LETTER TO THE EDITOR

Glutaminase GLS1 senses glutamine availability in a non-enzymatic manner triggering mitochondrial fusion

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Dear Editor,

The biological importance of glutamine lies in its being a major source of carbon and nitrogen for both catabolic and anabolic demands. Glutamine is converted to glutamate and then to α -ketoglutarate (α -KG), a catabolic process known as glutaminolysis. α -KG enters the tricarboxylic acid (TCA) cycle, referred to as anaplerosis, not only for the generation of ATP via oxidative phosphorylation, but also for the production of acetyl-coA as a critical precursor for the synthesis of lipids and nucleotides. This is particularly true in cancer cells where glutamine is even considered as a conditionally essential amino acid because its cellular demand often exceeds the rate of self-supply, owing to glucose being ineffectively utilized for energy production through aerobic glycolysis and diversion to biosynthesis.^{1,2} Glutamine also plays a vital role in clearing reactive oxygen species (ROS), not only by providing the precursors glutamate and cysteine for the synthesis of GSH, but also by promoting the production of NADPH via glutamate dehydrogenase (GLUD) or the aspartate/malate shuttle.³ In cancer cells, deprivation of glutamine results in a large increase of ROS, damaging the structure and function of mitochondria.⁴ When the glutamine level is low, mitochondria undergo fusion to maximize efficiency by diluting damaged mitochondrial proteins such as components of the respiratory chain complexes, and repair damage to preserve the integrity of mitochondrial DNA.^{5,6} Three dynamin-like GTPases, mitofusin 1 (MFN1) and mitofusin 2 (MFN2) on the mitochondrial outer membrane, and optic atrophy 1 (OPA1) anchored to the mitochondrial inner membrane, are known to be involved in mitochondrial fusion.^{6,7} However, little is known about how the signal of glutamine shortage is sensed and transmitted to maintain the quality of mitochondria.

In this study, we observed that mouse embryonic fibroblasts (MEFs) showed a significant increase of long tubular mitochondria or fused mitochondria within 2 h of culture in glutamine-deprived medium, whereas MEFs cultured in standard medium contained mostly fragmented mitochondria, as visualized by immunofluorescent staining of the mitochondrial marker TOM20 (Fig. 1a, b). At 4 h, >95% mitochondria had undergone fusion and re-addition of glutamine effectively reverted mitochondria to short tubular or fragmented ones within 2 h (Fig. 1a, b). Knockdown of *MFN2* or *OPA1* blocked mitochondrial fusion upon glutamine deprivation (Supplementary Information, Fig. S1a, b), indicating that the glutamine deprivation-induced mitochondrial fusion (GDIMF) is dependent on the mitofusins/OPA1 factors. As it is reported that a combined loss of nutrients by Earle's Balanced Salt Solution treatment induces mitochondrial fusion through the cAMP-PKA pathway,⁸ we also examined whether cAMP plays a role in GDIMF. We found that glutamine deprivation did not elevate the levels of cAMP (Fig. 1c), and activation of the cAMP-PKA pathway by forskolin did not trigger mitochondrial fusion (Supplementary Information, Fig. S1c, d), indicating that GDIMF is not mediated by the cAMP-PKA

pathway. As mitochondrial fusion still occurred in glutamine-free medium that contained glucose, pyruvate, and a full complement of other amino acids (Fig. 1a, b), glutamine itself or its glutaminolytic intermediate(s) was considered as the most likely regulator of mitochondrial fusion (Fig. 1d). To verify this, we manipulated the cellular levels of glutamine. First, glutamine, or the cell-permeable, dimethyl ester form of glutamate (dm-E) or α -KG (dm- α -KG) was added to MEFs in glutamine-free medium, and GDIMF was prevented (Supplementary Information, Fig. S1e, f). We then knocked down glutamate-ammonia ligase (*GLUL*) to block the conversion of glutamate to glutamine, and found that the effects of dm-E and dm- α -KG to suppress mitochondrial fusion were dampened (Fig. 1e; Supplementary Information, Fig. S1g, h). In addition, upon knockdown of all three transaminases expressed in MEFs, *GPT2*, *GOT1*, and *GOT2* (TA-TKD, Supplementary Information, Fig. S1i, j), to block the conversion of α -KG to glutamate (and then to glutamine by *GLUL*), or upon treating MEFs with the transaminase pan-inhibitor aminooxyacetate (AOA), the effect of dm- α -KG could no longer be observed, although its upstream metabolite, dm-E, sufficiently blocked GDIMF in these cells (Fig. 1f). These results together suggest that GDIMF can be rescued so long as glutamine can be generated from glutamate or α -KG. We also wondered whether ROS, increased after glutamine deprivation,⁴ could play a role in modulating mitochondrial fusion. Addition of the antioxidant *N*-acetyl-L-cysteine (NAC) to cells in glutamine-free medium did not prevent mitochondrial fusion, suggesting that ROS production following glutamine deprivation is not the cause of mitochondrial fusion (Supplementary Information, Fig. S1c, d). As an additional control, addition of dNTPs (deoxyribonucleotide triphosphates), the products of glutamine known to be able to rescue the K-Ras-transformed fibroblasts from abortive S-phase under glutamine deprivation,⁹ had no effect on mitochondrial fusion either (Supplementary Information, Fig. S1e, f). Taken together, we conclude that it is the absence of glutamine per se, but not its derivatives, acts as the signal for triggering mitochondrial fusion.

We next investigated which protein(s) could act as the glutamine sensor. In mammalian cells, glutamine is converted to glutamate by glutaminase (GLS), which functions as the rate-limiting enzyme, and *GLUL*, as mentioned above, catalyzes the reverse reaction. As glutamine is the substrate for GLS and the product of *GLUL*, we knocked down these enzymes in MEFs. There are two isozymes of GLS, GLS1, and GLS2; the *GLS1* gene, encoding two alternatively spliced forms in mammals, GAC and KGA, is ubiquitously expressed in various tissues and highly expressed in many types of cancer,^{10,11} while the expression of GLS2 is restricted to the liver and brain. It was found that deficiency of GLS1 virtually abolished mitochondrial fusion in cells incubated with glutamine-deprived medium (Fig. 1g, h; Supplementary Information, Fig. S2a), whereas knockdown of *GLUL* had no effect (Fig. 1e; Supplementary Information, Fig. S1e, f).

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Surprisingly, re-introduction of K294A or Y399A, mutants of the GAC isoform of GLS1 defective in catalyzing glutamine breakdown,^{12,13} into the *GLS1*-knockdown MEFs, restored mitochondrial fusion under glutamine deprivation, as efficiently as wild-type GLS1 did (Fig. 1i; Supplementary Information, Fig. S2b). Consistently, addition of BPTES, DON or 968, inhibitors of GLS1, into regularly cultured MEFs, did not trigger mitochondrial fusion (Supplementary Information, Fig. S2c, d). It has been reported that tetramerization of GLS1 is a prerequisite for constituting the binding site to glutamine.¹⁴ Re-introduction of the F394S mutant of GAC that is unable to be oligomerized to tetramers to *GLS1*-

knockdown MEFs did not restore GDIMF (Fig. 1i; Supplementary Information, Fig. S2b). As an additional control, single re-introduction of the K316Q and R322A mutants of GAC which lack the ability to form superoligomers (octamers or higher), to *GLS1*-knockdown MEFs, sufficiently restored GDIMF (Supplementary Information, Fig. S2e, f). Altogether, these data strongly suggest that GLS1 itself acts as the direct sensor of glutamine, which transmits the signal of glutamine absence to elicit mitochondrial fusion. To test whether GLS1 could also modulate mitochondrial fusion triggered by other metabolic alterations such as enhanced OXPHOS,¹⁵ we treated cells with glucose-free, galactose-

Fig. 1 Glutaminase GLS1 directly senses the absence of glutamine to trigger mitochondrial fusion. **a, b** Glutamine deprivation triggers mitochondrial fusion. MEFs were regularly cultured (NM) or deprived for glutamine (QD) for various time periods as indicated. Mitochondria were visualized using an antibody to TOM20. Representative images were shown in **(a)** (scale bars, 10 μ m) and quantification of the mitochondrial morphology of the cells was graphed in **(b)**. Data shown in **(b)** were mean $\pm$ SEM (three independent experiments with at least 100 cells counted for each technical replicate). Different colors indicate different morphologies of mitochondria (fragmented, short, or long). "Short" represents cells with a majority of mitochondria shorter than 10 μ m; "Long" represents cells in which the majority of mitochondria were longer than 10 μ m. "Fragmented" represents cells with a majority of granular mitochondria. *P*-value was determined by one-way ANOVA, *****P* < 0.0001; N.S., no significance, long mitochondria in MEFs incubated in glutamine-free medium were compared with those incubated in DMEM. **c** Glutamine starvation does not elevate cAMP levels. MEFs were incubated in glutamine-free medium for desired times, or were treated with 10 μ M forskolin (FSK), and the cellular cAMP levels were measured. Data were statistically analyzed and are shown in scatter plots. *P*-value was determined by two-way ANOVA, *****P* < 0.0001; N.S., no significance. **d** A schematic diagram of glutamine metabolism. Glutamine (Q) is converted to glutamate (E) by GLS (reverted by GLUL), then to α -ketoglutarate (α -KG) by transaminases such as GOT and GPT. α -KG enters the TCA cycle for the generation of ATP via OXPHOS, or for the synthesis of GSH that clears ROS. **e** Knockdown of *GLUL* impairs the effect of glutamate on the restoration of the fragmented state of mitochondria under glutamine deprivation. Cells were incubated in glutamine-free medium, or glutamine-free medium supplemented with 4 mM dm-E or dm- α -KG for 4 h. Mitochondria were visualized as in **(a)** and the representative images of this experiment were shown in Supplementary Information, Fig. S1f. The mitochondrial morphology was quantified as in **(a)**. *P*-value was determined by two-way ANOVA, *****P* < 0.0001, long mitochondria in *GLUL*-KD MEFs incubated in glutamine-free medium supplemented with 4 mM dm-E or dm- α -KG were compared with those in wild-type MEFs. **f** Knockdown of transaminases (TAs, including *GOT1*, *GOT2*, and *GPT2*) dampens the effect of α -KG on inhibition of mitochondrial fusion under glutamine deprivation. MEFs were incubated for 4 h in glutamine-free medium, or glutamine-free medium supplemented with 4 mM dm-E or 4 mM dm- α -KG with or without 100 μ M AOA. The mitochondrial morphology was determined and quantified as in **(a)**. *P*-value was determined by two-way ANOVA, *****P* < 0.0001, long mitochondria in TAs-knockdown or AOA-treated MEFs incubated in glutamine-free medium supplemented with 4 mM dm- α -KG were compared with those in wild-type MEFs. Note that *GPT1* was not expressed in MEFs, as determined in Supplementary Information, Fig. S1h. **g, h** Knockdown of *GLS1* blocks the glutamine deprivation-induced mitochondrial fusion. Mitochondria were visualized as in **(a)**, and the representative images were shown in **(f)**. The mitochondrial morphology was quantified and graphed in **(g)**. *P*-value was determined by two-way ANOVA, *****P* < 0.0001, long mitochondria in *GLS1*-KD MEFs incubated in glutamine-free medium were compared with those in wild-type MEFs. **i** The catalytic activity of GLS is dispensable in mediating glutamine sensing to mitochondrial fusion. Experiments were performed as in **(a)**, except that *GLS1*-KD MEFs stably expressing Gac-K294A, Gac-F394S, Gac-Y399A or its wild-type control were used. Representative images of this experiment were shown in Supplementary Information, Fig. S2b. The mitochondrial morphology was quantified as in **(a)**. *P*-value was determined by two-way ANOVA, *****P* < 0.0001; N.S., no significance. **j** GLS1 does not mediate galactose-induced mitochondrial fusion. Mitochondria were visualized as in **(a)** except that the glucose-free medium supplemented with 10 mM galactose was used. Representative images of this experiment were shown in Supplementary Fig. S2d. The mitochondrial morphology was quantified as in **(a)**. *P*-value was determined by two-way ANOVA, *****P* < 0.0001; N.S., no significance. **k, l** Elevated ROS levels in mitochondrial fusion-deficient MEFs under glutamine deprivation. MEFs were regularly cultured or deprived for glutamine for 4 h, and then collected for further flow cytometry assay. *P*-value was determined by two-way ANOVA, ****P* < 0.001, *****P* < 0.0001; N.S., no significance

containing medium and found that the galactose-induced mitochondrial fusion was not affected in *GLS1*-knockdown MEFs (Fig. 1j; Supplementary Information, Fig. S3a, b). Since GDIMF depends on dynamin-related GTPases, we wondered whether MFN2 acts as the downstream mediator of GLS1. Indeed, overexpression of MFN2 in *GLS1*-knockdown MEFs restored the fusion of mitochondria (Supplementary Information, Fig. S3c–e).

Finally, we determined whether the GLS1-mediated mitochondrial fusion plays a part in suppressing ROS under glutamine deprivation. Knockdown of *GLS1*, or *MFN2* as a control, led to a large increase of ROS under glutamine deprivation (Fig. 1k), albeit not to the same extent seen in cancer cells such as the 8988T cells.³ Moreover, re-introduction into *GLS1*-knockdown MEFs with WT GAC or the catalytically inactive K294A mutant, which all restored mitochondrial fusion, maintained low ROS levels under glutamine deprivation, while the substrate binding-defective F394S mutant of GAC failed to do so (Fig. 1l). These data indicate that GLS1-mediated GDIMF effectively prevents the increase of ROS during glutamine deprivation. In conclusion, we have identified that GLS1 specifically and directly senses glutamine availability and mediates the mitochondrial fusion process under glutamine deprivation to maintain mitochondrial integrity. It will be of great interest to identify the signaling factors that link the sensing of the lack of glutamine by GLS1 to mitofusins/OPA1-mediated mitochondrial fusion.

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ADDITIONAL INFORMATION

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