

1 **Carbonic anhydrase 8 (Car8) negatively regulates GLP-1 secretion**
2 **from enteroendocrine cells in response to long-chain fatty acids**

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23 **Running title:** Car8 suppresses LCFA-induced GLP-1 secretion
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28 **ABSTRACT**

29 Glucagon-like peptide-1 (GLP-1) is an incretin secreted from enteroendocrine
30 preproglucagon-expressing (PPG)-cells (traditionally known as L-cells) in response to
31 luminal nutrients that potentiates insulin secretion. Augmentation of endogenous GLP-1
32 secretion might well represent a novel therapeutic target for diabetes treatment in
33 addition to the incretin-associated drugs currently in use. In this study, we found that
34 PPG-cells substantially express carbonic anhydrase 8 (Car8), which has been reported
35 to inhibit inositol 1,4,5-trisphosphate (IP3) binding to the IP3 receptor and subsequent
36 Ca²⁺ efflux from the endoplasmic reticulum in neuronal cells. *In vitro* experiments using
37 STC-1 cells demonstrated that Car8 knockdown increases long-chain fatty acid
38 (LCFA)-stimulated GLP-1 secretion. This effect was reduced in the presence of
39 phospholipase C (PLC) inhibitor; in addition, Car8 knockdown increased the
40 intracellular Ca²⁺ elevation caused by α -linolenic acid, indicating that Car8 exerts its
41 effect on GLP-1 secretion via the PLC/IP3/Ca²⁺ pathway. Car8^{wdl} null mutant mice
42 showed significant increase in GLP-1 response to oral corn oil administration compared
43 to that in wild-type littermates, with no significant change in intestinal GLP-1 content.
44 These results demonstrate that Car8 negatively regulates GLP-1 secretion from
45 PPG-cells in response to LCFAs, suggesting the possibility of augmentation of
46 postprandial GLP-1 secretion by Car8 inhibition.

47 **NEW & NOTEWORTHY**

48 This study focused on the physiological significance of carbonic anhydrase 8
49 (Car8) in GLP-1 secretion from enteroendocrine preproglucagon-expressing (PPG)-cells.
50 We found an inhibitory role of Car8 in LCFA-induced GLP-1 secretion *in vitro* and *in*
51 *vivo*, suggesting a novel therapeutic approach to diabetes and obesity through
52 augmentation of postprandial GLP-1 secretion by Car8 inhibition.

53 **Keywords:** Incretin, glucagon-like peptide-1 (GLP-1), carbonic anhydrase 8 (Car8)

54 **INTRODUCTION**

55 Type 2 diabetes mellitus (T2DM) is a leading cause of morbidity and mortality and
56 is recognized as a worldwide problem. Obesity is strongly associated with insulin
57 resistance and increased risk of T2DM (22). Although various antidiabetic drugs have
58 been developed so far, there are still many cases in which existing drugs do not provide
59 a sufficient therapeutic effect. Thus, the development of more effective treatments for
60 diabetes and obesity is an urgent issue.

61 Glucagon-like peptide-1 (GLP-1) is one of the incretins, which are peptide
62 hormones released from the gastrointestinal tract into circulation in response to nutrient
63 ingestion that potentiate glucose-stimulated insulin secretion (4, 41). Besides its

64 insulintropic effect, GLP-1 also exerts a wide variety of anti-hyperglycemic functions
65 including inhibiting glucagon secretion (31), delaying gastric emptying (32, 46) and
66 reducing food intake through promotion of satiety (7, 44). Currently, inhibitors of
67 dipeptidyl peptidase-4 (DPP-4), an incretin-degrading enzyme, and DPP-4-resistant
68 GLP-1 mimetics are widely used for treatment of T2DM; in addition, high-dose
69 liraglutide, one of the GLP-1 mimetics presently in clinical use, is also licensed for the
70 treatment of obesity in European countries and the United States. Augmentation of
71 endogenous GLP-1 secretion might therefore be a novel therapeutic target for T2DM
72 and obesity treatment. Elucidation of the detailed GLP-1 secretory machinery is of
73 course essential for development of this novel therapeutic strategy.

74 GLP-1 is known to be secreted from enteroendocrine preproglucagon-expressing
75 (PPG)-cells in response to various stimuli including glucose (26), long-chain fatty acids
76 (LCFAs) (5, 15, 40), short chain fatty acids (SCFAs) (45), bile acid (25) and
77 oleoylethanolamide (OEA) (28). Transporters and receptors corresponding to each of
78 these have been identified. For example, the apical sodium glucose co-transporter 1
79 (SGLT1) contributes to GLP-1 secretion in response to glucose (10, 35). Bile acids
80 trigger GLP-1 secretion by activating G protein-coupled bile acid receptor 1 (GPBAR1)
81 (3). OEA, a physiological GPR119 ligand, increases GLP-1 release in rodent models

82 (28). The receptor most strongly linked to SCFA-triggered GLP-1 secretion is GPR43
83 (FFAR2) (45). The LCFA receptors GPR40 and GPR120 are both expressed in
84 GLP-1-expressing cells (5, 15); we have demonstrated that both GPR40 and GPR120
85 contribute to corn oil-induced GLP-1 secretion by using GPR120- and GPR40-double
86 knockout mice (40). A wide variety of stimuli induce GLP-1 secretion through
87 respective pathways but ultimately by a common mechanism, namely, increased
88 intracellular Ca^{2+} levels (8, 11, 25, 48). In fact, it has been reported that a number of
89 proteins involved in the Ca^{2+} -dependent exocytotic pathway are expressed in
90 GLP-1-expressing cells and involved in GLP-1 exocytosis (12, 29, 47). Thus, the search
91 for the molecules involved in the intracellular Ca^{2+} regulation that are expressed in
92 PPG-cells may reveal a novel mechanism of GLP-1 secretion.

93 Car8 is a member of the carbonic anhydrase family. However, unlike its other
94 family members, Car8 lacks the enzymatic potential to hydrate CO_2 due to the absence
95 of catalytic zinc-coordinating histidine residues within the active site (2, 24). Rather,
96 Car8 has been reported to allosterically inhibit inositol 1,4,5-triphosphate (IP3) binding
97 to the IP3 receptor (IP3R) and negatively regulate calcium efflux into the cytoplasm
98 from the endoplasmic reticulum (ER) via IP3R (17, 27, 49). Car8 is abundantly
99 expressed in cerebellar Purkinje cells; the waddle (Car8^{wdl}) mouse, which has a

100 spontaneous null mutation of Car8, exhibits gait ataxia and dystonia due to aberrant
101 synaptic morphology and impaired excitatory synaptic function in the cerebellum (16,
102 21). Besides the Purkinje cells of the cerebellum, dorsal root ganglia (DRG) neurons
103 also express Car8, and it has been reported that mechanical allodynia and thermal
104 hyperalgesia is observed concomitantly with increased activation of IP3R and enhanced
105 cytoplasmic Ca^{2+} release in DRG neuronal cells of Car8^{wdl} mutant mice (49). There
106 have been no reports on the expression and function of Car8 in non-neuronal cells. In a
107 recent report showing the data of single-cell RNA sequencing of PPG-cells, Car8 was
108 included in the list of genes possibly expressed in PPG-cells (9), but confirmation of the
109 expression by RT-PCR and the functional analysis were not performed.

110 In the present study, we confirmed high expression of Car8 in isolated PPG-cells
111 and investigated the physiological function of Car8 in PPG-cells, focusing on the
112 involvement of Car8 in GLP-1 secretion via the phospholipase C (PLC)/IP3 pathway
113 and subsequent intracellular Ca^{2+} change in GLP-1-expressing cells; we also explore the
114 significance of Car8 *in vivo* by comparing GLP-1 secretion in wild-type and Car8^{wdl}
115 null mutant mice.

116 MATERIALS AND METHODS

117 *Animals.* All animals used in this study were male C57BL/6J mice maintained under

118 conditions of a 12-h light and dark cycle and fed a control-fat diet (CFD; 10% fat, 20%
119 protein and 70% carbohydrate by energy)(Research Diets Inc., New Brunswick, NJ)
120 with free access to water and food unless otherwise indicated. All mice used in
121 individual experiments were age-matched at the beginning of experiments.
122 Glucagon-green fluorescent protein (GFP) knock-in ($Gcg^{gfp/+}$) mice in which the
123 PPG-cells are labeled by enhanced green fluorescent protein (EGFP) were generated as
124 reported previously (14), and backcrossed with the C57BL/6J strain for more than 10
125 generations. $Car8^{wdl}$ mutant mice, which have a spontaneous null mutation of *Car8*,
126 were purchased from The Jackson Laboratory (Bar Harbor, ME;
127 RRID:IMSR_JAX:004625). It has been reported that $Car8^{wdl}$ mutant mice exhibit gait
128 ataxia and dystonia due to aberrant synaptic morphology and impaired excitatory
129 synaptic function in the cerebellum (16, 21). All animal protocols were approved by the
130 Animal Care Committee of Kyoto University (Med Kyo 16584).

131 *Isolation of PPG-cells from intestinal epithelium of $Gcg^{gfp/+}$ mice.* The procedure of
132 PPG-cell isolation from murine intestinal epithelium was described previously (42).
133 Briefly, the lower small intestine of 10-week-old $Gcg^{gfp/+}$ mice was removed, cut into
134 several round pieces and tied on one side with a thread. This pouch-like portion of
135 intestine was injected with Hanks' balanced salt solution (HBSS) containing 0.5 mg/ml

136 collagenase, clamped, and incubated with CO₂ at 37 °C for 10 min in Krebs-Ringer
137 bicarbonate buffer (KRBB: 120mM NaCl, 4.7mM KCl, 1.2 mM MgSO₄, 1.2 mM
138 KH₂PO₄, 2.4 mM CaCl₂, 20 mM NaHCO₃). The digested intestinal epithelium was
139 cultured in a humidified incubator (95% air and 5% CO₂) at 37 °C for 1 h. Afterward, it
140 was centrifuged at 180 g for 5 min, resuspended in phosphate-buffered saline (PBS)
141 twice and filtered with a cell strainer (352340, Falcon cell strainer, BD Biosciences, San
142 Jose, CA, USA). The size of the openings of the strainer was 40 µm. GFP-positive cells
143 in the intestinal epithelium were isolated using BD FACS AriaTM flow cytometer (BD
144 Biosciences, San Jose, CA, USA). Sorted cells were collected into vials containing
145 medium. Total RNA was extracted from sorted cells of Gcg^{gfp/+} mouse intestinal
146 epithelium using PicoPure RNA isolation kit (Applied Biosystems, Inc., Alameda, CA)
147 and treated with DNase (Qiagen Inc., Valencia, CA).

148 *Quantitative reverse transcription (qRT)-polymerase chain reaction (PCR).*
149 Complementary DNA (cDNA) was prepared by reverse transcriptase (Invitrogen, CA,
150 USA) with an oligo (dT) primer (Invitrogen, CA, USA). Messenger RNA (mRNA)
151 levels were measured by qRT-PCR using ABI PRISM 7000 Sequence Detection System
152 (AB StepOne Plus Real Time PCR; Applied Biosystems, Foster City, CA, USA). PCR
153 analyses were carried out using oligonucleotide primers. SYBR Green PCR master mix

154 (Applied Biosystems, Foster City, CA, USA) was prepared for PCR runs. Thermal
155 cycling conditions were denaturation at 95 °C for 10 min followed by 50 cycles at 95 °C
156 for 15 s and 60 °C for 1 min. To quantify the results obtained by qPCR, we used 2^{-ΔΔCt}
157 method in the Car8 overexpression experiment in STC-1 cells and standard curve
158 method for the other evaluations. In standard curve method,
159 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.
160 Primer pairs for PCR are as follows: Car8 forward, 5' -aggtaactccttcgctgcaa-3' , and
161 reverse, 5' -gttcagatcgggaaggaaca-3' ; GAPDH forward, 5' -
162 -aaatggtgaaggtcggtgtg-3' , and reverse, 5' -tcgttgatggcaacaatctc-3' .

163 *Immunohistochemistry.* Lower small intestines of 10-week-old wild-type mice,
164 Car8^{wdl} mice and Gcg^{gfp/+} mice were fixed in Bouin's solution and transferred to 70%
165 ethanol before processing through paraffin. To confirm the specificity of the anti-Car8
166 antibody, rehydrated paraffin sections of wild-type mice and Car8^{wdl} null mutant mice
167 were incubated overnight at 4 °C with primary rabbit anti-Car8 antibody (Proteintech,
168 12391-1-AP, 1:100; RRID:AB_2066277). To examine the localization of Car8 in lower
169 small intestine, rehydrated paraffin sections of Gcg^{gfp/+} mice were incubated overnight
170 at 4 °C with primary mouse anti-GFP antibody (Santa Cruz Biotechnology, sc-9996,
171 1:100 ; RRID:AB_627695) and rabbit anti-Car8 antibody. In order to further

172 demonstrate that GLP-1-expressing cells indeed express Car8, rehydrated paraffin
173 sections of wild-type mice were incubated overnight at 4 °C with primary mouse
174 anti-GLP-1 antibody (abcam, ab23472, 1:100 ; RRID:AB_447455) and rabbit anti-Car8
175 antibody. After the overnight incubation with primary antibodies, the sections were
176 incubated for 1 h at room temperature with secondary antibody. Details of secondary
177 antibodies are as follows: Alexa Fluor[®] 546 Goat Anti-Rabbit IgG (H+L) (Life
178 Technologies Japan Corp., Tokyo Japan, A-11035) for anti-Car8 antibody and Alexa
179 Fluor[®] 488 Goat Anti-Mouse (Life Technologies Japan Corp., Tokyo Japan, A28175)
180 for anti-GFP antibody and anti-GLP-1 antibody. Images were taken using fluorescence
181 microscopy with BZ-8100 system (KEYENCE Corp., Osaka, Japan).

182 *Cell culture.* STC-1 cells, a mouse enteroendocrine cell line (RRID:CVCL_J405),
183 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, MO,
184 USA) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin
185 and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere (5% CO₂ and 95%
186 air).

187 *Car8 knockdown and overexpression.* Car8 small interfering (si)RNA (s63274) and
188 negative control siRNA (4390844) were synthesized by Thermo Fisher Scientific
189 (Thermo Fisher Scientific, Foster City, CA, USA). STC-1 cells were plated on 12-well

190 dishes (1×10^6 /well) and transfections were performed using Lipofectamine TM2000
191 according to the manufacturer's instructions (Invitrogen, CA, USA, 11668019). 48 h
192 after transfection, the medium was replaced with 1 ml of HBSS containing LCFAs (100
193 μ M oleic acid, 100 μ M linoleic acid and 100 μ M α -linolenic acid), 100 μ M bile acid, 50
194 μ M OEA, 22.2mM glucose, and SCFAs (100 μ M acetic acid, 100 μ M butyric acid and
195 100 μ M propionic acid). For the experiments using inhibitors, the concentrations of each
196 reagent were as follows: 10 μ M 2-aminoethyldiphenylborate (2APB) (Sigma-Aldrich,
197 D9754), 10 μ M U73122 (Abcam, ab120998), 10 μ M U73343 (Abcam, ab141764) and
198 10 μ M H89 (Abcam, ab120341). For the overexpression experiment, cDNA encoding
199 mouse Car8 was PCR-amplified and subcloned into pcDNA3.1+/C-(K)-DYK vector
200 (Genscript, Piscataway, NJ, USA). The empty pcDNA3.1+/C-(K)-DYK vector was used
201 as the control in the overexpression experiment. STC-1 cells were plated on 12-well
202 dishes (1×10^6 /well) and transfections were performed using Lipofectamine TM2000
203 according to the manufacturer's instructions (Invitrogen, CA, USA, 11668019). 48 h
204 after transfection, the medium was replaced with 1 ml of HBSS containing 100 μ M
205 α -linolenic acid. We determined the doses of the stimulants and inhibitors based on the
206 following previous reports: LCFAs (15), SCFAs (45), bile acid (3), OEA (28), U73122
207 and U73343 (15), 2APB (34) and H89 (43). All samples were collected after 1 h of

208 incubation and centrifuged (10,000 x g) for 5 min. The supernatant was then collected to
209 measure total GLP-1 concentration using electrochemiluminescence assay kit (Meso
210 Scale Discovery, Rockville, MD; RRID:AB_2801383).

211 *Measurement of intracellular Ca^{2+} concentration.* STC-1 cells were transfected with
212 siRNA in a 96-well dish (1×10^5 /well) as described above and cultured at 37°C for 48 h
213 before experiments. The STC-1 cells were loaded with 5 μ M fura-2 AM (Dojindo,
214 Japan) in HBSS for 30 min at 37°C. After incubation, STC-1 cells were washed twice
215 by HBSS, then placed on a microplate reader (SynergyH1; BioTek instruments, USA).
216 Images through the filter sets (340/11, 380/20 -DM 400- 508/20) were obtained every
217 30 sec at 37°C and the 340nm/380nm fluorescence ratio in each well was recorded.

218 *Oral glucose and corn oil administration.* Following overnight (16 h, standardized
219 between mice) fasting, 6g/kg glucose and 10ml/kg of corn oil was administered orally
220 to wild-type mice and Car8^{wdl} mutant mice (n=6, 12-week-old) by gavage. Blood
221 samples were collected via tail vein at 0, 15, 30, 60 and 120 minutes after oral glucose
222 administration and at 0, 30, 60 and 120 minutes after oral corn oil administration. Blood
223 glucose levels and GLP-1 levels were measured by the glucose oxidase method (Sanwa
224 Kagaku Kenkyusho, Nagoya, Japan) and total GLP-1 by electrochemiluminescence
225 assay, respectively. Anesthetic agent was not used in these tests due to concern for its

226 effects on GLP-1 secretion via diminished bowel movement. After collection, blood
227 samples were kept on ice and then centrifuged (1500 x g for 10 min at 4°C) and serum
228 was separated. The serum samples were used fresh or kept at -80°C until further
229 processing.

230 *Measurement of intestinal GLP-1 content.* Mice were killed by cervical dislocation
231 and the intestinal tract was quickly removed. The small intestine was divided into two
232 portions at the middle position and the oral and rectal portions were defined as the upper
233 small intestine and lower small intestine, respectively. Sections were resected from each
234 region of the intestine: between the pyloric ring and 4cm distal to the pyloric ring as
235 upper small intestine, between the ileocecal valve and 4cm proximal to the ileocecal
236 valve as lower small intestine and between the ileocecal valve to 4cm distal to the
237 ileocecal valve as colon. The mucosa from each of these sections was gently scraped off
238 and collected. The mucosal samples were homogenized in 1 ml of 0.2 N perchloric acid
239 (19) and were centrifuged (15,000 g) for 15 min. The supernatant was then collected
240 and diluted 10 times to measure total GLP-1 concentration using
241 electrochemiluminescence kit as described above.

242 *Total gastrointestinal transit rate.* Total gastrointestinal transit rate was measured by
243 administering a non-absorbed marker containing 10% charcoal suspension in 5% gum

244 Arabic as previously described (33). Briefly, after overnight fasting, mice (n=7 for
245 wild-type mice and n=8 for Car8^{w^{dl}} mutant mice) were given 0.2 ml of the suspension
246 by gavage through a straight blunt-ended feeding needle. After 20 min, the mice were
247 killed by cervical dislocation and the entire gastrointestinal tract was excised, dissected
248 medially and the distance from the pylorus to the front of the charcoal suspension was
249 measured. The rate of transit was determined as [(distance to charcoal front) / (length of
250 small intestine)]×100 (%).

251 *Statistical analysis.* All data are expressed as the mean ± standard deviation (SD) of
252 the mean. Statistical analysis was performed using Student t-test and one-way ANOVA
253 with Tukey's test using SPSS Statistics 20.0 software (IBM, Armonk, NY). P < 0.05
254 was considered statistically significant.

255 **RESULTS**

256 *Car8 is highly expressed in enteroendocrine PPG-cells.* We performed RT-PCR of
257 PPG-cells and immunohistochemistry of the lower small intestine from Gcg^{gfp/+} mice.
258 Car8 mRNA expression levels in PPG-cells were extremely higher compared to those in
259 non-PPG-cells (Fig. 1A). Using the Car8 antibody, we stained the small intestine of
260 wild-type mice and Car8 null mice as a negative control. In the small intestine of
261 wild-type mice, positive-staining cells were scattered in the epithelium, but no

262 positive-staining cells were found in the small intestine of Car8 null mice that were
263 stained using the same protocol, showing that the Car8 antibody we used was specific
264 for Car8 (Fig. 1B). Immunohistochemical staining of lower small intestine of Gcg^{gfp/+}
265 mice showed that Car8 and EGFP proteins were expressed in the same cell (Fig. 1C).
266 We performed double-staining of mouse small intestine with antibodies for GLP-1 and
267 Car8, demonstrating that GLP-1-expressing cells indeed express Car8 (Fig.1D). These
268 data clearly show the high expression of Car8 mRNA and protein in GLP-1-expressing
269 PPG-cells.

270 *Car8 negatively regulates GLP-1 secretion in response to LCFAs.* To evaluate the
271 effect of Car8 on GLP-1 secretion, knockdown experiments were conducted using
272 siRNA. STC-1 cells treated with Car8 siRNA showed approximately 70% reduction in
273 Car8 mRNA (Fig. 2A). GLP-1 content in STC-1 cells, total cell count and cell viability
274 were not affected by Car8 downregulation (data not shown). Car8 knockdown
275 significantly enhanced GLP-1 secretion from STC-1 cells in response to
276 polyunsaturated long-chain fatty acids (LCFAs), i.e., linoleic acid and α -linolenic acid
277 (ALA) (Fig. 2B), although GLP-1 secretion induced by oleic acid (OA), bile acid and
278 oleoylethanolamide (OEA) was not affected by Car8 downregulation (Fig. 2B and 2C).
279 The GLP-1 secretory responses of STC-1 cells to 22mM glucose and 100 μ M

280 short-chain fatty acids (acetic, butyric and propionic acid) were absent; in addition, the
281 effect of Car8 on GLP-1 secretion in response to glucose and SCFAs was not observed
282 (Fig. 2D). STC-1 cells transfected with Car8 expression vector showed a robust increase
283 in Car8 mRNA compared with that in empty vector-transfected cells (Fig. 2E). Car8
284 overexpression elicited no change in GLP-1 content in STC-1 cells, total cell count and
285 cell viability (data not shown). ALA-induced GLP-1 secretion in STC-1 cells was
286 significantly inhibited by Car8 overexpression (Fig. 2F).

287 *Enhancement of ALA-induced GLP-1 secretion by Car8 downregulation is dependent*
288 *on the PLC/IP3 pathway.* It has been reported that Car8 plays a role in regulation of
289 intracellular Ca^{2+} dynamics via the PLC/IP3 pathway in neuronal cells. We
290 hypothesized that Car8 modulates LCFA-induced GLP-1 secretion through a similar
291 mechanism. To verify this, we first evaluated the contribution of the PLC/IP3 pathway
292 to GLP-1 secretion induced by LCFAs. GLP-1 secretion from STC-1 cells stimulated by
293 ALA was significantly inhibited in the presence of the PLC inhibitor U73122 and the
294 IP3R antagonist 2-aminoethyldiphenylborate (2APB) but not by U73343, an inactive
295 analogue of U73122 (Fig. 3A), demonstrating the involvement of PLC/IP3/IP3R
296 signaling in LCFA-stimulated GLP-1 secretion. We then examined the effect of PLC
297 inhibition on enhancement of the GLP-1 response to LCFA stimulation by Car8

298 knockdown. GLP-1 secretion from STC-1 cells in response to ALA was not increased
299 by Car8 downregulation in the presence of U73122 but was significantly enhanced in
300 the presence of the PKA inhibitor H89 (Fig. 3B), demonstrating that Car8 knockdown
301 enhances GLP-1 secretion through a PLC-dependent pathway, not via a PKA-dependent
302 pathway.

303 *Car8 restrains the ALA-induced intracellular Ca^{2+} elevation in STC-1 cells.* As Car8
304 has been reported to allosterically inhibit IP3 binding to IP3R and to negatively regulate
305 calcium efflux from the ER via IP3R into cytoplasm in neuronal cells (17, 27, 49), we
306 evaluated the effect of Car8 knockdown on ALA-induced intracellular Ca^{2+} elevation in
307 STC-1 cells. We demonstrated that the ALA-mediated intracellular Ca^{2+} increase was
308 significantly enhanced by Car8 downregulation (Fig. 4A and 4B).

309 *Car8^{wdl} mice exhibit an augmented GLP-1 secretion in response to corn oil.* To
310 investigate the significance of Car8 in GLP-1 secretion *in vivo*, we performed oral
311 administration tests using Car8^{wdl} mutant mice, which possess a loss-of-function
312 mutation in the Car8 gene and exhibit a distinctive lifelong gait disorder due to
313 cerebellar ataxia (21). Plasma levels of glucose were similar between wild-type and
314 Car8^{wdl} mutant mice after oral glucose load and oral corn oil load (Fig. 5A, 5C). GLP-1
315 secretion was comparable between wild-type and Car8^{wdl} mutant mice in OGTT (Fig.

316 5B). In contrast, the plasma GLP-1 concentration 30 min after oral corn oil
317 administration in Car8^{wdl} mutant mice was significantly higher than that of their
318 wild-type littermates (Fig. 5D). In order to examine the effect of whole body Car8
319 depletion on GLP-1 storage in the intestine, we evaluated intestinal GLP-1 content;
320 there was no significant difference in GLP-1 content in the small intestine and colon
321 between wild-type and Car8^{wdl} mutant mice (Fig. 5E). We examined the total
322 gastrointestinal transit rate to exclude the possibility that Car8 is involved in neural
323 control of intestinal motility, which can affect GLP-1 secretion *in vivo*. The total
324 gastrointestinal transit rate of Car8^{wdl} mutant mice was comparable to that of wild-type
325 mice (Fig. 5F), suggesting that Car8 depletion per se has no effect on gastrointestinal
326 motility, although alteration of the overall enteric neuronal network and motility by
327 Car8 depletion could not be excluded by our results.

328 **DISCUSSION**

329 In this study, we demonstrate that Car8 is highly expressed in PPG-cells and
330 negatively regulates GLP-1 secretion in response to LCFAs both *in vitro* and *in vivo*.

331 The LCFA receptors GPR40 and GPR120 are expressed in GLP-1-expressing cells
332 (5, 15); we previously found that corn oil-induced GLP-1 release was intact in GPR120-
333 and GPR40-single knockout mice but was abolished in GPR120- and GPR40-double

352 current study, GLP-1 secretion stimulated by ALA was found to be significantly
353 attenuated in the presence of U73122. Hirasawa et al. used a specific GLP-1 (7-36)
354 amide Enzyme Immunoassay Kit (YK 160; Yanaihara Institute) ; we measured the total
355 GLP-1 concentration using electrochemiluminescence assay kit (Meso Scale
356 Discovery). It has been reported that DPP-4 expressed in STC-1 cells is involved in the
357 degradation of GLP-1 secreted into the medium (23). Thus, the discrepancy between
358 our findings and those of Hirasawa et al. may be due to the difference in the GLP-1
359 measurement kit used. As we confirmed reduced GLP-1 response to ALA by treatment
360 with IP3R antagonist as well as with PLC inhibitor in additional experiments, we
361 believe that our data establish involvement of the PLC/IP3 pathway in the mechanism
362 of ALA-induced GLP-1 secretion. Moreover, enhancement of GLP-1 secretion by Car8
363 knockdown was counteracted by inhibition of PLC, verifying that Car8 negatively
364 regulates GLP-1 secretion in response to LCFAs through the PLC-dependent pathway.

365 Car8 knockdown had no effect on GLP-1 secretion in response to bile acid and
366 OEA stimulation, possibly because Gpbar1 and GPR119, the receptors for bile acid and
367 OEA, respectively, have been reported to couple to Gs protein and not to Gq protein. In
368 addition, although it has previously been reported that certain GPR40 agonists also
369 activate Gs protein (13), the effect of Car8 knockdown on GLP-1 secretion was found

370 not to be affected by PKA inhibitor in the current study. Together, these data suggest
371 that the effect of Car8 on LCFA-stimulated GLP-1 secretion is independent of the
372 Gs/cAMP/PKA pathway.

373 In our *in vitro* experiment using STC-1 cells, there was no effect of Car8
374 downregulation on GLP-1 release in response to OA. The GLP-1 concentration in the
375 medium in the presence of OA was much lower than that of linoleic acid and ALA,
376 although it was yet significantly elevated compared to baseline. The reason for absence
377 of a significant increase in GLP-1 secretion by OA in conjunction with Car8 knockdown
378 could be that the mechanism of GLP-1 secretion in response to OA differs from that of
379 linoleic acid and ALA or that OA-induced GLP-1 secretion in STC-1 cells is too weak
380 to cause a difference. Iakoubov et al. has reported that OA significantly and strongly
381 stimulated GLP-1 secretion in GLUTag cells, another enteroendocrine cell line (18).
382 Similar knockdown experiments using GLUTag cells might help to clarify the effect of
383 Car8 on OA-induced GLP-1 secretion.

384 In any case, there are differences in the expression of molecules related to hormone
385 secretion between cell lines and enteroendocrine cells *in vivo*. Differences in magnitude
386 of GLP-1 secretion depending on types of fatty acids and the effect of Car8 deficiency
387 need to be verified by *in vivo* experiments.

388 Recent evidence has suggested that subpopulations of PPG-cells can express
389 transcripts for not only GLP-1 but also a number of different hormones including
390 peptide YY (PYY), cholecystokinin (CCK), glucose-dependent insulinitropic
391 polypeptide (GIP), secretin, and neurotensin(6). Indeed, the expression of LCFA
392 receptors and hormone secretion in response to LCFAs have been previously
393 demonstrated in GIP-expressing cells (20, 39) and CCK-expressing cells (36, 43). We
394 have previously shown that the LCFA response of GIP as well as that of GLP-1 was
395 abolished in GPR40 and GPR120 double-knockout mice (40), which is consistent with
396 the concept that PPG-cells secrete both GLP-1 and GIP through the common LCFA
397 sensing mechanism. Furthermore, in the immunohistochemistry of the lower small
398 intestine from $Gcg^{gfp/+}$ mice, nearly half of Car8-positive cells were GFP-negative,
399 indicating that Car8 expression is not completely specific to PPG-cells. In this study, we
400 focused on the significance of Car8 in GLP-1 secretion. However, further evaluation is
401 warranted to determine whether or not Car8 contributes to LCFA-stimulated secretion
402 of various gut hormones other than GLP-1 secreted from PPG-cells and non-PPG-cells

403 The physiological significance of Car8 as a negative regulator of GLP-1 secretion
404 remains unclear. One possibility is that Car8 alleviates the chronic ER Ca^{2+} depletion
405 associated with IP3R activation that might induce ER stress-mediated cell apoptosis

406 (37). Thus, it is necessary to examine whether Car8-depleted GLP-1-expressing cells are
407 more sensitive to ER stress and whether the number of GLP-1-expressing cells is
408 reduced in Car8^{wdl} mice compared with that in wild-type mice in old age or under
409 chronic high-fat diet feeding. Another possibility is that the regulation of
410 LCFA-stimulated GLP-1 secretion is only a part of the physiological function of Car8.
411 For example, the M1 cholinergic muscarinic receptor has been reported to be
412 abundantly expressed in PPG-cells and to be involved in regulation of postprandial
413 GLP-1 secretion (1). M1 receptors activate Gq proteins that in turn activate PLC (30).
414 Thus, Car8 could contribute to neuromodulatory regulation of GLP-1 secretion via the
415 PLC/IP3 pathway downstream of the M1 muscarinic receptor. This possibility warrants
416 further study.

417 In conclusion, our study provides several new findings. First, enteroendocrine
418 PPG-cells express functional Car8, which has not been reported previously in
419 non-neuronal cells. Second, the GLP-1 response to LCFAs in STC-1 cells was enhanced
420 by Car8 downregulation and the effect was abolished by PLC inhibition, indicating that
421 Car8 negatively regulates LCFA-induced GLP-1 secretion through a PLC-dependent
422 pathway. Finally, Car8^{wdl} null mutant mice exhibited an augmented GLP-1 secretion in
423 response to corn oil administration, supporting the physiological significance of Car8 in

424 GLP-1 secretion *in vivo*. These findings suggest a therapeutic approach to both diabetes
425 and obesity through augmentation of postprandial GLP-1 secretion by Car8 inhibition.

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437 **AUTHOR CONTRIBUTIONS**

438 Y.F. planned the study, researched data, contributed to discussions and wrote,
439 reviewed and edited the manuscript. S.Y. planned the study, contributed to discussions
440 and reviewed and edited the manuscript. N.H., E.I., R.U., T.N., K.I. and K.S. researched

441 data and contributed to discussions. D.Y. and Y.H. contributed to discussions. N.I.
 442 contributed to discussions and reviewed and edited the manuscript. All authors reviewed
 443 the results and approved the final version of the manuscript.

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613 **FIGURE LEGENDS**

614 **Fig. 1.** Expression of Car8 in enteroendocrine PPG-cells. A: Expression levels of Car8

616 mRNA in isolated PPG-cells and non-PPG-cells. Data are expressed as the ratio to that
617 of GAPDH in the same sample. n=3 for each group. Data are expressed as mean $\pm$ SD.

618 B: Immunohistochemical staining for Car8 (red) in the lower small intestine of a
619 wild-type mouse and Car8^{w^{dl}} mouse. C: Immunohistochemical staining for GFP (green)
620 and Car8 (red) in the lower small intestine of a Gcg^{gfp/+} mouse. D:
621 Immunohistochemical staining for GLP-1 (green) and Car8 (red) in the lower small
622 intestine of a wild-type mouse.

623 **Fig. 2.** Significance of Car8 in LCFA-induced GLP-1 secretion. A: Validation of Car8
624 gene knockdown in STC-1 cells using Car8 siRNA. Data are expressed as the ratio to
625 that of GAPDH in the same sample. STC-1 cells transfected with scramble siRNA
626 (black dot) and Car8 siRNA (white dot). *p<0.05 between scramble siRNA and Car8
627 siRNA. n=4 for each group. Data are expressed as mean $\pm$ SD. Scr-si; scramble siRNA,
628 Car8-si; Car8 siRNA. B: GLP-1 secretion from STC-1 cells transfected with scramble
629 siRNA (black dot) and Car8 siRNA (white dot) incubated in HBSS containing
630 long-chain fatty acids (LCFAs): oleic acid (100 μ M), linoleic acid (100 μ M) and α
631 -linolenic acid (100 μ M). *p<0.05 and **p<0.01 between scramble siRNA and Car8
632 siRNA. [#]p<0.05 versus STC-1 cells transfected with scramble siRNA incubated in the
633 control media. ^bp<0.05 versus STC-1 cells transfected with Car8 siRNA incubated in

634 the control media. n=3 for each group. Data are expressed as mean $\pm$ SD. OA; oleic
 635 acid, ALA; α -linolenic acid. C: GLP-1 secretion from STC-1 cells transfected with
 636 scramble siRNA (black dot) and Car8 siRNA (white dot) incubated in HBSS containing
 637 bile acid (100 μ M) and oleoylethanolamide (50 μ M). [#]p<0.05 versus STC-1 cells
 638 transfected with scramble siRNA incubated in the control media. ^bp<0.05 versus
 639 STC-1 cells transfected with Car8 siRNA incubated in the control media. n=3 for each
 640 group. Data are expressed as mean $\pm$ SD. OEA; oleoylethanolamide. D: GLP-1
 641 secretion from STC-1 cells transfected with scramble siRNA (black dot) and Car8
 642 siRNA (white dot) incubated in HBSS containing glucose (22.2mM), acetic acid
 643 (100 μ M), butyric acid (100 μ M) and propionic acid (100 μ M). n=3 for each group. Data
 644 are expressed as mean $\pm$ SD. E: Validation of Car8 gene overexpression using Car8
 645 expression vector. STC-1 cells transfected with empty vector (black dot) and Car8
 646 expression vector (white dot). **p<0.01 between empty vector and Car8 expression
 647 vector. n=3 for each group. Data are expressed as mean $\pm$ SD. Emp-v; empty vector,
 648 Car8-ev; Car8 expression vector. F: GLP-1 secretion from STC-1 cells transfected with
 649 empty vector (black dot) and Car8 expression vector (white dot). **p<0.01 between
 650 empty vector and Car8 expression vector. [#]p<0.05 versus STC-1 cells transfected with
 651 empty vector incubated in the control media. ^bp<0.05 versus STC-1 cells transfected

652 with Car8 expression vector incubated in the control media. n=3 for each group. Data
653 are expressed as mean $\pm$ SD.

654 **Fig 3.** Involvement of PLC/IP3/IP3R signaling in LCFA-stimulated GLP-1 secretion.

655 A: GLP-1 secretion in response to ALA from STC-1 cells treated with IP3R inhibitor
656 2-aminoethyldiphenylborate (2APB) and phospholipase C inhibitor U73122 and its
657 inactive analogue, U73343. **p<0.01. n=5 for each group. Data are expressed as mean
658 $\pm$ SD. B: ALA-induced GLP-1 secretion from STC-1 cells transfected with scramble
659 siRNA (black dot) and Car8 siRNA (white dot) in the absence and presence of PLC
660 inhibitor (U73122) and PKA inhibitor (H89). **p<0.01 between scramble siRNA and
661 Car8 siRNA. [#]p<0.05 versus STC-1 cells transfected with scramble siRNA incubated in
662 the control media. ^bp<0.05 versus STC-1 cells transfected with Car8 siRNA incubated
663 in the control media. ALA; α -linolenic acid. n=7 for each group. Data are expressed as
664 mean $\pm$ SD.

665 **Fig. 4.** Effect of Car8 gene knockdown on intracellular Ca²⁺ elevation in response to

666 ALA. A: Changes in intracellular Ca²⁺ concentrations ([Ca²⁺]_i) in STC-1 cells
667 transfected with scramble siRNA (Scr-si; black circle) and Car8 siRNA (Car8-si; white
668 circle) in response to ALA (0-5min, HBSS; 5-30min, HBSS+10 μ M ALA). n=4 for each
669 group. Data are expressed as mean $\pm$ SD. B: Increment of fura-2 ratio (Δ [Ca²⁺]_i) in

670 response to 10 μ M ALA in conditions as in A [(Max. value during 5–30 min) –
671 (Average value during 0-5 min)]. ** $p < 0.05$. Data are expressed as mean $\pm$ SD.

672 **Fig. 5.** Single oral administration of glucose and corn oil. Concentrations of blood
673 glucose (A,C) and total GLP-1 (B,D) after oral administration of 6g/kg glucose (A,B)
674 and 10 ml/kg corn oil (C,D). Wild-type mice (n=6; filled circle) and Car8^{wdl} mutant
675 mice (n=6; unfilled circle). ** $p < 0.01$ between wild-type mice and Car8^{wdl} mutant mice.
676 Intestinal GLP-1 content (E) (n=4 for each group) and intestinal transit rate (F) (n=7 for
677 each group). Wild-type mice (filled circle) and Car8^{wdl} mutant mice (unfilled circle).
678 Data are expressed as mean $\pm$ SD.

Figure 1

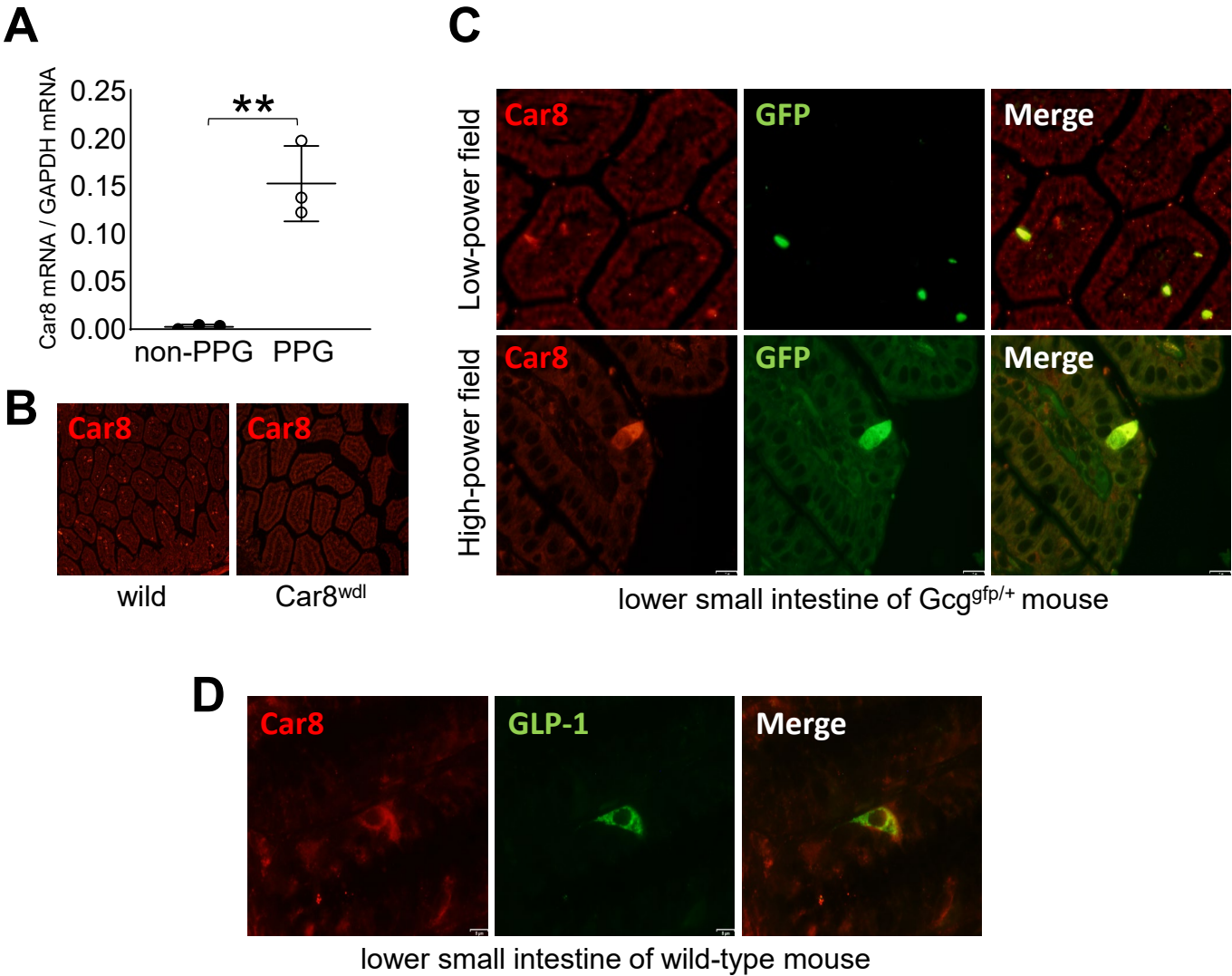


Figure 2

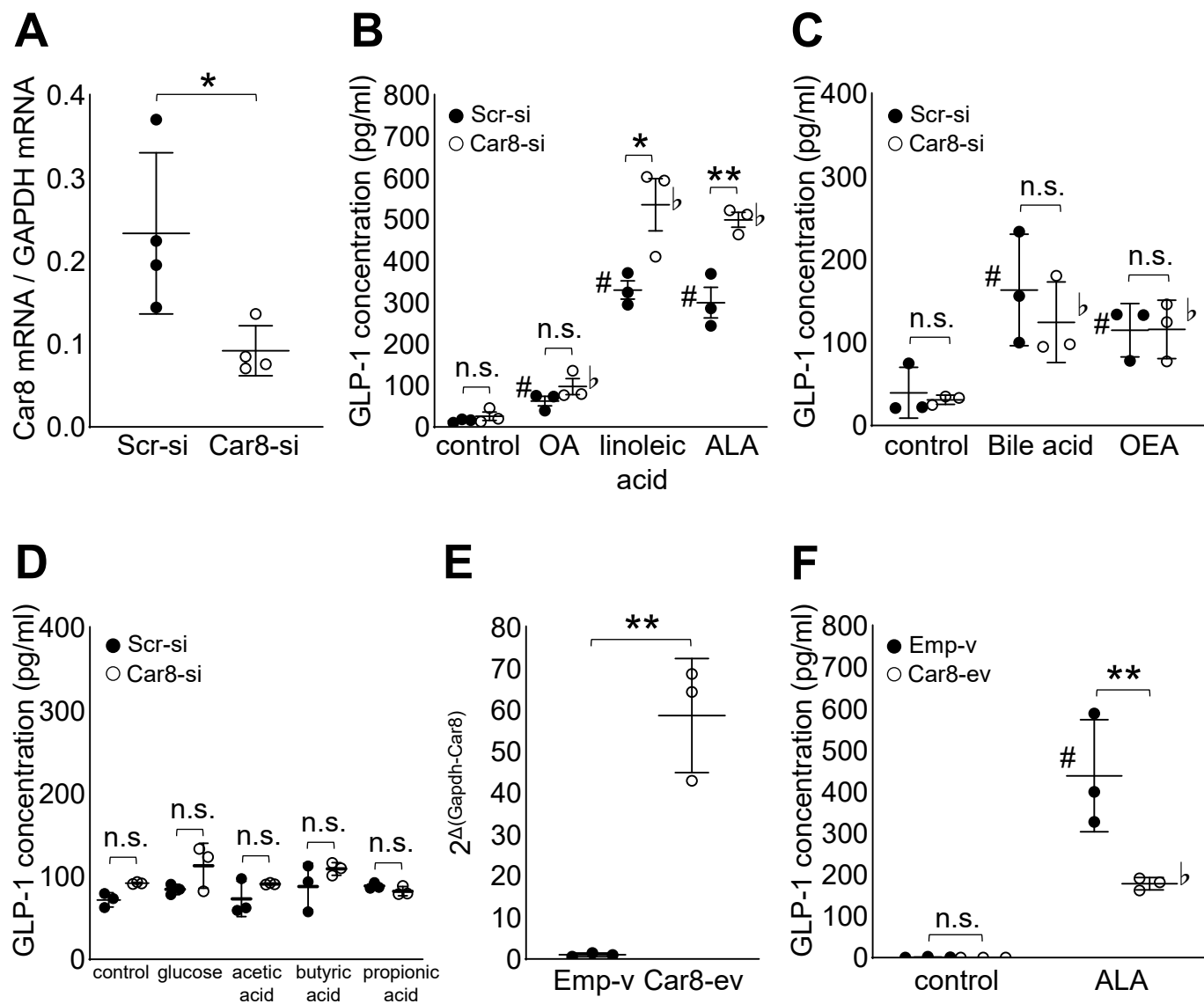


Figure 3

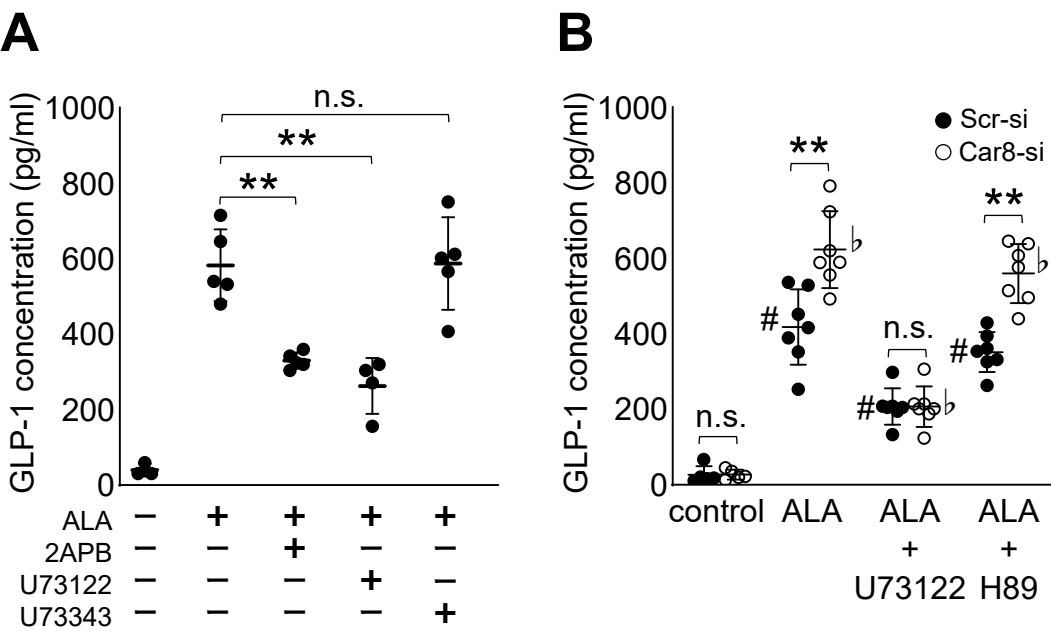


Figure 4

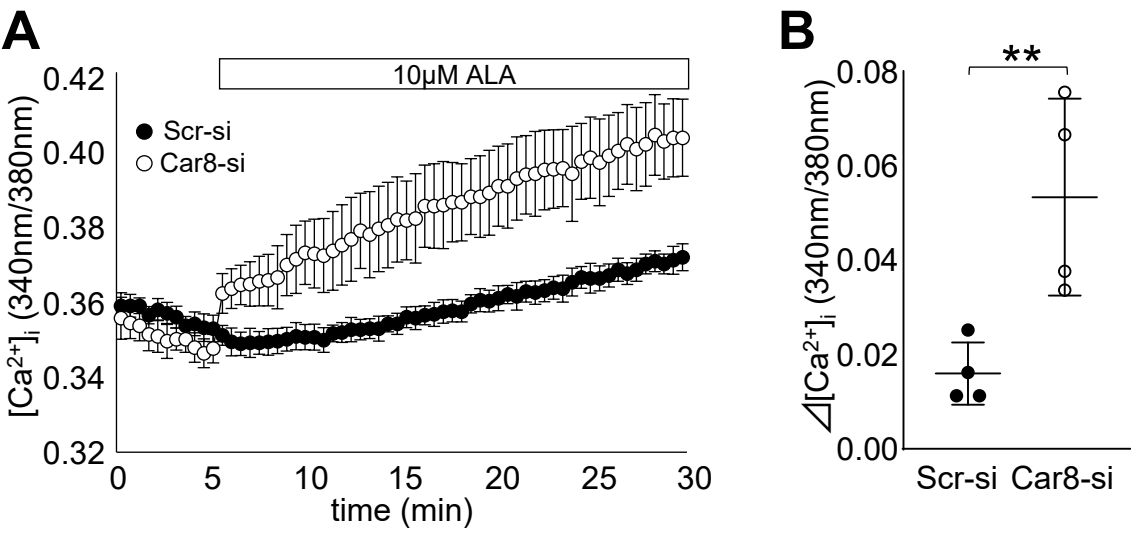


Figure 5

