A hyperfused mitochondrial state achieved at G₁–S regulates cyclin E buildup and entry into S phase

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Mitochondria undergo fission–fusion events that render these organelles highly dynamic in cells. We report a relationship between mitochondrial form and cell cycle control at the G₁–S boundary. Mitochondria convert from isolated, fragmented elements into a hyperfused, giant network at G₁–S transition. The network is electrically continuous and has greater ATP output than mitochondria at any other cell cycle stage. Depolarizing mitochondria at early G₁ to prevent these changes causes cell cycle progression into S phase to be blocked. Inducing mitochondrial hyperfusion by acute inhibition of dynamin-related protein-1 (DRP1) causes quiescent cells maintained without growth factors to begin replicating their DNA and coincides with buildup of cyclin E, the cyclin responsible for G₁-to-S phase blocks. The network is electrophysiologically continuous and has greater ATP output than mitochondria at any cell cycle stage. These cell cycle stages include a long growth phase (G₁), a DNA replicating phase (S), a short growth phase (G₂), and cell division (mitosis, M). Progression through these stages is controlled by cyclins and their partners, such as cyclin E, which regulates cyclin E buildup and entry into S phase (Fig. 1A and Movie S1). In mitosis, hundreds of fragmented mitochondria distribute throughout the cytoplasm. In G₀, both filamentous and fragmented mitochondria occur. In G₁–S, most surprisingly, mitochondria form a giant tubular network (Fig. 1A, G₁–S) with tubular elements undergoing fission and fusion (Movie S2). Similar cell cycle mitochondrial phenotypes are seen in synchronized cells released from G₀ by relief from serum starvation (Fig. 1B and Fig. S1), with fragmented/intermediate phenotypes in G₁ shifting to tubular in G₁–S, and back to fragmented/intermediate in S and G₂–M. The specific mitochondrial phenotypes are also seen in unsynchronized cells progressing through the cell cycle (Fig. 1C, Movie S3 and SI Methods for cell cycle marker description), including within a single cell passing through G₁–S (Fig. S2).

Morphometric analysis of mitochondrial volume, performed by outlining the volume of individual mitochondrial elements across a 3D stack of single optical slices, revealed mitochondrial volumes in cells in G₁, G₂, S, G₀, and mitosis distribute among many small elements (synchronization scheme in Fig. S3). By contrast, mitochondrial volume in cells at G₁–S primarily associate with a single element (Fig. 1D and E), similar to cells expressing a mutant (K38A) dynamin-related protein, DRP1m (also Movie S4), which prevents mitochondrial fission activity mediated by dynamin-related protein-1 (DRP1) (10). Mitochondria thus exhibit stage-specific cell cycle phenotypes, with mitochondria at G₁–S unique in being primarily a single tubular element.

Characteristics of Mitochondria at G₁–S. A single, tubular mitochondrial element has not been considered a normal mitochondrial phenotype because it is rarely seen within cells (11). Its specific occurrence at the narrow window of G₁–S transition, however, suggested it might serve particular cell cycle functions. So, we took a number of approaches to identify its specific characteristics.

We first investigated whether the tubular mitochondrial system at G₁–S has a continuous matrix. A rectangular box across the mitochondrial network of a mito-NRK cell in G₁–S or other stages/conditions was photobleached, and the kinetics of recovery of the expressed RFP-matrix molecule was measured (Fig. 2A and B). Fluorescence recovers within 2 min to >60% of the prebleached level from adjacent mitochondrial areas in G₁–S cells and cells expressing DRP1m. Virtually no recovery occurs in proliferating or mitotic cells. Mitochondria at G₁–S thus have their outer and inner membranes fused sufficiently to give rise to a continuous matrix to...
permit the matrix markers to diffuse freely between mitochondrial elements.

We next investigated whether the continuous mitochondrial system at G1–S has electrically continuous inner membranes. Mitochondria were loaded with tetramethylrhodamine ethylamine (TMRE), which incorporates specifically into mitochondrial membranes because of their transmembrane potential (12). A small region of interest (1 x 1 μm) of the TMRE-loaded mitochondria was irradiated by using a 2-photon laser. The cell was then monitored for TMRE loss from mitochondrial branches directly connected to the point of irradiation, indicative of spread of depolarization (13). In cells arrested at G1–S, depolarization at the irradiated point (Fig. 3A, arrow in preirradiation image) causes immediate TMRE loss in many mitochondrial elements throughout the cell (Fig. 3A, arrowheads in postirradiation image), indicating that these elements are electrically continuous. As mitochondrial elements undergo fusion and fission, the depolarization spreads to most remaining mitochondrial elements (Movie S5). Measuring the amount of TMRE loss in the outlined box over time reveals the extent of TMRE loss after irradiation (Fig. 3B). On microirradiation in cells whose mitochondria are irreversibly fused together through expression of DRP1m, a similar, and even faster, spread of mitochondrial depolarization is observed (Fig. 3A and B). By contrast, mitochondrial membrane depolarization is restricted to a small zone around the irradiated point in proliferating, mitotic, and G0 cells (Fig. 3A and B). Thus, only mitochondria in cells at G1–S or in cells expressing DRP1m show rapid spread of membrane depolarization.

We further examined mitochondrial membrane potential (assessed by TMRE uptake) per unit of mitochondrial mass (determined by using MitoTracker Green) at different cell cycle stages (Fig. 3C). Normalization of TMRE signal with MitoTracker Green in this assay is essential because mitochondrial mass increases during the cell cycle. A predictable decrease in mitochondrial potential after membrane depolarization by the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (14) is observed. Notably, when mitochondrial potential is monitored in cells at different cell cycle stages, the membrane potential is greatest at G1–S.

Oxygen consumption by mitochondria is reported to increase from early to late G1 (3). To test whether there is also a change in mitochondrial ATP production at G1–S, we measured levels of ATP at different cell cycle stages. Total ATP increases at G1–S (Fig. S4A), with mitochondrial ATP output (quantified as the fraction of total cellular ATP sensitive to oligomycin) significantly higher at G1–S compared with other stages of the cell cycle (Fig. 3D).

Mitochondrial Depolarization Specifically Blocks G1-to-S Cell Cycle Progression in a p53-Dependent Manner. Previous work in flies has shown that reduction of cellular ATP caused by a mutation in a mitochondrial electron transport chain component triggers the p53-dependent G1–S checkpoint (4). Therefore, we investigated whether depolarizing mitochondria by FCCP treatment, which prevents the G1–S-associated increased mitochondrial ATP output, will trigger the p53-dependent G1–S checkpoint. Cells released from G0 without treatment show BrdU incorporation and Aurora B expression (Fig. 4A and Fig. S5), indicative of S-phase entry. FCCP-treated cells, however, neither incorporate BrdU nor express...
Aurora B in the nucleus, similar to cells in G0 (Fig. 4A). When cells in late G1 are treated with FCCP, proliferating cell nuclear antigen (PCNA) foci (indicative of S-phase entry) do not form, even after 10 h, unlike that in untreated cells, which form PCNA foci within 4 h (Fig. 4B). FACS analysis of DNA content in the FCCP-treated cells reveal cell enrichment of the G1 population, indicative of a G1–S block (Fig. S6A). Cells treated with FCCP on exit from mitosis, by contrast, continue further into G1 (Fig. 4C), and cells in S phase treated with FCCP still progress through this phase (albeit more slowly than control cells) (Fig. S6B and C). This suggests that reducing mitochondrial potential by FCCP treatment specifically blocks progression from late G1 to S.

FCCP treatment of NRK cells reduces total ATP levels by ~30% (Fig. S4B), which could trigger the p33-dependent metabolic checkpoint at G1–S (15). To test whether FCCP-induced G1–S arrest was p33-dependent, we used isogenic HCT116 lines expressing or lacking p33 (16). Whereas both cell lines progress through S phase in a similar manner in the presence of FCCP (Fig. S6D), where FCCP is added after G0 release, G1–S arrest is observed in p33+/− cells but not in p33−/− cells (Fig. 4D). The p33/p21 stress-sensing module is known to induce a G1–S block in stressed cells by preventing cyclin E accumulation/activity (17), so we monitored cyclin E and p21 expression levels in FCCP-treated, p33−/− HCT116 cells. Both reduced cyclin E levels and increased p21 expression are observed (Fig. 4E and Fig. S7A for NRK cells). Hence, reducing mitochondrial transmembrane potential through FCCP treatment results in a p33-dependent G1–S arrest involving p21.

The Presence of Hyperfused Mitochondria Induces Cyclin E Buildup.

Levels of cyclin E rise in G1–S and then fall again in S phase in coordination with other cyclins (18). This allows cyclin E to play a specific role in S phase, including initiation of DNA replication. To investigate whether the transient formation of hyperfused mitochondria at G1–S and its subsequent breakdown into isolated tubular elements in S is linked to cyclin E regulation, we induced mitochondrial hyperfusion and then examined cyclin E levels within cells. Mitochondria were induced to become hyperfused through treatment with mdivi-1, a drug that tubulates mitochondria by specifically inhibiting the mitochondrial fission protein, DRP1 (19).
We found that \( \approx 50\% \) of cells in an asynchronous population exhibit highly tubular mitochondria within 3 h of treatment (Fig. S8A). Longer mdivi-1 treatment (>5 h) results in more cells with highly fused mitochondria, but there is also increased cell death, so we restricted our experiments to 4 h of treatment. Notably, immunoblot analysis of mdivi-1-treated cells reveals a time-dependent increase in cyclin E levels over 4 h, with no change in cyclin A levels (Fig. S4A). Cyclin E levels increase when hyperfused mitochondria are induced by DRP1m-GFP overexpression for 24 h in either NRK (Fig. S5) or HCT116 cells (Fig. S7B). Thus, cellular cyclin E levels rise whenever mitochondria are hyperfused.

**Hyperfused Mitochondria Induce G0 Cells to Enter S Phase.** We next investigated whether cyclin E buildup in response to hyperfused mitochondria affects cell cycle progression. Normally, serum-starved cells remain in G0 indefinitely unless growth factors are added to induce signaling pathways for cyclin E buildup (20). Because inducing hyperfused mitochondria leads to increased cyclin E levels, we tested whether this was sufficient to drive serum-starved G0 cells into S phase in the absence of growth factors.

NRK cells in G0 were treated with mdivi-1 to prevent mitochondrial fission and thereby maintain mitochondria in a highly fused form. The cells were then monitored for BrdU incorporation to determine initiation of DNA replication. A considerable fraction of the serum-starved cells incorporate BrdU within 4 h of mdivi-1 treatment, in contrast to untreated, serum-starved cells (Fig. 5C). The increase in cells incorporating BrdU under mdivi-1 treatment is similar to that observed after growth factor (FBS) replenishment for 4 h, as measured in HCT116 cells (Fig. 5D).

The increased BrdU incorporation in mdivi-1-treated cells is correlated with increased cyclin E levels in both HCT116 (Fig. 5E) and NRK cells (Fig. S8B). The MEK kinase inhibitor PD098059 (21) prevents cyclin E buildup in growth factor-stimulated cells but not in mdivi-1-treated cells (Fig. S5E), suggesting cyclin E accumulation in mdivi-1-treated G0 cells occurs downstream of growth factors.
factor signaling. Consistent with this, there is no increase in the growth factor cyclin, cyclin D, in mdivi-1-treated cells, in contrast to growth factor-stimulated cells (Fig. 5E).

Cell Cycle Defects Caused by Untimely Hyperfused Mitochondria. When cyclin E levels are forced to remain high before/during mitosis, defects in chromosome alignment arise (22). We investigated whether causing mitochondria to become hyperfused during mitosis has similar consequences. HCT116 cells were treated with mdivi-1 for 4 h to induce mitochondrial hyperfusion, and thereafter mitotic cells were examined. Treated metaphase cells have highly tubular mitochondria and severely misaligned chromosomes, unlike untreated metaphase cells, which have aligned chromosomes and fragmented mitochondria (Fig. 6H).

Constitutive overexpression of cyclin E throughout the cell cycle is also known to inhibit S-phase entry because of defects in licensing of replication origins occurring after the previous round of mitosis (23). We tested whether a similar phenotype occurs when mitochondria are perpetually hyperfused by DRP1m expression for 48 h. Significantly decreased BrdU incorporation and fewer Aurora B-positive nuclei are seen in these cells, whereas in cells overexpressing DRP1m for shorter periods (i.e., 24 h, before most DRP1m-positive cells undergo mitosis) no inhibition in BrdU incorporation occurs (Fig. 6B and C).

The S-phase entry delay induced by long-term DRP1m expression in DRP1m-expressing cells is linked to membrane potential of the highly fused state of mitochondria, we tested whether the delay is reversed by FCCP treatment. Supporting this, when cells expressing DRP1m for 48 h are treated with FCCP, BrdU-positive nuclei increase in number, indicative of S-phase entry (Fig. 6D). Furthermore, there is a drop in nuclear cyclin E levels (Fig. 6E), as typically occurs after initiation of DNA synthesis (18). Hence the S-phase entry delay induced by long-term DRP1m expression depends on mitochondrial membrane potential and occurs downstream of the p21-induced G1–S checkpoint.

Discussion

In this article, we identify a unique form of mitochondria at G1–S consisting of a giant, hyperfused network. Mitochondria in this state have higher ATP producing capacity than mitochondria at any other cell cycle stage. Depolarization of mitochondria triggered a specific p53/p21-dependent G1–S arrest, demonstrating that mitochondrial function is important for G1-to-S transition. Inducing mitochondria to become hyperfused leads to cell cycle defects characteristic of cyclin E overexpression. Based on these findings, we propose that a transient hyperfused mitochondrial state at G1–S is important for regulating G1–S transition by temporarily boosting cyclin E levels needed for S-phase entry.

Regulation of cyclin E levels by hyperfused mitochondria at G1–S could explain why knockouts of mitofusins (MFN1–2) phenocopy the effects of cyclin E knockouts (24, 25). Hyperfused mitochondria could boost cyclin E levels by augmenting cyclin E transcription, by preventing cyclin E degradation (through binding and sequestering cyclin E regulatory components), and/or by acting indirectly through increased ATP production for ubiquitination of cyclin E regulatory components.

The link between mitochondria and cyclin E buildups could well be regulated through p53, which is known to have a role in regulating mitochondrial respiration (5). p53 transcriptionally regulates SCO2, which regulates mitochondrial respiration by regulating cytochrome c oxidase complex. A potential role of p53 in mitochondrial regulation of cyclin E would provide a reason for why cell cycle defects caused by hyperfused mitochondria having cytochrome c oxidase complex. A potential role of p53 in mitochondrial regulation of cyclin E would provide a reason for why cell cycle defects caused by hyperfused mitochondria having
Methods

Reagents, Antibodies, and Immunoblotting. See SI Methods.

Cell Culture and Imaging. Cells were maintained by standard tissue culture techniques, and G418 (1 mg/ml) was used to generate stable lines. Immunofluorescent staining was performed following standard techniques. For BrdU incorporation experiments, cells were incubated with 100 mM BrdU for 10 min, which was detected by immunostaining with anti-BrdU antibody. Imaging was performed by using a laser-scanning confocal microscope (LSM510; Carl Zeiss Micro-Imaging). In live-cell experiments, cells were imaged in buffered medium on a preheated microscope stage (37 °C). Appropriate laser lines for each fluorophore were used.

High-resolution images were acquired by using the 63×, 1.4 N.A. Plan Neofluor oil-immersion objective. Optical slices were taken along the z axis covering the whole depth of the cell.

For fluorescence recovery after photobleaching (FRAP) analyses, the same 63× objective was used. The fluorescence was bleached and recovery was monitored every second for 2 min. Quantification of recovery kinetics was performed according to ref. 30.

The microirradiation experiment was performed using the 63× Plan Fluor oil objective of the Zeiss LSM 510 META system. Two-photon laser light at 800 nm and 25% power was used to irradiate a region of interest of 15×15 pixels (1 × 1 μm) on TMRE-loaded mitochondria. Images were acquired with the 543-nm line every 3 sec after irradiation until 18 sec or 2 min, as required.

Image processing was performed using the Zeiss LSM software (version 3.4). See SI Methods for details.

Analyses of Mitochondrial Properties. Cells were first incubated with MitoTracker Green (100 nM) for 15 min followed by TMRE (50 nM) for another 15 min. A 40× Plan Neofluor oil-immersion objective was used to acquire images. Mitochondrial potential per unit mass was assessed as a ratio of the TMRE/MitoTracker Green signal. Total cellular ATP was assayed using the ATP determination kit (Molecular Probes), and the mitochondrial contribution was assayed using oligomycin.

For morphometric analyses, a 3D stack of high-resolution confocal images was used for calculating the volume of individual mitochondrial elements within a single cell from different cell cycle stages. Within the merged 3D stack, mitochondrial elements were segmented (31). After segmentation, surface points were extracted for each identified mitochondrial object in the 3D image. The minimum distance between the surface points of the objects and a selective set of quantitative features was computed for each object and used for subsequent statistical analysis. Using all the extracted features, an unbiased volume quantification was used to quantify mitochondrial tubulation. See SI Methods for details.

Cell Cycle Synchronization and Identification of Cell Cycle Stage. A combination of synchronization methods including serum starvation, aphidicolin, and nocodazole treatment was used. See SI Methods and Fig. S3 for details.

In fixed cells, cell cycle stages were identified by BrdU incorporation, Aurora B staining, and propidium iodide staining. In live cells, cell cycle stages were identified through expression of cell cycle marker proteins, as described in detail in SI Methods.

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