Mitochondria Tether to Focal Adhesions During Cell Migration and Regulate Their Size.

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Abstract

Focal Adhesions (FA) couple the actin cytoskeleton to the extracellular matrix through transmembrane integrin receptors. FA assembly and disassembly regulate cell migration by controlling substrate interaction and the generation of intracellular contractile forces. Here we show that FA interact with mitochondria. Mitochondria are highly dynamic organelles that are now emerging as regulators of mammalian cell motility. We find that mitochondria infiltrate the leading edge of NIH3T3 fibroblasts during migration and tether to FA there. Importantly, we find that FA interacting with mitochondria are larger than those lacking mitochondrial interaction. In addition, inhibition of mitochondrial ATP generation reduces FA size and artificial tethering of FA to mitochondria concomitantly increases their size. Taken together this suggests that mitochondrial interaction with FA is a functional part of cell migration and adhesion.

Introduction

Mitochondria have important roles in aerobic energy generation\(^1\), cell death\(^2\) and aging\(^3\). In addition, mitochondria contribute to \(\text{Ca}^{2+}\) homeostasis, and the metabolism of amino acids, lipid and nucleotides\(^4,5\). Dysfunction of mitochondrial metabolism and dynamics contributes to cancer development\(^6\) and a broad spectrum of human diseases\(^7\).

Mitochondria are highly dynamic organelles with fission and fusion events continually reshaping their morphology\(^8\). Mitochondria vary in size from individual organelles in the submicron length range to large interconnected tubular networks spanning the cytoplasm. It is now recognized that contact between mitochondria and other organelles is an important part of cellular physiology and homeostasis\(^9,10\). For example, contact with the Endoplasmic Reticulum licenses mitochondrial fission\(^11,12\) and mitochondrial derived membranes are part of the peroxisome biogenesis pathway\(^13\).

Mitochondria are emerging as novel regulators of cell motility\(^14,15\). Cell migration regulates several important physiological processes, among them embryonic development, tissue morphogenesis and the immune response\(^16\). Dysregulated migration is often associated with cancer development and metastatic progression\(^17\). Fragmented mitochondria are found to infiltrate the leading edge of breast and ovarian cancer cell during migration\(^14,15\). Inhibition of either mitochondrial ATP generation\(^14\) or \(\text{Ca}^{2+}\) uptake through silencing of the Mitochondrial Calcium Uniporter (MCU)\(^15\) inhibits motility.

An unresolved question, however, is to what part of the cell migration machinery do mitochondria regulate and what motility structures do they move and tether to. In this report, we find that during fibroblast migration, mitochondria move to and tether to Focal Adhesions (FA). FA are multi-protein adhesive structures on the basal cell surface that couple the extracellular matrix (ECM) to intracellular actin fibers via clusters of transmembrane integrin receptors\(^18,19\). FA allow for the propagation of mechanical forces within the cell and into the external environment\(^18,19\). Here, we find that inhibition of mitochondrial ATP generation decreases FA size and that tethering FA to mitochondria increase FA size. Thus, we identify FA as a migratory structure regulated by mitochondrial contact and ATP generation.

Methods

Cell Culture. NIH3T3 cells were obtained from the ATCC. Cells were cultured at 37\(^\circ\)C, 5% CO\(_2\) in DMEM (Thermo Scientific, Burlington, Canada) supplemented with 10% FBS (Thermo Scientific, Burlington, Canada), 1mmol/L sodium pyruvate (Thermo Scientific), and penicillin-streptomycin.

Live cell imaging. For imaging, cells were trypsinized from 80-100% confluent plates and seeded at 10\(^5\) cells per 35mm µ-Dish high-wall (Ibidi) or in a 6-well plates (Corning) containing High Precision 1.5H cover slips (Deckglaser) and left to adhere to the surfaces overnight. The next day cells are transfected using Lipofectamine 2000 (11668-019) from Thermo Fisher at a 1:1.5 Lipo:DNA ratio for GFP-Cortactin (Addgene 50728) , mEmerald-Talin (Addgene 54266) or mCerulean3-Tomm20 ( Addgene55450). 1000 ng of cortactin and of talin was used for respective transfections. Cells were imaged in Gibco phenol red-free DMEM and mitochondria were stained with Thermo Fisher Mitotracker Red CMXRos (M7512) for 30 minutes. Live cell cortactin images are taken and deconvolved on the DeltaVision Elite-Olympus IX-71 with FemtoJet Microinjector using version 7 of Softworx. Deconvolution is done...
with 5 iterations. The Zeiss LSM880 AxioObserverZ1 inverted confocal microscope with AiryScan FAST mode at 63X objective was used for live-cell imaging of talin images.

Fixed Cell Microscopy. Cells grown on 18mm coverslips were incubated for 15 min at 37 °C with a Mitotracker™ Red CMXRos to a final concentration of 100-200 nM. Cells were fixed with 4% PFA/PBS for 10 min at room temperature, washed 3x with PBS and permeabilized with 0.1% Triton X-100/PBS for 15 min. Cells were then blocked for 1 hr at room temperature or overnight at 4 °C with 3% FBS/0.1% Triton X-100 in PBS. Cells were then stained for 1 hr at room temperature with antibodies for Talin (Sigma T3287), Tomm20 (Novus 9804), Vinculin (Sigma V9131) or phosphor-FAK (Thermo-Fisher 44-626G). Cells were washed 3X with PBS. Cells were then stained for 1 hr (RT) with secondary antibody, Alexa FluorTM Plus 488 (A32723, Thermo Fisher). Cells were then washed 3X with PBS. Cells were imaged on the Zeiss AxioObserver Z1 microscope using a 63X, 1.4 NA objective and deconvolved using Zen blue edition under fast iterative parameters and bad pixel correction. Spatial reconstruction of images were generated using surface rendering on Imaris software. The quantitation of focal adhesions immunostained for vinculin was performed using ImageJ.

Plasmids. Plasmids for GFP-Cortactin (50728), mEmerald-Talin (54266), mCerulean3-Tomm20 (55450) and mEmerald-Vinculin-N-21 (54304) were purchased from Addgene (Watertown, MA). The cBAK-GFP and cBAK-mEmerald-Talin plasmids were constructed by GenScript (Piscataway, NJ). For cBak-eGFP, a series of 12 glycine-glycine/serine-serine repeats followed by the mitochondrial targeting sequence, cBAK (TTGCCGTAGAGACCCCATCCTGACCACGTAATGGTGATTTTTGTGTGGTTCTGGGCAATTCGTTGACACAGATTTCTCAGATCATGA) was placed downstream of eGFP in the mammalian expression vector pcDNA3.1(+)-N-eGFP. cBak-Talin plasmid was constructed similarly by inserting the cBak sequence downstream of mEmerald in the Talin-mEmerald construct.

Results And Discussion

Mitochondria contact focal adhesions during migration. Previous reports indicate that regulation of calcium flux via the Mitochondrial Calcium Uniporter (MCU) and energy levels via the AMP-activated protein kinase (AMPK) are two pathways through which mitochondria affect cell motility. However, it is unknown what migratory structures mitochondria might be controlling with during migration.

We speculated that mitochondria might be interacting with Focal Adhesions (FA) since these adhesive structures position themselves at the periphery of a cell and are required for many forms of mammalian cell motility. To test this idea, we first used a confocal microscope to visualize mitochondria and FA in NIH3T3 fibroblasts (Fig. 1A and 1B). We visualized FA and mitochondria by staining cells with antibodies against Talin and Tomm20. Talin is a component of mature FA that links transmembrane integrin receptors to actin fibers in the cytosol. Tomm20 is an import receptor translocase found on the outer mitochondrial membrane. In fixed cells (Fig. 1A, arrowed), multiple FA in the cell periphery are observed tethered to mitochondria. Surface rendering of the fixed cells (Fig. 1B) also shows FA/mitochondrial tethering. Quantitation of this interaction (Fig. 1C) revealed that approximately one quarter (25.6 ± 6%) of all FA are in contact with mitochondria and approximately one fifth (20.4 ± 5%) of the total FA length is in contact with mitochondria. Approximately 8.0 ± 2.1% of all mitochondria and 7.3 ± 2% of the total cellular mitochondrial length is in contact with FA. Critically, FA in contact with mitochondria are larger than those not contacting them (Fig. 1D). FA tethering to mitochondria (n = 1,551) have, on average, an area of 5.77 ± 1.5 µm², significantly (t-test, p < 5 x 10⁻⁴) larger that the 5.37 ± 3.6 µm² area of FA not tethered to mitochondria (n = 4,774). Thus, FA represent a new addition to a growing list of cellular structures that are in physical contact with mitochondria.

Interaction between mitochondria and other cellular structures is now understood to be an important aspect of the homeostasis of multiple organelles. For example, mitochondria make important functional contacts with the endoplasmic reticulum (ER), vacuoles and peroxisome. Mitochondrial contact with the ER regulates mitochondrial division and also allows ER-derived lipids to move to mitochondria. The larger size of FA contacting mitochondria suggests that mitochondria could be controlling cell adhesion and migration through them.
Mitochondria move to FA during cell migration. To determine whether or not mitochondria at the leading edge were specifically migrating to FA, we imaged migrating cells for both FA (Talin) and mitochondria (Tom20). In these cells, we observe that as the cell moves forward, multiple mitochondria infiltrate the leading edge and interact with FA there (Fig. 3A, supplementary video 4). During this process, we also observe mitochondrial fission events prior to mitochondria moving to FA (Fig. 3A labelled “f”). To test this idea that mitochondrial interaction with FA might demonstrate leading edge/trailing edge polarization, we quantified the number of FA interacting with mitochondria relative to their position within the motile cell. We dived migrating cells (n = 5) into Leading Edge, Trailing Edge and Centre areas and measured the fraction of FA in each section contacting mitochondria. As shown in Fig. 3B, leading edge FA have almost eight times as many mitochondrial contacts per FA relative to the centre (25.6 ± 5% vs 3.27 ± 4%) and three times as many compared to the trailing edge (8.2 ± 4%). Taken together, the directional movement of mitochondria to FA during migration and the leading edge-dependent polarization of FA/mitochondrial contacts indicates that mitochondrial interaction with FA is a functional part of the cell migration program.

Inhibition of mitochondrial activity reduces Focal Adhesion size. To examine a functional role for mitochondrial interaction in FA structure, we treated NIH3T3 cells with oligomycin. Oligomycin inhibits mitochondrial ATP generation by preventing ATP synthase activity\(^{26}\) and has previously shown to retard migration of ovarian cancer cells\(^{14}\). We treated cells with oligomycin and used an image analysis program to quantitate FA length four hours later. The mean number of focal adhesions per cell is not affected by oligomycin: control NIH3T3 cells have an average of 29.8 ± 16 FA/cell (n = 177), not statistically different than the 29.2 ± 14 and 29.8 ± 21 FA/cell in 1µM and 2µM oligomycin treated cells (Fig. 4A, n = 190 and n = 160 respectively). However, oligomycin treated cells have shorter FA compared to controls. Cells treated with 1 µM or 2 µM oligomycin each have an average FA length of 2.33 ± 1.6 µm and 2.22 ± 1.8 µm respectively, significantly smaller than the average FA length of 2.79 ± 1.9 µm in untreated cells (Fig. 4A, t-test, \(p < 4 \times 10^{-5}\) and \(p < 5 \times 10^{-4}\)). Cumulative Distribution of FA length in cells shows that oligomycin significantly decreases FA length compared to controls (Fig. 4A, (Smirnov Kolmogorov test, \(p < 6 \times 10^{-4}\) and \(p < 1 \times 10^{-7}\)). In particular, 1 µM and 2 µM oligomycin treated cells have respectively 51 and 55% of FA shorter than 1.8 µm compared to 47% of control cells.

To further explore the relationship between mitochondrial contact and FA, we artificially tethered mitochondria to FA. We generated constructs containing the mitochondrial targeting sequence of the human Bak gene (cBak), a glycine spacer and an mEmerald tagged full length human Talin or GFP control (Fig. 4B). Similar constructs have been used to study interaction of vinculin and talin outside of endogenous FA\(^{27}\). We then transfected the constructs into NIH3T3 cells and stained the transfectants for Mitochondria (Tom20) and FA (phosphor-FAK, Fig. 4C). Both the GFP and Talin fusion proteins show predominant mitochondrial localization as well as some co-localization with FA. Cells transfected with the Talin construct show an increased number of FA/mitochondrial contacts compared to those transfected with the GFP control (Fig. 4D), indicating that the Talin fusion is functioning as expected. In cells transfected with cBak-Talin (n = 60), 26.9 ± 18% of FA per cell are in contact with mitochondria, significantly higher (t-test, \(p < 0.0001\)) that the 18.2 ± 11% in cells transfected with cBak-GFP (n = 62 cells). Importantly, FA in cells transfected with cBak-Talin (Fig. 4E; n = 3196 FA) have a mean area of 8.91 ± 5.7 µm\(^2\), significantly larger (t-test, \(p < 0.0001\)) that the mean FA area in cells transfected with cBak-GFP, 8.43 ± 5.5 µm\(^2\) (n = 3899 FA). Cumulative distribution analysis similarly indicates that cBak-Talin transfected cells have significantly larger FA than GFP controls (Smirnov Kolmogorov test, \(p < 3 \times 10^{-4}\)). In particular, cBak-Talin cells have 52% of their FA larger than ~7 µm\(^2\) in area, more than the 48% in GFP transfectants. Thus, tethering of mitochondria to FA increases their size.

Inflitrte into the leading edge lamellipodia during migration\(^{14,15,22}\). To determine whether or not this was the case in the non-transformed NIH3T3 cells, we used live cell microscopy to image mitochondria in freely migrating NIH3T3 mouse fibroblasts (Fig. 2a, Supplementary Video 1). In migrating cells, mitochondria can be observed moving towards the leading edge. To extend these findings, we tracked individual mitochondria in NIH3T3 cells expressing a fluorescently tagged Cortactin protein (Fig. 2b, Supplementary Video 2). Cortactin localises prominently to the lamellipodial edge in migrating cells and regulates cell migration by binding to the Arp 2/3 complex, stabilizing actin branches and activating multiple signaling pathways necessary for motility\(^{23}\). As shown in Fig. 2B, multiple mitochondria with various starting locations move towards the Cortactin polarized edge (red arrow) during migration (direction indicated by white arrow). In migratory NIH3T3 cells, as in other cell types\(^{24,25}\), mitochondria move along microtubules. To determine if this was the case during cell migration, we imaged mitochondria (Tom20) microtubules (tubulin) in migrating cells (Fig. 2c, Supplementary Video 3). Here, mitochondria can be seen to move along microtubules towards the leading edge during cell migration.

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The regulation of FA size by mitochondria suggests that full FA assembly requires mitochondrial action. A mature FA, generally 2 µm wide x 3–10 µm long, is created from smaller adhesive structures described as focal complexes or nascent adhesions\(^19\). Our observation that oligomycin decrease FA length is consistent with the idea that localized ATP generation by mitochondria is a key part of FA maturation. High local concentrations of ATP could support actin polymerization or the signaling processes necessary for full FA assembly. FA size has been shown to regulate cell speed, with shorter focal adhesions were associated with slower cell speeds through a biphasic relationship\(^20\). Thus, a requirement for mitochondria in FA maturation provides a plausible explanation for how mitochondrial metabolism might regulate motility.

**Declarations**

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**COMPETING INTERESTS**

The authors declare no competing interests.

**AUTHOR CONTRIBUTIONS.**

RD wrote the main manuscript text, designed and performed experiments, interpreted data, and contributed to revising it. PB, AM designed and performed experiments, interpreted data, drafted portions of the paper and contributed to revising it. KY designed and performed experiments and interpreted data. JML conceived experiments, interpreted data, drafted portions of the paper and contributed to revising it. All authors reviewed the manuscript.

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Figures
Mitochondria interact with Focal Adhesions. A) In a fixed NIH 3T3 fibroblast, mitochondria (Tomm20) can be seen interacting with Focal Adhesions (Talin). Lower images are an enlargement of the boxed rectangle. Scale bar is 1 μM. B) Surface reconstruction of the image in (B) shows contact between mitochondria and focal adhesions. Scale bar in the upper and lower image set are 4 and 1 μm respectively. C) Quantification of interaction between Focal Adhesions and Mitochondria. D) Focal adhesions in contact with mitochondria (n=1551) are significantly larger (t-test, p < 5 x 10^-4) than those not in contact (n=4774).
Figure 2

Mitochondria infiltrate the leading-edge during migration. Mitochondria (colored arrows) move towards the leading edge in migrating NIH3T3 fibroblasts. The direction of migration is indicated by the white arrow. The yellow dashed line indicates the plasma membrane edge from the DIC image (Supplementary Video S1). B) Mitochondria move towards the Cortactin edge (red arrow) during cell migration (white arrow) (Supplementary Video S2). C) Mitochondria move along microtubules during migration (Supplementary Video S3). Scale bar is 1 μm.
Figure 3

Mitochondria move to and tether to Focal Adhesions During Migration. A) During migration, a mitochondrion at the leading edge undergoes a fission event (f) and moves to one focal adhesin (Talin) and then another. Inset shows an enlarged image of the boxed rectangle (Supplementary Video 3). B) Mitochondrial interaction with Focal Adhesions show leading edge polarization. Migrating NIH3T3 cells (n=5) were divided into Leading Edge, Centre and Trailing Edge compartments (left panels) and the fraction of Focal Adhesions with mitochondrial contact was assessed in each section. Leading Edge Focal Adhesions have significantly greater interaction with mitochondria that Focal Adhesions in the Centre or Trailing Edge (t-test, p<0.007 and p<0.04 respectively).
Figure 4

Tethering mitochondria to FA increases FA size. A) FA length in cells treated with 1 or 2 µm oligomycin are significantly smaller (Smirnov Kolmogorov test, p < 6 x 10^-4 and p < 1 x 10^-7 respectively). Right panels show representative cells for each treatment. Focal Adhesion size is derived from three independent experiments each of 30 cells per condition. B) Constructs used to tether mitochondria to Focal Adhesions. C) Fixed cells are imaged for GFP or Emerald, Mitochondria (anti-Tomm20) and Focal Adhesions (anti-Vinculin). Arrows indicate constructs with colocalization with both mitochondria and Focal Adhesions. D) Cells transfected with cBAK-Talin show significantly more FA/mitochondrial contacts compared to those transfected with cBAK-GFP (t-test, p < 0.0001). Figure is the mean and standard deviation of triplicate independent experiments. (E) Focal Adhesions in cells transfected with cBAK-Talin show significantly larger FA compared to those transfected with cBAK-GFP (t-test, p < 0.0001). Solid red lines are the mean and dashed red lines are quartiles. Cumulative distribution similarly shows significantly larger FA in cBAK-Talin transfected cells (Smirnov Kolmogorov test, p < 3 x 10^-4). Figure is the mean and standard deviation of triplicate independent experiments.

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