Supplementary Information for

Proline mediates mitochondrial metabolic communication between RPE and retina

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Supplemental Methods

O_2 consumption

Human RPE cells were plated in Seahorse XF analyzer microplates (Agilent Seahorse Biosciences) and grown in RPE media. After maturation, RPE cells were switched to assay media (minimal DMEM (Agilent Seahorse Biosciences) containing 5 mM glucose, 5 mM glucose with 2 mM proline or 5 mM glucose with 2 mM glutamine and 1 mM pyruvate) before measurement. Oxygen consumption rates (OCR) were obtained and analyzed using the XF-24 Extracellular Flux Analyzer (Agilent Seahorse Biosciences) following injections of mitochondrial inhibitors from the XF Cell Mito Stress Kit at the following concentrations 1 μM oligomycin, 500 nM FCCP, and 2 μM Rotenone and 2 μM Antimycin A.

Cryosection immunohistochemistry (IHC)

After brief fixation of eyes in 4% PFA for 10 min, the cornea was removed and the eye cups were further fixed in 4% PFA for 50 min. After being washed three times in PBS (5 min each), the fixed eye cups were incubated first in 20% sucrose in PBS overnight at 4 ^{o}C and then in 1:1 20% sucrose in PBS and optimal cutting temperature compound (OCT) for 1h, at 30 min the lens was removed. Finally, eye cups were embedded in OCT and frozen in a dry ice/100% ethanol bath. For cryosection IHC, frozen sections were blocked in 10% normal goat serum and incubated with primary antibodies overnight, which was detected through secondary antibody incubation at 1h using Alexa-Fluor 488 or -594 conjugated secondary antibodies (1:1000, Invitrogen).

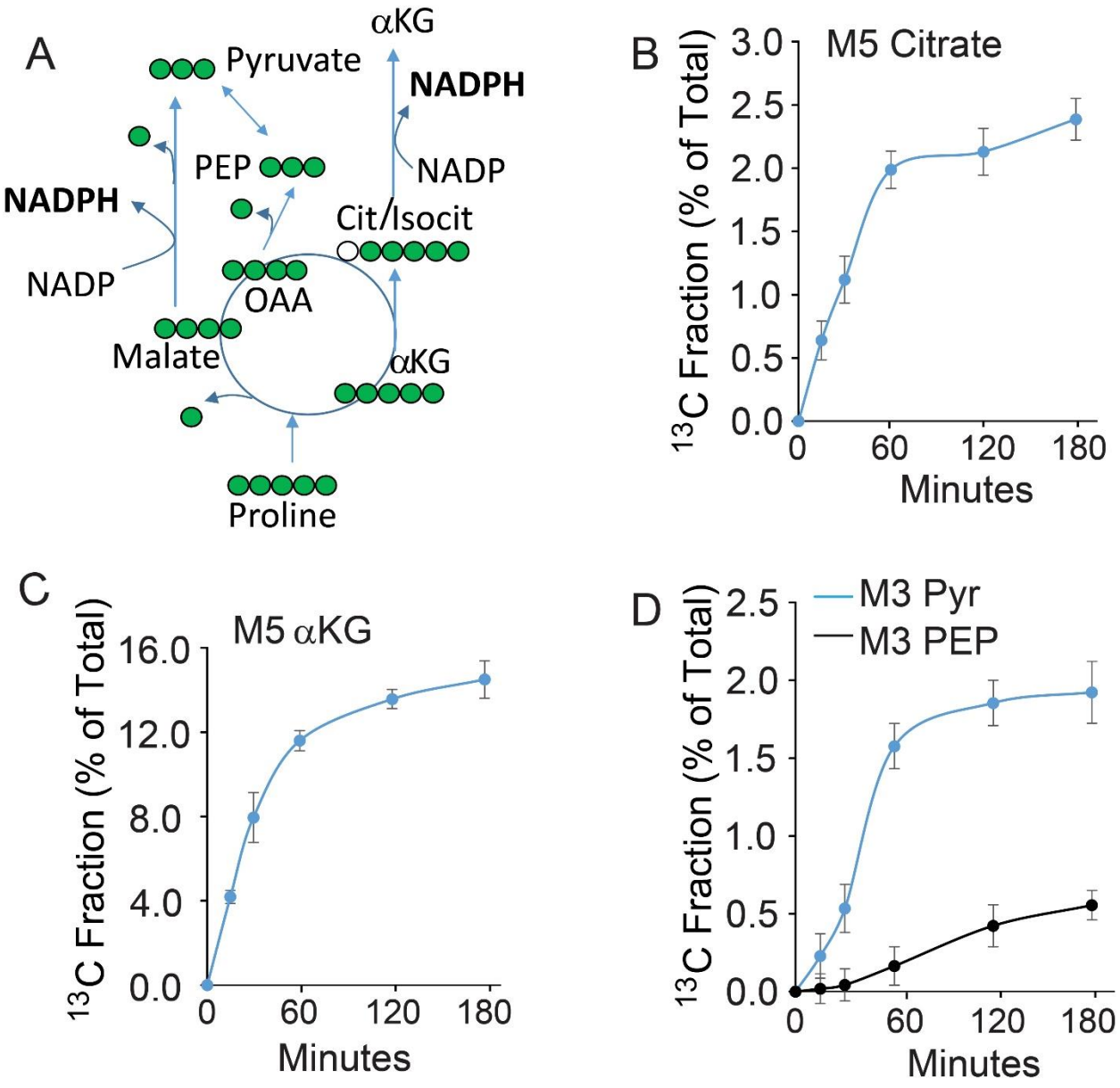

Fig S1. Proline increases reductive carboxylation and flux to produce pyruvate. (A) Schematic for the pattern of labeling by ^{13}C proline. Five carbon labeled proline (M5) can generate M5 citrate through reductive carboxylation and M3 pyruvate through malic enzyme. (B-D) Isolated mouse RPE/choroid was incubated with 5 mM glucose and 1 mM ^{13}C proline for different time and metabolites were analyzed by GC MS. Enrichment is the percent of labeled carbon of total isotopologues. N=4.

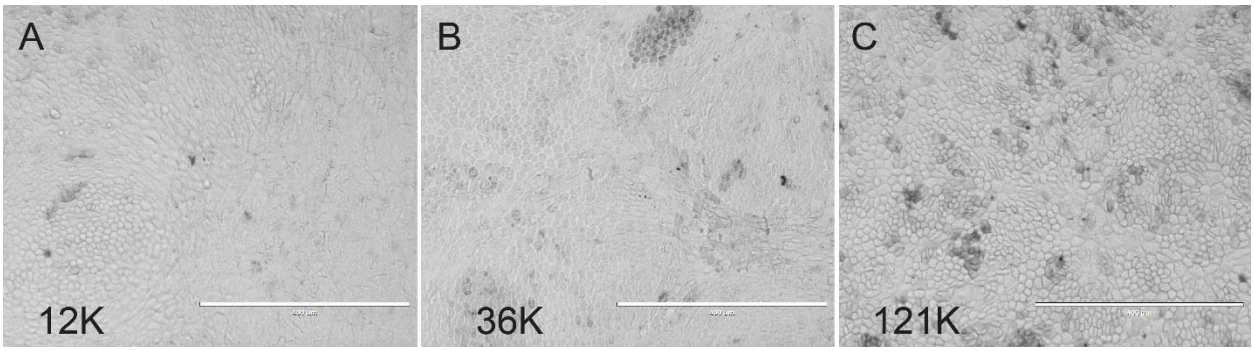

Fig S2. RPE maturation with different plating densities. (A-CI) RPE cells were plated at 12 thousands (K), 36K and 121K cells/per well in a 12-well plate and grown for a week. Scale bar = 400 μM.

Table S1. List of detected metabolites by LC MS/MS and GC MS.

Metabolite	Precursor (Da)	Product (Da)	Declustering potential	Collision energy	HMDB	Platform	Polarity
NAD	664	136	27	48	HMDB00902	LCMS	+
4-hydroxyproline	132.1	86	60	17	HMDB00725	LCMS	+
Proline	116.1	70.1	70	44	HMDB00162	LCMS	+
Arginine	175.1	70.1	45	33	HMDB00517	LCMS	+
Glutamine	147.1	84.1	45	23	HMDB00641	LCMS	+
ATP	507.9	136.1	14	60	HMDB00538	LCMS	+
Nicotinamide	123	80	111	20	HMDB01406	LCMS	+
N1-Methylnicotinamide	137	78	110	37	HMDB00699	LCMS	+
Nicotinic Acid 13C1 Positive	125	80	95	30	N/A	LCMS	+
UDP Glucose	611	449	40	29	HMDB00286	LCMS	+
Choline	104.1	60.1	95	37	HMDB00097	LCMS	+
O Acetyl L Carnitine	204.1	85	71	30	HMDB00201	LCMS	+
FAD	786.1	136.1	52	43	HMDB01248	LCMS	+
Riboflavin	377.1	243.1	14	29	HMDB00244	LCMS	+
Carnosine	227.1	110.1	157	32	HMDB00033	LCMS	+
Ophthalmic Acid	290.1	58	139	56	HMDB0005765	LCMS	+
coA	768.1	261	34	47	HMDB01423	LCMS	+
Acetyl coA	810.1	303.1	116	46	HMDB0001206	LCMS	+

Palmitoyl Coenzyme A	1006.3	499.5	45	53	HMDB0001338	LCMS	+
Ornithine	133	70	47	23	HMDB0000214	LCMS	+
Adenosine	268.1	136	95	38	HMDB00050	LCMS	+
1Methyladenosine	282.1	150.1	48	35	HMDB03331	LCMS	+
Creatine	132.1	90	170	17	HMDB00064	LCMS	+
Uridine	245	113	23	51	HMDB00296	LCMS	+
Cytidine	244	112.1	50	30	HMDB00089	LCMS	+
GDP	444	152	65	26	HMDB01201	LCMS	+
Betaine	118.1	58	166	56	HMDB00043	LCMS	+
Homoserine	120.1	74	37	16	HMDB00719	LCMS	+
Tryptophan	205	146	75	35	HMDB00929	LCMS	+
Aspartic Acid	134.1	74	50	20	HMDB00191	LCMS	+
Cytosine	112	95	97	23	HMDB0000630	LCMS	+
3 Aminoisobutyrate	104.1	58	55	38	HMDB03911	LCMS	+
Thiamine	265	122.1	67	40	HMDB00235	LCMS	+
Alanine	90	44	50	20	HMDB0000161	LCMS	+
Glycine	76	30.2	30	16	HMDB0000123	LCMS	+
Serine	106	60	40	22	HMDB0000187	LCMS	+
Valine	118.1	72	60	14	HMDB0000883	LCMS	+
Threonine	120.1	102	50	10	HMDB0000167	LCMS	+
Pyroglutamic Acid	130.1	84	100	20	HMDB0000267	LCMS	+
Leucine	132.1	86	60	16	HMDB0000687	LCMS	+
Asparagene	133.1	70.1	55	24	HMDB0033780	LCMS	+
Glutamic Acid	148.1	84.1	51	20	HMDB0003339	LCMS	+
Taurine	126	108	200	15	HMDB0000251	LCMS	+
Methionine	150.1	61	27	43	HMDB0000696	LCMS	+
Histidine	156.1	110	50	22	HMDB0000177	LCMS	+
Phenylalanine	166.1	120	50	18	HMDB0000159	LCMS	+
Tyrosine	182.1	136	40	17	HMDB0000158	LCMS	+
Lysine	147.1	84	38	22	HMDB0000182	LCMS	+
Trigonelline	138	92	60	27	HMDB00875	LCMS	+
CAMP	328	134	-87	-32	HMDB00058	LCMS	-
GSH	306	143.1	-61	-26	HMDB00125	LCMS	-
AMP	346	134	-82	-40	HMDB00045	LCMS	-
NADH	664	397	-7	-46	HMDB01487	LCMS	-
Creatinine	112	41	-67	-36	HMDB00562	LCMS	-
Kynurenine	207.1	144	-58	-33	HMDB00684	LCMS	-
Oxaloacetate	131	87	-30	-11	HMDB00223	LCMS	-
Glucose	179	89	-50	-15	HMDB00122	LCMS	-
GTP	521.9	159	-75	-57	HMDB01273	LCMS	-
Hypoxanthine	135	65	-108	-37	HMDB00157	LCMS	-

Phosphocreatinine	210	79	-42	-49	HMDB0041624	LCMS	-
Aminoadipic Acid	160.1	116.1	-47	-20	HMDB00510	LCMS	-
Citrulline	174.1	131.1	-32	-20	HMDB00904	LCMS	-
Adenine	134	107.1	-92	-25	HMDB00034	LCMS	-
Uracil	111	42	-51	-37	HMDB00300	LCMS	-
Xanthine	151	108	-70	-23	HMDB00292	LCMS	-
Hippurate	178	77.1	-61	-23	HMDB00714	LCMS	-
Biotin	243.1	200	-85	-23	HMDB0000030	LCMS	-
N Acetyl Glycine	116	74	-37	-14	HMDB00532	LCMS	-
Aconitate	173	85	-37	-18	HMDB00072	LCMS	-
Pentothenate	218.1	88.1	-80	-21	HMDB00210	LCMS	-
DHAP	169	79	-38	-37	HMDB01473	LCMS	-
Urate	167	124	-86	-19	HMDB0000289	LCMS	-
4-Hydroxyphenylpyruvate	179.1	97.1	-40	-17	HMDB00707	LCMS	-
Xanthosine	283.1	151	-92	-28	HMDB00299	LCMS	-
Citraconic Acid	129	85	-21	-12	HMDB00634	LCMS	-
Glutaric Acid	131	87	-45	-16	HMDB00661	LCMS	-
Guanosine	282.1	150	-67	-33	HMDB00133	LCMS	-
Xanthurenic Acid	204	160	-67	-19	HMDB00881	LCMS	-
Inosine	267	135	-123	-30	HMDB0000195	LCMS	-
ADP	426	79.1	-65	-84	HMDB01341	LCMS	-
GMP	362.1	79	-41	-61	HMDB01397	LCMS	-
Heptadecanoic Acid	269.1	135.1	-77	-35	HMDB02259	LCMS	-
Methylmalonate	117	73	-58	-14	HMDB00202	LCMS	-
G6P	259	79	-46	-62	HMDB01401	LCMS	-
IMP	347	79	-118	-86	HMDB00175	LCMS	-
UDP	403	79	-30	-73	HMDB00295	LCMS	-
Lactate	89	43	-60	-17	HMDB0000190	LCMS	-
Oxalic Acid	89	61	-45	-10	HMDB0002329	LCMS	-
Succinate	117	73	-54	-16	HMDB0000254	LCMS	-
a-Ketoglutarate	145	101	-56	-11	HMDB0000208	LCMS	-
Malate	133	115	-50	-14	HMDB0031518	LCMS	-
Phosphoenolpyruvic	167.1	79	-38	-20	HMDB0000263	LCMS	-
Pantothenic Acid	218.1	71	-70	-41	HMDB0000210	LCMS	-
Ribulose 5 Phosphate	229	79	-70	-45	HMDB0000618	LCMS	-
G3P	169	79	-74	-28	HMDB0000807	LCMS	-
G1P	259	79	-85	-60	HMDB0001586	LCMS	-
Carbamolyphosphate	151	108	-105	-25	HMDB0001096	LCMS	-
Myo Inositol	179	87	-105	-24	HMDB0000211	LCMS	-
Hypotaurine	108.1	64	-40	-17	HMDB0000965	LCMS	-

Nicotinic Acid 13C1 Negative	123	78	-50	-15	N/A	LCMS	-
2-hydroxyglutarate	433	NA	NA	NA	HMDB0059655	GCMS	+
3PG	585	NA	NA	NA	HMDB0000807	GCMS	+
4-hydroxyproline	314	NA	NA	NA	HMDB00725	GCMS	+
a-ketoglutarate	346	NA	NA	NA	HMDB0000208	GCMS	+
Alanine	158	NA	NA	NA	HMDB0000161	GCMS	+
Asparagine	417	NA	NA	NA	HMDB0000168	GCMS	+
Aspartate	418	NA	NA	NA	HMDB0000191	GCMS	+
Citrate	459	NA	NA	NA	HMDB0000094	GCMS	+
Cysteine	304	NA	NA	NA	HMDB0000574	GCMS	+
Fumarate	287	NA	NA	NA	HMDB0000134	GCMS	+
Glutamate	432	NA	NA	NA	HMDB0003339	GCMS	+
Glutamine	431	NA	NA	NA	HMDB00641	GCMS	+
Glycine	218	NA	NA	NA	HMDB0000123	GCMS	+
Isocitrate	591	NA	NA	NA	HMDB0000193	GCMS	+
Isoleucine	200	NA	NA	NA	HMDB0000172	GCMS	+
Lactate	261	NA	NA	NA	HMDB0000190	GCMS	+
Leucine	200	NA	NA	NA	HMDB0000687	GCMS	+
Lysine	300	NA	NA	NA	HMDB0000182	GCMS	+
Malate	419	NA	NA	NA	HMDB0031518	GCMS	+
Methionine	218	NA	NA	NA	HMDB0000696	GCMS	+
Myristic acid-D27	312	NA	NA	NA	N/A	GCMS	+
Ornithine	417	NA	NA	NA	HMDB0000214	GCMS	+
Phosphoenolpyruvate	453	NA	NA	NA	HMDB0000263	GCMS	+
Phenylalanine	302	NA	NA	NA	HMDB0000159	GCMS	+
Proline	184	NA	NA	NA	HMDB00162	GCMS	+
5-oxoproline	300	NA	NA	NA	HMDB0000267	GCMS	+
Pyruvate	174	NA	NA	NA	HMDB0000243	GCMS	+
Serine	390	NA	NA	NA	HMDB0000187	GCMS	+
Succinate	289	NA	NA	NA	HMDB0000254	GCMS	+
Taurine	296	NA	NA	NA	HMDB0000251	GCMS	+
Threonine	404	NA	NA	NA	HMDB0000167	GCMS	+
Tryptophan	244	NA	NA	NA	HMDB00929	GCMS	+
Tyrosine	302	NA	NA	NA	HMDB0000158	GCMS	+
Uracil	283	NA	NA	NA	HMDB00300	GCMS	+
Urate	567	NA	NA	NA	HMDB0000289	GCMS	+
Urea	231	NA	NA	NA	HMDB0000294	GCMS	+
Valine	186	NA	NA	NA	HMDB0000883	GCMS	+
Xanthine	437	NA	NA	NA	HMDB00292	GCMS	+

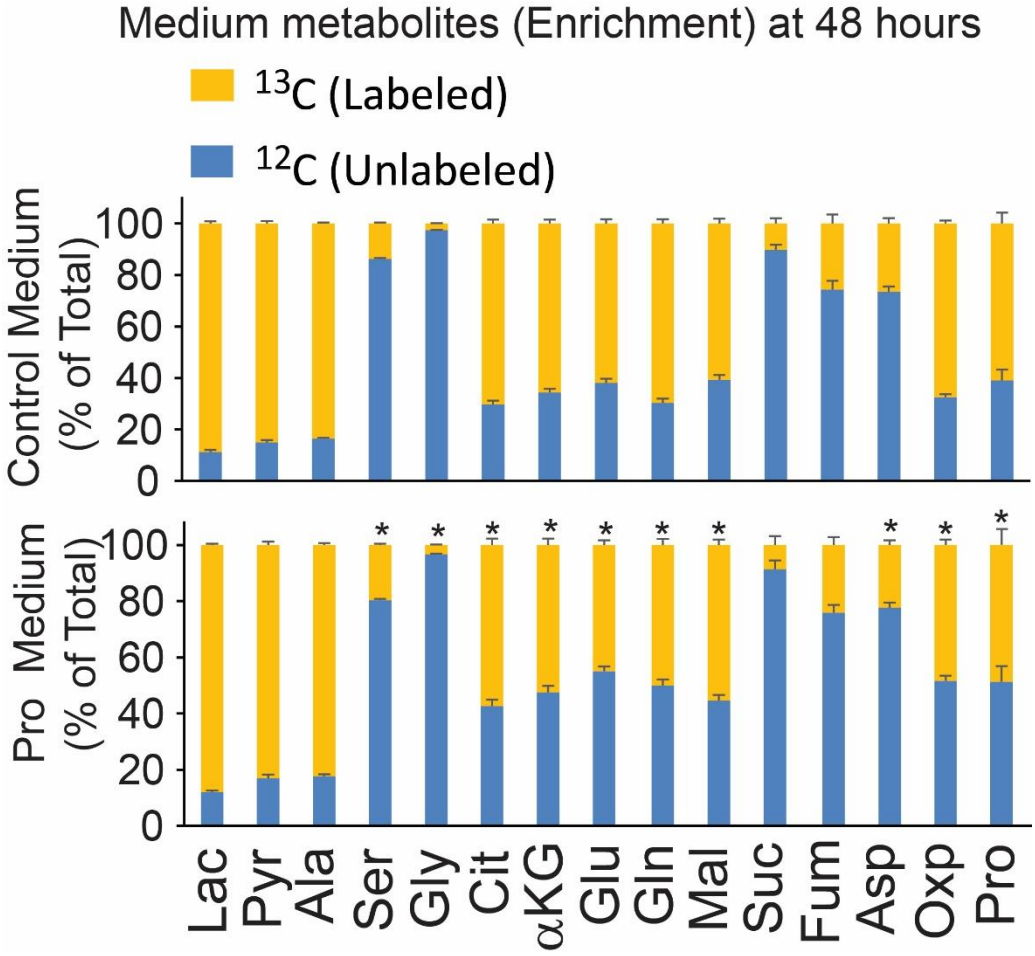

Fig S3. Proline regulates glucose metabolism and stimulates synthesis of serine and glycine in media. After cultured for 48 hrs in ^{13}C glucose with or without 1mM proline, medium metabolites were analyzed by GC MS. N=4. *P <0.05 vs Con without proline or Con at 48 hrs. Top panel was control and bottom panel was the medium with proline. N=4. *P<0.05 vs Con at 48 hrs. Lac, lactate; Pyr, pyruvate; Mal, malate; Suc, succinate; Fum, fumarate; Oxp, 5-oxoproline.

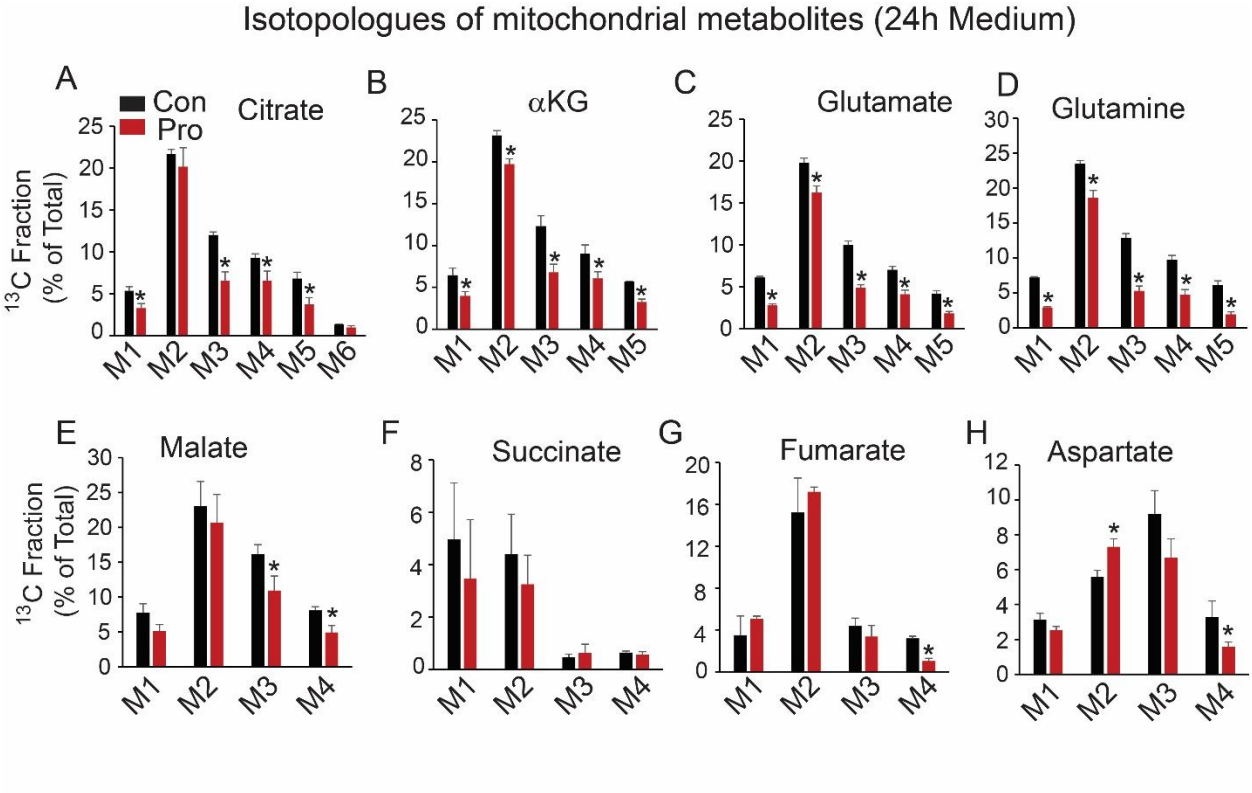

Fig S4. The labeling pattern of mitochondrial metabolites in media. (A-H) RPE cells were incubated in 5 mM ^{13}C glucose with or without 1 mM proline for 24 hrs. Culture media were analyzed by GC MS. N=4. *P<0.05 vs Con without proline.

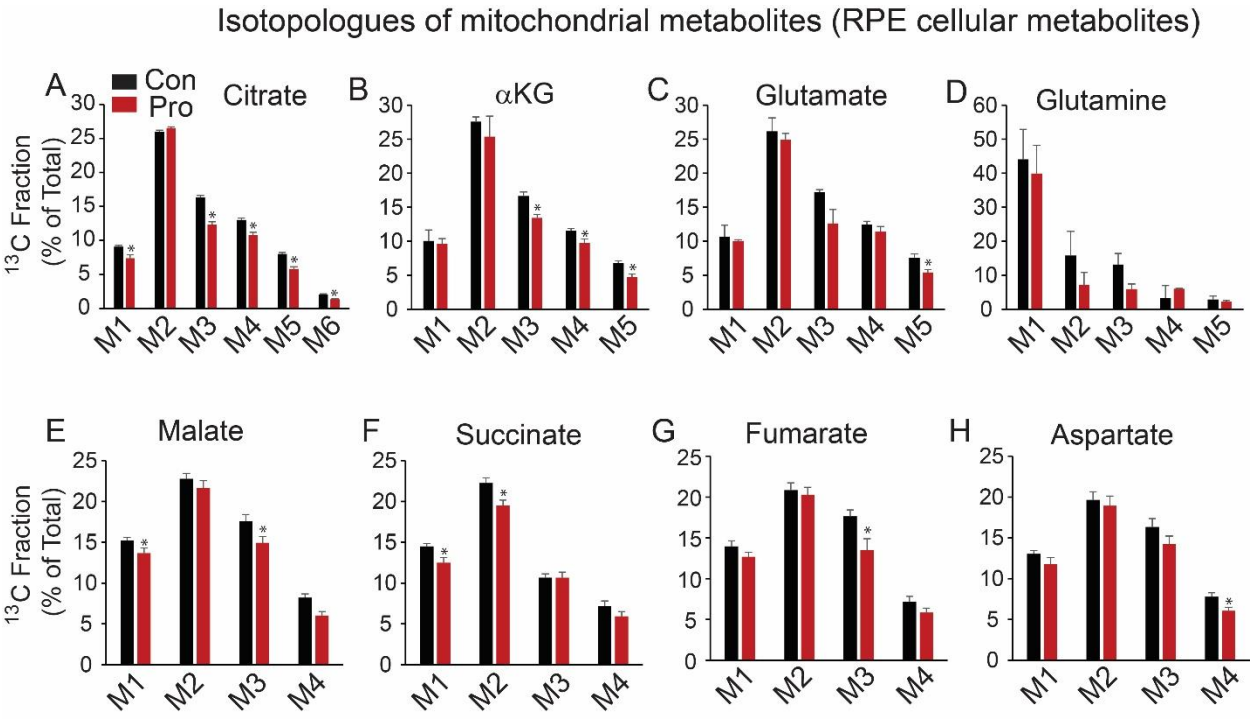

Fig S5. The labeling pattern of mitochondrial metabolites in RPE cells. (A-H) RPE cells were incubated in 5 mM ^{13}C glucose with or without 1 mM proline for 48 hrs. Culture media were analyzed by GC MS. N=4. *P<0.05 vs Con without proline.

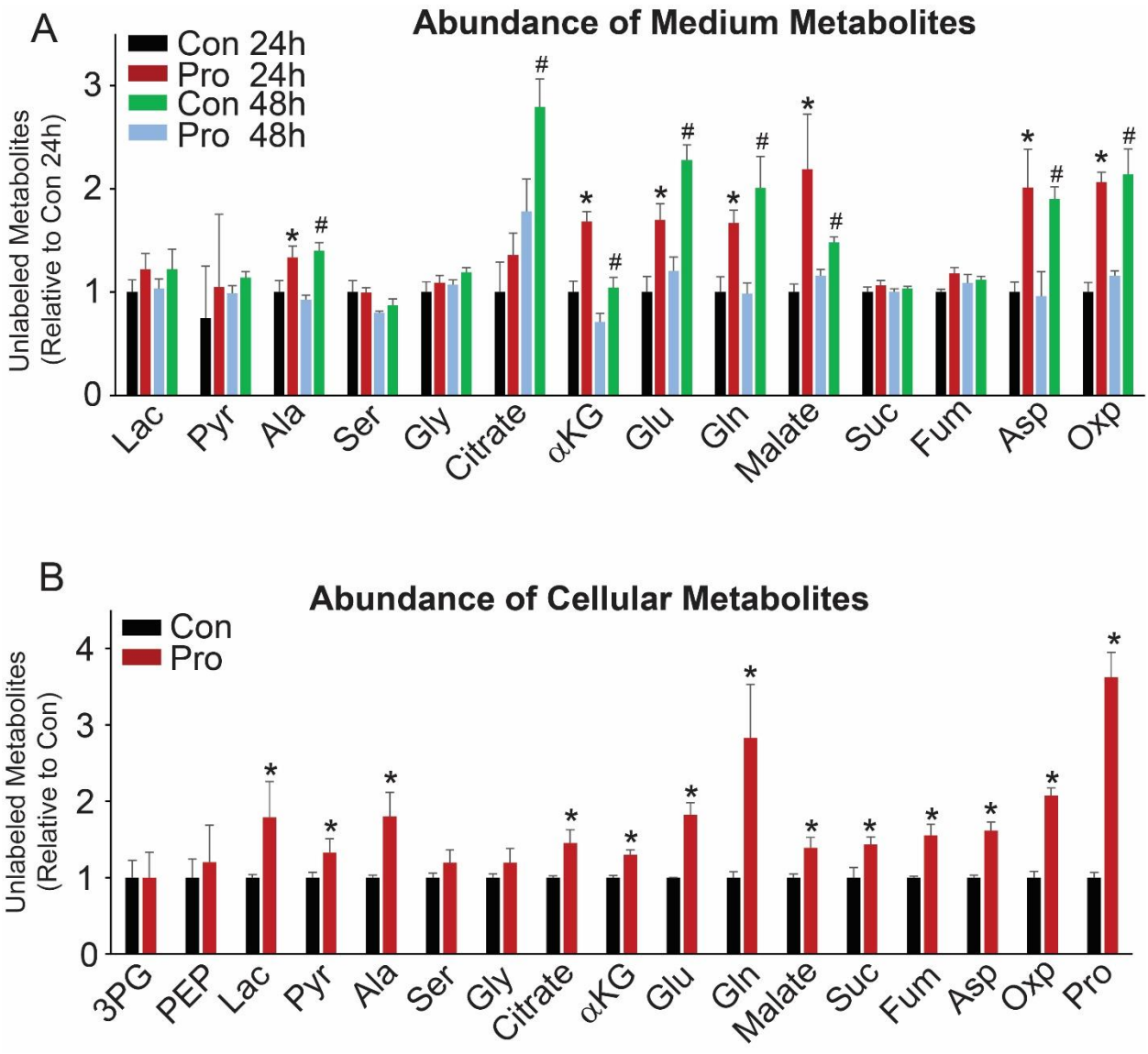

Fig S6 Proline increases glycolysis and mitochondrial intermediates. (A-B) Changes of unlabeled metabolites from proline in medium (A) and RPE cells (B), after cultured in ^{13}C glucose at 24 hr or 48 hrs. Cell metabolites were collected after 48 hrs. *P<0.05 vs Con medium at 24 hr or Con cells at 48 hrs. #P<0.05 vs Con medium at 48 hrs. Lac, Lactate, Pyr, pyruvate; Suc, succinate; Fum, fumarate; Asp, aspartate; Oxp, 5-oxoproline.

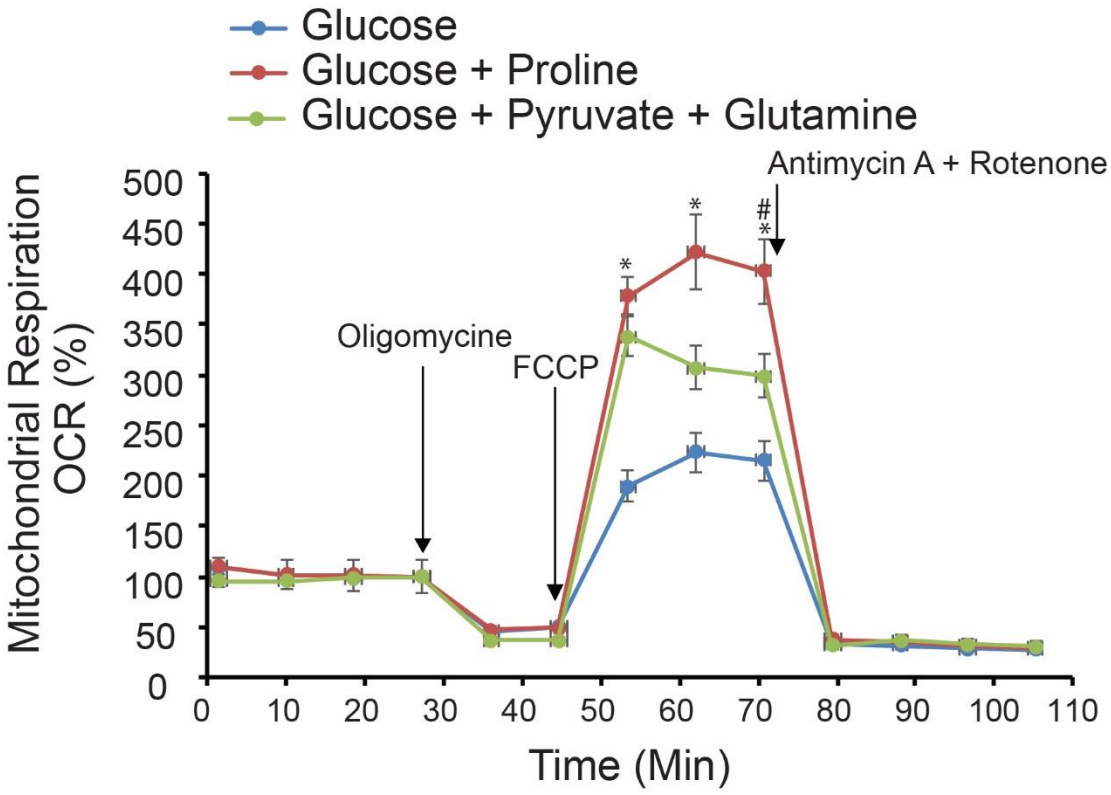

Fig S7. Proline increases mitochondrial oxygen consumption. RPE cells were incubated with different nutrients in DMEM (5 mM glucose; 5 mM glucose + 2 mM proline; 5 mM glucose +1 mM pyruvate + 2 mM glutamine). Oxygen consumption was measured by Seahorse XF24 analyzer. *P<0.05 vs glucose alone and #P<0.05 vs glucose + pyruvate + glutamine. OCR, oxygen consumption.

Table S2. Formula for high-proline diet and regular amino acid diet.

	Amino Acid (AA) Diet) g/Kg	High-Proline Diet g/KG
L-Alanine	3.5	2.9805
L-Arginine HCL	12.1	12.1
L-Asparagine	6	2.8
L-Aspartic Acid	3.5	4.0254
L-Cystine	3.5	3.5
L-Glutamic Acid	40	32.8645
Glycine	23.3	19.66
L-Histidine HCL, monohydrate	4.5	4.5
L-Isoleucine	8.2	8.2
L-Leucine	11.1	11.1
L-Lysine HCL	18	18
L-Methionine	8.2	8.2
L-Phenylalanine	7.5	7.5
L-Proline	3.5	20
L-Serine	3.5	3.9146
L-Threonine	8.2	8.2
L-Tryptophan	1.8	1.8
L-Tyrosine	5	5
L-Valine	8.2	8.2
Sucrose	344.98	341.935
Corn Starch	150	150
Maltodextrin	150	150
Soybean Oil	80	80
Cellulose	30	30
Mineral Mix, AIN-93M-MX (94049)	35	35
Calcium Phosphate, monobasic, monohydrate	8.2	8.2
Vitamin Mix, AIN-93-VX (94047)	19.5	19.5
Choline Bitartrate	2.7	2.7
TBHQ, antioxidant	0.02	0.02
Orange Food Color		0.1
Amino Acid (AA) Diet (#TD.01084)		
Nutrient Information		
	% By Weight	%kcal from
Protein	15.4	15.6

CHO	64.9	66
Fat	8	18.3
Kcal/g	3.9	
High-Proline Diet (#TD.170647) g/KG		
Nutrient Information		
	% By Weight	%kcal from
Protein	15.4	15.7
CHO	64.6	65.9
Fat	8	18.4
Kcal/g	3.9	

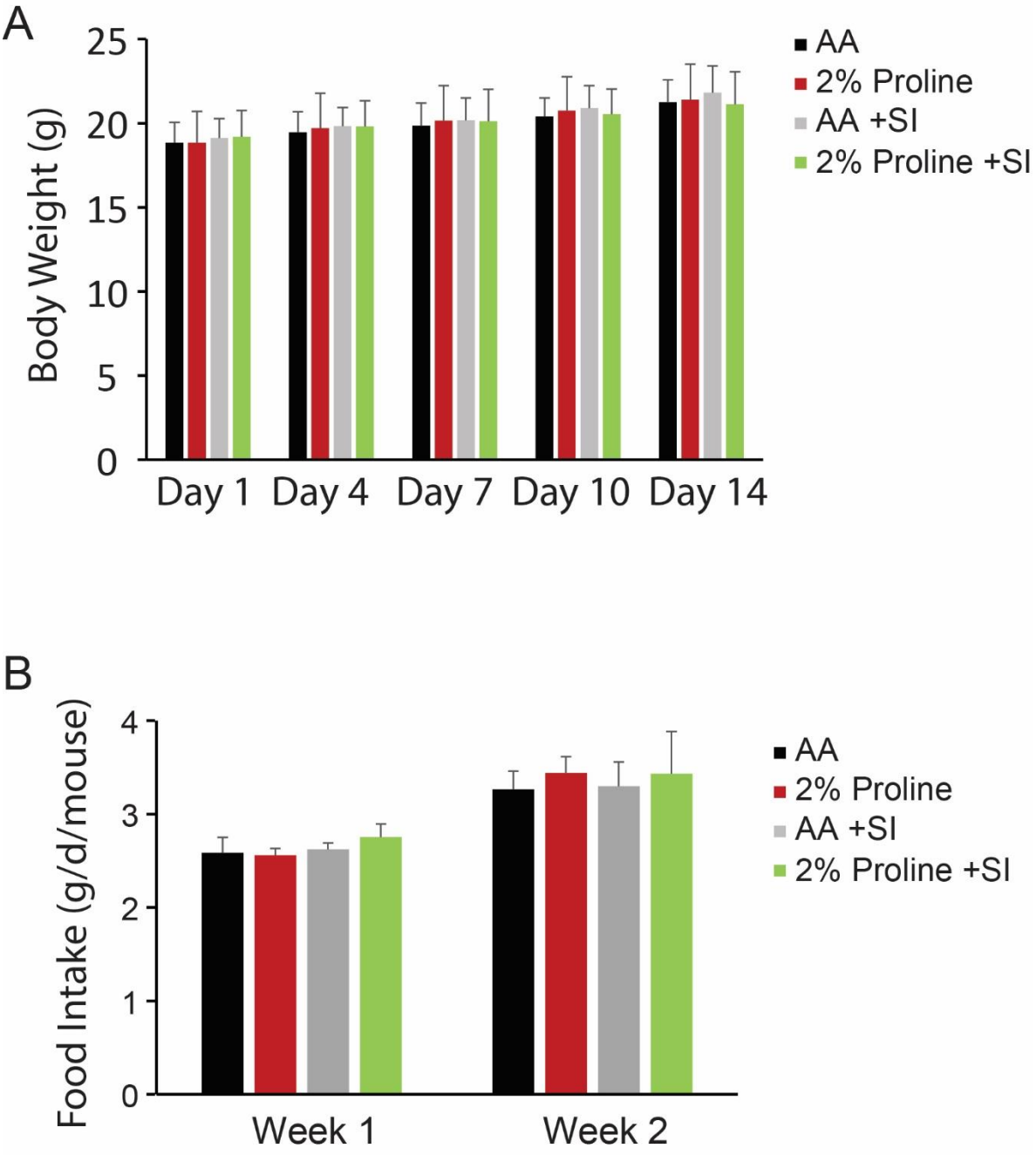

Fig S8. High-proline did not alter mouse body weight and food intake. (A) Body weight at different days with AA diet or high-proline diet. N=10. (B) Food intake was measured weekly. N=10. No significance difference in body weight and food intake among different groups (ANOVA).

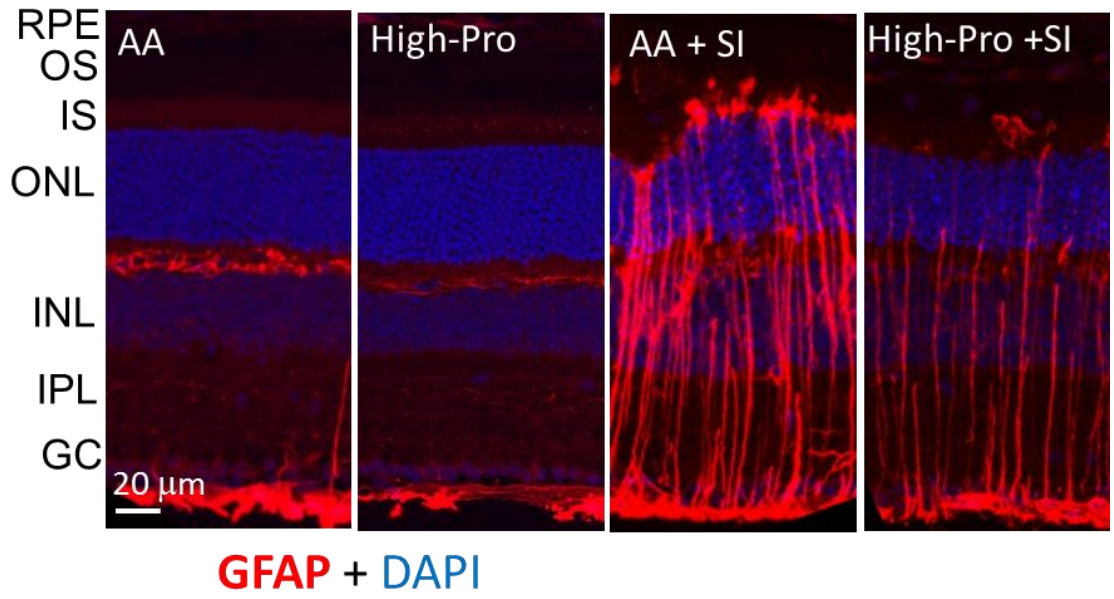

Fig S9. High-proline reduced the activation of Müller glial cells. Three days after injection with sodium iodate (SI), mouse eyes were fixed, sectioned and stained with antibody against glial fibrillary acidic protein (GFAP) antibody and counterstained with DAPI. N=3 for each group.

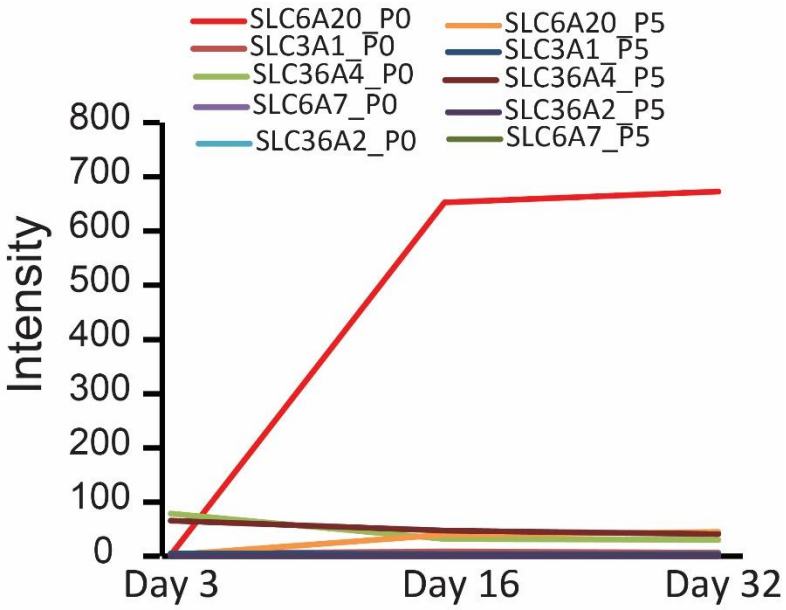

Fig S10. Expression of proline transporters in RPE maturation and differentiation. Transcriptional data were analyzed from published database. Early human RPE passage (P0) and late passage (P5) were cultured for different periods. After 32 days, P0 cells have typical RPE morphology but P5 cells lost the morphology due to dedifferentiation. Cells at different days were harvested for transcriptional study.

Table S3. **The reagents and key resources.**

Reagent or Media components	**SOURCE**	**IDENTIFIER**
U-^{13}C proline	Sigma-Alderich	604801-CONF
U-^{13}C Glucose	Cambridge-Isotope Laboratories	CLM-1396
L-Proline	Sigma-Aldrich	81709
Tetrahydro-2-furoic acid	Sigma-Aldrich	605166
Canaline	Cayman Chemical company	9002357
Halofuginone	Cayman Chemical company	13370
1,4-dihydrophenonthrolin-4-one-3-Carboxylic acid	Cayman Chemical company	71220
MEM a	Fisher	12561056
Non-essential amino acids	Life Technologies (Invitrogen)	1140-050
N1 medium supplement	Sigma-Alderich	N6530-5mL
FBS	Atlanta Biologicals	S11550
Taurine	Sigma-Alderich	T0625-10G
Hydrocortisone-water soluble bioreagent, suitable for cell culture	Sigma-Alderich	H0396-100MG
3, 3', 5-Triiodo-L-Thyronine	Sigma-Alderich	T6397-100MG
Penicillin-Streptomycin (5,000 U/mL)	Life Technologies (Invitrogen)	15070-063
DMEM	Fisher	10566016
DMEM Nutrient Mix F12 500mL	Fisher	11320033
Rock Inhibitor (Y-27632 dihydrochloride)	Tocris/R&D Systems	1254
HBSS	Fisher	14170112
Sodium iodate	Sigma-Alderich	S4007
Cytotoxicity experiments		
Clear DMEM (Gibco/Life Technologies)	ThermoFisher	A14430-01
H202 (30%w/w)	Sigma	H-1009
Pierce LDH cytotoxicity assay kit	ThermoFisher	88953
Lactate Dehydrogenase-SL (kit)	Genzyme Diagnostics	327-30
Cell lines		
A-RPE19 cells	ATCC	CRL-2302™

hRPE-1 cells	ATCC	CRL-4000™
HEK 293 T cells	Gift from Dr. Alexy Ivanov	
ERG		
GenTeal Tears, Severe Lubricant Eye Gel	NDC	0078-0429-47
Systane Ultra, High Percentage Lubricant Eye Drops	NDC	00065-1431-28
Paragon Phenylephrine Hydrochloride Hydrochloride, USP 2.5%	NDC	42702-102-15
Sandoz Tropicamide 1%	NDC	61314-355-02
Mass Spectrometry (LC MS and GC MS)		
Acquity UPLC BEH Amide 1.7 µm Vanguard pre-column 2.1 x 5mm column	Waters	186004799
Acquity UPLC BEH Amide 1.7 µm 2.1 x 50mm column	Waters	186004800
Water, Optima™ LC/MS Grade	Fisher Chemical	7732-18-5
Acetonitrile, Optima™ LC/MS Grade	Fisher Chemical	75-05-8
Ammonium Acetate	Sigma-Aldrich	431311
Ammonium hydroxide 28 - 30% in water ACS	VWR	AC42330-5000
Methoxyamine hydrochloride	Sigma-Aldrich	226904
Pyridine	Sigma-Aldrich	270970
N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide	Sigma-Aldrich	394882
DB-5ms GC Column, 30 m, 0.25 mm, 0.25 µm,	Agilent Technologies	122-5532
Animals		
C57 B6/J	Jackson Lab	664
Flat mount staining		
paraformaldehyde	Electron Microscopy Sciences	15710
Triton X-100	LabChem	LC262801
PBS	Research products international	33668-33747
PNA	Vector	ZC0606
ZO-1	ivitrogen	61-7300
flouromount	SoutnernBiotech	0100-01
Alexa Fluor 568 donkey anti-rabbit IgG	invitrogen	1826664