UCP3 plays a complementary role to UCP1 in brown adipose tissue mitochondrial bioenergetics

Christopher L. Riley[1], Edward M. Mills[2]

[1]Department of Cancer Biology, Dana Farber Cancer Institute

Boston, Massachusetts 02210 USA

[2]Department of Pharmacology and Toxicology, College of Pharmacy

The University of Texas at Austin, Austin, Texas 78712 USA

Abstract

Mitochondrial uncoupling protein 3 (UCP3) plays a complementary role in uncoupling protein 1 (UCP1) dependent uncoupling in mammalian thermogenesis. Using mouse strains lacking UCP1, UCP3, and a UCP1/UCP3 double knockout, we previously demonstrated that UCP3 is necessary for sustained heat production. However, how and where UCP3 is essential for heat production remains unknown. Using brown adipocyte mitochondria from UCP1, UCP3, and UCP1/UCP3 double knockout mice, we found that in the absence of UCP1, UCP3 does not act as a free fatty acid-inducible uncoupling protein; however, it is necessary for maximal GDP sensitive respiration. Additionally, we confirm that the loss of UCP1 is a dominant regulator of mitochondrial respiratory chain activity, assembly, and composition in brown adipose tissue. Our data suggest that in the absence of UCP1 brown adipose tissue is not a significant source of heat production and that UCP3-dependent heat generation plays a regulatory role outside of adipose tissues. Together these data suggest that UCP3's thermogenic function is independent of a role as a bona fide uncoupling protein in brown adipose tissue.

Introduction

Brown adipose tissue (BAT) mediates approximately 60% of adaptive non-shivering thermogenesis in small mammals and is an essential determinant of the arousal from torpor in hibernators[1]. Its recently identified presence in a significant percentage of adult humans and the identification of brown-like 'beige' adipocytes in white adipose tissue suggest that modulation of BAT function holds great promise for the treatment of human metabolic disease associated with overnutrition [2,3].

BAT mitochondria are unique because they oxidize substrates to generate heat instead of ATP, a metabolic property that antagonizes obesity. Mitochondrial uncoupling protein 1(UCP1) is the well-established thermogenic mediator of BAT and comprises approximately 20% of the BAT mitochondrial proteome. Stimulated by fatty acids, UCP1 'uncouples' substrate oxidation from ATP synthesis by physically transporting protons from the intermembrane space back into the mitochondrial matrix[4].

While UCP1 remains unquestioned as the dominant mitochondrial uncoupling protein, the close UCP1 homolog UCP3 (57% percent sequence identity to UCP1) is also expressed in BAT and has uncoupling properties *in vitro* and *in vivo*[5–7]. However, little is known about the function of UCP3 in BAT mitochondrial bioenergetics. UCP3 is expressed in skin[8], white and brown adipose tissue, skeletal muscle, and heart [9]. Most physiological and biochemical studies have examined UCP3 functions in skeletal muscle

mitochondria, where its role as a *bona fide* mitochondrial uncoupler remains in question [10–12]. Its expression in skeletal muscle is highly correlated with fatty acid oxidation and levels of reactive oxygen species. However, UCP3 expression in BAT is differentially regulated, suggesting that it may function differently in BAT versus skeletal muscle[13]. In contrast to the original reports, Hilse *et al.* demonstrated that UCP3 is expressed two-fold higher in BAT mitochondria than in skeletal muscle.

Nonetheless, and despite considerable interest for its role in metabolism in general, a role for UCP3 in murine brown adipose tissue mitochondrial oxidation has not been identified. We have previously established that UCP3 plays a significant role in the thermogenic responses to sympathomimetics and lipopolysaccharide *in vivo* [14,15]. In this report we sought to examine the bioenergetic role of UCP3 in mitochondrial function in BAT.

A recent report showed that UCP3 was expressed in BAT at higher levels that in SKM[9]. In combination with our findings *in vivo*, we hypothesized that UCP3 might act as a traditional uncoupler in brown adipose tissue. The generation of a UCP1/UCP3 double knockout allowed us to characterize brown adipose tissue mitochondrial uncoupling in the absence of UCP1 in BAT. Using this double knockout model along with single UCP1 and UCP3 null mice, we found that the loss of UCP3 in decreases GDP-sensitive mitochondrial respiration in a UCP1-dependent manner but does not regulate fatty acid-inducible uncoupling. We also confirmed previous reports that UCP1 is necessary for maximal and inducible

respiration, and controls mitochondrial respiratory chain functions in brown adipose tissue.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Aldrich unless otherwise noted.

Animals

All mice were males between the ages of 8-12 weeks of age at the time of sacrifice. All animals were fed standard chow and water ad libitum and maintained with a light dark cycle of 12hr in a room maintained at 23 +/- 2°C. All wild type, UCP1KO, UCP3KO, and UCP1/3KO animals were on C57B6/J background. UCP1KO mice were a gift from Dr. Leslie Kozak (Maine Medical Research Institute, Scarborough, ME, USA). UCP3KO were a gift from Mark Reitman, formerly of the National Institutes of Health. UCPDK (UCP1/UCP3 double knockout animals) were generated in house by breeding heterozygous UCP1KO with heterozygous UCP3KO. Every five generations animals from all genotypes were backcrossed with an in-house C57B6/J colony to prevent genetic drift. Animals were euthanized via isoflurane inhalation followed by cervical dislocation. The University of Texas IACUC approved all animal experiments.

Mitochondrial isolation

Mitochondria were isolated as described in [16]. Briefly, intrascapular brown adipose tissue was harvested from 2-5 mice male mice between the ages of 8-12 weeks, rinsed in ice cold PBS, and cleaned of surrounding white adipose tissue. Tissue was then minced with fine scissors and homogenized in 250mM sucrose. The homogenate was then filtered through a 40uM cell strainer and centrifuged at 8,500 x *g*. The fat layer and supernant were removed and the pellet resuspended in sucrose and centrifuged at 800 x *g*. The resulting supernant was then centrifuged and the pellet resuspended in sucrose supplemented with .3% fatty acid free BSA and pelleted at 8,500 x *g*. The resulting pellet resuspended in 200ul of 250mM sucrose and protein was measured using a BCA assay (Pierce). All steps were performed at 4°C or on ice unless otherwise noted. After preparation mitochondrial were either used directly for oxygen consumption measurements or frozen at -80°C for further analysis.

Mitochondrial respiration

Isolated mitochondria (0.25-0.5mg/ml) in respiration buffer 0.6mL (0.1% BSA, 125mM sucrose, 20mM TES, 1mM EDTA, 4mM KP_i, 2mM $MgCl_2$ pH 7.2) were added to a continuously mixed chamber kept at 37°C (Hanstech). After addition respiration was monitored for 15-30 seconds followed by the sequential addition of pyruvate (5mM) and malate (2.5mM), GDP (1mM), ADP (1mM), Oligomycin (1.3ug/mL), and FCCP (0.5-2uM). In a separate run, titration of respiratory activity by oleic acid (50% EtOH) was induced after mitochondrial respiration had

been inhibited by 1mM GDP and 1.3ug/mL oligomycin. Oxygen consumption was measured every second until the completion of the experiment. All mitochondrial respiratory chain inhibitors were solubilized in 95% EtOH unless otherwise noted and total EtOH up to 0.1% was found to have no effect on respiration.

Mitochondrial complex activity assays

Mitochondrial complex assays were run essentially as described by[17]. Frozen mitochondrial aliquots were thawed on ice. They were then freeze thawed an additional two times using a dry ice bath. Prepared mitochondria were used at a concentration of 4ug/ml (CS, CI, CIV) or 5ug/ml (CII, CI-III, CII-CIII). Absorbance was read on a Cary 40000 UV-Vis spectrometer at 37°C. Rates were calculated in Microsoft Excel from linear portions of the activity. All complex activities were normalized to citrate synthase activity.

Western blotting

Fresh mitochondrial lysates were prepared and diluted (2ug/ul) in sample buffer and incubated for 5 minutes at 95°C. Mitochondrial proteins were loaded and run on a Tris-glycine gel system and transferred to nitrocellulose. Membranes were then incubated with 5% milk in TBS-tween overnight followed by incubation with primary antibodies. Membranes were incubated with secondary antibodies for an additional hour, washed and developed using ECL (Thermo-scientific).

High resolution Clear Native PAGE

500ug of pelleted mitochondria was resuspended in 50ul of buffer (50mM NaCl, 50 mM imidazole, 2mM 6-aminohexanoic acid, 1mM EDTA, pH 7) and solubilized with digitonin (2g/g at 20%). Supernant was cleared by spinning at 20000 x *g* for 20 minutes. 2.5uL 50% glycerol was added and 10ul (~75ug) was loaded on to a 3-12% Bis-Tris native gel. Anode buffer (25mM imidazole pH 7), Cathode buffer (50 mm Tricine, 7.5 mm imidazole, 0.01% DDM pH 7). Gels were run until MW marker reached the end of the gel. Resulting gels were then subjected to sequential activity assays. Complex I-II activity: Gels were incubated for 10 minutes in Complex I buffer (2.5mg/ml nitrotetrazolium blue, 1 mg/ml NADH in 5mM Tris/HCl) transferred to 5mM Tris/HCl buffer and scanned. Gels were then incubated for an additional 20 minutes in Complex II buffer (2.5mg/ml nitrotetrazolium blue, 20mM Succinate, 2uM phenazine methanosulfate, 5mM Tris/HCl) and imaged. Complex III-IV activity: Gels were incubated for 30 minutes in Complex III buffer (.5mg/ml diaminobenzidine, 50mM sodium phosphate, pH 7.2) and scanned. After imaging gels were placed back in Complex III buffer and cytochrome c was added to 50uM final concentration and incubated for another 30 minutes and imaged.

Statistics

For all experiments a one-way ANOVA followed by a *Fisher LSD* test was used to compare multiple genotypes with significance set *a priori* at $p < .05$.

Results and Discussion

UCP1 is the dominant regulator of brown adipose tissue respiratory chain function, expression, and assembly in BAT mitochondria.

To better understand how UCP1/3 affects mitochondrial oxidation, we measured respiratory chain activities. We found that the loss of UCP1 in the presence or absence of UCP3 decreased all respiratory complex activities measured (Figure 1). The loss of UCP1 severely impacted complex I activity in both UCP1 and UCPDK, with only ~20% of WT activity remaining as opposed to complex II activities being ~50% of WT activity. Complex IV activity was also decreased in the absence of UCP1 to about 60% of WT activity. Interestingly, and in agreement with Liebig *et al.*, complex IV activity was found to be significantly increased in the UCP3KO mitochondria (Figure 1C) [18].

To explore why the respiratory chain activity was decreased, we chose to look at protein abundance via western blotting. We found that the loss of UCP1 decreased Ndufa9 (complex I), Uqcrc2 (complex III), and Coxiv(complex IV) protein abundance in mitochondria(Figure 2A). Coxiv expression was further decreased in the double knockout background. Interestingly Sdha(complex II) expression was not affected despite a reduction in activity(Figure 1B, 2A). Oscp (complex V) expression nor Mdh2(TCA cycle enzyme) expression was changed in any of the genotypes (Figure 2). As these enzymes only represent a selected few of the many numbers of components of each respiratory complex, we investigated the role of both UCP's on the formation of respiratory chain complex

assembly in BAT. Using activity-based high resolution clear native PAGE, we confirmed that in the absence of UCP1, complex I, complex III, and complex IV are depleted and disorganized (Figure 2C). The loss of UCP1 alone decreased complex III occupancy in supercomplexes (Figure 3C). However, there was no additional loss of mitochondrial complexes in the UCPDK when compared with the sole loss of UCP1 (Figure 3C).

Together these data unambiguously demonstrate that Ucp1 is necessary for thermogenically competent brown adipose mitochondria. Our data strongly support those of Kazak *et al.* and expand their finding that Ucp1 is essential for brown fat respiratory chain function at 4C to include mice that are raised at standard housing conditions[19]. The loss of Ucp1 extensively alters the brown adipose tissue proteome, and despite Ucp3 being expressed in this model, it is clear that UCP3 cannot compensate for its loss[9].

The discrepancies between Sdha protein abundance and complex II activity between WT and UCP1-null animals is interesting as it may suggest that there is a regulatory function that does not allow for the transfer of electrons between the matrix exposed Sdha-Sdhb complex and the membrane-embedded Sdhc-Sdhd complex. The confirmation of increased complex IV activity despite similar protein abundance and complex integrity in the UCP3-null mouse and the Ucp3-null hamster in Liebig's work suggests that this phenomenon is a compensatory mechanism for the loss of Ucp3 function that is superseded by the loss of Ucp1.

Loss of UCP3 decreases GDP sensitive respiration but does not affect FFA induced respiration in BAT mitochondria.

As mitochondria are an integrated system, we used high-resolution respirometry to probe further the bioenergetic function of UCP3 in brown adipose mitochondria. The protocol outlined in Figure 4A utilized continuous additions of substrates and inhibitors to elucidate GDP-sensitive (respiration after addition of substrate minus respiration after GDP addition), ADP-linked (respiration after addition of ADP minus respiration after oligomycin), and maximal respiration (CCCP induced) (Fig. 3A) [20]. In agreement with the complex activity data, we found that the loss of UCP1 was the dominant regulator of respiration (Fig. 3B-D). Interestingly, the loss of UCP3 only in the presence of UCP1 led to decreased GDP-sensitive respiration (Fig. 3C). ADP-linked respiration was equal between all genotypes despite the significant decrease in total respiratory capacity (Fig. 3E). We did not find a significant difference in free-fatty acid-mediated uncoupling in the presence or absence of UCP3(Fig. 3F).

The finding that ADP-linked respiratory capacity is the same between the UCP1-null and WT mitochondria is crucial as recently discovered ATP-linked thermogenic pathways would remain unaffected by the loss of UCP1. This finding means that proposed futile cycles that require ATP production (phospho-creatine and calcium) could run in the absence of UCP1 in brown adipose tissue[21,22]. However, total respiratory capacity in this tissue is likely compromised, and heat production would be severely impaired when compared with wild-type animals.

Previous reports suggested that the role Ucp3 plays in BAT is negligible. However, there are critical differences between these studies and the data presented in this report. Gong *et al.* used a mouse model on a mixed background, a model that is less reliant on brown adipose tissue thermogenesis [23]. Also, the authors used succinate as the oxidative substrate; succinate is not compatible with maximal oxidation in BAT mitochondria and may have underestimated the function of UCP3[16]. Additionally, the mice were housed at thermoneutrality when UCP3 expression and adrenergic tone in BAT are low [16]. Liebig *et al.* utilized a mutant hamster with BAT specific ablation of UCP3 and found that there was no significant effect on mitochondrial oxidation when respiration was driven with glycerol-3-phosphate(G3P). Here we used pyruvate and malate, a complex I linked substrate, requiring the transport of both substrates across the inner-membrane.

These discrepancies may be explained by UCP3's hypothesized transport function, through which UCP3 may act to increase substrate availability, pyruvate, or malate, during maximal UCP1 activation[11]. Interestingly, UCP2, which shares 72%, homology to UCP3, has been shown to directly transport C4 metabolites, controlling glycolysis, and glutamine oxidation in hepatocytes [24]. A recent study demonstrated that there are biochemical differences between UCP1 and UCP3 that point to differing transport roles[25]. Our data, in light of existing literature, suggest that UCP3 may complement UCP1's uncoupling function, not as an uncoupler but as a solute transporter.

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Figure Legend

Figure 1. *Loss of UCP1 is the dominant regulator of mitochondrial respiratory chain enzyme activity*. (A) Complex I activity (B) Complex II activity (C) Complex IV activity were all controlled for total mitochondria using citrate synthase activity. Freeze thawed mitochondria from WT (n=3) UCP1KO (n=3) UCP3KO (n=3) and UCPDK(n=4). *= p< .05, **=p<.001, ***= p<.0001, ****= p<.00001.*

Figure 2. *Loss of UCP1 is the dominant regulator of respiratory chain protein expression and organization in brown adipose tissue mitochondria.* (A) Western blots of mitochondrial extracts from indicated genotypes. Five micrograms of mitochondrial protein from three separate mitochondrial preparations per genotype. (B) Densitometry from data in *A* were quantified using ImageJ and controlled for Mdh2 expression. N=3 per genotype. (C) Representative high resolution clear native PAGE analysis of mitochondria from indicated genotypes.

Figure 3. *Regulation of brown adipose tissue mitochondrial respiration by uncoupling proteins 1 and 3*. (A) Scheme for mitochondrial respiration assays. (B) Mitochonrial respiration from 5-6 different preparations per genotype. (C) GDP-sensitive respiration (D) Maximal respiration (E) ADP-linked respiration (F) Oleate induced respiration. (A-E) n= 5-6 per genotype *= p< .05, **=p<.001, ***= p<.0001, ****= p<.00001.*

Figure 1

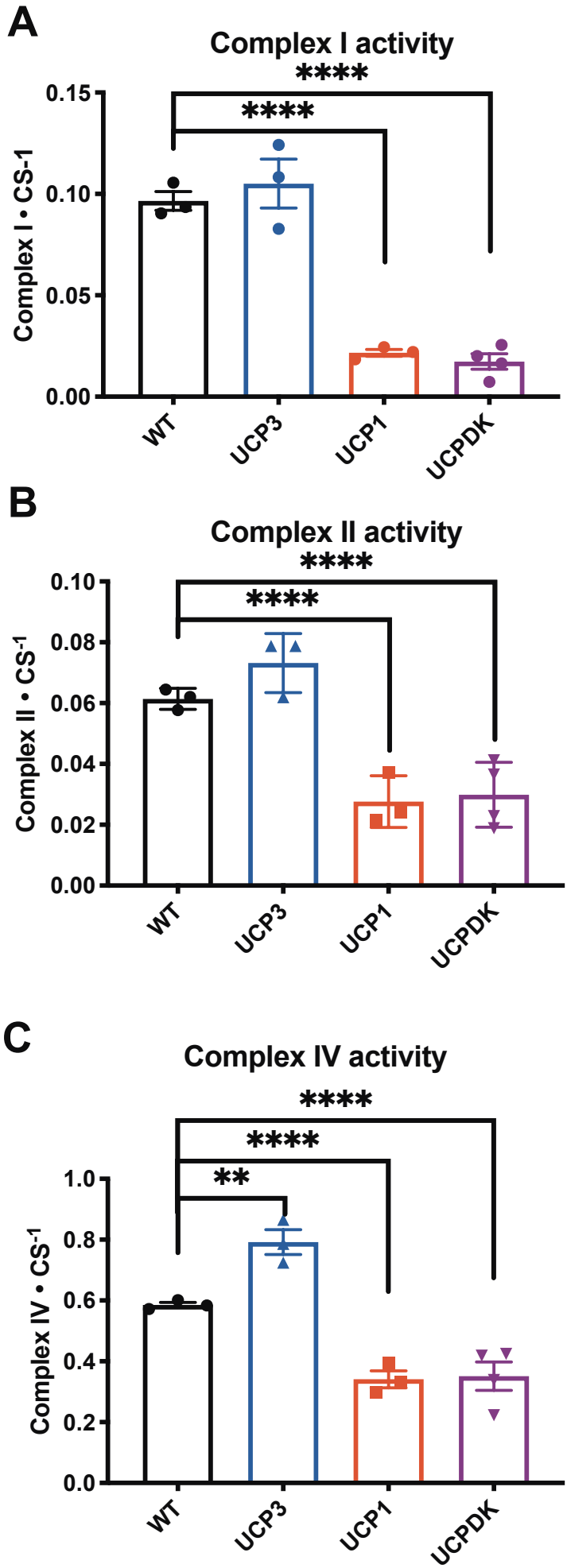
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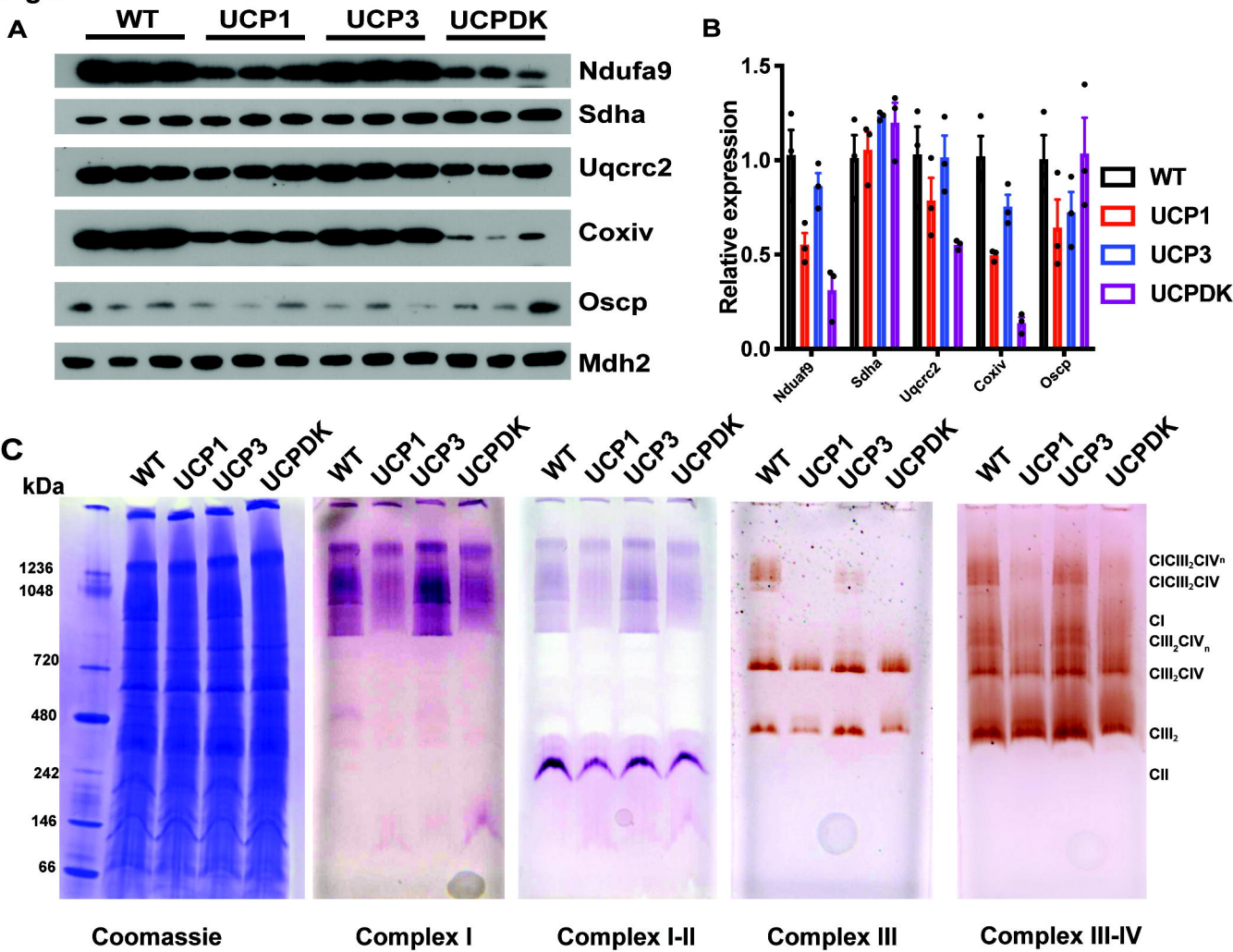
Figure 2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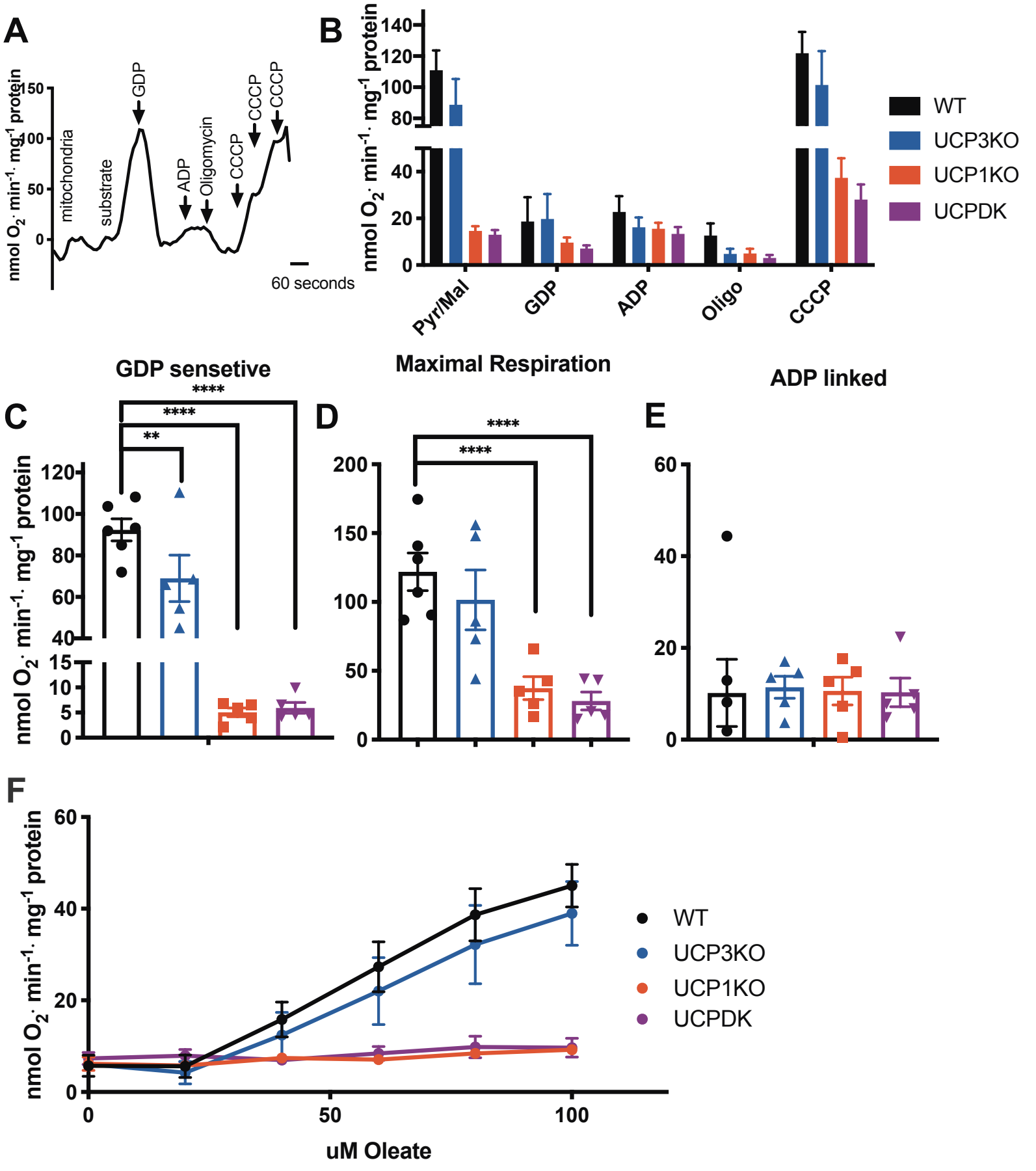
Complex III-IV

Figure 3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uM Oleate
WT
UCP3KO
UCP1KO
UCPDK