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Article

Thyroid Hormone Induces Ca^{2+}-mediated Mitochondrial Activation in Brown Adipocytes

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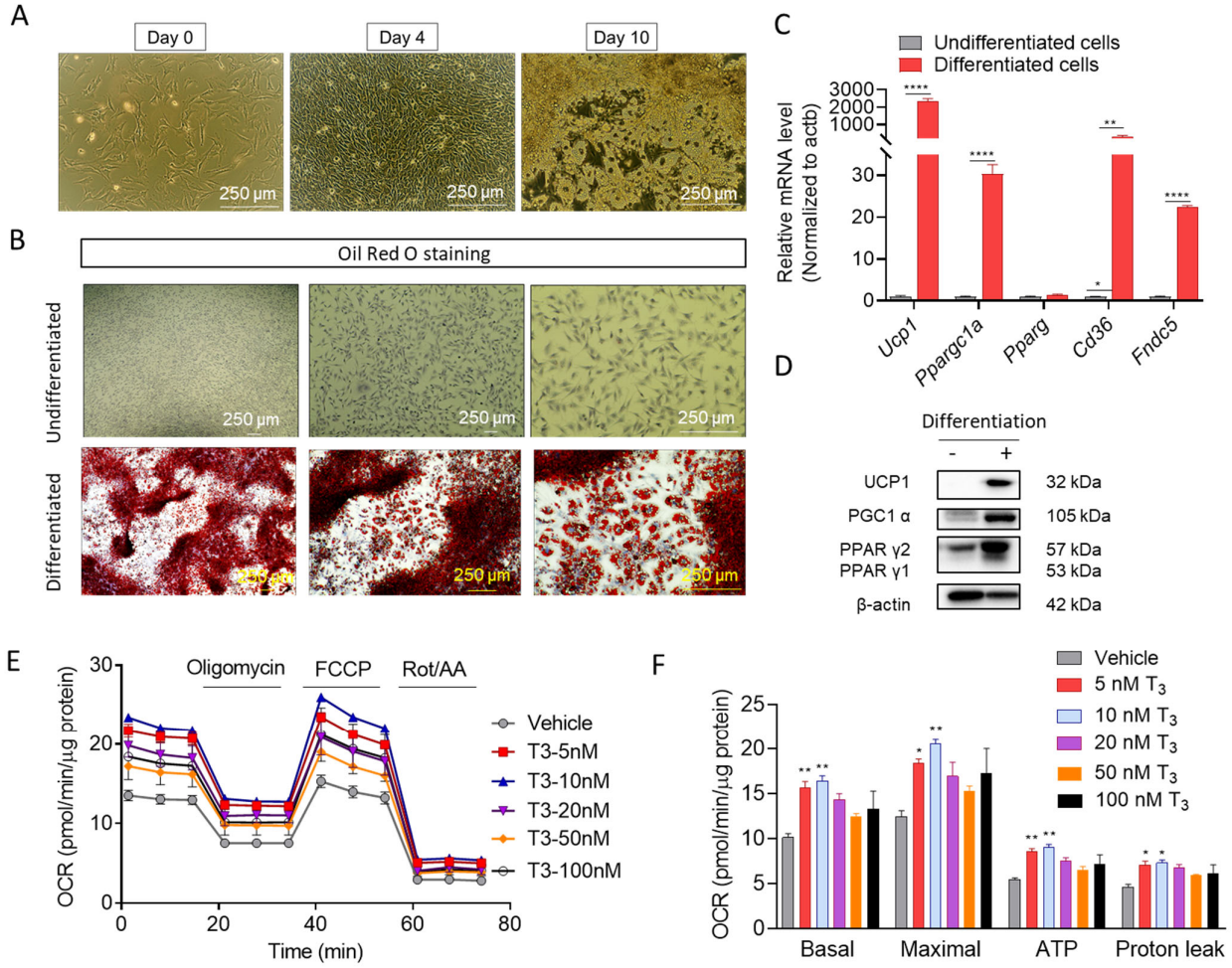

Figure S1. T_3 induces changes in brown adipocyte mitochondria functions within 30 min. (A) Immortalized brown adipocytes on day 0, 4, 10 of differentiation process. (B) Oil Red O staining of immortalized undifferentiated (upper) and differentiated (lower) brown adipocytes. (C) Relative mRNA levels of *Ucp1, Ppargc1a, Pparg, Cd36, Fndc5* (normalized to *actb*) (n=3) of immortalized brown adipocytes. (D) Western blot of UCP1, PGC1 α and PPAR γ on immortalized undifferentiated and differentiated brown adipocytes. (E) OCR measurement on immortalized brown adipocytes under the treatments of different concentrations of T_3 for 30 min. (F) Quantitative analysis of basal respiration, maximal respiration, ATP production, and proton leak (n=4). Data are presented as mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p < 0.0001$.

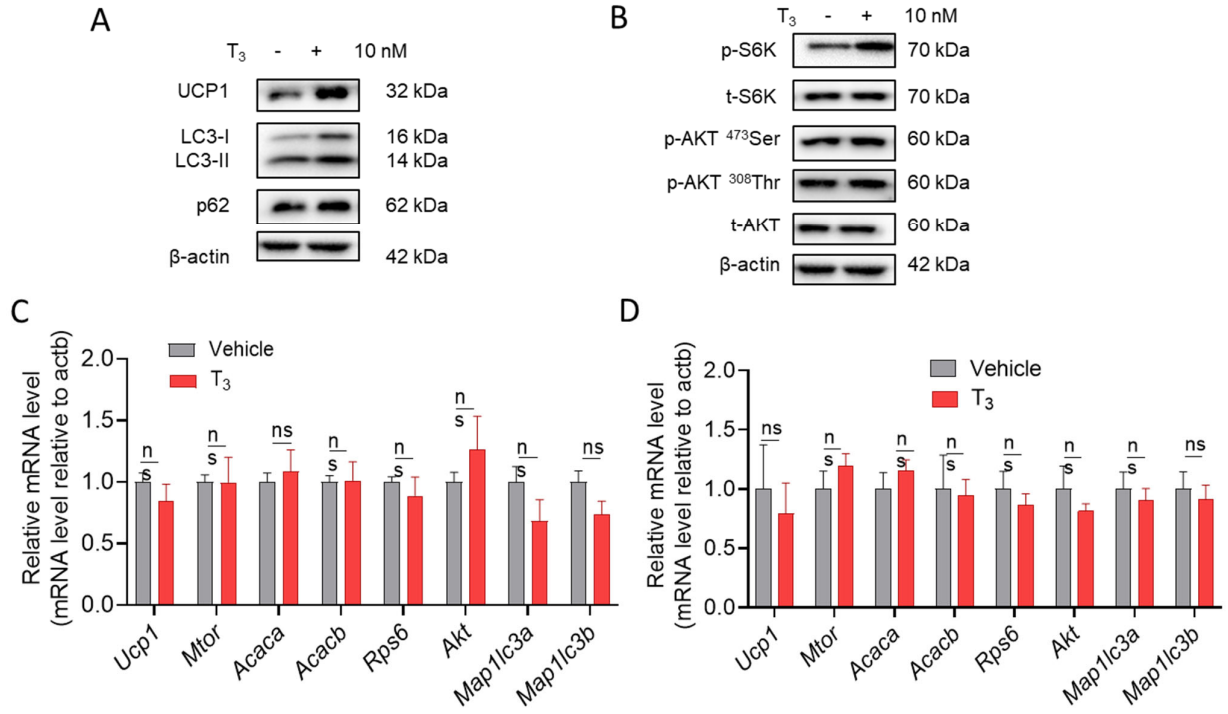

Figure S2. T3 induces changes in cell signaling within 30 min. Western blots of (A) UCP1 and autophagy-related proteins, and (B) mTOR and akt signaling proteins with 10 nM T3 treatment for 30 min in primary brown adipocytes. Relative mRNA levels of *Ucp1*, *Mtor*, *Acaca*, *Acacb*, *Rps6*, *Akt*, *Map1lca*, and *Map1lcb* with 30 min of T3 treatment in (C) immortalized, and (D) primary brown adipocytes. Data are presented as mean ± SEM, ns not significant.

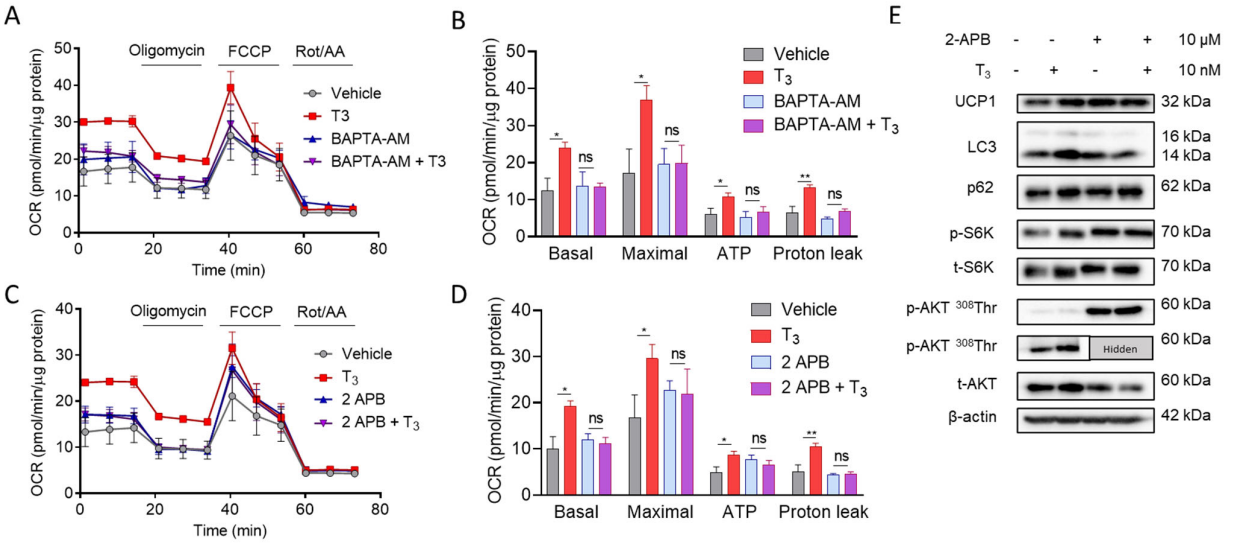

Figure S3. T3-induced cytosolic Ca^{2+} increase regulates OCR and cell signaling alterations. (A) OCR measurement in immortalized brown adipocytes under 10 μM BAPTA-AM treatment for 1 h prior to 10 nM T3 application for 30 min. (B) Quantitative analysis of basal respiration, maximal respiration, ATP production and proton leak. (C) OCR measurement in immortalized brown adipocytes under 10 μM 2-APB treatment for 1 h before 10 nM T3 treatment for 30 min. (D) Quantitative analysis of basal respiration, maximal respiration, ATP production, and proton leak (n=4). (E) Western blot of UCP1, autophagy related proteins, and mTOR signaling proteins with 10 μM 2-APB treatment for 1 h before 10 nM T3 treatment for 30 min in immortalized brown adipocytes. Data are presented as mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, ns not significant.

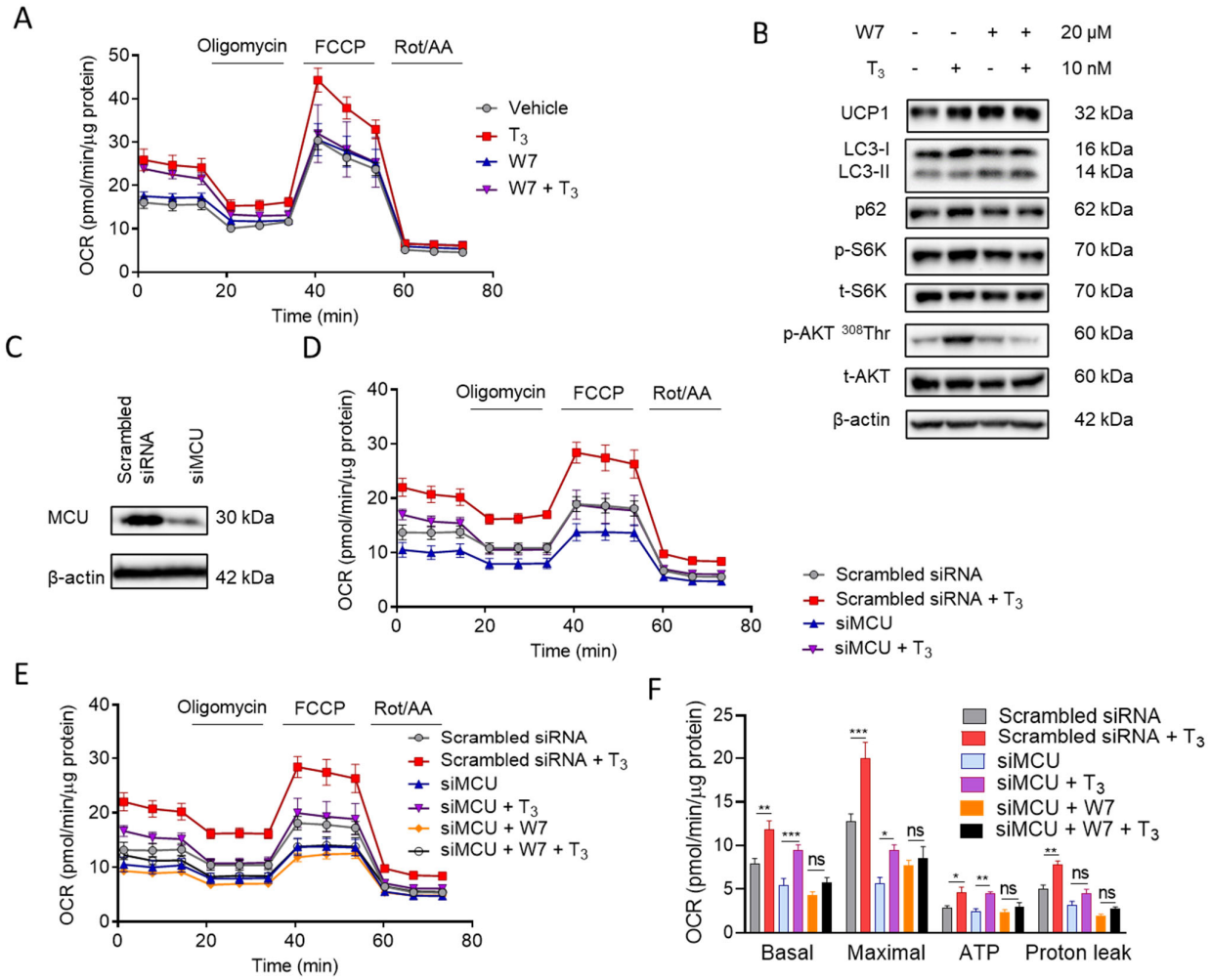

Figure S4. Both $[Ca^{2+}]_i$-CaM activation and mitochondrial Ca^{2+} influx are involved in T_3-induced OCR elevation. (A) OCR measurements in immortalized brown adipocytes with 20 μM W7 treatment for 1 h before T_3 treatment. (B)Western blot of several autophagy related proteins, and mTOR signaling pathway proteins in immortalized brown adipocytes with 20 μM W7 application for 1 hour before 10 nM T_3 treatment for 30 min. (C) Western blot of MCU under Scrambled siRNA and siMCU transfection in immortalized brown adipocytes. (D) OCR measurements of *Mcu* knockdown in immortalized brown adipocytes with 10 nM T_3 treatment for 30 min. (E) OCR measurements of *Mcu* knockdown immortalize brown adipocytes with 20 μM W7 treatment for 1 h before T_3 application. (F) Quantitative analysis of basal respiration, maximal respiration, ATP production, and proton leak. Data are presented as mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** < 0.001, ns not significant.

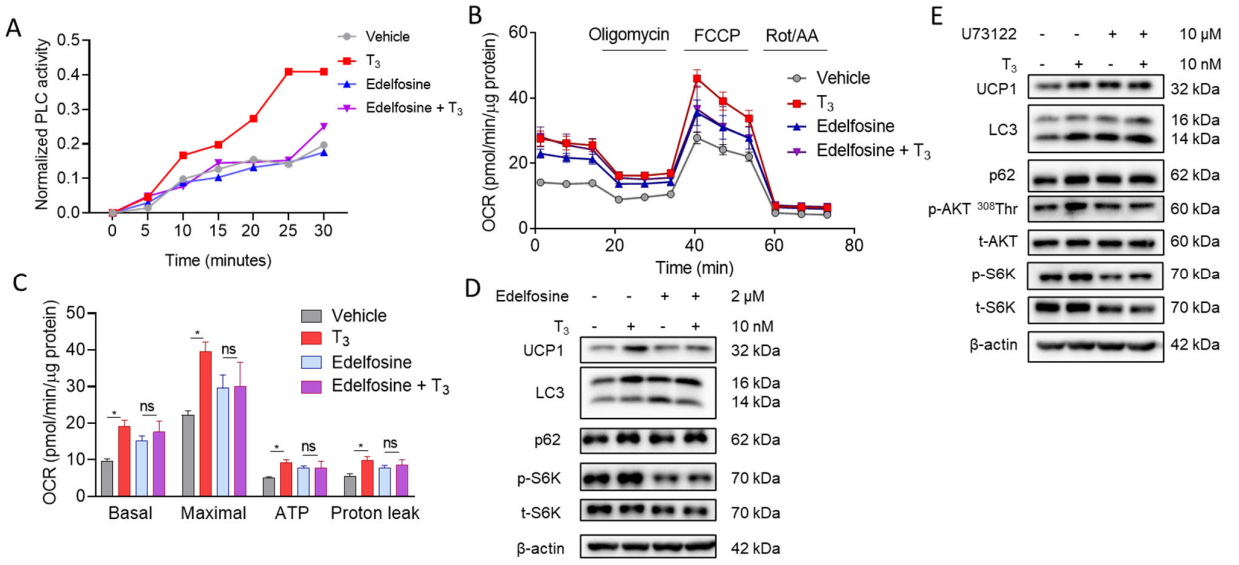

Figure S5. PLC inhibitors inhibit effects of T_3 in brown adipocytes. (A) PLC activity assay on immortalized undifferentiated brown adipocytes with 2 μM edelfosine treatment for 6 h prior 10 nM T_3 application for 30 min (B) OCR measurement in immortalized brown adipocytes with 2 μM edelfosine pretreatment. (C) Quantitative analysis of basal respiration, maximal respiration, ATP production and proton leak. (D)Western blot of UCP1, autophagy related proteins, and mTOR signaling proteins, with 2 μM edelfosine pretreatment in primary brown adipocytes. (E) Western blot of UCP1, autophagy related proteins, and mTOR signaling proteins, with 10 μM U73122 treatment for 6 h before 10 nM T_3 treatment for 30 min in immortalized brown adipocytes. Data are presented as mean ± SEM. * $p \leq 0.05$, ns not significant.

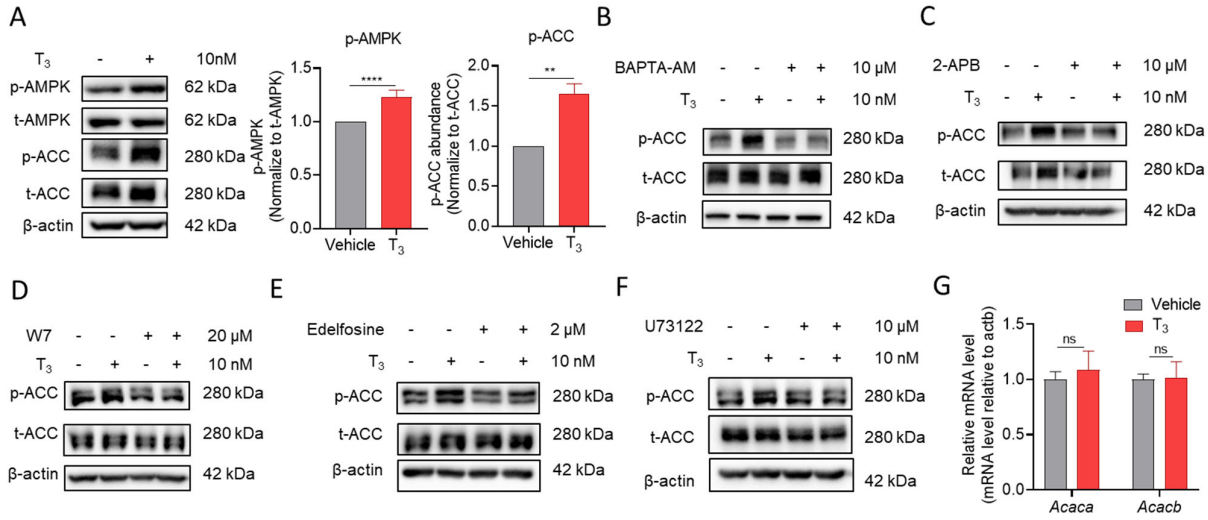

Figure S6. T_3 induces phosphorylations of AMP kinase (AMPK) and acetyl-coA carboxylase (ACC). (A) Western blot of AMPK and ACC with 10 nM T_3 treatment for 30 min in immortalized brown adipocytes. (B) Western blot of ACC in immortalized brown adipocytes with 10 μM BAPTA-AM treatment for 1 hour before 10 nM T_3 treatment for 30 min. (C) Western blot ACC with 10 μM 2-APB treatment for 1 h before 10 nM T_3 treatment for 30 min in immortalized brown adipocytes. (D) Western blot of ACC in immortalized brown adipocytes with 20 μM W7 treatment for 1 hour before 10 nM T_3 treatment for 30 min.(E) Western blot of ACC with 2 μM edelfosine treatment for 6 h before 10 nM T_3 treatment for 30 min in immortalized brown adipocytes. (F) Western blot of ACC with 10 μM U73122 treatment for 6 h before 10 nM T_3 treatment for 30 min in immortalized brown adipocytes. (G) Relative mRNA levels of *Acaca*, and *Acacb* with 30 min of T_3 in immortalized brown adipocytes. Data are presented as mean ± SEM. ** $p \leq 0.01$, **** $p < 0.0001$, ns not significant.

Supplemental Methods

1. Small interfering RNA transfection

Brown adipocytes were transfected with 30 nM SiGENOME Smart-pool small interfering RNA (siRNA) duplexes for mouse *Mcu* (siGENOME; catalog no. 062849-01, Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA) or negative control (catalog no. SN-1002, Bioneer, Daejeon, South Korea) mixed with DharmaFECT siRNA transfection reagent (catalog no. T-2001-03, Thermo Fisher Scientific, Waltham, MA, USA). The transfection efficacy was assessed after 72 h by western blotting.

2. PLC activity assay

Immortalized undifferentiated brown adipocytes were treated with 2 μM edelfosine for 6 h prior 10 nM T_3 application for 30 min. The PLC activity was measured with Phospholipase C activity assay kit (catalog no. ab273343, Abcam, Cambridge, UK), based on manufacturer's instruction. The cells were lysed with PLC Assay buffer and then centrifuged at 10,000 x g at 4 °C for 20 min to collect the supernatants. The supernatants were mixed with PLC Assay buffer and reaction mix following the manufacture's protocol and incubated at 37 °C along with standard curve. The reaction product was measured the OD at 405 nm every 5 min. The data was normalized using the formulation equation (1).

$$Normalized\ PLC\ activity = \frac{A_t - A_0}{A_0} \quad (1)$$

A_t, A_0: Amount of colorimetric product generated from the Standard Curve (nmol) at 0 (min) or t (min) respectively.