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MINI-REVIEW

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Phosphorylation of OXPHOS Machinery Subunits: Functional Implications in Cell Biology and Disease

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The complexes of the electron transport chain and ATP synthase comprise the oxidative phosphorylation (OXPHOS†) system. The reactions of OXPHOS generate the mitochondrial membrane potential, drive the majority of ATP production in respiring cells, and contribute significantly to cellular reactive oxygen species (ROS). Regulation of OXPHOS is therefore critical to maintain cellular homeostasis. OXPHOS machinery subunits have been found to be highly phosphorylated, implicating this post-translational modification as a means whereby OXPHOS is regulated. Multiple lines of evidence now reveal the diverse mechanisms by which phosphorylation of OXPHOS machinery serve to regulate individual complex stability and activity as well as broader cellular functions. From these mechanistic studies of OXPHOS machinery phosphorylation, it is now clear that many aspects of human health and disease are potentially impacted by phosphorylation of OXPHOS complexes. This mini-review summarizes recent studies that provide robust mechanistic detail related to OXPHOS subunit phosphorylation.

INTRODUCTION

Maintenance of bioenergetic homeostasis is a principle function of cellular metabolism as sufficient energy levels must be maintained for cells to thrive [1]. The complexes of the electron transport chain and ATP synthase comprise the OXPHOS system, through which the majority of cellular ATP is generated. It is clear that dysregulated bioenergetics play an important role in the etiology of many diseases, including diabetes, obesity, cardiovascular disease, aging and neurodegenerative diseases such as Alzheimer's and Parkinson's disease [1-4]. Additionally, several mitochondrial diseases directly result from deficient OXPHOS-dependent energy production [5-7]. Due to the critically important nature of OXPHOS, it is assumed that mechanisms exist that are

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†Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ATP, adenosine monophosphate; ATP5, ATP synthase subunit; cAMP, cyclic adenosine monophosphate ; Cdk1, cyclin dependent kinase 1; COX, cytochrome c oxidase; Cytc, cytochrome c; NADH, nicotinamide adenine dinucleotide; NDUF, NADH dehydrogenase subunit; NRTI, nucleoside analog reverse transcriptase inhibitor; OXPHOS, oxidative phosphorylation; PINK1, PTEN-induced kinase 1; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 12-acetate; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TNFα, tumor necrosis factor alpha; Tyr, tyrosine.

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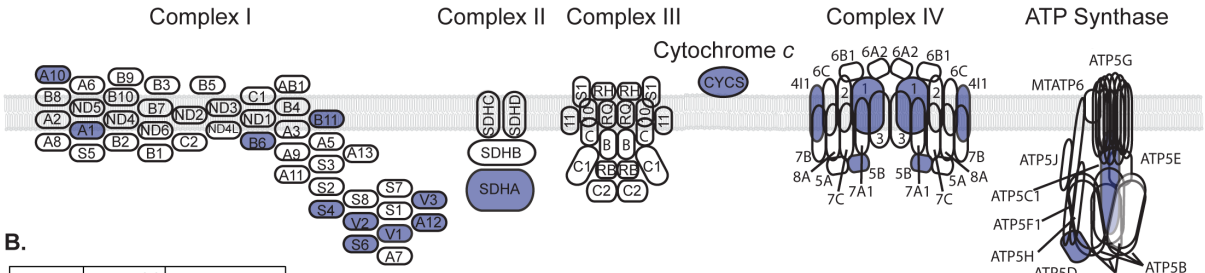
A.



B.

Subunit	Kinase(s)	References
NDUFA1	PKA	14
NDUFA10	PINK1	22
NDUFA12	CDK1	23
NDUFB6	CDK1	23
NDUFB11	PKA	14
NDUFS4	PKA	15, 16, 17, 18, 19
NDUFS6	CDK1	23
NDUFV1	CDK1	23
NDUFV2	Src	25, 27
NDUFV3	CDK1	23
SDHA	Fgr, Src	25, 29, 30
CYCS	AMPK	33
COX1	?	N/A
COX4I1	PKA	39
COX5B	PKA	40, 41
ATP5C1	PKCα	42, 43
ATP5D	PKCδ	44

Figure 1. **OXPHOS subunit phosphorylation**. 1A) Schematic representation of the OXPHOS complexes with subunits described in this review highlighted in blue. 1B) An index of subunits described in this review along with their purported associated kinases and references.

able to regulate OXPHOS both directly and indirectly. Post-translational modification of proteins is a common mechanism of regulatory control, and therefore it is unsurprising that cells regulate OXPHOS through multiple means, including phosphorylation of the OXPHOS machinery itself [8]. Dozens of kinases and phosphatases localize to mitochondria [9] and heavy phosphorylation of OXPHOS complex subunits is evident from the results of numerous phosphoproteomic analyses [10-12].

In this mini-review, selected studies which provide robust mechanistic details relevant to OXPHOS subunit phosphorylation in cells are summarized (Figure 1). From these studies, the picture emerges that phosphorylation of OXPHOS machinery is a common mechanism regulating OXPHOS complex assembly and stability, fine-tuning of bioenergetic control, and response to cellular stimuli both positively and negatively affecting OXPHOS and OXPHOS-dependent functions (such as ROS generation). Important cellular events such as progression through the cell cycle and cell differentiation are also governed in party in this way. Finally, the relevance of OXPHOS machinery phosphorylation to human health and disease becomes evident from these studies too, as diseases from early onset Parkinson's disease to Type 2 diabetes to cancer are associated with these events.

COMPLEX I

A large percentage of mitochondrial respiratory complex I subunits are phosphorylated (Figure 2), although the functional significance of only a minority of these phosphorylation events have been described. A major driver of complex I phosphorylation appears to be cAMP and PKA [13]. Both NDUFA1 and NDUFB11 contain putative PKA phosphorylation sites, and the functional significance of these sites were tested in cells harboring NDUFA1 and NDUFB11 knockouts which fail to both assemble functional complex I and respire [14]. While re-expression of wild type NDUFA1 and NDUFB11 rescues complex I assembly and respiration, the serine to alanine mutants exhibited both lower protein expression, complex I assembly, and respiration. This study also demonstrated a complete loss of complex I assembly upon expression of serine to aspartate or glutamine mutants. The results of this study could either implicate phosphorylation of two complex I subunits to be necessary to maintain or even initiate complex I assembly or they could implicate phosphorylation of a mature complex I to be a signal for complex disassembly and turnover. Alternatively, the results of the serine to alanine mutants may be due to the serine coordinating stabilizing hydrogen bonds in an assembled complex I while the glutamine mutant results may be due to ste-

COMPLEX I	
NDUFA1	S55/Y60/Y61/S63
NDUFA2	S7
NDUFA3	S36/Y41/S42
NDUFA4L2	S5/Y77
NDUFA5	Y28
NDUFA6	S11/T93
NDUFA7	S32/Y53/S63/S67/S85/T90/T96
NDUFA8	Y142/Y151/Y168
NDUFA9	S7/S78/204/T343
NDUFA10	S57/T61/T23 /S231/Y233/T241/S250/Y275/T345
NDUFA11	None Reported
NDUFA12	T120/T142
NDUFA13	T79/Y125
NDUFAB1	S11/S99/Y147
NDUFB1	S42/S52
NDUFB2	S9/T25
NDUFB3	S60
NDUFB4	Y23/S26/Y51
NDUFB5	S182
NDUFB6	T2/T5/S29/S55/Y95/T113
NDUFB7	T24/Y29/S73/Y89
NDUFB8	Y41/Y90/Y153/Y161/Y163/Y167/Y184
NDUFB9	Y73/Y80/S85/T89/Y91/Y115/Y118/S132/Y169
NDUFB10	S4/T17/Y26/Y55/Y56/Y57/Y137/Y143/S145
NDUFB11	T52/T53/Y128/S139
NDUFC1	None Reported
NDUFC2	S19/T54/Y80
NDUFS1	S14/S16/Y316/T317/S434/T439/Y440/T441/S448/ S478/S479/T503/S504/T507/T611/T697
NDUFS2	T272/S364
NDUFS3	S38/T43/T46/S59/Y64/T115/T121/T151/T189/ Y191/Y207/S237/S251
NDUFS4	T32/S34/S165/S173
NDUFS5	None Reported
NDUFS6	Y51/S78/T82/Y100
NDUFS7	S117/T125/T127
NDUFS8	Y38/T52/Y74
NDUFV1	T124/S251/S323/T324/T383/S464
NDUFV2	T15/T51/Y119/S147/T164/Y193/S220/S233
NDUFV3	S100/S101/S105
ND1	None Reported
ND2	T258Y298
ND3	S52/Y104
ND4	T8/T247/S290
ND4L	None Reported
ND5	S14/T565/Y587
ND6	None Reported

COMPLEX III	
CYB	T2/T7/T75
UQCR10	None Reported
UQCR11	None Reported
UQCRB	S8/S10/Y21/T37/Y39/Y56/Y90/Y94
UQCRC1	Y91/T101/S107/Y123/T125/Y131/Y132/T195/ S212/T222/Y224/T381/S401/S416/Y446/Y459/ Y468/S473
UQCRC2	Y55/S56/S59/T86/T100/S111/T113/Y182/Y191/ Y207/S226/S367/T369/T447
UQCRFS1	S16/T37/Y91/S99/T100/T122/S157/S193
UQCRH	T11/S61/T63
UQCRHL	T11
UQCRQ	S16/Y17/S20/T32
UQCC1	S291
UQCC2	Y67/Y72/Y88
CYC1	S103/Y174/Y179/S182/Y199/S278

COMPLEX II	
SDHA	T24/S177/Y215/S243/S321/Y365/Y436/S456/ S530/Y604/Y606/S628/Y629
SDHB	T119/Y216/S222
SDHC	S60
SDHD	None Reported

CYTOCHROME *c*	
CYCS	T29/T41/Y47/S48/Y49/T50/Y68/Y75/T79/Y98

COMPLEX IV	
COX1	T17/Y19/T31/S34/T316/S330
COX2	None Reported
COX3	Y67
COX4I1	S26/S30/S34/Y38/S72/S74/Y124/S132/S158/
COX4I2	None Reported
COX5A	T48/T76/T79/Y80/T141
COX5B	T39/Y62/S71/
COX6A1	None Reported
COX6A2	None Reported
COX6B1	Y34/T45/S52/T81
COX6B2	Y63/S65
COX6C	Y49/Y53/S73
COX7A1	S13/S16/T17
COX7A2	Y44/Y55/T71
COX7A3	None Reported
COX7A2L	S47/Y52/Y87
COX7B	None Reported
COX7C	S5/S17
COX8A	None Reported
COX8C	None Reported
NDUFA4	Y62/Y65/S66/Y71

ATP SYNTHASE	
ATP5A1	T48/S53/T64/S65/S76/S99/S100/T134/S166/S184/S 198/S220/T225/Y243/Y246/S254/T255/T264/ Y299/Y311/Y337/Y343/S419/T432/Y440/S451/ S462/S477/S502
ATP5B	T21/S23/S25/S51/S128/T140/T185/Y196/T213/ T240/Y247/Y269/T288/Y292/T312/S316/S319/S327/ T334/Y361/T374/Y395/S403/S415/Y418/S433/ S447/S465/T475/Y499/S528/S529
ATP5C1	T45/S73/S97/S108/S109/S116/T122/T144/S146/ S265/T284
ATP5D	S39/S44/T144
ATP5E	T29
ATP5F1	T31/Y116/S142/T152/ Y165/Y199/S226/T226/T229
ATP5G1	None Reported
ATP5G2	None Reported
ATP5G3	Y22
ATP5H	S30/T39/Y56/Y57/Y86/S100/S106/Y150/Y152
ATP5I	S8/Y25/T28/Y30/Y32/S66
ATP5J	S64/S65/Y67
ATP5J2	S3/S36
ATP5L	Y29/Y32/T42/S59/T62/S64
ATP5O	Y35/Y41/T43/Y46/S47/S77/Y82/S91/T96/S155/ S163/S166/T177/S180/Y193/S197/T200
ATP5S	T154/S162/T167
ATP6	None Reported
ATP8	T31/Y33/S38/Y47

Figure 2. A sample of OXPHOS machinery subunits reported to be phosphorylated [49]. For each complex, the left column indicates specific subunits and the right column indicates reported phosphorylated residues. Highlighted subunits are described in this mini-review.

ric hindrances considerations, both independent of any phosphorylation effects. Therefore, further mechanistic investigations into these proposed models are warranted. PKA also phosphorylates human NDUFS4 [15] and promotes accumulation of mature NDUFS4 in mitochondrial while alkaline phosphatase treatment inhibits the same [16,17]. Interestingly, NDUFS4, for which PKA-dependent phosphorylation is also required for proper complex I assembly, is not regulated at the level of mitochondrial import. PKA, therefore, appears to regulate mitochondrial respiration via complex I subunit phosphorylation at multiple levels. Importantly, fatal human NDUFS4 mutations inhibit cAMP-dependent NDUFS4 phosphorylation or destroy the cAMP/PKA-dependent phosphorylation site and prohibit complex I activation [18,19], underscoring the importance of this phosphorylatable residue to human health. Interestingly, evidence also exists for the ability of nucleoside analog reverse transcriptase inhibitors (NRTIs) to impair mitochondrial respiration. This effect has been linked to NRTI-mediated inhibition of cAMP-dependent phosphorylation of complex I and a resultant increase in superoxide production [20].

PINK1 is a kinase which regulates mitophagy in order to maintain healthy cellular pools of mitochondria, and PINK1 mutations confer one form of early-onset Parkinson's disease. In addition to its role in promoting mitophagy, PINK1 is hypothesized to regulate additional cellular functions not directly related to mitophagy [21], including mitochondrial respiration as some PINK1 mutations appear to impair complex I activity. This particular effect of PINK1 mutation was found to result from a lack of NDUFA10 phosphorylation at serine 250, which was necessary for efficient electron transfer to ubiquinone [22]. Expression of the phosphomimetic S250D rescued complex I activity and ATP production in cells from PINK1 patients, implicating complex I phosphorylation in some cellular phenotypes associated with this neurodegenerative disease.

During cellular proliferation, progression through the G2/M transition also appears reliant on complex I phosphorylation. During mitosis, a cyclin B1/Cdk1 complex localizes to the mitochondrial matrix and phosphorylates NDUFA12, NDUFB6, NDUFS6, NDUFV1, and NDUFV3 [23]. The respective alanine mutants in these proteins decrease complex I activity while the corresponding phosphomimetic glutamic acid mutations increase complex I activity. This cyclin B1/Cdk1-dependent phosphorylation of complex I subunits appears to be required for cells to produce the amount of ATP requisite to progress through the G2/M transition.

Under conditions of sepsis, phosphorylation of OXPHOS machinery is also relevant. In cardiac tissue, sepsis induces elevated ROS levels, an effect which appears to be mediated by SHP2 phosphatase-dependent dephosphorylation of complex I and complex II subunits and cytochrome C. Under normal conditions, these proteins are proposed to be phosphorylated by Src and treatment of mitochondrial fractions with active Src ameliorated some sepsis-associated effects [24]. Additional studies investigating the Src inhibitor, PP2, demonstrate reduced respiration. These effects are correlated with Src-mediated phosphorylation of NDUFV2 and SDHA (complex II) [25]. Cells harboring the tyrosine to phenylalanine phospho-defective mutants display decreased complex I activity, increased ROS generation, and decreased viability in normal and cancer cells. Src may also contribute to the bioenergetic capabilities of cancer cells. Src localizes to mitochondria in osteosarcoma and prostate cancer cells and leads to tyrosine phosphorylation of mitochondrial proteins, including complex I protein NDUFB10, to enhance mitochondrial bioenergetics [26]. Src activity is further proposed to *negatively* regulate complex I through phosphorylation of complex I subunit NDUFV2. In response to the adenosine receptor activator, 5'-(N-ethylcarboxamido), NDUFV2 phosphorylation Src activity and NDUFV2 phosphorylation correlated with reduced complex I activity, an effect that was reversed with an NDUFV2 tyrosine to phenylalanine mutant [27].

A particularly useful approach has recently provided a comprehensive analysis of complex I phosphorylation sites [28]. In this study, the authors utilized bioinformatics to predict both complex I phosphorylation sites and the potential associated kinases. Molecular modeling was also used to display the potential effects of subunit phosphorylation, and phosphoproteomics analyses were performed to validate their bioinformatic predictions. This approach identified many of the functionally relevant phosphorylation events described above.

COMPLEX II

SDHA is a known target of Fgr tyrosine kinase [29]. Studies of Fgr action on complex II demonstrate a ROS-dependent phosphorylation of SDHA at Y604 resulting from Complex I deficiencies. This phosphorylation was able to remodel and rescue mitochondrial respiration by elevating complex II activity [30]. In this context, compensatory complex II activity shifts cellular fuel sources from NADH to $FADH_2$ thereby elevating ROS production – possibly providing a mechanism for ROS signal amplification. On the other hand, under complex III-absent conditions, complex II cannot rescue energetic efficiency despite SDHA subunit phosphorylation from ROS accumulation seen in complex I deficiencies [31].

CI-deficient humans also express elevated ROS production from increased CII activity after Tyr604 phosphorylation, but Salvi *et al.* exclusively accredited decreased CI population for upregulated CII activity

– where absence of CIII and CIV had no influence on Complex II activity [29]. Similarly, Ogura *et al.* proposed additional phosphorylation sites of SDHA at Y215 and by c-Scr, as necessary regulatory sites for respiratory electron transfer processes, cell survival, and redox homeostasis in CII and CI, respectively [25]. In fact, perturbed phosphorylation of SDHA may affect efficient $FADH_2$ electron transfer, as Tyr215 is located at the FAD-binding domain intersecting SDHA and SDHB. Subsequently, phosphorylation-defective SDHA at Y215 was shown to induce a ROS-responsible regression in male mice B cell maturation, specifically inhibition of germinal center formation and proliferation and suppressed humoral immune responses [32]. These investigations highlight the versatility in metabolic remodeling as a consequence of complex II phosphorylation, and highlight the impacts of dysregulated OXPHOS subunit phosphorylation.

COMPLEX III

The majority of complex III subunits have been reported to be phosphorylated, with many phosphorylated at multiple residues (Figure 2). However, detailed, mechanistic studies describing the functional significance of these modifications have yet to be performed.

CYTOCHROME *c*

Cytochrome *c* (Cyt*c*) oxidizes complex III and reduces complex IV in addition to promoting apoptosis upon mitochondrial outer membrane permeabilization. Therefore, phosphorylation of Cyt*c* may impinge upon both or only one of these functions. One phosphorylation event that controls OXPHOS is Cyt*c* phosphorylation at threonine 28 which appears to ensure that OXPHOS does not become overactive and thus generate harmful ROS [33]. Phosphorylation at this putative AMPK site results in decreased ability of Cyt*c* to transfer electrons to complex IV and therefore acts as a throttle on OXPHOS. Cells expressing phosphomimetic Cyt*c* display both reduced ROS and mitochondrial membrane potential, lending support to the idea that phosphorylation at this site maintains "controlled" respiration.

Cyt*c* tyrosine phosphorylation at Y48 also imparts significant regulatory capacity of Cyt*c* function. Expression of non-phosphorylatable tyrosine to phenylalanine mutants decreases the redox potential of Cyt*c*; however, tyrosine to glutamic acid mutations had a similar effect [34,35]. A subsequent study used the phosphomimetic *p*-carboxy-methyl-L-phenylalanine as a substitute for Y48 for structural studies and found that Cyt*c* Y48 phosphorylation induces significant structural reorganization [36]. This study further suggests that Y48 phosphorylation would impair Cyt*c* mobility between complex III and complex IV.

COMPLEX IV

The majority of complex IV subunits are also phosphorylated (Figure 2). When cells are treated with TNFα, a cytokine that mediates many of the effects of systemic inflammation (such as sepsis), COX1 is tyrosine phosphorylated [37]. This phosphorylation correlates with reduced complex IV activity, a decrease in mitochondrial membrane potential, and reduced ATP generation, implicating complex IV phosphorylation in the glycolytic shift accompanying acute inflammation. Additional COX1 phosphorylation sites also appear to be functionally relevant in mammalian cells [38]. Notably, COX1 is encoded by mitochondrial DNA and therefore, phosphorylation must take place in the mitochondrial matrix, highlighting the importance of mitochondrially-localized kinases (and presumably phosphatases).

A detailed study of COX4I1 found that PKA-dependent phosphorylation of serine 58 does not profoundly alter complex IV conformation, yet this reversible phosphorylation site does affect that ability of ATP to negatively regulate complex IV activity [39]. Phosphorylation of S58 improves the ability of cells to respire in media that must rely on OXPHOS for ATP generation. This study provides some of the most compelling evidence that reversible phosphorylation of OXPHOS machinery can be utilized to fine tune bioenergetics.

Additional evidence of complex IV subunits being under the control of phospho-mediated regulation comes from ischemia/reperfusion models. Animal models implicate phosphorylation of multiple OXPHOS subunits to suppress OXPHOS following reperfusion [40]. In these models, reperfusion induces cAMP-independent, mitochondrially-localized, PKA to phosphorylate COX5B and suppress COX activity. PKA in this context is activated by ROS, and antioxidants and PKA inhibitors ameliorate this effect [41].

ATP SYNTHASE

Perhaps the clearest mechanistic studies related to ATP synthase phosphorylation focus on the effects of PKC isoforms. PKCα localizes to mitochondria in kidney cells following treatment with a kidney-damaging environmental toxin, and leads to the phosphorylation of ATP5C1 [42]. *In vitro*, PKCα directly phosphorylated this subunit and decreased ATP synthase activity. PKCα-dependent phosphorylation in this context diminishes the ability of renal cells to recover following injury with environmental toxins. However, later studies demonstrate that PKCα-dependent phosphorylation of ATP synthase improves mitochondrial function following cellular inju-

ry by exposure to toxins or hypoxia and reperfusion [43]. PKC isoform δ, on the other hand, associates with ATP5D following PKC stimulation by PMA or following exposure of cells to hypoxia [44]. Similar to the earlier PKCα reports, PKCδ interaction with and phosphorylation of ATP synthase appears to reduce ATP synthase activity.

CONCLUSIONS AND OUTLOOK

The studies summarized here demonstrate the functional relevance of OXPHOS machinery subunit phosphorylation (Figure 1). We reiterate that numerous other studies have demonstrated phosphorylation of OXPHOS machinery subunits, and in many instances changes in phosphorylation are observed in normal versus diseased cells/tissues. While these studies are certainly valuable, we chose to focus on those studies that reveal some mechanistic and/or functional insight into subunit phosphorylation. Like phospho-regulatory mechanisms of other proteins, phosphorylation can either promote or repress OXPHOS complex function. There is an obvious disparity between the high percentage of OXPHOS machinery subunits that are reported to be phosphorylated and those phosphorylation events that have assigned functional significance. Much of the reported phosphorylation comes from high throughput studies, and therefore additional validation studies are necessary. As described above, Gowthami *et al.* provide a useful approach to validating these phosphosites [28]. In addition, stoichiometric analyses measuring the ratio of phosphorylated:unphosphorylated respiratory complex proteins identified in both high throughput investigations and the mechanistic studies described above would have great value. Uncovering the stoichiometry at which biological effects are regulated by these phosphorylation events will be necessary to inform any potential therapeutic modulation of OXPHOS complex phosphorylation. While these measurements have been historically difficult or under-studied, recent advances now allow even large-scale stoichiometric determination of phosphorylation sites [45]. Therefore, stoichiometric determination of OXPHOS complex phosphorylation is a key and attainable future direction for the field.

In addition to validating the actual phosphorylation events, there is much room for future studies to determine the functional significance of OXPHOS machinery phosphorylation. Perhaps the most straight forward functional studies would address whether phosphorylation or prohibition of phosphorylation affects incorporation of specific subunits into complexes, the stability of the complexes themselves, and activity or reductive capacity of the complexes. Resolving these parameters would then help inform further assessment of respiration-dependent ATP production, mitochondrial membrane potential, and ROS generation and perhaps assembly and function of respiratory chain supercomplexes. Very closely related to these considerations are the contributions of specific kinases and phosphatases to phosphorylated OXPHOS machinery. The studies above employ gene deletions in cells and animal models, chemical inhibitors, and expression of constitutively active or dominant negative kinases to address this question. While some studies have assessed general features of mitochondrial function by screening groups of known mitochondrially-localized kinases and phosphatases [46], more direct investigations into the responsible regulators of OXPHOS subunit phosphorylation will provide greater clarity to these questions.

Important to this field are the data summarized above which demonstrate the high relevance of subunit phosphorylation to human health. An inability to phosphorylate and thus incorporate mutant NDUFS4 into complex I appears to contribute to a lethal form of mitochondrial disease [18,19], an inability of mutant PINK1 to phosphorylate NDUFA10 may contribute to early onset Parkinson's disease [22], and PKC-mediated phosphorylation of ATP synthase subunits appears to affect the ability of some tissues to recover from an ischemic insult [42-44]. Consideration of OXPHOS subunit phosphorylation is also relevant in human health as applied to maintaining cellular homeostasis (for example, balancing ROS and bioenergetic needs [30]) or even when treating a disease with drugs that may impinge upon these regulator mechanisms [20]. Therefore, it is reasonable to predict that regulation of additional phosphorylation events will also be involved in maintaining homeostasis to promote health while dysregulation contributes to disease. Indeed, mitochondrial dysfunction across many parameters including OXPHOS function is causally linked to many human diseases [47,48].

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