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Review

The regulation and turnover of mitochondrial uncoupling proteins

Vian Azzu [a], Martin Jastroch [b], Ajit S. Divakaruni [a,b], Martin D. Brand [b,*]

[a] Medical Research Council Mitochondrial Biology Unit, Hills Road, Cambridge CB2 0XY, UK
[b] Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945, USA

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ABSTRACT

Uncoupling proteins (UCP1, UCP2 and UCP3) are important in regulating cellular fuel metabolism and as attenuators of reactive oxygen species production through strong or mild uncoupling. The generic function and broad tissue distribution of the uncoupling protein family means that they are increasingly implicated in a range of pathophysiological processes including obesity, insulin resistance and diabetes mellitus, neurodegeneration, cardiovascular disease, immunity and cancer. The significant recent progress describing the turnover of novel uncoupling proteins, as well as current views on the physiological roles and regulation of UCPs, is outlined.

1. Introduction

Mitochondria are the centre of metabolism in cells, coupling the oxidation of substrates to ATP synthesis by an electrochemical proton gradient. Varying this protonmotive force allows for adjustments in energy metabolism to maintain metabolic homeostasis. For this reason, the coupling of substrate oxidation is incomplete, as protons can leak across the mitochondrial inner membrane independently of ATP production. This unregulated futile proton conductance is of considerable physiological relevance, as it can account for as much as 20–70% of cellular metabolic rate depending on cell type [1,2]. A majority of proton leak can be strictly attributed to the abundance, but not activity, of mitochondrial carrier proteins such as the adenine nucleotide translocase (ANT) and, in brown adipose tissue (BAT), uncoupling protein 1 (UCP1) [3,4].

Importantly, the regulation of proton leak allows for responses to fluctuations in energy demands and controls energy transduction to maintain cellular homeostasis and body function. The first proton leak mechanism was identified in BAT, where UCP1-catalysed proton conductance generates heat to defend body temperature during cold acclimation [5]. Sequence similarity allowed the identification of its paralogous proteins UCP2 and UCP3 [6,7]. These UCPs do not contribute to basal proton conductance *in vitro* in the absence of specific activators [8]. When activated, however, all UCPs (including avian and plant UCPs) can catalyse proton leak [9]. The precise mechanisms of activation and inhibition of both UCP2 and UCP3, as well as their physiological role, remain uncertain [10,11]. There has been considerable recent progress, however, in understanding the transcriptional and translational regulation that implicates UCP2 and UCP3 in adaptation to nutritional status and oxidative stress. More recently, the unique dynamic regulation of UCP2 reveals a new mechanism for the regulation of mitochondrial energy metabolism by the novel UCPs.

2. Acute activation of uncoupling protein activity

UCP1 activity is highly regulated at the molecular level by small molecules. It is inhibited by physiological concentrations of purine nucleoside di- and tri-phosphates and stimulated when fatty acids overcome nucleotide inhibition [12].

How fatty acids activate the net protonophoric activity of UCP1 is still debated. Broadly, there are three models that can explain the dependence on fatty acids. In the first, fatty acids act as co-factors by embedding their carboxyl groups in the core of the protein to bind and release protons as they access amino acid side chains during transport [13]. Evidence that UCP1 can translocate chloride and fatty acid anions suggests a second model. In this mechanism, protonated fatty acids

Abbreviations: ANT, adenine nucleotide translocase; ATF1, Cyclic AMP-dependent transcription factor; ATP, adenosine triphosphate; BAT, brown adipose tissue; GDP, guanosine diphosphate; ORF, open reading frame; PPAR, peroxisome proliferator-activated receptor; SREBP-1c, sterol regulatory element-binding protein-1c; TRE, thyroid response element; UCP, uncoupling protein; UTR, untranslated region

* Corresponding author. Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945, USA. Tel.: +1 415 493 3676; fax: +1 415 209 2232.
E-mail addresses: va@mrc-mbu.cam.ac.uk (V. Azzu), mjastroch@buckinstitute.org (M. Jastroch), adivakaruni@buckinstitute.org (A.S. Divakaruni), mbrand@buckinstitute.org (M.D. Brand).

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freely diffuse across the mitochondrial inner membrane. The pH gradient promotes their dissociation into fatty acid anions in the matrix, and the fatty acid anions are then exported from the matrix by UCP1 [14]. The net activity results in proton conductance across the inner membrane, though in this model UCP1 itself does not translocate protons. Thirdly, fatty acids themselves may not be directly required for UCP1 activity, but instead act as allosteric activators by promoting a conformation of the protein that is protonophoric (or that translocates hydroxide ions), since fatty acids and nucleotides appear to affect proton conductance in a manner described by simple competitive kinetics [15,16].

It remains unclear to what extent UCP2 and UCP3 are subject to the same acute molecular regulation as UCP1 (and the extent to which they share the same mechanism of uncoupling). Although they lack sequence homology in a matrix-localised region reportedly critical for fatty acid activation of UCP1 [17], proteoliposome studies show that UCP2 and UCP3 have similar fatty acid-activated proton conductance and purine nucleotide inhibition as UCP1 [18–20]. One difficulty has been the inability to directly compare UCPs in mitochondria, since UCP2 and UCP3 are expressed in different tissues and at hundred-fold lesser amounts than UCP1 [21–23]. Another difficulty relates to the fact that GDP has been shown to inhibit uncoupling via ANT [24,25] as well as by the UCPs. This complicates the calculations of UCP-mediated proton leak in tissues that express different amounts of UCP and ANT when activity is defined as GDP-sensitive uncoupling.

There is evidence that superoxide, both exogenous [26] and endogenous [27], and lipid peroxidation products such as hydroxynonenal [25,28,29] can activate uncoupling by all three UCPs, suggesting a model in which superoxide reacts with membrane phospholipids to generate the proximal activator, hydroxynonenal [28,30]. The physiological relevance of this model, which has not been reproduced in all laboratories, remains controversial [10,31–33].

3. Role and regulation of uncoupling proteins

The archetypal uncoupling protein, UCP1, is best known for its role in adaptive non-shivering thermogenesis and control of body weight, whereby a cold stimulus or over-feeding results in sympathomimetic stimulation of β_3-adrenergic receptors in BAT. This leads to upregulation of *Ucp1* mRNA expression via a BAT-specific enhancer box [34], activation of UCP1 by fatty acids [35] produced from lipolysis [36], and the transduction of the mitochondrial protonmotive force into heat [37]. Indeed *Ucp1* knockout results in the absence of non-shivering thermogenesis [38], loss of cold tolerance [39] and appearance of obesity at thermoneutrality [40]. Beyond thermogenesis, the role of UCP1 in thymus [41,42] and in ectotherms [43] remains speculative.

The UCP1 paralogues, UCP2 and UCP3, probably evolved from a duplication event in vertebrates. This is supported by their juxtaposition in the genome and their high sequence identity with each other (72–74% from fish to mammals). Sequence analysis shows that unlike UCP1, UCP2 and UCP3 are under strong purifying selection, suggesting that they have not changed function during evolution [44].

The literature varies on whether or not UCP2 and UCP3 are upregulated in response to cold in various organisms and tissues [45–47], but they are not thought to be significantly thermogenic [48], primarily because of their low abundance. However, rodent UCP3 can participate in thermogenesis under particular conditions [49,50]. UCP2 and UCP3 are also upregulated in response to starvation, and have been linked with a number of processes including insulin secretion from pancreatic β-cells [51] and insulin resistance [52] in peripheral tissues, as well as modulation of reactive oxygen species production and immune responses [10,53–55].

3.1. UCP2 function

An ever-increasing number of studies highlight the significance of UCP2 in a broad range of physiological and pathological processes, including cytoprotection [55–58], immune cell modulation [53,59] as well as the regulation of glucose sensing in the brain [60] and pancreas [51].

In thymocytes [61] and the intact INS-1E pancreatic β-cell model [62], UCP2 decreases the coupling between substrate oxidation and ATP production. Since mitochondrial ROS production is highly sensitive to decreases in protonmotive force [63–65], UCP2-mediated dissipation of the mitochondrial membrane potential and pH gradient results in decreased reactive oxygen species production [66,67], particularly during reverse electron transport [65].

In glucose-sensing cells in the pancreas and brain, UCP2 attenuates insulin secretion, likely acting in two ways. By lowering the coupling efficiency of oxidative phosphorylation, UCP2 decreases the ATP/ADP ratio, resulting in the decreased stimulation of K_{ATP} channels and lowered insulin secretion [51,68]. It may also function by decreasing ROS production [67], which is important signal in glucose-sensing systems [69,70].

As well as improving the diabetic phenotype via increased insulin secretion [51], UCP2 downregulation also improves insulin resistance in peripheral tissues such as white adipose [71]. Although much work indicates that UCP2 exacerbates the diabetic phenotype, recent work from the Collins group suggests that this effect is dependent on genetic background, and that the chronic absence of UCP2 causes persistent oxidative stress in general and impairs β-cell function [72]. However, it is unlikely that these findings simply invalidate all previous work demonstrating attenuation of glucose-stimulated insulin secretion by UCP2. For example, acute *in vivo* knockdown of UCP2 using antisense oligonucleotides in two animal models of diabetes and insulin resistance causes a significant improvement in insulin secretion and enhanced whole-body sensitivity to insulin [73]. In the light of the cytoprotective effects conferred by UCP2, however, Pi *et al.* [72] question the validity of the approach of inhibiting UCP2 function in order to improve glucose-stimulated insulin secretion in diabetes [74]. Numerous studies have shown that by attenuating oxidative stress, UCP2 promotes cell survival in pancreatic α- [58] and β- [55] cells and in neurones [57], and can regulate colon tumour formation [75] and atherosclerosis [56,76].

Newell and colleagues propose an interesting hypothesis in which a cell's ability to efficiently metabolise fat confers immune privilege. Specifically, they suggest that UCP2 is a part of the mechanism controlling the change from one metabolic strategy (glucose metabolism) to another (primarily lipid metabolism), and by doing this, UCP2 plays a role in preventing immune-mediated pathology [54,77]. This, of course, is in line with the cytoprotective effect of UCP2 described earlier. Bouillaud has recently proposed that this function could be explained by a uniport for anionic pyruvate that lowers the preference for pyruvate oxidation as membrane potential increases [78]. However, this hypothesis has yet to be experimentally verified and remains speculative.

3.2. Regulation of UCP2 concentration

UCP2 regulation occurs in a concerted manner by modulation of protein activity and protein content. Ligands (such as fatty acids and ROS derivatives) that stimulate UCP2 catalytic activity in isolated mitochondria [11] may also play a role in upregulating UCP2 content [79].

In hyperglycaemia and hyperlipidaemia, as occurs in diabetes mellitus, *Ucp2* gene transcription is activated by key regulatory proteins such as peroxisome proliferator-activated receptors (PPARs), forkhead transcription factors, and sterol regulatory element-binding protein-1c (SREBP-1c) [80]. Sirt1, a protein that has been implicated in metabolic stress resistance, suppresses the function of these proteins, thus decreasing *Ucp2* expression and promoting insulin secretion [81]. Additionally, reactive oxygen species and their products have been implicated in the upregulation of UCP2

expression, resulting in cellular defence via a negative feedback loop that decreases ROS production [82,83], although a direct interaction of ROS with ROS-responsive elements upstream of UCPs has never been demonstrated.

Ucp2 is also translationally regulated by an inhibitory upstream open reading frame (ORF) [84], which, when mutated, results in maximal *Ucp2* mRNA translation [84]. Glutamine, an amino acid that has been implicated in the insulin secretion pathway [85], overcomes ORF inhibition and increases *Ucp2* translation efficiency [86].

Recently we showed that in INS-1E pancreatic β-cells, UCP2 levels are dynamically regulated in response to nutrient supply, and this rapid fluctuation in content is permitted by variable synthesis rates coupled with rapid degradation [87]. Regulation of turnover is further discussed in Section 4.

3.3. UCP3 function

UCP3 tissue-specificity has been maintained during evolution: it is specific to skeletal muscle, although in mammals, notable protein levels are also found in BAT [7].

In mammals, cold-induced expression initially led to the conclusion that UCP3 may mediate thermogenesis in the same way as UCP1 [88]. However, the apparent upregulation during fasting and the lack of change in body temperature of *Ucp3*-ablated mice argue for no physiological thermogenic role for mammalian UCP3 [89,90]. Nonetheless, there is evidence that UCP3 may be involved in thermogenesis of some description, albeit not as its main function. Nau *et al.* have recently shown that a selective lack of UCP3 in BAT impairs non-shivering thermogenesis [50]. Other evidence points to this role of UCP3 in muscle: pharmacological intervention using the drug MDMA (3,4-methylenedioxymethamphetamine) in *Ucp3*-ablated mice results in a diminished thermogenic response [49]. These data do not necessarily suggest that UCP3 is directly involved in thermogenesis, but that it may, by currently unknown mechanisms, be necessary for the machinery that is. Additionally, there is a body of work showing that increased UCP3 levels do not always result in increased uncoupling [91], and work from our laboratory has reached the same conclusion [92]. As changes in protein concentration do not necessarily result in concomitant increases in protein activity [93], caution is required when interpreting these data.

Protection from ROS has been suggested as a putative role for UCP3. In this model, the protein can be activated with endogenous [27] and exogenous superoxide [26] as well as lipid peroxidation products [30], dissipating mitochondrial membrane potential and decreasing ROS production [64]. This theory is supported by work showing that UCP3 neutralises protein oxidation in skeletal muscle [94] and may mitigate ROS production during exercise [95]. Furthermore, UCP3 knockout mice have higher oxidative damage [96] and UCP3 over-expressing mice have reduced ROS production during aging [97].

Other work has suggested that UCP3 is involved in fatty acid metabolism. UCP3 over-expression has been shown to increase fatty acid transport and oxidation [98]. The hypothesis that UCP3 physiologically functions as a fatty acid transporter [99,100] has recently been refuted by Seifert *et al.*, who found that whilst UCP3 was necessary for the fasting-induced enhancement of fatty acid oxidation rate and capacity via mitigated mitochondrial oxidative stress, UCP3 was not itself a fatty acid transporter [101].

In addition to increased fatty acid oxidation and reduced ROS production, UCP3 over-expressing mice also have decreased diet-induced obesity [102] and are protected against insulin resistance. Insulin resistance in peripheral tissues, in particular skeletal muscle, is a major cause of type 2 diabetes mellitus [52]. Type 2 diabetes can be promoted by obesity and aging, which on the cellular level broadly equates to impaired fatty acid metabolism and oxidative damage from uncontrolled ROS production.

PPAR agonists such as rosiglitazone have been successfully used to treat insulin resistance. UCP3 expression is upregulated in response to PPAR stimulation, and UCP3 has been suggested as a potential therapeutic target to treat insulin resistance in skeletal muscle by dissipating energy of fat storage. Treatment of human patients with rosiglitazone upregulates UCP3 expression and improves insulin resistance in diabetic patients [103]. Although this effect can be explained by burning of excessive fats by mitochondrial uncoupling [104], some groups suggest that this occurs via a mechanism other than uncoupling [105,106], although the nature of this mechanism remains unspecified.

3.4. Regulation of UCP3 concentration

Although non-mammalian UCP3 has not been investigated extensively, fasting-induced gene expression appears conserved among vertebrates from fish and birds to mammals [46].

Coordinated *Ucp3* expression by fasting, cold, or high fat diet requires transcription factor binding to the *Ucp3* promoter region. MyoD and PPAR elements are responsible for muscle specificity and fatty acid responsiveness [107]. A region 1500 base pairs upstream from the *Ucp3* promoter that drives BAT-specific expression was recently found [108]. A role for UCP3 in thyroid metabolism has also been suggested [109] and, indeed, an active thyroid hormone response element was identified in the proximal promoter region of *Ucp3* [110]. The stimulation of *Ucp3* transcription by hormones that regulate energy expenditure and fat metabolism in skeletal muscle suggests (in line with other work) that UCP3 itself may also be involved in these processes. *Ucp3* transcription is well studied, but nothing is known about *Ucp3* mRNA translation efficiency. However, similar to *Ucp2*, there are pseudo-start codons in the *Ucp3* 5′UTR that can putatively trap ribosomes (MJ, unpublished data).

UCP3 activity can be further modulated via protein-protein interactions, activating/inhibiting ligands and by proteolysis. Speculatively, stress-responsive genes such as those of the 14.3.3 family have been implicated as having protein-protein interactions with UCP2 and UCP3, but not with UCP1 [111]. Although the biochemical significance of this interaction remains sketchy, it raises the question as to whether or how they are involved in UCP2 and UCP3 regulation.

Recently, we have made some headway in describing UCP3 turnover, which is more similar to UCP2 in half-life and mechanism of degradation than it is to UCP1 (discussed in Section 4). A non-exhaustive summary of UCP regulation is shown in Fig. 1.

4. Turnover of uncoupling proteins

Despite the fact that proteolysis is important in controlling overall levels of any given protein, the issue of UCP half-life and turnover has remained surprisingly neglected in the field, being described well over ten years after the discovery of each of the UCP homologues.

UCP1 half-life in BAT is in the order of hours to days and is significantly increased by administration of noradrenaline, which also upregulates UCP1 synthesis [112]. However, the mechanism of turnover remained uncertain until Desautels and colleagues showed that the proteolytic rates of other mitochondrial proteins parallel those for UCP1, and that the half-lives of UCP1 and other mitochondrial proteins are delayed by lysosomal inhibition [113,114].

The half-life and turnover mechanism of UCP1 differs from that of its homologues UCP2 and UCP3. These both have unusually short half-lives, which are at least an order of magnitude lower than that for UCP1. UCP2 has a half-life of one hour in a range of tissues [83,115], including pancreatic β-cell models [87]. We showed that this rapid half-life is not a general feature of mitochondrial inner membrane proteins like ANT, and is not recapitulated in isolated energised mitochondria, suggesting that an extramitochondrial factor may be required for efficient UCP2 degradation [87]. We further

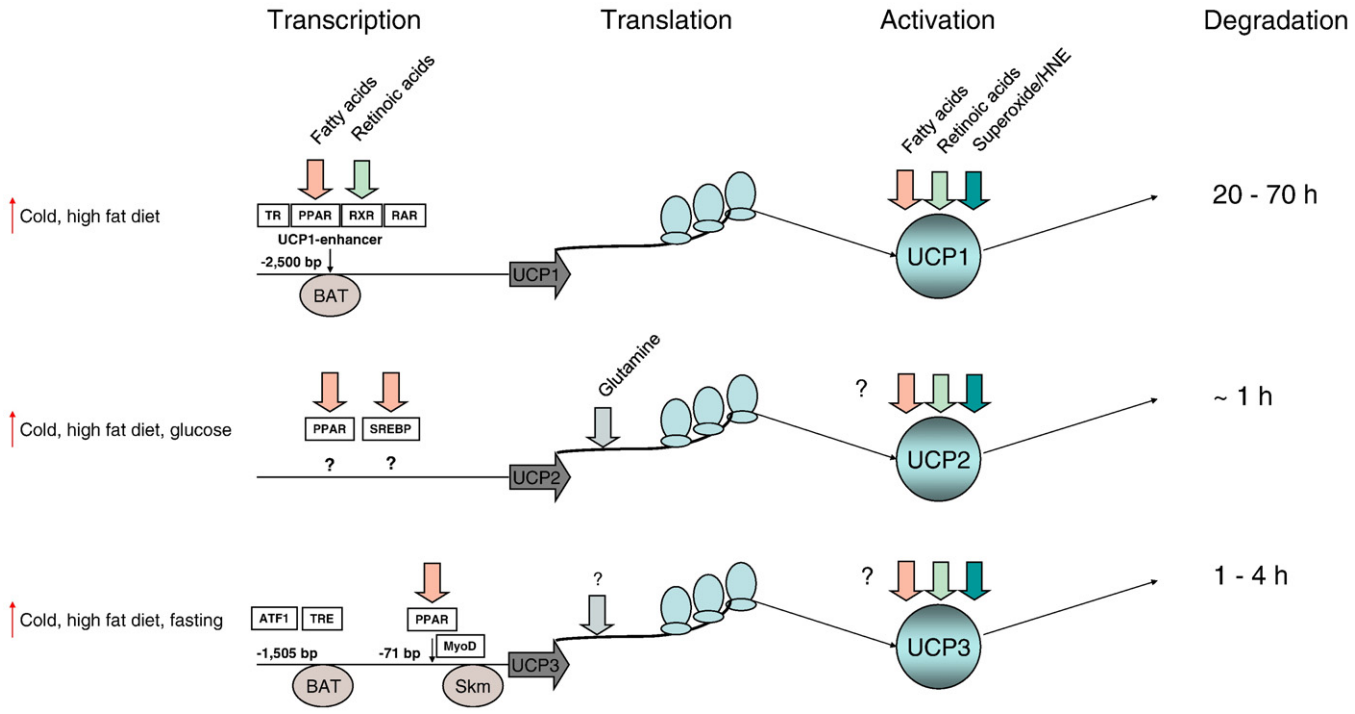

Fig. 1. Mammalian UCP gene expression and activity is regulated at multiple steps. Stimuli such as cold and over-feeding activate sympathomimetic pathways that act on the UCP1 enhancer box (2.5 kb upstream), thereby increasing *Ucp1* gene expression in BAT. These pathways also increase lipolysis resulting in fatty acids that stimulate UCP1 catalytic activity. Inhibition of lysosomal pathways that degrade UCP1 also contribute to optimising UCP1-mediated thermogenesis. UCP2 appears in various tissues. Its gene expression is regulated by various nutrients and cytokines/immunomodulators, which act via PPAR and SREBP at the transcription level. Translation efficiency is regulated by either the upstream ORF or pseudo-start codons in the 5′UTR, and this region appears to be responsive towards glutamine. UCP3 is targeted to skeletal muscle by coordination of PPAR and the MyoD element. A 1.5 kb upstream element controls BAT-specific expression. PPAR elements may transmit information about changes in fatty acid metabolism to *Ucp3* gene expression. The transcription factor ATF1 appears to regulate hypoxic-induced regulation of UCP3 while TREs mediate response to thyroid hormone. Translation efficiency has not been studied, but there are pseudo-start codons in the 5′UTR, putatively trapping ribosomes. UCP2 and UCP3 (but not UCP1) are rapidly turned over by the cytosolic proteasome.

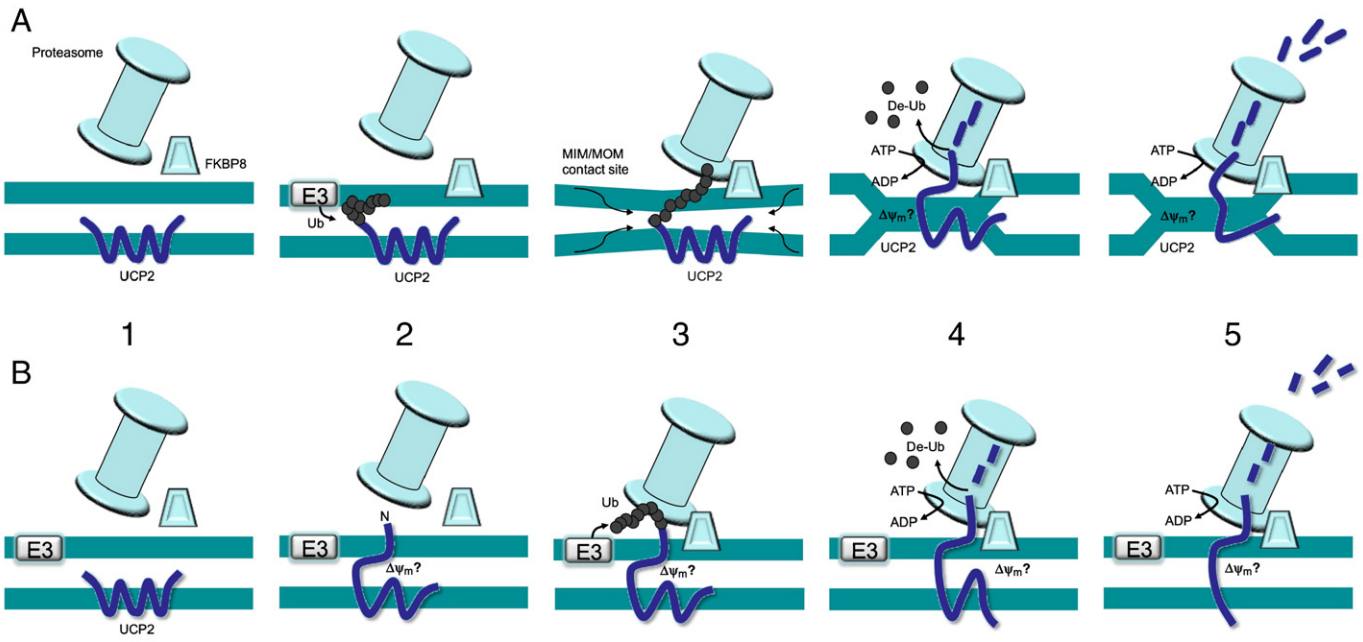

Fig. 2. Models of UCP2 degradation. In model A, UCP2 is ubiquitinated by an unidentified putative E3 ligase (A2) and unfolded from the mitochondrial inner membrane by processes that may be ATP- or $\Delta\psi$-dependent. At the mitochondrial outer membrane, the proteasome, perhaps tethered by FKBP8, recognises polyubiquitinated UCP2 (A4) and participates in its extraction from mitochondria in an ATP-dependent fashion, whereby the protein is subsequently degraded by the peptidase activity of the proteasome core (A5). However, firstly it remains unknown whether UCP2 can be ubiquitinated whilst still residing inside mitochondria: there are no known intramitochondrial E3 ligases, and it is widely believed that mitochondrially associated E3 ligases reside in the outer membrane and ubiquitinate proteins on the cytosolic face of mitochondria. Secondly, the speculative nature of model A also extends to the formation of mitochondrial inner and outer membrane contact sites (A3). Since there is evidence that the proteasome may be required for direct removal of UCP2 from mitochondria [116], the contact site feature was modelled to describe how the cytosolic proteasome might gain access to UCP2 given the interposition of the mitochondrial outer membrane. An alternative model B to explain the data from [116] is that a mitochondrial process ejects UCP2 only as far out as the mitochondrial outer membrane (B2), whereupon it is ubiquitinated by cytosolic-facing outer membrane-associated E3 ligases (B3), then retrotranslocated by the proteasome (B4) before being degraded in the cytosol (B5).

demonstrated that this extramitochondrial factor is the cytosolic proteasomal machinery [116]. Use of proteasome inhibitors, ubiquitin mutants and a novel cell-free reconstituted system showed that cytosolic proteasomal function is required for rapid UCP2 degradation in cells and in isolated mitochondria [116]. How this cytosolic machinery accesses inner membrane residing UCP2 despite the interposition of the mitochondrial outer membrane remains unknown, but our working models of how this might be achieved are shown in Fig. 2.

Using the same techniques, we found that UCP3 also has a half-life of between 1 and 4 h [117]. In contrast to UCP2 and UCP3, UCP1 and ANT had much longer half-lives and could not be degraded in the cell-free reconstituted system, suggesting their degradation is not mediated by the cytosolic proteasome [117].

We postulate that this fast turnover allows for rapid variations in UCP2 [87] and UCP3 levels in response to changes in nutrient fluxes, and the proteolytic pathway via the proteasome may allow the rapid regulation of these proteins in concert with other proteins involved in the same pathways. For example, in the pancreatic β-cell, the ubiquitin-proteasome system is responsible for regulating levels of other members of the insulin secretion pathway [118–120].

It is interesting to note that UCP1 protein levels are regulated by modulation of the synthesis and degradation in a concerted fashion [121]. The question arises as to whether UCP2 and UCP3 levels are also controlled in a concerted manner. Giardina *et al.* suggested that ROS not only increase UCP2 transcription but may also slow degradation [83]. However, we showed that the latter is in fact a confounding observation because only bioenergetic manipulations that increase ROS and simultaneously dissipate ATP and mitochondrial membrane potential result in slowing of UCP2 turnover [116]. This is not entirely unexpected since the proteasome-mediated degradation is ATP-dependent. Other than manipulation of ATP and mitochondrial membrane potential, to date, we have yet to find conditions that affect the rate of UCP2 degradation.

Interestingly, the literature suggests that cellular proteolysis via the proteasome increases under catabolic conditions [122], which promote upregulation of UCP2 and UCP3. As such, UCP2 and UCP3 may be subject to constant rapid turnover, with variable expression being dependent primarily on rates of synthesis. It would be noteworthy to further examine whether regulators of UCP2 transcription or translation can also influence turnover, as this remains an alternative possibility.

5. Concluding remarks

There is abundant evidence that UCPs are important metabolic regulators in permitting fat oxidation and in attenuating free radical production. Their levels and activity are regulated by modulators of cellular metabolism at multiple points, including transcription, translation, modulation of catalytic activity and protein degradation. Although UCP2 and UCP3 are not responsible for adaptive thermogenesis, they can, nevertheless be thermogenic when activated by appropriate effectors. Their broad effects on coupling efficiency, ROS production and fatty acid metabolism increasingly implicate them in body-wide pathology and physiology, making them prospective drug targets for the treatment of obesity, atherosclerosis, diabetes, immune disorders and neurodegenerative conditions. Intricate knowledge of UCP regulation and turnover, however, is only just beginning to materialise, and much work is required before we are able to develop therapies with maximal benefit and minimal side effects.

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