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MITOCHONDRIA AND THEIR ROLE IN CARCINOGENESIS

MITOCHONDRIE A JEJICH ROLE V KARCINOGENEZE

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This thesis was composed in the Institute of Biotechnology, Czech Academy of Sciences, in the Laboratory of Molecular Therapy. Experimental data included herein were published in four original research articles.

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree.

Prague, 2021 Mgr. Martina Bajžíková

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ABSTRACT (EN)

Mitochondria are the principal intracellular organelles responsible for fuel generation; however, they are not just cell powerhouses but are involved in a range of other intracellular functions including cell metabolism, proliferation, death, and immune responses. Loss of function in mitochondria will result in oxidative stress, which is one of the underlying causal factors for a variety of diseases including cancer.

Cancer cells can predominantly produce energy by glycolysis even in the presence of oxygen. This alternative metabolic behavior is known as the “Warburg Effect.” Linked to this, cancer cell mitochondria can switch between glycolysis and oxidative phosphorylation (OXPHOS) for their energy requirements and survival. The electron transport chain (ETC) function is pivotal for mitochondrial respiration, which is also needed for dihydroorotate dehydrogenase (DHODH) activity that is essential for *de novo* pyrimidine synthesis.

In our research, we have used respiration-deficient cancer cells to challenge the dogma that mitochondria with their DNA are constrained within cells in the body. Our results document that mitochondria move from normal cells within the tumor stroma to tumor cells without mitochondrial DNA (mtDNA), resulting in long-lasting recovery of mitochondrial functions and, consequently, efficient tumor formation. The evidence for our ground-breaking discovery comes from the identification of mtDNA polymorphisms, establishing the origin of mitochondria in the donor cells.

Our research shows that pyrimidine biosynthesis, which depends on respiration-linked DHODH, is required to overcome cell-cycle arrest, while tumor cells do not need mitochondrial ATP to form tumors. Based on the above considerations, we uncover the therapeutic implications of DHODH inhibition via two parallel inhibitory pathways with potential consequences for tumor therapy.

Keywords

cancer metabolism, mitochondria, electron transport chain, dihydroorotate dehydrogenase, mitochondrial transfer

ABSTRACT (CZ)

Mitochondrie jsou hlavním místem výroby buněčné energie; nejsou to však jen buněčné elektrárny, ale podílejí se na řadě dalších funkcí uvnitř buňky, včetně buněčného metabolismu, proliferace, smrti a imunitních reakcí. Ztráta mitochondriálních funkcí má za následek oxidační stres, který je jedním z hlavních faktorů pro řadu onemocnění a mimo jiné i rakoviny.

Rakovinné buňky produkují energii převážně glykolýzou a to i za přítomnosti kyslíku. Tento alternativní metabolický jev je znám také jako „Warburgův efekt“. V souvislosti s tím mohou mitochondrie rakovinných buněk přepínat mezi glykolýzou a oxidativní fosforylací (OXPHOS), pokud jde o jejich energetické požadavky a přežití. Funkce elektronového transportního řetězce je klíčová pro buněčné dýchání, které je také nutné pro aktivitu dihydroorotát dehydrogenázy (DHODH), nezbytnou pro de novo syntézu pyrimidinu.

V našem výzkumu jsme použili rakovinné buňky s poruchou dýchání, abychom vyvrátili dogma, že mitochondrie a jejich genom jsou omezeny uvnitř buněk v těle. Naše výsledky prokázaly, že mitochondrie jsou schopny pohybu v nádorovém stromatu a to z normálních buněk do nádorových buněk bez mitochondriální DNA (mtDNA), což má za následek dlouhotrvající obnovu dýchání a následně účinnou tvorbu nádoru. Důkaz našeho převratného objevu spočíval v identifikaci polymorfismů mtDNA, které stanovily původ mitochondrií z dárcovských buněk.

Náš výzkum odhalil, že tvorba pyrimidinů, která je závislá na DHODH spojeném s dýcháním, je nezbytná pro překonání zástavy buněčného cyclu, zatímco mitochondriální ATP k tvorbě nádorů potřeba není. Na základě výše uvedených skutečností jsme odhalili léčebné důsledky inhibice DHODH, a to prostřednictvím dvou paralelních inhibičních drah, a jejich potenciální využití pro nádorovou terapii.

Klíčová slova

rakovinný metabolismus, mitochondrie, elektron-transportní řetězec, dihydroorotát dehydrogenáza, mitochondriální přenos

LIST OF ABBREVIATIONS

α -KG	α -ketoglutarate
acetyl-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
ATM	ataxia-telangiectasia mutated gene
ATP	adenosine triphosphate
ATR	ataxia-telangiectasia and Rad3 related
CAFs	cancer-associated fibroblasts
Chk	checkpoint kinase
CoQ	coenzyme Q
CS	citrate synthase
CTC	circulating tumor cells
Cyt c	cytochrome c
Cytb	cytochrome b
DHODH	dihydroorotate dehydrogenase
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
EtBr	ethidium bromide
FMN	flavin mononucleotide
FADH ₂	flavin adenine dinucleotide
FH	fumarate hydratase
GPX	glutathione peroxidase
HIF1	hypoxia inducible factor 1
IDH	isocitrate dehydrogenase
IMM	inner mitochondrial membrane

IMS	inter-membrane space
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
MAPK	mitogen-activated protein kinase
MDH	malate dehydrogenase
MSC	mesenchymal stem cell
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
nDNA	nuclear DNA
nBFP	nuclear blue fluorescent protein
NFκB2	nuclear factor-κB2
NRF2	nuclear factor-like 2
NGS	next-generation sequencing
OAA	oxaloacetate
OSCP	oligomycin sensitivity–conferring protein
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PCC	pheochromocytoma
PDH3	prolyl hydroxylase 3
PGL	paraganglioma
Pi	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
PTEN	phosphatase and tensin homolog
qPCR	quantitative real-time PCR
ρ0	mtDNA depleted cells
ROS	reactive oxygen species
RPL5, 11	ribosomal Protein L5, 11

rRNA	ribosomal RNA
SC	supercomplex
SCL	lung metastatic tumor cells
SCS	succinyl-CoA synthetase
SDH	succinate dehydrogenase
SOD	superoxide dismutase
SQR	succinate ubiquinone reductase
TCA	tricarboxylic acid cycle
TFAM	mitochondrial transcription factor A
tRNA	transfer RNA
UbQ	ubiquinone
UbQH2	ubiquinol
UMP	uridine monophosphate
VDAC	voltage-dependent anion channel

LITERATURE OVERVIEW

1. Introduction

Cancer is the term for a broad spectrum of diseases with common features involving abnormal cell division without control. Malignant cells are well documented to rewire their metabolism to satisfy the biosynthetic demands of rapid proliferation, continuous growth, and long-term maintenance. Since the basic property of cancer cells is their constant proliferation, altered metabolism is now considered an important hallmark of cancer development. The most common postulated feature of this altered metabolism is increased uptake of glucose and its subsequent fermentation to lactate. Otto Warburg observed this phenomenon in the 1920s and proposed it to be the fundamental cause of cancer. This process, known as the Warburg effect, occurs even in the presence of fully functional mitochondria (Warburg, 1925; Warburg, 1956).

However, tumors arise, progress, and respond to therapy in the context of a complex interaction with the host immune system. Tumor formation can be controlled by the innate and adaptive immune system; nevertheless, after some time, the selective immune pressure develops cancer cells that involve different mechanisms that mimic peripheral immune tolerance, thus escaping tumoricidal attack (Gonzales et al., 2018). Furthermore, cancer cells are capable of modulating energy metabolism within the tumor microenvironment including immune cells, and inducing ‘metabolic anergy’ of the antitumor immune response.

Taken together, mitochondria gained a central and multifaceted role in cancer formation and progression, and targeting mitochondria provides therapeutic opportunities. This thesis reviews the fundamental influence of mitochondrial metabolism and signaling in malignant transformation and tumor progression.

2. Mitochondria in cancer development

Mitochondria supply energy, provide cellular building blocks and control redox homeostasis, cell death, immune and oncogenic signaling. These numerous functions of

mitochondria in normal physiology make them major sensors of environmental stress and allow for cellular adaptation to the environment (Kroemer and Poysssegur, 2008). Cancer is generally assumed to be a genetic disease involving mutations in nuclear oncogenes and tumor suppressor genes, and there are several mechanisms by which mitochondria are involved in cancer development (Hanahan and Weinberg, 2011). It was believed that altered mitochondrial metabolism is only a ‘cancer by-product’, but it has turned out that they play an intricate role in cancer cell viability (Weyandt et al., 2017).

Mitochondria are best known for harvesting and storing energy released during the oxidation of organic molecules by aerobic respiration. However, the biosynthetic functions of mitochondria are at least as important for tumor progression as ATP generation (Vander Heiden et al., 2017). ATP production is a response to the energy demand in the cell and varies depending on cellular conditions and the microenvironment (Sullivan et al. 2015, Birsoy et al., 2015). Mammalian cells rely on both OXPHOS and glycolysis to produce ATP. Nevertheless, the contribution ratio of OXPHOS versus glycolysis varies in different cell types, growth states, and microenvironments. Under normal conditions, about 70% of ATP is known to be synthesized by OXPHOS and the rest by glycolysis. Cancer cells use mainly glycolysis (Warburg effect) even in the presence of sufficient oxygen (Warburg, 1925, Warburg, 1956). Aerobic glycolysis in many cancers is a result driven by various factors, such as the activation of oncogenes, the loss of tumor suppressors, the hypoxic microenvironment, mtDNA mutations, and the tissue of origin (Porporato et al., 2018). It should be noted that aerobic glycolysis is not that efficient of a source of ATP as OXPHOS; glycolysis/OXPHOS cells generate 36 ATP molecules per glucose molecule, while aerobic glycolysis produces only 4 ATP molecules (Lehninger et al., 1993). The advantage of aerobic glycolysis is in terms of the formation of other required metabolites serving as biomass precursors for rapidly proliferating cells. Also, glycolysis, under conditions of sufficient levels of glucose, is kinetically superior to OXPHOS (Koppenol et al., 2011).

The predominance of the Warburg effect in cancer is more due to upregulated glycolysis suppressing OXPHOS rather than OXPHOS dysfunctions. Nowadays, it has also been recognized that mitochondrial respiration of most cancer cells is not severely impaired, and if glycolysis is inhibited, the function of mitochondrial OXPHOS can be

easily restored (Moreno-Sanchez et al., 2014). Moreover, the dysfunction in mitochondrial OXPHOS probably leads to decreased cancer cell proliferation, and in some cases, the function of OXPHOS in cancer cells is even higher than in normal cells (Zu et al., 2004). This is the case in transformed mesenchymal stem cells (MSCs), which are supposed to be sarcoma-initiating. Besides the increased OXPHOS, they have the ability to switch to glycolysis to adapt to their microenvironment. Another study showed the essential role of OXPHOS in tumorigenesis using a model of mitochondrial transcription factor A (TFAM) deficiency. The loss of TFAM protein abolished the tumorigenicity in an oncogenic Kras-driven mouse model (Weinberg et al., 2010).

A growing amount of experimental evidence indicates that OXPHOS affects the production of ATP more significantly in malignant cells than in normal cells because respiration is essential for cell proliferation. In addition, current findings point out that the main reason for the sustained respiration in rapidly proliferating cancer cells is the need for biosynthesis of nucleotides such as aspartate and pyrimidine and not ATP production (Birsoy et al., 2015; Mathur et al., 2017).

2.1. Mitochondrial energy metabolism and carcinogenesis

Mitochondria are organelles ranging from 0.5 to 2 μm in length, found in the cytoplasm of most eukaryotic cells. Mitochondria contain inner and outer membranes (IMM and OMM) separated by intermembrane space (IMS), and the space contained by the IMM is the mitochondrial matrix (mt-matrix).

The OMM serves as a signaling platform that connects mitochondria to other organelles within the cell, such as the endoplasmic reticulum (ER) and lysosomes, and the plasma membrane (Ellenrieder et al., 2017). The most abundant protein of the OMM is the voltage-dependent anion channel (VDAC), best known for its function as the gateway mediating the fluxes of metabolites, nucleotides, and ions between mitochondria and other cellular compartments (Shoshan-Barmatz et al., 2006).

The IMM contains a high protein fraction and allows only certain small molecules to pass through. The phospholipid composition of the OMM and IMM is unique for each membrane, and the IMM has an unusually high protein-to-lipid ratio of about 80:20,

compared to that of the OMM, where this ratio is 50:50 (Casares et al., 2019). The numerous dynamic invaginations of the IMM are called cristae, and they provide more surface area for cellular respiration (Mannella, 2006). The shape and number of these invaginations depend on the energy demand of the tissue; in muscle cells, for example, mitochondria are particularly rich in cristae (Nielsen et al., 2017).

The IMS is usually 60-80 Å in width and contains enzymes that use ATP molecules moving out of the mt-matrix to phosphorylate other nucleotides and proteins that initiate apoptosis (Johannes et al., 2010).

Finally, mt-matrix is extremely viscous and is the site of mtDNA replication, transcription, protein biosynthesis, and numerous enzymatic reactions, including those of the tricarboxylic acid (TCA) cycle, anaplerotic and cataplerotic reactions, transamination reactions, and others (Anderson et al., 2018).

2.1.1. Oxidative phosphorylation

The mitochondrial respiratory machinery is embedded in the IMM and consists of the ETC, which is composed of four large respiratory complexes (RCs; CI-CIV) and links the fifth complex, ATP synthase, and two mobile carriers ubiquinol (UbQH₂) and cytochrome c (Cyt c) (Lenaz and Genova, 2010). In the electron transport chain, electrons retrieved from substrates are passed from one molecule to another. CI, CIII and CIV use the energy released as these electrons transfer to move protons across the IMM, thus acting as proton pumps (Figure 1.). This generates the electrochemical gradient that is used to catalyze the condensation of ADP and inorganic phosphate into ATP by ATP synthase (Murphy, 2009).

CI is a multi-subunit complex consisting of 45 polypeptides of which 14 central or essential subunits harbouring the bioenergetic core functions. Only seven hydrophobic subunits (ND1, ND2, ND3, ND4, ND4L, ND5, ND6) are encoded by mtDNA (Signes et al., 2018). All other CI subunits and all assembly factors are encoded by nuclear DNA (nDNA) (Stroud et al., 2016). CI contains one non-covalently bound flavin mononucleotide (FMN) and eight iron-sulfur (Fe-S) clusters as cofactors. CI utilizes nicotinamide adenine dinucleotide (NADH) formed by glycolysis and the TCA cycle to pump protons out of the mt-matrix. The stepwise movement of electrons from NADH and FADH₂ to ubiquinone (UQ) through the FMN molecule and Fe-S clusters is coupled with the translocation of

protons from the mt-matrix to the IMS. This proton gradient generates the proton-motive force that powers ATP synthesis (Vinothkumar et al., 2014).

Mutations in mitochondrial and nuclear genes that encode CI subunits contribute to several pathological conditions such as neurodegenerative diseases, diabetes, and cancer. Mutations in mitochondrial genes that encode the CI subunits ND2 and ND4-6 were shown to be associated with CI deficiency and enhanced proliferation in a model of renal oncocytoma (Gasparre et al., 2008). Mutations of the ND5 subunit leading to its overexpression were shown to be associated with a drop in oxygen consumption, increased reactive oxygen species (ROS) production, potentiated glucose dependency, and enhanced tumor growth (Park et al., 2009).

CII (succinate dehydrogenase, SDH) is unique, as it links the ETC with the TCA cycle. SDH is composed of four nuclear-encoded subunits, SDHA and SDHB protrude into the mt-matrix and hydrophobic subunits SDHC and SDHD anchor the catalytic components to the IMM and produce the binding site for UQ (Signes et al., 2018). As a component of the TCA cycle, CII is responsible for oxidizing succinate to fumarate. The two electrons produced during the oxidation are directly transferred to UQ. This is coupled with the reduction of UQ to UbQH₂ in the ETC. UbQH₂ is oxidized by CIII, and the electrons are transferred to Cyt c (Lenaz and Genova 2010; Sun et al. 2005).

CII is linked to tumor progression by the fact that it is a major player in mitochondrial ROS generation (Raha et al., 2000). SDHx mutations in paraganglioma (PGL) and pheochromocytoma (PCC) lead to dysfunction of CII followed by accumulation of succinate, which acts as an oncometabolite (Papathomas et al., 2013).

CIII is also called Cytochrome c reductase based on its function. It is a multi-subunit transmembrane protein with three redox subunits, Cytochrome b (Cytb), Cytochrome c1, and the iron-sulfur protein, which all together form the catalytic core. Only Cytb is encoded by mtDNA and is fundamental for the assembly and function of CIII (Sousa et al., 2018). The additional supernumerary subunits have no well-established cellular function (Signes et al., 2018). This complex receives electrons from UQ and passes them to Cyt c (Lenaz and Genova, 2010; Trumpower, 2002).

CYTB gene mutations were reported in various tumor types, including colorectal (Polyak et al., 1998), ovarian (Liu et al., 2001), breast (Owens et al., 2011), and bladder

(Fliss et al., 2000) cancers. It was demonstrated that Cytb overexpression increases cell growth and invasion accompanied by increased ROS production both *in vitro* and *in vivo*. In this case, the elevated ROS production leads to upregulation of the nuclear factor- κ B2 (NF κ B2) signaling pathway, resulting in suppression of apoptosis (Dasgupta et al., 2008).

CIV, also known as cytochrome c oxidase (COX), consists of three core subunits, COX I, II, and III, encoded by mtDNA, and 10 accessory nuclear-encoded subunits (Signes et al., 2018). CIV is the terminal and rate-limiting step of OXPHOS, catalyzes oxidation of Cyt c and reduction of oxygen to water (Belevich et al., 2006). This electron transport-released energy is used to drive translocation of protons from the mt-matrix. Consequently, CIV generates the trans-membrane proton gradient, used by CV as an electrochemical proton-motive force to generate ATP (Breuer et al., 2013, Lenaz et al., 2010). Of particular interest is its feedback inhibition by ATP, known as the allosteric ATP-mediated inhibition of CIV, which depends on the ATP/ADP ratio (Arnold et al., 1997). This mechanism maintains low and ‘healthy’ mitochondrial membrane potential and prevents ROS formation (Kadenbach, 2021).

The link between CIV activity and cancer is controversial, in general, mtDNA-encoded subunits are considered to be tumor-suppressing while their nDNA-encoded counterparts are tumor-promoting (Gaude et al., 2014). Mutations of the COXI have been associated with ovarian (Permuth-Wey et al., 2011) and prostate (Petros et al., 2005) cancer.

Complex V (CV, mitochondrial ATP synthase, F1F0 synthase) is a large multi-subunit complex of ~600 kDa and consists of two distinct domains: F1 is its soluble part present in the mt-matrix, and F0 is bound to the IMM. F1 is composed of nine subunits, three copies of each of subunits α and β , and one each of subunits γ , δ and ϵ . F1 subunits γ , δ and ϵ constitute the central stalk of CV (Gibbons et al., 2000). F0 consists of the subunit c-ring and single copies of subunits a, b, d and F6; the oligomycin sensitivity-conferring protein (OSCP) and the accessory subunits e, f, g and A6L (Stock et al., 1999). Only a and A6L subunits are encoded by the mtDNA genes (ATP6 and ATP8, respectively) (Signes et al., 2018). ATP synthase is the complex where ATP is produced using adenosine diphosphate (ADP) and inorganic phosphate (Pi) in a reaction at the end of the OXPHOS process. As previously mentioned, the energy released by the movement of protons according to their

electrochemical potential is used as the proton-motive force for ATP synthase (Mitchell, 2011).

Homoplasmic mutations in both mitochondrial genes were found in patients with breast cancer (Parella et al., 2001). Additionally, a different role of CV in cancer progression is presented by the control of cell migration and metastasis. Increased activity of OXPHOS, leading to increased cell migration and invasiveness was observed in circulating cancer cells compared to primary tumor-derived cells in a 4T1 mouse adenocarcinoma model (LeBleu et al., 2004).

It is important to mention that recent data have suggested that ETC complexes are organized in higher structural assemblies called supercomplexes (SCs) (Schagger et al., 2000). These SCs increase substrate channeling and the efficacy of electron transfer through the ETC, reduce the rate of production of ROS, and prevent the aggregation of IMM proteins (Acin-Perez et al., 2008). CI associates with a dimer of CIII and one to several units of CIV, mediating direct transfer of electrons from NADH (Schagger et al., 2001). Compared with free complex, CI in the respirasome is more compact due to its associations with CIII and CIV (Acin-Perez et al., 2004). These higher-order complexes may be beneficial for maintaining the biochemical structure and increasing the physiological activity of individual complexes while improving the efficacy of electron movement.

2.1.2. Tricarboxylic acid cycle

The TCA cycle, also known as the Krebs or citric acid cycle, is the main source of cellular energy and an indispensable part of aerobic respiration (Figure 1.). It integrates the carbohydrate, protein, and lipid metabolism pathways. In a healthy cell, glycolysis is responsible for oxidation of the glucose molecule into pyruvate to produce ATP, which is then decarboxylated into acetyl coenzyme A (acetyl-CoA) as it enters mitochondria, allowing it to engage in the TCA cycle. The cycle harnesses the available chemical energy of acetyl-CoA into its reducing equivalents NADH and FADH₂ (Voet et al., 2016).

The enzyme citrate synthase (CS) combines the two-carbon acetyl-CoA, generated from fatty acids, amino acids, or pyruvate oxidation, with a four-carbon oxaloacetate (OAA) to generate the six-carbon compound citrate. Citrate loses one water molecule and gains

another as citrate is converted into its isomer, isocitrate. In the next step, α -ketoglutarate (α -KG), formed by oxidative decarboxylation of isocitrate catalyzed by isocitrate dehydrogenase (IDH), is converted into succinyl-CoA (SucCoA) by further decarboxylation through the α -KG dehydrogenase complex. SucCoA is then transformed to succinate by the succinyl-CoA synthetase (SCS). Fumarate is produced by succinate oxidation catalyzed by SDH (CII) with the production of a molecule of FADH_2 and hydration of fumarate to malate by fumarate hydratase (FH). Oxidation of malate is catalyzed by malate dehydrogenase (MDH) to regenerate OAA, which produces $\text{NADH} + \text{H}^+$, thus finally ensuring completion of the cycle (Voet et al., 2016).

Mutations in TCA cycle-related genes that encode SDH and FH lead to accumulation of their respective substrates, succinate and fumarate, in a subset of human cancers. These oncometabolites induce the stabilization of transcription factors as hypoxia-inducible factor 1 (HIF1) and nuclear factor-like 2 (Nrf2). They also inhibit p53 and prolyl hydroxylase 3 (PDH3) activity, regulate DNA/histone methylation, thereby activating cell growth signaling (Sajnjani et al., 2017). Moreover, they increase glutaminolysis, glycolysis, and production of ROS. These altered phenomena in cancer could, in turn, promote carcinogenesis by stimulating cell proliferation and survival. Overall, the inherited and acquired genetic alterations of TCA cycle enzymes lead to the generation of oncometabolite intermediates, which are important driving forces of neoplastic transformation (Raimundo et al., 2011). Understanding and applying the knowledge of these mechanisms is likely to open new therapeutic options.

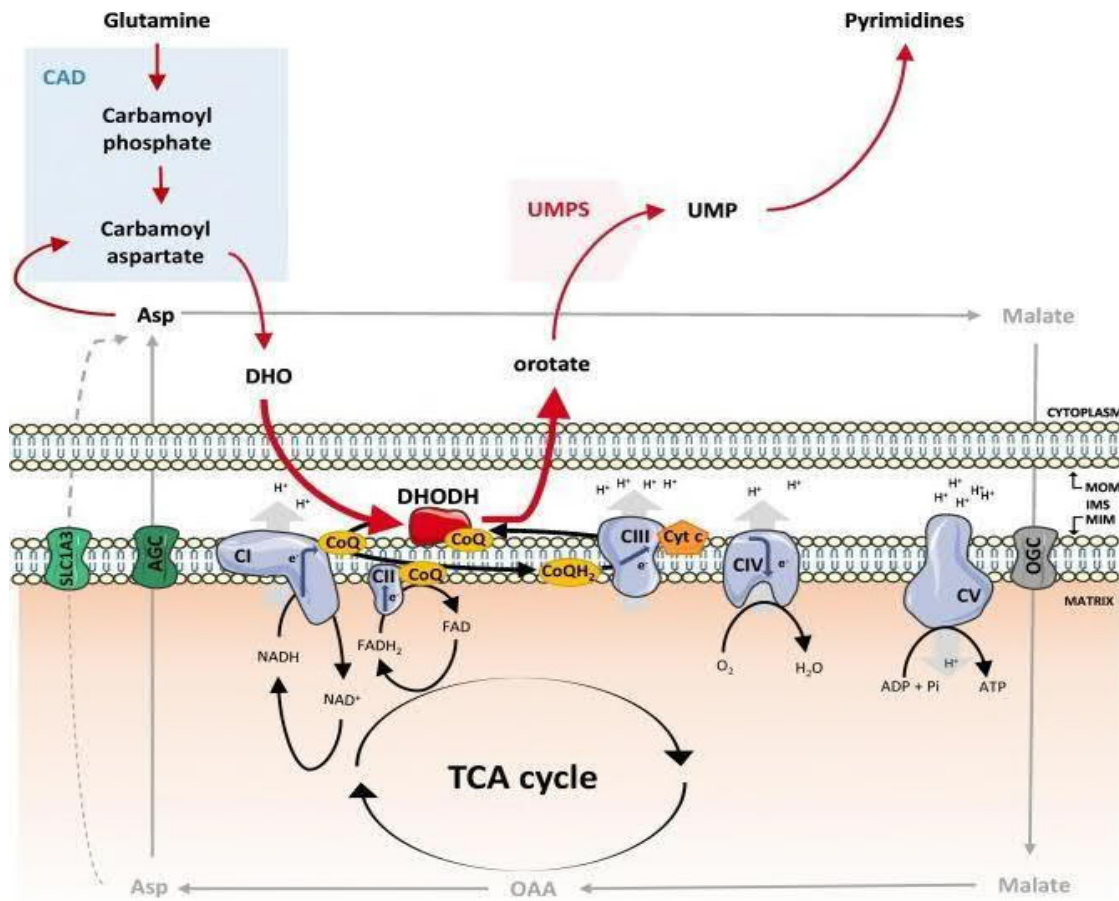


Figure 1. A schematic representation of the mitochondrial OXPHOS system and *de novo* pyrimidine synthesis pathway. Electrons transferred through the ETC originate at CI, which oxidises NADH from the TCA cycle, or at CII, which reduces FAD to FADH₂. Electrons are then transferred, via CIII and two mobile carriers UbQ and Cyt c, to CIV, where they reduce molecular oxygen to form water. These reactions are coupled to the creation of a proton gradient across the mitochondrial inner membrane, which is used to form ATP via ATP synthase (CV). DHODH converts dihydroorotate to orotate, generating electrons that are transferred via redox-cycling of ubiquinone to complex III. ADP (adenosine diphosphate); Asp (aspartate); AGC (aspartate-glutamate carriers 1/2); ATP (adenosine triphosphate); CI, CII, CIII, CIV, CV (respiratory complexes I, II, III, IV, V); CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, dihydroorotase); CoQ (coenzyme Q); Cyt c (cytochrome c); DHO (dihydroorotate); DHODH (dihydroorotate dehydrogenase); FADH₂ and FAD (reduced and oxidized forms of flavin adenine dinucleotide); IMS (intermembrane space); MIM (mitochondrial inner membrane); MOM (mitochondrial outer membrane); NADH and NAD⁺ (reduced and oxidized forms of nicotinamide adenine dinucleotide); OAA (oxaloacetate); OGC (2-oxoglutarate carrier); P_i (inorganic phosphate); SLC1A3 (sodium-dependent glutamate-aspartate transporter 1); UMP (uridine monophosphate); UMPS (uridine monophosphate synthetase). (Boukalova et al., 2020).

2.1.3. Reactive oxygen species

Cancer cells exhibit many characteristics that can contribute to tumor growth and progression. Mitochondria are important intracellular ROS generation organelles, and cancer cells produce increased levels of ROS compared to their healthy counterparts. Mitochondria possess at least nine different enzyme systems that can produce ROS (Starkov et al., 2008). ROS, including free radicals, oxygen ions and peroxides, are by-products of normal bioenergetic metabolism (Cross et al. 1987). ROS are engaged in a broad variety of redox-governing activities that are crucial to preserve redox homeostasis within the cell. Under physiological conditions, mitochondria utilize approximately 98% of the total amount of inhaled O₂, including 1-2% of mitochondrial ROS (mROS), and this amount of ROS is stable in a dynamic equilibrium (Cadenas and Davies., 2000). This redox balance is maintained by antioxidant proteins, such as superoxide dismutase (SOD), catalase, thioredoxin, glutaredoxin, glutathione peroxidase (GPX) (Trachootham et al., 2008). The cells essentially utilize mROS for modulating cell proliferation, the cell cycle or programmed cell death, with ROS acting as a secondary messenger in these signal-transduction pathways.

Because of its vicinity to the ETC, mtDNA is much more sensitive to oxidative stress-related damage from ROS than nDNA (Yakes et al., 1997). For decades it was believed that mtDNA had limited capacity for DNA proofreading, and it was observed only a few years ago that mitochondria possess many of the DNA repair pathways operational in the nucleus (Circu et al., 2010). mtDNA contains mainly genes encoding ETC proteins; once they are mutated as a consequence of ROS production, an 'endless' cycle commences in which they increase ROS production.

It was assumed that cancer cells produce more ROS than healthy cells, leading to further genomic instability that promotes neoplastic transformation (Ames et al., 1993; Weinberg et al., 2010). One of the most important signaling cascades activated by ROS is the mitogen-activated protein kinase (MAPK) cascade (Martindale and Holbrook, 2002). Four different signaling pathways responsible for cell proliferation and differentiation have been defined among MAPK cascades: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK5 (Corcoran et al., 2013). In this

context, Ras-ERK cascades are highly regulated cascades since they are a crucial trigger for the development of many types of cancer (Vos et al., 2003).

ROS-induced hyperactivation of the PI3K/Akt survival pathway has been shown to be activated in numerous cancers. ROS oxidize and inactivate phosphatases, such as the phosphatase and tensin homolog (PTEN), which act as negative regulators of PI3K/Akt signaling (Lee et al., 2002). PTEN is often inactivated in many types of human cancers, including glioblastomas, breast and prostate cancer (Li et al., 1997). Furthermore, the activated PI3K/Akt signaling pathway can increase the production of ROS to strengthen the effects on cancer cell survival and proliferation (Behrend et al. 2003).

ROS also promote tumor cell survival via activation of transcription factors including the Nrf2. Nrf2 was proposed as a master regulator that modulates the expression of antioxidant systems to evade ROS-mediated cancer cell death. Under normal conditions, Nrf2 is usually degraded in the cytoplasm by interaction with the Kelch-like ECH-associated protein 1 (Keap1) inhibitor as an adaptor for ubiquitination factors (Sajadimajd et al., 2018). Elevated ROS or signaling pathways such as ERK/MAPK and PI3K activate tyrosine kinases, lead to dissociation of Keap1 from Nrf2 (Itoh et al., 2003). Subsequently, Nrf2 translocates to the nucleus and binds to antioxidant-responsive elements (AREs) within the regulatory region of multiple antioxidant genes (Raghunath et al., 2018). Deregulation of the Nrf2/ARE signaling pathway leads to nuclear accumulation and constitutive activation of Nrf2, thereby inducing proliferation, metastasis, and chemoresistance (Jaramillo and Zhang, 2013). Since the ability of ROS to promote many aspects of tumor development and progression, their excessive levels have been documented in almost all cancers. It is important to highlight that not only the type of radical but also the location and local concentration are crucial factors influencing ROS impact on tumor development (Circu et al., 2010).

2.2. Mitochondrial DNA alterations in cancer

Mitochondria emerged because of endosymbiosis more than 1.45 billion years ago. During evolution, most mitochondrial genes have translocated to the nucleus, but a small number necessary for ATP generation remained encoded within mitochondria. While our

nuclear genome harbors about 22,000 genes inherited from both parents, mitochondria are maternally inherited and comprise only 37 genes. Mammalian cells typically contain 10^3 – 10^4 copies of mtDNA (Lightowlers et al., 1997). mtDNA is a circular, intron-free, double-stranded genome that is only 16.5 kb in size, comprising 13 protein-coding genes, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (Figure 2.) (Anderson et al., 1981). mtDNA also contains ~1 kb segment referred to as the displacement loop (D-LOOP), essential for the binding of proteins that control mtDNA packaging into nucleoids, unwinding, replication as well as translation, such as the TFAM, mtDNA-specific DNA polymerase (POLG), or the helicase TWINKLE (Attardi and Schatz, 1988). All the remaining mitochondrial proteins, including approximately 80 OXPHOS subunits and all 4 subunits of CII, are encoded by nDNA (Wallace, 2012). These proteins are expressed in the cytoplasm and folded upon entry through the OMM via the TOM/TIM complex (Chacinska et al., 2009). Afterwards, they translocate into their specific sites: the OMM, the IMM, the IMS, or the mt-matrix. Only the coordinated genetic interplay of nuclear and mitochondrial encoded genes is therefore essential to avoid biochemical and metabolic aberrations (Rodley et al., 2012).

Although mtDNA sequence alterations is quite frequent in cancer, it is not yet clarified whether they are modifier events involved in cancer progression or a consequence of carcinogenesis (Bogenhagen et al. 2008; Schon et al. 2012). mtDNA is a multicopy entity, which means that it may be redundant. mtDNA does not contain genes involved in DNA repair, and the rate of spontaneous mutations in mtDNA is high. The phenomenon when individual mtDNA copies slightly differ from one another is known as heteroplasmy (a mixture of mutant and wild-type mtDNA copies) (He et al., 2010; Stewart and Chinnery, 2015). Because of the large number of mitochondrial genome copies in each cell, the ratio of mutant and wild-type mtDNA is a significant factor in the phenotype acquisition (Jenuth et al., 1996). Typically, the threshold of heteroplasmy of certain mtDNA mutations is more than 60% (Wonnapijit et al., 2008), but there is evidence that even lower levels already significantly increase the risk of age-related chronic diseases such as left ventricular hypertrophy, atherosclerosis, coronary heart disease (Majamaa-Voltti et al., 2002), and diabetes (Zhang et al., 2009). Recent studies have shown that high heterogeneity of human mtDNA was found to be significantly amplified in tumors. Polyak et al. (1998) found

homoplasmic mtDNA mutations in 70% of all studied cell lines from patients with colorectal tumors. However, these mutations had almost no effect on mitochondrial function. Along with the previously mentioned facts the role mutations in the mitochondrial genome in tumorigenesis is of considerable interest.

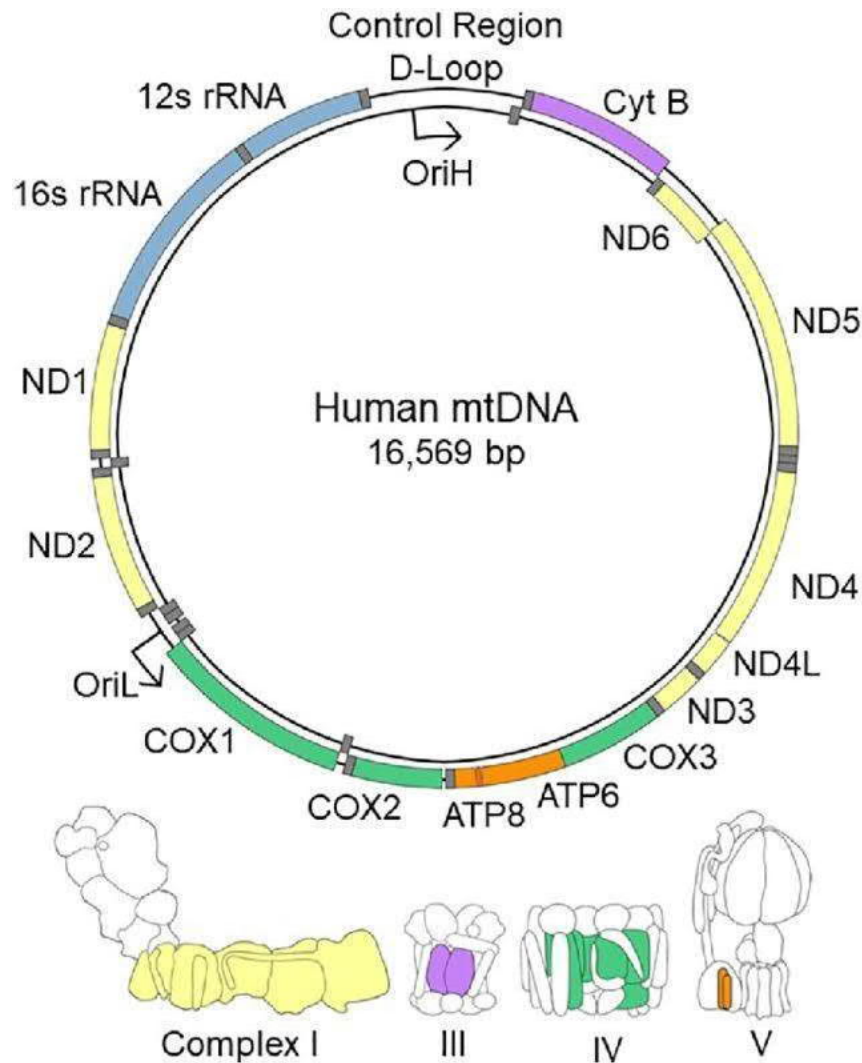


Figure 2. The human mtDNA structure: mtDNA is a circular molecule of 16,569 bp and contains 37 genes coding for two rRNAs, 22 tRNAs and 13 polypeptides. The mtDNA-encoded polypeptides are subunits of OXPHOS enzyme complexes (CI, CIII, CIV and CV). (Chocron et al., 2019).

2.3. Mitochondria in biomass synthesis

Due to the central role of mitochondria in metabolism, mitochondria are crucial for providing necessary building blocks, including nucleotides, fatty acids, and amino acids, for the growth of cancer cells (Eagle, 1955). In this respect, one essential role of ETC is enabling aspartase synthesis in proliferating cancer cells, ETC inhibition greatly reduces cancer cell growth, which can be partially rescued by supplementing aspartase in the medium (Birsoy et al., 2015). Key regulators of proline biosynthesis are found to be enhanced in different types of tumors, including carcinoma of the prostate and lymphoma (Liu et al., 2012). Glutamine is relatively abundant in plasma, and *de novo* glutamine synthesis in the liver and surrounding tissues is likely to be critical for tumor cell growth. Glutamine biosynthesis requires a supply of α -KG from mitochondrial metabolism to generate glutamate (a critical precursor of most non-essential amino acids) and subsequently glutamine via glutamine synthetase (GS) (Liaw et al., 1995). *De novo* glutamine biosynthesis in tumors has been detected *in vivo* using the infusion of [^{13}C] glucose into mice bearing human glioblastoma orthotopic tumors (Marin-Valencia et al., 2012). Furthermore, some breast epithelial cells can mediate glutamine independence via the expression of GS (Kung et al., 2011). Finally, glutamine, as well as other amino acids, may be scavenged via protein catabolism when unavailable in sufficient quantities (Weinberg et al., 2010).

2.3.1. Mitochondria and pyrimidine biosynthesis

It is important to keep in mind that only the ribose 5-phosphate moiety of nucleotides is generated in the cytosol and all the other components of pyrimidine biosynthesis are derived directly or indirectly from mitochondria (Voet et al., 2016). Taken together, balanced deoxynucleotide triphosphate (dNTP) pools are essential for both nuclear and mitochondrial genome replication and repair. Mitochondrial dNTP pools are separated from the cytosolic ones by the mitochondrial double membrane. Thus, serious mitochondrial diseases can be caused by mtDNA depletion and deletions as a consequence of imbalanced mitochondrial dNTP pools (Suomalainen and Battersby, 2018).

As mentioned above, pyrimidine nucleotides can play a significant role in tumor cell proliferation as precursors of both types of nucleic acids. There are two ways for the synthesis of pyrimidines: the salvage synthesis pathway, which is the main pyrimidine source for resting or fully matured cells, and the *de novo* synthesis pathway, which is necessary for strongly proliferating cells (Fang et al, 2013).

Human DHODH is the only enzyme of this pyrimidine biosynthesis pathway that is located in mitochondria. DHODH is a flavin-dependent oxidoreductase catalyzing the conversion of dihydroorotate into orotic acid, which is then converted into uridine monophosphate (UMP), the RNA nucleotide essential for ribosome biogenesis. Therefore, DHODH plays a substantial role in cell proliferation, mitosis, and cellular metabolism (Mathur et al., 2017). The functional link between DHODH and OXPHOS is via the electron acceptor ubiquinone. In the redox cycle of ubiquinone, DHODH transfers electrons from dihydroorotate to ubiquinone, which is reduced into ubiquinol and then re-oxidized again into ubiquinone by CIII, a process known as CoQ redox-cycling. Thus, inhibition of CIII results in suppressing the DHODH function by reducing the turnover of UQ, which is essential for DHODH activity. Cells with depleted mtDNA, OXPHOS-deficient cells, and cells with defective CIII display DHODH dysfunction. Despite the unique physical location of DHODH at the IMM, a direct association of DHODH was not identified in the structures of respiratory SCs (Fang et al., 2013).

DHODH has been reported to play an important role in various pathologies, including cancer (White et al., 2011; He et al., 2014). Thus, the effect of DHODH is more prevalent in fast-proliferating cancer cells, as DHODH is required to meet their enhanced need for nucleic acids and other cellular components. Malignant cells become even more sensitive to DHODH inhibitors in nutrient-deprived TME (Brown and Giaccia, 1998; Miyazaki et al., 2018). In recent years, many studies have shown that the interplay of oncogenic signaling and pyrimidine synthesis in tumorigenesis also affects other metabolic pathways and cellular signaling. Indeed, the oncogenic MAPK, KRAS, and mTOR pathways increase DHODH fuelling by inducing *de novo* pyrimidine biosynthesis, while MYC causes increased expression of DHODH (Liu et al., 2008).

DHODH inhibition could impair the biogenesis of ribosomes by UTP depletion, thereby activating the p53 tumor suppressor pathway, leading to cell cycle arrest. Inhibitors of

DHODH function, such as brequinar and leflunomide, have previously been demonstrated to be effective in several different cancers, such as melanoma (White et al., 2011), triple-negative breast cancer (Brown et al., 2017), skin tumors (Hosseini et al., 2018), and mutant KRAS-driven tumors (Koundinya et al., 2018), as well as glioblastoma (Echizenya et al., 2020), and prostate cancer (Numsen et al., 2010).

Since nucleotides play a significant role in cancer cell proliferation as precursors of synthesis of RNA and DNA, DHODH represents potential target for treatment of cancer.

2.4. Mitochondrial reprogramming by p53

Cancer is a gradual pathological process driven by mutations and leading to uncontrolled cell division. Cells escape growth control by targeting key oncogenes or tumor suppressors in various signaling pathways. These pathways have evolved to integrate both positive and negative growth signals according to cellular function and microenvironment during normal development and tissue repair. Thus, effective cancer suppression requires a controlled system with various abnormality sensors set in place to trigger a highly regulated form of programmed cell death, called apoptosis, to prevent uncontrolled proliferation and cancer development. Therefore, cancer development and progression are usually linked to a series of changes controlled by cell cycle regulators (Malumbres et al., 2001).

One example is the DNA damage response pathway, which upon DNA damage results in activation of several kinases that phosphorylate the transcription factor p53 (Canman et al., 1998; Tibbetts et al., 1999; Xiao et al., 2003; Hirao et al., 2000). The tumor suppressor p53 plays a central role in regulating cell cycle arrest, DNA repair, and apoptosis in response to cellular stress (Lowe, 2009). Mutations of the TP53 gene have been reported in ~50% of all human cancers, while the remaining 50% harbor mutations that inactivate the p53 pathway. It has recently been appreciated that the tumor suppressor function of p53 can also be reached via the regulation of genes involved in OXPHOS, demonstrating a functional role for mutant p53 in cancer metabolism (Kawauchi et al., 2008). While p53 plays a prominent role in the DNA damage response, it is also a sensor of altered ribosome biogenesis (Bursac et al., 2014).

Unstressed, non-transformed cells contain very low levels of p53 protein because of its short half-life and continual degradation by the E3 ubiquitin ligase MDM2 (Shieh et al., 1997). Under stress conditions, including DNA damage, activation of oncogenes, high ROS levels, and hypoxia, the level of p53 protein rises substantially (Cordon-Cardo et al., 1994). MDM2/p53 stress signalling pathway include also ribosomal proteins, such as RPL5 (Dai and Lu, 2004) and RPL11 (Bhat et al., 2004) which act as sensor and effector of ribosomal stress. Once stabilized, p53 modulates transcription of multiple target genes and ensures appropriate checkpoint maintenance, resulting in cell cycle arrest or apoptosis (Bieging et al., 2014).

Cell cycle checkpoints responding to DNA damage or incomplete DNA replication elicit cellular responses by inhibiting two main kinases involved in p53 phosphorylation: Chk1 (cell cycle checkpoint kinase-1) and Chk2 (cell cycle checkpoint kinase-2). If DNA damage occurs, p53 is phosphorylated by Chk1 and Chk2, which are activated by ATR (ataxia-telangiectasia and Rad3 related) (Tibbetts et al., 1999) and ATM (ataxia-telangiectasia mutated gene) (Canman et al., 1998), respectively. Thus, cells are arrested at the checkpoints until the damaged DNA is repaired. The p53-induced activation of target genes may result in the induction of growth arrest either before DNA replication in the G1 phase of the cell cycle or before mitosis in the G2 phase.

Oncogenic mitochondrial regulation by p53 is both through its transcription activity and direct regulation of the activity of mitochondrial proteins (Park et al., 2016). As p53 is a redox-sensitive protein with clusters of cysteine residues, it can be involved in ROS signaling. Increased levels of ROS have different effects on cell fate, depending on the p53 status: lower levels of ROS activate anti-oxidant genes, while higher levels of ROS pro-oxidant genes (Sablina et al. 2005). Under normal cellular conditions, p53 upregulates the transcription of several anti-oxidant genes, including manganese superoxide dismutase (MnSOD), GPX1, sestrins, glutaminase 2 (GLS2), and TIGAR, which increase the pentose phosphate pathway and NADPH production (Kotsantis et al., 2018). Under conditions of high ROS, p53 has been demonstrated to downregulate anti-oxidant proteins, including SOD2 (Drane et al., 2001) and the anti-oxidant transcriptional factor Nrf2 (Faraonio et al., 2006).

p53 inhibits glycolysis but promotes the transcription of genes involved in OXPHOS and fatty acid oxidation in cancer cells (Liu et al., 2014). Recently, it was also described that increased levels of α -KG in p53-mutated tumors can promote tumor cell differentiation and antagonize malignant progression (Morris et al., 2019). Also, it was described that p53 plays an important role in mtDNA homeostasis involving p53-nuclear transcriptional target genes as well as the non-nuclear direct effects of p53 protein in the cytosol and mitochondria (Bourdon et al., 2007).

Since mutations in the *TP53* gene are extremely widespread in human cancers, p53 signaling pathway represents one of the most studied oncogenic pathway.

3. Tumor microenvironment

The tumor mass consists not only of a heterogeneous population of cancer cells but also of a variety of resident and infiltrating host cells, including supporting stromal cells, immune cells, stem cells, secreted factors, and extracellular matrix proteins, collectively called the tumor microenvironment (TME) (Whiteside et al., 2007, Hanahan and Coussens, 2012). tumor progression is profoundly influenced by the constant and evolving interplay with microenvironmental players, which ultimately determines whether the primary tumor is eradicated, metastasizes, or establishes dormant micrometastases (Balkwill et al., 2012). Modulation of tumor mitochondria is an important mechanism that helps cancer cells to escape from immune surveillance and to develop drug resistance. In addition to cancer and immune cells, the TME contains many different cell types that can control the state of the mitochondria in a tumor both directly, by cell-cell contacts, and indirectly, by the secretion of soluble factors and a variety of extracellular vesicles (Bhowmick et al. 2004).

It is also important to note that the current knowledge of mitochondrial transfer *in vivo* is limited, and data from published studies are difficult to compare because of the use of different experimental systems. The most common mitochondrial donors are mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs) (Spees et al., 2006, Pasquier et al., 2013). MSCs are multipotent stromal cells that have the ability to migrate towards inflammatory sites and, importantly, to migrate towards tumors (Rattigan et al., 2010). They contribute to tumor growth and progression (Ye et al., 2012) and display several

remarkable properties, such as the capacity to regulate the immune response or angiogenesis and the ability to aid in the repair and regeneration of damaged tissues (Silva et al., 2005). In tumor stroma, MSCs have been shown to promote the epithelial-to-mesenchymal (EMT) transition to increase the metastatic potential of tumor cells by promoting their motility and invasiveness (Karnoub et al. 2007, Martin et al., 2009).

Similarly, MSCs increase cancer cell motility and can induce metastatic colonization of cancer cells at distant metastatic sites. MSCs have now been demonstrated to serve pro-malignant roles in several epithelial cancer subtypes, such as breast (Karnoub et al. 2007, Muehlberg et al. 2009), colon (Zhu et al. 2006, Shinagawa et al., 2010), lung (Hsu et al., 2012), and prostate cancer (Ye et al., 2012). Strikingly, MSCs have been reported to differentiate into CAFs (Spaeth et al., 2009, Zhang et al., 2009b). CAFs are a heterogeneous population of fibroblast-like cells that are implicated in various stages of tumor progression (Zeisberg et al., 2007). Genetically tagged bone-marrow-derived cells, injected into mice, were found at the tumor site with myofibroblast morphology, and they were co-expressing α -smooth muscle actin (α -SMA) and the $\alpha 1$ chain of type I (pro)collagen, which are conventionally identified as myofibroblast markers. Moreover, MSCs can be hijacked by malignant cells and manipulated for the tumor's advantage (Zhang et al., 2009b).

The above-described findings suggest that MSCs are recognized as important contributors to tumor progression, likely representing a critical component of the TME.

3.1. Mitochondrial transfer

As previously discussed, maintaining the quality and quantity of mitochondria is essential for tissue homeostasis and cell survival. Neighboring cells routinely exchange intracellular material making use of different processes, such as the formation of extracellular vesicles (EVs), tunneling nanotubes (TNTs) formed at the sites of physical contact, mitochondrial ejection, and cytoplasmic fusion (Rustom et al., 2004; Islam et al., 2012). Since mitochondria have a double protective membrane and the capacity to change their shape and length, intact mitochondria can be transported by TNTs and microvesicles (MVs) (Onfelt et al., 2006; Phinney et al., 2015).

TNTs are typically long, thin, dynamic filamentous cellular structures that have been identified as a novel mode of long-range intercellular communication (Rustom et al., 2004). They have been found in a variety of cell types: immune cells, neurons, endothelial cells, and MSCs. They can transfer different cargo, such as cellular vesicles, including mitochondria (Pasquier et al., 2013; Hayakawa et al., 2016), miRNAs (Thayanithy et al., 2014), and viral particles (Hashimoto et al., 2016). The TNT-mediated mitochondrial transfer has been described as the major mechanism by which the exchange of mitochondria takes place thereby correlating with cancer cell invasion and chemoresistance (Pasquier et al., 2013). The process of mitochondrial transfer through TNTs is thought to be mainly unidirectional from healthy/donor to damaged/recipient cells (Figure 3.) (Wang and Gerdes, 2015; Burt et al., 2019). The first evidence of mitochondrial transfer *in vitro* was documented by Spees et al. (2006) upon co-culture of MSCs with adenocarcinoma human alveolar basal epithelial cells with depleted mtDNA (A549 ρ 0).

It was assumed that the presence of this phenomenon *in vivo* is rare since the limitation of tracking transfer of mitochondria among cells *in vivo*. Nevertheless, several studies have been able to show mitochondrial trafficking under pathophysiological conditions. Astrocytes in mice have been observed to be the donor of healthy mitochondria, which entered neurons and contributed to endogenous neuroprotective and neuro-recovery mechanisms after stroke. Moreover, astrocytes were able to be the recipients of damaged mitochondria (Hayakawa et al., 2016). That horizontal mitochondrial genome transfer exists in nature is indicated by phylogenetic evidence from the canine transmissible venereal cancer (CTVT) genetics studies, documenting that this tumor originated ~10 thousand years ago. It is estimated that, due to the vulnerability of mtDNA to mutations, CTVT cells with heavily damaged mtDNA have previously been found to acquire mitochondria with high quality mtDNA from stromal cells (Strakova et al., 2016).

The above-described data from published reports collectively suggest the important role of functional mtDNA in cancer.

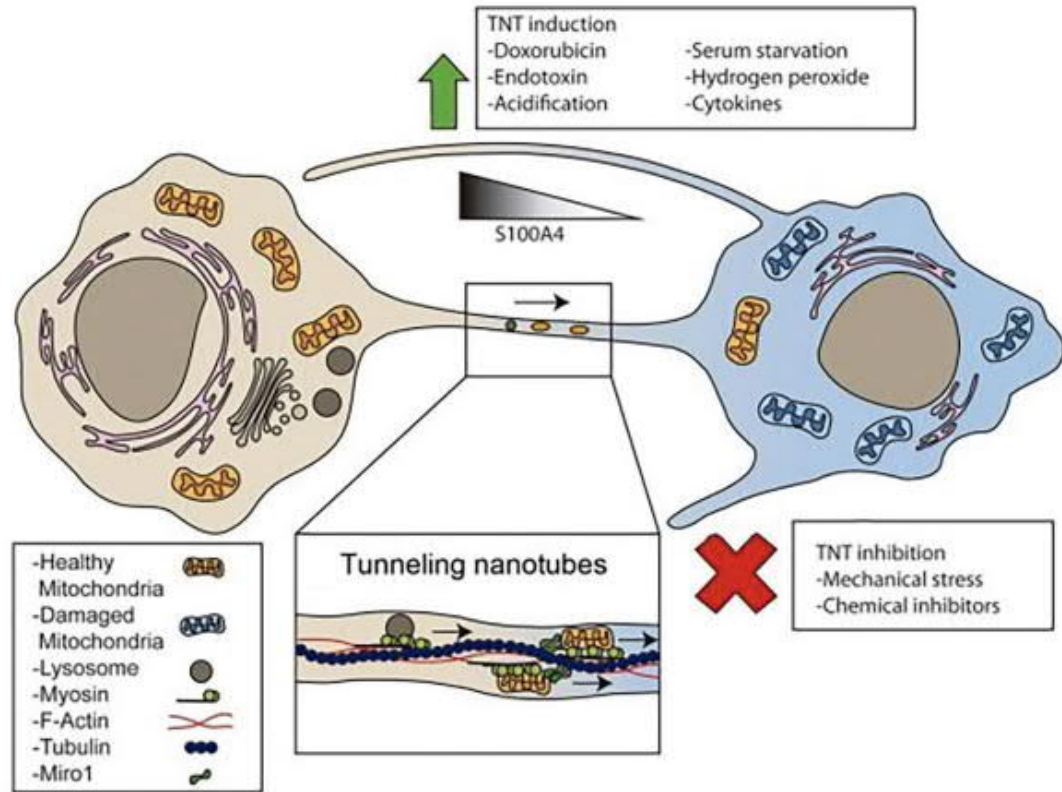


Figure 3. Intercellular mitochondrial transfer. Mitochondria can be released by donor (MSCs) cells and taken up by recipient (mtDNA depleted) cells through tunneling nanotubes (TNTs). TNTs are actin- or microtubule-based cytoplasmic extensions depending on the cell type. Myosin is a fundamental protein for organelle transfer. Miro1 regulates the transport of mitochondria across TNTs (adapted from Torralba et al. 2016).

AIMS

The overarching aim of the thesis was to describe the mechanisms underpinning the process of trafficking of mitochondria with their DNA payload from donor cells to cells with damaged mtDNA as well as the essential mechanisms which contribute to tumor formation and progression.

The specific questions of each paper were postulated:

1. Is there a link between OXPHOS and the initiation of tumorigenesis?
2. How can mtDNA be transferred from host cells to the tumor cells?
3. Is respiration recovery linked to metabolic remodeling in cancer cells?
4. Is there a link between the suppression of DHODH, the mitochondrial enzyme coupled to the respiratory chain, and cell death?

RESULTS

The list of this author's publications, which collectively forms the essence of the presented thesis.

Tan, A.S., Baty, J.W., Dong, L.F., Bezawork-Geleta, A., Endaya, B., Goodwin, J., **Bajzikova, M.**, Kovarova, J., Peterka, M., Yan, B., Pesdar, E.A., Sobol, M., Filimonenko, A., Stuart, S., Vondrusova, M., Kluckova, K., Sachaphibulkij, K., Rohlena, J., Hozak, P., Truksa, J., Eccles, D., Haupt, L.M., Griffiths, L.R., Neuzil, J., and Berridge, M.V. (2015). **Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA.** *Cell Metab* 21, 81-94.

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Dihydroorotate Dehydrogenase-Driven Pyrimidine Biosynthesis Restores Tumor Growth of Respiration-Deficient Cancer Cells. *Cell Metab* 29, 399-416.

Impact factor at the time of publishing 21.567; current impact factor 27.29.

Hubackova, S., Davidova, E., Boukalova, S., Kovarova, J., **Bajzikova, M.**, Coelho, A., Terp, M.G., Ditzel, H.J., Rohlena, J., and Neuzil, J. (2020). **Replication and ribosomal stress induced by targeting pyrimidine synthesis and cellular checkpoints suppress p53-deficient tumors.** *Cell Death Dis* 11, 110.

Impact factor at the time of publishing 6.3; current impact factor 7.57.

1. Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA

Mitochondria have many functions within cells: they are the site of OXPHOS, responsible for the generation of the majority of cell's ATP; they provide metabolic intermediates and are involved in many important signaling pathways. Mutations in mtDNA have also been described in cancer, but their contribution to tumor initiation, progression, and metastasis is less clear (Wallace, 2012; Schon et al., 2012). The reason is the difficulty of experimentally manipulating mtDNA of tumor cells and the lack of tumor models with mtDNA mutations (King and Attardi, 1989).

In the presented study, we used a model of extreme mtDNA damage that involves mitochondrial genome deletion (mouse metastatic breast cancer 4T1 $\rho 0$ cells). When we grafted these cells in Balb-c mice, they formed syngeneic tumors with a lag period in tumor initiation compared to their parental counterparts (4T1 cells). The cells isolated from these tumors recovered mitochondrial function, thus indicating mtDNA acquisition from the host. To confirm the absence of mtDNA in 4T1 $\rho 0$ cells, PCR analysis of the mitochondrial Cytb gene revealed no product in $\rho 0$ cells and the explicit proof for the host origin of mtDNA comes from sequencing of polymorphisms typical for Balb-c mouse and different for all polymorphisms from mtDNA in the parental cancer cells. Sublines derived from primary tumors grown from 4T1 $\rho 0$ cells (4T1 $\rho 0$ SC cells), and their circulating (4T1 $\rho 0$ CTL cells) and metastatic counterparts (4T1 $\rho 0$ SCL cells) revealed step-wise recovery of OXPHOS as well as assembly of mitochondrial SCs. This occurred at the level of transcription of mtDNA-encoded genes and translation of mtDNA-encoded proteins. We show a very similar paradigm for murine B16 melanoma cells.

Altogether, our model proposes that mtDNA-depleted cells acquire mitochondria from the host to restore their metabolic capacities to a 'threshold' level that enables them to form tumors.

Contribution to the publication: Experimental part (cell culture, evaluation of uridine and pyruvate auxotrophy, mtDNA level assay, qPCR, RT-PCR, respiration assays, mice experiments, ultrasound imaging of tumors), revision of the manuscript.

Mitochondrial Genome Acquisition Restores Respiratory Function and Tumorigenic Potential of Cancer Cells without Mitochondrial DNA

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SUMMARY

We report that tumor cells without mitochondrial DNA (mtDNA) show delayed tumor growth, and that tumor formation is associated with acquisition of mtDNA from host cells. This leads to partial recovery of mitochondrial function in cells derived from primary tumors grown from cells without mtDNA and a shorter lag in tumor growth. Cell lines from circulating tumor cells showed further recovery of mitochondrial respiration and an intermediate lag to tumor growth, while cells from lung metastases exhibited full restoration of respiratory function and no lag in tumor growth. Stepwise assembly of mitochondrial respiratory (super)complexes was correlated with acquisition of respiratory function. Our findings indicate horizontal transfer of mtDNA from host cells in the tumor microenvironment to tumor cells with compromised respiratory function to re-establish respiration and tumor-initiating efficacy. These results suggest pathophysiological processes for overcoming mtDNA damage and support the notion of high plasticity of malignant cells.

INTRODUCTION

Mitochondria are structurally dynamic organelles with a circular genome (mtDNA) of ~16.5 kb in mammals that encodes 13 subunits of the respiratory complexes (RCs), 22 tRNAs, and 2 rRNAs. Cells contain both discrete and networked mitochondria each with multiple mtDNA copies. Replication and transcription of mtDNA involves the regulatory D-LOOP region,

which binds the nuclear-encoded mitochondrial transcription factor TFAM and other regulatory proteins (Falkenberg et al., 2007). The mitochondrial proteome comprises ~1,500 proteins, most of which are encoded by nuclear DNA (nDNA) (Neupert and Herrmann, 2007; Wallace, 2012). A major role of mitochondria, ATP generation, is facilitated by oxidative phosphorylation (OXPHOS) comprising four complexes of the electron transport chain (ETC), complex I (CI), CII, CIII and CIV, and CV with ATP synthase activity. RCs form supercomplexes (SCs), such as the respirasome (Schägger and Pfeiffer, 2000; Acín-Pérez et al., 2008; Althoff et al., 2011) that transfers electrons from CI to CIV via CIII (Lapiente-Brun et al., 2013). RCs and SCs are assembled stepwise in a process involving specific factors (Moreno-Lazres et al., 2012; Lapiente-Brun et al., 2013; Ikeda et al., 2013).

Due to the central role of mitochondria in homeostasis, cells have conserved control systems to maintain mitochondrial integrity, appropriate mitochondrial mass, and bioenergetic and metabolic functions. In many pathologies, mtDNA mutations affect tissues with high demand for energy (Krishnan et al., 2006; Lu et al., 2009; Schon et al., 2012; Wallace, 2012). Cancer is characterized by altered energy metabolism (DeBerardinis et al., 2007; Kroemer and Pouyssegur, 2008; Vander Heiden et al., 2009; Ralph et al., 2010; Hanahan and Weinberg, 2011) involving not only genetic alterations in nDNA, but also mtDNA mutations and changes in mtDNA copy number (Surani, 2001; Wallace and Fan, 2010; De Carvalho et al., 2010; Plass et al., 2013). Certain mtDNA mutations compromise ETC function and contribute to a shift to aerobic glycolysis that typifies metastatic progression (Ishikawa et al., 2008; Ralph et al., 2010). Relevant studies are limited by the difficulty of experimentally manipulating mtDNA of tumor cells and the paucity of tumor models with mtDNA mutations. A model of severe mtDNA damage involves mitochondrial genome deletion (King and Attardi, 1988). These ρ^0 cells grow in culture with



uridine and pyruvate supplementation, but the question of whether they can form tumors following autologous transplantation has not been studied.

We have developed two stable murine tumor cell models lacking mtDNA and investigated whether these cells form tumors in syngeneic mice. Our results show delayed tumor growth and acquisition of mtDNA from host cells. Cells derived from primary tumors that grow from ρ^0 cells and from their circulating and metastatic counterparts progressively recovered mitochondrial respiratory function, and this was associated with assembly of mitochondrial SCs and correlated with tumorigenicity.

RESULTS

Tumor Cells Devoid of mtDNA Show Delayed Tumor Growth

To model severe mitochondrial genome damage, we deleted mtDNA in metastatic murine B16 melanoma and 4T1 breast carcinoma cells using low-dose ethidium bromide (EtBr), thereby generating B16 ρ^0 and 4T1 ρ^0 cells that have been maintained as stable auxotrophic cell lines without EtBr for more than 2 years. When injected subcutaneously (s.c.) into groups of five syngeneic C57BL/6 ($n = 3$ experiments) or NOD/scid ($n = 3$ experiments) mice, B16 ρ^0 cells (10^5) initiated tumors with a delay of more than 20 days compared to parental cells (Figure 1A). Intravenous (i.v.) injection of B16 ρ^0 cells into NOD/scid mice failed to generate lung tumors. However, cell lines derived from s.c. tumors and lung metastases that grew from B16 ρ^0 cells following long-term culture formed lung tumors at a similar frequency to B16 cells (Figure 1A). These cell lines were shown to be of B16, rather than mouse origin, because they produced melanin under stress. Similarly, 4T1 ρ^0 cells formed tumors with a lag of 20–25 days when 10^5 cells were injected s.c. ($n = 6$ experiments) or orthotopically into the mammary fat pad ($n = 1$ experiment) of Balb/c mice, but failed to form lung tumors following i.v. injection (Figure 1B) or following s.c. injection of $\leq 10^4$ cells (Figure S1 available online).

As 4T1 and 4T1 ρ^0 cells are resistant to 6-thioguanine (6TG), we used 20 μ M 6TG to select cell lines from primary tumors that formed from 4T1 ρ^0 cells (4T1 ρ^0 SC), circulating tumor cells (4T1 ρ^0 CTC), and lung metastases (4T1 ρ^0 SCL). When injected i.v., both 4T1 ρ^0 SC and 4T1 ρ^0 SCL cells, and a cell line derived from lung metastases that formed from an orthotopic tumor (4T1 ρ^0 MFPL), formed lung tumors that were comparable to 4T1 cells (Figure 1B). When injected s.c. into Balb/c mice, 4T1 ρ^0 SC cells formed tumors with a delay of ~ 10 days, whereas 4T1 ρ^0 SCL cells initiated tumors without a lag (Figure 1C; $n = 2$ experiments). 4T1 ρ^0 CTC cells formed tumors with a lag between that of 4T1 ρ^0 SC and 4T1 ρ^0 SCL cells.

To investigate whether acquisition of tumorigenic properties is associated with respiratory competence, tumors that formed from 4T1, 4T1 ρ^0 , and derived cell lines were assessed by high-resolution respirometry. Relative to cells from parental tumors, respiration by tumors that grew from 4T1 ρ^0 cells was reduced by 60%, whereas 4T1 ρ^0 SC, 4T1 ρ^0 CTC, and 4T1 ρ^0 SCL cell-derived tumors exhibited progressive recovery of respiration (Figure 1D). Histological examination of tumors derived from each cell line showed qualitative similarities (Figure 1E).

Tumors Derived from ρ^0 Cells Contain Host Mouse mtDNA and Exhibit Functional Mitochondrial Protein Synthesis

PCR analysis of the mtDNA-encoded *Cytb* gene revealed no product in B16 ρ^0 or 4T1 ρ^0 cells confirming the absence of mtDNA in these cells (Figure 2A). These results rule out possible contribution from nuclear mitochondrial DNA sequences (Schon et al., 2012). However, *Cytb* was present in B16 ρ^0 SC and B16 ρ^0 SCL cells. Similar results were obtained with 4T1 ρ^0 SC and 4T1 ρ^0 SCL cells (Figure 2A). To investigate the origin of the mtDNA in these cells, we sequenced the mtDNA region containing the polymorphic *tRNA^{Arg}* locus. Cells from both B16 ρ^0 - and 4T1 ρ^0 -derived tumors showed the polymorphism of the host mouse (Bayona-Safulay et al., 2003), rather than that of B16 and 4T1 cells (Figure 2B), demonstrating acquisition of mtDNA from host cells in the tumor microenvironment (eight consecutive adenines for C57BL/6 and nine for NOD/scid and Balb/c mice) to ρ^0 tumor cells in each model. To verify the host origin of mtDNA, we sequenced whole mtDNA from B16, B16 ρ^0 SC, 4T1, and 4T1 ρ^0 SC cells using next-generation Ion Torrent and Nanopore MinION methodologies. These approaches identified two additional polymorphisms in *16S rRNA* (site 1,576) and *D-loop* (site 16,076) that distinguished 4T1 from 4T1 ρ^0 SC cells (Table S1), verifying that mtDNA in tumors derived from 4T1 ρ^0 cells was from the recipient mouse. No additional polymorphisms were observed in B16 and B16 ρ^0 SC cells. Taken together, these findings unequivocally demonstrate that mtDNA in tumors derived from ρ^0 cells originate from the recipient mouse. Mitochondrial protein synthesis was shown to be functional in cell lines derived from tumors from B16 ρ^0 and 4T1 ρ^0 cells by the presence of COI, an mtDNA-encoded protein (Figure 2C).

Distinct Phenotypic Properties and Mitochondrial Morphology of 4T1 ρ^0 Cells following mtDNA Acquisition

Acquisition of host mtDNA by ρ^0 tumor cells prompted us to investigate the phenotypic and morphological properties of the 4T1 ρ^0 -derived sublines (4T1 ρ^0 SC, 4T1 ρ^0 CTC, and 4T1 ρ^0 SCL cells). We also investigated “matching” cells derived from s.c. tumors (4T1SC cells) and lung metastases (4T1SCL cells) that grew from parental 4T1 cells. The doubling times of these cell lines were as follows: 4T1, 18.7 hr; 4T1SC, 17.5 hr; 4T1SCL, 16 hr; 4T1 ρ^0 , 33.6 hr; 4T1 ρ^0 SC, 21.5 hr; 4T1 ρ^0 CTC, 23.1 hr; and 4T1 ρ^0 SCL, 16.1 hr. Light microscopy revealed morphological differences of individual sublines (Figure 3A). In agreement with the PCR analysis of mtDNA (Figure 2A), staining with EtBr confirmed the absence of mtDNA in 4T1 ρ^0 cells, showing only nucleolar staining. Costaining with MitoTracker Red showed high mtDNA heterogeneity in 4T1 ρ^0 SC and to a lesser extent in 4T1 ρ^0 CTC cells. Flow cytometric analysis revealed that $\sim 60\%$ of 4T1 ρ^0 SC cells contained little or no mtDNA, while almost 90% of 4T1 ρ^0 CTC and 100% of 4T1 ρ^0 SCL cells contained mtDNA (Figure 3B). 4T1SC cells showed similar mtDNA staining to that found in 4T1 and 4T1SCL cells (Figure S2A).

We tested individual sublines for their autophagic status, reasoning that 4T1 ρ^0 SC cells may exhibit higher mitophagy to remove respiration-defective mitochondria devoid of mtDNA. Initial acridine orange (AO) staining showed heterogeneous acidic vesicles in 4T1 ρ^0 SC cells, and western blotting (WB) revealed lower levels of the autophagy marker LC3B-II in 4T1 ρ^0

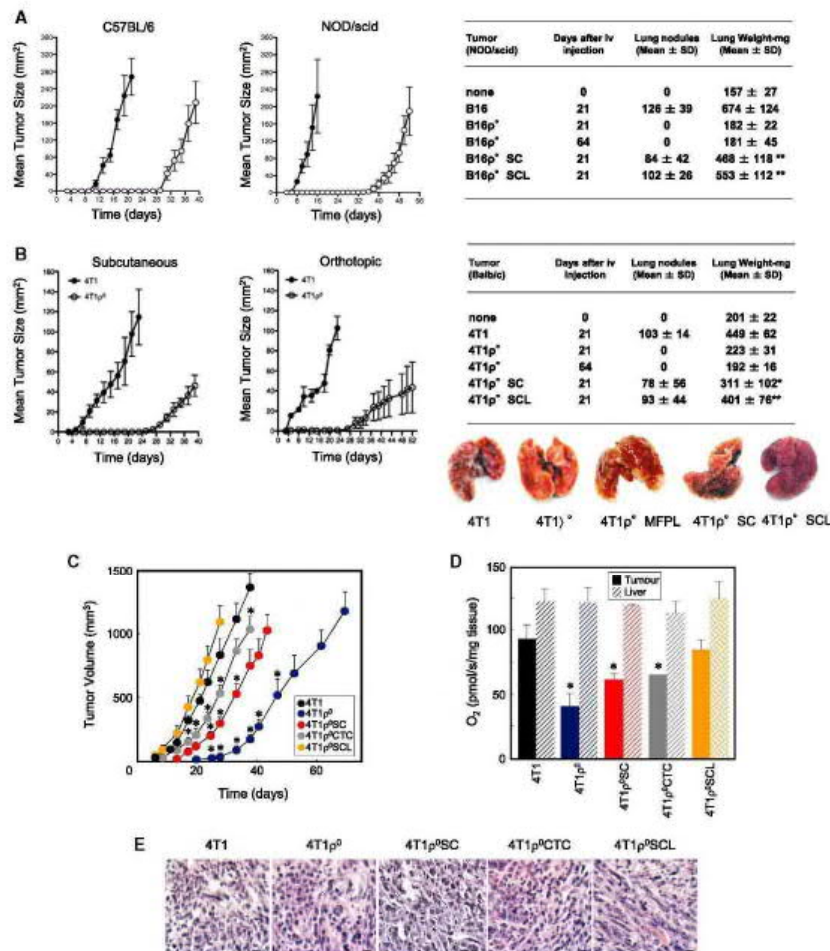


Figure 1. Tumor Cells without mtDNA Show Delayed Tumor Growth
 (A) B16 and B16p⁰ cells (10⁵) were injected s.c. into C57BL/6 or NOD/scid mice, and tumor growth was monitored. These cells and cell lines derived from s.c. tumors and lung metastases that grew from B16p⁰ cells were also injected i.v. into NOD/scid mice, and lung weight and tumor colonies were determined.
 (B) 4T1 and 4T1p⁰ cells were injected s.c. or orthotopically into the mammary fat pad of Balb/c mice, and tumor growth was monitored. These cells and 6TG-resistant cell lines derived from s.c. tumors and lung metastases that grew from 4T1p⁰ cells were also injected i.v. into Balb/c mice, and lung weight and tumor colonies were determined. Images of lungs from these mice are also shown.
 (C) 4T1, 4T1p⁰, 4T1p⁰SC, 4T1p⁰CTC, and 4T1p⁰SCL cells were injected into Balb/c mice, and tumor growth was monitored.
 (D) Oxygen consumption by tissue of tumors derived from cells as stated above (C) was compared with respiration of liver tissue obtained from the same mice.
 (E) Histological staining of tissue sections from (C) stained with H&E; scale bar, 20 μ m. Data are from three independent experiments, and the results are expressed as mean $\pm$ SD. The symbol * denotes significant differences with $p < 0.05$ and ** $p < 0.001$.

and 4T1p⁰SC cells than in 4T1p⁰CTC and 4T1p⁰SCL cells, and higher levels of p62 in 4T1p⁰SC cells, both with control cells and cells treated with the autophagy inhibitor bafilomycin A1

(Figure 3C). The mitophagy marker, Parkin, showed increased expression in cells derived from 4T1p⁰ and highest expression in 4T1p⁰SCL cells, but BNIP3 expression remained unaltered

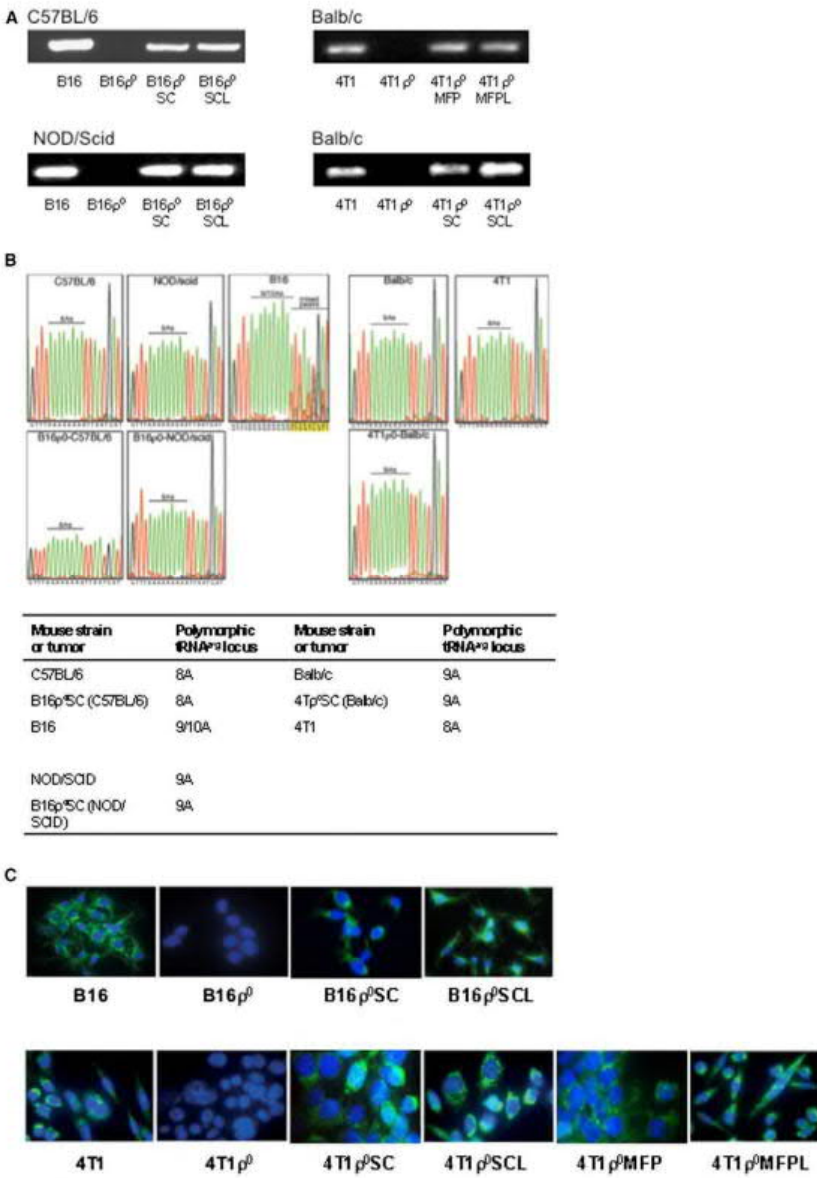


Figure 2. Tumors Derived from Cells without mtDNA Contain mtDNA of Host Origin
(A) Left panels: *Cytb* PCR analysis of B16, B16^{p0}, and B16^{p0}SC and B16^{p0}SCL tumor sublines from C57BL/6 (upper-left panel) and NOD/scid mice (lower-left panel). Right panels: *Cytb* PCR analysis of 4T1, 4T1^{p0}, and tumor sublines from orthotopic primary and metastatic tumors (upper-right panel, 4T1^{p0}MFP, 4T1^{p0}MFPL) and s.c. primary and metastatic tumors (4T1^{p0}SC, 4T1^{p0}SCL) that form in Balb/c mice.

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in the sublines. Since individual sublines were derived from different stages of metastatic tumor progression, we investigated markers of epithelial-mesenchymal transition (EMT). Figure 3D shows that the sublines differed coordinately in the level of expression of E-cadherin and vimentin consistent with their different origin.

Investigation of mitochondrial morphology by transmission electron microscopy (TEM) revealed that mitochondria of 4T1 ρ^0 cells were distended with low-density internal staining and were largely free of stacked cristae, but contained ring-like cristae (Figure 4). Intriguingly, 4T1 ρ^0 SC cells showed mitochondria with and without cristae, while 4T1 ρ^0 CTC cells contained more mitochondria with cristae. 4T1 ρ^0 SCL cells were indistinguishable from parental 4T1 cells and the matching tumor cells (Figures 4 and S2B).

Bioenergetics and Mitochondrial Function following mtDNA Acquisition

Cells devoid of mtDNA use glycolysis as a source of ATP. Compared to parental 4T1 cells, 4T1 ρ^0 , 4T1 ρ^0 SC, and 4T1 ρ^0 CTC cells showed elevated glucose uptake, increased production of lactate, and lower levels of ATP (Figures 5A–5C). In contrast, 4T1 ρ^0 SCL cells were less glycolytic and more similar to parental 4T1 cells, as were the matching sublines (Figures 53A–53C). Cellular bioenergetic status and the efficiency of mitochondrial respiration modulate production of reactive oxygen species (ROS). We used the mitochondrially targeted probe, MitoSOX, as an indicator of mitochondrial superoxide generation. Cell lines from primary tumors that developed from 4T1 ρ^0 cells when injected s.c. or into the mammary fat pad showed distinct intermediate and high fluorescence intensity peaks, while cells from lung metastases showed predominantly a high fluorescence intensity peak comparable with parental 4T1 cells (Figure 54A). Only the intermediate peak was present in 4T1 ρ^0 cells. Similar results were obtained with B16 cells (Figure 54B). Quantitation of MitoSOX staining showed low fluorescence in 4T1 ρ^0 cells with other sublines being comparable with parental 4T1 cells (Figure 54D). Dihydrodichlorofluorescein (DCF) measurement of cytosolic hydrogen peroxide showed a nonsignificant decrease in the signal in 4T1 ρ^0 , 4T1 ρ^0 SC, and 4T1 ρ^0 SCL cells relative to parental 4T1 cells (Figure 5E). Using TMRM as a probe, mitochondrial membrane potential ($\Delta\psi_m$) was found to be similar in 4T1 and 4T1 ρ^0 cells, elevated in 4T1 ρ^0 SCL and to a lesser extent in 4T1 ρ^0 SC cells, and was greatly reduced in 4T1 ρ^0 CTC cells (Figure 5F).

Respiration Recovery following mtDNA Acquisition by 4T1 ρ^0 Cells

4T1 ρ^0 cells showed barely detectable respiration, which was low in 4T1 ρ^0 SC cells and higher in 4T1 ρ^0 CTC cells, while 4T1 ρ^0 SCL cells respired at a similar rate to parental cells (Figures 5G and 55A). Respiration was similar in 4T1 and 4T1SC cells and slightly lower in 4T1SCL cells (Figure 53F). Respiration via CI and CII was

very low in 4T1 ρ^0 SC cells and higher in 4T1 ρ^0 CTC cells. Respiration via CI was similar in parental cells and 4T1 ρ^0 SCL, while respiration via CII was highly elevated in metastatic cells (Figures 5H and 55B). A similar respiration profile was found for isolated mitochondria (Figure 55C), indicating that respiration of the sublines is primarily governed by mitochondria, and not the extramitochondrial compartment. Collectively, these results show significant differences in respiration of individual 4T1 ρ^0 -derived sublines, which paralleled the efficacy of these cells to initiate tumor growth (Figure 1C).

mtDNA Recovery and Transcription following mtDNA Acquisition

To determine whether phenotypic differences in 4T1 ρ^0 -derived sublines following mtDNA acquisition were related to gradual restoration of mtDNA, we evaluated the mtDNA/nDNA ratio. While 4T1 ρ^0 cells did not contain mtDNA, we found a several-fold increase in the mtDNA/nDNA ratio in 4T1 ρ^0 SC compared to parental cells. This ratio was maintained over several months of cell culture (Figure 6A). The notion of no mtDNA is corroborated by no product in both B16 ρ^0 and 4T1 ρ^0 cells when amplifying whole cell mtDNA for NGS (Figure S6). 4T1 ρ^0 CTC and 4T1 ρ^0 SCL cells showed mtDNA/nDNA ratios more similar to those of parental cells as did the corresponding 4T1SC and 4T1SCL cells (Figure 53G). Transcripts of mtDNA-encoded genes were not detected in 4T1 ρ^0 cells, but were found in 4T1 ρ^0 -derived sublines. Relative to 4T1 cells, *Nd1*, *Nd2*, *Nd4*, *Nd4l*, *Nd5*, *Nd6*, and *mt-Co2* were elevated 2- to 4-fold in 4T1 ρ^0 SC cells, and *Nd1*, *Nd2*, *mt-Co2*, *Atp6*, and *Atp8* elevated 1.5- to 4.5-fold in 4T1 ρ^0 SCL cells. The level of *D-loop* transcript was similar in all 4T1 ρ^0 -derived sublines, indicating that the regulatory region of mtDNA does not hinder mtDNA replication or transcription in these cells (Figure 6B).

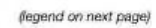
nDNA-encoded subunits of mitochondrial complexes, with the exception of *Sdhc*, showed a similar expression pattern indicating a common transcription mechanism (Figure 6C). Of the regulatory genes, relative expression of mRNA for the mitochondrial transcription factor *Tfam* was very low in 4T1 ρ^0 and almost 3-fold higher in 4T1 ρ^0 SC cells, but was detected at similar levels in 4T1, 4T1 ρ^0 CTC, and 4T1 ρ^0 SCL cells (Figure 6D). The *Tfam* transcript profile in the sublines mirrors the mtDNA/nDNA ratios, suggesting a causal relationship. *Nrf1*, *Nrf2*, *Tfb1m*, *Tfb2m*, and *Top1m* transcripts were depressed in 4T1 ρ^0 SC cells, while the assembly factor *Scaf1* (*Cox7a2l*) was decreased in 4T1 ρ^0 , 4T1 ρ^0 SC, and 4T1 ρ^0 CTC cells, and the assembly factor *Hig2a* was relatively unchanged. No differences in these transcripts were observed in the matching cells (Figure 53H).

Mitochondrial Protein Synthesis, Citrate Synthase, and CII Activity following mtDNA Acquisition

We next studied translation of mtDNA-encoded proteins. Figure 7A shows only background staining in 4T1 ρ^0 cells, with the proteins coordinately expressed 2- to 3-fold higher in the other

(B) Left panels: sequence analysis of the polymorphic region (9,817–9,836 C57BL/6 reference NC_005089.1) of the mitochondrial *tRNA^{Arg}* gene from C57BL/6 and NOD/scid mice and from s.c. tumors derived from B16 ρ^0 cells grown in C57BL/6 and NOD/scid mice. Right panels: comparable sequence analysis of the mitochondrial *tRNA^{Arg}* gene from Balb/c mice and from a s.c. tumor derived from 4T1 ρ^0 cells grown in Balb/c mice. A summary table is presented.

(C) COI immunocytochemical staining (green) of parental and ρ^0 cells and individual sublines from ρ^0 cells. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μ m.



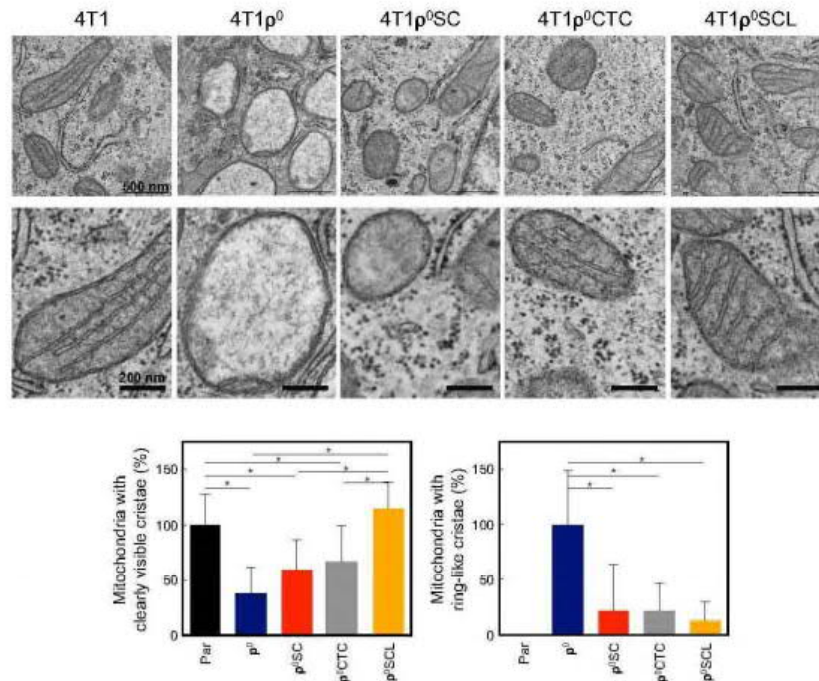


Figure 4. TEM Analysis of 4T1p⁰ Sublines Shows Mitochondrial Ultrastructural Differences
4T1 cells and derived sublines were grown on coverslips, processed for TEM, and examined using TEM. The lower graphs indicate percent mitochondria with visible cristae (left) and percent mitochondria with ring-like cristae (right) for each subline. The percentage of mitochondria with visible cristae in 4T1 cells was set as 100%, the percentage of mitochondria with ring-like cristae in 4T1p⁰ cells set as 100%. Data are from three independent experiments, and the results are expressed as mean ± SD. The symbol * denotes significant differences with $p < 0.05$.

sublines compared to parental cells. Activity of citrate synthase (CS), the first enzyme of the TCA cycle, was increased 2.5-fold in 4T1p⁰ and 4T1p⁰SC cells, while in 4T1p⁰CTC and 4T1p⁰SCL cells the activity was similar to that in parental cells (Figure 7B). This increase was not observed in the corresponding sublines (Figure S3I). We did not see a major difference in succinate dehydrogenase (SDH) activity in sublines derived from 4T1p⁰ cells (Figure 7C) or the matching cells (Figure S3J). Succinate quinone reductase (SQR) activity of CII was very low in 4T1p⁰, 4T1p⁰SC, and 4T1p⁰CTC cells, while in 4T1p⁰SCL cells it was 70% higher than in 4T1 cells (Figure 7D). SQR activity was about 2-fold

higher in the metastatic 4T1SCL cells than in 4T1 and 4T1SC cells (Figure S3K).

RC Assembly and Subunit Expression following mtDNA Acquisition

The expression of RC subunit proteins encoded by mtDNA and nDNA, as well as proteins with a regulatory or general role in mitochondria, was investigated in 4T1p⁰-derived cell lines and control cells (Figures 7E and S3L). Nuclear-encoded CI subunits NDUFS3 and NDUFA9 were not expressed in 4T1p⁰ cells, but were expressed in all other cell lines. The CII subunit

Figure 3. Parental 4T1- and 4T1p⁰-Derived Cell Lines Differ in Morphology and Phenotype

(A) 4T1 and derived sublines were evaluated using light microscopy.
(B) Sublines were stained with EtBr and MitoTracker Red, and confocal microscopy was used to identify yellow (EtBr) and red (MitoTracker Red) fluorescence. Nuclei were stained with Hoechst 33342 (blue). The percentage of cells with (upper-right quadrant) and without (left quadrants) mtDNA was evaluated by flow cytometry.
(C) Sublines were incubated with AO (red) to visualize acidic vacuoles and with Hoechst 33342 to show nuclei (blue). Cells were also probed by WB for LC3B-II, p62, Parkin, and BNIP3 with actin as loading control. Bafilomycin A treatment (20 nM, 16 hr) was included in the LC3BII analysis to determine autophagic flux.
(D) Sublines were incubated with E-cadherin (green) and vimentin (red) and counterstained with Hoechst 33342 and inspected by confocal microscopy.

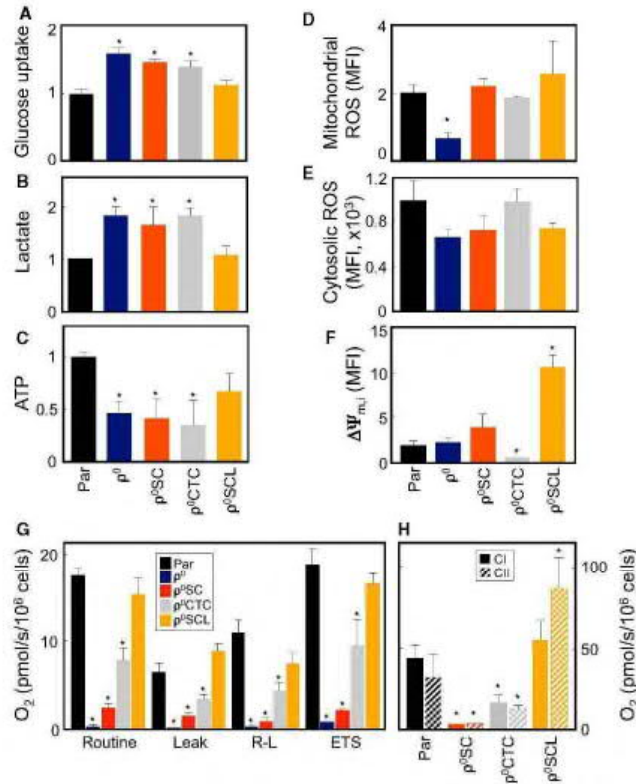


Figure 5. Bioenergetic Properties of 4T1_{p0} Sublines

4T1 cells and derived sublines were assessed for glucose uptake (A), lactate production (B), and ATP level (C). Mitochondrial superoxide generation was evaluated using MitoSOX (D), cytosolic hydrogen peroxide with DCF (E), and $\Delta\Psi_m$ using TMRM (F). (G) Cells were evaluated for routine, leak, net (R-L), and uncoupled (ETS) respiration using an Oxygraph respirometer. (H) Cells were permeabilized with digitonin and evaluated for respiration at the presence of substrates specific for CI and CII. Data are from three independent experiments, and the results are expressed as mean $\pm$ SD. The symbol * denotes significant differences with $p < 0.05$.

RCs and SCs (Figure 7F). Parental cells showed a high level of the fully assembled respirasome (CI+CI_{II}+CIV_h), which was absent in 4T1_{p0} cells. Lower respirasome levels were observed in 4T1_{p0}SC cells and 4T1_{p0}CTC cells, with full restoration in 4T1_{p0}SCL cells. Free CI, CII in the form of a dimer and in an SC with CIV, and CIV largely in the form of a monomer, were present at similar levels in all cell types except for 4T1_{p0} cells. CV was partially assembled in 4T1_{p0} cells due to the absence of structural mtDNA-encoded ATP6 and ATP8, while it was fully assembled and present as a dimer in all other cell types. CII was represented in 4T1_{p0} cells by the highly expressed SDHA subunit, SDHB, only observed in fully assembled CII, was lower in 4T1_{p0}, 4T1_{p0}SC, and 4T1_{p0}CTC than in 4T1 cells and elevated

SDHA was highly expressed in all cell lines, while SDHB was expressed at lower levels in 4T1_{p0}, 4T1_{p0}SC, and 4T1_{p0}CTC cells. The assembly factor SDHAF2 that flavinates SDHA was expressed in all cell lines, but was detected at higher levels in 4T1_{p0}SC cells, while the nuclear-encoded CIII protein Core2 was expressed in all sublines and was lowest in 4T1_{p0} cells. Nuclear-encoded CV ATPase β was expressed at similar levels in all sublines. The mtDNA-encoded COI and nDNA-encoded COIV subunits were not expressed in 4T1_{p0} cells, but COI was expressed highly in 4T1_{p0}SC cells. Nuclear-encoded proteins that are not part of RCs were expressed in all sublines with Cyt c being particularly high in 4T1_{p0} cells, and TIM23 and VDAC1 attenuated in these cells. PGC α/β that regulates the transcription factors NRF1/NRF2 was weakly expressed in 4T1_{p0} cells. OPA1, which modulates cristae formation and geometry, was also low in 4T1_{p0} and 4T1_{p0}SC cells, but progressively increased in 4T1_{p0}CTC and 4T1_{p0}SCL cells and was highly expressed in matching 4T1SC and 4T1SCL cells (Figure S3L).

Native blue gel electrophoresis (NBGE) using a mild detergent followed by WB was used to assess the assembly of individual

by 70% in 4T1_{p0}SCL cells. These findings demonstrate that transition from 4T1_{p0}CTC to 4T1_{p0}SCL cells yields a fully assembled respirasome and CII, associated with efficient respiration and tumor initiation.

DISCUSSION

Whole-genome approaches have deepened our understanding of the role of mutations in nDNA in different cancers (Kandoth et al., 2013; Khurana et al., 2013). Mutations in mtDNA have also been described in cancer, but their contribution to tumor initiation, progression, and metastasis is less clear (Brandon et al., 2006; Wallace, 2012; Schon et al., 2012). Functionally relevant mtDNA mutations may compromise mitochondrial respiration and promote aerobic glycolysis, a bioenergetic trait of many tumors. In breast cancer, mtDNA mutations and low mtDNA copy number are associated with increased metastasis and poor prognosis (Petros et al., 2005; Tseng et al., 2006), and a recent study showed that low mtDNA copy number promotes metastasis by inducing EMT via mitochondrial retrograde signaling (Guha and Avadhani, 2013).

Using metastatic murine tumor models with mtDNA deletion to simulate severe mtDNA damage, we have found a long lag to tumor formation for both B16 ρ^0 melanoma and 4T1 ρ^0 breast carcinoma cells in C57BL/6 and Balb/c mice, respectively, as well as in NOD/scid mice. Previous reports on grafting human ρ^0 tumor cells have provided variable results, depending on tumor type, the site of injection, and the recipient mouse (Hayashi et al., 1992; Morais et al., 1994; Cavalli et al., 1997; Magda et al., 2008; Kulawiec et al., 2008; Imanishi et al., 2011). In both models, we observed delayed tumor growth that was associated with mtDNA acquisition from the host suggestive of horizontal mtDNA transfer. This was demonstrated when cells derived from tumors that grew from B16 ρ^0 and 4T1 ρ^0 cells were found to be positive for both the *Cytb* gene and COI protein. We excluded the possibility of latent mtDNA being present in ρ^0 cells by showing that tumors growing from B16 ρ^0 and 4T1 ρ^0 cells contained the *tRNA^{Asp}* gene polymorphism of the recipient mouse (Bayona-Bafalluy et al., 2003), which is distinct from that of the original tumor. Two additional 4T1 polymorphisms, *D-loop* and *16S rRNA*, were identical in 4T1 ρ^0 SC cells and the recipient mouse. Although our results cannot formally disprove the possibility of residual latent mtDNA being present in ρ^0 cells, and that low-level heteroplasmy might occur at any particular polymorphic site in this latent mtDNA, the probability that latency and homoplastic conversion at all three sites could explain our results is considered to be exceedingly remote.

Because there is no known mechanism for intercellular transfer of mtDNA across both mitochondrial inner and outer membranes and the plasma membrane, transfer of whole mitochondria is most likely. This has previously been shown to occur in vitro (Spees et al., 2006; Cho et al., 2012; Gerdes and Carvalho, 2008; Gurke et al., 2008) and in a mouse model of acute lung injury, showing mitochondrial transfer from mesenchymal stem cells (MSCs) injected into the animal's lungs (Islam et al., 2012). Organelle transfer between cells has been reviewed recently, with a number of reports documenting mitochondrial transfer (Rogers and Bhattacharya, 2013). Phylogenetic evidence also supports mtDNA transfer between cells as shown in a transmissible canine venereal tumor (Rebbbeck et al., 2011).

To better understand the role of mitochondrial respiration in tumorigenesis and metastasis, we characterized malignant cells from different tumor microenvironments in the 4T1 ρ^0 model. To ensure that the results relate to mtDNA acquisition and not local microenvironment, we isolated matching cell lines from primary s.c. 4T1 tumors and lung metastases. Interestingly, we found that cells derived from primary tumors that grew from 4T1 ρ^0 cells still exhibited a considerable delay in tumor initiation, while the kinetics of growth of tumors derived from the metastatic 4T1 ρ^0 SCL cells showed no delay. These findings, initially acquired in our laboratory in Wellington and repeated with almost identical results in our laboratory in Prague 9 months later, indicate the robust nature of these results and high reproducibility.

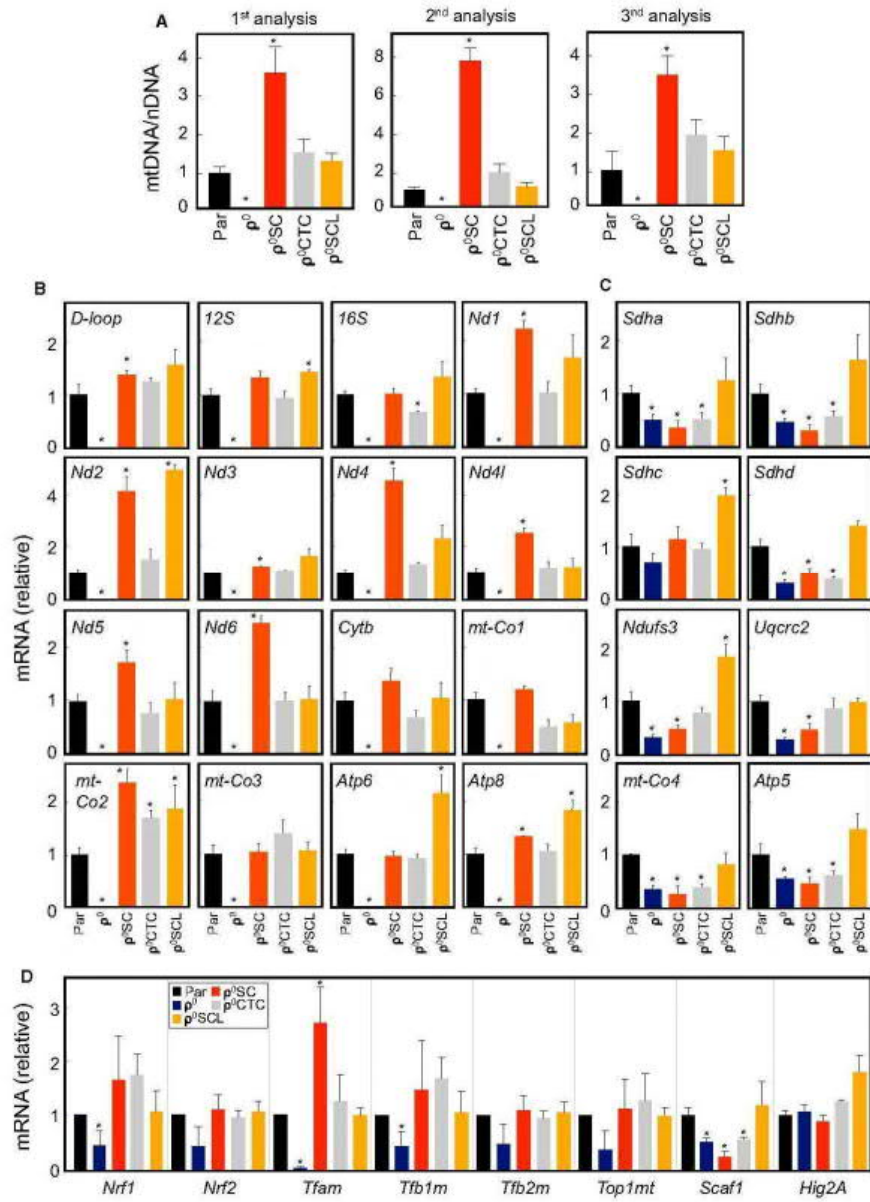
A striking finding is the stepwise recovery of mitochondrial respiration from undetectable levels in ρ^0 cells to low levels in primary tumor cells derived from ρ^0 cells, progressing to higher levels of respiration in CTCs and full restoration in metastatic cells. The phenotype of the individual cell types was maintained

in culture, as documented by sustained high mtDNA/nDNA ratio in cells from the primary tumor. This suggests that these cells are unable to restore full mitochondrial function during long-term maintenance in vitro and need microenvironmental exposure to progress to full respiratory competence. Our findings are consistent with earlier in vitro results (Spees et al., 2006) showing that mitochondrial transfer from MSCs and skin fibroblasts to human A549 ρ^0 lung adenocarcinoma cells restored their respiratory capacity.

We show detailed analysis of the individual stages of recovery of respiratory competence from parental cells to their metastatic counterparts, documenting each "recovery" stage. A crucial step of full respiration recovery is associated with the assembly of the respirasome and CII, demonstrating the need for fully functional OXPHOS. This is also supported by the high level of SQR activity in metastatic cells and agrees with the requirement for efficient OXPHOS in metastatic dissemination (LeBlau et al., 2014). In addition, we show that the level of the assembly factor SCAFI (Lapuente-Brun et al., 2013), but not HIG2A (Moreno-Lasvres et al., 2012), correlates with the extent of respirasome assembly in tumors derived from 4T1 ρ^0 cells. The key regulatory role of SCAFI and other respirasome assembly factors in the tumor microenvironment in metastatic progression needs further investigation. Similarly, the reasons for full assembly of CII in metastatic cells are not known, but are likely linked to retrograde mitochondrial signaling. That the assembly of RCs and SCs is crucial for the full recovery of mitochondrial respiration is demonstrated by our findings that except for ρ^0 cells, all sublines of these cells exhibited transcriptional and translational activity of mtDNA-encoded genes, with partially or fully assembled CI, CIII, and CIV. A key to understanding the different behavior of the sublines may relate to the high level of mitochondrial heterogeneity.

A fundamental question raised by our findings relates to how ρ^0 cells, having acquired mtDNA, progress from primary tumor via the circulation to lung metastases, and how this progression leads to full recovery of respiration. In our model, metastatic potential is encoded in the nuclear genome, but suppressed by the absence of mtDNA. Acquisition of "normal" mtDNA from the microenvironment enables 4T1 ρ^0 cells to grow as primary tumors and "unleashes" their metastatic potential. It is possible that rare cells acquire a few mitochondria that contain mtDNA, and that these cells replicate their mtDNA, build cristal structures of cristae, and multiply clonally at the expense of cells lacking mtDNA that are progressively eliminated by autophagy. Our finding that autophagic activity is low in 4T1 ρ^0 and 4T1 ρ^0 SC cells that also feature lower respiration is consistent with a report showing faulty autophagy in cells with suppressed ETC (Graef and Nunnari, 2011). Such cells do not appear to "clear" dysfunctional mitochondria, which is more likely to occur at the stage of CTCs when respiration is partially restored and autophagy higher.

Quantitation of mtDNA/nDNA ratios showed stable elevation in 4T1 ρ^0 SC cells, consistent with high levels of TFAM, a factor critical for replication, transcription, and packaging of mtDNA (Kukat and Larsson, 2013). Compromised respiration is correlated with lack of cristae in all 4T1 ρ^0 cells and poorly formed cristae in many 4T1 ρ^0 SC cells, consistent with low OPA1 levels (Cogliati et al., 2013). The reasons why ρ^0 cells that have



(legend on next page)

acquired mtDNA require conditioning by the local microenvironment to restore a parental intracellular distribution of mtDNA might relate to progressive changes in mitochondria-to-nucleus retrograde signaling that eventually normalizes both mtDNA distribution and respiratory function. The importance of the tumor microenvironment is corroborated by the finding that 4T1 cells show both epithelial and mesenchymal markers, highly localized within the cell and exhibiting different patterns of compartmentalization, with coordinate changes in E-cadherin and vimentin staining across 4T1 cells and derived sublines consistent with the different origins of the individual 4T1 sublines.

The signaling mechanism that ρ^0 cells employ to acquire mtDNA from stromal cells and how this process occurs is unclear. It has been proposed that cells form tunneling nanotubes to move biomaterial between cells (Rustom et al., 2004; Gardes and Carvalho, 2008), as shown for the mobilization of organelles (Rustom et al., 2004; Koyanagi et al., 2005; Lou et al., 2012; Islam et al., 2012; Pasquier et al., 2013), a process suppressed by cytochalasin B (Bukoreshtliev et al., 2009). Other mechanisms of organelle trafficking between cells have also been proposed including membrane vesicles, cell fusion, and various other cytoplasmic bridging processes. Of importance is the minimum level of mtDNA damage needed to initiate intercellular transfer of functional mitochondria. A gradual decrease in the number of mtDNA copies in human glioblastoma cells eventually resulted in a phenotype unable to recover in vitro, while injecting these cells into immunocompromised mice led to delayed formation of tumors and restoration of mitochondrial function (Dickinson et al., 2013). Another important question relates to how recovering mitochondrial signal to the nucleus to trigger transcription of genes needed for mitochondrial function. Retrograde mitochondrial signaling is poorly understood at present and may include the bioenergetic status of the cell (e.g., the AMP/ATP ratio), the level of ROS, release of metabolites and Ca^{2+} , etc. (Liu and Butow, 2006; Moreno-Loshuertos et al., 2006; Ryan and Hoogenraad, 2007; Guha and Avadhani, 2013).

In conclusion, we have shown that tumor cells devoid of mtDNA, a model of extreme mtDNA damage, can acquire mtDNA of host origin, resulting in stepwise recovery of respiration from primary to metastatic tumor cells. We show that a crucial aspect of full recovery of mitochondrial respiration is complete assembly of the respirasome and CII, which are directly linked to efficient tumor formation. This shows that tumor cells with compromised respiration due to mtDNA deletion are capable of in vivo mitochondrial genome acquisition and demonstrates that mitochondrial respiration is required for tumorigenesis in these models. Mitochondrial DNA mutations can also contribute to tumorigenesis, suggesting that optimum bioenergetic balance may relate to the individual tumor (Tan et al., 2014). Our results support the notion of tumor plasticity and the exceptional ability of

tumors to overcome unfavorable circumstances (Hanahan and Weinberg, 2011).

EXPERIMENTAL PROCEDURES

Cell Culture, Tumor Formation, and Establishment of Tumor-Derived Cell Lines

B16- and 6TG-resistant 4T1 cells from ATCC were used and maintained in culture as described in the Supplemental Information. For the preparation of ρ^0 cells, parental tumor cell lines were cultured for 10–12 weeks in low-dose EtBr (50–100 ng/ml) supplemented with 1 mM pyruvate and 50 $\mu\text{g}/\text{ml}$ uridine (King and Attardi, 1989) followed by transfer to medium lacking EtBr. Loss of mtDNA was monitored by sensitivity to 2 μM FCCP (Hirst and Berman, 2007; Hirst et al., 2011), PCR analysis of the mitochondrial *Cytb* gene, and by pyruvate/uridine auxotrophy. With 6TG-resistant 4T1 cells, cell lines were derived from primary s.c. tumors, mammary fat pad, circulating tumor cells, and from the lungs of mice 4–6 weeks after injection with 4T1 ρ^0 cells, by selection in the presence of 80 μM 6TG for 7–10 days. With the B16 model, cell lines were derived from primary s.c. tumors and from the lungs of mice injected with B16 ρ^0 cells by long-term culture. Tumor cells rapidly outgrew nontumor cells in these cultures, and B16 ρ^0 -derived cell lines have been maintained as stable lines for over 2 years.

Mitochondrial Isolation and Sequence Analysis

Mitochondria for respiration studies were isolated according to Schmitt et al. (2013), and mtDNA *rRNA*²⁰ sequencing was carried out on a PCR-amplified region using Sanger sequencing. Whole mtDNA sequencing used overlapping PCR-amplified products and both Ion Torrent and Nanopore MinION approaches using whole mtDNA sequencing.

Microscopic and Flow Cytometric Cell Evaluation

Standard methodology (see Supplemental Information) was used for bright field and fluorescence microscopy and TEM, while flow cytometry used BD FACSCalibur and FACSCanto instruments.

Respiration Assays

Oxygen consumption by dispersed tumor tissue and liver tissue from the same mice, and by B16, 4T1, B16 ρ^0 , and 4T1 ρ^0 and derived cell lines was evaluated with cells suspended in RPMI1640 medium without serum using Oxygraph-2K (Oroboros Instrumental) and published procedures (Postle and Gnaiger, 2012), as detailed in the Supplemental Information. Respiration by specific mitochondrial complexes was evaluated using digitonin-permeabilized cells as described in the Supplemental Information.

Mitochondrial Biochemistry Assays and Gene Expression Analysis

Mitochondrial membrane potential and ROS production were assessed by flow cytometry using the fluorescent probes TM/AM, DCF-DA, and MitoSOX. Glucose uptake was assessed using 2-NGDG, ATP using a luciferase-based assay, and lactate colorimetrically. *Cytb* was measured by PCR, while gene expression analysis used qRT-PCR. WB and NBGE used well-established methods, while newly synthesized mitochondrial proteins were evaluated using a Click-IT AHA kit followed by WB. CS and SDH/SQR activities were assessed using established protocols as described in the Supplemental Information.

Statistical Analysis

Unless stated otherwise, data are mean $\pm$ SD of at least three independent experiments and used groups of five mice. The two-tailed unpaired Student's *t* test was used to assess statistical significance with $p < 0.05$ being regarded

Figure 6. Mitochondrial DNA Content and Transcriptional Activity of 4T1 ρ^0 Sublines

(A) 4T1 cells and derived sublines were evaluated for mtDNA/nDNA ratio using qPCR and specific primers (Supplemental Information). Analyses were performed using cells maintained in culture for a short period of time (first analysis), 1 month (second analysis), and 4 months (third analysis). Sublines were also evaluated for the level of RC transcripts encoded by mtDNA (B) and nDNA (C) and for transcripts of mitochondrial regulatory genes (D) using qPCR and specific primers (Supplemental Information). Data are from three independent experiments, and the results are expressed as mean $\pm$ SD. The symbol * denotes significant differences with $p < 0.05$.

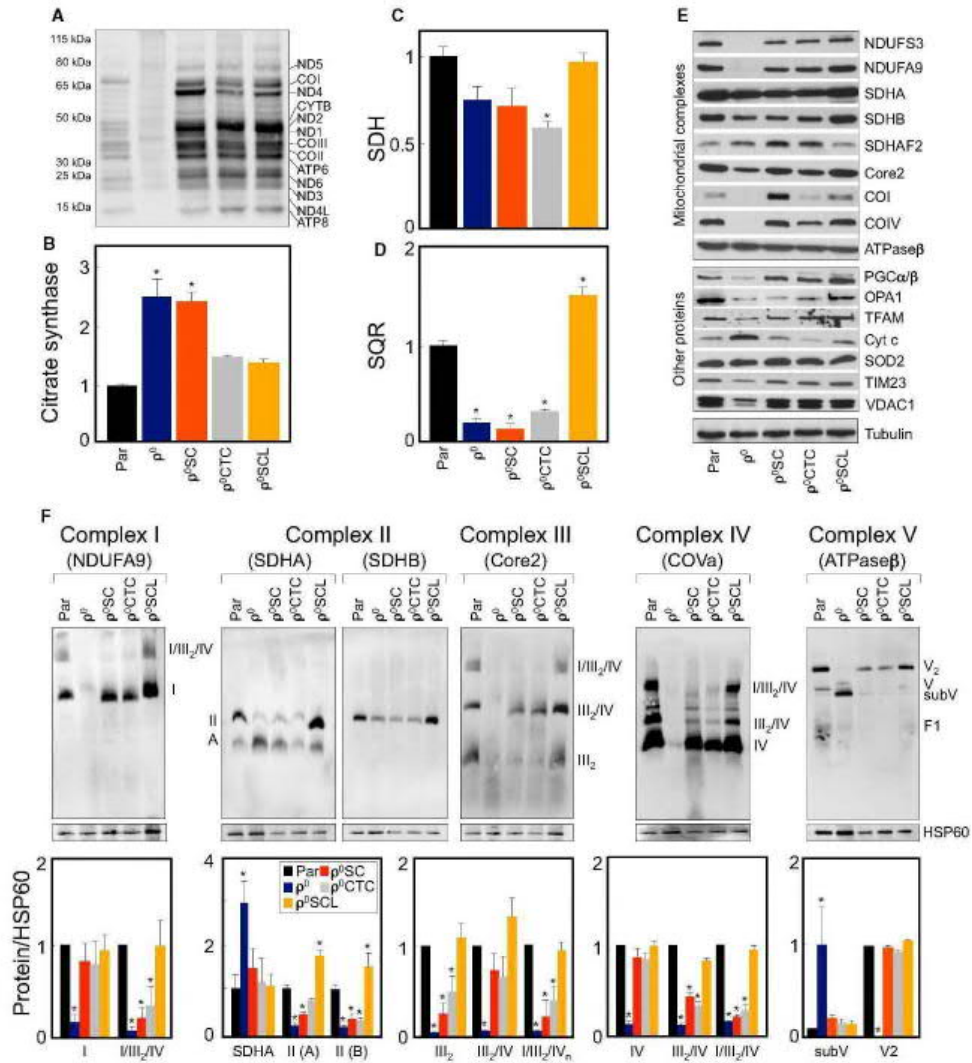


Figure 7. Mitochondrial Protein Synthesis, Respiratory Complex Formation, and Supercomplex Assembly in 4T1_{p0} Sublines
 (A) 4T1 cells and derived sublines were evaluated for newly synthesized proteins using the Click-IT AHA kit. Sublines were assessed for CS (B), SDH (C), and SQR activity (D) as detailed in *Experimental Procedures*, as well as for expression of specific proteins using WB (E). Tubulin was used as a loading control. (F) The mitochondrial fraction was lysed in the presence of digitonin and subjected to NBGE. Specific subunits of individual complexes were detected using the antibodies shown. HSP60 was used as a loading control. The level of expression of the individual bands representing RCs, subcomplexes, and SCs was evaluated by densitometry relative to HSP60. Data are from three independent experiments, and the results are expressed as mean ± SD. The symbol * denotes significant differences with $p < 0.05$.

as significant. Images are representative of three independent experiments. For TEM data, Tukey's multiple comparison procedure and statistical test was used at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2014.12.003>.

AUTHOR CONTRIBUTIONS

A.S.T. and M.V.B. established the mouse models of intercellular mtDNA transfer and together with J.W.B. performed the tRNAseq sequencing; L.-F.D. performed, analyzed, interpreted, and codirected a number of experiments; B.E., J.G., M.B., J.K., M.P., B.Y., E.A.P., M.V., and K.K. performed particular experiments; A.B.-G. and J.R. performed and analyzed experiments; M.S., A.F., and P.H. performed and interpreted TEM; J.R., J.T., D.E., S.S., L.M.H., and L.R.G. performed, analyzed, and interpreted mitochondrial genome sequencing; M.V.B. and J.N. designed and directed experiments and together wrote the manuscript.

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2. Horizontal transfer of whole mitochondria restores tumorigenic potential in mitochondrial DNA-deficient cancer cells

The idea that mitochondria move between cells has previously been shown to occur *in vitro* (Spees et al., 2006; Gurke et al., 2008; Wang and Gerdes, 2015) and in a mouse model of acute lung injury, showing mitochondrial transfer from MSCs injected into the mice lungs, presenting as a transition from *in vitro* to *in vivo* status (Islam et al., 2012). While this is close to an *in vivo* situation, it can be considered an ‘in vivo co-culture’ experiment, since the MSCs were injected into the animals’ lungs. These reports show that horizontal mitochondrial transfer, in general, occurs in the direction from donor cells to acceptor cells with a defect in mitochondria, in particular mtDNA, via TNTs. Our recent report is the only definitive proof of *in vivo* transfer of mtDNA/mitochondria, with functional consequences (Tan et al., 2015).

In this study, we further investigated the process of trafficking of mitochondria with their DNA from donor cells to cells with damaged (depleted) mtDNA. When we performed identical experiments as in the first paper with murine metastatic melanoma B16p0 cells, we found a complete recovery of respiration consistent with assembly of SCs already in B16p0SC cells, with no further increase in respiration in B16p0CTC and B16p0SCL cells. Next, we tested whether suppression of respiration interferes with tumor development and observed that B16p0 cells with suppressed levels of nDNA-encoded CI or CII impaired their ability to form tumors. Along with the previously mentioned hypothesis, we grafted B16p0nBFPcells in C57BL/6Nsu9-DsRed2 mice and observed transfer of DsRed mitochondria from MSCs into the recipient B16p0. We were able to isolate double-positive (nBFP plus DsRed) cells with fully formed mitochondrial cristae and mtDNA nucleoids.

The presented study provides a link between efficient tumor formation and recovery of mitochondrial respiration and shows that mtDNA acquisition occurs via trafficking of whole mitochondria between MCSs and cancer cells.

Contribution to the publication: Experimental part (cell culture, mtDNA level assay, qPCR, RT-PCR, respiration assays, mice experiments, ultrasound imaging of tumors, the isolation and characterization of double positive cell line), revision of the manuscript.

Horizontal transfer of whole mitochondria restores tumorigenic potential in mitochondrial DNA-deficient cancer cells

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Abstract Recently, we showed that generation of tumours in syngeneic mice by cells devoid of mitochondrial (mt) DNA (p^0 cells) is linked to the acquisition of the host mtDNA. However, the mechanism of mtDNA movement between cells remains unresolved. To determine whether the transfer of mtDNA involves whole mitochondria, we injected B16 p^0 mouse melanoma cells into syngeneic C57BL/6N^{suH-DsRed2} mice that express red fluorescent protein in their mitochondria. We document that mtDNA is acquired by transfer of whole mitochondria from the host animal, leading to normalisation of mitochondrial respiration. Additionally, knockdown of key mitochondrial complex I (NDUFV1) and complex II (SDHC) subunits by shRNA in B16 p^0 cells abolished or significantly retarded their ability to form tumours. Collectively, these results show that intact mitochondria with their mtDNA payload are transferred in the developing tumour, and provide functional evidence for an essential role of oxidative phosphorylation in cancer.

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Introduction

Mitochondria are vital organelles of eukaryotic cells responsible for energy production and other key biochemical functions. Many human diseases, including cancer, are characterised by mutations in mitochondrial DNA (mtDNA). This is linked to altered mitochondrial bioenergetics, such that cancer cells are more glycolytic than their non-malignant counterparts, as postulated almost a century ago by Warburg. Altered energy metabolism is now regarded as one of the hallmarks of cancer

(Koppenol et al., 2011; Hanahan and Weinberg, 2011; Vyas et al., 2016). In this context, the mitochondrial genome has been reported to play a role in tumorigenesis (Lee and St John, 2016; Patananan et al., 2016) and in metastatic cancer (Ishikawa et al., 2008; Hayashi et al., 2016).

Although many cancer cells are biased towards the glycolytic metabolism, they also need oxidative phosphorylation (OXPHOS) for their 'pathophysiological' requirements (Weinberg et al., 2010). Recent research has demonstrated a new concept of cancer metabolism, emphasising the importance of OXPHOS in the tumour environment beyond its role in bioenergetics (Gentric et al., 2016). A novel paradigm has emerged, according to which respiration is important for cancer cell proliferation, and also for tumour formation, progression and metastasis (LeBleu et al., 2014; Viale et al., 2014). This is probably linked to metabolic re-modelling (Birsoy et al., 2015; Sullivan et al., 2015). There are reports that some cancer cells retain OXPHOS capacity and have no obvious respiratory defects (Frezza and Gottlieb, 2009; Jose et al., 2011). Further, inhibiting glycolysis may restore higher rates of OXPHOS in neoplastic cells (Moreno-Sánchez et al., 2007; Michelakis et al., 2010). Hence, depriving cancer cells of their capacity to respire may preclude them from forming tumours.

We recently reported on the importance of respiration in tumour formation and progression (Tan et al., 2015). We showed that cancer cells without mtDNA (p^0 cells) form tumours after a considerable delay compared to their parental counterparts. Tumour progression was associated with mtDNA acquisition from the host, resulting in respiration recovery. While these findings point to a new phenomenon of horizontal transfer of mtDNA between mammalian cells in vivo (Berridge et al., 2015, 2016), direct evidence for the role of mitochondrial respiration in tumour formation as well as understanding the mode of mtDNA acquisition is lacking.

Here we provide a link between efficient tumour formation and recovery of mitochondrial respiration, and show that mtDNA acquisition occurs via trafficking of whole mitochondria.

Results

Cell lines derived from tumours that formed from B16 p^0 cells are homogeneous in mtDNA distribution and contain a fully assembled respirasome

We have shown that B16 p^0 cells injected subcutaneously into C57BL/6J mice formed syngeneic tumours with a 2–3-week delay compared to B16 cells, and next generation sequencing (NGS) indicated the host origin of mtDNA (Tan et al., 2015). Since NGS would not detect heteroplasmy of less than about 3%, a much more sensitive single cell/digital droplet (sc/dd) PCR method was used in the current study to document that the homoplasmic polymorphism at the *tRNA^{Arg}* locus of mtDNA of cell lines isolated from tumours grown subcutaneously from B16 p^0 cells (B16 p^0 SC cells) is of the host origin. The assay is able to detect heteroplasmy down to 0.5%, demonstrating with very high confidence that the mtDNA in B16 p^0 SC cells is of host origin, and that original B16 polymorphism is either completely absent or present below the detection limit of 0.5% of mtDNA (Figure 1A; see also Appendix 1—figure 1 for validation of sc/ddPCR).

We next analysed the properties of B16, B16 p^0 and B16 p^0 SC cells, as well as a B16 p^0 CTC sub-line derived from circulating tumor cells and a B16 p^0 SCL sub-line derived from lung metastases (Tan et al., 2015). Appendix 1—figure 2A documents confocal microscopy analysis of mtDNA in mitochondria, showing that B16 p^0 cells lack mtDNA, whereas mtDNA appears homogeneously distributed in mitochondria in all other sub-lines. Super-resolution stimulated emission depletion (STED) microscopy exerted similar levels and distribution of mtDNA nucleoids in B16, B16 p^0 SC, B16 p^0 CTC and B16 p^0 SCL cells, and no nucleoids in B16 p^0 cells, also showing largely unchanged levels of Tom20 and low level of TFAM (Figure 1B; see also Appendix 1—figure 2E). Native blue gel electrophoresis (NBGE) revealed that B16 p^0 cells do not contain the supercomplex/respirasome (formed by CI, CIII and CIV), but contain low amounts of sub-CV (Figure 1C). CII was found to be fully assembled in all sub-lines, which is reasonable considering that all four subunits in CII are encoded by nuclear DNA (nDNA) (Figure 1C). To test if cells replicate their mtDNA, we established the mitochondrial chromatin immunoprecipitation (mitoChIP) assay. This showed a high level of DNA polymerase- γ 1 (POLG1) binding to the D-LOOP region of mtDNA in all cells except B16 p^0 cells (Figure 1D). B16 p^0 SC, B16 p^0 CTC and B16 p^0 SCL cells showed similar respiration to B16 cells, but no respiration was observed with B16 p^0 cells (Figure 1E,F). Accordingly, B16 p^0 cells produced more

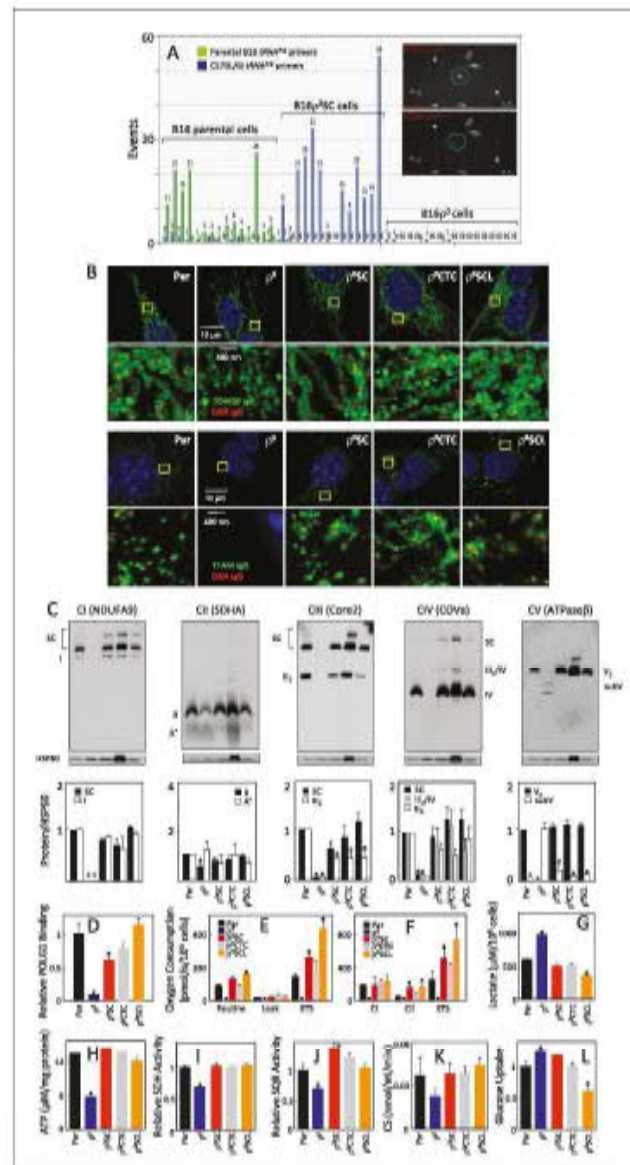


Figure 1. Cells derived from B16p0 cell-grown tumors feature mtDNA with host polymorphism, and recovered mitochondrial complexes and respiration. (A) B16, B16p0 and B16p0SC cells were assessed by sc/dd PCR for polymorphism of the *mtDNA* locus of mtDNA using specific probes (see Materials and methods). The insert shows a cell (circled) before (upper image) and after (lower image) withdrawn for analysis. (B) B16, B16p0, B16p0SC, Figure 1 continued on next page

Figure 1 continued

B16p⁰CTC and B16p⁰SCL cells were immunostained for anti-DNA (red) and anti-Tom20 or anti-TFAM IgGs (green). The upper panels represent lower resolution confocal images depicting a major part of a whole cell, the lower panels represent higher magnification STED images of the region of interest indicated by the yellow box. (C) Cells as above were subjected to NBGE followed by WB using antibodies against subunits of individual complexes. Below is a densitographic evaluation of three gels derived from individual experiments with HSP60 as the internal control. The cells were assessed for binding of POLG1 to the D-LOOP region of mtDNA using the mitoChIP assay (D), for routine respiration (E) and for respiration via CI and CII following their permeabilisation (F). The sub-lines were next assessed for lactate generation (G), ATP level (H), SDH (I), SQR (J) and CS activities (K) as well as for glucose uptake (L). The symbol '*' indicates statistically significant differences between individual sublines and B16 cells, the symbol '#' in panel C indicates statistically significant difference between individual sublines and B16p⁰ cells. The nature of the individual sublines derived from B16p⁰ cells is as follows: B16p⁰SC cells, cells derived from primary tumour grown in B57BL mice grafted with B16p⁰ cells; B16p⁰CTC cells, the corresponding circulating tumour cells; B16p⁰SCL cells, the corresponding cells isolated from lung metastases.

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lactate (Figure 1G) and less ATP (Figure 1H). B16p⁰ cells also had lower succinate dehydrogenase (SDH) (Figure 1I) and succinate quinone reductase (SQR) (Figure 1J) activity, as well as lower citrate synthase (CS) activity (Figure 1K). Finally, we observed higher glucose uptake in B16p⁰ and B16p⁰SC cells, and lower uptake in B16p⁰SCL cells (Figure 1L). Collectively, these results document that mitochondrial function is already fully restored in cells derived from the primary tumour.

We next analysed cells for their mtDNA levels and expression of selected transcripts. Appendix 1—figure 2B shows no mtDNA in B16p⁰ cells, while mtDNA was present at similar levels in other sub-lines. No mtDNA-encoded transcripts were present in B16p⁰ cells. Their levels were low in B16p⁰SC cells, while higher levels were seen for most transcripts in B16p⁰CTC and in B16p⁰SCL cells. Transcripts of the assembly factor SCAF1 were present at similar levels in all sub-lines, but TFAM transcripts were low in B16p⁰ cells relative to the other cells. Transcripts of nDNA genes coding for subunits of respiratory complexes were found to be present in all cells, with some being lower in B16p⁰ cells. WB revealed that most proteins investigated were relatively abundant in all sub-lines with LC3AII levels lower in B16p⁰ cells, indicating stalled autophagy (Appendix 1—figure 2C). Interestingly, although present in B16p⁰ cells, many nDNA-encoded mitochondrial proteins, including subunits of RCs, were unstable in these cells, as evidenced using cycloheximide treatment (Appendix 1—figure 2D). Exceptions were SDHA and ATPβ; a plausible reason is the absence of binding partners encoded by mtDNA, which could render the 'unassembled nDNA-encoded' subunits unstable. In summary, these results indicate that, in contrast to 4T1 cells (Tan et al., 2015), the respiratory function of B16 sublines is already fully recovered at the primary tumour stage.

Recovery of B16p⁰ cell respiration fully restores their propensity to form tumours

Given the rapid recovery of respiratory function in the B16 model, we next tested tumour-forming capacity of B16 and B16p⁰ cells, and of the sub-lines B16p⁰SC, B16p⁰CTC and B16p⁰SCL, derived from various tumour stages as described above. We found that all sub-lines formed tumours without delay except for B16p⁰ cells with >2 week delay (Figure 2A). Sectioning of tumours derived from all five sub-lines revealed melanomas with necrotic cells away from blood vessels (Figure 2B). Tumours derived from the sub-lines were assessed by NBGE, identifying full assembly of all complexes as well as the respirasome, with somewhat higher levels of the respirasome in tumours derived from B16p⁰ cells (Figure 2C). WB revealed similar levels of expression of most proteins tested in the five types of tumours (Figure 2D). No obvious differences in the expression of genes coding for transcripts of individual subunits of mitochondrial complexes encoded by mtDNA and nDNA were observed (Figure 2E). Finally, we tested respiration of tumour tissues derived from the sub-lines. Figure 2F reveals increased CI-dependent respiration and the maximum electron transfer capacity (ETS) in tumours derived from B16p⁰ and from B16p⁰SCL cells, and no change in CII-dependent respiration. Normal liver tissue from the same animals used as internal control showed no changes in respiration. The restoration of the tumorigenic potential fully correlates with the observed recovery of respiration.

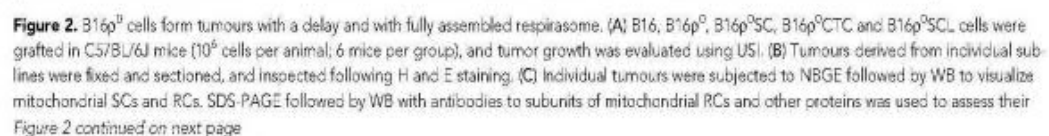


Figure 2 continued

levels (D), qPCR was used to assess the levels of representative mtDNA- and nDNA-coded mRNAs (E). (F) Tumor (left) and liver tissues (right) from mice grafted with individual B16 sub-lines were assessed for CI- and CII-dependent respiration, and for maximal uncoupled respiration (ETS, tumour only). The symbol '*' indicates statistically significant differences between tumours derived from individual sub-lines and tumours derived from B16 cells.

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Suppression of respiration interferes with efficient tumour formation

We have shown for B16 cells (Figures 1 and 2, Appendix 1—figure 2) and previously for 4T1 cells (Tan et al., 2015) that tumour formation from their respective p^0 variants correlates with the acquisition of mtDNA and recovery of respiration. However, direct evidence for the requirement of respiration for tumour formation has been missing. We therefore prepared B16 and B16 p^0 cells with suppressed levels of NDUFV1 (the catalytic subunit of CI) or SDHC (ubiquinone-binding CII subunit essential for its SQR activity) by RNA interference (RNAi) using two different shRNAs for each protein. Figure 3A shows that NDUFV1 shRNA#2 and SDHC shRNA#2 were rather efficient in knocking down the respective proteins. Figure 3A also reveals that NDUFV1 knock-down (KD) cells exhibit lower level of CI subunits (NDUFV1 and NDUF53) while CII subunits (SDHA and SDHC) were unaffected. Conversely, SDHC KD cells showed low levels of CII subunits and also decreased levels of CI subunits. We used cells stably transfected with NDUFV1 shRNA#2 or SDHC shRNA#2 in subsequent experiments. Both NDUFV1 KD and SDHC KD cells proliferated at a slower rate than parental cells (Figure 3B). NDUFV1 KD as well as SDHC KD cells showed lower routine respiration and lower ETS (Figure 3C). NDUFV1 KD cells respired less via CI, while CII-dependent respiration was largely unaffected; SDHC KD cells not only showed much lower CII-dependent respiration, but also significantly suppressed CI-dependent respiration (Figure 3D).

We next grafted B16, B16 p^0 and B16 cells, as well as their derived NDUFV1 KD and SDHC KD cells into C57BL/6J mice. Figure 3E shows that tumours started to grow from B16 p^0 cells shortly after day 20 post-grafting of B16 p^0 cells in all mice. 4 out of 6 mice grafted with B16 p^0 SDHC KD cells formed tumours with delays of 15 to 40 days compared to B16 p^0 cells. Only one out of 5 mice of the B16 p^0 NDUFV1 KD group formed a tumour, with a lag of about 40 days compared to B16 p^0 cells. A similar pattern was observed for B16 cells and their NDUFV1 KD and SDHC KD variants, though the lag for these sub-lines was considerably shorter (Figure 3F). On pathological examination of the different tumours, relatively subtle differences in morphology were observed (Figure 3G). B16 melanoma cells form tumours in control mice (NS) showed prominent nuclear pleomorphism. They appeared more aggressive than cells from p^0 tumours. Tumours derived from both B16 NDUFV1 KD and B16 SDHC KD cells showed better histological differentiation associated with eosinophilic cytoplasm, vesicular nuclei and frequent deposits of melanin pigments, indicating the KD cells are less aggressive than their parental counterpart. There were no obvious differences in morphology of the 3 types of p^0 melanoma cells. Both B16 p^0 NDUFV1 KD and B16 p^0 SDHC KD tumours showed focal areas of nuclear pleomorphism and frequent mitotic features.

These data clearly point to respiration recovery as essential for driving efficient tumour formation, since suppression of respiration completely deregulated this process. Interestingly, while with CII suppression the majority of mice formed tumours (albeit with additional lag time), most mice grafted with CI-compromised cells failed to form tumours within 100 days. In summary, we show that respiration is important for efficient tumour formation, which is consistent with recent reports (Weinberg et al., 2010; Birsoy et al., 2015; Sullivan et al., 2015).

B16 p^0 cells acquire mtDNA via transfer of whole mitochondria from the host

We have previously documented the host origin of mtDNA in cancer cells isolated from primary tumours derived from 4T1 p^0 and B16 p^0 cells based on NGS analysis (Tan et al., 2015), which we confirmed here for B16 p^0 SC cells using the more sensitive sc/dd PCR assay (Figure 1A). The only plausible explanation for this phenomenon is the transfer of mtDNA from host cells to tumour cells with compromised mtDNA. The question of how mtDNA moves between cells has not been addressed. An attractive scenario is that whole mitochondria with their payload of mtDNA are transferred, but alternative explanations such as cell fusion have also been suggested (Vitale et al.,

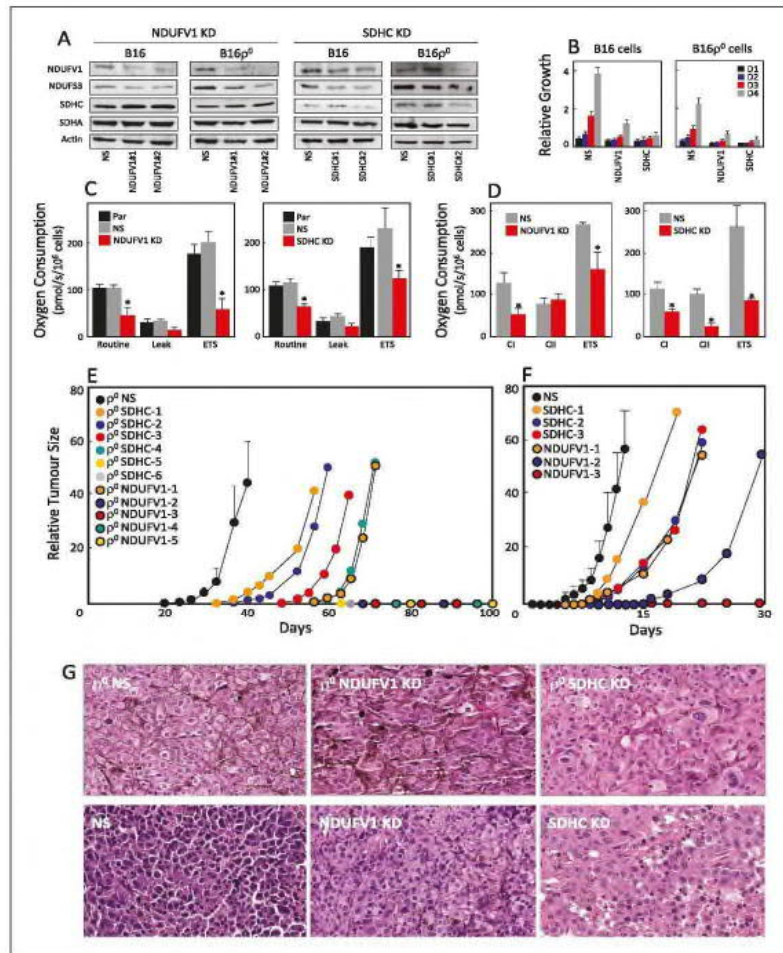


Figure 3. Suppression of respiration deregulates tumour growth. (A) B16 and B16p⁰ cells were stably knocked down for either NDUV1 or SDHC, or transfected with non-silencing (NS) shrRNA, and the expression of NDUFS3, NDUV1, SDHA and SDHC was assessed by WB. The sub-lines were next evaluated for proliferation on days 1 (D1), D2, D3 and D4 (B), for routine, leak and ETS respiration (C), and for respiration via CI and CII (D). Balb/c nude mice were injected s.c. with 5×10^5 B16p⁰ cells as well as cells with NDUV1 KD or SDHC KD cells (E), or with B16 cells and the derived NDUV1 KD or SDHC KD cells (F), with six mice per group except for the NDUV1 group with five animals. Individual mice were assessed for tumor volume using USI. Tumours derived from B16p⁰ and B16 cells were averaged and plotted as mean values, while tumours derived from knock-down cells were plotted individually. Circles on the X-axis represent individual mice in which tumours did not form within the duration of the experiment. (G) Mice with tumours derived from cell lines as shown in the Figure were sacrificed, and tumours fixed, sectioned and stained with H and E. The symbol '*' indicates statistically significant differences between B16 cells and NDUV1 KD or SDHC KD cells.

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2011; Maiuri and Kroemer, 2015). Mesenchymal stem cells (MSCs) have been proposed as donors of mitochondria due to their high levels of Miro-1, which is the adaptor protein responsible for mitochondrial association with the microtubule mobility complex (Ahmad et al., 2014; Mills et al., 2016). We performed co-culture of MSCs isolated from C57BL/6J mouse bone marrow with either B16 or B16 ρ^0 cells. Prior to co-culture, the mitochondria of the potential donor (MSC or B16) cells were labelled with mitochondrial dark red fluorophore (MitoDR). Carboxyfluorescein succinimidyl ester (CFSE) that binds covalently to amino acid residues inside cells was used for staining the potential recipient (B16 or B16 ρ^0) cells. Following co-culture, cells were assessed by confocal microscopy to determine the extent of mitochondrial transfer to recipient cells. Representative images and quantitative evaluation (Figure 4A, Appendix 1—figure 3A,B) reveal accumulation of MitoDR stained mitochondria in CFSE stained ρ^0 cells, as a result of their transfer from MitoDR stained MSCs. In similar experiments, pairs of either CFSE⁺B16 / MitoDR⁺MSC or CFSE⁺B16 ρ^0 /MitoDR⁺B16 cells did not show any evidence of MitoDR stained mitochondria in CFSE positive cells, thereby excluding dye diffusion. Collectively, this indicates that transfer of mitochondria between cells in a regulated process.

We next conducted a co-culture experiment, in which MSCs were isolated from transgenic C57BL/6N^{su9-DsRed2} mice with red fluorescent mitochondria in somatic cells. These cells were cultured with ρ^0 cells transfected with a plasmid coding for nuclear targeted blue fluorescence protein (nBFP) and plasma membrane green fluorescence protein (pmGFP). Appendix 1—figure 3C documents transfer of DsRed mitochondria from MSCs into the recipient ρ^0 cells via an intracellular bridge.

To investigate the origin of mtDNA and the manner of its transfer between host and cancer cells in vivo, we used transgenic C57BL/6N^{su9-DsRed2} mice with red fluorescent mitochondria in somatic cells. B16 ρ^0 cells transfected with a plasmid coding for nuclear-targeted blue fluorescent protein (nBFP) were injected subcutaneously into C57BL/6N^{su9-DsRed2} mice. Several days later, mice were sacrificed, the pre-tumour lesion excised, and single cell suspension sorted for double-positive (DP) cells with both red and blue fluorescence that on average were found with the frequency of 0.23 ± 0.18 in the BFP-positive population. Immediately after sorting, B16 ρ^0 DP cells were plated and inspected by confocal microscopy within about 12 hr before red fluorescence in mitochondria has been lost. Figure 4B shows an image of a B16 ρ^0 DP cell prepared from a day 11 pre-tumour lesion, identifying mouse stromal cells as a source of mitochondria that moved into a grafted B16 ρ^0 cell. The sorted DP cells were established as a sub-line. B16, B16 ρ^0 , and B16 ρ^0 DP cells were subjected to transmission electron microscopy and STED microscopy to show the presence of fully formed mitochondrial cristae (Figure 4C) and mtDNA nucleoids in DP cells (Figure 4D). DP cells also showed strong binding of POLG1 to the D-LOOP region of mtDNA (Figure 4E) as well as recovery of respiration (Figure 4F), and showed a propensity to form tumours without a lag phase (Figure 4G). These results document that mtDNA is transferred from stromal cells to B16 ρ^0 cells within intact mitochondria, resulting in the restoration of respiration and in efficient tumour formation.

Discussion

Horizontal gene transfer is a process that until recently had not been known in mammals (Keeling and Palmer, 2008), but has been described for lower eukaryotes (Gladyshev et al., 2008), affecting their phenotype (Boschetti et al., 2012). In mammals, the mitochondrial gene transfer has been inferred in a 10,000 year-old canine transmissible venereal tumour (CTVT) (Rebbek et al., 2011; Murchison et al., 2014; Strakova and Murchison, 2015). These predictions have now been confirmed, and a recent study showed at least 5 mtDNA transfers in CTVT within the last 1–2 thousand years (Strakova et al., 2016). Mitochondrial transfer was reported in vitro with functional consequences (Spees et al., 2006; Wang and Gerdes, 2015), as well as in mice with endogenously injected MSCs (Islam et al., 2012; Ahmad et al., 2014), but these results could also be explained by the association of membrane-bound particles or exosomes containing mitochondria with the damaged cells rather than functional mitochondria proliferating/dividing inside cells. A recent publication from our laboratory showed, for the first time, horizontal transfer of mitochondrial genes between mammalian cells in vivo, based on acquisition of host mtDNA by ρ^0 tumour cells and on the presence of host mtDNA markers in cell lines derived from these tumours (Tan et al., 2015). Several

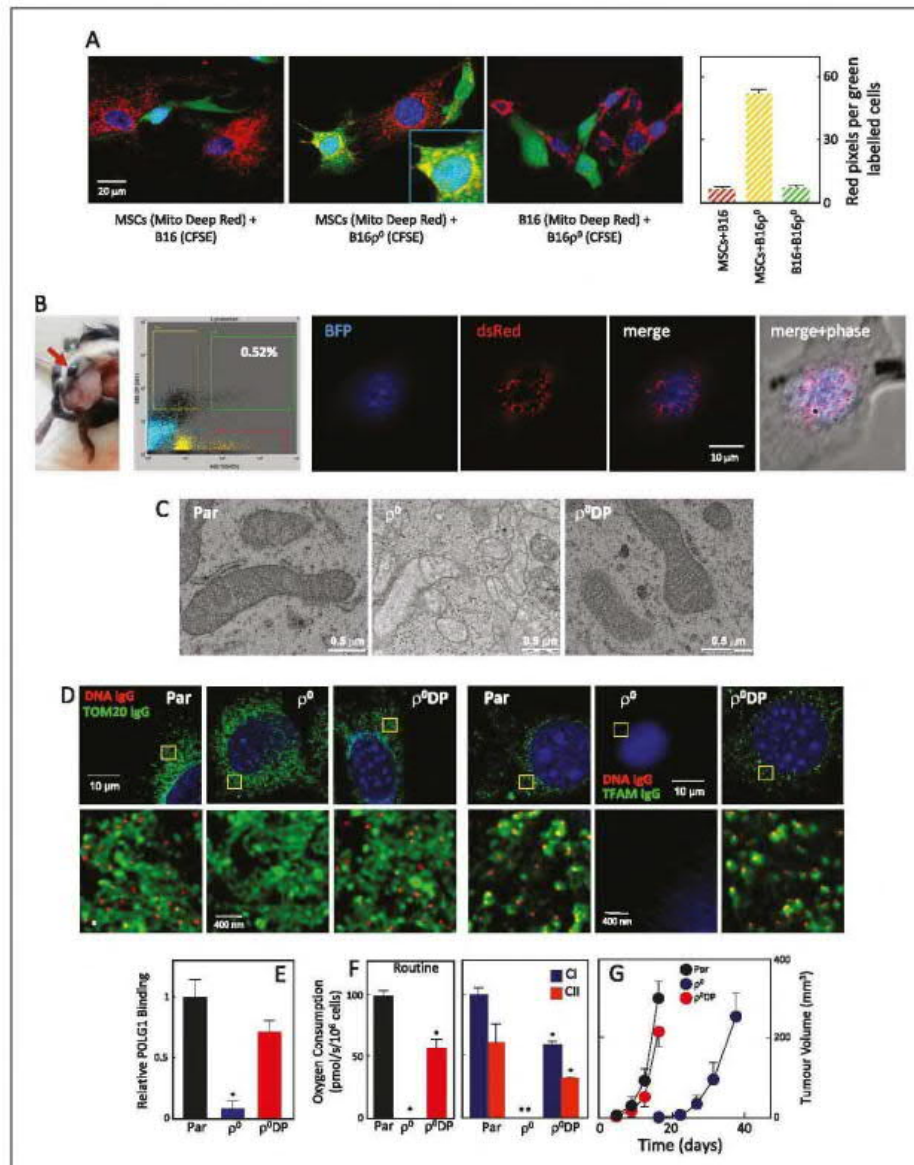


Figure 4. mtDNA transfers from host cells to B16p⁰ cells in whole mitochondria. (A) MSCs prepared from C57BL/6J mice were labelled with Mito Deep Red (MitoDR) targeted to mitochondria, B16 or B16p⁰ cells were labelled with CFSE. On the left, confocal micrographs are shown for MSCs labelled with MitoDR co-cultured with B16 or B16p⁰ cells, or B16 cells labelled with MitoDR co-cultured with B16p⁰ cells labelled with CFSE for 24 hr. On the right, evaluation of confocal microscopy is shown. (B) C57BL/6N^{trypD⁰DracD⁰} mice with red fluorescent mitochondria were grafted subcutaneously with 10⁶ Figure 4 continued on next page

Figure 4 continued

B16p⁰ cells stably transfected with nBFP. After 11 days, a mouse was sacrificed and the pre-tumour lesion excised and digested into the single cell population, which was sorted for double positive (DP) (red and blue fluorescent) cells. The cells were then inspected by confocal microscopy for blue nuclei and red mitochondria. The image shows maximum intensity Z-projection of a representative DP cell. (C) Parental B16, p⁰ and p⁰DP cells were evaluated for mitochondrial morphology using transmission electron microscopy. (D) Parental cells and their p⁰ and DP counterparts were immunostained for DNA, TFAM and Tom20, and inspected by STED microscopy for mitochondrial nucleoids. The upper panels show a confocal image of a major part of a whole cell, the lower images depict higher magnification of the regions of interest indicated above by the yellow box obtained by STED. (E) Parental cells and their p⁰ and DP counterparts were assessed for binding of POLG1 to the mtDNA D-LOOP region using mitoChIP. (F) Parental cells and their p⁰ and DP counterparts were evaluated for routine respiration or respiration via CI and CII. (G) B16 cells and their p⁰ and DP counterparts were grafted s.c. in C57BL/6J mice at 10⁶ per animal and tumour growth evaluated by USI. The symbol '*' indicates statistically significant differences between individual B16p⁰ or B16p⁰ DP cells and parental B16 cells.

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subsequently published papers also provide evidence for horizontal transfer of mitochondria under (patho)physiological conditions (Lei and Spradling, 2016; Hayakawa et al., 2016; Osswald et al., 2015; Moschoi et al., 2016), pointing to OXPHOS as a factor complicating cancer therapy (Osswald et al., 2015; Moschoi et al., 2016; Matassa et al., 2016). Hence, our data, along with those of other groups, point to mitochondrial transfer as a highly dynamic field of research, with important implications for the conceptual understanding of cancer.

Our recent findings (Tan et al., 2015) provoke a number of questions, such as whether respiration is essential for tumour formation, and what is the mode of mtDNA acquisition. To address these queries, B16 metastatic melanoma cells without mtDNA and with compromised respiratory function were used. We show that B16p⁰ cells do not form tumours unless they acquire mtDNA and that severe suppression of either CI- or CII-dependent respiration leads to impaired ability to form tumours, directly linking mitochondrial respiration and tumor growth. The reason(s) for the apparently greater importance of CI-dependent respiration in tumour formation (Figure 3E,F) remain to be explored, particularly when the role of SDH in metabolic re-modelling is taken into consideration (Cardaci et al., 2015; Lussey-Lepoutre et al., 2015). From a mechanistic point of view, CI is essential for respirasome assembly (Moreno-Lastres et al., 2012) that is needed for CI-dependent respiration (Acín-Pérez et al., 2008; Lapuente-Brun et al., 2013; Tan et al., 2015). Another indication of the importance of CI is the finding that tumours derived from B16p⁰ cells feature much higher CI-dependent respiration (Figure 2F).

We document here that the primary tumour-derived B16p⁰SC cells feature a fully assembled respirasome and complete recovery of respiration, while corresponding 4T1p⁰SC cells show only partial (20–25%) recovery of respiration (Tan et al., 2015). Consistent with this, 4T1p⁰SC cells formed tumours with a longer delay than parental 4T1 cells, while no delay was observed for B16p⁰SC cells. This is likely related to the higher requirement of B16p⁰ cells for respiration recovery to form tumours. This 'threshold', apparently lower for 4T1p⁰ cells, may be due to higher routine respiration of B16 cells of some 100 pmol O₂/s/10⁶ cells (Figure 1E) when compared to less than 20 pmol O₂/s/10⁶ cells for 4T1 cells (Tan et al., 2015). Additionally, the different genetic changes in B16 melanoma and 4T1 breast carcinoma cells could underpin altered respiration recovery. Whether there is a link to a requirement for 'threshold' recovery of respiration for cancer cells to initiate tumour formation has yet to be determined. We propose the term 'OXPHOS addiction' to describe the requirement for mitochondrial respiration across the landscape of tumours, and will investigate this in more detail in future research.

A key question has been the mode of movement of mtDNA between cells. Both selective transfer of mitochondria and cell fusion have been proposed in the past (Tan et al., 2015; Vitale et al., 2011). Using C57BL/6N^{suP-DsRed2} mice with red fluorescent mitochondria in somatic cells, we now provide evidence for acquisition of mtDNA by the trafficking of whole mitochondria from host donor cells to p⁰ cells both in vivo (Figure 4B), resulting in long-lasting respiration recovery and, consequently, efficient tumour formation (Figure 4E–G). The transient nature of dsRed expression in recipient B16p⁰ cell mitochondria in the in vivo model sheds additional light on the mechanism of mitochondrial transfer. When dsRed-containing mitochondria are selectively transferred from the host into the recipient cells, dsRed cannot be replenished in the donated mitochondria by de novo synthesis (not being encoded in the recipient's nuclear genome), and the red fluorescence in

donated mitochondria is quickly lost. On the other hand, if cell fusion were responsible for the observed acquisition of mitochondria from the donor cells (Maiuri and Kroemer, 2015), the donor's nuclear material would also be transferred, and mitochondrial dsRed expression would be maintained. As this is not the case, the selective transfer of whole, intact mitochondria remains the only possible explanation of our experimental data. The next step will be to investigate the mechanism of mitochondrial trafficking between cells in vivo, with tunnelling nanotubes being a plausible mode of intercellular transfer of the organelles (Rustom et al., 2004; Rogers and Bhattacharya, 2014; Rustom, 2016).

We conclude that recovery of respiration in tumor cells with damaged mtDNA is essential for efficient tumour formation and that this is accomplished by the intercellular transfer of whole mitochondria. Our findings are consistent with the emerging notion of the essential role of respiration in cancer cell proliferation and tumor progression (LeBleu et al., 2014; Viale et al., 2014; Birsoy et al., 2015; Sullivan et al., 2015; Cardaci et al., 2015; Lussey-Lepoutre et al., 2015; Berridge et al., 2015; Viale et al., 2015), and of a role for mitochondrial transfer in maintaining the bioenergetics balance (Sinha et al., 2016; Wu et al., 2016). From translational angle, recent studies on horizontal mitochondrial transfer indicate two tantalising, novel approaches to cancer therapy: targeting mitochondrial respiration and blocking transfer of mitochondria from stromal cells to cancer cells. While we have started exploring the first approach (Dong et al., 2011; Boukalova et al., 2016; Kluckova et al., 2015; Rohlenova et al., 2017), the other approach remains untested, with recent papers (Osswald et al., 2015; Moschoi et al., 2016) pointing to its plausibility. Finally, to the best of our knowledge, this paper is the first report to show lasting functional consequences of a well-documented mitochondrial transfer event.

Materials and methods

Cell culture

Cell lines were prepared and maintained as described (Tan et al., 2015). C57BL/6J mice were used as host animals for grafting B16 sub-lines as indicated (Tan et al., 2015). B16 lines formed syngeneic tumours in C57BL/6J mice indicating their authenticity. Parental and p^0 cells with BFP nuclei were prepared by stable transfection with the pTagBFP-H2B plasmid (Evrogen) followed by clonal selection. RNAi was used to knock down NDUFV1 or SDHC subunits, using two different shRNAs (OriGene) for each protein. In brief, cells were transfected with shRNA using a standard protocol and inspected by WB for protein levels. Cells with more efficient knock-down of NDUFV1 or SDHC were used in further experiments.

Isolation of mesenchymal stem cells and their co-culture with cancer cells

Mouse MSCs were isolated from C57BL/6J mice as described earlier (Ahmad et al., 2014). The primary cells were plated at the density of 10^6 cells/ml in T25 culture flasks and experiments were performed after the fourth passage. For co-culture experiments, MSCs were stained with Mito Deep Red (Ex/Em, 644/665 nm; Invitrogen) and B16 p^0 cells with the CFSE dye (Ex/Em, 492/517 nm; Invitrogen) for 15 min. The cells were then co-cultured for 24 hr and evaluated by flow cytometry (FACS Calibur) and confocal microscopy (63 x; Leica SP8). Mitochondrial transfer was primarily ascertained by a fraction of CFSE-positive cells that were also MitoDeepRed-positive. The quantitative mitochondrial transfer was primarily ascertained by pixel counts on Z-stack confocal images i.e. red pixels in cells with green background. Geometric mean intensity of MitoDeepRed fluorescence in double positive cells was additionally calculated, as a confirmatory measurement of the degree of mitochondrial transfer. Other co-culture combinations (MSCs with B16 cells, B16 cells with B16 p^0 cells) were performed in a similar fashion. Additionally, p^0 cells were transfected with a plasmid coding for nBFP and pmGFP followed by clonal selection. Transfected p^0 cells were seeded with MSCs prepared from C57BL/6N^{sl9DsRed2} mice in glass-bottom dishes (In Vitro Scientific) at 1:1 ratio and co-cultured for 24 hr. Live cells were inspected using the inverted fluorescence microscope Delta Vision Core with laser photo-manipulation. The acquired images were deconvolved by Huygens Professional software (Scientific Volume Imaging) and processed by Fiji ImageJ software.

Animals

C57BL/6J mice were used for most of the experiments. They were purchased from the Animal Resources Centre or produced by the animal breeding facilities of the Institute of Biotechnology and Malaghan Institute. In all cases, the mice were grafted subcutaneously with various cell lines at 5×10^5 cells per animal. Tumours were monitored by ultrasound imaging (USI) using the Vevo770 system (VisualSonics, Toronto, Canada). Transgenic mice expressing red fluorescent protein in somatic cell mitochondria (the CAG/su9-DsRed2 transgene) were generated in the Transgenic Unit of the Czech Centre for Phenogenomics, Institute of Molecular Genetics, Prague, Czech Republic, using a pronuclear injection from the construct provided by Prof. Masaru Okabe (Osaka University, Japan) (Hasuwa *et al.*, 2010) and C57BL/6N mice. The stable colony of transgenic mice was housed in the animal facility of the Faculty of Science, Charles University, Prague, Czech Republic, and food and water were supplied ad libitum. The mice used for the grafting experiments were healthy 10 weeks old animals with no sign of stress or discomfort. All animal procedures and experimental protocols were approved by the Animal Welfare Committee of the Czech Academy of Sciences (Animal Ethics Number 18/2015).

Single cell-digital droplet PCR

Details of the methodology are in the Supplemental information.

Microscopic and flow cytometric cell evaluation, cell sorting and STED microscopy

Details of the methodology are in the Supplemental information.

Mitochondrial biochemistry assays, gene expression analysis and respiration assays

Details of the methodology are in the Supplemental information.

Statistical analysis

Unless stated otherwise, data are mean values $\pm$ S.D. of at least three independent experiments. In mouse experiments, groups of 6 animals were used, unless stated otherwise. The two-tailed unpaired Student's *t* test was used to assess statistical significance with $p < 0.05$ being regarded as significant. Images are representative of three independent experiments.

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Animal experimentation: This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Czech Republic. All animal procedures and experimental protocols were approved by the Local Ethics Committee (Animal Ethics Number 18/2015).

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Appendix 1

Single cell-digital droplet PCR

Single cell samples were obtained by automated cell withdrawal using the CellCelector (ALS). Approximately 10,000 cells were seeded in a 6-well culture plate, nuclei were stained with DAPI and automated image analysis was performed to identify viable attached cells. Candidate cells were removed with a 30 μ m capillary aspirating 0.1 μ l per cell using automated bottom detection and scrape mode. Each removed cell was deposited into 10 μ l of nuclease-free water supplemented with 1 μ g/ μ l BSA (ThermoFisher) in a 384-well plate that was cooled during the cell picking. The plate was then sealed and placed on dry ice until use. Images were collected before and after picking, and only those samples that contained a single cell were used for further analysis. Negative controls were medium only. Half of the cell lysate (5 μ l) was used in 20 μ l ddPCR following the manufacturer's recommendations using ddPCR Mastermix for probes (BioRad) supplemented with 450 nM primers, 250 nM probes: tRNA 8A FAM and 9A HEX (Sigma Aldrich, sequences are in **Appendix 1—table 1** below). Annealing temperature of 57°C was used for the PCR run in a C1000 thermocycler (BioRad). Droplet analysis was performed using the QX200 instrument (BioRad), and data were analysed with QuantaSoft according to the digital MIQE guidelines (Huggett *et al.*, 2013).

Appendix 1—table 1. Primer and probe sequences used in sc/ddPCR. *Capital letters indicate LNA bases; polymorphism is shown in gray.

m_tRNA_Fw	gtcacaattctatctctagg
m_tRNA_Rv	ggttgaagaaggtagatg
m_tRNA_8A_FAM (host mouse)	taattagtTtAaAaAaAaTtAaTgattt
m_tRNA_9A_HEX (parental B16)	taattagtTtAaAaAaAaTtAaTgattt

*Polymorphism of the mtDNA tRNA^{Asp} locus of C57 mice and B16 cells has been published (Bayona-Bafaluy *et al.*, 2003; Tan *et al.*, 2015).

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The annealing temperature for the tRNA ddPCR assay was optimized using a gradient run (data not shown), and a simplified validation of the sensitivity of the tRNA assay was performed. To each 20 μ l ddPCR reaction mix about 20,000 copies of parental mtDNA were added ($\approx$ 1 ng of purified B16 DNA) and spiked with 1000, 100, 10 and 1 copy of host mtDNA. Loss of linearity was observed at a prevalence of 0.5% and 0.05% spiked in host DNA. Pure parental mtDNA gave a background corresponding to 0.18% of host mtDNA. This very low background is caused by cross reaction due to residual affinity of the 8A host probe to the 9A parental locus. The reciprocal cross reaction of 9A probe binding to 8A parental locus was not observed (**Figure 1A** and **Appendix 1—figure 1** for validation of sc/ddPCR). No template controls (NTC) as well as B16p⁰ cells were negative. A single B16 cell contains about 100 copies of mtDNA (not shown); hence our ddPCR sensitivity of 0.5% is sufficient to detect a single host mtDNA molecule.

Mitochondrial biochemistry and bioenergetics

The activity of SQR, SDH and CS were assessed as described (Tan *et al.*, 2015). Lactate production, ATP levels and glucose uptake were evaluated by standard procedures (Tan *et al.*, 2015).

Real-time (RT) PCR

Real time PCR was applied to assess the level of mtDNA, real time RT-PCR was used for evaluation of expression of selected transcripts. Details as well as the list of primers were published earlier (Tan et al., 2015).

Mitochondrial chromatin immunoprecipitation (mitoChIP) assay

Cells were crosslinked with 1% formaldehyde, harvested and cell pellets frozen. Pellets were then resuspended in the ChIP lysis buffer, sonicated for 12 min at cycles consisting of 15 s sonication at amplitude 5 (Qsonicaxx) and 30 s cooling in ice-water bath. Sheared chromatin was treated with RNaseA and proteinaseK, and subjected to agarose electrophoresis to confirm correct shearing. Dynabeads were pre-absorbed with salmon sperm DNA (0.2 mg/ml) and BSA (0.5%) overnight, and 20 μ l of bead slurry was combined with 5 μ g of sheared chromatin and 1 μ l of either anti-POLG1 IgG (Abcam, ab128899) or non-specific rabbit IgG, and incubated overnight on a rotating platform. After extensive washing, chromatin was eluted and treated with RNaseA and proteinaseK, followed by purification using PCR clean-up columns (MachereyNagel). qRT-PCR was then run using the Eva Green system (Solis Biodyne) at 95°C for 12 min, 40 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 35 s, followed by melt curve analysis using primers for mouse POLG1: forward - TGA TCA ATT CTA GTA GTT CCC AAA A, reverse - ACC TCT AAT TAA TTA GGC CAG G. Data were quantified against a non-specific IgG via the $\Delta\Delta C_t$ method.

Electrophoresis and western blotting

Cell as well as tumor lysates were prepared and electrophoresis/WB carried out by standard procedures.

Confocal microscopy, STED super-resolution microscopy and transmission electron microscopy (TEM)

For live cell imaging of mitochondria and mtDNA, cells were grown in chamber slides (LabTek) and incubated for 30 min with 100 nM MitoTracker Red (Molecular Probes) and 5 μ g/ml Hoechst 33342, and, subsequently for 4 min with 2 μ g/ml EtBr. Images were captured and analysed using the FV1000 confocal microscope and Fluoview software (Olympus).

STED microscopy was carried out as follows. Cells were grown on high precision coverslips over-night, fixed with 4% paraformaldehyde in PBS for 10 min at 37°C, permeabilised with the washing buffer (0.1 M glycine, 0.05% TWEEN, 0.05% Triton X-100, PBS), blocked with 5% normal goat serum (Vector Laboratories), and incubated with primary antibodies against DNA (PROGEN), TFAM (Abcam) or Tom20 (Santa Cruz Biotechnology). This was followed by appropriate secondary antibodies including Alexa Fluor555 goat anti-mouse IgM and Alexa Fluor488 goat anti-rabbit IgG (H+L) (Life Technologies). Nuclei were labelled with Hoechst 33342 (Sigma-Aldrich). The coverslips were mounted on glass slides using 90% glycerol mounting medium with 5% n-propylgalate. The samples were visualized using Leica TCS SP8 STED 3X microscope equipped with 660 nm STED depletion laser and fitted with the LAS X 64 bit package software with LAS AF SP8 Dye Finder, 3D visualization, deconvolution and co-localization module. The acquired images were

deconvolved by Huygens Professional software (Scientific Volume Imaging) and processed by Fiji ImageJ software.

TEM was performed using fixed cells via a standard procedure.

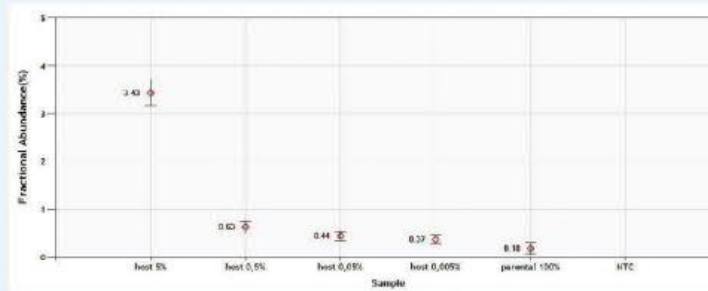
Generation of tumours in C57BL/6N^{su9-DsRed2} transgenic mice, cell sorting and confocal microscopy

B16p⁰ cells with BFP nuclei were suspended in 100 μ l PBS and grafted subcutaneously in C57BL/6N^{su9-DsRed2} transgenic mice at 10⁶ cells per animal. The experiment was repeated 4 times, each time with five mice, and cells were recovered 4, 7, 9, 11 or 12 days post implantation. The pre-tumor lesion was resected and digested enzymatically (collagenase/DNase, 20 min, 37°C). The isolated tumour cells were sorted for nBFP- and mitochondrial DsRed-positive cells using the BD Influx high speed cell sorter (BD Biosciences) in 8-chambered cover-glass system (In Vitro Scientific). 12 hr after sorting, the cells were viewed using the Leica TCS SP5 AOBs Tandem confocal microscope equipped with the LAS AF software. The acquired images were deconvolved using the Huygens Professional software (Scientific Volume Imaging) and processed by Fiji ImageJ software.

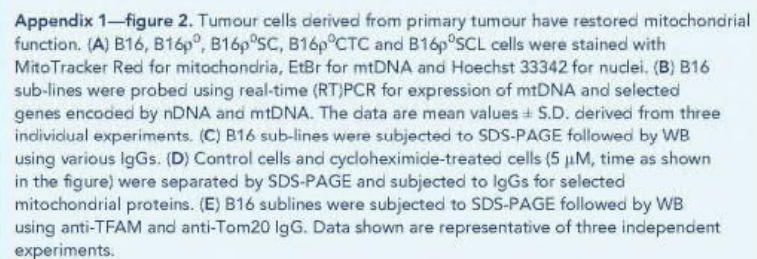
Respiration assays

Experiments evaluating intact cell respiration were performed using the Oxygraph-2k instrument (Oroboros) with cells suspended in RPMI medium without serum. Oxygen consumption was evaluated for cellular ROUTINE respiration, oligomycin-inhibited LEAK respiration, FCCP-stimulated uncoupled respiration capacity (ETS) and rotenone/antimycin-inhibited residual respiration (ROX). Respiration via mitochondrial complexes was evaluated using digitonin-permeabilised cells suspended in the mitochondrial respiration medium MiRO6, and oxygen consumption was evaluated for CI-linked respiration, (CI+CI_{II})-linked respiration, maximum uncoupled respiration, CI_{II}-linked uncoupled respiration as well as residual oxygen consumption. Respiration via CI and CI_{II} was evaluated in the presence of the proper substrates and inhibitors of the other complex. The results were normalized to the number of cells.

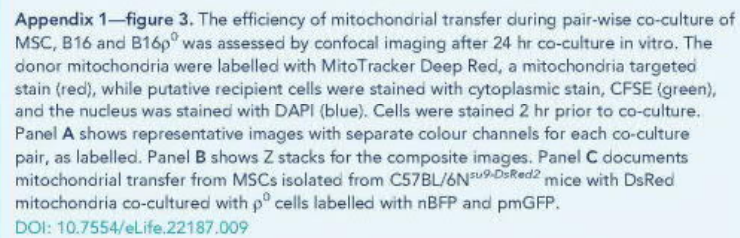
Respiration of tumour tissue was assessed basically as above. Following sacrifice of mice, tumours and livers (used as internal control) were excised and homogenized using the SG3 Shredder (Oroboros). The final cell suspension in the chamber contained 2 to 3 mg/ml of wet tissue. Mitochondrial respiration via CI or CI_{II} was evaluated as mentioned above.



Appendix 1—figure 1. Validation of the sc/ddPCR assay for the *tRNA* locus. Parental mtDNA (about 20,000 copies) was spiked with 1000; 100; 10 and 1 copy of host mtDNA. Respectively, this corresponds to the expected fractions of 5; 0.5; 0.05% and 0.005%. Red diamonds indicate the measured fractions. NTC is non-template control.
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3. Reactivation of dihydroorotate dehydrogenase-driven pyrimidine biosynthesis restores tumor growth of respiration-deficient cancer cells

Our recent research strongly suggests that respiration is crucially important for malignant cells. We showed that cancer cells with damaged (depleted) mtDNA acquire 'healthy' mtDNA from the host, and documented that subsequent recovery of respiration is linked to tumor formation (Tan et al., 2015). We also showed that these mtDNA depleted cancer cells, when grafted in mice, form tumors upon acquisition of mtDNA from the host *in vivo* by horizontal transfer of whole mitochondria (Dong et al., 2016). However, it remains unresolved, which aspect of OXPHOS activity is limiting for tumor growth.

For this study we used the same model of breast carcinoma as before. We grafted 4T1p0 cells mice to form syngeneic tumors, isolated cells from the tumors on various days after grafting and established stable cell lines from each time point (D5-D60 cells, 4T1p0 cells were referred to as D0 cells) to analyze temporal events preceding tumor formation. Sublines derived from 4T1p0 cells contained increasing levels of mtDNA from the host with >99.5% homoplasmic polymorphism. This was consistent with gradual appearance of cristae in mitochondria as well as gradual formation of mitochondrial nucleoids and binding of regulatory proteins in D0 to D20 cells. Surprisingly, mtDNA replication and transcription was very efficient already in D5 cells. Most subunits of RCs were expressed and SCs were assembled in D15 cells. To address the question why tumor formation requires recovery of respiration, we studied the bioenergetics of parental and D0-D60 sublines. ATP generation was not affected by respiration recovery as total cellular ATP was similar in parental cells and all sublines. It is widely accepted that p0 cells are auxotrophic for pyruvate and uridine, the latter being linked to low activity of DHODH (King and Attardi, 1989; Alcázar-Fabra et al., 2016). DHODH-dependent respiration and loss of auxotrophy for uridine was first observed in the D15 subline. This notion was supported by the failure of DHODH knock-out cells to form tumors. Intriguingly, reactivation of CoQ redox-cycling alone by transfecting the p0 cells with alternative oxidase (AOX) as an acceptor of electrons from reduced CoQ was found sufficient to drive DHODH and to allow cancer cells to form tumors.

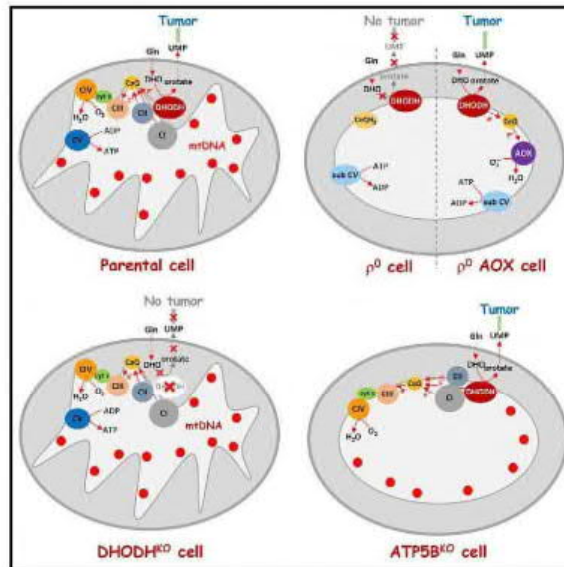
This together suggests that recovery of respiration in cancer cells with damaged mtDNA is necessary for unlocking the cells from auxotrophy, which prevents *de novo* pyrimidine synthesis, blocking tumor cell proliferation, linking respiration to metabolic reprogramming.

Contribution to the publication: Experimental part (cell culture, evaluation of uridine and pyruvate auxotrophy, evaluation of ATP level, glucose uptake and lactate formation, mtDNA level assay, qPCR, RT-PCR, respiration assays, confocal microscopy, mice experiments, ultrasound imaging of tumors, the isolation and characterization of sublines). Data analysis, manuscript writing.

Cell Metabolism

Reactivation of Dihydroorotate Dehydrogenase-Driven Pyrimidine Biosynthesis Restores Tumor Growth of Respiration-Deficient Cancer Cells

Graphical Abstract



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In Brief

Cancer cells without mitochondrial DNA (mtDNA) do not form tumors unless they can hijack host mitochondria. Bajzikova et al. show that the acquired mitochondrial electron transport is necessary to drive de novo pyrimidine synthesis to overcome cell-cycle arrest. Surprisingly, ATP generation is dispensable for tumorigenesis in this context.

Highlights

- Tumorigenesis depends on functional OXPHOS
- OXPHOS-derived ATP is not required for tumor formation
- DHODH-driven pyrimidine biosynthesis requires CoQ redox-cycling
- CoQ redox-cycling via OXPHOS drives tumorigenesis through pyrimidine biosynthesis



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CellPress

Reactivation of Dihydroorotate Dehydrogenase-Driven Pyrimidine Biosynthesis Restores Tumor Growth of Respiration-Deficient Cancer Cells

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SUMMARY

Cancer cells without mitochondrial DNA (mtDNA) do not form tumors unless they reconstitute oxidative phosphorylation (OXPHOS) by mitochondria acquired from host stroma. To understand why functional respiration is crucial for tumorigenesis, we used time-resolved analysis of tumor formation by mtDNA-depleted cells and genetic manipulations of OXPHOS. We show that pyrimidine biosynthesis dependent on respiration-linked dihydroorotate dehydrogenase (DHODH) is required to overcome cell-cycle arrest, while mitochondrial ATP generation is dispensable for tumorigenesis. Latent DHODH in mtDNA-deficient cells is fully activated with restoration of complex III/IV activity and coenzyme

Q redox-cycling after mitochondrial transfer, or by introduction of an alternative oxidase. Further, deletion of DHODH interferes with tumor formation in cells with fully functional OXPHOS, while disruption of mitochondrial ATP synthase has little effect. Our results show that DHODH-driven pyrimidine biosynthesis is an essential pathway linking respiration to tumorigenesis, pointing to inhibitors of DHODH as potential anti-cancer agents.

INTRODUCTION

Mitochondria are vital organelles for most eukaryotic cells (Karnikowska et al., 2016). They carry their own DNA (mtDNA) and are involved in a number of essential processes. The signature

feature of mitochondria is oxidative phosphorylation (OXPHOS), responsible for respiration and ATP formation. Respiration is performed by four respiratory complexes (RCs; i.e., CI–IV) that associate into supercomplexes (SCs) and generate a proton gradient across the inner mitochondrial membrane (IMM) that is used by ATP synthase (CV) to produce ATP (Acin-Perez et al., 2008; Althoff et al., 2011; Moreno-Lastres et al., 2012; Gu et al., 2016; Letts et al., 2016; Wu et al., 2016). Respiration also drives biosynthetic pathways directly or via the tricarboxylic acid cycle (Bezawork-Gelata et al., 2018).

Essential protein subunits of OXPHOS complexes are encoded by nuclear DNA and mtDNA. Therefore, when mtDNA is absent or damaged, OXPHOS is severely compromised (Brandon et al., 2006; Wallace, 2012). Recently we showed that cancer cells deficient in OXPHOS due to mtDNA depletion (ρ^0 cells) cannot form tumors unless they acquire functional mtDNA from host stroma (Tan et al., 2015) by transfer of whole mitochondria (Dong et al., 2017). Other researchers support our findings (Osswald et al., 2015; Lei and Spradling, 2016; Moschoi et al., 2016; Strakova et al., 2016). These observations suggest that functional OXPHOS is essential for tumorigenesis, a concept consistent with other reports (LeBlau et al., 2014; Viale et al., 2014). Furthermore, they conform to the notion that the Warburg effect is associated with altered biosynthetic needs of cancer cells rather than with cancer-linked mitochondrial damage (Vander Heiden et al., 2009; Vander Heiden and DeBerardinis, 2017).

However, important questions remain unresolved. Foremost, it is unclear which aspect of OXPHOS activity is limiting for tumor growth. ATP production is the best known function of OXPHOS, but proliferating cells also require respiration for its oxidizing power and to produce aspartate for pyrimidine biosynthesis (Birsoy et al., 2015; Sullivan et al., 2015; Titov et al., 2016). Further, OXPHOS directly drives the respiration-coupled mitochondrial enzyme dihydroorotate dehydrogenase (DHODH) that converts dihydroorotate (DHO) to orotate in the *de novo* pyrimidine synthesis pathway (Loffler et al., 2005).

Here we analyzed temporal events preceding tumor formation in ρ^0 cancer cells in the context of horizontal transfer of mtDNA *in vivo* and linked this to genetic manipulations of the OXPHOS system. Our results indicate that a key event facilitating tumor growth upon respiration recovery is reactivation of DHODH-driven pyrimidine synthesis.

RESULTS

mtDNA Is Replenished and Respiration Recovers Prior to Tumor Formation

Mouse breast cancer 4T1 ρ^0 cells form tumors with a 3-week lag compared with parental cells, with palpable tumors appearing on day 20–25 (Figures 1A and S1A). To understand the sequence of events leading to tumor growth, 4T1 ρ^0 cells (referred to as day 0, D0 cells) were grafted into BALB/c mice, tissue at the injection site was excised at various time points post injection (Figures 1B and 1C) and cancer cells were selected using 6-thioguanine (6TG) (Aslakson and Miller, 1992). Individual lines established in medium supplemented with pyruvate/uridine were stable over a long time in culture, maintaining their mtDNA status and growth properties. Analysis of the lines for respiration revealed

its recovery prior to tumor formation (Figure 1D), pointing to an association between respiration recovery and tumor growth.

We next assessed the mtDNA content in the lines using single-cell PCR (sc/qPCR) and probes that discriminate between the 16S rRNA polymorphism of host cells and that of 4T1 cells (Bayona-Bafaluy et al., 2003; Tan et al., 2015). We observed a progressive increase in the homoplasmic mtDNA of host origin in D5–D15 cells as well as absence of 4T1 mtDNA polymorphism in these cells (Figure 1E). The distribution profile of mtDNA was normalized in D15 cells (Figure 1F). The relative mtDNA copy number was verified by qPCR (Figure S1B), and the host origin of homoplasmic polymorphism was confirmed by DNA sequencing (Table S1).

To test whether the acquired mtDNA was functional, we assessed replication (mREP) and transcription (mTRANS) of mtDNA in single cells by the mTRIP method (Chatre and Ricchetti, 2013). Figure 1G shows relatively high mREP and mTRANS signal already in D5 cells. We also set up a specific mitochondrial “chromatin” immunoprecipitation (mitoChIP) assay (Figure 1H) that revealed a gradual increase in mtDNA binding of mitochondrial transcription factor TFAM and DNA polymerase- γ (POLG). The binding was very low in D5 cells, increased in D10 cells, and normalized in D15 and D20 cells. The protein levels of TFAM, POLG, and the mitochondrial single strand-binding protein (mtSSB) fully recovered in D15 cells that contain substantial mtDNA (Figures S1C and 1I).

Transcripts of mitochondrial genes encoding subunits of RCs were detectable in D5 cells and approached parental values in D15–D20 cells (Figures 1J and S1D). Protein subunits of RCs recovered in D15 cells (Figure 1K), where also fully assembled RCs and SCs appeared, and a switch from sub-CV to CV occurred (Figure 1L). Finally, we assessed the lines for mitochondrial morphology by transmission electron microscopy (TEM) (Figure S2) and for the presence of mitochondrial nucleoids using stimulated emission depletion microscopy (STED) (Figure S3). TEM detected mitochondria with cristae in D15 cells, consistent with the link between cristae formation and respiration (Cogliati et al., 2013), while STED microscopy revealed high numbers of TFAM-containing nucleoids in D15 cell mitochondria (Kukat et al., 2015).

In conclusion, mtDNA is acquired and amplified, and OXPHOS machinery is reconstituted in ρ^0 cancer cells during the long dormant period prior to tumor appearance.

Mitochondrial Function and Bioenergetics Are Normalized Early in Tumorigenesis and Are Unrelated to ATP Generated by OXPHOS

We investigated whether replenishment of mtDNA/reconstitution of OXPHOS components are reflected by normalization of the mitochondrial function. Figure 2A shows that mitochondrial membrane potential ($\Delta\Psi_m$), low in D0 to D10 cells, increased in D15 cells to parental cell level. Similarly, mitochondrial superoxide increased in D15 cells (Figure 2B), consistent with active electron transport through assembled RCs/SCs.

We next assessed the bioenergetics of D0–D60 lines, evaluating their basal respiration. Figure 2C shows little or no respiration in D0–D10 cells, with an increase to 50%–60% of the parental cell values in D15 cells and normalization in D20 cells. This was similar for CI- and CII-dependent respiration

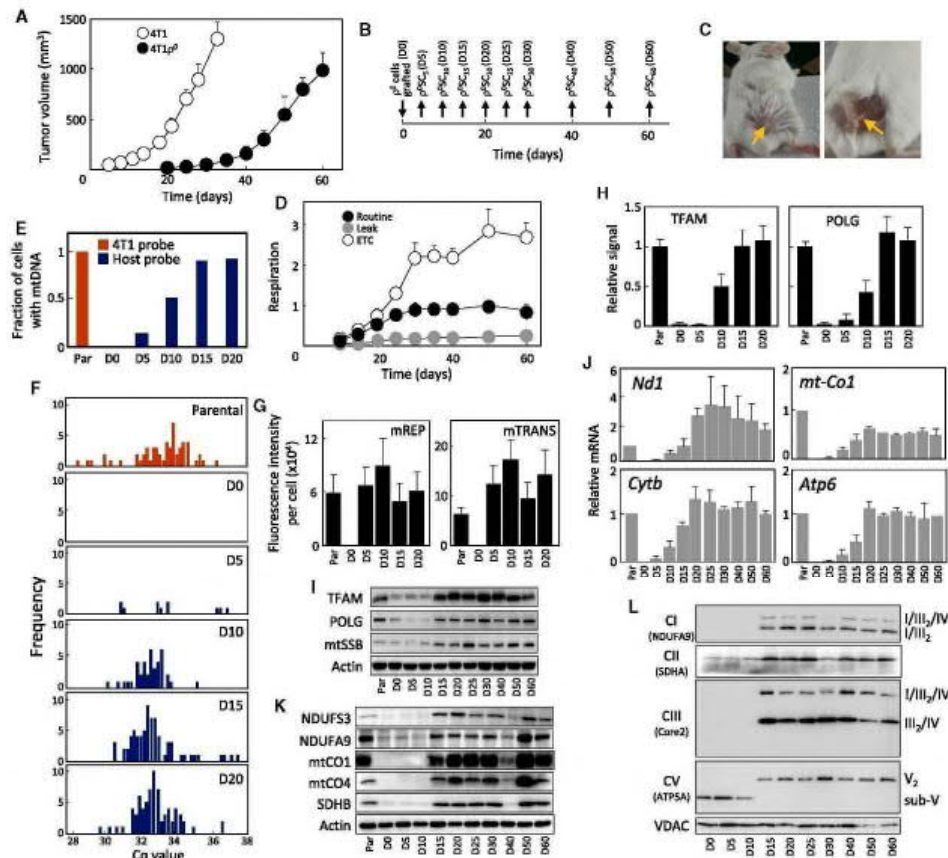


Figure 1. mtDNA Is Replenished and Respiration Recovers Early in Tumor Formation by 4T1p⁰ Cells
 (A) BALB/c mice were grafted subcutaneously (s.c.) with 4T1 or 4T1p⁰ cells at 10⁶ per animal, and tumor volume was assessed by ultrasound imaging (USI) (n = 6).
 (B and C) Time schedule of retrieval of pre-tumor plaques and tumors from BALB/c mice is shown in (B), as indicated in (C) for D5 tissue.
 (D) Individual lines retrieved from mice according to the schedule in (B) were assessed for uncoupled (ETC), routine, and leak respiration using the Oxygraph (n = 3).
 (E) Cell lines were evaluated for the presence of mtDNA using probes against a polymorphism in either 4T1 or host 16S rRNA by sc/qPCR; 92 cells per line were assessed.
 (F) Distribution of mtDNA polymorphism in 16S rRNA in single cells of lines shown in (E).
 (G) mTRIP assay in the presence of proteinase K was used to detect the mtDNA initiation of replication marker (mREP) and global mitochondrial transcripts (mTRANS) unmasked from proteins, in single cells (n = 3; 100 cells were assessed per condition).
 (H) Cell lines were assessed for binding of TFAM and POLG1 to the D LOOP region of mtDNA using a mitoChIP assay (n = 3).
 (I) Individual lines were probed for the level of mtDNA-processing proteins using WB.
 (J-L) qRT-PCR was used to assess the level of transcripts of selected subunits of mitochondrially encoded transcripts of RCs (n = 3) (J). Selected subunits of mitochondrial RCs were evaluated using WB in individual lines (K), which were then assessed by NBGE for the assembly of RCs and SCs using antibodies to relevant subunits, as shown (L).
 Data in (A), (D), (G), (H), and (J) are mean values ± SD. Representative images of three biological replicates are shown for (I), (K), and (L).

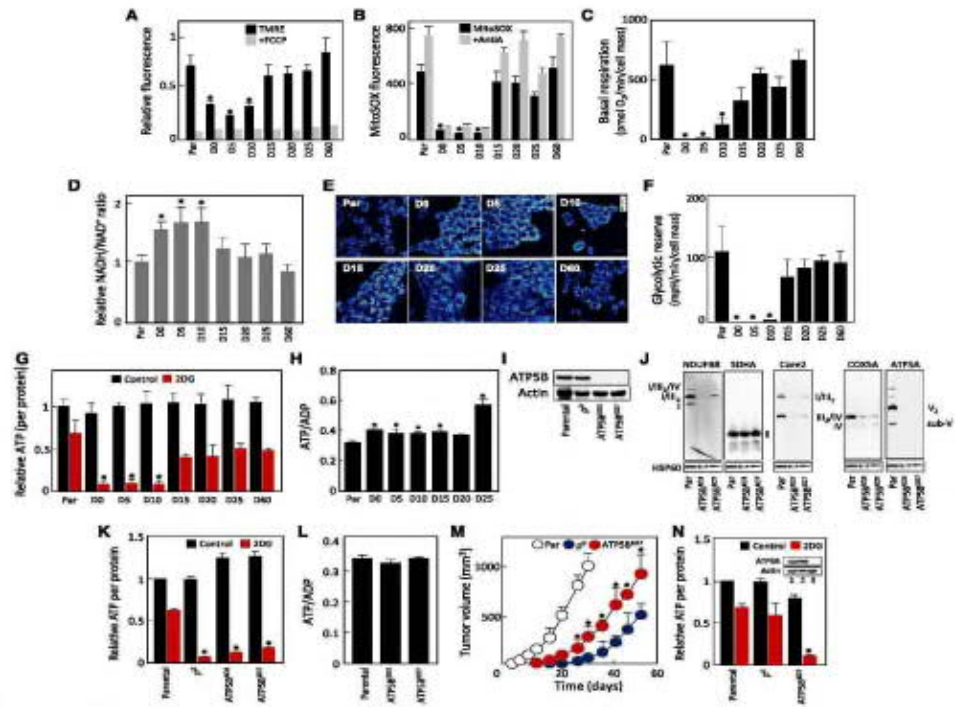


Figure 2. Mitochondrial Function and Bioenergetics Are Normalized Early in 4T1 ρ^R Cell Tumorigenesis and Are Unrelated to OXPHOS-Generated ATP
 (A–C) Individual lines derived from 4T1 ρ^R cells were assessed for $\Delta\psi_{m}$ using TMRE in the absence and presence of the uncoupler FCCP (A), for generation of mitochondrial superoxide using MitSOX in the absence and presence of antimycin A (AntA) (B), and for basal respiration using a Seahorse XF96 (C).
 (D) NADH/NAD⁺ ratio was assessed in cell lines using a luminescence kit.
 (E) Two-photon microscopy was used to visualize the level of mitochondrial NAD(P)H in individual lines.
 (F) Cell lines were assessed for glycolytic reserve using a Seahorse XF96.
 (G) Individual lines were evaluated for ATP levels in the absence and presence of 50 mM 2DG at 4.5 g/L glucose and 1 mM pyruvate, and the results were expressed relative to total ATP in parental cells.
 (H) The lines as shown were assessed for the ATP/ADP ratio using liquid chromatography-mass spectrometry.
 (I) Parental and ρ^0 cells and two clones of ATP5B^{KO} cells were probed for the level of ATP5B using WB.
 (J–L) Parental, ρ^0 , and ATP5B^{KO} cells were assessed by NBGE for assembly of RCs and SCs (J). Parental, ρ^0 , and ATP5B^{KO} cells were tested for the level of ATP5B at 4.5 g/L glucose in the absence or presence of 50 mM 2DG (K) and for the ATP/ADP ratio (L).
 (M) BALB/c mice were injected s.c. with 10⁶ cells per animal, and tumor volume was quantified by USI.
 (N) Cell lines derived on day 20 from tumors grown from parental, ρ^0 , and ATP5B^{KO} 4T1 cells were assessed for ATP as described in (G). The insert documents the level of ATP5B protein in the tumor-derived cell lines; 1, parental; 2, ρ^0 ; 3, ATP5B^{KO} cells.
 Data in (A)–(D), (F)–(H), (K), (L), and (N) are mean values $\pm$ SD (n = 3); those in (M) are mean values $\pm$ SEM (n = 5). The symbol *** in (A)–(D), (F)–(H), (K), and (N) indicates statistically significant differences from parental cells; in (M), statistically significant differences from tumors grown from ρ^0 cells; and in (L), statistically different from tumors grown from parental cells, with p < 0.05. (E), (I), (J), and (N) (insert) show representative images of three biological replicates.

(Figure S4A), with reserve respiratory capacity absent in D0–D10 cells (Figure S4B). Compromised respiration was confirmed by increased NADH/NAD⁺ ratio in D0–D15 cells evaluated by a luminescence kit (Figure 2D) and elevated mitochondrial NAD(P)H assessed by two-photon microscopy (Figures 2E and S4C). D0–D10 cells had no glycolytic reserve (Figure 2F), consistent with increased lactate production and glucose uptake compensating for the loss of OXPHOS-derived ATP (Figures S4D and S4E). Indeed, we found that glycolysis contributed to ATP generation by $\approx 30\%$ in parental and 90% in D0–D10 cells (Figure 2G). Total ATP was comparable in all cell lines and the ATP/ADP ratio was not reduced in respiration-deficient cells, suggesting that ATP availability is not the limiting factor (Figure 2H).

To test directly whether the onset of tumor formation is dissociated from ATP generation by OXPHOS, we prepared cells that cannot assemble CV by knocking out ATP5B (Figure 2I). Native blue gel electrophoresis (NBGE) followed by western blotting (WB) revealed that ATP5B^{KO} cells failed to assemble CV (Figure 2J), with other RCs/SCs also decreased. ATP5B^{KO} cells predominantly used glycolysis for ATP formation, their ATP content was comparable to that of parental cells (Figure 2K), and the ATP/ADP ratio was maintained (Figure 2L). When grafted into BALB/c mice, ATP5B^{KO} cells produced tumors somewhat slower than parental cells, but significantly faster than 4T1 ρ^0 cells (Figure 2M). To exclude the possibility that ATP5B^{KO}-derived tumors “acquire” ATP synthase and re-establish OXPHOS-dependent ATP production, we assessed ATP5B expression and ATP levels in lines isolated from tumors grown from 4T1, 4T1 ρ^0 , and 4T1 ATP5B^{KO} cells. We observed that ATP5B^{KO} tumor-derived cells were deficient in both OXPHOS-derived ATP and in ATP5B, whereas 4T1 ρ^0 cells re-established ATP production by mitochondrial transfer (Figure 2N).

To see whether these results are more broadly applicable, we deleted ATP5B in B16 cells. When grafted into C57BL/6 mice, B16 ρ^0 cells acquire mtDNA (Tan et al., 2015) via transfer of whole mitochondria, yielding B16 DP cells (Dong et al., 2017). We first assessed B16, B16 ρ^0 , B16 DP, and B16 ATP5B^{KO} cells by SDS-PAGE and NBGE for the level of subunits of RCs (Figure S5A) and their assembly (Figure S5B), and for ATP production (Figure S5C). These lines behaved similarly to their 4T1 counterparts. Also, B16 ATP5B^{KO} cells were more efficient in tumor formation than B16 ρ^0 cells (Figure S5D). These results indicate that OXPHOS-derived ATP is not essential for tumorigenesis.

Respiration Recovery Is Associated with Reactivation of Dihydroorotate Dehydrogenase

Cells devoid of mtDNA are auxotrophic for uridine and pyruvate (King and Attardi, 1988, 1989). OXPHOS defects may also increase the dependence of cells on otherwise non-essential nutrients such as pyruvate (Sullivan et al., 2015; Birsoy et al., 2015; Cardaci et al., 2015; Lussey-Lepoutre et al., 2015). We therefore tested the auxotrophy profile of 4T1 lines and observed that uridine and/or pyruvate are needed for proliferation of non-/low-respiring cells (D0–D10), while the dependence is lost in D15–D20 cells (Figures 3A and S4F).

We next investigated whether auxotrophy for uridine could explain the association between recovery of respiration and the onset of tumor growth. Since uridine auxotrophy is related

to the dysfunction of DHODH (King and Attardi, 1988; He et al., 2014), we investigated its role in D0–D60 lines and its link to the initiation of tumorigenesis. We found little difference in DHODH mRNA and protein contents in D0–D60 cells (Figure 3B). DHODH converts DHO to orotate, with the resulting two electrons transferred to the respiratory chain (Löffler et al., 2005). Figure 3C shows the extent of DHODH-dependent respiration in parental 4T1 cells and its suppression by leflunomide, a selective inhibitor of the enzyme (Greene et al., 1995). DHODH-dependent respiration was absent in D0–D10 cells and recovered in D15 cells (Figure 3D). We then assessed the orotate-to-DHO ratio, reflecting native DHODH activity. Figure 3E reveals low orotate/DHO ratio in D0–D10 cells, indicating little DHODH function in these cells, while its function recovered in D15 cells.

To link the level of respiration to the kinetics of tumor initiation, we grafted D0–D20 lines into BALB/c mice. D5 and D10 cells formed tumors with considerable lag, while D15 and D20 cells formed tumors with kinetics similar to parental cells (Figure 3F). We next evaluated tumors grown from parental and D0 cells for CII-, CII-, and DHODH-dependent respiration and found no significant difference (Figure 3G).

Finally, we tested whether respiration including the DHODH-dependent component was associated with proliferation of malignant cells after grafting 4T1 ρ^0 (D0) cells into BALB/c mice (cf. Figures 1B and 1C). The tissue excised from the grafted region was sectioned and assessed for proliferation using Ki67 staining. Figure 3H shows low proliferation in D5 tissue, with some increase in D10 and with parental tumor levels in D15 tissue, consistent with the delay in tumor formation shown in Figure 3F. These results suggest an important role for DHODH in tumorigenesis.

DHODH Is Essential for Tumor Growth

To directly examine whether DHODH is important for tumor formation/progression, we deleted the gene for DHODH in 4T1 cells using CRISPR/Cas9. WB in Figure 4A shows the absence of DHODH in two clones of DHODH^{KO} cells and its presence in DHODH-reconstituted (DHODH^{res}) cells. Consistent with this, DHODH-dependent respiration was undetectable in DHODH^{KO} cells and recovered in DHODH^{res} cells, while routine respiration was identical in all lines (Figure 4B). We also found no difference in mitochondrial superoxide in these cells (Figure S4G). In contrast, DHODH^{KO} cells showed stalled proliferation in the absence of uridine, which was restored by DHODH reconstitution (Figure 4C). No significant difference in the NADH/NAD⁺ ratio was detected in these lines (Figure 4D). NBGE revealed no discernible difference in the assembly of any of the five RCs or SCs in the cells, regardless of their DHODH status (Figure 4E), and also showed that DHODH does not associate with any of the complexes (Figure 4E, right panel). We then investigated DHODH^{KO} and DHODH^{res} cells for ATP generation as well as for the role of glycolysis. Figure 4F shows that there was virtually no difference when DHODH-manipulated cells were compared with parental cells. Further, DHODH^{KO} cells contain mitochondria with cristae, unlike ρ^0 cells (Figure 4G).

We next assessed the activity of DHODH in parental, DHODH^{KO}, and DHODH^{res} cells using an *in vitro* enzymatic assay independent of CIII/CIV. This revealed a similar pattern of DHODH activity to that of DHODH-dependent respiration

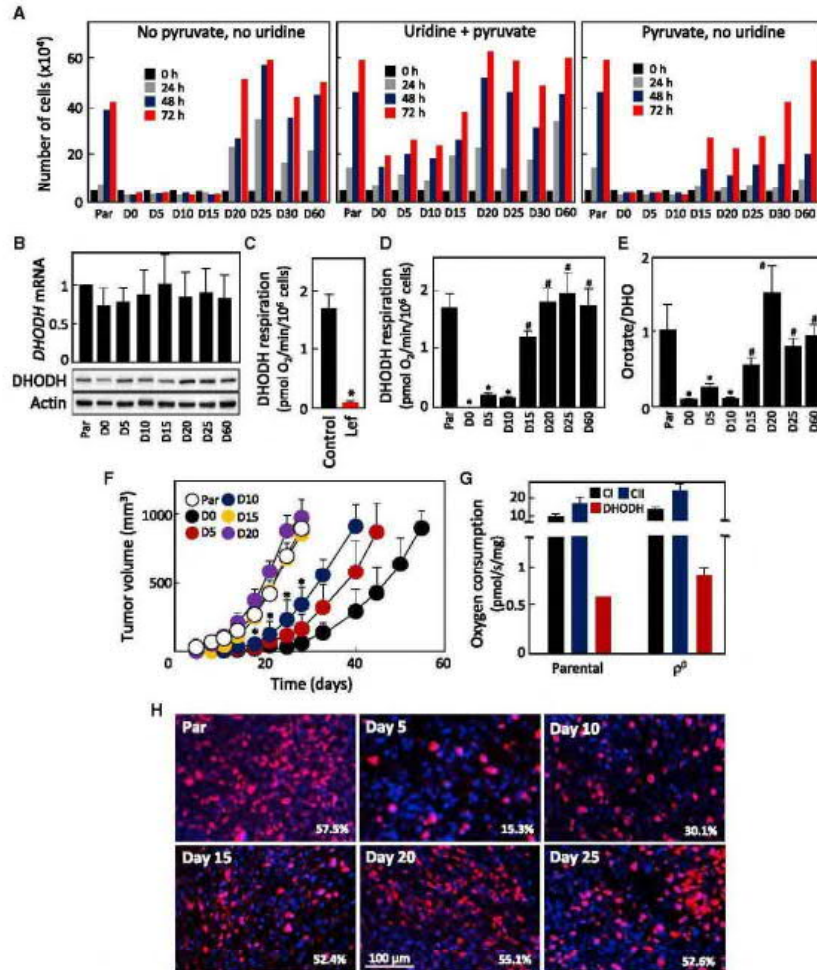


Figure 3. Respiration Recovery Is Associated with Reactivation of DHODH

(A) Cell lines as shown were evaluated for growth in the presence of uridine and pyruvate, or with pyruvate or uridine removed. (B) Cell lines were assessed for DHODH expression by qRT-PCR and WB. (C–E) DHODH-dependent respiration was assessed in parental 4T1 cells in the absence and presence of 30 μ M leflunomide (Lef) (C). Individual lines were assessed for DHODH-dependent respiration (D) and for the orotate-to-DHO ratio (E). (F) Parental, D0, D5, D10, D15, D20, D25, and D60 cells (10⁵) were grafted s.c. into BALB/c mice, and tumor growth was evaluated by USI. (G) Tumors grown from parental or 4T1p⁵ cells were excised and processed in a dedicated tissue shredder, and the homogenates were assessed for Cl⁻, Cl⁻, and DHODH-dependent respiration using an Oxygraph. (H) BALB/c mice were grafted s.c. with 10⁵ D0 cells. On the days indicated, animals were sacrificed and tissue from the grafted region excised, sectioned, and assessed by immunohistochemistry for proliferation using anti-Ki67 IgG followed by confocal microscopy. The numbers indicate percentage of Ki67-positive cells. Data in (A) are mean values derived from two biological replicates with differences less than 10%; data in (B) are mean values $\pm$ SD (n $\geq$ 3); data in (C–G) are mean values $\pm$ SEM (n $\geq$ 3); images in (B) and (H) are representative of three independent experiments. The symbol “*” indicates statistically significant differences from parental cells, and the symbol “#” indicates statistically significant difference from D0 cells, with p < 0.05.

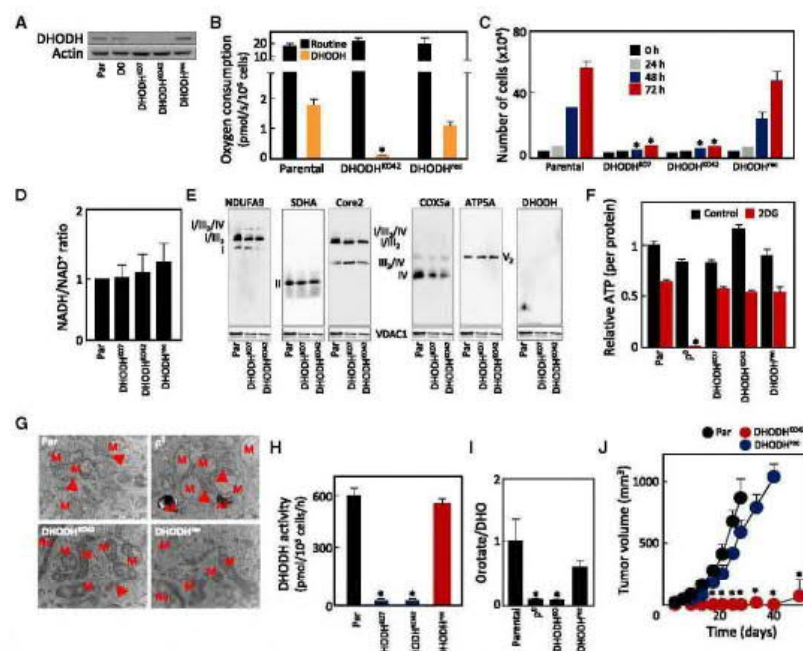


Figure 4. DHODH Is Essential for Tumor Growth

(A–I) WB shows the absence of DHODH in two clones of DHODH^{KO/1} 4T1 cells and its presence in DHODH^{res} cells (A). Parental, DHODH^{KO/1}, and DHODH^{res} cells were tested for routine and DHODH-dependent respiration (B), auxotrophy for uridine (C), NADH/NAD⁺ ratio (D), assembly of RCs and SCs (E), ATP generation in the absence or presence of 50 mM 2DG (4.5 g/L glucose) (F), mitochondrial morphology using TEM (G), DHODH activity (H), and orotate-to-DHO ratio (I). (J) Parental, DHODH^{KO/1}, and DHODH^{res} cells were grafted s.c. in BALB/c mice at 10⁵ per animal, and tumor formation was assessed by USI. Data in (C), (D), (F), and (I) are mean values ± SD (n ≥ 3); data in (B), (H), and (J) are mean values ± SEM (n ≥ 3 for B and H, n = 6 for J). The symbol “*” indicates statistically significant differences from parental cells, with p.

(Figure 4H; cf. Figure 4B). Consistent with these results, DHODH^{KO} cells showed low orotate/DHO ratio, which increased to parental values in DHODH^{res} cells (Figure 4I), verifying this parameter as a proxy for *in situ* DHODH activity. Finally, we found that DHODH^{KO} cells failed to produce tumors, while similar tumor-forming capacity was found for parental and DHODH^{res} cells (Figure 4J).

To verify whether the presence of DHODH is required for tumor growth in another model, we examined B16 DHODH^{KO} and B16 DHODH^{res} cells. Compared with the parental line, these cells had comparable levels of subunits of mitochondrial complexes (Figure S5A) and OXPHOS assembly (Figure S5B). The cell lines also had similar relative levels of ATP generated by OXPHOS and glycolysis (Figure S5C). We next found that B16 DHODH^{KO} cells failed to form tumors, while B16 DHODH^{res} cells formed tumors with similar rate to parental cells (Figure S5E). As expected, while DHODH^{res} cells grew in the absence of uridine, B16 DHODH^{KO} cells were auxotrophic (Figure S5F). Consistent with this, B16 DHODH^{KO} cells featured normal routine respiration but lacked DHODH-dependent respiration (Figure S5G). Finally, we tested

B16 lines for DHODH activity and found that it was absent in DHODH^{KO} cells (Figure S6H).

To summarize, DHODH is essential for tumor growth in 4T1 breast cancer and B16 melanoma models.

DHODH Links Respiration to De Novo Pyrimidine Synthesis and to Cell-Cycle Progression

De novo pyrimidine synthesis involves reactions that convert glutamine to uridine 5-monophosphate (UMP), with DHODH catalyzing the “mitochondrial” step (Figure 5A). To understand whether this pathway is operational in 4T1 lines, we measured the conversion of ¹³C₅¹⁵N₂-Gln into UMP. In agreement with low DHODH respiration, formation of the m+5 isotopomer of UMP was low in D0–D10 cells, while it increased in D15 cells (Figure 5B). We also evaluated the level of unlabeled pyrimidines and purines in parental and D0–D25 cells. Compared with parental cells, pyrimidine nucleotides were low in non-/low-respiring cells and increased in D15 cells, while the levels of purine nucleotides were only marginally affected (Figure S4H). We next assessed conversion of labeled glutamine to UMP in parental, DHODH^{KO},

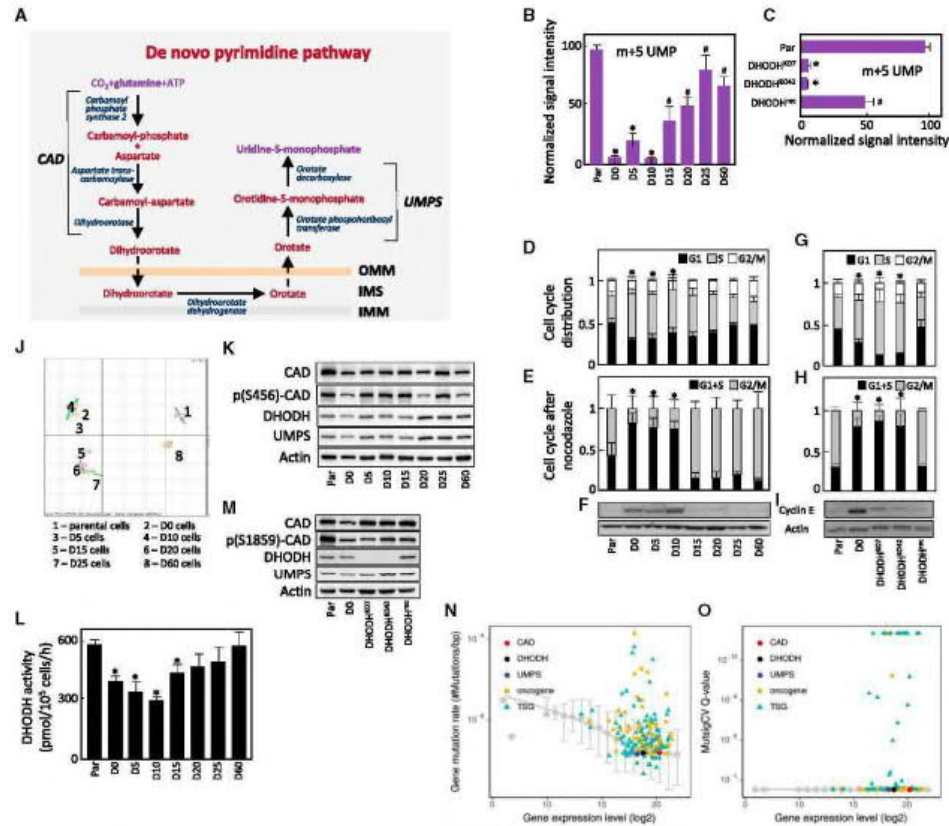


Figure 5. DHODH Links Respiration to De Novo Pyrimidine Synthesis and Cell-Cycle Progression

(A–L) *De novo* pyrimidine synthesis is catalyzed by the trifunctional CAD protein that includes the enzymatic activities of carbamoyl phosphate synthase 2, aspartate transcarbamoylase and dihydroorotase, the DHODH protein, the bifunctional UMPS protein with enzymatic activities of orotate phosphoribosyl transferase, and orotate decarboxylase (A). OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane. 4T1 lines (B) and 4T1 cells with different DHODH status (C) were analyzed by liquid chromatography-mass spectrometry with $^{13}\text{C}_5$ -glutamine as the substrate for generation of the m+5 isotopomer of UMP, the product of the *de novo* pyrimidine pathway shown in (A). 4T1 lines were assessed for cell-cycle distribution (D), arrest in G2 following nocodazole treatment (10 μM , 1 hr) (E), and the level of cyclin E (F). 4T1 cells with different DHODH status were assessed for cell-cycle distribution (G), arrest in G2 following nocodazole treatment (H), and the level of cyclin E (I). Parental, D0, D5, D10, D15, D20, D25, and D60 cells were used for transcriptome analysis using the mouse Affymetrix chip, and the data were assessed by PCA (J). Parental and D0–D60 cells were assessed for members of the *de novo* pyrimidine pathway by WB (K) and for the DHODH enzymatic activity using an *in vitro* assay (L). (M) Parental, D0, DHODH^{KO}, and DHODH^{KO}/UMPS cells were assessed for components of the *de novo* pyrimidine pathway by WB. (N) Raw mutation rates as a function of the average expression level. Mutations of oncogenes (in orange) and tumor suppressor genes (in blue) are compared with genes involved in the *de novo* pyrimidine biosynthesis. The baseline from median mutation rate of non-cancer genes is also shown as gray for comparison. (O) Statistical significance of mutation rates over expected values (MutSigCV Q-value) as a function of the average expression level for cancer genes and genes involved in *de novo* pyrimidine biosynthesis, as compared with the baseline from non-cancer genes. Data in (B)–(E), (G), and (H) are mean values $\pm$ SD; data in (L) are mean values $\pm$ SEM ($n \geq 3$). The symbol “***” indicates statistically significant differences from parental cells, and the symbol “***” indicates statistically significant difference from D0 cells, with $p < 0.05$. (F), (I), (K), and (M) show representative images of three biological replicates. Data in (N) and (O) represent analysis of more than 11,000 patients diagnosed with 33 different types of cancer listed in the Cosmic database.

and DHODH^{KO} cells. DHODH^{KO} cells contained lower UMP synthesis capacity compared with parental cells, and this was restored in DHODH^{resc} cells (Figure 5C).

Cancer cells are typified by rapid proliferation, which involves unhindered progression through the cell cycle. During the S phase, genomic DNA is replicated in a process that includes insertion of nucleotides produced by the *de novo* pyrimidine pathway into nascent DNA strands (Sigollet et al., 2003). To test if respiration is linked to cell-cycle progression, we evaluated D0–D60 lines for cell-cycle distribution. This revealed that more D0–D10 cells were in the S phase compared with parental and D20–D60 cells (Figure 5D). We also found that upon nocodazole synchronization, a higher proportion of parental and D15–D60 cells were arrested in G2 compared with D0–D10 cells (Figure 5E). Finally, D0–D10, but not parental and D15–D60 cells, accumulated cyclin E, a marker of early S phase (Figure 5F).

We next investigated a link between S-phase arrest and DHODH activity. Cell-cycle evaluation revealed increased number of DHODH^{KO} cells in S phase compared with parental cells, with the proportion of DHODH^{resc} cells in S phase being comparable to parental cells (Figure 5G). A similar pattern was observed for G2 arrest after nocodazole treatment (Figure 5H) and for the cellular content of cyclin E (Figure 5I), consistent with a previous report (Mohamad Fairus et al., 2017). Finally, we found that B16 DHODH^{KO} cells were also arrested in S phase, while B16 DHODH^{resc} cells showed cell-cycle distribution similar to parental cells (Figure S5I). Hence, absence of DHODH activity suppresses pyrimidines and interferes with cell-cycle progression.

De Novo Pyrimidine Pathway Is Primed in OXPHOS-Deficient Cells

To understand the status of the pyrimidine pathway in D0–D60 lines and in parental 4T1 cells, we performed transcriptome analysis. Principal component analysis (PCA) in Figure 5J shows clustering of D0–D10 and D15–D25 cells. A similar pattern was observed for mitochondrial protein transcripts sourced from MitoCarta 2.0 (Calvo et al., 2015) (Figure S6A). Given the link between respiration, DHODH, pyrimidine synthesis, and tumor progression observed above, we analyzed microchip data for transcripts relevant for the *de novo* pyrimidine pathway (cf. Figure 5A) to test if the clustering pattern was maintained. This revealed no trend in the expression of *CAD*, *DHODH*, and *UMPS* transcripts in parental, D0–D25, and D60 cells (Figure S6B), consistent with the DHODH mRNA and protein levels shown in Figure 3B.

We next examined expression of proteins of the *de novo* pyrimidine pathway. Similar to DHODH, there were few differences in CAD, phosphorylated CAD, and UMPS among the lines tested (Figure 5K). Next, DHODH activity was 30%–50% lower in D0–D10 than in parental cells (Figure 5L), yet considerably higher than the very low DHODH-dependent respiration and low orotate/DHO ratio in these lines (cf. Figures 3D and 3E). Also, cells with manipulated DHODH content did not show significant changes in the expression of CAD and UMPS (Figure 5M).

The above results suggest that the *de novo* pyrimidine pathway is primed and preserved in OXPHOS-deficient cells. To interrogate this notion with patient data, we compared mutation rates of known cancer genes to genes of *de novo* pyrimidine

synthesis (*CAD*, *DHODH*, and *UMPS*) as a function of average expression levels in >11,000 patients diagnosed with 33 different types of cancer listed in The Cancer Genome Atlas database. Both raw mutation rate (number of mutations per base pair in a gene) (Figure 5N) and statistical significance of mutations of a gene over expected mutations (Figure 5O) are shown for comparison. The median mutation rates (or significance) for other genes (not annotated as cancer genes) were utilized as a baseline. Raw mutation rates of *CAD*, *DHODH*, and *UMPS* were close to the baseline, and were statistically indistinguishable from the expected mutations according to MutsigCV (Q is nearly 1). By comparison, many known oncogenes and tumor suppressor genes had significantly higher mutation rates (20%, $Q < 0.1$). These results suggest that genes of the *de novo* pyrimidine pathway are rarely mutated in cancer.

To assess whether DHODH-dependent respiration and DHODH activity are conserved in different types of cancer, we examined breast, cervical, and pancreatic cancer cell lines as well as osteosarcoma cells. Routine and DHODH-dependent respiration varied between 30–80 and 2–4 pmol orotate/hr/10⁶ cells, respectively (Figure S6C), while DHODH activity ranged from 200 to 450 pmol O₂/hr/10⁶ cells (Figure S6D), indicating that DHODH/pyrimidine synthesis pathway function is preserved in cancer cells.

ATP5B^{KO} Cells Maintain Functional DHODH

Despite the absence of OXPHOS-derived ATP, ATP5B^{KO} cells form tumors in mice (Figures 2M and S5D) and should therefore maintain functional DHODH. We found that ATP5B^{KO} cells grew in the absence of uridine (Figure 6A), contained normal amounts of the DHODH protein (Figure 6A, insert), and featured DHODH-dependent respiration while routine respiration was decreased (Figure 6B). The relative NADH/NAD⁺ ratio increased by 50%–60% in ATP5B^{KO} cells compared with parental cells (Figure 6C). ATP5B^{KO} cells had mitochondria without cristae (Figure 6F), in agreement with the literature (Daum et al., 2013). The finding of normal DHODH protein and DHODH-dependent respiration in ATP5B^{KO} cells was corroborated by normal DHODH activity (Figure 6H) and conserved proteins of the *de novo* pyrimidine pathway (Figure 6I). We also tested ATP5B^{KO} B16 cells and found a similar pattern for the corresponding lines (Figures S5F–S5H). Consistent with this, we observed fewer cells in S phase in both 4T1 ATP5B^{KO} and B16 ATP5B^{KO} cells than in their non-respiring counterparts (Figures 6K and S5I).

Complexes III and IV Propel DHODH-dependent Respiration to Promote Tumor Formation

The above data indicate that DHODH presents a link between respiration and tumor formation, controlling *de novo* pyrimidine synthesis independently of mitochondrial ATP generation. As DHODH connects to the mitochondrial pool of coenzyme Q (CoQ) that is redox-cycled by CIII/CIV, DHODH might be stalled when respiration is deficient due to lack of its electron acceptor before it is reconstituted in D15–D20 cells. We therefore expressed alternative oxidase (AOX) in 4T1p⁰ and B16p⁰ cells. This protein is present in lower eukaryotes where it replaces CIII + CIV, facilitating direct electron transfer from CoQ to oxygen (Hakkaart et al., 2006; Perales-Clemente et al., 2008).

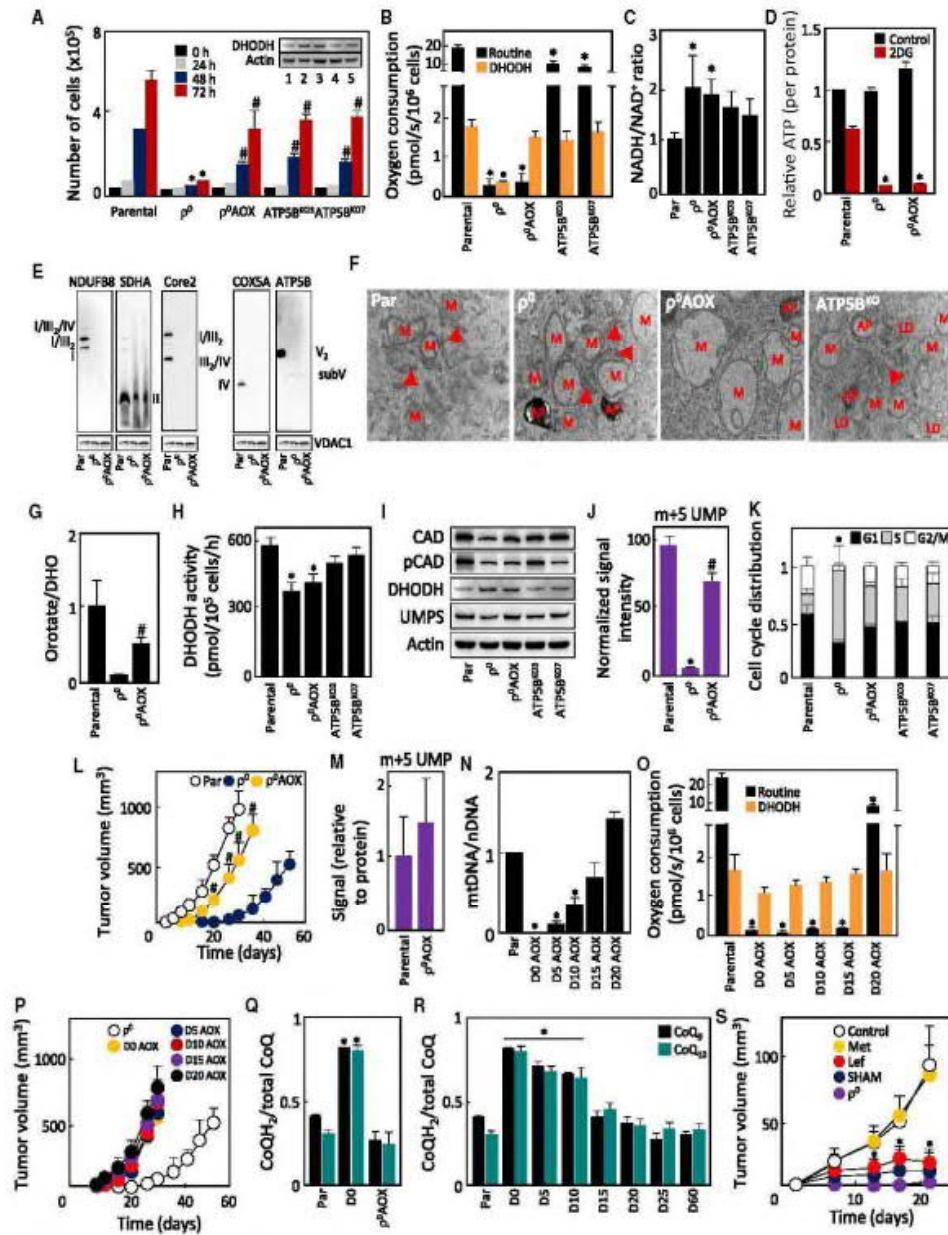


Figure 6. ATP5B Cells Maintain Functional DHODH, Propelled by CIII and CIV
 (A–D) Parental, p^0 , p^0 AOX, and ATP5B^{KO} cells were evaluated for growth in the absence of uridine (A), the level of the DHODH proteins by WB (A, insert), routine and DHODH-dependent respiration (B), NADH/NAD⁺ ratio (C), and ATP generation in the absence or presence of 50 mM 2DG (4.5 g/L glucose) (D). (E–K) Parental, p^0 , and p^0 AOX cells were evaluated by NBGE for the assembly of RCs and SCs using antibodies against subunits of individual RCs (E). Cells as shown were evaluated for mitochondrial morphology using TEM (F); the ratio of orotate-to-DHO (G); DHODH activity (H); level of the CAD, p(S185)-CAD, DHODH, and UMPS proteins (I); formation of m+5 UMP from ¹⁵N₂-Gln (J); and cell-cycle distribution (K).

(legend continued on next page)

4T1 ρ^0 AOX cells proliferated in the absence of uridine (Figure 6A), suggesting that AOX reactivates DHODH in ρ^0 cells. Consistent with this, 4T1 ρ^0 AOX cells contained normal DHODH protein (Figure 6A, insert) and DHODH-dependent respiration, while routine respiration was low and comparable to 4T1 ρ^0 cells (Figure 6B). The virtual absence of routine respiration was reflected by increased NADH/NAD $^+$ ratio in ρ^0 AOX cells, which was similar to 4T1 ρ^0 cells (Figure 6C). As with 4T1 ρ^0 cells, 4T1 ρ^0 AOX cells used glycolysis to maintain normal levels of ATP (Figure 6D), lacking assembled mitochondrial complexes (except for CII) (Figure 6E) and mitochondrial cristae (Figure 6F); 4T1 ρ^0 AOX cells converted DHO to orotate (Figure 6G), exerted normal DHODH activity (Figure 6H), maintained proteins of the *de novo* pyrimidine pathway (Figure 6I), and efficiently converted glutamine to UMP (Figure 6J). As a result, 4T1 ρ^0 AOX cells were unlocked from the S phase arrest (Figure 6K) and efficiently formed tumors in BALB/c mice (Figure 6L). To verify the AOX-driven reactivation of the *de novo* pyrimidine pathway in tumors *in vivo*, we performed a live labeling study, in which, $^{13}\text{C}_6$ - $^{15}\text{N}_2$ -Gln was infused via the jugular vein into BALB/c mice carrying tumors derived from 4T1 (17 days after grafting) and 4T1 ρ^0 AOX cells (14 days after grafting), and the abundance of the m+s isotopomer of UMP was evaluated in isolated tumor tissue. Conversion of Gln to UMP was comparable in 4T1 and 4T1 ρ^0 AOX cell-derived tumors (Figure 6M), consistent with normal flux through the *de novo* pyrimidine pathway in AOX-expressing tumors. B16 lines showed behavior similar to the corresponding 4T1 ρ^0 lines (Figures S5J–S5P), and B16 ρ^0 AOX cells formed tumors almost as efficiently as their parental counterparts (Figure S5Q).

To confirm that AOX acts *in vivo* by reactivating CoQ redox-cycling rather than by enhancing mitochondrial transfer into ρ^0 cells, we grafted 4T1 ρ^0 AOX cells into BALB/c mice and derived lines on days 5, 10, 15, and 20. The temporal increase in mtDNA in 4T1 ρ^0 AOX cells was similar to 4T1 ρ^0 lines (Figure 6N; cf. Figures 1E and S1B). However, D0–D15 AOX cells had very low routine respiration that increased in D20 AOX cells to $\approx$ 40% of parental cells, while DHODH-dependent respiration remained close to parental cell levels (Figure 6O). To examine their tumor-forming propensity, we grafted D0–D20 AOX cells into BALB/c mice, and observed that D5 AOX–D20 AOX cells formed tumors similarly to D0 (ρ^0) AOX cells (Figure 6P; cf. Figure 6L).

The link between CIII + CIV and electrons generated upstream in the OXPHOS systems is mediated by redox-cycling of CoQ (i.e., oxidation of CoQH $_2$ to CoQ). To assess whether CoQH $_2$ is

re-oxidized in 4T1 ρ^0 AOX cells, where the AOX protein substitutes CIII + CIV in redox-cycling CoQ, we determined the CoQ redox state in parental, 4T1 ρ^0 and 4T1 ρ^0 AOX cells. In rodents, CoQ $_9$ is the major form of CoQ with the remainder made up by CoQ $_{10}$. We therefore assessed the CoQH $_2$ /total CoQ (i.e., CoQH $_2$ +CoQ) for both forms of CoQ. In parental cells, $>$ 50% of CoQ was in the oxidized state, while in 4T1 ρ^0 cells most CoQ was in the reduced form (Figure 6Q). Expression of AOX in non-respiring cells shifted the redox state of CoQ to its more oxidized form (Figure 6Q), consistent with published data (Guarás et al., 2016). We next assessed the CoQH $_2$ /total CoQ ratio in D0–D60 cells and found it higher in D0–D10 and lower in D15–D60 cells, suggesting that restoration of respiration resulted in efficient oxidation of the CoQ pool (Figure 6R).

To confirm that restoration of DHODH function by reactivation of CoQ redox-cycling is a major consequence of AOX expression in ρ^0 cells that supports tumor formation, we tested the effect of the AOX inhibitor salicylhydroxamic acid (SHAM) (Martínez-Reyes et al., 2016), the DHODH inhibitor leflunomide (Lofler et al., 2005), and the CI inhibitor metformin (Wheaton et al., 2014) on tumor formation in BALB/c mice with grafted 4T1 ρ^0 AOX cells. Figure 6 shows the inhibitory effect of both SHAM and leflunomide but not that of metformin, pointing to the involvement of the AOX-DHODH axis in this process and excluding potential effects of AOX on the NADH/NAD $^+$ cycling via nascent CI.

Pyruvate May Not Be Limiting for Tumor Growth

Since low-respiring cells are auxotrophic for pyruvate and uridine, we tested whether the capacity of ρ^0 AOX cells to form tumors could be promoted by the presence of pyruvate in the microenvironment. By providing external oxidizing power, extracellular pyruvate could conceivably release the potential block in proliferation and tumor growth linked to defective aspartate biosynthesis due to the lack of NAD $^+$ recycling (Sullivan et al., 2015, 2018; Birsoy et al., 2015; García-Bermúdez et al., 2018). Pyruvate was suggested to be increased in malignant compared with normal tissues (Goveia et al., 2016). We thus analyzed pyruvate in tissue excised from the site of grafted 4T1 ρ^0 cells at time points used to establish the lines, in parental and D5–D25 cells (cf. Figure 1B). At all time points, pyruvate concentrations in the tumor lesions/tissue were $\approx$ 20%–40% higher than in the liver (Figure S7A), being 2–3 nmol/mg of tumor tissue. Assuming a tissue density close to 1 g/mL, this corresponds to concentration of $\approx$ 2 mM (i.e., higher than the 1 mM pyruvate that

(J) Parental, ρ^0 , and ρ^0 AOX cells were grafted s.c. in BALB/c mice at 10^6 per animal, and tumor formation was assessed by USI.

(M–O) BALB/c mice with 4T1 and 4T1 ρ^0 AOX cell-derived tumors ($\approx$ 250 mm ^{3}) were cannulated via the jugular vein and infused with $^{13}\text{C}_6$, $^{15}\text{N}_2$ -Gln, and the tumors were analyzed for the m+s UMP isotopomer as detailed in STAR Methods (M). ρ^0 AOX (D0 AOX) cells were grafted into BALB/c mice s.c. at 10^6 per animal. On days 5, 10, 15, and 20 post-grafting, the pre-tumor plaques were excised from the animals and D5 AOX, D10 AOX, D15 AOX, and D20 AOX lines were established. Parental and D0 AOX–D20 AOX cells were assessed for mtDNA using qPCR (N) and for routine and DHODH-dependent respiration using the Oxygraph (O).

(P) Parental, D0, and D0 AOX to D20 AOX cells were grafted in BALB/c mice (10^6 cells/per animal), and tumor volume was assessed by USI.

(Q) Parental, ρ^0 , and ρ^0 AOX cells were assessed for the ratio of CoQH $_2$ and total CoQ including the CoQ $_9$ and CoQ $_{10}$ analogs.

(R) Parental and ρ^0 (D0) cells as well as D5–D60 lines were assessed for the ratio of CoQH $_2$ and total CoQ.

(S) BALB/c mice were grafted with ρ^0 AOX cells at 10^6 per animal; tumors were treated with metformin (Met), leflunomide (Lef), and salicylhydroxamic acid (SHAM) as detailed in STAR Methods; and tumor volume was quantified.

Data in (A), (C), (D), (G), (J), (K), (M), (Q), and (R) are mean values $\pm$ SD ($n = 3$); those in (B), (H), and (O) $n \geq 3$; (L), (S) ($n = 5$), and P ($n = 3$) are mean values $\pm$ SEM. The symbol “***” indicates statistically significant differences from parental cells, with $p < 0.05$; the symbol “#” indicates statistically significant differences from ρ^0 cells, with $p < 0.05$. (A, insert), (E), (F), and (I) show representative images of three biological replicates.

supported proliferation of respiration-deficient cells in our *in vitro* experiments) (cf. Figure 3A) (King and Attardi, 1988, 1989).

To see whether pyruvate detected in tumors was extracellular (i.e., able to provide the oxidizing power for aspartate production), we analyzed serum (containing no intracellular pyruvate) from C57BL/6 and BALB/c mice with and without tumors as well as serum from cancer patients and healthy human subjects for pyruvate. The measured concentration was about 75–150 μ M in mice and about 200 μ M in human samples (Figure S7B), the latter being similar to published values (Landon et al., 1962).

To determine the effect of extracellular pyruvate on intracellular aspartate and on the proliferative potential of ρ^0 cells, we evaluated 4T1 ρ^0 and B16 ρ^0 cells maintained with uridine and increasing amounts of added pyruvate for intracellular aspartate. Figures S7C and S5R document $\approx$ 1.5-fold increase of intracellular aspartate with escalating extracellular concentrations of pyruvate over the range of 0–1 mM. Aspartate increased at extracellular pyruvate concentrations equivalent to those measured in mouse and human serum. Similarly, the same level of pyruvate as found in serum resulted in stimulation of cell proliferation (Figures S7D and S5S).

We next evaluated intracellular aspartate in the absence of extracellular pyruvate and uridine. While aspartate was reduced by $\approx$ 30% in non-respiring D0–D10 cells, it recovered in D15 cells (Figure S7E). Intracellular aspartate was unchanged in ATP5B^{KO} (Figure S7F) or DHODH-manipulated cells (Figure S7G). Further, AOX expression in 4T1 ρ^0 cells did not increase aspartate, which remained similar as in the ρ^0 cells (Figure S7H). This demonstrates that AOX overexpression does not increase intracellular aspartate in our system and should not support growth of ρ^0 cells in the absence of pyruvate. Accordingly, ρ^0 AOX cells, similarly as ρ^0 cells, were auxotrophic for pyruvate (Figure S7I).

Finally, we tested the effect of uridine on proliferation of 4T1 ρ^0 and B16 ρ^0 cells cultured in the presence of serum prepared from BALB/c and C57BL/6 mice instead of FCS. We observed uridine to significantly enhance cell growth under these conditions (Figures S7J and S5T). To further document the importance of uridine limitation in tumor growth, we tested the effect of uridine supplementation of mice with grafted 4T1 ρ^0 cells on tumor formation. Figure S7K shows that tumors appeared earlier in uridine-supplemented mice, and at a time that precedes mitochondrial transfer and the associated respiration recovery. Collectively, this further supports the notion that non-functional DHODH presents a severe limitation for tumor growth.

DISCUSSION

The importance of mitochondria for the initiation and progression of tumorigenesis is now emerging. It is evident that, despite the well-known Warburg effect, tumors have active mitochondrial bioenergetic metabolism (Marín-Valencia et al., 2012; Hensley et al., 2016). Disruption of the ETC shows promise in cancer therapy (Rohlena et al., 2011; Zhang et al., 2014; Rohlenova et al., 2017). Cells with deleterious mtDNA mutations fail to form tumors (Park et al., 2009), and genetic ablation of OXPHOS restrains tumorigenesis (Weinberg et al., 2010). mtDNA-depleted (ρ^0) tumor cells present a particularly instructive case. Having no functional OXPHOS, these cells cannot form tumors in mice unless they acquire host mtDNA (Tan et al., 2015) via horizontal

transfer of whole mitochondria from the stroma (Dong et al., 2017). In this way, ρ^0 cells give rise to palpable tumors only after a long initial lag period (Tan et al., 2015; Dong et al., 2017). While these studies suggest that functional OXPHOS is necessary for tumorigenesis, previously published data did not pinpoint exactly which aspect of OXPHOS function is essential. The key finding of the current study is that DHODH-driven pyrimidine biosynthesis, rather than OXPHOS-mediated ATP production, is essential for tumorigenesis.

To further explore this issue, we developed a unique model that allowed us to characterize the link between OXPHOS function and tumor formation in unprecedented temporal detail. We followed the events associated with tumorigenesis of mtDNA-depleted ρ^0 cells in mice during the initial lag period and throughout the various stages of tumor progression and demonstrate that the appearance of tumors coincides with OXPHOS reconstitution at days 15–25 post-grafting, after the end of a “dormant” period when mtDNA is replenished and OXPHOS machinery re-assembled. This detailed investigation was possible because cancer cells isolated at various time points in the 4T1 ρ^0 tumor model are remarkably stable in culture, and their properties, such as the level of mtDNA, respiration, or ETC assembly, did not change with time. This unexpected stability, observed by others in a different model (Picard et al., 2014), can be explained by the absence of selection pressure in the rich culture media containing uridine/pyruvate (see below).

Surprisingly, we found that the best known OXPHOS function, production of ATP by ATP synthase, is not essential for tumorigenesis. While increased mitochondrial contribution to total ATP production was concurrent with OXPHOS reconstitution, ATP synthase assembly and appearance of tumors, the total ATP content in the cells and energy charge were not, in general, significantly decreased, but maintained by glycolysis with its much faster kinetics compared with OXPHOS (Koppenol et al., 2011). Most strikingly, cells deficient in ATP synthase were found to readily produce tumors. This suggests that mitochondrial ATP production is not limiting for tumor growth, at least at its earlier stages. Instead, we found that the important OXPHOS-related feature that promotes tumorigenesis is *de novo* pyrimidine synthesis, directly driven by respiration via DHODH, which converts DHO to orotate. This was clearly demonstrated by the failure of DHODH-deficient cells to form tumors, despite the fact that these cells show otherwise fully functional OXPHOS and normal ATP levels. Furthermore, the DHO/orotate ratio was increased in DHODH^{KO} cells and non-respiring D0–D10 cells, and formation of UMP from glutamine was compromised. This indicates that the enzyme is inactive but is reactivated before tumors appear. The central role for DHODH in tumorigenesis is consistent with a recent report demonstrating upregulation of DHODH during the course of UV-induced skin tumorigenesis and its functional role therein (Hosseini et al., 2018).

The link between OXPHOS and DHODH is maintained by CoQ redox-cycling (Gregoire et al., 1984; Ayer et al., 2015). Electrons, removed from DHO by DHODH, are transferred to CoQ yielding CoQH₂, which is then re-oxidized at CIII. The CoQ redox cycle is broken in the absence of CIII/CIV activity when OXPHOS is non-functional due to mtDNA deficiency. This removes the only practical means of CoQ recovery in

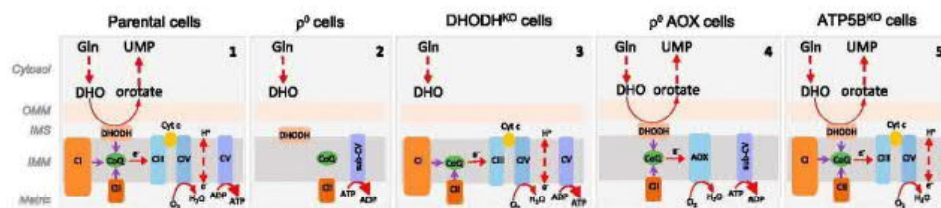


Figure 7. Scheme Depicting the Role of Respiration in Propelling *De Novo* Pyrimidine Synthesis
Individual schemes illustrating the role of respiration in *de novo* pyrimidine synthesis with central role of CoQ and DHODH, as documented using our cell models, including parental (1), ρ^0 (2), DHODH^{KO} (3), ρ^0 AOX (4), and ATP5B^{KO} cells (5).

mammalian cells, while maintaining upstream sources of electrons for CoQ reduction such as CII or DHODH itself. These sources of electrons reduce CoQ to the maximal attainable level before they stall due to the lack of the electron acceptor. Indeed, in non-respiring D0-D10 cells CoQ was present predominantly in the reduced form, whereas the CoQH₂/CoQ redox state decreased and approached parental cell values before the onset of tumor formation, coinciding with CIII/CIV reconstitution. AOX expression in ρ^0 cells was sufficient to reactivate DHODH-driven respiration, normalize the CoQH₂/CoQ and DHO/orotate ratios, reinitiate UMP synthesis both *in vitro* and *in vivo*, and restore tumorigenicity in the absence of mtDNA. Importantly, live labeling with ¹³C₅, ¹⁵N₂-Gln confirmed the *in vitro* results in a mouse model derived from 4T1 ρ^0 AOX cells 14 days after grafting the cells, i.e., at the stage when respiration is not recovered and tumor formation is largely dependent on AOX-propelled DHODH (Figures 6M–6P). It thus seems that lack of redox-cycling of CoQ, not OXPHOS deficiency per se, restricts tumorigenesis.

We therefore propose a scenario based on our experimental models and depicted schematically in Figure 7. In fully respiring cells, CI, CII, and DHODH transfer electrons to CoQ, which are then forwarded to CIII. This latter complex transfers electrons to CIV, which then produces water at the expense of molecular oxygen. CI, CIII, and CIV increase the proton-motive force in the form of $\Delta\Psi_{\text{m}}$, that, among other functions, drives formation of ATP catalyzed by CV. In ρ^0 cells, mtDNA is missing, resulting in the collapse of CI, CIII, and CIV, and in the assembly of CII and sub-CV. Under this scenario, DHODH cannot convert DHO to orotate, since CoQ cannot transfer electrons to CIII. ATP is generated by glycolysis and $\Delta\Psi_{\text{m}}$, maintained by ATP cleavage by the ATPase activity of sub-CV; ρ^0 cells form tumors only after acquisition of mtDNA from the host and restoration of respiration (Tan et al., 2015; Dong et al., 2017). DHODH^{KO} cells lack tumor-forming capacity and cannot be “repaired” by mtDNA acquisition, because the absence of DHODH precludes conversion of DHO to orotate. On the other hand, ρ^0 AOX cells have normal DHODH-dependent respiration, since electrons generated by conversion of DHO to orotate are captured by CoQ and transferred to AOX, which substitutes for the combined activity of CII and CIV. These cells efficiently form tumors. Finally, ATP5B^{KO} cells that are highly glycolytic have normal DHODH-dependent respiration and form tumors faster than ρ^0 cells. Together, our findings demonstrate an important role of DHODH

and CoQ redox-cycling in tumor formation in cells with damaged mtDNA, resulting in mtDNA acquisition and restoration of respiration independent of OXPHOS-derived ATP.

A typical consequence of OXPHOS dysfunction is the auxotrophy for pyruvate and uridine. Pyruvate as an exogenous electron acceptor is required to produce aspartate, a precursor of *de novo* pyrimidine biosynthesis, and uridine to complement defective DHODH-linked pyrimidine synthesis via salvage pathways (Loffler, 1980; King and Attardi, 1988, 1989; Birsoy et al., 2015; Sullivan et al., 2015). We observed auxotrophy in D0–D15 cells, which was relieved by OXPHOS reconstitution following mtDNA transfer and prior to tumorigenesis, or in the case of uridine, by AOX expression. Given the efficacy of AOX-mediated restoration of tumorigenicity, this suggests that the DHODH dysfunction-induced defect in *de novo* pyrimidine synthesis could be a major obstacle for *in vivo* growth of respiration-compromised tumor cells, and that pyruvate might not be the limiting factor. Indeed, we consistently measured substantial pyruvate at the site of tumor growth throughout the course of the experiment, and levels of pyruvate in serum of mice and cancer patients were sufficient to support proliferation of ρ^0 cells *in vitro*, although suboptimal. In addition, it has been reported that cancer cells deficient in the CII subunit SDHB are addicted to pyruvate (Cardaci et al., 2015; Lussey-Lepoutre et al., 2015), yet they readily form tumors in mice (Guzy et al., 2008; Bezawork-Geleta et al., 2018). Importantly, SDHB-deficient neoplasias are relatively common in humans and have unfavorable prognosis (King et al., 2011). These data suggest that pyruvate may not be limiting *in vivo* under all circumstances.

To begin to place our results into context, we suggest that *de novo* pyrimidine biosynthesis, driven by functional OXPHOS, is crucial for tumor growth. This notion is supported by the failure of DHODH^{KO} cells to form tumors, and by the recovery of tumorigenicity when AOX is expressed in ρ^0 cells. Our results also suggest that DHODH represents a bottleneck for pyrimidine synthesis in non-respiring cells, although they do not rule out other limitations that might constrain *in vivo* growth in the absence of functional OXPHOS. While a deficiency in DHODH disrupts the pyrimidine biosynthesis pathway in a defined manner, expression of AOX restores DHODH activity as well as reactivating CoQ redox-cycling. AOX might also impact on additional metabolic pathways converging on the CoQ pool. It seems unlikely, however, that AOX indirectly supports the synthesis of aspartate via nascent CI. AOX expression did not affect the NADH/NAD⁺

ratio or content of aspartate in ρ^0 cells, just as ρ^0 AOX cells are auxotrophic for pyruvate. Previous studies have reported that aspartate can be limiting for tumor growth *in vivo* due to its inefficient import into cells and proposed that CI inhibition constrains tumor growth by limiting aspartate biosynthesis (Garcia-Bermudez et al., 2018; Sullivan et al., 2018). However, while growth retardation by CI inhibition was complete *in vitro* in the absence of pyruvate, it was incomplete when CI was targeted *in vivo* (Sullivan et al., 2018). The remaining proliferation in that case could perhaps be supported by extracellular pyruvate. To summarize, while this and other reports clearly show that pyrimidine biosynthesis is essential for tumor growth in multiple cancer models, the identity of the rate-limiting steps in various conditions deserves further investigation, as it will likely be affected by the environment and by the experimental model employed.

What does this mean for cancer therapy? We found that components of the *de novo* pyrimidine synthesis pathway rarely mutate in cancer, clearly showing its importance. In our experimental models, the pathway, including DHODH itself, was primed to respond when the block in CoQ redox-cycling was removed. Inhibitors of DHODH are used in the clinic as anti-rheumatics (Olsen and Stein, 2004) and show efficacy in cancer settings either alone or in combination with anti-cancer agents (Brown et al., 2017; Mathur et al., 2017; Sykes et al., 2016; Shukla et al., 2017; Kim et al., 2017; Koundinya et al., 2018). A more effective therapeutic approach could involve intervention at the level of respiratory CIII. This would not only block DHODH activity and pyrimidine biosynthesis, but would also increase the generation of reactive oxygen species from the respiratory chain (Adam-Vizi and Chinopoulos, 2006; Murphy, 2009; Quinlan et al., 2012), striking the cancer cell on two fronts at once by way of combining the cell death inducing activity of excessive oxidative stress (Rohlena et al., 2011) with the cytostatic activity of DHODH inhibition. While anti-cancer agents directed at CIII exist (Le et al., 2007), their potential side effects on the hematopoietic and immune systems would need to be carefully evaluated (Anso et al., 2017).

In conclusion, we show here that cancer cells with dysfunctional OXPHOS due to mtDNA deficiency import mitochondria from the host to restore respiration and facilitate the DHODH-catalyzed conversion of DHO to orotate. Intriguingly, reactivation of CoQ redox-cycling is sufficient to drive DHODH and to allow cancer cells to form tumors. We propose that DHODH-mediated CoQ redox-cycling is an important link between *de novo* pyrimidine synthesis and respiration, and that it may be a promising target for broad-spectrum cancer therapy. This notion is corroborated by the emergence of tumor metabolism as a target for anti-cancer therapeutic strategies (Sullivan et al., 2016; Martinez-Outschoorn et al., 2017). Our reasoning is based on the concept that despite their high metabolic plasticity, that includes the ability to “import” mitochondria, cancer cells seem unable to “bypass” pyrimidine synthesis defects, presenting a novel aspect of cancer biology with potential therapeutic implications.

Limitations of Study

While we propose that targeting DHODH may present a novel, broad-spectrum anti-cancer strategy, we note that there are

mitochondrial inhibitors that are under clinical trial, such as inhibitors of complex I (Molina et al., 2018; Rohlenova et al., 2017) or α -ketoglutarate dehydrogenase (Alistar et al., 2017), which effectively suppress tumor growth without DHODH inhibition. In addition, it can be expected that DHODH inhibition will not be effective in all types of tumors, and that combined targeting involving DHODH and complex I may provide a broader and more effective anti-cancer strategy by simultaneously interfering with two crucial steps of pyrimidine biosynthesis. In addition, while we do not see a direct effect of AOX overexpression on aspartate biosynthesis *in vitro* in the absence of complex I activity, it is possible that AOX expression, by altering cellular redox balance, could stimulate tumor growth *in vivo* in a complex I-independent manner or that as yet uncharacterized factors in the tumor microenvironment could synergize with AOX to stimulate tumor growth.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <https://doi.org/10.1016/j.cmet.2018.10.014>.

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AUTHOR CONTRIBUTIONS

J.N., J.R., L.D., J.K., and M.V.B. designed the research; J.K., M.B., A.R.C., S.B., K.R., K.V., Z.E., A.N., L.K., Z.N., B.E., K.J., A.B.-G., K.K., K.D.-H., S.M.N., and R.Z. performed most *in vitro* and *in vivo* experiments; D.S. and M.K. performed and analyzed single-cell qPCR studies; S.H. and E.D. performed cell-cycle experiments; S.O., Y.J.A., and S.P. designed, performed, and interpreted metabolomics and fluxomics studies; T.C.-O., T.L.S., V.A.S., and P.J.O. performed and interpreted mitochondrial respiration, membrane potential, and superoxide anion experiments; L.G. and M.R. performed and analyzed mTRIP experiments; G.J.M. and R.S. performed and analyzed CoQ assays; M.S. and P.H. performed and interpreted TEM; M.O. performed and interpreted STED microscopy; H.S. performed and interpreted microchip analyses; T.Z. and Y.Z. provided and interpreted bioinformatics data. W.J.H.K. provided methodology; J.T. and C.S.-A. designed and performed mitoChIP

analyses. J.T. also performed and analyzed results of sequencing and revised the manuscript; T.H., K.P., and R.G. provided serum samples of healthy and neoplastic subjects; F.K. helped with *in vivo* labeling experiments; J.N., J.R., R.S., L.D., J.K., S.P., and M.V.B. wrote and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-BrdU-FITC IgG	BD Biosciences	340649; RRID: AB_400443
anti-TFAM IgG	Sigma-Aldrich	HPA040648; RRID: AB_10795635
anti-SSB IgG	Sigma-Aldrich	HPA002866; RRID: AB_1080092
anti-ATP5B IgG	Sigma-Aldrich	HPA001520; RRID: AB_1078243
anti-POLG IgG	Thermo Fisher Scientific	PA5-35163; RRID: AB_2552473
anti-cyclin E IgG	Thermo Fisher Scientific	MA5-14336; RRID: AB_10984356
anti-mtCO4 IgG	Thermo Fisher Scientific	PA5-29992; RRID: AB_2547466
anti-UMPS IgG	Santa Cruz Biotechnology	sc-398086; RRID: AB_2750932
anti-DHODH IgG	Santa Cruz Biotechnology	sc-166348; RRID: AB_2091729
anti-TOM20 IgG	Santa Cruz Biotechnology	sc-136211; RRID: AB_2207538
anti-CAD IgG	Cell Signaling	93925; RRID: AB_2750933
anti-p(S1859)-CAD IgG	Cell Signaling	12662; RRID: AB_2750934
anti-HSP60 IgG	Cell Signaling	12165S; RRID: AB_2636980
anti-Ki67 IgG	Cell Signaling	9129; RRID: AB_2687446
anti-NDUFS3 IgG	Abcam	ab110246; RRID: AB_10861972
anti-NDUFA9 IgG	Abcam	ab14713; RRID: AB_301431
anti-NDUFB8 IgG	Abcam	ab110242; RRID: AB_10859122
anti-mtCO1 IgG	Abcam	ab14705; RRID: AB_2084810
anti-COXVa IgG	Abcam	ab110262; RRID: AB_10861723
anti-SDHA IgG	Abcam	ab14715; RRID: AB_301433
anti-SDHB IgG	Abcam	ab14714; RRID: AB_301432
anti-Core2 IgG	Abcam	ab14745; RRID: AB_2213640
anti-ATP5A IgG	Abcam	ab14748; RRID: AB_301447
anti-VDAC IgG	Abcam	ab15895; RRID: AB_2214787
anti-TFAM IgG	Abcam	ab47517; RRID: AB_945799
anti-DHODH IgG	Proteintech	14877-1-AP; RRID: AB_2091723
anti-DNA IgG	PROGEN	61014; RRID: AB_2750935
Critical Commercial Assays/Kits		
NADH/NAD Glo Assay	Promega	G9071
ATP CellTiter Glo Assay	Promega	G9241
Seahorse XF Cell Mito Stress Test Kit	Agilent	103015-100
Seahorse XF Glycolysis Stress TestKit	Agilent	103020-100
Colorimetric pyruvate assay	Abcam	ab65342
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	#23225
Atto488 NT Labeling Kit	Jena Bioscience	PP-305S-488
Atto550 NT Labeling Kit	Jena Bioscience	PP-305S-550
Chemicals, Peptides and Recombinant Proteins		
ATP	Sigma-Aldrich	A1852
2-nitrobenzodeoxyglucose	Thermo Fisher Scientific	N13195
Dihydroorotic acid	Sigma-Aldrich	D7128
Orotic acid	TCI	50887-69-9
6-thioguanine	Sigma-Aldrich	A4882
Metformin	Metformin	04635
Lefunomide	Sigma-Aldrich	L5025

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Salicylhydroxamic acid	Sigma-Aldrich	S607
¹³ C ₅ , ¹⁵ N ₂ -glutamine	Sigma-Aldrich	607983
¹³ C ₅ , ¹⁵ N ₂ -glutamine	Cambridge Isotope Laboratories	CNLM-1275-H-0.1
Coenzyme Q ₉	Santa Cruz Biotechnology	sc-207451
Coenzyme Q ₁₀	Fluka	27595
Tetramethylrhodamine ethyl ester	Sigma-Aldrich	87917
Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrozone	Sigma-Aldrich	C2920
Carbonyl cyanide-3-chlorophenylhydrozone	Sigma-Aldrich	C2759
Oligomycin	Sigma-Aldrich	O4876
Antimycin A	Sigma-Aldrich	A8474
2-deoxyglucose	Sigma-Aldrich	D8375
4-(trifluoromethoxy) benzamidoxime	Sigma-Aldrich	422231
ADP	Sigma-Aldrich	A5285
Cytochrome c	Sigma-Aldrich	C7752
Rotenone	Sigma-Aldrich	R8875
Digitonin	Fluka	37008
K-lactobionate	Sigma-Aldrich	153516
Nocodazol	Sigma-Aldrich	M1404
Fluoroshield with DAPI	Sigma-Aldrich	F6057
MitoSOX	Thermo Fisher Scientific	M36008
Decylubiquinone	Sigma-Aldrich	D7911
Deposited Data		
Microarray Analysis Data	EBI ArrayExpress Database	ArrayExpress: MTAB-6150
Experimental Models: Cell Lines		
4T1	ATCC	CRL-2539
B16	ATCC	CRL-6323
MCF-7	ATCC	HTB-22
MDA-MB-231	ATCC	HTB-26
HeLa	ATCC	CCL-2
143B	ATCC	CRL-8303
P815	ATCC	TIB-64
PaTu8902	DSMZ	ACC-179
PANC-1	ATCC	CRL-1469
Experimental Models: Organisms/Strains		
C57BL/6 mice	Jackson Laboratories	C57BL/6
Balb-c mice	Jackson Laboratories	Balb-c
C57BL/6 ^{+/a9-DsRed2} mice	Our laboratory	Dong et al., 2017
Oligonucleotides		
Forward mouse DHODH cloning primer	Sigma-Aldrich	AAA GAA TTC CAC CAT GGC GTG GAG ACA GCT G
Reverse mouse DHODH cloning primer	Sigma-Aldrich	ATA AAG GAT CCT CAC CTG CGG TGA TCT ACT C
Forward guide mouse DHODH	Sigma-Aldrich	CAC CGT CAG GTA CTC GGC GTA GAA A
Reverse guide mouse DHODH	Sigma-Aldrich	AAA CTT TCT ACG CCG AGT ACC TGA C
Forward guide mouse ATP5B	Sigma-Aldrich	CAC CGC ACT GCC ACC GGG CGA ATC G
Reverse guide mouse ATP5B	Sigma-Aldrich	AAA CCG ATT CGC CCG GTG GCA GTG C
Primer for 4T1_12S forward primer	This paper	<i>ctt ggt gat agc tgg tta</i>
Primer for 4T1_12S forward primer	This paper	<i>ggc cta caa tgg tta aaa g</i>
Probe for 4T1_12S_mut_HEX	This paper	<i>aat aGt gAa CaA tTA aCa Aaa ca</i>

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Probe for 4T1_12S_mut_HEX	This paper	<i>aat aGt gAa TaA tTA aCa Aaa ca</i>
Primer for POLG binding site, forward	This paper	TGA TCA ATT CTA GTA GTT CCC AAA A
Primer for POLG binding site, reverse	This paper	ACC TCT AAT TAA TTA TAA GGC CAG G
Primers for q(RT-)PCR	This paper	Table S2
Forward mREP	Eurogentec	ATA TGA CTA TCC CCT TCC CG
Reverse mREP	Eurogentec	AGT TTA ATG GGC CCG GAG CG
Forward mTRANS (MT1, MT6, MT11 mix)_MT1	Eurogentec	GTT AAT GTA GCT TAA TAA
Reverse mTRANS (MT1, MT6, MT11 mix)_MT1	Eurogentec	TTC CAA GCA CAC TTT CCA
Forward mTRANS (MT1, MT6, MT11 mix)_MT6	Eurogentec	CCC ATT CCA ACT TGG TCT
Reverse mTRANS (MT1, MT6, MT11 mix)_MT6	Eurogentec	AAT GAT GGC TAC AAC GAT
Forward mTRANS (MT1, MT6, MT11 mix)_MT11	Eurogentec	GTC ATC TCA TAT AAT ATT
Reverse mTRANS(MT1, MT6, MT11 mix)_MT11	Eurogentec	TCG ACA AAT GTG TGT TAC
Recombinant DNA		
Salmon sperm DNA	Sigma-Aldrich	438545-06-3
pXPR_001	Addgene	49535
pCDH-CMV-MCS-EF1-Puro	SBI System Biosciences	CD610B-1
pWPI-AOX-IRES-GFP	Gift from N. Chandel	Martinez-Royes et al., 2016
Software and Algorithms		
Image J	Fiji	http://Fiji.sc/
Photoshop	Adobe	http://www.adobe.com/uk/products/photoshop.html
Prism	GraphPad	http://graphpad.com/scientific-software/prism/
SeqManPro	DNASTAR Lasergene 11	https://www.dnastar.com/software/
CFX Manager 3.1	BioRad	http://www.bio-rad.com/en-cz/product/cfx-manager-software?ID=aed9803d-cb4d-4ecb-9263-3efc9e650edb
Genes (MultiD)	MIQE guidelines	Bustin et al., 2009
Huygens Professional version software 17.04	Scientific Volume Imaging	https://svi.nl/HuygensSoftware
Classic Maximum Likelihood Estimation algorithm		https://svi.nl/ClassicMaximumLikelihoodEstimation
R	Open source	https://www.r-project.org/
Tidyverse R packages	Rstudio	https://www.tidyverse.org/
Analyst 1.6 Software	SCIEX	https://sciex.com/products/software/analyst-software
MassHunter Software	Agilent	https://www.agilent.com/en/products/software-informatics/masshunter-suite/masshunter/masshunter-software
IMARIS 8.0	BitPlane	http://www.bitplane.com/releasenotes/imaris800.aspx
Other		
DMEM	Lonza	BE12-708F
RPMI medium	Lonza	12-115Q
Fetal calf serum	Sigma-Aldrich	F7524
Penicillin/streptomycin	Sigma-Aldrich	P3032/S9137
Uridine	Sigma	U3750
Pyruvate	Sigma	P5280
Protease Inhibitor Cocktail	Roche	39102.01
Phosphatase Inhibitor Cocktail	Sigma-Aldrich	P5726-5ML
RNAzol reagent	MRC	RB190/100

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
HOT FIREPol Blend Master Mix	Solis BioDyne	08-36-00020
DNA clean-and-concentration kit	Zymo Research	D5205
AquaPure Genomic DNA Isolation Kit	BioRad	732-6340
2xRT ² SYBR Green qPCR Mastermix	Qiagen	330500
RNAeasy Mini Kit	Qiagen	74104
Revertaid First-Strand Synthesis System	Fermentas	K1621
EvaGreen	Solis BioDyne	08-24-00001
Grandmaster for probes	TATAA Biocenter	TA02-625
RNAse A	Sigma-Aldrich	R6513
Proteinase K	Sigma-Aldrich	P4032
Proteinase K	Eurobio	GEXPRK01-B05
PCR clean-up columns	Machery Nagel	740609
Affymetrix MouseGene 2.0 ST Array	Thermo Fisher	902119
Triton X-100	Sigma-Aldrich	X100
TWEEN-20	Sigma-Aldrich	P1379
Abberior Mount Liquid Antifade	Abberior Instruments	4-0100-004-3
Lipofectamine 3000	ThermoFisher	L3000008
FastDigest Esp3I	ThermoFisher	FD0454
FastDigest EcoRI	ThermoFisher	FD0274
FastDigest BamHI	ThermoFisher	FD0055
Puromycin	Invivogen	ant-pr-1
Q5 High-Fidelity DNA Polymerase	New England Biolabs	M0491S
XBridge Amide Column	Waters	Part# 186004868
SeQuant ZIC-pHILIC Column	Merck	Cat#1504600001

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed and will be fulfilled by the Lead Contact, Jiri Neuzil (j.neuzil@griffith.edu.au).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We have used female Balb/c mice, and male and female C57BL mice. Human cell lines were authenticated.

METHOD DETAILS

Cell Culture

4T1 mouse metastatic breast cancer cells were purchased from the ATCC and maintained in the RPMI medium with 10% FCS and antibiotics. B16 mouse metastatic melanoma cells were purchased from the ATCC cells and maintained in DMEM with 10% FCS and antibiotics. Unless stated otherwise, the cells were cultivated in the presence of 1 mM pyruvate and 50 µg/mL uridine. 4T1^{p0} and B16^{p0} cells were prepared as described (Tan et al., 2015). D5, D10, D15, D20, D25, D30, D40, D50 and D60 sublines were prepared following injection of D0 (p⁰) cells subcutaneously (s.c.) into Balb/c mice at 10⁶ per animal into groups of 5-6 mice. On days 5, 10, 15, 20, 25, 30, 40, 50 and 60, mice were sacrificed, the pre-tumor lesions or tumors excised, and cells isolated from the tissue in the presence of 6TG as described (Tan et al., 2015). The isolated cells were used to establish sublines for further study. D5-D20 AOX cells were prepared identically, following grafting of 4T1^{p0} AOX (D0 AOX) cells into Balb/c mice. The same procedure was applied to obtain cells derived from ATP5B^{R10} tumors. These cells were maintained in tissue culture media supplemented with uridine (50 µg/mL) and pyruvate (1 mM). Cells were moved into the same media without uridine/pyruvate, as indicated. B16 DP cells were derived from tumors grown in transgenic C57BL^{ins4-DsRed2} mice injected with B16^{p0} cells with blue fluorescent protein nucleus as reported (Dong et al., 2017). The human breast cancer MCF7 and MDA-MB-231 cells, human cervical cancer HeLa cells, human osteosarcoma 143B cells, mouse mastocytoma P815 cells and human pancreatic cancer PANC-1 cells (all from the ATCC), as well as human pancreatic cancer PaTu8902 cells (DMSZ) were maintained in the same media as B16 cells without uridine and pyruvate.

Evaluation of Uridine and Pyruvate Auxotrophy

Cells were seeded in 6-well plates at a density of 2.5×10^5 cells per well. Cells were then incubated in control RPMI medium or medium supplemented with 1 mM pyruvate (4T1 medium) or with 1 mM pyruvate and 50 $\mu\text{g}/\text{mL}$ uridine (ρ^0 medium) for 24, 48 or 72 h at 37°C in 5% CO_2 . At various time points, duplicates of cells were washed with PBS, harvested by trypsinization, and the number of cells counted using a Casy Cell Counter (Roche).

Preparation of Cells with Knocked Out or Reconstituted Genes

To prepare DHODH^{KO} and ATP5B^{KO} cells, we used CRISPR/Cas9 system (Shalem et al., 2014). Guide RNAs sequences from the GeCKO library (v2 09Mar2015) were synthesized and cloned into BsmBI-cleaved pXPR_001 vector containing the mammalian codon-optimized Cas9 nuclease expression cassette. Parental 4T1 or B16 cells were transiently transfected using Lipofectamine 3000 (Thermo Scientific), grown for 24 h and selected by puromycin for 48–72 h, or until all non-transfected control cells were eliminated. Surviving cells were sorted into 96 well plates, expanded, and clones unable to grow in media lacking uridine and pyruvate (for DHODH^{KO} cells) or glucose (ATP5B^{KO} cells) were selected. The absence of the targeted proteins was verified by WB. The guide RNA sequences used were as follows: DHODH, 5'-TCA GGT ACT CGG CGT AGA AA-3'; ATP5B, 5'-CAC TGC CAC CGG GCG AAT CG-3'.

To reconstitute DHODH protein expression, the DHODH coding sequence was PCR-amplified from cDNA obtained from mouse B16 cells. Primers used were 5'-ATA AAG AAT TCC ACC ATG GCG TGG AGA CAG CTG-3' (forward) and 5'-ATA AAG GAT CCT CAC CTG CGG TGA TCT ACT C-3' (reverse). Gel-purified PCR product was cleaved by fast-digest EcoRI and BamHI enzymes (Thermo Scientific) and ligated into the pCDH-CMV-MCS-EF1-Puro lentiviral vector (System Biosciences) digested in the same way. Lentiviral particles were produced as described (Blecha et al., 2017) and used to transduce 4T1 or B16 DHODH^{KO} cells, followed by puromycin selection. DHODH expression was verified by WB. To express AOX in 4T1 ρ^0 cells, we used pWPI-AOX-IRES-GFP vector (a kind gift of N.S. Chandel; Martinez-Reyes et al., 2016). Lentiviral particles were produced as above and transduced cells were selected by sorting for GFP. To express AOX in B16 ρ^0 cells, we used lipofectamine transfection as described above, as lentiviral transduction was ineffective in these cells. Transfected cells were grown for 10 days, and selected by two rounds of sorting for GFP.

Mitochondrial Membrane Potential and Mitochondrial Superoxide Evaluation

To assess $\Delta\Psi_{\text{mit}}$, cells were seeded at 80,000 per mL in 96-well plates and allowed to recover for 24 h. Tetramethylrhodamine ethyl ester (TMRE) at 50 nM was added to all wells and incubated for 30 min prior to analysis by flow cytometry (FACSclibur, Becton Dickinson). Prior to analysis, control cells were also pre-treated with 1 μM of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) for 10 min.

Mitochondrial superoxide was assessed in cells seeded at 80,000 per well in a 96-well plate and incubated with 5 μM MitoSOX for 90 min at 37°C . Fluorescence was assessed at the excitation of 510 nm and emission of 580 nm in a Biotek Cytation 3 microplate reader (Biotek Instruments). Mitochondrial superoxide production was further stimulated by incubation with 1 μM antimycin A for 90 min, and MitoSOX fluorescence was measured under the same conditions.

Evaluation of ATP Level, Glucose Uptake and Lactate Formation

ATP was determined in cells seeded at 10^4 per well in 96-well plates in media containing either 4.5 g/L glucose and 50 mM or no 2DG using a luciferase-based assay (CellTiter-Glo Luminescent Assay, Promega). Results were normalised to the total protein level in cell lysates assessed by the bicinchoninic acid (BCA) method.

For glucose uptake, cells were pre-incubated in low-glucose (1 g/L) RPMI medium for 24 h at 37°C and 5% CO_2 , followed by 15 min incubation in normal DMEM (4.5 g/L glucose) in the presence of 50 μM 2-nitrobenzooxyglucose (Life Technologies); glucose uptake was then analysed by flow cytometry.

For lactate evaluation, cells were seeded at 10^5 per well in 96-well plates. Aliquots of the medium were used for the analysis of lactate using a colorimetric kit (Trinity Biotech) following 24 h incubation. Results were normalised to the total protein level in cell lysates.

Evaluation of NADH/NAD⁺ Ratio

Oxidized and reduced nicotinamide adenine dinucleotides (NAD⁺ and NADH, respectively) were assessed in cells seeded at 1.5×10^4 per well in white-walled 96-well plates using the bioluminescent NADH/NAD⁺ Glo Assay (Promega). Luminescence intensity was acquired with a Tecan Infinite M200 Microplate Reader.

Analysis of Coenzyme Q

Cells were grown in 60 mm culture dishes, washed with PBS and scraped. Pellets were collected in conical-bottom cryotubes filled with argon and stored in liquid nitrogen. During the procedure, all solutions used were bubbled with argon and all sample preparation steps were carried out under an atmosphere of argon. Vials containing the cells were centrifuged briefly (1 min) at 800 rpm and 4°C , before 100 μL the HES buffer containing protease inhibitors (10 mM Hepes pH 7.4, 250 mM sucrose, 1 mM EDTA, 100 μM DTPA and 1:50 protease inhibitors) were added. Cell lysate (90 μL) was then added to 2 mL methanol containing 0.02% acetic acid, followed by 10 mL Milli Q water-washed hexane, mixed vigorously for 30 sec and the mixture centrifuged for 5 min at $900 \times g$ and 4°C . The resulting hexane layer was removed, evaporated under vacuum using a rotary evaporator, the vacuum released under argon, and the residue re-dissolved in 140 μL argon-flushed solvent mixture (73.3/5% ethanol, 22.5% methanol, 3.75% isopropyl alcohol,

0.375% MilliQ water and 5 mM LiClO₄·3H₂O). The re-dissolved lipids (100 µL) were subjected to isocratic HPLC performed using a C18 column (Supelcosil 250 x 4.6 mm, 5 µm; Supelco, Sigma-Aldrich) with guard column eluted with 65% ethanol, 30% methanol, 3% isopropyl alcohol and 2% ammonium acetate (1 M, pH 4.4) at 1 mL/min, with mobile phase being bubbled gently with a stream of argon gas. CoQ₉/CoQ₁₀ and CoQ₉H₂/CoQ₁₀H₂ were determined by HPLC with coulometric multi-electrode electrochemical detection (CoulArray, Thermo Fisher Scientific), with the oxidation and reduction potentials set at +700 and -700 mV, respectively, and the gain range set at 100 nC. The concentrations of all analytes were determined from calibration curves generated using authentic standards of CoQ₉ and CoQ₁₀ (Sigma-Aldrich). Standards of CoQ₉H₂ and CoQ₁₀H₂ were prepared from CoQ₉ and CoQ₁₀, respectively, by reduction with sodium borohydride (Frei et al., 1990). Concentrations of CoQ₉, CoQ₁₀, CoQ₉H₂ and CoQ₁₀H₂ were determined by UV-VIS spectrophotometry (Agilent Technologies Cary 100 UV-VIS) using $\epsilon_{275\text{ nm}}$ for CoQ₉ and CoQ₁₀ of 14,700 and 14,240 M⁻¹cm⁻¹, respectively, and $\epsilon_{290\text{ nm}}$ for CoQ₉H₂ and CoQ₁₀H₂ of 4,120 and 4,010 M⁻¹cm⁻¹, respectively (Podda et al., 1999).

Evaluation of Respiration and Glycolysis

As relevant, the high-resolution Oxygraph 2k respirometer (Oroboros) and the Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies) were used. Routine, CI- and CII-dependent respiration was assessed as published (Boukalova et al., 2016; Rohlenova et al., 2017). DHODH-dependent respiration was carried out as follows. In brief, cells were trypsinized, washed with PBS, re-suspended at 2x10⁵ cells per mL of the Mir05 medium (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 110 mM sucrose, 1 g/L essentially fatty acid-free bovine serum albumin, 20 mM Hepes, pH 7.1 at 30°C) and transferred to the chamber of the Oxygraph-2k instrument (Oroboros). Respiration measurements were performed at 37°C. Cells were permeabilized with 5 µg digitonin per 10⁶ cells and a CI inhibitor (0.3 µM rotenone) was added prior to the addition of the substrate. DHODH-mediated respiration was assessed by subtracting the residual respiration rate remaining after addition of 30 µM leflunomide from the respiration rate in the presence of 1 mM dihydroorotate (DHO), 3 mM ADP, and 10 µM cytochrome c.

For evaluation of CI-, CII- and DHODH-dependent respiration in tumors, freshly excised tissue was weighed, homogenised in a PBI-Shredder (Oroboros), re-suspended in Mir05 medium and transferred to the chamber of the Oxygraph-2k instrument. The total oxygen consumption was monitored in the absence and presence of specific inhibitors and substrates of CI (rotenone or glutamate/malate/pyruvate, respectively), CII (malonate or succinate, respectively) and DHODH (leflunomide and DHO, respectively). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was titrated to obtain maximal uncoupled respiration rate. Residual oxygen consumption after antimycin A addition was subtracted from all results to obtain mitochondria-specific rates.

The Seahorse XF96 Extracellular Flux Analyzer was used for assessment of both cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as described (Deus et al., 2017). Cells were seeded in XF96 cell culture microplates at 8x10⁴ per well in standard culture media. After 24 h, the medium was replaced by serum-free RPMI containing 5 mM glucose, 1 mM pyruvate, and 50 µg/mL uridine of the Mitostress kit and without supplementation for the Glycostress kit, with pH adjusted to 7.4. Cells were incubated at 37°C for 30 min to allow the temperature and pH of the medium to reach equilibrium before measurement. The assay protocol for mitochondrial respiration consisted of consecutive injection steps in which 1 µM oligomycin, 1 µM FCCP, and the combination of 0.5 µM rotenone and 0.5 µM antimycin A were added. For glycolytic measurements, 10 mM glucose, 1 µM oligomycin, and 50 mM 2-deoxy-D-glucose (2DG) were consecutively added. OCR and ECAR measurements were made using the 3 min mix and 5 min measure cycle. Results were analyzed by the XF Stress Test Report Generators (Agilent Technologies). The results were normalized to cellular protein.

DHODH Activity Assay

DHODH activity measurements were performed using a modified protocol according to Yin et al. (2017). In brief, cells were collected, washed with PBS, re-suspended in potassium phosphate buffer (0.1 M, pH 7.0) and lysed using three freeze-thaw cycles; the lysates were incubated in the solution of 160 mM K₂CO₃·HCl (pH 8.0), 400 µM DHO, 80 µM decylubiquinone at 37°C for 60 min. The reference sample was kept on ice. The reaction mixture was supplemented with 20 mM K₂CO₃, 2 mM K₃[Fe(CN)₆] and 1 mM 4-(trifluoromethoxy)benzamidoxime (4-TFMBAO), and heated at 80°C for 4 min. The reaction was stopped by cooling on ice and fluorescence intensity was measured using the Infinite M200 plate reader (Tecan); the excitation and emission wavelengths were 320 nm and 420 nm, respectively. The calibration curve was plotted using lysate-free samples containing 0.5, 0.75 and 1 µM orotic acid.

Cell-Cycle Analysis

For cell-cycle analysis, cells grown in medium without uridine for 48 h were pre-treated with 10 µM BrdU for 30 min 37°C, harvested and fixed with 500 µL of cold 80% EtOH added drop-wise during shaking, and incubated at -20°C. EtOH was then removed and 2M HCl was added for 30 min (room temperature) to denature DNA. HCl was removed and the pellet neutralised with 0.1M disodium tetraborate (pH 8.5) for 1 min. Cells were then incubated for 1 h with anti-BrdU-FITC IgG (BD Biosciences; 0.5 µg/mL of PBS) in the dark. Finally, cells were re-suspended in 150 µL of PSB containing 2.5 µg/mL propidium iodide and analysed by flow cytometry.

To assess G2 arrest, cells were grown in medium without uridine for 48 h and then treated with nocodazole (1 µg/mL) for 16 h. Cells (5x10⁴) re-suspended in 60 µL PBS were as above. After fixation, EtOH was removed and cells were re-suspended in 150 µL of PSB containing 2.5 µg/mL of propidium iodide and analysed by flow cytometry.

Western Blotting

Cells were lysed in the RIPA buffer supplemented with the Complete Protease Inhibitor Cocktail (Roche) and the Phosphatase Inhibitor Cocktail (Sigma-Aldrich). The lysate was cleared by centrifugation and total protein determined. 50 µg of total protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were then probed with the following antibodies: anti-TFAM IgG HPA040648, anti-mtSSB IgG HPA002866, anti-ATP5B IgG HPA001520 (all Sigma), anti-POLG IgG PA5-35163, anti-cyclin E IgG MA5-14336, anti-mtCO4 IgG PA5-29992 (all Thermo Fisher Scientific), anti-UMPS IgG sc-398086 (Santa Cruz Biotechnology), anti-CAD IgG 93925, anti-p(S1859)-CAD IgG 12662 (both Cell Signaling), anti-NDUFS3 IgG ab110246, anti-NDUFA9 IgG ab14713, anti-mtCO1 IgG Ab14705, anti-SDHB IgG ab14714 (all Abcam); anti-DHODH IgG was either from Santa Cruz Biotechnology (sc-166348) or from Proteintech (14877-1-AP).

Native Blue Gel Electrophoresis (NBGE)

NBGE was accomplished basically as published (Wittig et al., 2006). In brief, mitochondria were isolated using a Balch-style homogenizer as detailed elsewhere (Schmitt et al., 2013; Blecha et al., 2017). Digitonin-solubilised mitochondria were separated on NativePAGE Novex Bis-Tris 3%-12% gradient gels. After electrophoresis, the gels were incubated in transfer buffer containing 0.1% SDS for 10 min and proteins were transferred to PVDF membranes probed with specific antibodies against complex I (NDUFA9, ab14713, Abcam; or NDUFB8, ab110242, Abcam), CII (SDHA, 14715, Abcam), CIII (Core2, ab14745, Abcam), CIV (COXVa, ab110262, Abcam) and CV (ATP5A, ab14748, Abcam; or ATP5B, HPA001520, Sigma Aldrich), and VDAC1 (ab15895, Abcam) or HSP60 (12165S, Cell Signaling) as the loading control.

DNA Sequencing

Total DNA containing mtDNA was isolated via the DNeasy reagent (MRC) according to manufacturer's instructions. Full-length mtDNA was sequenced by amplifying ten 1.8 kb dsDNA PCR products using the 5x HOT FIREPol Blend Master Mix with 10 mM MgCl₂. PCR products were separated on agarose gels to confirm the specificity of the reaction and were purified using the DNA clean-and-concentrator kit (Zymoresearch). Purified products (200 to 400 ng) were combined with water and 0.5 µL of 100 µM primer stocks to yield 10 µL sequencing reaction mixture. Classical Sanger sequencing was performed via GACT Biotech sequencing service, and the resulting sequences aligned using the SeqManPro suite from the DNASTAR Lasergene 11 software.

mtDNA Level Assay

Total DNA was extracted using the AquaPure Genomic DNA Isolation Kit (BioRad). 50 ng of DNA was diluted with water to 4.5 µL, and 0.5 µL of the combined 10 µM forward and reverse primers added (for mtDNA, mMito1 primers; for nDNA mB2M1 primers; see Table S2 for sequences of primers) and 5 µL of 2xRT² SYBR Green qPCR Mastermix (Qiagen) added. The reaction was run on the Eco qPCR System (Illumina) with the following setting: one cycle of 50°C for 2 min; one cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min.

Quantitative Real-Time (Reverse Transcription) PCR

RNA was obtained in triplicate from 4T1- and 4T1⁰- derived sublines using the RNeasy Mini Kit (Qiagen). The Revertaid First-Strand Synthesis System plus random hexamer primers (Fermentas) were used to reverse-transcribe total RNA into cDNA. Primers specific for all mitochondrial and nuclear encoded genes (see Table S2 for sequences of primers) were then assessed with 2x EvaGreen (Solis BioDyne) using the Eco qPCR System. Target genes were normalized to the housekeeping gene GAPDH, and change in gene expression was determined using the ΔΔCt method.

Single Cell qPCR

Single cell samples were obtained by automated FACS sorting (Influx, BD Biosciences). The cell suspension was filtered and nuclei stained with DAPI to identify viable cells. Individual cells were placed in 96 well plate at 1 cell per well containing 5 µL of nuclease-free water supplemented with 1 µg/µL BSA (ThermoFisher). The plate was sealed and placed on dry ice until use. Positive samples contained 100 sorted cells. Negative controls were medium only. For mtDNA analysis 80% of cell lysate was used (4 µL) in 10 µL of qPCR reagent following the manufacturer's recommendations using Grandmastermix for probes (TATAA Biocenter) supplemented with 400 nM primers, 200 nM probes (Sigma Aldrich) (for sequences see Table below). We used the CFX 96 (BioRad) thermocycler as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, 57°C for 30 s with signal acquisition. Cq values were obtained using the subtracted baseline curve fit and the common threshold method with manual setting in the CFX manager 3.1. Data were analysed with Genex (MultiD) according to the MIQE guidelines (Bustin et al., 2009).

Table. Primer and probe sequences used in sc/qPCR. Capital letters indicate LNA bases; polymorphism is shown in gray.

m_4T1_12S_Fw	cttggtgatacctgggta
m_4T1_12S_Rv	ggcctacaatgggttaaaag
m_4T1_12S_mut_HEX (parental 4T1)	aataGtgAaCaAttAaCaAaaca
m_4T1_12S_WT_FAM (host mouse)	aataGtgAaTaAttAaCaAaaca

The annealing temperature for the 12S qPCR assay was optimized using a gradient run, validation of the FACS direct lysis for mtDNA was performed using 2-fold “serial dilution” sorting of 1, 2, 4, 8 up to 1024 cells per well and showed good linearity up to 128-256 cells (data not shown). Positive controls of 100 host cells, including all single cell samples showed no or negligible quantification of parental mtDNA. No template controls (NTC) as well as 4T1^{p0} cells were negative.

mTRIP Analysis

The fluorescence *in situ* hybridization analysis mTRIP (mitochondrial Transcription and Replication Imaging Protocol) was performed and analysed as previously described (Chatre and Ricchetti, 2013). Experiments were performed in the presence of proteinase K that reveals mitochondrial nucleic acids unmasked from proteins. Briefly, the mtDNA probes were labeled by nick translation of PCR products, incorporating either Atto488-dUTP or Atto550-dUTP (Jena Bioscience), using the commercial kit Atto488/Atto550 Nick Translation Labeling kit (Jena Bioscience). Mitochondrial coordinates of mREP correspond to the non-coding region 446-544, which is labelled when initiation of mtDNA replication is activated, and mTRANS to the coordinates of three distinct probes (1905-2866, which covers part of 16S rDNA; 7400-8518, which extends from COI end to ATP8; and 13416-14836 from ND5 end until the beginning of CYTB). Cells were cultured on slides, fixed, permeabilized, and denatured with formamide. Cells were treated with 5 µg/mL of proteinase K for 5 min at 37°C, then hybridized with the mREP and mTRANS probes for 16 h at 37°C. DNA was counterstained with 10 µg/mL Hoechst 33342. Confocal acquisition was performed using a spinning-disk Perkin-Elmer Ultraview RS Nipkow Disk, an inverted laser-scanning confocal microscope Zeiss Axiovert 200M with an Apochromat 63x/1.4 oil objective and a Hamamatsu ORCA II ER camera (Imagopole, CiTech, Institut Pasteur). Images were acquired using non-saturating settings, and the same imaging parameters were used for all samples. Optical slices were taken every 200-nm interval along the z-axis covering the whole depth of the cell, at resolution of 1024/1024 pixels. All acquisitions were post-treated using the same Intensity Range (Min/Max), Maximum Intensity Projection, and background correction using the Imaris software (Bitplane), which automatically performs pixel averaging. Three-dimensional (3D)-reconstruction was achieved using the IMARIS software. Original 2D images generated from 3D volume rendering were used for fluorescence quantification using a single-imaging frame collection and ImageJ software (post-acquisition analysis). Graphic settings were applied to 2D images for the purpose of visualization (panels in figures). For each condition, one hundred 3D-reconstructed cells were analysed from three independent experiments.

Mitochondrial Chromatin Immunoprecipitation (mitoChIP) Assay

Cells were crosslinked with 1% formaldehyde, harvested, and cell pellets frozen. Pellets were then resuspended in the ChIP lysis buffer, sonicated for 12 min at cycles consisting of 15 s sonication at amplitude 5 (Qsonica Q700) and 30 s cooling in ice-water bath. An aliquot of the sheared chromatin was treated with RNase A and proteinase K, and subjected to agarose electrophoresis to confirm correct shearing. Dynabeads were pre-absorbed with salmon sperm DNA (0.2 mg/mL) and BSA (0.5%) overnight, and 20 µL of bead slurry was combined with 5 µg of sheared chromatin and 1 µL of either anti-POLG1 IgG (Abcam, ab128899), anti-TFAM IgG (Abcam ab131607) or non-specific rabbit IgG, and incubated overnight on a rotating platform. After extensive washing, chromatin was eluted and treated with RNaseA and proteinase K to reverse the crosslinking, followed by purification using PCR clean-up columns (MachereyNagel). qRT-PCR was then run using the Eva Green system (Solis Biodyne) at 95°C for 12 min, 40 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 35 s, followed by melt curve analysis using primers for mouse mtDNA encompassing D-loop; the binding site of POLG: mPOLG ChIP F, TGA TCA ATT CTA GTA GTT CCC AAA A; mPOLG ChIP R, ACC TCT AAT TAA TTA TAA GGC CAGG. Data were quantified against a non-specific IgG via the $\Delta\Delta C_t$ method.

Microarray Analysis

Transcriptomic analysis of parental, D0, D5, D10, D15, D20, D25 and D60 4T1 cells was performed using a standard protocol and the MouseGene 2.0 ST array (Affymetrix). The data are deposited under the accession number ArrayExpress: MTAB-6150 in the EBI ArrayExpress database.

Stimulated Emission Depletion (STED) Microscopy

Cells were grown on high precision coverslips overnight, fixed with 4% paraformaldehyde for 10 min at 37°C, permeabilized with 0.05% Triton X-100, 0.05% TWEEN-20 and 0.1 M glycine in PBS, blocked with 5% normal goat serum, and incubated with primary antibodies against DNA (PROGEN), TFAM (Abcam) or TOM20 (Santa Cruz Biotechnology). This was followed by appropriate secondary antibodies conjugated with Abberior STAR RED or Abberior STAR 580 (Abberior Instruments). Nuclei were labelled with Hoechst 33342 (Sigma-Aldrich). The coverslips were mounted on glass slides using Abberior Mount Liquid Antifade (Abberior Instruments). STED images were acquired on commercial Abberior STED 775 QUAD Scanning microscope (Abberior Instruments) equipped with Nikon CFI Plan Apo Lambda objective (60 x Oil, NA 1.40). Abberior STAR 580 and STAR RED labelled proteins were illuminated by pulsed 561 nm and 640 nm lasers and depleted by pulsed 775 nm STED depletion laser of 2D donut. Confocal images were acquired by illuminating the sample by cw 405 nm laser, all channels were obtained in line-interleaved acquisition mode. Fluorescence signal was filtered (emission bandpasses: 500-550 nm, 580-630 nm and 650-720 nm; pinhole 35 µm) and detected on single photon counting modules (Excelitas Technologies). Z-stacks of three planes in distance of 250 nm were acquired for each channel, images were scanned with pixel size of 25 x 25 nm and pixel dwell time 6 µs. All images were stabilized for drift, deconvolved with Huygens Professional version software 17.04 (Scientific Volume Imaging) using Classic Maximum Likelihood Estimation algorithm and processed by Fiji ImageJ software. The maximal intensity projection of 500 nm z-stack is shown.

Mitochondrial NADH Assessment

Mitochondrial NADH was assessed by two-photon microscopy using Zeiss LSM880 NLO inverted microscope equipped with Chameleon Ultra II and Compact OPO MP lasers (Coherent, Santa Clara, CA, USA) as described (Blecha et al., 2017); 3 independent experiments were performed, with more than 150 cells per condition imaged in each experiment.

Transmission Electron Microscopy (TEM)

Cells were grown on cover-slips, fixed with 2.5% glutaraldehyde (Agar Scientific) and post-fixed with 1% OsO₄ made up in Sorensen's phosphate buffer (0.1 M, pH 7.2–7.4), dehydrated, and embedded in Epon-Durkupan (Sigma-Aldrich). Ultrathin sections (~70–90 nm) were cut, contrasted with uranyl acetate (Agar Scientific), and examined in the Morgagni 268 transmission electron microscope (FEI) at 80 kV and in the TECNAI G2 20 LaB6 electron microscope (FEI) at 200 kV. Images were captured with Mega View III CCD camera (Olympus Soft Imaging Solutions) and Gatan Model 894 UltraScan 1000 camera (Gatan Incorporated).

Measurement of Dihydroorotate and Orotate

For measurement of DHO and orotate, 5x10⁵ cells were seeded in a 100 mm cell culture dish and incubated with growth medium at 37°C in 5% CO₂. After 24 h, the cells were lysed for the metabolite extraction. All the cells were washed with DPBS two times and lysed with 2:1 methanol/chloroform mixture (total of 600 µL). The cell lysates were vortexed for 30 s, and incubated on liquid nitrogen for 60 s. It was then thawed at room temperature and 1:1 chloroform/water mixture (total of 400 µL) was added. After that, the lysates were centrifuged at 15,000 x g at 4°C for 30 min. The upper water phase was collected separately and evaporated using a centrifugal evaporator. The complete dried pellet samples were stored at -80°C until mass spectrometry analysis. Liquid chromatography-mass spectrometry (LC-MS) was done as described in Flux analysis of conversion of glutamine to UMP (see below).

Flux Analysis of Conversion of Glutamine to UMP

To evaluate UMP labeled with ¹³C₅, ¹⁵N₂ (M+5), 3x10⁵ cells were seeded in 100 mm cell culture dish with growth medium and incubated at 37°C in 5% CO₂. After 24 h incubation, media were changed to 1 mM L-glutamine (¹³C₅, 99%; ¹⁵N₂, 99%; Cambridge Isotope Laboratories) containing medium, and the cells were labeled overnight. After that, the cells were lysed for the metabolite extraction. The dried pellet samples were dissolved with 1:1 acetonitrile/water mixture for mass spectrometry analysis. The Agilent 1100 Series HPLC System and XBridge Amide Column (3.5 µm, 4.6 mm x 100 mm; Waters) were used for the chromatography, with 20 mM ammonium acetate (pH was adjusted to 9 with ammonium hydroxide) and acetonitrile as mobile phases. API 2000 Mass Spectrometer with Analyst 1.6 Software (AB/SCIEX, Framingham) was used for the mass spectrometry analysis to measure DHOA, orotate and labeled UMP.

Analysis of Aspartate and Nucleotides

To measure aspartate and other nucleotides, 5x10⁵ cells were seeded in a 6-well cell culture dish and incubated with growth medium at 37°C in 5% CO₂. The cells were lysed with 5:3:2 methanol/acetonitrile/water mixture (total of 200 µL). The lysates were centrifuged at 28,000 x g at 4°C for 30 min. The supernatant was collected separately and evaporated using a centrifugal evaporator. The Agilent 1290 Infinity LC System and SeQuant ZIC-pHILIC Column (5 µm, 150 mm x 2.1 mm; Merck KGaA, 64271 Darmstadt, Germany) were used for the chromatography, with 20 mM ammonium acetate (pH was adjusted to 9 with ammonium hydroxide) and acetonitrile as mobile phases. Agilent Technologies 6460 Triple Quad LC/MS with MassHunter Software (Agilent, United States) was used for the mass spectrometry analysis.

In Vivo Metabolic Studies

Balb/c mice with 4T1 and 4T1p⁰ AOX cell-derived tumors (volume of about 150 mm³) were infused with ¹³C₅, ¹⁵N₂-Gln (Sigma-Aldrich) according to a published protocol (Mashimo et al., 2014). Briefly, mice were anaesthetised with isoflurane and their jugular vein cannulated. The animals were then infused with labelled Gln at 0.28 mg/g body weight (in 0.3 mL saline) over 1 min and with Gln at 0.005 mg/g body weight (in 600 µL saline) at 200 µL/h for 180 min. After infusion, the mice were sacrificed and tumors excised. Tumor tissue was homogenised and assessed for labelled UMP as described above.

Mutation Rates of Genes in the De Novo Pyrimidine Biosynthesis Pathway

We obtained all somatic mutations from The Cancer Genome Atlas (TCGA), which contains 11,330 patients of 33 cancer types. Mutation maf files were downloaded from Firehose (<http://gdac.broadinstitute.org/>). The average gene expression levels (microarray RMA, log2ratio) for all genes in cancer cells were derived from 91 CCLE (Cancer Cell Line Encyclopedia) cell lines. Cancer genes were retrieved from CGC (cancer gene census, <http://cancer.sanger.ac.uk/census> COSMIC v81). To facilitate calculation of mutation rates, we excluded those cancer genes that are affected by translocation, amplifications or large deletions. This led to 262 cancer genes in which 67 are annotated as oncogenes, 100 as tumor suppressors. We calculated the raw mutation rate of a gene according to the number of mutations per base pair in the coding sequence (CDS) and normalised by sample size. For comparison, we used the mutation rates of 15,067 non-cancer genes as a background after excluding 1,090 genes lacking expression information and 2,440 putative cancer genes from CGC; uniprot oncogenes/Proto-oncogenes/Tumor suppressor genes, NCG (Network of Cancer Genes) and DISEASE. They were firstly divided into 20 bins according to gene expression levels. An error bar was then calculated for each bin, marking 75 quantile, 25 quantile and median mutation rates, respectively. We then fitted a baseline for mutation rates of

non-cancer genes using the generalised additive model (GAM). Because different genes have different background (random) mutation rates, we utilised MutsigCV, which employs a sophisticated model for estimating gene-specific and patient-specific background mutation rate and calculating significantly mutated genes (Q-score, the smaller Q-score, the higher significance). For MutsigCV calculation, the combined pan-cancer mutation maf file was used as input and all other input files and default options were kept. Data analysis and visualisation were conducted in R using packages dplyr and ggplot2.

Evaluation of Cell Proliferation in Tumor Tissue

Immunohistochemistry was performed using paraffin-embedded tissue section (7 μ m). Sections were dehydrated, heated in citrate buffer (70 mM, pH 6.0) for antigen retrieval, and blocked with PBS containing 10% normal goat serum (Jackson Immuno Research Labs) and 0.1% Tween 20 (Sigma). Anti-Ki67 IgG was purchased from Cell Signaling, the secondary antibody coupled to AlexaFluor 594 from Jackson Immuno Research Labs. To detect cell nuclei, sections were mounted on histology mounting medium Fluoroshield with DAPI (Sigma). The sections were imaged with fluorescence microscope Nikon Eclipse E400.

Evaluation of Pyruvate

The levels of pyruvate in tumor and liver tissue were measured using a colorimetric assay (Abcam) according to the manufacturer's instructions. In brief, serum from mice with and without tumors, and from cancer patients and healthy subjects, as well as mouse tumor and liver tissue (10 mg) was collected, washed with cold PBS, and lysed in the pyruvate assay buffer; the supernatant was collected by centrifugation at 20,000 $\times$ g for 5 min at 4°C. The reaction assay mixture (50 μ L), prepared according to the manufacturer's instructions, was then added to each well containing 50 μ L of the sample. The colorimetric signal was evaluated at 570 nm using the Tecan Infinite M200 plate reader. The following cancer patient serum was used in the analysis: colorectal cancer (7 patients), pancreatic cancer (9 patients), gastric cancer (1 patient), B-cell lymphoma (1 patient).

Formation of Syngeneic Tumors

4T1, 4T1^{p0} (D0), D5, D10, D15 and D20 cells, D0 AOX, D5 AOX, D10 AOX, D15 AOX and D20 AOX cells, DHODH^{KO/Δ2} and DHODH^{res} cells, and p⁰ AOX and ATP5B^{KO/Δ7} cells were injected subcutaneously (s.c.) into the right flank of female Balb/c mice at 10⁶ cells per animal. To examine the effect of uridine on tumor formation, 10 Balb/c mice were grafted with 10⁶ 4T1^{p0} cells. Two days later, uridine (1 g/kg) was administered by intra-peritoneal injection to 5 mice 3 times per week (Peters et al., 1988), with the remaining 5 mice kept as control animals. To examine the effect of Met, Lef and SHAM on growth of tumors derived from 4T1^{p0} AOX cells, the respective agent was administered by intra-peritoneal injection twice a week for 28 days as soon as USI-detectable tumors appeared in the mice. The following dosing was used: SHAM, 200 mg/kg in 5% DMSO in corn oil; leflunomide, 20 mg/kg in corn oil containing 4% ethanol; metformin, 125 mg/kg in PBS. Tumors were visualized and their volume precisely calculated using the USI device Vevo770 or Vevo3100 (Fuji Film). The experiments were terminated when tumor volume reached about 1,000 mm³. Animal studies were performed according to the guidelines of the Australian and New Zealand Council for the Care and Use of Animals in Research and Teaching, as well as the Czech Animal Ethics Committee, and were approved by the respective local Animal Ethics Committee.

Statistical Analysis

Unless stated otherwise, data are mean values $\pm$ S.D. of at least three independent experiments. In mouse experiments, groups of 6 animals were used, unless stated otherwise. The two-tailed unpaired Student's t test was used to assess statistical significance with p<0.05 being regarded as significant, using GraphPad Prism software. Anova was used for multiple comparisons where applicable. Images are representative of at least three independent experiments.

DATA AND SOFTWARE AVAILABILITY

The accession number for the microarray data reported in this paper is ArrayExpress: MTAB-6150.

4. Replication and ribosomal stress induced by targeting pyrimidine synthesis and cellular checkpoints suppress p53-deficient tumors

We have recently demonstrated the crucial importance of DHODH-dependent *de novo* pyrimidine synthesis for the ability of cancer cells to proliferate and form tumors (Bajzikova et al., 2019). mtDNA-depleted cells did not form tumors unless they acquire mtDNA by transferring whole mitochondria from the tumor microenvironment. DHODH reactivation upon this mtDNA acquisition and mitochondrial respiration recovery was crucial for tumor growth. Recent data further show that inhibition of DHODH leads to activation of the tumor suppressor p53 (Khutornenko et al., 2014; Ladds et al., 2018b).

We used the same model as in previous papers, i.e. 4T1 sublines derived on various days after grafting of mtDNA-depleted 4T1p0 cells in Balb/c mice. We can cluster separately sublines D0-D10 and D15-D25, before and after restoring mitochondrial functions, respectively. This clustering was also observed in the restoration of DHODH activity as well as in increased levels of transcripts regulating cell cycle arrest in D0–D10 sublines and this pattern changed after mtDNA acquisition. Since replenishment of the dNTP pool could also impair the biogenesis of ribosomes, thereby causing replication stress, observed increased RPL levels promoted p53 stabilization via interfering with ubiquitin ligase MDM2. p53 is often suppressed in cancer, using DHODH inhibited p53-deficient cancer cells revealed that the block in cell cycle progression upon DHODH inhibition can be p53-independent. When we treated p53-deficient cancer cells with a Chk1 inhibitor and found a stronger effect compared to wild-type p53 cancer cells. Next, we compared the effect of DHODH inhibition or its combination with Chk1 inhibition *in vivo*. The combined DHODH/Chk1 inhibition was more efficient in p53-deficient tumors compared to DHODH inhibition alone, with no additional benefit in wild-type p53 tumors.

Taken together, there are two pathways contributing to cell cycle arrest, RPL/MDM2-modulated p53 stabilization and Chk1 activation. Since around 50% of human cancers possess a mutated form of p53, combined inhibition appears as a promising therapeutic strategy.

Contribution to the publication: Experimental part (cell culture, mice experiments), revision of the manuscript.

ARTICLE

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Replication and ribosomal stress induced by targeting pyrimidine synthesis and cellular checkpoints suppress p53-deficient tumors

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Abstract

p53-mutated tumors often exhibit increased resistance to standard chemotherapy and enhanced metastatic potential. Here we demonstrate that inhibition of dihydroorotate dehydrogenase (DHODH), a key enzyme of the de novo pyrimidine synthesis pathway, effectively decreases proliferation of cancer cells via induction of replication and ribosomal stress in a p53- and checkpoint kinase 1 (Chk1)-dependent manner. Mechanistically, a block in replication and ribosomal biogenesis result in p53 activation paralleled by accumulation of replication forks that activate the ataxia telangiectasia and Rad3-related kinase/Chk1 pathway, both of which lead to cell cycle arrest. Since in the absence of functional p53 the cell cycle arrest fully depends on Chk1, combined DHODH/Chk1 inhibition in p53-dysfunctional cancer cells induces aberrant cell cycle re-entry and erroneous mitosis, resulting in massive cell death. Combined DHODH/Chk1 inhibition effectively suppresses p53-mutated tumors and their metastasis, and therefore presents a promising therapeutic strategy for p53-mutated cancers.

Introduction

Mitochondria are essential organelles with fundamental role in cellular metabolism and energy homeostasis. Besides being a major source of ATP, mitochondria also regulate cell death and differentiation, as well as cell cycle progression¹. The mitochondrial oxidative phosphorylation system (OXPHOS) comprises respiratory complexes I–IV (CI–CIV) that are coupled to ATP generation catalyzed by complex V (CV). OXPHOS also involves dihydroorotate dehydrogenase (DHODH), a flavoprotein localized in the inner mitochondrial membrane. DHODH converts dihydroorotate (DHO) to orotate within the de novo pyrimidine synthesis pathway, generating electrons

that are transferred, via redox-cycling of ubiquinone, to CIII². Since DHODH is functionally linked to CIII activity, impairment of CIII function results in reduced activity of DHODH³. Hence, DHODH is an important link between de novo pyrimidine biosynthesis and mitochondrial respiration.

DHODH catalyzes conversion of DHO to orotate, a precursor of uridine monophosphate (UMP)⁴ that can yield all other pyrimidines. Since constant replenishment of the pyrimidine pool from glutamine via UMP is required for replication of nuclear DNA and cell growth, dysfunction of DHODH may result in genomic instability⁵, replication stress⁶, and cell cycle arrest⁷. This is particularly relevant for rapidly proliferating cancer cells that are not able to efficiently replenish their pyrimidine pool by salvage pathways and are therefore ‘addicted’ to DHODH-dependent synthesis of pyrimidines. Indeed, several DHODH inhibitors have been shown to suppress proliferation and to induce cell death in various types of tumors^{6,8,9}.

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While the tumor suppressor protein p53 plays a key role in DNA damage response, it is also a sensor of aberrant ribosomal biogenesis¹⁰. Its stability is controlled by the E3 ubiquitin ligase MDM2, which ubiquitinates p53 to mark it for degradation. Under stress conditions, a number of ribosomal proteins, like RPL5 and RPL11, act as a p53 stabilizer^{11,12}. Once stabilized, p53 modulates transcription of multiple target genes and ensures appropriate checkpoint maintenance, resulting in cell cycle arrest or apoptosis¹³. Since p53 serves as a tumor suppressor, the loss of wild-type (wt) p53 is beneficial for cancer cells. Notwithstanding the notion that more than 50% of human cancers lack functional p53, they are able to adapt to various types of stress¹⁴. Therefore, targeting p53-independent stress response pathways within p53-mutated background may be beneficial for cancer therapy.

Cell cycle checkpoints responding to DNA damage or incomplete DNA replication elicit cellular responses by inhibiting cyclin-dependent kinases that drive cell cycle progression, with checkpoint kinase 1 (Chk1) being a major checkpoint kinase in mammals¹⁵. Chk1 can be activated by ataxia telangiectasia and Rad3-related kinase (ATR) in response to DNA damage¹⁶ and regulates S phase entry and G2/M progression. As a consequence, cells are arrested at the checkpoints until damaged DNA is repaired¹⁷. Therefore, disruption of checkpoint signaling leads to re-entry of damaged cells into the cell cycle, resulting in cell death due to erroneous mitosis¹⁸. Besides its role in the maintenance of DNA integrity, Chk1 is a major regulator of cell cycle arrest after replication stress, independent of DNA damage¹⁹.

Here we demonstrate that Chk1 is activated in tumor cells as a consequence of replication/ribosomal stress after pyrimidine synthesis inhibition. Simultaneous inhibition of Chk1 and DHODH results in efficient elimination of p53-dysfunctional tumor cells *in vitro* as well as in suppression of tumor growth and metastatic dissemination *in vivo*. Such combination therefore represents a promising treatment strategy for patients with p53-dysfunctional cancer poorly responding to standard chemotherapy.

Results

Pyrimidine synthesis inhibition induces replication stress and cell cycle arrest

Recently we showed that reactivation of DHODH-linked pyrimidine biosynthesis is essential for tumor growth from mtDNA-depleted cancer cells. Restoration of DHODH activity, induced by respiration recovery following a horizontal transfer of mitochondria from the stroma, reinitiates pyrimidine synthesis, leading to the release of cell cycle arrest and to tumor proliferation²⁰. To understand the molecular basis of cell cycle reactivation

and its possible therapeutic potential, we performed transcriptome analysis of 4T1 sublines derived on various days after grafting of mtDNA-depleted 4T1p⁰ cells (referred to as day 0, D0 cells) in Balb/c mice from pre-tumor lesions (for detailed description see ref. ²⁰). D5 and D10 cells were found to contain low levels of mtDNA derived from the host, reflected by compromised respiration. However, mitochondrial function as well as mtDNA levels was normalized in D15–D25 cells. Transcriptome analysis of cell cycle regulators revealed that D0–D10 cells, which are auxotrophic for uridine due to the lack of DHODH-dependent pyrimidine biosynthesis, clustered separately from D15 to D25 cells featuring fully restored DHODH-dependent *de novo* pyrimidine synthesis²⁰ (Fig. 1a). Further analysis revealed an increased level of transcripts regulating cell cycle arrest (ATM, ATR, Chk1 or Cdkn1a) in D0–D10 time points as well as a decrease of transcripts linked to mitosis progression (Fig. 1b, Supplementary Tables 1 and 2). In addition, we observed a switch in this pattern when cells that are no longer auxotrophic for uridine (D15–D25 cells) were compared to D0–D10 cells (Fig. 1c, Supplementary Tables 1 and 2).

To understand the molecular mechanism of cell cycle arrest, we treated MCF7 and HCT116 cells with a DHODH inhibitor leflunomide for 72 h (Supplementary Fig. 1B, C), where we detected cell cycle arrest as well as increased β -galactosidase activity and a switch to senescence (Supplementary Fig. 1A). We observed decreased accumulation of cells in the G2/M phase in leflunomide-treated cells following synchronization with nocodazole (Fig. 1d), activation of the checkpoint kinases Chk1 and Chk2 by their phosphorylation, activation of the p53/p21 signaling pathway, decreased histone H3 phosphorylation on serine 10 (Fig. 1e), and increased β -galactosidase activity (Supplementary Fig. 1D). We observed similar results after silencing of DHODH using siRNA (Supplementary Fig. 1E–I). Overall, this points to a decrease in cell proliferation after DHODH inhibition. Supplementation of leflunomide-treated cells with uridine completely restored the parental phenotype, pointing to a link between suppression of pyrimidine synthesis and cell cycle arrest. Further, accumulation of cyclin E in leflunomide-treated cells indicates their arrest in early S phase (Fig. 1f), which is also supported by pulse BrdU incorporation in leflunomide-treated or DHODH-silenced cells (Fig. 1g, Supplementary Fig. 1F–H).

Checkpoint activation is critical for the induction of DNA damage response. However, we did not detect any DNA damage after DHODH inhibition, as assessed using the DNA damage markers 53BP1 and γ H2AX (Fig. 1h, i, Supplementary Fig. 1J, K). The activation of checkpoints may therefore result from perturbation of the replication machinery, for example, as a consequence of pyrimidine

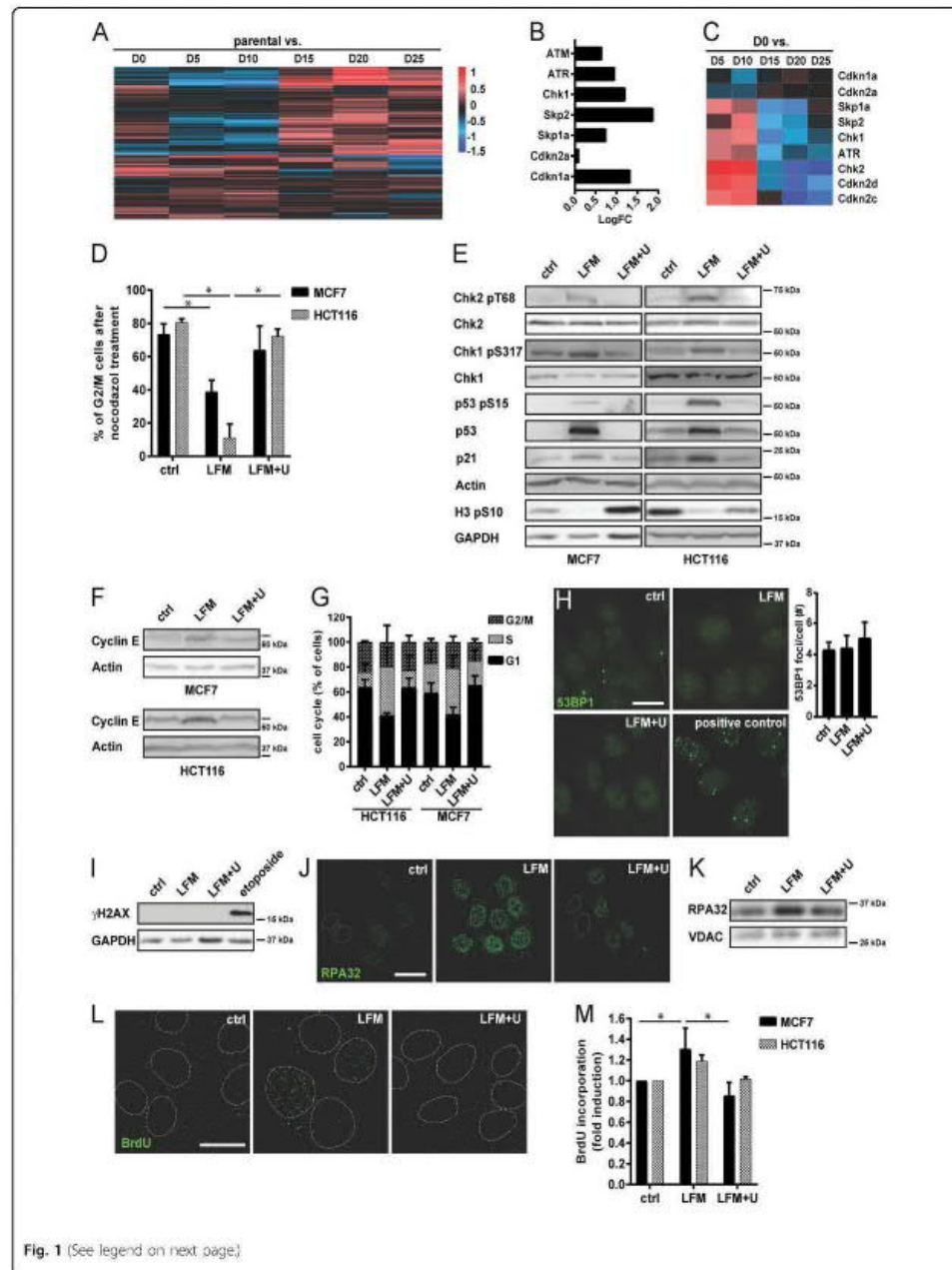


Fig. 1 (See legend on next page.)

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Fig. 1 DHODH inhibition induces replication stress and cell cycle arrest. **a–c** Parental 4T1 cells and D0–D25 sublines were subjected to microchip analysis and heat map for the 194 transcripts involved in cell cycle and p53 signaling pathway was constructed. **d–m** MCF7 and HCT116 cells were exposed to leflunomide (LFM; 50 μ M) for 72 h in the presence or absence of uridine (U; 50 μ g/l). **d** Cells were incubated with nocodazole (10 μ M, o/n) and the G2/M fraction was detected by flow cytometry using propidium iodide (2.5 μ g/ml). **e** Immunoblotting detection of Chk2 pT68, Chk2, Chk1 p5317, Chk1, p53 p515, p53, p21, and H3 p510 and **f** cyclin E protein level was determined. β -Actin and GAPDH were used as a loading control. **g** MCF7 and HCT116 cells were assessed for cell cycle distribution. **h** Immunofluorescent detection of 53BP1 in MCF7 cells was performed. MCF7 cells treated with Etoposide (10 μ M) for 24 h were used as a positive control. Scale bar, 10 μ m. **i** Immunoblotting detection of γ H2AX signal was performed in MCF7 cells. GAPDH was used as a loading control. **j** Accumulation of the RPA32 protein on DNA in MCF7 cells was evaluated by immunofluorescence staining and **k** by immunoblot analysis. VDAC was used as a loading control. **l** Accumulation of BrdU staining on non-denatured ssDNA in MCF7 cells was evaluated by immunofluorescence staining and **m** by FACS. In **d**, **g** and **m**, data are shown as mean $\pm$ SEM, $n=3$. * $P<0.05$, two-way ANOVA. In other panels, representative experiment (from a total number of three experiments) is shown.

depletion²¹. Using a pre-extraction protocol to remove unbound proteins, we observed increased association of single-strand DNA (ssDNA)-binding replication protein A (RPA²²) with DNA in leflunomide-treated cells, which was reduced upon uridine supplementation (Fig. 1j, k, Supplementary Fig. 1L). Quantification of ssDNA in leflunomide-treated cells, detected by incorporation of BrdU under non-denatured conditions, confirmed induction of replication stress following DHODH inhibition (Fig. 1l, m).

Interestingly, we recapitulated the same findings by silencing uridine monophosphate synthase (UMPS; Supplementary Fig. 2A), an enzyme catalysing formation of UMP from orotate, confirming the involvement of pyrimidine depletion and excluding non-specific effects of DHODH dysfunction in the OXPHOS system.

Pyrimidine synthesis inhibition stabilizes the p53 tumor suppressor protein

Persistent DNA replication stress²³ and, importantly, depletion of the pyrimidine nucleotide pool, especially UTP, resulting from DHODH inhibition, could impair biogenesis of ribosomes, which activates the p53 pathway leading to cell cycle arrest¹⁰. Similarly as for the cell cycle, we detected decreased levels of ribosomal transcripts in uridine-dependent D0–D10 cells and their normal levels in proliferating D15–D25 cells (Fig. 2a, Supplementary Table 3). We examined ribosomal synthesis by measuring 45S rRNA, a precursor rRNA which is processed into mature 28S, 18S, and 5.8S rRNAs that form ribosomal subunits. A decreased level of 45S rRNA and 18S rRNA was observed in MCF7 and HCT116 cells after their treatment with leflunomide (Fig. 2b, c) or after UMPS silencing (Supplementary Fig. 2B). Consistent with this, we found reduced mRNA and protein level for RPS6, a crucial ribosomal component responsible for docking of mRNAs onto the ribosome (Fig. 2d, Supplementary Fig. 2C; see also Supplementary Table 3 for the RPS6 transcript). To demonstrate the importance of functional ribosomes for cell survival, we silenced RPS6 in MCF7 cells, which resulted in increased

cell death (Supplementary Fig. 2D, E) and p53 stabilization (Supplementary Fig. 2F). Moreover, increased nucleolar fusion connected with ribosomal stress (Fig. 2e for MCF7, Supplementary Fig. 2G for HCT116) confirmed defects in ribosomal biogenesis in leflunomide-treated cells²⁴. Similarly, as for the cell cycle arrest, uridine supplementation completely reverted the phenotype. Together, these results point to the induction of ribosomal stress after inhibition of DHODH.

Perturbation of ribosome biogenesis can sometimes lead to disruption of nucleoli²⁵, followed by translocation of ribosomal proteins such as RPL5 and RPL11 from the nucleolus to the nucleoplasm, where they bind to MDM2 and inhibit its ubiquitin ligase activity required for p53 degradation²⁶. To investigate the role of RPL5 and RPL11 in p53 stabilization after DHODH inhibition, we silenced both proteins either alone or in combination. This resulted in reduced leflunomide-induced p53 stabilization and decreased induction of its downstream target p21 in the silenced cells (Fig. 2f for MCF7, Supplementary Fig. 2H for HCT116; for siRNA efficacy see Supplementary Fig. 2I).

MDM2 is normally degraded by the proteasome together with the p53 protein. To demonstrate this process, blocking of MDM2 binding to p53 by Nutlin3, an MDM2 inhibitor, resulted in stabilization of both proteins and cell cycle arrest in our experimental system (Supplementary Fig. 2J–L). Since MDM2 was increased in leflunomide-treated cells (Fig. 2g), we tested its ability to bind p53. Using immunoprecipitation, we detected decreased ubiquitination of p53 and MDM2 after leflunomide treatment rather than changes in the MDM2–p53 interaction (Fig. 2h, i). Moreover, we confirmed binding of the RPL5 protein to MDM2 after inhibition of DHODH (Fig. 2j). Silencing of RPL5 and RPL11 then restored ubiquitination of p53 (Fig. 2k, l) and its subsequent degradation (Fig. 2f, Supplementary Fig. 2H), documenting that these RPL proteins released by ribosomal stress induced by DHODH inhibition interfere with MDM2-mediated ubiquitination of p53 and promote its stabilization.

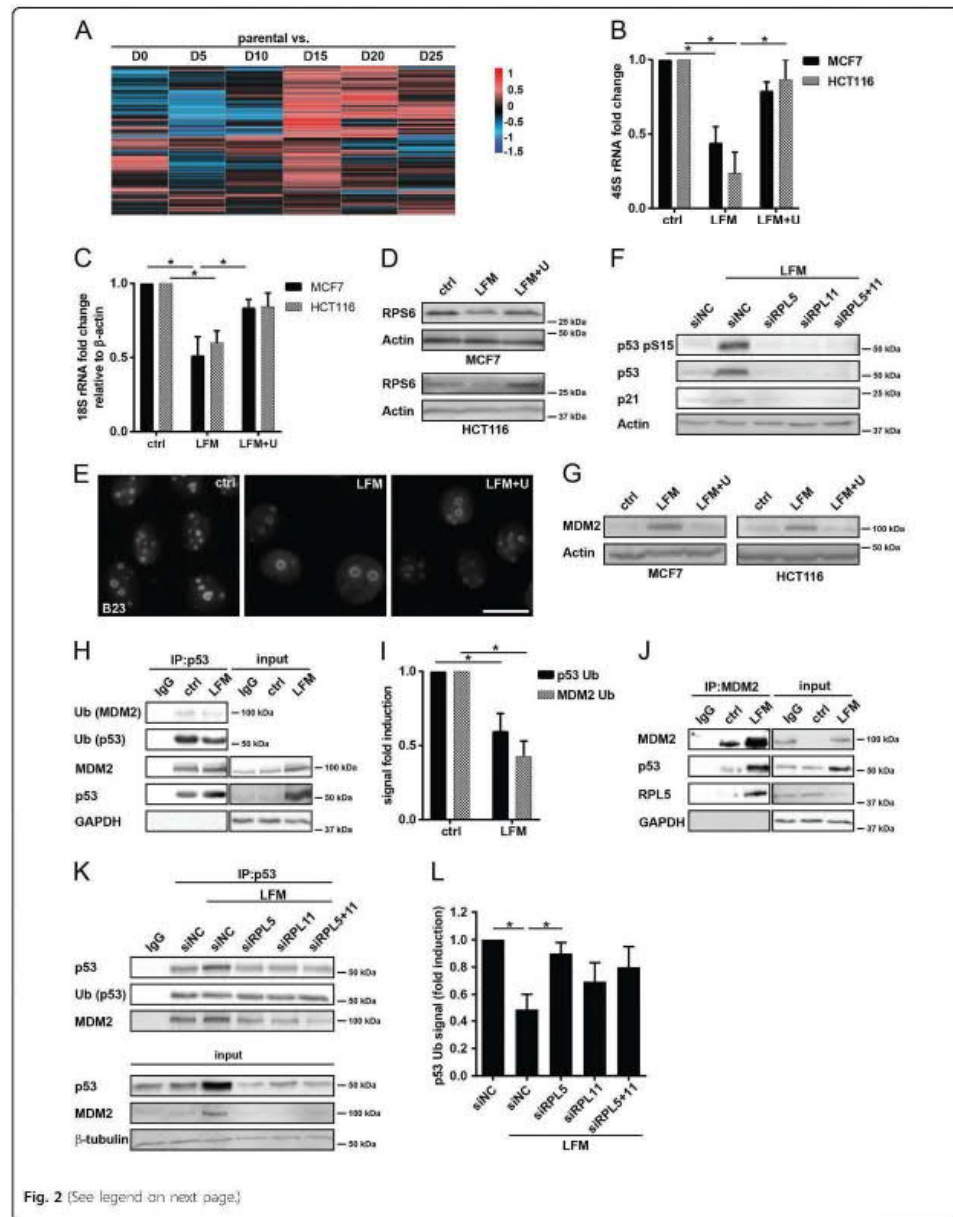


Fig. 2 (See legend on next page.)

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Fig. 2 inhibition of DHODH stabilizes the p53 tumor suppressor protein. **a** Parental 4T1 cells and D0–D25 sublines were subjected to microchip analysis and a heat map for the 119 transcripts involved in ribosome biogenesis was constructed. **b–e** MCF7 and HCT116 cells were exposed to leflunomide (LFM, 50 μ M) for 72 h in the presence or absence of uridine (U; 50 μ g/l). Expression of 45S rRNA (**b**) and 18S rRNA (**c**) was assessed by qRT-PCR. **d** Immunoblotting detection of RPS6 was performed. β -Actin was used as a loading control. **e** Immunofluorescent detection of B23 in MCF7 cells was performed. **f** Immunoblotting detection of p53 pS15, p53, and p21 in MCF7 cells treated 72 h with LFM (50 μ M) after downregulation of RPL5 and RPL11 alone or in combination using specific siRNA. Non-targeting siRNA (siNC) was used as a control. β -Actin was used as a loading control. **g** Immunoblot detection of MDM2 in MCF7 and HCT116 cells. β -Actin was used as a loading control. **h** Co-immunoprecipitation of p53 followed by immunoblot analysis of p53, MDM2, ubiquitinated p53, and ubiquitinated MDM2 in MCF7 control and LFM (50 μ M, 72 h)-treated cells. GAPDH was used as an input control. Immunoprecipitated samples and input represent one membrane with different intensity of signal. **i** Levels of ubiquitinated p53 and MDM2 related to their immunoprecipitated total forms were evaluated from three independent experiments. **j** Co-immunoprecipitation of MDM2 followed by immunoblot analysis of MDM2, p53, and RPL5 protein in MCF7 control and LFM (50 μ M, 72 h)-treated cells. GAPDH was used as an input control. Immunoprecipitated samples and input represent one membrane with different intensity of signal. **k** Co-immunoprecipitation of p53 followed by immunoblot analysis of total and ubiquitinated p53 and MDM2 in MCF7 cells treated with LFM (50 μ M, 72 h) after downregulation of RPL5 and RPL11 alone or in combination using specific siRNA. Non-targeting siRNA (siNC) was used as a control. p53, MDM2, and β -tubulin were used as an input control. **l** The level of ubiquitinated p53 related to its immunoprecipitated total form was evaluated from three independent experiments. In **b**, **c**, **i**, and **l**, data are shown as mean $\pm$ SEM, $n = 3$. * $P < 0.05$, two-way ANOVA. In other panels, representative experiment (from total number of three experiments) is shown.

p53 deficiency sensitizes to cell death upon leflunomide treatment

Aberrant execution of the cell cycle in the absence of effective checkpoints can lead to cell death. Searching for the role of RPL in cell death, we observed that RPL silencing increased cell death upon leflunomide treatment (Fig. 3a, b, Supplementary Fig. 3C–E; see Supplementary Fig. 3A, B for siRNA efficacy). To provide direct evidence that p53, stabilized by RPL proteins, promotes cellular viability in leflunomide-treated cells, we knocked down p53 in MCF7 cells and used these (Fig. 3c, Supplementary Fig. 3F, G) as well as HCT116 p53^{KO} cells (Fig. 3d, Supplementary Fig. 3H) to show that in both cases, p53 deficiency promotes cell death upon leflunomide treatment. We conclude that the loss of functional p53 synergizes with DHODH inhibition in cell death induction, indicating an important role of p53 in regulating cellular decision between cell cycle arrest and cell death after impairment of de novo pyrimidine synthesis.

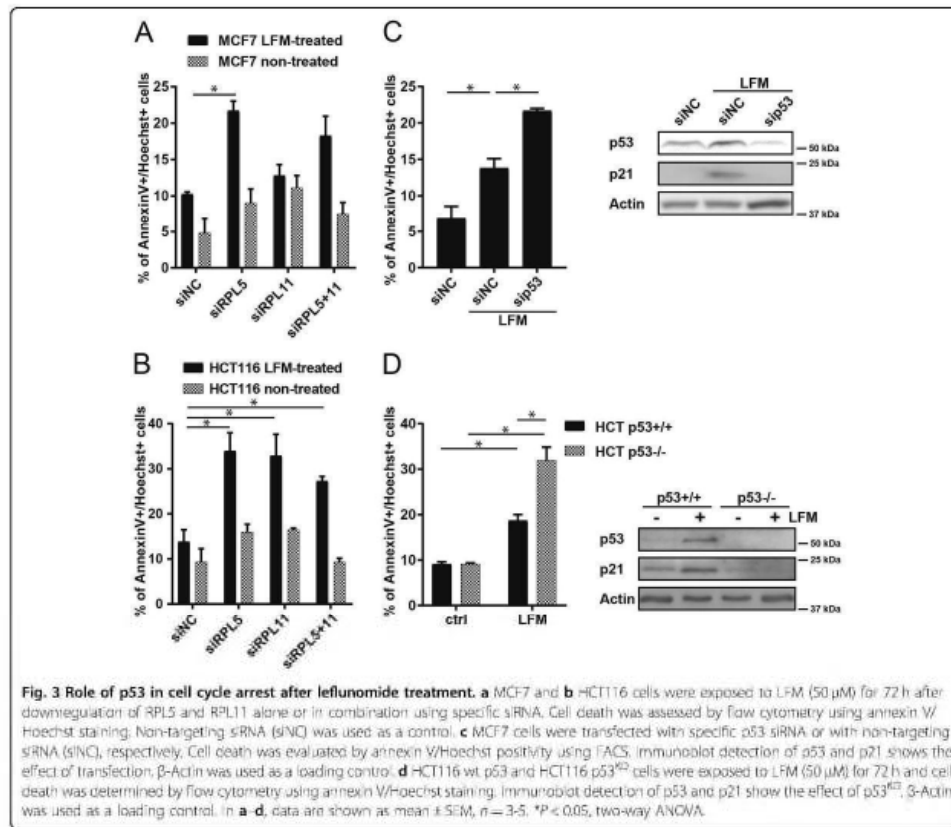
Pyrimidine synthesis inhibition induces cell cycle arrest independently of p53

Using p53-mutant MDA-MB-231 breast cancer cells treated with leflunomide and p53-deficient 4T1 breast cancer cells with ablated DHODH (DHODH^{KO} cells; see Supplementary Fig. 4A, B for DHODH activity), we observed a similar effect of pyrimidine depletion on the cell cycle as found for wt p53 cells (Fig. 4a; see Fig. 1a for wt p53 cells) with arrest in early S-phase (Fig. 4b, c, Supplementary Fig. 4C), increased β -galactosidase activity (Supplementary Fig. 4D), and checkpoint activation (Fig. 4d). Similarly, we detected accumulation of the RPA protein (Fig. 4e, f) and BrdU (Fig. 4g) on ssDNA as a result of impaired replication, as well as perturbation of ribosomal biogenesis and ribosomal stress (Supplementary Fig. 4G–I) independent of DNA damage, which was not

detected (Supplementary Fig. 4E, F). Uridine supplementation or re-expression of DHODH in DHODH^{KO} 4T1 cells restored the parental phenotype. These results reveal that while p53 stabilization induces cell cycle arrest, the block in cell cycle progression upon DHODH inhibition can be p53-independent, pointing to an additional mechanism of cell cycle inhibition.

Inhibition of Chk1 promotes cell death in DHODH-inhibited p53-deficient tumor cells

Chk1 activation after DHODH inhibition (Figs. 1e and 4d) as well as the ability of p53-deficient or p53-mutant cells to induce cell cycle arrest indicates its potential role in the response to pyrimidine depletion. Using MCF7 and MDA-MB-231 cells, we observed increased cell death upon Chk1 inhibition by LY2603618 in leflunomide-treated cells (Fig. 5a, Supplementary Fig. 5B; for Chk1 inhibition efficacy see Supplementary Fig. 5A). Surprisingly, this effect was much stronger in p53-mutant MDA-MB-231 cells compared to p53-wt MCF7 cells. To investigate whether p53 deficiency sensitizes cells to Chk1 inhibition, we silenced p53 in MCF7 cells (Supplementary Fig. 5C), which resulted in elevated cell death upon Chk1 inhibition in response to leflunomide treatment (Fig. 5b, Supplementary Fig. 5D). To verify these findings in an independent model, we examined HCT116 p53^{KO} cells (Supplementary Fig. 5E, F). Furthermore, we compared mouse p53-deficient 4T1 and NeuTL cells with wt p53 B16 mouse cells (Supplementary Fig. 5G, H). Using these additional models, we also observed a strong correlation between p53 deficiency and sensitization to combined Chk1/DHODH inhibition. Finally, to control for possible off-target effects of leflunomide and to demonstrate directly that DHODH deficiency sensitizes to Chk1 inhibition, DHODH^{KO} 4T1 cells were tested. Similarly to leflunomide-treated cells, we detected increased cell death



in DHODH^{KO} cells exposed to the Chk1 inhibitor (Fig. 5c, Supplementary Fig. 5I), which was reverted when DHODH activity was restored by DHODH reconstitution (Supplementary Fig. 4A).

Activation of checkpoints prevents damaged or stressed cells to progress through the cell cycle. Inhibition of these checkpoints could therefore induce a mitotic catastrophe arising as a result of aberrant re-entry of tumor cells into the cell cycle²⁷. While phosphorylation of histone H3 at serine 10 (H3 pS10), an epigenetic mitotic marker²⁸, was found downregulated in tumor cells treated with leflunomide independent of the p53 status (Fig. 5d), its increased levels were observed in p53-deficient cells (4T1 cells and p53^{KO} HCT116 cells) or p53-mutant MDA-MB-231 cells after Chk1 inhibition (Fig. 5d), indicating re-entry of these cells into the cell cycle. Also, while nuclei of wt p53 cells (MCF7 and B16 cells) remained unchanged, we

observed robust nuclear fragmentation in p53-mutant or p53-deficient cells (MDA-MB-231 and 4T1 cells, respectively) after combined treatment with leflunomide and the Chk1 inhibitor (Fig. 5e, f) as a consequence of cell cycle progression without replicated DNA.

We therefore demonstrate here that depressed pyrimidine synthesis caused by inhibition of DHODH activates, via p53 and Chk1, two alternative checkpoint pathways that contribute to cell cycle arrest. This explains sensitivity of p53-deficient cells to Chk1 inhibition resulting in aberrant mitosis and cell death.

Simultaneous Chk1 and DHODH inhibition sensitizes p53-deficient tumors to cell death and blocks metastases

To establish the effect of combined DHODH/Chk1 inhibition on tumor growth in vivo, FVB/N *c-neu* mice were injected with murine breast cancer NeuTL p53-deficient cells and administered with leflunomide and the

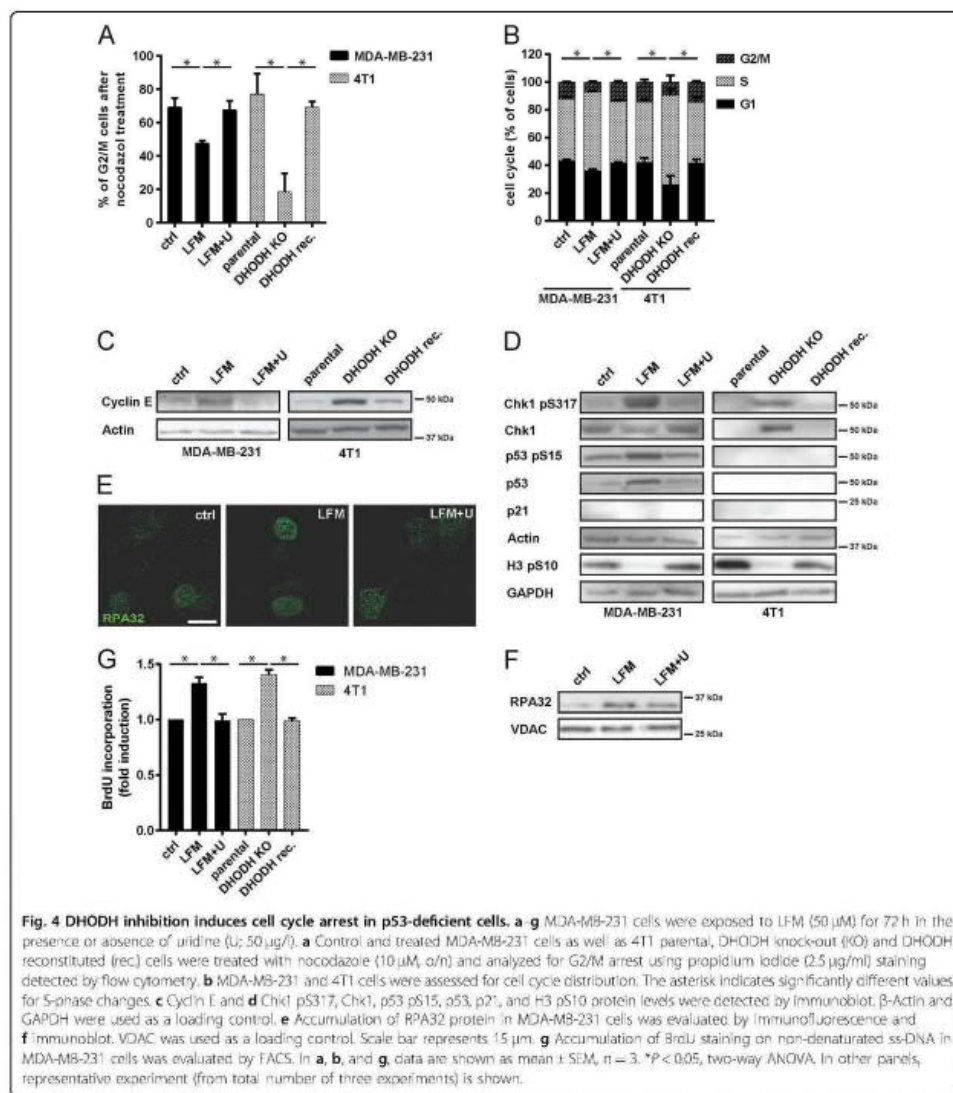
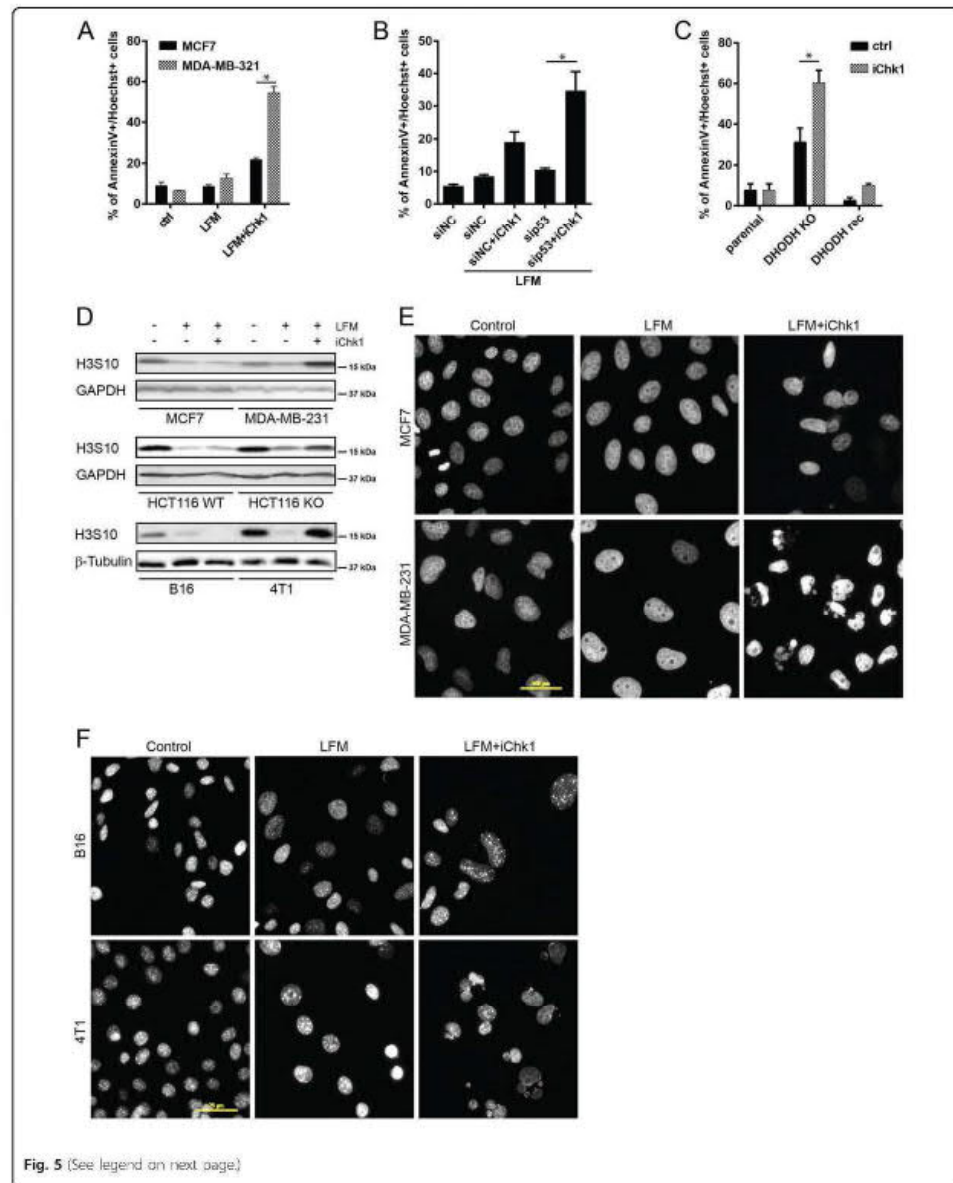


Fig. 4 DHODH inhibition induces cell cycle arrest in p53-deficient cells. **a–g** MDA-MB-231 cells were exposed to LFM (50 μ M) for 72 h in the presence or absence of uridine (U; 50 μ g/l). **a** Control and treated MDA-MB-231 cells as well as 4T1 parental, DHODH knock-out (KO) and DHODH reconstituted (rec.) cells were treated with nocodazole (10 μ M, o/n) and analyzed for G2/M arrest using propidium iodide (2.5 μ g/ml) staining detected by flow cytometry. **b** MDA-MB-231 and 4T1 cells were assessed for cell cycle distribution. The asterisk indicates significantly different values for S-phase changes. **c** Cyclin E and **d** Chk1 pS317, Chk1, p53 pS15, p53, p21, and H3 pS10 protein levels were detected by immunoblot. β -Actin and GAPDH were used as a loading control. **e** Accumulation of RPA32 protein in MDA-MB-231 cells was evaluated by immunofluorescence and **f** immunoblot. VDAC was used as a loading control. Scale bar represents 15 μ m. **g** Accumulation of BrdU staining on non-denatured ss-DNA in MDA-MB-231 cells was evaluated by FACS. In **a**, **b**, and **g**, data are shown as mean $\pm$ SEM, $n = 3$. * $P < 0.05$, two-way ANOVA. In other panels, representative experiment (from total number of three experiments) is shown.

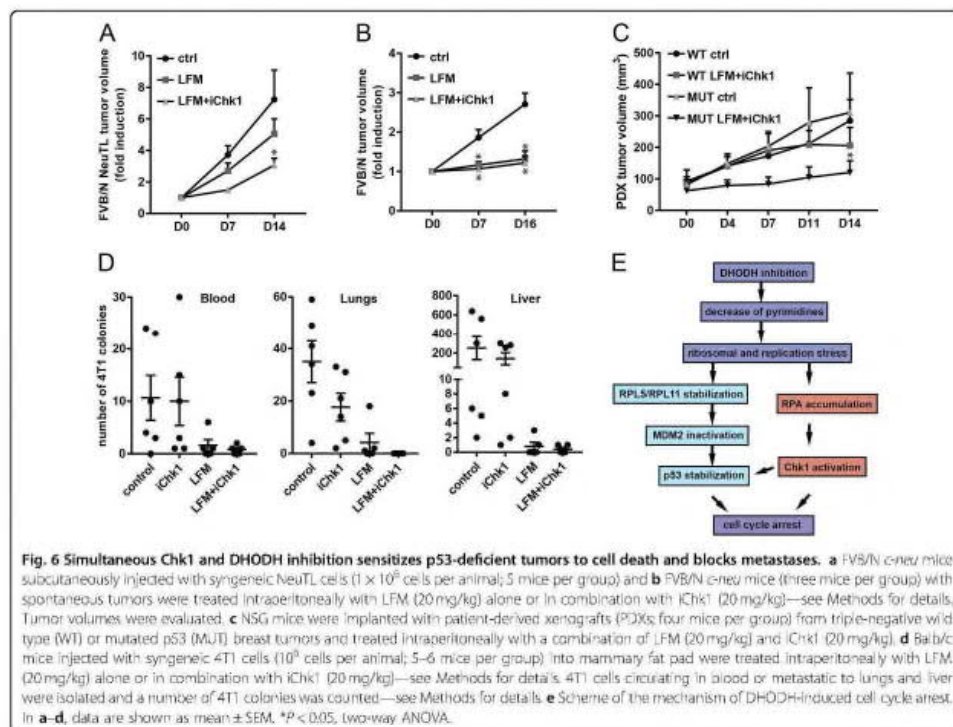
Chk1 inhibitor intraperitoneally twice a week for 2 weeks (see Methods for details). In parallel, transgenic FVB/N *c-neu* mice with spontaneous Her2^{high}, wt p53 breast carcinomas (Supplementary Fig. 5f) were treated using the same regimen. Similarly as for in vitro experiments, we observed reduced growth of p53-deficient tumors treated

with the combination of leflunomide and the Chk1 inhibitor compared with the leflunomide treatment alone (Fig. 6a), while spontaneous wt p53 tumors did not show any additional benefit of combined administration (Fig. 6b). To corroborate these findings in a clinically relevant model, we used mice with patient-derived



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Fig. 5 Inhibition of Chk1 promotes cell death in DHODH-inhibited p53-deficient tumor cells. **a** MCF7 and MDA-MB-321 cells were pre-treated with Chk1 kinase inhibitor LY2603618 (Chk1, 5 μ M, 30 min before LFM) followed by LFM treatment (50 μ M, 48 h) and cell death was evaluated by annexin V/Hoechst staining using flow cytometry. **b** MCF7 cells were pre-treated with Chk1 (5 μ M, 30 min before LFM) after downregulation of p53 using specific siRNA followed by LFM treatment (50 μ M, 48 h). Cell death was detected by annexin V/Hoechst staining using FACS. **c** 4T1 parental, DHODH knock-out (KO), and DHODH reconstituted (rec) cells were treated with Chk1 (5 μ M, 48 h) and cell death was evaluated by annexin V/Hoechst staining using FACS. **d–f** MCF7, MDA-MB-321, HCT116 wt p53, HCT116 p53^{KO}, 816, and 4T1 cells were pre-treated with Chk1 (5 μ M, 30 min before LFM) followed by LFM treatment (50 μ M, 24 h). **d** Protein level of H3 pS10 was detected by immunoblot. GAPDH or β -tubulin was used as a loading control. **e, f** Nuclear morphology was detected by a fluorescent microscope using DAPI staining. Scale bar represents 100 μ m (**e**) or 25 μ m (**f**). In **a–c**, data are shown as mean $\pm$ SEM, $n = 3$. * $P < 0.05$, two-way ANOVA. In other panels, representative experiment (from total number of three experiments) is shown.



xenografts (PDXs) originating from triple-negative breast cancers (TNBC) with either wt p53 or mutant p53 (Supplementary Fig. 5K). With either wt p53, we observed strong inhibition of p53-mutant tumors treated together with leflunomide and Chk1 inhibitor, while only moderate effect was apparent for the wt p53 tumors (Fig. 6c).

Loss of functional p53 often results in promotion of metastases. Therefore, Balb/c mice were transplanted with metastatic breast cancer 4T1 cells into the mammary fat pad and treated with the agents twice a week for

2 weeks. At the end of the treatment, circulating tumor cells in blood and metastases in lungs and liver were evaluated (see Methods for details). In contrast to untreated and Chk1 inhibitor-treated mice, very few metastases were observed in the lungs and the liver in mice treated with leflunomide alone, while none were observed in mice undergoing the combinatorial treatment (Fig. 6d, Supplementary Fig. 6A; see Supplementary Fig. 6B for primary tumor growth). Further, DHODH inhibition resulted in the induction of senescence

(Supplementary Figs. 1A, D, 4D and 6C), and the associated increase of pro-inflammatory cytokines, in tumor cells (Supplementary Fig. 6D). Decreased motility associated with senescence³⁹ could explain the ability of leflunomide to effectively reduce metastasis, but not solid tumors (Supplementary Fig. 6A, B).

Discussion

Pyrimidine nucleotides play a critical role in cellular physiology and metabolism, mainly by providing precursors for RNA and DNA synthesis³⁰. While pyrimidine nucleotides can be synthesized via the de novo pyrimidine biosynthetic pathway dependent on the respiration-linked DHODH, they can be also formed by salvage pathways in a DHODH-independent manner. The relative contribution of de novo pyrimidine synthesis to maintaining the pyrimidine pool depends on the cell type and the developmental stage. It is less important in resting or fully differentiated cells, where salvage pathways are sufficient³¹. In contrast, de novo pyrimidine synthesis gains prominence in rapidly proliferating cells due to an increased demand for nucleic acid precursors resulting from a high rate of DNA replication and nucleic acid biosynthesis³².

We have recently demonstrated the crucial importance of DHODH-dependent de novo pyrimidine synthesis for the ability of cancer cells to proliferate and form tumors³⁰. Similarly, administration of leflunomide in mice reduced tumor growth in this report, in agreement with the findings of others^{6,33}.

Cell cycle arrest induced by DHODH inhibition/deficiency is likely to prevent proliferation while not inducing cell death, which may compromise its effect on cancer therapy. Therefore, it is warranted to define factors that promote cell death upon DHODH inhibition. Indeed, despite high expectations, targeting DHODH was shown to be therapeutically effective only in some types of cancer. For example, such intervention was successful in patients with TNBC, where inhibition of de novo pyrimidine synthesis sensitized the tumors to genotoxic therapy⁸, similar as reported for patients with acute myeloid leukemia^{9,33} or multiple myeloma³⁴. Better understanding of molecular mechanisms sensitizing to DHODH inhibition may therefore help to translate DHODH-directed therapy into more efficient clinical outcomes.

In the present study, we identified the combination of stalled replication and ribosomal stress, induced either by DHODH deficiency/inhibition or respiratory insufficiency, both of which result in the failure to produce pyrimidines³⁰, as major factors regulating both cell cycle arrest and cell death in response to DHODH inhibition/deficiency. The lack of balanced dNTP pool can induce delay of replication fork progression and DNA replication as well as inhibition of RNA synthesis and proper

assembly of ribosome^{35,36}. In accordance with previous reports^{5,7,37,38}, we have observed cell cycle arrest in the early S phase after inhibition of DHODH activity by leflunomide, a clinically used agent in patients with rheumatoid arthritis and multiple sclerosis^{39,40}. Surprisingly, we did not observe an increase in DNA damage, unlike reported previously⁴¹. In contrast, we observed accumulation of RPA as well as BrdU on ssDNA, indicating replication stress triggered by defective replication fork recovery as a consequence of pyrimidine deficiency. Replication stress explains the presence of activated checkpoints and induction of p53 in the absence of DNA damage. The mechanism of activation relies on binding of RPA proteins to ssDNA, which is recognized by its binding partner ATR-interacting protein (ATRIP) and is activated by topoisomerase II β -binding protein 1 (TopBP1). This complex then recruits and activates ATR, which phosphorylates and activates ATM^{42,43}. ATR/ATM activation after replication stress was shown to arrest cells in S/G2⁴². Indeed, leflunomide treatment inhibits transcriptional elongation of genes required for melanoma growth⁴⁴, which corresponds to our observation of stalled replication.

Uncontrolled proliferation of cancer cells requires extensive protein synthesis dependent on constant supply of newly formed ribosomes. Therefore, depletion of the pyrimidine nucleotide pool leads to ribosomal stress since UTP is essential for ribosome biogenesis. Our results confirmed decreased expression of ribosome-related genes and proteins in DHODH-dysfunctional cells. Aberrant expression of ribosomal genes results in nucleolar stress with ensuing cell cycle arrest⁴⁵. This is linked to the nucleolus as a stress sensor, coordinating p53 activation via a number of proteins such as nucleophosmin (B23), RPL5, and RPL11, which directly bind to MDM2 and prevent ubiquitin-mediated p53 degradation⁴⁶. While p53 can be pro-apoptotic, stabilization of p53 after DHODH inhibition represents an important checkpoint ensuring cell cycle arrest and cell survival after pyrimidine depletion. Removal of this checkpoint leads to increased cell death, a consequence of damage induced by attempts to proceed in the cell cycle without complete replication.

Inactivation of the p53 tumor suppressor function is a frequent event in tumorigenesis, giving rise to cell cycle progression and uncontrolled proliferation. Since suppression of p53 led only to a modest increase in cell death and we observed cell cycle arrest also in p53-deficient cells, it is likely that inhibition of de novo pyrimidine synthesis leads to p53-independent cell cycle arrest. Conventional chemotherapeutics have been combined with Chk1 inhibition in order to increase cytotoxicity^{47,48}. Chk1, phosphorylated by ATR, prevents replication of damaged DNA and prevents premature chromosome condensation and cell division⁴⁹. Ineffective activation of

the ATR/Chk1 pathway, as in PTEN-deficient tumors, therefore increases their sensitivity to depletion of pyrimidine pool⁶. Using a selective Chk1 inhibitor LY2603618, currently undergoing phase II clinical trial⁴⁸, we have observed increased elimination of tumor cells lacking de novo pyrimidine synthesis, especially those with p53-deficient status.

Importantly, we observed a synergistic effect of DHODH and Chk1 inhibition on tumor progression in vivo as well as its effect on metastases. Such treatment was also highly effective in PDX tumor models derived from patients with p53-mutant triple-negative breast cancer, contrary to those derived from wt p53 breast cancer showing its clinical relevance. Decreased motility associated with senescence induced after DHODH inhibition in tumor cells could explain the anti-metastatic effect of leflunomide. However, increased production of pro-inflammatory cytokines by senescent cells increase the risk of chronic inflammation and secondary tumor formation⁵⁰ (Supplementary Fig. 6C), and their persistence in the organism is detrimental. Elimination of tumor cells, not only inhibition of proliferation leading to senescence, is therefore preferred for effective therapy. Combination of DHODH and Chk1 inhibitors is clearly superior to DHODH inhibition alone in this respect and represents a novel approach to treatment of tumors that are poorly responding to established therapies.

In our in vivo experiments, the combination of DHODH/Chk1 inhibition was non-toxic. However, previous studies using teriflunomide, the biologically active metabolite of leflunomide, showed high toxicity in mice indicating that the use of teriflunomide in patients may be unrealistic⁵¹. It should be noted that leflunomide is gradually converted to teriflunomide ablating the toxic effects of the latter when administered at high doses⁵¹. Moreover, mitochondrial localization of DHODH allows for targeting of leflunomide to mitochondria using a specific anchor, which could further increase the tumor sensitivity to this drug while minimizing its effects on normal cells.

In conclusion, we present in detail the complex machinery linked to cell cycle inhibition after suppression of pyrimidine synthesis. It comprises activation of two parallel inhibitory pathways, RPL/MDM2-modulated p53 stabilization and Chk1 activation, in response to replication and ribosomal stress induced by the depletion of pyrimidines (Fig. 6e). This explains why p53-deficient cells are more sensitive to Chk1 inhibition, since absence of both pathways allows for tumor cells to re-enter the cell cycle without replicated DNA, which in turn results in aberrant mitosis and cell death. Since p53 is mutated in more than 50% of tumors, simultaneous inactivation of DHODH and inhibition of the Chk1 kinase appears as a promising therapeutic modality for patients with hard-to-manage p53-mutant tumors.

Methods

Chemicals and antibodies

Leflunomide (L5025), nocodazole (M1404), uridine (U3003), etoposide (E1383), and nutlin 3 (N6287) were purchased from Sigma (St. Louis, MO, USA). The Chk1 inhibitor LY2603618 (S2626) was purchased from Selleckchem (Munich, Germany).

For immunoblotting, the following antibodies were used: anti-RPS6 (#2217), anti-p53 pS15 (#9282), anti-H3 pS10 (#3642), anti-Chk1 pS317 (#2344), anti-Chk2 pT68 (#2661) (all from Cell Signaling Technology, Danvers, MA, USA), anti-Chk2 (sc-9064), anti-Chk1 (sc-8408), anti-RPA32 (sc-56770), anti-Ub (sc-8017), anti-p53 (sc-126), anti-DHODH (sc-166348), anti-UMPS (sc-398086) (all from Santa Cruz Biotechnology, Dallas, TX, USA), anti-VDAC (ab15895) (Abcam, Cambridge, UK), anti-cyclin E (MA5-14336), anti-p21 (701151), anti-MDM2 (700555), B23 (32-5200) (all from ThermoFisher, Waltham, MA, USA). HRP-conjugated β -actin (PA1-183-HRP), HRP-conjugated α -tubulin (MA5-16308-HRP) (ThermoFisher, Waltham, MA, USA), anti-GAPDH (#5174) (all from Cell Signaling Technology, Danvers, MA, USA) or anti-TFIIH (sc-271500) (Santa Cruz Biotechnology) were used as loading controls. All antibodies were diluted 1:1000 in 2.5% non-fat milk. IgG-HRP anti-rabbit (170-6515) and anti-mouse (170-6516) secondary antibodies were purchased from BioRad Laboratories (Hercules, CA, USA). Secondary antibodies were diluted 1:10,000 in 2.5% non-fat milk. The following antibodies were diluted 1:100 in phosphate-buffered saline (PBS): anti- γ H2AX (05-636) (Millipore, Billerica, MA, USA), anti-53BP1 (sc-22760) (Santa Cruz Biotechnology) and anti-RPA32 (ab-2175) (Abcam). Secondary antibodies Alexa 488 (anti-rabbit) and Alexa 568 (anti-mouse) were purchased from ThermoFisher (Waltham, MA, USA) were diluted 1:1000 in PBS.

Cell culture

Human breast carcinoma cells MCF7 and MDA-MB-231, colon adenocarcinoma cells HCT116 (wt p53 and KO p53 cells) and wt p53 mouse melanoma cells B16 were cultivated in DMEM containing 4.5 g/l glucose (Biochrom, Berlin, Germany). All cells are from ATCC. Breast cancer NeuTL cells derived from tumors of transgenic FVB/N *c-neu* mice and 4T1 mouse breast carcinoma cells (ATCC) were cultivated in the RPMI medium containing 4.5 g/l glucose (Biochrom, Berlin, Germany). Media was supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin sulfate (Sigma). The RPMI medium was supplemented with sodium pyruvate (1 mM). Cells were kept at 37 °C under 5% CO₂. All cells were tested for mycoplasma contamination.

Animal studies

Transgenic FVB/N *c-neu* mice that develop spontaneous tumors at 6–8 months of age were treated with leflunomide (20 mg/kg dissolved in 4% EtOH in corn oil) alone or in combination with Chk1 inhibitor (LY2603618; 20 mg/kg dissolved in 5% DMSO in corn oil) given intraperitoneally twice a week for 2 weeks. In case of combined treatment, leflunomide was applied 24 h before the Chk1 inhibitor. Control mice were treated with the same volume of the excipient (4% ethanol in corn oil or 5% DMSO in corn oil) as was described above for combined treatment. We randomized mice according to the tumor volume before treatment.

Balb/c mice were injected subcutaneously (s.c.) with 10^6 4T1 cells in PBS. FVB/N *c-neu* mice (6 weeks old) were injected s.c. with 10^6 NeuTL cells in PBS before they developed spontaneous tumors. After 1 week (when tumors reached on average volume of 100 mm^3), mice were treated with leflunomide and the Chk1 inhibitor as described above. At the end of the experiment lungs, liver and blood from Balb/c mice were removed and processed according to the protocol described by Pulaski et al.⁵² to analyze metastases. We randomized mice according to the tumor volume before treatment.

NOD/SCID gamma (NSG) mice were implanted with patient tumor tissue, grown as first-generation xenografts in the mammary fat pad. In brief, mice were anesthetized, the mammary fat pad was surgically exposed and injected with 50 μl of the Matrigel extracellular matrix (Corning, Wiesbaden, Germany). When Matrigel solidified, tumor pieces ($\sim 2 \text{ mm}^3$) were implanted into a pocket excised in the mammary fat pad and secured with an internal stitch. The incision was closed by suture and mice were left on a heated pad until awoken. When tumors reached the volume of $\sim 50 \text{ mm}^3$, mice ($n = 3$ per group for control mice treated, $n = 4$ per group for treated mice) were treated with the combination of leflunomide (20 mg/kg dissolved in 4% EtOH in corn oil) and the Chk1 inhibitor LY2603618 (20 mg/kg dissolved in 5% DMSO in corn oil) or the excipient (4% ethanol in corn oil and 5% DMSO in corn oil, 100 μl per dose) given intraperitoneally twice a week for 2 weeks. Leflunomide was applied 24 h before the Chk1 inhibitor. We randomized mice according to the tumor volume before treatment.

All experiments were approved by the Czech Academy of Sciences Ethics Committee and performed according to the Czech Council guidelines for the Care and Use of Animals in Research and Teaching.

Evaluation of reactive oxygen species

To assess ROS production, cells were incubated with 2',7'-dichlorofluorescein (DCF; 10 μM ; Sigma) for 30 min prior to analysis by flow cytometry using the LSR Fortessa instrument (BD, San Jose, CA, USA). Cells without added

DCF probes were used as a control of non-specific signal. Hoechst 33258 (5 $\mu\text{g}/\text{ml}$; Invitrogen, Carlsbad, CA, USA) was added to cells prior to the measurements to exclude dead cells from the analysis.

Assessment of cell death

The medium containing dead cells was collected into a clear tube, adherent cells were trypsinized, resuspended in the medium containing dead cells and centrifuged at $1000 \times g$ for 3 min. The pellet was resuspended in 200 μl of annexin V-binding buffer containing 0.3 μl of annexin V-Dyomics 647 (Apronex, Vestec, Czech Republic), and incubated for 20 min at 4°C . Hoechst 33258 (5 $\mu\text{g}/\text{ml}$) was added before the cells were then analyzed using the LSR Fortessa flow cytometer. Cell death was expressed as the percent of the annexin V-positive/Hoechst-positive fraction.

SDS-PAGE and immunoblotting

Cells were washed twice with PBS, harvested into Laemmli SDS sample lysis buffer (2% SDS, 50 mM Tris-Cl, 10% glycerol in double distilled H_2O) and sonicated ($2 \times 10 \text{ s}$ at 1 μm amplitude with 10 s cooling interval) using the Soniprep 150 instrument (MSE, London, UK). Protein concentration was estimated using the BCA method (Pierce Biotechnology, IL, Rockford, USA). Cell lysates were supplemented with 100 mM DTT (Sigma) and 0.01% bromophenol blue (Sigma) before separation by SDS-PAGE. The same amount of protein (50–70 μg) was loaded into each well. The protein was transferred onto a nitrocellulose membrane using wet transfer and detected by specific antibodies combined with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse). Peroxidase activity was detected using the ECL Western Blotting Substrate or the SuperSignal West Femto Extended Duration Substrate (Thermo Fisher).

For RPA protein detection, cells were incubated for 10 min in the pre-extraction buffer (0.5% Triton X-100, 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl_2 , 1 mM PMSF) before harvesting into Laemmli SDS sample lysis buffer.

Immunoprecipitation

Cells grown to near confluency in 10 cm^2 plates were washed twice with cold PBS, harvested into 300 μl of the harvest buffer (100 mM HEPES pH 7.5, 500 mM NaCl, 0.5% Nonidet P40, 2% glycerol, 2 mM EDTA, protease and phosphatase inhibitor cocktail) and incubated for 30 min on ice. The suspension was centrifuged for 15 min/ 4°C /13,200 r.p.m., the lysate was transferred into a clean tube and protein concentration was assessed using the BCA kit (Pierce Biotechnology). Protein A/G-sepharose beads (10 μl) were gently washed in 500 μl of

the harvest buffer and centrifuged (1 min/room temperature (RT)/3000 r.p.m.). Clear beads were mixed with 500 µg of the lysate and incubated using a vertical shaker for 1 h at 4 °C to remove unspecific binding. After pre-incubation, beads were removed from the sample (1 min/RT/3000 r.p.m.), and the samples were incubated with the primary antibody overnight at 4 °C. Thirty microlitres of clear beads were then incubated with the sample for 3 h using a vertical shaker. After incubation, beads were centrifuged (1 min/RT/3000 r.p.m.), the supernatant was removed, beads were mixed with 2× Laemli Buffer (see SDS-PAGE) and 100 mM DTT + bromophenol blue, and denatured at 95 °C for 5 min. The samples were centrifuged, the supernatant transferred into a clean tube and the samples were then processed as described in the section on SDS-PAGE and immunoblotting.

Detection of senescence-associated β-galactosidase activity

SA-β-gal activity was detected as previously described⁵³ with slight modifications. Cells were washed with PBS, fixed with 0.5% glutaraldehyde (in PBS; pH 7.2; Sigma) and washed with PBS (pH 6.0) supplemented with 1 mM MgCl₂. Cells were stained with the X-gal solution (1 mg/ml X-gal, 0.12 mM K₃Fe[CN]₆, 0.12 mM K₄Fe[CN]₆, 1 mM MgCl₂ in PBS at pH 6.0) at 37 °C for 3–5 h. The signal was detected using the Leica DMIL Led microscope.

Indirect immunofluorescence

Cells grown on glass coverslips were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 in two consecutive steps, each at room temperature for 15 min. After washing with PBS, cells were incubated in 10% FBS (diluted in PBS) for 30 min to block unspecific signals. After this step, cells were incubated with diluted primary antibodies (1 h, RT), washed extensively with PBS/0.1% Tween 20 and incubated with secondary antibodies (1 h, RT). To counterstain nuclei, coverslips were mounted in Mowiol containing 4',6-diamidino-2-phenylindole (DAPI; Sigma) and viewed in a confocal microscope (Leica SP8). For detection of the bound RPA protein, cells were washed for 15 min in the pre-extraction buffer (0.5% Triton X-100, 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 1 mM PMSE, 0.01 M β-glycerol phosphate) before fixation in formaldehyde to wash out all unbound RPA.

siRNA-mediated gene knock-down

Cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. siRNAs against RPL5 (sense sequence: 5'-GAC GAG AGG GUA AAA CUG Att-3'), RPL11 (sense sequence: 5'-GGU GCG GGA GUA UGA GUU Att-3'),

RPS6 (sense sequence: 5'-CCU UAA AUA AAG AAG GUA Att-3') and p53 (sense sequence: 5'-GGU GAA CCU UAG UAC CUA Att-3'), DHODH#1 (sense sequence: 5'-GGU AUG GAU UUA ACA GUC Att-3'), DHODH#2 (sense sequence: 5'-CGG GAU UUA UCA ACU CAA Att-3'), UMPS#1 (sense sequence: 5'-GCA GAU GCU UUA GGA CCU Att-3'), and UMPS#2 (sense sequence: 5'-GUA UGA AGG AGG UAU CUU Utt-3') were purchased from ThermoFisher (Waltham, MA, USA). Non-targeting siRNA (Silencer® Select Negative Control No. 1, #4390843) was used as a negative control (siNC).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using RNeasy (400 µl for a 4 cm² dish; Molecular Research Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 µg of total RNA with random hexamer primers using Revert Aid First strand cDNA Synthesis Kit (Thermo Fisher). qRT-PCR was performed using the Eco Real-Time PCR System (Illumina, San Diego, CA, USA) with 5× HOT FIREPol Evagreen qPCR Supermix GreenE dye (Solis Biodyne, Tartu, Estonia). The relative quantity of cDNA was estimated by the ΔΔCT method; data were normalized to β-actin. The following primers were purchased from Sigma: RPL5: 5'-CCA AAT ACA GGA TGA TAG TTC GTG-3', 5'-TTG GCA GTT CGT GTG CAT ACG C-3'; RPL11: 5'-AGA GTG GAG ACA GAC TGA CGC G-3', 5'-CGG ATG CCA AAG GAT CTG ACA G-3'; 45S: 5'-CTC CGT TAT GGT AGC GCT GC-3', 5'-GCG GAA CCC TCG CTT CTC-3'; 18S: 5'-ACC CGT TGA ACC CCA TTC GTG A-3', 5'-GCC TCA CTA AAC CAT CCA ATC GG-3'; mouse IL6: 5'-TAC CAC TTC ACA AGT CGG AGG C-3', 5'-CTG CAA GTG CAT CAT CGT TGT TC-3'; mouse IL8: 5'-CTC TAT TCT GCC AGA TGC TGT CC-3', 5'-ACA AGG CTC AGC AGA GTC ACC A-3'; mouse TNFα: 5'-GCC TCT TCT CAT TCC TGC TTG-3', 5'-CTG ATG AGA GGG AGG CCA TT-3'; mouse β-actin: 5'-CAT TGC TGA CAG GAT GCA GAA GG-3', 5'-TGC TGG AAG GTG GAC AGT GAG G-3'; β-actin: 5'-CCA ACC GCG AGA AGA TGA-3', 5'-CCA GAG GCG TAC AGG GAT AG-3'. Data are expressed as mean values ± SEM of a minimum of three independent experiments performed in triplicates. The *P* values were calculated using two-way ANOVA; differences with *P* < 0.05 were considered statistically significant.

Knock out and reconstitution of DHODH in 4T1 cells

To prepare DHODH^{KO} cells, we used the CRISPR/Cas9 system⁵⁴. Guide RNAs sequences from the GeCKO library (v2 09Mar2015) were synthesized and cloned into *Bsm*BI-cleaved pXPR_001 vector containing the mammalian codon-optimized Cas9 nuclease expression cassette. Parental 4T1 cells were transiently transfected using Lipofectamine

3000 (Thermo Fisher), grown for 24 h and selected with puromycin for 48–72 h, or until all non-transfected control cells were eliminated. Surviving cells were sorted into 96-well plates, expanded, and clones unable to grow in media lacking uridine and pyruvate (for DHODH^{KO} cells) were selected. The absence of DHODH was verified by western blotting. The guide RNA sequence used was as follows: 5'-TCA GGT ACT CGG CGT AGA AA-3'.

To reconstitute DHODH protein expression, the DHODH coding sequence was PCR-amplified from cDNA obtained from mouse B16 cells. Primers used were 5'-ATA AAG AAT TCC ACC ATG GCG TGG AGA CAG CTG-3' (forward) and 5'-ATA AAG GAT CCT CAC CTG CGG TGA TCT ACT C-3' (reverse). The gel-purified PCR product was cleaved by fast-digest *EcoRI* and *BamHI* enzymes (Thermo Fisher) and ligated into the pCDH-CMV-MCS-EF1-Puro lentiviral vector (System Biosciences) digested in the same way. Lentiviral particles were produced as described⁵⁵ and used to transduce 4T1 DHODH^{KO} cells, followed by puromycin selection. Expression of the DHODH protein was verified by western blotting.

DHODH activity assay

DHODH activity was evaluated using a modified protocol according to Yin et al.⁵⁶. In brief, cells were collected, washed with PBS, re-suspended in potassium phosphate buffer (0.1 M, pH 7.0) and lysed using three freeze-thaw cycles; the lysates were incubated in the solution of 160 mM K₂CO₃/HCl (pH 8.0), 400 μM dihydroorotate, 80 μM decylubiquinone at 37 °C for 60 min. The reference sample was kept on ice. The reaction mixture was supplemented with 20 mM K₂CO₃, 2 mM K₃[Fe(CN)₆] and 1 mM 4- (trifluoromethoxy)benzamidoxime (4-TFBAO), and heated at 80 °C for 4 min. The reaction was stopped by cooling on ice, and fluorescence intensity was measured using the Tecan Infinite M200 plate reader (Schoeller, Prague, Czech Republic); the excitation and emission wavelengths were 320 and 420 nm, respectively. The calibration curve was plotted using lysate-free samples containing 0.5, 0.75 and 1 μM orotic acid.

Cell cycle analysis

For cell cycle analysis, cells were pre-treated with 10 μM nocodazole for 16 h at 37 °C to allow their accumulation in G2/M. Cells were trypsinized, harvested into 500 μl of PBS and centrifuged (2500 r.p.m./3 min). Pellets were resuspended in 70 μl of PBS, fixed with 500 μl of cold 80% EtOH added drop-wise during shaking and incubated at -20 °C for 30 min. EtOH was then removed, cells were re-suspended in 200 μl of PSB containing 2.5 μg/ml propidium iodide (for 10⁶ cells) and the samples were analyzed by flow cytometry. For BrdU detection, 2 M HCl was added for 30 min (room temperature) after fixation to denature DNA. HCl was removed and the pellet neutralized with

100 μl of 0.1 M disodium tetraborate (pH 8.5) for 1 min. Cells were then incubated for 1 h with anti-BrdU-FTIC IgG (BD Biosciences; 0.5 μg/ml of PBS) in the dark. Finally, cells were re-suspended in 200 μl of PSB containing 2.5 μg/ml propidium iodide and analyzed by flow cytometry.

Microarray analysis

Transcriptomic analysis of parental, D0, D5, D10, D15, D20 and D25 4T1 cells was performed using a standard protocol and the MouseGene 2.0 ST array (Affymetrix; Thermo Fisher). The data are deposited under the accession number MTAB-6150 in the EBI ArrayExpress database.

Statistical analysis

Unless stated otherwise, data are mean values ± SEM of at least three independent experiments. In mouse experiments, groups of six animals were used, unless stated otherwise. Two-way Anova presented as mean ± standard error of means (SEM) was used to assess statistical significance with *P* < 0.05 being regarded as significant, using GraphPad Prism software. Images are representative of at least three independent experiments.

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GENERAL DISCUSSION AND CONCLUSIONS

Mitochondria are small cell organelles that originated when specialized bacteria were engulfed by other primitive cells two billion years ago. They produce the requisite energy for various cellular processes, including cellular growth, which is the fundamental characteristic of tumor tissue. This energy production is dependent on mitochondrial respiration, i.e. oxygen consumption. Mitochondria are often present in hundreds or thousands of copies, depending on the cell type and bioenergetic requirements (Veltri et al., 1990). The number of mitochondria in a particular cell type can change depending on many factors, including the stage in the cell cycle, cellular redox environment and homeostasis, the stage of differentiation, and a number of cell signaling mechanisms.

An important property of mitochondria is the presence of its own genetic information, encoded by mtDNA. mtDNA is subject to a high level of mutations since the mitochondrial genome lacks histone protection and resides in close proximity to the sites of generation of ROS; coupled with the limited fidelity of mtDNA replication and repair machinery causing an estimated 10–17-fold higher mutation rate than nDNA (Weissig et al. 2015). mtDNA heteroplasmy was originally viewed as being associated with pathological states and rare in healthy individuals. However, in recent years, studies have indicated that heteroplasmy also occurs in asymptomatic humans (Stewart and Chinnery, 2015). Moreover, reports of an increase in somatic mtDNA mutations with aging and the incidence of tissue-specific mutations have been published (Wang et al., 2001). In general, the integrity and amount of mtDNA influence mitochondrial function and correlates with OXPHOS activity. In many pathologies, mtDNA mutations affect tissues with high energy demand (Wallace, 2012; Schon, 2012). One of the essential characteristics of cancer is altered metabolism of mitochondria, which is triggered by genetic alterations not only in nuclear DNA but also in mtDNA (Kroemer and Poyseur, 2008; Vander Heiden et al., 2017). However, mutations in mtDNA and their contribution to cancer development are less clear and, moreover, no correlation between the ability to form tumors and lack of energy is yet to be shown.

Cell lines lacking mtDNA are very effective tools for studying the consequences of mtDNA mutations/deletions. Our previous unpublished data demonstrated that grafting human p0 cells to immunosuppressed mice is limited by a strong species-dependent

relationship between mitochondria, and mtDNA- and nDNA-coded proteins. Therefore, we were restricted to mouse cancer cells and syngeneic mice. To analyze the contribution of mtDNA to tumorigenesis, we deleted mtDNA in murine B16 melanoma and 4T1 breast carcinoma cells using low-dose ethidium bromide (EtBr), thereby generating two stable murine tumor cell models that lacked mtDNA, and we investigated whether these cells could form tumors in syngeneic mice. These mtDNA-deficient cells formed syngeneic tumors with a delay compared to their parental counterparts, but once tumors emerged, the growth curve was similar. This lagged tumor growth was associated with mtDNA acquisition from the host. When we established cell cultures from $\rho 0$ cell-derived tumors at different stages of tumor progression (primary tumors, circulating tumor cells, and lung metastases), next-generation sequencing (NGS) revealed that sublines derived from tumors grown from $\rho 0$ cells share mtDNA polymorphism with the host. Our results, for the first time reporting on horizontal transfer of genes (mtDNA) in mammalian cells *in vivo*, clearly documented the essential role of OXPHOS in tumor initiation and progression (Tan et al., 2015). We have challenged the dogma that genes are the property of individual cells by showing unequivocally that mitochondria with their DNA payload are acquired by tumor cells with compromised mtDNA *in vivo*, a process that was previously considered almost exclusively restricted to prokaryotes (Keeling et al., 2008; Gladyshev et al., 2008).

First, we performed these experiments with mouse metastatic mtDNA depleted 4T1 breast cancer cells, discovering a step-wise recovery of OXPHOS in isolated 4T1 $\rho 0$ SC, 4T1 $\rho 0$ CTC, and 4T1 $\rho 0$ SCL cells. However, performing identical experiments with murine metastatic melanoma B16 $\rho 0$ cells, we found a complete recovery of respiration already in B16 $\rho 0$ SC cells, with no further increase in respiration in B16 $\rho 0$ CTC or B16 $\rho 0$ SCL cells. Moreover, the re-gained respiratory function was in agreement with assembly of SCs and shortening of the lag phase during tumor growth initiation. The above-mentioned findings indicate that fully assembled mitochondrial complexes and respiration are needed for efficient tumor initiation. There is therefore a link between respiration of parental cells and the level of recovery of respiration after tumor initiation by the originally mtDNA-depleted cells following mtDNA acquisition. The B16 $\rho 0$ cells seem to have higher requirements for the respiration recovery connected with tumor formation than 4T1 $\rho 0$ cells, indicating that different types of cancer cells need to recover their respiration to different ‘threshold’ levels

to be able to initiate tumor formation. Our hypothesis was corroborated by Viale et al., (2014) showing that the subpopulation of dormant malignant cells surviving KRAS ablation responsible for tumor relapse strongly rely on OXPHOS rather than glycolysis.

We next tested the tumor-forming capacity of parental cells, p0 cells, and cells derived from various tumor stages. In the case of 4T1p0 cells, the sublines formed tumors with different latencies; tumors originating from lung metastasis cultures grew faster than those originating from circulating tumor cells, which in turn grew faster than those from primary tumors. Furthermore, these tumors also showed gradual recovery of mitochondrial respiratory function and restoration of OXPHOS, which are both directly linked to efficient tumor formation. Conversely, B16p0 sublines formed tumors without lag phase except for B16p0 cells; moreover, instead of a gradual pattern in recovery of mitochondrial respiratory function as observed in 4T1p0 cells, their restoration of the tumorigenic potential fully correlated with the observed recovery of mitochondrial aerobic respiration.

Then, we searched for direct evidence that respiration is crucial in the phase of initiation of tumor growth. We grafted B16p0 cells with compromised CI or CII cells into C57BL/6J mice. CII suppression resulted in tumor formation but with an additional lag phase compared to B16p0 cells. While mice grafted with CI-compromised cells failed to form tumors within 100 days. These data indicate that respiration restoration is an indispensable element for tumorigenesis since suppression of respiration completely deregulated this process. This finding is consistent with several recent studies (Weinberg et al., 2010; Birsoy et al., 2015; Sullivan et al., 2015; Cardaci et al., 2015; Lussey-Lepoutre et al., 2015)

Based on the aforementioned considerations, maintaining or restoring respiration is a crucial requirement for cancer cells, and inter-cellular movement of mitochondria appears highly relevant in this context. It was previously demonstrated that inter-cellular distribution of mitochondria can occur via several modes. One possibility is cellular fusion, as shown for example may occur during the process of oncogenesis (Vitale et al., 2011) but we did not observed polyploidy in our system. Another possibility relates to exosomes that have been shown to promote OXPHOS under specific conditions (Panfoli et al., 2016) or drive cancer resistance (Boelens et al., 2014), however, not necessarily carrying whole mitochondria (Panfoli et al., 2016). Trafficking via inter-cellular bridges referred to as TNTs is a plausible mode of moving mitochondria with their DNA payload between cells, a

process whose partial understanding is derived from studies on neuronal cells, where mitochondria move along tubulin fibers to and from synapse (MacAskill et al., 2010).

The hypothesis of intact mitochondrial transfer via TNTs was verified by grafting B16p0 nBFP cells into C57BL/6Jsu9DsRed2 mice. After 11 days, we prepared a single cell suspension from a pre-tumor lesion tissue that was sorted for nBFP and DsRed positive cells. This hypothesis could be supported by the fact that dsRed protein is encoded by nuclear DNA of the donor cell thus it cannot be replenished by *de novo* synthesis in the recipient cancer cell; in the case of cell fusion, the dsRed fluorescent signal would be stable. This was proved when we inspected DP cells by confocal microscopy as the fluorescent signal of dsRed protein was gradually lost after the cell isolation from tumor. In addition to our results, this mechanism of mitochondrial movement was observed to rescue impaired mitochondrial functions in different models (Vallabhaneni et al., 2012; Liu et al., 2014b). Moreover, there is evidence that mitochondrial transfer via TNTs can promote resistance to chemotherapy in mouse models of brain tumors (Osswald et al., 2015).

It remained unclear what happens in the grafted p0 cells before a tumor starts to form. We thus prepared relevant cellular tools by grafting 4T1p0 cells into Balb/c mice and generated cell lines from isolated tumor lesion tissue on different days post grafting. These cell lines were stable in culture and their properties, such as the level of mtDNA, respiration, ETC assembly, did not change with time, similarly as reported (Picard et al., 2016). Based on the results mentioned above, we designed a unique tool to study *in vitro* temporal events that occur in cells grafted into the host, recapitulating what happens *in vivo*, since they are derived from the p0 cells at different times after their grafting. To answer the question why tumor formation requires restoration of respiratory function and capacity for oxidative metabolism, we studied the bioenergetics of parental and D0-D60 cell lines. This revealed that D0-D10 cells lack respiratory capacity and glycolytic reserve, generating ATP exclusively by glycolysis. However, total cellular ATP was comparable in parental cells and D0-D60 sublines, suggesting that recovery of respiration is not linked to increased ATP production and cellular bioenergetics, but must have another function associated with tumor formation.

As described in the literature overview chapter, there is a link between mitochondrial respiration and metabolic remodeling (Sullivan et al., 2015; Birsoy et al., 2015) as p0 cells

were shown to be auxotrophic for pyruvate and uridine, the latter being linked to low activity of DHODH (Alcázar-Fabra et al., 2016). DHODH, controlling the fourth step of pyrimidine *de novo* synthesis, has been reported to play an important role in various pathologies, including cancer (White et al., 2011; He et al., 2014), although its precise role is unclear. Linked to this, we evaluated the time when the grafted 4T1p0 cells lost auxotrophy for uridine, which appeared to be between 15 and 20 days post grafting. This was consistent with the recovery of DHODH-dependent respiration by D15, which was low in D0-D10 cells. Intriguingly, the orotate-to-dihydroorotate ratio was low in D0-D10 cells and recovered to parental levels in D15-D60 cells, indicating stalled conversion of DHO to orotate and suboptimal pyrimidine synthesis in D0-D10 cells.

To evaluate the involvement of DHODH in tumor formation and progression, we knocked out the gene for this enzyme that consequence in failure of tumor formation. Nevertheless, coupling respiration with mitochondrial ATP production is not essential for tumor growth. To study further whether respiration is needed for tumor growth via its link to DHODH rather than ATP generation, we prepared cancer cells lines with non-functional ATP synthase. These cells were able to grow tumors and contained normal amounts of the DHODH protein as well as featured DHODH-dependent respiration. The results discussed above indicate that DHODH presents a link between respiration and tumor formation, controlling *de novo* pyrimidine synthesis independently of mitochondrial ATP generation.

As UbQH2 oxidation is an essential activity of mitochondrial complex III that allows complex I, II, and DHODH to function, we transfected p0 cells with AOX that transports electrons from UbQH2 directly to oxygen, bypassing the ETC complex III and IV activities (Hakkaart et al., 2006). We found that in 4T1p0 AOX, the alternative oxidase caused reactivation of DHODH-dependent respiration as well as restoration of tumor formation in the absence of mtDNA. Taken together, a major consequence of AOX expression in p0 cells is the reactivation of CoQ redox-cycling leading to restoration of the DHODH function.

Thus, we could hypothesize that inhibiting the enzyme necessary for DNA synthesis and cell proliferation, i.e. DHODH, but not mitochondrial energy production, could stop tumor growth (Mathur et al., 2017; Koundinya et al., 2018). A link between inhibition of CIII leading to pyrimidine deficiency and induction of the main cell cycle regulators p53

was found at early time points in a cancer cell (Khutornenko et al., 2010, He et al., 2014, Ladds et al., 2018b). According to the fact that DHODH seemed to be a remarkably frequent target for activators of p53, we decided to assess the level of transcripts regulating p53-driven cell cycle arrest (ATM, ATR, Chk1 or Cdkn1a) in 4T1p0 sublines derived from different days post grafting. We observed increased levels of these transcripts in D0–D10 sublines, and this pattern switched when cells were no longer auxotrophic for uridine, as mentioned before. To describe the involvement of DHODH in the molecular mechanism of cell cycle arrest, once we inhibited the DHODH function, the checkpoint kinases Chk1 and Chk2 and the p53/p21 signaling pathway became activated. As we did not detect any DNA damage, the underlying mechanism of DHODH inhibition was linked to an unbalanced dNTP pool. Specifically, pyrimidine synthesis inhibition resulted in delay of the replication fork progression and DNA replication as well as inhibition of RNA synthesis and proper assembly of ribosomes. It is important to emphasize that pyrimidine deficiency can induce cell cycle arrest independent of p53, since we have found a similar effect for p53-deficient DHODH-dysfunctional and wild-type p53 cancer cells. Chk1 activation after DHODH inhibition as well as the ability of p53-deficient cells to induce cell cycle arrest indicates its potential role in the response to pyrimidine depletion. The combined administration of DHODH and Chk1 inhibitors increases cytotoxicity in p53-dysfunctional cancer cells through increased cell death. Therefore, this promising therapeutic strategy can be used for p53-mutated tumors and their metastasis.

The presented thesis relates to the topic of ‘multifaceted’ mitochondria and extends our knowledge about these organelles and their role in cancer. With the recent advancement in molecular biology and molecular medicine, researchers are discovering new phenomena and paradigms, sometimes breaking the years-long dogmas that are bases of our understanding of physiological and pathological processes. A discovery that can be considered as breaking one of the dogmas on which our understanding of cell biology stands, is our finding of horizontal transfer of mitochondria and mitochondrial genes *in vivo*, using a syngeneic mouse model of cancer. Before our discovery was published in *Cell Metabolism* in 2015 (Tan et al., 2015), it had been shown that mitochondria move between cells in cultured cells, using monoculture and, more plausibly, co-culture of two or more cell types (Moschoi et al., 2016). For a number of researchers, this inter-cellular movement

was, at least to some extent, driven by the rather artificial conditions of cultured cells that do not well reproduce what is going on in a whole animal. Therefore, this discovery can be considered as ground-breaking, which is also documented by close to 500 citations since the publication of the paper. Further, we showed that whole mitochondria move between cells (Dong et al., 2017) and, finally, we found an explanation for the need of cancer cells without mtDNA to import mitochondria from other cells in order to restore respiration that, rather than the anticipated recovery of OXPHOS, it is needed to fuel *de novo* pyrimidine synthesis (Bajzikova et al., 2019). Linked to this, we showed that the mitochondrial component of pyrimidine synthesis, DHODH, is also associated with ribosomal stress in cancer cells (Hubackova et al., 2020).

Thus, our findings point to the ever expanding knowledge on the unprecedented plasticity of tumor cells and tumors. Similar to hijacking of the immune system to their advantage and blood vessels to supply tumors with oxygen and nutrients, and with the ability of malignant cells to differentiate and de-differentiate, we add an intriguing, novel paradigm for cancer cells, further conferring the notion of unprecedented plasticity of cancer cells. In this case, their ability to switch from OXPHOS-related bioenergetics to glycolysis, and their unexpected propensity to restore respiration in order to form pyrimidines. We believe that this is a paradigm-shifting discovery that has opened a rather new aspect of cancer biology research, linked to horizontal transfer of mitochondria.

Our quest to better understand cancer biology, while driven by our curiosity of adding new knowledge pertinent to basic (cancer) cell biology, has yet another angle of importance, which is the clinical translation of our research. Conceivably, we present DHODH, a crucial player in a very important (in fact, indispensable) pathway, as a relatively neglected target for cancer therapy. This stems from the notion that one of the features of cancer cells is their overwhelming capability and need to proliferate, for which they critically need constant supply of the building block of nucleic acids, making the *de novo* pyrimidine pathway of utmost importance and a broad-spectrum target for cancer therapy.

In summary, the novelty of the presented thesis mainly pertains to numerous discoveries that describe the importance of mitochondrial bioenergetics and metabolism in cancer formation and progression.

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