Metabolic reprogramming in the OPA1-deficient cells

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Received: 24 May 2022 / Revised: 12 August 2022 / Accepted: 31 August 2022
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Abstract
OPA1, a dynamin-related GTPase mutated in autosomal dominant optic atrophy, is essential for the fusion of the inner mitochondrial membrane. Although OPA1 deficiency leads to impaired mitochondrial morphology, the role of OPA1 in central carbon metabolism remains unclear. Here, we aim to explore the functional role and metabolic mechanism of OPA1 in cell fitness beyond the control of mitochondrial fusion. We applied [U-¹³C]glucose and [U-¹³C]glutamine isotope tracing techniques to OPA1-knockout (OPA1-KO) mouse embryonic fibroblasts (MEFs) compared to OPA1 wild-type (OPA1-WT) controls. Furthermore, the resulting tracing data were integrated by metabolic flux analysis to understand the underlying metabolic mechanism through which OPA1 deficiency reprograms cellular metabolism. OPA1-deficient MEFs were depleted of intracellular citrate, which was consistent with the decreased oxygen consumption rate in these cells with mitochondrial fission that is not balanced by mitochondrial fusion. Whereas oxidative glucose metabolism was impaired, OPA1-deficient cells activated glutamine-dependent reductive carboxylation and subsequently relied on this reductive metabolism to produce cytosolic citrate as a predominant acetyl-CoA source for de novo fatty acid synthesis. Prevention of cytosolic glutamine reductive carboxylation by GSK321, an inhibitor of isocitrate dehydrogenase 1 (IDH1), largely repressed lipid synthesis and blocked cell proliferation in OPA1-deficient MEFs. Our data support that, when glucose oxidation failed to support lipogenesis and proliferation in cells with unbalanced mitochondrial fission, OPA1 deficiency stimulated metabolic anaplerosis into glutamine-dependent reductive carboxylation in an IDH1-mediated manner.

Keywords OPA1 dysfunction · Oxidative metabolism · Reductive carboxylation · Citrate · De novo lipogenesis · Cell growth

Abbreviations
OPA1-WT OPA1 wild-type
OPA1-KO OPA1-knockout
MEFs Mouse embryonic fibroblasts
MFA Metabolic flux analysis
IDH1/2 Isocitrate dehydrogenase 1/2
MPC1/2 Mitochondrial pyruvate carrier 1/2
PDH Pyruvate dehydrogenase
LDH Lactate dehydrogenase
CTP Citrate transport protein
DIC Mitochondrial dicarboxylate carrier
FASN Fatty acid synthetase
TFAM Mitochondrial transcription factor A
MIDs Mass isotopologue distributions
SSR Sum-of-squared residuals
α-KG α-Ketoglutarate
OXPHOS Oxidative phosphorylation
OCR Oxygen consumption rate

Introduction
The mitochondrion undergoes dynamic changes of morphology by fusion and fission, which are critical for its function [1, 2]. Mitochondrial fusion is crucial to ensure the distribution of mitochondrial (mt)DNA and enhance respiratory efficiency [3, 4]. The dynamin-related GTPase OPA1, a casual gene product of human autosomal dominant optic atrophy, plays a central role in mitochondrial fusion and inner membrane remodeling [5, 6]. A growing body of
evidences suggest OPA1 deficiency caused fragmentation of the mitochondrial network, accompanied by an oxygen consumption defect [7–11], loss of membrane potential [10], and repressed mitochondrial ATP production [11, 12]. Additionally, OPA1-deficient cells show metabolic impairment, with the elevation of glutamine but a severe decrease of aspartate and glutamate [9, 13, 14], along with active phospholipid remodeling [13–15]. However, how OPA1-deficiency reprograms cellular metabolism is not fully clear.

Citrate is a crucial metabolite that bridges mitochondrial energetics and cytosolic lipid biosynthesis [16]. Within normal cells, glucose usually acts as the predominant source to produce citrate for de novo lipogenesis (DNL) through oxidative metabolism. In contrast, in cancer cells under hypoxia [17] or mitochondrion dysfunction [18], glutamine has been proven to be an alternative source to contribute to citrate production through reductive carboxylation that largely supports DNL and the subsequent cancer cell growth. Moreover, this reductive glutamine pathway would be dependent on isocitrate dehydrogenase 1 (IDH1) or IDH2 to generate citrate within cytosol and mitochondria, respectively [16–18].

To achieve a better understanding of the impact of OPA1 dysfunction on cellular metabolism, here we applied [U-13C] glucose and [U-13C] glutamine isotope tracing techniques to OPA1-knockout (OPA1-KO) mouse embryonic fibroblasts (MEFs), comparing the results with those of OPA1-wild-type controls (OPA1-WT). We show that loss of OPA1 repressed oxidative glutamine metabolism and stimulated an alternative glutamine-dependent reductive pathway to produce citrate, which subsequently provided more acetyl-CoA substrates for DNL. We further observed that the OPA1-deficiency-stimulated reductive carboxylation was predominantly dependent on isocitrate dehydrogenase 1 (IDH1) activity, and IDH1 inhibition subsequently blocked the cell growth of OPA1-deficient cells. Together, our work expands the understanding of mitochondrial metabolism underlying OPA1 dysfunction and implies a repressed oxidative metabolism but a compensatory reductive metabolism to maintain lipid synthesis and cell growth.

**Materials and methods**

**DNA constructs and cell culture**

OPA1-KO and OPA1-WT MEFs, and plasmids carrying human OPA1 variants (OPA1-v1, OPA1-v1∆S1, and OPA1-v5) were previously described [5]. The OPA1 variants were transfected to OPA1-KO MEFs, and stable MEF clones were isolated by puromycin selection (2 μg/mL). Cells were maintained in complete media (DMEM high-glucose medium with 10% fetal bovine serum FBS; HyClone, CA, USA), 1% penicillin/streptomycin, 1% HEPES, and 4 mM l-glutamine at 37 °C in a humidified atmosphere containing 5% CO2.

**Mito-tracker staining**

Cells were seeded on imaging chambers (Ibidi, WI, USA) overnight. Mitochondria were labeled with Mito-tracker Green (100 nM; Invitrogen, CA, USA) for 15 min at 37 °C. Complete DMEM medium was used for imaging performed on a Zeiss Imager equipped with an N-Achroplan 40 X/0.75 water immersion lens and an AxioCAM MRM digital camera. Images were captured using AxioVision 4.8 and Zeiss Zen software. The Z-stack images were processed using Image J software (NIH, MD, USA).

**Stable isotope tracing**

Stable isotope tracing experiments to determine isotope distributions in soluble metabolites were performed as described [19, 20]. Glucose and glutamine labeled with 13C were both purchased from Cambridge Isotope Laboratories (MA, USA). The 13C-tracing studies of OPA1-WT and OPA1-KO MEFs were performed in DMEM medium containing 10% dialyzed FBS. Subsequently, either the glucose or glutamine pool within these cells was 100% labeled, and the other pool was unlabeled. DMEM lacking glucose and glutamine was prepared from powder (Sigma, MO, USA), then supplemented with 10 mM D[U-13C]glucose and 3 mM unlabeled glutamine as glucose-tracing medium or supplemented with 10 mM unlabeled glucose and 3 mM D[U-13C] glutamine as the glutamine-tracing medium. To examine OPA1 deficiency on glucose and glutamine metabolism, cells were grown in 60 mm dishes until 80% confluency and then incubated with the above 13C-labeled medium for 4 h. Subsequently, cells were extracted by freeze-thawing three times in 0.9 mL of a cold 1:1 mixture of methanol and water. Macromolecules and debris were removed by centrifugation (12,000 x g) for 15 min at 4 °C. Afterward, the supernatants with aqueous metabolites were evaporated, derivatized for 2 h at 42 °C in 50 μL of methoxyamine hydrochloride (Sigma) and 100 μL N-tert-butyl(dimethyl)silyl-N-methyltrifluoroacetamide (Sigma) for 1.5 h at 72 °C. To examine the impact of OPA1 disruption on DNL, OPA1-WT and OPA1-KO MEFs with 80% confluency were pre-treated with DMSO and 5 μM GSK321 (IDH1 inhibitor, IDH1i) [21] for 4 h and then incubated with the 13C-labeled glutamine medium containing the corresponding treatments for 20 h. For metabolic flux analysis of DNL, 1 mL of the medium in each dish was collected into a 1.5 mL tube and
frozen at − 80 °C for later analysis. The cells were collected in 1 mL of a cold 1:1 mixture of methanol and water. All the metabolites were analyzed using an Agilent 7890B gas chromatograph (Agilent, CA, USA) networked to an Agilent 5977B mass selective detector. Retention times and mass fragmentation signatures of all metabolites were validated using pure standards. To determine the relative metabolite abundance across samples, the area of the total ion current peak for the metabolite of interest was compared to that of the internal standard and normalized for protein content. The mass isotopomer distribution analysis measured the fraction of each metabolite pool that contained every possible number of $^{13}$C atoms: a metabolite could contain 0, 1, 2, ..., $n$ $^{13}$C atoms, where $n$ = the number of labeled carbons in the metabolite. For each metabolite, an informative fragment ion-containing all carbons in the parent molecule was analyzed by MATLAB software (MathWorks, CA, USA). The abundance of all mass isotopomers was integrated from $m + 0$ to $m + n$, where $m$ = the mass of the fragment ion without any $^{13}$C. The abundance of each mass isotopomer was then corrected mathematically to account for natural abundance isotopes and finally converted into a percentage of the total pool.

**Cell growth assay**

Approximately $1 \times 10^4$ OPA1-WT and OPA1-KO MEFs were seeded in 60 mm dishes. After 4 h, when cells were attached to plates, every 3 dishes of OPA1-WT and OPA1-KO MEFs were counted as the basal cell number (day 0). Once attached to the plates, OPA1-WT and OPA1-KO MEFs treated with DMSO and 5 µM GSK321 were counted after 48 h (day 2). The collected cells were stained by 0.4% trypan blue (Gibco, Thermo, MA, USA) in a 1:1 ratio on a hemocytometer and incubated for 2 min. Subsequently, the live (unstained) and dead (stained) cells were counted. Each treatment was repeated three times.

**Immunoblot analyses**

The protein concentrations of cell lysates were determined by the BCA protein assay using bovine serum albumin as a standard (Thermo, MA, USA). An equal amount of protein (10 µg) per sample was separated on 10% or 15% SDS polyacrylamide gels and then transferred onto PVDF membranes (Millipore, MA, USA). The membranes were blocked with 4% non-fat milk in TBST buffer at room temperature for 2 h, followed by incubation at 4 °C overnight with primary OPA1 (Cell signaling technology, Cat. No. 67589S; dilution 1:2000), IDH1/2/3A (IDH1 and IDH2 from Cell signaling technology, Cat. No. 8137S and 56439S; IDH3A from Abcam, Cat. No. ab154886; dilution 1:1000), mitochondrial pyruvate carrier MPC1 and MPC2 (Cell Signaling Technology; Cat. No. 14462S and 46141S; dilution 1:1000), pyruvate dehydrogenase PDH (Cell Signaling Technology; Cat. No. 3205S; dilution 1:1000), lactate dehydrogenase LDH (Cell Signaling Technology; Cat. No. 2012S; dilution 1:1000), citrate transport protein CTP (Santa Cruz Biotechnology; Cat. No. sc86392; dilution 1:1000), mitochondrial dicarboxylate carrier DIC (Invitrogen; Cat. No. RH214410; dilution 1:1000), fatty acid synthetase FASN (Cell Signaling Technology; Cat. No. 3189S; dilution 1:1000), mitochondrial transcription factor A TFAM (Cell Signaling Technology; Cat. No. 8076S; dilution 1:1000), and β-actin (Cell Signaling Technology; Cat. No. 4970L; dilution 1:2000) antibodies. After washing with TBST three times, the membranes were incubated with secondary goat-anti-rabbit IgG (Bio-Rad Laboratories; Cat. No. 170-6515 dilution 1:3000) or goat anti-mouse IgG (Bio-Rad; Cat. No. 170-6516; dilution 1:3000) conjugated with horseradish peroxidase in 4% non-fat milk for 4 h at 37 °C. Membranes were then washed with TBST three times and incubated with enhanced chemiluminescence Western blotting substrate (Thermo), followed by visualization using an Amersham Imager 680 system (GE Healthcare, MA, USA). The level of β-actin was used as an internal control.

**Metabolic flux analysis (MFA)**

Steady-state metabolic fluxes were calculated by combining extracellular flux rates (glucose/glutamine utilization, lactate/alanine/pyruvate/glutamate secretion) and $^{13}$C mass isotopologue distributions (MIDs) for citrate, glutamate, malate, aspartate, glutamine, glutamate, α-ketoglutarate and palmitate, using the INCA software package [22], which applies an elementary metabolite unit framework to efficiently simulate MIDs [23, 24]. We developed reaction networks describing the stoichiometry and carbon transitions of central carbon metabolism, with assumptions as summarized below:

1. During the experiments, cells are at a metabolic steady state.
2. $^{13}$CO$_2$ produced during oxidation reactions is not reincorporated via carboxylation reactions.
3. Cells are given 20 h to metabolize $^{13}$C substrates. After 20 h, it is assumed that the isotopic labeling has reached steady state.
4. The metabolites succinate and fumarate are symmetrical and their metabolism through the TCA cycle does not produce a particular orientation.
5. The metabolites pyruvate, acetyl-CoA, citrate, α-ketoglutarate, malate, fumarate, and oxaloacetate are metabolically active in both the cytosol and mitochon-
drion. Malate and α-ketoglutarate are allowed to freely mix between the compartments.

6. During the extraction process, intracellular pools of metabolites are homogenized. Therefore, GC–MS analysis of the isotopic enrichment of these metabolites reflects the mixture of distinct metabolic pools. By employing the INCA platform to perform metabolic flux analysis, it is possible to extract meaningful information from these mixed pools. To do this, the model employs parameters to account for the mixing of mitochondrial (m) and cytosolic (c) metabolites. Mitochondrial and cytosolic fractions of the same metabolite are separately considered in the compartmented reactions in the model, and the enrichment of such metabolite matches the mixture (.mix) of mitochondrial (.m) and cytosolic (.c) fractions, which are listed as v38–v52 in Table S2 and S3.

7. For flux calculations in OPA1-WT and OPA1-KO MEFs treated with IDH1 inhibitor (IDH1i, GSK321), it is assumed that citrate generated from cytosolic reductive carboxylation is very low (0.0011). To achieve adequate fits, it is essential to keep the velocity of mitochondrial citrate into α-ketoglutarate the same as the upper bound of those in each cell line (OPA1-WT and OPA1-KO MEFs). Parallel labeling data from cultures fed 13C-glucose and 13C-glutamine (OPA1-WT and OPA1-KO MEFs) were used to simultaneously fit the same network model to estimate intracellular fluxes. The labeling data from cultures fed 13C-glutamine (OPA1-WT and OPA1-KO MEFs with IDH1i treatment) were used to simultaneously fit the same network model to estimate intracellular fluxes. To ensure that a global minimum of fluxes was identified, flux estimations were initiated from random values and repeated a minimum of 50 times. A chi-square test was applied to test goodness-of-fit, and accurate 95% confidence intervals were calculated by assessing the sensitivity of the sum-of-squared residuals to flux parameter variations. Table S1 and Table S2 contain the degrees of freedom and sum-of-squared residuals (SSR) for the best fit model and the lower and upper bounds of 95% confidence intervals for all fluxes.

### Metabolic assays of some metabolites

Glucose and lactate in culture medium were measured using a kit (D-glucose kit, K-GLUHK-110A, Megazyme; l-lactic acid kit, K-LATE, Megazyme). Pyruvate, alanine, glutamine, and glutamate in the culture medium were analyzed using an Agilent 7890B gas chromatograph networked to an Agilent 5977B mass selective detector. We made standard curves of each metabolite with different concentrations.

### Statistical analysis

Significance in metabolite enrichment (and relative levels of metabolites) and relative cell growth rate in OPA1-WT and OPA1-KO MEFs between DMSO and GSK321 treatment groups were determined using multiple t-tests in each cell line. For OPA1-WT and OPA1-KO MEFs and different OPA1 variants, metabolite enrichment was analyzed using one-way ANOVA with Tukey test for multiple comparisons. The metabolic data of OPA1-KO MEFs were compared to OPA1-WT controls using multiple t-tests. A p value less than 0.05 was considered significant. All statistical tests were two-tailed and calculated using Prism software (GraphPad, CA, USA).

### Results

**OPA1-deficiency repressed oxidative glucose metabolism**

OPA1-deficient MEFs displayed complete mitochondrial fragmentation, compared with elongated mitochondrial tubules in OPA1-WT cells (Fig. 1A), which is consistent with the previous studies [5, 7–9]. As a major energy source, glucose is normally metabolized through glycolysis into pyruvate, which subsequently enters mitochondria to be oxidized through the TCA cycle. As illustrated in Fig. 1B, the fully 13C-labeled m+6 glucose produces m+3 labeled pyruvate via glycolysis, which releases a molecule of m+2 labeled acetyl-CoA within mitochondria; this 13C-labeled acetyl-CoA further produces m+2 labeled citrate by combining with oxaloacetate, and m+2 labeled citrate is further metabolized into m+2 labeled α-ketoglutarate (α-KG), succinate, fumarate, and lactate through the TCA cycle. For simplicity, we focused on the m+2 TCA metabolites in the first round of the TCA cycle from the fully 13C-labeled glucose tracer (Fig. 1B). The m+2 labeled TCA cycle intermediates were significantly reduced in OPA1-KO MEFs compared with OPA1-WT controls (Fig. 1C). Moreover, the intracellular level of citrate was depleted in OPA1-KO MEFs (Fig. 1D), indicating that OPA1 deficiency highly repressed oxidative glucose metabolism and reduced the citrate level.

In comparison to the reduced enrichment of TCA cycle intermediates, the enrichment of glycolytic intermediates was less affected in OPA1-KO MEFs. The m+3 enrichment of pyruvate was very mildly decreased in OPA1-KO MEFs (Fig. S1A), and OPA1 disruption did not affect m+3 enrichment of lactate (Fig. S1B). The intracellular level of lactate was much higher in OPA1-KO MEFs (Fig. S1C). These data indicate that OPA1 deficiency induces glycolysis, which is in line with the impaired mitochondrial oxidative phosphorylation (OXPHOS), as previously reported.
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Additionally, OPA1-KO MEFs have lower levels of mitochondrial DNA [7, 8], and we found that OPA1-KO MEFs expressed lower levels of TFAM (Fig. S1D). We also observed mitochondrial ROS was significantly higher in OPA1-KO MEFs (Fig. S1E). Together, we confirm that OPA1 deficiency severely impairs mitochondrial function.

**OPA1 deficiency induced glutamine-dependent reductive carboxylation**

Glutamine emerges as another major energy source, especially for cells with impaired glucose oxidation [17, 25]. As illustrated in Fig. 2A, glutamine carbons enter the TCA cycle as the fully labeled \( m+5 \) α-KG (containing all five carbons from \([U-^{13}C]\)glutamine tracer), which can be oxidized through the forward TCA cycle into \( m+4 \) labeled fumarate, malate, and citrate (gray dots). By contrast, \( m+5 \) α-KG could alternatively generate five-carbon-labeled \( m+5 \) isocitrate/citrate through reductive carboxylation (catalyzed by cytosolic IDH1 and mitochondrial IDH2), which can be further metabolized into acetyl-CoA and three-carbon-labeled \( m+3 \) oxaloacetate, malate, and fumarate. Compared to OPA1-WT MEFs, OPA1-KO MEFs had lower \( m+3 \) labeled citrate from \([U-^{13}C]\)glutamine (Fig. 2B). Together with the lower \( m+2 \) labeled citrate from \([U-^{13}C]\)glucose (Fig. 1C), these results are consistent with reduced flux of the TCA cycle in the forward direction. In the meantime, there was a significant induction of \( m+5 \) labeled citrate, as well as \( m+3 \)
labeled fumarate and malate, from [U-13C]glutamine in OPA1-KO MEFs (Fig. 2C). These data indicate that OPA1 deficiency indeed induced reductive carboxylation.

Furthermore, we determined the metabolic alterations in OPA1-KO MEFs overexpressing short- (S-) and long- (L-) forms of OPA1. Immunoblots and mito-tracker staining confirmed the proper expression of these OPA1 isoforms (Fig. S2A and S2B), as previously reported [5, 7]. We observed that $m+4$ enrichment of citrate in L-plus S-, L-, and S-OPA1 cells were completely restored to a similar or slightly higher level than those in OPA1-WT cells; on the contrary, $m+5$ citrate fraction in the three cell lines was

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**Fig. 2** OPA1 deficiency induced glutamine-dependent reductive carboxylation. A An illustration of the metabolic flow of reductive glutamine metabolism in [U-13C]glutamine cultured cells. The empty dots represent the natural carbon 12, and the solid red and gray dots represent the carbon 13 isotope. The full five-carbon-labeled ($^{13}$C) glutamine produces five-carbon-labeled α-ketoglutarate (α-KG) ($m+5$), which further produces five-carbon-labeled citrate ($m+5$) by mitochondrial IDH2 and cytosolic IDH1. After the release of two-carbon-labeled acetyl-CoA, the five-carbon-labeled citrate would generate one molecule of two-carbon-labeled acetyl-CoA for palmitate synthesis and another molecule of cytosolic three-carbon-labeled oxaloacetate ($m+3$) as well as malate ($m+3$) and fumarate ($m+3$). Mass isotopologue analysis of citrate (B) and the other three TCA cycle intermediates including α-KG, fumarate, and malate (C) in OPA1-WT and OPA1-KO MEFs cultured with [U-13C]glutamine and unlabeled glucose for 4 h. D Mass isotopologue analysis of citrate and palmitate as well as the relative contribution of glucose oxidation/glutamine reduction to lipogenic AcCoA in OPA1-WT and OPA1-KO MEFs cultured with [U-13C]glucose and unlabeled glutamine for 20 h or cultured with [U-13C]glutamine and unlabeled glutamine for 20 h. The data in 2B–D were analyzed by multiple t-test and the significant level was set as $*p<0.05$ (OPA1-KO vs. OPA1-WT). The experiments were repeated at least three times and $n=3$ cultures from a representative experiment.
significantly decreased compared to OPA1-KO cells but was not fully as low as that of OPA1-WT counterparts (Fig. S2C). The alteration of \( m + 3 \) enrichment of malate among the five groups was in line with their changing patterns of \( m + 5 \) citrate enrichment (Fig. S2C). These data overall further confirmed that L- and S-OPA1 alone or together could similarly restore oxidative metabolism in comparison with the OPA1-null energetic phenotype. Thus, L- and S-OPA1 alone or together reduced the reductive carboxylation. Importantly, this regulation of mitochondrial oxidative and reductive metabolism appears to be independent of the role of OPA1 in mitochondria fusion.

Reductive carboxylation supports DNL in cancer cells under hypoxia [18, 19]. To analyze fatty acid synthesis, we next incubated OPA1-WT and OPA1-KO MEFs in the presence of \([U,^{13}C]\)glucose and \([U,^{13}C]\)glutamine tracers for 20 h. Similar to the above short-time incubation (4 h) results, the enrichment of polar metabolites after 20 h incubation confirmed that OPA1 deficiency reduced glucose oxidation and induced glutamine-dependent reductive metabolism (Fig. S3). \([U,^{13}C]\)glucose tracing showed that palmitate was heavily enriched (peaked at \( m + 12 \)) in OPA1-WT MEFs. In comparison, \([U,^{13}C]\)glutamine tracing showed that palmitate was heavily enriched (peaked at \( m + 12 \)) in OPA1-KO MEFs (Fig. 2D). Furthermore, the calculated lipogenic acetyl-CoA contribution showed that, in OPA1-KO MEFs, glutamine contributed to 70% of the lipogenic acetyl-CoA, while glucose only contributed about 10% of the lipogenic acetyl-CoA (Fig. 2D). In contrast, in OPA1-WT MEFs, glucose contributed to 70% of the lipogenic acetyl-CoA. These data show that OPA1-deficient MEFs relied on reductive glutamine metabolism for DNL.

**Metabolic flux analysis of OPA1 deficiency**

To systemically interpret the metabolic reprogramming upon the loss of OPA1, we next performed metabolic flux analysis (MFA) by incorporating isotope enrichment data from parallel \([U,^{13}C]\)glucose and \([U,^{13}C]\)glutamine tracers in OPA1-WT and OPA1-KO cells. Net rates of glucose and glutamine consumption; net rates of lactate, alanine, pyruvate, and glutamate secretion; and \(^{13}C\) mass isotopologue distributions (MIDs) of pyruvate, citrate, malate, aspartate, glutamate, glutamine, \(\alpha\)-KG, and palmitate were used to constrain fluxes related to central carbon metabolism and fatty acid synthesis (Fig. 2, Fig. S3, and Table S1). The MFA modeling largely confirmed the experimental observations in OPA1-KO MEFs: glycolysis was more active; lactate and alanine secretion was increased; less pyruvate was oxidized through the mitochondrial TCA cycle; cytosolic reductive carboxylation was induced (Fig. 3A, and Table S2). Moreover, the expression levels of LDH, catalyzing pyruvate to lactate reaction, were significantly higher in OPA1-KO MEFs (Fig. 3B). The expression level of IDH1/2, mediating reductive carboxylation, was also higher in OPA1-KO MEFs (Fig. 3B). In comparison, the expression levels of PDH, MPC1/2, CTP, and FASN were lower in OPA1-KO MEFs (Fig. 3B), indicating less active pyruvate oxidation and lipogenesis. Together, OPA1 deficiency reprogrammed cellular metabolism from glucose-dependent oxidation toward glutamine-dependent reductive carboxylation.

**OPA1-deficient MEFs rely on cytosolic reductive carboxylation for proliferation**

Lipogenesis is essential for proliferation [26]. Since OPA1-KO MEFs predominantly depended on cytosolic IDH1 to produce citrate for fatty acid synthesis, we further tested whether IDH1 inhibition impaired the proliferation of OPA1-KO MEFs. Indeed, OPA1-KO MEFs do not grow in the presence of IDH1 inhibitor (IDH1i, GSK321, 5 μM), although IDH1i also partially impaired the growth of OPA1-WT MEFs (Fig. 4A). \([U,^{13}C]\)glutamine tracing confirmed that IDH1 inhibitor completely blocked palmitate synthesis in OPA1-KO MEFs (Fig. 4B). These data indicate that OPA1-KO MEFs depend on IDH1 to synthesize fatty acids for cell growth.

While the lowered \( m + 3 \) labeled malate and citrate were in line with the impaired fatty acid synthesis, the \( m + 5 \) labeled citrate was surprisingly unchanged in IDH1i-treated OPA1-KO MEFs (Fig. 4C). Next, we compared the modelings of OPA1-KO MEFs in the presence or absence of IDH1 inhibitor. Since 5 μM IDH1i GSK321 almost completely blocks IDH1 activity [21], we assumed the cytosolic IDH1-mediated reaction from \(\alpha\)-KG to citrate is 0.0011 in our MFA model. Furthermore, MFA indicated that, compared to DMSO control, IDH1 inhibition induced IDH2-dependent reductive carboxylation, and enhanced glucose-dependent fatty acid synthesis in OPA1-KO MEFs (Fig. S4, and Table S3). It is worth noting that this compensatory induction of glucose metabolism failed to rescue the growth defect in IDH1i-treated OPA1-KO MEFs, further proving the essential role of cytosolic reductive carboxylation in cells with extreme mitochondrial fission. Altogether, OPA1 deficiency stimulates glutamine anaplerosis for lipogenesis to maintain cell growth (Fig. 5).

**Discussion**

OPA1, which encodes a dynamin-related GTPase, is responsible for the fusion of mitochondrial inner membrane [5]. Previous work defined the association between OPA1-related disruption and mitochondrial energetics [7–9, 27]. In this study, we assessed the effects of OPA1 deficiency on metabolic flux alterations in MEFs using parallel stable glucose
and glutamine isotope tracing techniques. We showed that OPA1 deficiency stimulated a switch from mitochondrial oxidative glucose metabolism to reductive glutamine metabolism to maintain citrate production, which is essential for DNL and cell growth in MEFs. Moreover, IDH1 was predominantly involved in the glutamine-dependent reductive metabolism. Cell growth was impaired, when this enzyme activity was inhibited in OPA1-KO MEFs. Our work provides more understanding of how OPA1 deficiency regulates mitochondrial metabolism.

The prominent dysfunction in OPA1-deficient MEFs included reduced oxidative phosphorylation and enhanced glycolytic metabolism. Increased glycolysis was directly reflected as higher glucose consumption and lactate secretion via LDH and indirectly as elevated secretion of alanine and pyruvate. Our MFA convincingly proved OPA1-KO MEFs had a large decrease in flux through MPC, and at least modest decreases mediated by a set of enzymes in the TCA cycle. Reduced TCA cycle flux was likely due to a combination of factors, including reduced mitochondrial carbon entry via MPC1, MPC2 and PDH, glutaminolysis, and malate/succinate transport through DIC; and increased carbon efflux via α-KG export. The finding that OPA1 deficiency highly repressed oxidative metabolism is completely in line with the much lower oxygen consumption rate (OCR) in OPA1-KO MEFs compared to OPA1-WT controls [7, 8, 11]. Other observations indicating an OXPHOS defect within OPA1-KO cells included the loss of mitochondrial respiratory complexes and the disassembly of supercomplexes of the electron transport chain [7, 8], as well as the reduction of mitochondrial potential (ΔΨm) [28] and mitochondrial biogenesis [7, 8]. Because NAD+ could act as a direct cofactor of various cytosolic and mitochondrial enzymes including PDH, IDH3, α-KG dehydrogenase and malate dehydrogenase in the TCA cycle, the lower NAD+ production in OPA1-KO MEFs [9] may also account for the repressed oxidative metabolism. The elevation of mitochondrial ROS in OPA1-deficient MEFs [29] might also contribute to the depression of mitochondrial oxidative metabolism. Furthermore, lower production of ATP was observed in OPA1-KO MEFs [11], supporting our finding of reduced oxidative metabolism in OPA1-deficient MEFs.

Another striking feature of OPA1-KO cells in MFA displayed an elevated reductive glutamine metabolism, mainly mediated by IDH1 and partially via IDH2. Indeed, a recent study forced OPA1-deficient MEFs oxidative...
metabolism by replacing glucose with galactose culture, and observed that glutamine was the only one amino acid found relatively increased among 20 amino acids [13]. This finding might also support glutamine as a potential energy source in certain circumstances. A previous study established that the α-KG to citrate/isocitrate ratio was positively correlated with the magnitude of the reductive glutamine contribution to citrate [30]. Here the remarkable ratio of α-KG to citrate/isocitrate in OPA1-KO MEFs suggests that this ratio might be the principal driving force for

Fig. 4 OPA1-deficient MEFs rely on cytosolic reductive carboxylation for growth. A Relative live cell number of OPA1-WT and OPA1-KO MEFs after treatment with the medium containing DMSO and 5 µM IDHi for 48 h (vs. OPA1-WT-DMSO). The live cells were counted with Trypan blue staining. The data were analyzed by multiple t-test and the significant level was set as *p < 0.05 (IDH1i vs. DMSO) in each cell line. The experiments were repeated at least three times and n = 3 cultures from a representative experiment.

Mass isotopologue analysis of palmitate (B), citrate and malate (C) in OPA1-KO MEFs cultured with [U-13C]glutamine and unlabeled glucose medium containing DMSO or 5 µM IDH1 inhibitor (IDH1i, GSK321) for 20 h. The data were analyzed by multiple t-test and the significant level was set as *p < 0.05 (IDH1i vs. DMSO) in each cell line. The experiments were repeated at least three times and n = 3 cultures from a representative experiment.

Fig. 5 The schematic model of OPA1-deficiency-induced glutamine-dependent reductive carboxylation for DNL that is essential for cell growth
the switch from oxidative glucose to reductive glutamine metabolism as the primary carbon source for citrate synthesis. A set of studies concluded the different dependency on IDH1 and IDH2 of their reductive glutamine metabolism in different conditions and cell types [16–18, 21, 25, 30]. Our MFA data distinguish their roles of IDH1 and IDH2 in the reductive production of citrate that IDH1 is predominantly involved and IDH2 somehow partially participates in this process. Meanwhile, the much lower expression of CTP, a transporter promoting citrate translocation from mitochondria to cytosol, indirectly supports that the IDH2-mediated route only accounts for a very small contribution of cytosolic citrate production under OPA1 deficiency. Another interesting aspect is that when IDH1 activity was blocked, the m + 5 citrate labeling in OPA1-null cells did not show any reduction, while the m + 3 enrichment of citrate was decreased as expected. We assume that under IDH1 inhibition, IDH2 activity could be further stimulated to a relatively high level that compensates for the m + 5 citrate production. Moreover, this may have some other indications, which require further explorations.

Within cells, citrate can be derived from glucose and glutamine, and then be used as a lipogenic precursor [17, 18, 21]. The NADPH-dependent reversible reactions between cytosolic citrate and α-KG could be primarily catalyzed by IDH1 [16]. Our MFA also shows that OPA1 loss-induced TCA cycle anaplerosis could be compensated by glutamine-dependent reductive carboxylation primarily via IDH1, reflected by the increased contribution of reductive glutamine metabolism-derived lipid synthesis. Comparing the lipidomic signatures of OPA-WT and OPA-KO MEFs [15], they observed that the proportion of sphingomyelins was lower while the triglycerides were at increased levels in Opa1-KO MEFs than those in OPA1-WT cells. Our finding of this switch from oxidative glucose metabolism into reductive glutamine metabolism to produce citrate for DNL might be, at the very least, involved in the active lipid remodeling. Previous studies implied that in cancer cells especially under hypoxia, glutamine-dependent reductive metabolism could be a primary way to generate citrate that ensures lipid synthesis and supports rapid proliferation of cancer cells [16, 17]. These findings support that OPA1 deficiency may exert similar effects on MEFs as those cancer cells. Furthermore, we show the high deficiency of aspartate, which may be due to the enhanced flux into asparagine and its secretion outside the cells as MFA showed. These data are consistent with the findings that aspartate was highly deficient within cells along with the intracellular high asparagine production [9, 13, 14]. Aspartate is proven to be a limiting metabolite for cancer cell proliferation under hypoxia and in tumors [20]. Thus, we assume that high aspartate deficiency may be also associated with the repression of cell growth in OPA1-KO MEFs [9].

In conclusion, OPA1 deficiency reprograms central carbon metabolism in MEFs, namely stimulating the metabolic anaplerosis into IDH1-mediated glutamine-dependent reductive carboxylation for citrate production, which further maintains the lipid synthesis and cell growth. Further work is required to determine the specific correlations between metabolic enzyme regulations and cell growth in this process.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00180-022-04542-5.

Acknowledgements We thank the Light Microscopy Core at City of Hope Medical Center for technical assistance.

Author contributions WTD and LJ conceptualized the study, designed the experiments, interpreted the data, and revised the manuscript. WTD performed the experiments and wrote the original draft. ZCW helped with the INCA modeling analysis. QAW and DC helped revise the manuscript. LJ supervised the whole study. All authors approved the manuscript.

Funding The work was supported by Caltech-City of Hope Biomedical Research Initiative awarded to Lei Jiang, David Chan, and Qiong A. Wang, grant NIH R35 GM127147 to David Chan, and P30CA033572 to City of Hope. Qiong A. Wang was also supported by National Institutes of Health grants R01AG063854, R01HD096152, R01DK128907, and the American Diabetes Association Junior Faculty Development Award 1-19-JDF-023.

Availability of data and materials All the metabolic alterations data generated by GC–MS are shown in Supplementary Information.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

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