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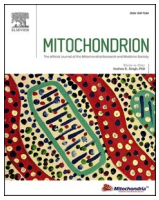

Review

Muscle-UCP3 in the regulation of energy metabolism

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ABSTRACT

Uncoupling protein-3 (UCP3) is a mitochondria-regulatory protein with potential energy- homeostatic functions. This study explores the role of UCP3 in the regulation of muscle- and energy metabolism. UCP3 is critical for tuning substrate utilization, favoring lipid oxidation, particularly in conditions of high-fat availability. While UCP3 is non-essential for lipid oxidation during energy excess, it proves vital during fasting, indicating an energy-homeostatic trait. Preliminary evidence indicates UCP3′ promotion of glucose uptake and oxidation, at least in conditions of high glucose/low fat availability. However, the dynamics of how fats and glucose differentially influence UCP3 remain undefined. UCP3 exhibits inducible proton transport and uncoupling activity, operating in a dual manner: *a resting state* with no/low activity and an *activated state* in the presence of activators. Uncoupling may enhance thermogenesis in specific conditions and in the presence of activators such as fatty acids, thyroid hormones, and catecholamines. This energy-dissipative activity adapts to varying energy availability, balancing energy dissipation with fatty acid oxidation to optimize whole-body energy homeostasis: fasting triggers UCP3 upregulation, enhancing lipid utilization while suppressing uncoupling. Additionally, UCP3 upregulation induces glucose and lipid disposal from the bloodstream and decreases tri-/diglyceride storage in muscle. This process improves mitochondrial functionality and insulin signaling, leading to enhanced systemicgluco-metabolic balance and protection from metabolic conditions. Reviewed evidence suggests that UCP3 plays a crucial role in adapting the system to changing energy conditions. However, the precise role of UCP3 in regulating metabolism requires further elucidation.

1. Introduction

Uncoupling proteins (UCPs) are a family of five structurally-homologous transmembrane proteins located in the inner mitochondrial membrane (Codella et al., 2023) that control different aspects of mitochondrial function. The primary role of UCPs is to reflux protons into the mitochondrial matrix, uncoupling substrate oxidation from the ATP synthesis and dissipating transmembrane proton potential in the form of heat (Codella et al., 2023). UCPs have also protective effects against oxidative stress, mitochondrial dysfunction and are implicated as energy regulators (Codella et al., 2023). Among the five members of UCPs family, the most studied and well-characterized for their proton transporter and uncoupling properties are UCP1, UCP2 and UCP3.

Uncoupling protein-1 (*UCP1*), also known as "thermogenin" is expressed in brown adipose tissue (BAT) and white adipocytes, which shares cell linage and metabolic characteristics with brown adipocytes (*beige* or *bright* adipocytes) (Bouillaud et al., 2016; Hirschenson et al., 2022). UCP1 possesses high proton transport activity and remains the prototype of proton uncouplers among the UCP family. It creates a shunt through which protons are redirected from the mitochondrial intermembrane space into the mitochondrial matrix, generating heat in response to proper stimuli (such as fatty acids, cold exposure, and increased sympathetic outflow) (fatty acids, cold exposure, augmented sympathetic outflow) (Bouillaud et al., 2016; Hirschenson et al., 2022). This mechanism is essential for thermogenesis in newborn mammals and is critical in small rodents during adult life to ensure acclimation to cold conditions (Bouillaud et al., 2016; Hirschenson et al., 2022). The uncoupling/thermogenic activity of UCP1 partly accounts for the metabolic regulatory properties of BAT in animals and likely in humans, suggesting that upregulation of UCP1 could be a potential strategy to combat obesity and related metabolic disorders (Giordano et al., 2016; Maurizi et al., 2018; Maurizi et al., 2018). All UCP isoforms have tissue-specific expression distinct from UCP1 while possessing a certain degree of structural homology with UCP1.

UCP2 is an anion transporter located in the mitochondrial inner membrane with 59% sequence homology with UCP1. Its gene is closely located to UCP3 (chromosome 11 in humans), suggesting gene

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Nomenclature

Abbreviations

AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocator
BAT	Brown adipose tissue
DAG	Diacylglycerol
HFD	High Fat Diet
KO	Knock-Out, mice with inactivated ("knocked out") gene/s
LPL	Lipoprotein lipase
PPAR	Peroxisome proliferator-activated receptor
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1-α
RC	Regular Chow
ROS	Reactive Oxygen Species
SERCA	Sarcoplasmic Reticulum Ca^{2+} ATPase
T2D	Type 2 Diabetes mellitus
TCA	Tricarboxylic Acid Cycle
TG	Transgenic
TGC	Triglycerides
UCP	Uncoupling Protein/s
WAT	White adipose tissue
WT	Wild Type

duplication and similar physiological roles (Bouillaud et al., 2016; Hirschenson et al., 2022). Unlike UCP1, UCP2 is expressed at various levels in many body tissues, with particularly high abundance in skeletal muscle and the heart. Its broad distribution in body tissues suggests a non-exclusive thermogenic effect of the molecule (Bouillaud et al., 2016; Hirschenson et al., 2022). UCP2 appears to play important roles in lipid oxidation, reactive oxygen species (ROS) scavenging, and regulating tissue energy utilization and whole-body energy homeostasis. However, the activating stimuli, proton transport activity, and uncoupling properties of UCP2 are largely undefined. Although it is known that UCP2 is induced by fatty acids, the exact mechanism by which fatty acids stimulate proton transport, as well as the potential involvement of UCP2 in the regulation of metabolic balance, remains elusive (Bouillaud et al., 2016; Hirschenson et al., 2022).

UCP3 is 6-domain peptide chain, exhibiting 55% homology with UCP1 and 73% with UCP2 (Pohl et al., 2019). It is expressed in various tissues including skeletal muscle, heart, pancreas, spleen, BAT and white adipose tissue (WAT) (Kutsche et al., 2022). Although BAT is the principal reservoir of UCP3, it represents the dominant UCP isoform in skeletal muscle (Kutsche et al., 2022; Hilse et al., 2016). UCP3 can operate as a regulator of energy homeostasis at both tissue and whole-body levels. Changes in UCP3 expression are accompanied by variations in energy substrate utilization and energy balance in animals (Codella et al., 2023; Pohl et al., 2019; Bevilacqua et al., 2005). Mice overexpressing UCP-3 are generally leaner and protected from weight gain and obesity compared to wild-type (WT) mice (Codella et al., 2023). UCP3 expression is induced by fatty acids, and UCP3 modulates their transport and oxidation in mitochondria (Seifert et al., 2008). Several studies have demonstrated a true uncoupling activity of UCP3, which may be critical for whole-body thermogenesis (Codella et al., 2023; Nabben et al., 2011). Moreover, early observations in animals and humans suggest that specific UCP3 genotype polymorphisms, influencing the expression/activity of the protein, may be involved in the genesis of metabolic disorders such as obesity and type-2 diabetes (T2D) (Liu et al., 2013; Costford et al., 2008). However, the lack of suitable experimental models and the functional overlap with cognate uncoupling proteins pose significant challenges to the selective assessment of UCP3 function (Bouillaud et al., 2016; Pohl et al., 2019). Therefore, the precise physiological role of UCP3 in energy metabolism remains largely undetermined (Pohl et al., 2019).

This review critically discusses available evidence in an effort to elucidate the role of muscle-UCP3 in the regulation of energy metabolism and analyzes the putative effects of UCP3 in obesity-related metabolic conditions.

2. UCP3 proton-transport activity

The ability of UCP3 to transport protons has been highlighted in various investigations (Pohl et al., 2019; Parker et al., 2008; Gong et al., 1997). UCP3's proton-transporting function was evidenced in liposomes and bilayer membrane systems (Macher et al., 2018), showing a rate of proton transport comparable to UCP2 and about 5-fold lower than UCP1 (Macher et al., 2018). UCP3 demonstrates increased proton conductance upon superoxide stimulation (Esteves and Brand, 2005) and its activity in presence of fatty acids increases at physiological mitochondrial membrane potential (Lombardi et al., 2010). This underscores the pivotal role of the mitochondrial membrane potential in regulating UCP3-mediated proton leakage at a consistent concentration of fatty acids. Importantly, ex vivo studies have shown no differences in proton conductance in mitochondria between UCP3-KO and WT mice unless stimulated (Nabben et al., 2011; Lombardi et al., 2010; Cadenas et al., 2002; Criscuolo et al., 2006). For instance, Lombardi et al. found no difference in proton conductance between UCP3-KO and WT mice in non-stimulated muscle mitochondria. However, when challenged with arachidonic acid, mitochondria from WT mice exhibited higher proton conductance compared to KO mice (Lombardi et al., 2010). These findings suggest that UCP3's proton transport activity is inducible rather than constitutive. (Fig. 1).

3. Regulation of UCP3 in skeletal muscle

Skeletal muscle harbors all five isoforms of UCPs, though UCP3 is the most abundant (Kutsche et al., 2022). Within skeletal muscle, UCP3 is believed to serve various functions, including the promotion of fatty acid oxidation and energy dissipation through uncoupling activity (Pohl et al., 2019). At the cellular level, UCP3 gene transcription is stimulated by AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptors (PPARs) (Kutsche et al., 2022; Putman et al., 2003).

Various biological stimuli can induce UCP3 expression in muscle, although a specific inducer of UCP3 has not yet been identified. Potent activators of muscle UCP3 include non-esterified fatty acids and the superoxide anion (Kutsche et al., 2022; Echtay et al., 2002). Thyroid hormones and 9-cis retinoic acid serve as stimuli for UCP3 activation, upregulating UCP3 mRNA by directly acting on the promoter region of the gene as well as indirectly, via stimulating PPARs (Gong et al., 1997; Shaw et al., 2003; Nagase et al., 1999; Tawfik et al., 2022). Leptin markedly increases UCP3 expression in skeletal muscle (Henry et al., 2011; Nozhenko et al., 2015), with no effect on UCP1 (Henry et al., 2011). Irisin has been also shown to enhance UCP3 expression in myocytes *in vitro* and *in vivo* (Vaughan et al., 2014; Tekin et al., 2018). Energy restriction is associated with increased expression of UCP3 in skeletal muscle (Samec et al., 1999; Collins et al., 2002). Conversely, UCP3 can be fully inhibited by purine nucleotides and phosphate independently, unlike UCP1 (Macher et al., 2018). The distinct inhibitory signals for UCP1 and UCP3 suggest that the two proteins may have evolved to fulfill complementary functions (Macher et al., 2018). Additionally, conditions such as chronic stress, inactivity, and mild cold exposure are linked to lower expression of UCP3 in muscle mitochondria (Kutsche et al., 2022; Liu et al., 2013; Blaylock et al., 2004).

3.1. Muscle-UCP3 expression is differently modulated by acute and chronic exercise

A significant upregulation of UCP3 in response to an acute exercise

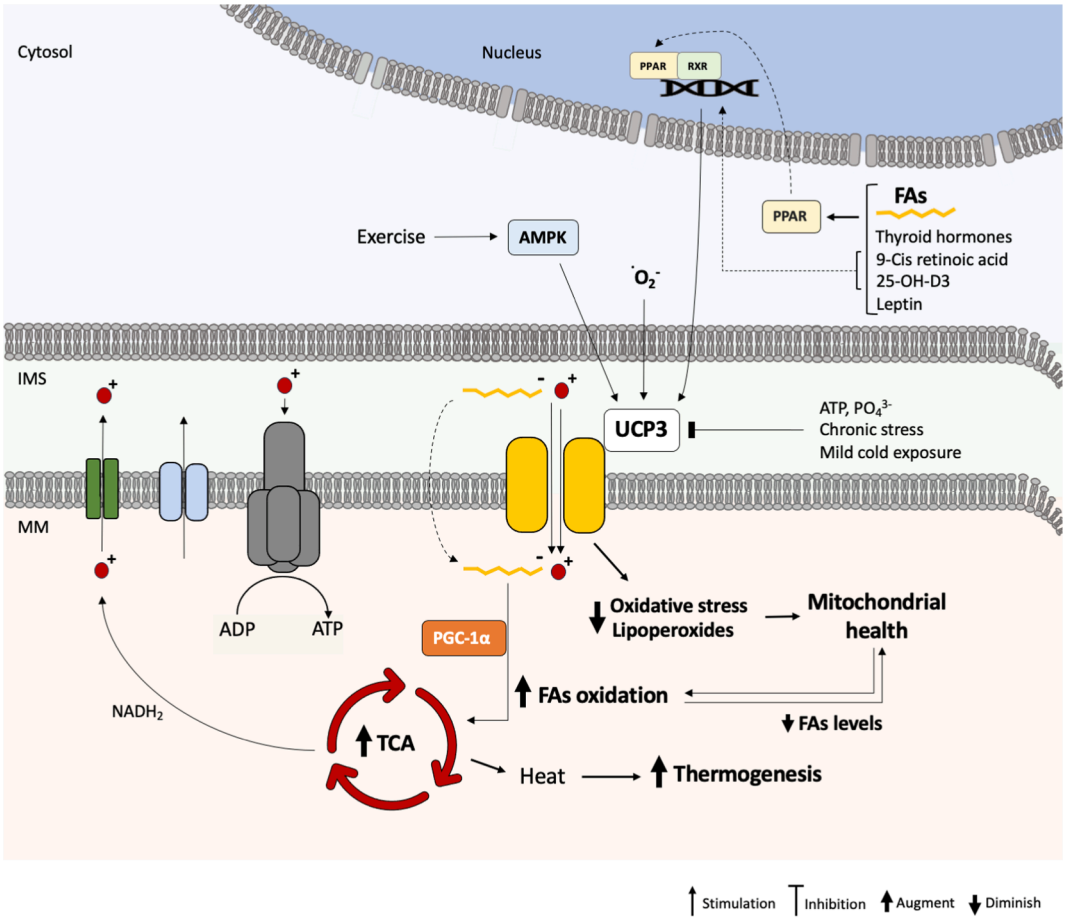

Fig. 1. UCP3 proton transport, fatty acid metabolism and thermogenesis in skeletal muscle. Fatty acids (FAs) induce UCP3 expression via activating PPAR, and likely directly. Thyroid hormones and leptin directly activate UCP3 gene transcription and/or indirectly, via PPAR activation. 9-cis retinoic acid and vitamin D induce UCP3 direct binding to the promoter. Purine nucleotides and stress conditions inhibit UCP3 expression. UCP3 exhibits inducible proton conductance and uncoupling activity upon fatty acid stimulation. UCP3 mediates FAs translocation across the inner mitochondrial membrane (IMM) and induces FAs oxidation, especially during fasting conditions, partly via activating PGC-1α. This results in lower levels of FAs in the mitochondrial matrix. Fatty acids and probably other modulators (such as catecholamines, thyroid hormones, and cis retinoic acid) increase proton shunt, resulting in an increased thermogenetic effect, which can be enabled exclusively in the presence of fatty acids. *FAs, fatty acids; IMS, intramembrane space; MM, mitochondrial matrix; PPAR,* Peroxisome proliferator-activated receptor; *PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1-α; TCA, tricarboxylic acids cycle.*

bout has been described in different studies (De Queiroz et al., 2012; Kogure et al., 2005), likely driven by AMPK activation (Thomson et al., 2009). The increased plasma concentration of fatty acids in response to acute exercise likely stimulates the expression of UCP3 in the skeletal muscle (Kutsche et al., 2022). The elevation of UCP3 during acute exercise may serve as a mechanism to enhance fatty acid oxidation and/or to buffer ROS produced under higher oxidation rates. In contrast to acute exercise, chronic training has been shown to have no effect on UCP3 expression (Cortright et al., 1999) or even downregulate UCP3 levels (Gong et al., 1997; De Queiroz et al., 2012; Boss et al., 1998; Wolf et al., 2021). This suggests a metabolic adaptation in muscle mitochondria aimed at decreasing energy dissipation to optimize energy substrate utilization during periods of high energy demand (Fig. 2) (Cortright et al., 1999). Interestingly, the decrease of UCP3 upon chronic exercise was accompanied by an increase in protein expression in WAT (Cortright et al., 1999), suggesting i) a positive adaptive mechanism to chronic training occurring outside the skeletal muscle; ii) a potential role of UCP3 in the mobilization/utilization of stored triglycerides (TGC) in WAT, in chronically trained animals (Wolf et al., 2021). Alongside the decrease in UCP3 expression in skeletal muscle (Gong et al., 1997); chronic endurance training was also found to elevate UCP1/UCP3 mRNA in rats' BAT (Gong et al., 1997) as possible metabolic adaptation (Della Guardia and Codella, 2021). These data suggest a mutual regulation of the UCP homologues in response to changes in whole-body energy conditions, which deserves further elucidation (Shabalina et al., 2010).

4. UCP3 regulates energy substrates oxidation in skeletal muscle

4.1. *UCP3 expression is stimulated by fatty acids*

A substantial body of evidence supports the pivotal role of UCP3 in the utilization of energy substrates, particularly lipids (Pohl et al., 2019; García-Martínez et al., 2001). The transcription of UCP3 gene is regulated by fatty acids (Bouillaud et al., 2016), which compete with ATP for binding to UCP3, a major suppressor of UCP3 activity (Macher et al., 2018). In rodents, the administration of fatty acids significantly enhances UCP3 mRNA compared to saline-infused controls (Weigle et al., 1998; Schrauwen et al., 2003), and UCP3 activation increases with the level of unsaturation and the length of fatty acids chain (Macher et al., 2018). Muscle-specific UCP3 expression was shown to increase in response to lipid availability both upon consumption of high fat diet (HFD) (Chou et al., 2001) and under energy restriction (Samec et al., 1999). Mice fed a low-calorie low-fat diet exhibited lower mRNA levels of UCP3 (Samec et al., 1999). In contrast, re-feeding with an HFD increased UCP3 expression, even under conditions of suppressed energy

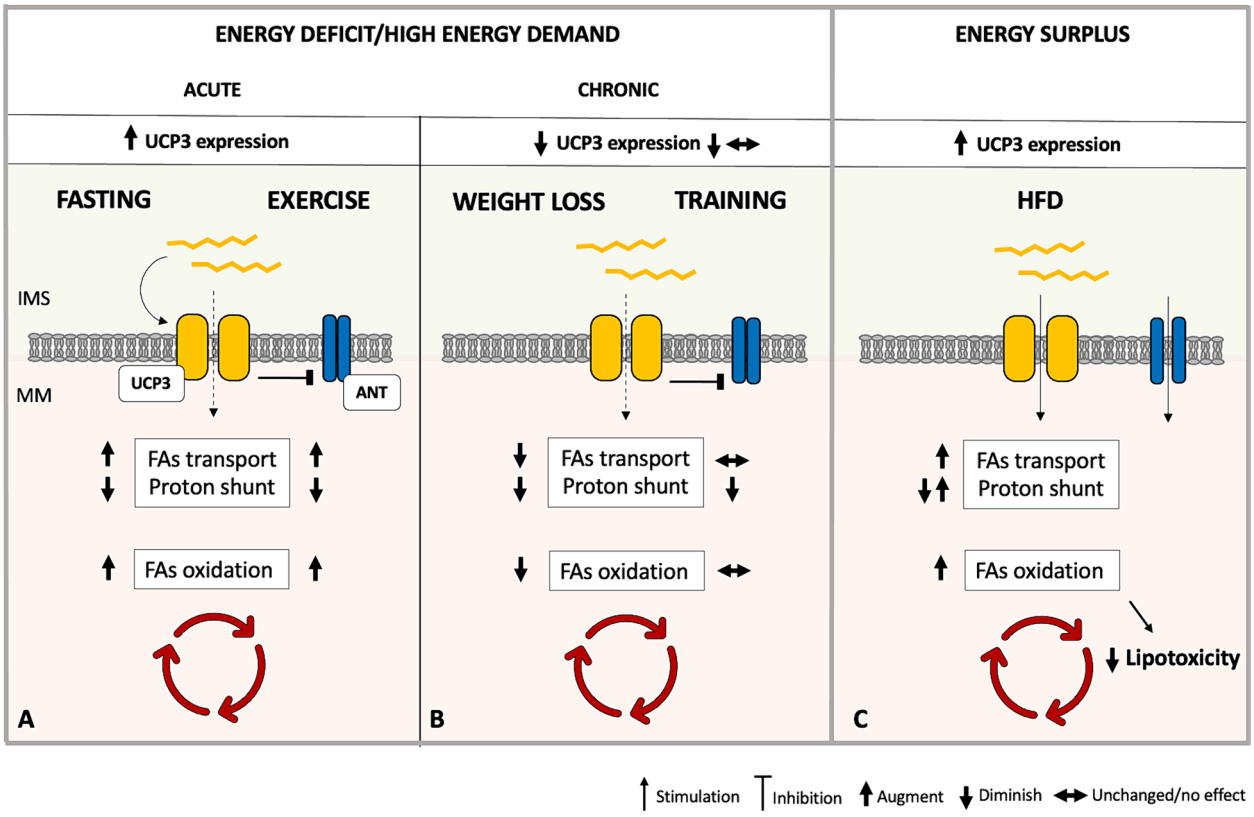

Fig. 2. Hypothetical scheme illustrating the differential effect of fatty acid availability on UCP3 in skeletal muscle according to energy status. (A) In energy-deprived conditions or during transient high energy demand, UCP3 is overexpressed in response to fatty acid stimulation and exhibits enhanced fatty acid transport properties. This condition is associated with increased fatty acid oxidation. However, the uncoupling activity of UCP3 is partially suppressed to prevent excessive energy dissipation. This suppression of uncoupling is achieved, in part, through the inhibition of cognate uncouplers such as ANT. (B) UCP3 adapts to conditions of prolonged energy deficit or sustained energy demand. Following endurance training, the expression of UCP3 remains unchanged or may even decrease in skeletal muscle, likely due to optimized fatty acid oxidation. During calorie restriction, UCP3 expression is downregulated, leading to a suppression of fatty acid utilization over time with the aim of reducing whole-body metabolic rate. Similar to energy-deprived conditions, uncoupling activity is suppressed to prevent excessive energy dissipation. (C) In conditions of high-fat feeding, UCP3 is upregulated to promote lipid utilization, thereby protecting cells and mitochondria from lipid accumulation and lipotoxicity. In this scenario, the uncoupling activity of UCP3 remains unchanged, although it may vary depending on the presence of activators or inhibitors such as catecholamines, thyroid hormones, or retinol. However, the physiological signals that regulate this differential activity of UCP3 are not yet fully defined. *ANT, adenine nucleotide translocator*; *HFD, high fat diet; FAs, fatty acids. IMS; intramembrane space; MM, mitochondrial matrix.*

expenditure (Samec et al., 1999; Crescenzo et al., 2003), suggesting that UCP3 responds to elevated lipid levels, independent from the condition determining lipid availability (De Queiroz et al., 2012; Kogure et al., 2005).

4.2. *UCP3 enhances fatty acids oxidation*

UCP3 promotes fatty acids oxidation within mitochondria (Fig. 2) (García-Martínez et al., 2001; Darcy MacLellan et al., 2005) (Fig. 2). In mouse skeletal muscle, activation of PPAR-δ has been shown to enhance mitochondrial fatty acid utilization through UCP3 activation, (Koh et al., 2020). Pharmacological blockade of fatty acid transportation in myocytes mitochondria suppresses UCP3-induced fatty acids oxidation (García-Martínez et al., 2001). Molecules that activate oxidative phosphorylation and β-oxidation, such as peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) and PPAR-β enhance UCP3 expression in cultured myocytes during myogenesis and in response to palmitate stimulation (Lima et al., 2019). Additionally, UCP3 is essential for the full expression of PGC-1α-induced oxidative capacity and the adaptive mitochondrial response to fatty acid challenge (Lima et al., 2019). Given that PGC-1α-induced mitochondrial biogenesis is directly influenced by intracellular energy status, it is plausible that processes involving increased energy demand stimulate UCP3 expression.

Skeletal muscle mitochondria in UCP3-KO mice exhibit a lower ability to oxidize fatty acids compared to WT mice (Lombardi et al., 2019). UCP3 overexpression in cultured myocytes has been demonstrated to stimulate the oxidation of palmitate and oleate (Darcy MacLellan et al., 2005), irrespective of glucose concentration (García-Martínez et al., 2001; Darcy MacLellan et al., 2005), consequently reducing the concentration of fatty acids in mitochondrial matrix (Schrauwen et al., 2001). The muscle-specific overexpression of UCP3 enhances the transport of lipids into mitochondria and increases fatty acid oxidation (Bezaire et al., 2005; Wang et al., 2003), partly via reducing lipoperoxidation (Schrauwen et al., 2001; Hesselink et al., 2003) and mitochondrial oxidative stress (Seifert et al., 2008). UCP3 mRNAs were found to be upregulated in mice overexpressing lipoprotein lipase (LPL) in skeletal muscle, while down-regulated in LPL-deprived mice, regardless of the feeding status or of plasma fatty acids concentrations (Kratky et al., 2001). These findings suggest that increased LPL activity during starvation mat contribute to inducing UCP3 expression (Kratky et al., 2001). Interestingly, significant weight loss in obese humans was accompanied by a gradual decline in UCP3 expression, correlated with decreased fatty acid oxidation (Schrauwen et al., 2000). This indicates that UCP3 is likely downregulated by low lipid availability as weight loss progresses. The gradual downregulation of UCP3 during weight loss may represent muscle adaptation to a slowed metabolic rate aimed at preserving fat mass, a mechanism commonly observed in calorie restriction (Knuth et al., 2014). Additionally, it is

noteworthy that the body's adaptation to calorie restriction is partly driven by reduced concentrations of leptin, a hormone secreted by white adipose tissue with thermogenic properties, which also acts as a UCP3 inducer (Knuth et al., 2014). Importantly, while UCP3 was found to be necessary for fasting-induced enhancement of fatty acid oxidation rate in muscle (Seifert et al., 2008), Seifert *et al.* demonstrated that UCP3 is not strictly required for fatty acid oxidation in muscle under fed conditions (Seifert et al., 2008).

4.3. UCP3 activation lowers TGC/DAG accumulation protecting from lipotoxicity

In UCP3-TG mice, the upregulation of markers of fatty acid oxidation is accompanied by decreased intramuscular TGC accumulation (Bezaire et al., 2005). Under low-fat feeding conditions, Costford *et al.* observed that UCP3-TG mice exhibited decreased TGC storage in muscles compared with both WT and UCP3-KO mice (Costford et al., 2006). Similar results were observed under HFD feeding conditions (Lombardi et al., 2019): UCP3-KO mice showed increased accumulation of intramyocellular TGC compared with WT mice (Lombardi et al., 2019; Costford et al., 2006), which in turn presented greater TGC stores compared to UCP3-TG (Costford et al., 2006). This suggests a critical role for UCP3 in preventing accumulation of TGC, in muscle. Consistent with these findings, muscle-UCP3 overexpressing mice showed marked reduced accumulation of diglycerides (DAG) in both muscle and liver (Cheol et al., 2007). These results suggest that UCP3 activation can protect against the accumulation of long-chain fatty acids and consequent lipotoxicity, especially during prolonged high-fat feeding (Schrauwen et al., 2003; Schrauwen et al., 2001). Elevated levels of saturated fatty acids (e.g., palmitate) induce mitochondrial dysfunction (Schrauwen et al., 2001) and insulin resistance in skeletal muscle (Della Guardia and Codella, 2021). Indeed, Lima et al. demonstrated that PGC-1α overexpression in myocytes prevented palmitate-induced cell death, whereas UCP3 knockdown exacerbated this effect, indicating that UCP3 is involved in this protective mechanism (Lima et al., 2019). A recent study by Koh et al. showed that the activation of UCP3-stimulated fatty acid utilization (Koh et al., 2020), contributed to protect mitochondria against lipotoxicity and insulin resistance in skeletal muscle secondary to alcohol intake (Koh et al., 2020). On the other hand, in several works, Nabben et al. questioned the protective role exerted by UCP3 against lipotoxicity (Nabben et al., 2011; Nabben et al., 2011; Nabben et al., 2014).

4.4. UCP3 stimulates glucose uptake and oxidation in muscle

Embryonic evidence indicates a stimulatory activity of UCP3 on glucose utilization in skeletal muscle (Fig. 3) (Darcy MacLellan et al., 2005; Wang et al., 2003). Rodents overexpressing UCP3 in muscle

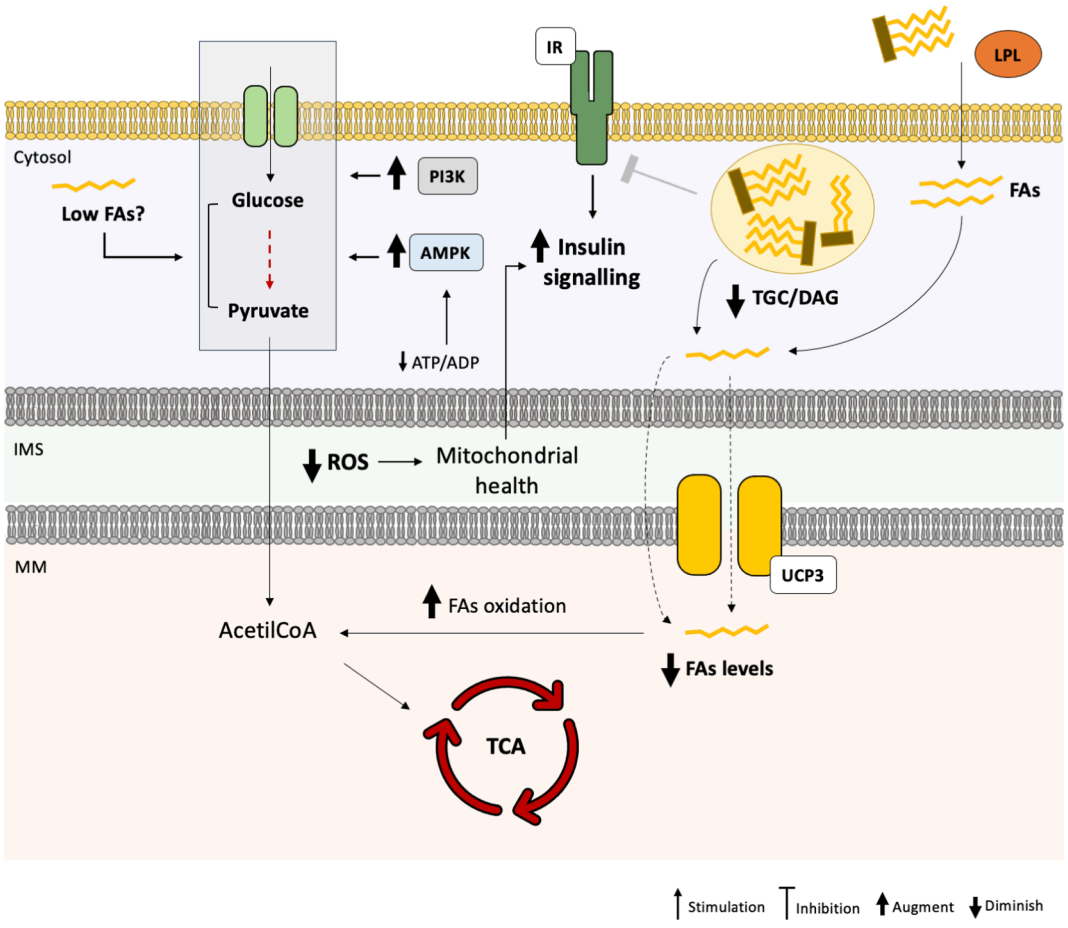

Fig. 3. Theoretical model depicting potential mechanisms underlying UCP3 regulation of skeletal muscle metabolism. UCP3 plays a crucial role in promoting fatty acid (FAs) oxidation within muscle tissue, facilitating the disposal of FAs from circulation. This activity leads to a reduction in the storage of triglycerides (TGC) and diglycerides (DAG), thereby protecting against mitochondrial lipotoxicity. The decreased accumulation of stored lipids within myocytes likely contributes to improved insulin sensitivity in muscle tissue. Additionally, UCP3 activation promotes glucose oxidation, potentially through the upregulation of PI3K and AMPK pathways, facilitating the disposal of glucose from circulation. These effects collectively contribute to the amelioration of systemic insulin sensitivity and glucose balance, thereby aiding in the maintenance of metabolic health. *DAG, diglycerides; FAs, fatty acids; IMS; intramembrane space; IR, insulin receptor; LPL, lipoprotein lipase. MM, mitochondrial matrix; PI3K, phosphoinositide 3-kinase; TCA, tricarboxylic acids cycle; TGC, triglycerides.*

exhibit lower whole-body glucose concentrations (Wang et al., 2003). In cultured myocytes, UCP3 overexpression stimulates a slight increase of glucose uptake and elevated the glycolytic rate by upregulating phosphoinositide 3-kinase (PI3K) (García-Martínez et al., 2001; Huppertz et al., 2001). In mice skeletal muscle, GLUT4 overexpression and the increased glucose influx in muscle cells were accompanied by augmented UCP3 expression (Tsuboyama-Kasaoka et al., 1999). Similarly, in trained animals exhibiting low levels of UCP3, feeding a high sugar diet promoted muscle UCP3 mRNA upregulation, suggesting a stimulatory effect of glucose, particularly in conditions of high concentrations (Gong et al., 1997). The increased activity of AMPK observed in skeletal muscle of mice overexpressing UCP3 has been suggested to mediate the enhanced glucose uptake/utilization (Schrauwen et al., 2004). In apparent contrast with these findings, Wang *et al.*, in mice overexpressing mucle-UCP3, observed no changes in glucose uptake in myocytes (Wang et al., 2003). To further understand the complex regulatory effect of UCP3 on glucose metabolism, Garcia-Martinez *et al.* showed that glucose oxidation in UCP3-overexpressing myocytes was stimulated only in the presence of low fat concentrations (García-Martínez et al., 2001). Similarly, pyruvate oxidation was increased by approximately 1.5-fold in the presence of fatty acid in myocytes of mice overexpressing UCP3 selectively in muscle. Altogether, these findings suggest that glucose uptake/oxidation can vary according to the availability of energy substrates. Conditions of high glucose and low lipids stimulate glucose uptake/utilization, and vice versa. However, the dynamics by which fatty acids and glucose mutually interact to differentially modulate UCP3 expression/activity remain undefined. Mailloux et al. showed that UCP3 was required to upregulate aerobic metabolism in glucose-treated cells and in the muscle of fed mice (Mailloux et al., 2011). In contrast, the knockout of UCP3 produced a metabolic shift that favored anaerobic glycolytic metabolism and increased glucose uptake. These findings suggest that i) UCP3 optimizes metabolic adaption by promoting aerobic metabolism; ii) glucose uptake can be observed when anaerobic metabolism is enhanced (Mailloux et al., 2011). Data on the effect of UCP3 on carbohydrate metabolism are limited and do not provide clear insights into the regulatory mechanisms, mainly due to the scarce and poorly deepened evidence on the subject.

5. UCP3 exhibits inducible uncoupling activity

5.1. UCP3 uncoupling activity: Evidence from -KO and -TG models

A substantial body of evidence supports the presence of true uncoupling activity for UCP3. In isolated myocytes, UCP3 knockout correlated with reduced proton leak, lower chain complex I-induced respiration, and maximal coupled respiration (McBride et al., 2019), resulting in increased ATP production (De Marchi et al., 2011). Similarly, in rodents, *capsiate*-induced UCP3 downregulation led to elevated ATP and phosphocreatine levels at rest and during muscle activation (Faraut et al., 2007). Muscle mitochondria from UCP3-KO mice, exhibited decreased state 4 respiration and decreased concentrations of ADP, a pivotal regulator of ATP production (Vidal-Puig et al., 2000). UCP3 also seems to impede the kinetics of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), an uncoupler that dissipates heat by increasing ATP hydrolysis. Indeed, in cultured cells, UCP3 knockout led to increased mitochondrial ATP production and accelerated endoplasmic reticulum Ca^{2+} refilling activity, suggesting enhanced SERCA activity (De Marchi et al., 2011). These observations indirectly support the existence of true uncoupling activity of UCP3 and indicate that SERCA and UCP3 may have redundant activity regulated by mutual inhibitory feedback, likely governed by ATP availability in the cell (De Marchi et al., 2011). However, the dynamic regulating this inhibitory feedback is undefined.

Studies on -TG animals corroborate the existence of a UCP3 true uncoupling activity. Muscle mitochondria from *ad libitum* fed UCP3 transgenic mice exhibited higher basal uncoupling activity, with unchanged maximal oxidative phosphorylation compared to pair-fed WT mice (Estey et al., 2012). Isolated mitochondria from muscle-UCP3-TG mice demonstrated increased state-4 of respiration and increased proton leak across the inner membrane (Clapham et al., 2000). Likewise, muscle-UCP3-TG mice exhibited reduced efficiency in oxidative phosphorylation (Codella et al., 2023; Cline et al., 2001) augmented respiration rate and proton conductance (Cadenas et al., 2002). Importantly, in absence of appropriate activators of proton transport, UCP3 does not seem to express uncoupling (Mozo et al., 2006). In this respect, MacLellan *et al.* demonstrated that mitochondrial membrane potential decreased only in presence of dinitrophenol (a UCP3 uncoupling stimulator), while no changes were observed following UCP3 overexpression (Darcy MacLellan et al., 2005). Consistent with this observation, UCP3 overexpression was associated with augmented uncoupling activity during thyroid hormone stimulation (Jucker et al., 2000) and PPARδ activation (Koh et al., 2020). However, some investigations have questioned the uncoupling capacity of UCP3 (Nabben et al., 2011; Cadenas et al., 2002; Bezaire et al., 2005; Jucker et al., 2000). Pair-fed UCP-KO mice showed no change in maximal leak-dependent respiration in muscle, while paradoxically exhibiting increased proton conductance compared to WT (Cadenas et al., 2002; Bevilacqua et al., 2010). Notably, this was accompanied by an increased adenine nucleotide translocator (ANT) activity (a cognate uncoupler), suggesting that the heightened proton conductance in the KO models can be a result of ANT activation (Bevilacqua et al., 2010). Collectively, these findings suggest that UCP3 exhibits uncoupling selectively *in vivo,* in the presence of activators (Cadenas et al., 2002; Darcy MacLellan et al., 2005; Mozo et al., 2006).

5.2. UCP3 uncoupling in energy-restricted conditions

In energy-restricted conditions, muscle-specific UCP-TG mice undergoing calorie restriction lose as much weight as WT littermates (Estey et al., 2012), indicating that UCP3 uncoupling and energy-dissipating activity are likely not active during calorie restriction (Fig. 2) (Estey et al., 2012). As a possible explanation, Bevilacqua *et al.* noted that UCP3 increase upon calorie restriction, was paralleled by a drop in mitochondria maximal respiration (Bevilacqua et al., 2010) and UCP3-TG mice under calorie restriction showed reduced proton conductance in muscle (Estey et al., 2012). Additionally, starvation was found to elevate UCP3 levels in skeletal muscle mitochondria, while no change in proton conductance was observed (Cadenas et al., 1999), suggesting energy conservation. Jucker *et al.* measured the Tricarboxylic Acid Cycle (TCA) cycle flux and ATP synthesis in mice subject to days of fasting: although fasting increased UCP3 mRNA and protein levels in muscle, there were no changes in mitochondrial energy coupling activity (as measured by the ratio of ATP synthesis to TCA flux) (Jucker et al., 2000). Similarly, WT mice subjected to calorie restriction (40% less energy compared to a standard diet) exhibited a decrease in maximal leak-dependent respiration and increased UCP3 expression in muscle mitochondria, without alterations in proton conductance (Bevilacqua et al., 2010). These experiments suggested that UCP3 likely suppresses energy-dissipative mechanisms during food restriction (Bevilacqua et al., 2010), partially through the inhibition of adenine nucleotide translocator (ANT) activity in skeletal muscle (Fig. 2) (Bevilacqua et al., 2010).

6. UCP3 activation enhances thermogenesis

6.1. Thermogenic activity in UCP3-KO and UCP3-TG models

Uncoupling proteins dissociate the proton gradient for the synthesis of ATP, leading to heat production. This mechanism is well defined for UCP1 and it is essential for inducing the thermogenesis in rodents during early and adult life (Van Den Berg et al., 2011). Several observations suggest that UCP3 activity can contribute to increase energy expenditure (Table 1). In obese animals and humans, for example, the expression of UCP3 is markedly suppressed (Cortes de Oliveira et al., 2018;

Table 1
Studies evaluating changes in energy expenditure upon variation of UCP3 expression in muscle.

Study	Model	UCP3 expression	Effect
Lima et al. (2019)	PGC-1α over-expressing myocytes	UCP-KO (myocytes)	↓ Max respiration rate ↑ TGC storage
Darcy MacLellan et al. (2005)	Myocytes	UCP-TG (myocytes)	No effect
Codella et al. (2023)	RC mice	UCP-TG (2–3 folds; muscle)	↑ Basal metabolic rate ↑ Energy expenditure
Son et al. (2004)	RC mice HFD-fed mice	UCP-TG (18 folds; muscle)	No effect ↑ Energy expenditure

HFD, high fat diet. PGC-1α, proliferator-activated receptor-γ coactivator 1-α. RC, regular chow. TGC, triglycerides.

Schrauwen et al., 2001; Son et al., 2022). Also, muscle-specific overexpression of UCP3 mice increased mitochondrial uncoupling activity and rendered the animals resistant to HFD-induced obesity (Tiraby et al., 2007).

Mice overexpressing UCP3 in muscle have been found to possess higher metabolic rates than pair-fed WT controls, with the increased energy expenditure correlating with augmented uncoupling activity, strongly suggesting the presence of a UCP3-induced thermogenic effect (Codella et al., 2023). In support, after long-term HFD feeding, UCP3-TG resulted to be less metabolically efficient (higher ratio gram weight gain/kcal intake) and accumulated less WAT than both WT and UCP3-KO pair-fed littermates (Costford et al., 2008). However, it is important to note that data on uncoupling activity derived from UCP-TG animals may be subject to a model-intrinsic bias (Cadenas et al., 2002), and therefore should be interpreted cautiously. While the concept of a thermogenic role for UCP3 is intriguing, other studies in isolated myocytes or mice have reported no changes in thermogenesis following artificially-induced variations in UCP3 expression or alterations in energy substrate availability (see Table 1) (Darcy MacLellan et al., 2005; Son et al., 2004). As for other UCP3 functions, the thermogenetic activity may be ancillary or only activated in specific physiological conditions. Indeed, as suggested for the uncoupling activity, the presence of specific activators of UCP3 is probably critical for the induction of a relevant thermogenetic activity, *in vivo*. For example, in a set of chow-fed and HFD-fed UCP3-TG mice, only the HFD-fed animals exhibited higher energy expenditure compared to WT (Son et al., 2004), implying that thermogenesis can probably be activated only in presence of fatty acids (see Figs. 1 and 3).

6.2. *The modulation of thyroid- and catecholaminergic pathways suggests a thermogenic role of UCP3*

Experimental models manipulating thyroid or catecholamine pathways highlight the role of UCP3 in thermogenesis. Thyroid hormones, known for their thermogenic properties, strongly stimulate UCP3 expression in skeletal muscle (Tawfik et al., 2022). In mice lacking β-adrenergic receptors, both cold tolerance and UCP3 expression in skeletal muscle increased following exogenous triiodothyronine administration, suggesting that thyroid hormone-induced thermogenesis in skeletal muscle partly depends on UCP3 (Flandin et al., 2009; Jimenez et al., 2002). Supporting this, the removal of fatty acids (potent UCP3 activators) was sufficient to eliminate proton leak in muscle mitochondria in hyperthyroid rodents (Sprague et al., 2007). According to a proposed model, the thermogenic effect of thyroid hormone in skeletal muscle may be mediated by fatty acids released from white adipose tissue in response to increased catecholaminergic signaling (Gong et al., 1997; Sprague et al., 2007; Solmonson and Mills, 2016).

Evidence supporting the pivotal role of UCP3 in thyroid-induced thermogenesis includes observations that triiodothyronine-administered UCP3-knockout mice exhibited a lower thermogenic response than WT mice and were unresponsive to thermogenic stimulation by sympathomimetic agents such as methamphetamines (Flandin et al., 2009). Conversely, selective overexpression of UCP3 in skeletal muscle restored the thermogenic response to methamphetamine in these mice (Riley et al., 2016). Additionally, mice lacking both UCP1 and UCP3 failed to increase heat production in response to methamphetamine, and exhibited accelerated loss of body temperature and lower survival rates after cold exposure compared to UCP1-knockout mice (Riley et al., 2016); these mice also showed an accelerated loss of body temperature and lower survival rate after cold exposure, compared to UCP1-KO mice (Riley et al., 2016). These findings underscore the critical role of muscle-specific UCP3 and catecholamine stimulation in inducing thermogenesis, suggesting that UCP1 alone may be insufficient for an adequate physiological thermogenic response in animals.

7. UCP3 activation prevents metabolic alterations: Evidence *in vitro*, in animals and in humans

Studies conducted in cultured cells and animals suggests a relationship between UCP3 expression and improved insulin sensitivity and glucose homeostasis (Fig. 3). In cultured myocytes, UCP3 overexpression stimulates GLUT4 translocation, glucose uptake (García-Martínez et al., 2001) and glycolysis (García-Martínez et al., 2001; Huppertz et al., 2001), likely by activating the PI3K (Huppertz et al., 2001; Schrauwen et al., 2004). Higher UCP3 mRNA levels in rodent muscle have been associated with improved glucose tolerance (Samec et al., 1999). In mice, varying degrees of UCP3 overexpression in the skeletal muscle (ranging from 5 to 20 folds) were associated to an increased glucose clearance rate and lowered fasting glucose and insulin levels, compared to WT littermates (Wang et al., 2003; Clapham et al., 2000). Administration of vitamin D3 has been found to protect against HFD-induced obesity by activating the UCP3 gene and increasing protein expression in muscles (Fan et al., 2016). Increased UCP3 activity has been shown to reduce TGC/DAG accumulation in the cytoplasm and mitochondrial matrix of muscle cells as well as decreases ROS/lipoperoxide production, which is critical for maintaining proper insulin sensitivity in muscle cells (Hirschenson et al., 2022; Della Guardia and Codella, 2021; Schrauwen et al., 2001). AMPK-mediated UCP3 activation has been observed to stimulate fatty acid utilization, protecting against TGC accumulation and insulin resistance in skeletal muscle secondary to alcohol intake (Koh et al., 2020). Similarly, mice overexpressing UCP3 in muscle resulted protected from DAG accumulation and the development of insulin resistance in liver and muscle upon HFD feeding, compared to WT littermates (Cheol et al., 2007; Tiraby et al., 2007).

In humans, decreased UCP3 protein expression has been reported in skeletal muscle of subjects with T2D (Schrauwen et al., 2001). Similarly, UCP3 protein content was found to be reduced in skeletal muscle of obese-prediabetic and T2D subjects compared to age- and BMI-matched healthy controls (Schrauwen et al., 2006). Interestingly, in these patients, short-term treatment with the insulin sensitizer – *rosiglitazone* - significantly increased insulin sensitivity and restored UCP3 expression (Schrauwen et al., 2006). Mogesten *et al.* found that maximal ADP-stimulated respiration and the electron transport chain were markedly suppressed in skeletal muscle mitochondria of T2D subjects, while UCP3 levels remained within the normal range (Mogensen et al., 2007). The effect of UCP3 on metabolic health is supported by the observation that specific polymorphisms in the UCP3 gene (e.g. −55C/T) are associated with increased risk of developing T2D, obesity and -related metabolic conditions in humans (Liu et al., 2013; Cassell et al., 2000; Meirhaeghe et al., 2000). These early insights collectively indicate a critical involvement of UCP3 in the regulation of energy balance and a potential

role in the prevention of metabolic alterations.

8. Concluding remarks and future perspectives

UCP3 plays a significant role in regulating energy balance, primarily by modulating the translocation and oxidation of fatty acids in mitochondria, particularly under conditions of elevated lipid concentration or low-calorie intake (e.g., during exercise or fasting). Additionally, UCP3 appears to be associated with increased glucose uptake and oxidation in muscle cells, particularly when lipid availability is low. However, the precise interaction between fatty acids and glucose in modulating UCP3 expression and activity remains unclear.

The evidence reviewed suggests that UCP3 exhibits true uncoupling activity, which is regulated by a dual dynamic: a *resting state* with minimal or no uncoupling activity in the absence of activators and in the presence of inhibitors, and an *activated state* with significant uncoupling activity. This uncoupling activity is modulated by the availability of energy substrates and the need to balance energy preservation and dissipation to optimize homeostasis under varying energy conditions. Under appropriate stimulation, UCP3 uncoupling may also contribute to increased thermogenesis in animals, although this phenomenon is not yet fully understood. Furthermore, UCP3 improves mitochondrial health and may enhance insulin sensitivity and metabolic balance by promoting the disposal of lipids, and possibly glucose, from circulation and reducing tri-/diglyceride storage in muscle.

Despite significant advancements, our understanding of UCP3 physiology and its contribution to systemic metabolic homeostasis remains limited. Several factors contribute to this:

I) The limited reliability of animal models: knocking out constitutive genes like UCP3 leads to forced adaptations in biological systems, resulting in biased physiological responses. Transgenic UCP-TG models may also have limitations due to possible artifacts related to transgenic expression.
II) Interactions with cognate uncoupling proteins: UCP3 interacts with other uncoupling proteins, such as UCP1, ANT, and SERCA, which may have redundant functions, making it challenging to selectively interrogate the role of UCP3.
III) Lack of knowledge about specific physiological stimuli that activate UCP3 to enable its specific functions, such as proton transport, uncoupling, and thermogenesis.

Establishing experimental models to selectively explore UCP3 physiology remains a challenge. Further studies are needed to clarify UCP3's role in energy metabolism. Several areas warrant deeper investigation:

- Fatty acids and glucose metabolism: understanding UCP3's role as a fatty acid translocator, its modulation by glucose, and its effects on glucose uptake and oxidation, as well as the metabolic signals driving these processes.
- Mechanistic links: investigating the role of lipid load in regulating UCP3 expression and uncoupling, the drivers of AMPK upregulation in the context of UCP3 overexpression, and the regulation of this phenomenon by other activators.
- UCP3 and energy expenditure: exploring the presence and regulation of UCP3's thermogenic activity in animals and humans, the mechanisms underlying its regulation, and its interaction with cognate uncoupling proteins to maintain energy homeostasis under varying energy conditions.

Despite the significant attention UCP3 has received in the last three decades, much remains unknown, and research on UCP3 physiology is still in its infancy. Identifying UCP3 functions and its activators may be key to considering UCP3 as a potential target for mitigating obesity-associated metabolic conditions.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lucio Della Guardia Livio Luzi and Roberto Codella. Lucio Della Guardia wrote the manuscript and prepared the figures; all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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