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Mitochondrial Dysfunction and Mitochondrial Dynamics-The Cancer Connection

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Abstract

Mitochondrial dysfunction is a hallmark of many diseases. The retrograde signaling initiated by dysfunctional mitochondria can bring about global changes in gene expression that alters cell morphology and function. Typically, this is attributed to disruption of important mitochondrial functions, such as ATP production, integration of metabolism, calcium homeostasis and regulation of apoptosis. Recent studies showed that in addition to these factors, mitochondrial dynamics might play an important role in stress signaling. Normal mitochondria are highly dynamic organelles whose size, shape and network are controlled by cell physiology. Defective mitochondrial dynamics play important roles in human diseases. Mitochondrial DNA defects and defective mitochondrial function have been reported in many cancers. Recent studies show that increased mitochondrial fission is a pro-tumorigenic phenotype. In this paper, we have explored the current understanding of the role of mitochondrial dynamics in pathologies. We present new data on mitochondrial dynamics and dysfunction to illustrate a causal link between mitochondrial DNA defects, excessive fission, mitochondrial retrograde signaling and cancer progression.

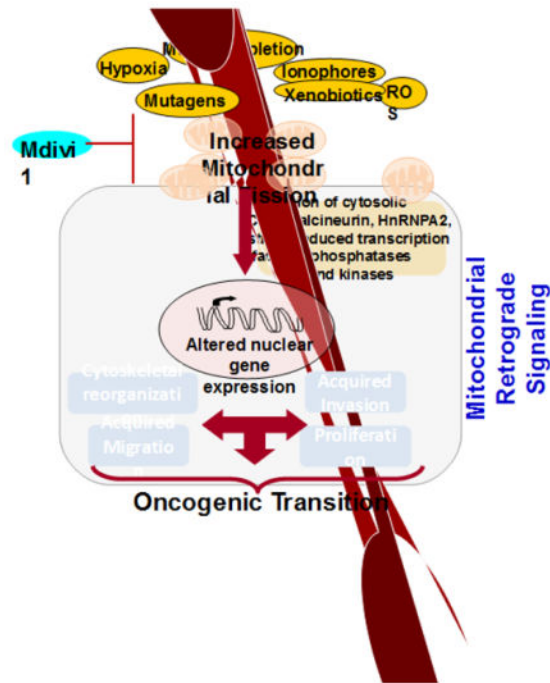
Graphical abstract

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I. Introduction

An essential function of mitochondria in mammalian cells is to supply most of the cellular ATP through oxidative phosphorylation. In addition, mitochondria integrate various metabolic pathways and through this process synthesize intermediates needed for the synthesis of cellular biomass. The more specialized functions of mitochondria include maintenance of Ca^{2+} homeostasis and regulation of apoptosis. These organelles also produce reactive oxygen species as byproducts of respiration coupled oxidative metabolism, which in turn act as physiological signaling molecules, but induce toxicity when produced in excess under pathological conditions. It has been proposed that mitochondria evolved from symbiotic aerobic bacteria engulfed by pre-eukaryotic cells more than a billion years ago (1;2). While this symbiosis likely gave a survival advantage to the host cell in an oxygen rich environment, it was necessary for the cell to stringently control function of the endosymbiont, not only to meet its energy needs but also to protect itself from any toxic metabolites produced by the organelle. This was achieved during the course of evolution by transferring most of the bacterial DNA to the host nucleus (2). The mitochondrial DNA (mtDNA) encodes only 13 polypeptide subunits, all of which are part of the electron transport chain. However, normal mitochondrial function requires well over 1000 proteins (3) and large number of metabolic substrates, which is maintained by constant bidirectional communication with the nucleus.

II. Factors affecting mitochondrial functions

Generally, mitochondrial function is designed to meet the cell's energy and metabolic demands. The efficiency of the process however varies widely due to genetic polymorphism

of mtDNA and tissue specific variations in nuclear encoded mitochondrial proteins. Several pathologies and adverse environmental conditions disrupt mitochondrial function in multiple ways. MtDNA mutations, deletions or impaired DNA replication are the most common cause of mitochondrial dysfunction (4;5). Mitochondria contain multiple copies of their own DNA ranging from 100 to 10,000 per cell. However, many diseases such as mtDNA depletion syndrome, several cancers including breast, prostate and colon cancers, and age related pathologies are characterized by significantly low mtDNA copy number that affect normal mitochondrial function (6–13). MtDNA is susceptible to damage and mutation due to both its proximity to a high concentration of toxic metabolites and also relatively inefficient repair mechanism (14). The overall effect on mitochondrial function depends on the extent of heteroplasmy of mtDNA (15;16). Mitochondrial dysfunction can also result from exposure to xenobiotics and adverse environmental conditions.

Hypoxia is a common factor in many pathological conditions such as the interior of solid tumors, tissue ischemia and inflammation (17;18). Hypoxia not only depletes oxygen, which is the terminal acceptor of electrons from the electron transport chain, but also causes permanent damage to the proteins, lipids and mitochondrial nucleic acid components by inducing oxidative stress. Xenobiotics or their metabolites can cause mitochondrial dysfunction by multiple mechanisms including disruption of membrane potential, direct interaction with mitochondrial DNA, and proteins, affecting their function (19–21). The mitochondrial dysfunctions range from reduced ATP production by oxidative phosphorylation, inability to modulate production of excessive reactive oxygen and nitrogen species, dysregulation of calcium to opening of permeability transition pore and initiation of apoptosis.

Many chemotherapeutic drugs used for the treatment of primary tumors, including doxorubicin, cisplatin, other DNA damaging anthracycline derivatives, damage mtDNA and impair mitochondrial functions in cancer patients (22). Doxorubicin, has been shown to cause cardiomyopathy by targeting mitochondria. It increases ROS production, reduces ATP synthesis and inhibits CcO by directly binding to the complex (23). Environmental toxin TCDD is shown to disrupt mitochondrial membrane potential and mitochondrial transcription translation mechanisms in a time-dependent manner and induce stress signaling (24). Similarly, benzo[a]pyrene a common cigarette smoke carcinogen has been shown to be metabolized in mitochondria and form mtDNA adducts. Benzo[a]pyrene treatment disrupts mitochondrial respiration and induces oxidative stress (25). Anti-retroviral drugs such as Zidovudine affect mitochondrial function by inhibiting mtDNA polymerase and causing mtDNA depletion (26). Defects in mitochondrial DNA and membrane components affect mitochondrial function depending on the extent of damage. Reduced ATP production from oxidative phosphorylation is seen in mtDNA depletion and heteroplasmic mutations in mtDNA or components of electron transport chain.

Excessive production of reactive free radicals is one of the common effects of mitochondrial dysfunction. Mitochondrial electron transport chain is a source of reactive oxygen and nitrogen species that are produced as byproducts of oxidative phosphorylation. Hypoxia and mutations in subunits have been associated with increased ROS production from complex I. Mitochondria play an important role in Calcium homeostasis. Dysregulation of calcium can

be attributed to both overload of mitochondria with calcium or their inability to uptake calcium. Mitochondrial calcium overload has been shown to be one of the hallmarks of reperfusion injury after ischemic episode. Excessive mitochondrial calcium and reactive oxygen species have been shown to be important triggers for mitochondrial permeability transition. Loss of membrane potential causes decreased calcium uptake and increased cytosolic calcium levels $[Ca^{2+}]_c$.

III. Mitochondrial Morphological Alterations in response to stress

Mitochondrial morphology in different cells can vary from long tubular structures seen in fibroblasts and myocytes to smaller spherical and ovoid structures found in macrophages. Mitochondrial stress and dysfunction resulting from hypoxia, exposure to mitochondrial toxins and metabolic diseases affect mitochondrial morphology. Excessive ROS production induced by photo-excitation of cytochrome c oxidase in ASTC-1 and COS7 cells resulted in increased fragmentation of mitochondria (27). Similarly, hypoxia-reoxygenation stress in H9C2 cells resulted in fragmented, donut-shaped mitochondria (28). We observed fragmented, circular mitochondrial structures in C2C12 cells expressing shRNA to cytochrome c oxidase subunits (29). As shown in Figures 1A–C, electron micrographs of C2C12 cells with more than 80% depleted mtDNA content show small, circular mitochondria. In control cells mitochondria are densely packed in the periphery of the nuclear envelope, whereas in cells with reduced mtDNA content (either by ethidium bromide treatment or silencing *Tfam* mRNA), the mitochondria are fewer in number, markedly smaller in size, appear fragmented and distributed throughout the cellular cytoplasm (Fig 1B). In contrast to control cells, the mitochondrial cristae appear disorganized in mtDNA-depleted cells. Furthermore, we observed that loss of *Tfam* mRNA has more severe effect on mitochondrial structural integrity indicated by fragmented mitochondria (Fig 1C) possibly due to the function of TFAM as a mitochondrial nucleoid component.

IV. Mitochondrial defects deregulate mitochondrial dynamics

Mitochondrial dynamics is regulated by cellular bioenergetic demands. Although several studies report effect of mitochondrial dysfunction on mitochondrial morphology (27–30), the role of mitochondrial dynamics in cell signaling has not been well studied. Healthy mitochondria are dynamic and morphologically fluid which facilitate both the efficiency of mitochondrial function and turnover. The mitochondrial morphology is the result of the interplay between rapid fusion and fission events and is brought about by large dynamin family GTPases (31). Mitochondrial fusion is a two-step process that begins with the initial joining of outer membrane, followed by fusion of the inner membrane. Outer membrane fusion is mediated by the mitofusin proteins (*Mfn1* and *Mfn2*), anchored on the mitochondrial outer membrane (31–33). OPA1 mediates the fusion of inner membrane (31;32). The mechanism of fusion is similar for both the outer and inner membrane, in which the fusion proteins on the opposing membranes form interlocking coiled coils, which are then powered to fuse by GTP hydrolysis (31). Under normal conditions, the fusion of outer and inner membranes is coordinated (31). However, mutations targeted to these genes show that the two events can be independent of each other with the inner membrane fusion

dependent on mitochondrial membrane potential (34;35). Mitochondrial fission is mediated by a highly conserved dynamin related GTPase, Drp1 (36). Drp1 is a cytoplasmic protein that is maintained in the cytosol by PKA mediated phosphorylation at Ser 637 (37). Drp1 is recruited to the outer membrane of mitochondria by membrane anchored proteins Mff and Fis1, where it forms a constricting spiral around the outer and inner membrane to fragment the mitochondrion (38–40). Mitochondrial fusion results in extended mitochondrial networks, which provides advantage to cells under high energy demands and disruption of mitochondrial fusion has been shown to result in mitochondrial dysfunction.

In immortalized as well as primary cells, in response to induction of mitochondrial dysfunction, we observed lower expression of the mitochondrial fusion marker protein OPA1 (Fig 2A, B). This correlated with increased expression of the mitochondrial fission marker protein DRP1 (Fig 2C). Additionally inhibiting mitochondrial fission by Mdivi1 (Drp1 inhibitor) treatment reverses cellular morphology in mtDNA-depleted cells (Fig 2D). Reorganization of cytoskeleton is associated with cell migratory potential and aggressive tumor cells are highly migratory in nature. Using a scratch-wound healing assay we tested the migration pattern of mtDNA depleted C2C12 cells as a function of their metastatic potential. We observed that mtDNA depleted C2C12 cells acquired migratory capacity which was markedly reduced in response to treatment with Mdivi1 (Fig 3 and Movie files 1–3). The Windrose plots of cell movement, where all cell tracks are placed at the same starting point, clearly demonstrate a markedly different migratory pattern of mtDNA-depleted cells as compared to control cells (Fig 3A and B). Treatment with with mDivi1 reduced the migration rates of mtDNAdepleted cells (Fig. 3C). The mtDNA depleted cells exhibited unorganized trajectory, typical of cells with high metastatic potential. These results suggest the contribution of higher mitochondrial fission towards cellular migration and therefore tumor progression. (41)This suggests that mitochondrial dysfunction induces alterations in mitochondrial fusion-fission dynamics, which by a feedback loop further modulates mitochondrial integrity and functions, exacerbating the effects of mitochondrial dysfunction and promoting oncogenesis.

V. Retrograde signaling by dysfunctional mitochondria

Functional interaction between mitochondria and nucleus is maintained by signaling network that controls both the biogenesis and functioning of mitochondria. The anterograde signals from nucleus to mitochondria control import of proteins, availability of substrates, mtDNA maintenance and gene expression in accordance with the energy and growth requirements of the cell (42–45). In a healthy cell, under physiological conditions, retrograde feedback from mitochondria to nucleus is likely to be normal mitochondrial output of ATP, metabolic intermediates and basal reactive oxygen radicals, and serve to fine-tune the metabolic flux to meet cellular energy demands. On the other hand, mitochondrial retrograde signaling (MtRS), which is activated by mitochondria under stress, has received considerable attention in recent years due to its ability to bring about global changes in nuclear gene expression and phenotypic changes in cells (5;46). Of the multiple retrograde signaling pathways, at least three are triggered directly by mitochondrial stress (47–50). Interestingly all three pathways suggest important role for retrograde stress signaling in

cellular transformation and cancer cell survival implicating a role of mitochondrial dysfunction in cancer development(47–50).

V.1 Reactive oxygen species initiated signaling

Reactive oxygen species (ROS) in mitochondria are result of premature oxidation of electrons in the mitochondrial electron transport chain. It is estimated that 2–5% of oxygen consumed by mitochondria is converted to ROS. In healthy mitochondria, ROS is rapidly detoxified by antioxidant defenses (51). Excess ROS produced under hypoxia or by increased inflammatory cytokines, on the other hand, activates MtRS leading to cellular adaptations (52–54). ROS produced by complex III under hypoxia was shown to activate AMPK and inhibit mTOR to conserve energy at the same time increase ATP production (55). Similarly, TNF alpha induced mitochondrial ROS production and triggered cell death by activation of JNK pathway (56). In RAW 264.7 macrophages, hypoxia alone or synergistically with RANKL, produces mitochondrial ROS, triggering retrograde signaling and differentiating the cells into bone resorbing osteoclasts (57;58). In the case of macrophages however, we observed that the initial ROS signal resulted in the activation of calcium-calcineurin mediated MtRS signaling pathway discussed in detail below (57;58). Some reports showed that mitochondrial ROS generated under hypoxia stabilize HIF1alpha and facilitate cellular transformation (48). Similarly, high ROS levels in cancers characterized by loss of tumor suppressor mutations could activate proliferation, angiogenic and antiapoptotic pathways aiding in cancer progression (59–62).

V.2 Unfolded protein response (mtUPR) initiated signaling

Mitochondrial stress and dysfunction affects protein homeostasis. Oxidatively damaged proteins and unfolded or mis-folded proteins are degraded by mitochondrial proteases for quality control (63–65). In mammalian cells mitochondrial ability to communicate the matrix unfolded protein response to the nucleus involve activation of CHOP and CREB (66;67). Experimental targeting of misfolded ornithine transcarbamoylase to mitochondrial matrix resulted in upregulation of several heat shock proteins like HSP60, HSP10 and chaperones without affecting cytosolic or ER stress response (67). Similarly, presence of mutant EndoG in intermembrane space resulted in Akt phosphorylation mediated activation of estrogen receptor alpha and induction of quality control proteases localized to IMS (47). Direct evidence for retrograde signaling by unfolded protein response has been shown in *C. elegans*, in which excessive accumulation of unfolded proteins resulted in their degradation and efflux of peptides (68). The peptides activate a transcriptional response mediated by ATFS-1 that localizes to nucleus and along with two other factors DVE-1 and Ubl-5 induce expression of genes involved in mitochondrial quality control and cellular metabolism (68–71). Although there is no direct evidence, it is likely that the retrograde signaling by unfolded protein response may play a major role in cancer cell survival. Aberrant growth and exposure to adverse conditions like hypoxia are common feature of cancer cells and likely result in very high rates of oxidatively damaged and misfolded proteins inside mitochondria, conditions that could initiate unfolded protein response. In this context, it is interesting that expression of HSP60 and HSP90 related protein TRAF1 is elevated in many tumors. It is however unclear if this is an effect of retrograde signaling by UPR.

V.3 Calcium-calcineurin mediated retrograde signaling

Calcium-calcineurin mediated signaling is activated in response to mtDNA depletion, loss of electron transport chain proteins and disruption of mitochondrial membrane potential (24;49;50;72;73). Mitochondria play an important role in Ca^{2+} homeostasis by transiently removing Ca^{2+} released by intracellular stores or from outside the cell and releasing back to the cytosol to regulate calcium dependent (74–76) signaling. Mitochondrial dysfunction due to loss of membrane potential from either mtDNA defects or exposure to xenobiotic toxins impairs mitochondrial calcium uptake (49;73;77). Interestingly, in models of mtDNA depletion by ethidium bromide treatment and membrane potential disruption by CCCP treatment, the cells exhibited elevated expression of Ryanodine receptor (RYR1, 2 or 3), which is a calcium leak channel in the endoplasmic reticulum membrane (24;49;73). Collectively, inability of mitochondria to take up calcium, excessive calcium leak through upregulated RYR channels and likely reduced activity of the ATP dependent Endoplasmic Reticulum calcium pump together result in sustained elevation of $[\text{Ca}^{2+}]_c$. Increased steady state $[\text{Ca}^{2+}]_c$ activates calcineurin, a cytosolic phosphatase, which in turn activates NFAT and a unique I κ B dependent NF κ B pathway (29;49;78). Calcium dependent kinases PKC, CAMKIV, JNK and MAPK are also activated, which leads to activation of transcription factors CEBP/δ and CREB (5). An important factor activated by this pathway is the heterogeneous nuclear ribonuclear protein hnRNPA2, which acts as a transcriptional co-activator (79;80). It assembles the stress induced transcription factors in enhanceosome complexes at promoter sequences leading to synergistic activation of several stress response genes (46;79–81).

Recently we reported that hnRNPA2 is a novel mitochondrial stress activated protein lysine acetyltransferase that acetylates histone H4 at lysine K8 on target gene promoters. This H4K8 acetylation is essential for the transcriptional activation of the mitochondrial stress-responsive target genes (82). In ethidium bromide treated C2C12 cells, mtDNA depletion results in induced expression of more than 120 genes that bring about phenotypic changes like altered morphology, increased invasiveness, metabolic shift to glycolysis and resistance to apoptosis (50;83–85). Similar activation of Calcium-calcineurin mediated MTRS signaling was seen in mtDNA depleted immortalized RAW264.7 and MCF10A cells as well as transformed cells such as A549, MCF7 and HCT116 (29;73;81;86).

VI. Mitochondrial defects in cancer

Altered mitochondrial function is a hallmark of many cancers although the nature of functional modification depends on the type of cancer (87–91). Nearly 80 years ago Warburg noted that cancer cells have damaged respiration and increased lactate production, a phenomenon referred to as aerobic glycolysis or Warburg Effect (92). Since then, several studies have shown reduced mitochondrial respiration in a wide range of cancers (93). Supporting evidence for the defective mitochondria in cancer tissue has been shown by both genetic screening for mtDNA defects and by providing experimental support by using cybrids (91;94;95). Low copy number of mtDNA has been observed in many cancers including breast, colon, hepatocellular carcinomas, astrocytomas and prostate cancers (6;8;11;96;97). Experimentally induced mtDNA depletion in colorectal and prostate cancer

cells promotes aggressive cancers (98;99). Similarly, increased tumor growth is seen in the intestine of mice with adenomatous polyposis coli intestinal neoplasia when crossed with mice heterozygous for the mitochondrial transcription factor A (TFAM) with reduced mtDNA copy number (100). Mutations in mitochondrial DNA has been reported in several cancers including breast, renal adenocarcinoma, thyroid tumors, colon cancer, head and neck cancer and prostate cancer (95;97;101;102). Although the causal role of mitochondrial DNA defect in tumorigenesis has not been clearly established, cybrid cell lines generated by fusing cytoplasts and nuclei from different cell lines provide a means for testing the role of WT and mutant mtDNA under the same nuclear genetic background. Mutations in complex I subunit ND6 increased the metastatic potential by producing excessive ROS, whereas an ND5 mutation promoted tumorigenesis by oxidative stress and Akt activation (94;95).

Mitochondrial function is affected by changes in nuclear coded proteins. Mutations in SDH subunits and FH genes have been observed in paragangliomas, pheochromocytomas, multiple cutaneous and uterine leiomyomas and aggressive forms of renal cell cancer (103–107). In both these instances, loss of function causes accumulation of substrates succinate and fumarate, which have been shown to activate specific stress pathways with roles in tumor development (103;107–109). Heterozygous missense mutations in IDH have been shown in gliomas, chondromas and astrocytomas (110–112). Heterodimers of mutant IDH1 and IDH3 have been shown to increase accumulation of 2-hydroxyglutarate that has been shown to affect methylation and other epigenetic modifications (110;111;113;114).

In addition to mutations, levels of mitochondrial proteins can also be affected by environmental changes. Long term hypoxia as commonly seen in solid tumors has been shown to specifically degrade subunits IV and Vb of cytochrome oxidase (115;116). Using stable cell lines expressing shRNA to subunits IV and Vb, we showed that mitochondrial dysfunction results in activation of several genes that have role in tumor development (29). The causal role of this loss of CcO subunits in the observed phenotype was further confirmed by rescue of CcO activity WT subunit cDNAs, which reverted most of the phenotypic changes (29).

Mitochondrial dysfunction is also shown to play important role in metastasis. Depletion of mtDNA resulting in mitochondrial dysfunction induced an epithelial to mesenchymal transition in multiple cell lines including MCF 10A breast cancer cells (81). Mutation in complex I subunit ND6 was shown to increase the metastatic potential of a mouse lung carcinoma cell line (95). In human cervix squamous cell carcinoma and murine melanoma cell lines, clonally selected cells with high metastatic potential produced elevated levels of superoxide (117). Furthermore, experimental inhibition of electron transport chain functions seemed to attenuate the metastatic potential of these cells suggesting the causal role of superoxide in conferring metastatic potential. Superoxide was shown to promote tumor cell migration by activating Pyk2, the focal adhesion kinase in Src mediated pathway. Moreover, partial inhibition of ETC resulted in prometastatic phenotype, which was reversed by treatment with mitochondria targeted antioxidants (117) clonal selection of high metastatic cells over successive generations could have attributed to the phenotypic drift commonly reported in transformed cell lines (118–120)(119–121). It is also likely that different mitochondrial outputs are needed for cells at different stages of metastatic progression.

A more recent focus of research in the field has been on the contribution of mitochondrial dynamics towards tumor initiation and progression. Although the exact mechanism and the signaling of defective mitochondrial dynamics in cancer development is not known, it has been observed that excessive fission and reduced fusion is a feature of many tumors (121–123). Interestingly most of the available literature shows dysregulated Drp1 action as responsible for excessive fission (124–126). In human pancreatic cancer, expression of oncogenic *Ras* or activation of MAPK pathway leads to *Erk2* mediated phosphorylation of Drp1 on Ser 616 that leads to increased mitochondrial fragmentation (126). Moreover, inhibition of this phosphorylation in xenografts is sufficient to block tumor growth (126). Similarly, both A549 lung adenocarcinoma cell lines and human tissues exhibit higher expression of Drp1 with increased phosphorylation (121). Interestingly this was accompanied by reduced Mfn2 expression. Increasing mitochondrial networking by Mdivi1 treatment resulted in higher apoptosis and reduced proliferation in A549 cells and reduced tumor growth in xenografts expressing Mfn2 delivered by adenoviral vector (121). The trigger and the mechanism resulting in Drp1 overexpression and phosphorylation are not well understood. In high stage neuroblastoma, Survivin, an antiapoptotic protein recruits Drp1 to mitochondria and induces mitochondrial fragmentation (127). Survivin overexpressing cells were shown to have reduced complex I and IV activities with a shift to aerobic glycolysis (127).

As mentioned earlier, mtDNA depletion in C2C12 cells induces mitochondrial retrograde signaling that transforms them to tumorigenic cells. We recently observed in these cells that mitochondrial dysfunction caused by mtDNA depletion leads to increased Drp1 mitochondrial localization and reduced OPA1 expression, accompanied with fragmented mitochondria. As shown in Fig 1 and 2, mtDNA depletion (either by EtBr or knock down of Tfam mRNA) induced higher levels of mitochondrial fission. The mtDNA-depleted cells also showed remarkably altered cytoskeleton and pseudopodia like structures, characteristic of tumor cells (Fig. 2). Interestingly, the treatment with Mdivi1 (128), a specific inhibitor of fission protein DRP1 attenuated these tumorigenic morphological changes suggesting a connecting link between mtDNA depletion, mitochondrial dynamics, altered morphology and tumor-like phenotype. However, the mechanistic link between increased mitochondrial fragmentation and phenotypic transformation remains to be investigated.

VII. Alterations in Cellular Metabolism effected by mitochondrial dysfunction

As the hub of metabolic integration, mitochondrial metabolome consists of hundreds of intermediates generated in metabolic pathways like TCA cycle, fatty acid metabolism, amino acid oxidation and oxidative phosphorylation. Dysfunctional mitochondria, either due to specific defects in mitochondrial enzymes or general effects like hypoxia, loss of membrane potential or mtDNA depletion exhibit characteristic accumulation of metabolites. Aberrant levels of metabolites are not only signatures of pathologies, but also responsible for signaling and disease phenotype. For example, high fat diet induced mitochondrial dysfunction in metabolic syndrome and inflammation is characterized by presence of medium and long chain acyl carnitines (129). One of the metabolites, lauryl carnitine was

shown to increase expression of pro inflammatory cytokines in bone marrow derived macrophages (129). Similarly, in mouse models of Alzheimer s disease, differential levels of intermediates of nucleotide metabolism, TCA cycle and amino acid metabolic pathways accumulate (130). These data provide evidence of causal effect of defective energy metabolism. As detailed in an earlier section, altered mitochondrial function is a feature of several cancers. Mutations in enzymes of TCA cycle, components of the electron transport chain and mtDNA defects affect mitochondrial metabolism and alter the metabolome. Metabolic profiling of hereditary paraganglioma and pheochromocytoma harboring mutations in succinate dehydrogenase showed increased levels of succinate, which has been shown to inhibit prolyl hydroxylases (PHD) and stabilize HIF1 (107;109;112). Hereditary leiomyomatosis and renal cell cancers that have a high frequency of mutation in Fumarate hydratase similarly accumulate millimolar levels of fumarate, which has been shown to also inhibit PHDs and histone demethylases (108;109;112).

Fumarate has also been shown to react with the thiol group of glutathione by a process called succination and increase oxidative stress (131). Isocitrate dehydrogenase is another enzyme of the TCA cycle that is mutated in many human cancers like colon cancer, gliomas, AML and osteosarcoma. Mutations in IDH generates a neomorphic enzyme that converts isocitrate to 2-hydroxyglutarate (2-HG) instead of oxo-glutarate (110;114). 2-HG has been shown to be oncogenic through its action on PHDs, and can bring about epigenetic changes (110;114). mtDNA depletion mediated mitochondrial dysfunction induces stress signaling that transforms non-tumorigenic cells to acquire tumorigenic phenotype.

To understand the metabolic shift by partial mtDNA depletion, we carried out a metabolic profile analysis on control and mtDNA depleted C2C12 cells (Fig 4). The depleted cells showed between 3–4 fold higher lactate levels indicating a metabolic shift to glycolysis. Interestingly mtDNA depleted cells also showed higher levels of 2-HG and Fumarate, although their roles in cancer promotion and signaling need to be investigated.

VIII. Mitochondrial dysfunction modulates cellular morphological changes

It is well documented that mitochondrial dysfunction influences pathological phenotypes and morphological changes has been postulated to be an adaptive cellular response to the mitochondrial stress. Reports from our laboratory and others have shown that immortalized mammary cells harboring mitochondrial defects undergo cellular morphological reprogramming similar to an epithelial-mesenchymal transition (81;132;133). Actin microfilaments are critical components of cellular cytoskeleton. It is known that actin filaments acquire conformational changes in response to changes in nucleotide binding or cellular stressors (134–136). The interaction between actin filaments and various actin-binding proteins are critical for the functional differentiation of actin filaments *in vivo* (134;135;137). Cell polarity is dynamic and dependent on various physiological processes such as cell division, migration, and morphogenesis. Only recently, the critical role of cell polarity in regulating cancer phenotype has gained interest (138–140). We observed mtDNA-depleted C2C12 and HEK293T cells have extensive actin reorganization with long stretched F-actin filaments shown by phalloidin staining (Fig 5A, B). These are characteristics of a highly invading tumor cell.

In primary esophageal cells derived from *Tfam*^{fl/fl} mice, we observed, cells transduced *ex vivo* with adenoviral Cre contained >95% reduced *Tfam* mRNA and also with reduced mtDNA content compared to cells transduced with the adenoviral vector expressing GFP (Fig 6A,B). We observed that mtDNA depleted primary esophageal cells (*Tfam*^{fl/fl/+Cre}) were ~20% larger in size (Fig 6C). Similar to immortalized cells, primary esophageal mtDNA depleted cells also exhibit higher levels of F-actin and stretched F-actin filaments (Fig. 6D) suggesting that mitochondrial stress signaling regulate cell polarity. Turnover of actin filament and dynamic regulation of actin structures are critical for cell migration, cell adhesion and protrusion, important processes during oncogenesis (141–143). Moreover, cell polarity regulating proteins also modulate microtubule dynamics, which is significant because dysregulation of microtubule dynamics is used in cancer therapy (144). Therefore, the changes in actin reorganization we observed in response to mitochondrial stress are of relevance to tumor cell physiology, morphology and cell growth characteristics.

The 3D organoid cultures of esophageal epithelial cells (EEC) are called “mini organs” because of their close similarity to the esophageal tissue in terms of cell types, overall organization and function (145). These organoids therefore provide an excellent *ex vivo* model for studying the pathophysiology of esophagi. Using 3D organoid cultures of primary EEC we demonstrate that mitochondrial functions are necessary for normal organoid development (Fig 7). Organoids derived from *Tfam* knockout *Tfam*^{fl/fl/+Cre} murine primary esophageal cells as described in the above section, were fewer in numbers and showed reduced basal and parabasal cells. Additionally these organoids also showed higher keratinization as indicative of terminal differentiation, supporting the role of mitochondria in regulating cellular development and morphology.

Summary and Conclusions

The Warburg hypothesis proposed nearly 80 years ago still remains an enigma in cancer biology and tumor development. The first part of the Warburg hypothesis on markedly increased glycolysis and lactate production by fast growing tumors in culture or *in vivo* in tumors is universally accepted. The second part of the hypothesis suggesting a role for defective mitochondrial function as a possible cause of cancer initiation or progression remains contentious, despite intense investigations in many laboratories. Although clinical studies show strong correlation between mitochondrial dysfunction, including respiratory defects, mtDNA mutations and low mtDNA contents in a variety of human cancers, the cause or effect relationship remains unclear. Many studies including ours have shown that partial depletion of mtDNA, heteroplasmic mtDNA mutations, or disruption of Complex I and complex IV induce tumorigenic phenotypes in immortalized epithelial cells. However, *in vivo* models of tumor production specifically induced by mitochondrial dysfunction are currently limited, and more research efforts and more models would be needed to address this important question.

It is widely known that anapleurotic metabolic processes including the activity of TCA cycle and metabolism of carbon skeletons of Gln and Asn that are used as preferred fuel sources by tumor cells are essential for the production of biomass (146;147). This has led to the concept that a full mitochondrial function is essential for tumor development. Some studies

show that metastasizing tumors and those resistant to chemotherapeutic drugs, show more robust respiration and TCA cycle activity. It should be noted that mitochondrial dysfunction induced by partial mtDNA depletion or disruption of ETC complexes retain nearly full ability to metabolize amino acids and generate citrate as their citrate synthase activities are equal to or even higher than control cells (148). However, the metabolic status in Rho zero cells used in some studies (149) is quite different than that we have observed in partial mtDNA depleted cells. Furthermore, the role of mitochondrial dysfunction in early stages of tumor initiation and progression observed in our and others studies are likely to be different from that reported in tumor metastasis and drug resistance (150) where cellular signals from the microenvironment might play more critical roles. The details of these differences need to be investigated to gain a firm understanding of the second part of Warburg hypothesis. Our observations over the years clearly show that mitochondrial dysfunction and associated mtRS plays a critical role in inducing oncogenic phenotypes in immortalized cells.

It is becoming increasingly clear that mitochondrial fission and fusion play a critical role in quality control and mitochondrial damage repair (151). Our results show extensive mitochondrial fission and reduced fusion by partial mtDNA depletion and shRNA mediated knock down of CcO subunits. Thus, altered fusion and fission are closely linked to mitochondrial dysfunction and mtRS activation. The Drp1 inhibitor Mdivi-1 inhibits colony formation, MtRS and other tumorigenic characteristics. A recent study also showed that Mdivi-1 treatment inhibited tumor growth in a KRAS induced mouse tumor model (126). It is therefore likely that altered quality control associated with mitochondrial dysfunction plays a role in tumor induction and progression, while these processes are partly or wholly repaired in tumor metastasis and drug resistance.

Methods

Electron Microscopy

Cell Pellets for electron microscopic examination were fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer, pH7.4, overnight at 4°C. After subsequent buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 hour at room temperature, and then washed again in buffer followed by DH₂O. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMBED-812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software.

Esophageal Three Dimensional Organoid Cultures

Three dimensional organoid cultures were grown as described in Tanaka et al (152). Briefly, 1×10^3 cell suspension in 50 μ l Matrigel were seeded per well in 24 well plates. Cells were grown in organoid growth medium supplemented with 1X B27, 0.1 mM N-acetyl-L-cysteine (Sigma-Aldrich), mouse recombinant epidermal growth factor (R&D Systems, Minneapolis, MN), 2.0% Noggin/R-Spondin-conditioned media and 10 μ M Y27632 (Tocris Biosciences, Bristol, UK). For H&E sections, organoids were recovered by digesting Matrigel® (BD Biosciences, San Jose, CA) with Dispase I (BD Biosciences, San Jose, CA; 1 U/ml) and

fixed overnight in 4.0% paraformaldehyde. Samples were embedded in 2.0% Bacto-Agar: 2.5% gelatin prior to paraffin embedding.

Cell Migration (wound-healing) Assay

Migration assay was performed as described before (81). Confluent monolayer of C2C12 cells were scratched using a pipette tip, and cells migrating into this area were observed at 5-min intervals for 20 h under an inverted bright-field microscope. For quantitative analysis, individual cells were tracked and their direction of movement, velocity and distance covered in the direction of the wound were measured using Volocity software (Perkin Elmer, Waltham, MA, USA).

Metabolite analysis

For metabolomic analysis, metabolites were extracted in 80% methanol in water solution. Samples were analysed using an Orbitrap mass spectrometer coupled to reverse phase ion pairing chromatography (153). Data was normalized to the median and fold change were calculated using control C2C12 cells as reference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mitochondrial defects affecting μ_m induce stress signaling
- MtDNA depletion induces reorganization of actin cytoskeleton and regulates cell polarity
- MtDNA depletion induces mitochondrial fission and alters dynamics
- Inhibition of mitochondrial fission by Mdivi1 abrogates the acquired migratory potential of mtDNA depleted cells
- MtDNA depletion affects 3D organoid formation by primary esophageal epithelial cells

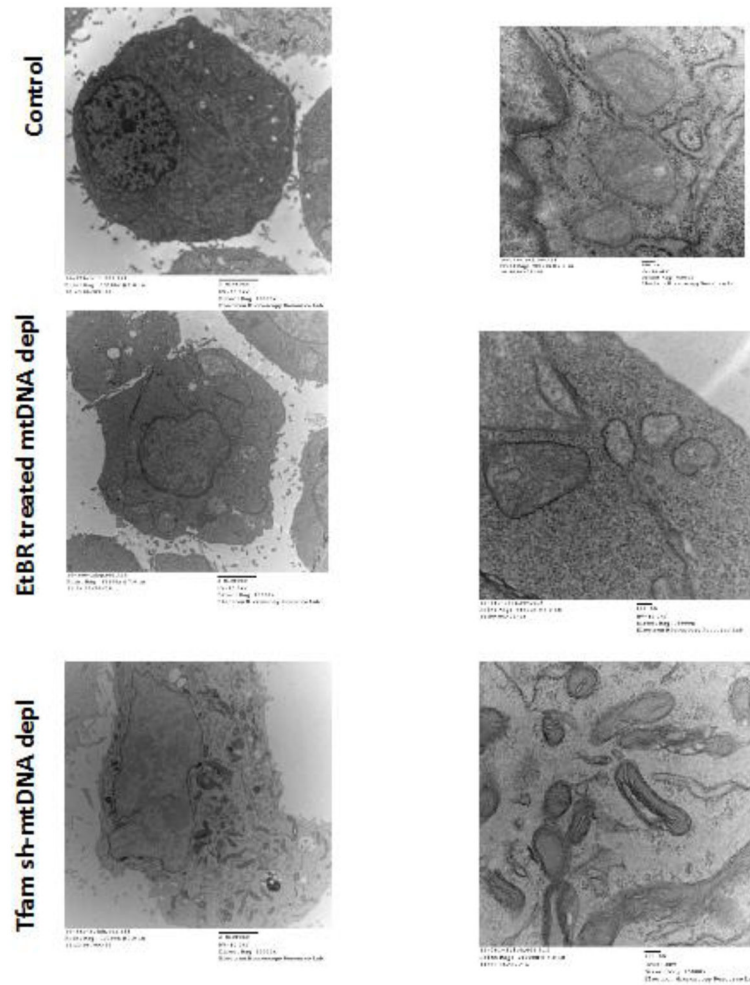


Figure 1. Altered mitochondrial ultrastructure in response to mtDNA depletion
 Electron micrographs of control, EtBr-treated mtDNA-depleted and *Tfam* shRNA-mtDNA depleted cells. *Left panel*: Scale Bar 2µm, magnification 10000x; *Right panel*: Scale Bar 100nm, magnification 75000x.

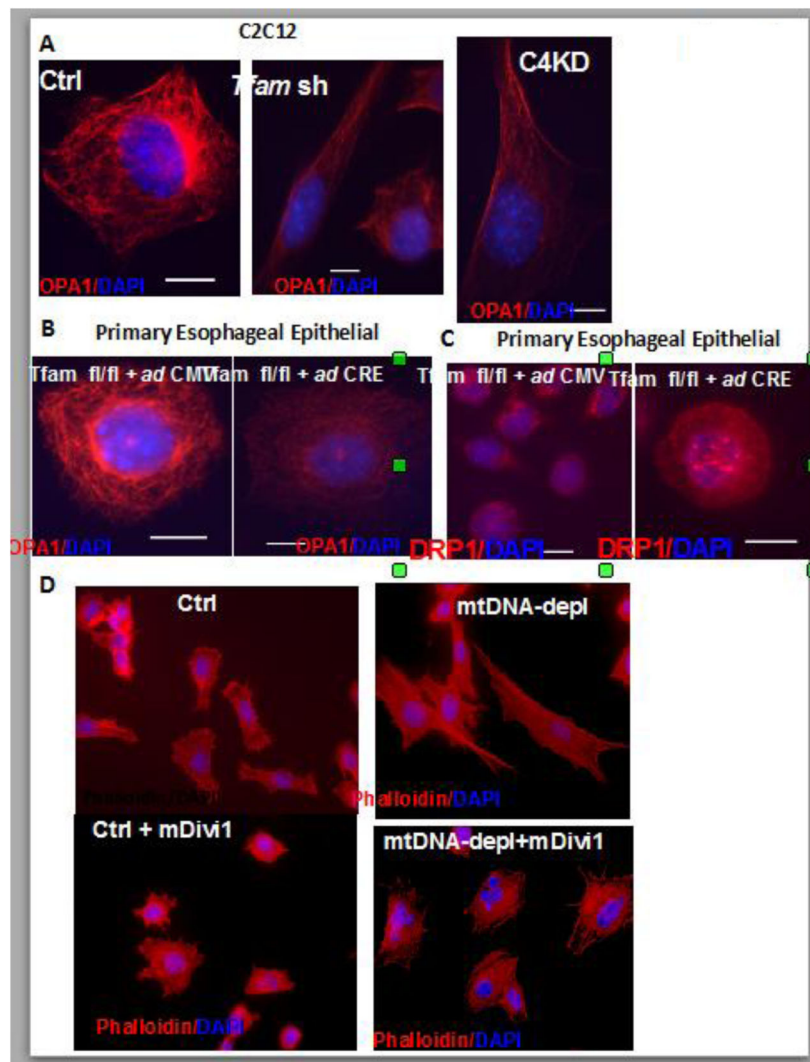


Figure 2. Altered mitochondrial fission and fusion dynamics in mtDNA depleted cells
(A) Immunofluorescence images showing OPA1 (mitochondrial fusion marker in red) and DAPI (nuclei in blue) staining pattern in control, mtDNA depleted (*Tfam* sh) and CcOIVi1kd C2C12 cells. **(B)** Primary esophageal epithelial cells derived from *Tfam*^{fl/fl} mice expressing either *Adeno GFP* (control) or *AdenoL2- Cre* (*Tfam* KO) stained with OPA1 (mitochondrial fusion marker in red) and DAPI (nuclei in blue). **(C)** Primary esophageal epithelial cells derived from *Tfam*^{fl/fl} mice expressing either *Adeno GFP* (control) or *AdenoL2- Cre* (*Tfam* KO) stained with DRP1 (mitochondrial fission marker in red) and DAPI (nuclei in blue). **(D)** Primary esophageal epithelial cells derived from *Tfam*^{fl/fl} mice expressing either *Adeno GFP* (control) or *AdenoL2- Cre* (*Tfam* KO) treated with or without mDivi1 (10 μ M, 48h) stained with Texas-Red Phalloidin (red, actin) and DAPI (blue, nuclei). Images are captured under 40x objective of a NIKON E600 microscope.

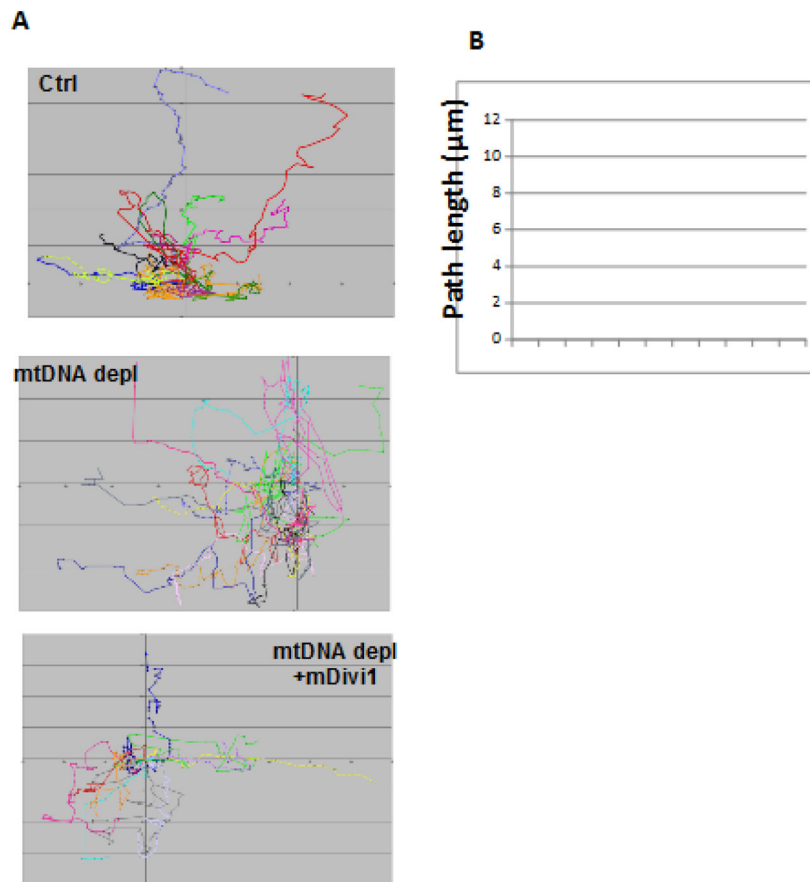


Figure 3. Acquired Migratory pattern of C2C12 cells

(A) Windrose plot showing the directionality of migration of Control, mtDNA depleted and mtDNA depleted cells treated with mDivi1 as indicated in the figure. Individual cells tracked are indicated by different colors. (B) Individual cells in each category were tracked using the Volocity software (Perkin Elmer) to estimate the maximum distance covered during the 10h migration. **Movie Legends:** Time lapse recordings for “scratch wound healing” migration assay: Control (Video 1), mtDNA- depleted (Video 2) and mtDNA-depleted + mDivi1 treated (Video 3) C2C12 cells were grown to confluence and wounded with a pipette tip. Wound healing as a measure of cell motility and images were captured every 5 mins and followed for 10 hours.

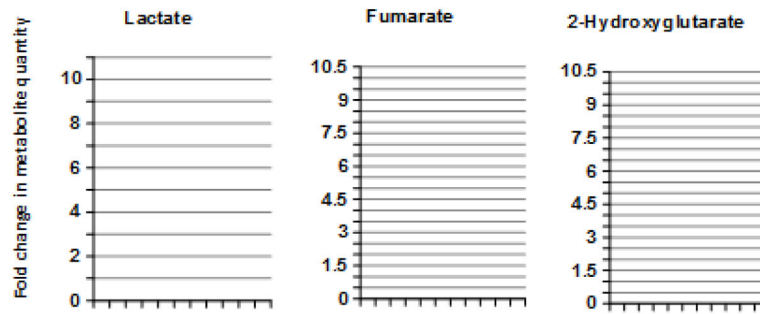


Figure 4. Metabolic changes in response to mtDNA depletion

Intracellular lactate, fumarate and 2-hydroxyglutarate were quantified by reverse phase LC coupled to an Orbitrap Mass Spectrometer. Graphs show fold change in indicated metabolites between control and mtDNA depleted C2C12 cells. Values normalized to control cells.

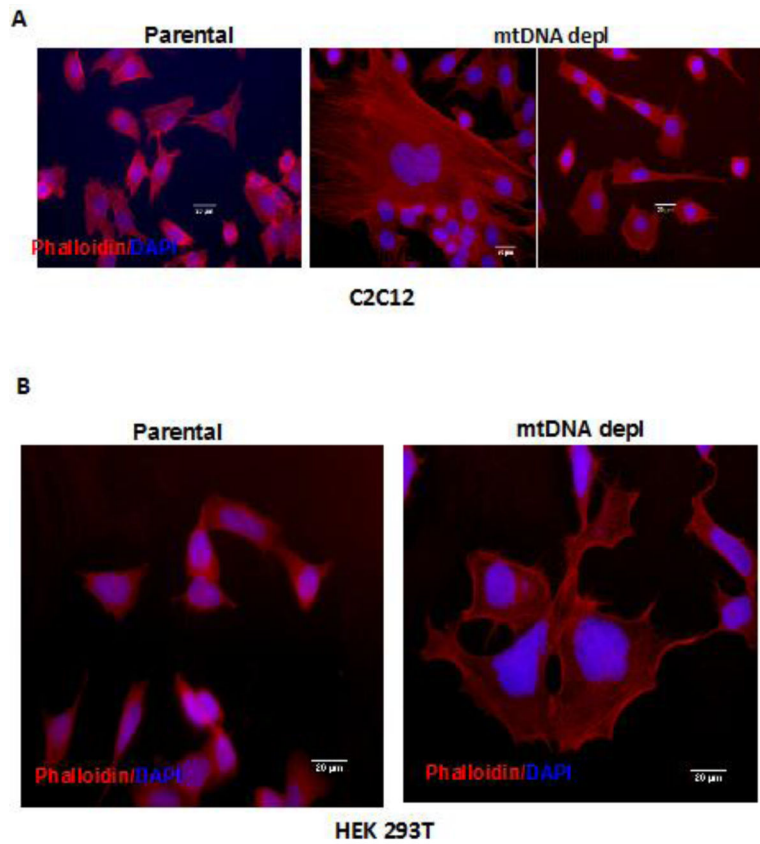


Figure 5. MtDNA depletion induced cytoskeletal reorganization in immortalized cells Control and mtDNA depleted C2C12 (**A**) and HEK293T (**B**) stained with Texas-Red® conjugated Phalloidin (Molecular Probes) for gamma Actin (red) and DAPI for nuclei (blue). Phalloidin staining was performed according to manufacturer's suggested protocol. Cells were imaged under Nikon E600 microscope 40x Objective. Scale Bar: 20µm.

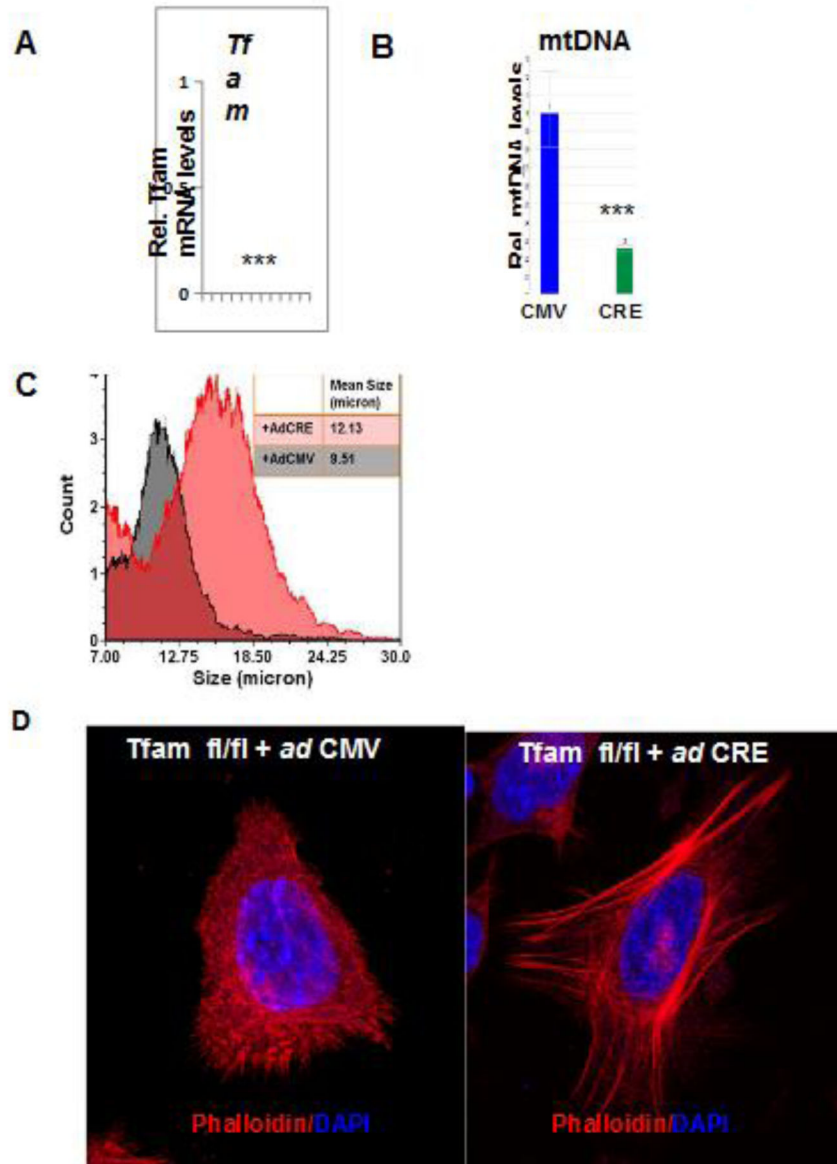


Figure 6. Alterations in cellular morphology in *Tfam*^{fl/fl/Cre}-primary esophageal cells Real Time PCR quantitation of relative *Tfam* mRNA levels (**A**) and mtDNA content (**B**) in primary esophageal epithelial cells derived from *Tfam*^{fl/fl} mice expressing either *Adeno GFP* (control) or *AdenoL2- Cre* (*Tfam* KO). (**C**) Cell Size distribution in primary esophageal epithelial cells derived from *Tfam*^{fl/fl} mice expressing either *Adeno GFP* (control) or *AdenoL2- Cre* (*Tfam* KO) assessed on Nexcelom Vision CBA. (**D**) Primary esophageal epithelial cells derived from *Tfam*^{fl/fl} mice expressing either *Adeno GFP* (control) or *AdenoL2- Cre* (*Tfam* KO) stained with Texas-Red Phalloidin (red, actin) and DAPI (blue, nuclei) imaged under 40x objective of a NIKON E600 microscope.

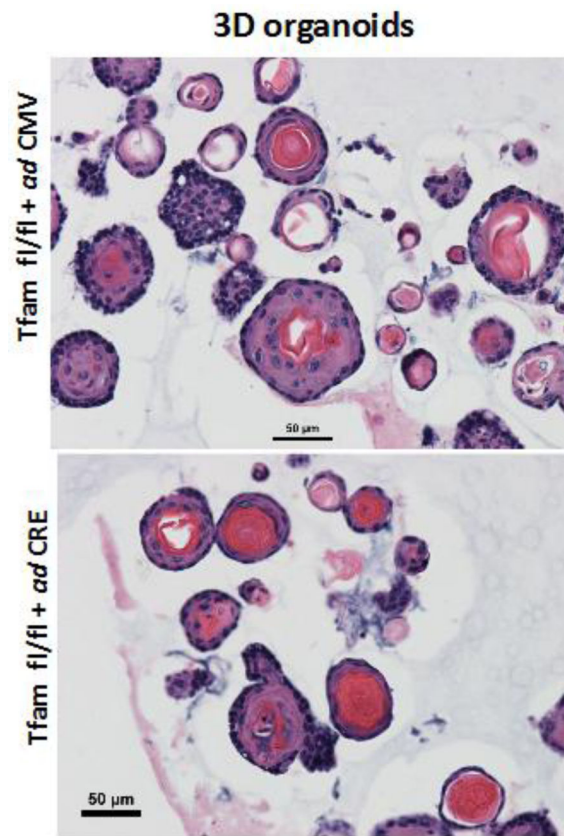


Figure 7. Three dimensional organoids in *Tfam*^{fl/fl/Cre}-primary esophageal cells
Bright Field images of Hematoxylin-Eosin stained sections of 3D organoids from primary esophageal epithelial cells derived from *Tfam*^{fl/fl} mice expressing either *Adeno GFP* (control) or *AdenoL2-Cre* (*Tfam* KO).