuming process which in many cell types is inhibited during low oxygen and nutrient availability. On the other hand, mechanisms that allow (and even promote) protein synthesis during hypoxia have also been described as stabilization of HIF2α promotes mTORC1 activation during low amino acid availability by increasing the expression of the amino acid carrier Lati. In fact, in vivo data in lung and liver during hypoxia have indicated that HIF2α mediated activation of mTORC1 can prevail over HIF1α dependent mTORC1 inhibition. It is therefore likely that control of mTORC1 oxygen sensitive mechanisms is dependent on the cellular context. Here, we describe that the oxygen sensor PHD1 promotes mTORC1 activation upon leucine stimulation. We found that PHD1 interacts with the leucine sensor LRS and controls LRS stability. This interaction is promoted under conditions of hypoxia and amino acid starvation when PHD1 hydroxylates LRS and protects LRS from degradation. Consequently, loss of Phd1 reduces the stability of LRS, impairs leucine-mediated activation of mTORC1 and leads to lower muscle mass in vivo.

We found that PHD1KO mice have lower muscle mass, muscle fiber size, and force production. This was not caused by increased muscle proteolysis as we did not find upregulation of autogenes nor activation of autophagy. Instead, we observed a blunted mTORC1 activation in response to leucine stimulation leading to lower protein synthesis. mTORC1 plays a central role in the regulation of muscle mass: activation of mTORC1 induces muscle hypertrophy, whereas skeletal muscle specific inactivation results in low muscle mass coinciding with dystrophy. Furthermore, the fact that sustained activation of mTORC1 in muscle also induces myopathy characterized by muscle atrophy and weakness, indicates that mTORC1 activity should be tightly controlled in order to prevent muscle dysfunction. We did not find any evidence for muscle dystrophy or myopathy, showing that the ability of PHD1 to control mTORC1 activity is modest, and is restricted to modulating upstream anabolic events that activate mTORC1. Several anabolic signals such as leucine, growth factors, but also eccentric contractions are potent regulators of mTORC1 in muscle. The activation of mTORC1 in response to growth factors (such as insulin) as well as contractions was however preserved in PHD1KO mice. Instead, there was a selective “resistance” of PHD1KO muscle toward leucine, showing that PHD1 controls mTORC1 via events upstream of mTORC1 which are specifically linked to the sensing and transmission of amino acid signals.

Ample literature has shown the contribution of LRS and SESNs to leucine sensing in vitro. However, their functional role as leucine sensors in vivo tissues such as skeletal muscle, has been questioned since the Kd dissociation constant for leucine of both SESN2 and LRS is approximately tenfold lower than the leucine concentration observed in skeletal muscle of humans. This implies that both enzymes are completely saturated at physiological leucine concentrations and has led to the suggestion that alterations in protein levels of leucine sensors impose an additional level of control in modulating mTORC1 activity. On the other hand, in vitro experiments in HEK293T cells demonstrated that leucylation of RagA, considered as a readout of LRS activity, increases in an almost linear manner with leucine doses up to 30-fold of the normal physiological values. The exact mechanisms through which leucine sensors control mTORC1 still remain to be fully elucidated. We show here that altering LRS levels suffices to increase leucine mediated mTORC1 activation in myotubes. Moreover, we found decreased LRS levels and lower leucylation of RagA in PHD1KO animals, showing that LRS protein levels and LRS activity can be modulated in vivo to/and control mTORC1 activation.

It has been reported that LRS levels remain stable during amino acid starvation. This is in agreement with our data. In addition, we show that PHD1 is required to maintain LRS protein stability during conditions of oxygen and amino acid deprivation. The increased stability of LRS is in sharp contrast to most amino acid tRNA synthetases, which are rapidly degraded during amino acid starvation induced autophagy. These observations support the idea that the maintenance of LRS protein levels may not be related to its role as a tRNA synthetase but with other noncanonical LRS functions. Instead, keeping LRS levels high during episodes of low oxygen and/or amino acid availability may allow the cell to ensure a fast and efficient reactivation of mTORC1 as soon as oxygen and nutrient levels are restored. Our data also shows active modulation of LRS levels by metabolism.

We did not observe differences in SESN1–2 protein content upon loss of Phd1, nor did we observe a rescue of leucine mediated mTORC1 activation upon knockdown of SESN1–2 in PHD1KO cells. It is important to mention that these data do not exclude a role for SESNs in regulating leucine mediated mTORC1 activation in muscle. SESN2 knockout mice also show preserved mTORC1 activation in response to insulin, but the response to leucine stimulation has not been evaluated. Moreover, while recent evidence indicates that SESN1, rather than SESN2, is abundantly expressed in muscle and dissociates from GATOR2 in response to oral leucine administration, its functional role in mediating mTORC1 activation upon leucine administration is not known. Whether or not SESNs directly interact with other members of the oxygen sensing machinery remains an outstanding question.
PHDs are key proteins mediating oxygen sensing in cells\(^3\).\(^1\),\(^3\).\(^2\) Besides oxygen, PHDs also use \(\alpha\)-KG, ascorbic acid and Fe\(^2+\) as cofactors to hydroxylate their targets at specific proline residues\(^3\).\(^1\).\(^3\) The enzymatic activity of PHDs is lost when one of these cofactors is insufficiently available, such as during hypoxia and/or nutrient deprivation\(^7\).\(^4\),\(^5\).\(^6\).\(^7\). While HIFs are the best characterized targets of PHD hydroxylation activity, more PHD (hydroxylation dependent or independent) targets have been reported\(^3\).\(^4\),\(^6\).\(^7\). Photosynthetic organisms, which produce but do not oxidize oxygen for energy production, have been reported to lack HIFs but not PHDs\(^7\) demonstrating potential HIF-independent functions of PHDs\(^7\).

Our data indicates that PHD1 controls leucine mediated mTORC1 activation in a HIF-independent fashion. First, we did not see transcriptional activation of \(\text{Redd1}\) in PHD1KO muscle, a previously described HIF1\(\alpha\)-target gene which inhibits mTORC1\(^1\),\(^3\),\(^6\),\(^3\) nor did we find differences in phosphorylation of its downstream target TSC2. Second, most described hypoxia dependent mechanisms which inhibit mTORC1 have been shown to act via TSC2 (or in the case of AMPK, via RAPTOR).
Preserved activation of mTORC1 by insulin (acting via TSC2) as well as contractions (acting via TSC2 or RAPTOR directly) strongly argues against involvement of TSC2 or RAPTOR itself. And last, in agreement with our observations, in vitro reports have shown that PHD dependent control of mTORC1 upon leucine stimulation can occur independent of HIFs/TSC2. Indeed, DMOG treatment of amino acid starved MEFs did not result into accumulation of HIF1α or an increase in HIF transcriptional activity. In addition, DMOG treatment also prevented mTORC1 activation in both TSC2 knockout MEFs, suggesting that PHDs act through the Rag GTPases. The ability of PHD1 to ensure mTORC1 activity upon leucine stimulation was independent of its hydroxylation activity, since reintroduction of the catalytic dead PHD1 in PHD1KO myotubes completely restored mTORC1 activity. Second, we found that PHD1 interacted with LRS and this interaction also did not require PHD1 hydroxylation activity because the enzymatic dead mutant PHD1 also interacted with LRS. Due to different levels of overexpression for wild type vs. mutant PHD1 within our immunoprecipitation experiments, it is difficult to make a clear statement on whether inhibition of the enzymatic activity of PHD1 suffices to actively promote the interaction or whether other unknown processes are involved. Nonetheless, we found that reducing PHD activity by using DMOG as well as amino acid starvation increased the interaction with LRS and preserved LRS stability in WT cells. Consequently, loss of Phd1 prevented LRS stabilization under conditions of amino acid and oxygen shortage, and resulted into a rapid decline of LRS protein levels. The detailed mechanisms underlying this improved interaction require further investigation. Indeed, our data does not allow us to conclude that the interaction between LRS and PHD1 is actively promoted when PHD1 hydroxylation activity is inhibited. An alternative explanation could be that reduced interaction of PHD1 with its canonical target HIF during hypoxia increases the availability of PHD1 to interact with other substrates. Nonetheless, similar observations were made in breast cancer cells where the interaction between PHD1 and NRF1/PGC1α to preserve mitochondrial function during tumor growth was enhanced during hypoxia and was also independent of PHD1’s hydroxylation activity. Our findings show that PHDs can exert different functions during normoxia/nutrient availability when they hydroxylate HIFs (and potentially other targets) vs. hypoxia/nutrient depletion when they execute an autonomous role within the hypoxia-dependent program.

**Fig. 6 PHD1 levels and LRS activity decline during aging.** a Representative picture (left panel) and quantification (right panel) of western blot analysis of PHD1 and LRS protein levels in m. vastus lateralis biopsies from young (white bars) and old (gray bars) volunteers. Representative immunofluorescent pictures (b) and quantification (c) of PHD1 (red), Hoechst nuclear staining (blue) and wheat germ agglutinin (WGA, white) in young and old skeletal muscle. Arrows indicate nuclei. d Representative picture (left panel) and quantification (right panel) of western blot analysis of RagA leucylation (KLeu142) levels in m. vastus lateralis biopsies from young (white bars) and old (gray bars) volunteers. Statistics: unpaired t test (a) (*p < 0.05; **p < 0.01; ***p < 0.001; ns not significant). Dots represent values from different volunteers. Bar graphs represent mean ± SEM (error bars). Data is presented as fold change to young (a, d). Source data are provided as a Source Data file.
Altogether, PHD1 integrates oxygen and nutrient availability to modulate the activation of mTORC1 in response to leucine. The in vivo relevance of our data is underscored by the lower muscle mass of PHD1KO animals. Moreover, we observed that PHD1 levels and LRS activity are lower in muscle of elderly with ana- bolic resistance. To the best of our knowledge, we did not find any report showing reduced PHD1 levels in aging muscle. The upstream mediators of altered PHD1 levels during aging are not known and warrant further research. But since we show that modulating PHD1/LRS levels can alter the muscular response to leucine, our data raises the question whether PHD1/LRS levels could be therapeutically targeted to improve the anabolic effect of leucine and to prevent the development of age-related sarcopenia.

Methods

Reagents and cell culture. HEK293T cells (DSMZ, ACC 633) were maintained in DMEM (ThermoFisher Scientific; 41960052) supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific, 10270-106). Freshly isolated myogenic progenitors (MPs) were cultured in a 1:1 ratio of DMEM (ThermoFisher Scientific, 12200302) and Ham’s F-10 (1×) nutrient mix (ThermoFisher Scientific, 22390058) supplemented with 10% horse serum (ThermoFisher Scientific, 16050-122), 20% FBS and 10 ng/ml basic-FGF (ThermoFisher Scientific, PHG0266). All media were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were routinely cultured in 21% O2 and 5% CO2 (normoxic conditions). For hypoxic progenitors (MPs) were cultured in a 1:1 ratio of DMEM (ThermoFisher Scientific) supplemented with 10% dialyzed FBS (dFBS) (ThermoFisher Scientific) and 0.1% P/S. MPs were fully differentiated after 3 days.

For FACS, MPs were sorted based on positive alpha 7-integrin (1:100) and absence of CD31. In vitro pull-down assay

Leucine stimulation: differentiated myotubes were starved for 1 h in low-glucose amino acids free DMEM (US Biology, D9800-13) and kept in a homozygous mating system for 24 h. Myotubes were backcrossed to C57BL6/J mice for 6 generations, to generate Phd1−/− mice. The Phd1−/− mouse was built in a pPNloxp2 plasmid with loxp sites flanking the neomycin resistance (neo) cassette, and contained from 5′ to 3′: a 5.3-kb EcoRV-HindIII fragment comprising exons 2 and 3 at the 3′ homology arm, a 1.8-kb floxed neomycin resistance cassette, a 2.5-kb HindIII-EcoRV fragment comprising exon 4, in which a third loxp site was introduced downstream of exon 4 along with a novel EcoRI site for genotyping purposes, an SV40 thymidine kinase expression cassette for negative selection purposes. The introduced third loxp site together with the loxp site located 3′ of the neo cassette thus flanked the Phd1 gene segment comprising exons 3 and 4, which encode part of the catalytic domain conferring the prolyl hydroxylase activity. The construct was linearized with NotI and electroporated into G4 ES cells of 129SvEv/C57BL6 origin (kind gift from A. Nagy, Toronto). After positive-negative drug selection with 200 µg/ml G418 (Invitrogen) and 2 µg/ml Ganciclovir (Sigma Aldrich), resistant clones were analyzed for correct homologous recombination by appropriate Southern blotting and PCR. Correctly targeted ES cells were then transiently electroporated with the pOG231 Cre-recombinase expressing plasmid to excise the floxed neo cassette. Clones with exclusive excision of the neo cassette and retention of the floxed genomic fragment, were identified by PCR and Southern blot screens based on the retention of the newly introduced EcoRI site. The resulting Phd1−/− ES cells were then used for diploid aggregation with Swiss morula embryos to obtain chimeric mice, and germline Phd1−/− offspring from crosses of male chimeras with C57BL6/J females were backcrossed to C57BL6/J mice for 6 generations, to generate Phd1−/− mice with a genetic background of >98% C57BL6/J. To generate muscle-specific Phd1 knockout (PHD1mKO) animals, Phd1−/− mice (C57BL6/J background) were crossed with transgenic mice expressing Cre under the control of HSA promoter and in a homozygous mating system for iil and heterozygous for HSA.Cre-negative littermates were used as controls. At the age of 10 weeks, mice were injected with 1 mg tamoxifen for 5 days and a washout period of 9 days was allowed before experiments were initiated. To generate HSA.Cre; Rosa26Tm2mice, transgenic mice expressing Cre under the control of HSA promoter were crossed with heterozygous Rosa26Tm mice.

Human muscle samples. Participants were informed of the purpose and methodology of the study prior to providing written informed consent. Ethical approval was obtained through the NHS Black country Research Ethics Committee (13/WM/0429). The study was performed in agreement with the standards set by the Declaration of Helsinki (seventh edition). Muscle samples from old (n = 8, 4 women and 4 men, 72.6 ± 2.3 y) and young (n = 8, 4 women and 4 men, 26.1 ± 1.1 y) volunteers were obtained from m. vastus lateralis after an overnight fast. Details from biopsy procedure and freezing method can find elsewhere.

Animals. Whole body Phd1 knockout (PHD1mKO) mice (50% Swiss/50% 129S1 background) were previously generated. Phd1−/−/− mice were generated using homologous recombination in embryonic stem (ES) cells. The targeting vector was built in a pPNloxp2 plasmid with loxp sites flanking the neomycin resistance (neo) cassette, and contained from 5′ to 3′: a 5.3-kb EcoRV-HindIII fragment comprising exons 2 and 3 at the 3′ homology arm, a 1.8-kb floxed neomycin resistance cassette, a 2.5-kb HindIII-EcoRV fragment comprising exons 4 and 6, in which a third loxp site was introduced downstream of exon 4 along with a novel EcoRI site for genotyping purposes, an SV40 thymidine kinase expression cassette for negative selection purposes. The introduced third loxp site together with the loxp site located 3′ of the neo cassette thus flanked the Phd1 gene segment comprising exons 3 and 4, which encode part of the catalytic domain conferring the prolyl hydroxylase activity. The construct was linearized with NotI and electroporated into G4 ES cells of 129SvEv/C57BL6 origin (kind gift from A. Nagy, Toronto). After positive-negative drug selection with 200 µg/ml G418 (Invitrogen) and 2 µg/ml Ganciclovir (Sigma Aldrich), resistant clones were analyzed for correct homologous recombination by appropriate Southern blotting and PCR. Correctly targeted ES cells were then transiently electroporated with the pOG231Cre-recombinase expressing plasmid to excise the floxed neo cassette. Clones with exclusive excision of the neo cassette and retention of the floxed genomic fragment, were identified by PCR and Southern blot screens based on the retention of the newly introduced EcoRI site. The resulting Phd1−/− ES cells were then used for diploid aggregation with Swiss morula embryos to obtain chimeric mice, and germline Phd1−/− offspring from crosses of male chimeras with C57BL6/J females were backcrossed to C57BL6/J mice for 6 generations, to generate Phd1−/− mice with a genetic background of >98% C57BL6/J. To generate muscle-specific Phd1 knockout (PHD1mKO) animals, Phd1−/− mice (C57BL6/J background) were crossed with transgenic mice expressing Cre under the control of HSA promoter and in a homozygous mating system for iil and heterozygous for HSA.Cre-negative littermates were used as controls. At the age of 10 weeks, mice were injected with 1 mg tamoxifen for 5 days and a washout period of 9 days was allowed before experiments were initiated. To generate HSA.Cre; Rosa26Tm mice, transgenic mice expressing Cre under the HSA promoter were crossed with het- erozygous Rosa26Tm mice.

Experimental procedures. All animal procedures were approved by the Veterinary office of the Canton of Zürich (licence nr. ZH255-16), by the local ethics committee of the KU Leuven, Belgium (P174-2014) and were executed in compliance with the institutional and national guidelines and regulations. Sample size was determined based on previous experiments in our labs and similar studies reported in the literature. All mice used for the experiments were housed 3–4 litters per cage in individually ventilated cages at standard housing conditions (22 °C, 12 h light/dark cycle, dark phase starting at 7 pm), with ad libitum access to food. Health status of all mouse lines was regularly monitored according to FELASA guidelines.

Leucine and insulin administration in vivo. The morning (8:00 A.M.) of the experiments, mice were fasted for 4 h. A suspension of 40 g L-leucine/L in distilled water was freshly prepared and mice were administered 0.4 g L-leucine kg−1 body weight i.p. Control mice were injected with an equal volume of saline (0.9% NaCl). Exactly 30 min after saline or leucine administration, mice were sacrificed and GAS, SOL, TA, and EDL, were harvested and either snap frozen in liquid N2 overnight. After washing 3 times in lysis buffer supplemented with 50 mM NaCl, the precipitates were dissolved in laemmli buffer.
CHAPS containing primary antibodies were incubated for 15 min, the sections were then incubated in solution A (PBS containing 10% goat serum, was applied for goat anti-mouse Alexa Fluor 488,350,568 and 647 (1:250) (ThermoFisher Scientific, 12130-08A). Afterwards a secondary antibody cocktail, diluted in solution B (PBST supplemented with 0.05% triton (PBST) and subsequently blocked for 60 min in PBST + 10% goat serum) was applied for goat anti-rabbit IgG secondary antibody (1:250, ThermoFisher Scientific). The next morning, after three 5 min washes in PBS, samples were incubated for 1 h in solution A with the appropriate 488-conjugated and 568-coupled antibodies before and 3, 6, 10, 15, 20, and 30 min after administration of the solution. Once the final blood sample was taken, mice were sacrificed and GADPH and β-Actin were quantified using a fluorometric microscope (Zeiss Axio Observer Z.1). The rate of L-leucine uptake (Kin) was calculated by the equation Kin = total dpm muscle/AUC 0–30 min.

Statistical analysis. A two-way ANOVA design was used to assess the statistical significance of differences between mean values over phenotype and treatment. When appropriate, Tukey post hoc test was used. To determine statistical significances between two groups, an unpaired student’s t test was used. Level of significance was set to α = 0.05. Results are shown as mean ± SEM.

Data availability. The data presented in this study are available from the corresponding authors upon reasonable request. The source data underlying Figs. 1–6 and Supplementary Figs. 1–5 are provided as a Source Data file.

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References

1. Hughes, V. A. et al. Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health. J. Gerontol. A. Biol. Sci. Med. Sci. 56, B209–B217 (2001).
2. Sharples, A. B et al. Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging Cell 4, 511–523 (2015).
3. Fried, L. P. et al. Handgrip strength and cause-specific and total mortality in older disabled women: exploring the mechanism. J. Am. Geriatr. Soc. 51, 636–641 (2003).
4. Grooten, M., Montgomery, H. & Vercuiel, A. High-altitude physiology and pathophysiology: implications and relevance for intensive care medicine. Crit. Care 11, 203 (2007).
5. Baldi, S. et al. Fat-free mass change after nutritional rehabilitation in weight losing COPD: role of insulin, C-reactive protein and tissue hypoxia. Int. J. Clin. Exp. Pathol. 10, 29–39 (2010).

6. Prado, C. M. et al. Central tenet of cancer cachexia therapy: do patients with advanced cancer have exploitable anabolic potential? Am. J. Clin. Nutr. 98, 1012–1019 (2013).

7. Norman, K. & Otten, L. Financial impact of sarcopenia or low muscle mass—a short review. Clin. Nutr. 4, 1489–1495 (2018).

8. Burd, Na, T., Moore, D. R. & Phillips, S. M. Exercise training and protein metabolism: influences of contraction, protein intake, and sex-based differences. J. Appl. Physiol. 106, 1692–1701 (2009).

9. Phillips, S. M., Hartman, J. W. & Wilkinson, S. B. Dietary protein to support anabolism with resistance exercise in young men. J. Am. Coll. Nutr. 24, 320–326 (2005).

10. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–292 (2012).

11. Jacobs, B. L. et al. Eccentric contractions increase the phosphorylation of the transforming growth factor-beta (TGF-beta) signaling pathway. J. Appl. Physiol. 107, 1403–1409 (2009).

12. Bonaldo, P. & Sandri, M. Cellular and molecular mechanisms of muscle atrophy. Dis. Model. Mech. 6, 25–39 (2013).

13. Sancak, Y. et al. The rag GTPases bind raptor and mediate amino acid sensing and the mechanistic target of rapamycin to the lysosome. J. Biol. Chem. 283, 2893–2904 (2008).

14. Sestrin2 is a Leucine Sensor for the mTORC1-signaling pathway. Trends Cell Biol. 38, 311–317 (2018).

15. Aragonés, J. et al. Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. Nat. Genet. 40, 170–180 (2008).

16. Thomas, A. et al. Hypoxia-inducible factor prolyl hydroxylase 1 (PHD1) deficiency promotes hypoxic stress tolerance and liver-specific insulin resistance in mice. Sci. Rep. 6, 24618 (2016).

17. Bar-peled, L. & Sabatini, D. M. Regulation of mTORC1 by amino acids. Trends Cell Biol. 24, 400–406 (2014).

18. Olsen, L. A., Nicoll, J. X. & Fry, A. G. The skeletal muscle fiber: a mechanically sensitive cell. Eur. J. Cell Biol. 92, 333–349 (2013).

19. He, X. Di et al. Sensing and transmitting intracellular amino acid signals through the mechanistic target of rapamycin signaling pathway in skeletal muscle following functional overloading. J. Cell Physiol. 232, 1073–1082 (2017).

20. Zhang, Q. et al. Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. J. Biol. Chem. 283, 29364–29372 (2008).

21. He, X. Di et al. Sensing and transmitting intracellular amino acid signals through the mechanistic target of rapamycin signaling pathway in skeletal muscle following functional overloading. J. Cell Physiol. 232, 1073–1082 (2017).

22. Zhang, Q. et al. Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. J. Biol. Chem. 283, 29364–29372 (2008).

23. Mccarthy, J. J., Srikuea, R., Kirby, T. J., Peterson, C. A. & Esser, K. A. Inducible Cre Transgenic Mouse Strain for Skeletal Muscle-specific Gene Targeting. Skelet. Muscle 3, 1–7 (2012).

24. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-labeled Cre reporter mouse. Genesis 45, 393–605 (2007).

25. Schimmel, P. Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. Ann. Rev. Biochem. 56, 125–158 (1987).

26. He, X. Di et al. Sensing and transmitting intracellular amino acid signals through the mechanistic target of rapamycin (mTORC1) signaling pathway. Cell Metab. 22, 29–30 (2015).

27. Wang, Y. L. & Sabatini, D. M. Mammalian target of rapamycin: A target for translation elongation inhibitors? Nat. Rev. Mol. Cell Biol. 6, 802–810 (2005).

28. Zhang, Q. et al. Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. J. Biol. Chem. 283, 29364–29372 (2008).

29. Mccarthy, J. J., Srikuea, R., Kirby, T. J., Peterson, C. A. & Esser, K. A. Inducible Cre Transgenic Mouse Strain for Skeletal Muscle-specific Gene Targeting. Skelet. Muscle 3, 1–7 (2012).

30. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-labeled Cre reporter mouse. Genesis 45, 393–605 (2007).

31. Schimmel, P. Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. Ann. Rev. Biochem. 56, 125–158 (1987).

32. He, X. Di et al. Sensing and transmitting intracellular amino acid signals through the mechanistic target of rapamycin (mTORC1) signaling pathway. Cell Metab. 22, 29–30 (2015).

33. Wang, Y. L. & Sabatini, D. M. Mammalian target of rapamycin: A target for translation elongation inhibitors? Nat. Rev. Mol. Cell Biol. 6, 802–810 (2005).

34. Mccarthy, J. J., Srikuea, R., Kirby, T. J., Peterson, C. A. & Esser, K. A. Inducible Cre Transgenic Mouse Strain for Skeletal Muscle-specific Gene Targeting. Skelet. Muscle 3, 1–7 (2012).

35. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-labeled Cre reporter mouse. Genesis 45, 393–605 (2007).

36. Schimmel, P. Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. Ann. Rev. Biochem. 56, 125–158 (1987).

37. He, X. Di et al. Sensing and transmitting intracellular amino acid signals through the mechanistic target of rapamycin (mTORC1) signaling pathway. Cell Metab. 22, 29–30 (2015).

38. Wang, Y. L. & Sabatini, D. M. Mammalian target of rapamycin: A target for translation elongation inhibitors? Nat. Rev. Mol. Cell Biol. 6, 802–810 (2005).

39. Mccarthy, J. J., Srikuea, R., Kirby, T. J., Peterson, C. A. & Esser, K. A. Inducible Cre Transgenic Mouse Strain for Skeletal Muscle-specific Gene Targeting. Skelet. Muscle 3, 1–7 (2012).

40. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-labeled Cre reporter mouse. Genesis 45, 393–605 (2007).

41. Schimmel, P. Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. Ann. Rev. Biochem. 56, 125–158 (1987).

42. He, X. Di et al. Sensing and transmitting intracellular amino acid signals through the mechanistic target of rapamycin (mTORC1) signaling pathway. Cell Metab. 22, 29–30 (2015).

43. Wang, Y. L. & Sabatini, D. M. Mammalian target of rapamycin: A target for translation elongation inhibitors? Nat. Rev. Mol. Cell Biol. 6, 802–810 (2005).

44. Mccarthy, J. J., Srikuea, R., Kirby, T. J., Peterson, C. A. & Esser, K. A. Inducible Cre Transgenic Mouse Strain for Skeletal Muscle-specific Gene Targeting. Skelet. Muscle 3, 1–7 (2012).

45. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-labeled Cre reporter mouse. Genesis 45, 393–605 (2007).

46. Schimmel, P. Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. Ann. Rev. Biochem. 56, 125–158 (1987).
63. DeYoung, M. P., Horak, P., Sofer, A., Sgroi, D. & Ellisen, L. W. Hypoxia regulates TSC1/mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev. 22, 239–251 (2008).

64. Bodine, S. C. et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat. Cell Biol. 3, 1014–1019 (2001).

65. Ogasawara, R., Jensen, T. E., Goodman, C. A. & Hornberger, T. A. Resistance exercise-induced hypertrophy: a potential role for rapamycin-insensitive mTOR. Exerc. Sport Sci. Rev. 47, 188–194 (2019).

66. Castets, P. et al. Sustained activation of mTORC1 in skeletal muscle inhibits constitutive and starvation-induced autophagy and causes a severe, late-onset myopathy. Cell Metab. 17, 731–744 (2013).

67. Chantranupong, L., Wolfson, R. L. & Sabatini, D. M. Nutrient-sensing mechanisms across evolution. Cell 161, 67–83 (2015).

68. Zhang J. et al. EglN2 associates with the NRF1-PGC1α complex and regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev. 34, 349–358 (2020).

69. Lee, J. H. et al. Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. Cell Metab. 16, 311–321 (2012).

70. Kimball, S. R., Gordon, B. S., Moyer, J. E., Dennis, M. D. & Jefferson, L. S. Leucine induced dephosphorylation of Sestrin2 promotes mTORC1 activation. Cell Signal. 28, 896–906 (2016).

71. Kristensen, A. R. et al. Ordered organelle degradation during starvation-induced autophagy. Mol. Cell. Proteom. 7, 2419–2428 (2008).

72. Lee, J. H. et al. Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. Cell Metab. 16, 311–321 (2012).

73. Ogasawara, R., Jensen, T. E., Goodman, C. A. & Hornberger, T. A. Resistance exercise-induced hypertrophy: a potential role for rapamycin-insensitive mTOR. Exerc. Sport Sci. Rev. 47, 188–194 (2019).

74. Xu, D. et al. Evidence for A Role for Sestrin1 in mediating leucine-induced activation of mTORC1 in skeletal muscle. AJP Endocrinol. Metab. 262, 21 (2019).

75. Kimball, S. R., Gordon, B. S., Moyer, J. E., Dennis, M. D. & Jefferson, L. S. Leucine induced dephosphorylation of Sestrin2 promotes mTORC1 activation. Cell Signal. 28, 896–906 (2016).

76. Giretmezler, C. et al. Degradation of protein translation machinery by amino acid starvation-induced macroautophagy. Autophagy 13, 1064–1075 (2017).

77. Tomas, A. R. et al. Ordered organelle degradation during starvation-induced autophagy. Mol. Cell. Proteom. 7, 2419–2428 (2008).

78. Lee, J. H. et al. Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. Cell Metab. 16, 311–321 (2012).

79. Duran, R. V. et al. HIF-independent role of prolyl hydroxylases in the cellular response to amino acids. Oncogene (2012).

80. MacKenzie, E. D. et al. Cell-permeating-ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. Mol. Cell. Biol. 27, 3282–3289 (2007).

81. Epstein, A. C. et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107, 54 (2001).

82. Keskiaho, K., Hieta, R., Sormunen, R. & Myllyharju, J. Chlamydomonas reinhardtii has multiple prolyl 4-hydroxylases, one of which is essential for proper cell wall assembly. Plant Cell 19, 256–269 (2007).

83. Rennie, M. J. et al. Muscle protein synthesis measured by stable isotope techniques in man: the effects of feeding and fasting. Clin. Sci. 63, 519–523 (1982).

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Author contributions
G.D. and I.S.-A. designed the study, conducted the experiments, performed the laboratory analysis, wrote and edited the paper. E.M., K.V., P.K. and G.F. conducted the experiments and performed the laboratory analysis. B.S., and L.B. provided the human muscle samples. P.K. performed the in vitro hydroxylation experiments. S.K., L.D., B.B., P.C. and S.-M.Z. provided the reagents/materials and experimental protocols. K.D.B., conceptualized and designed the study and wrote the paper.

Competing interest
The authors declare no competing interests.

Additional information
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