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1 **The role of mitochondria linked fatty-acid uptake-driven adipogenesis in**  
2 **Graves' Orbitopathy**

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13 **Short title:** The essential role of mitochondria in Graves' Orbitopathy

14 **Key words:** Graves' Orbitopathy; Orbital Adipose Tissue; fatty acid uptake;  
15 adipogenesis; mitochondria OXPHOS; ATP production.

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23

24 **Abstract**

25 **Context**

26 Depot-specific expansion of orbital-adipose-tissue (OAT) in Graves' Orbitopathy (GO,  
27 an autoimmune condition producing proptosis, visual impairment and reduced quality  
28 of life) is associated with fatty-acid (FA) uptake-driven adipogenesis in  
29 preadipocytes/fibroblasts (PFs).

30 **Objective**

31 A role for mitochondria in OAT-adipogenesis in GO.

32 **Design/Setting/Participants**

33 Confluent PFs from healthy OAT (OAT-H), OAT from GO (OAT-GO) and white-  
34 adipose-tissue in culture-medium compared with culture-medium containing a mixed  
35 hormonal-cocktail as adipogenic-medium (ADM); or culture-medium containing FA-  
36 supplementation, oleate:palmitate:linoleate (45:30:25%) with/without different  
37 concentration of mitochondrial bio-substrate ADP/GDP, AICAR (adenosine-analog)  
38 or inhibitor oligomycin-A for 17 days.

39 **Main outcome measures**

40 Oil-Red-O staining and foci-count of differentiated adipocytes for in-vitro  
41 adipogenesis; flow-cytometry, relative-QPCR, MTS-assay/ $10^6$  cells, total cellular-  
42 ATP detection kit and Seahorse-XFe96-Analyzer for mitochondria and  
43 OXPHOS/Glycolysis-ATP production analysis.

44 **Results**

45 During early adipogenesis before adipocyte formation (day-0,4&7), we observed  
46 OAT-specific cellular ATP-production via mitochondrial-OXPHOS in PFs from both  
47 OAT-H/OAT-GO, and substantially disrupted OXPHOS-ATP/Glycolysis-ATP  
48 production in PFs from OAT-GO, e.g. 40% reduction in OXPHOS-ATP and trend-

49 increased Glycolysis-ATP production on day-4&7 compared with day-0, which  
50 contrasted with the stable levels in OAT-H.

51 FA-supplementation in culture-medium triggered adipogenesis in PFs from both  
52 OAT-H/OAT-GO, which was substantially enhanced by 1mM GDP reaching 7-18%  
53 of ADM-adipogenesis. The FA-uptake-driven adipogenesis was diminished by  
54 oligomycin-A but unaffected by treatment with ADP or AICAR. Furthermore, we  
55 observed significant positive correlation between FA-uptake-driven adipogenesis by  
56 GDP and the ratios of OXPHOS-ATP/Glycolysis-ATP through adipogenesis of PFs  
57 from OAT-GO.

## 58 **Conclusions**

59 Our study confirmed that FA-uptake can drive OAT-adipogenesis and revealed a  
60 fundamental role for mitochondria-OXPHOS in GO development, which provides  
61 potential for therapeutic interventions.

62

## 63 **Introduction**

64 Graves' Orbitopathy (GO), also called thyroid eye disease, is a disfiguring disease of  
65 the orbit with a higher incidence in women (80%) (1,2). The uncontrolled expansion of  
66 orbital adipose tissue (OAT) contributes to proptosis, double vision and in some cases  
67 visual loss. GO develops mainly in the context of an autoimmune condition, Graves'  
68 disease, in which thyrotropin receptor (TSHR) activation by thyroid stimulating  
69 antibodies (TSAB) mimics the action of TSH producing hyperthyroidism (1,2). The  
70 TSHR is also detected and increased in OAT in GO, and is an essential cellular target  
71 for both GO and Graves' disease (3-6). Previous GO studies have examined the cross-  
72 talk of signalling pathways via two cell-surface receptors, TSHR and insulin like growth  
73 factor 1 receptor (IGF1R), focusing on disease targeted preadipocytes/fibroblasts (PFs)  
74 embedded in OAT (7,8).

75 OAT-PFs are mesenchymal stem cell (MSC) with multi-differentiation potentials, as  
76 has been described by ourselves and others (9-11). In GO patients, the excessive  
77 adipogenesis via lineage-specific differentiation of PFs in OAT occurs rapidly (1,12). By  
78 contrast, WAT (white adipose tissues) from the same individual typically shrinks in  
79 Graves' disease due to hyperthyroidism (2). Previously we described a cell specific  
80 signalling network (PI3K/Akt/mTORC1/FOXOs) in PFs from human OAT not present in  
81 WAT (13,14). The identified pathways interact with TSHR/IGF1R signalling to play  
82 essential roles in the depot-specific OAT expansion in GO (2). Our recent work has  
83 demonstrated that OAT is a distinctive metabolic-quiescent fat depot which neither  
84 stores additional triglyceride in obesity (15) nor burns fatty acid (FA) (11), in contrast  
85 to WAT and BAT (brown adipose tissue)/BRITE (BRown in whITE), respectively. OAT  
86 also displays a unique FA-uptake-driven adipogenesis mechanism, which occurs in  
87 addition to the hyperplastic expansion (increased adipocyte number) of PFs in OAT

88 in GO (11). In particular, lower lipolytic activity with similar (low) FA-synthesis  
89 accompanied by increased expression of a depot-specific FA-transporter (SLC27A6)  
90 were observed in OAT from healthy individuals (OAT-H) and GO patients (OAT-GO)  
91 compared with WAT (11).

92 Involvement of mitochondrial dysfunction in the orbital fat expansion in GO is  
93 suggested by increased expression of the uncoupling protein UCP1 in OAT from GO.  
94 This has been observed both in human models - ex-vivo analysis of OAT-GO (11),  
95 GO-targeted PFs by TSHR activation (16,17) - and a mouse model of TSHR-induced  
96 GO (18). UCP1 expression in mitochondria is a known feature of BAT, which dissipates  
97 energy as heat by uncoupling mitochondrial oxidative-phosphorylation (OXPHOS) from  
98 ATP production and also plays an important role in mitochondrial function (19).  
99 Hyperplastic expansion of adipocytes in human BAT caused by mitochondrial  
100 dysfunction via mutation of *MFN2* has recently been reported (20). Substantially  
101 increased expression of *MFN2* has also been observed in OAT from GO patients  
102 compared with OAT-H in our recent study (11). Overexpression of adiponectin which is  
103 also a mitochondrial function modulator has been shown to induce expansion of both  
104 OAT and BAT in a mouse model (21,22).

105 These factors, together with the identified specific molecular signatures of OAT (e.g.  
106 Sirtuin/Wnt/Ca<sup>+</sup> signaling pathways) from our recent study suggest a role for  
107 mitochondria in the development of GO (11). Apart from being a cellular 'power-  
108 house', mitochondrial-OXPHOS and its bio-substrates, e.g. ATP/ADP, GTP/GDP,  
109 play fundamental roles in the regulation of cell metabolism through interacting with  
110 complex molecular cascades, e.g. cell proliferation, differentiation (23,24).

111 Our current study investigated the hypothesis that dysfunction of mitochondria plays a  
112 role in the FA-uptake-driven adipogenesis in OAT expansion in GO. Our investigation

113 demonstrated mitochondrial dysfunction in PFs from OAT-GO, which linked with the  
114 confirmed FA-uptake-driven adipogenesis from in vitro adipogenesis analysis.

## 115 **Materials and Methods**

116 All reagents were obtained from Sigma-Aldrich (U.K.) and tissue culture components  
117 from Cambrex (U.K.) unless otherwise stated.

### 118 **Adipose Tissue Collection & Preparation**

119 Adipose Tissue was collected with informed written consent and local research  
120 ethics committee approval. WAT (subcutaneous) was from 10 patients undergoing  
121 elective open abdominal or breast surgery for non-metabolic conditions. OAT from  
122 GO patients (n=13) were from 10 inactive GO patients with a CAS (clinical activity  
123 score) $<2$ , 3 active GO patients with CAS $\geq 3$  undergoing 2-wall or 3-wall orbital  
124 decompression surgery. Most of the GO patients had carbimazole treatment, RAI  
125 and/or thyroidectomy in the past; two GO patients received no anti-thyroid treatment  
126 and two were receiving carbimazole treatment while the OAT samples were obtained.  
127 OATs from non-GO patients (n=11) who were free of thyroid or other inflammatory  
128 eye disease and were undergoing augmented upper eyelid blepharoplasty surgery.  
129 OAT-PFs were obtained from adipose tissue explants and WAT-PFs were obtained  
130 by collagenase digest, both as previously described (17). Cells were used at low  
131 passage number ( $<5$ ), hence not all samples were analyzed in all experiments.

### 132 **Preadipocytes/fibroblasts culture and in vitro adipogenesis**

133 Preadipocytes/fibroblasts (PFs) were cultured in DMEM/F12 10% FCS (complete  
134 medium, CM). Adipogenesis was induced in confluent cells by replacing with ADM  
135 (adipogenic medium) containing 10% FCS, biotin (33 $\mu$ M), panthothenate (17 $\mu$ M), T3  
136 (1nM), dexamethasone (100nM), thiazolidinedione (1 $\mu$ M) and insulin (500nM) for 17

137 days. Adipogenesis was assessed by microscopic examination to detect the  
138 characteristic morphological changes (cell rounding, accumulation of lipid droplets),  
139 acquisition of lipid filled droplets (oil-red-O staining) and transcript measurement of  
140 terminal adipogenic marker (lipoprotein lipase, LPL) by QPCR as described  
141 previously (17).

142 For experiments using exogenous fatty acid (FA), a fatty acid mixture (200 $\mu$ M)  
143 comprising oleate:palmitate:linoleate (45:30:25%) bound to BSA was added to CM  
144 throughout the whole time culture as previous described (25), with/without ADP  
145 (Adenosine 5'-diphosphate sodium salt), GDP (Guanosine 5'diphosphate sodium  
146 salt), oligomycin (mitochondrial inhibitor) or AICAR (adenosine analog) for 17-19  
147 days. Adipogenesis was analysed by foci of differentiation (groups of cells with lipid  
148 droplets), which were counted in ten different fields for each experimental condition  
149 as described before (16).

#### 150 **Triglyceride (TAG) extraction from differentiated PFs and iLAB analysis**

151 Confluent PFs were cultured in ADM with/without the supplementation of FA,  
152 oleate:palmitate:linoleate (45:30:25%), for 10 days in 24-well plate. Cellular TAG  
153 from the differentiated PFs were analysed as previously described (25). In brief, cell  
154 lysates were obtained using lysis buffer (1% IGEPAL CA-630, 150 mM NaCl and  
155 50 mM Tris-HCl (pH8.0)) and sonication. Some of the lysates were used for protein  
156 quantification using BCA assay (Bio-Rad, DC protein assay kit). The lysates used for  
157 TAG analysis were heated at 95°C for 30 minutes and centrifuged at 12,000 g for 10  
158 minutes after cooling. Cellular TAG concentration was measured using enzymatic  
159 assay (TAG assay, Instrumentation Laboratory UK) with glycerol standards, and run  
160 on an ILAB 650 clinical analyzer (Instrumentation Laboratory UK). The normalised



161 TAG content per unit protein were obtained using the following calculation: TAG ( $\mu\text{M}$ )  
162  $\div$  protein (mg / ml).

### 163 **NAD(P)H and ATP measurement**

164 Confluent PFs were cultured in CM or ADM for 4 days and changed to CM before  
165 the following experiments. Cell number was counted using Cellometer from  
166 Nexcelom. MTS assay was performed (indicating the production of NAD(P)H (26))  
167 using CellTiter 96(R) AQueous One Solution Assay from Promega according to the  
168 manufacturer's instructions, 490nm absorbance was measured after 2h incubation  
169 and normalised by cell number. Cells were harvested after culture in CM or ADM for  
170 4 days, total cellular ATP ( $\mu\text{M}$ ) was measured using standard ATP dilutions by  
171 luminescent ATP detection assay kit from Abcam according to the manufacturer's  
172 instructions. Each condition had four repeats for the above experiments.

### 173 **Mitochondria number analysis by relative QPCR**

174 DNA was extracted from confluent PFs from OAT-H, OAT-GO or WAT using  
175 standard protocol and QPCR was conducted using SYBR Green incorporation  
176 measured on a Stratagene MX 3000 as previous described (16). Comparative QPCR  
177 was measured and expressed relative to a reference DNA RPL13A for mitochondria  
178 DNA cytochrome b (Cytob) detection to determine relative mitochondria number  
179 using the primers as follows: RPL13A, forward 5'- CTCAAGGTCGTGCGTCTG-3'  
180 and reverse 5'-TGGCTTTCTCTTTCCTCTTCT-3'; Cytob, forward 5'-  
181 GCGTCCTTGCCCTATTACTATC-3' and reverse 5'-  
182 CTTACTGGTTGTCCTCCGATTC-3' as described before (27).

### 183 **Flow cytometry analysis**

184 Confluent PFs were cultured in CM (as day 0), and changed to CM or ADM for 4  
185 days. Cells number were counted by Cellometer from Nexcelom, and followed the

186 procedures using flow cytometry (FACS) (BD FACSCanto II systems) with  
187 FACSDiva 6.0 software from Becton Dickinson and Co. (Mountain View, CA) as  
188 described before (28). In brief, cells were fixed with 100% methanol and  
189 permeabilized with 0.1% PBS-Tween20 for 20 min. Cells were then incubated with  
190 10% normal goat serum/0.3M glycine to block non-specific protein-protein  
191 interactions followed by primary antibody of mitochondrial-cytochrome-oxidase  
192 (MtCO2) (ab3298 from Abcam (RRID:AB\_303683), 1 $\mu$ g/1x10<sup>6</sup> cells) or isotype  
193 control antibody (mouse IgG) for 30 min. The secondary antibody used Alexa Fluor  
194 488 anti-mouse IgG (1:500 dilution) for 30 min, and then fluorescence emissions  
195 were collected for 10,000 cells by FACS analysis. Flow cytometric intensity of positive  
196 MtCO2 staining referenced to negative control was analysed using FlowJo software  
197 version 10.0.5 (Tree Star, Inc., Ashland, OR).

#### 198 **Mitochondrial functional assays by Seahorse XFe96 Analyzer**

199 Basal Assay medium and XFe96 consumables were purchased from Agilent  
200 Technologies; Draq5 was purchased from Abcam for DNA staining. Oxygen  
201 consumption rate (OCR) and extracellular acidification rate (ECAR) were measured  
202 with the Seahorse XFe96 (Agilent Technologies) using Mito Stress kit according to  
203 the manufacturer instructions as described before (29). Briefly, cells were seeded at  
204 1  $\times$  10<sup>4</sup> per well in Seahorse plate. 48 h post-seeding, confluent PFs were then  
205 transferred into non-buffered Seahorse Assay medium containing 17.5 mM glucose,  
206 1.5 mM sodium pyruvate and 2.5mM of L-Glutamine with 3% FCS. Basal cellular  
207 respiration rate was first measured followed by oligomycin A injection (1  $\mu$ M) to  
208 inhibit ATP synthase. Maximal respiration capacity was determined in 3.5  $\mu$ M FCCP.  
209 Finally, non-mitochondrial respiration rate was determined using a combination of  
210 rotenone (0.5  $\mu$ M) and antimycin A (1  $\mu$ M). Measurements were performed with 3

211 cycles including 4 min of medium mixing followed by 3 min of measurements. For  
212 data normalization, cells were fixed for 20 min with 4% PFA. Cell nuclei were then  
213 stained for 1 h with Draq5 (ab108410 from Abcam (RRID:AB\_2892715)) 1/10000  
214 diluted in PBS +0.1% Tween-20. Stained cells were then detected with Odyssey  
215 Scanner (Li-Cor). Fluorescent intensity per well was used to normalize respiration  
216 values per well. The average of the non-mitochondrial respiration measures was  
217 then subtracted from each corresponding condition/time-point per well; all similar  
218 condition measurements per well were then averaged. On day 0 (48 h post-seeding),  
219 PFs from OAT, OAT-GO and WAT (n=4 for each) in CM were measured with 5  
220 repeats of each condition from one 96-well plate; day 4 (or day 7) PFs (n=4) in CM  
221 and ADM were measured from two 96-well plates. PFs from WAT (n=4), OAT (n=8)  
222 and OAT-GO (n=9) were used, and two sets of independent experiments were  
223 performed for OAT (n=4 & 4) and OAT-GO (n=4 & 5), respectively.  
224 Cellular ATP production rates by mitochondria-OXPHOS and glycolysis were  
225 calculated using obtained OCR and ECAR from Seahorse analysis, taking into  
226 account of the acidification rates due to mitochondrial CO<sub>2</sub> production as described  
227 previously (30,31).

## 228 **Statistics**

229 Results were analysed using Prism 5 (version 5.02), data normality was initially  
230 analysed using the Kolmogorov-Smirnov test. To compare groups we used the T-test  
231 for variables normally distributed and the Mann Whitney test was used for non-  
232 normal distributed data. Differences between groups were analysed using one way  
233 ANOVA. We applied Dunnett's multiple comparison post hoc test for multiple  
234 comparisons when identifying statistically significant differences. In all cases  $p < 0.05$   
235 was considered significant. Data are presented as mean  $\pm$  SEM.

236 For correlation analysis, the percentage increased FA-uptake adipogenesis by GDP  
237 and measured OXPHOS-ATP/Glycolysis-ATP of the cells were analysed for  
238 Normality using the Kolmogorov-Smirnov test. All the data were normally distributed  
239 and correlation was analysed using Pearson's correlation.

## 240 **Result**

### 241 **Depot-specific mitochondrial-ATP production by PFs from OAT in early** 242 **adipogenesis**

243 We induced in vitro adipogenesis using ADM (adipogenic medium) with a mixed  
244 hormonal-cocktail in normal culture medium (CM) on human primary PFs for 17 days.  
245 The differentiated cells displayed cell rounding and accumulation of lipid droplets  
246 (triglyceride formation) with positive Oil-Red-O staining and the induction of LPL  
247 expression (marker of late adipogenesis) as previously reported (17).

248 We compared the early stages of adipogenesis, prior to the formation of adipocytes,  
249 in confluent PFs from WAT, OAT-H or OAT-GO cultured in CM (non-differentiating)  
250 or ADM (differentiating) conditions. We analysed mitochondria numbers by relative  
251 QPCR of mitochondrial DNA, cytochrome b (Cytob) to a reference genomic DNA,  
252 RPL13A. Figure 1A demonstrates lower ( $35.3\pm 3.2\%$ ) mitochondrial number in OAT-  
253 H or OAT-GO, either in CM or ADM-condition on day 4 compared to PFs from WAT.  
254 Interestingly, mitochondrial-cytochrome-oxidase (MtCO<sub>2</sub>) levels were significantly  
255 higher ( $52.8\pm 2.5\%$ ) in OAT-H and OAT-GO compared with PFs from WAT using flow  
256 cytometry analysis (Figure 1B). MtCO<sub>2</sub> is a necessary component of the respiratory  
257 chain of mitochondria for OXPHOS-ATP production (32).

258 Furthermore, we detected a substantially higher production of NAD(P)H ( $1.23\pm 0.06$   
259 fold-increase) indicating increased mitochondrial-OXPHOS activity (MTS assay/ $10^6$   
260 cells (26), Figure 1C) and increased total cellular-ATP ( $1.97\pm 0.03$  fold-increase) of

261 differentiating-PFs from OAT-H cultured in ADM compared with CM-condition (non-  
262 differentiating-PFs) (Figure 1D), which was not observed in WAT. The increased  
263 production of NAD(P)H and total cellular-ATP were also observed in differentiating-  
264 PFs from OAT-GO compared with non-differentiating-PFs (supplemental Figure S1),  
265 however significantly lower fold-increases were observed when compared with OAT-  
266 H. There was no significant difference in cell number between PFs cultured in CM  
267 and ADM on day 4 (supplemental Figure S2).

### 268 **Dysfunction of live ATP-production via mitochondrial-OXPHOS in PFs from** 269 **OAT-GO.**

270 We next explored live ATP-production rates by mitochondrial-OXPHOS or cellular-  
271 Glycolysis according to oxygen consumption rate (OCR) and extracellular acidification  
272 rate (ECAR) measured by 96-well Seahorse analyser, as previously described (30,31).  
273 Analyses were performed at basal-day-0 in CM-condition or on day 4 and 7 in CM &  
274 ADM conditions at early adipogenesis, i.e. before observation of any differentiated  
275 adipocytes. All the obtained data were referenced to cell DNA staining at each time  
276 point and condition.

277 During early adipogenesis, an increased OXPHOS-ATP level of differentiating-PFs was  
278 observed compared to non-differentiating-PFs on day 4 from WAT, OAT-H and OAT-  
279 GO, and further increased on day 7 from WAT and OAT-H, but not OAT-GO (Figure  
280 2A). We then looked detail into changes in OXPHOS & Glycolysis-ATP levels in PFs  
281 from OAT-H and OAT-GO separately.

282 From OAT-H, non-differentiating-PFs had substantially decreased Glycolysis-ATP level  
283 on day 4 compared with day 0 and day 7 (Figure 2B), accompanied by unchanged  
284 levels of OXHPOS-ATP (Figure 2A). Consequently, there was a higher OXPHOS-  
285 ATP/Glycolysis-ATP ratio on day 4 compared with day 0 and day 7 in non-

286 differentiating-PFs (Figure 2C). Interestingly, in differentiating-PFs (through  
287 adipogenesis), significantly higher levels ( $28.8\pm 11.7\%$ ;  $46.9\pm 15.3\%$ ) of OXPHOS-ATP  
288 production (Figure 2A) and OXPHOS-ATP/Glycolysis-ATP ratio ( $56\pm 10.8\%$ ;  $68.9\pm 23\%$ )  
289 (Figure 2C) with unchanged Glycolysis-ATP (Figure 2B) were observed on day 4 or 7  
290 when compared with day 0, respectively.

291 From OAT-GO, non-differentiating-PFs showed a trend to increased Glycolysis-ATP  
292 levels (Figure 2B) contrasting to a sharp and consistent drop ( $39.7\pm 7.5\%$ ;  $40.8\pm 10.7\%$ )  
293 of OXPHOS-ATP levels (Figure 2A) on day 4 or day 7 when compared with day 0,  
294 respectively. Consequently, there was a trend to decreased ratio of OXPHOS-  
295 ATP/Glycolysis-ATP on day 4 & 7 compared with day 0 in non-differentiating-PFs  
296 (Figure 2C). Through adipogenesis (Differentiating PFs) in OAT-GO, unchanged levels  
297 of OXPHOS-ATP/Glycolysis-ATP & its ratio on day 0, 4 & 7 were observed contrasting  
298 with our findings in OAT-H (Figure 2).

299 In summary, there is a reduction in ATP production by OXPHOS in OAT-GO non-  
300 differentiating-PFs, which recovers somewhat in differentiating-PFs. By contrast this  
301 reduction of OXPHOS-ATP production is absent in healthy OAT non-differentiating-PFs  
302 and levels increase as PFs differentiate.

### 303 **FA-uptake-driven adipogenesis and the link with mitochondrial in OAT**

304 Cells with lipid droplets and positive Oil-Red-O staining were identified as  
305 differentiated adipocytes, and a foci count was performed as described before (16).

306 1) In order to investigate FA-uptake-driven adipogenesis in vitro, we cultured  
307 confluent PFs from OAT-H or OAT-GO with extra free FA using a mixture of three  
308 major triglyceride FA, oleate:palmitate:linoleate (45:30:25%) as described in the  
309 investigation of FA-uptake in PFs from WAT (25). As expected, differentiated  
310 adipocytes were apparent in ADM after 17 days full-differentiation protocol with

311 adipogenesis hormonal-cocktail, while no adipogenesis was observed in CM-  
312 condition (Figure 3A). Addition of the FA-supplement to ADM, led to the  
313 differentiated adipocytes as early as day 10, which had about 3-fold higher cellular  
314 triglyceride per protein unit compared with adipocytes in ADM alone which were still  
315 at the early-middle stage of adipogenesis (supplemental Figure S3).

316 The effect of FA and the role of mitochondria in OAT-adipogenesis were further  
317 investigated by culturing confluent PFs from OAT-H and OAT-GO with FA-  
318 supplement in CM without adipogenesis hormonal-cocktail and with addition of  
319 different concentrations of mitochondrial bio-substrates, ADP (0.05, 0.1, 0.2mM) and  
320 GDP (0.1, 0.5, 1mM) for 17 days. To our surprise, we observed differentiated  
321 adipocytes by feeding FA only in CM for 17 days to both OAT-H and OAT-GO PFs  
322 (OAT-H n=1 and OAT-GO n=4, Figure 3A). This type of FA-uptake-driven  
323 adipogenesis was substantially enhanced by 1mM GDP in CM reaching to 7-18% of  
324 ADM-hormonal-cocktail induced adipogenesis (Figure 3A). In contrast, no significant  
325 changes in the FA-uptake-driven adipogenesis were observed by ADP-supplement  
326 in PFs from both OAT-H and OAT-GO (Figure 3A).

327 2) We then analysed the effect of the optimal 1mM concentration of GDP and FA  
328 supplement in FA-uptake-driven adipogenesis in additional samples. We also tested  
329 the effect of ADP and chemical adenosine-analogue/inhibitor of mitochondria  
330 (AICAR/oligomycin, respectively). The results are summarised in Figure 3B; we  
331 observed differentiated adipocytes from OAT-H (n=1 out of 4) and OAT-GO PFs  
332 (n=7 out of 8) by feeding FA in CM. Furthermore, substantially increased FA-uptake-  
333 driven adipogenesis in PFs from all OAT-H and OAT-GO was observed upon  
334 addition of 1mM GDP (Figure 3B). In contrast, addition of the adenosine-analogue  
335 AICAR or ADP had no effect on FA-uptake-driven adipogenesis, and the use of

336 different concentrations of mitochondrial inhibitor, oligomycin, abolished the induced  
337 FA-uptake-driven adipogenesis of all PFs from OAT-H and OAT-GO (Figure 3B).

338 3) We have replicated the effects of GDP, ADP with FA-supplement in CM on OAT-  
339 PFs in further experiments (supplemental Figure S4).

340 In summary, FA-triggered adipogenesis was observed in OAT-H PFs from 4 out of 5  
341 individuals and 8 out of 10 OAT-GO PFs samples from GO patients. In all cases, this  
342 was substantially enhanced by addition of GDP-supplement.

### 343 **Correlation of FA-uptake-driven adipogenesis by GDP with increased** 344 **OXPHOS/Glycolysis capacity of PFs from OAT-GO**

345 In 6 OAT-GO samples we had data from both seahorse analysis (Figure 2) and FA-  
346 induced adipogenesis (Figure 3).The further enhanced FA-uptake-driven  
347 adipogenesis by GDP treatment (percentage increase versus FA-feeding only)  
348 significantly and positively correlated with the measured OXPHOS-ATP levels ( $r =$   
349  $0.946$ ,  $p = 0.004$ ) (Figure 4A) and OXPHOS-ATP/Glycolysis-ATP ratio ( $r = 0.840$ ,  $p =$   
350  $0.036$ ) (Figure 4C), but not with the levels of Glycolysis-ATP ( $r = -0.75$ ,  $p = 0.086$ )  
351 (Figure 4B) in non-differentiating-PFs at basal-day-0. The further enhanced FA-  
352 uptake-driven adipogenesis by GDP treatment significantly correlated negatively with  
353 the measured Glycolysis-ATP levels ( $r = -0.966$ ,  $p = 0.002$ ) (Figure 4E) and  
354 positively with OXPHOS-ATP/Glycolysis-ATP ratio ( $r = 0.857$ ,  $p = 0.029$ ) (Figure 4F),  
355 but no significant correlation with levels of OXPHOS-ATP ( $r = 0.096$ ,  $p = 0.857$ )  
356 (figure 4D) in differentiating-PFs on day 4.

### 357 **Discussion**

358 Our study demonstrated increased cellular ATP-production via mitochondrial-  
359 OXPHOS during adipogenesis of PFs from OAT but not WAT. Mitochondrial  
360 dysfunction was observed with disrupted levels of OXPHOS-ATP/Glycolysis-ATP



361 and its ratio in PFs from OAT-GO compared with OAT-H. Furthermore, FA-  
362 supplementation was able to trigger in vitro adipogenesis in PFs from both OAT-H  
363 and OAT-GO, which was substantially enhanced by addition of GDP and diminished  
364 by mitochondrial inhibitor. The enhanced FA-uptake-driven adipogenesis by GDP  
365 was significantly and positively correlated with ratios of OXPHOS-ATP/Glycolysis-  
366 ATP at basal non-differentiating-PFs or early-adipogenesis of differentiating-PFs  
367 from OAT-GO. Taken together, these observations suggested that an essential role  
368 of mitochondrial OXPHOS-ATP production and its bio-substrate GDP in the depot-  
369 specific FA-uptake-driven adipogenesis of OAT-H and exacerbated in GO by  
370 mitochondrial dysfunction.

371 Our investigation employed a well-established cell model, heterogeneous population  
372 (PFs) derived from the entire stromal-vascular fraction of human orbital (OAT) and  
373 WAT (10,33). The key findings of this study were confirmed by using more than one  
374 independent technique and the observations from our recent ex vivo OAT analysis  
375 (11) as discussed below. The low availability of human orbital PFs precludes the use  
376 of a model comprising a homogeneous cell type, although this would be preferable.

377 We and others have described the multi-differentiation MSC potential of adipose  
378 tissue derived-PFs (9-11,34). Classically, in vitro adipogenesis is triggered by ADM  
379 with a mixed hormonal cocktail to activate key transcriptional factors, e.g. PPAR,  
380 CEBP, etc. (35,36), as routinely used in our lab (17). Our current study demonstrated  
381 that FA alone were able to induce in vitro adipogenesis in PFs from OAT-H and  
382 OAT-GO. This confirms the depot-specific FA-uptake-driven adipogenesis in OAT,  
383 which was suggested by ex vivo analysis of human adipose tissue from our recent  
384 study (11). Furthermore, we previously demonstrated an abundant expression of the  
385 FA-transporter, SLC27A6, with limited FA-synthesis/lipolysis in OAT in GO

386 supporting the excessive FA-uptake-driven adipogenesis in GO (11). Thyroid  
387 hormone, PPAR $\gamma$  ligand and cytokines (e.g. TNF or IL6) play important roles in the  
388 pathogenesis of GO reviewed in (2), which may also contribute to OAT adipogenesis  
389 through its role in the regulation of lipid metabolism via FA transporter (37,38).  
390 Further investigation is needed to dissect the important role of the FA transporter  
391 system, in the specific context of the MSC phenotype of OAT-PFs, in the FA-uptake-  
392 driven adipogenesis in GO (9-11).

393 Our study revealed that inhibition of mitochondrial-OXPHOS by oligomycin abolished  
394 the FA-uptake-driven adipogenesis of OAT, which supports the important function of  
395 mitochondria in adipogenesis as reported in other fat depots (20,39). However  
396 AICAR (adenosine-analog) or ADP, the activator of AMP kinase (AMPK), had no  
397 effect on the FA-uptake-driven adipogenesis of OAT thereby eliminating the AMPK-  
398 pathway, which plays important roles in other fat depots (40). By contrast, our  
399 current study demonstrated that the depot-specific FA-uptake-driven adipogenesis  
400 was substantially enhanced by supplementing GDP in PFs from both OAT-H and  
401 OAT-GO. The important role of GDP in mitochondrial function and cellular  
402 metabolism has been reported in OXPHOS-ATP production (41) or via GTP/GDP  
403 exchange (42). Our study suggested that the reduced mitochondrial-OXPHOS  
404 results in more available GDP to form a feedback loop to regulate the FA-uptake-  
405 driven adipogenesis in OAT.

406 The current study demonstrated that mitochondrial function in OAT-PFs during  
407 adipogenesis is linked with an inducible total cellular-ATP production via  
408 mitochondrial-OXPHOS. It contrasts with WAT-PFs, or other human stem cells, that  
409 have unchanged or even decreased levels of total cellular ATP, apart from increased  
410 OXPHOS-ATP through adipogenesis as observed in this study and by others (39,43).

411 Our investigation observed a depot-specific higher level of mitochondrial  
412 cytochrome-C oxidase (32) with low mitochondria number in PFs from OAT  
413 compared with WAT suggesting increased mitochondrial activity rather than numbers.  
414 Furthermore, in OAT-H, early adipogenesis (days 0-4-7) is accompanied by a U-  
415 shaped distribution (high, low, high) of Glycolysis-ATP and unchanged OXPHOS-ATP  
416 levels. In contrast, levels of OXPHOS-ATP fell sharply with compensated high level of  
417 Glycolysis-ATP from non-differentiating-PFs from OAT-GO indicating sustained  
418 mitochondrial dysfunction of PFs in GO.

419 Our recent study highlighted the key role of enhanced proliferation of PFs synergized  
420 with FA-uptake-driven adipogenesis in the hyperplastic expansion of OAT in GO (11).  
421 Studies have shown that the Warburg phenotype of proliferating cells is important to  
422 have enhanced Glycolysis with suppressed mitochondrial-OXPHOS, however non-  
423 proliferating cells display higher OXPHOS-ATP production with inhibited Glycolysis  
424 (44,45). Our data clearly demonstrate OXPHOS-ATP levels falling sharply with  
425 compensated high-level of Glycolysis-ATP production in non-differentiating-PFs from  
426 OAT-GO, which may trigger the proliferation of PFs in GO (44,45). Once proliferation  
427 is induced, we see higher levels of OXPHOS-ATP and low levels of Glycolysis-ATP  
428 through adipogenesis in differentiating-PFs from OAT-H reported here, which may in  
429 turn lead to inhibition of proliferation and drive PFs to differentiate (44,45). This  
430 study provides further support in the positive correlation noted between FA-uptake-  
431 driven adipogenesis by GDP and the ratios of OXPHOS-ATP/Glycolysis-ATP  
432 through adipogenesis of PFs from OAT-GO. We hypothesise that the levels of  
433 mitochondrial OXPHOS-ATP and metabolites (GDP) play central roles in triggering  
434 PFs proliferation and excessive FA-uptake-driven adipogenesis of OAT in GO. The  
435 uncorrected dysfunction of mitochondria in PFs from OAT-GO leads to maintained

436 levels of low-OXPHOS/high-Glycolysis causing excessive proliferation, and more  
437 available GDP promoting FA-uptake-driven adipogenesis in OAT expansion in GO.  
438 The OAT depot-specific cell signalling cascades (13,14) play a central role in the  
439 pathogenesis of OAT expansion in GO through the interplay of TSHR/PKA,  
440 IGF1R/PI3K/Akt, mTORC1, and the downstream nucleus factor, FOXOs, (7,46-49),  
441 which are also important in mitochondrial function (42). More work is needed to  
442 clarify how TSHR/IGF1R interfere with mitochondria via GDP and OXPHOS-ATP  
443 production in triggering proliferation and FA-uptake-driven adipogenesis of OAT, e.g.  
444 via pathological activation of UCP-1 (11), or interference with the adenylate cyclase  
445 system of GTP/GDP exchange to allow the activation of GO targeted TSHR/IGF1R  
446 (50,51). However, we hypothesise that the resultant mitochondrial dysfunction in  
447 association with FA-uptake mechanism drives OAT expansion in GO (summarized in  
448 Figure 5).

#### 449 **Ethical approval**

450 Human Adipose Tissue was collected with informed written consent and approved by  
451 the South East Wales Research Ethics Committee (30 May 2006) with registry  
452 number (06/WSE03/37).

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#### 458 **Author contributions**

459 LZ designed the study, performed experiments, prepared the figures, and drafted the  
460 manuscript; PR, SM and DMT supported LZ for Seahorse technique and analysis;  
461 SM supported LZ for OXPHOS-ATP/Glycolysis-ATP calculation using OCR/ECAR;  
462 MSD, DAR, ASH, DM supported sample collections with ethical approval and  
463 obtained consent forms from patients; ART supported LZ for relative QPCR analysis  
464 of mitochondria number; LZ, ML, DMT & CMD interpreted data and finalised the  
465 manuscript; all authors revised the manuscript and approved the submitted version.

466 **Reference:**

- 467 1. Bahn RS. Graves' ophthalmopathy. *N Engl J Med.* 2010;362(8):726-738.
- 468 2. Taylor PN, Zhang L, Lee RWJ, Muller I, Ezra DG, Dayan CM, Kahaly GJ,  
469 Ludgate M. New insights into the pathogenesis and nonsurgical management  
470 of Graves orbitopathy. *Nat Rev Endocrinol.* 2020;16(2):104-116.
- 471 3. Bahn RS, Dutton CM, Natt N, Joba W, Spitzweg C, Heufelder AE. Thyrotropin  
472 receptor expression in Graves' orbital adipose/connective tissues: potential  
473 autoantigen in Graves' ophthalmopathy. *J Clin Endocrinol Metab.*  
474 1998;83(3):998-1002.
- 475 4. Crisp MS, Lane C, Halliwell M, Wynford-Thomas D, Ludgate M. Thyrotropin  
476 receptor transcripts in human adipose tissue. *J Clin Endocrinol Metab.*  
477 1997;82(6):2003-2005.
- 478 5. Gerding MN, van der Meer JW, Broenink M, Bakker O, Wiersinga WM,  
479 Prummel MF. Association of thyrotrophin receptor antibodies with the clinical  
480 features of Graves' ophthalmopathy. *Clin Endocrinol (Oxf).* 2000;52(3):267-  
481 271.
- 482 6. Khoo DH, Ho SC, Seah LL, Fong KS, Tai ES, Chee SP, Eng PH, Aw SE, Fok  
483 AC. The combination of absent thyroid peroxidase antibodies and high

- 484 thyroid-stimulating immunoglobulin levels in Graves' disease identifies a group  
485 at markedly increased risk of ophthalmopathy. *Thyroid*. 1999;9(12):1175-1180.
- 486 7. Zhang L, Grennan-Jones F, Draman MS, Lane C, Morris D, Dayan CM, Tee  
487 AR, Ludgate M. Possible targets for nonimmunosuppressive therapy of  
488 Graves' orbitopathy. *J Clin Endocrinol Metab*. 2014;99(7):E1183-1190.
- 489 8. Krieger CC, Place RF, Bevilacqua C, Marcus-Samuels B, Abel BS, Skarulis  
490 MC, Kahaly GJ, Neumann S, Gershengorn MC. TSH/IGF-1 Receptor Cross  
491 Talk in Graves' Ophthalmopathy Pathogenesis. *J Clin Endocrinol Metab*.  
492 2016;101(6):2340-2347.
- 493 9. Brandau S, Bruderek K, Hestermann K, Gortz GE, Horstmann M, Mattheis S,  
494 Lang S, Eckstein A, Berchner-Pfannschmidt U. Orbital Fibroblasts From  
495 Graves' Orbitopathy Patients Share Functional and Immunophenotypic  
496 Properties With Mesenchymal Stem/Stromal Cells. *Invest Ophthalmol Vis Sci*.  
497 2015;56(11):6549-6557.
- 498 10. Kozdon K, Fitchett C, Rose GE, Ezra DG, Bailly M. Mesenchymal Stem Cell-  
499 Like Properties of Orbital Fibroblasts in Graves' Orbitopathy. *Invest*  
500 *Ophthalmol Vis Sci*. 2015;56(10):5743-5750.
- 501 11. Zhang L, Evans A, von Ruhland C, Draman MS, Edkins S, Vincent AE,  
502 Berlinguer-Palmini R, Rees DA, Haridas AS, Morris D, Tee AR, Ludgate M,  
503 Turnbull DM, Karpe F, Dayan CM. Distinctive Features of Orbital Adipose  
504 Tissue (OAT) in Graves' Orbitopathy. *Int J Mol Sci*. 2020;21(23).
- 505 12. Kumar S, Coenen MJ, Scherer PE, Bahn RS. Evidence for enhanced  
506 adipogenesis in the orbits of patients with Graves' ophthalmopathy. *J Clin*  
507 *Endocrinol Metab*. 2004;89(2):930-935.

- 508 13. Zhang L, Grennan-Jones F, Lane C, Rees DA, Dayan CM, Ludgate M.  
509 Adipose tissue depot-specific differences in the regulation of hyaluronan  
510 production of relevance to Graves' orbitopathy. *J Clin Endocrinol Metab.*  
511 2012;97(2):653-662.
- 512 14. Zhang L, Ji QH, Ruge F, Lane C, Morris D, Tee AR, Dayan CM, Ludgate M.  
513 Reversal of Pathological Features of Graves' Orbitopathy by Activation of  
514 Forkhead Transcription Factors, FOXOs. *J Clin Endocrinol Metab.*  
515 2016;101(1):114-122.
- 516 15. Smolders MH, Graniewski-Wijnands HS, Meinders AE, Fogteloo AJ, Pijl H, de  
517 Keizer RJ. Exophthalmos in obesity. *Ophthalmic Res.* 2004;36(2):78-81.
- 518 16. Draman MS, Stechman M, Scott-Coombes D, Dayan CM, Rees DA, Ludgate  
519 M, Zhang L. The Role of Thyrotropin Receptor Activation in Adipogenesis and  
520 Modulation of Fat Phenotype. *Front Endocrinol (Lausanne).* 2017;8:83.
- 521 17. Zhang L, Baker G, Janus D, Paddon CA, Fuhrer D, Ludgate M. Biological  
522 effects of thyrotropin receptor activation on human orbital preadipocytes.  
523 *Invest Ophthalmol Vis Sci.* 2006;47(12):5197-5203.
- 524 18. Schluter A, Horstmann M, Diaz-Cano S, Plohn S, Stahr K, Mattheis S,  
525 Oeverhaus M, Lang S, Flogel U, Berchner-Pfannschmidt U, Eckstein A,  
526 Banga JP. Genetic immunization with mouse thyrotrophin hormone receptor  
527 plasmid breaks self-tolerance for a murine model of autoimmune thyroid  
528 disease and Graves' orbitopathy. *Clin Exp Immunol.* 2018;191(3):255-267.
- 529 19. Kazak L, Chouchani ET, Stavrovskaya IG, Lu GZ, Jedrychowski MP, Egan DF,  
530 Kumari M, Kong X, Erickson BK, Szpyt J, Rosen ED, Murphy MP, Kristal BS,  
531 Gygi SP, Spiegelman BM. UCP1 deficiency causes brown fat respiratory

- 532 chain depletion and sensitizes mitochondria to calcium overload-induced  
533 dysfunction. *Proc Natl Acad Sci U S A*. 2017;114(30):7981-7986.
- 534 20. Sawyer SL, Cheuk-Him Ng A, Innes AM, Wagner JD, Dymont DA, Tetreault M,  
535 Care4Rare Canada C, Majewski J, Boycott KM, Screatton RA, Nicholson G.  
536 Homozygous mutations in MFN2 cause multiple symmetric lipomatosis  
537 associated with neuropathy. *Hum Mol Genet*. 2015;24(18):5109-5114.
- 538 21. Combs TP, Pajvani UB, Berg AH, Lin Y, Jelicks LA, Laplante M, Nawrocki AR,  
539 Rajala MW, Parlow AF, Cheeseboro L, Ding YY, Russell RG, Lindemann D,  
540 Hartley A, Baker GR, Obici S, Deshaies Y, Ludgate M, Rossetti L, Scherer PE.  
541 A transgenic mouse with a deletion in the collagenous domain of adiponectin  
542 displays elevated circulating adiponectin and improved insulin sensitivity.  
543 *Endocrinology*. 2004;145(1):367-383.
- 544 22. Kusminski CM, Scherer PE. Mitochondrial dysfunction in white adipose tissue.  
545 *Trends Endocrinol Metab*. 2012;23(9):435-443.
- 546 23. Bouchez C, Devin A. Mitochondrial Biogenesis and Mitochondrial Reactive  
547 Oxygen Species (ROS): A Complex Relationship Regulated by the  
548 cAMP/PKA Signaling Pathway. *Cells*. 2019;8(4).
- 549 24. Cheng Z, Tseng Y, White MF. Insulin signaling meets mitochondria in  
550 metabolism. *Trends Endocrinol Metab*. 2010;21(10):589-598.
- 551 25. Todorovic M, Hilton C, McNeil C, Christodoulides C, Hodson L, Karpe F,  
552 Pinnick KE. A cellular model for the investigation of depot specific human  
553 adipocyte biology. *Adipocyte*. 2017;6(1):40-55.
- 554 26. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-  
555 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular



- 556 localization, substrate dependence, and involvement of mitochondrial electron  
557 transport in MTT reduction. *Arch Biochem Biophys*. 1993;303(2):474-482.
- 558 27. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver  
559 P. mTOR controls mitochondrial oxidative function through a YY1-PGC-  
560 1alpha transcriptional complex. *Nature*. 2007;450(7170):736-740.
- 561 28. Draman MS, Grennan-Jones F, Zhang L, Taylor PN, Tun TK, McDermott J,  
562 Moriarty P, Morris D, Lane C, Sreenan S, Dayan C, Ludgate M. Effects of  
563 prostaglandin F(2alpha) on adipocyte biology relevant to graves' orbitopathy.  
564 *Thyroid*. 2013;23(12):1600-1608.
- 565 29. Russell OM, Fruh I, Rai PK, Marcellin D, Doll T, Reeve A, Germain M, Bastien  
566 J, Rygiel KA, Cerino R, Sailer AW, Lako M, Taylor RW, Mueller M,  
567 Lightowlers RN, Turnbull DM, Helliwell SB. Preferential amplification of a  
568 human mitochondrial DNA deletion in vitro and in vivo. *Sci Rep*.  
569 2018;8(1):1799.
- 570 30. Mavin E, Verdon B, Carrie S, Saint-Criq V, Powell J, Kuttruff CA, Ward C,  
571 Garnett JP, Miwa S. Real-time measurement of cellular bioenergetics in fully  
572 differentiated human nasal epithelial cells grown at air-liquid-interface. *Am J*  
573 *Physiol Lung Cell Mol Physiol*. 2020;318(6):L1158-L1164.
- 574 31. Mookerjee SA, Brand MD. Measurement and Analysis of Extracellular Acid  
575 Production to Determine Glycolytic Rate. *J Vis Exp*. 2015(106):e53464.
- 576 32. Rak M, Benit P, Chretien D, Bouchereau J, Schiff M, El-Khoury R, Tzagoloff A,  
577 Rustin P. Mitochondrial cytochrome c oxidase deficiency. *Clin Sci (Lond)*.  
578 2016;130(6):393-407.

- 579 33. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC,  
580 Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of  
581 multipotent stem cells. *Mol Biol Cell*. 2002;13(12):4279-4295.
- 582 34. Fraser JK, Wulur I, Alfonso Z, Hedrick MH. Fat tissue: an underappreciated  
583 source of stem cells for biotechnology. *Trends Biotechnol*. 2006;24(4):150-  
584 154.
- 585 35. Sarjeant K, Stephens JM. Adipogenesis. *Cold Spring Harb Perspect Biol*.  
586 2012;4(9):a008417.
- 587 36. Spiegelman BM, Flier JS. Adipogenesis and obesity: rounding out the big  
588 picture. *Cell*. 1996;87(3):377-389.
- 589 37. Dube E, Gravel A, Martin C, Desparois G, Moussa I, Ethier-Chiasson M,  
590 Forest JC, Giguere Y, Masse A, Lafond J. Modulation of fatty acid transport  
591 and metabolism by maternal obesity in the human full-term placenta. *Biol*  
592 *Reprod*. 2012;87(1):14, 11-11.
- 593 38. Sinha RA, Singh BK, Yen PM. Direct effects of thyroid hormones on hepatic  
594 lipid metabolism. *Nat Rev Endocrinol*. 2018;14(5):259-269.
- 595 39. Zhang Y, Marsboom G, Toth PT, Rehman J. Mitochondrial respiration  
596 regulates adipogenic differentiation of human mesenchymal stem cells. *PLoS*  
597 *One*. 2013;8(10):e77077.
- 598 40. van Dam AD, Kooijman S, Schilperoort M, Rensen PC, Boon MR. Regulation  
599 of brown fat by AMP-activated protein kinase. *Trends Mol Med*.  
600 2015;21(9):571-579.
- 601 41. Woyda-Ploszczyca AM, Jarmuszkiewicz W. Different effects of guanine  
602 nucleotides (GDP and GTP) on protein-mediated mitochondrial proton leak.  
603 *PLoS One*. 2014;9(6):e98969.

- 604 42. Demine S, Renard P, Arnould T. Mitochondrial Uncoupling: A Key Controller  
605 of Biological Processes in Physiology and Diseases. *Cells*. 2019;8(8).
- 606 43. Trayhurn P, Beattie JH. Physiological role of adipose tissue: white adipose  
607 tissue as an endocrine and secretory organ. *Proc Nutr Soc*. 2001;60(3):329-  
608 339.
- 609 44. Harvey A, Caretti G, Moresi V, Renzini A, Adamo S. Interplay between  
610 Metabolites and the Epigenome in Regulating Embryonic and Adult Stem Cell  
611 Potency and Maintenance. *Stem Cell Reports*. 2019;13(4):573-589.
- 612 45. Maldonado EN, Lemasters JJ. ATP/ADP ratio, the missed connection  
613 between mitochondria and the Warburg effect. *Mitochondrion*. 2014;19 Pt  
614 A:78-84.
- 615 46. Chen CC, Jeon SM, Bhaskar PT, Nogueira V, Sundararajan D, Tonic I, Park  
616 Y, Hay N. FoxOs inhibit mTORC1 and activate Akt by inducing the expression  
617 of Sestrin3 and Rictor. *Dev Cell*. 2010;18(4):592-604.
- 618 47. Krieger CC, Neumann S, Place RF, Marcus-Samuels B, Gershengorn MC.  
619 Bidirectional TSH and IGF-1 receptor cross talk mediates stimulation of  
620 hyaluronan secretion by Graves' disease immunoglobins. *J Clin Endocrinol*  
621 *Metab*. 2015;100(3):1071-1077.
- 622 48. Kumar S, Nadeem S, Stan MN, Coenen M, Bahn RS. A stimulatory TSH  
623 receptor antibody enhances adipogenesis via phosphoinositide 3-kinase  
624 activation in orbital preadipocytes from patients with Graves' ophthalmopathy.  
625 *J Mol Endocrinol*. 2011;46(3):155-163.
- 626 49. Smith TJ, Kahaly GJ, Ezra DG, Fleming JC, Dailey RA, Tang RA, Harris GJ,  
627 Antonelli A, Salvi M, Goldberg RA, Gigantelli JW, Couch SM, Shriver EM,  
628 Hayek BR, Hink EM, Woodward RM, Gabriel K, Magni G, Douglas RS.

- 629 Teprotumumab for Thyroid-Associated Ophthalmopathy. *N Engl J Med*.  
630 2017;376(18):1748-1761.
- 631 50. Johnston CA, Siderovski DP. Receptor-mediated activation of heterotrimeric  
632 G-proteins: current structural insights. *Mol Pharmacol*. 2007;72(2):219-230.
- 633 51. Schneyer CR, Pineyro MA, Kirkland JL, Gregerman RI. Stimulation of human  
634 fat cell adenylate cyclase by GDP and guanosine 5'-O-(2-thiodiphosphate). *J*  
635 *Biol Chem*. 1984;259(11):7038-7044.
- 636 52. Harper ME, Seifert EL. Thyroid hormone effects on mitochondrial energetics.  
637 *Thyroid*. 2008;18(2):145-156.
- 638 53. Le Moli R, Muscia V, Tumminia A, Frittitta L, Buscema M, Palermo F, Sciacca  
639 L, Squatrito S, Vigneri R. Type 2 diabetic patients with Graves' disease have  
640 more frequent and severe Graves' orbitopathy. *Nutr Metab Cardiovasc Dis*.  
641 2015;25(5):452-457.
- 642 54. Starkey K, Heufelder A, Baker G, Joba W, Evans M, Davies S, Ludgate M.  
643 Peroxisome proliferator-activated receptor-gamma in thyroid eye disease:  
644 contraindication for thiazolidinedione use? *J Clin Endocrinol Metab*.  
645 2003;88(1):55-59.

646 **Supplementary data** (4 supplemental figures) 10.6084/m9.figshare.14578311  
647 Figure S1 Fold-increase on day 4 ADM-adipogenesis compared with CM condition.  
648 Figure S2 Cell number count on day 4 adipogenesis.  
649 Figure S3 Increased adipogenesis by FAs supplement in ADM.  
650 Figure S4 Induced adipogenesis by FAs and GDP treatment in CM.

651 **Figure 1. Mitochondria analysis & cellular ATP production of OAT-PFs in ADM-**  
652 **adipogenesis compared with WAT.** Confluent PFs from OAT-H, OAT-GO or WAT  
653 were cultured in complete medium (CM) or ADM (adipogenic medium) for 4 days. **A)**

654 DNA extracted from OAT (n=3) & OAT-GO (n=3) or WAT-PFs (n=4) on day-4, relative  
655 QPCR of cytochrome b (Cytob, mitochondrial DNA) to RPL13A (reference genomic  
656 DNA) was performed. **B**) MtCO2 antibody (mitochondria cytochrome oxidase) was  
657 analysed by FACS from confluent OAT (n=4), OAT-GO (n=4) & WAT-PFs (n=7) at day-  
658 0 in CM or day-4 in CM & ADM, the percentage of flow cytometric (FL) intensity, positive  
659 MtCO2 staining, was shown referenced to negative control. **C**) MTS assay (indicating  
660 the production of NAD(P)H and mitochondria-OXPHOS activity (26)) was performed on  
661 PFs from OAT-H (n=3) & WAT-PFs (n=4) on day 4 in CM & ADM, 490nm absorbance  
662 normalized by cell number. **D**) Total level of cellular ATP (Luminescent ATP detection  
663 assay kit) was measured from OAT-H (n=7) & WAT-PFs (n=4) on day 4 in CM & ADM.  
664 Histograms = mean±SEM of all samples studied. T-test was used for statistical  
665 analysis. \*\*\*p≤0.001, \*\*\*\*p<0.0005.

666 **Figure 2. Live ATP production from OXPHOS/Glycolysis in differentiating- or**  
667 **non-differentiating PFs from WAT, OAT-H & OAT-GO.** Confluent PFs were cultured  
668 in 96-well plates, Oxygen Consumption Rate (OCR) as OXPHOS-ATP and  
669 extracellular acidification rate (ECAR) as Glycolysis-ATP were measured using Mito  
670 Stress kit from WAT (n=4), OAT (n=8) and OAT-GO (n=9) using Seahorse analyzer.  
671 OXPHOS-ATP (**A**), Glycolysis-ATP (**B**) were measured as pmol ATP/min on day (D)0  
672 plate in complete culture medium (CM, white square), or after PFs in CM (non-  
673 differentiating) or adipogenic medium (ADM, black square) (differentiating-PFs) for 4  
674 or 7 days and referenced to DNA staining. **C**) Percentage ratio of OXPHOS vs  
675 Glycolysis on D0, 4 & 7 with CM or ADM condition were presented. Basal-day-0  
676 levels of ATP production by OXPHOS (4.5 pmol ATP/min), Glycolysis(16.6 pmol  
677 ATP/min) and its ratio (31.4%) of PFs from OAT-H were displayed and indicated with  
678 dash lines. Histograms = mean±SEM of all samples studied. Data were normally

679 distributed, a one-way ANOVA with Dunnett's multiple comparison test or t-test was  
680 used to compared D4 or D7 time points with D0 (^); or t-test was used to compared  
681 between CM and ADM on D4 and D7 (\*). \*p<0.5; \*\*p≤0.01; \*\*\*p<0.005; \*\*\*\*p<0.0005.

682 **Figure 3. The link of mitochondrial-OXPHOS in FA-uptake-driven adipogenesis**

683 **of PFs from OAT. A)** Confluent cells from OAT-H (n=1) and OAT-GO (n=4),  
684 passage 2-4, were cultured in CM or ADM (black square), or with FA supplement in  
685 CM (square with dot) for 17 days with treatment of ADP (0.05mM, 0.1mM or 0.2mM),  
686 or GDP (0.1mM, 0.5mM or 1mM); **B)** Confluent cells from OAT-H (n=4, white column)  
687 or OAT-GO (n=8, black dot column), passage 2-3 were cultured in CM with FA  
688 supplement for 19 days, with/without 10ng/ml oligomycin-A (Olig-10), 100ng/ml  
689 oligomycin-A (Olig-100), 0.1mM ADP, 0.1mM AICAR, 1mM GDP. Cells were then  
690 fixed and stained using oil-red-O technique. Differentiated adipocytes (with lipid  
691 droplets) were observed and counted in ten different fields. Representative photos  
692 were shown with arrows indicating differentiation adipocytes (x10 mignification).  
693 Histograms = mean±SEM of all samples studied. Normal (t-test) or non-normal  
694 (Mann Whitney test) distributed data was analysed accordingly. \*p<0.05, \*\*\*p<0.005.

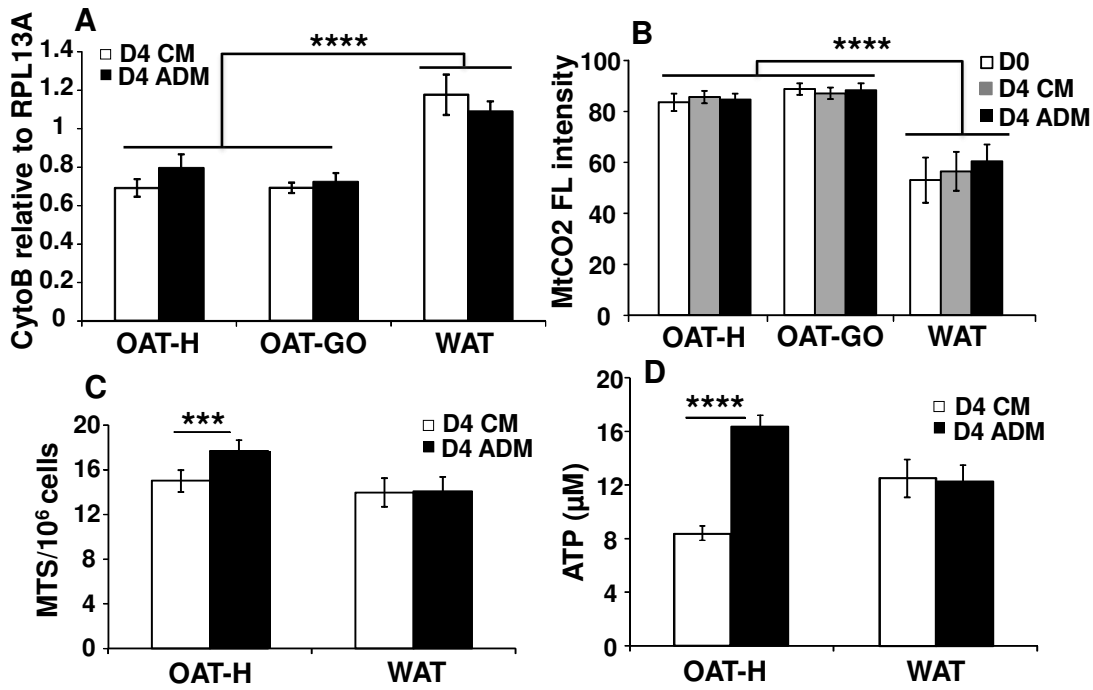
695 **Figure 4. Correlations of FA-uptake-driven adipogenesis by GDP treatment**

696 **with levels of OXPHOS/Glycolysis-ATP in PFs from OAT-GO.** The scatterplot  
697 showing the relationship between percentage induced FA-uptake-driven  
698 adipogenesis by GDP+FA vs FA-only, and levels of **A)** OXPHOS-ATP (dot), **B)**  
699 Glycolysis-ATP (triangle) and **C)** the percentage ratio of OXPHOS/Glycolysis (square)  
700 in non-differentiating-PFs at basal day 0; or **D)** OXPHOS-ATP levels, **E)** Glycolysis-  
701 ATP levels and **F)** the percentage ratio of OXPHOS/Glycolysis of differentiating-PFs  
702 on day 4 adipogenesis. Statistically Pearson's correlations for the normally  
703 distributed data have shown (significant correlations were underlined).

704 **Figure 5. The essential role of mitochondrial-OXPHOS for FA uptake-driven**  
705 **adipogenesis in the hyperplastic OAT expansion.** Our study demonstrated a  
706 depot specific cellular ATP production via mitochondrial-OXPHOS through  
707 adipogenesis in OAT-PFs. Dysfunction of mitochondria (dys-Mt) with disrupted  
708 OXPHOS-ATP production was observed in PFs from OAT-GO due to GO factors, e.g.  
709 TSHR/IGF1R etc. (11,17,18,21,52-54). **Hypothesis:** Mitochondrial OXPHOS-ATP  
710 production through adipogenesis is important in maintaining OAT stability by forming a  
711 beneficial relationship with the available GDP in PFs to maintain a healthy (low) level of  
712 proliferation/adipogenesis in OAT-H (highlighted in dash blue-square). The pathological  
713 fall in OXPHOS-ATP with compensated high-level of Glycolysis-ATP by GO factors  
714 triggered the proliferation of PFs in OAT in GO, the kept low-level of mitochondrial-  
715 OXPHOS and consequent higher GDP availability drive excessive fatty-acid uptake-  
716 driven adipogenesis in OAT in GO (highlighted in red-square). Dashed lines indicate  
717 proposed mechanism scheme.

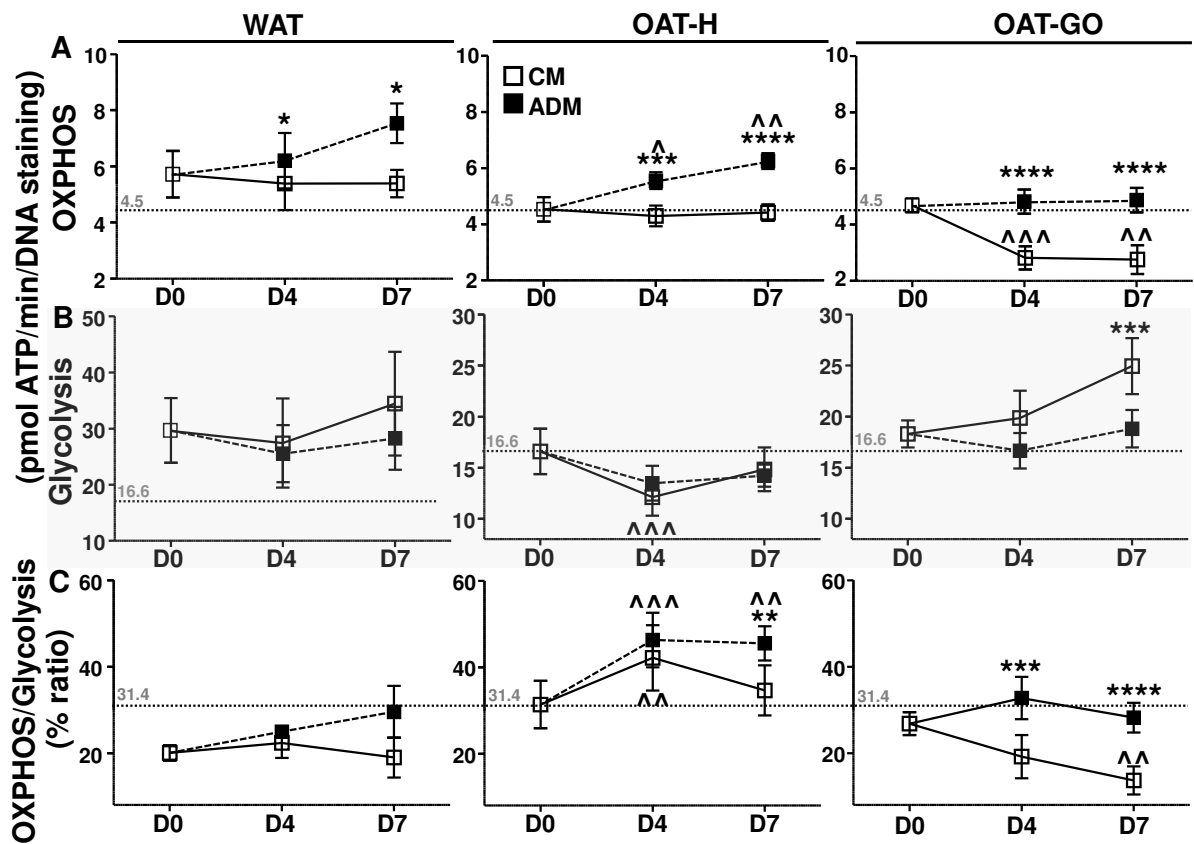
718

719 **Figure 1**



720

721 **Figure 2**

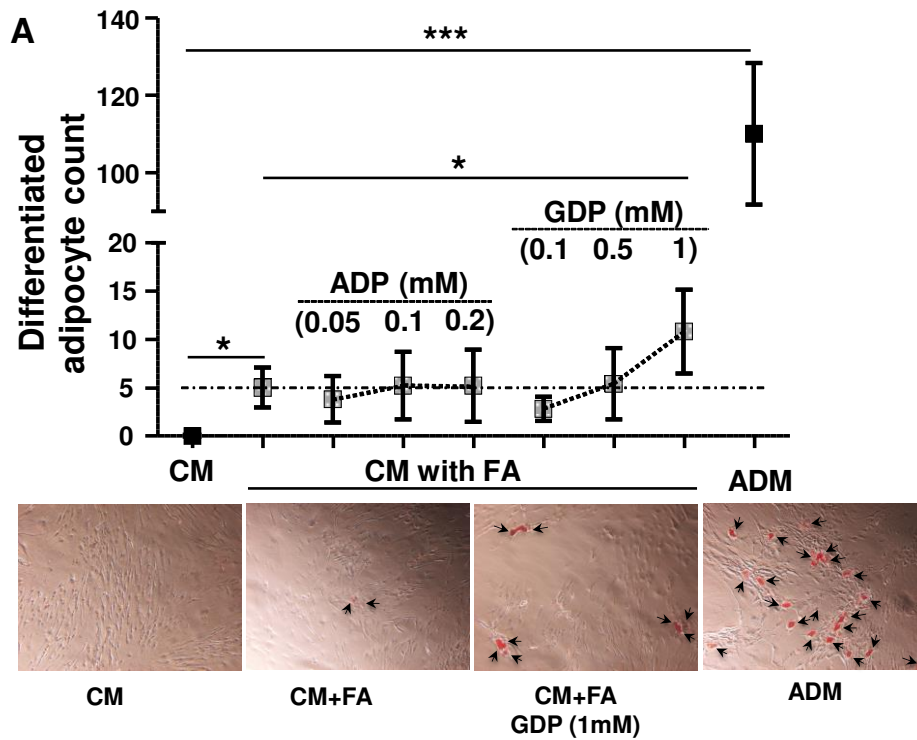


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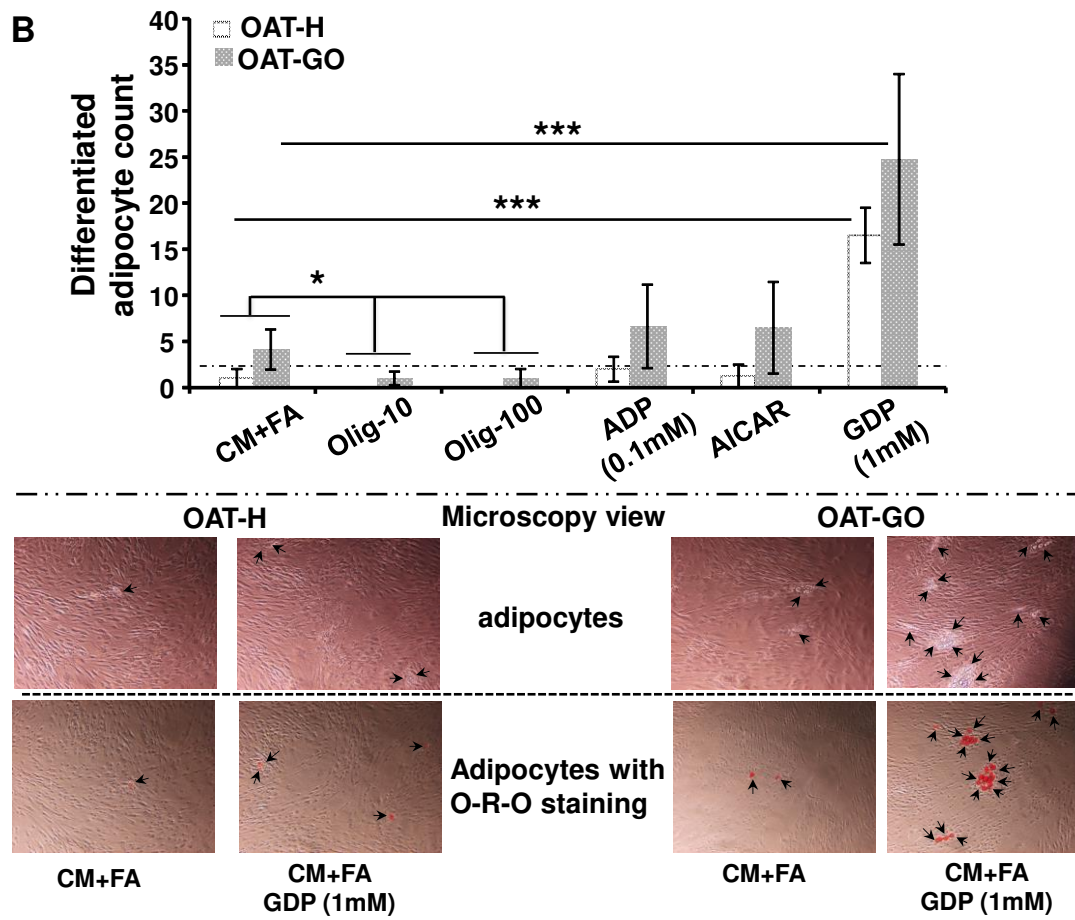


724 **Figure 3**



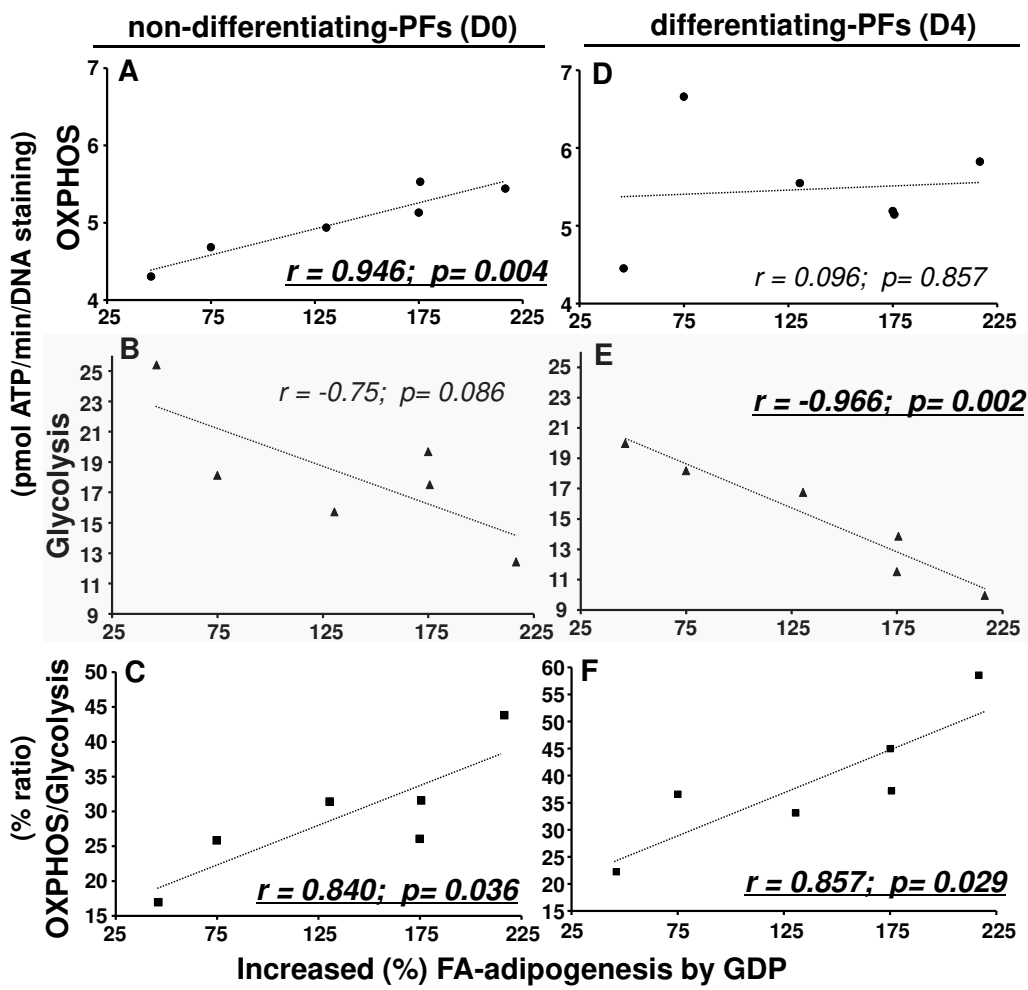
Differentiated adipocytes with O-R-O staining

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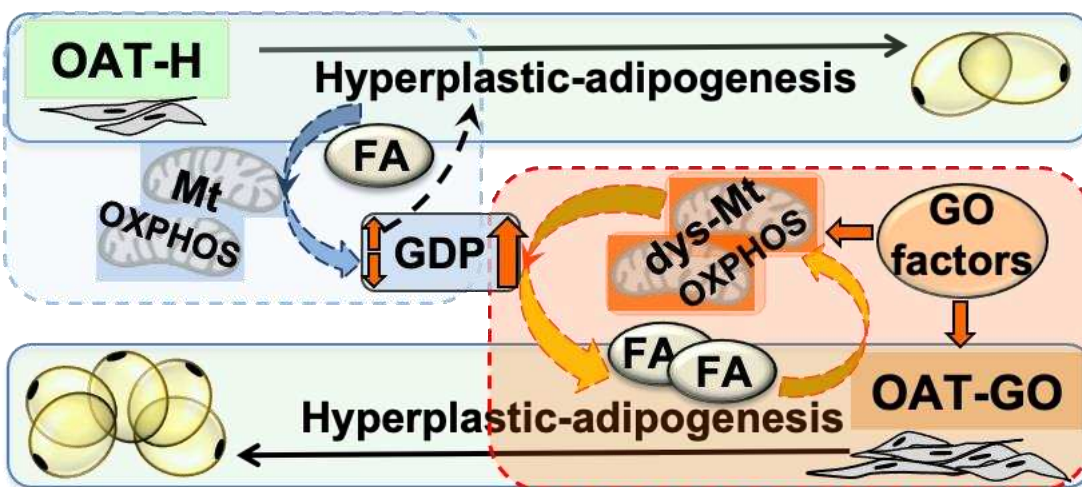
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728 **Figure 4**



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730 **Figure 5**



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