Regulation of mitochondrial oxidative phosphorylation through cell signaling

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Abstract

The mitochondrial oxidative phosphorylation (OxPhos) system plays a key role in energy production, the generation of free radicals, and apoptosis. A lack of cellular energy, excessive radical production, and dysregulated apoptosis are found alone or in combination in most human diseases, including neurodegenerative diseases, stroke, cardiovascular disorders, ischemia/reperfusion, and cancer. In the context of its relevance to human disease, this article reviews current knowledge about the regulation of OxPhos with a focus on cell signaling and discusses identified phosphorylation sites with the aid of crystal structures of OxPhos complexes. Several recent studies have shown that all OxPhos components can be phosphorylated; even the small electron carrier cytochrome c is tyrosine phosphorylated in vivo. We propose that in higher organisms, in contrast to bacteria, cell signaling pathways are the main regulator of energy production, triggered for example by hormones. Pathways that have been identified to act on OxPhos include protein kinases A and C and growth factor activated receptor tyrosine kinase signaling. Present knowledge about kinases and phosphatases that execute signals at the level of the mitochondrial OxPhos system, and newly emerging concepts, such as the translocation of kinases to the mitochondria upon stimulation of a signaling pathway, are discussed.

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1. Introduction

Energy homeostasis is a main determinant of survival in all higher organisms. It has been estimated that an average person consumes 100 kcal/h at rest, roughly corresponding to the energy output of a 100 W light bulb or the production of about 65 kg of ATP/day [1]. During exercise these values can increase many fold. The initial pathway for breaking down carbohydrates from food to pyruvate via glycolysis in the cytosol produces only a small amount of ATP, whereas the oxidation of pyruvate in the mitochondria provides more than 15 times the ATP produced by anaerobic glycolysis [2]. In contrast to many unicellular organisms including yeast, which have evolved mechanisms allowing them to grow and survive in the absence of oxygen, animals fully rely on cell respiration. This is so because the roughly 10¹⁴ cells present in a human are not only highly specialized and energy expensive but cell density is also high, requiring efficient delivery of food and oxygen and removal of carbon dioxide through the body’s circulation. Thus, provision of a constant energy supply through glycolysis alone is not feasible in animals under healthy conditions (theoretically, one would have to eat constantly), but there are conditions in which energy is primarily derived from glycolysis, such as seen in the fetus during the first half of the pregnancy [3] and in cancer, where it is known as the Warburg effect [4,5]. In both examples, the rest of the body provides glucose and disposes of the end product of the glycolytic pathway, lactate, to sustain growth as long as possible. For its own survival and that of the mother, the fetus later must switch to aerobic energy production, whereas cancer if not stopped would eventually starve its host to death.

Cell respiration takes place in the mitochondria, organelles the size of bacteria (2–4 μm). The number of mitochondria varies significantly among cell types. For example it was estimated that mammalian hepatocytes and oocytes contain 800 and 100,000 mitochondria, respectively [6]. Mitochondria have two membranes and contain their own DNA encoding 13...
proteins, all of which are located in the inner mitochondrial membrane and are part of the oxidative phosphorylation (OxPhos) machinery.

OxPhos is a key functional unit in the mitochondria, and combines electron transport with cell respiration and ATP synthesis (Fig. 1). It not only produces the vast majority of cellular energy, but is also involved in radical production and apoptosis. OxPhos consists of the electron transport chain (ETC), which comprises NADH-dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone, bc1 complex (complex III), cytochrome c (Cyt c), and cytochrome c oxidase (CcO; complex IV). The majority of electrons transferred by the ETC are derived from NADH and enter the chain via complex I. In addition, complex II feeds electrons directly into the ubiquinone/ubiquinol pool, and further input sources include the electron-transferring flavoprotein (ETF), ubiquinone oxidoreductase, and glycerol-3-phosphate dehydrogenase. These electrons are transferred through the ETC to oxygen, while the mitochondrial membrane potential is generated by pumping protons across the inner mitochondrial membrane. Finally, the proton gradient is utilized by ATP synthase (complex V), which couples the backflow of those protons previously pumped by the ETC to the production of ATP (Fig. 1).

Cellular energy demand ranges widely depending on the function and activity of the cell. Thus adjustment of energy production to physiological demand is essential to all higher organisms. The first mechanism identified by which OxPhos is controlled is called respiratory control as was shown with isolated mitochondria [7]. Here, OxPhos activity is limited by the availability of the substrates for ATP synthesis, ADP and phosphate. Thus, OxPhos activity increases when ATP utilization generates ADP and phosphate. In addition to this basic substrate limitation mechanism there are several further levels of control, and nature has cultivated three major regulatory means, which are found alone or in combination (Fig. 1). There are 1) tissue-specific and/or developmental expression of isozymes, which equip a cell with variants that perform the same chemistry but are adapted to tissue-specific requirements, 2) allosteric control, mediated through small molecules that bind to the enzyme, changing its kinetic properties, and 3) cell signaling. The latter two mechanisms can be viewed as a hierarchy: allosteric control represents an intracellular response, for example triggered by changes in the concentration of substrate or product molecules. In contrast, cell signaling primarily serves the purpose of higher-order communication between cells or organs. For example, glucagon and insulin, released by the pancreatic alpha and beta cells, respectively, reach their target organs such as liver and muscle through the bloodstream. There, they bind to specific receptors and trigger an amplification cascade, which eventually adjusts cell function to the physiological circumstances the whole organism faces at the time of hormone release. In the example given glucagon, a starvation signal, is present after extended periods of food deprivation, e.g., after overnight rest, and triggers a major metabolic response through the cAMP-dependent pathway in liver. The liver, as an altruistic organ, then provides food in the form of glucose for other organs such as the brain. After ingestion of food insulin is released, signaling that metabolic building blocks have become available and have to be taken up by the cells. The effect of insulin is mediated through the phosphoinositol-3 kinase pathway.

Within the cell there is a sequence of kinases and phosphatases, which add or remove phosphate groups from downstream kinases and/or phosphatases or endpoint targets. It is now clear that the traditional view of linear, i.e., hierarchical, signaling cascades is an oversimplification because there is considerable crosstalk between different signaling pathways. This crosstalk sometimes complicates the interpretation of experimental results and, when overlooked, may also account for some discrepancies reported in the literature.

Several recent review articles have summarized data on phosphorylated proteins identified within the mitochondria and on signaling pathways that act on mitochondria [8–11]. The goal

Fig. 1. Regulation of mammalian oxidative phosphorylation (OxPhos). At the functional level OxPhos is regulated through a hierarchy of regulatory mechanisms: organs express tissue-specific isoforms, hormones mediate signals from the outside of the cell to the mitochondria through signaling cascades, and allosteric regulation allows fine-tuning of energy production within the cell IMS: intermembrane space.
of this article is to provide a focused view of current knowledge about regulation of the mitochondrial OxPhos system as an endpoint of cell signaling due to its emerging recognition as an important factor in human pathophysiology.

2. Cell signaling targets all mitochondrial OxPhos components in mammals

Partial or complete crystal structures from mammals and/or homologous structures from bacteria are now available from all components of the OxPhos system (Fig. 2). They are not only essential for a better understanding of the catalytic mechanisms of the individual enzymes but also for the interpretation of regulatory events. For example, after a phosphorylation site has been mapped the structural information can be used to interpret changes in enzymatic properties. Several different types of data have been acquired in recent years, such as proteomic studies analyzing protein phosphorylation on a larger scale. Often, however, the information obtained represents just one piece of the puzzle. In order to understand the complex regulation of aerobic energy metabolism the approach must be integrative, linking a molecular event, such as the attachment of a phosphate group to a specific amino acid, to functional change, such as changes in enzyme activity.

We here discuss current knowledge of the regulation of OxPhos complexes and the physiological relevance of such regulation. Although many open questions remain, CcO is the best-studied complex with respect to regulation through isoform expression, allosteric control, and reversible phosphorylation. Thus, CcO serves as an example of what can be expected to be revealed for the entire OxPhos system in the future.

2.1. NADH-dehydrogenase (NADH-DH) (complex I)

With 46 subunits and a molecular weight close to 1 MDa [12], NADH-DH is the most complex OxPhos member and also the least understood. Recently, a partial crystal structure containing eight subunits from the hydrophilic arm of complex I from *Thermus thermophilus* was resolved [13] (Fig. 2A). This bacterial enzyme has a smaller number of only 15 subunits, 14 of which have homologs in mammals. Bacterial complex I presumably lacks several regulatory mechanisms that are yet to be discovered in mammals, including additional regulatory subunits, similar to CcO as discussed below.

2.1.1. cAMP-dependent signaling to the mitochondria

The majority of studies of cell signaling targeting ETC complexes investigated the effect of the cAMP-dependent pathway. Most effects of increased intracellular cAMP are mediated through phosphorylation reactions catalyzed by protein kinase A (PKA), a serine/threonine-specific kinase [14]. However, it remains to be clarified whether cytoplasmic PKA can localize to the mitochondrial matrix and/or intermembrane space. Several studies have concluded that there was PKA activity in the mitochondria. For example, incubation of cow heart mitochondria with cAMP in the presence of \(\gamma^{-32}\text{P}\) ATP showed labeling of four proteins [15]. Other studies also suggested the presence of PKA in mitochondria of yeast, mammalian cell lines, and a variety of mammalian tissues [16–24]. Other studies have provided a different model of action of cAMP-dependent signaling in the mitochondria. For example, A-kinase anchoring proteins (AKAPs) have recently gained much attention because they localize PKA to cellular compartments in close proximity to its targets [25]. Several recent studies have indicated that AKAPs localize PKA to the outer side of the outer mitochondrial membrane. In germ cells AKAP84 has been shown to control the migration of mitochondria from the cytoplasm in round spermatids to the cytoskeleton in elongating spermatids [26], and AKAP1-mediated cellular localization of PKA to the mitochondria is required during oocyte maturation in mice, which starts 1.5 h after initiation of the maturation process [27]. Similarly, stimulation of the cAMP pathway in the human embryonic kidney cell line HEK293 leads to a sub-mitochondrial localization of PKA to the outer side of the outer mitochondrial membrane, mediated by AKAP84 and AKAP121 [28,29]. At the same sub-cellular location PKA phosphorylates the proapoptotic protein BAD on serine 112, a process that is AKAP-mediated [30,31]. In contrast, a recent study on rat heart mitochondria concluded that AKAP121 was localized within the mitochondria [32].

Possibly, both models co-exist and PKA can modify other signaling compounds on the outer side of the mitochondria, but it can also translocate into the mitochondria and directly phosphorylate mitochondrial proteins (Fig. 3). In addition, tissue-specific differences may favor one mode of action over the other.

The action and localization of certain kinases, such as PKA, and the importance of scaffolding proteins, such as AKAPs, for the convergence and specific targeting of phosphorylation events to recipient proteins, should always be addressed by in vivo studies. For example, the combination of a purified OxPhos complex with a commercially available kinase should be interpreted cautiously and should be validated in the physiological context in vivo (see also section ‘Cytochrome c oxidase’).

cAMP-dependent phosphorylation of complex I was first studied by the Papa group. They suggested that PKA directly phosphorylates complex I in heart tissue, leading to enzyme activation [33,34]. A radioactively labeled band was obtained in the 18 kDa size range and it was proposed that the N-terminal sequence containing the AQDQ-18 kDa subunit was targeted for phosphorylation. cAMP-dependent phosphorylation of an 18 kDa band was also shown in rat C6 glioma cells in cell culture after treatment with forskolin or dibutyryl-cAMP [35]. However, mapping of phospho-epitopes by mass spectroscopy was not performed in those studies. Later, using heart sub-mitochondrial particles and isolated complex I with an in vitro PKA phosphorylation approach, the Walker lab showed via mass spectroscopy that under similar conditions, instead of the AQDQ subunit the N-terminal sequence containing ESSS-18 kDa subunit of complex I was phosphorylated on serine 20 in addition to serine 55 of the 10 kDa-MWFE subunit [36] (Fig. 2A). Although the membrane-containing arm of complex I that contains the ESSS and MWFE subunits has not yet been crystallized, the phosphorylation sites are likely to be located on
the matrix and intermembrane space sides, respectively [36]. No experiments were performed to address functional consequences of the dual phosphorylation of complex I.

In order to assess in vivo phosphorylation of complex I, the Gibson and Capaldi labs isolated the cow heart enzyme, separated the sample by SDS-PAGE, and stained the gel with the
phosphate-specific fluorescent probe Pro-Q-Diamond [37]. Numerous bands were stained even after alkaline phosphatase treatment, a commonly used control, which nonspecifically removes phosphate groups; thus the specificity of the probe has to be further evaluated in future studies. The authors tried to identify phosphorylated epitopes by tandem mass spectrometry (MS/MS). Although the ultimate proof through MS/MS sequencing was difficult to achieve, they concluded through indirect controls that the NDUFA10 (42 kDa) subunit was phosphorylated on serine 59. This residue is not evolutionarily conserved in mammals and functional implications of its phosphorylation have not been addressed yet. Recently, serine 59 phosphorylation in cow heart was confirmed by another proteomic study using isolated complex I followed by mass spectrometry [39] (see also below). For complex I seven subunits showed Pro-Q-Diamond staining: the 75 kDa Fe–S protein 1, flavoprotein 1, 39 kDa 1α subcomplex, 24 kDa subunit, 23 kDa subunit (mitochondrial precursor), 22 kDa 1β subcomplex, and the 15 kDa subunit. Although no phosphate groups were assigned to specific amino acids and no functional studies were undertaken for any of the OxPhos complexes, this study provides strong evidence that the input point of the OxPhos system is targeted for regulation via cell signaling cascades, which would be expected for a key metabolic enzyme. Phosphorylation of the 39 kDa subunit on a yet-to-be-identified tyrosine residue was also inferred based on studies on isolated rat brain mitochondria [41].

An interesting finding was that complex I subunit ND2 seems to have a second role that, at first sight, appears not to be related to electron transfer and does not take place in the mitochondria. In rat ND2 was shown to interact with Src tyrosine kinase at excitatory synapses, localizing Src to N-methyl-D-aspartate receptors [42]. The authors found that ND2 was not targeted for phosphorylation and thus proposed that it functions as an adaptor protein. It has been shown that Src also localizes to the mitochondria, in particular to the intermembrane space [43], and phosphorylates CcO subunit II [44] (Fig. 3). It remains elusive whether ND2 can also function as a scaffolding protein in the mitochondria, in which case one would expect that a subunit near ND2 should be targeted for phosphorylation. A regulatory action mediated by Src kinase at complexes I and IV would be physiologically sensible, because it would allow a concerted change of enzyme activities both at the input point and the endpoint of the electron transport chain.
Table 1

<table>
<thead>
<tr>
<th>OxPhos complex</th>
<th>Species and tissue</th>
<th>Phosphorylated amino acid</th>
<th>Phospho-epitope</th>
<th>Reference</th>
<th>Method and comments</th>
<th>Kinase prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-DH</td>
<td>Cow heart</td>
<td>Ser95, B14.5a subunit</td>
<td>ASEKAVSPAPIKR</td>
<td>[38]</td>
<td>MS</td>
<td></td>
</tr>
<tr>
<td>NADH-DH</td>
<td>Cow heart</td>
<td>Ser59, 42 kDa subunit</td>
<td>GNICSGKSLAKEIA</td>
<td>[37,38]</td>
<td>MS</td>
<td>No known motif</td>
</tr>
<tr>
<td>NADH-DH</td>
<td>Cow heart</td>
<td>Ser20, ESSS (18 kDa) subunit</td>
<td>TLAGKRSEPTLRWQ</td>
<td>[36]</td>
<td>Edman degradation/MS</td>
<td>PKA, medium confidence</td>
</tr>
<tr>
<td>NADH-DH</td>
<td>Cow heart</td>
<td>Ser55, MWFE (10 kDa) subunit</td>
<td>MERDRVSGVNRSSYVS</td>
<td>[36]</td>
<td>Edman degradation/MS</td>
<td>PKA, medium confidence</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cow heart</td>
<td>Tyr97</td>
<td>EREDLYLKKATNE</td>
<td>[62]</td>
<td>MS</td>
<td>No known motif</td>
</tr>
<tr>
<td>CeO</td>
<td>Cow liver</td>
<td>Tyr304, subunit I</td>
<td>MDVDTLYFSTSATMI</td>
<td>[83]</td>
<td>MS, cAMP-dependent</td>
<td>No known motif</td>
</tr>
<tr>
<td>CeO</td>
<td>Rabbit heart</td>
<td>Ser115 and Ser116, subunit I</td>
<td>SLHLAGVSSLGAINF</td>
<td>[118]</td>
<td>MS, after ischemia/reperfusion</td>
<td>No known motif</td>
</tr>
<tr>
<td>CeO</td>
<td>Rabbit heart</td>
<td>Thr52, subunit IV</td>
<td>KAPWGIKEYDEKVEL</td>
<td>[118]</td>
<td>MS, after ischemia/reperfusion</td>
<td>DNA PK, low confidence</td>
</tr>
<tr>
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<td>Rabbit heart</td>
<td>Ser40, subunit Vb</td>
<td>MLPPKASGKTEDPN</td>
<td>[118]</td>
<td>MS, after ischemia/reperfusion</td>
<td>No known motif</td>
</tr>
<tr>
<td>CeO</td>
<td>Human skeletal muscle</td>
<td>Thr11, subunit Vla</td>
<td>ACGDHGTTARTWRF</td>
<td>[120]</td>
<td>Crystal structure</td>
<td>No known motif</td>
</tr>
<tr>
<td>ATP synthase</td>
<td>Mouse brain</td>
<td>Ser76(33)α subunit</td>
<td>EETGVRILSIGDGIAR</td>
<td>[134]</td>
<td>BEMAD/MS</td>
<td>Akt, Medium confidence</td>
</tr>
<tr>
<td>ATP synthase</td>
<td>Human NIH3T3 cells</td>
<td>Thr75, 6 subunit</td>
<td>EDGTTSKYFVSSGSR</td>
<td>[133]</td>
<td>Indirect, PDGFR-dependent</td>
<td>PDGFR kinase, low confidence</td>
</tr>
</tbody>
</table>

Taking a side glance at plant chloroplasts, enzyme activity of the analogous plastid NADH-DH can be strongly increased upon stimulation with H₂O₂ or Ca²⁺. This is mediated through threonine phosphorylation of the NDH-F subunit and maximal enzyme activity correlates with full phosphorylation [45].

So far, no tissue-specific subunit isoforms of complex I have been identified, although there is evidence of functional tissue-specific differences. Recently, the Villani group has shown variability of the P/O ratio (moles of ATP synthesized from ADP per 2 electrons flowing through the ETC), a measure of the efficiency of OxPhos. Comparison of rat brain, heart, and liver mitochondria revealed P/O values of 2.0, 2.8, and 2.6, respectively with complex I substrates but no differences were observed with complex II substrates suggesting that the site responsible for P/O variability is complex I [46]. The authors hypothesized that the low P/O ratio found in the brain might convey neuro-protection, because a higher rate but lower yield would accommodate the high energy demand of the brain and at the same time lower intracellular oxygen concentrations, the substrate for ROS production. Although the underlying mechanisms for P/O variability are unclear, differences in complex I phosphorylation in addition to tissue-specific subunit isoforms might account for those observations.

2.2. Succinate dehydrogenase (SDH, complex II)

The four-subunit SDH complex is part of the Krebs cycle and oxidizes succinate to fumarate, feeding the released electrons into the ETC. In contrast to the other ETC complexes, SDH does not pump protons across the inner mitochondrial membrane and therefore does not contribute to the generation of the mitochondrial membrane potential. Very little is known about the regulation of complex II, which is surprising since it integrates Krebs cycle metabolism into the ETC. Indeed, complex II seems to be regulated through cell signaling, as was recently indirectly demonstrated in plant and animal mitochondria by assigning complex II subunits that can be phosphorylated. 2D gel electrophoresis of isolated mitochondrial porcine proteins, followed by Pro-Q-Diamond staining, and identification of phosphorylated proteins via mass spectrometry, suggested phosphorylation of the flavoprotein precursor subunit and the iron sulfur cluster containing B subunit [39] (Fig. 2B). In potato tubers 14 mitochondrial phospho-proteins were identified after incubating intact isolated mitochondria with [γ-³²P]ATP, followed by 2D gel electrophoresis and MS/MS analysis of labeled spots [40]. Among those proteins identified were no subunits of complex I whereas the flavoprotein (A subunit) of complex II was radioactively labeled (Fig. 2B). A third study with the goal of identifying posttranslational modifications on complexes II and III reported several modifications, including N-terminal acetylation and oxidation of methionines [47]. However, the study failed to detect a single phosphorylated peptide, which may be explained by the omission of the calcium chelator EGTA and phosphatase inhibitors during mitochondria isolation. Under such conditions calcium activated phosphatases appear to prevail, leading to dephosphorylation of most mitochondrial proteins [39].
2.3. Cytochrome c reductase (complex III; bc₁ complex)

Complex III catalyzes the transfer of electrons from reduced ubiquinone to oxidized Cyt c via a Q-cycle mechanism, which leads to the translocation of protons across the inner mitochondrial membrane. The mammalian enzyme contains 11 subunits per monomer but functions as a dimer, which was confirmed by the X-ray crystal structure [48]. Hardly anything is known about regulatory mechanisms acting on this enzyme. A phospho-proteomic study already discussed in the previous section indicated phosphorylation of the β-mitochondrial processing peptidase (β-MPP) subunit in potato [40], and the porcine heart core protein I precursor, core protein II, Rieske iron sulfur protein precursor, and ubiquinone binding protein [39] (Fig. 2C). Phosphorylated amino acids, relevant signaling pathways, and effects of those phosphorylations remain elusive.

2.4. Cytochrome c (Cyt c)

The one-electron carrier Cyt c is located in the mitochondrial intermembrane space and shuttles electrons from bc₁ complex to cytochrome c oxidase. Cyt c from human, cow, and mouse contains 104 amino acids and a covalently linked heme group. The heme iron is further coordinated by histidine 18 and methionine 80. Cyt c is highly positively charged with a pI of 9.6. Cyt c is essential for aerobic energy production and Cyt c knockout mice die around midgestation [49]. This is plausible because at that developmental stage energy production that relies only 5% on aerobic metabolism before gestation day 9 switches to aerobic energy metabolism relying 95% on OxPhos after gestation day 11, as was demonstrated with cultured rat embryos [3].

In addition to its essential role in aerobic energy metabolism, Cyt c also participates in type II apoptosis that involves the release of Cyt c into the cytosol, which binds to Apaf-1, leading to activation of procaspase-9 to caspase-9 [50]. Although not understood in detail, the release of Cyt c into the cytosol is considered a crucial proapoptotic signal [51,52]. Interestingly, Cyt c has a third function as a cardiolipin peroxidase at early stages during apoptosis, where it selectively oxidizes cardiolipin [53], a lipid of the mitochondrial inner membrane that binds to Cyt c. This mechanism may play an important role in the release of Cyt c after the induction of apoptosis, which requires dissociation from the inner mitochondrial membrane. In contrast, a fourth function as a reactive oxygen species scavenger has been proposed for Cyt c under normal (non-apoptotic) conditions [54].

Cyt c has a testes-specific isoform in rodents [55], which is mainly expressed in spermatocytes and cells in the later stages of spermatogenesis, whereas Sertoli cells, spermatogonia, and interstitial cells express the otherwise ubiquitously expressed, so-called somatic Cyt c isoform [56], and both isoforms are gradually expressed in intermediate cells during sperm maturation [57]. Testes-specific Cyt c has an interesting feature in that it shows a threefold increased activity to reduce hydrogen peroxide compared with the somatic isoform, but also shows a three to fivefold activity to induce apoptosis [58]. The authors concluded that the first effect protects sperm from radical damage whereas the second effect is part of a suicide mechanism of male germ cells, which selects for functional and undamaged sperm. However, the syntenic region of testes Cyt c in the human genome contains a non-transcribed pseudogene [59], and it remains elusive whether humans converge the functions of the somatic and sperm-specific Cyt c variants found in rodents into a single protein expressed in all human tissues.

One means of Cyt c regulation has been known for three decades: ATP binds to Cyt c at physiological concentrations, which affects the binding of Cyt c to CcO, leading to an inhibition of the reaction between Cyt c and CcO, and the elimination of the low Km phase of the otherwise biphasic kinetics with CcO [60]. An ATP binding site was later mapped to the conventional “left side” of the molecule [61]. A similar regulatory mechanism is present in CcO as discussed below that also leads to an inhibition of respiration.

A novel regulatory means mediated by phosphorylation was recently discovered by our group [62]: Cyt c isolated from cow heart tissue under conditions that preserved the physiological phosphorylation state revealed phosphorylation on tyrosine 97, as shown by tandem-MS. Clear differences were observed in respiration with CcO, which was inhibited in the presence of tyrosine 97-phosphorylation and showed pronounced sigmoidal kinetics [62]. The inhibitory effect of tyrosine 97-phosphorylation on respiration with CcO was surprising because tyrosine 97 does not directly participate in Cyt c–CcO binding [63]. In addition, there are many studies where basic residues of Cyt c were modified into neutral or acidic moieties [64–66], with the most dramatic changes in enzyme kinetics being observed when amino acids were altered that directly participate in binding. However, the localization of tyrosine 97 in the midst of the only relatively hydrophobic region on Cyt c [67] might explain far-reaching structural disturbances ranging to the other side of the molecule, as was deduced from a spectral shift of the heme iron–methionine 80 absorption band from 695 to 687 nm [62].

Several questions related to Cyt c phosphorylation remain open due to its other functions. We have proposed that tyrosine 97 phosphorylation may affect apoptosis [62] because an essential residue for the interaction with Apaf-1, lysine 7, is spatially located next to tyrosine 97 [68]. Based on structural considerations the formation of a salt bridge is possible that would neutralize the positive charge of lysine 7, which might prevent apoptosome formation. In addition, a conformational change in Cyt c was earlier observed in apoptotic cells [69], which could be explained with a change of the phosphorylation status.

Tyrosine 97 phosphorylation might also affect the cardiolipin peroxidase activity of Cyt c. The Kagan lab has shown that this mechanism involves a tyrosine radical [53]. In addition, lipid binding capability would be required that could be provided by this relatively hydrophobic area [67,70]. However, there are three additional conserved tyrosine residues in mammalian Cyt c as potential candidates for the chemistry involved, and more experiments are needed to decide this issue.

2.5. Cytochrome c oxidase (complex IV; CcO)

CcO is the terminal enzyme of the electron transport chain. It accepts electrons from reduced Cyt c and transfers them to
molecular oxygen, which is reduced to water. This reaction requires protons that, taken up from the matrix, contribute to the generation of the mitochondrial membrane potential. In addition to those chemical protons, CcO pumps protons across the inner mitochondrial membrane, a process that is still not fully understood and is a matter of intense debate [71–75]. Mammalian CcO has been crystallized as a dimer, which is composed of 13 subunits per monomer [76]. In addition to the 3 largest mitochondrial encoded subunits, which contain the 4 catalytic redox centers, the mammalian enzyme contains 10 nuclear encoded subunits. The function of the nuclear encoded subunits and their isoforms remains largely unknown. However, several studies have started to shed light on some of those, and thus regulation of CcO has been studied in more detail compared to the other OxPhos complexes. These studies provide a preliminary taste of what may be revealed in the future for the entire OxPhos system. CcO shows all three regulatory features found in key metabolic enzymes: isoform expression, allosteric control, and phosphorylation (Fig. 1).

2.5.1. Isoforms

There are three liver- and heart-type isoform pairs of subunits VIa, VIIa, and VIII, which have been known for several decades (reviewed in [77]). Liver-type CcO is expressed in organs such as liver, kidney, and brain, whereas the heart-type isozyme is expressed in heart and skeletal muscle. In addition, three more isoforms were discovered recently. These are a lung-specific isoform of CcO subunit IV, a testes-specific isoform of subunit VIIb, and a third isoform of subunit VIII [59,78,79]. There is only one published knockout mouse model, which lacks the subunit VIa heart-type isoform. The mice are viable under no-stress conditions, but showed only 23% CcO activity in heart tissue compared to the wild-type, which correlated with reduced CcO amount (23%) [80].

Functional data including enzyme kinetics have so far been reported only for the better-studied liver- and heart-type CcO isoenzymes. Older studies tried to analyze differences between the two major isozymes (e.g., [81,82]) but no consistent results were obtained. The Avadhani lab used sub-mitochondrial particles and reported a $K_m$ for Cyt $c$ of 26 and 8 $\mu M$ and maximal turnover values of 495 and 750 s$^{-1}$ at high ionic strength for heart and liver CcO, respectively. These data are in agreement with the idea that heart-type CcO is found in tissues with a high aerobic capacity and a large number of mitochondria, whereas other tissues including the brain cannot afford such a high mitochondrial mass due to their specialized functions, but still fully depend on aerobic energy production, which requires a more active enzyme. However, CcO kinetics may have to be re-evaluated for two reasons: 1) as discussed below, phosphorylation of CcO that decisively regulates enzyme activity has to be considered; this requires isolation conditions that preserve the physiological phosphorylation status [83], and 2) some components commonly used during enzyme isolation or during activity measurements have to be carefully considered. Examples include the detergent cholate that strongly binds to and activates CcO, which has to be removed by dialysis [76]; the detergent dodecylmaltoside that was suggested to monomerize CcO [84]; and $N,N,N',N'$-tetramethyl-p-phenylenediamine (TMPD), which facilitates electron transfer between molecules and thus alters enzyme kinetics because Cyt $c$ does not have to dissociate from CcO in order to be re-reduced [60]. Therefore, it would be desirable to develop a generally accepted and applied protocol.

2.5.2. Allosteric control

The largest nuclear encoded subunit, CcO IV, has been shown to bind ATP at the matrix side, leading to an allosteric inhibition of enzyme activity at high intra-mitochondrial ATP/ADP ratios, adjusting energy production to the physiological demand [85,86]. Computer modeling of an ATP molecule into a pocket comprised of subunit IV and the catalytic subunits I and II supported these findings [78]. It was then postulated that CcO IV is a pivotal regulator of CcO activity in higher organisms and yeast in contrast to bacteria, which lack this subunit [87].

Subunit Va, which has no transmembrane helix and localizes to the matrix site, was shown to bind the thyroid hormone T2, releasing the allosteric ATP inhibition, and allowing a high turnover even at a high ATP/ADP ratio [88].

The muscle specific isoform of subunit VIa was suggested to decrease the pumped proton to electron stoichiometry at high ATP/ADP ratios [89]. In contrast, CcO containing the VIa liver-type isoform pumps protons at a high ratio in the presence of cardiolipin independently of the ATP/ADP ratio, whereas the addition of palmitate leads to a decreased proton/electron stoichiometry [90].

There are several studies using protein interaction techniques that suggest additional regulatory means, which need further investigation. CcO subunit II was shown to bind to the epidermal growth factor receptor (EGFR) by phage display screening and co-immunoprecipitation [91]. EGFR signaling is involved in important cellular processes, such as survival, proliferation, and migration. Binding of EGFR to CcO subunit II requires tyrosine 845 phosphorylation on EGFR, which is mediated by c-Src, suggesting that EGFR and c-Src act synergistically (Fig. 3). This idea is further supported by the fact that CcO subunit II is a target of c-Src [44], as detailed below.

Another physical partner of CcO subunit II is cyclin G1, which was demonstrated by yeast two-hybrid work and confirmed by co-immunoprecipitation in an osteosarcoma cell line that overexpresses cyclin G1 [92]. The authors proposed that cyclin G1, which is induced by tumor suppressor p53, might participate in the induction of apoptosis through its interaction with CcO.

Subunit Vb, which similarly to subunit Va localizes to the mitochondrial matrix side, was shown by a yeast two-hybrid screen to interact with the cytosolic human androgen receptor [93]. In addition, it was reported for the Rho regulatory subunit of PKA that it can also bind to CcO subunit Vb [94]. The authors concluded that activation of the cAMP pathway using adenylyl cyclase activator forskolin leads to CcO inhibition through this direct interaction. As discussed below, it is now evident that the cAMP-dependent pathway leads to phosphorylation of CcO, which strongly inhibits enzyme activity [83].
2.5.3. Competitive inhibition of CcO by nitric oxide (NO)

Over the last two decades the work of many labs has established the importance of NO action on mitochondrial function, which cannot be addressed here in detail (for reviews see references [95–97]). NO is synthesized by three types of NO synthases (NOS), a neuronal (nNOS) and an endothelial NOS (eNOS) isoform that are constitutively expressed, and an inducible form (iNOS). Interestingly, a subtype of nNOS localizes to the mitochondria and was therefore denoted mitochondrial NOS (mtNOS) [98]. mtNOS can directly interact with CcO on the matrix side by binding to subunit Va [99], which increases local concentrations of NO at its target enzyme. Mechanistically, NO competes with oxygen at the binuclear heme $a_2$-Cu$\beta$ binding site, which leads to reversible CcO inhibition [100,101]. Increased cellular NO levels coincide with many human diseases, including arthritis [102], sepsis and septic shock [103], trauma [104], cancer [105], obesity, and diabetes [106]. Despite other actions of NO in the cell its inhibitory action on CcO might at least in part explain some aspects of NO-related pathological conditions, because inhibition of oxidative energy production would decrease cellular energy levels. The inhibitory effect on cell respiration has also been proposed as a general mechanism underlying neurodegenerative diseases mediated by an over-production of NO [107].

2.5.4. Calcium and zinc binding sites

Mammalian CcO contains a calcium–sodium exchange site in subunit I [108], whereas in bacteria this site contains a strongly bound calcium [109]. The function of reversible calcium binding is unknown. There have been reports indicating that CcO can bind at least two zinc ions at two different sites, which leads to enzyme inhibition and particularly to inhibition of the proton pumping process [110–112]. It is unclear where variation of zinc concentrations may affect CcO activity, and whether zinc really constitutes a physiological regulator. Perhaps it might play a role for the regulation of sperm motility before and after ejaculation in testes, which have higher zinc levels compared to other tissues. That would be sensible because CcO activity would be down-regulated during periods of quiescence whereas zinc ions would be diluted after ejaculation and allow aerobic energy production when needed. Interestingly, reduction of zinc concentration clearly improves sperm motility, and it was concluded that this effect was related to improved mitochondrial performance [113].

2.5.5. Phosphorylation of CcO

Only during the last few years has it become evident that CcO can be phosphorylated at several subunits and it is likely that this mechanism plays the pre-eminent role in regulating enzyme activity.

The first report indicating CcO phosphorylation was published by Steenaart and Shore [114]. The authors labeled mitochondrial proteins phosphorylated by endogenous kinases using [$\gamma$-$^{32}$P]ATP and concluded that CcO subunit IV can be phosphorylated. However, the identification of a phosphorylated residue and possible functional effects still remain elusive. The Kadenbach lab isolated CcO from cow heart and incubated the solubilized enzyme with commercially available PKA, cAMP, and [$\gamma$-$^{32}$P]ATP [115]. After SDS-PAGE and autoradiography they observed a signal for subunit Vb and a faint signal for subunits II or III. Later, applying the same in vitro approach they presented data suggesting that subunit I was also phosphorylated [116]. These experiments demonstrate the modification of heart CcO by PKA in vitro both on the intermembrane and matrix side of the enzyme. Functional studies showed that the in vitro phosphorylated CcO was inhibited [115]. The Avadhani lab recently reported phosphorylation of CcO subunits I, IV, and Vb after ischemia in rabbit hearts, and they proposed that PKA directly phosphorylates CcO under those conditions because phosphorylation was abolished in the presence of PKA inhibitor H89 [117]. They later assigned the phosphorylated amino acids by mass spectrometry to serines 115 and 116 of subunit I, threonine 52 of subunit IV, and serine 40 of subunit Vb [118]. The sites identified (Table 1) are not PKA consensus sites, suggesting that PKA acts upstream or, more likely, that the inhibitor H89 also targets one or more other kinases that mediate those phosphorylations.

Since in vivo phosphorylation sometimes requires auxiliary proteins for specific targeting of the signal, such as AKAPs in case of PKA, other labs have started to evaluate cAMP-dependent signaling in a more physiological context. To address this point, our group initially focused on liver instead of heart tissue. The starvation hormone glucagon is the strongest physiological signal for increasing intracellular cAMP levels in liver without affecting other signaling pathways [119]. In heart there is no such exquisite trigger, and G-protein linked receptor-mediated increase of cAMP often parallels an increase in calcium. Therefore, liver CcO was isolated in the presence or absence of theophylline, a phosphodiesterase inhibitor that creates high cAMP levels, and the phosphorylation status was preserved by the addition of phosphatase inhibitors [83]. High cAMP produced an enzyme that was tyrosine phosphorylated on subunit I in vivo whereas the control enzyme did not show a signal. Subunits II or III and IV were also tyrosine phosphorylated, but there was no change after theophylline treatment. The site of phosphorylation was identified by mass spectrometry to be CcO subunit I tyrosine 304 (Fig. 2D). Phosphorylated CcO was fully or strongly inhibited up to 10 $\mu$M Cyt c substrate concentrations, even in the presence of allosteric activator ADP [83]. Treatment of liver tissue or human liver cancer HepG2 cells with the physiological hormone glucagon or the adenyl cyclase activator forskolin, which both increase intracellular cAMP, produced a similar response. Interestingly, theophylline, which is one of the oldest anti-asthma drugs, also led to CcO inhibition and lowered ATP levels in the lung at concentrations used in therapy. It was then proposed that this mechanism might explain the effect of theophylline on asthma relief because airway constriction during asthma requires energy [83]. Although the above studies clearly show that cAMP signaling leads to CcO inhibition, in vivo tyrosine 304 phosphorylation of liver CcO contrasts with in vitro phosphorylation of heart CcO using PKA, which is a serine/threonine kinase and
Thus, mitochondrial ATP synthase utilizes the proton gradient generated by the ETC in order to produce ATP from ADP and phosphate. The enzyme is a nano-motor consisting of a soluble component, $F_1$, and a membrane bound $F_0$ part that couples proton backflow to ATP synthesis through a peripheral and a rotating central stalk [126,127]. The $F_1$ part consists of five subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ and the core $F_0$ part contains subunits $\alpha\beta_{10-14}\gamma$; the so-called oligomycin sensitivity-conferring protein (OSCP), and subunits $d$, $e$, $f$, $F_0$, and $A_6$. Several partial crystal structures including the $F_1$ component from mammals and parts of $F_0$ from bacteria have been resolved and are presented as a combined structure in Fig. 2F.

The activity of ATP synthase is dependent on $\Delta \Psi_m$ and maximal rates of ATP synthesis in bacteria occur at $\Delta \Psi_m = 100-120$ mV, as has been demonstrated for ATP synthases from $E. coli$ and $P. modestum$ [128].

In mammals there is so far no evidence for tissue-specific or developmentally regulated isoforms. However, three isoforms of the $\beta$ subunit have been identified in tobacco plants, two of which seem to be ubiquitously expressed and the third one exclusively expressed in pollen [129]. Since tissue-specific isoforms of ATP synthase have hardly been studied, and considering the numerous isoforms of CcO subunits, the presence of isoforms in plants probably just represents the tip of the iceberg and much more remains to be learned about tissue-specific energy requirements at the level of this central enzyme.

In order to prevent wasteful ATP hydrolysis a small 10 kDa inhibitory protein IF$_1$ binds to the soluble $F_1$ component [130]. In addition to the transmembrane subunits $e$ and $g$, IF$_1$ also mediates ATP synthase dimer formation [131].

In 1995 the first study was published showing that ATP synthase can be phosphorylated on the $\delta$ subunit in cultured mouse cortical neurons, triggered by platelet-derived growth factor (PDGF) [132]. The $\delta$ subunit is part of the central rotating stalk, and phosphorylation can be expected to lead to changes in enzyme activity, but additional experiments are needed. The Pedersen lab later extended those initial data and demonstrated that PDGF-mediated phosphorylation of the $\delta$ subunit also occurs in NIH3T3 and kidney cells, and the authors provided indirect evidence that this phosphorylation occurs on the only tyrosine residue present in this subunit, tyrosine 75 [133] (Fig. 2F). Growth hormone receptor-triggered signaling to the mitochondria may also affect other OxPhos complexes, because it leads to proliferation and cell survival, and Cytc might be a first target to look at due to its participation in apoptosis and the fact that it can be phosphorylated.

Using mouse brain tissue, a recent phospho-proteomic study applying an improved protocol called BEMAD ($\beta$-Elimination/ Michael addition with DTT labeling) for the detection of phospho-peptides identified Ser76 of the alpha subunit as being phosphorylated [134] (Fig. 2F).

There is plenty of evidence that phosphorylation can occur on additional sites of ATP synthase in mammals, yeast, and plants. After in vitro incubation of potato tuber inside-out sub-mitochondrial particles with $[\gamma^{32}P]ATP$ the $\delta$ subunit was labeled in addition to the $b$ subunit, which is part of the peripheral stalk [135]. A similar approach using intact potato tuber mitochondria revealed phosphorylation of the $\alpha$ and $\beta$ subunits [40], which comprise the catalytic $F_1$ part. A recent study of thylakoid ATP synthase from barley resolved six variants of the $\beta$ subunit on 2D gels caused by differences in serine/threonine phosphorylation.

### 2.6. ATP synthase (complex V)

ATP synthase utilizes the proton gradient generated by the ETC in order to produce ATP from ADP and phosphate. The enzyme is a nano-motor consisting of a soluble component, $F_1$, which is in the matrix and contains the catalytic ATP/ADP/P$_i$ binding sites, and a membrane bound $F_0$ part that couples proton...
In humans the β subunit can also be phosphorylated, and four phosphorylated variants were identified after incubation of cultured skeletal myoblasts with [γ-32P]ATP, followed by 2D gel analysis [137]. The authors provided some evidence that threonine 213, which is located in the catalytic nucleotide-binding site, is one phosphorylation site (Fig. 2F). The study also showed that, at least under cell culture conditions, the unphosphorylated β subunit was undetectable.

Other studies using Western blot analysis and anti-phospho tyrosine antibodies on rat brain mitochondria indicated phosphorylation of the α and γ subunits [41], whereas Pro-Q-Diamond staining of porcine mitochondrial proteins pointed to phosphorylation of the mature peptides of the α and OSCP (oligomycin sensitivity-conferring protein) subunits [39].

Interestingly, even for yeast, which was long considered a “zero background” organism for tyrosine phosphorylation studies, it was recently shown to contain phosphorylated tyrosine residues on the α and β subunits (yeast nomenclature: ATP1p and ATP2p) in addition to phosphorylated serine and threonine residues [138]. Using MALDI-FTICR-MS (matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry), the authors were able to map specific phosphorylation sites to tyrosine 434 and serines 413 and 426 of the α subunit, and tyrosine 7 of the β subunit. However, there was some ambiguity because anti-phospho antibodies did not show a signal for the α subunit [138]. Such results might be explainable because mass spectrometry and immunological approaches show varying sensitivity to differentiating phosphorylated and unphosphorylated peptide species. For example, ionization during mass spectrometry often leads to loss of a phosphate group and antibodies may require adjacent amino acids in addition to the phosphorylated amino acid in order to bind, a problem especially encountered with anti-phospho-serine and -threonine antibodies, whereas the phospho-tyrosine group appears to be a big enough epitope for specific recognition.

Clearly, comprehensive mapping of phosphorylation sites and functional studies are necessary to understand regulation of ATP synthase in light of cell signaling.

3. OxPhos supercomplexes

There is now ample evidence that the OxPhos machinery is organized into higher-order complexes. ATP synthase from rat liver has recently been shown to co-purify with the phosphate and the adenine nucleotide carriers, which provide ATP synthase with its substrates ADP and phosphate [139,140]. Another interesting aspect of ATP synthase is its occurrence in dimeric and oligomeric states, which was demonstrated for several organisms including yeast and mammals [141–144], and it was proposed that cristae morphology is dependent on these states. Yeast mutants of the e and g subunits, in which the dimeric and oligomeric states of ATP synthase are destabilized, showed reduced levels of mitochondrial membrane potential, which could not be explained through uncoupling of the membrane or activity changes of OxPhos complexes [145]. However, the phosphorylation state of ATP synthase and of the other OxPhos complexes was not preserved during mitochondria isolation, which might explain those findings. The Lippe group recently speculated that under physiological conditions monomeric and oligomeric states might be regulated through phosphorylation and they performed 2D gel analysis of monomeric and dimeric ATP synthase followed by Western analysis with anti-phospho tyrosine antibodies [146]. The authors found that the γ subunit was tyrosine phosphorylated only in the monomeric but not in the dimeric state. Although the phosphorylated tyrosine residue remains unknown this work suggests that not only oligomeric states of ATP synthase but also aggregation of the other members of the OxPhos machinery into supercomplexes may be regulated through cell signaling. Interestingly, tyrosine 304 phosphorylation of CcO subunit I resulted in enhanced sigmoidal kinetics and resulted in a Hill coefficient of about 4 in contrast to a value of 2 for the tyrosine 304 unphosphorylated enzyme [83]. One interpretation of an increased Hill coefficient would be an increase in monomer–monomer interaction in the CcO dimer, and it will therefore be interesting to see if such phosphorylation affects the stoichiometry of CcO in OxPhos supercomplexes.

Many studies on larger aggregates of OxPhos complexes employed methods developed by Schägger and coworkers, blue native and more recently clear-native gel electrophoresis [147,148], which allows the separation of higher-order supercomplexes under comparably gentle conditions using mild detergents, such as digitonin. Various stoichiometries of OxPhos components in supercomplexes have been observed in yeast, plants, and mammals, including the following variants: I–III2, I–III2–IV, I–III2–IV2, I–III2–IV3, I–III2–IV4 (for current reviews see [149–151]). The arrangement of OxPhos complexes into assembly lines would make possible a more efficient electron transfer due to substrate channeling. This would be especially beneficial to speed up energy production in conditions that require increased ATP production, such as during exercise. Not only electron transfer might be affected but also the backflow of protons through the OxPhos complexes (intrinsic uncoupling) and the production of reactive oxygen species (ROS) as a byproduct of the OxPhos machinery might be altered. It has been postulated that intrinsic uncoupling is a mechanism involved in processes such as thermogenesis [152], and ROS are important in cell signaling including the induction of apoptosis [153]. Thus, changes in supercomplex stoichiometry, probably regulated by cell signaling, and their functional consequences seem to be an interesting new avenue for future research.

Cardiolipin is an important mitochondrial lipid, which constitutes about 20% of lipids in the inner mitochondrial membrane. It was shown for bc1 complex and CcO that cardiolipin stabilizes supercomplexes in yeast [154,155]. In humans there is a rare recessive X-linked disease, Barth syndrome, with variable symptoms including motor delays, hypotonia, delayed growth, and cardiomyopathy, which is caused by mutations in the cardiolipin synthesis pathway [156]. A recent study on lymphoblasts from patients with Barth syndrome carrying mutations in the TAZ1 gene revealed labile supercomplexes of complexes I, II, and IV [157].
4. Mitochondrial kinases and phosphatases that may act on OxPhos complexes

Cell signaling affects mitochondria through direct phosphorylation and dephosphorylation of mitochondrial proteins, or through changes in regulation of gene expression of proteins that localize to the organelle. An example of the latter case is contractile activity in skeletal muscle, which is paralleled by increased calcium fluxes, and leads to activation of a series of kinases including PKC, extracellular signal-regulated kinases ERK-1 and ERK-2, and ribosomal S6 kinase (reviewed in [158]). The important role of calcium in gene regulation was demonstrated in myotubes using calcium ionophore A23187, which induced the expression of Cyt c and CeO subunit Vβ genes and was mediated by a PKC-dependent pathway [159]. Contractile activity also leads to the induction of peroxisome proliferator activated receptor co-activator 1α (PGC-1α), a key nuclear factor that coordinates the expression of nuclear encoded genes that function in the mitochondria [160]. Another independent mechanism altering mitochondrial biogenesis after chronic contractile activity is an acceleration of protein import into the mitochondria [161].

However, little is known about mitochondrial kinases and phosphatases, their OxPhos substrates, and the transduction of the signal from the cytosol to the mitochondria. Based on selected examples we will outline emerging principles of such signaling relevant to OxPhos. For recent comprehensive reviews of kinases and phosphatases that have been associated with mitochondria see references [8–10].

4.1. Translocation of signaling molecules to the mitochondria

Apparently the static picture of kinases and phosphatases with a fixed localization to cellular compartments has to be at least in part modified to a more dynamic model. There are increasing numbers of signaling components that change their sub-cellular localization upon alterations in the signaling state: ubiquitously expressed tyrosine kinase c-Abl, which localizes to the nucleus and cytoplasm, translocates from the endoplasmic reticulum (ER) to the mitochondria, where it participates in the induction of apoptosis; this was demonstrated after cells were subjected to ER stress triggered with the calcium ionophore A23187, brefedin A, or tunicamycin [162].

Increased respiration and an increase of the mitochondrial transverse membrane potential Δψm is paralleled by the translocation of AKAPs to the outer side of the outer mitochondrial membrane. AKAPs not only provide a platform for PKA signaling, but they also appear to be a hub for other signaling pathways, converging signals to the mitochondria. Livigni and coworkers [163] have recently shown that protein tyrosine phosphatase PTPD1, and c-Src share the same scaffold with PKA (Fig. 3). This model raises an interesting question related to other studies discussed above that inferred localization of PKA and c-Src to the inner compartments of the mitochondria. A co-existence of both models is possible, particularly in a tissue-specific context. For example, c-Src localization to the mitochondrial intermembrane space was demonstrated in cells involved in bone formation that overexpress this kinase. In other cell types with lower expression, c-Src might instead be part of the signaling hub and stay on the outer side of the mitochondria.

Akt (protein kinase B) localization to all compartments in the mitochondria upon stimulation of the phosphoinositol-3-kinase pathway was shown to be dynamically regulated [164]. Stimulation of cells with insulin-like growth factor-1, insulin, and stress resulted in phosphorylation and activation of Akt, followed by a fast accumulation in the mitochondria. The study concluded that the β subunit of ATP synthase and glycogen synthase kinase-3β were targets for phosphorylation by Akt.

Protein kinase Cδ (PKCδ) is a stress-activated kinase involved in triggering the production of free radicals and the induction of apoptosis. Translocation of PKCδ to the mitochondria in mouse keratinocytes was shown after stimulation with 2-O-tetradecanoylphorbol-13-acetate (TPA), which induced cell death [165]. Translocation of PKCδ to the mitochondria does not occur during ischemia, but was observed with 5 min of reperfusion applied after 30 min ischemia in rat hearts, followed by a decrease in mitochondrial respiration and an increase in superoxide radical production [166].

Tyrosine phosphorylation in the mitochondria has recently gained attention, in part because tyrosine kinases and phosphatases known to play pivotal functions in the cytosol or nucleus also localize to the mitochondria [9]. Based on studies with rat brain, Src and other Src family kinases Lyn, Fyn, Csk, and Fgr were shown to localize to the mitochondrial intermembrane space [9,43].

The presence of those tyrosine kinases not only raises the question of their involvement in OxPhos regulation but also implies the presence of tyrosine phosphatases. Protein phosphatase Shp-2 was the first identified tyrosine phosphatase that localizes to the mitochondrial intermembrane space and the outer mitochondrial membrane [167]. It represents another example of dual localization because it is also a component of the signaling scaffold located on the inner side of the plasma membrane as part of the Ras pathway. Interestingly, mutations in the PTPN11 gene, which encodes Shp-2, account for about 50% of cases with Noonan syndrome [168], an autosomal dominant disorder characterized by dysmorphic facial features, congenital heart disease, short stature, webbed neck, chest deformity, and mental retardation. In Noonan patients Shp-2 is constitutively active [169], and it remains to be seen if disease pathology has a mitochondrial component. Mitochondrial targets of Shp-2 are currently unknown but due to their sub-mitochondrial co-localization CeO tyrosine 304 [83] and Cyt c tyrosine 97 [62] are possible candidate epitopes with known function, both leading to activation of respiration in the unphosphorylated state. Interestingly, calcium is believed to be a major activator of mitochondria, including OxPhos, and Shp-2 mutations were recently shown to enhance calcium oscillations in cardiomyocytes [170].

Direct dephosphorylation of OxPhos components by Shp-2 or indirect activation of OxPhos through calcium would lead to increased respiration and an increase of the mitochondrial

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membrane potential followed by an increase in reactive oxygen species (ROS), which are produced at elevated mitochondrial membrane potential levels [153,171,172]. ROS play an important role in organ development but the amount has to be well controlled because fetal antioxidant activities such as superoxide dismutase are depressed until just prior to parturition [173], which might account for the increased ROS in fetal tissue in certain pathological conditions [174]. The importance of a narrow window of ROS production for proper heart development and function was shown in a recent study using knockout mice lacking the mitochondrial thioredoxin reductase, another protective weapon against ROS [175]. The ubiquitous knockout of the gene caused embryonic death after mid-gestation and organ maldevelopment, including the heart, whereas cardiac tissue-restricted inactivation of the gene resulted in fatal dilated cardiomyopathy. Another relevant example is diabetes, where oxidative damage caused by ROS was found in fetuses of diabetic rats, leading to congenital abnormalities, including abnormalities of the heart and great vessels, and neuronal damage [176].

5. Cancer, apoptosis, free radicals, and neurodegenerative diseases

Mitochondria seem to play an essential role in cancer proliferation. Most solid tumors show a reduction in mitochondrial mass of 25–60% compared to normal proliferating tissue [177]. This disequilibrium appears to signal the cell that it is in an undifferentiated state, comparable to embryonic cells, which show similarly low mitochondrial amounts [177]. Compared to differentiated cells tumors rely less on respiration and more on glycolysis, which is known as the Warburg effect [4,5].

The reasons cancer cells utilize glycolysis even in the presence of oxygen – what is known as aerobic glycolysis – are currently being elucidated. One obvious factor appears to be an enormous demand for energy in rapidly growing cells. Under normal conditions mitochondria produce most cellular ATP. However, respiration and in particular the shuttling of NADH from the cytosol into the mitochondria are slow processes (Fig. 4). Therefore, as long as glucose supply is unlimited, glycolysis allows considerably faster ATP production compared to OxPhos [178].

Another key aspect of increased glycolysis results from its linkage to the pentose phosphate pathway, which provides essential building blocks for biosynthesis, such as the five-carbon sugar ribose 5-phosphate and NADPH. Ribose 5-phosphate and its derivatized forms are components of DNA, RNA, and the co-factors ATP, NADH, FAD, and coenzyme A. NADPH is required for biosynthetic pathways, including fatty acid, cholesterol, and nucleotide synthesis. The cell can adjust the amount and ratio of pentose and NADPH production according to its needs, because the pentose phosphate pathway is linked to glycolysis through transketolase and transaldolase reactions, which as a result allows the interconversion between five- and six-carbon sugars. The increased utilization of the pentose phosphate pathway in cancer cells compared to normal cells has been elegantly demonstrated through metabolic studies using [1-14C]glucose and [6-14C]glucose followed by the determination of [14C]CO2 production [179]. The authors showed that utilization of the pentose phosphate pathway was increased fourfold in prostate tumors compared to normal cells, and more than 12 times elevated in a poorly differentiated urothelial tumor compared to normal urothelium, whereas a well-differentiated urothelial tumor showed an intermediate, more than threefold increase. One could speculate that the ratio of the two linked pathways is a marker for cancer differentiation, staging, and aggressiveness (Fig. 4).

Cancer cells require these major metabolic changes to develop a rapid and unrestricted proliferation, and the reduced amount of mitochondria and the reduced capacity of the remaining mitochondria to respire might be interpreted as markers for de-differentiation in cancers: the less differentiated the more aggressive and the less reliant on mitochondrial respiration. Interestingly, cancer therapy approaches, including ionizing radiation and many of the chemical differentiation enhancing agents, may act at least in part by increasing the mitochondrial complement, and thereby returning the cell to the differentiated, respiring, and non-proliferating state. This is so because such agents aim at a transient nuclear cell cycle inhibition, which suppresses mitochondrial DNA synthesis much less efficiently [180].

Mutations in mitochondrial DNA (mtDNA) have been identified in many tumors (reviewed in [181]), and have been proposed as a possible tool for cancer diagnosis [182]. Once present, such mutations may enhance tumorigenesis as was recently shown with cybrid cells containing the same nuclear DNA in combination with or without mutant mtDNA [183]. However, in most cases mtDNA mutations are probably a secondary event and not the cause of cancer.

It is now well established that cell signaling pathways are dysregulated in cancers. Classic examples, which pointed to the importance of signal transduction, are cancers caused by viruses, such as the Rous sarcoma virus that infects chickens and expresses a kinase, v-Src, homologous to the cellular kinase
The converging principle seems to be the release of Cyt c, and was shown to directly inter-
stitute recessive neurodegenerative disease caused by mutations also contribute to the pathology of Friedreich ataxia, an auto-
mitochondrial respiration[184], perhaps through decreased assembly factor, and that deletion of either p53 or SCO2 represses mitochondrial respiration[184], thereby decreased amounts of fully assembled CcO.

Mitochondria are emerging as a target for cancer therapy for another reason: they are involved in apoptosis, a process that cancer cells manage to evade. Mitochondrial or type II apoptosis involves several proteins that are mostly localized on the outside of the mitochondria such as anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1, and proapoptotic proteins Bax and Bak. Bax and Bak are involved in pore formation after the induction of apoptosis, which leads to the release of proteins located in the mitochondrial intermembrane space including apoptosis inducing factor (AIF) and OXPhos component Cyt c. Several studies are underway testing chemical compounds that enhance apoptosis (reviewed in[185]). For example Genasense™ is an antisense oligonucleotide that targets and down-regulates anti-apoptotic protein Bcl-2. Cells are then more susceptible to apoptosis, which can be exploited by co-administration of a standard chemotherapeutic drug.

A number of other anti-neoplastic compounds currently in use or in (pre-)clinical testing including topoisomerase II inhibitor etoposide, anti-microtubule agent taxol, and steroid mimetic CD437 also appear to directly target mitochondria[186]. The converging principle seems to be the release of Cyt c and the induction of apoptosis, although the exact mechanisms remain elusive in most cases.

Another mechanism by which the mitochondrial OXPhos system appears to be involved in the carcinogenesis process is increased free radical production. Free radicals are a byproduct of the ETC, and increased levels lead to enhanced damage of DNA, proteins, and lipids. Free radicals were thus proposed to contribute to genomic instability and cancer progression. Increased free radical production can derive from dysfunctional ETC complexes: this was recently shown for mutations in genes encoding complex II subunits B, C, and D, which have been associated with human parangliomas that include head, neck, and adrenal cancers[187]. It was shown that the introduction of such a mutation of the C subunit into cultured B6 cells results in significantly increased superoxide and hydrogen peroxide levels and a fivefold increase in glucose uptake, another hallmark of cancer metabolism[188].

The capability of complex II to produce free radicals may also contribute to the pathology of Friedreich ataxia, an auto-
somal recessive neurodegenerative disease caused by mutations in the frataxin gene. The frataxin protein is associated with the inner mitochondrial membrane and was shown to directly interact with complex II[189]. Dysfunctional frataxin might induce free radical production, perhaps by interfering with complex II assembly or by inducing structural changes in complex II. Increased amounts of free radicals produced by the mitochondrial ETC are emerging as common factor in neurodegenerative disease, including Parkinson’s disease, Alzheimer’s disease, Friedreich ataxia, and multiple sclerosis. This can be at least in part explained by the fact that neuronal tissue is highly aerobic but at the same time very sensitive to oxidative damage due to relatively low levels of antioxidants and antioxidant enzymes (reviewed in[190]).

Dysfunctional mitochondria play an important role in various neurodegenerative diseases. Among OXPhos complexes, significantly decreased activity of CcO above all was found in Alzheimer disease (AD) patients[191,192]. It was recently shown that amyloid precursor protein accumulates in mitochondria of AD patients, clogging the protein import machinery of the mitochondria[193]. This mechanism can also explain decreased protein levels of the core protein 1 of bc1 complex and the β-chain of ATP synthase in AD patients[194], a pattern which is also observed in patients with Parkinson’s disease (PD). PD is the most common neurodegenerative movement disorder. Several PD mutations have been identified in genes that encode proteins involved in mitochondrial function and regulation, including α-synuclein, parkin, the mitochondrial kinase PINK1, and the mitochondrial protease OMI (reviewed in[195]). Interestingly, α-synuclein, the major component of Lewy bodies found in PD patients, was shown to interact with CcO by yeast two-hybrid and co-immunoprecipitation studies[196]. A general mechanism underlying dopaminergic cell loss as found in PD patients might be increased cellular stress and ROS production leading to cell death over time.

6. Concluding remarks

A large and still increasing number of human diseases are associated with a dysfunctional OXPhos machinery. The two distinct key factors involved are a lack of energy and increased free radical production. Because of the central role of OXPhos in cellular function the activity of its components has to be tightly regulated and fine-tuned, as is clear from considering developmental and tissue-specific energy requirements, and as is also seen in the expression of subunit isoforms in CcO. Moreover, higher organisms have to adapt to a constantly changing meta-
abolic environment, as seen in distinct states such as during starvation, feeding, and exercising. Adaptation to those conditions requires communication between cells and organs and is mediated through hormones and electrical signals that activate cell signaling cascades. Since the first reports on the phosphorylation of OXPhos components more than a decade ago the importance of this new field is increasingly recognized because it is the link between physiology at the organismal level and molecular changes within the mitochondria. Only 14 phosphorylation sites have been mapped to date (Table 1) and the functional consequences of most of these events have yet to be studied. One would expect co-regulation of OXPhos components under certain conditions, and we recently proposed that this may be the case for Cyt c and CcO, which share 5 out of 10 amino
acids in their phoso-epitopes (Table 1). To map further phosphorylation sites and to study mitochondrial function in a physiologically meaningful context, standard protocols to isolate mitochondria have to be modified in order to prevent dephosphorylation during the isolation of mitochondria and OxPhos enzymes. Such protocols are available [62,83], although isolate mitochondria have to be modified in order to prevent physiologically meaningful context, standard protocols to phosphorylation sites and to study mitochondrial function in a 

To map further work on non-mammalian OxPhos complexes was not included. 

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