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Mitochondrial uncoupling protein 1 expression in thymocytes

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ABSTRACT

Using an antibody specific and selective to mitochondrial uncoupling protein 1 (UCP1) peptide, this study confirms the observation that UCP 1 is present in thymocytes isolated from UCP 1 wild-type, but not UCP 1 knock-out mice. UCP 1 is also shown to be present in thymocytes isolated from rat. It was also demonstrated that an antibody raised to the full-length UCP 1 protein appears to be non-specific for UCP 1, as it detects protein in UCP 1 wild-type and UCP 1 knock-out mice, protein in mitochondria isolated from brown adipose tissue of both UCP 1 wild-type and UCP 1 knock-out mice, as well as detecting protein in mitochondria isolated from rat spleen, kidney, skeletal muscle and liver, tissues that do not express UCP 1. We were also able to show that CIDEA, a soluble protein with a suggested role in regulating UCP 1 function, is equally abundant in thymocytes from UCP 1 wild-type and UCP 1 knock-out mice. Taken together our data demonstrate that (a) UCP 1 is present in rat and mouse thymocytes, (b) that the antibody to full-length UCP 1 is not specific for UCP 1 and (c) that the absence of UCP 1 does not affect native expression of CIDEA in thymocytes.

1. Introduction

UCP 1 is usually only associated with brown adipose tissue (BAT) where it uncouples mitochondria, therein, resulting in the production of heat in a process called non-shivering thermogenesis [1–3]. We have recently discovered UCP 1 in thymus mitochondria [4–8]. Expression of UCP 1 in thymus was shown by multiple techniques [4–8], including: (i) reverse transcriptase-polymerase chain reaction detection of RNA transcripts for UCP 1 in whole thymus and in isolated thymocytes of rats and mice, (ii) anti-UCP 1 peptide antibody detected protein of appropriate molecular mass in mitochondria isolated from whole thymus and thymocytes of rats and mice, but not in thymus mitochondria from UCP 1 knock-out mice, (iii) UCP 1 was purified from thymus mitochondria and identified by mass spectroscopy and (iv) confocal images of UCP 1 detection and association with mitochondria in thymocytes, expressing the thymocyte marker Thy-1, isolated from UCP 1 wild-type, but not UCP 1 knock-out mice.

The objective of this investigation was to pave the way for investigations into the role of UCP 1 in thymus. To that end, we investigated whether we could visualize UCP 1 expression in thymocytes from another species, namely rat. As there are data refuting the existence of UCP 1 in thymus/thymocytes, we addressed discrepancies in these studies. Using an antibody to full-length UCP 1, Frontini et al. [9] have produced histological images of protein detection which they describe as BAT in the vicinity of thymus tissue from mouse and rat. These authors suggest that any detection of UCP 1 in thymus is solely due to associated BAT. Thus, we assessed the sensitivity of the antibody to full-length UCP 1, as used by Frontini et al. [9], relative to the antibody raised against a UCP 1 peptide used in our previous studies [4–8].

Interestingly, there have been other reports, in the literature, of the immunodetection of UCP 1 in tissues other than BAT. Nibbelink et al. [10] reported detecting UCP 1 protein in uterine longitudinal smooth muscle cells, an observation later attributed by Rousset et al. [11] as being due to UCP 2. Recently, Mori et al. [12] reported UCP 1 expression in human skin, with immunohistochemistry, placing UCP 1 in the granular layer of the epidermis, sweat glands, hair follicles, and sebaceous glands of various sites in the human body. Of course these latter observations require further scrutiny.

In addition we also looked at the level of expression of the *C*ell death *I*nducing *D*NA fragmentation factor (DFF), alpha subunit-like *E*ffector *A* (CIDEA) in UCP 1 wild-type and UCP 1 knock-out mice. Zhou et al. [13] have proposed a role for CIDEA in directly regulating UCP 1 in BAT. CIDEA has been shown to activate apoptosis. This activation of apoptosis is inhibited by the DNA fragmentation factor DFF45. Mice that lack functional CIDEA have higher metabolic rates, higher lipolysis in brown adipose tissue and higher core body temperatures when subjected to cold. Consequently, we set out to determine whether native CIDEA expression was affected by UCP 1 expression in mouse thymocytes.

Abbreviations: BAT, brown adipose tissue; BSA, bovine serum albumin; FBS, foetal bovine serum; PBS, phosphate buffered saline; UCP, uncoupling protein; CIDEA, *C*ell death *I*nducing *D*NA fragmentation factor alpha subunit-like *E*ffector *A*; CO III, cytochrome oxidase subunit III

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2. Materials and methods

2.1. Animal source

Wistar rats were bred in-house (BioResources Unit, School of Biochemistry and Immunology, Trinity College Dublin). UCP 1 knock-out mice on a C57BL/6J background, originally provided by Dr. Leslie Kozak (Pennington Biomedical Research Center, Baton Rouge, Louisiana, US) were bred in-house. UCP 1 knock-out mice were confirmed to be homozygous by PCR genotyping of tail DNA. All animals were housed in a specific pathogen-free facility and fed ad libitum. Both male and female mice were used.

2.2. Thymocyte isolation

Thymocytes were isolated from UCP 1 knock-out/wild-type C57BL/6 mice and Wistar rats, essentially as described by Buttgereit and Brand [14]. The thymus was removed, trimmed clean of connective tissue and brown fat (if present) and transferred into Hanks' Balanced Salt Solution (Sigma) containing 10% (w/v) foetal bovine serum (FBS). A single cell suspension was prepared by passage through a 70 μm nylon sieve (Falcon).

2.3. Antibodies and fluorescent probes

Rabbit anti-mouse UCP 1 peptide IgG antibody was obtained from Calbiochem. The antibody was raised against position 145–159 [(C)SHLHGIKPRYTGTYN] of mouse UCP 1. Mouse anti-rat Thy-1 was obtained from Abcam, and mouse anti-rat FITC-conjugated anti-Thy-1 was obtained from Serotec. The sheep anti-mouse antibody to full-length UCP 1 was a gift from Daniel Ricquier [15]. CMXRos (Mitotracker Red), DNA stain (Hoechst 33342), Alexa 488- and Alexa 647-labelled secondary antibodies were obtained from Invitrogen. Cy3 and Cy5 were obtained from Jackson Immunoresearch. Rabbit anti-mouse CIDEA IgG was obtained from Millipore. Rabbit anti-bovine cytochrome oxidase subunit III (CO III) had been raised against position 1–11 [MTHQTHAYHMV] of rat CO III by Eurogentec (Herstal, Belgium).

2.4. Immunofluorescence

To visualize the mitochondria, CMXRos (Mitotracker Red, 200 nM; Invitrogen) was added directly to the thymocyte suspension for 60 min at 37 °C. Following this, 0.1% (v/v) paraformaldehyde was added to the thymocyte suspension and incubated on ice for 5 min. The thymocytes were allowed to dry completely on poly-L-lysine slides and were subsequently fixed with cold methanol (−20 °C) for 5 min and permeabilized with cold acetone (−20 °C) for 5 min. Finally, thymocytes were rinsed briefly with Phosphate Buffered Saline (PBS) buffer containing sodium azide (15 mM).

Thymocytes were blocked with PBS containing 5% (w/v) bovine serum albumin (BSA) and 0.1 M methylamine for 1 h at room temperature. Following this, thymocytes were incubated with primary antibody in 5% (w/v) BSA in PBS overnight at room temperature, followed by incubation with the secondary antibody in 5% (w/v) BSA in PBS for 3 h at room temperature. After a brief rinse in PBS, the slides were mounted with coverslips and sealed. The mounting media used was 50% (v/v) glycerol in PBS containing 2% (w/v) *p*-phenylenediamine. The DNA stain, Hoechst 33342, was added to the mounting media at 1:10,000 dilution. Primary antibodies and dilutions were used as follows: rabbit anti-mouse UCP 1 peptide at 1:50, full-length sheep anti-mouse UCP 1 at 1:100, mouse anti-Thy-1 at 1:100, rabbit anti-mouse CIDEA at 1:50. Alexa 488 and Alexa 647 were used at a dilution of 1:1000 dilution and Cy3 and Cy5 at a dilution of 1:250. Altogether, thymocyte preparations for each analysis were from 3–16 preparations. At least 6 samples from each preparation were distributed into wells (all identical) and each well was analyzed over at least 8 fields. The data presented are one of many examples of "best representative" data. Confocal microscopy was performed on an Olympus FV1000 laser scanning confocal microscope. A UPlanSAPO 60X/1.35 NA oil objective was used and the images were acquired with the accompanying software. Brightness and contrast adjustments and necessary cropping was performed using Photoshop Elements 5.0 (Adobe).

2.5. Polyacrylamide gels and immunoblot analysis

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was used to separate proteins prior to immunoblot analysis. Following SDS-PAGE, resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P^{SQ}; Millipore), as described by Cunningham et al. [16]. UCP 1 was detected using the rabbit anti-mouse UCP 1 peptide antibody (1:1,000 dilution) or the sheep antiserum to full-length rabbit UCP 1 protein (1:10,000 dilution). Following blocking and a 1 h primary antibody incubation, the blots were incubated with a horseradish peroxidase (HRP) conjugated goat anti-rabbit, or donkey anti-sheep, secondary antibody (1:10,000 dilution) in PBS/0.5% (v/v) Tween 20/ 5% (w/v) milk powder for 1 h at room temperature. Blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia) and immunoreactions were visualized by exposure to Kodak X-Omat LS film.

3. Results

Confocal microscopy of cells from the thymus of UCP 1 wild-type and UCP 1 knock-out mice were confirmed by a fluorescent detection of the thymocyte surface antigen Thy-1 (CD-90; green) to be thymocytes (Fig. 1). Furthermore, the size of Thy-1 positive cells is consistent with thymocytes (~5 μm in diameter) when compared to other cells such as hepatocytes (~20 μm in diameter) [17] and crucially brown adipocytes (~40 μm in diameter) [18]. Using the anti-UCP1 peptide antibody, magenta staining, we confirm that UCP 1 is associated with mitochondria in thymocytes from UCP 1 wild-type mice, but not thymocytes from UCP 1 knock-out mice (Fig. 1).

The detection of UCP 1 in situ in mitochondria of thymocytes is not unique to mouse. Fig. 2 demonstrates visual evidence for the presence of UCP 1 in Thy-1 positive (green staining) thymocytes isolated from rats. Mitochondrial location in the thymocytes was determined using Mitotracker Red. Using the UCP 1 peptide antibody, (magenta), we show that in the merge image UCP 1 is associated with the mitochondria therein.

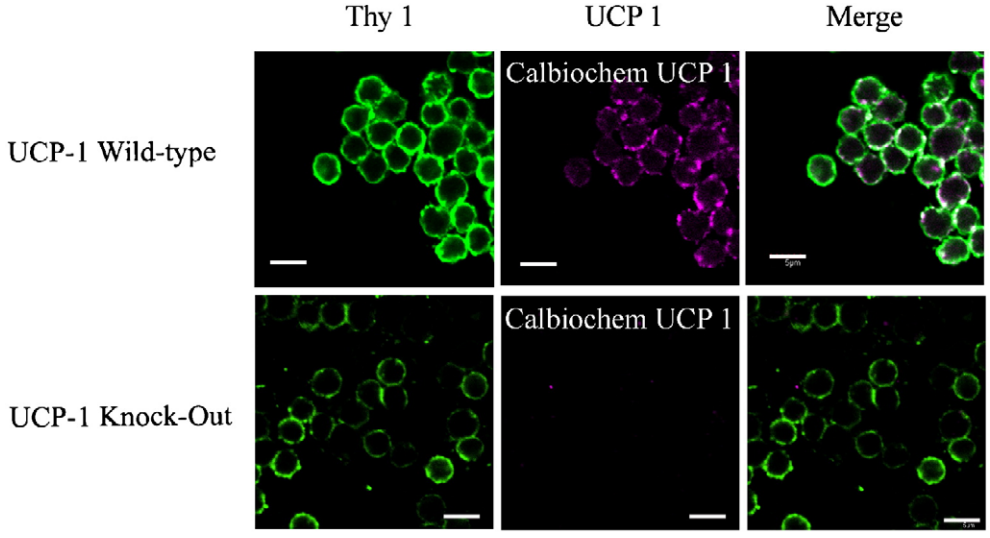


Fig. 1. Identification of UCP 1 in thymocytes. In situ identification of UCP 1 in thymocytes from UCP 1 wild-type mice using a primary antibody specific to UCP 1 peptide (Calbiochem). UCP 1 is not detected in thymocytes isolated from UCP 1 knock-out mice. The anti-UCP 1 peptide antibody was detected using an Alexa 647 (magenta) labelled secondary antibody. Thymocytes were detected using an anti-Thy1 primary antibody and detected using an Alexa 488 (green) labelled secondary antibody. (×2400 zoom; Bar 5 μm).

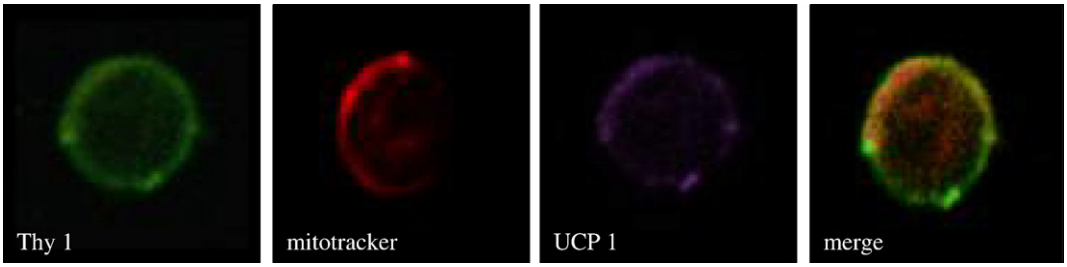
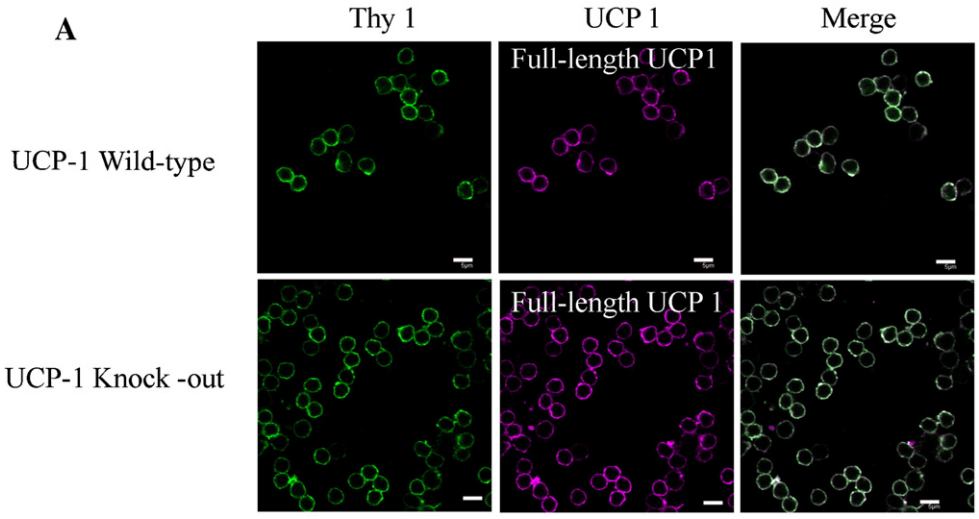


Fig. 2. In situ identification of UCP 1 in thymocytes from rat. In situ identification of UCP 1 in thymocytes from a Wistar rat using the anti-UCP 1 peptide antibody (Calbiochem). The anti-UCP 1 peptide antibody was detected using a Cy5 (magenta) labelled secondary antibody. Thymocytes were detected using an anti-rat Thy1 primary antibody and detected using an Alexa 488 (green) labelled secondary antibody. (×4200 zoom; Bar 5 μm).

In an attempt to resolve the discrepancy between data reported by our group [4–8] and data reported in studies by others [9], we compared the specificity of the respective UCP 1 antibodies used in these studies. We predicted that the discrepancy could be due to our use of a UCP 1 peptide-specific antibody rather than an antibody raised against the whole UCP 1 protein. Confocal analysis showed that using an antibody



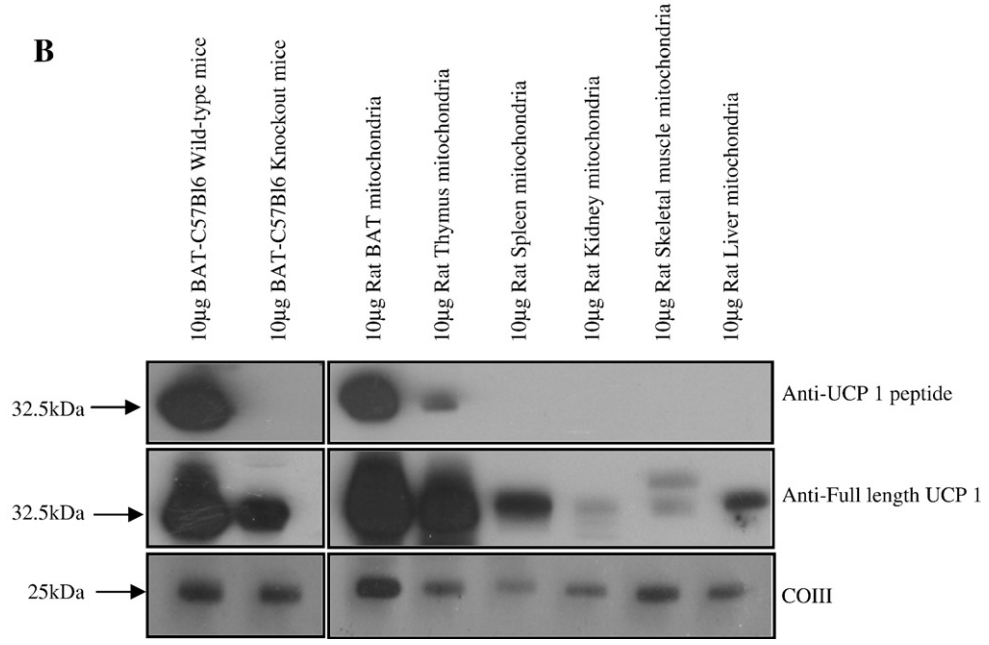


Fig. 3. Non-specific in situ detection of protein in thymocytes using antibody to full-length UCP 1. (A) Thymocytes were isolated from the thymus of UCP 1 wild-type and UCP 1 knock-out mice using an antibody raised to full-length UCP 1. Thymocytes were identified using a Thy1 primary antibody and detected using an Alexa 488 (green) labelled secondary antibody. The antibody to full-length UCP 1 was detected using a Cy3 (magenta) labelled secondary antibody. (×2400 zoom; Bar 5 μm); (B) Immunoblotting was performed on mitochondria samples prepared from (i) BAT of UCP 1 wild-type *and* (ii) BAT of UCP 1 knock-out mice, (iii) BAT of rat, (iv) thymus of rat, (v) spleen of rat, (vi) kidney of rat, (vii) skeletal muscle of rat and (viii) liver of rat. The antibodies used were the anti-UCP 1 peptide antibody (Calbiochem) (1:1000 dilution), the antibody to full-length UCP 1 (1:10,000 dilution) and the antibody to CO III (1:1000 dilution).

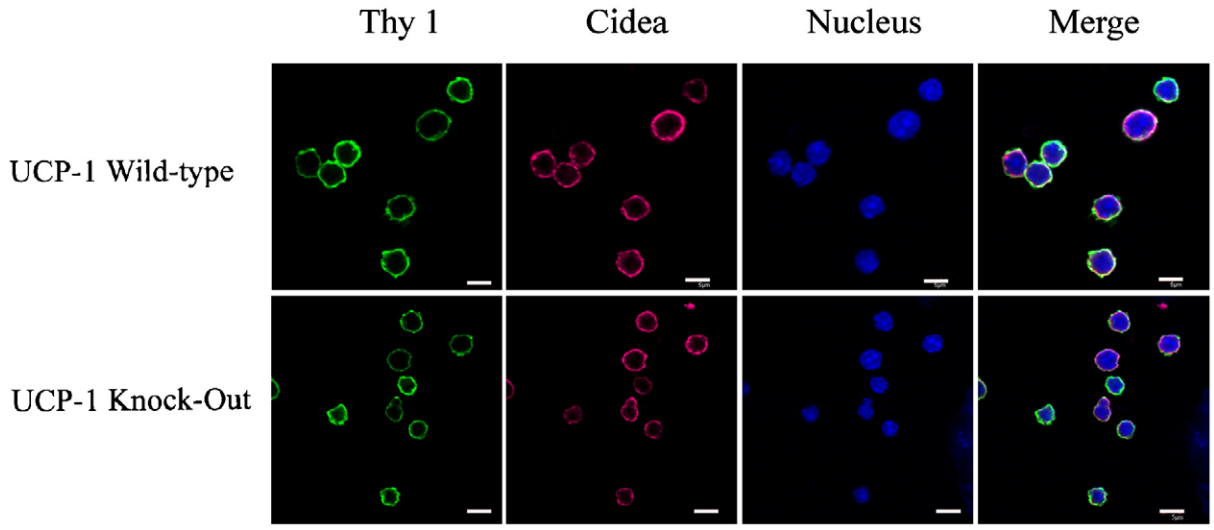


Fig. 4. Identification of CIDEA in thymocytes from wild-type and UCP 1 knock-out mice thymocytes. Thymocytes were fixed and permeabilized as described in Materials and methods, probed with anti-Thy1 IgG and anti-CIDEA IgG antibody. Thy 1 was detected using a secondary labelled Alexa 488 (green) secondary antibody and CIDEA was detected using a secondary labelled Alexa 647 (red) secondary antibody. The nucleus was detected using Hoechst stain. (×3600 zoom; Bar 5 μm).

raised to full-length UCP 1, a protein is detected (magenta staining) in Thy-1 positive thymocytes from UCP 1 wild-type mice and also from UCP 1 knock-out mice (Fig. 3A). Western immunoblotting was used to draw further comparison between the specificity of the UCP 1 peptide-specific antibody to UCP 1, relative to the antibody raised to full-length UCP 1 (Fig. 3B). The immunoblot shows detection of protein at ~32.5 kDa by the antibody raised to full-length UCP 1 in mitochondria isolated from (i) BAT of UCP 1 wild-type and (ii) BAT of UCP 1 knock-out mice, (iii) mitochondria isolated from BAT of rat, (iv) mitochondria isolated from thymus of rat, (v) mitochondria isolated from spleen of rat, (vi) mitochondria isolated from kidney of rat, (vii) mitochondria isolated from skeletal muscle of rat and (viii) mitochondria isolated from liver of rat. In marked contrast, the anti-UCP 1 peptide antibody only detects protein at ~32.5 kDa in BAT mitochondria of UCP 1 wild-type mice, BAT mitochondria from rat and thymus mitochondria from rat, with no staining of BAT mitochondria from UCP 1 knock-out mice or mitochondria from other tissue tested (Fig. 3B).

We were also able to demonstrate that CIDEA, a protein suggested to be directly associated with UCP 1 in brown adipose tissue, was also present in mouse thymocytes (Fig. 4). However, CIDEA was not associated with UCP 1 in thymocytes, with equivalent abundance of CIDEA in thymocytes from UCP 1 wild-type *and* UCP 1 knock-out mice (Fig. 4).

4. Conclusion

In this study we have confirmed the expression of UCP 1 in mouse and rat thymocyte, as determined by the Thy-1 marker surface antigen and their size (~5 μm in diameter), mitochondria by confocal imagery. Nevertheless, the findings in this study contrast with those of Frontini et al. [9] who presented evidence that UCP 1 is in BAT within the thymus. One marked disparity between these studies is the antibody used to probe tissue or Western blots. Our data also highlight the specificity of the commercial antibody raised against UCP 1 peptide, used in our published studies [4–8,16], which reacted against a ~32.5 kDa protein, consistent with the size of UCP 1, in BAT and thymus mitochondria and not other tissue. In striking contrast, there was marked lack of specificity in reactivity of the antibody raised against the full-length UCP 1 protein, with a ~32.5 kDa protein detected in rat BAT and thymus mitochondria and also mitochondria from spleen, kidney, skeletal muscle and liver. Crucially, the antibody to full-length UCP 1 protein reacted against BAT from mice deficient in UCP 1. Clearly, the antibody to full-length UCP 1 is detecting other proteins at ~32.5 kDa and possibly at other masses. These studies confirm the expression of UCP 1 in mouse and rat thymocytes and also highlight the importance of full characterization of the specificity of antibodies against target proteins.

Our data also clearly demonstrate that CIDEA is expressed in thymocytes. However, using UCP 1 deficient mice we have shown that the absence of UCP 1 does not affect native expression of CIDEA. It would still be interesting to discover whether the absence of CIDEA affects mitochondrial function in thymocytes as has been demonstrated for BAT [13].

Confirmation of UCP 1 expression in thymocytes from mice and rats highlights the need to address the biological functions of UCP 1 in such cells. Our on-going studies are addressing alterations in metabolic and physiological functions of UCP 1 deficient mice. We have already provided circumstantial evidence that UCP 1 does not play a thermogenic role in thymus [8]. One teleological approach that we are investigating currently in thymocytes is whether UCP 1 deficiency manifests itself as an increase reactive oxygen species production as has been observed in macrophages from UCP 2 deficient mice [19].

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