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Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel?

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Abstract

Mitochondria cooperate with their host cells by contributing to bioenergetics, metabolism, biosynthesis, and cell death or survival functions. Reactive oxygen species (ROS) generated by mitochondria participate in stress signalling in normal cells but also contribute to the initiation of nuclear or mitochondrial DNA mutations that promote neoplastic transformation. In cancer cells, mitochondrial ROS amplify the tumorigenic phenotype and accelerate the accumulation of additional mutations that lead to metastatic behaviour. As mitochondria carry out important functions in normal cells, disabling their function is not a feasible therapy for cancer. However, ROS signalling contributes to proliferation and survival in many cancers, so the targeted disruption of mitochondria-to-cell redox communication represents a promising avenue for future therapy.

The relationship between mitochondria and their host cells began approximately 2 billion years ago, when an antecedent of modern-day mitochondria was engulfed by an archezoan cell, forming the first primitive eukaryote[1,2]. This relationship evolved over time, as gene transfer with other prokaryotes occurred or as genes were transferred from the endosymbiont to the nucleus[3,4]. The original symbiotic relationship probably succeeded because of the mutual benefits derived from the complementary roles in cellular energy production. For the host cell, oxidative phosphorylation, whereby ATP is generated from ADP and inorganic phosphate, is likely to have been the principal benefit. In exchange, the antecedent mitochondria enjoyed an intracellular environment that was rich in nutrients and protected from extremes of pH that could undermine their membrane transport functions. These symbiotic interactions persist in modern-day cells, but the relationship has grown more complex in terms of the number of shared responsibilities involved in a wide range of functions. Modern-day mitochondria now participate in the biosynthesis of haem and iron-sulphur centres, regulation of cytosolic calcium ion concentrations, regulation of cellular redox status, and the generation of substrates for protein and lipid biosynthesis. Mitochondria also facilitate cellular stress responses, including the response to hypoxia and the activation of programmed cell death via the release of pro-apoptotic molecules from the intermembrane space (IMS) to the cytosol. Under normal conditions, mitochondria trigger

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redox signalling in the cell through the release of reactive oxygen species (ROS) from the electron transport chain (ETC). Under pathophysiological conditions, ROS generation from mitochondria can also contribute to the initiation of cancer and to an amplification of the tumour cell phenotype. At the same time, mitochondrial ROS may render the tumour cell vulnerable to therapies that further stress their ability to regulate redox homeostasis, thereby opening opportunities for novel therapies.

This Review considers how mitochondria generate ROS, how these reactive molecules contribute to the transformation of healthy cells into tumours, and how redox signalling in established tumour cells can amplify the phenotypic behaviour in terms of proliferation, survival and migration. Although tumour cells rely on increased mitochondrial ROS signalling to regulate their phenotype, this characteristic puts them in dangerous territory, in terms of their vulnerability to therapeutic interventions that further stress their redox homeostasis. How this characteristic could be exploited represents both a major challenge and an important opportunity in the treatment of this disease.

Sources of mitochondrial ROS in cancer

Cancer cells are characterized by a need for ATP, which is required to support the anabolic processes involved in growth and proliferation. Mitochondria generate ATP by oxidizing lipids, amino acids and glucose, and by transferring the electrons derived from those reactions to the ETC, which ultimately delivers them to molecular O_2. Free energy conserved in this process is then used to generate ATP. The oxidation and reduction steps in these reactions involve a diverse set of metalloproteins, quinones, flavin groups and haem moieties that function as electron ‘way-stations’, analogous to stepping-stones across a river. Collectively, these discrete sites constitute a discontinuous electrical conduction system, as electrons are routed from one site to the next. For the most part, this system is designed to limit the ability of electrons to engage in interactions that would divert them from the intended pathway. However, several factors undermine the ability of the system to prevent electron escape. First, the movement of electrons from one site to the next occurs sequentially, so a transient delay at one location generates a traffic backup of electrons at earlier sites. This delay creates opportunities for electrons that are stalled at a site to interact with O_2, generating superoxide, a free radical. In addition, electrical charges moving within the mitochondrial inner membrane are subjected to a strong electrical field, arising from the potential difference between the matrix and intermembrane compartments. For example, a normal membrane potential of −180 millivolts across the inner membrane of 7-nanometre thickness produces an electrical field strength that is equivalent to 257,000 volts per centimetre. This considerable field strength exerts a powerful influence on the movement of ions in the inner membrane, potentially diverting electrons from their intended path. In addition, any superoxide anions formed within the lipid bilayer would be forcefully accelerated towards the IMS by this field, thus facilitating their access to the cytosol.

While several electron transfer sites in the mitochondria have been identified as potential sources of ROS generation, definitive measurements from every possible site have not been made and it is safe to assume that many sites can potentially generate ROS. The reactivity of reactive oxygen intermediates ranges from relatively low (superoxide) to moderate

(hydrogen peroxide) to extremely high (hydroxyl radical). Excessive ROS generation, or failure of oxidant scavenging systems, can disrupt cellular function by causing oxidation of lipids, proteins and DNA[5]. Lower levels of oxidants act as signal transduction messengers in redox signalling pathways, which have important roles in the regulation of cell function, including proliferation. It has been estimated that about 2% of the O_2 that is consumed by mitochondria is involved in ROS generation[6], but the assumptions required for that calculation limit its usefulness. Cancer-inducing mutations in mitochondrial enzymes can augment the generation of ROS from multiple sites, as discussed later. The following sections summarize the known and potential sites of ROS generation within mitochondria.

The tricarboxylic acid cycle

The tricarboxylic acid cycle (TCA cycle) is comprised of a series of enzymes located in the mitochondrial matrix that remove electrons from intermediary metabolites and pass these to the ETC (FIG. 1). Several enzymes in the mitochondria use flavin-containing prosthetic groups — either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) — as electron way-stations. These enzymes include NADH dehydrogenase, pyruvate dehydrogenase, α-ketoglutarate and succinate dehydrogenases, acyl-CoA dehydrogenase involved in fatty acid oxidation and branched-chain 2-oxoacid dehydrogenase; all of which can potentially generate ROS[7]. Many other mitochondrial proteins that do not directly participate in the TCA cycle also incorporate flavin groups. Some flavoproteins undergo two-electron transfers, but others transfer only a single electron at a time, forming a semiquinone radical in the process. Electrons that become stranded on the flavin group have the potential to generate superoxide, making them important potential sites of ROS generation. Other enzymes incorporate metals, such as iron in the form of iron–sulphur clusters, which can also contribute to ROS generation. Mitochondrial aconitase, which contains a $[4Fe–4S]^{2+}$ iron–sulphur cluster, is potentially capable of generating ROS but is better known for its inactivation by superoxide[8]. Inactivation of any site in the TCA cycle can augment ROS generation from earlier sites by stalling electrons on flavin-linked or iron–sulphur clusters.

Most dehydrogenases in the TCA cycle use nicotinamide adenine dinucleotide (NAD) or NADP as electron carriers. These cofactors bind to the dehydrogenase and are then released to carry the reducing equivalents to the ETC. Although they function as electron carriers, NADH and NADPH do not directly participate in ROS generation, because the reducing equivalents are bound covalently. This covalent bond prevents the direct oxidation of the reduced carrier by O_2.

The ETC

In mitochondria, electrons that are derived from the TCA cycle are passed down the ETC and transferred to O_2 at complex IV. The electron transfer steps at complexes I, III and IV are coupled with proton translocation across the inner mitochondrial membrane, which drives ATP synthesis at complex V. As with TCA cycle enzymes, electrons can escape from flavin groups or iron–sulphur clusters in the ETC and be captured by O_2, forming ROS (FIG. 2). Ubiquinone (also known as coenzyme Q), is a quinone with an isoprenoid side chain. It resides in the inner membrane and delivers pairs of electrons that are derived from

multiple sources — including complexes I and II — to complex III. When the first electron is donated to complex III, a free radical, ubisemiquinone, is transiently created. Any delay in the release of its second electron increases the probability that the electron will instead be donated to O_2, generating superoxide[9]. ETC sites that are implicated in ROS generation include complexes I, II, III and ubisemiquinone. Complex IV transfers electrons to O_2, so one might expect that it could generate ROS. However, upon transfer of the first electron, the binding affinity for O_2 increases markedly, assuring that release cannot occur until all four electrons have reduced the O_2 to H_2O (REF. 10). Complex V does not participate in electron transport, and there is no evidence that it directly generates ROS. However, changes in complex V activity can produce reciprocal changes in the membrane potential, which in turn can markedly affect ROS generation from the ETC.

Different subcellular compartments can be affected by ETC-derived ROS, depending on where the superoxide is generated. When generated at sites within the inner mitochondrial membrane, superoxide is driven towards the IMS by the strong electric field within the membrane. ROS that are derived from prosthetic groups of matrix-oriented subunits are released into the matrix compartment. ROS that are released in the matrix are scavenged by the antioxidant systems in that compartment; although high levels of matrix ROS may overwhelm that capacity and escape to the IMS and the cytosol. ROS that reach the IMS or the cytosol can then participate in redox signalling or cause oxidative damage. ROS have some ability to cross membranes, as H_2O_2 can travel through aquaporins and superoxide can pass through anion channels[11,12]. However, the cytosol, IMS and matrix contain their own collections of antioxidant enzymes, which allow the independent control of redox homeostasis in these sub-compartments[13,14].

Antioxidant machinery

Scavenging of ROS is mediated by a set of ‘antioxidant’ enzymes that are expressed in various subcellular compartments. By degrading ROS, these systems limit their signalling effects and prevent oxidative damage. Superoxide dismutases (in the cytosol, the IMS and the matrix) redistribute electrons between two superoxide molecules to form hydrogen peroxide (FIG. 2). The degradation of hydroperoxides is achieved primarily by enzymes that supply electrons to reduce them to water. These enzymes include glutathione peroxidase[15], which acts in concert with glutathione, a tripeptide containing a reactive cysteine residue. Reduced glutathione (GSH) is present in millimolar concentrations in the cell and is used in the scavenging of ROS. Glutathione peroxidase reduces H_2O_2 to water while it oxidizes GSH to form a dithiol (GSSG). Oxidized glutathione that is generated by these reactions is sequestered within vacuoles to maintain a high GSH/GSSG ratio in the cytosol[16]. GSSG is subsequently reduced by glutathione reductase in the cytosol and the mitochondria, using electrons obtained from NADPH.

Peroxiredoxins[17,18] are a family of H_2O_2-scavenging enzymes that incorporate reactive cysteines, which become oxidized during the scavenging of H_2O_2; peroxiredoxins are re-reduced primarily by thioredoxins[19]. Oxidized thioredoxins in turn are restored to a reduced state by thioredoxin reductases, which use NADPH as the source of reducing equivalents.

NADPH has an important role in cancer cells because it is required for maintaining cellular redox balance. In certain cancer cells, futile redox cycling results in excessive consumption of NADPH, resulting in a chronic state of oxidative stress caused by limitations in the ability to maintain a sufficient pool of NADPH. Maintenance of NADPH levels in subcellular compartments is therefore essential for the regulation of redox in those environments. An important source of NADPH in the cytosol is the pentose phosphate pathway, which generates NADPH from the oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase (FIG. 2). This pathway diverts the glycolytic flux towards the synthesis of purines and the generation of NADPH in the cytosol. NADPH is also derived from folate metabolism involving methylene tetrahydrofolate oxidation to 10-formyl-tetrahydrofolate, generating NADPH. Recent studies suggest that this is a major source of NADPH in the cell[20]. In a separate study, Lewis *et al.*[21] compared the fate of serine conversion to glycine in mitochondria and cytosol, by following the fate of NADPH subsequently generated by methylene tetrahydrofolate oxidation. They found that this mechanism is primarily operative in the mitochondria. Other sources of mitochondrial NADPH include isocitrate dehydrogenase 2 (IDH2) and the transhydrogenase system that exchanges reducing equivalents between NADH and NADPH (FIG. 2).

Hydrogen peroxide that escapes clearance by antioxidant systems can affect cellular signalling, principally by causing protein thiol oxidation. Protein oxidation is normally reversed by thioredoxin, glutaredoxin[22] and sulphiredoxin[23]. These enzymes rely on thioredoxin reductase and GSH for reduction, both of which are in turn reduced by NADPH. The glutaredoxin and thioredoxin systems are expressed independently in the cytosol (GRX, TRX1 and TRX1 reductase) and the mitochondria (GRX2, TRX2 and TRX2 reductase), allowing independent regulation of redox status in these subcellular compartments[22]. Some tumours exhibit increased expression of TRX and TRX reductase, which may contribute to resistance to chemotherapies[24].

Some cancer cells upregulate their ability to deal with oxidative stress by augmenting their expression of enzymes responsible for degrading ROS. Nuclear respiratory factor 2 (NRF2) is a transcriptional activator of genes that mitigate oxidant stress; its activity is suppressed by kelch-like ECH-associated protein 1 (KEAP1). Some tumours exhibit constitutive activation of NRF2 (REF. 25), either through gain-of-function mutations or through inactivation of KEAP1. NRF2 gain-of-function confers resistance to therapies that act by augmenting oxidant stress[26,27]. In some tumours, inactivation of KEAP1 by post-translational modifications leads to the activation of NRF2 (REF. 28), which may help to enhance their resistance to chemotherapeutic agents that augment oxidant stress.

Although increases and decreases in antioxidant systems have been found in various tumour cell lines, a comprehensive study of redox status across a wide range of tumour cell types has not been carried out. Hence, our understanding of how redox shifts contribute to phenotype across a broad spectrum of tumour cell types is incomplete. Conceivably, selective pressures have led to the emergence of redox alterations that promote growth and survival in each cell line. When a better understanding of redox status is achieved across a wide range of tumour cell types, it may be possible to link redox disruptions with phenotypic behaviour, resistance to therapy, originating tissue, or metastatic potential.

Hypoxia and ROS generation in cancer

Although many tumours undergo a Warburg-type shift towards glycolytic metabolism[29], many still maintain substantial mitochondrial oxygen consumption. This oxygen consumption seems to represent a metabolic shift in glucose utilization towards biosynthetic and redox regulatory functions, rather than a compensation for dysfunctional oxidative phosphorylation. For example, using a novel fluorescent protein dual reporter to detect the activation of hypoxia-inducible factors (HIFs) and proliferation, Le *et al.*[30] detected a sub-population of non-cycling cells lacking HIF activation that had high expression levels of genes involved in mitochondrial function. These cells exhibited relatively high oxygen consumptions and mitochondrial capacity while they maintained the ability to form new tumours[30]. In general, many tumours develop profound hypoxia when growth outpaces their vascular supply. In the mitochondrial matrix, non-specific ROS generation decreases during hypoxia and increases when cells are made hyperoxic[31,32]. However, release of ROS from complex III to the IMS increases paradoxically during hypoxia[13,33]. Evidence for this response comes from a large number of studies from multiple laboratories using diverse methods to assess oxidant signalling[13,34–38], targeted antioxidant studies[39,40], genetic suppression or deletion models[31,36–38] and studies of *in vivo* tumour growth[39,41,42]. Although the specific mechanism has not been described, a likely source is the outer ubiquinone binding site (Qo) of complex III, in which lowered oxygen concentrations may prolong the lifetime of the semiquinone radical. This could enhance superoxide generation by augmenting the conditions favouring its generation, even though the oxygen concentration is lower[9]. Targeting a hydrogen peroxide scavenger specifically to the mitochondrial IMS abolishes hypoxia-induced increases in oxidant stress in that compartment and the cytosol, and abrogates the downstream stabilization of HIF1α in the cytosol[14]. The enhanced generation of mitochondrial ROS in the hypoxic tumour microenvironment represents an important mechanism promoting growth, metabolic reprogramming and survival, as these oxidants can act both as signalling molecules and by damaging DNA.

Mitochondrial DNA and ROS in cancer

Mitochondrial DNA as a target of ROS

In the context of cancer initiation, an important target of mitochondrial ROS is mitochondrial DNA (mtDNA), as mutations in mtDNA seem to be capable of promoting tumorigenesis[43,44]. Human mitochondria contain redundant copies of a circular loop of DNA comprised of ~16.6 kilobases, encoding 13 polypeptide components of the oxidative phosphorylation system (OXPHOS system), 12S and 16S ribosomal RNAs, and 22 transfer RNAs. The polypeptides include seven of the nearly 45 subunits of complex I, one subunit of complex III, three subunits of complex IV and two subunits of complex V, the ATP synthase. The remaining ~1,500 proteins in the mitochondria are encoded by nuclear DNA. Each mitochondrion potentially carries dozens of mtDNA copies and each cell contains scores of mitochondria, so each cell can potentially contain many hundreds of mtDNA copies. mtDNA is maternally inherited, and mutations in mtDNA that contribute to cancer

can either be inherited as germline mutations or appear as somatic mutations in specific tissues. Functionally, mutations can either be neutral, pathogenic or beneficial.

Unlike nuclear DNA, mtDNA is not protected by histones; therefore, the proofreading capacity is limited and ROS that is generated in the matrix can attack it[45]. Accordingly, the rate of mitochondrial mutation is much greater than for nuclear DNA[46]. In general, mutations can include deletions, insertions, point mutations and changes in mtDNA copy number. When a mutation arises, it can be passed to the daughter cells along with the normal mtDNA, resulting in a state of heteroplasmy. For reasons that are not fully understood, non-dichotomous segregation of mutant and wild-type mitochondria can occur during cell division, causing a subset of the tumour cell population to drift towards a state of homoplasmy, in which all mtDNA copies are of the mutant form. Both normal and cancer cell mitochondria seem to contain considerable amounts of homoplasmic and heteroplasmic mutations in mtDNA in different tissues of the same individual[47], although the importance of this in cancer is not fully clear.

mtDNA mutations and the generation of ROS in cancer

Alterations in mtDNA were described in leukaemic cells even before the age of DNA sequencing[48,49]. Germline and somatic mtDNA mutations have been linked to colorectal cancer[50], renal, lung, bladder, head and neck, gastric, thyroid, prostate and ovarian cancers, glioblastomas and hepatocellular carcinomas[51] and others, as reviewed previously[43,52]. There is good evidence suggesting that at least some mtDNA mutations are tumour-specific[43]. In some cases the somatic mtDNA mutations identified in tumours represent a natural progression that is synonymous with the neutral mutations that developed in the general population during human evolution[53]. It is also possible that these changes represent selection against detrimental mutations[54]. In support of this idea, a progressive shift in haplotype has been tracked in a patient with myelodysplastic syndrome, during progression to acute leukaemia. The slow progress of this transition made it possible to document a shift from the original germline haplotype to a heteroplasmic state, and finally to a homoplasmic state[55]. Similar shifts over time have also been noted in other studies[56]. Somatic mutations that are rarely seen in the general population are sometimes seen in tumours, suggesting that the functional changes they introduce may confer some beneficial effect on the tumour growth and survival[43].

More compelling evidence for the role of mtDNA mutations in cancer comes from the observation that the introduction of mutant mtDNA into a tumour cell line can alter its phenotype. Petros *et al.*[57] compared the *in vivo* growth of PC3 prostate cancer cells that were homoplasmic for the mtDNA mutation T8993G to those with wild-type mtDNA (T8993T). The T8993G mutation was previously shown to result in an amino acid substitution in complex V that significantly decreased its activity[58] and increased mitochondrial ROS generation[59]. The mutant mtDNA-containing cells grew faster than wild-type cells in immunocompromised mice, suggesting that mutations in mtDNA have the potential to alter the phenotype in an existing tumour cell line. Additional insight comes from the study of cybrids. These are cells generated by fusing enucleated cytoplasts containing mutant mitochondria with tumour cells that have been depleted of their

endogenous mtDNA (ρ^0 cells). The effects of different mitochondrial haplotypes on the tumorigenic behaviour of the cybrids can then be compared in the same nuclear genetic background. Shidara *et al.*[60] studied cybrid lines containing mutations in the mitochondrial ATP synthase subunit VI, T8993G and T9176C, and found that these mutations conferred a growth advantage and apoptotic resistance when grown in nude mice. Collectively, these findings indicate that at least some mitochondrial mutations can markedly increase the tumour phenotype, potentially through the generation of ROS.

How do mtDNA mutations contribute to tumorigenicity? Missense and nonsense mtDNA mutations can potentially alter the function of the ETC or the ATP synthase. One conceivable mechanism is that the mutation causes a decrease in mitochondrial function that promotes a Warburg-like shift towards glycolysis. However, there are clear counter-examples of that explanation[61]. More likely, the decrease in activity caused by the mutation produces a shift in mitochondrial membrane potential or the redox status of electron carriers upstream or downstream from the altered site. Changes such as these do not necessarily inactivate oxidative phosphorylation, but they may introduce shifts in function that alter ETC redox status and thus ROS generation. For example, mutations that decrease but do not abolish activity of complex IV can shift the reduction state of electron carriers upstream in the chain, thereby augmenting the probability of superoxide generation from complex I, II or III[60]. To the extent that the ROS generated by this change affects cytosolic redox signalling involved in the regulation of proliferation, this could amplify the growth rate. The magnitude of these effects in cells with heteroplasmic versus homoplasmic mtDNA is not known, but it seems likely that the answer would vary depending on the specific mutation involved.

A clear illustration of how alterations in mtDNA can drive tumorigenesis comes from the study of Woo *et al.*[42], who noted that mitochondrial transcription factor A ($Tfam$)$^{+/-}$ mice develop a modest decrease in mtDNA copy number and an increase in mtDNA oxidative damage, suggestive of increased basal mitochondrial ROS generation. When bred with the adenomatous polyposis coli (Apc)$^{Min/+}$ mouse tumour model, the mice developed increased numbers of intestinal tumours that grew faster. The tumour-promoting effect was linked to an increase in mitochondrial ROS by breeding the $Apc^{Min/+}$ mice with transgenic mice that overexpress catalase targeted to the mitochondrial matrix. Those mice showed fewer polyps in the intestine in association with a decrease in mitochondrial oxidant stress. These results provide an important link between mtDNA stability and tumorigenesis that is mediated by increases in mitochondrial ROS generation.

The understanding of how specific mtDNA alterations regulate the tumorigenic phenotype is still incomplete. Difficulty in addressing this question arises because it is often difficult to judge how a specific alteration affects mitochondrial function. One approach to solving this has been to introduce similar changes into simpler model systems that express orthologous complexes, and to use these to investigate function. For example, introduction of a G171D mutation in subunit I of the cytochrome oxidase in *Rhodobacter sphaeroides* produced a structural shift resulting in a decrease in activity arising from slowed intramolecular electron transfer, along with a specific proton leak across the inner membrane[62]. Another approach has been to replace the mtDNA in a cancer cell with a mutant form to determine the

consequences in terms of growth, survival or metastatic behaviour. For example, Ishikawa *et al.*[44] replaced the endogenous mtDNA in a poorly metastatic mouse tumour cell line (P29) with mtDNA carrying a loss-of-function mutation in complex I subunit 6 (NOD6) from a highly metastatic line (A11). This transfer conferred the highly meta-static phenotype to the recipient cells, in association with an increase in cellular ROS generation that was required for the altered metastatic behaviour. Examples such as this provide a proof-of-principle that some mtDNA mutations have the potential to shape tumour cell behaviour and survival, potentially through the generation of ROS. However, it seems likely that many other mutations, although correlated with oncogenic transformation, are merely innocent events that do not drive behaviour.

Mitochondrial ROS and genomic instability

Mitochondrial oxidants also contribute to genomic instability. In fibroblasts that are deficient in mitochondrial superoxide dismutase (SOD2; also known as MnSOD), Samper and colleagues[63] detected increases in double-strand breaks and chromosomal translocation, along with loss of cell viability and proliferative capacity. Mice lacking *Sod2* die shortly after birth, whereas heterozygotes (*Sod2*$^{+/-}$) survive but develop mammary tumours as they age[64]. In single-cell zygotes, Liu and colleagues[65] introduced mitochondrial dysfunction using the uncoupling agent carbonylcyanide-4-trifluorometh-oxyphenylhydrazone (FCCP) and observed increases in ROS, as well as telomere loss and chromosome damage that was prevented by antioxidants. Loss of caveolin 1, a structural component of membrane caveolae, occurs in response to transformation in cancer-associated fibroblasts, and its downregulation in breast cancer is a negative prognostic indicator. Loss of caveolin 1 in cancer-associated fibroblasts leads to mitochondrial dysfunction that is mediated by increased nitric oxide generation, leading to increased mitochondrial ROS generation, which in turn contributes to genomic instability in adjacent cancer cells[66]. In patients with Fanconi anaemia, a genetic disorder that is associated with bone marrow failure and increased risk of cancer, increased levels of DNA oxidative damage correlate with increased generation of ROS, apparently from mitochondria[67]. In lymphocytes from patients with Fanconi anaemia, Ponte *et al.*[68] found that antioxidants significantly improved genomic stability. Finally, upregulation of matrix metalloproteinase 3 (MMP3) is frequently observed in cancer. It induces epithelial–mesenchymal transition (EMT), and its expression in transgenic mice leads to the formation of mammary tumours with genomic instability. Interestingly, MMP3 induces the expression of an alternatively spliced form of the small GTPase RAC1, which stimulated ROS generation from the mitochondria[69]. The resulting oxidant stress then contributes both to signalling by induction of the transcription factor SNAIL (also known as SNAI1), and to genomic instability. Collectively, these studies reveal that mitochondrial oxidants in cancer cells have the capacity to enhance the tumour phenotype by inducing genomic damage, and that mitochondrial oxidants in other disorders can promote oncogenic transformation, EMT and other tumour-inducing behaviours through the induction of genomic instability.

ROS as death inducers in tumour cells

Excessive mitochondrial oxidant stress can induce cell death, both in tumours and in healthy cells. However, the mechanism underlying this process has been controversial, with some studies implicating apoptosis and others involving the mitochondrial permeability transition pore (MPTP). Evidence for the involvement of apoptosis comes from studies showing that cytochrome *c* is released from the mitochondria to the cytosol after inducing oxidant stress, and that caspase activation ensues[70]. Other studies implicate the MPTP, a small yet incompletely defined channel that permits the transit of small solutes (<1.5 kDa) from the mitochondrial matrix to the cytosol[71]. Its molecular characterization has been controversial but, minimally, it seems to include a dimer of complex V and cyclophilin D[72]. Opening of the MPTP is crucial for cell death in ischaemia–reperfusion injury in the heart, as genetic deletion of cyclophilin D confers significant protection[73]. In the heart, reperfusion after an ischaemic insult causes the generation of excessive mitochondrial ROS[74]. These oxidants synergize with Ca^{2+} to cause opening of the MPTP, which depolarizes the mitochondria and permits redistribution of solutes such as NAD^+ and NADH to escape to the cytosol[75]. ROS reaching the nucleus cause DNA damage, leading to poly(ADP-ribose) polymerase (PARP) activation and DNA repair. If left unchecked, however, PARP activity consumes cytosolic and/or mitochondrial NAD^+, which halts glycolysis and causes a lethal bioenergetic crisis[75]. Many hours later, mitochondrial swelling in the injured cells triggers mitochondrial outer membrane rupture and cytochrome *c* release, but that process occurs in a cell that is already committed to death by a different pathway[75]. Indeed, excessive oxidant stress in cultured cells also causes MPTP opening as well as cytochrome *c* release, and significant protection is conferred by PARP inhibition. However, loss of either BAX or cytochrome *c* expression confers no protection in that model[74]. Collectively, these findings suggest that excessive mitochondrial oxidant stress triggers a death pathway involving MPTP activation. Although later release of pro-apoptotic factors from the mitochondria may occur, this represents a late event whose inhibition is not required for the death response. In tumours, a similar cell death process may occur when intermittent blood flow causes transient ischaemia in the interior core, followed by reperfusion and an associated burst of mitochondrial ROS generation[76]. This occurrence may help to explain why some tumours demonstrate necrotic cores even though genetic mutations may have inactivated their ability to undergo apoptosis.

ROS as signalling agents in cancer

ROS and signalling

Oncogenic transformation is frequently associated with a shift in cytosolic thiol redox balance to a more oxidized state, which may enhance the proliferative phenotype[77]. This shift may also contribute to genomic instability, as ROS in the cytosol can enter the nucleus during DNA replication to cause additional mutations. Although excessive oxidant stress is lethal, low levels of mitochondrial ROS have crucial roles in signalling and the regulation of protein function. Important targets of ROS signalling include reactive cysteine groups on proteins, which can be oxidized by H_2O_2 but not by superoxide. Protein thiol oxidation can cause conformational and functional changes and can also result in intra- or inter-molecular dithiol linkages. An important group of proteins subject to ROS attack are the lipid and

protein phosphatases, which are inactivated by oxidation of the reactive cysteine thiol at their catalytic site[78,79]. Oxidative inactivation of phosphatases can cause major changes in the phosphorylation of protein targets of the MAPK–ERK and AKT kinase pathways that contribute to the proliferation and survival of cancer cells. In a broader sense, it is possible that the effects of genetic mutations in certain genes could be mimicked in tumour cells that develop continuous increases in basal ROS generation. For example, the continuous oxidation-mediated inactivation of PTEN by constitutive increases in ROS generation could potentially mimic the phenotypical changes induced by genetic inactivation of that gene. In that regard, oxidant stress-induced enzyme dysfunction could synergize with genetic mutations to amplify tumorigenic behaviour in some cancer cells. This might explain why growth inhibition in response to antioxidant treatment has been observed in some studies[80].

ROS and hypoxia

The hypoxic environment within tumours promotes the stabilization of HIFs[81–83]. Destabilization of the HIFα subunits of HIF1 and HIF2 is mediated by prolyl hydroxylases (PHDs), which hydroxylate conserved proline residues located within an oxygen-dependent degradation domain[84] in a 2-oxoglutarate- and oxygen-dependent reaction[85,86]. That modification then facilitates interaction with the E3 ubiquitin ligase, von Hippel–Lindau tumour suppressor protein (VHL), targeting the HIFα subunit for proteasomal degradation[87]. Whereas PHD activity is abolished in the absence of oxygen (anoxia), regulation of PHD activity under more physiological hypoxia is regulated by a paradoxical increase in ROS generation by mitochondria[33,88]. During physiological hypoxia, ROS originate from complex III (FIG. 2), as knockdown of the Rieske iron–sulphur protein (UQCRFS1), a subunit required for electron flux in that complex, attenuates the hypoxia-induced ROS signalling and the inhibition of PHD activity and HIF1α stabilization in hypoxia[36,37,89]. However, exogenous ROS can rescue HIF1α stabilization in the absence of the complex. Hypoxia-induced mitochondrial ROS signals are released from the Qo site of complex III to the mitochondrial IMS, and they subsequently enter the cytosol. Accordingly, fluorescent redox reporter sensors targeted to the mitochondrial matrix, the IMS or the cytosol detect increases in protein thiol oxidation in the IMS and the cytosol during hypoxia, while oxidant stress in the matrix simultaneously decreases[14]. Expression of a peroxide scavenger, peroxiredoxin 5, within the IMS attenuates hypoxia-induced thiol oxidation in that compartment and in the cytosol, while it also attenuates hypoxia-induced HIF1α stabilization[14]. These observations demonstrate that during hypoxia, ROS that are released from the inner mitochondrial membrane cross the IMS to reach the cytosol, where they inhibit PHDs and thereby contribute to the activation of HIF transcription factors. Although the mechanism of PHD inhibition is not known, it may involve oxidation of the Fe^{2+} within PHDs. Importantly, hypoxia-induced ROS signalling by mitochondria is a characteristic of both primary and malignant cells[14,31] and seems to function as a normal oxygen-sensing mechanism[9]. Hence, the enhancement of HIF activation by ROS signalling within the tumour is an example of how mitochondria carrying out their normal functions are co-opted by the tumour cells to promote growth and survival through the induction of HIF target genes.

Although oxidant stress in malignant cells probably contributes to the acquisition of additional mutations, some studies suggest that the amplifying effects on HIF regulation are also important. In a study assessing genomic instability in tumours, Gao *et al.*[80] administered *N*-acetyl cysteine (NAC) in a mouse model of MYC-dependent human B cell lymphoma and found that growth was inhibited, whereas genomic instability was unaffected. The beneficial effect of NAC was traced to its inhibition of HIF activation in a PHD- and VHL-dependent manner. Moreover, expression of an oxygen-independent mutant HIF1α abolished the protective effect of NAC. These findings reveal the importance of hypoxia-induced ROS for the regulation of hypoxia-induced responses that determine the tumour phenotype.

Pseudohypoxic activation of HIF in cancer

HIFα stabilization occurs when PHD activity declines, as occurs during hypoxia. Pseudohypoxic stabilization of HIFα refers to conditions in which PHD is inhibited not by a lack of O_2, but rather by a lack of 2-oxoglutarate, or chelation of its adducted iron, or by pharmacological inhibitors such as dimethyloxallyl-glycine (DMOG) (FIG. 3). Low levels of exogenous ROS can also inhibit PHD during normoxia, presumably by oxidizing its iron to the ferric state[36]. Succinate, a TCA cycle intermediate with structural similarities to 2-oxoglutarate, can also theoretically inhibit PHD[90]. Depending on their mechanism of action, pseudohypoxic activators can also trigger other responses that can alter the phenotype of tumour cells (FIG. 3).

Succinate dehydrogenase (SDH) is a TCA cycle enzyme comprised of four nuclear-encoded subunits (A–D). This heterotetramer links the TCA cycle to the ETC by oxidizing succinate to fumarate and passing the electrons to ubiquinone, a mobile electron carrier that delivers them to complex III (FIG. 2). Mutations in the A subunit of SDH (SDHA) diminish enzymatic activity and thereby impair mitochondrial oxidative phosphorylation. The mutation-induced loss of function causes a bioenergetic deficiency that manifests as encephalopathy, myopathy and Leigh syndrome[91]. However, with one exception[92], SDHA mutations have not been associated with cancer. By contrast, mutations in the SDHB, SDHC or SDHD subunits produce a tumorigenic phenotype that is associated with pheochromocytomas and paragangliomas in humans[93–96]. Paragangliomas are benign, highly vascular tumours the incidence of which is increased in populations living at high altitude[97]. It was suggested that succinate accumulation caused by decreased SDH activity inhibits PHD, thereby stabilizing HIFα[90]. But that cannot explain why HIF is activated by SDHB, SDHC or SDHD subunit mutations but not by those in SDHA. A more likely mechanism is that defects in the SDHB, SDHC or SDHD subunits trigger HIFα by augmenting ROS generation at the SDHA subunit[98]. SDHA catalyses the oxidation of succinate to fumarate, using an FAD group as an electron carrier. Other electron carriers within SDH include three iron–sulphur clusters and a haem moiety in the SDHB, SDHC and SDHD subunits. Mutations in the SDHA subunit interfere with catalytic activity and prevent oxidation of succinate. However, if SDHA is normal and there are defects in the SDHB, SDHC or SDHD subunits, then the oxidation of succinate proceeds but the shuttling of electrons within the complex to the electron carrier ubiquinone is impaired[28,98]. Hence, the electron obtained from succinate remains on the FAD group, where it can react with O_2 to

form superoxide. That event clears the FAD group and permits SDHA to oxidize another succinate, creating a futile redox cycle yielding superoxide. Consistently, Guzy and colleagues[98] showed that genetic suppression of SDHB but not SDHA expression results in an increase in ROS signalling in tumour cells, leading to an increase in HIF1α stabilization, an increase in cell proliferation and increased tumour xenograft growth. Cells with stable suppression of SDHB exhibited HIF1α stabilization in normoxic conditions and this suppression was inhibited by antioxidants, whereas no increase in HIF1α was observed in cells with SDHA knockdown. Interestingly, mutations in SDHD have also been shown to contribute to genomic instability through the increased generation of superoxide and H_2O_2 (REF. 99). This provides an attractive mechanism to explain how a mutation in a single gene encoding SDHB, SDHC, or SDHD could, over time, lead to the ROS-mediated accumulation of additional genomic mutations resulting in the tumour phenotype. In a broader sense, it shows how a somatic or germline mutation could cause a small but continuous increase in ROS generation that undermines genomic stability progressively over time, thereby pushing the cell (or cells) towards neoplastic transformation.

A parallel story has emerged with respect to fumarate hydratase (FH), a TCA cycle enzyme that converts fumarate to malate (FIG. 4). Humans with mutations in *FH* are predisposed to hereditary leiomyomatosis and renal clear cell cancer (HLRCC)[100]. Individuals with a germline mutation in one allele of *FH* typically undergo somatic loss of heterozygosity, often in the kidney. Tissue analysis in HLRCC tumours revealed excessive HIF1α levels in the cytosol and nucleus[101], consistent with the idea that biallelic loss of *FH* contributes to tumour formation through the activation of HIF1 (REF. 102). Increased oxidant stress seems to explain this phenotype. Loss of FH activity abolishes oxidative phosphorylation, forcing the cells to rely entirely on glycolysis for energy production[103]. Although the TCA cycle is blocked at FH, it can still operate in reverse to metabolize glutamate to citrate by reductive carboxylation[104]. This reaction yields citrate, which is exported to the cytosol for use in the biosynthesis of lipids[105]. TCA cycle inhibition in this manner can potentially increase the generation of ROS by halting the flux of electrons at iron–sulphur centres or flavin groups. Trapped in the ‘traffic gridlock’, these electrons may be captured by O_2 to generate superoxide that is sub-sequently dismuted to H_2O_2. Indeed, Sudarshan *et al.*[103] found that ROS levels were constitutively elevated in HLRCC-derived tumour cells and in renal epithelial cells in which FH had been genetically suppressed. Importantly, the increase in HIF1α was dependent on ROS generation, presumably because oxidants inhibit the activity of PHDs.

Further insight into the mechanism by which FH loss augments ROS signalling and HIF activation comes from Sullivan *et al.*[28], who observed that mitochondria lacking FH oxidize glutamine to fumarate in the TCA cycle. As fumarate accumulates, it reacts with GSH to yield succinated glutathione (GSF), a cancer-associated metabolite that is subsequently metabolized by glutathione reductase at the expense of NADPH. As NADPH levels become depleted by the recursive generation of GSF, the ability of glutathione reductase to reduce GSSG becomes compromised. This causes a redox shift in the cell towards a more oxidized state, leading to an increase in ROS-dependent signalling, PHD inhibition and HIF1α stabilization[28]. Thus, fumarate constitutes a proto-oncometabolite by undermining cellular

antioxidant defences, thereby favouring oxidant-driven pathways that are involved in tumorigenic and metastatic behaviour. These findings reveal that disrupted mitochondrial function can promote tumour progression through the activation of HIF in a ROS-dependent manner.

Oncogenes and mitochondrial ROS

Oncogenic transformation promotes tumorigenesis by increasing mitochondrial ROS generation[106]. For example, KRAS activation increases oxidant stress in the mitochondrial matrix as detected by a fluorescent redox sensor[39]. Mitochondria-targeted nitroxide scavengers of superoxide attenuated the mitochondrial ROS levels and abolished anchorage-independent cell growth, compared to cells treated with chemically similar untargeted nitroxides. The mitochondria-targeted antioxidants also induced cell proliferation arrest. These findings link the matrix ROS generation with the ability to proliferate in soft agar[39].

To determine the mitochondrial site that is responsible for ROS generation, 143B osteosarcoma ρ^0 cells were reconstituted with either normal mitochondria or with others containing a mutation in the mtDNA-encoded cytochrome *b* gene[39]. The ρ^0 cells lack mtDNA, their ETC is dysfunctional, they do not respire, and their mitochondrial ROS levels were undetectable. The cytochrome *b* mutants also lacked respiration but could still generate ROS because they sustain electron transport into complex III. Unlike ρ^0 cells, the cytochrome *b* mutants demonstrated anchorage-independent cell growth, which was abolished by genetic suppression of complex III. To test this idea *in vivo*, these investigators used an inducible KRAS-driven mouse model of lung cancer (LSL-*Kras*-G12D$^{+/fl}$ mice) combined with genetic deletion of *Tfam.* The loss of TFAM leads to loss of mtDNA, mitochondrial transcription and inactivation of the ETC. In that model, loss of *Tfam* led to a decrease in the number and size of lung tumours and in tumour cell proliferation. These results underscore the importance of the ETC and mitochondrial ROS for the growth and proliferation in KRAS-G12D-induced tumorigenesis.

Other oncogenes also manipulate mitochondrial ROS generation to support proliferation and survival. The transcription factor MYC is activated by extra-cellular mitogenic signals that regulate the MAPK–ERK signalling and normal cell proliferation[107]. In lymphomas, oncogenic MYC activation causes an acceleration of mitochondrial glutaminolysis[108], enhanced mitochondrial biogenesis[109], and activation of lactate dehydrogenase A (LDHA) expression[110] to drive glycolytic metabolism. These changes are associated with an increase in ROS signalling that renders the cells more susceptible to exogenous oxidant stress[77]. These ROS signals also contribute to proliferation by activating transcription factors such as HIF or nuclear factor-κB[111], and by activating AKT, possibly through the oxidative inactivation of the phosphatase PTEN[79].

Summary and therapeutic implications

Are mitochondrial ROS the initiators, amplifiers or the Achilles' heel in cancer? The answer is all of the above. Mitochondrial ROS can alter cellular redox regulation, induce nuclear DNA and mtDNA damage and influence the activation of cancer-promoting transcription factors, thereby contributing in diverse ways to the initiation and progression of cancer. At

least some cancer cells exhibit increased basal levels of oxidant stress. This oxidative shift may render the cells vulnerable to chemotherapeutic agents that act by augmenting oxidant generation[112] or inhibiting antioxidant capacity[113]. Indeed, the selective toxicity of some chemotherapeutics in cancer cells relative to normal cells may arise because the former sit closer to the 'precipice', in terms of their ability to defend against excessive oxidant stress. Given the importance of mitochondrial ATP production in normal tissues, pharmacological inhibition of the ETC as a means of limiting ROS generation represents an unlikely therapeutic target for the treatment of cancer. However, many cancer cells benefit from mitochondrial ROS generation through its effects on redox signalling. Therefore, one possibility would be to develop small molecules that interfere with the ability of mitochondria to release oxidant signals, thereby preventing their activation of protective mechanisms such as HIF and AMP-activated protein kinase (AMPK). Clinical trials of 'antioxidants' have been uniformly unsuccessful in the prevention[114] or the treatment of cancer[115]. This is perhaps not surprising, as the broad range of redox-dependent processes in cells make it just as likely that non-specific antioxidants will disrupt as protect a cell. However, it is conceivable that therapeutics aimed at intercepting the redox signals between the mitochondria and the host cell could be achieved without disrupting oxidative phosphorylation, thereby inactivating an important aspect of the communication between them[116]. Such an approach might not be sufficient as a single agent, but it could help to 'tip the scale' in the battle between the patient and the cancer cells by limiting the ability of mitochondria to function as initiators or amplifiers, thereby turning them into 'innocent bystanders'.

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Glossary

Mitochondria	Organelles within eukaryotic cells that participate in energy production, biosynthetic processes, redox regulation, cell survival, signalling and cell death pathways
ATP	(Adenosine triphosphate). A high-energy molecule that is hydrolysed by enzymes to provide the exergonic free energy required to carry out endergonic reactions
Hypoxia	A condition in which the molecular oxygen concentration is decreased relative to normal physiological levels
Reactive oxygen species	(ROS). Reactive molecules generated by the reduction of O_2 with a single electron (superoxide), two electrons (hydrogen peroxide) or three electrons (hydroxyl radical)
ROS signalling	(Reactive oxygen species signalling). A cellular signal transduction mechanism involving oxidation–reduction reactions, usually resulting in a reversible alteration of protein structure and function that elicits a subsequent cellular response. ROS signalling

	frequently involves redox alterations of cysteine thiol (SH) groups in proteins
Free radical	A molecule or atom containing an unpaired valence electron that renders it chemically reactive. Free radicals can potentially oxidize or reduce other molecules
Tricarboxylic acid cycle	(TCA cycle). A system within mitochondria that participates in intermediary metabolism involved in energy production, inter-conversion of metabolites, and synthesis of small molecules needed for lipid or protein synthesis
NADPH	A cofactor that is used by enzymes mediating electron transfer steps in energy production, lipid and nucleic acid synthesis, and the maintenance of intracellular oxidation–reduction status
Superoxide dismutases	A family of enzymes that redistribute electrons between two superoxide anions to form a single molecule of hydrogen peroxide
Hypoxia-inducible factors	(HIFs). A family of heterodimeric transcription factors that become activated during hypoxia or pseudohypoxia in a cell, and are responsible for potentially altering the expression of hundreds of genes involved in regulating cellular responses to hypoxia
Mitochondrial DNA	(mtDNA). Circular loops of DNA containing ~16.6 kilobases, located in the matrix of mitochondria. This DNA encodes 13 proteins, ribosomal RNAs and transfer RNAs that are required for a functional oxidative phosphorylation system
Cybrids	Experimental cells that are formed by fusing a cell lacking mitochondrial DNA with an enucleated cytoplast containing mutant mitochondria
Pseudohypoxic activators	Stimuli that trigger activation of cellular responses to hypoxia, even though the O_2 level in the cell is normal
AMP-activated protein kinase	(AMPK). A complex consisting of three proteins that has a central role in the regulation of cellular energy production and energy utilization

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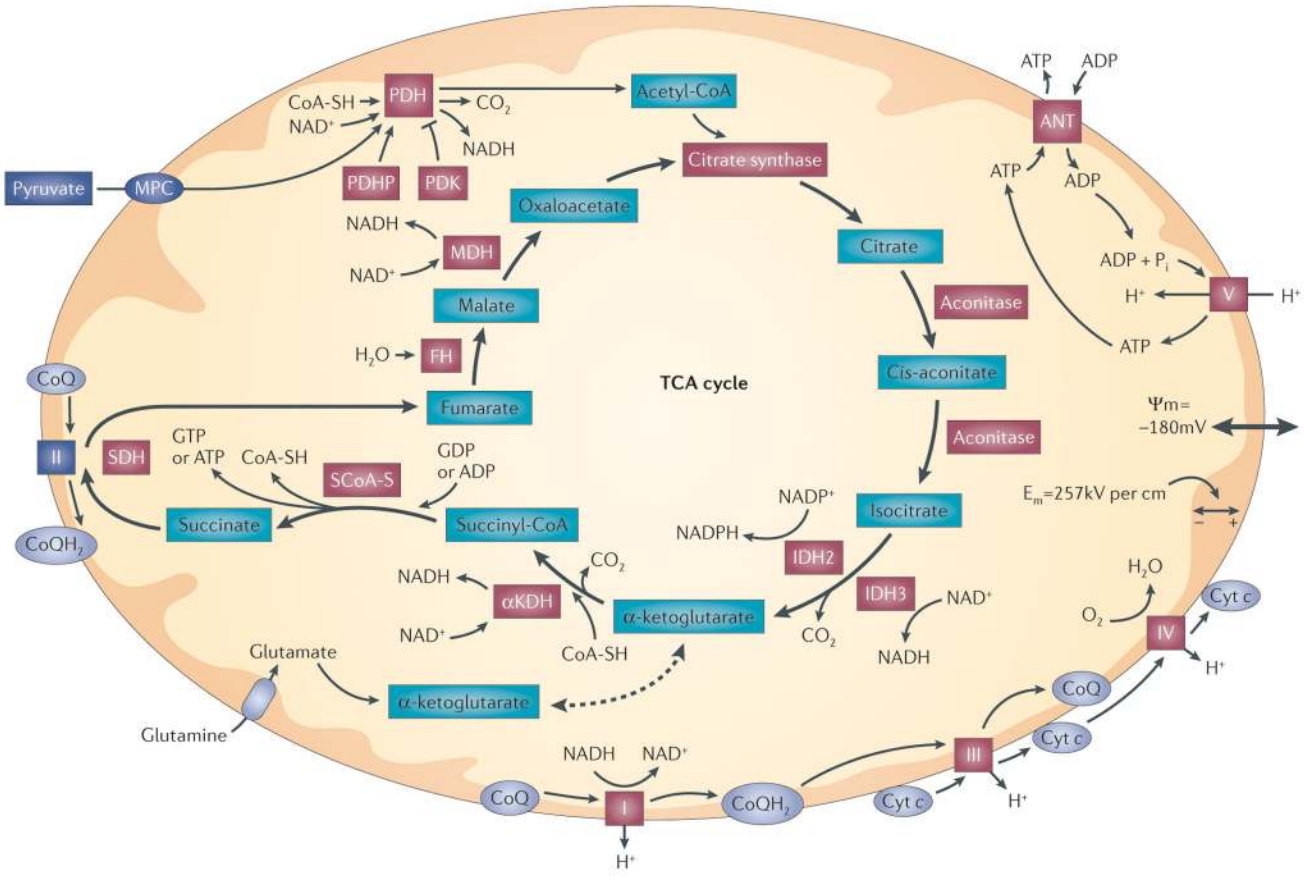

Figure 1. Mitochondrial bioenergetic function

Pyruvate enters mitochondria via the mitochondrial pyruvate carrier (MPC), where it is decarboxylated and oxidized to acetyl-CoA by pyruvate dehydrogenase (PDH). Acetyl-CoA enters the tricarboxylic acid (TCA) cycle at citrate synthase. Subsequent steps in the cycle lead to the generation of reducing equivalents (NADH and NADPH) at dehydrogenase steps. Amino acids can also enter the TCA cycle by conversion to α-ketoglutarate, succinyl-CoA, fumarate, oxaloacetate or acetyl-CoA. For example, glutamine enters the TCA cycle after conversion to glutamate and subsequently to α-ketoglutarate. Reducing equivalents generated by the TCA cycle enter the electron transport chain and are eventually transferred to O_2. Reducing equivalents generated at complex I (NADH–ubiquinone oxidoreductase) and complex II (succinate dehydrogenase (SDH)) are transferred to complex III by ubiquinol ($CoQH_2$), a lipophilic quinone that carries a pair of electrons within the membrane. Electrons are transferred between complex III and complex IV by cytochrome *c* (cyt *c*), which carries a single electron coordinated to a haem group. Electrons that are transferred to complex IV (cyt *c* oxidase) are sequentially transferred to O_2, generating H_2O. Electron transfer steps at complexes I, III and IV are associated with proton translocation from the matrix to the intermembrane space, resulting in the generation of an electrochemical gradient across the inner membrane (ΔΨm). Complex V (ATP synthase) uses this gradient to catalyse the phosphorylation of ADP, yielding ATP. Exchange of ATP for ADP across the inner membrane is mediated by the adenine nucleotide transporter

(ANT). Increases in ADP availability (as a result of cellular metabolic activity) tend to decrease ΔΨm slightly, which facilitates electron transfer at the steps involving proton extrusion. Hence, increases in cellular use of ATP cause an increase in mitochondrial respiration and ATP synthesis. By contrast, when ATP utilization falls, complex V activity decreases and the electron transport flux and oxygen consumption fall as a consequence of an increase in ΔΨm. Red highlighted proteins are known targets of reactive oxygen species (ROS). αKDH, α-ketoglutarate dehydrogenase; E_m, electrical field within the membrane; FH, fumarate hydratase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; PDHP, pyruvate dehydrogenase phosphatase; PDK, pyruvate dehydrogenase kinase; P_i, inorganic phosphate; SCoA-S, succinyl-CoA synthase; SH, cysteine thiol.

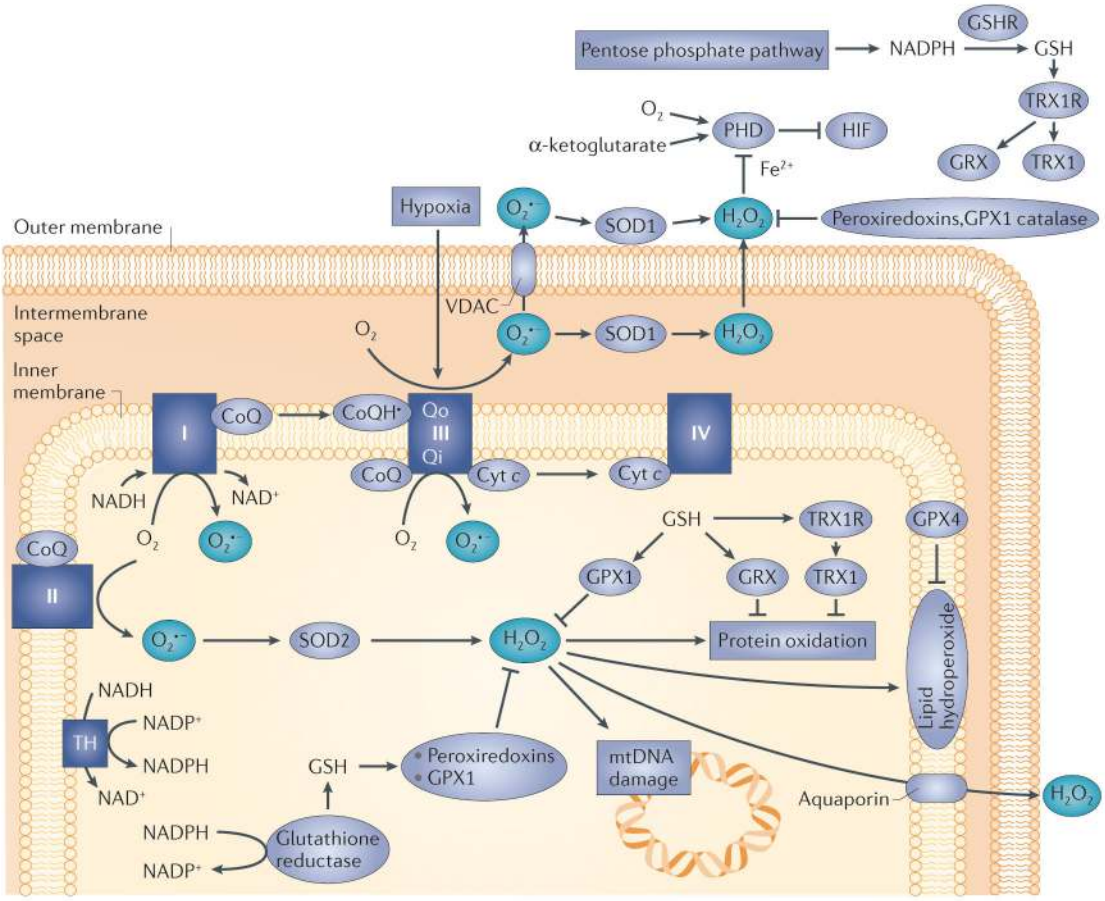

Figure 2. Mitochondrial reactive oxygen species (ROS) generation

Electrons derived from the oxidation of metabolic intermediates can lead to the generation of ROS at specific sites in mitochondria. Transfer of a single electron to O_2 yields superoxide ($O_2^{\bullet -}$) which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase in the matrix (SOD2; also known as MnSOD), or in the intermembrane space (SOD1; also known as CuZn–SOD). The H_2O_2 is degraded in the matrix by glutathione peroxidase 1 (GPX1) or peroxiredoxins (PRDX3 or PRDX5) using reducing equivalents obtained from the oxidation of reduced glutathione (GSH)[22–24]. Oxidized glutathione (GSSG) is reduced by glutathione reductase, which obtains its equivalents from NADPH oxidation. H_2O_2 generated in the matrix can oxidize proteins, lipids or mitochondrial DNA (mtDNA). Oxidized proteins are repaired by thioredoxin 2 (TRX2) or glutaredoxin (GRX). TRX2 and GRX are subsequently reduced by thioredoxin reductase 2 or by glutathione. Lipid hydroperoxides are reduced by GPX4. Ultimately, all ROS removal depends on the availability of GSH, which is maintained by the availability of NADPH in the respective compartments. H_2O_2 can potentially leak to the intermembrane space and the cytosol when excessive ROS generation occurs or antioxidant mechanisms fail. Complexes I, II and III can potentially generate ROS in the matrix compartment. Superoxide can be released to the intermembrane space from complex III, owing to generation from ubisemiquinone at the outer ubiquinone binding site (Qo) of complex III. The electrical gradient across the inner membrane (−180 mV) creates a strong electrical field within the membrane (257 kV per cm)

that accelerates superoxide anions from the membrane into the intermembrane space. Paradoxically, cellular hypoxia augments the rate of ROS generation at that site, leading to the production of H_2O_2 in the intermembrane space[13,14,33,36,37]. Subsequent diffusion to the cytosol triggers redox-dependent inhibition of prolyl hydroxylases (PHDs), negative regulators of hypoxia-inducible factor-α (HIFα) stabilization. Thus, mitochondria-derived ROS can promote cancer initiation through oxidative stress, and cancer cell progression through the activation of transcription by HIF. CoQ, ubiquinone; cyt *c*, cytochrome *c;* GSHR, glutathione reductase; Qi, inner ubiquinone binding site on complex III; TH, mitochondrial *trans*-hydrogenase; TRX1R, TRX1 receptor; VDAC, voltage-dependent anion channel.

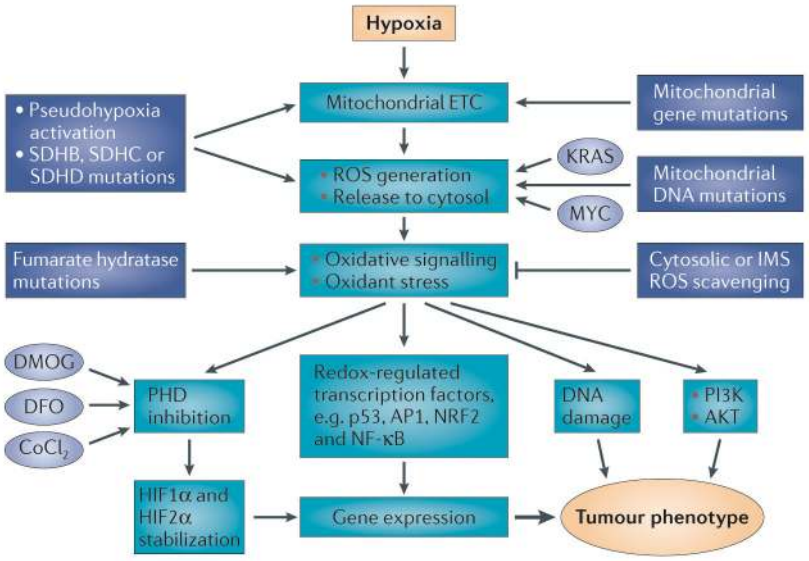

Figure 3. Hypoxia and pseudohypoxia activate mitochondrial reactive oxygen species (ROS) generation and oxidant signalling that drives the tumour cell phenotype

Hypoxia triggers ROS generation by the mitochondrial electron transport chain (ETC), leading to activation of hypoxia-inducible factor 1 (HIF1) and HIF2 through prolyl hydroxylase (PHD) inhibition[88]. Hypoxia also activates other responses and transcription factors through ROS-dependent signalling. Pseudohypoxia mimetics trigger the effects of hypoxia by activating or inhibiting steps in the hypoxia response pathway. Oncogenes including *KRAS* and *MYC* can further drive ROS generation. Mutations in mitochondrial DNA (mtDNA)- or nuclear DNA-encoded proteins can augment mitochondrial ROS generation and mimic the effects of hypoxia[57,59,63]. Succinate dehydrogenase (SDH) mutations in subunits SDHB, SDHC or SDHD augment ROS generation from the ETC. Fumarate hydratase loss-of-function mutations lead to oxidative stress that activates redox signalling. Cobalt chloride ($CoCl_2$) increases oxidant generation by redox cycling, thereby augmenting ROS generation[33]. Depending on the site of action, pseudohypoxic activators may only mimic some of the responses seen during authentic hypoxia. For example, chemical inhibitors of PHD (dimethyloxallyl glycine (DMOG) and desferrioxamine (DFO)) only trigger the activation of HIF1 and HIF2 without activating other pathways. AP1, activating protein 1; IMS, intermembrane space; NF-κB, nuclear factor-κB; NRF2, nuclear respiratory factor 2.

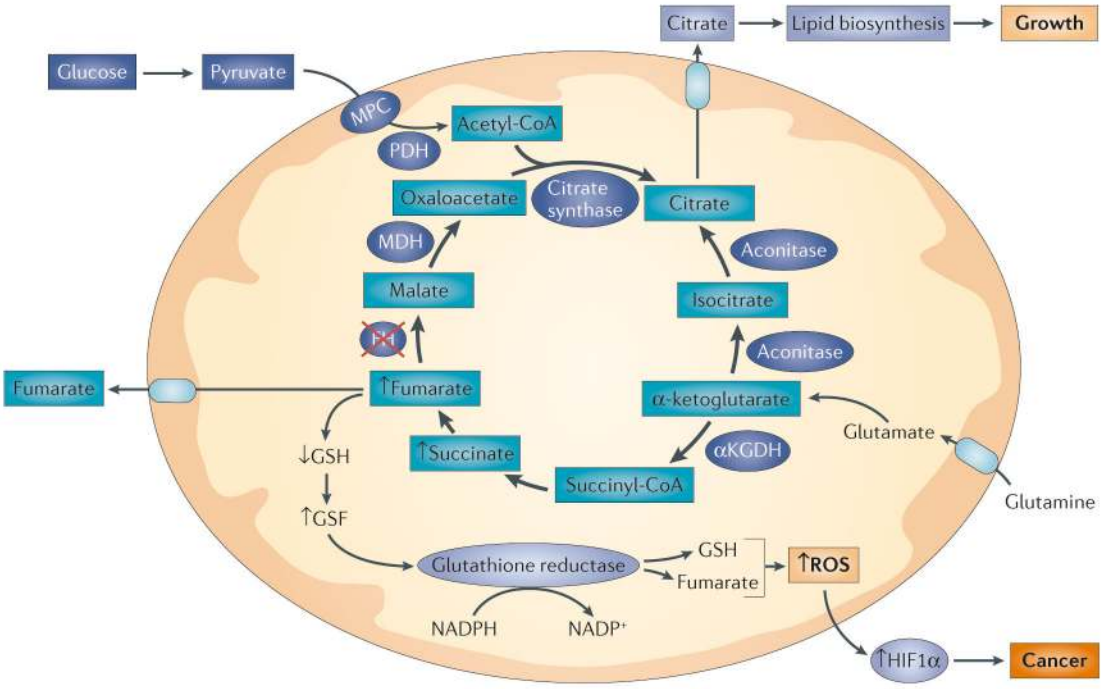

Figure 4. Mutations in tricarboxylic acid (TCA) cycle enzymes drive tumour cell progression through the generation of oxidant signalling

Inactivating mutations in fumarate hydratase (FH) block TCA cycle function, causing accumulation of fumarate and succinate. Fumarate reacts with reduced glutathione (GSH) to produce succinated glutathione (GSF), an oncometabolite that is degraded by glutathione reductase at the expense of NADPH[28]. Released GSH is then available to recombine with fumarate in a futile cycle that consumes NADPH. This impairs the reactive oxygen species (ROS) detoxifying capacity of mitochondria, leading to an increased ROS release to the cytosol that inhibits prolyl hydroxylase and increases the stabilization of hypoxia inducible factor 1α (HIF1α). Mitochondria continue to metabolize α-ketoglutarate to produce citrate through reverse carboxylation[104]. The supply of α-ketoglutarate is maintained by glutamate derived from glutamine deamination. Citrate that is produced by this reverse operation is then exported to the cytosol, where it is used to generate acetyl-CoA that is needed for lipid biosynthesis. Thus, TCA cycle inhibition can produce diverse effects in a tumour cell by driving ROS-dependent and ROS-independent signalling. αKGDH, α-ketoglutarate dehydrogenase; Acon, aconitase; KEAP1, kelch-like ECH-associated protein 1; MDH, malate dehydrogenase; MPC, mitochondrial pyruvate carrier; NRF2, nuclear respiratory factor 2; PDH, pyruvate dehydrogenase.