Biochemical adaptations of the retina and retinal pigment epithelium support a metabolic ecosystem in the vertebrate eye

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1 **ABSTRACT**
2 Here we report multiple lines of evidence for a comprehensive model for retinal
3 energy metabolism. Metabolic flux, locations of key enzymes and our finding that
4 glucose enters the neural retina almost entirely through photoreceptors support a
5 conceptually new model for retinal metabolism. In this model, glucose from the
6 choroidal blood supply passes through the retinal pigment epithelium to the retina
7 where photoreceptors convert it to lactate. Photoreceptors then export the lactate
8 as fuel for the retinal pigment epithelium and for neighboring Müller glial cells. A
9 key feature of this model is that aerobic glycolysis in photoreceptors produces
10 lactate to suppress glycolysis in the neighboring retinal pigment epithelium. That
11 enhances the flow of glucose to the retina by minimizing consumption of glucose
12 within the retinal pigment epithelium. This framework for metabolic relationships
13 in retina provides new insights into the underlying causes of retinal disease, age-
14 related vision loss and metabolism-based therapies.

15

INTRODUCTION

16

17 Mutations in any of more than140 genes can cause photoreceptors in a
18 vertebrate retina to degenerate (1). The relationship between photoreceptor
19 degeneration and the diversity of biochemical functions and expression patterns
20 of those genes is enigmatic. The diversity suggests that the consequences of
21 loss or gain of function of these genes may converge onto a few essential
22 metabolic processes (2, 3). Much has been gained by studying specific functions
23 of the genes. Based on those studies therapeutic strategies for specific genetic
24 deficiencies are being developed (4). Nevertheless, a more general approach to
25 understanding what photoreceptors need to survive could lead to more broadly
26 applicable therapeutic strategies. With that in mind, we have been investigating
27 the fundamental nature of energy metabolism in the retina and the retinal
28 pigment epithelium (RPE) (5-11).

29 Glucose that fuels the outer retina comes from the choroidal blood. Before it can
30 reach the retina, however, the glucose first must traverse the RPE. The RPE is a
31 monolayer of polarized cells between the choroid and retina that functions as a
32 blood-retina barrier. The cells in the RPE are bound together by tight junctions
33 and they express specific transporter proteins on their basolateral and apical
34 surfaces (12). Glucose from the choroid passes through transporters on the
35 basolateral surface, and then wends its way through the cytoplasm of the RPE
36 cell. If metabolic enzymes within the RPE cell do not consume it, glucose moves
37 down a concentration gradient toward the opposite side of the RPE cell where it
38 exits to the retina through transporters on the apical surface of the RPE.

39 When the glucose reaches the retina most of it is consumed by glycolysis and
40 converted to lactate. Retinas and tumors were the two tissues first identified in
41 the 1920's by Warburg (13) as relying mostly on "aerobic glycolysis". This type of
42 metabolism can release massive amounts of lactate from a cell even when O_2 is
43 available. Evidence indicates that photoreceptors in the outer retina are the site
44 of aerobic glycolysis (8, 13-17). The importance of aerobic glycolysis for survival
45 and function of photoreceptors has not been established, but it is thought to
46 enhance anabolic activity within the photoreceptor (3, 18-20).

47 In RPE cells energy metabolism is strikingly different than in the retina. In
48 particular, RPE cells are specialized for a type of energy metabolism called
49 reductive carboxylation (9) that aids in redox homeostasis. In general, RPE cells
50 rely more on their mitochondria.

51 Recent reports described genetic manipulations that explored the effects of
52 qualitatively altering energy metabolism either in photoreceptors or in RPE cells
53 *in vivo*. Glycolysis in rods was enhanced in one study by blocking expression of
54 SIRT6 (3). Another study enhanced glycolysis in rods by activating mTORC1 (21).
55 Those studies showed that making photoreceptors more glycolytic makes them
56 more robust. Both strategies slowed degeneration of rods caused by mutations
57 associated with retinitis pigmentosa (3, 21). However, making RPE cells more
58 glycolytic *in vivo* has the opposite effect; it causes neighboring photoreceptors to
59 degenerate. When glycolysis in the RPE was enhanced by knocking out VHL

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60 (22) or by knocking out an essential mitochondrial transcription factor in RPE
61 cells *in vivo* (23) the neighboring photoreceptors died.

62 The findings of those *in vivo* studies appear puzzling and seemingly contradictory
63 when considered only from a cell autonomous perspective. Why does enhancing
64 glycolysis help some cells and endanger others? Here we propose that those
65 findings make more sense when interpreted in the context of metabolic
66 relationships between the retina and the RPE. We describe evidence that the
67 retina and RPE function as a metabolic ecosystem. We show that photoreceptors
68 are the main point of entry for glucose into the retina. The photoreceptors convert
69 glucose to lactate, which then serves as a fuel for other cells in the retina. We
70 show here that lactate also can enhance the ability of RPE cells to pass glucose
71 from the blood to the retina. The model that we propose based on these findings
72 predicts that each cell in the retina and RPE contributes an essential metabolic
73 function that promotes survival of the complete retina-RPE ecosystem.

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75 **RESULTS**
76 **Photoreceptors express a glucose transporter.** Uptake of glucose into cells
77 requires a protein that can transport glucose. We used immunoblotting of mouse
78 tissues to evaluate expression of glucose transporters (**Fig. 1A**) and confirmed
79 previous findings that GLUT1 is the primary glucose transporter isoform in retina
80 (24) and RPE (25). The protein immunoreactive with the GLUT1 antibody was
81 confirmed to be membrane associated (**Fig. 1B**). GLUT3 was detected only in
82 brain. GLUT4 was detected in heart and muscle as expected, but not in the retina.

83 Immunohistochemistry shows that GLUT1 immunoreactivity overlaps with
84 cytochrome oxidase subunit 1 (MT-CO1) (**Fig. 1C**), which identifies rod inner
85 segments by the unique elongated shape of their mitochondria (**Fig 1E)**. These
86 mitochondria extend beyond the ends of the Müller glial cell (MGC) apical
87 processes (**Fig 1E)** . There are no mitochondria within these fine MGC apical
88 processes. Instead, small spherical-shaped mitochondria line up within the
89 MGCs along the outer limiting membrane just beneath the apical processes (**Fig.
90 1F and arrowheads in Fig. 1C**). MGCs extend from the outer limiting membrane
91 to the ganglion cell side of the retina. Most of the GLUT1 immunoreactivity in
92 MGCs is in the inner retina (**Fig. 1D).** GLUT1 immunoreactivity also overlaps with
93 a marker specific for rod photoreceptors, rod arrestin (**Fig. 1G**) and it overlaps
94 with GFP expressed from the rod-specific Nrl promoter (**Fig. 1H**). Taken
95 altogether, the distribution of GLUT1 immunoreactivity supports the idea that
96 photoreceptors can take up glucose released from the apical side of the RPE.

97 **Dietary glucose enters the retina primarily through photoreceptors.** Next we
98 asked which cells in the retina take up glucose in the context of an eye within a
99 living animal. We used oral gavage to introduce a fluorescent derivative of 2-
100 deoxy glucose (2-NBDG) (26) into stomachs of mice. We harvested the retinas
101 20 or 60 minutes after gavage, mounted them on filter paper and cut slices for
102 imaging by confocal microscopy (27). **Fig. 2A** shows that 2-NBDG fluorescence
103 is strongest in the photoreceptor layer, suggesting that photoreceptors are the

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104   first cells in the retina to take up glucose from the blood. Surprisingly, 2-NBDG
105   fluorescence is stronger in the outer retina than in the inner retina even though
106   mouse inner retinas are vascularized. We noted that 2-NBDG fluorescence does
107   not overlap with Müller Glial Cells (MGC's), which were labeled in these
108   experiments by transgenic expression of tdTomato (28), though in rare instances
109   there was overlap at a MGC end foot.. These results are summarized and
110   quantified in **Fig. 2C**. They show that glucose that reaches the outer retina is
111   taken up primarily by photoreceptors and not MGC's.

112   The images in **Fig. 2A** were made from live, unfixed mouse retinas. Most
113   photoreceptors in mouse retinas are rods. It is difficult in these images to resolve
114   whether cones also import 2-NBDG. To address this we also introduced 2-NBDG
115   by oral gavage into adult zebrafish, whose retinas are more enriched with cones
116   (29). **Fig. 2B** shows that cones become intensely fluorescent 30 minutes after
117   gavage. As in mouse retinas, there was no indication of glucose uptake into
118   MGCs, which in these retinas were marked with tdTomato expressed from a
119   GFAP promoter (30). **Fig. 2D** reports quantification and summarizes the
120   zebrafish retina results.

121   **Carbons from glucose are metabolized in RPE cells more slowly than in**
122   **retina.** Previous studies showed that most of the glucose taken up into a retina is
123   used to make lactic acid (8, 13-17). Within the eye of a living animal, glucose
124   from the choroidal blood first must pass through the monolayer of RPE cells
125   before it can reach the retina. We hypothesized that the energy metabolism of
126   RPE cells might be adapted to minimize consumption of glucose in order to
127   maximize the amount of glucose that can pass through the RPE to reach the
128   retina.

129   To examine glucose metabolism in RPE versus in retina, we compared two
130   preparations, mouse retina (mRetina) and cultured human fetal RPE cells
131   (hfRPE). The mouse retinas were freshly dissected from mouse eyes. The hfRPE
132   cells were grown 4-6 weeks in culture to form a monolayer with tight junctions
133   and a trans-epithelial resistance similar to native human RPE ($\geq$200 $\Omega\bullet cm^2$). This
134   hfRPE preparation has been widely used to study RPE metabolism and to model
135   RPE-related diseases such as age-related macular degeneration due to its
136   similarity to native RPE cells (31-35). We added ^{13}C labeled glucose to both
137   preparations and then used mass spectrometry (7) to compare incorporation of
138   ^{13}C into glycolytic and TCA cycle intermediates. For these experiments we used
139   1,2 ^{13}C glucose because the pattern of ^{13}C labeling from this isotopomer can be
140   used to distinguish metabolites generated by glycolysis from metabolites
141   generated by the pentose phosphate pathway (36). Metabolites with one ^{13}C
142   ("m1") are generated from glucose that flows through the oxidative reactions of
143   the pentose phosphate pathway whereas metabolites with two ^{13}C ("m2") are
144   produced when glucose enters glycolysis directly. In a previous report we used
145   this labeling method to show that < 2% of the metabolic flux from glucose goes
146   through the pentose phosphate pathway in both mRetina and in hfRPE (9).

147   **Fig. 3A** shows the total pmoles per μg protein of several metabolites in mRetina
148   and in hfRPE. There are several striking differences. Lactate and succinate are

149 more abundant in mRetina than in hfRPE, whereas citrate and α-ketoglutarate
150 are more abundant in hfRPE than in mRetina. **Fig. 3B** shows the time course of
151 incorporation of ^{13}C into several key metabolites. The initial rate at which ^{13}C
152 from glucose incorporates into intracellular lactate in mRetina is at least 8 times
153 faster than in hfRPE. We also noted that the citrate and α-ketoglutarate pools are
154 larger and fill more gradually in hfRPE cells than in retina indicating a large
155 oxidative metabolic capacity of RPE mitochondria.

156 These findings support our hypothesis that a slow rate of conversion of glucose
157 to lactate in the RPE minimizes consumption of glucose so that more glucose
158 can reach the retina. In a previous study we showed that RPE cells further
159 minimize their use of glucose by using an alternative pathway known as
160 reductive carboxylation to make NADPH (9). We propose that limited production
161 of lactate and the use of reductive carboxylation to make NADPH are adaptations
162 of RPE cells that minimize their consumption of glucose so that more glucose
163 reaches the retina.

164 **RPE cells can use lactate as a fuel.** The retina converts most of the glucose it
165 consumes into lactate so we considered the possibility that RPE cells can use
166 retinal lactate as an alternative fuel source to compensate for their minimal
167 consumption of glucose, or even to further suppress glycolysis. We incubated
168 hfRPE cells either with U-^{13}C glucose or with U-^{13}C lactate for 5 or 10 minutes
169 and quantified incorporation of ^{13}C into glycolytic and TCA cycle metabolites. **Fig.**
170 **4A** shows that ^{13}C is incorporated rapidly into the pyruvate pool from both
171 glucose and lactate. However, in the citrate pools, ^{13}C from lactate accumulates
172 at least 20 times faster than ^{13}C from glucose. We also noted that substantial
173 amounts of m3 malate are formed. This shows that carboxylation of pyruvate is a
174 major metabolic pathway in hfRPE cells. **Fig. 4B** reports the time course of
175 incorporation of ^{13}C from lactate into TCA cycle intermediates in hfRPE cells.
176 Carboxylation of pyruvate occurs at about half the rate of decarboxylation of
177 pyruvate.

178 **Lactate can suppress consumption of glucose by RPE cells.** The results in
179 **Fig. 3** and **Fig. 4** show that glucose is consumed more slowly by RPE cells than
180 by retina. They also show that RPE cells efficiently use lactate for fuel as an
181 alternative to using glucose. Based on these findings we next asked whether
182 lactate can suppress consumption of glucose by RPE cells. We hypothesized
183 (**Fig. 5A**) that lactate dehydrogenase (LDH) in RPE cells depletes cytosolic NAD$^+$
184 when it oxidizes lactate to pyruvate. Since NAD$^+$ is required for glycolysis,
185 depletion of NAD$^+$ by lactate and LDH would suppress glycolysis so that RPE
186 cells consume less glucose.

187 We incubated hfRPE cell monolayers with 5 mM U-^{13}C glucose either in the
188 absence or presence of 20 mM unlabeled lactate. We then harvested the cells
189 and used GC-MS to determine if lactate suppresses incorporation of ^{13}C from
190 glucose into glycolytic and TCA cycle intermediates.

191 The results of this experiment (**Fig. 5B**) show that unlabeled lactate substantially
192 increases the amounts of unlabeled pyruvate, citrate, isocitrate, fumarate and

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193   malate (left panel of **Fig. 5B**). This is consistent with the results in **Fig. 4** showing
194   that carbons from lactate are incorporated rapidly into TCA cycle metabolites
195   through both carboxylation and decarboxylation of pyruvate.

196   Addition of lactate also causes accumulation of GAP, the triose phosphate
197   immediately upstream of the glyceraldehyde-3-phosphate dehydrogenase
198   (GAPDH) reaction, that requires NAD^+. Consistent with this, added lactate
199   diminishes the amount of ^{13}C from glucose incorporated into intermediates
200   downstream of the GAPDH reaction (right side of **Fig. 5B**). Lactate does not
201   decrease incorporation of ^{13}C from glucose into m2 citrate and m2 isocitrate,
202   most likely because of anapleurotic supplementation of unlabeled TCA cycle
203   intermediates through pyruvate carboxylation enhanced TCA cycle activity (see
204   left side of **Fig. 5B**). Overall, lactate substantially inhibits glycolysis in hfRPE cells.

205   **Lactate can enhance transport of glucose across a monolayer of hfRPE**
206   **cells.** Next we asked whether lactate from photoreceptors can enhance the flow
207   of glucose across the RPE. We reasoned that suppression of glycolysis by
208   lactate minimizes consumption of glucose, so more glucose would cross from the
209   basolateral to the apical side of the RPE.

210   To test this idea, we measured the influence of lactate on transport of glucose
211   across a monolayer of hfRPE cells. We grew hfRPE cells on transwell filters to
212   confluence with a transepithelial resistance ≥200 $\Omega \bullet cm^2$. We then added ^{13}C
213   glucose to the basolateral side, where the RPE normally would face the choroid
214   in an eye, and used mass spectrometry of the apical medium to quantify
215   transport of ^{13}C glucose to the apical side, where the RPE normally would face
216   the lactate-rich retina. We performed this experiment either with no added lactate
217   or with 1, 5, or 10 mM unlabeled lactate added to medium on the apical side (**Fig.**
218   **6A**). Consistent with our hypothesis, lactate added to the apical medium
219   substantially enhances transport of ^{13}C glucose from the basolateral to the apical
220   side of the hfRPE cells (**Fig. 6B**). Unlabeled lactate also suppresses the release
221   of ^{13}C lactate on the apical side (**Fig. 6C**).

222   **Long-term exposure to lactate enhances maturation of hfRPE cells**. Culture
223   conditions for hfRPE cells have been optimized for differentiation and for growth
224   (35). High concentrations of lactate generally are not included in culture media
225   for RPE cells. However, in their native environment within a living eye, RPE cells
226   normally would be exposed to constantly high concentrations of lactate released
227   from the retina.

228   Since lactate is metabolized efficiently by hfRPE cells we hypothesized that the
229   presence of lactate in the culture medium might influence their differentiation
230   state. To test this we incubated hfRPE cells in standard medium containing
231   glucose until they were confluent and had achieved a trans-epithelial resistance
232   ≥200 $\Omega \bullet cm^2$. We then lowered the concentration of glucose to 0.5 mM and
233   added 10 mM lactate. Remarkably, cell survival was excellent. After 21 days
234   pigmentation of the lactate supplemented hfRPE cells increased substantially
235   compared to control hfRPE cells provided with standard 5 mM glucose sans
236   lactate. (**Fig 7A**). **Fig. 7B** shows that the levels of several key metabolic enzymes

237   decreased, whereas the ratio of IDH3 to other IDH isoforms increased. Exposure
238   to lactate also alters metabolic flux through decarboxylation and carboxylation of
239   pyruvate (**Fig. 7C**) and reductive carboxylation (**Fig. 7D**). A more complete
240   analysis of the effects of lactate on RPE cell differentiation is needed, but our
241   initial findings, shown here, demonstrate that lactate not only supports RPE
242   metabolism, but it also influences the differentiation state of RPE cells.

243   **Metabolic specializations of the retina and RPE decline with age**. The
244   analyses of RPE metabolism in this study focused on the cultured hfRPE, which
245   is a well characterized model that has been used to evaluate RPE metabolism
246   (32). hfRPE cells have been used as a cell culture model for studying various
247   diseases, including age-related macular degeneration (34). The RPE cultures
248   used in the experiments reported here are of a similar age in culture as the ones
249   used in other published studies, including those modeling AMD.

250   However, to confirm that the metabolic differences between retina and hfRPE are
251   similar to the differences between retina and RPE in an eye, we used a mouse
252   eyecup preparation with retinas removed to confirm the basic metabolic features
253   that we identified in this study. We incubated freshly separated retinas and
254   eyecups in medium containing glucose and glutamine and then analyzed
255   metabolites by GC-MS. **Fig. 7E** compares the ratio of total lactate to total citrate
256   in the retina vs. in the eyecup. Similar to the ratio we found for mouse retina and
257   hfRPE, the lactate/citrate ratio is ~30 times higher in retina than in the eyecup.

258   Based on our findings we speculated that degeneration of photoreceptors in
259   response to mutations or to aging could be influenced by the metabolic
260   relationship between the retina and RPE. The eyecup preparation allowed us to
261   examine the effect of aging on the lactate/citrate ratio in retina and RPE. **Figs.**
262   **7E and 7F** show that lactate/citrate in the retina *decreases* substantially as mice
263   age from 6 months to 32 months. In contrast, lactate/citrate *increases* with age in
264   the eyecup. In other words the retina becomes less glycolytic with aging while the
265   eyecup, containing the RPE layer, becomes more glycolytic. These results
266   suggest that the specialized metabolic features, that are fundamental to the
267   retina-RPE ecosystem, decline with age both in the retina and in the RPE and
268   that this decline could contribute to age-related vision loss (37, 38).

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DISCUSSION

Model for a network of metabolic interdependence between the retina and RPE. Fig. 8 summarizes our model for the retina-RPE metabolic ecosystem. Lactate from photoreceptors suppresses glycolysis in the RPE so more glucose can reach the retina.

Previous evidence that cells in the retina have specific metabolic roles. The distributions of metabolic enzymes in mouse retina indicate that photoreceptors have the enzymes and transporters they need for glycolysis, but MGCs do not. Glycolysis requires pyruvate kinase (PK). The M2 isoform of PK (PKM2) is highly enriched in photoreceptors (10, 18-20, 39), but MGCs do not express substantial amounts of any PK isoform (10). MGCs also do not express hexokinase (20). Furthermore, lactate, rather than glucose, is the most effective source (10) of carbon for glutamine synthesis by MGCs (40) in mouse retinas. Based on these observations, we proposed that MGCs in a retina primarily use lactate produced by photoreceptors (41). Together with the results described in this report it appears that the central role of photoreceptors in retinal energy metabolism is to convert glucose to lactate, which then is used as fuel by both RPE and Müller cells.

Significance of aerobic glycolysis in the retina. Enhanced capacity for anabolic metabolism has been proposed as the purpose of aerobic glycolysis in photoreceptors (10, 18, 19), but our model suggests an additional purpose. We propose that the laminated structure of the eye, in which the RPE separates the retina from its source of nutrients, requires photoreceptors to produce and release lactate to suppress glycolysis so that sufficient glucose can flow through the RPE.

The "retinal ecosystem" model helps to explain recent findings from genetic manipulations of photoreceptors and RPE. Engineering photoreceptors to be more glycolytic makes them more robust, whereas engineering RPE cells to be more glycolytic causes photoreceptors to degenerate (3, 21-23). The model shown in **Fig. 8** provides an explanation for those seemingly opposing findings. When photoreceptors are engineered to be more glycolytic they produce more lactate that can more substantially suppress glycolysis in the RPE. In this way more glucose reaches the retina, thereby enhancing photoreceptor survival. When the RPE is more glycolytic it consumes more glucose, leaving less glucose available for the retina so photoreceptors starve and become stressed.

The concept of a metabolic ecosystem and its relationship to retinal disease. The "retina ecosystem" model in **Fig. 8** suggests an explanation for the linkage between Age-Related Macular Degeneration and accumulation of mitochondrial DNA damage in RPE cells (42). Photoreceptors may starve when RPE mitochondria fail because the RPE becomes more dependent on glycolysis, which prevents glucose from reaching the retina.

The concept of a metabolic ecosystem also has implications for other types of retinal disease. Mutations that affect genes expressed only in rods can cause

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315 rods to degenerate. However, cones subsequently degenerate as a
316 consequence of the loss of rods, even though the cones are not affected directly
317 by the mutant gene (43). One reason for this is that loss of a cone viability factor
318 that normally is produced by rods may contribute to cone degeneration in this
319 type of disease state (44). The model in **Fig. 8** suggests another factor that also
320 can contribute to the secondary loss of cones when rods degenerate. A retina
321 without rods makes less lactate (8). We have shown in this report that, without
322 lactate to suppress glycolysis, RPE cells oxidize more glucose and transport less.
323 In a rod-less retina the loss of lactate production would limit the rate at which
324 glucose can be supplied to cones. This can explain why cones starve (2) and
325 why 2-NBDG accumulates in RPE cells (45) when rods degenerate. Consistent
326 with this explanation, cones in these retinas can be rescued from degeneration
327 by supplementation with an alternative supply of glucose (45).

328 **Other fuels also may contribute in the metabolic ecosystem.** This study
329 highlights one way that RPE, photoreceptors and MGCs can work together as an
330 ecosystem of metabolically specialized interdependent cells. Our investigation
331 focused on glucose and lactate, but it is very likely that fatty acids, ketone bodies,
332 (46-48) and metabolites from other metabolic pathways (20) also contribute
333 significantly to this metabolic ecosystem. For example, oxidation of fatty acids by
334 the RPE can supply the retina with ketone bodies (46). Furthermore, a recent
335 study showed that RPE oxidizes fatty acids from photoreceptor phagocytosis,
336 (48) which also can suppress the need for RPE cells to consume glucose.
337 Together these studies suggest that energy homeostasis in retina and RPE relies
338 on a specialized metabolic interplay that could form the basis of future therapies
339 for a range of retinal degenerative diseases.

340

341 **MATERIAL AND METHODS**
342 **Animals**. All research was authorized by the University of Washington
343 Institutional Animal Care and Use Committee. Mice in the C57BL6 background
344 were maintained in the University of Washington South Lake Union vivarium at
345 27.5 ºC on a 14h/10h light-dark cycle. Transgenic mice expressing eGFP under
346 the Nrl promoter (49) (RRID:IMSR_JAX:021232), or tdTomato under the Rlbp-
347 CRE promoter (28) were described previously. Aged mice (6 month and 32
348 month) were C57BL/6J. This strain does not carry the rd8 mutation in the Crb1
349 gene (50).

350 Transgenic heterozygote zebrafish in the AB background were maintained in the
351 University of Washington South Lake Union aquatics facility at 27.5 ºC on a
352 14h/10h light-dark cycle. Fish used for experiments were male and female
353 siblings between 12-24 months old. A transgenic line stably expressing tdTomato
354 in Müller cells (GFAP:tdTomato; RRID:ZDB-FISH-150901-17843) was described
355 previously (30). Prior to gavage experiments, fish were fasted >18 h and dark-
356 adapted >12 h.

357 **Antibodies**.
358 Arrestin1, D9F2 (from Larry Donoso and Cheryl Craft)
359   IHC: 1:200

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360   GLUT1, (AbCam, ab115730; RRID:AB_10903230)
361   IB: 1:200,000, 0.86 ng/ml;
362   IHC 1:1000, 0.17 mg/ml
363   GLUT3, (AbCam, ab41525; RRID:AB_732609)
364   IB: 1:5000, 0.136 ug/ml
365   GLUT4, (AbCam, ab654; RRID:AB_305554)
366   IB: 1:5000
367   Glutamine synthetase, (Millipore, MAB302; RRID:AB_2110656)
368   IHC: 1:1000
369   MTCO1 (Abcam, ab14705; RRID:AB_2084810)

370   **Tissue preparations for immunoblotting.** Frozen tissue samples were
371   homogenized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.05% sodium
372   deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 with a mixed
373   phosphatase/protease inhibitor cocktail (ThermoFisher 88668), briefly sonicated,
374   then rocked at 4°C for 30min. Samples were then spun at 13,300 RPM at 4°C for
375   15 min, and the supernatant was normalized for loading by BCA assay to 20
376   mg/tissue. RPE protein lysate was prepared according to a described protocol
377   (51).

378   To prepare membrane fractions, frozen tissue samples were homogenized in
379   PBS (0.14 M, pH 7.4) with a mixed phosphatase/protease inhibitor cocktail, then
380   rocked at 4°C for 30 min. Samples were then spun at 45,000 rpm at 4°C, the
381   supernatant (cytosolic fraction) drawn off and saved, and the pellet (membrane
382   fraction) was resuspended in an equal volume of PBS. After mixing with 5X
383   Laemmli loading buffer, 1 ml benzonase (Millipore 70746) was added. Each
384   tissue was then loaded with equal volumes of cytosolic and membrane fraction.

385   **Immunoblotting**. Samples were run on 12%, self-cast acrylamide gels and
386   transferred onto   PVDF membranes (Millipore IPFL00010). Following protein
387   transfer, membranes were blocked with LI-COR Odyssey Blocking Buffer (LI-
388   COR, 927-40000) for 1 h at room temperature. Primary antibodies were diluted in
389   blocking buffer and incubated overnight at 4°C. Membranes were washed,
390   incubated with secondary antibody (LI-COR IRDye 800CW, 926-32210,
391   (RRID:AB_621842), and 926-32211, (RRID:AB_621843),1:5000 1h at room
392   temperature, and washed again. Imaging was performed using the LI-COR
393   Odyssey CLx Imaging System (RRID:SCR_014579).

394   **Immunohistochemistry:** Retinal eyecups were micro-dissected from C57BL6J
395   mice and were fixed in 4% paraformaldehyde in PBS, rinsed with PBS, incubated
396   in a sucrose gradient (5%, 10%, and 20%), embedded into OCT and
397   cryosectioned at 20µm. Mouse sections were washed in PBS, then blocked in
398   IHC buffer (5% normal donkey serum diluted in PBS with 2 mg/mL BSA and
399   0.3% Triton X-100) for 1 h. Primary antibodies were diluted in IHC blocking buffer
400   as specified, and applied to blocked cryosections overnight at 4°C. Secondary
401   antibodies were diluted at 1:3000 in IHC blocking buffer, and applied to mouse
402   retina sections for 1 h in darkness. Sections were washed in PBS three times,
403   and mounted with SouthernBiotech Fluoromount-G (Fisher Scientific) under
404   glass coverslips and visualized using a Leica SP8 confocal microscope with a

405 63X oil objective. Images were acquired at a 4096x4096 pixel resolution with a
406 12-bit depth using Leica LAS-X software (RRID:SCR 013673).

407 **RPE cell culture.**   Human fetal eyes with a gestational age of 16-20 weeks
408 were harvested and shipped overnight on ice in RPMI media containing
409 antibiotics from Advanced Bioscience Resources Inc. (Alameda, CA).
410 Dissections of fetal tissue were performed within 24 hours of procurement and
411 followed a modified version of the dissection protocol in order to isolate the
412 retinal pigment epithelium (RPE) (35). The fetal RPE sheets were incubated at
413 37°C with 5% CO_2 and cultured in RPE media. The RPE media consisted of
414 Minimum Essential Medium alpha (Life Technologies) supplemented with 5%
415 (vol/vol) fetal bovine serum (Atlanta Biologicals), N1-Supplement (Sigma-Aldrich),
416 Nonessential Amino Acids (Gibco), and a Penicillin-Streptomycin solution (Gibco).
417 Isolated fetal RPE reached confluency about 3-4 weeks after dissection and was
418 then passaged using a 0.25% Trypsin-EDTA solution (Gibco) and passed
419 through a 40 mm nylon cell strainer (BD Falcon) in order to collect a suspension
420 of single cells. After counting, the RPE cells were plated onto 0.3 cm^2 cell culture
421 inserts (Falcon) coated with Matrigel (Corning) at a seeding density of 100,000
422 cells per insert. Cells grown on these inserts were cultured in RPE media
423 containing 1% (vol/vol) FBS. Transepithelial resistance was measured weekly
424 after 2 weeks in culture using a Millicell ERS-2 Epithelial Volt-Ohm Meter
425 (Millipore).

426 **Oral Gavage**. Mice were fasted overnight in the dark, and gavaged the next
427 morning in ambient light. A micro-syringe fitted with a 22 gauge 1.5" straight 1.25
428 mm ball-tip needle was used to orally administer 100 μl of 50 mM 2-NBDG
429 (Invitrogen) dissolved in water. Successfully gavaged mice were returned to
430 darkness during the 2-NBDG incubation period.

431 Zebrafish were gavaged using methods described previously(52) under red light.
432 Briefly, overnight fasted adult zebrafish were anaesthetized > 1 min with 150
433 mg/mL MS-222 in fish water. Fish were placed in a slit cut in a cellulose sponge
434 soaked with MS-222 solution, and the sponge was rotated to orient the fish
435 mouth up. A micro-syringe fitted with thin, flexible 1 mm OD plastic tubing was
436 used to orally administer 5 μL of either fish water or 30 mM 2-NBDG (Invitrogen).
437 Gavaged fish were immediately placed into a recovery tank of fresh fish water
438 and monitored briefly using a UV flashlight for regurgitation of 2-NBDG.
439 Successfully gavaged fish were returned to darkness during the 2-NBDG
440 incubation period.

441 **Tissue slicing and imaging**. Gavaged mice were euthanized by asphyxiation
442 with CO_2. Zebrafish were euthanized in an ice bath followed by cervical
443 dislocation. Euthanized animals were enucleated, and the retinas dissected away
444 under red light into cold Ringer's solution (133 mM NaCl, 2.5 mM KCl, 1.5 mM
445 NaH_2PO_4, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, 1 mM
446 sodium lactate, 0.5 mM L-glutamine, 0.5 mM reduced glutathione, 0.5 mM
447 sodium pyruvate, 0.3 mM sodium ascorbate, pH 7.4). Isolated retinas were
448 mounted on filter paper (0.45 μm pore, mixed cellulose, Millipore) and flattened
449 with gentle suction. After peeling away remaining RPE, flat-mounted retinas were

450 sliced into 300-400 μm slices using a tissue slicer (Stoelting). Slices were rotated
451 90° and the filter paper edges buried in strips of wax on a coverslip for imaging at
452 room temperature. Fresh retinal slices were imaged at room temperature using a
453 Leica SP8 confocal microscope with a 40X water objective; excitation/emission
454 wavelengths were 488/525-575 nm for 2-NBDG, and 559/580-630 nm for
455 tdTomato. Leica LAS-X (RRID:SCR_013673) software was used to acquire
456 images at 2048 x 2048 pixel resolution with 12 bit depth, and Z-stacks imaged
457 every 0.5 μm over a tissue depth of 10-30 μm.

458 **Image analysis**. ImageJ software (RRID:SCR_002285) was used for
459 quantification of 2-NBDG fluorescence in fresh retinal slices. 10 slices of each Z-
460 stack were maximum intensity projected, and retinal layers were identified by
461 morphology and expression of transgenic markers. For every slice, 3 small
462 uniformly sized rectangular regions of interest (ROIs) were placed randomly in
463 each retinal layer, and mean fluorescence intensity of each ROI was measured.
464 Average 2-NBDG fluorescence in each layer was divided by the
465 autofluorescence of corresponding retinal layers from animals gavaged with
466 saline or water.

467 **Metabolic flux analysis**: Isolated mouse retina or confluent human fetal RPE
468 cells were changed into pre-warmed Krebs-Ringer Bicarbonate Buffer containing,
469 depending on the experiment, [1,2] ^{13}C glucose, U-^{13}C glucose, U-^{13}C lactate or
470 U-^{13}C glutamine (Sigma) as described elsewhere (5, 7, 9). Both retinas and RPE
471 cells were incubated for 5 min, 30 min, 60 min and 120 min. Metabolites from
472 each time point were extracted and analyzed by gas chromatography mass
473 spectrometry (GC-MS, Agilent 7890/5975C) as described in detail (5, 6).

474 **Measurement of U-^{13}C glucose transport across hfRPE cells on transwell
475 filters**. After maturation for 4-6 weeks in culture, hfRPE cells grown on transwell
476 filters were changed into 500 ml of DMEM containing 1% FBS on each side. 5.5
477 mM U-^{13}C glucose (Cambridge Isotope Laboratories) was included in the
478 medium in the basolateral side while various concentrations of sodium lactate
479 was added to the apical side, while maintaining a constant pH. Apical side
480 medium was collected at 8 and 24 h to analyze the transported U-^{13}C glucose by
481 liquid chromatography coupled with triple quadrupole mass spectrometry (Waters
482 Xevo TQ Tandem mass spectrometer with a Waters ACQUITY system with
483 UPLC) as reported in detail (7).

484 **Serial Block Face Scanning SEM**
485 Mouse eyes were enucleated, the anterior half was dissected away, and the
486 eyecup was cut in half. Tissue was fixed in 4% glutaraldehyde in 0.1 M sodium
487 cacodylate buffer, pH 7.2, at room temperature (RT), then stored overnight at
488 4°C. Samples were washed 4 times in sodium cacodylate buffer, postfixed in
489 osmium ferrocyanide (2% osmium tetroxide/3% potassium ferrocyanide in buffer)
490 for 1 h on ice, washed, incubated in 1% thiocarbohydrazide for 20 min, and
491 washed again. After incubation in 2% osmium tetroxide for 30 min at RT,
492 samples were washed and en bloc stained with 1% aqueous uranyl acetate
493 overnight at 4°C. Samples were finally washed and en bloc stained with Walton's
494 lead aspartate for 30 min at 60°C, dehydrated in a graded eth- anol series, and

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495 embedded in Durcupan resin. Serial sections were cut at 60 nm thickness and
496 imaged with 6 nm pixel size using a Zeiss Sigma VP scanning electron
497 microscope fitted with a Gatan 3View2XP ultramicrotome apparatus. Imaged
498 stacks were concatenated and aligned using TrakEM2 (RRID:SCR_008954).
499 Unless stated otherwise, five washes with water were used for all wash steps.

500 **Statistical analyses**.
501 R (RRID:SCR_001905) with R Commander was used to perform one-way
502 ANOVA for NBDG gavage experiments.

503 **Reproducibility**. All data shown here have been reproduced at least three times
504 by the authors.

505 **Data availability**. All data supporting the findings of this study are available
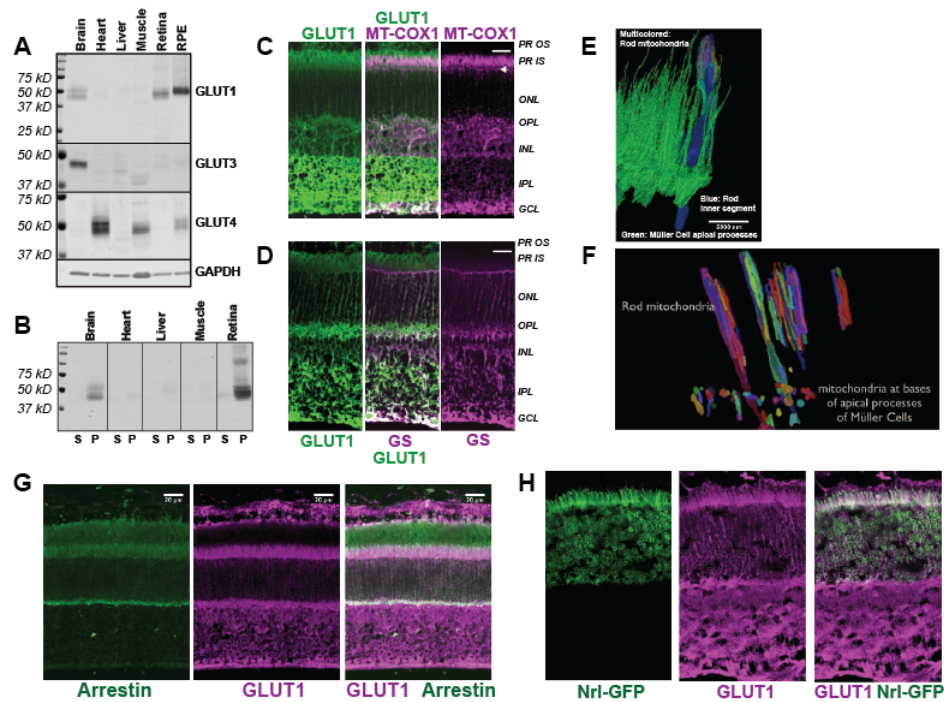506 within the paper.
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Fig. 1. Distribution of GLUT1 in retina. **A.** Immunoblot analysis of mouse tissue homogenates confirms that GLUT-1 is the primary glucose transporter in mouse retina and RPE. 1 μg total homogenate protein was loaded in each lane. No antibodies that we could validate were available for GLUT2. However, the Human Protein Atlas reports no expression of GLUT2 in retina (53). **B**. Evidence that the protein immunoreactive with the GLUT-1 antibody is membrane associated. Homogenates were centrifuged and equivalent percentages of total supernatant (S) and total pellet (P) were probed with the GLUT-1 antibody. **C.** GLUT1 in mouse retina. Rods inner segments are identified by the unique morphology of their mitochondria labeled with mitochondrial cytochrome oxidase 1 antibody (MT-COX1). White arrowhead indicates the layer of Müller cell mitochondria. **D**. Müller cells are identified by glutamine synthetase (GS) immunoreactivity. Scale bars represent 20 μm. **E**. Serial Block Face Scanning Electron Microscopy. The inner segment of one rod cell is shown in blue with its mitochondria shown as multi-colored. The green structures are apical processes of MGCs. **F**. Differences in location and morphology between rod mitochondria and MGC mitochondria in mouse retina. For clarity not all of the mitochondria are shown. MGC mitochondria are located just below the outer limiting membrane. **G**. The left panel shows labeling of rods in a partially light-adapted mouse retina with a rod arrestin antibody. The middle panel shows labeling with a GLUT1 antibody and the right panel shows the merge **H.** The left panel shows expression of GFP from the rod-specific Nrl promoter. The middle panel shows labeling with a GLUT1 antibody and the right side shows the merge. PR OS, photoreceptor outer segment; PR IS photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL inner nuclear layer; IPL inner plexiform layer; GCL, ganglion cell layer.
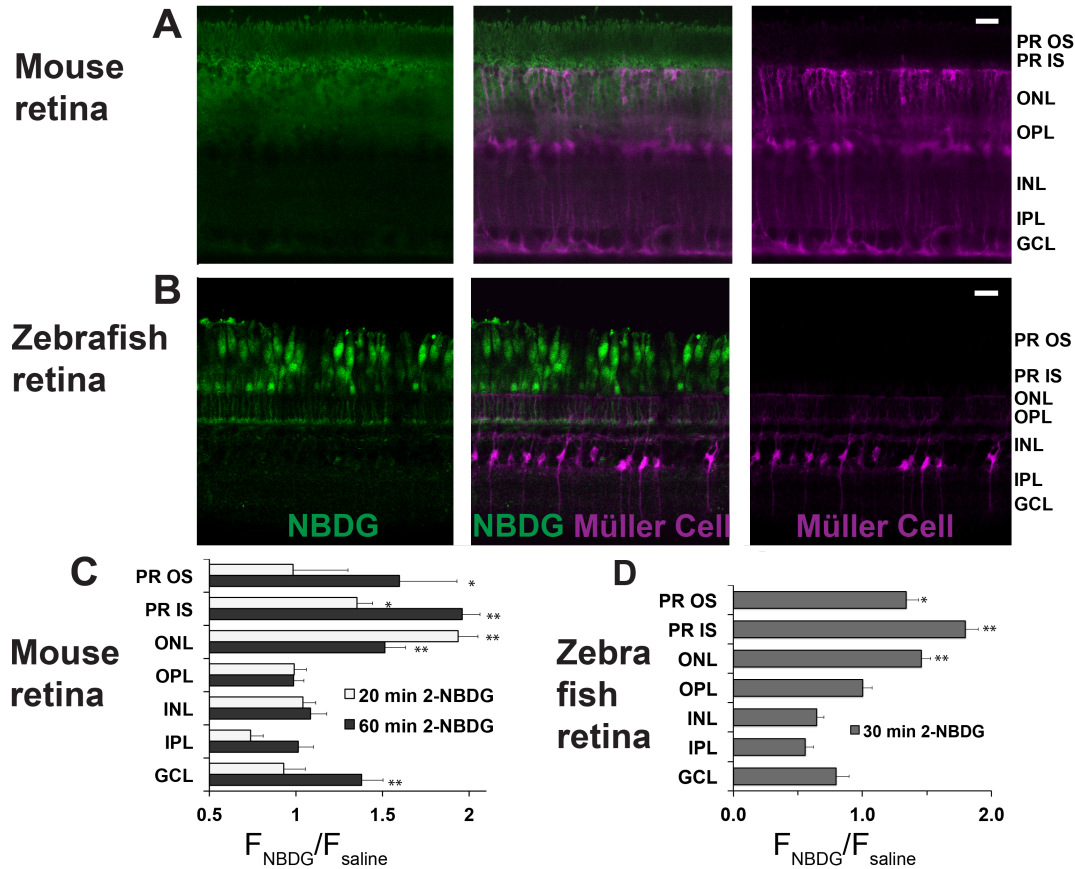
Fig. 2. Fluorescent glucose (2-NBDG) accumulates in photoreceptors after oral gavage. **A.** 2-NBDG (green) accumulation in a mouse retina 20 min. after oral gavage. MGCs are identified by tdTomato in cells in which the Rlbp1 promoter is active. **B**. 2-NBDG accumulation in a zebrafish retina 30 min after oral gavage. MGCs are identified by tdTomato expressed from the GFAP promoter. Labels on the right of panels A and B represent approximate positions of the retinal layers, **C.** Quantification of 2-NBDG fluorescence from mouse retinas (n = 5 animals, 17 slices for 20 min 2-NBDG; 3 animals, 8 slices for 1 h 2-NBDG; 3 animals, 8 slices for saline). F_{NBDG}/F_{saline} compares fluorescence from retinas of mice gavaged with 2-NBDG vs. with saline. Error bars report SEM. **D.** Quantification of 2-NBDG fluorescence from zebrafish retinas (3 animals, 8 slice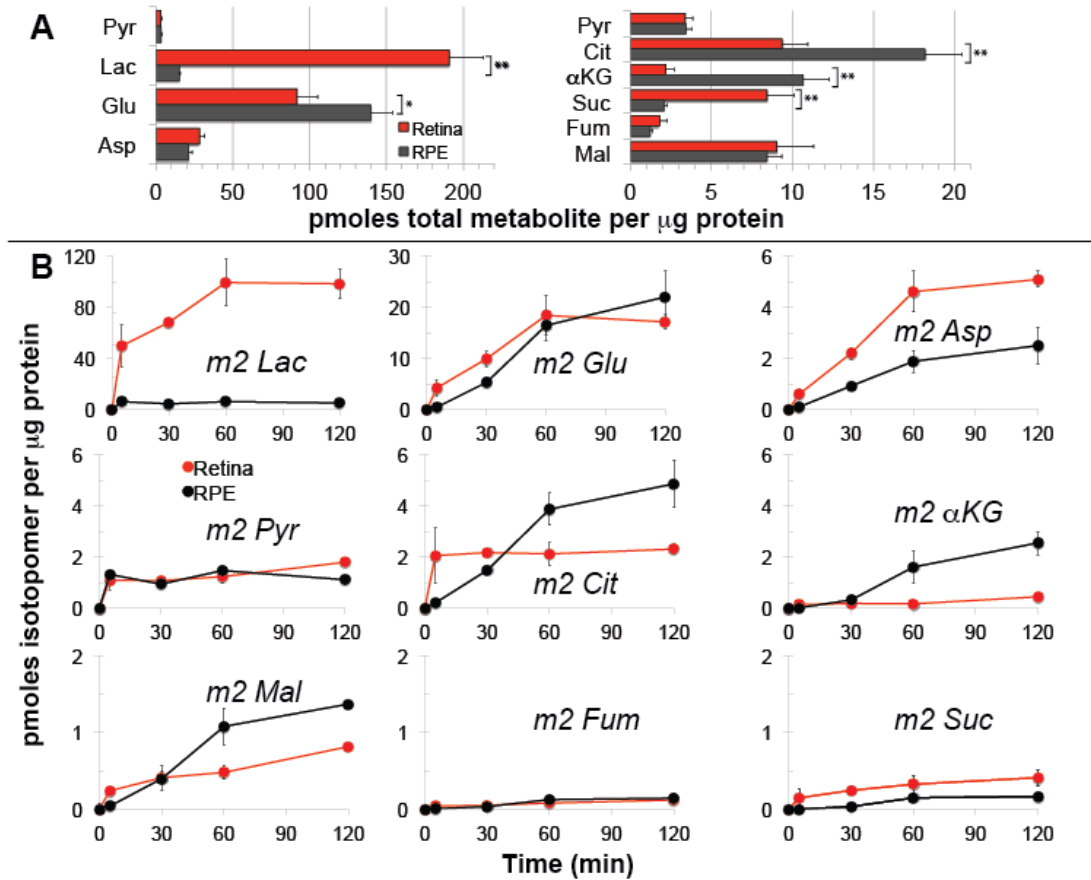s for 30 min 2-NBDG; 2 animals, 3 slices for saline). PR OS, photoreceptor outer segments; PR IS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars represent 20 μm. * indicates $p < 0.05$ and ** indicates $p < 0.01$ for the comparison of F_{NBDG} to F_{saline}.

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562
563 **Fig. 3. Differences in metabolic flux in retina and RPE**. **A**. Total metabolite
564 levels (pmoles per μg protein) in mRetina (red) and hfRPE (black). (n = 11) Note
565 the different scales for the left and right panels. **B**. Incorporation of ^{13}C from [1,2]
566 ^{13}C glucose into metabolites in mRetina and hfRPE cells (pmoles per μg protein).
567 Each of the isotopomers shown is derived from glucose metabolized by
568 glycolysis. Note the different scales for the top, middle and bottom panels. (n = 3
569 for each time point; error bars represent standard deviation).
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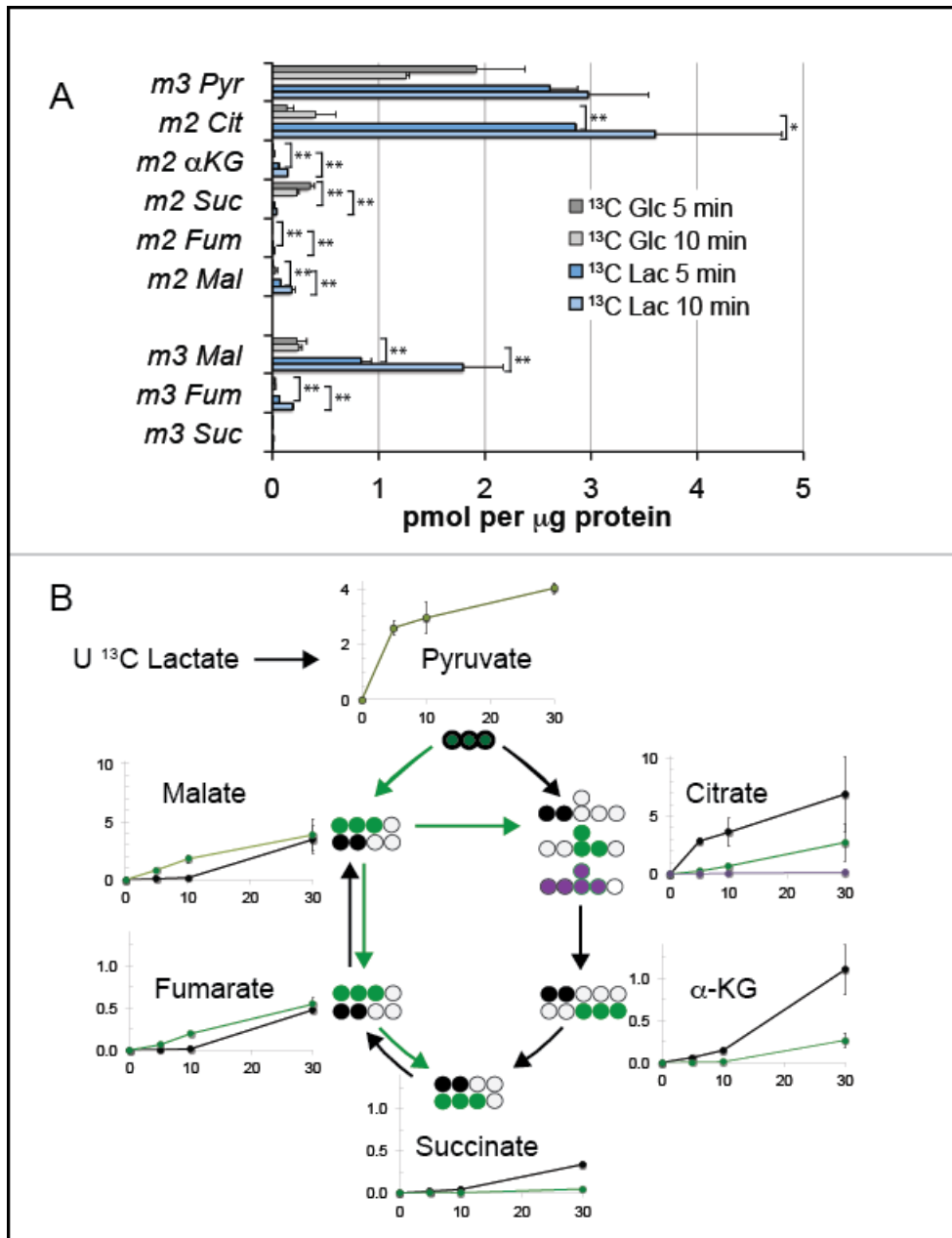
Fig. 4. Incorporation of ^{13}C from lactate into metabolic intermediates in hfRPE cells. **A.** Comparison of initial rates of labeling (at 5 and 10 minutes after introduction of labeled fuel) from U-^{13}C glucose vs. from U-^{13}C lactate. Citrate and malate take up label faster from lactate than from glucose. **B**. Time courses of incorporation of ^{13}C from U-^{13}C lactate into hfRPE metabolites accompanied by schematic illustrations of the labeled species in the context of the TCA cycle. (n = 2-3 for each time point; error bars represent range or standard deviation).

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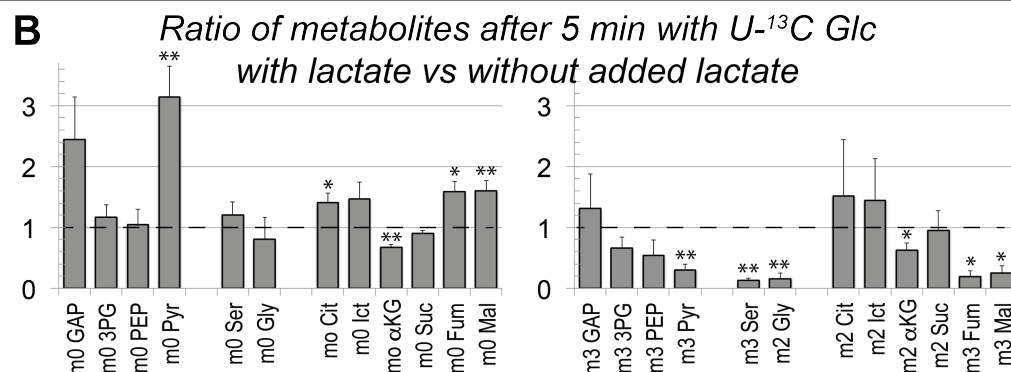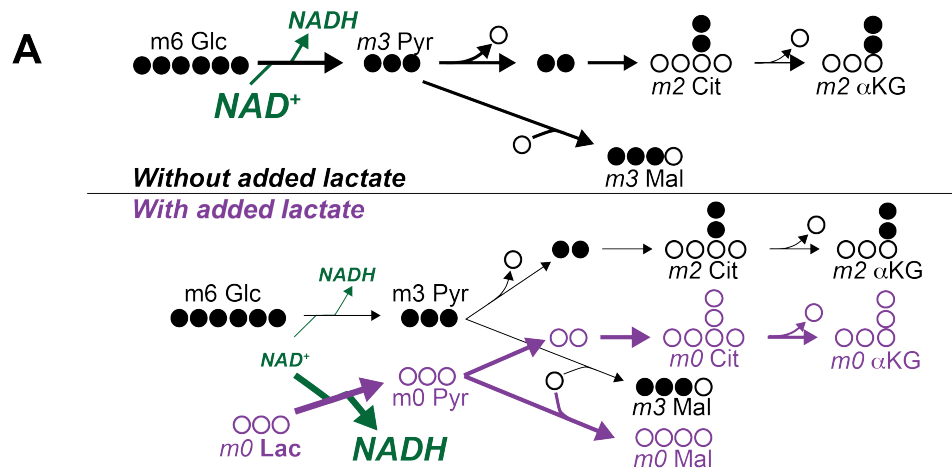
Fig. 5. **Lactate suppresses oxidation of glucose by hfRPE cells**. **A**. Schematic prediction of how U-^{13}C Glc ("m6 Glc") would be metabolized without lactate (top) vs. with lactate (bottom). We hypothesized that lactate would suppress glycolysis of m6 Glc by depleting NAD$^+$. The model also predicts that unlabeled (m0) pyruvate and TCA cycle intermediates become more abundant. **B**. Experimental results showing that lactate substantially increases the total amounts of unlabeled (m0) GAP, pyruvate, citrate, isocitrate, fumarate and malate (left panel) in hfRPE cells. The right panel shows that 20 mM lactate suppresses the incorporation of ^{13}C from 5 mM ^{13}C Glc into glycolytic and TCA intermediate. (n = 3; error bars represent SEM, * indicates p < 0.05 and ** indicates p < 0.01 for the comparison of with vs. without added unlabeled lactate.

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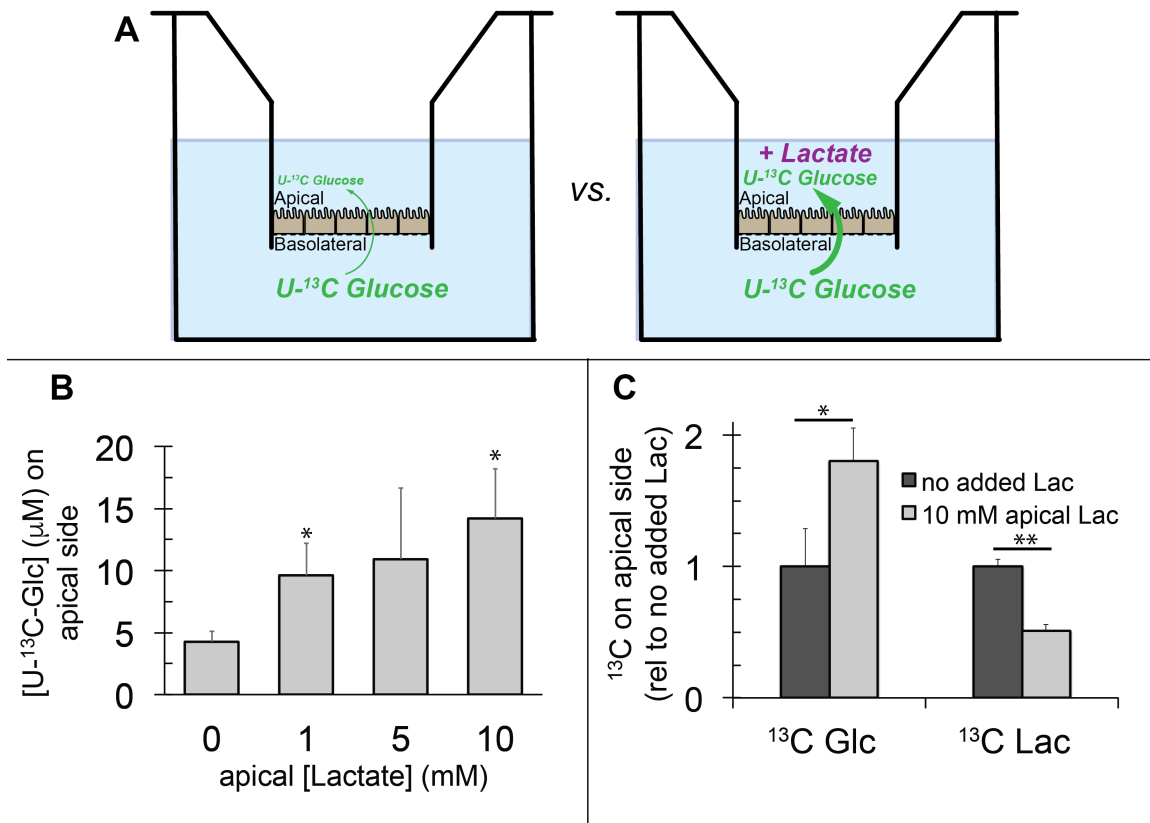
Fig. 6. Lactate can enhance transport of glucose across a monolayer of RPE cells. A. Strategy to evaluate the effect of lactate on transport of glucose across a monolayer of RPE cells. Without lactate (left) glycolysis consumes glucose before it can cross the RPE cell monolayer. With lactate on the apical side (right) glycolysis is partially suppressed so more glucose can cross the monolayer without being consumed by glycolysis. **B.** Lactate (unlabeled) on the apical side enhances transport of U-^{13}C glucose (U-^{13}C Glc) (measured after 24 hours) from the basolateral to apical side of the RPE cell monolayer (n = 3 for each condition; representative of 3 separate experiments, error bars represent StDev * indicates p < 0.05 compared to no added lactate). **C.** Addition of lactate (unlabeled) to the apical side enhances transport of ^{13}C Glc from the basolateral side to the apical side (left) (measured after 8 hours) and it suppresses the formation and/or release of ^{13}C lactate (right) into the medium on the apical side. (n = 3 for each data point, error bars represent StDev).

608
609 **Fig. 7**. Effect of long-term exposure to lactate on hfRPE cells. A. hfRPE cells
610 were grown on standard media to confluence and with a transepithelial
611 resistance >200 $\Omega \bullet cm^2$. They then were treated with either media containing 5
612 mM glucose (top) or 0.5 mM glucose/10 mM lactate (bottom) for 14 days. The
613 pigmentation of the hfRPE cells treated with lactate increased substantially. **B.**
614 Immunoblot quantification of protein extracted from hfRPE cells revealed that
615 several key metabolic enzymes decreased in abundance with lactate
616 supplementation (normalized to total protein) (n = 4; error bars represent SEM).
617 The ratio of LDH3 to other LDH isoforms increased substantially. **C**.
618 Quantification of metabolite ratios from hfRPE cell monolayers incubated with U-
619 ^{13}C lactate for 10 min using GC-MS. The data show that lactate pretreatment
620 alters metabolic flux through specific reactions. **D**. Lactate treatment enhances
621 reductive carboxylation. The graph shows quantification of newly synthesized
622 metabolites from hfRPE cell monolayers incubated with U-^{13}C glutamine for 1 h
623 using GC-MS. The percentage of newly synthesized metabolite made by
624 reductive carboxylation was determined from m5 citrate and isocitrate or m3
625 malate, fumarate and aspartate relative to all other isotopomers of those
626 metabolites that had incorporated ^{13}C. **E**. Comparison of total Lactate/Citrate
627 ratios in retina and eyecups from young (6 month) and old (32 month) mice after
628 incubating the retinas or eyecups with 5 mM Glc and 2 mM Gln for 1 hour. **F**.
629 Same data as in (E) normalized to the Lac/Cit ratio for young mice. (n = 2 for 6
630 month and n = 4 for 32 month; error bars represent StDev.)

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Fig. 8. A working model that describes the flow of metabolic energy in the retina-RPE ecosystem. Photoreceptors convert glucose into lactate and release the lactate into the interphotoreceptor matrix. Lactate suppresses glycolysis in RPE cells by depleting NAD^+. Lactate also fuels metabolic activity in Müller cells, which lack key enzymes that would be required for glycolysis.

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