Glucose starvation induces mitochondrial fragmentation depending on the dynamin GTPase Dnm1/Drp1 in fission yeast

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ABSTRACT
Mitochondria undergo morphological and dynamic changes in response to environmental stresses. Few studies have focused on addressing mitochondrial remodeling under stress. Using the fission yeast Schizosaccharomyces pombe as a model organism, here we investigated mitochondrial remodeling under glucose starvation. We employed live-cell microscopy to monitor mitochondrial morphology and dynamics of cells in perfusion chambers under glucose starvation. Our results revealed that mitochondria fragment within minutes after glucose starvation and that the dynamin GTPase Dnm1 is required for promoting the mitochondrial fragmentation. Moreover, we found that glucose starvation enhances Dnm1 localization to mitochondria and increases the frequency of mitochondrial fission, but decreases protein kinase A (PKA) activity. We further demonstrate that low PKA activity enhances glucose starvation-induced mitochondrial fragmentation, whereas high PKA activity confers resistance to glucose starvation-induced mitochondrial fragmentation. Moreover, we observed that AMP-activated protein kinase (AMPK) is not involved in regulating mitochondrial fragmentation under glucose starvation. Of note, Glucose starvation–induced mitochondrial fragmentation was associated with enhanced reactive oxygen species (ROS)
Mitochondrial fragmentation under stress

Carbon sources are fundamental for life. Generally, cells consume glucose to produce ATP through glycolysis in the cytoplasm and/or through oxidative phosphorylation in mitochondria. Mitochondria serve as not only the power plants of the cell but also a metabolic hub. Therefore, proper mitochondrial function is crucial for maintaining cellular energy hemostasis. Many studies have revealed that nutrient availability dictates mitochondrial morphology and dynamics in different types of cells, including budding yeast, pancreatic β cells, and mouse embryonic fibroblasts (1-5). However, it has remained controversial whether poor nutrient conditions lead to fragmented mitochondria because seemingly opposite conclusions have been made in different studies (1,3-5). For example, on the one hand, excessive glucose, along with high fat, was reported to cause mitochondrial fragmentation in β cells (4); on the other hand, glucose depletion was reported to enhance mitochondrial fragmentation in mouse embryonic fibroblasts (5). Therefore, the response of mitochondria to glucose availability is still unclear.

Mitochondria are dynamic organelles undergoing constant fusion and fission within the cell. The conserved dynamin GTPase Drp1 (Dnm1 in yeasts) is responsible for mitochondrial fission and is recruited to mitochondria by the mitochondrial adaptor proteins Fis1, MFF, MiD49, and MiD51 (6,7), while Mitofusins (MFN1/2) and the dynamin-family GTPase Opa1 are responsible for mitochondrial fusion (7,8). The balance between mitochondrial fusion and fission gives rise to specific morphological patterns of mitochondria in a cell-type dependent manner. Generally, enhanced mitochondrial fission and/or inhibited mitochondrial fusion leads to mitochondrial fragmentation, while the opposite causes elongated mitochondria. Despite some progress that has been made to establish the connection between mitochondrial dynamics and glucose availability (4,5), how mitochondria respond to the changes of glucose availability is unclear.

The fission yeast Schizosaccharomyces pombe is an excellent model organism for studying mitochondrial dynamics because of the relatively simple organization of the mitochondria network and the availability of many genetic tools (9). In addition, most of the proteins regulating mitochondrial dynamics are conserved through evolution. Moreover, fission yeast has been developed as a model organism to characterize glucose metabolism (10), and glucose concentration at 4.4-5.5 mM has been identified as the critical concentration for promoting cell growth (11). These findings make it convenient to delineate how mitochondria respond to the availability of glucose.

In this present work, we employed live-cell microscopy to monitor mitochondrial morphology in response to glucose starvation. Our work demonstrated that mitochondrial fragmentation takes place within minutes in a Dnm1-dependent manner after glucose starvation. We further showed that PKA activity, but not AMPK, is involved in regulating the glucose starvation-induced mitochondrial fragmentation. Therefore, this work provides mechanistic insights into understanding mitochondrial fragmentation that is triggered by glucose starvation.

**Results**

**Glucose starvation leads to mitochondrial fragmentation**

To examine the effect of glucose starvation on mitochondria, we employed live-cell microscopy
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To monitor mitochondria (marked by Cox4-GFP, a mitochondrial matrix protein). Fission yeast cells were first grown in the defined medium EMM (Edinburgh Minimal Medium) containing 2% glucose (111.1 mM) and the cells at exponential phase were then attached to the coverslip of perfusion chambers for imaging. Images were acquired upon replacement of the EMM medium containing 111.1 mM glucose (referred to as glucose-rich EMM medium) with glucose-free EMM medium. As shown in Fig. 1A, 2~3 tubular mitochondrial structures were seen in cells grown in the glucose-rich EMM medium (i.e. 0 minute) while mitochondrial fragmentation became noticeable after 10 minutes of growing the cells in the glucose-free EMM medium. The mitochondrial fragmentation was enhanced over time and was able to be reversed by glucose replenishment within ~10 minutes. This result suggests that glucose starvation induces mitochondrial fragmentation within minutes in fission yeast cells.

To be unbiased in analysis of imaging data, we took an automated method to quantify mitochondria number by using the algorithm MiNA developed previously (12). Briefly, maximum projection images of mitochondria were first binarized and then skeletonized for quantification of mitochondria number (Fig. 1B). The quantification results confirmed that mitochondria number increased gradually after glucose starvation and restored after glucose replenishment (Fig. 1C). It has been shown that fission yeast cells in media containing $\geq$ 4.4 mM glucose grow equally fast as in media containing 111.1mM glucose but divide stochastically in length in the medium containing 1.1mM glucose (10). We then asked whether the identified critical concentration of glucose (i.e. 4.4 mM) is also a critical factor in determining mitochondrial morphology. To address this question, we examined mitochondria in cells in EMM media containing 111.1, 11.1, 4.4, 1.1 and 0 mM glucose, respectively. As shown in Fig. 1D and 1E, mitochondrial fragmentation was not detected in cells cultured in EMM media containing 111.1, 11.1, and 4.4 mM glucose but became apparent in cells cultured in EMM media containing 1.1 and 0 mM glucose. These results suggested that 4.4 mM is a critical concentration of glucose in maintaining tubular mitochondrial structures.

Glucose starvation increases the frequency of mitochondrial fission, but not mitochondrial fusion

Glucose starvation-induced mitochondrial fragmentation could be caused by enhanced mitochondrial fission and/or inhibited mitochondrial fusion. To test this hypothesis, we quantified mitochondrial fusion and fission events in each cell within the first 20 minutes after the replacement of the glucose-rich EMM medium (111.1mM glucose) with the glucose-free EMM medium. Intriguingly, mitochondria in cells growing in the glucose-free EMM medium became more dynamic and underwent more frequent fission than mitochondria in cells growing in the glucose-rich EMM medium (Fig. 2A). Quantification confirmed that glucose starvation causes a 2-fold increase of the frequency of mitochondrial fission but does not appear to affect mitochondrial fusion (Fig. 2B and 2C). Hence, we concluded that glucose starvation-induced mitochondrial fragmentation is due to enhanced mitochondrial fission.

Dnm1 is required for mediating mitochondrial fragmentation upon glucose starvation

The dynamin-related GTPase Dnm1 (Drp1 in...
mammalian cells) is the master regulator of mitochondrial fission and is recruited to mitochondria by Fis1 via Caf4 (13,14). Therefore, we further asked whether and how Dnm1 contributes to glucose starvation-induced mitochondrial fragmentation. Similarly, we employed live-cell microscopy to monitor mitochondrial morphology in dnm1Δ cells upon the replacement of the glucose-rich EMM medium with the glucose-free EMM medium. In the glucose-rich EMM medium, mitochondria in dnm1Δ cells displayed an intact interconnected structure due to the impairment of mitochondrial fission (Fig. 3A, 0 min). Throughout the period of glucose starvation, mitochondria in dnm1Δ cells did not appear to fragment but shrank over time (Fig. 3A). Intriguingly, many furrows appeared along the shrinking mitochondria, indicative of incomplete mitochondrial constriction in the absence of Dnm1 during glucose starvation (Fig. 3A). Further, quantification confirmed that mitochondria number remained largely unchanged during glucose starvation (Fig. 3B). These results suggested that Dnm1 is required for promoting mitochondrial fragmentation during glucose starvation.

Next, we examined the localization of Dnm1 to mitochondria. Fluorescently tagging Dnm1 either at the N terminus or at the C terminus appeared to affect its function since cells expressing fluorescently tagged Dnm1 displayed defective mitochondrial fission, a phenotype similar to the one caused by the absence of Dnm1 (See Fig. S1). To overcome this issue, we kept the endogenous Dnm1 and ectopically expressed Dnm1-TagRFP from an ase1 promoter. Since Dnm1 functions as oligomers to constrict mitochondria (15,16), the presence of both the endogenous Dnm1 and Dnm1-TagRFP in Dnm1 oligomers may enable the complex to be functional and meanwhile be visible. Indeed, mitochondria in cells expressing endogenous Dnm1 and Dnm1-TagRFP underwent fission and fusion, indicative of proper function of Dnm1 oligomers. By using this co-expression strategy and live-cell microscopy, we monitored the localization of Dnm1 during glucose starvation. As shown in Fig. 3C, multiple Dnm1 foci were present on mitochondria before glucose starvation and the signals of the Dnm1 foci became much brighter after glucose starvation. To ascertain the observation, we quantified the average Dnm1 intensity on mitochondria (i.e. dividing the integrated intensity of Dnm1 on mitochondria by mitochondria area). Consistently, most of the cells displayed stronger average intensity of Dnm1 on mitochondria after glucose starvation (Fig. 3D).

Taken together, these results suggest that glucose starvation likely enhances the localization of Dnm1 to mitochondria for promoting mitochondrial fragmentation.

Protein kinase A is involved in regulating mitochondrial fragmentation under glucose starvation

Protein kinase A (PKA) regulates the activity of Drp1 (the ortholog of Dnm1 in mammalian cells) by phosphorylation in response to stresses (Cribbs and Strack, 2007; Mishra and Chan, 2016). We then asked whether the fission yeast PKA is also involved in regulating glucose starvation-induced mitochondrial fragmentation.

It has been established that a glucose-sensing PKA pathway is present in fission yeast to regulate glucose metabolism in response to the altered availability of extracellular glucose (17-19). In such pathway, the adenylate cyclase Cyr1 mediates production of the second messenger cAMP, and the association of cAMP with the inhibitory subunit of PKA (i.e. Cgs1) subsequently separates Cgs1 from the catalytic subunit of PKA (i.e. Pka1), allowing activation of Pka1 (20-22). By using the β-galactosidase assay as reported previously (17,23,24), we first confirmed that the absence of
Cyr1 reduces Pka1 activity whereas the absence of Cgs1 elevates Pka1 activity (Fig. 4C). We then assessed the effects of altered Pka1 activity on glucose starvation-induced mitochondrial fragmentation by live-cell microscopy. As shown in Fig. 4A and 4B, mitochondria appeared to be resistant to fragmentation in cgs1Δ cells (high Pka1 activity) whereas mitochondria appeared to be prone to fragmentation in cyr1Δ cells (low Pka1 activity). These results established a connection between Pka1 activity and glucose starvation-induced mitochondrial fragmentation. It is likely that low Pka1 activity promotes mitochondrial fragmentation. Indeed, β-Galactosidase assays showed that Pka1 activity decreased significantly after glucose starvation (Fig. 4D). In mammalian cells, PKA phosphorylates Drp1 at Ser637 to prevent Drp1 from localizing to mitochondria, inhibiting mitochondrial fission (3,5,25-27). Interestingly, Ser637 is not conserved in Dnm1, the fission yeast counterpart of Drp1. Nevertheless, multiple PKA phosphorylation sites (i.e. Thr75, Ser203, Ser398, Ser643, and Ser768) were predicted to be present in Dnm1 by using the bioinformatics tool GPS (Group-based prediction system) (28). Therefore, it is possible that the decreased Pka1 activity caused by glucose starvation enhances the localization of Dnm1 to mitochondrial through phospho-regulation of Dnm1 for promoting mitochondrial fission (Fig. 3C and 3D). Consistently, we found that even in the glucose-rich EMM medium, cyr1Δ cells (i.e. low Pka1 activity) showed fragmented mitochondria structures, whereas similar to WT cells, cgs1Δ cells displayed tubular mitochondrial structures (Fig. 4E and 4F). Taken together, we concluded that PKA signaling plays a critical role in regulating mitochondrial dynamics under glucose starvation.

AMPK is not involved in regulating mitochondrial fragmentation under glucose starvation

The adenosine monophosphate (AMP)–activated protein kinase (AMPK) is another important metabolic sensor, which is activated when the intracellular ATP level decreases. It has been reported that AMPK promotes mitochondrial fission by phosphorylating Mff, the mitochondrial receptor of Drp1 (29). AMPK comprises three subunits: the regulatory subunits Amk2 and Cbs2 and the catalytic subunit Ssp2 (30).

To assess if AMPK is also involved in regulating mitochondrial fragmentation under glucose starvation, we performed time-lapse imaging to examine mitochondrial morphology in WT, amk2Δ, cbs2Δ and ssp2Δ cells after glucose starvation, respectively. As shown in Fig. 5A, the changes of mitochondrial morphology were similar within 40 min of glucose starvation in the three mutant and WT cells (Fig. 5A and 5B). Indeed, no noticeable change of mitochondrial morphology and altered mitochondria number were found in the three mutant cells cultured in the glucose-rich EMM medium (Fig. 5C and 5D). Therefore, it is unlikely that AMPK is involved in regulating mitochondrial morphology under glucose starvation, at least in fission yeast.

Glucose starvation increases intracellular ROS levels

To appreciate the biological significance of mitochondrial fragmentation caused by glucose starvation, we assessed ROS (reactive oxygen species) levels within the cell with DCDHF-DA, a ROS indicator (31). Of interest, ~50% WT cells displayed DCDHF-DA staining after 1 hour of glucose starvation, whereas few WT cells were stained by DCDHF-DA in the glucose-rich medium (Fig. 6A and 6B), suggesting that ROS production is significantly enhanced by glucose starvation. This enhanced ROS production may be due to mitochondrial fragmentation induced by glucose starvation. To test this idea further, we also stained...
dnm1Δ cells, in which glucose starvation-induced mitochondrial fragmentation was inhibited (Fig. 3A and 3B), cultured in the glucose-rich and glucose-free media with DCDHF-DA. As shown in Figs 6A and 6B, ROS production under glucose starvation was reduced, but not abolished, in the absence of Dnm1 since only ~25% of dnm1Δ cells were DCDHF-DA positive after glucose starvation. Because mitochondria are the main source of ROS production through the respiratory chain (32), it is likely that glucose starvation enhances oxidative phosphorylation by promoting mitochondrial fragmentation. The DCDHF-DA staining result for dnm1Δ cells also suggests the existence of a Dnm1/fission-independent mechanism underlying ROS production induced by glucose starvation.

Discussion

Mitochondria undergo morphological and dynamic changes in response to environmental stresses and the molecular mechanisms underlying such mitochondrial changes have begun to be revealed (25). However, few studies focused on addressing the rapid changes of mitochondrial morphology and dynamics under stress and most studies instead focused on the ultimate effects of stresses on mitochondrial morphology. Here, we employed live-cell microscopy to examine the morphological and dynamic changes of mitochondria under glucose starvation in profusion chambers (Fig. 1 and 2). Our work reveals that mitochondria fragment in a Dnm1-dependent manner within minutes after the removal of glucose (Fig. 1 and 3). We further demonstrate that PKA, but not AMPK, is involved in regulating the glucose starvation-induced mitochondrial fragmentation (Fig. 4 and 5) and that the glucose starvation-induced mitochondrial fragmentation is associated with elevated ROS production (Fig. 6).

Mitochondria respond to environmental stresses by changing their shape and dynamics. Interestingly, many studies have shown that mitochondria form interconnected and elongated structures under stress, indicative of attenuated fission and/or enhanced fusion (3,5,33,34). Similarly, in budding yeast, mitochondria become more complex with the tubular structure forming many branches in cells grown in medium containing the non-fermentable carbon source glycerol (2). Mitochondria are sensitive organelles. Therefore, culture media containing complex ingredients and culture conditions could have profound effects on mitochondrial morphology and dynamics, which may cause complications in data interpretation. To determine the response of mitochondria to glucose starvation, we cultured fission yeast cells in Edinburgh Minimal Media (i.e. EMM) containing glucose at the indicated concentration and imaged cells, which were at the exponential phase, at room temperature in a profusion chamber. Under such carefully controlled conditions, our experiments revealed that fission yeast mitochondria respond to glucose starvation by increasing the fission frequency, leading to rapid fragmentation (within minutes) (Fig. 1A, 1C, and 2). The consequences are reversible as glucose replenishment restores normal tubular mitochondrial morphology within minutes (Fig. 1A and 1C). This underscores the robustness of the mechanisms orchestrating glucose sensing and mitochondrial remodeling.

The concentration of glucose in cultured media is a critical factor in dictating the response of mitochondria. We found that glucose at a concentration <4.4 mM, which is close to the blood glucose concentration in human before breakfast, is required for promoting mitochondrial fragmentation (Fig. 1D and 1E). Such concentration is the threshold concentration of glucose required for normal cell division of fission yeast cells as shown previously (10,11,35). It is therefore conceivable that glucose starvation-induced
mitochondrial fragmentation has a detrimental effect on cell growth. In support of this hypothesis, we found that ROS production is greatly enhanced after glucose starvation (Fig. 6). Whether the critical glucose concentration (i.e. 4.4 mM) in human cells also promotes mitochondrial remodeling and affects cell proliferation awaits further study.

Several kinases have been reported to be involved in regulating mitochondria remodeling in response to stresses (19,25,29,36). Among the kinases, PKA phosphorylates Drp1 at Ser637 to sequester Drp1 in the cytoplasm in order to inhibit mitochondrial fission (25,37). Our work showed that PKA, but not AMPK, is involved in regulating mitochondrial fragmentation under glucose starvation (Fig. 4 and 5). First, glucose starvation significantly decreases Pka1 activity (Fig. 4D). Second, consecutive inactivation of Pka1 in cyr1Δ cells enhances mitochondrial fragmentation under glucose starvation and causes mitochondrial fragmentation even in a glucose-rich condition whereas consecutive activation of Pka1 in cgs1Δ cells confers resistance to mitochondrial fragmentation under glucose starvation (Fig. 4A-E). Dnm1 is required for promoting mitochondrial fragmentation under glucose starvation (Fig. 3A and 3B). Moreover, the localization of Dnm1 to mitochondria is enhanced by glucose starvation (Fig. 3C and 3D), correlating well with the decreased Pka1 activity under glucose starvation (Fig. 4D), and Drp1/Dnm1 is a substrate of PKA as mentioned in the result section. These findings suggest a possible model that glucose starvation enhances the mitochondrial localization of Dnm1 by inhibiting the activity of Pka1. Nonetheless, it is worthwhile to test if Dnm1, like its human orthologue Drp1, is a substrate of Pka1 by biochemical approaches.

Four mitochondrial receptor proteins are present in human cells: Fis1, Mff, MiD49, and MiD51 (6,7). MFF, MiD49, and MiD51, but not Fis1, play major roles in recruiting Drp1 to mitochondria. By contrast, Fis1 is the only reported receptor protein for recruiting Dnm1 to mitochondria membrane in yeasts. It is established that AMPK phosphorylates Mff to promote mitochondrial fission by recruitment of Drp1 to mitochondria (29). This present work shows that the absence of the three subunits of AMPK (i.e. Amk2, Cbs2, and Ssp2) does not appear to affect mitochondrial fragmentation under glucose starvation and in a glucose-rich growth condition (Fig. 5). We interpreted the ineffectiveness of the AMPK mutants on glucose starvation-induced mitochondrial fragmentation as the lack of a counterpart of Mff in fission yeast.

In conclusion, our work demonstrates that mitochondria respond to glucose starvation by rapid fragmentation and that glucose starvation-induced mitochondrial fragmentation depends on Dnm1 and requires proper regulation of PKA activity.

Experimental procedures

Plasmids and yeast strains

Plasmids were created with restrictive enzymes purchased from NEB (New England Biolabs, MA, USA). Yeast strains were created either by random spore digestion or tetra-dissection analysis, as described before (38). Gene deletion was achieved using the PCR-based homologous recombination method (39). All strains used for glucose starvation are prototrophic to Adenine, Leucine, uracil, Lysine and Histidine (the 5 supplements generally used in EMM media). Therefore, Edinburgh minimal medium without the 5 supplements were used in this study. The plasmids and strains used in this study are listed in the supplemental tables 1 and 2, respectively.

β-Galactosidase activity assays

Strains containing the fbp1-LacZ plasmid
were cultured in EMM medium containing 111.1 mM glucose at 30°C overnight until the cells reached the exponential phase. For normal conditions, the exponential cells were collected for measuring β-Galactosidase activity using the Yeast β-Galactosidase Assay Kit (Thermo Scientific, MA, USA) in a 96-well plate as previously described (40). For the starvation conditions, the exponential cells were washed for three times with the glucose-free medium and then cultured again in the glucose-free medium at 30°C for the indicated time. Cells collected at the indicated time points were kept in liquid N₂ and used later for simultaneous measurements of β-Galactosidase activity with the Yeast β-Galactosidase Assay Kit.

**ROS measurement**

ROS assays were performed as described previously (31). Briefly, the starved and control cells were stained with the ROS indicator dye 2',7'-Dichlorodihydrofluorescein diacetate (DCDHF-DA; Molecular Probes, Eugene, OR) at a working concentration of 10 µg/ml by incubation of the cells in dark at 30°C for 80 min. The stained cells were then collected by centrifugation and were washed twice with icy PBS buffer (pH 7.0), followed by microscopy analysis.

**Imaging and data analysis**

Time-lapse imaging in the study was performed in profusion chambers. The profusion chambers were prepared with double-sticky tape and coverslips that were coated with 5 mg/ml poly-L-Lysine, as described previously (41). Profusion chambers were washed with 40 µl EMM medium containing 111.1 mM glucose before cells were injected. After injection of cells that were grown in the EMM medium containing 111.1 mM glucose, profusion chambers were placed upside down in a 30°C incubator for 15 min to allow attachment of the cells onto coverslips. Unattached cells were washed away with 40 µl EMM medium containing 111.1mM glucose. Images were acquired immediately after replacement of the glucose-rich EMM medium with 80 µl glucose-free EMM medium. Regular imaging was performed on EMM agarose pad slides, as described previously (42).

All imaging data were collected with a PerkinElmer UltraVIEW Vox spinning-disk microscope equipped with a Hamamatsu C9100-23B EMCCD camera and a CFI Apochromat TIRF 100x objective (NA=1.49). For time-lapse imaging, stack images containing 11 planes (0.5µm/5µm) were acquired every 30 seconds. For maximum projection images, stack images containing 11 planes (0.5 µm/5µm) were acquired. All imaging experiments were performed at room temperature. Data was analyzed with MetaMorph 7.7 (Molecular Devices, Sunnyvale, CA) and Fiji Image J (National Institutes of Health, Bethesda, MD). Mitochondria number analysis was conducted with the algorithm MiNA (12). Graphs and plots were generated using KaleidaGraph 4.5 (Synergy Software, Reading, PA). Statistical analysis was performed with Microsoft Excel and KaleidaGraph 4.5.
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FOOTNOTES
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Abbreviations: AMPK, The adenosine monophosphate (AMP)–activated protein kinase; PKA, Protein kinase A; EMM, Edinburgh Minimal Media; ROS, reactive oxygen species
Figure 1. Mitochondria number increases during glucose starvation. (A) Maximum projection time-lapse images of WT cells expressing GFP-Cox4. Cells inoculated in the EMM medium containing 111.1 mM glucose (without supplements) were attached to the coverslip coated with 5 mg/ml Poly-L-lysine in a profusion chamber. Cells were then washed one time with the EMM medium containing glucose at the indicated concentration before medium replacement. Time-lapse imaging was performed to monitor mitochondrial dynamics. Note that mitochondria number increased upon glucose starvation and decrease after the replenishment of glucose. DIC, differential interference contrast images. Scale bar, 5 µm. (B) Diagram illustrating the quantification of mitochondria number using the algorithm MiNA in ImageJ. Red
arrows indicate two tubular mitochondria. (C) Plot of mitochondria number against time. Time-lapse movies as indicated in (A) were used for analysis, and 22 cells at each time point were analyzed. Data represents mean ± SD. Statistical analysis was performed by one-way repeated measurements ANOVA (P<0.001), followed by the Tukey HSD (honest significance test) test (* P<0.01, ** P<0.001, vs. 0-min group). (D) Maximum projection time-lapse images of WT cells expressing GFP-Cox4. Cells grown in EMM containing glucose at the indicated concentration in profusion chambers were imaged. Note that mitochondria number increased significantly when cells were cultured in the EMM medium containing glucose at 0 mM and 1.1 mM, but not at 4.4 mM, 11.1 mM and 111.1 mM. Scale bar, 5 µm. (E) Plots of mitochondria number against time. Time-lapse movies as indicated in (D) were used for analysis, and 18 cells at each time point were analyzed. Data represents mean ± SD. Statistical analysis was performed by two-way repeated measurements ANOVA (** P<0.001, vs. 0 mM; n.s. indicates not significant).
Figure 2. Glucose starvation enhances mitochondrial fission. (A) Maximum projection time-lapse images of WT cells expressing GFP-Cox4. Cells were cultured in the EMM medium containing 111.1 mM and 0 mM glucose, respectively. Green and red arrows indicate mitochondrial fusion and fission, respectively. Shown on the left are time-lapse images at 5-min intervals. Time-lapse images on the right are the images between the time points 10 min and 15 min (red dashed rectangle). Scale bar, 10 μm. (B and C) Dot plots of mitochondrial fission (B) and fusion (C) frequencies. Time-lapse images as indicated in (A) were used for analysis. P values were calculated by student’s t-test, and n indicates cell number.
Figure 3. Glucose starvation-induced mitochondrial fragmentation depends on Dnm1. (A) Maximum projection time-lapse images of *dnm1Δ* cells expressing GFP-Cox4. The *dnm1Δ* cells were cultured in the EMM medium lacking glucose in perfusion chambers. One cell (highlighted by the dashed rectangle in the DIC image) was shown at the bottom panel at 2-min intervals. Note that mitochondria in *dnm1Δ* cells resisted fragmentation induced by glucose starvation but incomplete mitochondrial constriction took place in *dnm1Δ* cells after prolonged glucose starvation. Scale bar, 10 µm. (B) Plot of mitochondria number against time. Time-lapse images as indicated in (A) were used for quantification, and 18 cells were analyzed at each time point for each group. Data represents mean ± SD. For comparison, the same WT plot (0 mM) in Figure 1E was used, and statistical analysis was performed by two-way repeated measurements ANOVA (*P*<0.001). (C) Maximum projection images of WT cells expressing GFP-Cox4 and Dnm1-TagRFP. WT cells, initially cultured in EMM medium containing 111.1 mM glucose, were washed with and then cultured in the glucose free EMM medium, followed by imaging at the indicated time. Note that Dnm1-TagRFP signals within the cells increased after glucose starvation. Scale bar, 10 µm. (D) Dot plots of the average...
pixel intensity of Dnm1-TagRFP that localized along mitochondria at the indicated time points. The $P$ values (* $P<0.01$, vs. 0 min) were calculated by student’s t-test, and cell number was indicated.
Figure 4. Protein kinase A (PKA) is involved in regulating glucose starvation-induced mitochondrial fragmentation. (A) Maximum projection time-lapse images of WT, cgs1Δ and cyr1Δ cells expressing GFP-Cox4. Cells initially inoculated in EMM medium containing 111.1mM glucose were washed with the
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glucose free EMM medium, and then live-cell imaging was performed to monitor mitochondrial dynamics of the cells in the glucose-free EMM medium. Note that mitochondria became fragmented rapidly after glucose starvation in cyr1Δ cells whereas mitochondria number increased more slowly in cgs1Δ cells after glucose starvation. Scale bar, 10 µm. (B) Plots of mitochondria number against time. Time-lapse images as indicated in (A) were used for quantification, and 18 cells were analyzed at each time point for each group. Data represents mean ± SD. For comparison, the same WT plot (0 mM) in Figure 1E was used, and statistical analysis was performed by two-way repeated measurements ANOVA (* P=0.011, ** P<0.001, vs. WT). (C) β-galactosidase activity of WT, cgs1Δ and cyr1Δ cells. Note that the β-galactosidase activity of the mutant cells was normalized to the β-galactosidase activity of WT cells (displayed as fold change). Five independent experiments were performed. Error bars represent confidence intervals. Single-group student t-test was used to calculate the P values of log fold change for each group. (D) β-galactosidase activity of WT cells starved in the glucose-free EMM medium for the indicated time. Three independent experiments were performed. Note that the β-galactosidase activity was normalized to the β-galactosidase activity of the cells measured at 0 min (displayed as fold change). Error bars are confidence intervals. Single-group student t-test was used to calculate the P values of log fold change for each group. (E) Maximum projection images of WT, cgs1Δ and cyr1Δ cells expressing GFP-Cox4. Cells were cultured in the EMM medium containing 111.1 mM glucose. Scale bar, 10 µm. Magnified images (1.9X) for the cells indicated by red dashed lines are shown on the right, and the white scale bars indicate 5 µm. (F) Quantification of mitochondria number in the indicated cells. The P values were determined by student’s t-test, and n.s. indicates not significant. Cell number analyzed is indicated on the top of the graph.
Figure 5. AMPK is not involved in regulating glucose starvation-induced mitochondrial fragmentation. (A) Maximum projection time-lapse images of WT, amk2Δ, cbs2Δ and ssp2Δ cells expressing GFP-Cox4. The cells, initially inoculated in the EMM medium containing 111.1mM glucose, were washed with the glucose-free EMM medium, and time-lapse imaging was performed to monitor mitochondrial dynamics for the cells in the glucose-free EMM medium. Scale bar, 10 µm. (B) Plots of mitochondria number against time. Time-lapse images as shown in (A) were used for quantification, and 18 cells were analyzed at each time point for each group. Data represent mean ± SD. Statistical analysis was performed by two-way repeated measurements ANOVA (n.s. indicates not significant, vs. WT). (C)
Maximum projection images of WT, amk2Δ, cbs2Δ and ssp2Δ cells expressing GFP-Cox4. Cells were cultured in EMM medium containing 111.1mM glucose medium. Scale bar, 10 µm. (D) Quantification of mitochondria number in the indicated cells. The $P$ values (n.s., not significant) were determined by student’s t-test. Cell number analyzed is indicated on the top of the graph.
Figure 6. Glucose starvation elevates ROS levels. (A) Maximum projection images of WT and dnm1Δ cells stained with DCDHF-DA (a ROS indicator). Cells were incubated with DCDHF-D or DMSO (control, the solvent of DCDHF-D). Scale bar, 10 µm. (B) Quantification of the percentage of DCDHF-D stained cells in (A). Three independent experiments were performed and error bars represent SD. Two-way repeated measurements ANOVA was performed to determine the $P$ values between WT and dnm1Δ groups.
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