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Inhibition of eIF5A hypusination reprogrammes metabolism and glucose handling in mouse kidney

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29 **Abstract.**

30 Inhibition of the eukaryotic initiation factor 5A activation by the spermidine analogue GC7 has
31 been shown to protect proximal cells and whole kidneys against an acute episode of ischemia.
32 The highlighted mechanism involves a metabolic switch from oxidative phosphorylation toward
33 glycolysis allowing cells to be transiently independent of oxygen supply. Here we show that GC7
34 decreases protein expression of the renal GLUT1 glucose transporter leading to a decrease in
35 transcellular glucose flux. At the same time, GC7 modifies the native energy source of the proximal
36 cells from glutamine toward glucose use. Thus, GC7 acutely and reversibly reprograms function
37 and metabolism of kidney cells to make glucose its single substrate, and thus allowing cells to be
38 oxygen independent through anaerobic glycolysis. The physiological consequences are an
39 increase in the renal excretion of glucose and lactate reflecting a decrease in glucose reabsorption
40 and an increased glycolysis. Such a reversible reprogramming of glucose handling and oxygen
41 dependence of kidney cells by GC7 represents a pharmacological opportunity in ischemic as well
42 as hyperglycemia associated pathologies from renal origin.

43

44 **keywords:** metabolic switch, GC7, GLUT, glycolysis, glucose reabsorption, kidney

45

46 **Introduction**

47 The eukaryotic initiation factor eIF5A is the only protein known to be activated by the post
48 translational transformation of a specific lysine residue to hypusine through the so-called
49 hypusination process¹ which is mediated by the successive catalytic action of deoxyhypusine
50 synthase (DHS) and deoxyhypusine hydroxylase (DOHH)². A unique feature of eIF5A is that it
51 seems necessary for translation of a limited number of mRNA through its ability to bind RNA in a
52 sequence specific manner³. eIF5A was also shown to be involved in the synthesis of only 5% of
53 the total protein content in mammalian cells⁴ and particularly those containing polyproline

54 sequences⁵. eIF5A is present as two isoforms: eIF5A1 which is ubiquitously expressed and
55 eIF5A2 which is restricted to few tissues and is a hallmark of numerous types of cancer⁶. eIF5A
56 and its hypusination step has been also described as involved in HIV-1 replication⁷, malaria
57 disease⁸ or diabetes^{9,10}. Interestingly, a strong relationship between hypoxia tolerance and the
58 hypusination of eIF5A has been highlighted in drosophila model¹¹. Considering this new concept
59 we have recently shown in mammals that the specific inhibition of eIF5A hypusination by the
60 spermidine analogue N1-guanyl-1,7-diamine-heptane (GC7)¹² is able to enhance the ischemic
61 tolerance both at the cellular and tissue level in a rat kidney model of ischemia/reperfusion injury¹³.
62 Based on these results, we successfully used GC7 both in preclinical model of kidney
63 transplantation in pig¹⁴ and in a transient focal cerebral ischemia (tFCI) model in mice¹⁵. Indeed,
64 acute systemic administration of GC7 in the donor allowed better functional recovery of the kidney
65 graft in the first case^{13,14} and reduced the infarct volume, and motor and cognitive post-stroke
66 deficits in the second¹⁵. At the cellular level one of the first observations reported on cultured
67 mouse kidney cells treated with GC7 was a metabolic shift from aerobic oxidative phosphorylation
68 toward anaerobic glycolysis decreasing consequently oxygen consumption. In parallel, GC7
69 treated kidney cells displayed a mitochondrial remodeling characterized by a downregulation of
70 respiratory chain complexes expression and activity while the ATP content remained high¹³. These
71 observations have been recently corroborated in macrophages¹⁶.

72 In vertebrates the ischemia tolerance depends in part upon their ability to urgently use
73 anaerobic glycolysis to ensure their energy supply. This switch is time dependent and not adapted
74 to an acute episode of ischemia. Thus, the ischemic tolerance reported through GC7 treatment
75 suggests a pharmacological conditioning metabolic effect. Nonetheless, the molecular mechanism
76 that support this process remains to be unraveled. Proximal cells manage glucose reabsorption
77 while they do not use it as a source of energy for their own¹⁷. The transepithelial transport of
78 glucose occurs in two steps¹⁸ : 1) an apical membrane entry via SGLT1 and SGLT2, two sodium-

79 linked cotransporters 2) a basolateral export mediated by the facilitated glucose transporters
80 GLUT1 and GLUT2. In this study, we aimed to understand how kidney cells conciliate glucose
81 reabsorption function and anaerobic glycolysis under GC7 treatment. We finally propose that the
82 pharmacological inhibition of eIF5A hypusination by GC7 reversibly reprograms the use of glucose
83 by the proximal cells toward glycolysis parallelly to an impairment of renal glucose reabsorption
84 due to GLUT1 misexpression. This leads to a deep and reversible cell metabolism remodeling
85 allowing survival of kidney cells to oxygen deprivation. These results highlight a therapeutic
86 opportunity in conditions of glucose homeostasis unbalance.

87

88 **Material & Methods.**

89 **Reagents**

90 N-guanyl-1,7-diaminoheptane (GC7) was synthesized by AtlanChim Pharma (Saint-Herblain,
91 France) according to the methods described by Jasiulionis et al.¹⁹ Canagliflozin was purchased
92 from InVitrogen (Cergy Pontoise, France). Culture media, buffer solutions, fetal bovine serum
93 (FBS) and other culture reagents were from Sigma-Aldrich (Saint-Quentin Fallavier, France).

94 **Animals**

95 The experiments were conducted in accordance with the French and European regulations
96 (2010/63/EU directive) for the care and use of research animals and were approved by national
97 experimentation committees (MESR N°: APAFIS#22670-2019101811258232).

98 10-week-old C57BL/6 male mice from Janvier Laboratory (France) were maintained at housing
99 temperature (22°C) and 12:12-hour light-dark cycles, with ad libitum access to food and water.
100 Mice were daily treated with GC7 (3 mg/kg in saline solution, intraperitoneal injection, n=12, "GC7"
101 group) or with vehicle only (saline solution NaCl 0.9% w/v, intraperitoneal injection, n=12, "ctrl"
102 group) for three days. After the first injection, mice were individually housed and acclimated alone

103 in calorimetric cages (Oxylet-Physiocage Panlab, Bioseb, Vitrolles, France) for 24 hours before
104 experimental measurements.

105 **Cell Culture**

106 Renal proximal convoluted tubule cells (PCT) were obtained from primary cultures of murine
107 proximal tubule segments, immortalized with pSV3neo vector and were cultured as previously
108 described^{20,21}. Cultures were classically maintained in a 5% CO₂/95% air water-saturated
109 atmosphere in M1 medium (DMEM/F12, Glutamine, SVF, EGF, T3, dexamethasone, ITS, G418).
110 All experiments were performed the day after cell confluence.

111 **Cell metabolism analysis**

112 The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of PCT cells
113 were determined using an XF24 Extracellular Flux Analyser (Seahorse Bioscience). Uncoupled
114 and maximal OCR were determined using oligomycin (1.2 µM) and FCCP (1 µM). Rotenone and
115 Antimycin-A (2 µM each) were used to inhibit mitochondrial respiration. All parameters were
116 calculated as described previously²².

117 **Cell survival analysis**

118 The Fluorescent LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit (Invitrogen, France) was
119 used on PCT cells (24-well plates), according to the manufacturer's protocol. Fluorescent
120 micrographs were recorded using an observer D1 microscope (Zeiss, Germany) and analysed
121 using imageJ software.

122 **Protein analysis**

123 Whole proteins from cells and tissues were prepared using TNET lysis buffer (25 mM Tris-Cl
124 (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P40, 1x protease inhibitor
125 cocktail and 1x Phosphostop mix (Roche Diagnostics, Meylan, France)). In addition, tissues were

126 solubilized using Precellys tissue homogenizer in ice-cold buffer and using CK14 beads (Bertin
127 Technologies, Ozyme). For membrane protein enriched lysates, cells were washed twice with cold
128 PBS, once with cold water and finally incubated in hypotonic buffer (Tris 10 mM, EDTA 1 mM,
129 pH7.5, 1x protease inhibitor cocktail) 10 min on ice. Cells were scratched and disrupted using a
130 25G needle. Crude lysate was centrifuged first at 2000 g (10 min, 4°C) to eliminate undisturbed
131 cells and nuclei, and then at 10000 g (30 min, 4°C). Pellet containing membranes was re-
132 suspended in hypotonic buffer containing protease inhibitors.

133 Protein concentration was evaluated by BCA assay (PIERCE, Thermo Scientific, France) and
134 blotted using SDS-PAGE basic protocol. Primary antibody incubation was performed overnight at
135 4°C (eIF5A, Abcam #32443; GLUT1, Abcam #ab54460; GLUT2, Cell Signaling #54460; hypusine-
136 eIF5A, ¹³; β -actin, sigma # A5441) and then with adequate HRP-conjugated secondary antibodies
137 (Jackson ImmunoResearch, Interchim, France) (30 minutes, 1:10000, RT). Detection was
138 performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Molsheim,
139 France) and Fuji apparatus. Band intensities were evaluated using PCBas Software.

140 Lactate dehydrogenase (#K2228, APExBIO, Clinisciences) and glyceraldehyde 3-phosphate
141 dehydrogenase (#K680, BioVision, Clinisciences) cells content were evaluated using activity
142 assay and following manufacturer's instructions.

143 **Biochemical parameter analysis**

144 Urinary creatinine was assayed by colorimetric reaction (NaOH 0.75N/Picric acid 0.04 M V/V)
145 detected at 520 nm. Glucose was evaluated directly in plasma, urine and cell supernatant using
146 Glucose-Glo Assay (Promega) and following manufacturer's instructions. Cell glucose efflux was
147 directly measured in glucose free media at indicated time. Cell glucose consumption was
148 evaluated by assay of glucose decrease in full media containing 4.5 mg/L glucose at indicated
149 time and normalized by glucose efflux measured at the same time.

150 Glucose uptake was measured using 2-deoxy-d-[³H]-glucose (2-DG). After two washes with
151 PBS, cells were incubated 10 min with 100 μM 2-DG and 1 μCi 2-deoxy-D-[³H] glucose. Culture
152 plates were put on ice and rinsed with ice-cold PBS. Cells were scraped in 0.5N NaOH, neutralized
153 with 0.5N HCl and 2-deoxy-d-[³H]-glucose uptake was measured by liquid scintillation counting of
154 cell lysate with a beta-counter.

155 Ion concentrations (Cl⁻, lactate, Na⁺, K⁺) were evaluated by ion chromatography analysis. All
156 biological samples (plasma, urine and cell supernatant) as well as ion standard solutions were
157 previously deproteinized by addition of acetonitrile (dilution 1:1 volume). Samples were strongly
158 mixed and centrifuged at 12.000g (10min at 4°C). Ion concentrations of the supernatant were
159 determined using an ion chromatography Dionex ICS-5000 plus system (Thermo Scientific). The
160 system included an autosampler, pumps, eluent generator and conductivity detectors. The system
161 was equipped with 2 eluent generator cartridges (Dionex EGC500KOH; Dionex EGC500MSA),
162 an anion column (IonPac CS17, 2 mm) and a cation column (IonPac AS-11 HC, 2mm). Ion
163 concentrations were determined using Chromeleon software (Thermo Scientific) by measuring
164 surface area of the peaks and were compared to the corresponding ion standard profiles.

165 **mRNA analysis**

166 Procedures follow MIQE recommendations ²³. Total RNA was extracted using TRIzol
167 (Invitrogen) according to the manufacturer's instructions. In addition, tissues were solubilized
168 using Precellys tissue homogenizer in TRIzol reagent and using CK14 beads. Reverse
169 transcription-polymerase chain reaction (RT-PCR) was performed using M-MLV-RT (Promega).
170 SYBR qPCR premix Ex TaqII from Takara (Ozyme, France) was used for quantitative PCR
171 (qPCR), and assays were run on a StepOne Plus ABI real-time PCR machine (PerkinElmer Life
172 and Analytical Sciences, Boston). The expression of selected genes was normalized to that of the
173 TATA-box binding protein (TBP) and β-actin housekeeping genes, and then quantified using the
174 comparative-ΔCt method. Primer sequences are available upon request.

175 **Histology and immunohistochemistry**

176 Freshly sampled tissues were fixed in 10% buffered formalin overnight at RT and then paraffin-
177 embedded. Embedded tissues were cut in 5 µm sections and dried 30 min at 55°C. For
178 immunohistochemistry, sections were then deparaffinized in xylene, rehydrated through alcohol,
179 and washed in phosphate-buffered saline (PBS). Antigen retrieval was performed using Vector
180 unmasking reagent (Vector Laboratories). Sections were blocked with MOM blocking solution
181 (MOM kit, Vector Laboratories), then incubated with mouse monoclonal primary antibody anti-
182 GLUT1 (Abcam, #ab40084) diluted in MOM diluent for overnight at 4°C. Detection was performed
183 with Alexa647-conjugated donkey anti-mouse secondary antibody (ThermoFisher Scientific).
184 GLUT1 stained kidney sections were sequentially scanned for far red signal allowing imaging of a
185 large area of the sections. GLUT1 signal analysis was then performed using ImageJ software
186 (Supplemental Fig. 4).

187 **Statistical analysis**

188 Data were analysed using GraphPad Prism 6 software. Calorimetry data were analysed using
189 two-way ANOVA multiple comparison and activity using multiple t-test. Other data were analysed
190 by Mann-Whitney (2 groups) or Kruskal-Wallis (more than 2 groups) test to assess statistical
191 differences between experimental groups. Differences were considered statistically significant
192 with $p < 0.05$. Data were displayed as scatter plot of independent values and group mean values \pm
193 SD.

194

195 **Results**

196 **GC7 promotes anaerobic glycolysis.**

197 We firstly show that GC7, which induced a decrease of eIF5A hypusination in the kidney *in*
198 *vivo*¹³, had the same effect in our *in vitro* model of PCT cells. Fig. 1a shows that GC7 treatment
199 (24 h, 30 μ M) induced a decrease of eIF5A hypusination in these cells issued from proximal
200 tubules²⁴. The dose of 30 μ M we used was previously shown to be the lowest dose with a maximal
201 protective effect on the anoxia induced cell death¹³. Since the metabolic switch observed involves
202 a mitochondrial down-regulation¹³ we investigated how GC7-induced switching from oxidative
203 phosphorylation to glycolysis affects glucose and the overall metabolism of PCT cells treated 24
204 hours with GC7. Mitochondrial oxygen consumption rate (OCR) of GC7 treated cells maintained
205 in glucose free medium was very low and not modulated by addition of 10 mM glucose. By
206 contrast, PCT control cells exhibited a higher rate of basal O₂ consumption but remained also
207 insensitive to the addition of glucose (Fig. 1b). Using a substrate free medium, we demonstrated
208 that PCT control cells increased their mitochondrial O₂ consumption upon addition of 2 mM
209 glutamine instead of glucose, and that GC7 treated cells were unable to increase their O₂
210 consumption whatever the substrate used (Fig. 1c). The pH analysis of both types of cells revealed
211 an increase in extracellular acidification upon glucose addition (Fig. 1d, e) and not with that of
212 glutamine (Fig. 1e). However, this increase was three-fold higher in GC7 treated cells (Fig. 1d, e).
213 This metabolic analysis demonstrated that GC7 treatment switched PCT cells from amino-acids
214 dependent oxidative phosphorylation and used glucose instead of glutamine as energetic
215 substrate through the anaerobic glycolysis pathway.

216 **GC7 enhances glucose consumption**

217 We then analysed the kinetic of lactate efflux and glucose consumption in PCT cells after 24
218 hours of GC7 treatment (Fig. 2a-b). Correlatively to media acidification analysis, the lactate efflux
219 was increased (three-fold more at 8 hours) in GC7 treated cells (Fig. 2a). This was associated to
220 a significant increase in glucose consumed by the cells (Fig. 2b). Looking for a modification in
221 glucose flux, we analysed the glucose uptake using 2-deoxy-D-[³H] glucose. Such analysis

222 revealed a significant decrease of glucose uptake in GC7 treated cells (Fig. 2c). This modification
223 was not linked to a decrease in Na⁺ dependent glucose transporters SGLT1 and SGLT2
224 (responsible of glucose uptake from nephron lumen by proximal cells *in vivo*) mRNA expression
225 (Fig. 2d).

226 **SGLT2 glucose entry is essential to GC7 treated cell metabolism and survival**

227 As glucose enters proximal cells mainly by the Na⁺-glucose transporters SGLT2, and in a lower
228 extent by SGLT1¹⁸ in proximal tubule cells, we used the SGLT2 inhibitor canagliflozin to disrupt
229 this crucial step in control and GC7 treated cells (30 μM, 24h). Pretreatment (4 h) as well as acute
230 treatment with 10 μM canagliflozin blunted medium acidification of PCT cells upon addition of
231 glucose and consequently anaerobic glycolysis (Fig. 3 a-b). Moreover live/dead analysis after 24
232 hours of treatment with 10 μM canagliflozin revealed a cytotoxic effect of the SGLT2 inhibition
233 leading to a mortality of approximately 75% in GC7 treated cells, but not in control canagliflozin
234 treated PCT cells and untreated cells that both remained alive (Fig. 3c). Thus, glucose entry via
235 SGLT2 transporter is required for survival of GC7 treated PCT cells contrary to control cells.

236 **Glycolysis is the exclusive energy source in GC7 treated cells**

237 To evaluate the importance of glucose as energy source for GC7 treated PCT cells, we treated
238 or not PCT cells with GC7 (30 μM, 24h) and analysed their metabolism by sequential addition of
239 glucose and 2-deoxy-glucose (2-DG) (Fig. 4a-b). As previously shown, the respiratory capacity of
240 PCT cells were not modified after glucose addition and were insensitive to 2-DG (Fig. 4a).
241 Differently, glucose addition increased slightly extracellular acidification rate (ECAR) in control
242 cells and strongly in GC7 treated ones whereas 2-DG inhibited it close to the basal level (Fig. 4b).
243 This is confirmed by the measurement of lactate efflux that showed that 2-DG treatment inhibited
244 the release of lactate in control and GC7 treated PCT cells (Fig. 4c). Thus, the weak glycolytic
245 activity displayed by control cells was notably increased by GC7. Survival of PCT cells, which

246 displayed a basal glycolysis, was not affected by addition of 2-DG (Fig. 4d). Indeed, these cells
247 use glutamine-fueled oxidative phosphorylation as energy source (Fig. 1). By contrast, GC7
248 treated cells displayed more than 90 % of mortality under 2-DG treatment (Fig. 4d), demonstrating
249 clearly that glucose dependent glycolysis is the unique energy source of the GC7 treated cells.

250 **GC7 inhibits glucose efflux in proximal cells and represses GLUT1 expression**

251 As proximal cells mediate glucose reabsorption into the interstitial milieu *in vivo*, we looked
252 for their *in vitro* glucose efflux capacity. As shown in Fig. 5a, the glucose efflux was decreased in
253 GC7 treated PCT cells and reaches a more than two-fold difference after 8 hours of treatment. In
254 proximal cells the glucose efflux corresponds to a passive transport through both the GLUT1 and
255 GLUT2 facilitated transporters expressed at the basolateral membrane, so we analysed the effect
256 of GC7 on their expression. The GLUT1 protein expression was impaired in GC7 treated PCT
257 cells whereas that of GLUT2 was not significantly modified (Fig. 5b).

258 **LDH and PDH activities are not involved in the GC7-induced metabolic shift.**

259 The lack of oxidative phosphorylation could be due to a high activity of lactate dehydrogenase
260 (LDH) reducing pyruvate availability to enter mitochondria. We did not find any difference between
261 untreated and GC7 treated cells in LDH activity in whole cell lysates (Supplemental Fig. 1a).
262 Moreover, we used oxamate, a noncompetitive inhibitor of LDH. Oxamate treatment (100 mM) of
263 control and GC7 treated cells inhibited strongly the lactate efflux (Supplemental Fig.1b) and in a
264 lesser extent media acidification (Supplemental Fig.1c). Analysis of oxygen consumption did not
265 reveal any effect of oxamate in control and GC7 treated cells (Supplemental Fig.1d). Thus,
266 increasing pyruvate availability in PCT cells did not favor its use by mitochondria. Moreover,
267 oxamate treatment did not lead to cell death neither in control nor in GC7 treated cells
268 (Supplemental Fig. 1e) suggesting that cell survival was not affected under oxamate treatment
269 contrarily to canaglifozin or 2DG. These results demonstrate that GC7-induced metabolic shift was
270 not linked to LDH activity.

271 Pyruvate dehydrogenase (PDH), a key enzyme of OXPHOS, can be inactivated by multiple
272 phosphorylations of serine residues (Ser232, Ser239 and Ser300) catalyzed by pyruvate
273 dehydrogenase kinases (PDK). In control PCT cells PDH was highly phosphorylated and these
274 phosphorylations were decreased in the presence of 5 mM dichloroacetate (DCA) (Supplemental
275 Fig. 2a) an inhibitor of PDK, which maintained PDH in an active form. Surprisingly, in GC7 treated
276 cells all residues were found dephosphorylated close to the values found after DCA treatment
277 (Supplemental Fig. 2a). As DCA promotes pyruvate entry into the Krebs cycle, we checked for
278 oxygen consumption in GC7 treated and untreated cells and submitted to DCA (5 mM, 24h). As
279 expected, DCA increased OCR in control cells allowing activation of PDH. In contrast no change
280 was observed in GC7 treated cells (Supplemental Fig. 2b) demonstrating that pyruvate cannot be
281 used as substrate for oxidative phosphorylation and is preferentially reduced in lactate. In the
282 same way no change by DCA treatment was found in lactate efflux (Supplemental Fig. 2c) as well
283 as the cell survival rate (Supplemental Fig. 2d) of GC7 treated cells.

284 **The GC7 induced metabolic switch is fully reversible.**

285 We next analysed the recovery of the OXPHOS pathway following GC7 removal. Confluent
286 PCT cells were treated for 24 h with 30 μ M GC7. Afterward GC7 was removed and cells were
287 maintained in culture medium for an additional period of 72 h. Seahorse experiments revealed
288 that GC7 pre-treated PCT cells recovered a basal OCR close to the one measured in control
289 condition (Fig. 6a) in the presence of 10 mM glucose. In the same way the ECAR of these cells
290 returned to the values observed in control cells (Fig. 6b). This indicates that PCT cells are able to
291 recover an OXPHOS confirming the reversion of the GC7 effect. From a functional point of view,
292 Fig. 6c shows that 72 h after GC7 removal the glucose efflux of PCT cells, which is fully impaired
293 by GC7, is hugely recovered. Parallely the glucose consumption tends to return toward the value
294 observed in control cells (Fig. 6d). These data demonstrate that the mitochondrial “silencing”
295 induced by GC7 is reversible as far as both the metabolic and functional features of PCT cells are

296 concerned. This conclusion was reinforced by the fact that GLUT1 expression which is fully
297 repressed by GC7 is back following the recovery period (Fig. 6e).

298 **Impact of GC7 treatment on mice physiological parameters**

299 We looked *in vivo* for the metabolic and main physiological effects associated to the overall
300 modifications observed *in vitro*. Mice were treated daily with GC7 (IP, 3mg/kg/day; “GC7” group)
301 or with vehicle only (NaCl 0.9% w/v; “ctrl” group) for 72 hours, according to the protocol
302 demonstrated to have beneficial effect in rodent and pig models^{13,14,15}. No difference was found
303 between control and GC7 treated mice for sodium, potassium and chloride ion contents in plasma
304 and urine (Supplemental Fig. 3a). Interestingly, while glucose and lactate plasma levels were
305 unchanged upon GC7 treatment (Supplemental Fig. 3b), urinary glucose and lactate were
306 significantly higher in GC7 compared to control mice when reported to urinary creatinine (Fig. 7a).
307 Moreover, urinary creatinine was not significantly different between the two groups suggesting an
308 unaltered renal function (Fig. 7b). To verify that these phenotypes were linked to GC7 effect, we
309 confirmed that eiF5A hypusination was decreased in the kidney after 3 days of GC7 treatment
310 (Fig. 7c).

311 **GLUT1 expression is impaired in kidneys from GC7 treated mice.**

312 As the increase in glucosuria and lactaturia found in mice could be due to the altered
313 expression of glucose transporters shown *in vitro* (Fig. 6e), we studied the effect of GC7 treatment
314 on the expression of glucose transporters in mice kidneys. As observed in PCT cells we retrieved
315 a decrease in the protein level of GLUT1, but not of GLUT2, in whole kidney protein extract from
316 GC7 treated mice (Fig. 7c). To confirm the GLUT1 misexpression in kidney, we evaluated its
317 expression in renal cortical region by immunohistochemistry using sagittal kidney sections from
318 control and GC7-treated mice. As displayed in Fig. 7d, the expression of GLUT1 is clearly
319 noticeable in control kidneys at the basolateral membrane of the tubules while its expression is
320 impaired in term of labeling intensity (Fig. 7d) and number of positive tubules in kidneys from GC7-

321 treated mice as evaluated by quantifying the labelled areas of each sections (Fig. 7e,
322 Supplemental Fig. 4 and 5).

323

324 **Discussion**

325 We have previously demonstrated that inhibition of eIF5A hypusination, through conditioning
326 treatment with the spermidine analogue GC7, led to anoxic tolerance in kidney cells^{13,14} and
327 neurons¹⁵ both *in vitro* and *in vivo*. Tolerance to anoxia due to GC7 treatment has been shown to
328 be linked to an inhibition of mitochondrial activity together with an increase in anaerobic glycolysis
329 that allowed cell survival by maintaining basal ATP synthesis and membrane integrity. Here we
330 are advancing in understanding this mechanism by demonstrating a phenotypical switch of the
331 proximal tubule cells treated with GC7, from a full functional state to a survival state based on the
332 fate of glucose. In *in vivo* control condition proximal convoluted tubular cells uptake glucose *via*
333 SGLT2 transporters family in the S1 segment and drive it out to the interstitial space *in vivo* using
334 the high affinity GLUT1 and low affinity GLUT2 facilitated glucose transporters²⁵. Moreover, these
335 cells do not use this glucose as an energy source for their own and rather generate ATP from
336 oxidative phosphorylation of glutamine^{17,26}. Herein, we demonstrate that GC7 treatment inhibits
337 the efflux of glucose (i.e. glucose reabsorption) and mitochondrial oxidative phosphorylation,
338 which leads to the establishment of anaerobic glycolysis to ensure the energy demand necessary
339 for cell survival (Fig. 8). Interestingly, while under GC7 treatment the cells were unable to use
340 another source of energy than glucose, this capacity was not lost as the cells retrieve normal
341 metabolism and function after GC7 removal^{13,14}.

342 This functional and metabolic shift allows cells to become transiently independent of the
343 environmental oxygen and thus to resist to the deep decrease in oxygen concentration resulting
344 either from anoxia *in vitro* or ischemia *in vivo*¹³. With regards to kidney transplant where the GC7-
345 conditioning lasts a couple of days after treatment, cells resist to the high deleterious

346 reoxygenation step characterizing the reperfusion step *in vivo*¹⁴. Anoxia tolerance of proximal
347 tubular cells is a crucial challenge as they are highly dependent on oxidative metabolism and they
348 do not use glucose as energy source¹⁷. Thus, opportunistically the GC7 takes advantage of this
349 high availability of glucose to make these cells independent of oxygen by imposing the
350 implementation of an anaerobic glycolytic metabolism.

351 The functional and metabolic shift observed *in vitro* in proximal tubule cells treated by GC7
352 leads to a decrease in glucose reabsorption by the kidney *in vivo*. Indeed, the increase in
353 glucosuria highlights a deficit in glucose reabsorption and the increase in lactaturia is linked to the
354 anaerobic glycolysis setting up along the nephron. This is reinforced by the fact that lactate plasma
355 level is not modified by GC7 treatment. Surprisingly the decrease in glucose uptake found *in vitro*
356 was not linked to sglT expression alteration, while sglT glucose transport is required for PCT cells
357 survival. SGLT co-transporters are electrogenic due to their coupling with Na⁺ and their activities
358 depend both on the plasma membrane potential and the inward Na⁺ gradient, thus we suspect
359 that GC7 treatment could modify the membrane potential and/or the inward directed sodium
360 gradient resulting in an apparent independence between their expressions and transport
361 capacities. We cannot exclude a more general effect of GC7 on whole body metabolism which
362 could be compensated by other physiological mechanisms. Hepatic clearance is the main
363 mechanism regulating hyperlactatemia, in addition to other tissues as skeletal muscles, heart and
364 kidney proximal tubule which clear lactate by converting it to pyruvate²⁷. *In vitro* we demonstrate
365 that proximal cells are unable to use lactate as substrate, suggesting that kidney cells are not
366 involved in lactate clearance. We know that GC7 can perform this metabolic switch in other organs
367 and cells such as the brain and neurons¹⁵. Therefore, considering our findings in kidney cells, it is
368 tempting to assume that an epithelium with equivalent glucose metabolism may also be targeted
369 and thereby protected by GC7.

370 *In vitro* we demonstrated that inhibition of eIF5A hypusination leads to a shift from
371 mitochondrial oxidative phosphorylation to anaerobic glycolysis. Nevertheless, the pathway
372 governing GC7 effect still needs to be clarified. Indeed, inhibition of mitochondrial activity could
373 promote metabolic shift and favor anaerobic glycolysis leading to inhibition of glucose efflux to
374 preserve cell integrity. The low level of PDH phosphorylation found in GC7 treated cells,
375 demonstrating the inability of mitochondria to perform oxidative phosphorylation even in the
376 presence of pyruvate, could suggest that the mitochondria "silencing" occurred before the onset
377 of glycolysis. Another way would be that the increase in glucose availability, due to GLUT1
378 misexpression, enhances glucose metabolism concomitantly to inhibition of mitochondria activity,
379 which ultimately leads to an anaerobic metabolism. In the context of an increased glycolysis a
380 parallel point has also to be studied in the future: it is the effect of GC7 on the pentose pathway
381 involving glucose 6 phosphate that is produced early in glycolysis and that provides carbon
382 skeleton for the synthesis of nucleic acids. So many questions that deserve to be studied in order
383 to fill in the gaps of the molecular mechanisms linking eIF5A hypusination inhibition to metabolic
384 shift. Puleston and collaborators demonstrated that GC7 treatment of macrophages leads to an
385 inhibition of several mitochondrial proteins explaining a shift to anaerobic glycolysis¹⁶. These
386 authors explained the decrease in a subset of mitochondrial proteins by a specific defect of their
387 translation due to a specific motif hypersensitive to eIF5A hypusination¹⁶. These interesting results
388 obtained *in vitro* involves that all cells displaying mitochondrial metabolism must be sensitive to
389 GC7 treatment, which may not be the case especially *in vivo* were GC7 treatment did not imply a
390 general shift toward anaerobic glycolysis in our model and others^{9,13,14,15,28} .

391 It is known that *dhps* deficiency (the gene encoding DHS) or chronic treatment with DHS
392 inhibitors, as GC7, ameliorate glucose tolerance and glycemia in various mouse models of
393 diabetes (HFD^{29,30}, STZ^{9,31}, humanized mouse model of T1D³², db/db³³; NOD³⁴). All these exciting
394 studies relied hypusination inhibition effect to beta cell mass protection and decreased pancreas

395 inflammation. Unfortunately, none of these works studied peripheral effects of GC7 and especially
396 its impact on intestine and renal functions, as we demonstrated in this work. Thus, it will be very
397 interesting to further analysed GC7 effect on glucose reabsorption functions in mouse models of
398 diabetes in order to determine if eIF5A hypusination inhibition in this context could participate to
399 glucose tolerance improvement. One of the more striking result of this study is the drastic inhibition
400 of GLUT1 expression consecutive to GC7 treatment, an effect that is fully reversible in a couple
401 of days and devoid of side effects in our time frame. Indeed, the physiological consequence of this
402 misexpression leads to an inhibition of glucose reabsorption and an enhancement of glucosuria.
403 This could be of importance in clinic since it has been shown that an antisense GLUT1 transgene
404 could protect mesangial cells from glucose induction of GLUT1 and fibronectin expression that
405 could be beneficial in the setting of diabetes³⁵. The figure 8 summarizes the molecular effects we
406 evidenced and that are driven by GC7 treatment regarding glucose handling. The remaining
407 question is the link between eIF5A hypusination and GLUT1 expression. Indeed, GLUT1 amino
408 acid sequence does not include proline rich regions and its encoding mRNA sequence does not
409 contain consensus sequences described up to now as having a link with eIF5A^{3,36}. Thus, future
410 investigations are warranted to establish additional target motifs and recognition sites of
411 hypusinated eIF5A.

412 An important point is the complete reversion of the GC7 effect that demonstrates a non-
413 harmful effect on the mitochondrial network but rather a transitory “silencing” effect. In conclusion
414 such a reversible pharmacological reprogramming of glucose handling and oxygen dependence
415 by GC7 represents a pharmacological opportunity in ischemic as well as hyperglycemia
416 associated pathologies from renal origin.

417 **Authors contributions**

418 Michel Tauc, Didier Pisani: Equal contribution, conceptualization, project administration,
419 interpretation, planning administration, writing, funding acquisition, supervision.

420 Marc Cougnon, Romain Carcy, Nicolas Melis, Karine Dumas, Christophe Duranton, Isabelle
421 Rubera: Investigation, data acquisition and curation, visualization, methodology
422 Catherine Pons, Nicolas Soubeiran, Marina Shkreli: Investigation, methodology.
423 Nicolas Blondeau, Jean-François Tanti, Sébastien Giraud: investigation
424 Thierry Hauet, Luc Pellerin : interpretation, data analysis

425

426

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434 **Competing interests**

435 The authors declare no competing financial interests

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534

535 **Figure legends**

536 **Fig. 1. GC7 induces a switch in cell metabolism and substrate preference.**

537 Confluent PCT cells were treated or not with 30 μ M GC7 for 24 hours and then used for different
538 analysis. a/ Representative western blot displaying inhibition of eIF5A hypusination and ratio
539 between optical density of hypusine and eIF5A band, MW : Molecular weight. b to e/ Cells were
540 analysed using Seahorse technology to evaluate oxygen consumption (b, c) and extracellular
541 acidification (d, e) after addition of 10 mM glucose (b-e) or 2 mM glutamine (c, e). Addition of other
542 compounds are indicated. Dots displayed mean \pm SD of 3 independent experiments. * $p < 0.05$
543 evaluated using Mann-Whitney (b, d) or Kruskal-Wallis (c, e) tests.

544

545 **Fig. 2 GC7 increases glucose consumption and lactate efflux.**

546 Confluent PCT cells were treated or not with 30 μ M GC7 for 24 hours. a/ Measurement of lactate
547 media content related to whole cell proteins content. b/ Evaluation of cell glucose consumption
548 corresponding to the difference between measurement of the media glucose content decrease (in
549 the presence of glucose in media) and media glucose content increase (after glucose deprivation).

550 Dots displayed mean \pm SD of 3 independent experiments. * $p < 0.05$ evaluated using Mann-Whitney
551 (a, b) tests. c/ Glucose uptake measured using 2-deoxy-d-[³H]-glucose. Results displayed cpm of
552 2-deoxy-d-[³H]-glucose measured in cell lysate. d/ RT-qPCR analysis of glucose transporters from
553 SGLT family. Graphic displayed scatter plot of 9 (c) or 5 (d) independent values and mean \pm SD.
554 * $p < 0.05$ evaluated using Mann-Whitney test.

555

556 **Fig. 3. SGLT2 glucose entry is essential to GC7 treated cell metabolism and survival.**

557 a-b/ Confluent PCT cells were treated or not with 30 μ M GC7 for 24 hours and then glycolysis in
558 response to glucose addition (10 mM) was evaluated using Seahorse technology. Canagliflozin
559 (10 μ M) was added 4 hours before analysis or along analysis as indicated. Dots displayed mean
560 \pm SD of 3 independent experiments. c/ Mortality evaluation using live/dead cells fluorescence
561 assay of PCT cells co-treated with 10 μ M canagliflozin and 30 μ M GC7. Representative pictures
562 for each group are displayed and show live (green, calcein-FITC) and dead (red, ethidium bromide
563 homodimer) cells. Scale bar is indicated. Graphic displayed scatter plot of 4 independent values
564 and mean \pm SD. * $p < 0.05$ evaluated using Kruskal-Wallis (a, c) or Mann-Whitney (b) tests.

565

566 **Fig. 4. Glycolysis is essential to GC7 treated cell metabolism and survival.**

567 a-b/ Confluent PCT cells were treated or not with 30 μ M GC7 for 24 hours and then (a) oxygen
568 consumption (OCR) and (b) extracellular acidification (ECAR) rates were measured using
569 Seahorse technology. Analysed was performed first without glucose and then glucose (10 mM)
570 and 2-deoxy-glucose (2-DG, 25 mM) were sequentially added as indicated. c/ Confluent PCT cells
571 were treated or not with 30 μ M GC7 for 24 hours and by 25 mM 2-DG the last 8 hours. Graphic
572 displayed measurement of lactate media content after 8 hours related to whole cell proteins
573 content. d/ Confluent PCT cells were treated or not with 30 μ M GC7 and 25 mM 2-DG for 24 hours.

574 Mortality was evaluated using live/dead cells fluorescence assay. Representative pictures for each
575 group are displayed and show live (green, calcein-FITC) and dead (red, ethidium bromide
576 homodimer) cells. Scale bar is indicated. Graphics displayed scatter plot of 8 (a-b), 4 (c), 11 (d)
577 independent values and mean \pm SD. * $p < 0.05$ evaluated using Kruskal-Wallis.

578

579 **Fig. 5. GC7 modulates glucose flux in PCT cells.**

580 Confluent PCT cells were treated or not with 30 μ M GC7 for 24 hours and then used for different
581 analysis. a/ At the end of treatment cells were deprived in glucose and the efflux of glucose was
582 measured in the next 8 hours. Dots displayed mean \pm SD of 3 independent experiments. b/
583 Western blot analysis of GLUT1 and GLUT2 expressions in membrane enriched protein lysate
584 from PCT cells. β -Actin was used as loading control. MW: molecular weight. * $p < 0.05$ evaluated
585 using Mann-Whitney test.

586

587 **Fig. 6. The effect of GC7 is fully reversible.**

588 Confluent PCT cells were treated or not with 30 μ M GC7 for 24 hours and then GC7 was removed
589 and cells were maintained for an additional period of 72 h and analysed. a, b/ Cells were analysed
590 using Seahorse technology to evaluate oxygen consumption (a) and extracellular acidification (b)
591 in the presence of 10 mM glucose. Addition of other compounds are indicated. Dots displayed
592 mean \pm SD of 3 independent experiments. * $p < 0.05$ evaluated using Mann-Whitney. c/ At the end
593 of treatment cells were deprived in glucose and the efflux of glucose was measured in the next 8
594 hours. d/ Measurement of cell glucose consumption corresponding to the difference between
595 measurement of the media glucose content decrease (in the presence of glucose in media) and
596 media glucose content increase (after glucose deprivation). Dots displayed mean \pm SD of 3
597 independent experiments. * $p < 0.05$ evaluated using Mann-Whitney tests. 'Ctrl rev' are control cells

598 at 72 h without GC7 treatment and 'GC7 rev' correspond to cells 72 h after GC7 removal. 'Ctrl'
599 and 'GC7' are cells analysed after 24 h. (e) Western blot analysis of GLUT1 in membrane enriched
600 protein lysate from PCT cells following a 24h GC7 treatment (30 μ M) and after 72h of GC7 washout
601 (72h rev). β -Actin was used as loading control. MW: molecular weight. * $p < 0.05$ evaluated using
602 Mann-Whitney test.

603

604 **Fig. 7. Impact of a 3-days GC7 treatment on kidney glucose metabolism.**

605 Mice were treated 3 days with GC7 (3 mg/kg, i.p., "GC7" group) or vehicle only (NaCl, "ctrl" group)
606 and metabolic parameters were analysed at the end of the treatment. a/ Glucosuria
607 (glucose/creatinine) and lactaturia (lactate/creatinine) measured in bladder urine. b/ urinary
608 creatinine (mg.dL⁻¹). c/ Hypusination of eIF5A and GLUT1 and GLUT2 protein levels evaluated by
609 western blot using four kidney protein extract for each group. MW: molecular weight. Graphic
610 displayed hypusine/total eIF5A ratio and GLUT/ β -Actin ratio using optical density band values. d/
611 Mosaic reconstruction of whole kidney sections from untreated and GC7 treated mice displaying
612 GLUT1 signals in cortical region. Magnification of a representative cortical regions from each
613 group. Scale bars are indicated. e/ quantification of GLUT1 immunofluorescence. Graphics
614 displayed scatter plot of 4 (c), 6 (a, b) or 10 (e) independent values and mean \pm SD. * $p < 0.05$
615 evaluated using Mann-Whitney test.

616

617 **Fig. 8. Schema of GC7 effects on glucose handling in proximal cells.**

618 Schematic representation of normal and GC7 treated proximal cells regarding the glucose
619 handling. GC7 represses the oxidative phosphorylation mediated by the mitochondrial function
620 and enforces the cell to shift toward a glycolytic pathway to maintain energetic status. The apical
621 flux of glucose mediated by the SGLT2 transporter is thus necessary to feed this pathway and cell

622 survival becomes then largely independent of ambient oxygen. This reversible shift is
623 accompanied by a strong inhibition of the glucose reabsorption through the basolateral membrane
624 due to the down-regulation of the facilitated transporter GLUT1.

625

Figure 1

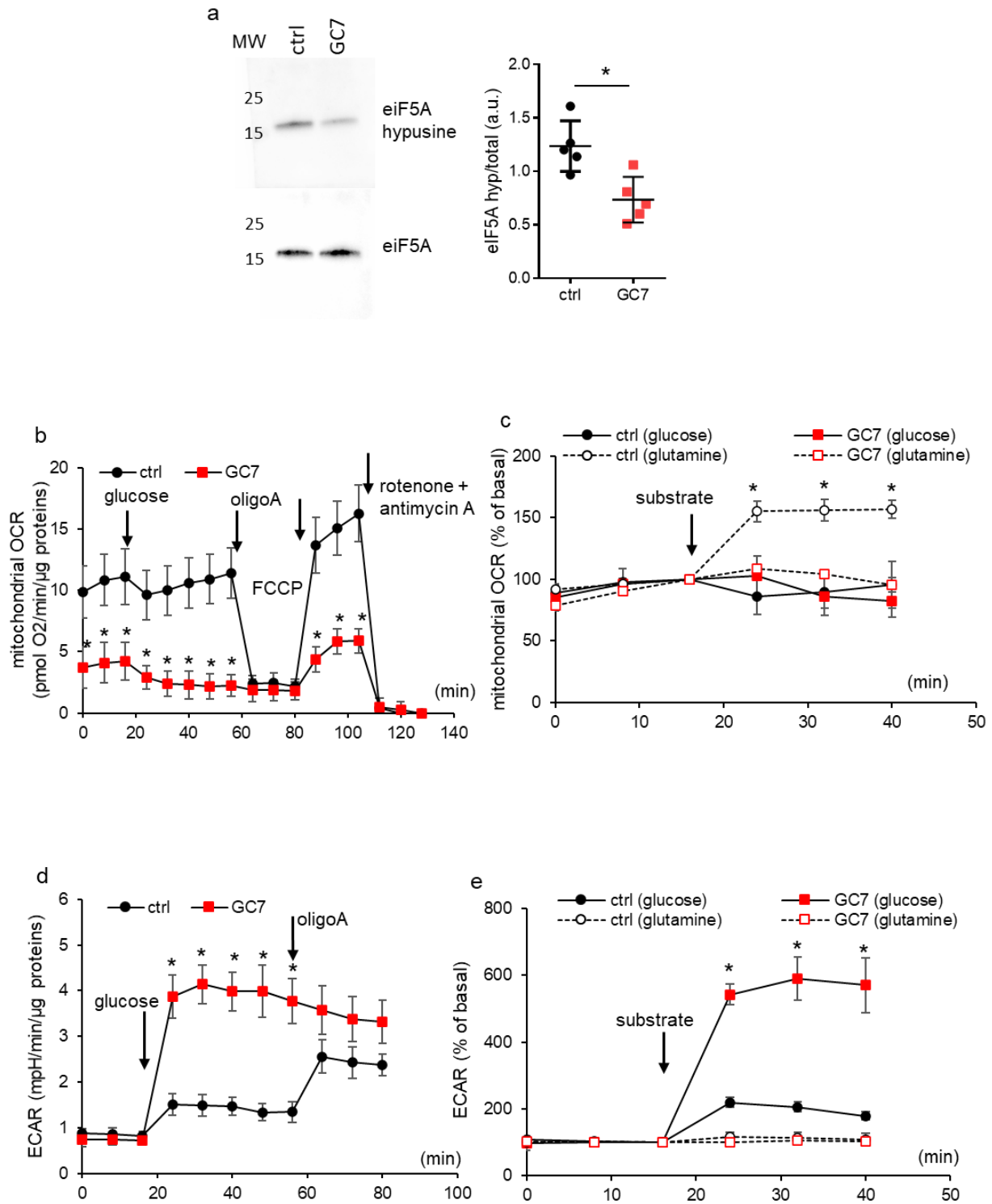
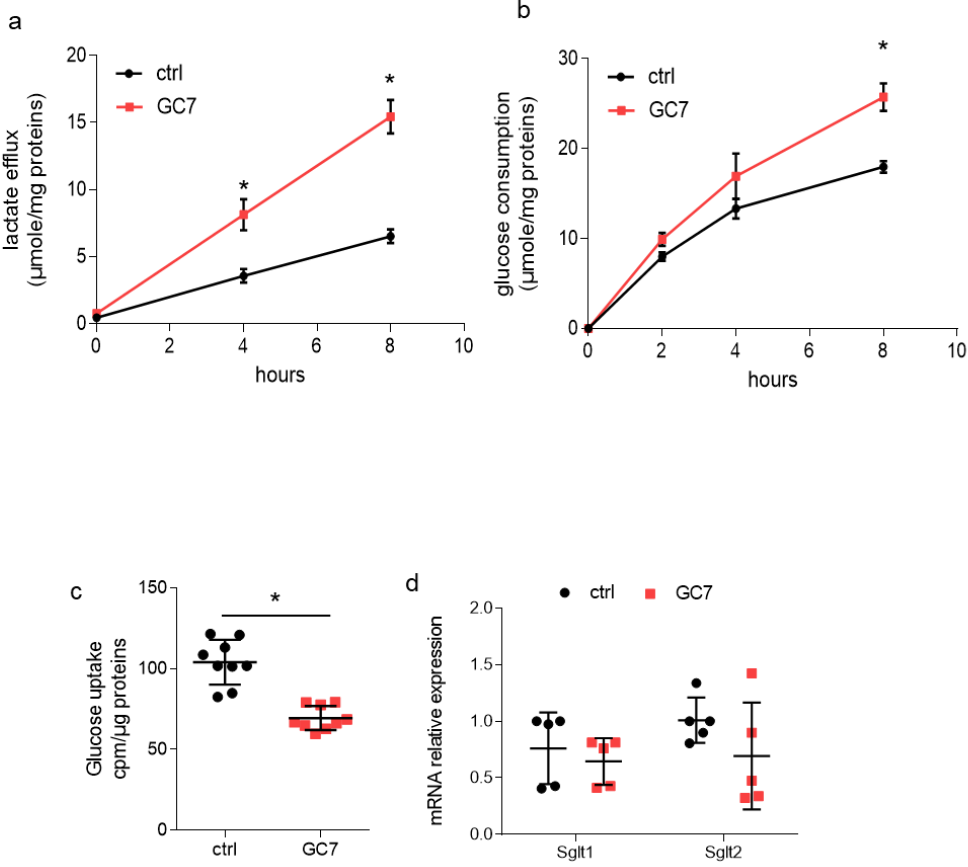
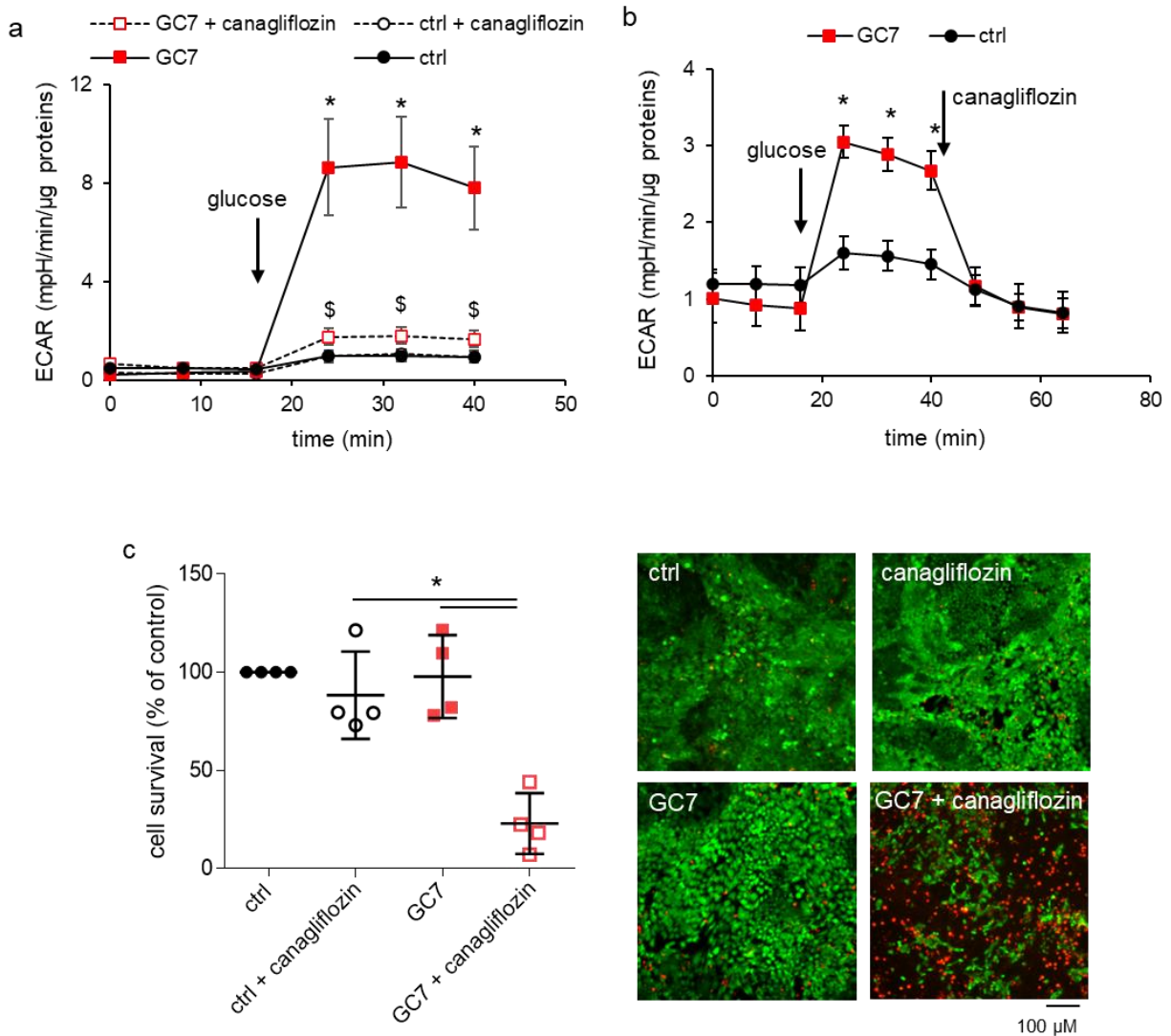


Figure 2



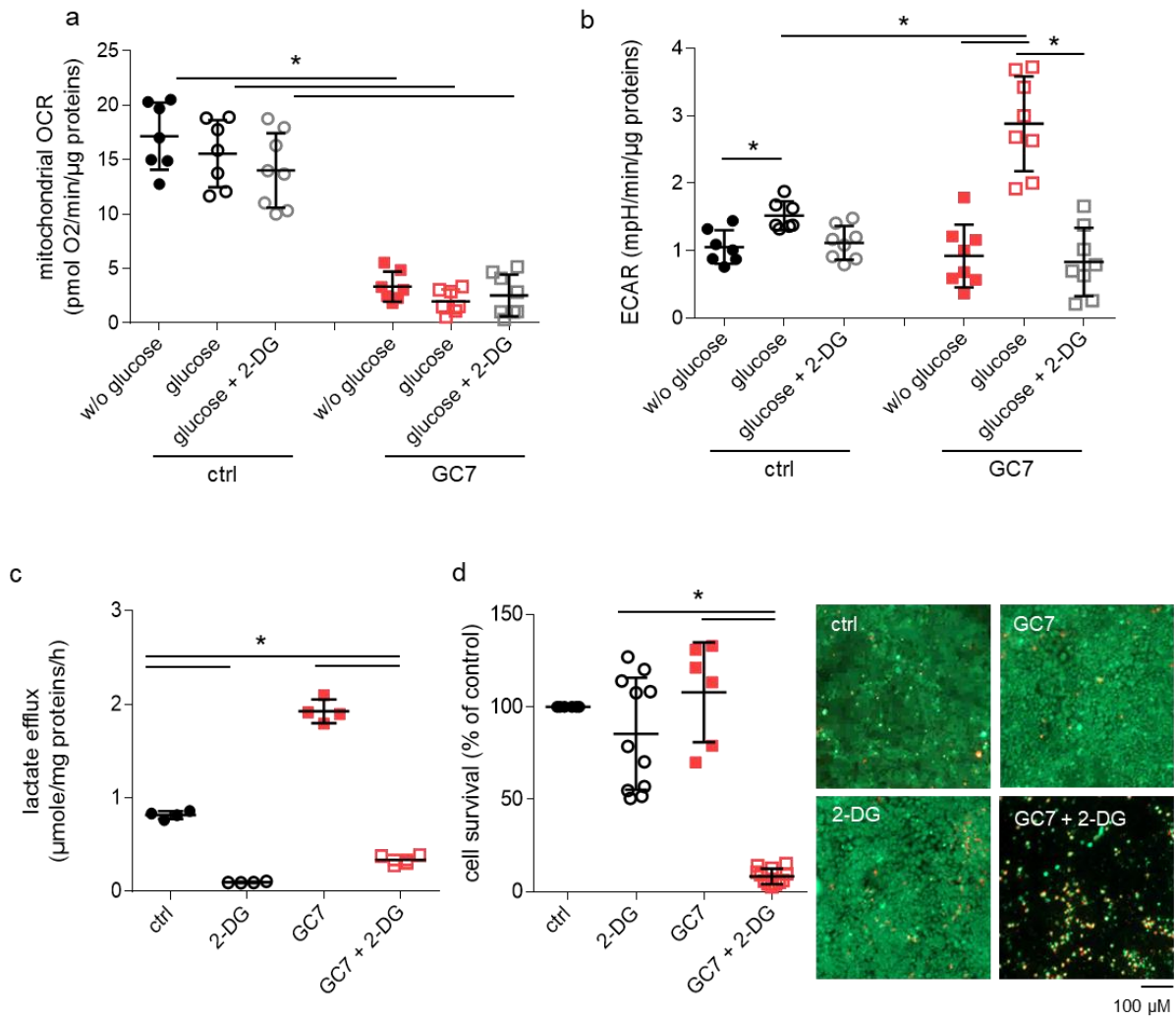
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Figure 3



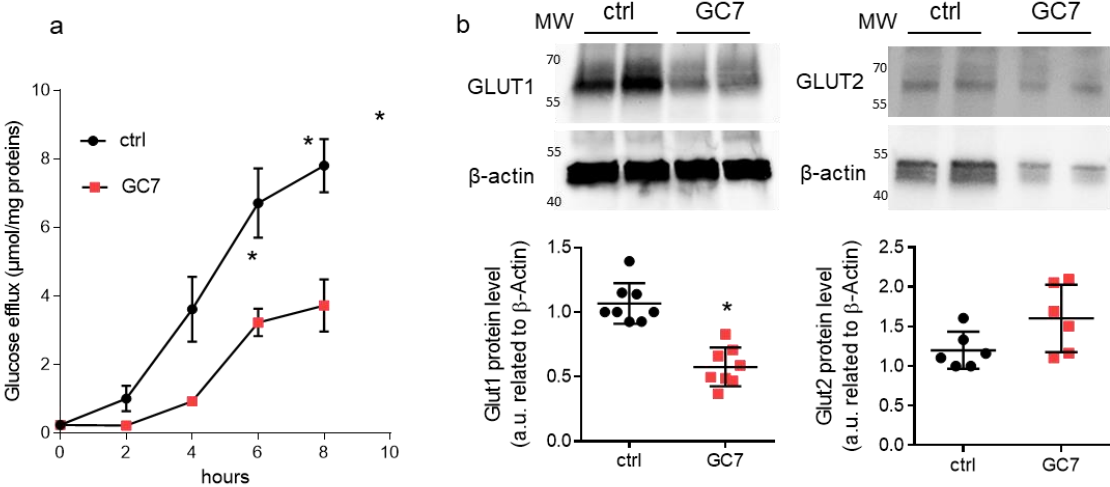
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Figure 4



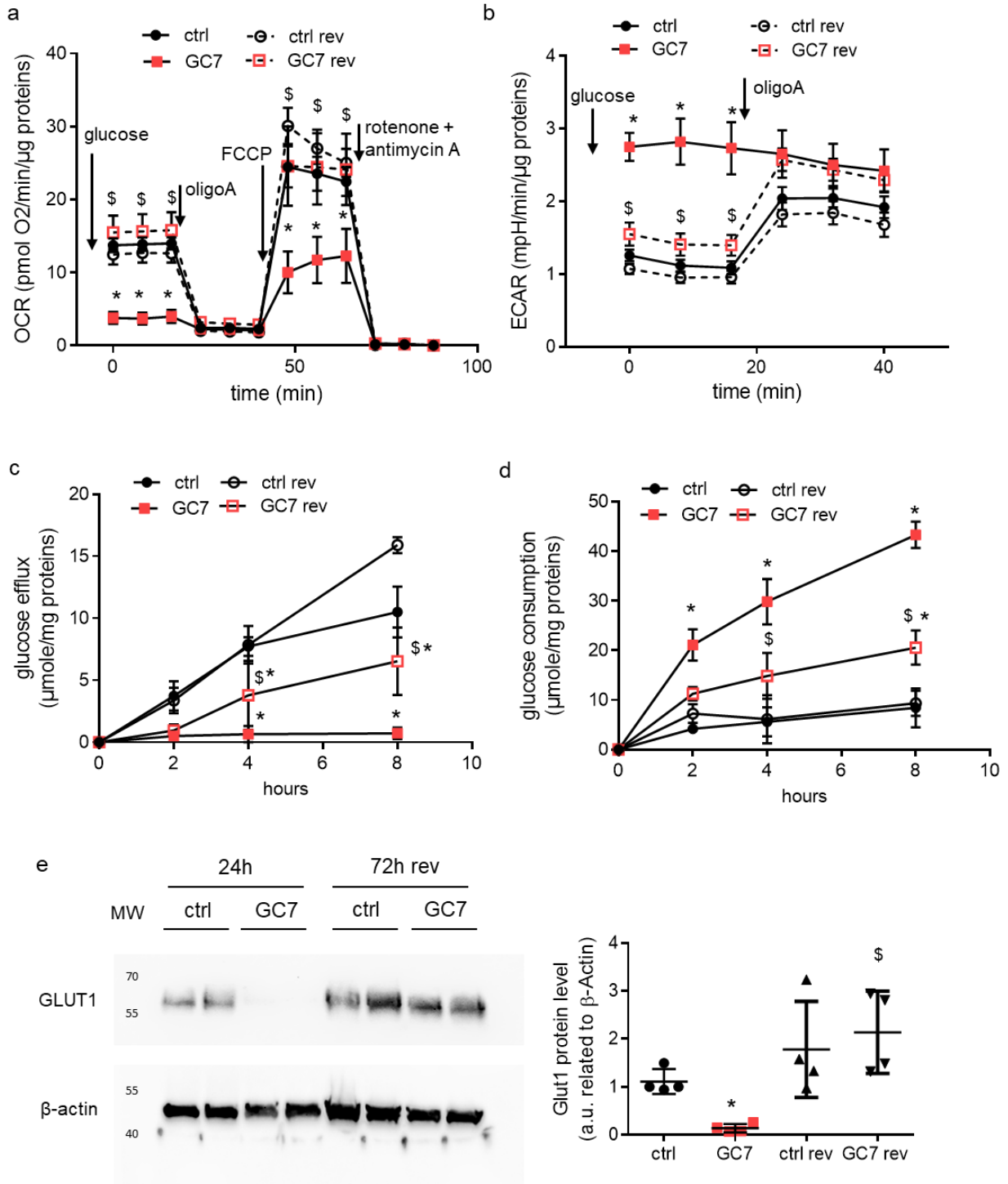
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Figure 5



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Figure 6



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Figure 7

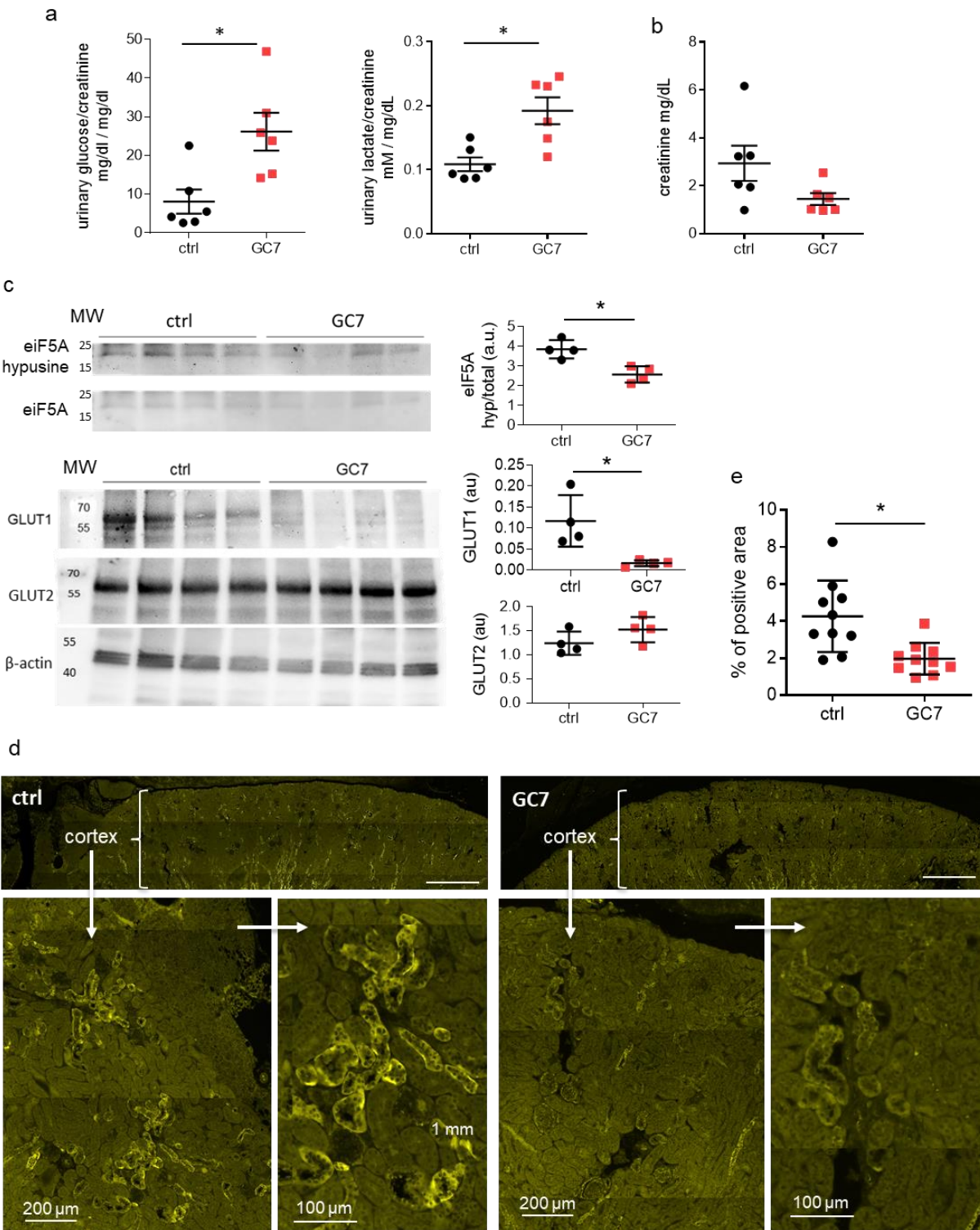
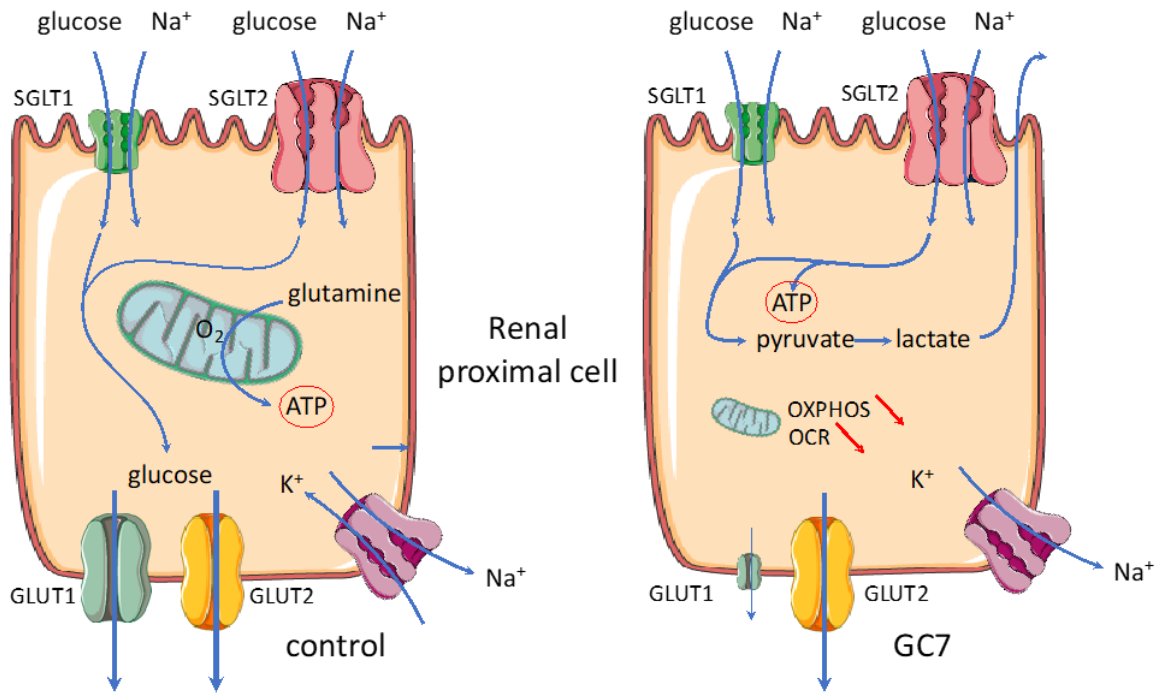


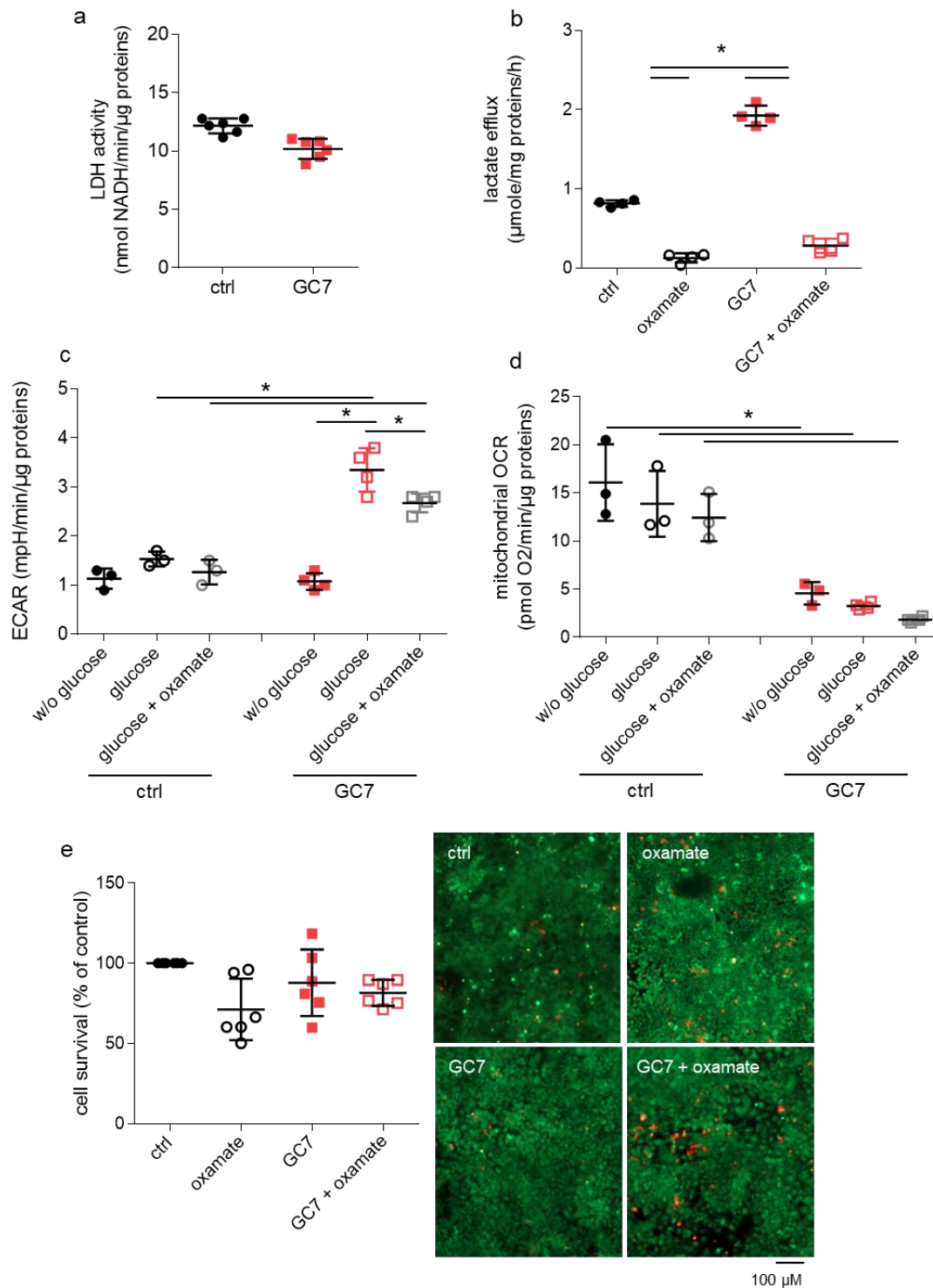
Figure 8.



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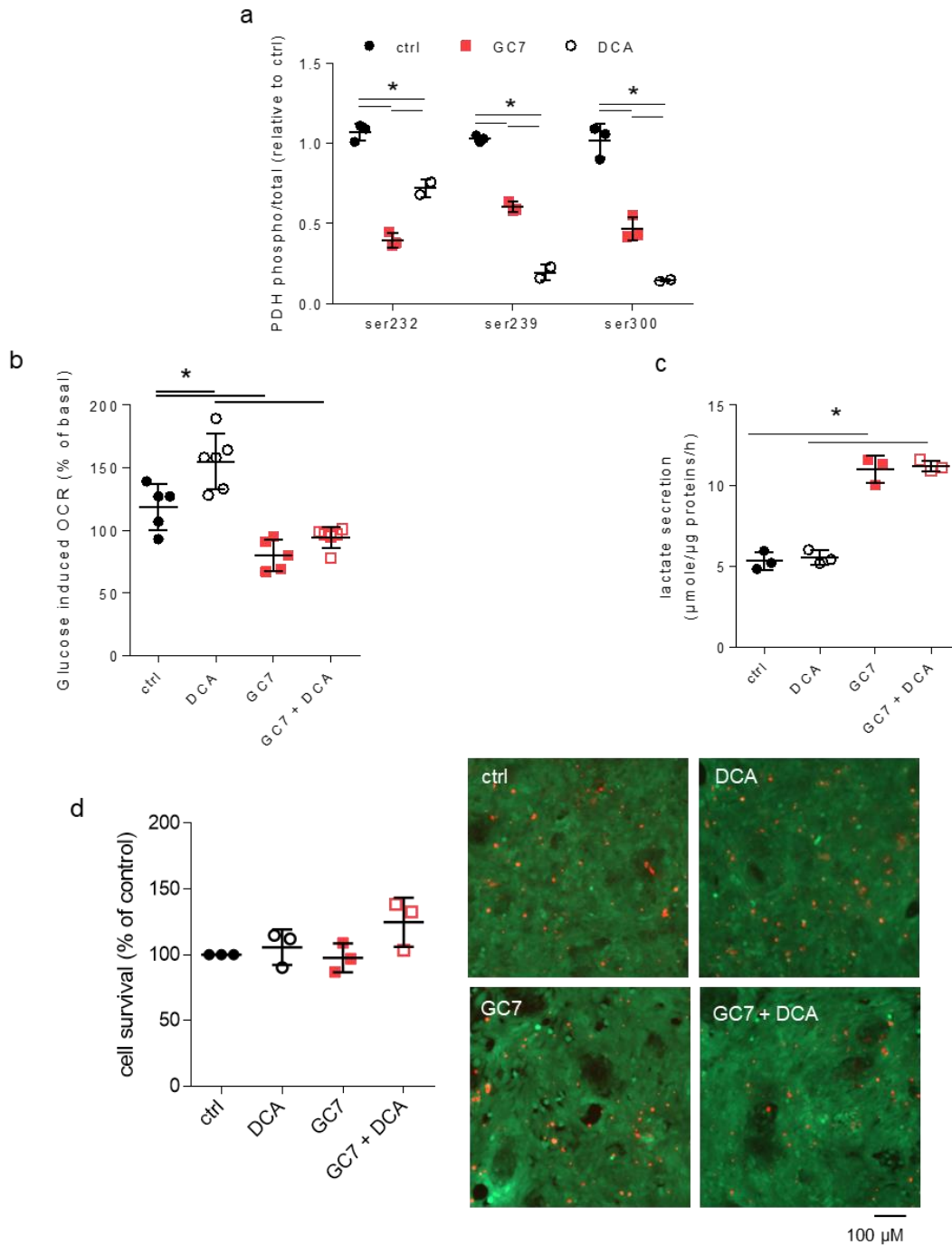
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Supplemental Figure 1.



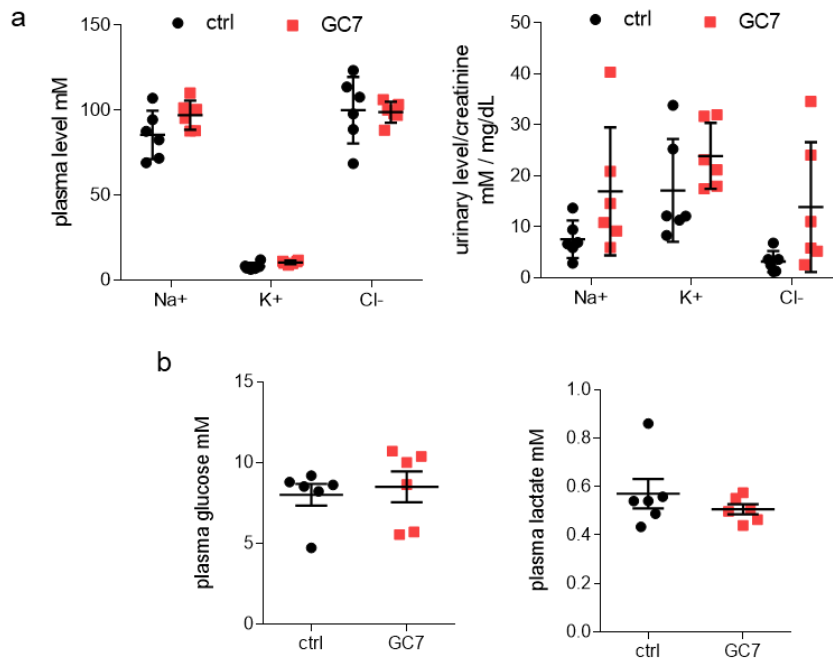
Supplemental Fig. 1 a/ Evaluation of LDH protein content in confluent PCT cells treated or not 24 hours with 30 μ M GC7. b/ measurement of lactate media content after 8 hours of GC7 treatment. c/ extracellular acidification (ECAR) and d/ Oxygen consumption (OCR) rates in different conditions. e/ effect of 100mM oxamate on GC7 treated cells 100 mM oxamate for 24 hours. Scale bar is indicated. Graphic displayed scatter plot of 3 to 6 independent values (as indicated by the number of dots) and mean \pm SD. * $p < 0.05$ evaluated using Mann-Whitney test.

Supplemental Figure 2.



Supplemental Fig. 2. a/ Evaluation of PDH phosphorylations in whole cell protein extract using an ELISA kit in different conditions. b/ Oxygen consumption rate (OCR). c/ Lactate media content measured 8 hours after media renewal. e/ Mortality evaluated using live/dead cells fluorescence assay. Scale bar is indicated. Graphic displayed scatter plot of 3 to 6 independent values (as indicated by the number of dots) and mean \pm SD. * $p < 0.05$ evaluated using Mann-Whitney test.

Supplemental figure 3.

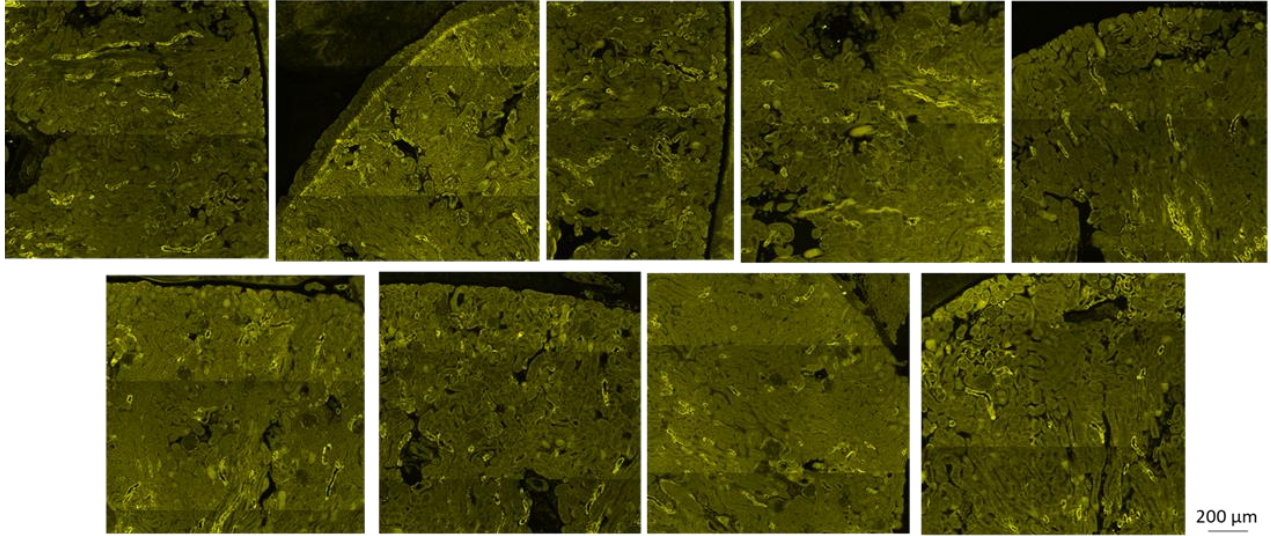


Supplemental Fig. 3. Mice were treated 3 days with GC7 (3 mg/kg, i.p., “GC7” group) or vehicle only (NaCl, “ctrl” group) and metabolic parameters were analysed at the end of the treatment. a/ Ionic composition (Na⁺, K⁺ and Cl⁻) in plasma (mM) and urine (mM.mg⁻¹.dL⁻¹) of mice. b/ Plasma glucose (mM) and lactate levels (mM). Graphics displayed scatter plot of 6 independent values and mean ± SD. * p<0.05 evaluated using Mann-Whitney test.

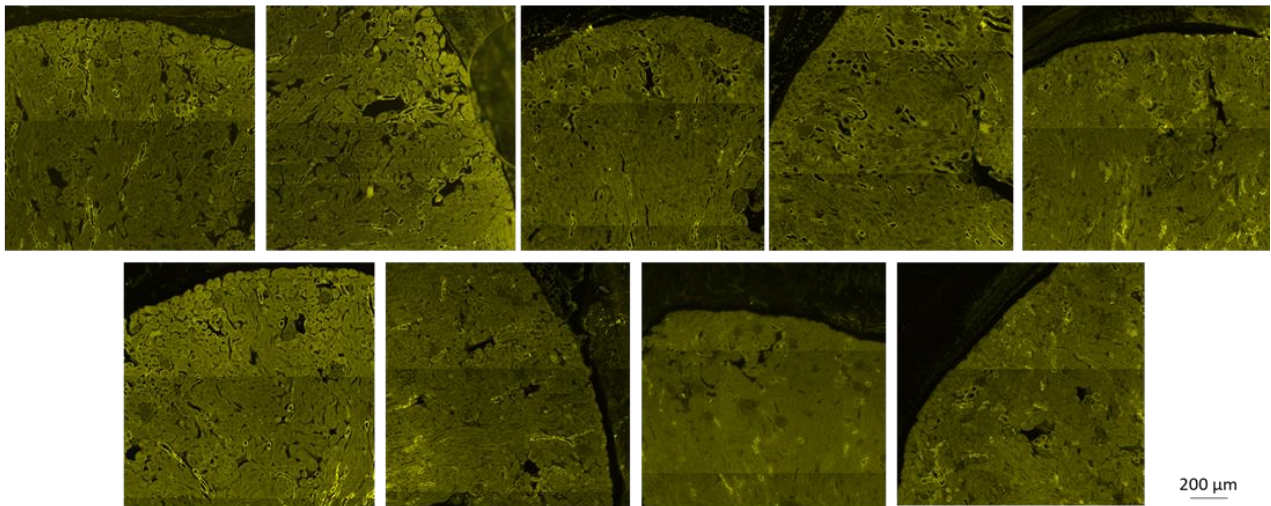
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Supplemental Figure 4.

a

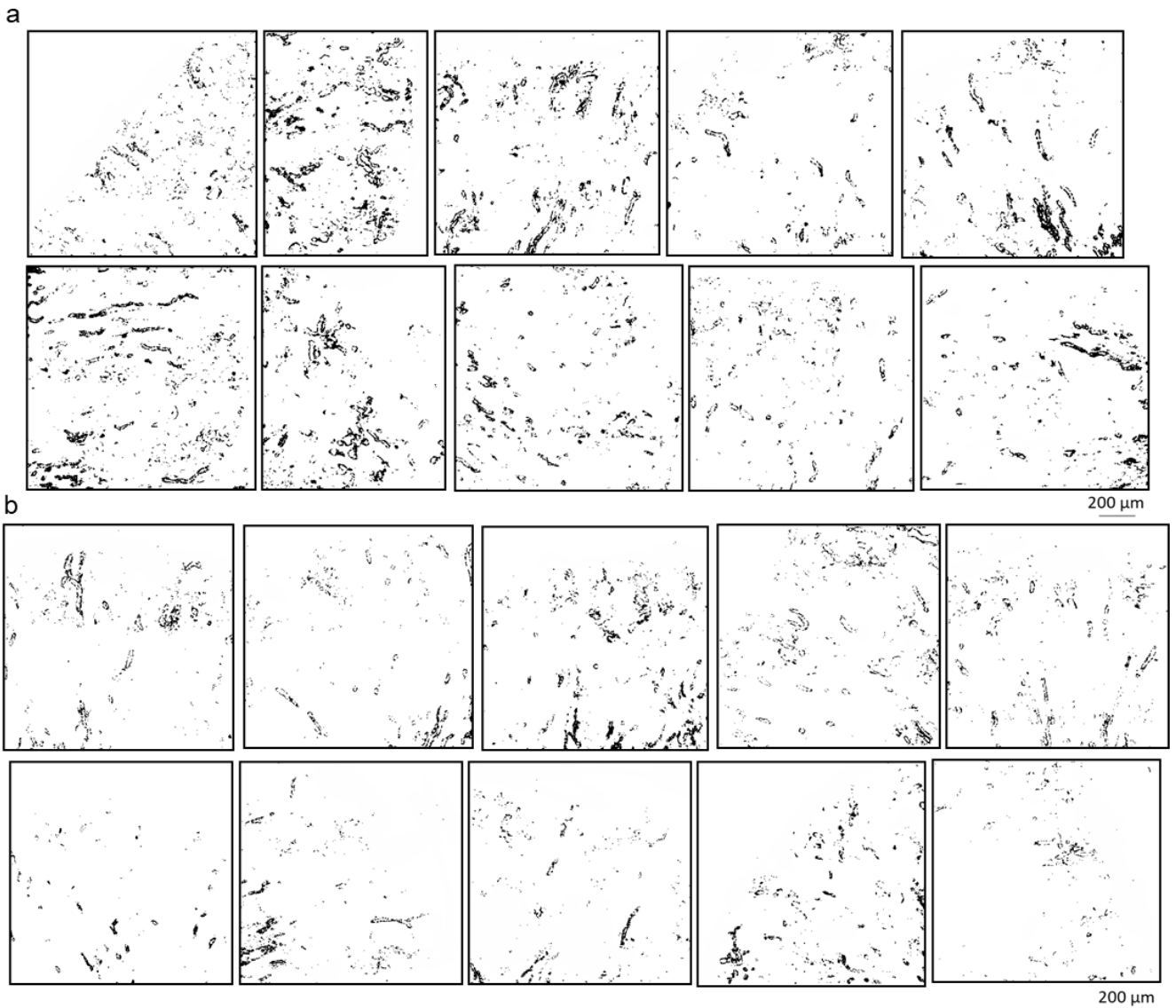


b



Supplemental Fig. 4. Mosaic reconstruction of cortical regions sequentially scanned after immuno-histofluorescence GLUT1 targeting. Six kidneys from NaCl- (control group) (a) or GC7-treated (b) mice were simultaneously stained for GLUT1. Scale bars are indicated.

Supplemental Figure 5.



Supplemental Fig. 4. For GLUT1 signal analysis, area to be analyzed were defined by outlining the kidney cortical area, excluding area with no tissue. Conversion of images to 8-bit then allowed automatic quantification of pixels displaying GLUT1 signal within the cortical area for each animal. Final quantification showing the percentage of cortical area that is positive for GLUT1 signal in NaCl- (control group) (a) or GC7-treated (b) mice is presented in Fig. 7c. Scale bars are indicated.

639

640