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1 **GC-globulin/vitamin D-binding protein is required for pancreatic α cell**
2 **adaptation to metabolic stress**
3

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24
25

26 **ABSTRACT**

27 GC-globulin (GC), or vitamin D-binding protein, is a multifunctional protein involved in
28 transport of circulating vitamin 25(OH)D and fatty acids, as well as actin-scavenging. In the
29 pancreatic islets, the gene encoding GC, *GC*, is highly-localized to glucagon-secreting α cells.
30 Despite this, the role of GC in α cell function is poorly understood. We previously showed that
31 GC is essential for α cell morphology, electrical activity and glucagon secretion. We now show
32 that loss of GC exacerbates α cell failure during metabolic stress. High fat diet-fed *GC*^{-/-} mice
33 have basal hyperglucagonemia, which is associated with decreased α cell size, impaired
34 glucagon secretion and Ca²⁺ fluxes, and changes in glucose-dependent F-actin remodelling.
35 Impairments in glucagon secretion can be rescued using exogenous GC to replenish α cell
36 GC levels, increase glucagon granule area and restore the F-actin cytoskeleton. Lastly, GC
37 levels decrease in α cells of donors with type 2 diabetes, which is associated with changes in
38 α cell mass, morphology and glucagon expression. Together, these data demonstrate an
39 important role for GC in α cell adaptation to metabolic stress.

40 **Brief Summary:** GC-globulin/vitamin D-binding protein protects pancreatic α cells from
41 metabolic stress, maintaining glucagon secretion.

42

43 INTRODUCTION

44 During metabolic stress, α cell function becomes dysregulated, leading to inappropriate
45 glucagon secretion and exacerbation of blood glucose levels (1), as well as impaired counter-
46 regulatory responses (2). The mechanisms involved are multifactorial and include changes in
47 α cell glucose-sensing, α cell de-differentiation, paracrine feedback, hyperaminoacidemia, as
48 well as α cell mass (3-8). The importance of glucagon during metabolic stress and diabetes is
49 exemplified by studies showing that deletion or blockade of the glucagon receptor protects
50 against diabetes (9; 10), and that immunoneutralization of glucagon restores glucose
51 homeostasis in STZ-treated rats (11) or obese mice (12).

52 GC globulin (GC), also known as vitamin D-binding protein, is an ~58 kDa glycosylated
53 α protein that transports vitamin D metabolites and fatty acids in the circulation (13; 14). GC
54 is also amongst the most potent actin scavengers in the body and acts in concert with gelsolin
55 to sequester actin filaments released from lysed cells (15; 16). *GC/Gc*, the gene encoding GC,
56 was thought to be almost exclusively expressed in the liver, where sterol-derivatives such as
57 cholecalciferol are converted into pre-hormone 25-OH vitamin D (25(OH)D) (17). However,
58 recent studies have shown that human and mouse α cells express equally high levels of
59 *GC/Gc* (18; 19), whereas the gene is absent from β cells (19; 20). Notably, GC constitutes an
60 α cell signature gene, since its promoter region is enriched for cell type-selective open
61 chromatin regions (18). Moreover, a large-scale Mendelian randomization study of European
62 and Chinese adults has revealed associations between GC single nucleotide polymorphisms
63 (SNPs) and T2D risk (21). Despite this, the tissue-specific mechanisms by which GC
64 influences metabolic traits are poorly characterized.

65 Recently, we showed that GC contributes to the maintenance of α cell function (22;
66 23). Deletion of GC leads to smaller and hyperplastic α cells, which display abnormal Na^+
67 conductance, Ca^{2+} fluxes and glucagon secretion. This effect was found to be mediated by
68 changes in the F-actin cytoskeleton, with a large increase in microfilament thickness and

69 density in GC^{-/-} islets. Notably, up until this point, actin-binding properties of GC had only been
70 described in the circulation. These studies were recently corroborated by Patch-seq studies
71 where GC was shown to inversely correlate with peak Na⁺ current in human α cells (8). Despite
72 the role of GC in maintaining α cell identity, it remains unknown how GC impacts α cell
73 responses to metabolic stress. Studies have suggested that manipulation of GC levels is able
74 to restore glucose homeostasis in obese mouse models, although the exact cellular
75 mechanisms are unclear, as GC is upregulated in de-differentiated β cells (24). Although GC
76 is an α cell signature gene, it is also unclear if abundant circulating levels of GC may also
77 impact glucagon secretion and α cell function.

78 In the present study, we set out to understand the role of GC in α cell function during
79 metabolic stress. By combining GC^{-/-} mice and pancreas sections from type 2 diabetes (T2D)
80 donors with immunohistochemistry, live imaging and high-resolution microscopy, we show that
81 GC is required for α cell function and glucagon secretion under diabetogenic conditions.
82 Pertinently, α cell dysfunction can be rescued by restoring GC levels using exogenous protein.

83

84 RESEARCH DESIGN AND METHODS

85 Experimental design

86 No data were excluded unless the cells failed to respond to positive control, responded
87 inappropriately to negative control, or displayed impaired viability. All individual data points
88 are reported. The measurement unit is animal or batch of islets or pancreas section, with
89 experiments replicated independently. Animals and islets were randomly allocated to
90 treatment groups to ensure that all states were represented in the different experiment arms.
91 Animals and pancreas sections were coded to allow blinded analysis.

92 Mouse models

93 GC^{-/-} mice harboring deletion of exon 5 of the GC gene were backcrossed to C57BL/6J for 10
94 generations (25). These mice have undetectable circulating GC, as well as 25(OH)[3H]D3
95 binding (22; 25). Mice were housed in a specific pathogen-free facility with ad lib access to
96 food and water. Vitamin D sufficiency was ensured by using chow supplemented with 1000
97 U/kg cholecalciferol. Mice were fed standard chow (SC) or high fat diet (HFD) containing 60%
98 fat (Research Diets, Catalog# D12492), and body weight checked weekly from 0 to 12 weeks.
99 Male and female mice were placed on SC or HFD from 8 weeks of age (numbers reported in
100 the figure legends).

101 Human donors

102 Formalin-fixed paraffin-embedded pancreas sections were obtained from the Alberta Diabetes
103 Institute IsletCore. Quality control and phenotyping data is available for each preparation via
104 www.isletcore.ca.

105 Glucose and insulin tolerance tests

106 Mice were fasted for 4-5 hours (8:00 am- 12:30 pm) before intraperitoneal injection of 1.5g/kg
107 of sterile filtered D-glucose. Tail vein sampling was performed at 0, 15, 30, 60, 90 and 120

108 mins post injection. Glucose levels were measured using a Contour XT glucometer (Bayer).
109 For plasma glucagon measures, mice were fasted for 4-5 hours (8:00 am- 12:30 pm) before
110 intraperitoneal injection of 0.75U/kg of insulin (Lilly, Humulin S). Blood was collected at 0 and
111 30 mins post injection and stored at -80°C pending ELISA for serum glucagon (Merckodia,
112 Catalog# 10-1281-01). Values lower than the assay detection limit were interpolated from the
113 standard.

114 **Islet isolation and culture**

115 Mice were humanely euthanized by rising CO₂ and cervical dislocation (Schedule 1), before
116 bile duct injection and inflation of the pancreas with 1 mg/ml SERVA NB8 collagenase
117 (Amsbio, Catalog# 17456.02). Islets were purified using Ficoll-Paque (Cytiva, Catalog#
118 17144003) or Histopaque (Sigma-Aldrich, Catalog# 11191, Catalog# 10831) gradient
119 separation and maintained at 37°C and 5% CO₂ in RPMI 1640 medium (Gibco, Catalog#
120 21875034) containing 10% FCS (Sigma-Aldrich, Catalog# F9665), 100 units/mL penicillin, and
121 100 µg/mL streptomycin (Gibco Catalog# 15140122).

122 **Immunostaining of mouse pancreases**

123 Pancreases harvested before overnight incubation with 10% formalin overnight and
124 dehydration and wax embedding. Sections were cut at 5 µm using a Leica microtome, before
125 de-paraffinization and blocking with PBS-T + 1% BSA for 1 hour. Sections were incubated
126 with primary antibodies overnight at 4°C, before washing in PBS-T + 0.1% BSA (Sigma-
127 Aldrich, Catalog# A3803) and incubation with secondary antibodies for 2 hours at room
128 temperature.

129 Primary antibodies used were: rabbit anti-insulin 1:500 (Cell Signaling Technology, Catalog#
130 3014, RRID:AB_2126503); mouse monoclonal anti-glucagon 1:2000 (Sigma-Aldrich,
131 Catalog# G2654, RRID:AB_259852); mouse anti-somatostatin 1:1000 (Thermo Fisher
132 Scientific, Catalog#14-9751-80, RRID:AB_2572981) and rabbit anti-DBP 1:1000 (Sigma-
133 Aldrich, Catalog# HPA019855, RRID:AB_1849545). Secondary antibodies were: goat anti-

134 rabbit Alexa Fluor 633 (Thermo Fisher Scientific, Catalog# A-21052, RRID:AB_2535719), goat
135 anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, Catalog# R37116, RRID:AB_2556544),
136 goat anti-guinea pig Alexa Fluor 488 (Thermo Fisher Scientific, Catalog# A-11073,
137 RRID:AB_2534117), goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific, Catalog# A-
138 11029, RRID:AB_138404) and goat anti-guinea pig Alexa Fluor 568 (Thermo Fisher Scientific,
139 Catalog# A-11075, RRID:AB_2534119), applied at 1:1000. Specificity of antibodies was
140 based upon known cell type co-localizations, overlap with insulin, glucagon or somatostatin
141 reporters, or loss of staining in knockout tissue. F-actin and G-actin were visualized using
142 Phalloidin-488 (Abcam, Catalog# ab176753) or DNase1-594 (Invitrogen, Catalog# D12372),
143 respectively.

144 Imaging was performed using either Zeiss LSM780 or LSM880 meta-confocal microscopes,
145 equipped with sensitive GaAsP spectral detectors. Alexa Fluor 488, Alexa Fluor 568 and Alexa
146 Fluor 633 were excited at $\lambda = 488$ nm, $\lambda = 568$ and $\lambda = 633$ nm, respectively. Emitted signals
147 for Alexa Fluor 488, Alexa Fluor 568 and Alexa Fluor 633 were detected at $\lambda = 498$ – 559 , nm
148 $\lambda = 568$ – 629 and $\lambda = 633$ – 735 nm, respectively. Super-resolution images of F-actin were
149 acquired using a Nikon N-SIM S microscope, SR HP Apo TIRF 100x 1.49 NA/oil immersion
150 objective and ORCA-Flash 4.0 sCMOS camera, with online deconvolution. Alexa Fluor 488
151 was excited at $\lambda = 500$ – 550 nm, with emitted signals detected at $\lambda = 570$ – 640 nm.

152 **Intracellular Ca²⁺ imaging**

153 The ratiometric Ca²⁺ dye, Fura2 (HelloBio, Catalog# HB0780-1mg), was loaded into islets
154 using 20% pluronic acid dissolved in DMSO (Thermo Fisher Scientific, Cat# P3000MP) at
155 37°C for 40 mins. For islets treated with GC, 5 μ M GC (East Coast Bio, Catalog# LA166) was
156 added during Fura2 incubation. Islets were transferred to the heated chamber (34 C) of a
157 Nikon Ti-E microscope coupled to a 10 x / 0.3 NA air objective (Nikon Plan Fluor), allowing
158 simultaneous cell resolution imaging of multiple islets (lateral resolution = 910 nm). A Cairn
159 Research FuraLED system provided excitation at $\lambda = 340$ nm and $\lambda = 385$ nm. Emitted signals

160 were captured at $\lambda = 470\text{--}550$ nm using a Photometric Delta Evolve EM-CCD. Intracellular
161 Ca^{2+} levels were shown as the emission ratio at 340 nm and 385 nm. A number of experiments
162 were repeated using the non-ratiometric Ca^{2+} dye Fluo-8 (AAT Bioquest, Catalog# 20494).
163 Confocal excitation was delivered at 470 nm (emission $\lambda = 500\text{--}550$ nm) by a North 89 LDI
164 Illuminator, CrestOptics V2 X-light spinning disk and 20 x / 0.75 NA air objective (Nikon Plan
165 Apo λ). Intracellular Ca^{2+} levels were quantified as F/F_{\min} where F = fluorescence at any given
166 timepoint, and F_{\min} = mean minimum fluorescence. All experiments were performed in
167 HEPES-bicarbonate buffer was used, containing (in mmol/L) 120 NaCl, 4.8 KCl, 24 NaHCO_3 ,
168 0.5 Na_2HPO_4 , 5 HEPES, 2.5 CaCl_2 , 1.2 MgCl_2 , and supplemented with 0.5–17 mM D-
169 glucose.

170 **Insulin and glucagon secretion assays**

171 HEPES-bicarbonate buffer was used for all assays, containing (in mmol/L) 120 NaCl, 4.8
172 KCl, 24 NaHCO_3 , 0.5 Na_2HPO_4 , 5 HEPES, 2.5 CaCl_2 , 1.2 MgCl_2 + 0.1% BSA.

173 For glucagon secretion, batches of 10 islets were pre-incubated in buffer supplemented with:
174 1) 10 mM glucose, before incubation with either 10 mM glucose, 0.5 mM glucose or 0.5 mM
175 glucose + 5 μM epinephrine (Sigma-Aldrich, Catalog# E4250) or 5 μM GC for 1 hr at 37°C; or
176 2) pre-incubated in 17 mM glucose before incubation with either 17 mM glucose, 2 mM glucose
177 or 2 mM glucose + 5 μM epinephrine. Glucagon released into the supernatant was then
178 measured using either HTRF ultrasensitive assay (Cisbio, Catalog# 62CGLPEG) or Lumit
179 bioluminescent immunoassay (Promega, Catalog# CS3037A06) (26).

180 For insulin secretion, batches of 10 islets were pre-incubated in buffer supplemented with 3
181 mM glucose, before sequential incubation in 3 mM glucose, 17 mM glucose and 17 mM
182 glucose + 10 mM KCl or 5 μM GC for 30 mins at 37°C. Insulin was measured using HTRF
183 ultrasensitive assay (Cisbio, Catalog# 62IN2PEG) or Lumit bioluminescent immunoassay
184 (Promega, Catalog# CS3037A01). Total glucagon and insulin were extracted from islets lysed
185 in acid ethanol.

186 **Image analysis**

187 Ca^{2+} imaging: α cells were identified in an unbiased manner by their characteristic Ca^{2+} spiking
188 activity at low glucose, as well as responses to epinephrine, as reported (22; 27). Signal
189 contributions from β cells are unlikely given that they are electrically silent at low glucose and
190 inhibited by epinephrine. The proportion of low glucose-responsive α cells was calculated as
191 the area occupied by identified α cells normalized to total islet area. Ca^{2+} spike amplitude was
192 calculated for individual cells using $\Delta 340/385 \text{ nm}$ or F/F_{\min} .

193 Immunostaining: GC, F-actin, G-actin and glucagon were analyzed using corrected total cell
194 fluorescence (CTCF), as previously described. CTCF accounts for the effect of cell size on
195 fluorophore intensity by taking the integrated density and subtracting area of the selected cell
196 x mean background fluorescence (28; 29). α cell, β cell and δ cell area and size were analysed
197 using ImageJ (NIH) Particle Analysis plugin applied to binarized and thresholded images.
198 Linear adjustments to brightness and contrast were applied to representative images, with
199 intensity values maintained between samples to allow cross-comparison.

200 **Statistics**

201 Statistical details for each experiment are reported in the corresponding figure legend. The n
202 number represents animal, batch of islets or donor. No data were excluded unless the cells
203 displayed a clear non-physiological state (i.e. impaired viability), and all individual data points
204 are reported in the figures. Data normality was assessed using D'Agostino-Pearson test. All
205 analyses were conducted using GraphPad Prism 9.0 software. Pairwise comparisons were
206 made using two-tailed unpaired t test (parametric) or Mann-Whitney test (non-parametric). To
207 assess multiple interactions, one-way or two-way ANOVA were used, adjusted for repeated
208 measures where needed. Post hoc comparisons were made using Bonferonni's, accounting
209 for degrees of freedom. Linear regression was used to assess strength of association between
210 explanatory and dependent variables, with slopes compared using analysis of covariance.
211 Data represent mean \pm SEM or SD, with individual datapoints shown where possible. Where

212 a large number of datapoints obscure mean \pm SEM or SD, violin plots are instead used
213 (showing median and interquartile range).

214 **Study approval**

215 Mouse studies were regulated by the Animals (Scientific Procedures) Act 1986 of the U.K.
216 (Personal Project Licences P2ABC3A83 and PP1778740). Approval was granted by the
217 University of Birmingham's Animal Welfare and Ethical Review Body (AWERB).

218 Human pancreas sections were obtained from Alberta Diabetes Institute IsletCore (30).
219 Procurement of human pancreases was approved by the Human Research Ethics Board at
220 the University of Alberta (Pro00013094). All donors' families gave informed consent for the
221 use of pancreatic tissue in research. Studies with human tissue were approved by the
222 University of Birmingham Ethics Committee, as well as the National Research Ethics
223 Committee (REC reference 16/NE/0107, Newcastle and North Tyneside, UK). Donor
224 characteristics are reported in Table S1. Anonymized donor IDs can be cross-referenced
225 against the IsletCore database (www.isletcore.ca) including information about cold ischemia
226 time, total islet equivalents isolated, tissue purity, insulin content and stimulation index.

227 **RESOURCE AVAILABILITY**

228 All data generated or analyzed during this study are included in the published article (and its
229 online supplementary files). Source data files generated and/or analyzed during the current
230 study are available from the corresponding author upon reasonable request.

231 **RESOURCE AVAILABILITY**

232 Non-commercially available reagents are available from the corresponding author upon
233 reasonable request.

234 **RESULTS**

235 **Deletion of GC increases basal glucagon secretion during high fat diet**

236 Mice with global GC deletion were used, since: 1) GC is exclusively expressed in α cells and
237 liver (18; 22); 2) Gcg-Cre lines have variable recombination efficiency and specificity (31); 3)
238 recently reported Gcg-Cre^{ERT} knock-in mice require tamoxifen induction (32), which interferes
239 with hepatic triglyceride accumulation and hence GC levels; and 4) two patients with
240 homozygous inactivating mutations in GC have been described (33; 34). The GC^{-/-} mice used
241 here are phenotypically well-validated, do not possess detectable GC/Gc expression, and
242 have 50% reduced and 90% reduced 25(OH)D and 1,25(OH)D levels, respectively (22; 25).

243 GC^{-/-} and littermate control GC^{+/+} mice were placed on high fat diet (HFD) for up to 12
244 weeks, with glucose tolerance tested every 4 weeks. The GC^{+/+} cohort included some
245 heterozygous (GC^{+/-}) animals as controls, since we did not see any differences versus wild-
246 types (GC^{+/+}). As expected from our previous studies, glucose tolerance was similar in GC^{+/+}
247 and GC^{-/-} mice under standard diet (i.e. 0 weeks HFD) (Figure 1A and B). No significant
248 differences were observed in glucose tolerance in GC^{+/+} and GC^{-/-} mice at 4 weeks, 8 weeks
249 and 12 weeks HFD (Figure 1C-H). Confirming efficacy of the preclinical obesity model, 4-week
250 HFD-fed GC^{+/+} and GC^{-/-} mice were glucose intolerant versus age-matched controls fed
251 standard chow (Figure 1I). Body weight gain was similar in female and male GC^{+/+} and GC^{-/-}
252 mice during HFD (Figure 1J and K).

253 As a similar phenotype was observed in both female and male mice, we combined
254 both sexes for subsequent studies. Plasma glucagon levels were assessed at 0 and 30 mins
255 post-injection of insulin. While glucose levels were lowered to a similar extent in GC^{+/+} and
256 GC^{-/-} mice (Figure 1L), basal fasted glucagon secretion was significantly (2-fold) elevated in
257 GC^{-/-} mice after 4 weeks of HFD (Figure 1M-O). Glucagon:glucose ratios, calculated using
258 measures from the same animal, provided further evidence of dysregulated basal but not
259 stimulated glucagon secretion (Figure 1M and P).

260 In summary, GC^{-/-} mice are glucose tolerant during HFD, but display elevated basal
261 glucagon levels, indicative of dysregulated α cell function.

262 **HFD GC^{-/-} mice have aberrant α -, β - and δ -cell morphology**

263 Pancreata isolated from HFD-fed GC^{+/+} mice showed a 2-fold increase in GC protein levels
264 versus standard chow (SC) controls (Figure 2A and B). GC protein was undetectable in
265 pancreata from HFD-fed GC^{-/-} mice, further demonstrating the reliability of the antibody and
266 immunostaining approach used (22) (Figure 2A and B). We have previously shown that
267 pancreata from SC-fed GC^{-/-} mice possess decreased α cell mass and α cell size (22). We
268 thus performed high resolution morphometric analysis in pancreata from HFD-fed mice.

269 HFD feeding itself did not affect islet area occupied by α cells, nor α cell size, as
270 compared to age-matched SC controls (Figure 2C-E). However, a large reduction in α cell size
271 was observed in HFD-fed GC^{-/-} mice versus GC^{+/+} littermates (Figure 2C-E). By contrast to its
272 effects on α cells, HFD increased β cell size in GC^{+/+}, with a further increase detected in GC^{-/-}
273 islets (Figure 2C, F and G). Analysis of δ cells revealed a HFD-induced increase in their
274 proportion, a change that was partly reversed by deletion of GC (Figure 2H-J).

275 In summary, these data suggest that, during HFD, GC restrains β cell size, while
276 promoting α cell size and δ cell expansion to support normal plasticity.

277 **Glucagon secretion and α cell Ca²⁺ responses are impaired in HFD GC^{-/-} islets**

278 Islets were isolated from HFD-fed GC^{+/+} and GC^{-/-} mice and their age-matched standard chow
279 controls for detailed in vitro analyses. As reported previously (22), SC GC^{-/-} islets presented
280 with impaired low glucose- and low glucose + epinephrine-stimulated glucagon secretion
281 versus GC^{+/+} littermates (Figure 3A). Similar impairments were detected for HFD, although
282 responses to epinephrine remained intact, suggesting that the defect is upstream of the
283 exocytotic machinery (Figure 3A). At glucose concentration sub-maximal for alpha cell
284 function (i.e. 2 mM), glucagon secretion still tended to be reduced in HFD GC^{-/-} islets (Figure

285 3B). Glucose-stimulated insulin secretion tended to be increased in SC GC^{-/-} islets and this
286 trend became significant during HFD (Figure 3C). A tendency toward increased basal
287 glucagon secretion was also noted in HFD GC^{-/-} islets (Figure 3B), which might partly explain
288 the increased insulin secretion, since glucagon is insulinotropic when beta cells are active
289 (35). No significant differences in total glucagon or insulin content could be detected between
290 GC^{-/-} islets and GC^{+/+} controls (Figure 3 D and E).

291 Given the apparent changes in glucagon and insulin secretion, we next investigated
292 upstream Ca²⁺ fluxes, with α cells identified by their characteristic responses to low glucose
293 (0.5 mM) as well as epinephrine (27). Confirming our previous findings, proportion active α
294 cells (i.e. % cells displaying Ca²⁺ spikes; a measure of recruitment into Ca²⁺ activity) was
295 decreased in SC GC^{-/-} islets (Figure 3F-I). By contrast to our previous results, we also
296 observed a significant decrease in Ca²⁺ amplitude in SC GC^{-/-} islets (Figure 3G-I). The most
297 likely explanation for this discrepancy is the relatively advanced age of the SC mice used in
298 the study here, which were age-matched with those receiving HFD, and suggests that age
299 might exacerbate the in vitro phenotype following GC deletion. Nonetheless, HFD decreased
300 both the proportion of active α cells, as well as the amplitude of their Ca²⁺ spikes (Figure 3F-
301 I). The effect of HFD on Ca²⁺ spike amplitude, but not proportion active α cells, was
302 exacerbated following loss of GC (Figure 3F-I) (Supplementary Movies 1 and 2). Ca²⁺ imaging
303 results were validated using a second Ca²⁺ probe (Fluo8), confocal microscopy and a higher
304 magnification objective (Figure 3J-K) (Supplementary Movies 3 and 4).

305 **GC-dependent actin cytoskeleton remodelling occurs during HFD**

306 During stimulation, the F-actin cytoskeleton undergoes rearrangement to facilitate exocytosis
307 of hormone vesicles (36-38). In line with the actin-scavenging function of GC, we previously
308 showed that F-actin density was increased in GC^{-/-} islets, leading to changes in glucagon
309 granule morphology and distribution, suggestive of sequestration and trapping (22). Directly
310 implicating the F-actin cytoskeleton in glucagon release, incubation of GC^{-/-} islets with

311 Latrunculin B was able to restore function (22). We therefore investigated whether restoration
312 of GC level and ergo F-actin cytoskeletal structure might rescue the phenotype of HFD GC^{-/-}
313 islets. Following acute (10 mins) stimulation with low glucose, F-actin density was decreased
314 in GC^{-/-} islets but unchanged in islets from GC^{+/+} islets (Figure 4A-C). F-actin remained low in
315 GC^{-/-} islets after chronic (60 mins) stimulation, but was increased ~2-fold in GC^{+/+} islets (Figure
316 4A-C).

317 As we previously showed (22), deletion of GC from standard chow islets led to increased F-
318 actin density, concomitant with a decrease in G-actin monomers, presumably due to their
319 involvement in forming polymerized actin (Figure 4D-F). On the other hand, F-actin density
320 and fiber thickness increased by almost 3-fold in HFD GC^{+/+} islets (Figure 4D-F).
321 Unexpectedly, given its actin scavenging function, deletion of GC led to a decrease in F-actin
322 density in HFD GC^{-/-} islets versus GC^{+/+} controls (Figure 4D-E). By contrast, monomeric G-
323 actin was increased in HFD GC^{-/-} islets, suggesting that G-actin is sequestered away from
324 sites of F-actin polymerization following deletion of GC (Figure 4D-F). In all cases, changes in
325 F-actin and G-actin were detected throughout the islet (Figure 4E and F) as well as in
326 individual α cells (Figure 4G and H) and β cells (Figure 4I and J), suggesting that glucagon
327 granule-resident GC acts in a paracrine manner to influence cytoskeletal structure throughout
328 the islet i.e. by severing and depolymerizing F-actin into G-actin (22; 38).

329 **GC supplementation restores F-actin cytoskeletal structure and glucagon release**

330 We next investigated whether exogenous GC could modify F-actin levels in GC^{-/-} islets to
331 restore α cell activity. Using a published RNA-seq dataset (19), transcripts for the endocytic
332 receptors responsible for GC uptake, megalin (*Lrp2*) and cubilin (*Cubn*) (14; 39-41), were
333 found to be expressed in purified α cells at a similar level to the gastric inhibitory polypeptide
334 receptor (*Gipr*) (normalized expression: 8.9 ± 5.3 versus 9.7 ± 3.4 versus 8.0 ± 7.0 for *Lrp2*
335 versus *Cubn* versus *Gipr*, respectively) (taken from (GSE76017)). As expected from this, GC
336 levels could be restored in HFD GC^{-/-} islets following incubation with exogenous protein (Figure

337 5A-C). Confirming the directionality of F-actin changes, treatment of HFD GC^{-/-} islets with GC
338 restored F-actin levels to wild-type levels (Figure 5D), as seen throughout the islet as well as
339 in individual α cells (Figure 5C-E).

340 As expected, low glucose (G0.5)-stimulated glucagon secretion was impaired in HFD GC^{-/-}
341 islets (22). Pertinently, application of GC restored normal glucagon secretion in HFD GC^{-/-}
342 islets, without influencing the function of HFD GC^{+/+} islets (Figure 5F). The effects of GC on
343 glucagon secretion were not associated with increases in intracellular Ca²⁺ concentration,
344 which was slightly but significantly decreased in GC-treated islets (Figure 5G and H).
345 Reflecting either the lowered glucagon tone or decreased F-actin in GC^{-/-} islets, insulin
346 secretion failed to shut off at low glucose (0.5 mM) (Figure 5I), an effect remarkably similar to
347 that reported when the small GTPase and actin polymerizer, RhoA, is inhibited in α cells (42).
348 GC treatment was unable to restore this defect or influence basal insulin levels in either GC^{+/+}
349 or GC^{-/-} islets (Figure 5I). By contrast, GC treatment led to a large (~ 10-fold) amplification of
350 glucose-stimulated insulin secretion, with a greater effect in HFD GC^{-/-} islets (Figure 5J)

351 Finally, as a proof of principle, we were able to show that GC could be supplemented in human
352 islets, leading to increases in glucagon granule area as well as F-actin density (Figure 5K-M),
353 visualized at ~110 nm resolution using structured illumination microscopy.

354 **GC expression is decreased in islets of T2D donors**

355 In pancreas sections from non-diabetic (ND) donors, GC expression was only present in α
356 cells, as expected (18; 22; 43) (Figure 6A). While a similar staining pattern was observed in
357 pancreas sections from T2D donors, GC expression levels were markedly (~ 2-fold) reduced
358 (Figure 6A). Some inter-individual variability was observed, but reduced GC expression
359 appeared to be a remarkably consistent feature of T2D (Figure 6B and C). Reflecting findings
360 in HFD mice, analysis of individual α cells in T2D donors revealed a decrease in cell size
361 (Figure 6D and E). While proportion of islet area occupied by δ cells was unchanged during
362 T2D, δ cell size was slightly but significantly reduced (Figure 6F-I).

363 Linear regression showed a strong correlation between GC and glucagon expression in α cells
364 from donors without diabetes (Figure 6J). Whilst a significant linear correlation was also
365 detected for individuals with T2D, the strength of correlation was much lower (Figure 6K),
366 consistent with the reported decrease in GC expression (Figure 6B), as well α cell glucagon
367 expression (Figure 6L). As expected from this, the regression slopes were significantly
368 different between ND and T2D samples ($P < 0.001$). Together, these analyses show that
369 glucagon expression co-varies with GC expression and that this relationship is partly lost
370 during T2D.

371

372 **DISCUSSION**

373 In the present study, we show that deletion of GC in HFD-fed animals leads to basal
374 hyperglucagonemia and impaired low glucose-stimulated glucagon secretion. These
375 secretory defects are associated with changes in Ca²⁺ fluxes, α cell, β cell and δ cell size and
376 mass, as well as F-actin and G-actin abundance. α cell function can be restored in GC^{-/-} islets
377 by using exogenous GC, which is taken up into cells following culture. Lastly, islets from
378 donors with T2D show decreases in GC expression, with concomitant changes in α cell and δ
379 cell size and mass. Together, these results expand our previous findings on GC by revealing
380 its regulatory role in glucagon secretion during metabolic stress, and further suggest that GC
381 is a pivotal component of the α cell phenotype in health and disease. While GC is a signature
382 gene expressed in α cells, the current study shows that α cells also have the potential to
383 acquire GC via megalin-mediated endocytosis. This raises the possibility that circulating levels
384 of GC may also contribute to α cell GC-actin dynamics and phenotype.

385 *In vivo* metabolic phenotyping demonstrated that GC^{+/+} and GC^{-/-} mice possess similar glucose
386 excursion curves in response to intraperitoneal glucose injection. However, basal plasma
387 glucagon concentrations were consistently raised in GC^{-/-} mice, in line with a tendency toward
388 elevated glucagon secretion from isolated islets at 17 mM glucose, which would be expected
389 to increase hepatic glucose output. One possible explanation for the apparently normal
390 glucose homeostasis is that the increase in glucagon levels is not sufficient to influence insulin
391 counter-regulation, or might even act to prime β cells for insulin secretion (44; 45).
392 Alternatively, recent studies have shown that *Gc* is upregulated in de-differentiated β cells and
393 deletion of *GC* increases glucose-stimulated insulin secretion and liver insulin sensitivity at 12
394 weeks HFD (24). In any case, these data show that HFD-induced basal hyperglucagonemia
395 (46) is further aggravated following GC deletion. We were unable to reliably detect significant
396 increases in *in vitro* insulin secretion or β cell function at 4-8 weeks HFD, arguing against this
397 possibility here, although we concede that clamp studies are needed to properly assess this.

398 Moreover, GC expression was variably upregulated in β cells, remaining much lower than the
399 levels seen in α cells.

400 Plasma glucagon levels, stimulated by insulin injection, were almost identical in HFD-fed GC^{+/+}
401 and GC^{-/-} mice, despite impaired glucagon release from isolated islets incubated in low (0.5
402 mM) glucose. We found, however, that the effect of GC deletion was milder in islets exposed
403 to sub-maximal (2 mM) glucose concentration, which would be closer to that achieved in vivo.
404 Together, these data suggest that GC is relatively more important in alpha cells operating
405 close to their functional ceiling, with the caveat that in vitro glucagon secretion assays might
406 be less sensitive at 2 mM glucose due to the relatively smaller magnitude change. Along
407 similar lines, HFD-fed GC^{-/-} mice presented with basal hyperglucagonemia at blood glucose
408 concentrations ~ 10-11 mmol/L, whilst in isolated islets basal glucagon secretion was similar
409 in GC^{-/-} and GC^{+/+} mice at high glucose. One explanation for this discrepancy might lie in the
410 finding that glucose-stimulated insulin secretion was almost 2-fold increased in HFD-fed GC^{-/-}
411 islets. In vivo, relative hyperinsulinemia would be expected to drive hyperglucagonemia to
412 maintain blood glucose levels, which were not different between HFD-fed GC^{+/+} and GC^{-/-}
413 mice. Another explanation might lie in the changes in α cell morphology observed in pancreas
414 sections taken from HFD-fed GC^{-/-} animals. A decrease in α cell size might lead to an increase
415 in α cell membrane juxtaposed with the islet capillaries, favoring release of glucagon into the
416 circulation.

417 During standard diet, we showed that loss of GC leads to a large increase in the density of
418 the F-actin cytoskeleton (and concomitant decrease in G-actin), acting as a physical barrier
419 against exocytosis of glucagon granules during low glucose stimulation (22). Changes in the
420 F-actin and G-actin cytoskeleton occur throughout the islet, since ~ 50% of GC is present in
421 glucagon granules and can readily be taken up by neighboring cells by endocytosis, as shown
422 here following application of exogenous GC. Following 4-8 weeks HFD, F-actin density was
423 increased almost 2-fold in GC^{+/+} islets. On a background of metabolic stress, deletion of GC
424 did not further increase F-actin density. In fact, HFD GC^{-/-} islets showed a surprising reduction

425 in F-actin density, contrary to our previous findings in standard diet islets (22). A similar
426 decrease in F-actin density was seen in GC^{-/-} islets stimulated with low glucose for 60 mins.
427 Notably, treatment with exogenous GC replenished intracellular GC and F-actin levels in HFD
428 GC^{-/-} islets, confirming that GC acts to increase F-actin density during metabolic stress. Given
429 that GC is a potent actin scavenger, what might be the mechanisms involved in this apparent
430 decrease in F-actin? A likely mechanism revolves around G-actin, which was virtually
431 undetectable in HFD GC^{-/-} islets. Without G-actin to supply monomers, polymerized F-actin
432 cannot be formed. Indeed, previous reports by us have shown a similar decrease in F-actin in
433 trophoblasts depleted for GC, which was associated with an increase in G-actin monomers in
434 the nucleus where they are unavailable for assembly into polymerized F-actin (39). Another
435 mechanism might be a large compensatory upregulation in gelsolin, which severs F-actin into
436 G-actin (36; 47), although we would expect this to be associated with an increase in G-actin
437 levels.

438 Kuo *et al.* recently reported that GC^{-/-} islets show an insulin signaling/sensitivity defect, but
439 exhibit a normal glucagon phenotype under both standard diet and high fat diet conditions
440 (24). Since our studies used islets from animals on 4 and 8 week HFD, we cannot exclude
441 that glucagon secretion in GC^{-/-} animals/islets normalizes in line with improved β cell function
442 at 12 weeks, the feeding period used by Kuo et al. We also used a different GC knockout
443 mouse line, which might give rise to a different phenotype. However, it should be noted that
444 these animals are well-validated by multiple groups and show complete loss of GC in α cells
445 and the liver, undetectable circulating GC based upon LC-MS, and a 90% reduction in
446 circulating 25(OH)D in homozygotes (22; 25). Suggesting that GC plays a critical role in α cell
447 biology: 1) GC is an α cell signature gene; 2) GC protein expression is upregulated during
448 HFD, remaining 10-100-fold higher than that in β cells; and 3) defects in α cell function have
449 a clear mechanistic basis, including changes in cell morphology, cell mass, cytoskeletal
450 structure and ionic fluxes, shown also by recent Patch-seq studies (8). Nonetheless, these
451 studies together posit that, depending on duration of metabolic stress, effects of GC deletion

452 (and GC supplementation) can be seen on both the insulin and glucagon axes. Further studies
453 using conditional GC deletion in α cells and β cells are warranted.

454 Studies in human donor pancreas sections revealed that GC and glucagon expression are
455 positively associated, with levels co-varying across hundreds of individual cells examined, a
456 relationship that was lost during T2D. Mechanistically, this observation likely reflects changes
457 in the alpha cell transcription factor network, since GC possesses cell type-selective open
458 chromatin regions (18). Ultimately, however, altered gene regulation must impact functional
459 protein targets, and our in vitro findings support the notion that the disrupted relationship
460 between GC and glucagon might contribute to impaired glucagon secretion during T2D.
461 Further studies are warranted in isolated human islets to investigate the effects of silencing
462 GC on glucagon expression and secretion in α cells.

463 Changes in the actin cytoskeleton could be rescued using exogenous GC. In the kidney, GC-
464 bound 25(OH)D is taken up by facilitated endocytotic uptake via the megalin-cubulin complex
465 (14; 41; 48), where liberated 25(OH)D is then converted to 1,25(OH)₂D. Immunostaining
466 clearly showed dose-dependent uptake of GC into islets, demonstrating that similar transport
467 mechanisms also exist in the pancreas, as suggested by published RNA-seq data (49). These
468 data suggest that, unusually, decreases in expression of a key cell signature gene can be
469 offset by supplementing its protein product and warrant further investigation of the uptake
470 mechanisms involved. While these results point to GC as a therapeutic target, caution should
471 be extended due to opposing effects of GC on both the α cell and β cell compartments (24).
472 However, it should be noted that high glucose levels have been shown to inhibit megalin-
473 mediated endocytosis, which might differentially affect GC uptake into α cells and β cells (50).
474 Moreover, molecular addresses such as V1BR could be used to target GC specifically to α
475 cells (51-53). In any case, we envisage that GC administration during type 2 diabetes could
476 maintain α cell function, whilst restraining β cell proliferation and hyperinsulinemia, known to
477 drive insulin resistance (54; 55). Our data in human pancreas sections supports a reduction

478 in GC during type 2 diabetes, lending further weight to this argument. Nonetheless, careful
479 preclinical studies in mice at various timepoints are required to assess this.

480 There are a number of limitations in the present study. Firstly, we used a well-phenotyped
481 global GC^{-/-} mouse model in which GC is undetectable in the circulation. Whilst GC^{-/-} mice are
482 vitamin D sufficient (22), we cannot exclude that loss of circulating GC influences α cell
483 phenotype and function. In the future, it will be worthwhile conditionally deleting GC in the α
484 cell or liver to explore the role of circulating GC in α cell and more widely islet function.
485 Secondly, we decided to investigate HFD at 4 and 8 weeks, since longer feeding periods did
486 not lead to further changes in glucose tolerance. In any case, this length of HFD allowed α cell
487 function to be determined without any confounding effects caused by GC upregulation in the
488 β cell compartment, as shown by our immunohistochemical analyses. Thirdly, the mild in vivo
489 phenotype seen in HFD-fed GC^{-/-} mice might reflect compensation, especially since the gene
490 was (presumably) deleted during development. Finally, whilst GC supplementation increased
491 glucagon granule area/density in human α cells, it should be noted that the decrease in GC
492 levels seen in samples from T2D donors is associative and might be a consequence rather
493 than a cause of changes in α cell morphology and function.

494 In summary, we show that α cells lacking GC fail to adapt properly to metabolic stress,
495 displaying a range of defects leading to impaired basal and low glucose-stimulated glucagon
496 release. Given its role under both normal and obesogenic conditions, GC should thus be
497 considered as a key regulator of α cell function and glucagon secretion.

498

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515 D.J.H. receives licensing revenue from Celtarys Research. K.V., M.H. and D.J.H. are named
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517 **AUTHOR CONTRIBUTIONS**

518 K.V., D.N., J.A., A.H., F.C., L.J.B.B. and S.H. performed experiments and analyzed data.
519 D.J.H. and M.H. conceived and designed the studies. D.J.H. and M.H. supervised the studies.
520 D.J.H., M.H. and K.V. wrote the paper with input from all authors. M.H. and D.J.H are the
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526

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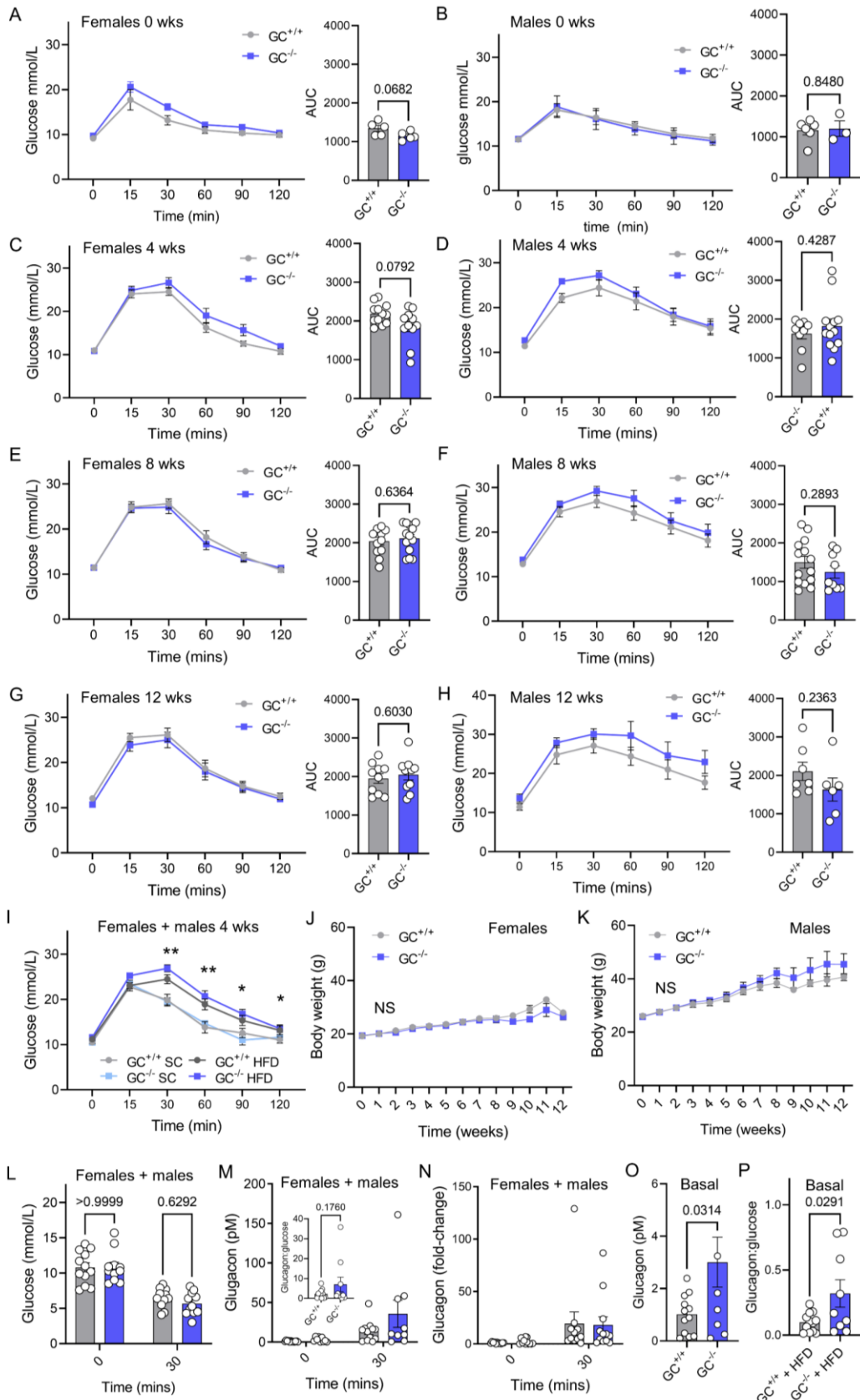
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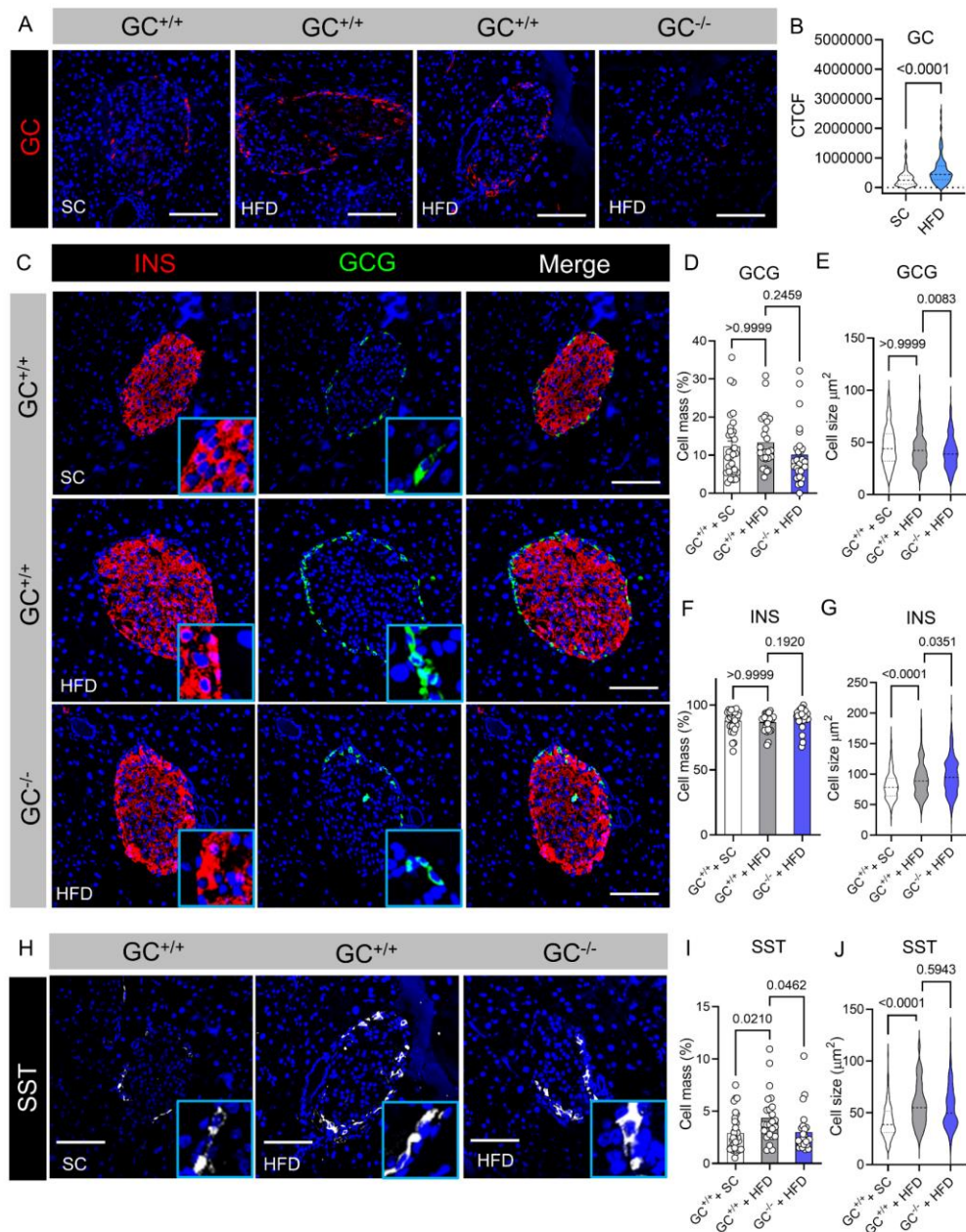
701 **Figure 1: Glucose tolerance, body weight and glucagon secretion in GC^{+/+} and GC^{-/-}**

702 **mice during high fat diet.** A-H) Intraperitoneal glucose tolerance is similar in female and
703 male GC^{+/+} and GC^{-/-} mice during 0 weeks (A, B), 4 weeks (C, D), 8 weeks (E, F) and 12 weeks
704 (G, H) HFD, as shown by bar graphs and AUC (0 weeks, n = 3-6 animals; 4 weeks, n = 9-13
705 animals; 8 weeks, n = 9-14 animals; 12 weeks, n = 6-10 animals) (two-way RM ANOVA with
706 Sidak post-hoc test for line graphs; Mann-Whitney test or unpaired t-test for AUC). I) Pooled
707 data from age-matched male and female mice fed SC or HFD for 4 weeks (n = 5-25 mice)
708 (two-way RM ANOVA with Tukey's post-hoc test). J and K) Body weight gain is similar in
709 female (J) and male (K) GC^{+/+} and GC^{-/-} mice during 0-12 weeks HFD (two-way ANOVA with
710 Sidak post-hoc test). L) Glucose responses to intraperitoneal injection of insulin, used to
711 stimulate glucagon release, are not significantly different in GC^{+/+} and GC^{-/-} mice at 0 mins and
712 30 mins (n = 11-12 animals) (two-way RM ANOVA with Sidak post-hoc test). (M and N) Basal
713 but not insulin-stimulated plasma glucagon concentrations are significantly higher in GC^{-/-}
714 versus GC^{+/+} mice, shown by raw values (M) fold-change (N) (n = 9-12 animals) (unpaired t
715 test) (inset in M is glucagon:glucose ratio). O and P) Basal glucagon levels from (M) shown in
716 a separate graph for clarity, alongside glucagon:glucose ratio at t = 30 mins post insulin
717 injection (P). Glucose and glucagon readings in L-P are from the same mice, albeit with
718 dropout of two samples in which glucagon was undetectable by ELISA. AUC, area-under-the-
719 curve. SC, standard chow; HFD, high-fat diet.

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724 **Figure 2: α cell, β cell and δ cell morphometry in $GC^{+/+}$ and $GC^{-/-}$ mice during high fat**

725 **diet. A, B) GC expression levels are increased 2-fold following 8 weeks HFD compared to SC**

726 (A; left two image panels), quantified in (B) using corrected total cell fluorescence (CTCF).

727 Note that immunopositivity is detected in HFD-fed $GC^{+/+}$ and not $GC^{-/-}$ mice, thus validating

728 the antibody under the conditions used here (A; right two image panels). C-G) Proportion α

729 cells per islet (C, D) ($n = 26-32$ islets from 3-4 mice), as well as α cell size (C, E) ($n = 233-301$

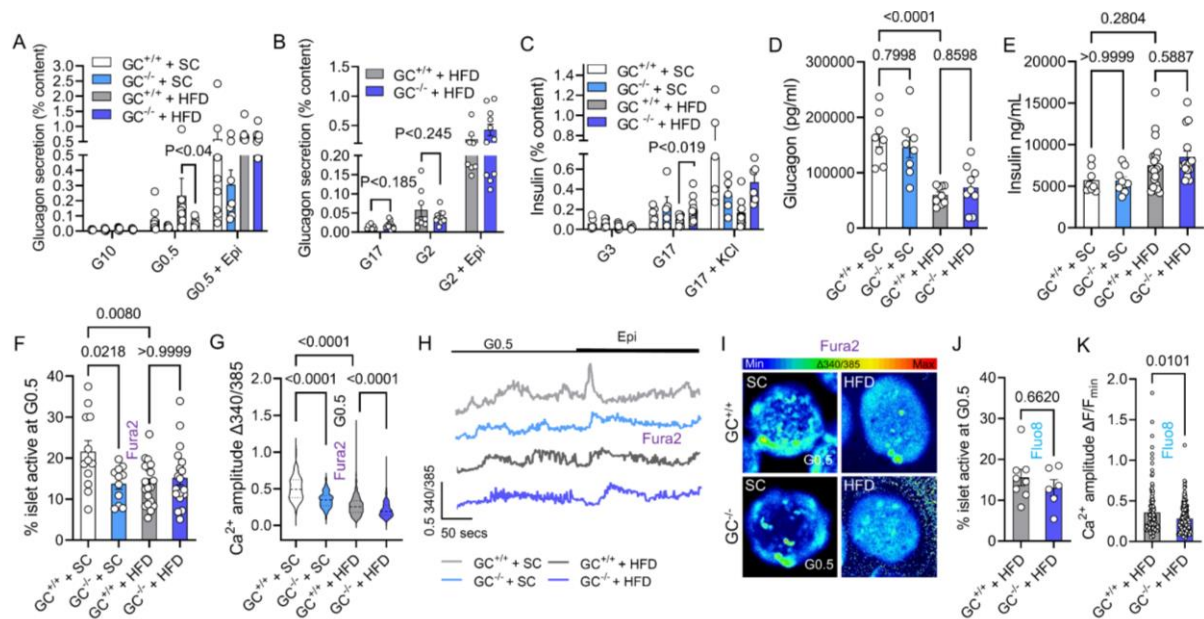
730 cells from 3-4 mice), is not affected by 8 weeks HFD in $GC^{+/+}$ mice. Deletion of GC ($GC^{-/-}$)

731 leads to fewer and smaller α cells per islet (C-E) (proportion α cell per islet, $n = 26-32$ islets

732 from 4 mice) (α cell size, $n = 233-301$ cells from 3-4 mice). HFD (8 weeks) does not increase
733 proportion β cells per islet (C, F) ($n = 26-32$ islets from 3-4 mice), but increases β cell size, an
734 effect accentuated following deletion of GC ($GC^{-/-}$) (C,G) (proportion α cells per islet, $n = 26-$
735 32 islets from 3-4 mice) (β cell size, $n = 240-320$ cells from 3-4 mice) (one-way ANOVA with
736 Bonferroni's post-hoc test). H-J) HFD (8 weeks) increases proportion δ cells per islets, as well
737 as δ cell size in $GC^{+/+}$ but not $GC^{-/-}$ islets ($GC^{-/-}$) (proportion δ cells per islet, $n = 24-31$ islet from
738 3-4 mice) (δ cell size, $n = 117-294$ cells from 3-4 mice) (one-way ANOVA with Bonferroni's
739 post-hoc test). Scale bar = $85 \mu\text{m}$. GC, GC-globulin, GCG, glucagon, INS, insulin, SST,
740 somatostatin. SC, standard chow; HFD, high-fat diet.

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744 **Figure 3: Hormone secretion and ionic fluxes in islets isolated from high fat diet-fed**

745 **GC^{+/+} and GC^{-/-} mice.** A) Low glucose (0.5 mM)-stimulated glucagon secretion is impaired in

746 SC and HFD islets isolated from GC^{-/-} versus GC^{+/+} mice (SC, n = 4-5 animals; HFD, n = 7

747 animals) (Mann-Whitney test). B) As for A) but showing a tendency toward elevated basal

748 glucagon secretion and impaired low glucose-stimulated glucagon secretion in HFD islets from

749 GC^{-/-} versus GC^{+/+} mice (n = 3-4 animals) (Student's t-test). C) Glucose-stimulated insulin

750 secretion is significantly increased in GC^{-/-} islets isolated from HFD- but not SC-fed animals

751 (SC, n = 7-8 replicates from 3-5 animals; HFD, n = 7-8 replicates from 4-5 animals) (Mann-

752 Whitney test). D and E) Total glucagon (D) and insulin (E) concentration is similar in GC^{+/+} and

753 GC^{-/-} islets isolated from SC and HFD-fed mice (n = 4-8 animals) (two-way ANOVA with

754 Bonferroni's post-hoc test). F) Proportion of α cells active at low glucose (0.5 mM) was reduced

755 in GC^{-/-} islets from SC-fed but not HFD-fed mice (versus GC^{+/+} littermate controls) (n = 11-19

756 islets from 3-4 animals) (one-way ANOVA with Bonferroni's post-hoc test). G-I) Amplitude of

757 Ca²⁺ spikes (at 0.5 mM glucose) was reduced in GC^{-/-} versus GC^{+/+} islets from mice on SC.

758 HFD-alone reduced Ca²⁺ spike amplitude and baseline Ca²⁺ concentration, an effect

759 accentuated by deletion of GC (GC^{-/-}). Bar graphs (G) show summary data, traces (H) and

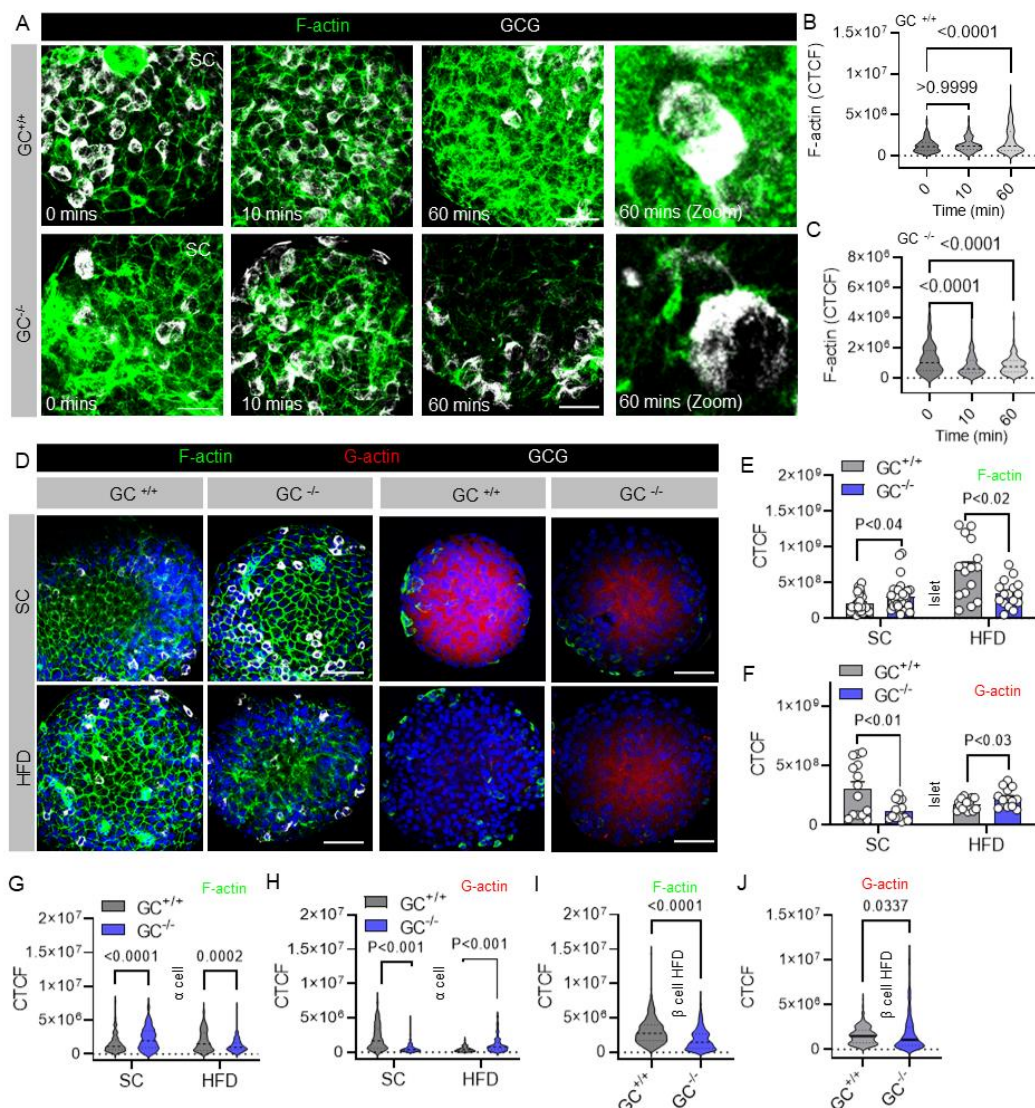
760 images (I) are representative (one-way ANOVA with Bonferroni's post-hoc test) (n = 184-339

761 cells from 3-4 animals). J and K) As for F and G, but using the non-ratiometric Ca²⁺ indicator,

762 Fluo8 (J, n = 6-8 islets from 2-3 animals; K, n = 50-79 cells from 2-3 animals) (Mann-Whitney
763 test). GC, GC-globulin; G0.5, 0.5 mM glucose; G2, 2 mM glucose; G3, 3 mM glucose, G10,
764 10 mM glucose; G17, 17 mM glucose. SC, standard chow; HFD, high-fat diet.

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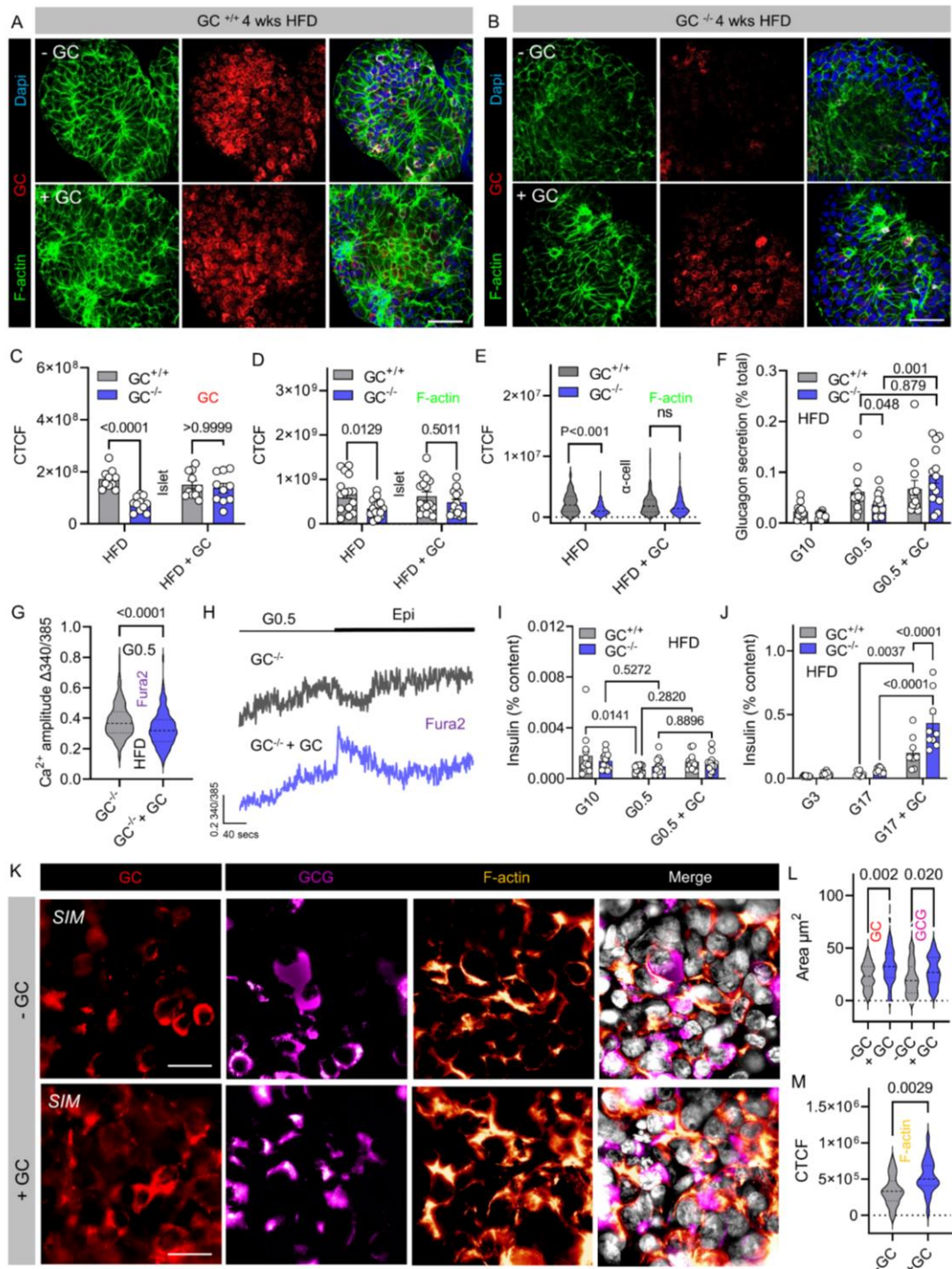
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768 **Figure 4: F-actin and G-actin levels in islets isolated from high fat diet-fed GC^{+/+} and GC^{-/-}**
 769 **mice.** (A-C) Polymeric actin (F-actin) levels increase and decrease in SC GC^{+/+} and GC^{-/-}
 770 islets, respectively, following 60 mins stimulation with low (0.5 mM) glucose concentration, as
 771 shown by representative images (A) and summary bar graphs (B and C) (n = 110-145 α cells
 772 from 3 animals) (one-way ANOVA with Bonferroni's post-hoc test) (scale bar = 34 μ m). D-F)
 773 F-actin density is increased in GC^{-/-} islets from SC-fed mice. HFD induces a large increase in
 774 F-actin density in GC^{+/+} islets, which can be partly reversed by deletion of GC (GC^{-/-}).
 775 Monomeric G-actin, which is liberated following F-actin disassembly, shows the opposite
 776 trend. Representative images show F-actin and G-actin levels in the islet (D), analyzed in (E

777 and F) using corrected total cell fluorescence (CTCF) (Mann-Whitney test or unpaired t test)
778 (n =15-30 islets from 3 animals) (scale bar = 53 μ m).G-J), As for D-F, but CTCF analysis of F-
779 actin and G-actin in HFD α cells (G and H) and β cells (I and J) (two-way ANOVA with
780 Bonferroni's post-hoc test) (n = 159-176 cells from 3 animals). SC, standard chow; HFD, high-
781 fat diet.

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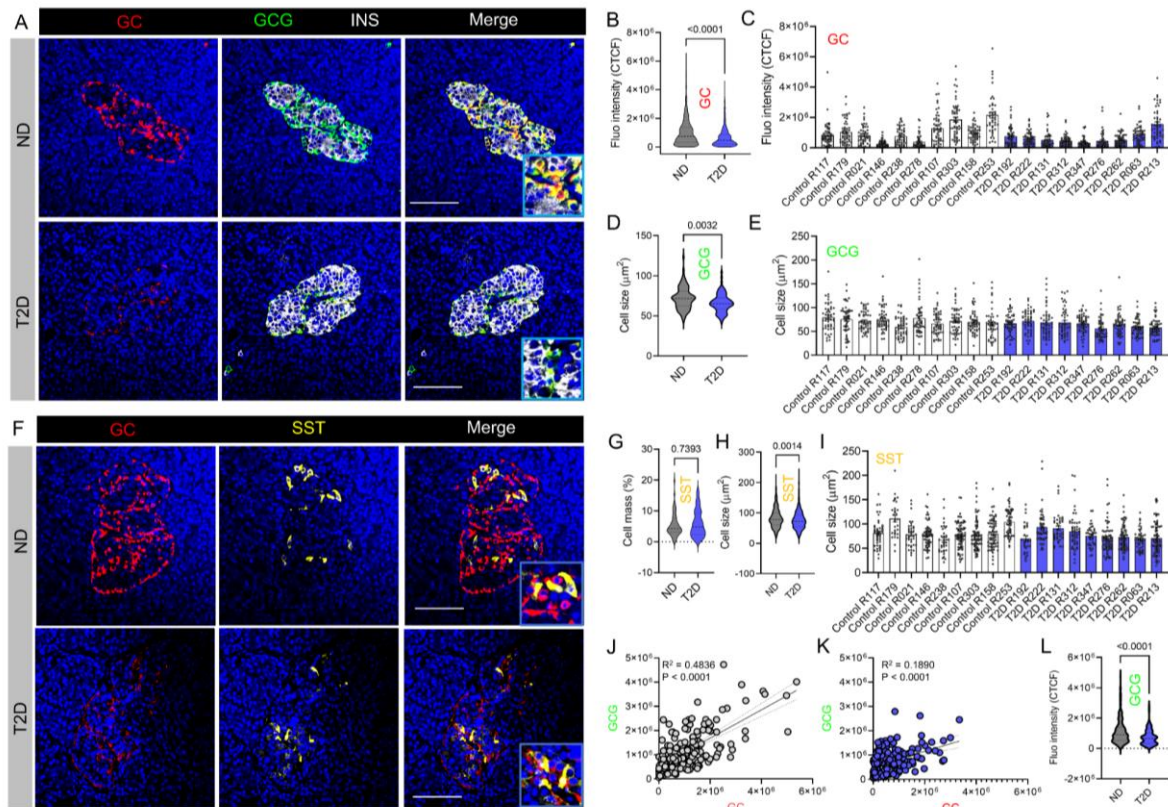
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785 **Figure 5: Effects of exogenous GC supplementation in islets from high fat diet-fed GC^{+/+}**
 786 **and GC^{-/-} mice.** A and B) Representative images showing that GC expression and F-actin
 787 levels can only be modified in HFD GC^{-/-} islets following incubation with GC (scale bar = 53
 788 μm). C and D) Corrected total cell fluorescence (CTCF) analysis showing a significant
 789 increase in GC (C) and F-actin (D) levels in GC-treated HFD GC^{-/-}, but not GC^{+/+}, islets (n =

790 10-11 islets from 3 animals) (two-way ANOVA with Bonferroni's post-hoc test). E) As D, but
791 F-actin levels in individual α cells (n = 10-11 islets from 3 animals) (two-way ANOVA with
792 Bonferroni's post-hoc test). (F) Exogenous GC restores low glucose (G0.5)-stimulated
793 glucagon secretion in HFD GC^{-/-} islets (n = 14-16 repeats from 5-7 animals) (Mann-Whitney
794 test). G and H) Exogenous GC does not affect low glucose (G0.5)-stimulated Ca²⁺ rises in
795 HFD GC^{-/-} islets, shown by amplitude (G) and representative traces (H) (n = 174-205 cells
796 from 4 animals). I) HFD GC^{-/-} islets fail to shut off insulin secretion at low glucose, although
797 GC treatment itself is unable to influence basal insulin release (n = 14 repeats from 4 animals).
798 J) GC treatment amplifies glucose-stimulated insulin secretion, with a larger effect in HFD GC^{-/-}
799 ^{-/-} compared to GC^{+/+} islets (n = 9 repeats from 2-3 animals) (two-way ANOVA with Bonferroni's
800 post-hoc test). K-M) GC levels can be supplemented in human islets (K, L), leading to an
801 increase in glucagon granule area (L) and F-actin density (M). Note that the GC images are
802 not from the same islets as those showing glucagon and F-actin. Scale bar = 15 μ m. GC, GC-
803 globulin; GCG, glucagon; G0.5, 0.5 mM glucose; HFD, high-fat diet.

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807 **Figure 6: GC and glucagon expression in islets from donors with and without type 2**
 808 **diabetes.** A) Representative images from non-diabetic (ND) and type 2 diabetes (T2D) donors
 809 showing a large reduction in GC expression, as well as decrease in α cell size. B and C)
 810 Quantification using corrected total cell fluorescence (CTCF) reveals a highly significant
 811 decrease in GC expression in T2D versus ND donors (B), which is consistent across individual
 812 donors (C) (ND, n = 89 cells from 10 donors; T2D, n = 82 cells from and 9 donors) (Mann-
 813 Whitney test). D and E) α cell size is significantly decreased in T2D versus ND donors (D),
 814 again consistent across donors (E) (ND, n = 495 cells from 10 donors; T2D, n = 450 cells from
 815 and 9 donors) (Mann-Whitney test). F) Representative images from non-diabetic (ND) and
 816 type 2 diabetes (T2D) donors showing a large reduction in GC expression, as well as decrease
 817 in δ cell size. (G-I) δ cell mass (G) is similar in ND and T2D donors, whereas δ cell size is
 818 reduced during T2D (H), consistent across individual donors (I) (ND, n = 174 islets from 9
 819 donors; T2D, n = 210 islets from and 9 donors). (J and K) GC and glucagon expression are
 820 strongly correlated in α cells of ND (J), but not T2D donors (K) (linear regression). (L) Glucagon

821 expression is significantly decreased in T2D versus ND donors (ND, n = 200 cells from 10
822 donors; T2D, n = 180 cells from 9 donors). Scale bar = 85 μ m. GC, GC-globulin, GCG,
823 glucagon, INS, insulin, SST, somatostatin.

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