KU LEUVEN

GROEP BIOMEDISCHE WETENSCHAPPEN

FACULTEIT BEWEGINGS- EN REVALIDATIEWETENSCHAPPEN

Can L-leucine counteract the negative role of a PHD1 KO in protein metabolism?

door Christel VAN BEIJSTERVELD
en Anneleen VAN BULCK

masterproef aangeboden, tot het behalen van de graad van Master of Science in de revalidatiewetenschappen en kinesitherapie

o.l.v.
prof. dr. L. Deldicque, promotor
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WOORD VOORAF

Langs deze weg willen wij graag onze oprechte dank betuigen aan iedereen die ons begeleid en gesteund heeft bij het realiseren van deze masterproef. Zonder de gewaardeerde hulp van bepaalde personen was het niet mogelijk geweest deze thesis te schrijven. Allereerst willen wij onze co-promotor, drs. Gommaar D'Hulst bedanken voor de professionele begeleiding en ondersteuning. Wij kijken terug op een zeer fijne samenwerking de voorbije 2 jaren waarin hij ons vele nieuwe inzichten heeft verschaft, kennis heeft laten maken met lab technieken en altijd bereikbaar was als we door de bomen het bos niet meer zagen. Daarnaast willen wij ook onze promotor prof. dr. Louise Deldicque bedanken voor haar deskundige en kritische feedback alsook het mogelijk maken van de experimenten in het laboratorium. Verder willen wij nog een woord van dank richten aan prof. dr. Peter Carmeliet voor zijn vriendelijke bereidheid tot het ter beschikking stellen van de muizen voor onze studie. Daarnaast mogen wij dhr. Patrick Peeters ook niet vergeten te bedanken voor het uitgebreid nalezen en corrigeren van onze thesis. Tot slot willen wij de personen die ons goede raad en steun hebben gegeven gedurende de afgelopen 2 jaren, namelijk onze ouders, broers, zussen, vrienden en huisgenoten, van harte bedanken.

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Dongen, mei 2015 C.V.B.

Halle, mei 2015 A.V.B.

SITUERING

Deze masterproef is gekaderd binnen het project 'Effecten van hypoxie op proteïnemetabolisme' van de onderzoeksgroep Inspanningsfysiologie van de faculteit Bewegings- en Revalidatiewetenschappen aan de Katholieke Universiteit Leuven. Deze onderzoeksgroep bestudeert onder andere de rol van hoogte/hypoxie en voeding op de fysiologische respons via *in vitro* (spiercelculturen) en *in vivo* (knaagdieren of mensen) experimenten.

Langdurig verblijf op hoogte of een pathologische aandoening (zoals een longaandoening) leiden tot verlies van spiermassa (1-3). Het is algemeen geaccepteerd dat langdurige hypoxie leidt tot een negatieve regulatie van het protëinemetabolisme, ondanks publicaties met tegenstrijdige resultaten (4,5). Met behulp van moderne technieken o.a. het manipuleren van een specifiek gen genaamd Prolyl Hydroxylase Domain 1 (PHD1), kunnen we de respons van hypoxie simuleren (6). Dit model is dus uiterst geschikt voor het bestuderen van de langdurige effecten van hypoxie op cellulair niveau. Aan de andere kant zijn er ook mechanismen die het verlies van spiermassa tegengaan, één daarvan is leucine, een essentieel aminozuur dat de aanmaak van nieuwe proteïnen in de spiercel stimuleert. Op deze manier zorgt leucine op lange termijn voor een verhoging van spiermassa (7-9). Uit het bovenstaande kunnen we opmaken dat hypoxie en leucine een tegengestelde invloed hebben op de regulatie van proteïnesynthese.

Deze masterproef onderzoekt het effect van L-leucine toediening op de proteïnesynthese in PHD1 KO muizen. De moleculaire mechanismen hierachter zijn, naar ons weten, nooit eerder *in vivo* bestudeerd.

Dit onderzoek is maatschappelijk relevant binnen de sportwereld, zo zou men op deze manier de positieve effecten van zowel een hoogtestage alsook proteïnesupplementatie kunnen combineren. Ook voor patiënten met chronische long- of bloedaandoening zou men hierin een oplossing kunnen vinden voor de toegenomen spierzwakte en mortaliteit bij deze patiëntengroep (3,10).

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Can L-leucine counteract the negative role of a PHD1 KO in protein metabolism?

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Key words: hypoxia, hypoxia-inducible factor (HIF), mammalian target of rapamycin (mTOR), protein synthesis, transgenic mice, prolyl hydroxylase domain (PHD), gene knock out (KO), leucine.

Background: L-leucine administration and PHD1 KO in mice, two opposite regulators of protein synthesis, are examined *in vivo*.

Results: Markers of protein synthesis are not altered after L-leucine administration in PHD1 KO mice.

Conclusion: Neither L-leucine nor PHD1 KO prevail on the regulation of protein synthesis.

Significance: Benefits of leucine administration on chronic hypoxia induced skeletal muscle atrophy.

ABSTRACT

Chronic hypoxia induced by environmental or pathological conditions negatively affects protein metabolism and is associated with muscle atrophy. Opposite effects are seen when the amino acid L-leucine is administered. Whether L-leucine counteracts the negative role of hypoxia in protein metabolism *in vivo* is open for investigation. Wild type (WT) and mice lacking the gene that encodes for oxygen sensor PHD1 (PHD1 KO) were orally administered with saline (CTRL) or L-leucine (LEU). The mice were randomly assigned to one of the four groups; (1) WT with saline (WT/CTRL), (2) WT with L-leucine (WT/LEU), (3) PHD 1 KO with saline (KO/ CTRL) and (4) PHD1 KO with L-leucine (KO/LEU). Skeletal muscles (m. soleus, m. tibialis anterior, m. gastrocnemius) were extracted in anesthetized mice, thirty minutes after gavage. Immunoblotting was performed for the markers for cellular stress (AMP-activated protein kinase (AMPK) and regulated in development and DNA damage response 1 (Redd1)) and for protein synthesis (mTOR and ribosomal protein S6Kinase-1 (S6K1). We hypothesized that oral administration of L-leucine stimulates protein synthesis despite a PHD 1 KO. No significantly differences were seen in the hypoxic markers between the different groups and between the muscles. S6K1 was significant increased after L-leucine administration in WT mice in m. gastrocnemius ($p = 0.005$) but no statistically significant difference was found in the PHD1 KO group in all three muscle groups. These results suggested that L-leucine administration did not predominate on PHD1 KO in regulating protein synthesis.

Introduction

Leucine is an essential amino acid (AA) in humans, meaning it cannot be produced by the organism itself. Therefore, it is a required component in our diet, especially found in high quality protein food like eggs, beans, rice, chicken, nuts etc. Leucine, together with isoleucine and valine, is one of the branched-chain amino acids (BCAA) and has varied functions. First, it modulates leptin synthesis and secretion from adipose tissue and thereby influences weight gain, food intake, fat deposition, body temperature, energy consumption, protein metabolism and insulin function (1). The second function of leucine is regulation of glucose homeostasis by influencing insulin release (2). The last but most important function for our research is the stimulating effect of leucine on protein synthesis in skeletal muscle. Leucine exerts these effects of protein synthesis by influencing some important steps of the translation (3).

Protein synthesis contains two steps, transcription and translation, and each occurs in approximately three phases (initiation, elongation and termination). The mammalian target of rapamycin complex 1 (mTORC1) is a serine/threonine protein kinase (Ser/Thr kinase) that controls a number of components that are involved in the initiation and elongation stages of translation (4). Besides protein synthesis, mTORC1 regulates other anabolic processes like lipid biogenesis and limits the catabolic processes such as autophagy (5). External stimuli like growth factors, nutrients, oxygen levels and energy status influence the mTORC1 activity and signalling. This study will investigate the response of mTORC1 and proteinsynthesis *in vivo,* after L-leucine administration in a hypoxic condition. Before elaborating on mTORC1 and the effects of the above mentioned stimuli on its regulation, we first describe the adaptions of the cell in response to hypoxia.

HIF - regulation in hypoxia

When oxygen demand exceeds supply, cellular stress occurs and this is called hypoxia (6). This state of lowered oxygen tension (pO_2) in tissue can be created by environmental conditions such as high altitude or by pathological conditions such as chronic obstructive pulmonary disease, obstructive sleep apnoea or anaemia (7). In these cases pO_2 serves as an accurate physical measure of the state of oxygenation (8). To deal with hypoxic conditions, cells have evolved complex mechanisms for adaptation and survival. One well preserved mechanism is the stabilisation of Hypoxia Inducible Factor (HIF), which acts as transcription factor to prepare the cell for conditions of low oxygen. This is achieved by translating genes involved in angiogenesis, oxygen transport and inhibition of cell growth via mTORC1 (9).

HIF is a heterodimer complex that consists of an oxygen-sensitive labile α-subunit (HIF-α) located in the cytoplasm and a stable β-subunit (HIF-β), originally known as the aryl hydrocarbon receptor nuclear translocator (ARNT), located in the nucleus. HIF-α and HIF-β both have three subunits: HIF-1α, HIF-2α, HIF-3α and ARNT 1, 2 and 3 respectively (10) (figure 1). Both α and β subunits belong to the bHLH-PAS family containing an N-terminal basis helix-loop-helix (bHLH) domain followed by two PAS domains for DNA binding and dimerization (11). The α-subunits also contain an O_2-dependent degradation domain (ODDD), which harbors the two conserved prolines (Pro402 and Pro564) and two transactivation domains: N-terminal activation domain (NTAD) and C-terminal activation domain (CTAD) (12-14).

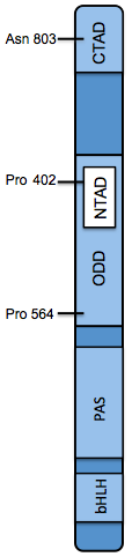

Figure 1: Structure Hypoxia Inducible Factor- α (HIF-α)

The HIF-β subunits are constitutively expressed and are thereby not influenced by changes in oxygen levels. In contrast, the HIF-α subunits are highly unstable in the presence of oxygen and will be hydroxylated by HIF hydroxylases. This causes binding of the von Hippel-lindau tumour suppressor protein (pVHL) (6). HIF-α is now marked for subsequent proteasomal degradation through ubiquitylation at several sites (15) (figure 2).

The oxygen dependent hydroxylases that modify HIF-α are prolyl hydroxylases (PHDs) and factor-inhibiting HIF (FIH), both are active on different sites of the HIF-α. Important note is that in a normoxic situation PHD is more determined for the direct degeneration pathway of HIF-α (16). Both belong to a group of non-equilibrium enzymes because they only catalyse the hydroxylation of their substrate and not the reverse reaction (8).

The first hydroxylation reaction of proline (Pro-OH) is catalysed by PHDs (6,17). Three distinct forms have been characterizes named: PHD1, PHD2 and PHD3 (17). They differ in their expression patterns, tissue distribution, subcellular localization and their ability to hydroxylate HIFs (18). In the presence of oxygen, the PHDs use molecular oxygen to hydroxylate the conserved Pro-564 and Pro-402 residues within the ODD (17,19). PHD1 and PHD2 hydroxylate both residues, only PHD3 recognises pro- 564 (8). Chan et al. found that hydroxylation of pro- 564 enhances the hydroxylation of proline 402, thus providing pVHL two recognition sites to target HIF-α for degradation. The second hydroxylation is performed by FIH which hydroxylates an asparaginyl residue (Asn-803) in the CTAD (carboxy-terminal transactivation domain) of HIF-1α (17,20,21). This prevents the interaction of the coactivator p300/CREB-binding protein, and leads to a reduction in transcription activity of the HIF-1

target genes (17). Similarly with PHD, FIH also belongs to a group of non-equilibrium enzymes because they only catalyse the hydroxylation of their substrate and not the reverse reaction (8).

In contrast, in a situation of hypoxia HIF hydroxylase activity is reduced because less oxygen is available for hydroxylation and HIF-α escapes recognition by pVHL (6). This results in protein stabilization and HIF-α can translocate into the nucleus, where it associates with HIF-β and with the cysteine/histidine-rich domain 1 (CH1) of co-activators p300/CBP (6,12). This leads to gene expression by binding to the conserved hypoxia responsive elements (HRE) on the promotor of target genes (6). By doing this it will either induce or suppress gene expression. These activated HIF-target genes fall into two categories whose functions aim to restore energy and O_2 homeostasis within the cell. This through stimulation of anaerobic energy production by adapting glycolytic enzymes and improving tissue oxygenation via stimulated angiogenesis, vasodilation and erythropoiesis (8,9).

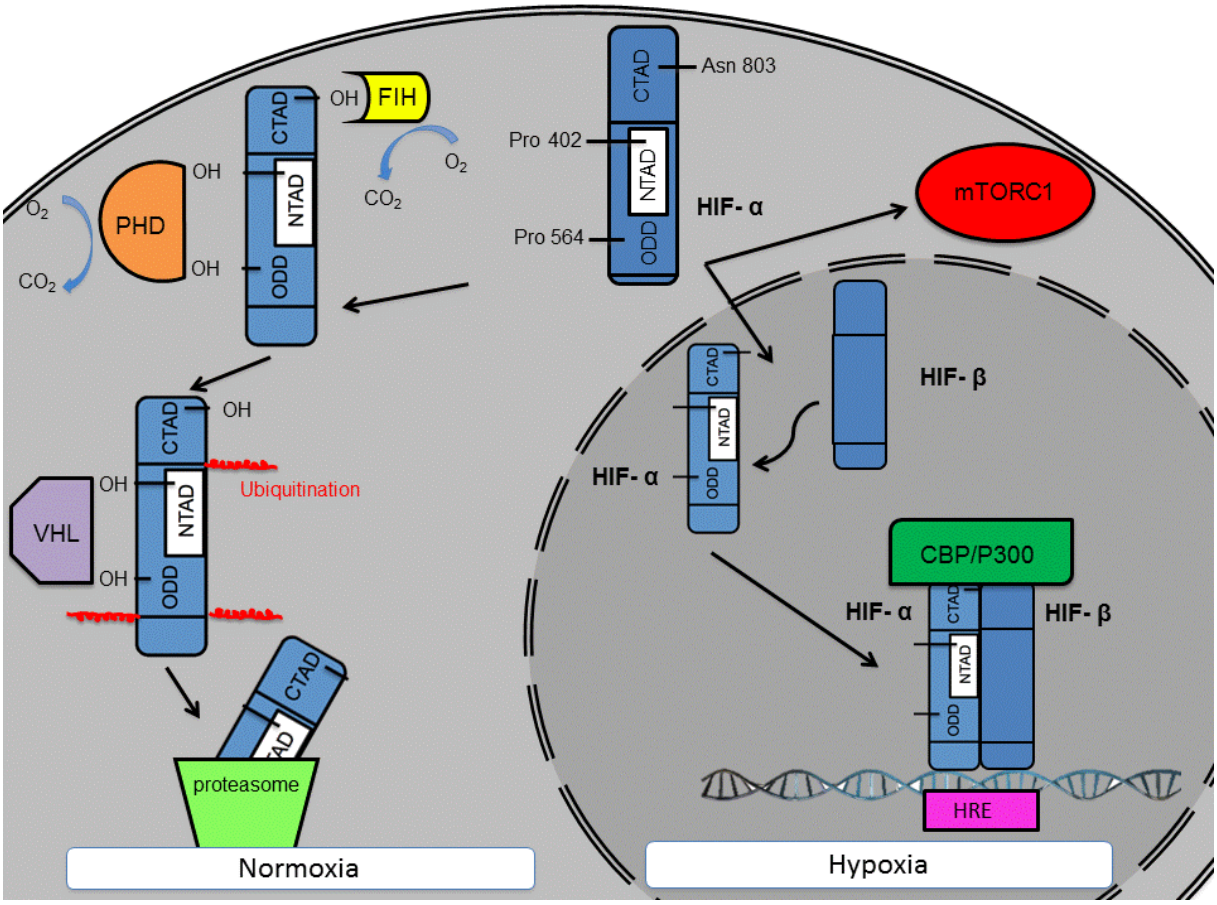

Figure 2: HIF regulation during normoxia and hypoxia (based on Fandrey et al. (8))

In hypoxic conditions, two pathways control the amount of PHDs and FIH. First, hypoxia will lead to an accumulation of Siah E3 ubiquitin protein ligase 2 (Siah2). This protein controls the proteasome-mediated degradation of PHD1, PHD3 and FIH and thereby plays a role in stabilization of HIF-α through targeting PHD and FIH (22,23). It remains unclear if a similar mechanism exists for PHD2 (22). Research by Nakayama showed that Siah2 knockout mice decreased metabolism but failed to increase the rate of ventilation, whereas the wild-type mice responded to the acute phase of hypoxia by increasing ventilation and decreasing metabolism (22). Secondly, there is a negative feedback loop in response to hypoxia. Although, hypoxia decreases overall PHD activity, up regulation of HIF-1α induces the expression of PHD 2 and PHD 3 (24). The reason, being in a more severe or a longer period of hypoxia, a more rapid rate of degradation of HIF-1α to a normoxic situation will occur.

Like mentioned before, HIF signalling is not the only pathway linking declining pO_2 with DNA expression but it is considered as the major component in survival of cells (8). The mammalian target of rapamycin complex 1 (mTORC1) is also influenced by hypoxia which affects protein synthesis and muscle mass. Indeed, the HIF-1 is most important in the cellular response for oxygen homeostasis and interferes with mTORC1 in the response to hypoxia (25).

mTORC1

The mammalian target of rapamycin (mTOR) consists of two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2); both complexes are involved in cell growth. In broad terms, mTORC1 regulates when the cell grows and mTORC2 regulates where it grows (26). Since many cellular processes are regulated by mTOR it is also involved in conditions such as diabetes, cancer and ageing (27). Our primary focus here is mTORC1 because this is activated by amino acids (AAs) through different pathways.

mTORC1 is an homodimer and consists in addition to mTOR of four components: regulatory-associated protein of mTOR (raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL), proline rich AKT substrate 40 kDA (PRAS40) and DEP-domain-containing mTOR interacting protein (Deptor) (5). Raptor is essential for recruitment of downstream kinase substrates like 4E-binding protein 1 (4E-BP1), while the function of mLST8 for mTORC1 remains unclear (28,29). On the other hand, PRAS40 and Deptor inhibit the activity of the mTORC1. When the mTOR component of the mTORC1 is activated, it phosphorylates both inhibitors to enhance the kinase activity of the complex. This will affect protein synthesis via modulation of translation initiation and elongation (30,31).

Upstream regulation of mTORC1

As mentioned above, mTORC1 is a central molecular node controlled by four major input signals: growth factors, energy status, oxygen levels and AAs (figure 3). Nutrients, containing AAs, and growth factors have a stimulating effect while low energy levels and low oxygen levels inhibit the protein kinase activity (32). Central component in the relation between the four factors and mTORC1 activity, is the tuberous sclerosis complex (TSC 1/2) that contains TSC1 (also known as hamartin) and TSC2 (also known as tuberin) (5). TSC 1/2 inhibits mTOR through functioning as a GTPase-activating protein (GAP) toward the small G-protein Rheb (33). Rheb binding to mTOR enables activating the protein kinase (34). TSC 1/2 regulates Rheb-GTP levels, thereby is Rheb a direct target of TSC ½ complex, (35-37). In addition, other cellular conditions and/or signals such as inflammation, Wnt ligand, genotoxic stress also have an influence on mTORC1 regulation but will not further be discussed here (5).

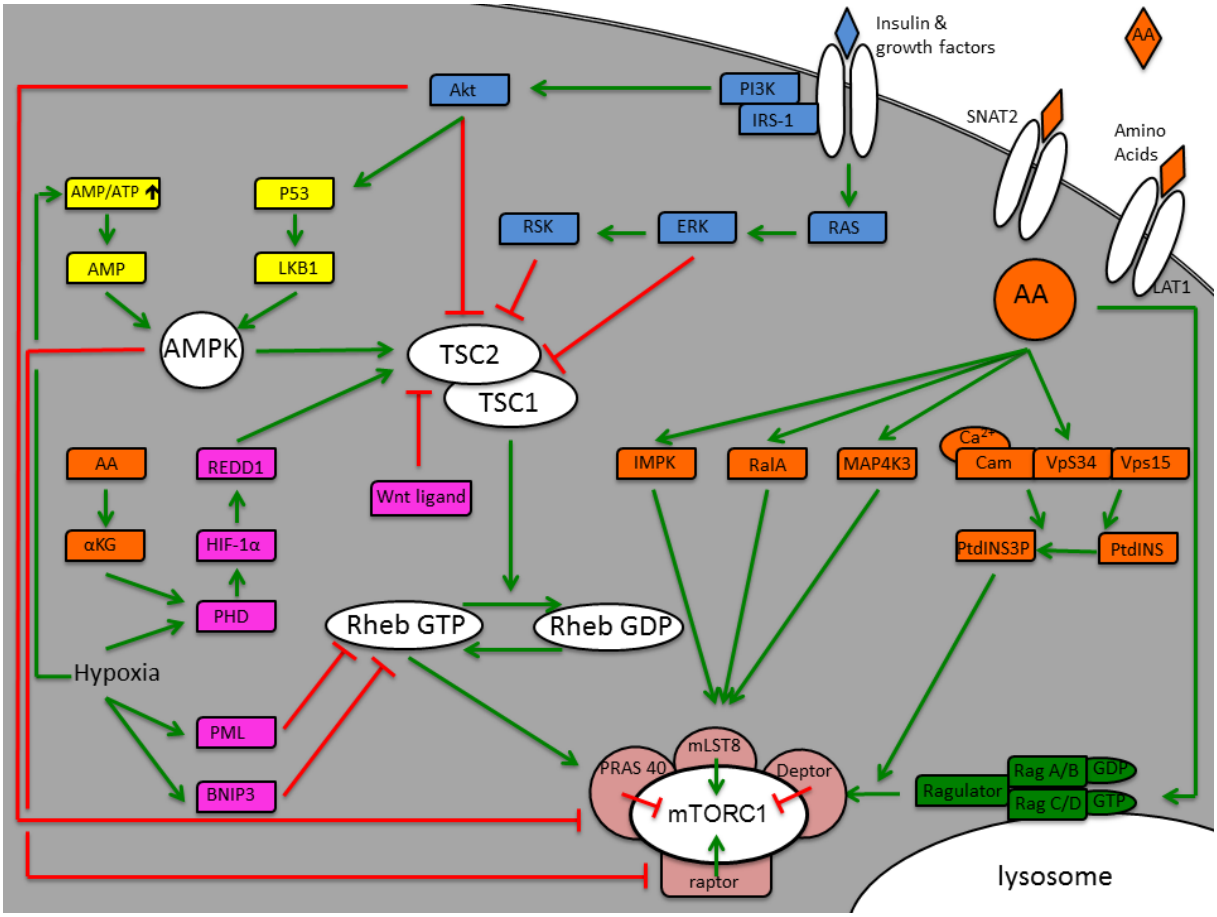

Figure 3: Upstream regulation of mTORC1 by four major input factors; growth factors (blue), energy status (yellow), oxygen levels (pink) and amino acids (orange).

The distinct upstream signals regulate mTORC1 through different pathways. The regulation occurs through direct modification of mTORC1 components or through interference with the TSC1/2 and the related Rheb GTPase. First, blood concentration of growth factors such as insulin and IGF-1 regulates anabolic cell processes such as translation, lipid biosynthesis and nutrient storage via mTORC1 through the phosphoinositide 3-kinase (PI3K) pathway and Ras-MAPK signalling pathway (via ERK and RSK) (31). Binding of insulin (or insulin-like growth factor IGF) recruits the insulin receptor substrate (IRS) and PI3K, to the insulin receptor and activates Akt (also called protein kinase B (PKB)) (27). Akt directly phosphorylates TSC2 on multiple sites and thereby suppress TSC2 function, thus promoting mTORC1 activation (38). In addition Akt can, independent of TSC2, activate mTORC1 by promoting phosphorylation and causes inhibition of PRAS40 from mTORC1 (39).

Secondly, cellular stress results in a lowered ATP level, which inhibits mTORC1. This inhibition is regulated by AMP-activated protein kinase (AMPK), an intracellular energy sensor that takes care of the cell energy balance. Indeed, low energy levels, expressed as increased ADP/ATP ratios stimulate AMPK to switch off anabolic processes and to activate catabolic processes as reviewed by Hardie et al. (40,41). As a consequence, mTORC1 signalling is blocked due to phosphorylation of TSC2 (increasing its GAP activity) in an AMPK-dependent manner. Furthermore, Gwinn et al. showed that AMPK can also directly phosphorylate raptor resulting in inhibition of mTORC1 without interference of TSC2 (42).

Oxygen levels are the third mechanism in regulating mTORC1 signalling through multiple mechanisms. In a state of low oxygen levels, cells shift their metabolic program from oxidative phosphorylation to anaerobic glycolysis and as a consequence ATP levels decrease (31). This activates AMPK and causes mTORC1 inhibition through TSC1/2 as mentioned above. Redd1 (regulated in development and DNA damage response 1) can also influence TSC 1/2 in response to hypoxia and thereby influence mTOR activity (43). Redd1 binds to 14-3-3 of the TSC2 and separates them from each other and activates TSC 1/2 complex (44). The expression of Redd1 can be upregulated through HIF-1α in response to hypoxia (45). TSC 1/2 independent mechanism of mTORC1 regulation in response to hypoxia occurs by promyelocytic leukemia (PML) tumour suppressor and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) as reviewed by Wouters et al., but these will not be further discussed here (46).

Finally, Hara et al. showed that AAs influence translation control through phosphorylation of mTOR, independent of the growth factor pathways (47). AAs are transported in the cell with a glutamate system using two transporters; L-transporter 1 (LAT1) and sodium coupled amino acid transporter 2 (SNAT2) as reviewed by Dodd et al. (30). This relation between SNAT2 and LAT1 is called "tertiary active transport" and described by Baird et al. (48). Poncet et al. demonstrated the relationship between the LAT1 transporter and the control of muscle mass by confirming that AA delivery by LAT1 is important for activation of the mTOR-S6K1 signalling (49). The mechanism by which AAs influence mTORC1 signalling remains unclear in the literature; different mechanisms have been proposed. One mechanism is the regulation through the four Rag proteins expressed in mammals: RagA, RagB, RagC and RagD. These four can combine into heterodimers consisting of GTP-bound Rag A or B complexed with GDP-bound Rag C or D (50). Sancak et al. found that when AAs are available in the cell, the Rag proteins will bind to raptor (50). In response, they will promote the relocalization of mTOR to a perinuclear region that contains its activator Rheb (50). The results from a study of Durán et al., are more than relevant; they found that PHD activity is required for mTORC1 activation by AAs through the Rag GTPases (51). PHDs are dependent on α- ketoglutarate (αKG) for their function in the prolyl hydroxylation of HIF-α. αKG in its turn is related to AA metabolism. Under AAs starvation there is a lack of αKG and PHD activity is inhibited resulting in inhibition of mTORC1 (51). The loss of PHD activity did not lead to HIF stabilization and therefore PHD could also be seen as nutrient sensors (51).

Another possible mechanism of AAs to stimulate mTOR is the increased expression of Vacuolar protein sorting 34 (Vps34) after leucine administration (52). Vps3 binds to active complex Vps15, Cam and Ca^{2+} to increase mTORC1 signalling (30). Besides the Rag proteins and Vps53, Mitogen-activating protein kinase kinase kinase kinase-3 (MAP4K3), Ral protein A (RalA) and inositol polyphosphate multikinese (IMPK) have also been suggested as interfering factors in mediating mTORC1 signalling. Increased phosphorylation of 4E-BP1 and S6K1 is found through overexpression of the MAP4K3 kinase, suggesting it plays an important role in mTORC1 signalling (53).

Downstream regulation of mTORC1

Once mTORC1 is stimulated, one of its functions is the regulation of protein synthesis by activating the downstream substrates 4E-BP1 and S6K1 (5). Both are involved in the initiation and elongation of mRNA translation and thereby influence the process of protein synthesis (figure 4).

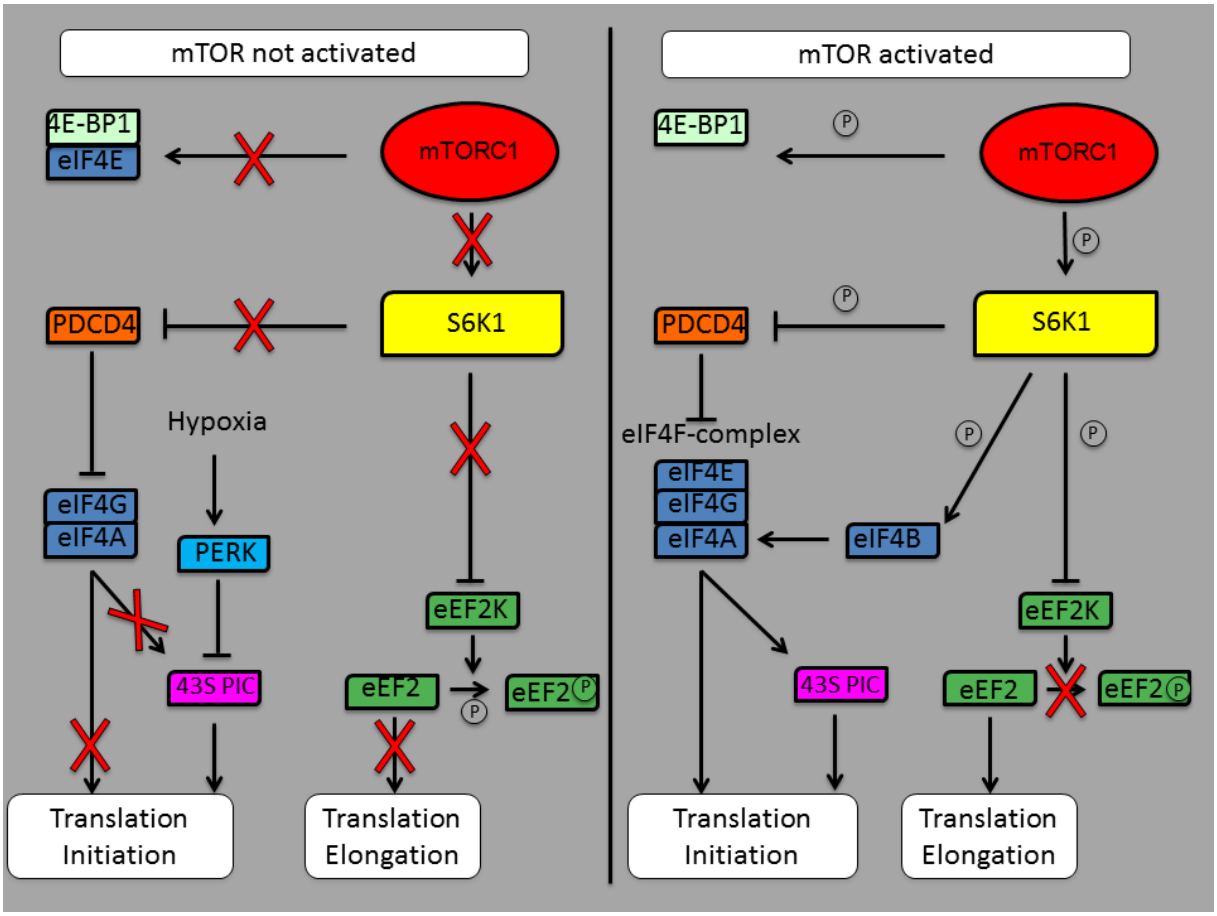

Figure 4: Processes downstream of mTORC1. Left: when mTORC1 is not activated. Right: when mTORC1 is activated.

The initiation of mRNA translation is a complex process, but two steps in this phase are crucial: the formation of the eIF4F Complex and the formation of the 43S Pre-initiation Complex (43S PIC). The eIF4F complex consists of the cap- binding protein eukaryotic Initiation Factor 4E (eIF4E), a scaffold protein eukaryotic Initiation Factor 4G (eIF4G) and an ATP-dependent helicase protein eukaryotic Initiation Factor 4A (eIF4A) (20). The formation of this complex can be prevented by binding of 4E-BP1 to eIF4E. In hypophosphorylated state, 4E-BP1 binds to eIF4E and inhibits the formation of the eIF4F Complex. mTORC1 phosphorylates 4E-BP1, leading to the release of eIF4E and the formation of the eIF4F complex (54). This complex now stimulates translation initiation.

Anthony et al. investigated the protein synthesis response of skeletal muscles after an orally administered dose of leucine in male rats. They found a stimulatory effect of leucine on the formation of the eIF4F complex. This was assessed by the phosphorylation status of eIF4E binding protein and the association of the eIF4E with 4E-BP1 and eIF4G (55). In contrast, hypoxia leads to a hypophosphorylated state of mTOR and its effector 4E-BP1 and thereby prevents translation initiation (56). Also S6K1 influences proper eIF4F Complex formation. First, this is achieved by

phosphorylating the eukaryotic translation Initiation Factor 4B (eIF4B) that stimulates the activity of eIF4A (57). Secondly, it is completed by phosphorylating the tumour suppressor PDCD4 and thereby preventing negative regulation of eIF4A (58).

Assembly of the eIF4F facilitates the second major control point in the regulation of the translation initiation: the formation of the 43S PIC, which includes the binding of methionyl-tRNAi (met-tRNAi) to the 40S ribosomal subunit (59). This binding step is mediated by eukaryotic Initiation Factor 2 (eIF2), a GTP binding protein. This eIF2 interacts with Met-tRNAi in GTP bounded state and thus forms a ternary complex, eIF2-GTP-Met-tRNAi(60). This complex binds to a 40S ribosomal subunit and then translocates to the AUG start codon where a hydrolysis occurs of the eIF2-GTP into GDP (60). The eIF5 protein stimulates the hydrolysis of the eIF2-bound GTP into GDP and eIF2B ensures the reverse reaction. A phosphorylation of the α-unit of EIF2 prevents the exchange of GDP into GTP but not the binding of eIF2B to eIF2. As a result, eIF2B remains bound to the phosphorylated and inactive eIF2 complex (59). This phosphorylation of eIF2-α could occur in a state of hypoxia where protein kinase RNA-like ER kinase (PERK) could be responsible for the phosphorylation and thereby inhibits protein translation (59).

The translation elongation is controlled through the phosphorylation of eukaryotic elongation factor 2 (eEF2). mTORC1 regulates the phosphorylation of eEF2 by acting on the eukaryotic Elongation Factor 2 kinase (eEF2K) which phosphorylates and inhibits eEF2 (61). Wang et al. showed that eEF2K is a substrate for S6K1; this protein phosphorylates eEF2K at different sites and thereby inactivates the kinase (62). Elongation is now continued forming novel proteins.

In vivo experiments have shown that leucine administration promotes phosphorylation of 4E-BP1 and S6K1 and thereby enhances muscle protein synthesis (63). Crozier et al. examined the effect of different leucine doses on the biomarkers of protein synthesis. They concluded that low doses effectively stimulate 4E-BP1 and S6K1 and their targets. Important remark: administration of high doses of leucine produces a slight increase in circulating insulin levels and may further stimulate mRNA translation in the skeletal muscle, because of involvement of the PI3K-pathway (64). Therefore they recommend that more research is necessary to prove the right dose that ensures no insulin increase. It is possible that insulin secretion by small doses of leucine compared to high doses happens quicker and that therefore the increase in insulin could be induced due to a transient leucine induced increase (64).

Based on the current knowledge in the literature, leucine and hypoxia have opposite effects on mTOR regulation and protein synthesis in the cell. The aim of our study is to analyse the effects of L-leucine administration in PHD1 KO mice on the regulation of protein synthesis. Aragonés et al. showed that in the absence of PHD1 the oxygen consumption is reduced in skeletal muscle *in vivo* (65). This PHD1 KO induces a reprogramming of the metabolism, by increasing HIF stabilization. This occurs only in muscle tissue while other tissues will not be affected.

We hypothesize that administration of L-leucine will upregulate mTOR signalling in PHD1 KO mice and thereby promote protein synthesis. We think that L-leucine will prevail on PHD1 KO because previous reports have shown that individual silencing of PHDs does not block mTORC1 activation by AAs (51). In our study we only inhibit PHD1 so PHD2 and PHD3 will remain available for mTORC1 stimulation after AA administration through increased intracellular αKG concentration. Only when all three different PHDs are silenced the ability of AAs to activate mTORC1 signalling will be blocked (51). This master thesis is unique because it measures the response of both L-leucine administration (an anabolic stimulus) and a PHD1 gene KO (catabolic stimulus) on protein synthesis in one mouse model. We aim to formulate an answer of the following question; "Will L-leucine counteract the negative effects of a PHD1 KO on protein metabolism?"

Materials and methods

Ethics statement. All mice experiments were performed under the guidance of the Department of kinesiology, Exercise Physiology Research group, Faber, KU Leuven. The experimental procedures were approved by the local Ethical Committee for Animal Experimentation of the KU Leuven, in accordance with the Belgian and European laws, guidelines and policies for animal experimentation, housing and care.

Experimental animals. 63 C57BL/6 mice (PHD1 (cKO) tm Cond KO L/L, CKmm.cre TG WW or Tg/WW (main exp. C57BL/6)) were maintained in the Central Animal Facility of KU Leuven under standard conditions with 12-h dark/light cycles and normal temperature (20°, ± 2°). They were housed in cages per 3-5 mice and received, ad libitum, water and standard chow. We used wild type (WT) mice and PHD1 knock out (KO) mice (referred to as PHD-1 $^{-}/^{-}$) kindly donated by prof. Peter Carmeliet. Briefly, the PHD-1 $^{-}/^{-}$ is a conditional KO, a mouse with a "floxed" gene sequence is crossed with mice expressing Cre gene under control of a promotor specific for muscles.

Experimental protocol. The mice were assigned randomly to one of the following four groups; (1) WT matched controlled with saline (WT/CTRL) n = 17, (2) WT with L-leucine (WT/LEU) n = 12, (3) PHD-1 $^{-}/^{-}$ (KO) matched controlled with saline (KO/ CTRL) n= 12 and (4) PHD-1 $^{-}/^{-}$ (KO) with L-leucine (KO/LEU) n= 19. Blinding of the experiment was not performed. The mice were food deprived for 10 hours and then randomly saline (0.9% sodium chloride (NaCl), 10 µl/10g BW) or L-leucine (0.2 g/kg BW) was administered by oral gavage. Thirty minutes later, the mice were anesthetized and muscle tissue (m. soleus, m. gastrocnemius and m. tibialis anterior) from hind limbs was extracted. All collected tissue samples were snap frozen in liquid nitrogen and stored at -80° until needed.

Western Blot. Details of the immunoblotting procedures were previously described by D'Hulst et al. (7). Briefly, frozen muscle tissue (± 20 mg) was homogenized (3 cycles of 5 sec) with a Polytron mixer in ice-cold buffer (1 : 10, w/v) [50 mM Tris–HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride,1 mM DTT, 0.1% Triton-X 100 and a complete protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium)]. Homogenates were then centrifuged at 10 000 g for 10 min at 4 °C. The supernatant was collected and immediately stored at -80 °C. The protein concentration was measured using the DC protein assay kit (Bio-Rad laboratories, Nazareth, Belgium). 30–80µg of proteins was separated by SDSPAGE (8–12% gels) and transferred to PVDF membranes.

Subsequently, membranes were blocked with 5% non-fat milk for 1 h and afterwards incubated overnight (4 °C) with the following antibodies (1 : 1000, Cell Signaling, Leiden, the Netherlands): Phospho-AMPKα Thr^{172}, total AMPK, Redd1, phospho-mTOR ser^{2448}, total mTOR, phospho-S6K1 $Thr^{389,}$ total S6K1 and total eEF2 (Cell signaling, Leiden, The Netherlands). Appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, Bornem, Belgium) were used for chemiluminescent detection of proteins. Membranes were scanned and quantified with Genetools and Genesnap softwares (Syngene, Cambridge, UK) respectively. Then, membranes were stripped and reprobed with the antibody for the total form of the respective protein to ascertain the relative amount of the phosphorylated protein compared with the total form throughout the whole experiment. The results are presented as the ratio phosphorylated/total forms of the proteins and the ratio interested protein/ eEF2. A value of 1.0 was assigned to the mean value of the WT/CTRL group to which all other values from the respective condition were reported.

Statistical analysis. Outliers were removed when the difference was greater than four Standard Error of Measurements (SEMs) above or under the mean. All other data were analysed by SigmaStat statistical software package for Windows version 3.5. For statistical comparison of age and weight between groups, a non-paired *t*-test was used. A one-way Analysis of Variance (ANOVA) was performed to assess main (protein synthesis) vs. interaction effects with L-leucine administration and PHD-1 $^{-}/^{-}$ as independent variables. A Bonferroni post hoc test was performed when group effects were observed. For comparison of the different muscle groups, we used a two-way ANOVA. For all statistical tests a p-value < 0.05 was considered to represent a significant difference. Results are expressed as the means ± SEM.

Results

Age and weight in KO- and WT-mice

There was no statistically significant difference between the age and weight of the two groups (p = 0,887 and p = 0,519 respectively) (figures 5a. and 5b.). PHD1 KO mice were not older or heavier than WT mice. Even if weight is controlled for age there is no statistically significant difference between the two groups (figure 5c.) (p = 0.981).

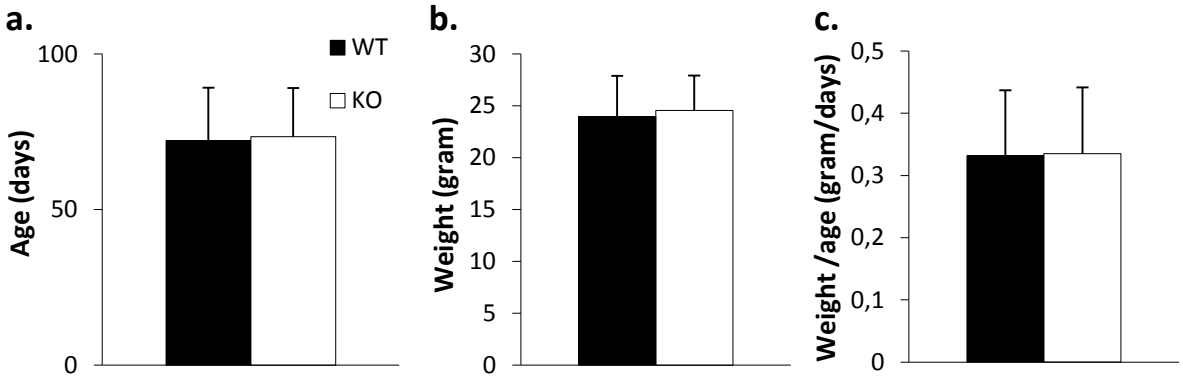

Figure 5: Age (a.), Weight (b.) and Weight to age ratio (c) for WT (black bar) and PHD1 KO (white bar) mice.

Phosphorylation of AMPK subunit α at Thr^{172}

No significant differences (p= 0,492) were observed between the different groups, measuring the phosphorylated AMPK / total AMPK ratio in m. gastrocnemius (figure 6).

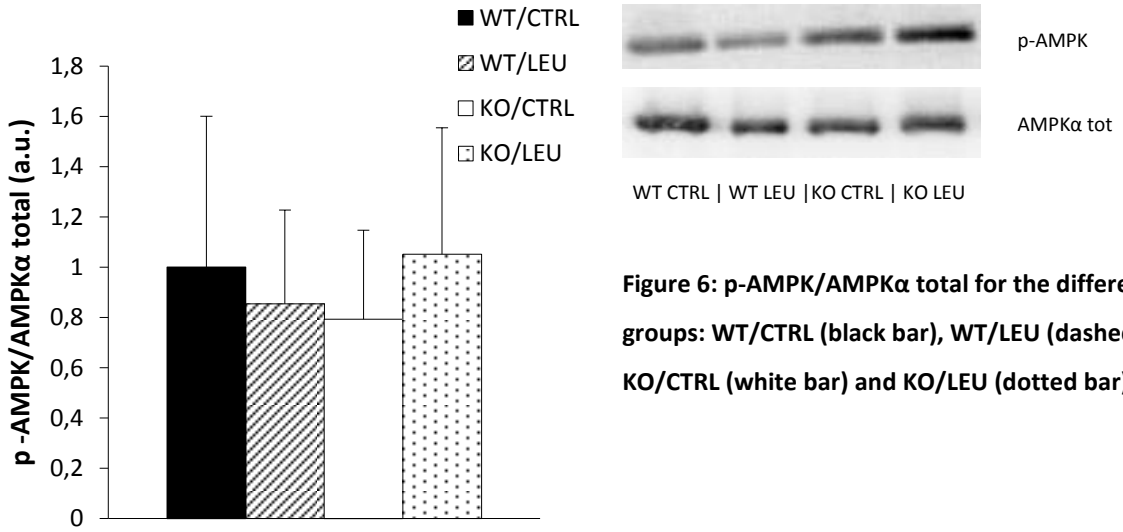

Figure 6: p-AMPK/AMPKα total for the different groups: WT/CTRL (black bar), WT/LEU (dashed bar), KO/CTRL (white bar) and KO/LEU (dotted bar).

Redd1

In m. soleus, the KO/CTRL group showed an increase in Redd1 (by 116%) in comparison with the control group (WT/CTRL) but no significant differences could be remarked (p = 1,000) (figure 7a.). Remarkably, administration of L-leucine in WT mice showed a non-significant increase of Redd1 (by 175%) (p = 0,491). In m. tibialis anterior no significant differences were seen among the four groups (p = 0,883) (figure 7b.). Also, no significant differences were seen between the different muscle groups (p = 0,118).

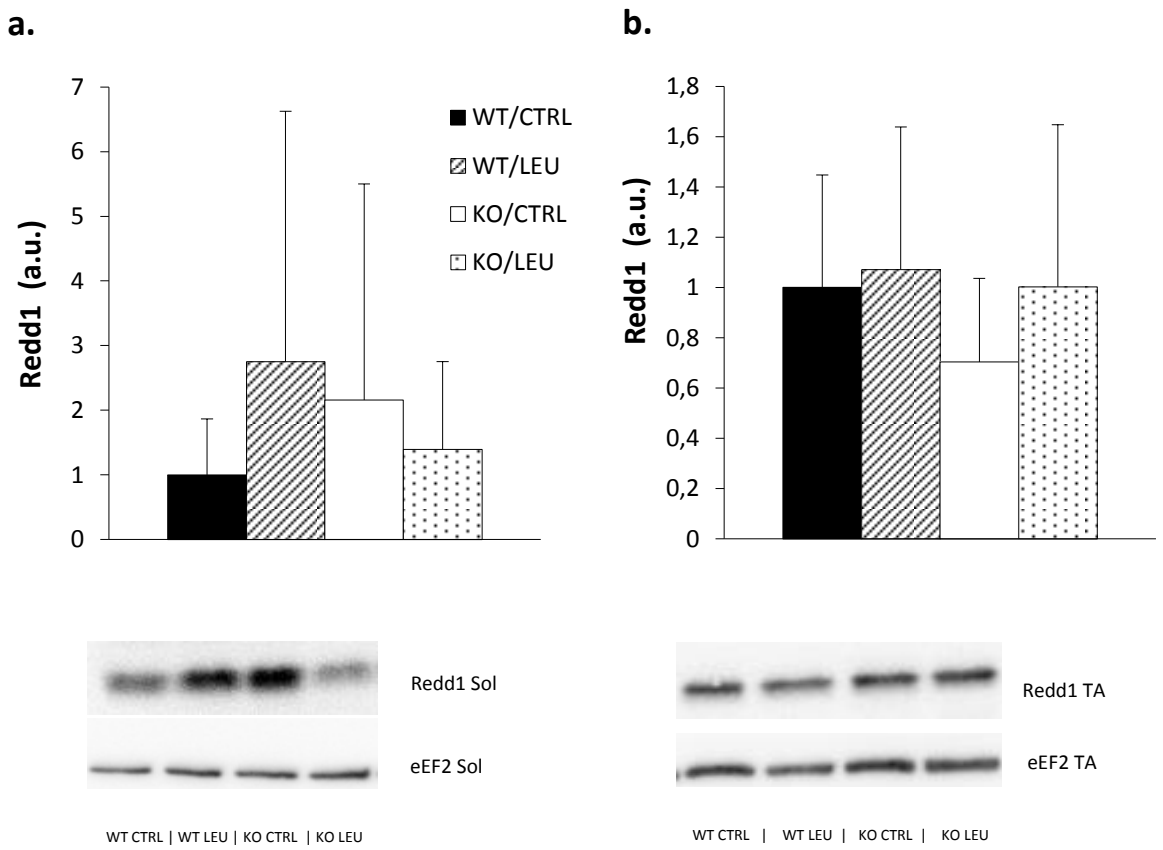

Figure 7: Redd1 for the different groups: WT/CTRL (black bar), WT/LEU (dashed bar), KO/CTRL (white bar) and KO/LEU (dotted bar) in both m. soleus (a.) and m. tibialis anterior (b.).

Phosphorylation of mTORC1 at Ser^{2448}

Oral administration of L-leucine in WT mice did not increase phosphorylation of mTOR in m. soleus (p = 1,000) and m. gastrocnemius (p = 0,431) (figure 8a. and 8c.). In m. tibialis anterior, this trend was not seen (figure 8b.). There is not a statistically significant difference when comparing the four different groups in each muscle. Also, no significant differences were seen between the different muscle groups (p = 0,121).

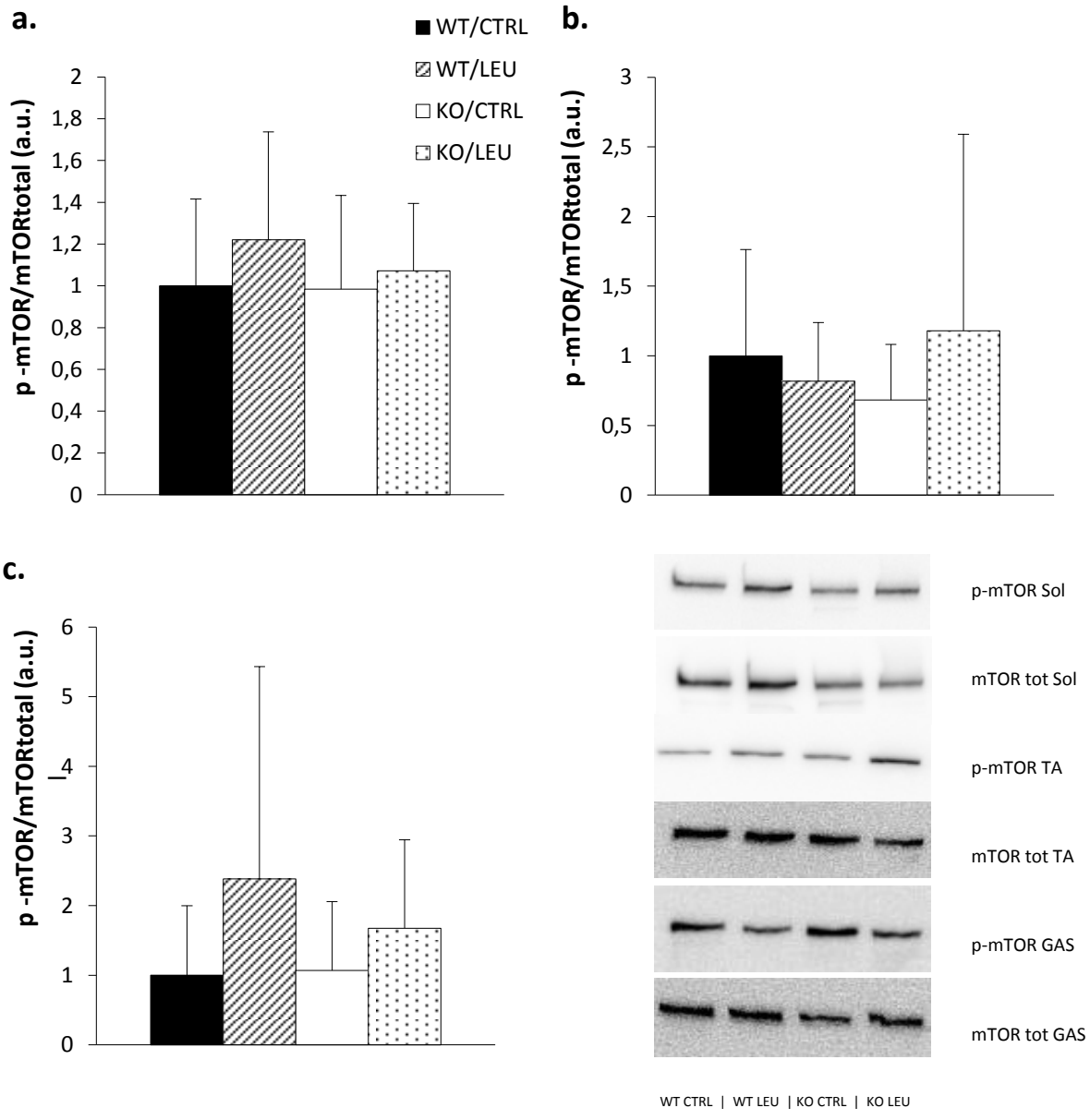

Figure 8: p-mTOR/mTORtotal for the different groups: WT/CTRL (black bar), WT/LEU (dashed bar), KO/CTRL (white bar) and KO/LEU (dotted bar) for m. soleus (a.), m. tibialis anterior (b.) and m. gastrocnemius (c.).

Phosphorylation of S6K1 at Thr^{389}

L-leucine administration in WT mice augmented the phosphorylation of S6K1 in m. tibialis anterior (by 65,7%) and m. gastrocnemius (by 400%) (p = 1,000 and p = 0,005 respectively) (figures 9b. and 9c.). In m. soleus, this augmentation was not seen (p =1,000) (figure 9a.). Remarkably, in all three muscles, the KO/CTRL group showed an increase in phosphorylation status of S6K1 (figure 9a. with 94,8% (p = 0,034); b. with 137% (p = 0,160) and c. with 377% (p = 0,021)). In m. gastrocnemius there was a significant difference between KO/CTRL versus KO/LEU group (figure 9c. p = 0.031). No statistically significant difference was found between the WT/CTRL group versus the KO/LEU group in all three muscles. No significant differences were seen between the different muscle groups (p = 0,101).

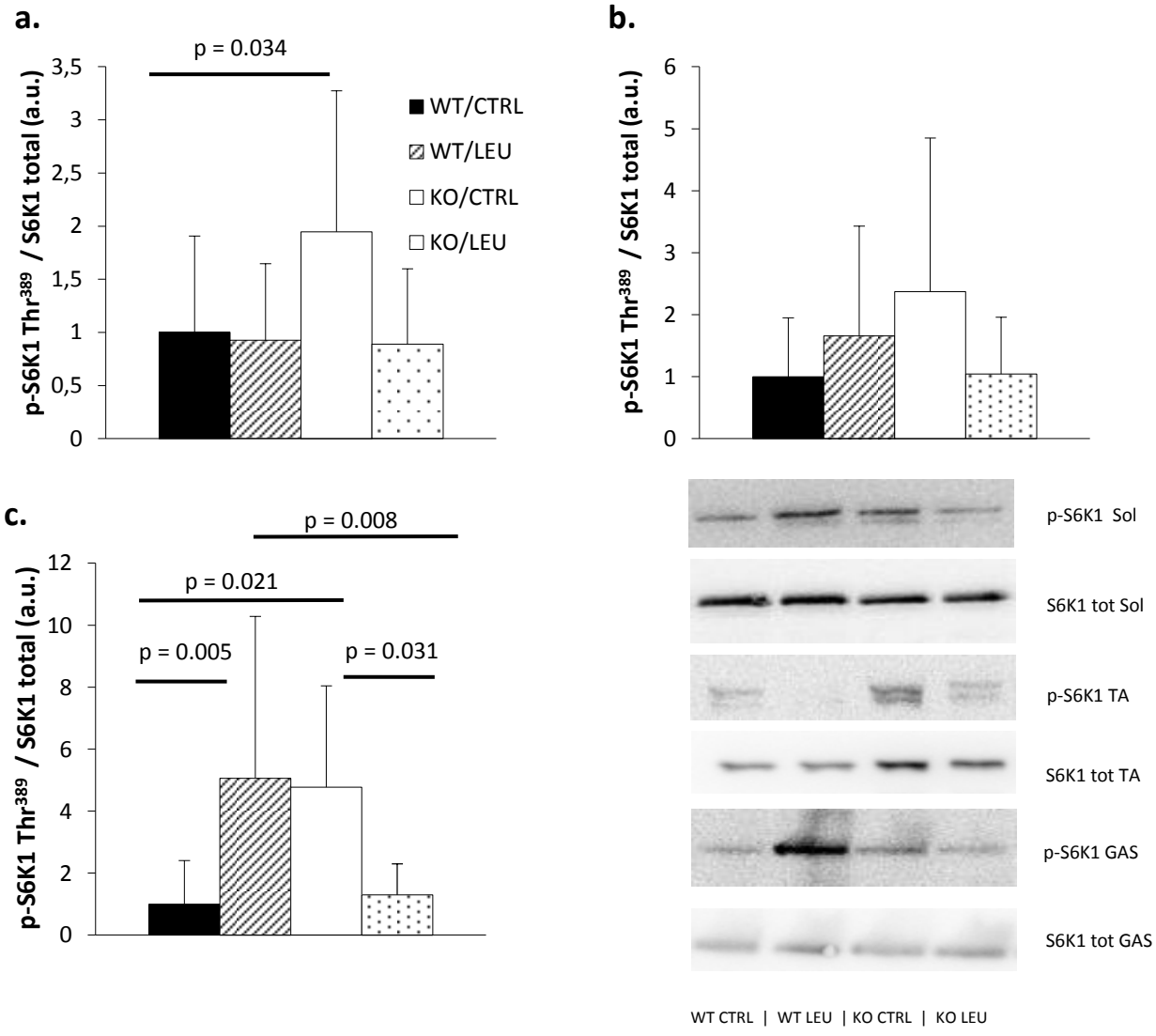

Figure 9: p-S6K1/ S6K1 total for the different groups: WT/CTRL (black bar), WT/LEU (dashed bar), KO/CTRL (white bar) and KO/LEU (dotted bar) for m. soleus (a.), m. tibialis anterior (b.) and m. gastrocnemius (c.).

Discussion

Leucine, one of the BCAAs, plays an important role in promoting skeletal muscle protein synthesis. Animal studies have shown that the beneficial effects of leucine administration on protein synthesis in skeletal muscle are linked with mTOR signalling (3,55,63,64). On the other hand, in hypoxia, protein synthesis is downregulated and the mTOR-pathway is one of the key pathways by which this is achieved (46). We have studied whether L-leucine can counteract the negative effects of hypoxia in protein metabolism *in vivo*. Therefore we have analysed the effects of L-leucine administration in mice with a KO of the gene that encodes for PHD1, a controller of hypoxia tolerance. We hypothesized that despite a PHD1 KO, L-leucine will upregulate the downstream substrates of mTORC1 and thereby stimulate protein synthesis. Our hypothesis was based on a study by Durán et al. (51). They demonstrated that inhibition of all three PHDs blocks the ability of AAs to activate mTORC1 (51). Individual silencing of one of the PHDs did not block mTORC1 activity (51). In our study we have only performed a KO of PHD1 and so PHD2 and PHD3 can still stimulate mTORC1 after L-leucine administration. Our data could not confirm this hypothesis; not one of these two conditions, L-leucine and a PHD1 KO, predominates in regulating mTORC1 activity and its downstream substrates. We believe that other contributing factors - other activated pathways - may play a role in the investigated signalling pathways (figure 10).

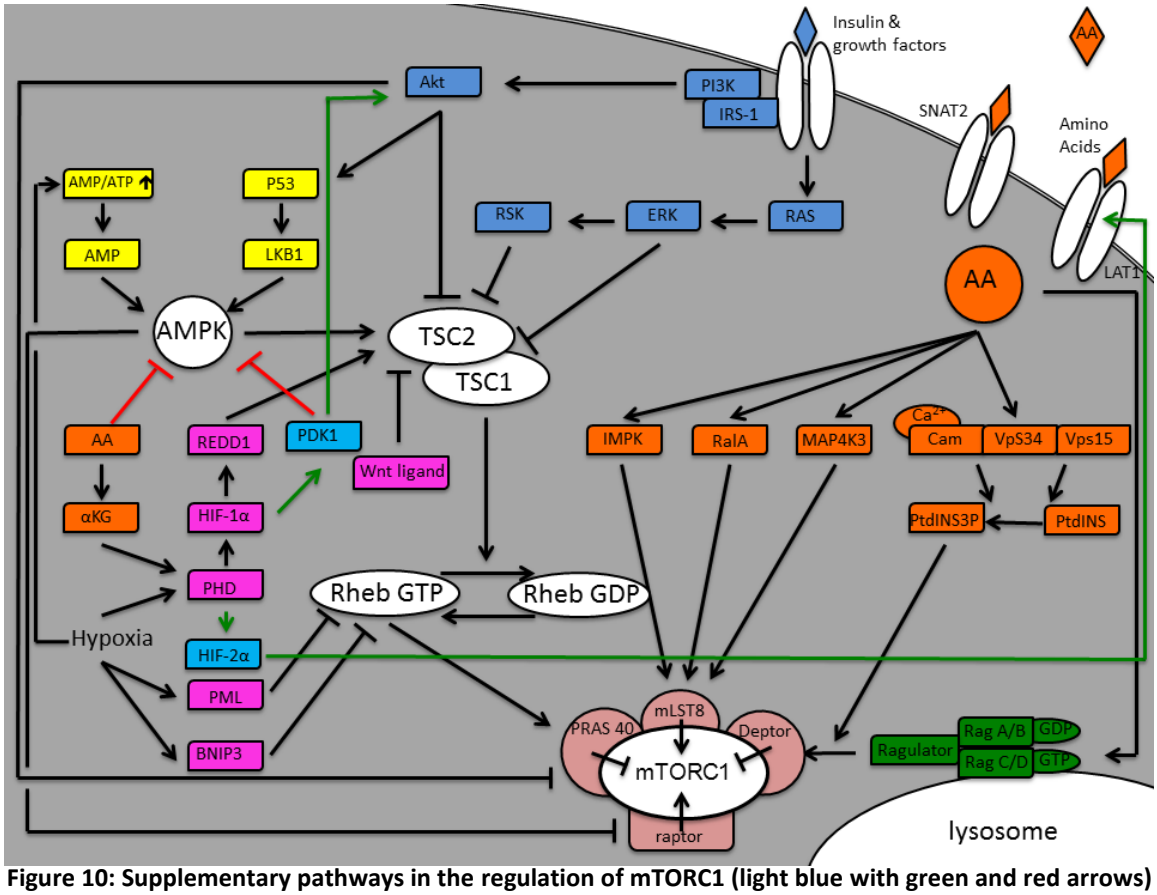

Figure 10: Supplementary pathways in the regulation of mTORC1 (light blue with green and red arrows)

Upstream mTORC1

Cellular stress such as hypoxia inhibits the production of ATP which increases ADP to ATP ratio, leading to hyperphosphorylation and activation of the α-subunit of AMPK (41). TSC2 is directly phosphorylated by AMPK on Thr^{1227} and Ser^{1345} and represses mTORC1 activation (37). Furthermore, in hypoxia, Redd1 can be upregulated through two distinct mechanisms and inhibits mTORC1 signalling. First, Redd1 is transcriptionally upregulated in the presence of HIF-1α (45,66). As a consequence, De Young et al. showed that Redd1 binds to 14-3-3 which induces dissociation from the TSC2 and this allows the TSC1/2 complex to inhibit mTORC1 (44). Secondly, Redd1 can be upregulated during ER stress due to the UPR response (67). UPR signalling consists of three ER sensors: PERK, inositol-requiring protein 1 (IRE1) and activating transcription factor-6 (ATF6), as reviewed by Ron en Wouters et al. (68). Activation of PERK through hypoxia leads to phosphorylation of eIF-2α and inhibits translation initiation (69). This mechanism has not yet been confirmed in *in vivo* experimental conditions. Interestingly, in our mouse model, this activation of AMPK and Redd1 could not be confirmed in the PHD1 KO group. None of the investigated hindlimb muscles showed higher phosphorylation of AMPK or increased protein content of Redd1. Undeniably, the KO of PHD1 is not sufficient enough to induce cellular stress and thereby activation of both molecules, AMPK and Redd1. Further, HIF-α stabilisation induces the stimulation of the enzyme pyruvate dehydrogenase kinase 1 (PDK1), a critical kinase involved in maintaining constant ATP levels (70). Therefore, due to a compensatory mechanism, AMPK activation could remain unaffected due to unaltered ATP to ADP ratio in the KO mice (70).

In the present study, L-leucine administration in the WT group did not show a decrease in phosphorylated AMPK. Although, in the literature it is known that leucine administration could suppress AMPK activity concomitantly with the increase in mTOR/p70S6K signalling, demonstrated by Saha et al. (71). Leucine has been shown to increase the lactate-to-pyruvate ratio in the muscle (71). This causes a decrease in the NAD/NADH-ratio, which is associated with a decrease in histone/protein deacetylase silent information regulator T1 (SIRT1) and contributes to a decrease in AMPK activity (71,72). Since we have not measured metabolites, this question remains open and it requires further investigation.

Durán et al. showed that PHDs are not only oxygen sensors but also nutrient sensors (51). Interestingly, AA administration influences PHD through αKG and thereby suppresses HIF-1α and Redd1. On the other hand, it is well established that Redd1 is one of the main HIF-α targets (45). These opposing effects could provide an answer to why Redd1 protein content is unaffected in our

study. Further analysis of mRNA content of Redd1 should provide more insight to differentiate between post-translational modifications and transcription.

We have to be careful with extrapolation of the above-mentioned conclusion to our results because Redd1, like many other enzymes involved in cell growth, is a very transient protein. For example, in mice, Redd1 was rapidly induced after five days of tail suspension, but after fourteen days Redd1 expression was reduced to baseline (73). Therefore, it is certainly possible that Redd1 was upregulated acutely in response to PHD1 KO, but that we have missed it due to imperfect timing.

mTORC1 and dowstream substrate S6K1

Once stimulated, mTORC1 increases translation through regulation of key enzymes 4E-BP1 and S6K1 (5). *In vivo* experiments by Yoshizawa et al. have shown that oral administration of leucine in rats promotes activation of mTORC1 and phosphorylation of S6K1 and 4E-BP1 and thereby enhances muscle protein synthesis (3). The same results were found in earlier studies (55,63). This is in contrast with a condition of hypoxia where a downregulation of downstream substrates of mTORC1 is seen. Indeed, Arsham et al. described the influence of hypoxia on the phosphorylation status of 4E-BP1 by affecting the activity of mTOR and Sofer et al. drew the same conclusion for the downstream substrate S6K1 (56,74). This study is unique due to the fact that we have measured an anabolic stimulus (L-leucine) and a potential catabolic stimulus (PHD1 KO) in one mouse model. L-leucine administration alone increases phosphorylation of S6K1 in the WT group but this was only seen in the oxidative m. soleus. On the other hand, mTORC1 phosphorylation at Ser^{2448} was unaffected over all muscles in all conditions. This unchanged mTORC1 could be explained by the sensitivity of mTORC1. Recent reports have shown different sensitivities for the different Thr-sites of mTOR (75,76). This could influence the magnitude of our results.

Interestingly, phosphorylation of S6K1 at Thr^{389}, an established read-out of mTORC1 activity, was upregulated in our PHD1 KO group. This phenomenon was replicated over the three investigated muscle groups. As a result of PHD1 KO, HIF-1α is upregulated and activates PDK1. Besides its influence on AMPK, PDK is likely to mediate the phosphorylation of Akt on Thr^{308} resulting in the promotion of downstream signalling (77). This could be one of the explanations for the augmented S6K1 in the PHD1 KO group. In addition to HIF-1α, HIF-2α also plays a role in the contrasting results. The study of Aragonés et al. showed that the upregulation of HIF-2α was greater than HIF-1α expression in the muscle after a PHD1 KO (65). This could be explained by the fact that in general in a normoxic situation PHD1 and PHD3 are responsible for HIF-2α and PHD2 for HIF-1α (78). The preferences of the PHDs for HIF-1α and HIF-2α might be explained by the differential actions of the

PHD proteins on the different binding sites of HIF-1α and HIF-2α (78). Therefore in our PHD 1 KO mice HIF-2α may also be upregulated. Of note, we did not measure HIF-1α and HIF-2α protein contents in this work because the set-up of the primary anti-bodies was in full progress. Elorza et al. found that HIF-2α activation increases the LAT1 transporter and thereby influences mTORC1 activity (79). This may be another explanation why we have found an upregulation of S6K1 in the PHD1 KO group. More LAT1 expression ensures more transporters in the membrane and a greater capacity to transport leucine in the cell. This could lead to more AAs stimulating mTORC1 despite HIF-1α upregulation in a state of hypoxia. At last, earlier research has elegantly shown that a PHD3 KO leads to elevated plasma insulin levels and an upregulation of protein synthesis (80). An increase in blood insulin levels activates the PI3K pathway leading to augmented levels of S6K1. Insulin data is beyond the scope of this thesis, but it is clear that augmented insulin levels, despite the fasted state of the mice, could explain increased p-S6K1 in the KO without L-leucine group.

To further clarify these unexpected results, we have repeated the experiment in another mouse strain (50% swiss/ 50% 129s), lacking PHD1 gene in all tissues including skeletal muscle tissue. This is in contrast with the mice (100% C57BL/6) in the main experiment, which were muscle specific KOs (*see materials and methods section)*. Interestingly, these pilot data did not show this increased phosphorylated S6K1 in the KO CTRL group. This is in contrast with the above mentioned main experiment data (figure 11).

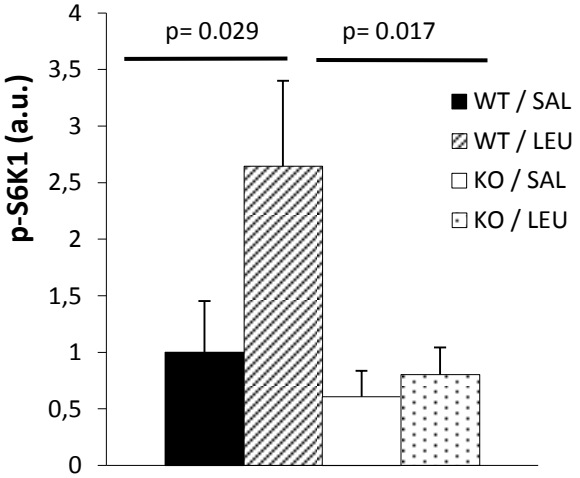

Figure 11: p-S6K1 measured in m. tibialis anterior for the different groups: WT/CTRL (black bar), WT/LEU (dashed bar), KO/CTRL (white bar) and KO/LEU (dotted bar).

These pilot data show that L-leucine administration increases the phosphorylation of S6K1, which is completely according to current knowledge of this topic. Thus we can state that L-leucine administration augments the phosphorylation of the downstream substrates of mTOR and thereby stimulated protein synthesis. Moreover, these data show that L-leucine administration in PHD1 KO mice does not cause an increase in S6K1 phosphorylation compared to L-leucine administration in WT mice. This lack of S6K1 phosphorylation could clearly state that there is an anabolic resistance in the KO mice after L-leucine administration.

Earlier studies confirmed that there are major differences in metabolic response when the same genetic mutations are introduced in different strains. For example, Kulkarni et al. demonstrated that after KO of the insulin receptor and IRS-1, different metabolic responses were seen in different genetic backgrounds of mice (81). The C57BL/6 mice strain showed greater glycemia, larger fat mass and hyperinsulinemia compared to the 129s mice strain (81). This could explain why we have found differences between the pilot data mice (50% swiss/ 50% 129s) and the mice used in our main experiment (100% C57BL/6).

Limitations of the present study

The major limitation of this study is that we have created a chronic state of hypoxia by a conditional KO in mice muscles. Therefore generalizing our results to mice in hypoxia or to pathological conditions like COPD is not possible. Secondly, we did not measure serum leucine. So we could not control whether the amount of AA that was administered was in proportion with serum leucine concentrations. Another limitation is that we could only make an estimation of protein synthesis by measuring the downstream marker S6K1. Protein synthesis itself was not directly measured. At last, all groups consisted of relatively small numbers of mice and experiments were not blinded. These small sample sizes decrease the power of the statistical analysis and increase the chance of a type II error. Hence we have to be cautious when interpreting our results.

Conclusion

In conclusion, we cannot confirm that administration of L-leucine effectively counteract the negative effects of a PHD1 KO. When L-leucine administration and PHD1 KO are combined, neither predominates. In line with other studies we can conclude that administration of L-leucine effectively stimulates protein synthesis by enhancing phosphorylation of S6K1. Further research studies will be required to define to what extent a PHD1 KO alone and after L-leucine administration affects the upstream and downstream components of the mTOR signalling pathway.

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Appendix

1. Populaire samenvatting: Kan leucine de negatieve effecten van chronisch zuurstoftekort op het proteïnemetabolisme tegengaan?
2. Guidelines for authors

1. Populaire samenvatting

Kan leucine de negatieve effecten van chronisch zuurstoftekort op het proteïnemetabolisme tegengaan?

Situering Chronisch zuurstoftekort (hypoxie) door langdurig verblijf op hoogte of door een pathologische long- of bloedaandoening leidt tot een vermindering in spiermassa. In deze situaties is de normale balans tussen spieraanmaak en spierafbraak verstoord. Wij onderzoeken of het mogelijk is deze balans positief te beïnvloeden en spieraanmaak te stimuleren in een situatie van hypoxie door het toedienen van één specifiek aminozuur.

Eiwitten of proteïnen bestaan uit verschillende aminozuren die aan elkaar gekoppeld zijn zoals een ketting. Een essentieel aminozuur is leucine, dat naast zijn functie als bouwsteen ook een belangrijke stimulator van de proteïnesynthese zelf is. Deze synthese is een proces dat bestaat uit twee stappen: ten eerste de transcriptie, het overschrijven en kopiëren van genetische informatie van het DNA en vervolgens de translatie, het vertalen van deze genetische informatie naar een volgorde van aminozuren. Een enzyme in dit proces is de “mammalian target of rapamycin complex 1” (mTORC1). Dit complex beïnvloedt verschillende signaalwegen die instaan voor de aanmaak van proteïnen door te zorgen voor een structuurverandering in enkele markers voor de proteïnesynthese. De activiteit van mTORC1 is afhankelijk van de input die het krijgt. Zo zullen groeifactoren, insuline en aminozuren (o.a. leucine) leiden tot een stimulatie van het complex; hypoxie en gebrek aan energie zullen leiden tot een negatieve activiteit van mTORC1. Hieruit kunnen we dus concluderen dat leucine en hypoxie een tegengestelde invloed op de proteïnesynthese hebben. Wij willen dan ook onderzoeken wat beide samen tot gevolg hebben in de cel en of leucine de negatieve effecten van hypoxie kan tegengaan.

Methoden Voor onze studie gebruikten we muizen die genetisch gemanipuleerd waren om een toestand van chronische hypoxie in de spieren na te bootsen. Deze muizen missen het gen dat codeert voor “prolyl hydroxylase domain 1” (PHD1); een “PHD1 knock out” (PHD1 KO) werd gecreëerd. Kort gezegd, zien we in afwezigheid van PHD1, een stimulatie van “hypoxia inducible factors” (HIFs). Dat zorgt potentieel voor een negatieve regulatie van mTORC1 en een verminderde proteïnesynthese.

Deze muizen vergeleken we vervolgens met gezonde, wild type (WT) muizen en we creëerden zo vier groepen door toediening van ofwel leucine ofwel een zoutoplossing (saline) als controle (zie tabel). Op deze manier konden we de effecten van een toediening van aminozuren nagaan. We namen spierstalen om veranderingen in de markers van de proteïnesynthese op te merken.

Groep	Type muis	Type oplossing	Hypothetisch effect op markers van proteïnesynthese
1	WT	Saline	=
2	WT	Leucine	↑
3	KO	Saline	↓
4	KO	Leucine	?

Resultaten Leucine toediening liet een verhoging zien van de markers voor proteïnesynthese in WT muizen. Interessant genoeg, en in tegenstelling tot onze hypothese, zorgde de KO voor een verhoging van de markers van proteïnesynthese (mTORC1) zonder externe stimulus (KO + Saline). Dit effect werd grotendeels tenietgedaan bij toediening van leucine (KO + Leucine).

Conclusie en interpretatie Uit onze data blijkt dat de uitschakeling van een gen (PHD1) zorgt voor potentieel meer eiwitsynthese in de skeletspier. Meer onderzoek is nodig om specifiek te bepalen of proteïnesynthese *per se* wordt beïnvloed en welke signaalwegen hiervan de oorzaak zijn.

2. Guidelines for authors

Deze thesis is opgesteld volgens de richtlijnen van *The Journal of Biological Chemistry*.

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Correspondence of functional and microscopic pK_a values in a ribozyme

The pH Dependence of Hairpin Ribozyme Catalysis Reflects Ionization of an Active Site Adenine*

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*Running title: *Correspondence of functional and microscopic pK_a values in a ribozyme*

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Keywords: RNA catalysis; ribozyme; fluorescence

Background: The hairpin ribozyme uses active site functional groups for catalysis.
Results: Self-cleavage activity decreases as the fraction of the protonated form of an active site adenosine increases.
Conclusion: Unprotonated adenosine facilitates formation of the 5'-oxygen-phosphorus bond in the transition state.
Significance: Learning how RNA functional groups participate in catalysis is crucial for understanding RNA-mediated processes in biology.

ABSTRACT

Understanding how self-cleaving ribozymes mediate catalysis is crucial in light of compelling evidence that human and bacterial gene expression can be regulated through RNA self-cleavage. The hairpin ribozyme catalyzes reversible phosphodiester bond cleavage through a mechanism that does not require divalent metal cations. Previous structural and biochemical evidence implicated the amidine group of an active site adenosine, A38, in a pH dependent step in catalysis. We developed a way to determine microscopic pKa values in active ribozymes based on the pH-dependent fluorescence of 8-azaadenosine. We compared the microscopic pKa for 8azaA38 ionization to the apparent pKa for the self-cleavage reaction in a fully functional hairpin ribozyme with a unique 8-azaadenosine at position 38. Microscopic and apparent pKa values were virtually the same, evidence that A38 protonation accounts for the decrease in catalytic activity with decreasing pH. These results implicate the neutral, unprotonated form of A38 in a transition state that involves formation of the 5'-oxygen-phosphorus bond.

Hairpin ribozymes (Hp Rzs) belong to one of several families of small self-cleaving RNAs that serve as useful models of RNA catalysis because they are relatively simple and amenable to chemogenetic analyses (1). The Hp Rz remains functional in the absence of divalent metals, relying exclusively on nucleotide functional groups for catalytic chemistry (2-5). High resolution structures of the Hp Rz bound to transition state mimics show two active site purines, G8 and A38, positioned in a similar manner to the two histidines in RNase A that mediate general acid base catalysis of the same reaction (6-8) (Fig. 1A), leading to the proposal that the hairpin ribozyme uses the same concerted general acid base mechanism (9) (Fig. 1B). A38(N1) is near the 5'-oxygen of the reactive phosphodiester, and A38(N6) lies within hydrogen bonding distance of the *pro*-R_P non-bridging oxygen. Exogenous nucleobase rescue experiments confirmed that the amidine group of A38 interacts with the transition state but its precise role remained unclear (10). In a general acid base model, the cationic, protonated form of A38 would act as a general acid during cleavage

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Formatting requirements:

- 8.5-by-11-inch paper size (standard US letter).
- Single-spaced text throughout.
- Two-column format for capsule/abstract through discussion sections. Single-column format for title, references, footnotes, figure legends and tables. Click the image above to see an example. See below for help converting text to columns in Microsoft Word.
- One-inch left and right margins and 0.25-inch spacing between columns.
- 11-point Times New Roman font.
- Number all pages, including those with figures. **Manuscripts without page numbers will be returned to authors for correction before review, thereby delaying the review process.**

Converting text to columns in Microsoft Word:

- Finish writing and editing the text.

- Select text in the capsule/abstract through discussion sections.
- Click on "Format," and then "Columns" from the drop-down menu.
- Select two columns and equal column width, and then change the spacing to 0.25 inches.

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Text Organization

Order of Sections

Manuscripts should be arranged in the following order:

a. title, author(s), complete name(s) of institution(s), running title and keywords
b. capsule
c. abstract
d. introduction
e. experimental procedures
f. results
g. discussion
h. acknowledgments
i. conflict of interest
j. author contributions
k. references
l. footnotes
m. figure legends
n. tables
o. figures

Title

- Should be intelligible to JBC readers who are not specialists in the field and should convey your essential points clearly.
- Should be short and informative (less than two lines).
- Should avoid acronyms or abbreviations aside from the most common biochemical abbreviations (*e.g.*, ATP). A complete list of abbreviations that are acceptable in titles can be found on the abbreviations page. Other acronyms or abbreviations should either:
  - be introduced in their full form (*e.g.*, Visualization of Polarized Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) Activity in Live Cells by Fluorescence Resonance Energy Transfer (FRET) Imaging); or
  - be clarified by use as a modifier of the appropriate noun (*e.g.*, FOX1 transcription factor, ACC dopamine receptor).

Authors

- All authors are responsible for the content of the manuscript.
- Provide the complete names of all authors.
- Identify which author will receive correspondence regarding the manuscript.
- Provide the corresponding author's name, telephone and fax numbers, and current e-mail address.
- Non-Latin characters can be used for author's names, as long as the characters can be encoded in Unicode (*e.g.*, Chinese, Japanese, Korean, Arabic). These characters can only be used for author names, not author affiliations or titles. Non-Latin characters should be enclosed in parentheses after the transliterated version (see example below).

Zi-Zhen Wu (吴子桢)[‡1], De-Pei Li (李德培)[‡1], Shao-Rui Chen (陈少瑞)[‡1], and Hui-Lin Pan (潘惠麟)[‡§2]

Running title

- Should be at the top of each page and may not exceed 60 characters and spaces.

Keywords

You must choose at least five keywords from this list. You may also choose up to five keywords that are not on the list. You may not choose more than ten keywords in total.

Capsule

A capsule reports the core findings of a paper to a broad spectrum of readers in a way that makes clear how those findings significantly advance understanding of a biological process. The capsule and the abstract have different purposes and audiences, so the capsule is not simply a truncated version of the abstract. A well-crafted capsule increases the impact of a JBC article by:

- Providing scientists outside your specialty the chance to be inspired by your work or to start an interdisciplinary collaboration.
- Communicating the value of your research to the public and policymakers.
- Helping teachers identify papers suitable for classroom instruction.
- Offering a succinct summary of your work that can be easily accessed from mobile devices.

How to write a Capsule

The capsule is a highly visible part of a JBC article so you should write it with care and precision. The capsule:

- Must not exceed 60 words.
- Should use language with which all readers are familiar, including the broad scientific community and the general public.
- Use only approved acronyms. All others must be spelled out on first reference.
- Must have the following four sections:

Background: A complete sentence that explains the impetus and context of the work.
Results: A complete sentence that summarizes the major findings.
Conclusion: A complete sentence that summarizes the interpretation of the findings.

Significance: A complete sentence about the paper's impact on the field and its long-term implications.

- Each capsule section should add information, not repeat what has already been said.
- After you write the capsule, you should evaluate and revise it using these strategies:
  - Honestly ask yourself whether non-specialists will understand your findings and their significance.
  - Ask a non-specialist to read your capsule and then ask if they understood the essence of your work.

Capsule Example 1
Background: Amyloid fibrils are protein aggregates associated with numerous neurodegenerative diseases.
Results: A theoretically consistent, two-parameter model is proposed describing very distinct amyloid fibrillization kinetics.
Conclusion: Amyloid fibril formation takes place by a general mechanism involving supersaturation-dependent nucleation and growth steps.
Significance: This mathematically simple model can be routinely used to characterize the action of new targets for disease therapeutics.

Capsule Example 2
Background: Galectins from peritoneal cells of conger eel contribute to the encapsulation of nematode.
Results: A new galectin from peritoneal cells, congerin P (Con-P), shows unusual sequence, specificity, and allosteric regulation by mannoside.
Conclusion: Con-P is a new type of galectin with allosteric carbohydrate-binding ability.
Significance: Con-P is the first known lectin allosterically modulated by its ligands.

Capsule Example 3
Background: Metabolite binding to riboswitch RNAs regulates expression of metabolic genes.
Results: Inhibitory and activating ligands interact with the same riboswitch.
Conclusion: Riboswitches integrate information about the overall metabolic state of the cell.
Significance: This might be the first sign of a complex RNA-metabolite interactome.

Click here to return to the top of the page.

Abstract

- Should succinctly and clearly describe the major findings reported in the manuscript.
- Avoid specialized terms and use only approved acronyms. All others must be spelled out on the first reference.
- Must not exceed 250 words.

Introduction

- Presents the purpose of the study and its relationship to earlier work in the field.
- Should not be an extensive review of the literature.
- Usually less than one formatted page.

Experimental procedures

- Brief but sufficiently complete to permit a qualified reader to repeat the experiments.
- Only truly new procedures should be described in detail.
- Previously published procedures should be referenced.
- Modifications of previously published procedures should not be given in detail except where necessary to repeat the work.
- If the study characterizes the activity of new compounds, compound structures must be provided.
- Quantification of gel or blot intensities must be performed with data obtained within a linear range of exposure.

Results

- Presented in figures, tables, or text.

Discussion

- Concise (usually less than two formatted pages).
- Focused on the interpretation of the results
- Should not repeat information in the "Results" section.

Acknowledgments

- Funding sources.
- Database names and accession codes *(if applicable)*.
- Brief note(s) of thanks to people who helped with the study or preparation of the paper *(optional)*.

Conflict of interest

- Statement disclosing whether there are any actual or perceived conflicts of interest on the part of any author.
- If there are no conflicts of interest, insert the following statement: The authors declare that they have no conflicts of interest with the contents of this article.
- Review the Conflicts of interest section of the JBC Editorial Policies.

Author contributions

- Statement describing each author's contributions to the manuscript.
- Authorship credit should be based on the following:
  1. substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data;
  2. drafting the article or revising it critically for important intellectual content;
  3. final approval of the version to be published

Acquisition of funding, collection of data, or general supervision of the research group alone does not

constitute authorship. Corporate authorship is not accepted.

- Review the Authorship criteria section of the JBC Editorial Guidelines.
- Identify each author by his or her initials, as in these examples:
  - Example 1: PFG conceived and coordinated the study and wrote the paper. AKR and JRG designed, performed and analyzed the experiments shown in Figures 2 and 4. NFJ designed, performed and analyzed the experiments shown in Figure 3. THP provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.
  - Example 2: MAF and GFG designed the study and wrote the paper. GFG purified and crystallized AnsT protein and determined its X-ray structure. NFJ characterized AnsT enzyme activity in vitro. THP designed and constructed vectors for expression of mutant proteins and analyzed the mutant phenotypes in bacteria. All authors analyzed the results and approved the final version of the manuscript.

References

- Cited in text by number only.
- References should include article titles.
- Numbered consecutively in the order of appearance.
- If you use EndNote version 5 or earlier, you must update the JBC EndNote style. (*See Updating EndNote, below, for instructions.*)
- When using the EndNote plugin in Microsoft Word, you will have to manually add the DOI after the journal title for all e-pub references (*see reference example 3*).
- Journal names are abbreviated according to PubMed. (*After updating your JBC style in EndNote, in order to ensure proper journal name abbreviations, see Updating EndNote, below, for instructions.*)
- Authors are responsible for the accuracy of the references.

Examples:

1. MacDonald, G. M., Steenhuis, J. J., and Barry, B. A. (1995) A difference Fourier transform infrared spectroscopic study of chlorophyll oxidation in hydroxylamine-treated photosystem II. *J. Biol. Chem.***270**, 8420-8428
2. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. **References appearing as e-pubs should be in the following style**: Aphasizheva, I., Aphasizheva, R., and Simpson, L. (April 1, 2004) RNA editing terminal uridylyl transferase 1: identification of functional domains by mutational analysis. *J. Biol. Chem.* 10.1074/jbc.M401234200
4. Farrell, C. (1992) *The Role of SecB during Protein Export in Escherichia coli*. Ph.D. thesis, The Johns Hopkins University

Updating EndNote:
EndNote versions 5 and earlier do not contain the current JBC reference style with titles. To obtain the current EndNote style:

- Visit http://endnote.com/downloads/style/journal-biological-chemistry.
- Download the file **J Biological Chem.ens**. Save it in your EndNote Style folder so that it replaces the current JBC style.
- Full instructions for installing the new *ens* file can be found on the right-hand side of the Web page http://endnote.com/downloads/styles.
- For the journal name abbreviations to appear correctly as EndNote renders them in your manuscript text document (*e.g.*, "J.Biol.Chem."), you will need to ensure that the correct term list is imported. Follow the steps listed at http://endnote.com/kb/82228.

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Unpublished observations and personal communications

- Must be cited using footnotes.
- Authors must obtain written approval for all personal communications, exactly as written in the text, to be provided to the Editor upon request.

Abbreviations

- Must be defined in a single footnote inserted after the abbreviation is first used.
- Some common biochemical abbreviations — *e.g.*, ATP, NADH, DNA, and amino acids in proteins — need not be defined. A complete list of abbreviations that are acceptable in the text is available on the abbreviations page.
- Names of enzymes are usually not abbreviated except in terms of the substrates for which there are accepted abbreviations — *e.g.*, ATPase and RNase.
- The trivial and systematic names of enzymes should be those recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB).
- Table I lists the abbreviations for units of measurement and certain physical and chemical quantities that can be used without definition. Also listed are the prefixes that can be added to names of units and the multipliers indicated by each prefix.

Table I: Abbreviations of units of measurement and of physical and chemical quantities.

Genetic nomenclature

Organisms should be described using standard nomenclature following the guidelines developed by the American Society for Microbiology (http://mcb.asm.org/site/misc/journal-ita_nom.xhtml#04).

Experimental uncertainty and reproducibility

- Authors should include information on the uncertainty and reproducibility of data in the figure legends.
- Authors should state the number of independent samples (biological replicates) and the number of replicate samples (technical replicates) and report how many times each experiment was repeated.
- Scatter plots are recommended for comparisons of small data sets, or box and whisker plots for comparisons of large data sets because they provide more information about the variability within the data.
- Statistical analyses of variation and precision for establishing differences between experimental groups should be reported using the standard deviation (SD), confidence intervals (CI) or standard error of the mean (SEM).

Chemical and mathematical usage

- Numerical data should be reported with the number of significant digits that corresponds to the magnitude of experimental uncertainty.
- Chemical equations, structural formulas and mathematical equations should be placed between successive lines of text.
- For lipids, please refer to the classification, nomenclature, and structural representation of lipids used by the LIPID MAPS Initiative (see Fahy et al. J. Lipid Res. 2005 46: 839–862). You can download lipid structures directly from the LIPID MAPS Structure Database. This database draws structures de novo, allowing you to insert them into your documents.
- In general, the rules and recommendations of the IUBMB and the International Union of Pure and Applied Chemistry (IUPAC) will be used for abbreviation of chemical names, nomenclature of chemical compounds, enzyme nomenclature, isotopic compounds, optically active isomers, and spectroscopic data. Table II lists references to publications of the rules and recommendations of the International Scientific Unions that may be consulted for detailed information.

Table II: Tentative rules and recommendations of international scientific unions.

Many of the documents in this table can be obtained at http://www.chem.qmw.ac.uk/iupac/index.html.

Chemicals

Papers describing the activity of synthetic or natural chemical entities must include the chemical structures of such molecules, either as systematic names or as drawn structures. For synthetic chemical entities, a protocol for synthesis should be provided, generally as an allowable data supplement. Alternatively, reference to a publication or issued patent that includes such a synthetic scheme and details can be provided. For natural chemical entities, methods for extraction/purification of the chemical entity, plus determination of the chemical structure, should be provided in the Materials and Methods section.

Enzyme activity data

Papers reporting kinetic and thermodynamic data concerning enzymes and other catalytic proteins and nucleic acids should include the identity of the enzymes, additional biological information (*e.g.*, species and tissue normally found in, post-translational modification), preparation and criteria of purity, assay conditions, methodology, activity, and any other information relevant to judging the reproducibility of the results. See the Beilstein Institut/STRENDA (standards for reporting enzymology data) commission Web site (http://www.beilstein-institut.de/en/projekte/strenda/guidelines/) for more details and suggestions.

Enzyme activity (steady-state) generally should be reported in terms of V_{max} (nmol or μmol product formed per amount ((enzyme)) per time) or, when possible, as k_{cat} (V_{max} divided by molar enzyme concentration), in min^{-1} or s^{-1}. Km units are given in molarity.

Any other units of activity (absorbance, % change) should be converted to units of molarity to express k_{cat} or V_{max}. Values of k_{cat} (V_{max}) and K_m should be estimated using nonlinear fitting (and the software system cited).

Parameters should include estimates of error (*e.g.*, SE). The use of linear transformation for Michaelis–Menten parameters is recognized to be inaccurate and use of an alternate method should be justified (*e.g.*, graphical presentation of inhibition).

A lack of activity should be defined in terms of a limit of detection. In a series of comparisons to a basal or "control" level of activity (*e.g.*, set as unity or "100%"), this activity should be indicated, in the units mentioned above, along with estimates of error. The inclusion of examples of some of the raw data is encouraged. Please refer to the STRENDA Web site regarding enzyme inhibition (http://www.beilstein-institut.de/en/projekte/strenda/guidelines/). K_i values are preferred to IC_{50}.

Biological materials

Following the recommendation in the Proposed Principles and Guidelines for Reporting Preclinical Research that emerged from the Reproducibility Workshop sponsored by the Nature Publishing Group (NPG), Science/AAAS and the National Institutes of Health (NIH) on June 2, 2014, descriptions of biological materials should include enough information to uniquely identify the materials, such as:

- Repository accession numbers when available
- Antibodies – source, dilutions, and validation criteria
- Cell lines – source, authentication, derivation, and contamination (such as mycoplasma) status
- Animals – source, species, strain, sex, age, husbandry
- Transgenic animals – genetic background

Animal and preclinical research studies

The JBC publishes basic mechanistic studies using animal models and we adhere to the principals of transparency in reporting of all animal research data. We strongly encourage the reporting of animal data using the nomenclature and standards outlined in the ARRIVE (Animal in Research: Reporting In Vivo Experiments) guidelines, *Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research PLoS Biol. 2010 Jun 29;8(6):e1000412*.

- State that the research has been reviewed and approved by an Institutional Animal Care and Use Committee.
- State how often each animal experiment was done and whether the results were substantiated by repetition. Unambiguous information about sample collection must be provided to distinguish between independent biological data points and technical replicates.
- State the statistical test(s) used, exact value of N, definition of center, dispersion and precision measures (e.g., mean, median, SD, confidence intervals).
- State whether or not the animals and/or analyzed samples were randomized. If randomized indicate the specific method of randomization.
- State whether the studies were blinded or not blinded. If blinded, then indicate the method of blinding.
- State whether any animal experimental data were excluded. If data were excluded, then indicate the reason and/or criteria for exclusion

Immunoblots (Western blotting)

Antibody validation

- Authors should define the specificity, species of origin and source of all antibodies used, including catalog numbers, in Experimental Procedures.
- Where novel antibodies are used, describe how the antibody was generated, including the epitope/antigen
- Provide evidence of specificity, including specificity for post-translational modifications or neoepitopes when appropriate.
- When possible, data showing loss of immunoreactivity in samples following genetic or other molecular modification to the antigen is preferred.

Immunoblot data

- Generally, immunoblots should be cropped in a way that retains information about antigen size and antibody specificity.
- The cropped images should to retain sufficient area around the band(s) of interest, ideally including the positions of at least one molecular weight marker above and below the band(s).
- Avoid assembling immunoblot figures by splicing lanes from different gels, or different sections of a gel. If blots must be spliced, borders between separate sections must be clearly marked, and explained in the figure legend.

For studies reporting semi-quantitative analyses of immunoblots, authors should clearly explain how quantitative data were obtained, whether signal intensity has a linear relationship with antigen loading, and how protein loading was normalized among lanes. Note that some detection methods including detection of enhanced chemiluminescence using X-ray film have a very limited linear range. Normalization of signal intensity to total protein loading (assessed by staining membranes using Coomassie blue, Ponceau S or other protein stains) is preferred. “House-keeping” proteins should not be used for normalization without evidence that experimental manipulations do not affect their expression. Signals obtained using antibodies specific for phosphorylated epitopes should be normalized to the total protein level of the target protein.

During the editorial review process, authors may be asked to provide high resolution images of the original immunoblots , quantification details, and antibody validation data.

Microscopic imaging data

The JBC has adopted the policy of the Journal of Cell Biology in requiring the following information regarding microscope image acquisition:

- Make and model of microscope
- Type, magnification, and numerical aperture of the objective lenses
- Temperature
- Imaging medium
- Fluorochromes
- Camera make and model
- Acquisition software
- Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.)

If you export files from a microscope or other acquisition device, be sure to use consistent file formats (8 bit, 16 bit, etc.).

Micrographs must include a bar to indicate the scale.

Protein and nucleic acid sequences

Newly determined nucleotide or protein sequences must be deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), EMBL (http://www.ebi.ac.uk), or the DNA Databank of Japan (http://www.ddbj.nig.ac.jp). Accession numbers must be reported in the manuscript and data must be available upon acceptance and publication of the manuscript as a Paper in Press. No data are to be withdrawn following publication.

Genomic and proteomic data

Authors of papers that include functional genomics data such as microarray, ChIP seq, RNA-seq, or other high-throughput data are required to deposit the data in a MIAME-compliant database such as GEO (http://www.ncbi.nlm.nih.gov/geo), ArrayExpress (http://www.ebi.ac.uk/arrayexpress) or CYBEX (http://cibex.nig.ac.jp/data/index.html) and to provide accession numbers. Data must be publicly accessible upon acceptance and publication of the manuscript as a Paper in Press. No data are to be withdrawn following publication.

Authors of papers that include proteomics data should comply with the guidelines developed by Molecular and Cellular Proteomics (http://www.mcponline.org/site/misc/CheckList.pdf).

Database accession hyperlinks

The journal provides direct hyperlink access to entries in databases like GenBank. Authors must provide database accession numbers for all new genomics and proteomics data reported in their manuscripts. In addition, authors are strongly encouraged to include accession numbers for any database information that would aid a reader in understanding the paper, regardless of who deposited the information.

For database hyperlinks to be generated automatically, the citation must appear as a footnote and be written as follows:

1. GenBank = GenBank Accession Number XYYYYY
2. Molecular Modeling Database = MMDB # XYYY
3. NCBI Protein Database = NCB Accession # XXXXX
4. UniProtKB = UniProt # XXXXXX
5. Enzyme Collection Number = xx.yy.zz.bb
6. Research Collaboratory for Structural Bioinformatics Protein Databank = PDB # XXXX

The molecule or structure for an accession number can be identified through the NCBI Entrez utility at

http://www.ncbi.nlm.nih.gov/sites/gquery.

Example:
The nucleotide sequence for the artificial sperm whale myoglobin gene has been deposited in the GenBank database under GenBankAccession Number (Reference). The amino acid sequence of this protein can be accessed through NCBI Protein Database under NCBI Accession # 2311060 (Reference). The atomic coordinates for the crystal structure of this protein are available in the Molecular Modeling Database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=structure) under MMDB # 5MBN(Reference).

Authors may not include homepage URLs or use URLs to refer readers to unpublished data.

Structural studies

For papers describing structures of biological macromolecules, the atomic coordinates and the related experimental data (structure factor amplitudes/intensities and/or NMR restraints and chemical shifts) must be deposited in the PDB at a member site of the Worldwide Protein Data Bank, RCSB PDB, PDBe, PDBj, or BMRB.

For papers describing structures of biological macromolecules from electron microscopy (all averaging methods that produce 3D maps, including sub-tomogram averaging), the 3D maps must be deposited in EM Data Bank (EMDB; emdatabank.org). Any structure models fitted to EM maps must also be deposited in the PDB. For electron tomographic studies with no averaging, deposition of one or more representative tomograms in EMDB is strongly recommended.

The PDB ID and/or EMDB accession codes assigned after deposition should be included in the manuscript, together with a brief descriptive title. In cases where PDB models have been fitted to EMDB maps, the correspondences between them should be clearly stated. Authors must also submit the PDB Summary Validation Report (provided after annotation by the wwPDB) to JBC for review at the time of submission.

PDB and/or EMDB data must be ready for release before final acceptance of the manuscript. No data are to be withdrawn from PDB once a paper has been accepted and published as a Paper in Press article. As of December 2006, PDB no longer accepts coordinates for model structures determined by computational methods. The coordinates must be included as a supplement to the online paper and formatted just as if it were a PDB submission.

Click for additional guidelines for structural studies.

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Preparing tables & figures

Introduction

The number of tables and figures used to present data essential to illustrate or prove a point should be kept to a minimum. Read the following instructions and the guidelines at http://art.cadmus.com/da/jbc/guidelines.html carefully to avoid publication delays. If you require further information, please contact jbc@asbmb.org.

Tables

Tables should have titles and sufficient experimental detail in a legend immediately following the title to be understood without reference to the text. Each column in a table must have a heading. Abbreviations, when necessary, should be defined in the legend. Complex tables, such as sequence alignments, should be submitted as figures. Very large tables that cannot fit on a single page should be submitted as supplemental information in an appropriate format, such as Excel.

Figures

Policy on image manipulation

While certain modifications of primary data are often needed for clarity and/or brevity, image manipulation for deceptive purposes, to unfairly enhance or eliminate or otherwise obscure data, is misconduct and will be addressed as such.

For graphic material, we have adopted the policy of The Journal of Cell Biology:
*"No specific feature within an image may be enhanced, obscured, moved, removed, or introduced. The groupings of images from different parts of the same gel, or from different gels, fields or exposures must be made explicit by the arrangement of the figure (*e.g.*, using dividing lines) and in the text of the figure legend. Adjustments of brightness, contrast, or color balance are acceptable if they are applied to every pixel in the image and as long as they do not obscure, eliminate, or misrepresent any information present in the original, including the background. Nonlinear adjustments (*e.g.*, changes to gamma settings) must be disclosed in the figure legend."*

File formats

You will initially submit the figures in a single PDF file with the manuscript text for review. If your paper is accepted, you will need to submit the figures as high resolution TIFF or EPS files. For tips on how to prepare high resolution TIFF or EPS files, visit http://art.cadmus.com/da/jbc/index.jsp.

It is your responsibility to verify the quality of the graphics and confirm that compression of the files during the submission process does not distort the images.

We cannot accept figure files in certain applications, such as Microsoft Office (PowerPoint, Word, Excel, Access), Corel Perfect Office (WordPerfect, Quattro Pro, Presentations), and Lotus SmartSuite (Freelance Graphics, 1-2-3, Approach, and WordPro). These applications are not intended for high-resolution imaging.

Color figures

We encourage the use of color figures where they will enhance the presentation of the data. Note that each color figure carries a $50 publication charge if the corresponding author is not a member of ASBMB, but will be published

at no additional charge if the corresponding author is an ASBMB member. Any figure submitted in color will be reviewed and processed with the understanding that the figure will be published in color.

Color figures must be prepared in RGB color mode, not CMYK. JBC uses the RGB format to optimize color performance for online publication.

Multipanel figures

The parts of multipart figures (*i.e.*, figures with parts labeled a, b, c, d, etc.) should be assembled into a single figure that fits on one page and should be provided as a single file. That is, rather than providing separate files for each panel (*e.g.*, Fig1a, Fig1b, Fig1c, Fig1d, etc.), please send just one file for each composite figure.

Figure dimensions

Figures should be properly sized and cropped so that no unnecessary white space is left bordering the figure. Figures are sized to fit 1, 1.5 or 2 column widths. See Table III for column dimensions. The maximum height should be 6 inches (15 cm).

Table III: Maximum figure widths

Units	1-column width	1.5-column width	2-column width
cm	8.9	12.7	18.2
inch	3.5	5	7.2
pica	21	30	43

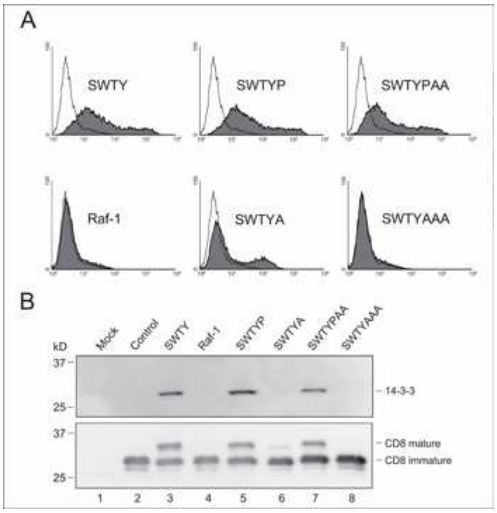
Titles and legends

Figures should have titles and legends containing sufficient detail to make the figure easily understood. Legends should be organized consecutively in a separate section of the manuscript. Indicate the figure number on each figure.

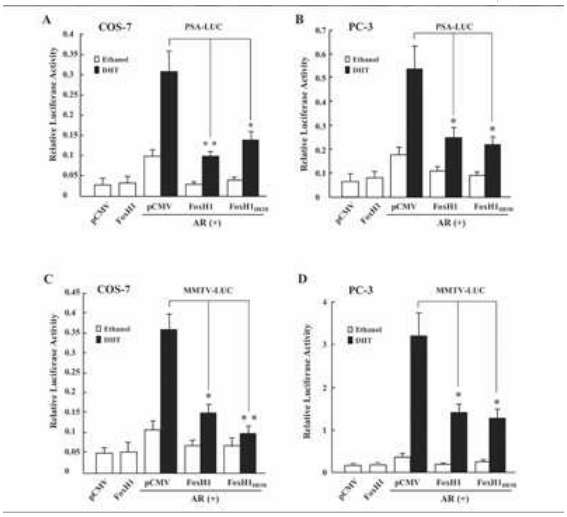
Letters, numbers and symbols

It is important to choose letters with the appropriate proportion and size. Figures in the Journal are sized in 1-column, 1.5-column, or full 2-column widths (see Table III above for dimensions). Your figures will be sized so that the smallest type size on the figure will be at least 2mm.

Numbers, letters and symbols used in multi-paneled figures must be consistent. In the examples below, the poor quality figure has a wide range of text sizes and the good quality figure has all the text in proportion.



Poor quality figure
(inconsistent text size and unlabeled graph axes)
Click on the image to see a larger version



Good quality figure
(consistent text and labeled graph axes)
Click on the image to see a larger version

The following fonts are recommended for use in figures:

European PI
Helvetica
Mathematical PI
Times Roman
Symbol
Arial

Graphs

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