Human retinal pigment epithelial cells prefer proline as a nutrient and transport metabolic intermediates to the retinal side

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Jennifer R. Chao†, Kaitlen Knight†, Abbi L. Engel‡, Connor Jankowski‡, Yekai Wang†, Megan A. Manson†, Haiwei Gu**, Danijel Djukovic**, Daniel Raftery**, James B. Hurley†‡, and Jianhai Du†‡

From the Departments of††Ophthalmology and ‡‡Biochemistry and **Northwest Metabolomics Research Center, Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, Washington 98109 and the Departments of ††Ophthalmology and ‡‡Biochemistry, West Virginia University, Morgantown, West Virginia 26506

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Metabolite transport is a major function of the retinal pigment epithelium (RPE) to support the neural retina. RPE dysfunction plays a significant role in retinal degenerative diseases. We have used mass spectrometry with 13C tracers to systematically study nutrient consumption and metabolite transport in cultured human fetal RPE. LC/MS-MS detected 120 metabolites in the medium from either the apical or basal side. Surprisingly, more proline is consumed than any other nutrient, including glucose, taurine, lipids, vitamins, or other amino acids. Besides being oxidized through the Krebs cycle, proline is used to make citrate via reductive carboxylation. Citrate, made either from 13C proline or from 13C glucose, is preferentially exported to the apical side and is taken up by the retina. In conclusion, RPE cells consume multiple nutrients, including glucose and taurine, but prefer proline, and they actively synthesize and export metabolic intermediates to the apical side to nourish the outer retina.

The retinal pigment epithelium (RPE) in the vertebrate eye is a monolayer of polarized pigmented epithelial cells that is situated between the photoreceptors and the choroidal blood supply. The RPE provides critical support for the function of the neural retina. It has many long microvilli at its apical side that wrap around the photoreceptor outer segments. On its basal side, the RPE forms convoluted microinfolds that increase its surface area. It transports nutrients and metabolites, recycles retinoids, and engulfs shed outer segment (1, 2). Failure of the RPE leads to photoreceptor degeneration in diseases including age-related macular degeneration (AMD), bestrophinopathy, and Sorsby fundus dystrophy (3–7).

A major function of the RPE is to transport metabolites between the choroid and retina. Photoreceptors have a high demand for energy and are highly glycolytic; like many cancer cells, they metabolize about 90% of the glucose they consume into lactate (8, 9). RPE cells directionally transport glucose to the retina and lactate into the blood through highly expressed glucose transporters and monocarboxylate transporters on both the apical and basal membranes (1, 10, 11). Loss of monocarboxylate transporters causes retinal dysfunction and degeneration (12, 13). However, less is known about the transport of other nutrients and metabolites through the RPE.

The RPE requires an active metabolism to support its multiple functions. Either suppression of its mitochondrial metabolism or activation of glycolysis can cause RPE dysfunction to induce an AMD-like phenotype in mouse models (14, 15). How the RPE imports nutrients to support its own energy demands is still unclear.

Human fetal RPE (hRPE) cultures have similar morphological and physiological characteristics as native RPE (16). These cultures have been well-characterized as a useful model for evaluating RPE metabolism (17) and RPE diseases, including AMD (18). The RPE cultures used in the experiments conducted in this manuscript are of a similar age in culture as the ones used in studies published previously, including investigations of the cause of AMD.

MS provides a sensitive, quantitative, and high-throughput platform to measure metabolites. Transport of metabolites labeled with a stable isotope and biochemical transformations of those metabolites can be monitored. In this report, we use both LC/MS-MS and GC-MS coupled with 13C metabolic flux analysis to investigate how nutrients are consumed and how metabolites are transported through cultured hRPE cells. Surprisingly, we found that proline is the most preferred fuel for RPE cells. They convert proline into ornithine and mitochondrial intermediates through multiple mitochondrial pathways. We also found that RPE transports metabolic intermediates, including citrate, glutamate, serine, and glycine, to its apical side and that, when these metabolites are released from the
Apical RPE, they can be taken up by the retina. We report here a comprehensive study of nutrient utilization and metabolite transport in RPE. Our findings provide new insights into RPE biochemistry and physiology and its role in the pathogenesis of retinal diseases, including AMD.

**Results**

RPE preferentially consumes proline, glucose, and taurine, and it exports metabolic intermediates from both its apical and basal surfaces

To examine how the RPE consumes and metabolizes nutrients, we cultured hRPE cells on filter membrane inserts (Fig. 1A) and quantified metabolites in RPE culture medium from both the apical and basal chambers 0, 8, and 24 h after changing the culture to fresh medium (Fig. 1B). RPE cells cultured on filter inserts developed the polarity seen in native tissue, including apical microvilli, basal infoldings, and tight junctions, as seen by transmission electron microscopy (supplemental Fig. 1). For these experiments, we used a rich RPE medium containing all 20 amino acids, glucose, taurine, pyruvate, and other supplements (the formulation is listed in supplemental Table 1). We quantified 202 metabolites, covering most major metabolic pathways, and detected 110 metabolites in the medium (supplemental Table 2). Of these metabolites, 53 changed substantially between 0 and 8 h (-fold change $>1.3$ or $<1.3$) (Fig. 1). Surprisingly, more proline was consumed than any of the other nutrients.

**Figure 1. The nutrient consumption of hRPE cells from the apical and basal sides. A, schematic illustrating hRPE cultured on transwell filter membranes with 500 µl of RPE medium on each side. MEM α, minimum Eagle’s medium α. B, metabolite consumption and generation in RPE. 40 µl of medium was sampled from each side at different time points, and metabolites were analyzed by LC/MS-MS. Green represents consumed metabolites, and red represents generated metabolites. Data are the average -fold change ± S.D. over the intensity at 0 h from the apical side with the exception of acetoacetate. Acetoacetate is undetectable at 0 h, and its change is represented as -fold over intensity at 8 h from the apical side. *, $p < 0.05$ versus 0 h control ($n = 4$).
other metabolites we measured. After 24 h, the amount of proline decreased 15.3-fold on the apical side and 2.1-fold on the basal side. As expected, RPE also consumed substantial amounts of glucose (\(^{13}C\)) and taurine (\(^{2}H_{11}002\)). Other metabolites decreased by less than 2-fold. Nutrients in the medium were consumed through multiple pathways, including the tricarboxylic acid (TCA), \(\beta\)-oxidation, ketogenesis, and pentose phosphate pathways. Intermediates in those pathways accumulated in the medium at 8 and 24 h. For example, lactate, 3-hydroxybutyrate, citrate, and 1-methyladenosine increased 10-fold on the apical side (Fig. 1B). In general, these intermediates increased earlier and more substantially on the apical side compared with the basal side, suggesting that RPE may actively transport these metabolites. To exclude the influence of filter coating on metabolite transport, we quantified the ratios of metabolites from the two sides from wells with coated inserts without RPE after 24-h incubation. The metabolites reached equilibrium with ratios between the two sides of less than 1.2 (supplemental Table 3).

**Figure 2. Proline is utilized to generate intermediates through both mitochondrial oxidative and reductive pathways.** A, schematic of tracing proline metabolism in RPE cells. hfRPE cells were grown in 6-well plates and incubated with [U-\(^{13}C\)]proline (\(^{13}C\) carbon in blue) and glucose (\(^{12}C\) carbon in black). B, [U-\(^{13}C\)]proline-labeled metabolites in hfRPE. hfRPE cells were incubated with 2 mM [U-\(^{13}C\)]proline in the presence of 5 mM glucose in KRB for 1 h. C, schematic of mitochondrial oxidative and reductive pathways. Shown in blue is \(^{13}C\) (labeled), and shown in black is \(^{12}C\) (unlabeled). D, [U-\(^{13}C\)]proline labeled both oxidative and reductive pathways. M4 citrate/isocitrate derives from the oxidative pathway, and M5 citrate/isocitrate derives from the reductive pathway. E and F, [U-\(^{13}C\)]proline-labeled intermediates that are exported into the medium (n = 3).

**Proline is converted into ornithine and TCA cycle intermediates through both oxidative and reductive pathways**

To determine how proline is metabolized, we used [U-\(^{13}C\)]proline to trace its metabolism through known pathways. For example, proline can be reduced to form pyrroline-5-carboxylate (P5C). P5C can be converted into ornithine or glutamate, which can feed into the TCA cycle (Fig. 2A). To avoid interference from other nutrient sources, we changed the medium to KRB containing only 5 mM glucose and 2 mM [U-\(^{13}C\)]proline. After 1 h, the intensity of [U-\(^{13}C\)]proline dropped 20% in the medium (supplemental Fig. 2), consistent with our finding that RPE cells prefer proline. Even in the presence of unlabeled glucose, about half of the pool of ornithine, glutamate, and TCA cycle intermediates was labeled with \(^{13}C\) from [U-\(^{13}C\)]proline (Fig. 2B). Only ~5% of pyruvate, alanine, and lactate became labeled, possibly through malic enzyme activity. Proline can also be hydroxylated to form hydroxyproline. However, we did not detect any \([^{13}C]\)hydroxyproline.
after 1 h, indicating that free proline may not be hydroxylated into hydroxyproline or that hydroxyproline turnover is slow.

Recently, we reported that reductive carboxylation is a major metabolic pathway in RPE cells (19). When [U-13C]proline is used to generate citrate by reductive carboxylation, all five carbons from citrate are labeled with 13C (M5); if it goes through the classic TCA cycle, then one carbon is removed by decarboxylation catalyzed by α-ketoglutarate (αKG) dehydrogenase, so the citrate produced is M4 (Fig. 2C). We found that M5 citrate/isocitrate is the predominant isotopomer after 1-h labeling of hfRPE cells with [13C]proline. M5 citrate is 2.5-fold more enriched than M4 citrate/isocitrate (Fig. 2D). The M3 and M2 isotopomers are derivatives of M5 and M4, respectively, after a second round of reactions after citrate lyase. Proline-derived citrate and other intermediates are exported into the medium (Fig. 2E). M5 citrate also is the predominant isotopomer in the medium (Fig. 2F).

**RPE exports glucose-derived intermediates preferentially to the apical side**

Glucose is considered to be an essential nutrient source for RPE (20). We confirmed this by showing that glucose is consumed significantly in hfRPE culture (Fig. 1B). To study how intermediates derived from glucose are exported from RPE cells, we added [U-13C]glucose at either the apical or basal side with unlabeled glucose on the opposite side and quantified the labeled intermediates from both sides at 8, 24, and 48 h (Fig. 4, A and E). GC-MS data showed a time-dependent increase of intermediates from glucose metabolism in the medium from both apical and basal sides. Lactate, pyruvate, alanine, serine, and glycine are generated from glycolysis, and citrate, malate, glutamate, and glutamine are from the mitochondrial TCA cycle (Fig. 4 and supplemental Fig. 3). When [13C]glucose is added to the apical side, the apical intermediates reach a steady state by 24 h, whereas accumulation of most intermediates on the basal side is delayed (Fig. 4, B and C). The concentration difference of labeled metabolites between apical and basal compartments was highest at 8 h, with ~10-fold more on the apical side (Fig. 4D). When [13C]glucose is provided on the basal side (Fig. 4E), intermediates were labeled more slowly with lower enrichment. The concentration difference also is less obvious for most intermediates, with the exception of glutamine (Fig. 4, F–H). Serine and glycine labeled with 13C also are exported (supplemental Fig. 3). Because there were high concentrations of unlabeled serine and glycine (0.4 mM) in the DMEM, the apparent enrichment of serine and glycine was diluted to less than 5%. Remarkably, no matter the side to which [13C]glucose was added, citrate, glutamate, and glutamine were at higher...
levels on the apical side than on the basal side, suggesting that export of these metabolites can supply energy to the outer retina.

**Mitochondrial function enhances transport of some metabolic intermediates**

We noted that mitochondrial intermediates are exported into the medium. To test the importance of mitochondrial energy metabolism in metabolite transport, we used rotenone to block mitochondrial respiratory complex I in hRPE cells (Fig. 5A). When complex I (NADH dehydrogenase) is inhibited, NADH should accumulate in the mitochondrial matrix. Malate can be exported to the cytoplasm, where its reducing power can be transferred through NADH to convert pyruvate to lactate. Pyruvate is drawn away from entering mitochondria, and oxaloacetate is drawn out of the matrix as malate. Together, these would decrease the matrix concentrations of the substrates for synthesizing citrate (Fig. 5A). Consistent with this interpretation, we found that 8 h of rotenone treatment caused relative increases of lactate and malate and a relative decrease of pyruvate and citrate. We noted that glycine and serine decreased more substantially on the basal side (Fig. 5, B and C). We also noted
that inhibition of mitochondrial metabolism abrogated the apical preference for citrate, malate, glutamate, and alanine (Fig. 5D). To monitor cell death, we tested the release of lactate dehydrogenase from the cells into the medium. There was little evidence of cell death in these experiments (supplemental Fig. 4).

**The retina imports metabolites exported by the RPE**

To determine whether metabolites from the RPE can be imported as nutrients by the retina, we co-cultured mouse retina with photoreceptors in contact with the apical side of RPE cells grown on transwell filters. We then added [13C]glucose to the basal side (Fig. 6A). The retina has a high rate of aerobic glycolysis that metabolizes glucose to lactate. As expected, the retina enhanced accumulation of labeled lactate in both the apical and basal chambers. The retina caused depletion of both citrate and glutamate from the apical medium, indicating that they were taken up by the retina. Glutamine accumulated on the apical side but decreased on the basal side. Labeled serine and glycine were depleted only on the basal side (Fig. 6, B and C). To examine how retina co-culture affected RPE metabolism, we quantified the intracellular metabolites in RPE cells. Only lactate was increased in RPE cells co-cultured with retina, supporting the idea that RPE imports retina-derived lactate. These results suggest that the retina does not significantly affect intracellular glucose metabolism in RPE under these conditions (Fig. 6D).

To further examine whether metabolites released into the medium by RPE cells are used by the retina, we compared labeled metabolites in retina from [13C]glucose with or without RPE. Strikingly, co-culture with RPE markedly increased lactate, pyruvate, citrate, αKG, alanine, glutamine, serine, and glycine (Fig. 6E), corroborating our hypothesis that RPE exports intermediates to support the retina (Fig. 6F).

**Discussion**

RPE culture is an excellent *in vitro* model to study RPE function and AMD (17, 21). In this study, we used targeted metabolomics to study how the RPE consumes nutrients. Unexpect-
edly, we found that RPE cells prefer proline as an energy substrate. We also developed a stable isotope-based approach to trace metabolite transport out of the RPE to either its apical or its basal side, and we showed that metabolites exported from the RPE can support retinal metabolism.

Proline is consumed by RPE cells faster than any other nutrients in the culture medium. Proline is a non-essential amino acid that is not normally included in standard DMEM preparations. However, proline ranging from 10–115 mg/liter is typically included in most of the widely used protocols for human RPE culture media (16, 21–27). Proline can be provided within the RPE by synthesis either from glutamate by P5C synthase or from ornithine by ornithine aminotransferase (OAT). Deficiency of OAT can cause gyrate atrophy, an inborn error of metabolism characterized by lobular loss of RPE and choroid (28, 29). RPE has been identified as the major and most early damaged site in gyrate atrophy (29). Intriguingly, supplementation with proline rescues ornithine cytotoxicity induced by inhibition of OAT in RPE cells (30, 31). Under our experimental conditions, glutamate and arginine (the precursor of ornithine) are not significantly used in 24 h (Fig. 1B), indicating that proline in RPE cells is more dependent on exogenous supply.

Proline imported into RPE cells can be catabolized into glutamate for mitochondrial intermediates and into ornithine for urea cycle activity. We found that $^{13}$C from proline replaced 50% of the endogenous glutamate, ornithine, and mitochondrial intermediates within 1 h (Fig. 2B). Labeled glutamate and ornithine accumulated at 24 h, even in the presence of abundant glutamate, glutamine, and arginine in the RPE culture medium (Fig. 2B). These results demonstrate that proline is an important nutrient source for RPE metabolism. In addition to being oxidized through the TCA cycle, proline fuels the active reductive carboxylation pathway we reported previously in RPE cells (19). Reductive carboxylation increases mitochondrial bioenergetics and cellular resistance against oxidative damage. Both mitochondrial dysfunction and oxidative stress are major contributors to the pathogenesis of AMD. Our findings highlight the need to elucidate how proline catabolism contributes to RPE metabolism in vivo and how it is influenced in diseased RPE cells.

Figure 6. Retina uptake of the metabolites released by RPE. A, top panel, schematic illustrating hRPE cultured on filter inserts with and without mouse retina overlay. The medium consisted of DMEM with 1% FBS and 2 mM glutamine with unlabeled 5.5 mM glucose on the apical side and 5.5 mM [U-$^{13}$C]glucose on the basal side. B and C, the intensity of labeled metabolites in apical or basal medium. *, $p < 0.05$ versus RPE cells only ($n = 3$). D, the intensity of labeled metabolites in RPE cells. *, $p < 0.05$ versus retina only ($n = 3$). E, schematic of metabolite transport between the RPE and retina.
Metabolite transport in RPE

Proline and its hydroxylated form, hydroxyproline, make up 25% of collagen (32), which is the most abundant protein in extracellular matrix (ECM) and in the collagenous zones of the Bruch membrane (BrM). The BrM is located between the RPE and the choroid, and ECM remodeling plays a critical role in the deposition of drusen in the BrM in AMD (33). Mutations of ECM metabolism genes have been identified in AMD patients (34). RPE cells control collagen synthesis for the BrM (35). Both [14C]proline and [3H]proline were incorporated in newly synthesized collagen in feline RPE cells and aged primate RPE cells (36, 37). In our preparations, RPE cells have abundant free hydroxyproline; however, it is not labeled by [13C]proline in 1 h (Fig. 2B). This indicates that the hydroxylation of proline occurs after nascent collagen synthesis (38). It also suggests that the hydroxyproline turnover in collagen synthesis and degradation is a very slow process. Interestingly, one proline transporter, SLC6A20, is one of 154 RPE signature genes that is specifically and highly expressed in human RPE by a comparative study of gene expression from 78 tissues (39). Additional investigations will be required to show how carbons from proline are distributed to various metabolic pathways, how proline is transported, and how deprivation of proline in culture impacts RPE differentiation and function.

Besides proline, RPE also consumes substantial amounts of taurine and glucose. Photoreceptors are enriched with taurine, and they use glucose for aerobic glycolysis. RPE expresses glucose transporters and taurine transporters and is enriched with these two nutrients (10, 40, 41). Taurine supplementation promotes RPE proliferation and suppresses cell death in RPE culture (42, 43). As a well-known essential nutrient source, glucose is included in almost all RPE culture protocols. Deprivation of glucose reduces RPE viability, and attempts to rescue glucose-deprived RPE using other energy substrates have not been successful (20). These reports are consistent with our finding that the RPE needs these two basic nutrients under standard culture conditions.

We found that RPE cells export metabolic intermediates other than lactate and β-hydroxybutyrate into the culture medium. Citrate, glutamate, and glutamine are predominantly enriched in the culture medium on the apical side. Little is known about plasma membrane transporters for citrate and glutamine in RPE. Glutamate transporters have been found in cultured RPE cells, but their distribution is unknown (44, 45). Citrate is a key component of the TCA cycle, an important substrate for lipid biosynthesis, and a chelator forivalent cations like Ca2+, Zn2+, Fe2+, and Mg2+ (46). Citrate is produced in the mitochondria and exported into the cytosol through the mitochondrial citrate transporter (SLC25A1). Alternatively, citrate can also be synthesized in the cytosol from αKG through reductive carboxylation by isocitrate dehydrogenase 1 (47). Three citrate transporters (SLC13A2, SLC13A3, and SLC13A5) are responsible for intracellular citrate transport or for import of citrate from blood (48). The citrate concentration in cerebrospinal fluid is about 0.4 mM (49). 13C NMR spectroscopy has shown that astrocytes, but not neurons, are capable of exporting citrate (46, 49). Microvilli from the RPE surround photoreceptors outer segments. Photoreceptor uptake of citrate derived from the RPE might facilitate glycolysis by supplying oxaloacetate to shuttle reducing power into mitochondria, provide acetyl-CoA for fatty acid synthesis, be utilized directly for the TCA cycle, and/or regulate divalent cations in the outer segment. Our RPE/retina co-culture experiments showing increased lactate, pyruvate, αKG, and glutamine in the mouse retina (Fig. 6E) support this hypothesis.

In the retina, glutamine is synthesized in glia cells and transported into photoreceptors to generate glutamate. We have reported a neuron-glial metabolism model in which lactate, together with neuron-derived aspartate, is used for glutamine synthesis in Müller cells in the retina (50). Our RPE/retina coculture experiments revealed increased labeled glutamine in the retina and the apical medium with no change in RPE cells. Under our culture conditions, it appears that lactate produced either by the RPE or by photoreceptors in the retina contributes to glutamine synthesis within Müller cells. Additionally, glutamate is depleted from the apical medium when retina is present, suggesting that RPE cells might contribute to the glutamine-glutamate cycle.

Serine and glycine are synthesized from 3-phosphoglycerate, a glycolytic intermediate. Surprisingly, RPE causes an ~7-fold increase in incorporation of 13C from glucose into serine and glycine (Fig. 6F). Serine is used for biosynthesis of glycerophospholipids, sphingosine, and ceramide. These phospholipids are in high demand for the daily renewal of shed outer segments (51). Glycine is essential for purine biosynthesis. Purines like cGMP, ATP, and hypoxanthine are required for phototransduction (8). Additionally, metabolism of serine and glycine is an important source of NADPH, which is needed for anti-oxidative stress and lipid synthesis (52). Recent genome-wide analyses have shown that several key enzymes in the serine and glycine pathways have common variants associated with macular telangiectasia type 2, a neurovascular degenerative retinal disease (53).

In summary, we have shown that proline is a preferred nutrient source for cultured RPE cells. Proline is used to generate mitochondrial intermediates through both oxidative and reductive pathways. We found that RPE cells transport glucose-derived citrate, glutamate, serine, and glycine from their apical surface to be used by the retina. These findings reveal how RPE utilizes substrates and provide insights into RPE biochemistry and retinal diseases. It is important to note that we have shown in this study how RPE cells have these preferences when grown in culture. Additional experiments will be required to confirm that RPE cells in the eye of a live animal have similar preferences for metabolic fuels.

Experimental procedures

Reagents

Unless otherwise specified, all reagents were obtained from Sigma.

RPE cell culture

Human fetal RPE was isolated from fetal tissue (16–18 weeks of gestation) as reported previously (19, 26) and cultured for 4 weeks to form a confluent, pigmented monolayer of hexagonal cells. For nutrient transport experiments, RPE cells were passaged and grown on 0.4-μm transparent polyethylene tereph-
thale membrane inserts (Corning, 353180) precoated with Matrigel (BD Biosciences) at \(1 \times 10^5\) cells/insert for 4–6 weeks. The cells were cultured in RPE medium consisting of a minimum Eagle’s medium (Life Technologies), non-essential amino acids (Life Technologies), N1 supplement (Life Technologies), 1% (v/v) FBS (Atlanta Biologicals), taurine, hydrocortisone, and triiodo-thyronine (the detailed formulation of the medium can be found in supplemental Table 1). Transepithelial resistance (TER) was measured with a Millicell ERS-2 epithelial volt-ohm meter (Millipore). The pigmented RPE cells were cultured on filter membranes with a TER \(\geq 200\) ohm/cm\(^2\) were changed into DMEM containing 1% FBS and [\(\mathrm{U}-^{13}\mathrm{C}\)]glucose (Cambridge Isotope Laboratories Inc.) or [\(\mathrm{U}-^{13}\mathrm{C}\)]proline. 20 \(\mu\)l of medium was collected for metabolite analysis.

For the intracellular proline labeling experiments, RPE cells were cultured in precoated 6-well plates for 4–6 weeks. Prior to each experiment, the cells were changed into Krebs-Ringer bicarbonate buffer (KRB) (54) containing 5 \(\text{mM}\) glucose and 2 \(\text{mM}\) [\(\mathrm{U}-^{13}\mathrm{C}\)]proline and incubated for 1 h before collection of medium and cells for metabolite analysis.

**Retina and RPE co-culture**

RPE cells grown on Millicell-HA filters (Millipore) for 4–6 weeks with TER \(\geq 200\) ohm/cm\(^2\) were used for experiments. Isolated mouse retinas were laid on top of the RPE cells, with the photoreceptors facing the apical side of the RPE. Four cuts were made to the retina to relieve the curvature, and minimal medium was left in the apical chamber to make the retina flat. After 1 h, the medium was carefully changed into DMEM containing 1% FBS and 2 \(\mu\)M glutamine with unlabeled glucose on the apical side and [\(\mathrm{U}-^{13}\mathrm{C}\)]glucose on the basal side.

**Sample preparation for metabolite analysis**

20 \(\mu\)l of medium was mixed with 80 \(\mu\)l of cold methanol on ice for 15 min to precipitate proteins. The mixture was centrifuged at 13,300 rpm for 15 min at 4 °C, and the supernatant was lyophilized for analysis by either LC/MS-MS or GC-MS. RPE cells, after removing the medium, were quickly rinsed with cold 0.9% NaCl and placed on dry ice to quench metabolism (54).

**Metabolite analysis by LC/MS-MS**

The LC/MS-MS method was described in detail previously (8, 55, 56). Briefly, dried metabolites were reconstituted in 200 \(\mu\)l of 5 \(\text{mM}\) ammonium acetate in 95% water, 5% acetonitrile, and 0.5% acetic acid and filtered through 0.45-\(\mu\)m PVDF filters. The metabolites were separated by a ethylene bridged hybrid Amid column (1.7 \(\mu\)m, 2.1 mm \(\times\) 150 mm, Waters) with an Agilent 1260 LC (Agilent Technologies, Santa Clara, CA) and detected using an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, ON, Canada) system. Targeted data acquisition was performed in multiple reaction monitoring modes for 202 transitions listed in supplemental Table 2. The extracted peaks were integrated using MultiQuant 2.1 software (AB Sciex).

All stable isotope-labeled metabolites were analyzed by GC-MS as reported previously (8, 19, 54). Dried samples were derivatized by methoxymine (Sigma) and N-terbutyldimethylsilyl-N-methyltrifluoroacetamide (Sigma) and analyzed on an Agilent 7890/5975C GC-MS system (Agilent Technologies) using an HP-5MS column (30 m \(\times\) 0.25 mm \(\times\) 0.25 \(\mu\)m, Agilent). The peaks were analyzed using Agilent Chemstation software, and the measured distribution of mass isotopomers was corrected for natural abundance with IsoCor software. Methyl-succinate was added to each sample as a reference. Enrichment was calculated by dividing the labeled ions by the total ion intensity.

**Statistics**

Data are expressed as the mean \(\pm\) S.D. The significance of differences between means was determined by unpaired two-tailed t tests or analysis of variance with an appropriate post hoc test. \(p < 0.05\) was considered to be significant.

**Author contributions**—Conceptualization, J. R. C., J. B. H., and J. D.; Investigation, J. R. C., K. K., A. L. E., C. J., Y. W., M. A. M., H. G., D. D., D. R., and J. D.; Writing, J. D., J. B. H., and J. R. C.; Funding Acquisition, J. R. C., J. B. H., and J. D.; Supervision, J. R. C., J. B. H., and J. D.

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**References**

1. Lehmann, G. L., Benedicto, I., Philp, N. J., and Rodriguez-Boulan, E. (2014) Plasma membrane protein polarity and trafficking in RPE cells: past, present and future. Exp. Eye Res. 126, 5–15
2. Strauss, O. (2005) The retinal pigment epithelium in visual function. Physiol. Rev. 85, 845–881
3. Sparrow, J. R., Hicks, D., and Hamel, C. P. (2010) The retinal pigment epithelium in health and disease. Curr. Med. Res. Opin. 26, 802–823
4. Ambati, J., and Fowler, B. J. (2012) Mechanisms of age-related macular degeneration. Neuron 75, 26–39
5. Rattner, A., and Nathans, J. (2006) Macular degeneration: recent advances and therapeutic opportunities. Nat. Rev. Neurosci. 7, 860–872
6. Guziewicz, K. E., Sinha, D., Gómez, N. M., Zorych, K., Dutor, E. V., Dingra, A., Mullins, R. F., Stone, E. M., Gamm, D. M., Boesze-Battaglia, K., and Aguirre, G. D. (2017) Bestrophinopathy: an RPE-photoreceptor interface disease. Prog. Retin. Eye Res. 58, 70–88
7. Weber, B. H., Vogt, G., Pruett, R. C., Stöhr, H., and Felbor, U. (1994) Mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3) in patients with Sorsby’s fundus dystrophy. Nat. Genet. 8, 352–356
8. Du, J., Rountree, A., Cleghorn, W. M., Contreras, L., Lindsay, K. J., Sadilek, M., Gu, H., Djukovic, D., Raftrey, D., Satrústegui, J., Kanow, M., Chan, L., Tsang, S. H., Sweet, I. R., and Hurley, J. B. (2016) Phototransduction influences metabolic flux and nucleotide metabolism in mouse retina. J. Biol. Chem. 291, 4698–4710
9. Hurley, J. B., Lindsay, K. J., and Du, J. (2015) Glucose, lactate, and shuttling of metabolites in vertebrate retinas. J. Neurosci. Res. 93, 1079–1092
10. Sugawara, K., Deguchi, J., Okami, T., Yamamoto, A., Omori, K., Uyama, M., and Tashiro, Y. (1994) Immunocytocchemical analyses of distributions of Na, K-ATPase and GLUT1, insulin and transferrin receptors in the developing retinal pigment epithelial cells. Cell Struct. Funct. 19, 21–28
11. Philp, N. J., Wang, D., Yoon, H., and Hjelmeland, L. M. (2003) Polarized expression of monocarboxylate transporters in human retinal pigment
epithelium and ARPE-19 cells. *Invest. Ophthalmol. Vis. Sci.* 44, 1716–1721.
28. O'Donnell, J. J., Sandman, R. P., and Martin, S. R. (1978) Gyrate atrophy of the retina. *J. Clin. Invest.* 62, 2079–2090.
29. Wang, T., Milam, A. H., Steel, G., and Valle, D. (1996) A mouse model of gyrate atrophy of the choroid and retina: early retinal pigment epithelial damage and progressive retinal degeneration. *J. Clin. Invest.* 97, 2753–2762.
30. Ueda, M., Masu, Y., Ando, A., Maeda, H., Del Monte, M. A., Uyama, M., and Ito, S. (1998) Prevention of ornithine cytotoxicity by proline in human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 39, 820–827.
31. Ando, A., Ueda, M., Uyama, M., Masu, Y., Okumura, T., and Ito, S. (2000) Heterogeneity in ornithine cytotoxicity of bovine retinal pigment epithelial cells in primary culture. *Exp. Eye Res.* 70, 89–96.
32. Wang, T., Milam, A. H., Steel, G., and Valle, D. (1996) A mouse model of gyrate atrophy of the choroid and retina: early retinal pigment epithelial damage and progressive retinal degeneration. *J. Clin. Invest.* 97, 2753–2762.
33. O'Donnell, J. J., Sandman, R. P., and Martin, S. R. (1978) Gyrate atrophy of the retina. *J. Clin. Invest.* 62, 2079–2090.
34. Duvvari, M. R., van de Ven, J. P., Geerlings, M. J., Saksens, N. T., Bakker, B., Henkes, A., Neveling, K., del Rosario, M., Westra, D., van den Heuvel, L. P., Schick, T., Fauser, S., Boon, C. J., Hoyng, C. B., et al. (2016) Whole exome sequencing in patients with the cuticular drusen subtype of age-related macular degeneration. *PLoS ONE* 11, e0152047.
35. Nita, M., Strzalka-Mrozik, B., Grzybowski, A., Mazurek, U., and Romaniuk, W. (2014) Age-related macular degeneration and changes in the extracellular matrix. *Med. Sci. Monit.* 20, 1003–1016.
36. Li, W., Stramm, L. E., Aguirre, G. D., and Rockey, J. H. (1984) Extracellular matrix production by cat retinal pigment epithelium *in vitro*: characterization of type IV collagen synthesis. *Exp. Eye Res.* 38, 291–304.
37. Hirata, A., and Feeney-Burns, L. (1992) Autoradiographic studies of aged primate retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 33, 2079–2090.
38. Mitsubuchi, H., Nakamura, K., Matsumoto, S., and Endo, F. (2008) Inborn errors of proline metabolism. *J. Nutr.* 138, 2016S–2020S.
39. Strunnikova, N. V., Maminishkis, A., Barb, J. J., Wang, F., Zhi, C., Sergeev, Y., Chen, W., Edwards, A. O., Stambolian, D., Abecasis, G., Swaroop, A., Munson, P. J., and Miller, S. S. (2010) Transcriptome analysis and molecular signature of human retinal pigment epithelium. *Hum. Mol. Genet.* 19, 2468–2486.
40. Hillenkamp, J., Hussain, A. A., Jackson, T. L., Constable, P. A., Cunnigham, J. R., and Marshall, J. (2004) Compartmental analysis of taurine transport to the outer retina in the bovine eye. *Invest. Ophthalmol. Vis. Sci.* 45, 4099–4105.
41. El-Sherbeny, A., Naggar, H., Miyauuchi, S., Ola, M. S., Maddox, D. M., Martin, P. M., Ganapathy, V., and Smith, S. B. (2004) Osmoregulation of taurine transporter function and expression in retinal pigment epithelial, ganglion, and Muller cells. *Invest. Ophthalmol. Vis. Sci.* 45, 694–701.
42. Gabrielián, K., Wang, H. M., Ogden, T. E., and Ryan, S. J. (1992) *In vitro* stimulation of retinal pigment epithelium proliferation by taurine. *Curr. Eye Res.* 11, 481–487.
43. Udawatte, C., Qian, H., Mangini, N. J., Kennedy, B. G., and Ripps, H. (2008) Taurine suppresses the spread of cell death in electrically coupled RPE cells. *Mol. Vis.* 14, 1940–1950.
44. Mäenpää, H., Gegelashvili, G., and Tähti, H. (2004) Expression of glutamate transporter subtypes in cultured retinal pigment epithelial and retinoblastoma cells. *Curr. Eye Res.* 28, 159–165.
45. Miyamoto, Y., and Del Monte, M. A. (1994) Na+-dependent glutamate transporter in human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 35, 3589–3598.
46. Westergaard, N., Waagepetersen, H. S., Belhage, B., and Schouboe, A. (2011) Citrate, a ubiquitous key metabolite with regulatory function in the CNS. *Neurochem. Res.* 36, 1583–1588.
47. Jiang, L., Boufersaoufi, A., Yang, C., Ko, B., Rakhsha, D., Guevara, G., Hu, Z., and DeBerardinis, R. J. (2016) Quantitative metabolic flux analysis reveals an unconventional pathway of fatty acid synthesis in cancer cells deficient for the mitochondrial citrate transport protein. *Metab Eng.* 10.1016/j.ymben.2016.11.004.
48. Pajoor, A. M. (2014) Sodium-coupled dicarboxylate and citrate transporters from the SLC13 family. *Pflugers Arch.* 466, 119–130.
49. Sonnewald, U., Westergaard, N., Krane, J., Ungsgård, G., Petersen, S. B., and Schouboe, A. (1991) First direct demonstration of preferential release of citrate from astrocytes using [13C]NMR spectroscopy of cultured neurons and astrocytes. *Neurosci. Lett.* 128, 235–239.
50. Lindsay, K. J., Du, J., Sloat, S. R., Contreras, L., Linton, J. D., Turner, S. J., Sadilek, M., Satrústegui, J., and Hurley, J. B. (2014) Pyruvate kinase and aspartate-glutamate carrier distributions reveal key metabolic links between neurons and glia in retina. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 15579–15584

51. Ruggiero, L., and Finnemann, S. C. (2014) Lack of effect of microfilament or microtubule cytoskeleton-disrupting agents on restriction of externalized phosphatidylserine to rod photoreceptor outer segment tips. *Adv. Exp. Med. Biol.* **801**, 91–96

52. Ducker, G. S., and Rabinowitz, J. D. (2017) One-carbon metabolism in health and disease. *Cell Metab.* **25**, 27–42

53. Scerri, T. S., Quaglieri, A., Cai, C., Zernant, J., Matsunami, N., Baird, L., Scheppke, L., Bonelli, R., Yannuzzi, L. A., Friedlander, M. S., Egan, C. A., Fruttiger, M., Leppert, M., Allikmets, R., and Bahlo, M. (2017) Genome-wide analyses identify common variants associated with macular telangiectasia type 2. *Nat. Genet.* **49**, 559–567

54. Du, J., Linton, J. D., and Hurley, J. B. (2015) Probing metabolism in the intact retina using stable isotope tracers. *Methods Enzymol.* **561**, 149–170

55. Zhu, J., Djukovic, D., Deng, L., Gu, H., Himmati, F., Chiorean, E. G., and Raftery, D. (2014) Colorectal cancer detection using targeted serum metabolic profiling. *J. Proteome Res.* **13**, 4120–4130

56. Gu, H., Carroll, P. A., Du, J., Zhu, J., Neto, F. C., Eisenman, R. N., and Raftery, D. (2016) Quantitative method to investigate the balance between metabolism and proteome biomass: starting from glycine. *Angew. Chem. Int. Ed. Engl.* **55**, 15646–15650