Krüppel-like factor 4 (KLF4) induces mitochondrial fusion and increases spare respiratory capacity of human glioblastoma cells

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Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor critical for the regulation of many cellular functions in both normal and neoplastic cells. Here, using human glioblastoma cells, we investigated KLF4’s effects on cancer cell metabolism. We found that forced KLF4 expression promotes mitochondrial fusion and induces dramatic changes in mitochondrial morphology. To determine the impact of these changes on the cellular functions following, we analyzed how KLF4 alters glioblastoma cell metabolism, including glucose uptake, glycolysis, pentose phosphate pathway, and oxidative phosphorylation. We did not identify significant differences in baseline cellular metabolism between control and KLF4-expressing cells. However, when mitochondrial function was impaired, KLF4 significantly increased spare respiratory capacity and levels of reactive oxygen species in the cells. To identify the biological effects of these changes, we analyzed proliferation and survival of control and KLF4-expressing cells under stress conditions, including serum and nutrition deprivation. We found that following serum starvation, KLF4 altered cell cycle progression by arresting the cells at the G2/M phase and that KLF4 protected cells from nutrition deprivation-induced death. Finally, we demonstrated that methylation-dependent KLF4-binding activity mediates mitochondrial fusion. Specifically, the downstream targets of KLF4-mCpG binding, guanine nucleotide exchange factors, serve as the effector of KLF4-induced mitochondrial fusion, cell cycle arrest, and cell protection. Our experimental system provides a robust model for studying the interactions between mitochondrial morphology and function, mitochondrial dynamics and metabolism, and mitochondrial fusion and cell death during tumor initiation and progression.

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This article contains Figs. S1–S7.

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Each year, there are ~700,000 people living with a primary brain tumor in the United States, one-tenth of which are newly diagnosed. Gliomas make up about half of the primary brain tumors. Grade IV glioma, glioblastoma (GBM), is the most aggressive primary brain tumor in adults with median survival of less than 2 years even after a combination treatment of surgical resection, chemotherapy, and radiotherapy (1). Tumor recurrence after initial treatment is nearly certain, and currently there is no therapy proven to prolong the survival of GBM patients. The dismal prognosis associated with GBM has fostered aggressive investigations into alternative therapeutic paradigms. Radical improvements in clinical outcomes will require a better understanding of the molecular and cellular bases of GBM propagation and recurrence (2).

One of the hallmarks of GBM is the uncontrolled cell proliferation fueled by abnormal cellular metabolism that provides the energetic and anabolic demands of cell growth (3). Cancer cells are able to support their proliferation and survival by acquiring necessary nutrients from the tumor microenvironment. Like many other solid tumor cells, metabolic alterations in brain tumor cells include increased aerobic glycolysis, decreased oxidative phosphorylation, and increased pentose phosphate pathway that provides biosynthetic intermediates to build new tumor biomass (4).

Mitochondria are complex cellular organelles that play a central role in energy metabolism. Recognized as the energy powerhouse of a cell, the major tasks of the mitochondria are the production of ATP and the metabolites necessary to fulfill the bioenergetic and biosynthetic demands of the cell. Mitochondria are dynamic organelles that supply energy required for driving key cellular processes, such as survival, proliferation, and migration. Critical to all of these processes are changes in

The abbreviations used are: GBM, glioblastoma; GEF, guanine nucleotide exchange factor; CID, CID 1067700; Dox, doxycycline; ROS, reactive oxygen species; H2DCFDA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; KLF, Krüppel-like factor; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; G6PD, glucose-6-phosphate dehydrogenase; RNA-seq, RNA-sequencing; ND, nutrition deprivation; OXPHOS, oxidative phosphorylation; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; BSS, buffered balanced salt solution.
mitochondrial architecture, also referred to as mitochondrial dynamics, a mechanical mechanism encompassing both fusing (fusion) and dividing (fission) of the mitochondrial network (5). Changes to mitochondrial shape, size, and localization occur in a regulated manner to maintain energy and metabolic homeostasis (6), whereas deregulation of mitochondrial dynamics is associated with the onset of metabolic dysfunction and disease (7). In tumors, many factors, such as limited nutrient supply, increased intracellular stress, and oncogenic signals that drive excessive proliferation, are able to alter the bioenergetic and biosynthetic requirements of cancer cells. Consequently, mitochondrial function and shape rapidly adapt to these hostile conditions to support cancer cell proliferation and evade activation of cell death programs (8). In another words, mitochondria have to alter their morphology and functions to meet the metabolic demands of cancer cells.

Mitochondrial fusion and fission are evolutionarily conserved behaviors that allow cells to respond to their constant changing conditions. In general, fusion and fission antagonize each other: a shift toward fusion favors the generation of tubular-like, interconnected mitochondria that are detected in metabolically active cells (5). By contrast, mitochondrial fission produces smaller, fragmented mitochondria that are important for mitochondrial movement to regions of high-energy demand or to allow for equal mitochondrial distribution to daughter cells during mitosis (9). In mammals, three GTPases of the mitochondrial dynamin family members have been identified to control mitochondrial dynamics: mitofusins (Mfn 1 and 2) mediate mitochondrial outer membrane fusion; Opa1 mediates mitochondrial inner membrane fusion; Drp1, which cycles between the cytosol and the mitochondrial outer membrane, mediates mitochondrial fission. The upstream signaling that regulates mitochondrial fusion and fission is far from clear.

Krüppel-like factors (KLFs) belong to the zinc finger family of transcription factors. Only a small number of studies have reported members of the KLF family as critical regulators of metabolism-related enzymes and transporters, including ATP2B4, SLC2A3, and SLC9A3R1, were significantly up-regulated by KLF4 (13). We validated our large-scale RNA-seq analysis by real-time PCR (RT-PCR). Several metabolism-related enzymes and transporters, including ATP2B4, SLC2A3, and SLC9A3R1, were significantly up-regulated by KLF4 (Fig. S1C). This prompted us to focus on the effect of KLF4 on the metabolism of GBM cells.

As mitochondria are the centerpiece of cellular metabolism, we investigated whether KLF4 affected mitochondrial morphology changes following Dox expression (14). We validated our large-scale RNA-seq analysis by real-time PCR (RT-PCR). Several metabolism-related enzymes and transporters, including ATP2B4, SLC2A3, and SLC9A3R1, were significantly up-regulated by KLF4 (Fig. S1C). This prompted us to focus on the effect of KLF4 on the metabolism of GBM cells.

### Results

**KLF4 expression induces mitochondrial morphology changes in glioblastoma cells**

In our previous studies, we generated a human GBM U87 cell line to express Tet-On KLF4 WT (13). Upon doxycycline (Dox) treatment (1 μg/ml), the KLF4 protein level was induced in U87 cells (Fig. S1A). Prominent immunocytochemistry staining confirmed KLF4 induction in the nucleus (Fig. S1B). Our previous RNA-seq analysis indicated that many genes related to metabolism were affected by KLF4 (13). We validated our large-scale RNA-seq analysis by real-time PCR (RT-PCR). Several metabolism-related enzymes and transporters, including ATP2B4, SLC2A3, and SLC9A3R1, were significantly up-regulated by KLF4 (Fig. S1C). This prompted us to focus on the effect of KLF4 on the morphology of GBM cells.

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KLF4 promotes mitochondrial fusion

cating distinct small spheres or short rods of mitochondrial morphology. In contrast, in KLF4-expressing cells (KLF4), mitochondria became much longer and, most strikingly, the majority of the mitochondria formed network-like structures, consistent with mitochondrial fusion. The newly-formed mitochondrial network no longer crowded around the nucleus but spread over the whole-cell body, even to distal areas of the cytosol (Fig. 1A, lower panels, arrowheads). We marked the cytoplasm of the control cells (asterisks in Fig. 1A and Fig. S2) and confirmed that the observed mitochondrial fusion in KLF4-expressing cells was not due to cell morphology change, e.g. a flat or more spreading cell phenotype.

We quantified the percentage of cells with fused mitochondria (network-like) and fragmented mitochondria (dotted) based on the ATP synthase staining. Mitochondria in 90% of the KLF4-expressing cells were fused together. In contrast, 90% of the control cells showed punctate staining around the nucleus (Fig. 1C). These data further supported that KLF4 induced mitochondrial fusion in GBM cells.

To further analyze the mitochondrial morphology change, we took microphotographs using confocal microscopy and superimposed ~40 adjacent z-stack images (Fig. 1A, right panels). Compared with control, KLF4 induced dramatic mitochondrial fusion by forming interconnected and thick tubes over the entire cell bodies (Fig. 1A, lower right panel). We used ImageJ (National Institutes of Health) to quantify the average length of mitochondria in U87 cells before and after Dox treatment. Relative to control, there was an ~3.2-fold increase in the average length of mitochondria per cell in U87 KLF4-expressing cultures (Fig. 1D, p < 0.001). To further analyze the mitochondrial network, we compared the number of branches and junctions of the network using ImageJ. KLF4-expressing cells showed an extensively branched mitochondrial network (Fig. 1, E and F).

KLF4 has been reported to promote mitochondrial biogenesis in cardiac cells (11). With the dramatic mitochondrial morphology change induced by KLF4, we asked whether the mitochondrial fusion induced by KLF4 was associated with an increase in mitochondrial mass (14). KLF4 expression did not alter mitochondrial mass, as evidenced by immunoblotting analysis showing no change in the expression level of mitochondrial proteins Tom 20 and ATP synthase following KLF4 expression (Fig. 1G).

Mitochondrial fusion is a new biological function of KLF4

To further confirm the mitochondrial morphology change, we stained the cells with additional markers specific for mitochondria, including MitoTracker Red, cytochrome c, and the translocase of mitochondrial outer membrane Tom 20. When the cells were stained with these markers, similar patterns of mitochondrial morphology change were observed in KLF4-expressing cells. Specifically, in control cells, disconnected mitochondria with the shape of small spheres or short rods resided in the perinuclear areas; in KLF4-expressing cells, interconnected mitochondria formed massive networks covering the whole-cell bodies (Fig. 2A).

To determine the time course of KLF4-induced mitochondrial fusion, we treated U87 cells with Dox at different time points and found that as early as 16 h following Dox treatment, there was a distinct mitochondrial fusion (Fig. 2B, upper panels). We also examined mitochondrial staining after Dox withdrawal. Mitochondrial fusion disappeared 16 h after Dox withdrawal (Fig. 2B, lower panels). Immunocytostaining showed KLF4 induction and reduction following Dox treatment and withdrawal, respectively (Fig. S3). This suggested that mitochondrial fusion happened simultaneously with KLF4 expression.

It is noteworthy that Dox itself is an antibiotic that may affect mitochondrial function (15). To rule out the nonspecific effect of Dox on mitochondrial morphology in our Tet-On system, we treated the parental U87 cells with Dox as an additional control for KLF4 function. We did not observe any formation of mitochondrial network in parental U87 cells treated with the same dosage and length of Dox (Fig. 2C), further supporting that KLF4’s function drove the mitochondrial morphology change in GBM cells.

Baseline cell metabolism is not affected by KLF4

Mitochondria are the energy source of a given cell, and any mitochondrial morphology change may suggest alterations in fundamental cellular metabolic pathways. Three metabolic pathways were measured in KLF4-expressing cells: glycolysis, glucose oxidation, and the pentose phosphate pathway. First, we examined whether KLF4 changed glucose uptake in U87 cells. Glucose uptake analysis using 2-[3H]deoxyglucose showed no difference between control and KLF4-expressing cells, indicating that KLF4 did not change glucose transporters to affect glucose uptake rate (Fig. 3A). Next we examined glycolysis by measuring the rate of lactate secretion into the culture medium. We did not observe dramatic changes in the rate of lactate production in control and KLF4-expressing cells (Fig. 3B). We also measured the rate-limiting enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase, and we found no differences in the production of NADPH by control or KLF4-expressing cells (Fig. 3C). We next assayed the rate of oxidation of [U-13C]glucose to 13CO2; this process requires the integration of several pathways, including glycolysis, the tricarboxylic acid cycle, and the electron transport chain/oxygen phosphorylation (OXPHOS). We detected a slight but not significant decrease in glucose oxidation in KLF4-expressing cells (Fig. 3D). Finally, we measured ATP content in control and KLF4-expressing cells and did not detect a significant increase in ATP levels in KLF4-expressing cells (Fig. 3E). All this suggested that mitochondrial fusion induced by KLF4 did not cause dramatic alteration in mitochondrial function. In other words, KLF4 had minimal effect on cell metabolism when cells were under normal culture conditions.

KLF4 increases the spare respiratory capacity and reactive oxygen species in GBM cells

To further understand the biological impact of KLF4-induced mitochondrial fusion, we asked whether KLF4 changed cancer cell metabolic profiling under stressed conditions using a Cell Mito stress test kit (herein referred as Mito stress). Cell metabolism was measured by using a Seahorse XF instrument. We examined metabolic changes when the cells were treated by
the following four drugs: oligomycin, FCCP, and rotenone/antimycin A, all of which affect mitochondrial function (Fig. 4A). Consistent with our detailed metabolism analyses, Seahorse XF analysis showed that KLF4 did not affect glycolysis and oxidative phosphorylation of U87 cells under basal conditions (Fig. 4B and Fig. S4). However, KLF4 dramatically increased the spare (or reserve) respiratory capacity of U87 cells (Fig. 4C), which measures the capability of the cells’ response to an energetic demand, as well as how closely the cell is able to respire to its theoretical maximum. Seahorse XF analysis revealed no significant change in ATP level in KLF4-expressing cells (Fig. 4D), yet there was a significant increase in the nonmitochondrial oxygen consumption (Fig. 4E), an indicator of increased reactive oxygen species (ROS). To confirm this, we performed flow cytometry analysis of ROS in cells using the ROS detection dye 2′,7’-dichlorofluorescin diacetate (H2DCFDA). Compared with control, a dramatic increase in ROS production relative to control (Fig. 4G, p < 0.001), consistent with our Seahorse XF analysis.

The energy phenotype from Seahorse XF analysis further indicated that KLF4-expressing cells were more energetic when mitochondrial function was impaired (Fig. 5A). Detailed analysis showed that following drug treatment, KLF4 cells had more metabolic potential by showing increased oxygen consumption rate (OCR) (Fig. 5B). Compared with baseline conditions, KLF4 promoted a higher oxygen consumption rate (Fig. 5C) as well as a higher extracellular acidification rate under Mito stress (ECAR, Fig. 5D). All these data suggested that even though there was no difference in metabolic profiling under normal culture conditions, KLF4-expressing cells were more fit when mitochondrial function was impaired.

**KLF4 changes cell cycle progression after serum deprivation**

To determine the biological implication of mitochondrial morphology and function changes, we analyzed how KLF4 affected cellular function. Cell growth analysis indicated that
compared with control, KLF4 did not change cell proliferation in normal cultures (Fig. 6A). Because Seahorse XF analysis showed that KLF4 mainly affected mitochondrial functions under Mito stress conditions, we examined cell cycle progression in control and KLF4-expressing cells after serum withdrawal, followed by serum repletion at different time points. Serum withdrawal for 24 h synchronized the cell cycle at G0/G1 (~86%) in both control and KLF4-expressing cells (Fig. 6, B and C). Twelve hours following serum repletion, KLF4 promoted a nearly 3-fold increase in cells entering S phase, from 5.8 to 14.9% (Fig. 6D). Eighteen to 24 h following serum repletion, KLF4-expressing cells seemed to be halted at the G2/M phase (~40% versus 20% in control, Fig. 6, E and F), suggesting that even though KLF4 promoted cells to enter the S phase quickly, slowing at G2/M prevented these cells from proliferating faster than control cells. We quantified cell proliferation in KLF4-expressing cells following serum deprivation/repletion for 4 days. KLF4 did not change cell proliferation under these conditions (Fig. S5), consistent with our cell growth analysis under normal culture conditions.

**KLF4 protects GBM cells from death induced by nutrition deprivation**

Furthermore, we hypothesized that KLF4-induced mitochondrial fusion played a protective role in cells undergoing metabolic stress such as nutrition deprivation (ND), in which normal culture medium was replaced with a buffered balanced salt solution without serum, glucose, glutamine, and other amino acids (16). We explored the effect of KLF4 on cell survival under ND. After KLF4 was induced for 48 h, cells were placed in nutrition-deprived medium. Control U87 cells changed their morphology 48 h after nutrition deprivation (Fig. 7A, upper left panel), and they completely died at day 3 (Fig. 7B). Anti-ATP synthase staining indicated that under nutrition dep-
KLF4 promotes mitochondrial fusion

Finally, we wanted to determine the molecular mechanisms involved in KLF4-induced mitochondrial fusion. First, we examined whether KLF4 up-regulated any of the three GTPases involved in mitochondrial fusion, namely Mfn1, Mfn2, and Opa-1. RT-PCR showed that KLF4 did not change their expression at the mRNA level (Fig. S6A). We also examined the SIRT family members, which induced deacetylation of Mfn1 and Mfn2 as reported by Oanh et al. (17). RT-PCR showed that the four SIRT family members were not altered by KLF4 (Fig. S6B).

We turned to our site-specific mutant form of KLF4, KLF4 R458A, which lacks KLF4 binding to methylated CpG (mCpG) but retains its binding to nonmethylated CpG (13). In cells expressing Tet-On KLF4 R458A, upon Dox treatment the KLF4 R458A protein was detected by immunoblotting (Fig. S7). We examined mitochondrial morphology in KLF4 R458A-expressing cells (KLF4-mut) by immunostaining for ATP synthase. Mitochondrial morphology with scattered small spheres or short rods surrounding the nucleus was observed in KLF4-mut expressing cells (KLF4-mut) by immunostaining for ATP synthase. Compared with control, significantly more KLF4-mut-expressing cells survived at days 2 and 3 following nutrition deprivation. There was no protection of KLF4-mut expressing cells from death under nutrition-deprived conditions (Fig. 8A). These findings suggested that mitochondrial fusion induced by KLF4 was involved in cell protection under nutrition-deprived conditions.

Because KLF4 WT and KLF4 mutant differ in their ability to bind to methylated CpGs, our results indicated that methylation-dependent KLF4 downstream targets mediated the observed mitochondrial fusion and cell protection effect of KLF4. Our previous RNA-seq analysis identified ~116 genes up-regulated by KLF4 via interactions with mCpGs in cis-regulatory elements of the targets genes (13). Some of the 116 targets were involved in GTPase regulation, such as the guanine nucleotide exchange factors (GEFs) that positively regulate small GTPase activation. Members in the GEFs family, including neuronal guanine nucleotide exchange factor, Rho/Rac guanine nucleotide exchange factor 2 (ARHGEF2), and RAB
guanine nucleotide exchange factor 1 (RABGEF1), were up-regulated by KLF4 WT but not by KLF4 R45A mutant from our RNA-seq data (Table 1), indicating the involvement of KLF4-mCpG–binding activity in the activation of these genes. RT-PCR validated the significant up-regulation of these genes by KLF4 only, but not by KLF4-mut (Table 1). Because the modulators of mitochondrial dynamics are GTPases such as Mfn1/2 and Opa1, we hypothesized that KLF4-mCpG binding-induced regulation of these GEFs promotes mitochondrial fusion. We used a Ras superfamily GTPase GTP/GDP-binding antagonist (18), CID 1067700 (CID) to challenge the KLF4-expressing cells. In KLF4-expressing cells CID treatment (5 μmol/liter) decreased the percentage of cells with fused mitochondria from 83 to 57% (Fig. 8, C and D). We examined cell survival and cell cycle progression in KLF4-expressing cells in the presence of CID. CID treatment significantly blocked KLF4-induced cell protection under nutrition-deprived conditions (Fig. 8E). Moreover, 24 h after serum repletion, G_{2}/M blockage induced by KLF4 was completely reversed by CID (Fig. 8F). Thus, our study showed for the first time that GEF proteins regulate mitochondrial fusion, and we confirmed that KLF4-induced mitochondrial fusion is the direct cause of cell protection and cell cycle alteration induced by KLF4.

Discussion
Mitochondria are not only the powerhouse of a cell but are also important organelles in regulating cellular functions. Many functions of mitochondria have been closely linked to their morphology, which is shaped by ongoing events of fusion and fission of outer and inner mitochondrial membranes (6). Mitochondrial morphology varies tremendously across cell types and tissues, changing rapidly in response to external insults and

Figure 7. KLF4 protects GBM cells from death induced by nutrition deprivation. A, left panels, phase contrast of control (Con) and KLF4-expressing cells under nutrition-deprived conditions for 48 h. Right panels, ATP synthase staining of control and KLF4-expressing cells under nutrition-deprived conditions showing mitochondrial fusion in KLF4-expressing cells. B, cell viability analysis of cells grown under nutrition-deprived conditions for 24–72 h. At 48 h following nutrition deprivation, U87 cells started to die, and KLF4 significantly protected cells from cell death. C, measurement of mitochondrial membrane potential by JC-1 staining and flow cytometry. D, quantification of percentage of cells with mitochondrial membrane depolarization under normal and nutrition deprivation for 24 h.
metabolic cues, such as nutrient status. Although mitochondrial dynamics has been reported previously with extensive investigation of direct mediators of these processes, the upstream regulators that control mitochondrial fusion and fission remain elusive. Our work has revealed that the transcription factor KLF4 promoted substantial mitochondrial fusion in human brain tumor cells and protected cells from death induced by nutrition deprivation. Our study also showed for the first time that GEFs regulate mitochondrial dynamics and confirmed that KLF4-induced mitochondrial fusion is the direct cause of cell protection and cell cycle alteration induced by KLF4.

KLF4 has been reported previously to promote mitochondrial biosynthesis in cardiac cells, but the effect of KLF4 on mitochondrial dynamics has not been reported. Here, we try to understand why U87 glioblastoma cell mitochondria fuse upon KLF4 expression. Mitochondria undergo fusion when they are facing stressful conditions (19); and one of the biological functions of mitochondrial fusion is to repair damaged mitochondria (20). For example, during normal metabolism and ATP production, mitochondria produce a large quantity of ROS that damage the mitochondrial genetic material. Mitochondrial fusion is critical for the exchange of their genetic material that can repair damaged DNA through recombination (21). In our model, we detected a dramatic increase in ROS generation in KLF4-expressing cells, which exhibited an extensive branching mitochondrial network by fusing with each other. It remains to be determined whether ROS production is the cause or result of mitochondrial fusion in our system. It is noteworthy that oxidative and other stresses frequently produce fission rather than fusion of mitochondria (22).

Many cells operate at a basal level that only requires a part of their total bioenergetic capability. The difference between ATP produced at basal and that at maximal activity is termed “spare respiratory capacity” or “reserve respiratory capacity,” which describes the ability of a cell to produce an extra amount of ATP by oxidative phosphorylation under certain conditions that require a sudden burst of additional cellular energy in response to stress or increased workload. Depletion of the reserve respiratory capacity has been related to a range of pathologies, including senescence or cell death in normal tissues (23). Our Seahorse XF analysis revealed that KLF4 promoted a more energetic status of cancer cells via increased spare respiratory capacity when mitochondrial fusion was impaired. Several scenarios could explain the increased spare respiratory capacity observed in our system: more live cells, more mitochondria per cell, increased protein expression of mitochondrial complexes, or increased activity of mitochondrial complexes. In our study, results from Seahorse XF analysis were normalized by cellular DNA content, and we did not detect an increase in mitochondrial mass following KLF4 expression cells using immunoblotting analysis of mitochondrion-specific proteins. These experiments eliminated the first two possibilities that may increase the spare respiratory capacity. Pfleger et al. (24) has reported that the main source of spare respiratory capacity in cardiac cells is the mitochondrial complex II succinate dehydrogenase, which is also involved in ROS generation. However, our RNA-seq analysis did not find an increase in the expression of succinate dehydrogenase (data not shown). Thus, post-translational modification of mitochondrial complexes or other mechanisms may be involved in KLF4-induced increase in spare respiratory capacity and ROS generation. In our study, we noticed rotenone/antimycin-insensitive respiration in KLF4-expressing cells. The source of this phenomenon could be due to nonmitochondrial respiration, which involves various pathways, including but not limited to ROS, very-long-chain fatty acid catabolism in peroxisomes, etc. Higher nonmitochondrial OCR may suggest the existence of ROS, which has been confirmed in our study.

### Table 1

| Gene symbol | Full name | Fold change from RNA-sequence | Fold change from RT-PCR |
|-------------|-----------|-------------------------------|-------------------------|
| NGEF        | Neuronal GEF | KLF4: 8.77, 0.83 | KLF4-mut: 2.91, 1.57 |
| ARHGEF2     | Rho/Rac GEF 2 | KLF4: 3.81, 0.69 | KLF4-mut: 8.78, 0.83 |
| RABGEF1     | RAB GEF 1 | KLF4: 3.08, 0.83 | KLF4-mut: 2.87, 0.95 |

* *p < 0.05.*
KLF4 promotes mitochondrial fusion

The biological function of mitochondrial fusion may be related to cancer cell metabolism and the cancer stem cell phenotype. During tumorigenesis, cells undergo metabolic reprogramming and switch from mitochondrion-dependent oxidative phosphorylation to glycolysis, a phenomenon called the Warburg effect (4). Although cancer cells dominantly utilize aerobic glycolysis rather than mitochondrial metabolism, accumulating evidence has reported that many cancer cells, especially cancer stem cells, still depend on mitochondrial metabolism to survive under harsh conditions such as low oxygen levels, limited nutrients, and low pH values (4). It has been postulated that cancer stem cells are more dependent on OXPHOS (25). Here, we provided evidence that KLF4 regulated mitochondrial dynamics and homeostasis in U87 cells under normal conditions and increased spare respiratory capacity for cells to survive when nutrition was deprived. KLF4 is one of the four reprogramming factors to induce somatic cells to pluripotent cells (26), and KLF4 has been shown to be significantly up-regulated during growth factor-induced reprogramming of cancer cells (27). We speculate that KLF4’s influence on mitochondrial fusion supports a more OXPHOS-dependent cell metabolism and stem cell-like phenotype of cancer cells, which may contribute to tumor recurrence as cancer stem cells are usually resistant to cell death. The protective effect of KLF4 on cancer cell death induced by nutrition deprivation in our model supports our hypothesis, and it may explain why stem-like cancer cells are more resistant to cell death, possibly via mitochondrial fusion. This is consistent with published work in cardiac cells, where a hyper-fused network of mitochondria serves to counteract metabolic insults, preserve cellular integrity, and protect against autophagy (5).

Mitochondrial shape change, brought about by molecules that promote either fission or fusion between individual mitochondria, has been documented in several model systems. However, the deeper significance of mitochondrial shape change has only recently begun to emerge. Among others, it appears to play a role in the regulation of cell proliferation. Regulation of mitochondrial shape may modulate mitochondrial metabolism and/or energetics to promote cross-talk between signaling components and the cell cycle machinery. In our cell model, we did not observe cell proliferation changes induced by KLF4 under normal and serum-deprivation/repletion conditions, but we detected a significant cell cycle difference in cells under serum starvation in KLF4-expressing U87 cells. Even though KLF4 induced early entering to the S phase in U87 cells, the cells were halted at the G2/M phase, possibly because mitochondrial fission is required prior to mitosis so that mitochondria can be divided into daughter cells. Thus, our model has revealed that besides a protective role, mitochondrial fusion also contributed to cell cycle regulation. Although KLF4 changed the cell cycle, it had no effect on cell proliferation. The impact of cell cycle arrest induced by KLF4 may drive cells into a quiescent state when facing nutrition deprivation. This may partially explain our observation that KLF4-expressing cells survived longer when nutrition was completely deprived in the culture medium.

Currently, much of the regulation of mitochondrial fusion is unknown. Interpreting the mechanism of mitochondrial morphology change is complicated by the functional redundancy and additional roles of mitochondrial regulators within and outside mitochondria (28). In U87 cells, we found that KLF4 did not directly regulate the expression or modification of the three major modulators of mitochondrial fusion: Mfn1, Mfn2, and Opa1. Many possibilities can be changed directly or indirectly by KLF4 without changing the expression level of the three fusion regulators, such as protein modifications and enzyme activity. The exact mechanism and detailed downstream signaling pathways by which KLF4 regulates mitochondrial dynamics will be the focus of our future work. By using a KLF4 site-specific mutant that lacks KLF4 binding to methylated DNA, we found that KLF4’s effect on mitochondrial fusion is methylation-dependent. It is worthwhile to point out that the effect of KLF4 on mitochondrial fusion may be cell type-specific because the overall biological function of KLF4 is contextual and cell type-dependent (29).

In summary, we reported here a novel function of KLF4 on regulation of mitochondrial dynamics. Cancer hijacks the mitochondrial machinery to drive cell proliferation and survival. The identification of KLF4 as a novel regulator of mitochondrial fusion may have implications beyond those we detailed in this work. In all, our system provides a unique model to study the relationship between mitochondrial morphology and function, cancer cells and metabolism, mitochondrial fusion, and cell death for targeted cancer therapy in the future.

Experimental procedures

Cell culture

All reagents were purchased from Sigma unless otherwise stated. The human GBM cell line U87 was purchased from the ATCC and cultured as reported previously (30). Briefly, cells were cultured in minimum essential media supplemented with sodium pyruvate (1%), sodium bicarbonate (2%), nonessential amino acid (1%), and 10% fetal calf serum (FCS). Cells were normally incubated in a humidified incubator containing 5% CO2, 95% air at 37 °C and passaged every 4–5 days in a ratio of 1:4–8. Cells were free from mycoplasma and were authenticated with short tandem repeat profiling by The Johns Hopkins Genetic Resources Core facility using the Promega GenePrint 10 system (Madison, WI).

To express KLF4 in U87 cells, a Dox-inducible TripZ lentiviral vector expressing KLF4 WT and KLF4 R458A mutant was introduced into U87 cells via viral infection as we previously reported (13). Stable U87-KLF4 cell lines were established through puromycin selection (1 μg/ml). KLF4 expression was initiated by Dox treatment (1 μg/ml).

Immunocytochemistry

For immunofluorescent staining (31), U87 cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 1% BSA for 30 min. The cells were then incubated with primary antibodies at 4 °C overnight and then incubated with appropriate secondary antibodies conjugated with Alexa Fluor® 488 or cy3 for 1 h at room temperature. Slides were mounted with solution containing 4,6-diamidino-2-phenylindole and observed under fluores-
cent microscopy. Immunofluorescent images were taken and analyzed using Axiosvision software (Zeiss, Oberkochen, Germany). Antibodies used for staining were as follows: anti-ATP synthase β chain (Millipore, Burlington, MA); anti-cytochrome c (BD Biosciences); anti-Tom 20 (Santa Cruz Biotechnology, Dallas, TX); and anti-KLF4 (Santa Cruz Biotechnology).

**Image processing and analyzing**

All image processing and analyses were performed using ImageJ software (National Institutes of Health). Multiple z-stack section images were captured with Axiosvision software (Zeiss). For 3D analysis, the z-stacks were loaded into ImageJ and image/stacks/3D projection was performed to rebuild the 3D structure of mitochondria (32). For skeleton analysis, the z-stack sections were opened with Fiji software (National Institutes of Health), and image/type was performed to adjust photounit to 8 bits. Then Plugins/Skeleton/Skeletonize (2D/3D) was performed to assess branching and mitochondrial volume. Regions of interest were selected in the mitochondrion-rich parts of the cytoplasm. For mitochondria network analysis, the objects were skeletonized first, which involved an intensity threshold, followed by thinning and then pruning of the objects. The results of mitochondrial skeleton analysis were used to count and measure branches and junctions of the mitochondrial network.

**Mitochondrial stress test**

To determine how KLF4 affected mitochondrial function of GBM cells, the OCR and extracellular acidification rate (ECAR) were analyzed using the Seahorse XF96 Analyzer and The Seahorse XF Cell Mito stress test kit (Agilent/Seahorse Bioscience, Santa Clara, CA). Protocols were optimized according to previously published papers (33). The day before assay, the sensor cartridge was hydrated according to the protocol provided by the manufacturer. Prior to seedling cells, Seahorse XF96 cell culture microplates were coated with poly-d-lysine (50 μg/ml, 100 μl) for 1 h to allow the cells to attach. U87 cells were plated at a density of 10,000 cells per well and incubated overnight in 80 μl of normal culture medium with or without Dox. The next day, cells were washed with warm PBS and incubated with fresh assay media. The cartridge was loaded with three metabolic inhibitors, which were sequentially added to the plate according to the settings of the assay program: oligomycin (an ATP synthase inhibitor, 2 μM), followed by FCCP (an uncoupler of mitochondrial oxidative phosphorylation, 0.375 μM), followed by the combination of rotenone (an inhibitor of mitochondria complex I, 1 μM) and antimycin A (an inhibitor of mitochondria complex III, 1 μM). The compounds were serially injected to measure basal OCR and ECAR, as well as the changes in OCR caused by addition of metabolic inhibitors. ATP content, maximal respiration, and nonmitochondrial respiration were also measured, respectively. Proton leak and spare respiratory capacity were then calculated using these parameters and basal respiration. Basal OCR and ECAR were measured, as well as the changes in OCR caused by the addition of the metabolic inhibitors described above. At the end of the assay, cells in the plate were stained with CyQuant (Thermo Fisher Scientific, Waltham, MA) to quantify DNA for measurement normalization.

**ROS measurement**

U87 cells were cultured with complete medium in a six-well plate at 37 °C and 5% CO₂. When reaching 70–90% confluency, cells were washed two times with PBS and treated with 2 μM H2DCFDA (Thermo Fisher Scientific) in PBS for 1 h (34). Cells were harvested by trypsinization, suspended in PBS at a density of 1 × 10⁶ cells/ml, and subjected to flow cytometry (BD Biosciences) for ROS detection using the 488-nm laser for excitation and detected at 535 nm.

**Reverse-transcriptase PCR and quantitative real-time PCR**

Total RNA was extracted using an RNeasy mini kit (Qiagen, Mansfield, MA). RNA (1 μg) was reverse-transcribed using the oligo(dT)₁₂–₁₈ primer and High-Capacity RNA transcriptase (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using SYBR Green PCR Mix (Applied Biosystems) and the IQ5 detection system (Bio-Rad) (35). Primer sequences are listed in Table S1. Relative gene expression was normalized to GAPDH.

**Immunoblotting**

Total cellular protein was extracted with radioimmunoprecipitation assay buffer (36) containing protease and phosphatase inhibitors (Millipore, Billerica, MA) and sonicated for 15 s; the suspensions were centrifuged at 3000 × g for 10 min. SDS-PAGE was performed with 30–60 μg of total proteins using 4–12% gradient Tris-glycine gels (LI-COR Biosciences, Lincoln, NE). Western blot analysis was performed using the quantitative Western blotting system, with secondary antibodies labeled by IRDye IR dyes (LI-COR Biosciences) (37). Anti-KLF4 was purchased from Santa Cruz Biotechnology, and anti-β-actin was purchased from Cell Signaling Technology (Danvers, MA).

**Glucose uptake and glucose oxidation**

The protocol for glucose uptake assay was optimized according to a previously published paper (38, 39). Briefly, U87 cells were plated at a density of 1 × 10⁴/cm² in 12-well plates; KLF4 was induced by treating the cells with Dox for 2 days. On the day of assay, cells were washed twice with warm PBS and incubated with 0.1% FCS medium for 1 h in a CO₂ incubator, followed by incubation with warm KRH buffer (0.5% BSA, 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 1.3 mM KH₂PO₄) for 20 min at 37 °C. 2-[³H]Deoxyglucose (0.1 μCi; Moravek Biochemicals, Brea, CA) was made in KRH buffer, and 0.5 ml of labeling solution was added to each well and incubated for 10 min in a 37 °C room. Ice-cold labeling solution was used for the control plate. The wells were washed twice with ice-cold PBS, replenished with 250 μl of 0.8% Triton X-100, and sonicated in 37 °C water bath for 15 min. The reaction buffer (150 μl) from each well, including control, was collected for radioactivity determination by liquid scintillation counting. The protein content of duplicate wells was quantified using the method of Lowry protein assay method (40) to normalize assay results.

For glucose oxidation assays, cells were incubated in T-25 flasks with 0.1 μCi/flask D-[U-¹⁴C]glucose (Moravek Biochem-
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ics) in serum-free DMEM containing 2.5 mM glucose, 2 mM glutamine, and 0.5 mM pyruvate. Flasks were capped with serum stoppers fitted with plastic center wells containing a filter paper wetted with 15 µl of 1 n NaOH and incubated for 4 h at 37 °C. The reaction was stopped by injecting 200 µl of 70% perchloric acid through the serum stopper into the culture medium, and 14CO2 was trapped by incubating the flasks at 55 °C for 1 h. Radioactivity on the filter paper was measured by liquid scintillation counting and normalized to total protein.

Lactate assay

Quantitation of lactate secreted into the culture medium was performed according to a previously published method (41). U87 cells were plated at a density of 1 × 10^4/cm² in 12-well plates for 48 h ± Dox. Cells were washed with PBS two times and continuously cultured in DMEM without phenol red for 6 h. A portion of the medium (80 µl) was collected from each well at 0, 1, 2, 4, and 6 h for lactate dehydrogenase assay. To determine lactate concentration, 80 µl of collected medium was mixed with 900 µl of assay buffer (1.875 g of glycine, 1.3 g of hydroxide sulfate, 0.05 g of EDTA, 4.3 ml of 6 n NaOH in a total of 25 ml of solution), and 10 µl of NAD+ (40 µg/ml). Lactic dehydrogenase (~100 units) was added to the mixture and incubated for 10 min at room temperature, and absorbance at 340 nm was measured. After the 6-h time point, cells were washed with PBS and harvested, and the protein concentration was measured for normalization.

ATP assay and G6PD assay

ATP amount in U87 cells was measured using CellTiter-Glo® 2.0 assay kit (Promega). Briefly, U87 cells were plated at a density of 1 × 10^4 cells/well in white-walled 96-well plates and cultured for 24 h in normal U87 medium ± Dox. Background luminescence was determined by control wells containing medium without cells. An equal volume of CellTiter-Glo® 2.0 reagent to the volume of cell culture medium was added to each well, and cells were lysed by mixing for 2 min. After incubation for 10 min at room temperature, luminescence was recorded using a luminometer (Molecular Probes). Proteins from an external well were quantified to normalize assay results.

G6PD activity was measured by the method described by Bergmeyer et al. (42). Briefly, U87 cells were plated at a density of 1 × 10^4/cm² in a 10-cm-diameter dish for 48 h ± Dox. Cells were harvested with trypsin, washed twice with PBS, and resuspended in STE buffer (0.25 M sucrose, 10 mM Tris, pH 7.5, and 1 mM Na2EDTA). Cells were lysed by addition of Tween 20 to a final concentration of 0.5%. Lysate cell sample (20 µl) was mixed with 175 µl of assay buffer containing (final concentrations) 50 mM triethanolamine, 5 mM EDTA (Na2) (in 1% NaHCO3), 0.5 mM MgCl2, and 0.5 mM NADP in a black-walled 96-well plate. Fluorescence was read at 340:420:450 nm (λ_ex/λ_cutoff/λ_em) for 3 min at 30-s interval as baseline. Glucose 6-phosphate (5 µl, 0.7 mM final concentration) was added to the mixture, and fluorescence was read at 340:420:450 nm for 20 min at 30-s intervals. Protein was determined by Lowry protein assay protocol (40) to normalize the data.

Nutrition deprivation (ND)

U87-KLF4 cells were plated at a density of 1 × 10^4/cm² in 6-well plates. The next day, cells were treated with Dox for 48 h to induce KLF4 expression. To start nutrition deprivation, normal culture medium was replaced with a buffered balanced salt solution (BSS) that contained (in mM): 3.1 KCl, 134 NaCl, 1.2 CaCl2, 1.2 MgSO4, 0.25 KH2PO4, 15.7 NaHCO3. The pH was adjusted to 7.2, and the solution was equilibrated with 5% CO2 at 37 °C. Osmolarity was verified at 290–310 mosm with a Wescor vapor pressure osmometer (16). KLF4 expression was sustained through Dox addition in BSS buffer. Cells were collected and analyzed after 24–72 h of starvation.

Mitotracker Red and JC-1 staining

MitoTracker Red staining was performed according to the manufacturer-provided protocol (Cell Signaling Technology). Briefly, cells were treated with 0.25% trypsin/EDTA to obtain single cells, resuspended into PBS, and incubated with 200 nM MitoTracker Red at 37 °C for 30 min. Cells were washed once by PBS and subjected to flow cytometer with 488 nm excitation and emission filters appropriate for Alexa Fluor® 488 dye.

For JC-1 staining, U87 cells were harvested with 0.25% trypsin/EDTA and resuspended in 1 ml of medium at ~1 × 10^6 cells/ml, followed by incubation with 10 µl of 200 µM JC-1 (2 µM final concentration) at 37 °C, 5% CO2, for 20 min. For the control tube, 1 µl of 50 mM FCCP was used to treat the cells at 37 °C for 5 min. Cells were washed once by PBS followed by flow cytometry analysis with 488 nm excitation and emission filters appropriate for Alexa Fluor® 488 dye and (R)-phycocerythrin.

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, La Jolla, CA). Post hoc tests included the Student’s t test and Tukey multiple comparison tests as appropriate. All experiments reported here represent at least three independent replications. All data were represented as mean value ± S.E.; significance was set at p < 0.05.

The raw data of our large-scale RNA-Seq have been deposited in NCBI Gene Expression Omnibus (GEO) under accession no. GSE97632. The methods to generate these data have been described in detail in Wan et al. (13).

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