Biochemistry and Cell Biology
Biochimie et biologie cellulaire

Adaptations to Excess Choline in Insulin Resistant and Pcyt2 Deficient Skeletal Muscle

Journal:	*Biochemistry and Cell Biology*
Manuscript ID	bcb-2016-0105.R1
Manuscript Type:	Article
Date Submitted by the Author:	29-Jul-2016

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Keyword:	Pcyt2, choline, skeletal muscle, insulin resistance, triglycerides

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1 **Adaptations to Excess Choline in Insulin Resistant and Pcyt2 Deficient Skeletal Muscle**

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19 **Abstract**

20 It was hypothesized that choline supplementation in insulin resistant (IR) 21 CTP:phosphoethanolamine cytidylyltransferase deficient ($Pcyt2^{+/-}$) mice would ameliorate 22 muscle function by remodeling glucose and fatty acid (FA) metabolism. $Pcyt2^{+/-}$ mice either 23 received no treatment or were allowed access to 2 mg/ml choline in drinking water for 4 weeks. 24 Skeletal muscle was harvested from choline treated and untreated mice. Lipid analysis and 25 metabolic gene expression and signaling pathways were compared between untreated $Pcyt2^{+/-}$ 26 mice, treated $Pcyt2^{+/-}$ mice and $Pcyt2^{+/+}$ mice. The major positive effect of choline 27 supplementation on IR muscle was the reduction of glucose utilization for FA and TAG 28 synthesis and increased muscle glucose storage as glycogen. Choline reduced the expression of 29 genes for FA and TAG formation (Scd1, Fas, Srebp1c, Dgat1/2), upregulated the genes for FA 30 oxidation (Cpt1, Pparα, Pgc1α) and had minor effects on phospholipid and lipolysis genes. 31 $Pcyt2^{+/-}$ muscle had reduced insulin signaling (IRS1), autophagy (LC3) and choline transport 32 (CTL1) proteins that were restored by choline treatment. Additionally, choline activated AMPK 33 and Akt while inhibiting mTORC1 phosphorylation. These data established that choline 34 supplementation could restore muscle glucose metabolism by reducing lipogenesis and 35 improving mitochondrial and intracellular signaling for protein and energy metabolism in insulin 36 resistant Pcyt2 deficient mice.

37 **Key words:** Pcyt2, choline, skeletal muscle, insulin resistance, triglycerides

38 **Abbreviations:** Pcyt2-CTP:phosphoethanolamine cytidylyltransferase; CEPT-choline 39 ethanolamine phosphotransferase 1; PE-phosphatidylethanolamine; FA-fatty acid; CHO-choline 40 treated; PC-phosphatidylcholine; ACh-acetylcholine; TAG-triglyceride; IR-insulin resistance; PI

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41 phosphatidylinositol; FC-free cholesterol; PC-phosphatidylcholine; CE-cholesterol ester; DAG-
42 diacylglycerol; CTL1-choline transporter like protein 1; *Pcyt1*-CTP:phosphocholine
43 cytidylyltransferase; *Pss1* and *Pss2*-phosphatidylserine synthase 1 and 2; *Psd*-phosphatidylserine
44 decarboxylase; *Srebp1*-sterol regulatory binding protein 1; *Fas*-fatty acid synthase; *Scd1*-
45 stearoyl-CoA desaturase 1; *Dgat1* and *Dgat2*-diacylglycerol acyltransferase 1 and 2; *Lpl*-
46 lipoprotein lipase; *Hsl*-hormone-sensitive lipase; *Atgl*-adipocyte triglyceride lipase; *Pparα*-
47 peroxisomal proliferation activation receptor α; *Pgc1α*-Ppar-gamma coactivator 1α; *Acc*-acyl
48 CoA carboxylase; *Cpt1*-carnitine palmitoyltransferase 1; *Gapdh*-glyceraldehyde-3-phosphate
49 dehydrogenase

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50 **Introduction**

51 The nutrient choline is required for the formation of membrane phospholipids
52 phosphatidylcholine (PC) and sphingomyelin, the neurotransmitter acetylcholine (ACh)
53 (Niculescu 2013) and the methyl group donor betaine (Zeisel 2012). Choline is a vital
54 component of skeletal muscle function and choline deficiency is directly implicated in the
55 development of muscular dystrophy (Sher et al. 2006). Choline specific transporter
56 CTL1/SLC44A1 is abundantly present in skeletal muscle (Yuan et al. 2004) and upregulated in
57 numerous mitochondrial and muscle myopathies (Yuan et al. 2006). Inherited mutations in
58 choline kinase beta result in the development of muscular dystrophy in both mouse and humans
59 (Sher et al. 2006 and Mitsuhashi et al. 2011). ACh is essential for muscle contraction (Yamada et
60 al. 2012) and both neuron-specific (Haga 1971) and ubiquitous choline transporters (Michel and
61 Bakovic, 2012) supply choline for muscle ACh synthesis (Nassenstein et al. 2015). Choline
62 deficiency compromises muscle cell function by decreasing mitochondrial abundance, increasing
63 TAG accumulation and perturbing phospholipid and FA metabolism (Michel et al. 2011).

64 The ATTICA study (Detopoulou et al. 2008) as well as the Nurses' Health Study (Zeisel and da
65 Costa 2009) showed that diets rich in choline and betaine could reduce inflammatory biomarkers,
66 including C-reactive protein, interleukin-6 and tumor necrosis factor alpha. Adequate intakes of
67 choline and betaine have been frequently associated with lower plasma homocysteine, an
68 independent risk factor for cardiovascular disease. In experimental animals as well as humans,
69 choline deficiency results in the formation of non-alcoholic fatty liver and an increased
70 sensitivity to inflammation (Tryndyak et al. 2016). Choline and betaine are named lipotropic
71 dietary agents because they can prevent or reduce the accumulation of TAG (fat) in the liver.

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72 Betaine has been shown to curb liver fat accumulation and it has been broadly recognized as a
73 safe and effective therapy for alcoholic fatty liver disease (Purohit et al. 2007). The beneficial
74 role of choline and betaine was also clearly observed with respect to non-alcoholic
75 steatohepatitis (which develops in obesity) when oral supplements improved homocysteine
76 levels, lipid profile (attenuation of fatty liver and proper export and formation of triglycerides
77 and VLDL), steatosis, inflammation and fibrosis (Cordero et al. 2013). Therefore, it is clear that
78 a diet enriched in choline is beneficial in the prevention and treatment of obesity related
79 disorders. However, it is not known if dietary choline could regulate muscle function and
80 mitigate muscle IR and lipotoxicity.

81 It is becoming increasingly evident that skeletal muscle phospholipids are important mediators of
82 insulin sensitivity and muscle contraction. Muscle-specific knockout models for
83 CTP:phosphoethanolamine cytidylyltransferase/Pcyt2 (Selathurai et al. 2015) and
84 choline/ethanolamine phosphotransferase 1/CEPT1 (Funai et al. 2016) have been recently
85 developed. These enzymes catalyze the rate-regulatory step and the final step respectively in the
86 CDP-ethanolamine (Kennedy) pathway for phosphatidylethanolamine (PE) biosynthesis. The
87 two knockout models established that impaired PE biosynthesis has a direct impact on muscle
88 lipid metabolism and function (Selathurai et al. 2015 and Funai et al. 2016). The muscle-specific
89 knockout (Selathurai et al. 2015) accumulated diacylglycerol (DAG) and TAG but did not
90 develop insulin resistance (IR), probably because of upregulated mitochondrial FA oxidation.
91 CEPT1-deleted muscle (Funai et al. 2016) was on the other hand protected from high-fat diet IR
92 but was exercise intolerant, which has been attributed to PE dependent regulation of endoplasmic
93 reticulum calcium handling. We have generated a heterozygous Pcyt2 mouse (Pcyt2$^{+/-}$) which

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94 has systemically reduced PE biosynthesis (Basu et al. 2015). Young Pcyt2$^{+/-}$ mice are
95 asymptomatic but gradually develop IR, obesity and metabolic syndrome, including the
96 accumulation of DAG and TAG in skeletal muscle (Fullerton et al. 2007 and Schenkel et al.
97 2015). Choline treatment reduced Pcyt2$^{+/-}$ obesity and fatty liver disease (Schenkel and Bakovic
98 2014). Here, we hypothesise that choline will also aid in mitigating Pcyt2$^{+/-}$ muscle pathologies
99 by restoring FA and general energy homeostasis, altogether resulting in improved muscle insulin
100 signalling and mitochondrial function.

101

102 **Methods**

103 *Animals and choline treatments*

104 Generation of Pcyt2 deficient (Pcyt2$^{+/-}$) mice and genotyping were described previously (Basu et
105 al. 2015). All procedures were approved by the University of Guelph's Animal Care Committee
106 and were in accordance with guidelines of the Canadian Council on Animal Care. Mice were
107 exposed to a 12-h light/12-h dark cycle beginning with light at 7:00 a.m. Mice were fed a
108 standardized chow diet (Harlan Teklad S-2335) *ad libitum* and had free access to water. Pcyt2$^{+/-}$
109 mice progressively develop obesity and insulin resistance starting at 2.5 months of age (Fullerton
110 et al. 2007). In the present study, 8-10 month old mice are divided into three groups (n = 6-10 in
111 each group): Pcyt2$^{+/-}$ treated (Pcyt2$^{+/-}$ CHO), and untreated (Pcyt2$^{+/-}$) with choline, and wildtype
112 littermate controls (Pcyt2$^{+/+}$). Treated mice were kept in separate cages and had free access to
113 water containing 2 mg/ml choline (Sigma) for 4 weeks. Choline supplementation did not
114 influence food and water intake, as determined on a daily basis. The choline intake from drinking
115 water was ~240 µg/g/day as determined from the choline concentration (2 mg/ml), water intake

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116 (5 ml/day/mouse), and mouse weight (Schenkel et al. 2015). Thus, in addition to 17 mg/day
117 obtained from diet, the Pcyt2$^{+/-}$ CHO group obtained additional 10 mg choline/day from water**,**
118 which was a 59% increase in total choline intake. The recommended adequate intake (AI) for
119 choline is 425 mg/day for women and 550 mg/day for men (Penry and Manore 2008).

120

121 *Analysis of phospholipids and cholesterol*

122 The analysis of muscle phospholipids and cholesterol was performed by HPLC (Agilent
123 Technologies, Santa Clara, CA, USA) as before (Schenkel et al. 2015). Briefly, 1 mg of skeletal
124 muscle homogenates from Pcyt2$^{+/-}$ CHO, Pcyt2$^{+/-}$ and Pcyt2$^{+/+}$ mice (n = 3) was extracted in the
125 presence of an internal standard (50 mg dipalmitoyl-phosphatidyl dimethyl ethanolamine) using
126 a method of Folch *et al* (1957). The extracted lipids were dried, resuspended in
127 chloroform:isooctane (1:1) and separated by HPLC using a 3-solvent gradient and a normal
128 phase column (Onyxmonolithic silica; Phenomenex Incorporated, Torrance, CA, USA). The
129 amounts of PC, PE, phosphatidylinositol (PI), sphingomyelin (SM), cholesterol (FC), cholesterol
130 ester (CE) and free fatty acids (FA) were determined using appropriate standards and expressed
131 in mg of lipid/mg of protein.

132

133 *Analysis of DAG and TAG*

134 Muscle samples (50 mg, n = 3 per group) were homogenized in 200 µl 5% PBS-T, the
135 homogenates twice heated to 100°C and cooled to room temperature and centrifuged to remove
136 protein debris. Total TAG content (µg/mg of muscle) was determined using Wako Diagnostics
137 Kit. In addition, 50 mg muscle samples (n = 3 per group) were extracted by the method of Bligh
138 and Dyer (Bligh and Dyer 1959). The lower organic phase was dried and resuspended in 100 µl

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139 of chloroform. DAG and TAG were resolved by TLC using a solvent system of
140 heptane/isopropyl ether/acetic acid (60:40:3). Authentic standards were spotted in parallel to the
141 samples, the plates were dried and exposed to iodine vapour for visualization. DAG and TAG
142 spots were scanned and quantified with densitometry (Fullerton et al. 2007).
143
144 *Analysis of muscle glycogen*
145 Skeletal muscle samples (50 mg; n = 3 per group) were hydrolyzed in 500 µl 30% KOH.
146 Homogenates were heated to 100°C for 30 min with frequent vortexing. Glycogen was
147 precipitated with 600 µl of 95% ethanol, kept on ice for 45 min and centrifuged at 840 x g for 30
148 min. Glycogen pellet was dissolved in 1 ml of water by vortexing. The glycogen solution was
149 mixed with 1 ml of 5% phenol and 5 ml of 98% sulfuric acid was then added and kept at room
150 temperature for 10 min. Samples were cooled on ice for 30 min and developed colour which was
151 determined spectrophotometrically at 490 nm. A standard curve was generated using pure
152 glycogen (Roche) (Lo et al. 1970).
153
154 *Immunoblotting*
155 Muscle samples (50 mg; n = 3 per group) were homogenized in cold lysis buffer (10 mM Tris-
156 HCl [pH 7.4], 1 mM EDTA and 10 mM NaF) containing protease (1/10) and phosphatase
157 (1/100) inhibitor cocktails (Sigma). The lysates were centrifuged for 20 minutes at 10,000 x g at
158 4°C to remove cell debris and 50 µg of lysate was used for analysis. For CTL1 protein, the
159 lysates were mixed with a non-denaturing loading buffer (62 mM Tris-HCl and 0.01%
160 bromophenol blue in 10% glycerol) and resolved with an 8% native gel at 90V for 2 h. All other

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161 proteins were assessed under denaturing conditions with a loading buffer comprised of 400 mM
162 Tris-HCl, 50% glycerol, 10% SDS, 0.25% (w/v) bromophenol blue and 3% (w/v) DTT. AMPKα,
163 pAMPKα, ACC, pACC, PKCα, PI3K, IRS-1, Akt, p^{308}Akt, p^{473}Akt, mTORC1 and
164 p^{2443}mTORC1 were resolved with a 10% denaturing gel. LC3I and LC3II were resolved with a
165 15% denaturing gel. All proteins were transferred to PVDF membranes (VWR). Membranes
166 were blocked with 5% skim milk in PBS-T and incubated with the C-terminal CTL1 antibody
167 (ENS-627; 1:500). Membranes were also incubated with AMPKα, pAMPKα, PI3K, Akt,
168 p^{308}Akt, p^{473}Akt, mTORC1 and p^{2443}mTORC1 (Cell Signaling), LC3 I/II (Abcam), IRS-1
169 (Millipore) antibodies as a 1:1,000 dilution with 5% BSA in PBS-T overnight at 4°C.
170 Membranes were washed 2 times with PBS-T and incubated with horseradish peroxidase-
171 conjugated anti-rabbit IgG secondary antibody (NEB) (1:5,000) in 5% skim milk in PBS-T for 1
172 h. After washing with PBS-T, the proteins were visualized using a chemiluminescent substrate
173 (Sigma). β-tubulin (Sigma) was used as a loading control.

174

175 *Analysis of gene expression*

176 mRNA was extracted from $Pcyt2^{+/-}$ CHO, $Pcyt2^{+/-}$ and $Pcyt2^{+/+}$ skeletal muscle (n = 5-8 per
177 group) using TRIzol reagent (Invitrogen). cDNA was prepared from 2 µg of total mRNA using a
178 3-AP primer and SuperScript II reverse transcriptase (Invitrogen). For the phospholipid
179 pathways, the expression of the choline transporter like protein 1 (*Ctl1*), CTP:phosphocholine
180 cytidylyltransferase (*Pcyt1*), phosphatidylserine synthase 1 and 2 *(Pss1 and Pss2*) and
181 phosphatidylserine decarboxylase (*Psd*) were analyzed. Genes involved in FA and TAG
182 synthesis included sterol regulatory binding protein 1c (*Srebp1c*), FA synthase *(Fas)*, stearoyl-

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183 CoA desaturase 1 *(Scd1)*, and diacylglycerol acyltransferase 1 and 2 (*Dgat1 and Dgat2*). The
184 expression of the lipid degradation genes lipoprotein lipase (*Lpl*), hormone-sensitive lipase (*Hsl*)
185 and adipocyte triglyceride lipase (*Atgl*) was also analyzed. The expression of the mitochondrial
186 regulatory genes peroxisomal proliferation activation receptor α (*Pparα*), Ppar gamma
187 coactivator 1α (*Pgc1α*), and the FA genes acyl CoA carboxylase (*Acc*) and carnitine
188 palmitoyltransferase 1 (*Cpt1*) were used to assess mitochondrial FA metabolism.
189 Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was used as a control. The primers
190 and PCR conditions are in Table 1 and as described (Basu et al. 2015, Schenkel et al. 2015,
191 Schenkel and Bakovic 2014).

192

193 *Statistical analysis*

194 Data are represented as mean ± SEM. One way ANOVA with post-hoc Tukey's test was
195 performed to compare means between WT, Pcyt2$^{+/-}$ and Pcyt2$^{+/-}$ CHO groups. A difference at
196 95% confidence interval ($p \leq 0.05$) was considered as significant. All statistical tests were
197 performed with GraphPad Prism 4 software.

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198

199 **Results**

200 *Choline modifies membrane composition and reduces TAG accumulation in Pcyt2$^{+/-}$ muscle*

201 The analysis of the membrane lipids in choline treated and control muscle is shown in Fig. 1A.
202 The content of the major phospholipids PE and PC in the Pcyt2$^{+/-}$ muscle was similar to the
203 content in the control Pcyt2$^{+/+}$ muscle. The IR Pcyt2$^{+/-}$ muscle however had reduced levels of the

204 cell signalling phosphatidylinositol (PI) and the 'lipid raft' free cholesterol (FC). Choline
205 treatments (Pcyt2$^{+/-}$ CHO) did not improve the PI content but significantly increased PC,
206 sphingomyelin (SM) and FC, reaching the levels present in the control muscle. Additionally**,** free
207 FA content was dramatically (60%) reduced by choline treatment (Fig. 1A). Overall, choline
208 treatment increased the membrane phospholipid ratio (PC/PE, Fig. 1B) but did not improve the
209 "lipid rafts' ratio (SM/FC, Fig. 1C).

210 The levels of Pcyt2$^{+/-}$ muscle TAG were elevated relative to control muscle, and became
211 significantly reduced by choline treatment. Pcyt2$^{+/-}$ muscle had 60% (Fig. 1D) more TAG than
212 the control muscle and choline reduced muscle TAG by 40%. A similar trend was observed in
213 TAG and DAG relative ratios (Fig. 1E); Pcyt2$^{+/-}$ muscle DAG was elevated by 30% relative to
214 control Pcyt2$^{+/+}$ muscle and choline (Pcyt2$^{+/-}$ CHO) returned the elevated Pcyt2$^{+/-}$ DAG to the
215 control levels. Taken together, the lipid analysis established that the major positive effects of
216 choline diet on the IR Pcyt2$^{+/-}$ muscle were the increased quantities of the choline-containing
217 membrane phospholipids (PC and SM) and cholesterol and the reduced quantities of the energy
218 producing FA, DAG and TAG.

219

220 *Choline restores Pcyt2$^{+/-}$ muscle glycogen*

221 Choline treatment had a positive effect regarding the restoration of Pcyt2$^{+/-}$ muscle glycogen
222 levels (Fig. 1F). Glycogen was reduced in Pcyt2$^{+/-}$ muscle in relation to Pcyt2$^{+/+}$ wildtype muscle
223 by 50%. One month of dietary choline supplementation was able to increase Pcyt2$^{+/-}$ muscle
224 glycogen levels by 60% ($p < 0.05$). Taken together, the data in Figures 1A-F demonstrated that

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225 choline supplementation dramatically remodelled Pcyt2$^{+/-}$ muscle TAG and glycogen
226 metabolism to be more similar to the lean phenotype of Pcyt2$^{+/+}$ muscle.

227

228 *Choline does not modify phospholipid gene expression*

229 Choline is incorporated into PC by the CDP-choline (Kennedy) pathway. Choline transporter
230 *Ctl1* and the main regulatory gene for PC synthesis *Pcyt1* did not differ among the three groups.
231 The expression of *Pss1*, which catalyzes the degradation of PC and the formation of PS from PC,
232 was decreased by 15% ($p < 0.05$) with choline treatment. This was also observed with *Pss2*,
233 which catalyzes the formation of PS from PE (20% reduction, $p < 0.05$). The expression of *Psd*,
234 which catalyzes the mitochondrial formation of PE from PS, was also not altered with choline
235 treatments and it was similar across the three groups (Fig. 2A). Taken together, these data
236 demonstrated that dietary choline did not significantly modify the genes for phospholipid
237 synthesis in muscle but had a modest reducing effect on PC and PE metabolic genes.

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238

239 *Choline down-regulates Pcyt2$^{+/-}$ muscle lipogenesis*

240 Since choline treatment reduced FA, DAG and TAG levels in muscle of obese mice (Fig.1. A, D
241 and E), we then investigated the effect of choline on the expression of genes involved in FA and
242 TAG synthesis (Fig. 2B). The main regulators of lipogenesis, *Srebp1c*, and its target genes *Fas*
243 and *Scd1* were elevated in Pcyt2$^{+/-}$ relative to Pcyt2$^{+/+}$ control muscle and they were reduced by
244 choline treatment (25%, 15% and 15% respectively, $p < 0.05$), to become similar to the
245 expression observed in the wildtype muscle. The expression of *Dgat1* and *Dgat2*, which catalyze

246 the formation of TAG from FA and DAG, were elevated in Pcyt2$^{+/-}$ relative to Pcyt2$^{+/+}$ muscle
247 and both decreased with choline treatment (20% and 35% respectively, $p < 0.05$). Reduced
248 muscle lipolysis is linked to IR and obesity (Kase et al. 2015). Choline supplementation
249 modestly increased the expression of *Lpl* (10%, $p < 0.05$). *Hsl* and adipose triglyceride lipase
250 (*Atgl*) were only slightly different from wildtype muscle and unaffected by choline treatments.
251 Taken together, the gene expression data and the metabolic analysis in Fig. 1 established that in
252 the IR Pcyt2$^{+/-}$ muscle, choline supplementation primarily reduced *de novo* FA synthesis and
253 lipogenesis while having a minor effect on DAG/TAG degradation by lipolysis.

254

255 *Choline improves AMPK activity and FA oxidation in Pcyt2$^{+/-}$ muscle*

256 As shown in Fig. 3A and 3C, AMPK activity (pAMPK:AMPK) was dramatically reduced in
257 Pcyt2$^{+/-}$ muscle relative to the wildtype muscle (3 fold, $p < 0.05$). AMPK activity was
258 significantly increased (2 fold) by choline treatment, indicating improvements in Pcyt2$^{+/-}$
259 mitochondrial energy production. Additionally, ACC phosphorylation (pACC:ACC) which was
260 diminished in Pcyt2$^{+/-}$ mice (2 fold, $p < 0.05$) was increased with choline supplementation (2
261 fold, $p < 0.05$), indicating that choline inhibited the first committed step in *de novo* FA synthesis
262 (Fig. 3B and 3C). In addition to decreased FA synthesis and increased pAMPK activity, there
263 was an increase in FA oxidation with choline treatment. The expression of genes involved in FA
264 oxidation - *Pparα*, *Cpt1* and *Pgc1α* (Fig. 3D) were reduced 20-35% in Pcyt2$^{+/-}$ muscle relative to
265 Pcyt2$^{+/+}$ control muscle. Choline (Pcyt2$^{+/-}$ CHO) stimulated their expression by 20-25%, back to
266 the levels observed in the control muscle. ACC mRNA expression was similar between the

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267 groups, however the inhibition of ACC activity through increased phosphorylation (AMPK
268 activation) is described in Fig. 3A-C.

269

270 *Choline improves nutrient signaling in Pcyt2$^{+/-}$ muscle*

271 To support improvements in FA formation (Fig. 2) and mitochondrial oxidation (Fig 3), we then
272 assessed the effect of choline on the insulin signaling pathway. As shown in Fig. 4A, the most
273 dramatic effect of choline was the restoration of IRS1 protein levels and Akt phosphorylation
274 (Fig. 4B-E). The amount of IRS1 in Pcyt2$^{+/-}$ muscle was diminished by 80% and choline
275 recovered IRS1 protein to the control levels (Fig. 4B). Downstream from IRS1, the most affected
276 was the mTORC2 regulated phosphorylation of Akt at Ser^{473} (p^{473}Akt:Akt). Pcyt2$^{+/-}$ p^{473}Akt was
277 reduced 50% and choline increased p^{473}Akt levels 3 fold (Fig. 4E). Interestingly, Akt
278 phosphorylation at Thr^{308} (p^{308}Akt:Akt, Fig. 4D) was not modified by Pcyt2 deficiency or
279 affected by choline treatment. Finally, choline dramatically reduced (50%) the amount of PKCα
280 (Fig. 4F), the well-established inhibitor of muscle insulin signalling (Mellor and Parker 1998).

281 Because choline improved Pcyt2$^{+/-}$ muscle AMPK activity and insulin signaling, we also probed
282 the regulation of mTORC1 (protein synthesis) and LC3 (autophagy), downstream of both AMPK
283 and insulin signaling (Fig. 5A-D). Indeed, there was a 60% increase in mTORC1
284 phosphorylation at Ser^{2448} in Pcyt2$^{+/-}$ muscle with respect to wildtype muscle, and choline
285 completely prevented this increase in phosphorylation (p^{2448}mTORC1:mTORC1 in Fig 5B).
286 Since mTORC1 is a negative regulator of autophagy, we also probed the level of lipidation of the
287 autophagy marker LC3 (LC3II:LC3I in Fig 5C). The lipidation of LC3 was reduced in Pcyt2$^{+/-}$

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288 muscle and choline increased LC3 lipidation to the level present in the control muscle. As there
289 are alterations in autophagy occurring with choline treatment, it was also of interest to determine
290 the amount of the choline transporter CTL1, previously shown to be degraded by the
291 autophagosomal-lysosomal pathway in palmitate treated muscle cells (Schmitz-Peiffer 2002).
292 The CTL1 protein as well as the total LC3 protein were however dramatically reduced (by 70%)
293 in Pcyt2$^{+/-}$ muscle and almost completely recovered by choline treatment, implying elevated
294 proteosomal degradation in Pcyt2$^{+/-}$ muscle (Fig. 5D). Taken together, the above data established
295 that choline was able to restore not only insulin signalling but also protein synthesis and turnover
296 in Pcyt2$^{+/-}$ muscle.

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297 **Discussion**

298 This study assessed the effects of dietary choline on IR Pcyt2$^{+/-}$ muscle. Choline decreased
299 muscle TAG accumulation by reducing expression of FA and TAG biosynthetic genes and by
300 increasing mitochondrial genes for energy utilization. Choline raised muscle glycogen levels and
301 restored the activity of the metabolic regulators AMPK, Akt and mTORC1. Overall, choline
302 improved glucose and lipid balance and insulin signaling in IR Pcyt2$^{+/-}$ muscle.

303 One-month of choline supplementation in Pcyt2$^{+/-}$ mice increased the content of choline-
304 containing phospholipids (PC and SM) and cholesterol (FC). This increased the membrane
305 phospholipid ratio but did not modify the lipid raft ratio in the skeletal muscle. The major effect
306 of choline supplementation was the reduction in FA, DAG and TAG content that were otherwise
307 elevated in the IR Pcyt2$^{+/-}$ muscle. Based on previous studies (Kase et al. 2015), one may
308 anticipate that choline facilitated TAG degradation by lipolysis, which was not the case. Choline
309 mediated effects were primarily via reduction in FA synthesis by lipogenesis and FA
310 esterification/TAG formation regulated by Srebp1c and Dgat1/2 respectively. The expression of
311 Srebp1 and its target genes Fas and Scd1 as well as Dgat1/2 were significantly reduced while the
312 lipolysis genes Lpl and Atgl were not affected by choline treatment. Srebp1 is positively
313 regulated by insulin signaling and downstream mTORC1 but it is not known how it became
314 upregulated in IR states to cause elevated lipogenesis and an accumulation of TAG in the liver
315 and perhaps other tissues. On the other hand, it has been established that AMPK mediated
316 mTORC1 inhibition decreases the ability for Srebp1c to localize to the nucleus in IR skeletal
317 muscle (Liu et al. 2012). Here we established that together with impaired insulin signaling,
318 mTORC1 was upregulated and AMPK was down regulated in IR Pcyt2$^{+/-}$ muscle. Choline

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319 supplementation improved insulin signaling, upregulated AMPK and downregulated mTORC1,
320 consequently reducing the expression of Srebp1c and lipogenesis.

321 We previously established by NMR metabolomic studies that the majority of choline
322 supplied to Pcyt2$^{+/-}$ mice became oxidized to betaine, which enters the one-carbon cycle and
323 stimulates mitochondrial oxidation. The increased flux through the one-carbon cycle prevents the
324 accumulation of choline and betaine in the circulation (Schenkel et al. 2015). Plasma acyl-
325 carnitine composition after choline supplementation to Pcyt2$^{+/-}$ mice further supports this
326 possibility (Schenkel et al. 2015). Therefore, the main positive effect of choline on Pcyt2$^{+/-}$
327 muscle could be attributed to its action as a methyl group donor, and subsequently stimulating
328 mitochondrial FA oxidation. High dietary choline and betaine intakes are significantly associated
329 with favorable body composition in humans (Gao et al. 2016) and Sachan et al. 2005
330 demonstrated that choline supplementation lowers oxidative stress during exercise. Recently, 600
331 mg per day of alpha glycerylphosphorylcholine has been recommended to supplement the diets
332 of speed and power athletes to enhance their muscle performance (Bellar et al. 2015). In
333 addition, betaine has been used as an ergogenic reagent to improve muscle performance and to
334 increase body mass (Cholewa et al. 2014), presumably by increasing circulating IGF levels
335 leading to increased PI3K/Akt signaling (Senesi et al. 2013). More recently, it has been
336 demonstrated that betaine supplementation reduces obesity and systemic IR by upregulation of
337 Fgf21 (Ejaz et al. 2016).

338 In addition to the effects on muscle FA oxidation through the betaine/one-carbon cycle,
339 choline could have influenced acetylcholine signaling, which is essential for muscle contraction.
340 Reduction in acetylcholine production has a marked influence on muscle performance and most

341 sport nutrition products are supplemented with choline to maintain acetylcholine levels (Penry
342 and Manore 2008). The mechanism of muscle contraction after acetylcholine stimulation
343 primarily depends on AMPK activation and is independent of insulin (Koh et al. 2008). IR is
344 associated with muscle weakness and exercise ameliorates IR via AMPK activation and Ca^{2+}
345 signaling (Funai et al. 2016 and Park et al. 2015). Therefore, the activation of AMPK (Merlin et
346 al. 2010) by choline supplementation improved the metabolic condition of $Pcyt2^{+/-}$ muscle and
347 indicated that choline had exercise mimetic properties for IR muscle. The question remains if
348 muscle Ca^{2+} signaling is also activated by choline supplementation and will require further
349 investigation.

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350 The general role of AMPK is to stimulate FA oxidation and to diminish TAG synthesis
351 by lipogenesis, and they are both in effect in choline supplemented $Pcyt2^{+/-}$ muscle. Deactivation
352 of ACC with choline treatment is beneficial because ACC catalyzes the formation of malonyl-
353 CoA, the rate limiting step in FA biosynthesis. Additionally, choline increased the expression of
354 the mitochondrial regulators, Pparα, Pgc1α and Cpt1. Pparα stimulates FA oxidation and Pgc1α
355 regulates mitochondrial biogenesis, while Cpt1 is the rate limiting step in FA transport in the
356 mitochondria.

357 Choline supplemented $Pcyt2^{+/-}$ muscle demonstrated improvements in insulin signaling.
358 There was an increase in IRS1, an increase in p^{473}Akt (mTORC2 site) and increased muscle
359 glycogen content with choline treatment. Glycogen synthesis and content are typically decreased
360 in IR. This occurs because elevated GSK3 phosphorylation leads to the inhibition of glycogen
361 synthase activity (Liu et al. 2015). Furthermore, GSK phosphorylates Raptor, an mTORC1
362 associated scaffold protein, which is important in mTORC1 activation (Stretton et al. 2015).

363 mTORC1 was hyperactivated in Pcyt2$^{+/-}$ muscle and there was a decrease in activation with
364 choline treatment. mTORC1 is a regulator of protein synthesis (Wullschleger et al. 2006), and
365 the activation of this protein was normalized with choline treatment. AMPK activation has been
366 shown to inhibit the activation of mTORC1 (Hong-Brown et al. 2010), and is likely most
367 responsible for the normalization of protein turnover in choline supplemented Pcyt2$^{+/-}$ muscle.
368 Why was mTORC1 more active in IR Pcyt2$^{+/-}$ muscle? Chronic mTORC1 activation has been
369 shown to promote insulin resistance via S6K-dependent phosphorylation of IRS1 leading to its
370 degradation and the inhibition of insulin signalling pathway (Um et al. 2004). Typically,
371 mTORC1 is activated via Akt phosphorylation but this is not the case in IR muscle. mTORC1
372 hyperactivation was likely a consequence of increased nutrient availability/elevated proteolysis
373 in IR Pcyt2$^{+/-}$ muscle, and choline supplementation restored this balance (Bond 2016).

374

375 *Conclusion*

376 Choline supplementation has the ability to affect muscle metabolism and improve insulin
377 signaling in mice that have a reduced proficiency to synthesize the membrane phospholipid PE.
378 The mechanisms that link choline with improvements in IR muscle signaling and energy
379 metabolism involve multiple parameters. These include the normalization of gene expression to
380 favour less FA formation and more FA oxidation, causing depression of lipogenesis, increases in
381 muscle glycogen and activation of AMPK. These data suggest that choline supplemented in the
382 diet could normalize muscle FA and TAG homeostasis in order to diminish the IR displayed by
383 perturbed PE biosynthesis.

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384 **Acknowledgements**

385 This work was supported by an Ontario Graduate Scholarship (AT) and an operating grant (MB)
386 and USRA award (MY) from the National Sciences and Engineering Research Council of
387 Canada.

388

389 **Competing interests**

390 The authors have declared that no competing interests exist.

391

392 **Author contributions**

393 Conceived and designed the experiments: AT and LCS. Performed the experiments: AT,
394 MY. Analyzed the data: AT, MB. Wrote the paper: AT, MB.

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518 **Table 1**: Primer sequences, fragment size and melting temperature for the amplification of genes

519 involved in lipolysis, lipogenesis, mitochondrial function and phospholipid synthesis.

520

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	T_M (^OC)	Size (bp)
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	57	452
Pss2	GAGTGGCTGTCCCTGAAGAC	TCGTAGATCTCACGCATGGC	59	305
Atgl	CAACGCCACTCACATCTACGG	GGACACCTCAATAATGTTGGCAC	57	106
Hsl	ACGCTACACAAAGGCTGCTT	TCGTTGCGTTTGTAGTGCTC	59	125
Lpl	GCTCGCACGAGCGCTCCATT	CCTCGGGCAGGGTGAAGGGAA	59	351
Pgc1α	TTGACTGGCGTCATTCGGG	GAAGGACTGGCCTCGTTGTC	59.5	396
Fas	AGATGGAAGGCTGGGCTCTA	GAAGCGTCTCGGGATCTCTG	59	268
Ctl1	GAACGCTCTGCGAGTGGCTGC	CGGCTTTAGCTCTCGGGCGT	49	376
Pcyt1	ATGCACAGAGAGTTCAGCTAAAG	GGGCTTACTAAAGTCAACTTCAA	50	170
Pss1	CTGTTGTGCAATGGTGGTGG	GGCTGGCTTGGAACACAAAG	59.5	283
Psd	GTTTGCTGTCACGTGCCTGTG	CAGTGCAAGCCACATACGGG	59.5	205
Dgat2	GGCGCTACTTCCGAGACTAC	TCTTTAGGGTGACTGCGTTC	58.5	255
Scd1	CGCATCTCTATGGATATCGCCCC	CTCAGCTACTCTTGTGACTCCCG	57	279
Dgat1	ATCCAGACAACCTGACCTACCG	GACCGCCAGCTTTAAGAGACGC	57	257
Cpt1	CCCATGTGCTCCTACCAGAT	GGTCTCATCGTCAGGGTTGT	57	396
Pparα	CGCATGTGAAGGCTGTAAGGGC	GGCTTCGTGGATTCTCTTGCCC	57	289
Srebp1c	TCACAGGTCCAGCAGGTCCC	GGTACTGTGGCCAAGATGGTCC	57	438
Acc	CGCATCTCTATGGATATCGCCCC	CTCAGCTACTCTTGTGACTCCCG	57	295

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522 **Figure Legends**

523

524 **Fig. 1 Choline modifies muscle glycerolipid and glucose metabolism.** *A. Choline*
525 *supplementation modified choline-containing phospholipids and cholesterol.* The IR Pcyt2$^{+/-}$
526 muscle contained reduced FC and PI relative to the control Pcyt2$^{+/+}$ muscle. CE and PE were not
527 significantly modified in Pcyt2$^{+/-}$ muscle with respect to the other two groups. Pcyt2$^{+/-}$ CHO
528 muscle had decreased FA content and increased PC and SM content with respect to Pcyt2$^{+/-}$
529 muscle. *(B)* The relative phospholipid ratio of PC/PE increased with choline supplementation,
530 and (C) the relative SM/FC ratio was not affected with choline treatment and remained increased
531 as in Pcyt2$^{+/-}$ muscle. *D. and E. Choline reduced muscle TAG and DAG.* Total TAG content *(D)*
532 and both relative TAG and DAG ratios *(E)* were decreased with choline supplementation
533 (Pcyt2$^{+/-}$ CHO) relative to Pcyt2$^{+/-}$ and they were similar to the control Pcyt2$^{+/+}$ mice. *F. Choline*
534 *increases muscle glycogen content.* Pcyt2$^{+/-}$ mice have dramatically reduced glycogen content
535 that was increased with choline treatment, approaching the value of the Pcyt2$^{+/+}$ mice. (A-F)
536 number of mice per each group: n = 4; one-way ANOVA: *$p < 0.05$ compared to Pcyt2$^{+/+}$ and #p
537 < 0.05 compared to Pcyt2$^{+/-}$.

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539 **Fig. 2 Choline regulates expression of genes involved in muscle fatty acid and triglyceride**
540 **metabolism.** A. *The phospholipid biosynthetic genes are not significantly altered across groups.*
541 *Ctl1, Pcyt1 and Psd* were unmodified while *Pss1* and *Pss2* expression were modestly decreased
542 with choline treatment with respect to Pcyt2$^{+/+}$ and Pcyt2$^{+/-}$ muscle. B. *Choline normalizes the*
543 *expression of genes involved in lipogenesis.* (a) The expression of FA biosynthetic genes *Scd1,*
544 *Fas* and *Srebp1c* were elevated in IR Pcyt2$^{+/-}$ muscle and choline normalized their levels; (b)
545 Choline also normalized the expression of genes involved in TAG synthesis. The expression of
546 *Dgat1* and *Dgat2* were elevated in Pcyt2$^{+/-}$ relative to Pcyt2$^{+/+}$ muscle and became decreased with
547 choline treatment. C. *Choline has no major effect on muscle lipolysis.* The expression of Lpl and
548 Atgl were modestly increased in Pcyt2$^{+/-}$ muscle relative to the wildtype Pcyt2$^{+/+}$ muscle but not
549 affected by choline treatment. The expression of Hsl that was slightly reduced in Pcyt2$^{+/-}$ muscle

550 was increased with choline treatment. The intensity of bands were normalized to Gapdh gene
551 control and expressed in arbitrary units; (A-C) number of mice per group: $n = 4$; one-way
552 ANOVA: $*p < 0.05$ compared to Pcyt2$^{+/+}$ and $\#p < 0.05$ compared to Pcyt2$^{+/-}$.

553

554 **Fig. 3 Choline improves mitochondrial energy production.** *AMPK activation (A) and ACC*
555 *inhibition (B) with choline.* Pcyt2$^{+/-}$ muscle showed a dramatic reduction in AMPK and ACC
556 phosphorylation (pAMPK:AMPK and pACC:ACC ratio) and choline treatment (Pcyt2$^{+/-}$ CHO
557 muscle) increased the phosphorylation of both species. C. The amounts of AMPK, pAMPK,
558 ACC and pACC proteins was assessed using β-tubulin as a control. The intensity of bands was
559 quantified by densitometry and expressed in arbitrary units. $*p < 0.05$ compared to Pcyt2$^{+/+}$ and
560 $\#p < 0.05$ compared to Pcyt2$^{+/-}$ D. *Mitochondrial gene activation with choline***.** The expression of
561 Cpt1, Pparα and Pgc1α was increased with choline treatment whereas the expression of Acc was
562 unchanged. The intensity of PCR bands were quantified using ImageJ software and expressed as
563 arbitrary units. (A-C) Each experiment was repeated 3 times with $n = 3$ per group. D. The
564 number of mice per group: $n = 4$; one-way ANOVA $n = 4$ per group). $*p < 0.05$ compared to
565 Pcyt2$^{+/+}$ and $\#p < 0.05$ compared to Pcyt2$^{+/-}$.

566

567 **Fig. 4 Choline reduces PKCα and restores muscle insulin signaling**. A. Representative
568 immunoblots showing differences in insulin signalling pathway. The amounts of IRS1, PI3K,
569 Akt, pAkt and PKCα proteins were assessed using β-tubulin as a control.. B. IRS1 protein was
570 diminished in Pcyt2$^{+/-}$ muscle and completely restored with choline treatment. C. PI3K levels
571 were elevated in Pcyt2$^{+/-}$ muscle and decreased with choline treatments. D. pThr^{308}Akt:Akt ratio
572 was unchanged across all groups whereas (E.) muscle pSer^{473}Akt:Akt increased with choline
573 treatment relative to Pcyt2$^{+/-}$ muscle. F. PKCα that was elevated in IR Pcyt2$^{+/-}$ muscle
574 dramatically decreased with choline treatments. The intensity of bands were normalized relative
575 to β-tubulin and expressed in arbitrary units relative to the normal Pcyt2$^{+/+}$ muscle. (A-F) Each
576 experiment was repeated 3 times with $n = 3$ per group; one-way ANOVA: $*p < 0.05$ compared to
577 Pcyt2$^{+/+}$ and $\#p < 0.05$ compared to Pcyt2$^{+/-}$.

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578 **Fig. 5 Choline reduces mTORC1 activity and stimulates autophagy.** A. Representative
579 immunoblots for mTORC1, p^{2448}mTORC1, LC3I, LC3II, CTL1, and β tubulin in choline treated
580 and untreated groups. B. *Choline reduces muscle mTORC1 activation.* Total mTORC1 content
581 was unchanged across groups but p^{2448}mTORC:mTORC1 ratio was decreased with choline
582 treatment in relation to $Pcyt2^{+/-}$ C. *Choline increases muscle autophagy.* Choline treatment
583 increased $Pcyt2^{+/-}$ LC3 lipidation shown as elevated LC3II:LC3I ratio. D. *Choline restores the*
584 *amount of choline transporter CTL1.* CTL1 protein was diminished in $Pcyt2^{+/-}$ muscle but
585 became restored with choline treatment. The intensity of all protein bands and control β tubulin
586 were quantified by densitometry and expressed as arbitrary units (fold change) relative to wild
587 type muscle. (A-D): Each experiment was repeated 3 times (n = 3 per group); one-way ANOVA:
588 *$p < 0.05$ compared to $Pcyt2^{+/+}$ and #$p < 0.05$ compared to $Pcyt2^{+/-}$.

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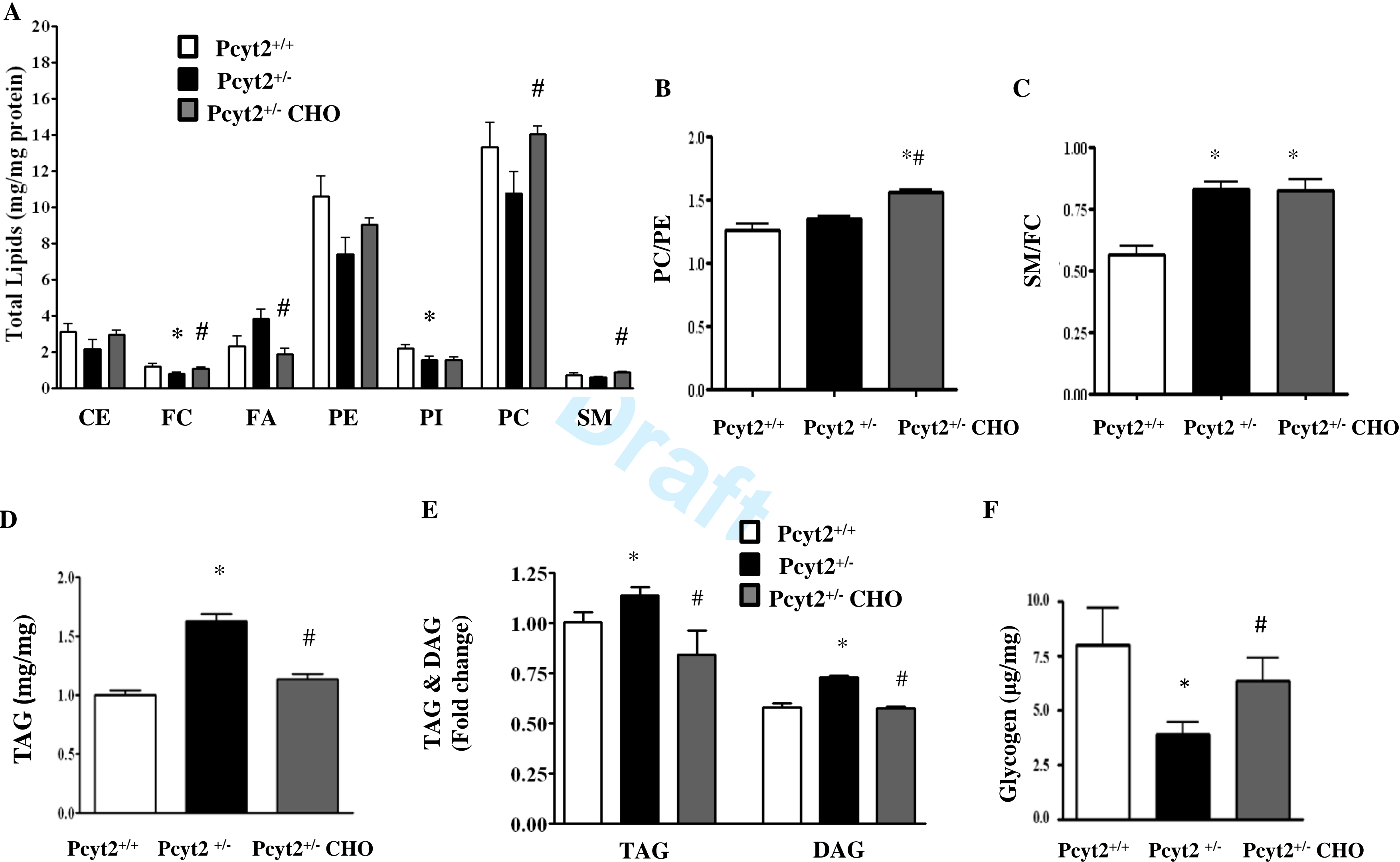

Figure 1

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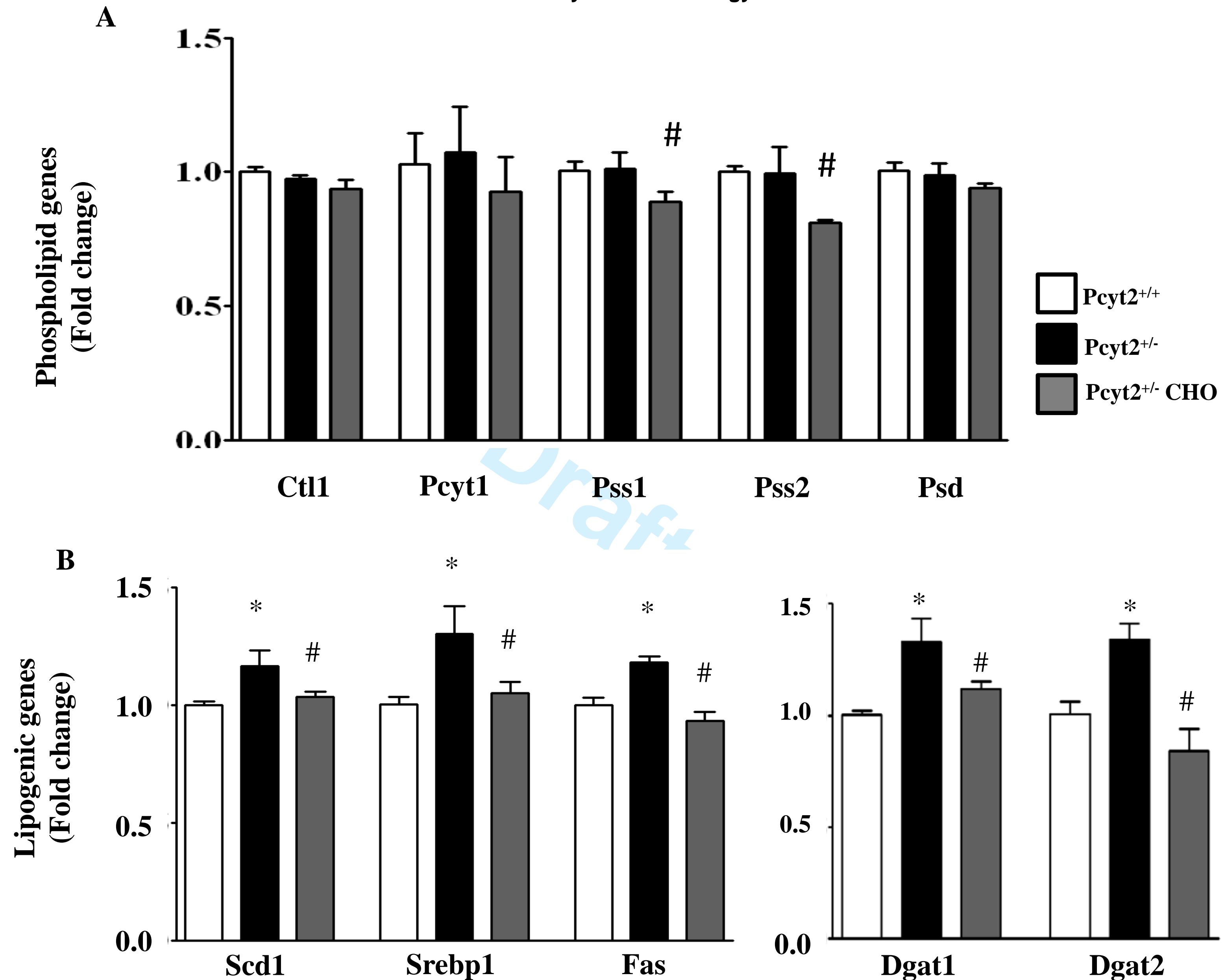

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Figure 2 A&B

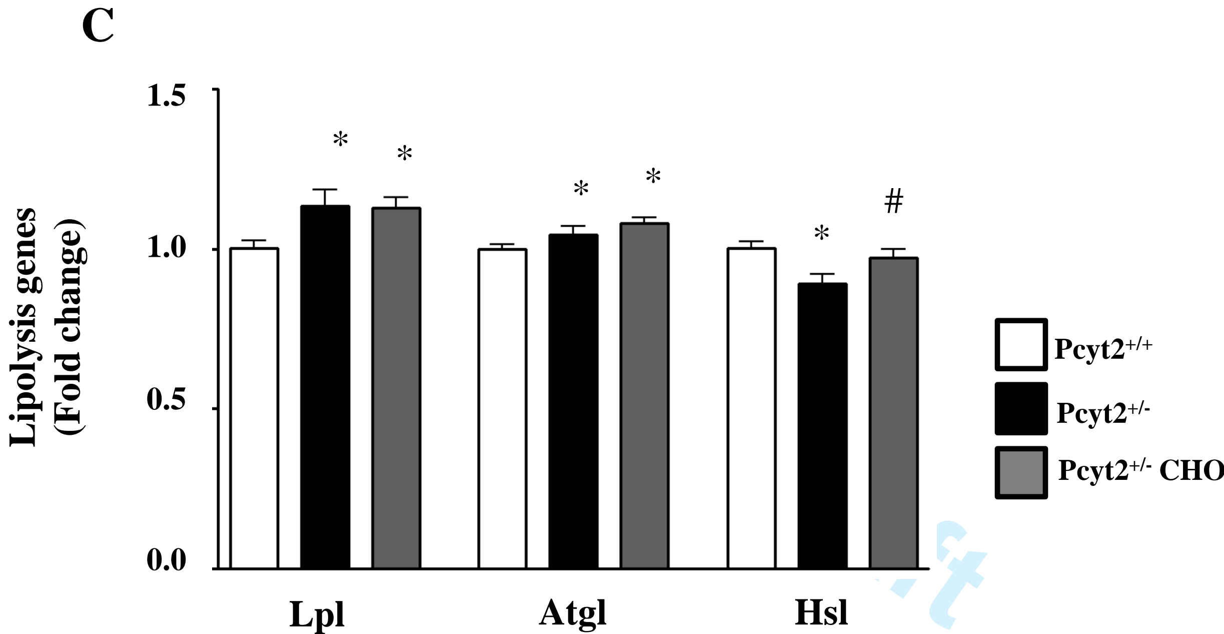

Figure 2C

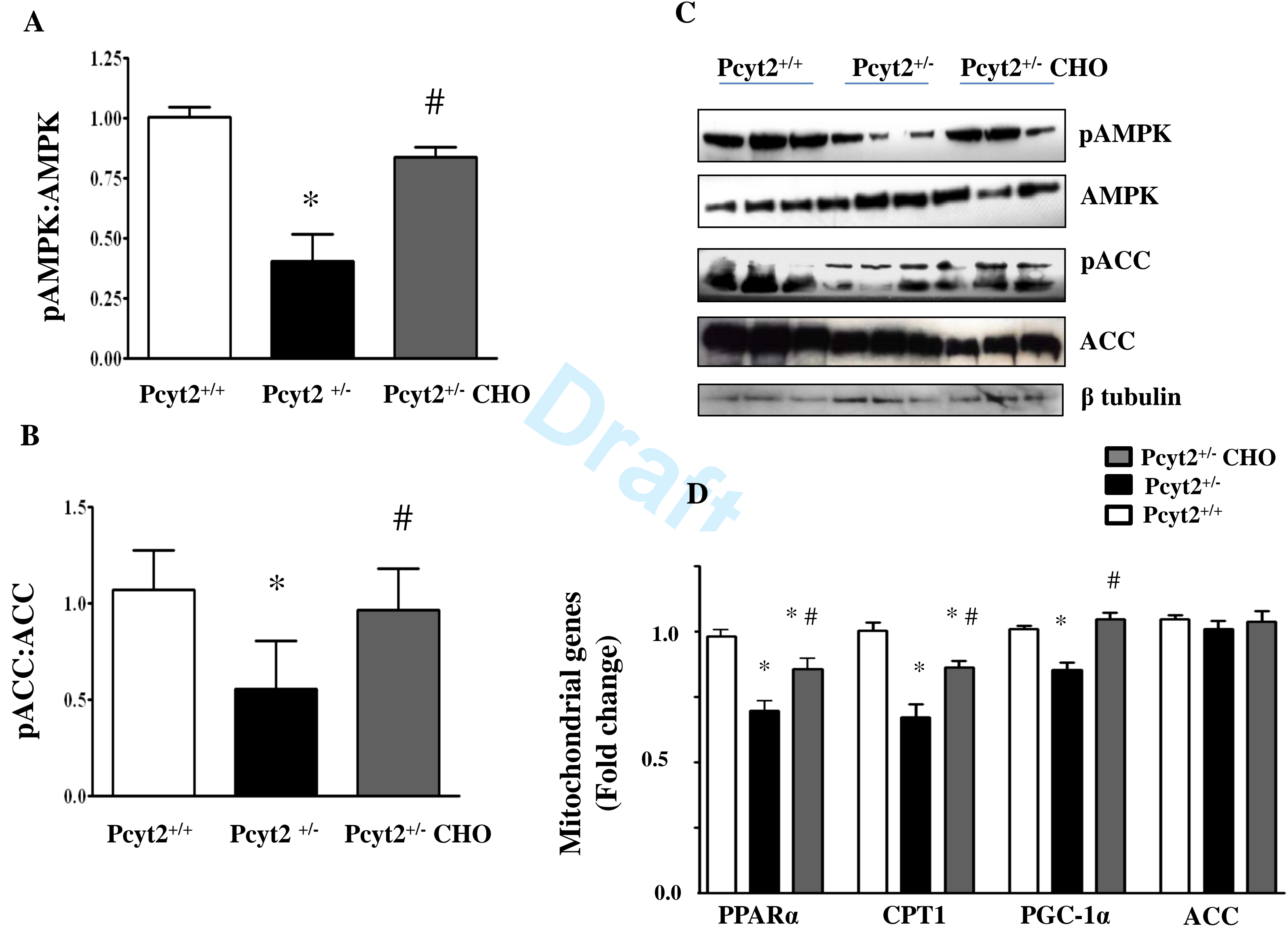

Figure 3

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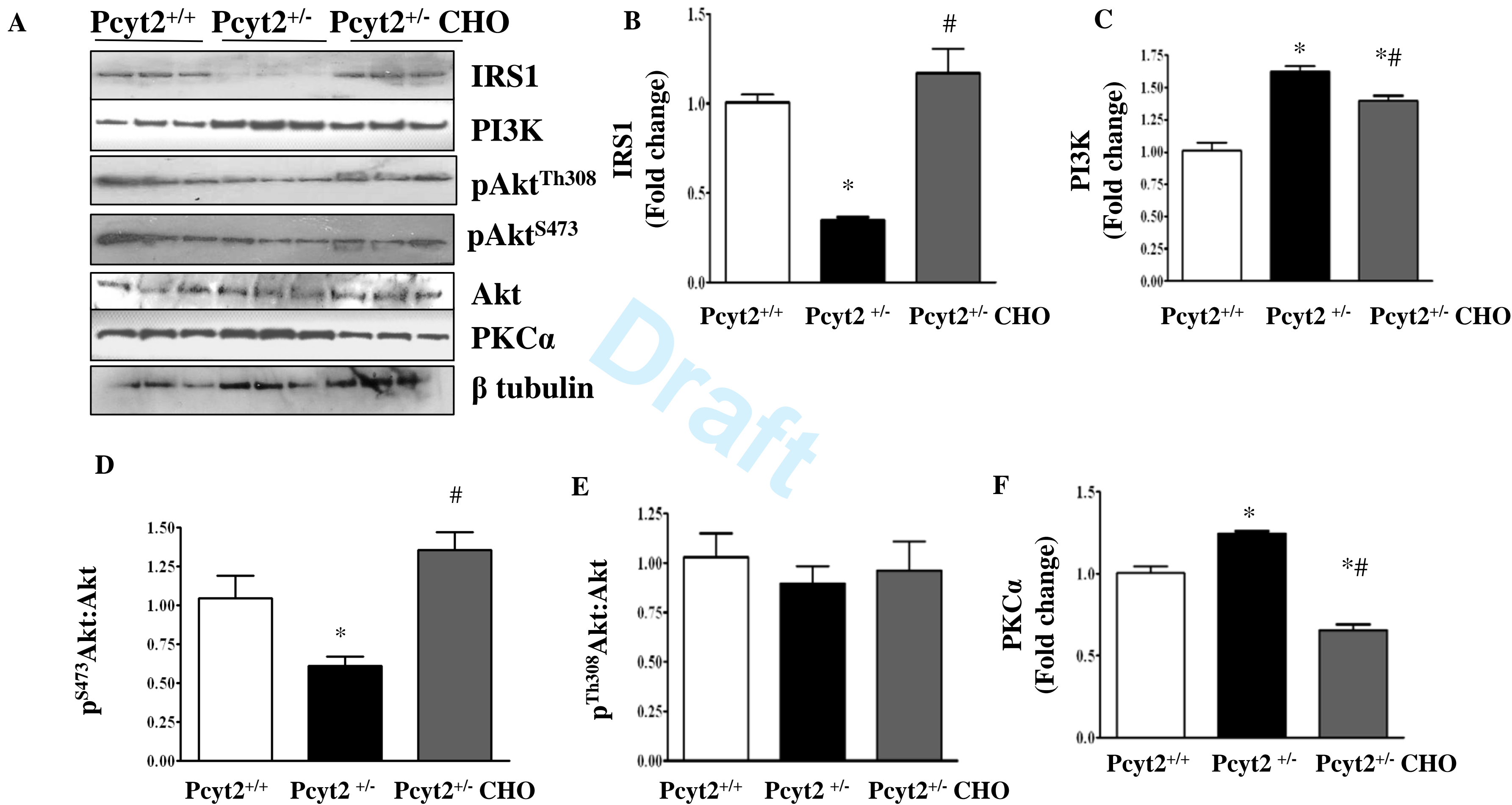

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Figure 4

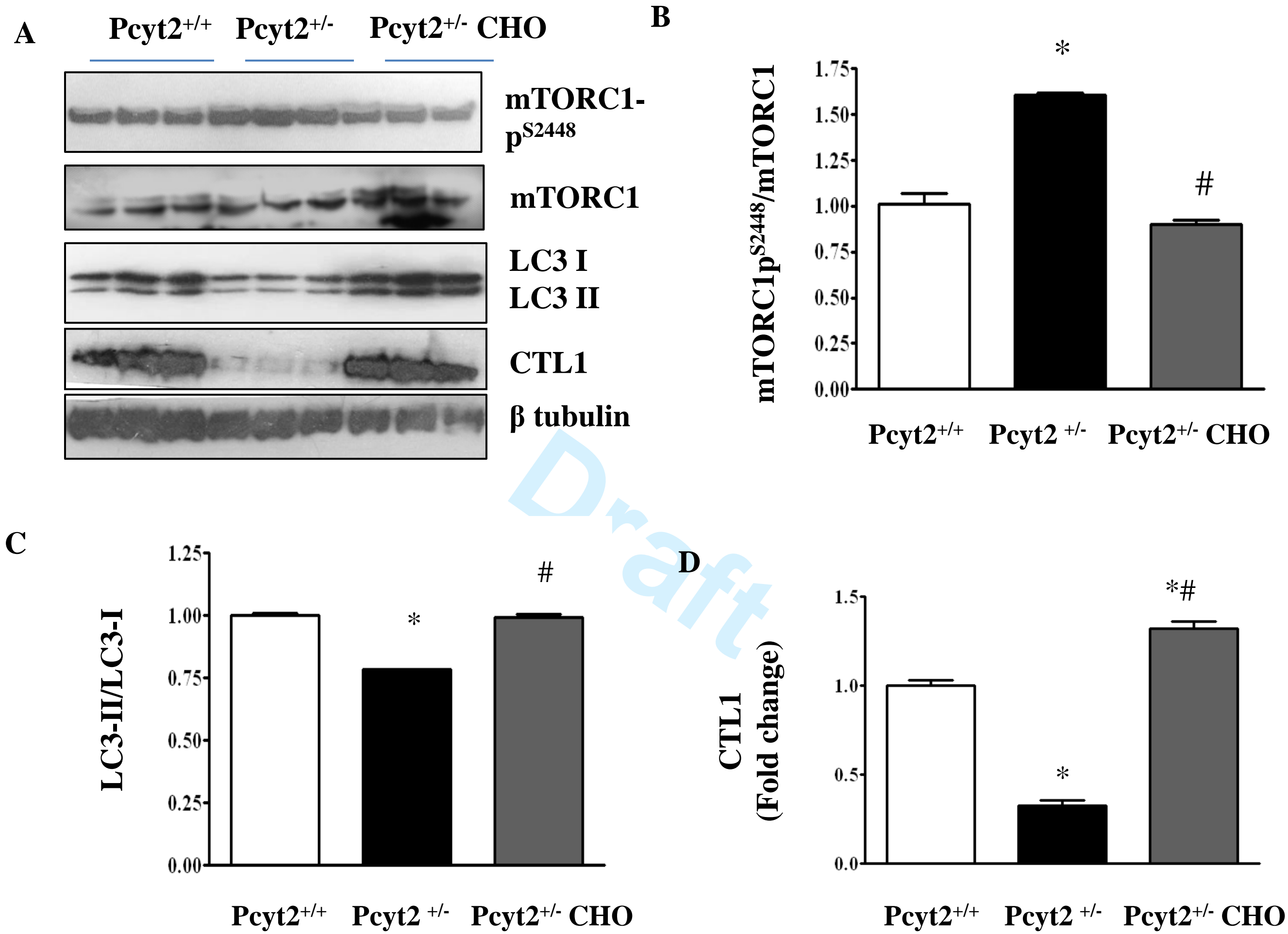

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Figure 5