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Pharmacological approaches to restore mitochondrial function

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Abstract

Mitochondrial dysfunction is not only a hallmark of rare inherited mitochondrial disorders, but is also implicated in age-related diseases, including those that affect the metabolic and nervous system, such as type 2 diabetes and Parkinson's disease. Numerous pathways maintain and/or restore proper mitochondrial function, including mitochondrial biogenesis, mitochondrial dynamics, mitophagy, and the mitochondrial unfolded protein response. New and powerful phenotypic assays in cell-based models, as well as multicellular organisms, have been developed to explore these different aspects of mitochondrial function. Modulating mitochondrial function has therefore emerged as an attractive therapeutic strategy for a range of diseases, which has spurred active drug discovery efforts in this area.

Introduction

Mitochondria — the intracellular powerhouse in which energy from nutrients is converted into ATP — are thought to be evolutionarily derived from alphaproteobacteria that operated within cells in endosymbiosis[1]. Consequently, mitochondria have unique characteristics that pose several challenges to the host cell. During evolution, most of the genes of the endosymbiont were transferred to the nucleus (nuclear DNA; nDNA) of the host cell. In human cells, mitochondrial DNA (mtDNA) — in the form of multiple copies of circular double-stranded DNA molecules — encodes only 13 key proteins, which require separate transcription and translation machinery. Furthermore, as ~1,500 additional nDNA-encoded proteins[2] are essential for proper mitochondrial function, a complex system is required for importing, processing and surveying these other proteins[3–6].

To perform their key roles in cellular energy production, mitochondria use an intricate system that encompasses the breakdown of fatty acids and glucose, which is coupled to oxidative phosphorylation. Mitochondria are highly dynamic structures that undergo rapid remodeling through fusion and fission to adapt to changes in the cellular context[7]. When mitochondria are damaged, mitophagy — a specific autophagic response confined to mitochondria — regulates their controlled degradation[8]; furthermore, following extensive damage or specific triggers, mitochondria are central to the initiation of apoptosis[9]. Given the complex balance between the nuclear and mitochondrial genome, and the fact that mitochondria are the site of metabolic transformation and hence a hotspot of metabolic stress, it is not surprising that mitochondrial dysfunction is involved in a broad spectrum of diseases, both inherited and acquired. Prototypical inherited mitochondrial diseases can be caused by mutations in either mtDNA or nDNA, and typically result in very severe multisystem disease from birth. Conversely, mitochondrial dysfunction is important, or at

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least implicated, in a diverse range of acquired diseases, including cancer, metabolic diseases and neurodegenerative disorders, which are often associated with ageing. Here, we first provide an overview of diseases that affect mitochondria and then present key mitochondrial pathways that are amenable to therapeutic intervention, focusing on mitochondrial biogenesis and quality control circuits as the most tractable targets. Finally, we discuss state-of-the-art screening strategies that can be applied to identify drugs targeting these pathways.

Mitochondrial diseases

Mitochondrial diseases can narrowly be defined as inherited disorders resulting from mutations in mtDNA or nDNA that impair mitochondrial function. However, in a broader sense, ageing-associated disorders in which defective mitochondrial function has been pathophysiologically could also be considered as mitochondrial diseases. Below, we briefly discuss these different aspects of mitochondrial dysfunction in diseases, which have recently been extensively reviewed in the literature (see REFS 10–12).

Inherited mitochondrial diseases

Many inborn errors in metabolism are characterized by a primary defect in mitochondrial processes, such as fatty acid oxidation, haem biosynthesis or oxidative phosphorylation[13]. Most of these mitochondrial diseases follow a Mendelian mode of inheritance, meaning that a mutation in a single genetic locus is responsible for the phenotype in either a dominant or recessive fashion (BOX 1). For example, defects in oxidative phosphorylation can be caused by mutations in genes encoding subunits of the electron transport chain (ETC), as well as by mutations in genes involved in mtDNA replication, maintenance and repair, mitochondrial translation, respiratory complex assembly and processes that affect mitochondrial biogenesis, dynamics and homeostasis in general. The pleiotropic origin of defects in oxidative phosphorylation is illustrated by cytochrome c oxidase (complex IV) deficiency, which can be caused by mutations in over 15 different genes encoding complex IV subunits or its assembly proteins[14] (TABLE 1).

Another hallmark of inherited mitochondrial diseases is that their phenotypic presentation is highly heterogeneous, with some diseases affecting single tissues (such as the cochlea in maternally inherited deafness) and others affecting multiple systems (such as encephalomyopathies), and with disease onset ranging from the neonatal phase to adulthood[15]. In the case of diseases caused by mutations in mtDNA, this heterogeneity can be partially explained by the phenomenon of heteroplasmy — in which both native and mutated mtDNA coexist within the same cell — which can arise because mitochondria contain multiple copies of mtDNA[16]. This means that the mutational load could vary substantially among tissues or among members of the same family[17].

Despite this variability, tissues that rely heavily on mitochondria for the generation of ATP — such as the sensory, nervous, cardiac and muscle systems — are more frequently affected than others[18] (TABLE 1). Furthermore, the segregation capacity of mtDNA varies in different tissues. In fact, studies have reported that heteroplasmy is maintained in the brain, muscle and heart of NZB-BALB/c or NZB-129S6 heteroplasmic mice, whereas DNA segregation occurs over time in the ovary, liver and spleen[16,17]. It was also demonstrated that simply carrying two types of normal mtDNA from different inbred strains — that is, NZB-BALB/c and NZB- 129S6 mice — leads to abnormal metabolism, activity and behaviour[16]. Although further work is necessary to understand the underlying genetic causes of this phenomenon, these observations indicate that reducing heteroplasmy in patients with mtDNA mutations could be a therapeutic objective (reviewed in REFS 19,20).

Mitochondrial dysfunction in common diseases

Although the origin of many inherited mitochondrial diseases has been identified, this is not straightforward for common diseases in which mitochondrial dysfunction is involved. Such common diseases are rarely caused by single genetic defects; rather, they result from a combination of factors, including the quantitative contribution of several genes (so-called quantitative trait loci; QTLs) and environmental factors such as exercise, stress, diet and age[21] (BOX 1). Deregulation of various signalling pathways, such as the insulin–IGF1 (insulin-like growth factor 1) pathway[22], the mammalian target of rapamycin (mTOR) pathway[23], the AMP-activated protein kinase (AMPK) pathway[24] and the sirtuin[25] pathways, can induce mitochondrial dysfunction[26]. The phenotypic signs of reduced mitochondrial function — such as impaired fitness, metabolism, cognitive function and memory — that herald underlying organ dysfunction also typify ageing[27–30]. The multiscalar regulatory network that governs mitochondrial homeostasis may therefore be involved in the development of some of the most common age-related diseases, including type 2 diabetes, obesity, Alzheimer's disease and Parkinson's disease.

Metabolic disorders—Type 2 diabetes is a complex multisystem metabolic disease that is characterized by elevated glucose levels. A common early feature in the pathogenesis of type 2 diabetes is the accumulation of lipids in skeletal muscle, adipose tissue and the liver owing to mitochondrial dysfunction[31,32]. This is likely to be caused by an inability to maintain metabolic flexibility — that is, the ability to switch from one fuel source to another. In fact, several studies have shown that there is a global reduction in oxidative capacity within the skeletal muscle of patients with type 2 diabetes[33,34]. This was linked to reduced expression and activity of PPARγ coactivator 1α (PGC1α) and its target genes[35–37]. Similarly, the mitochondrial content of white adipose tissue is lower in patients with insulin resistance, type 2 diabetes and severe obesity[38,39]. The situation is less clear in the liver, as both increased and decreased (as well as unaltered) expression of oxidative phosphorylation genes has been observed in the liver of humans and mice with metabolic disease[40–42].

In addition, a reduction in the oxidative capacity of mitochondria seems to be linked to the development of insulin resistance in the context of type 2 diabetes and obesity[11,43]. This hypothesis goes hand in hand with the fact that exercise and caloric restriction both promote mitochondrial biogenesis and oxidative capacity as well as improving insulin sensitivity in patients who are obese and/or have type 2 diabetes[44,45]. Conversely, moderate dysfunction in oxidative phosphorylation following the deletion of apoptosis-inducing factor (AIF) in the liver and muscle, or deletion of mitochondrial transcription factor A (TFAM) in adipose tissue, was shown to protect mice from diet-induced obesity and type 2 diabetes[46,47]. Despite these apparent opposing effects on oxidative phosphorylation overall, these examples support the general concept that mitochondrial function has a role in type 2 diabetes. Further work is needed to establish whether the link between dysfunctional oxidative phosphorylation and type 2 diabetes is causal or consequential, which could clarify how mitochondrial metabolism might be modulated to prevent or treat type 2 diabetes.

Neurodegenerative disorders—Abnormal protein folding homeostasis and subsequent protein aggregation — a phenomenon termed proteotoxicity — is a common feature of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease[48]. Senile plaques, composed of amyloid-β (Aβ) peptide in the case of Alzheimer's disease and Lewy bodies that stain positive for α-synuclein and ubiquitin in the case of Parkinson's disease, are examples of this phenomenon[10]. Defective mitochondrial proteostasis has also been implicated in such diseases[48]. In addition, several proteins associated with early-onset neurodegenerative disease have been directly or indirectly connected to mitochondrial function[10] (TABLE 2). Two genes associated with familial Parkinson's disease, the E3

ubiquitin protein ligase Parkinson's disease protein 2 (PARK2; also known as parkin) and PTEN-induced putative kinase 1 (PINK1)[49,50], have a crucial role in mitophagy, partly by modulating mitochondrial fusion and fission[51,52] (TABLE 2). Likewise, the proteotoxic α-synuclein and the Parkinson's disease-associated protein DJ1 (also known as PARK7) control mitochondrial morphology as well as fusion and/or fission events in the same pathway as parkin and PINK1 (REFS 53–55).

The potential involvement of mitochondrial dysfunction in Alzheimer's disease is currently less clear. Aβ was shown to translocate to mitochondria[56], resulting in reduced ETC activity. This may occur following the induction of mitochondrial fragmentation[57], an increase in mitochondrial membrane viscosity[58] and/or direct inhibition of complex IV activity[59], and involve mitochondria-associated endoplasmic reticulum membranes (MAMs) in the processing of amyloid precursor protein[60]. Additional evidence supporting the potential involvement of mitochondria in Alzheimer's disease has come from mtDNA haplogroup association studies, in which mtDNA haplogroups that have a greater likelihood of predisposing individuals to Alzheimer's disease seem to be associated with defects in oxidative phosphorylation[61].

Following these observations, it was suggested and shown that boosting mitochondrial function by dietary restriction or using the caloric restriction mimetic resveratrol can counteract neurodegeneration in worms and mice (reviewed in REF. 48). Conversely, inhibiting mitochondrial function by knocking out the cytochrome c oxidase assembly protein (Cox10) gene — which encodes an enzyme that is required for the function of mitochondrial cytochrome c oxidase (COX) — in the brain[62] delayed the onset of neurodegeneration in a mouse model of Alzheimer's disease. Even though the approach to knock out Cox10 appears to be counterintuitive, these two types of interventions may be connected through a common induction of protective mitochondrial quality control systems, as discussed below.

Pathways to restore mitochondrial function

The treatment of inherited mitochondrial diseases typically involves general measures, such as the optimization of nutrition and administration of vitamins and food supplements, along with symptom-based management. Other specific approaches are focused on establishing a heteroplasmic shift towards a higher proportion of nonmutated mtDNA in diseases that are caused by mutations in mtDNA rather than nDNA (reviewed in REFS 19,20). Specific novel interventions to improve mitochondrial function in rare and common diseases are in their infancy but could provide benefit in several disease settings. With the growing understanding of mitochondrial function, it has become clear that several aspects could be therapeutically targeted. We focus here on the main pathways that have been demonstrated to maintain and/or restore proper mitochondrial function, such as: mitochondrial biogenesis and metabolic flexibility; mitochondrial dynamics, including fusion and fission; and mitochondrial quality control through proteostasis and mitophagy. We do not cover apoptosis, which is another important aspect of mitochondrial function, but this topic has been extensively reviewed in REFS 63,64.

Mitochondrial biogenesis and metabolic flexibility

Mitochondrial biogenesis is a complex process, driven by a set of nuclear-encoded transcription factors and assisted by transcriptional co-factors, through which the cell equilibrates its energy-harvesting capacity to meet its energetic demands (BOX 2). Considering this transcriptional network, one can distinguish two approaches to induce mitochondrial biogenesis: targeting the upstream regulators (for example, sensors), or targeting their downstream effector pathways (for example, transcription factors and

cofactors) (FIG. 1). It should be noted, however, that for most of the therapeutic strategies it is impossible to discriminate between beneficial effects that arise within the mitochondria and those that arise from other subcellular compartments.

Upstream sensors - caloric restriction, AMPK, mTOR, NAD^+ boosters and sirtuins—Caloric restriction is the only physiological intervention that has so far been shown to increase lifespan in a range of species[65]. Although it is uncertain whether this effect also occurs in primates, caloric restriction does improve metabolic health and prevent pathological decline associated with ageing in such species[66,67]. The molecular network underlying the effects of caloric restriction comprises the key nutrientsensing pathways, such as those involving insulin–IGF1, mTOR, AMPK and the sirtuins[26]. As a caloric restriction diet is very difficult to apply and maintain, the search for caloric restriction mimetics has intensified in the past decade.

The sirtuin family of histone deacetylases has emerged as a prime target to mimic caloric restriction[25]. This was based on the critical dependence of sirtuins on the metabolic co-factor NAD^+ (REF. 68), and on the fact that sirtuin 1 (SIRT1) controls the activity of various transcription factors and co-factors — such as the tumour suppressor p53 (REF. 69), myocyte-specific enhancer factor 2 (MEF2)70, forkhead box O (FOXO) proteins[71–73] and PGC1α[74] — which are known to govern mitochondrial biogenesis and function. The best-characterized sirtuinactivating compound is resveratrol, which is present in low levels in red grapes and red wine[75]. Resveratrol, however, does not directly activate SIRT1; rather, it acts indirectly via the activation of AMPK and the subsequent induction of NAD^+ levels, which stimulate the activity of SIRT1 (REFS 76–80). Regardless of its mechanism of action, resveratrol enhances mitochondrial biogenesis and oxidative capacity in rodent models of diet-induced obesity, which translates into improved muscle function and protection against obesity and insulin resistance, ultimately improving healthspan[81,82].

In humans, resveratrol improved mitochondrial function and lipid levels in obese patients at a 200-fold lower dose than that used in mice (150 mg per day)[83], although differences in the study design and the resveratrol dose used may lead to variable results[84–86]. Resveratrol also improved glycaemic control in patients with type 2 diabetes when it was administered alone or in combination with other hypoglycaemic agents[84,87]. Together, these clinical trials indicated the therapeutic potential of resveratrol in the context of metabolic diseases, although further pharmacokinetic studies will be needed to optimize resveratrol's therapeutic efficiency[88].

Spurred by these beneficial effects of resveratrol, a search for more potent SIRT1-activating compounds (STACs) that are structurally unrelated to resveratrol led to the identification of SRT1720 and SRT2104 (REFS 89,90). The exact mechanism of action of these compounds is the subject of intense controversy, as studies have shown that these STACs only seem to be active on artificial substrates that contain a fluorophore tag[79,91,92]. However, it was recently shown that STACs also activate SIRT1 when certain structural features that mimic the large fluorophore are present in natural SIRT1 substrates (for example, on PGC1α-K778 and FOXO3A-K290)[93]. Despite the controversy regarding the capacity of STACs to directly induce the deacetylase activity of SIRT1, SRT1720 has been reported to improve glucose homeostasis and insulin sensitivity in rodents[89], which translated into improved healthspan and increased lifespan in dietinduced obese (DIO) mice; these findings are consistent with SIRT1 activation[94]. SRT2104, which has similar beneficial effects in DIO mouse models[95,96], was recently assessed for its safety and pharmacokinetics in humans[97]. Besides SRT1720 and SRT2104, several new potent SIRT1 agonists have been reported[98–100]. The outcomes of clinical efficacy studies of these STACs in various diseases are eagerly awaited.

Considering the crucial dependence of sirtuins on the enzymatic co-factor NAD^+ (REF. 101), modulation of NAD^+ levels should activate SIRT1 signalling and promote mitochondrial biogenesis and function. NAD^+ levels can be increased by directly stimulating NAD^+ synthesis or by inhibiting NAD^+-consuming enzymes[102]. Indeed, dietary supplementation with the NAD^+ precursors nicotinamide mononucleotide (NMN) or nicotinamide riboside increased NAD^+ levels in several tissues in mice[103,104]. This effect on NAD^+ levels led to SIRT1-dependent mitochondrial activation and improved exercise endurance and insulin sensitivity[103,104]. Strikingly, nicotinamide riboside also activated the mitochondrial SIRT3 (REF. 103). Although SIRT1 and SIRT3 both regulate important aspects of mitochondrial metabolism, non-mitochondrial targets of these regulators may also contribute to the beneficial phenotype. Similarly, NAD^+ levels and SIRT1 activity were increased in the tissues of mice treated with inhibitors of poly(ADP-ribose) polymerases (PARPs) or ADPribosyl cyclase 1 (also known as CD38), two enzymes that consume NAD^+, leading to a boost in mitochondrial function and metabolic fitness[105,106]. The activation of AMPK, which enhances mitochondrial energy production following an increase in the AMP/ATP ratio[107], also mimics caloric restriction[24]. In healthy animals, the AMPK agonist 5-aminoimidazole- 4-carboxamide riboside (AICAR) increases exercise endurance by activating a gene programme that is normally induced after exercise[108]. AMPK activation by AICAR also rescued mitochondrial dysfunction and the limited exercise capacity of mice deficient in cytochrome c oxidase[109]. Alternatively, inhibition of mTOR, a conserved kinase that promotes cell growth and anabolism in the presence of nutrients and growth factors[110], simulates a state of energy crisis and thereby activates AMPK and boosts mitochondrial activity through an as yet unknown mechanism (BOX 2). In fact, the mTOR inhibitor rapamycin increases lifespan in various organisms including mice[111–113]. Importantly, the beneficial effects of mTOR inhibition in mice seem to be tissuespecific, as adipose-specific knockout of the mTOR complex protein Raptor (regulatory associated protein of mTOR) enhances mitochondrial respiration[114], whereas muscle-specific knockout of Raptor results in muscular dystrophy[115].

Downstream effectors – nuclear receptors, NRF1, TFAM—Nuclear receptors are popular targets for drug discovery as many nuclear receptors can be activated by small-molecule ligands. Ligand binding changes the conformation of nuclear receptors, facilitating the exchange of transcriptional co-repressors for co-activators and inducing the transcription of their target genes. The three peroxisome proliferator-activated receptors (PPARs), PPARα, PPARδ (also known as PPARβ) and PPARγ, constitute a small subfamily of the large nuclear receptor gene family[116–118]. Although fatty acids are natural PPAR ligands, several synthetic PPAR ligands have been identified. PPARα has a crucial role in the adaptive response to fasting, by inducing the expression of genes that are involved in fatty acid oxidation[119]. Consequently, activation of PPARα by fibrates has been shown to reduce hyperlipidaemia and improve insulin sensitivity in patients with type 2 diabetes[116,117]. Similarly, synthetic PPARδ agonists such as L-165041 or GW501516 improve hyperlipidaemia and insulin sensitivity and reduce adiposity in mice[120,121] and obese patients[122], an effect that is linked to enhanced mitochondrial biogenesis and activity[108,121].

Recently, it was proposed that this effect of PPARδ on mitochondria could improve fatty acid oxidation in the context of inherited mitochondrial disorders. Cells derived from patients with a deficiency in very-longchain-acyl-CoA dehydrogenase (VLCAD; also known as ACADVL) were treated with the PPARδ agonist bezafibrate, which restored VLCAD gene expression[123,124]. In patients with carnitine O-palmitoyltransferase 2 (CPT2) deficiency, bezafibrate substantially increased the rates of fatty acid oxidation and CPT2 gene expression in muscle[125]. When administered to mouse models of complex IV deficiency, bezafibrate was unable to fully restore complex IV activity[109]. The final member of the PPAR family, PPARγ, also has a pleiotropic role in metabolic homeostasis[126].

Although potent PPARγ agonists such as rosiglitazone have been successfully used to treat type 2 diabetes, they became sidelined owing to their potential adverse effects[127]. It became clear that less potent PPARγ modulators that selectively modify the recruitment of specific co-factors exhibit fewer side effects than the traditional PPARγ agonists but maintain beneficial effects on energy homeostasis[128,129] (reviewed in REF. 130).

Interestingly, one of the heterodimerization partners of PPARs, the retinoid X receptor-α (RXRα), was recently shown to be involved in mitochondrial retrograde signalling in cybrid cells containing different loads of mt3243, an mtDNA carrying a mutation in the gene encoding tRNALeu (tRNA recognizing a triplet codon for leucine)[131]. The oxidative phosphorylation deficiency in these cybrid cells was accompanied by a decrease in the expression of RXRα and of mitochondrial genes, and was reversed by the RXR agonist retinoic acid[131]. The decrease in RXRα activity was also dependent on JUN N-terminal kinase (JNK) activation by reactive oxygen species (ROS) and reversed by JNK inhibitors[131]. These data suggest that increasing the transcriptional activity of RXRα, either using a direct activator such as retinoic acid or using JNK inhibitors, might restore the efficiency of oxidative phosphorylation. Further work is required to determine whether this also applies to other types of mtDNA mutations or nDNA mutations.

Oestrogen-related receptor-α (ERRα) and ERRγ also regulate mitochondrial biogenesis[132], as shown by genome-wide location analyses identifying ERRα- and ERRγ-binding sites in several genes encoding mitochondrial proteins[133], and by studies in mouse models showing that these receptors coordinate many aspects of oxidative metabolism in muscle tissue[134,135]. Given the beneficial effects of these receptors on mitochondrial function, efforts to develop specific ligands for ERRs — which have long been considered to be constitutively active orphan nuclear receptors[132] — have intensified. Indeed, the finding that 4-hydroxytamoxifen can specifically antagonize ERRγ shows that it is possible to modulate the activity of these receptors using exogenous compounds[136]. ERRγ agonists have been identified[137,138], but no data on their effects in mitochondrial disease models are yet available. However, only inverse agonists have been described for ERRα[139–141], which is congruent with the outcome of structural activity studies indicating that it is challenging to activate ERRα using small molecules[142,143].

Interestingly, a diaryl ether-based ERRα inverse agonist improved glucose homeostasis in rodent models of type 2 diabetes and stimulated the expression of genes involved in mitochondrial metabolism and insulin sensitization in the liver[141]. In the same vein, treatment of leptin-receptor-deficient (db/db) mice with GSK5182, an inverse agonist of ERRγ, reduced hyperglycaemia through inhibition of hepatic gluconeogenesis[144]. These observations could possibly be explained by the promiscuity of ERRα and ERRγ for target genes and their compensatory induction when one ERR is inhibited[133]. Further studies are required to elucidate the crosstalk among the ERRs and to define whether inverse agonists or agonists are desired to modulate mitochondrial function and manage metabolic diseases.

Finally, nuclear respiratory factor 1 (NRF1) and TFAM are two nDNA-encoded transcription factors that have a crucial impact on mitochondrial function. NRF1 promotes, among others, the transcription of ETC subunits and nDNA-encoded mitochondrial transcription factors, including TFAM[145]. The stabilization and activation of NRF1 was achieved by inhibiting its natural antisense transcript[146]. A recombinant form of human TFAM was designed, in which an exogenous amino-terminal domain allows rapid translocation across cell membranes, whereas its mitochondrial targeting signal stimulates mitochondrial uptake[147]. Treatment of aged mice with recombinant human TFAM stimulated oxidative metabolism and improved memory[148]. In addition, treatment of cells derived from patients with Leber's hereditary optic neuropathy (LHON) and Leigh

syndrome using recombinant human TFAM and non-mutated mtDNA restored mitochondrial biogenesis and respiration[149]. However, although these studies indicate that TFAM activation may represent a promising strategy for the treatment of mitochondrial diseases[147–149], it should be noted that aged Tfam-transgenic mice displayed an increased mtDNA copy number that was associated with unexpected mtDNA deletions and ETC deficiency[150]. Further work is therefore required to determine the safety window of this approach.

Downstream effectors – cofactors—The activity of transcription factors results from the delicate balance between inhibitory effects of co-repressors and stimulatory effects of co-activators. This balance can be exploited for therapeutic purposes by fine-tuning or directing the activity of co-factors towards specific transcription factors, pathways or cells and tissues. For instance, selective recruitment of the co-factor steroid receptor co-activator protein (SRC1; also known as NCOA1) instead of its family members SRC2 (also known as NCOA2 or GRIP1)[151] or SRC3 (also known as NCOA3)[152] orients PPARγ activity towards an oxidative programme, resulting in enhanced thermogenesis and protection against obesity and type 2 diabetes in mice[151,153]. Specific PPARγ ligands such as Fmoc-l-Leu induce a particular conformational change in PPARγ that translates into selective SRC1 recruitment and insulin sensitization, without the induction of the weight gain that is commonly associated with PPARγ activation[128].

The similar effects observed with SR1664 and SR1824, two PPARγ ligands that lack classical transcriptional agonist activity but mediate their effects via the inhibition of cyclin-dependent kinase 5 (CDK5) phosphorylation, were probably also due to selective recruitment of the oxidative co-factor PGC1α[129,154]. The induction of PGC1α levels has also been explored as a strategy to boost mitochondrial biogenesis. In a screen performed in mouse white adipocytes, antagonists of transient receptor potential vanilloid 4 (TRPV4) induced PGC1α expression, mitochondrial biogenesis and fat browning[155]. Similarly, irisin, a hormone (and so-called myokine) that is secreted by muscle tissue following exercise, stimulated a broad PGC1α-mediated gene expression programme leading to the browning of white fat[156]. Bile acids can also stimulate oxidative metabolism in brown adipose tissue and skeletal muscle through the activation of G protein-coupled bile acid receptor 1 (GPBAR1; also known as TGR5)[157]. Activated TGR5 induces the cyclic AMP-dependent thyroid hormone-activating enzyme type 2 iodothyronine deiodinase (DIO2), which leads to activation of the thyroid hormone receptor and subsequent induction of PGC1α[157].

Inhibition of transcriptional co-repressors of the PPARs and ERRs also enhances mitochondrial function. For example, in muscle-specific nuclear receptor corepressor 1 (Ncor1)-mutant mice there was an observed increase in both muscle mass and in mitochondrial number and activity, a phenotype that is also conserved in worms[70]. In line with these results, reduced NCOR1 function in mice has been linked to ketogenesis in the liver[158] and to reduced fat accumulation in adipose tissue[159], in a manner reminiscent of PGC1α activation. Furthermore, these tissue-specific loss-of-NCOR1-function studies suggest that inhibiting mTOR complex 1 (mTORC1)[158] and/or insulin signalling[70] may be a pharmacologically viable way to attenuate NCOR1 signalling. Alternatively, inhibition of deacetylases (such as histone deacetylase 3 (HDAC3)), which interact with NCOR1, can result in similar beneficial effects on oxidative metabolism, as indicated by the improved muscle function observed after pharmacological inhibition of HDAC3 in mice[160], reduced adipogenesis after pharmacological inhibition of HDAC3 in cells[161] and reduced gluconeogenesis in liver-specific Hdac3-knockout mice[162].

Other co-factors that are capable of affecting oxidative metabolism include receptor-interacting protein 140 (RIP140; also known as NRIP1), which functions as a corepressor

for several nuclear receptors such as PPARs and ERRα[163]. Genetic ablation of RIP140 increases mitochondrial biogenesis and oxidative metabolism in muscle[164] and adipose tissue[163], and protects mice against metabolic diseases[165,166]. The retinoblastoma protein Rb can also have a major impact on oxidative metabolism through its repressive actions on the transcription factor E2F1, which is a key regulator of cell proliferation and metabolism[167]. In fact, the absence of Rb favours the development of more oxidative programmes in brown adipose tissue[161,168–170] and muscle[167], as evidenced through both in vitro and in vivo studies. Intervening in either the RIP140–nuclear receptor or E2F1–Rb pathways may therefore represent another approach to modulate mitochondrial function, but the potential tumour-promoting effects of Rb inhibition as well as the induction of sterility resulting from the loss of RIP140 function in female mice[171] must be considered.

Mitochondrial quality control

The central position of mitochondria in metabolism makes them vulnerable to damage. First, mitochondria are a site of oxidative stress generation, in particular at the level of complex I and III of the ETC, where electrons might react prematurely with oxygen, leading to the accumulation of ROS[172]. Although ROS may have a signalling function in healthy cells[173], they can also damage lipids, proteins and DNA. Mammalian cells have therefore developed a battery of protective systems to tightly regulate ROS levels. This includes the presence of uncoupling proteins, which dissipate the proton gradient across the mitochondrial inner membrane[174], the production of antioxidant enzymes that dampen ROS accumulation and the induction of mitophagy or even apoptosis if ROS levels become too high (FIG. 2).

Second, the import of proteins into mitochondria involves the unfolding and refolding of the ~1,500 nuclearencoded mitochondrial proteins when they pass through the two mitochondrial membranes. This poses a major challenge for maintaining proteostasis. Furthermore, the number of proteins encoded by both the nDNA and mtDNA needs to be stoichiometrically matched in the complexes and supercomplexes of the ETC. Both of these challenges make the mitochondria particularly vulnerable to proteotoxic stress. In the mitochondria, proteostasis is maintained by fine-tuning the expression of mitochondrial chaperones in the mitochondrial unfolded protein response (UPR). Abnormal ROS production or proteostasis render the mitochondria vulnerable to the accumulation of damage. Several mitochondrial quality control pathways, which include fusion, fission, mitophagy and protein folding homeostasis, have evolved to efficiently protect mitochondria against these insults. These mitochondrial quality control pathways also open up new opportunities for drug discovery, as summarized below.

Fusion and fission—Live-imaging technology has revealed the dynamic nature of mitochondria[175]. Their shape results from the balance between fusion and fission events and involves concerted and balanced actions of fusion proteins (such as mitofusin 1 (MFN1), MFN2 and optic atrophy protein 1 (OPA1)) and fission proteins (such as dynaminrelated protein 1 (DRP1) and mitochondrial fission 1 protein (FIS1))[7,9,176]. In humans, mutations in fusion proteins lead to the development of neurodegenerative diseases; an OPA1 mutation is associated with autosomal dominant optic atrophy, whereas MFN2 mutations lead to Charcot–Marie–Tooth disease type 2A[177,178] (TABLE 1). Diseases caused by mutations in fission proteins are extremely rare in humans[179], probably because these proteins are crucial for embryonic development, as illustrated by the embryonic lethality observed as a result of knocking out Drp1 in mice[180]. Likewise, suppression of either fusion or fission in cultured cells is associated with a deficiency in oxidative phosphorylation, mtDNA loss and mitochondrial heterogeneity (that is, concomitant presence of both abnormal and functional mitochondria)[181,182]. These data underline the importance of a proper balance between mitochondrial fusion and fission.

Furthermore, there is some evidence to indicate that fusion has a role in physiological processes. MFN2 expression is induced in skeletal muscle and brown adipose tissue of mice exposed to cold temperatures, following the administration of β3-adrenergic receptor agonists or after exercise — conditions that induce energy expenditure[183,184]. The extent to which the changes in mitochondrial biogenesis, which also occur in these conditions, translate into the modulation of mitochondrial dynamics, however, requires further investigation. Although our understanding of the regulation of fusion and fission is still in its infancy, novel compounds have been identified that are capable of either promoting fusion (that is, M1 hydrazone) [185] or inhibiting fission (that is, the DRP1 inhibitor MDIVI-1)[186] (FIG. 3). These two compounds have been mostly tested for their effects on apoptosis in the context of neurodegenerative diseases[185,187] and further results on their potential beneficial effects in disease models of fusion and fission are awaited.

Mitophagy—In response to prolonged nutrient deprivation, cells start to digest themselves to recycle intracellular components — a process known as autophagy[188]. Non-selective macroautophagy is one particular type of autophagy that is triggered by energy stress. During this process, proteins and organelles such as endoplasmic reticulum (ER) or mitochondria are targeted to autophagosomes for subsequent fusion with lysosomes, where they are degraded via acidic lysosomal hydrolases[189].

By contrast, mitochondrial autophagy — also called mitophagy — is a type of selective macroautophagy that occurs under nutrient-rich conditions and is central to controlling the number and quality of mitochondria[8]. The specific recruitment of autophagosomes to mitochondria is mediated by different proteins in mammals according to the cell type and process. For instance, during the maturation of reticulocytes into red blood cells, the expression of the mitochondrial outer membrane NIP3-like protein X (NIX) increases, and NIX directly binds to microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3) to allow the removal of mitochondria by mitophagy[190].

In many cultured cell lines, including neurons and HeLa cells, PINK1 and parkin are the key mediators of mitophagy. The loss of mitochondrial membrane potential leads to PINK1 accumulation at the surface of the mitochondria and the recruitment of parkin, which in turn ubiquitylates mitochondrial outer membrane proteins for their recognition by autophagosomes[55,191] (FIG. 3). Mutations in parkin or PINK1, which are found in Parkinson's disease, disrupt parkin recruitment and parkin-induced mitophagy at distinct steps and therefore result in the accumulation of dysfunctional mitochondria[191–194]. Conversely, in a mouse model of Parkinson's disease in which Tfam is specifically knocked out in dopaminergic neurons, defective mitochondria accumulate and are not cleared by mitophagy[195], although it should be noted that the mitochondrial dysfunction in this model may not fully reflect the pathophysiology of Parkinson's disease.

Other studies have also demonstrated how mitophagy is dependent on mitochondrial dynamics by showing that overexpression of PINK1 or parkin promotes fission, most probably via the ubiquitylation of MFN1 or MFN2 (REFS 196,197). Although our understanding of this pathway is still evolving, these data suggest that modulation of fusion and fission may be a way to modulate mitophagy. Unravelling the exact mechanisms and links between mitophagy and fusion or fission will probably allow the identification of novel targets that are more specific to mitophagy.

Protein folding homeostasis—Proteotoxicity is often associated with the accumulation of misfolded or unfolded proteins. Cells respond to this accumulation by increasing the level of protein quality control effectors, including chaperones and proteases[198]. Depending on

the subcellular localization of the damage, an organelle-specific UPR is activated: for instance, UPR in the ER (often referred to as ER stress), and UPR in mitochondria[3,4].

The mitochondrial UPR reduces the amount of unfolded proteins in the mitochondria by stimulating the transcription of nuclear-encoded mitochondrial chaperones such as heat shock 70 kDa protein 9 (HSPA9; also known as mtHSP70) and the mitochondrial chaperonin heat shock protein 60 (HSP60; also known as HSPD1)[3,4] (FIG. 3). In *Caenorhabditis elegans*, the triggering signal for the mitochondrial UPR is the cleavage of unfolded proteins by the CLPP-1 protease into smaller peptides, which are exported by the HAF-1 transporter in the cytosol. These peptides, through an as yet unknown mechanism, activate the transcription factor ATFS-1 (activating transcription factor associated with stress 1; also known as ZC376.7)[199], which — together with co-factors such as ubiquitin-like protein 5 (UBL-5) and DVE-1 (REF. 200) — induces the transcription of genes encoding mitochondrial chaperones and proteases: that is, *hsp-6* and *hsp-60* (FIG. 3). The mitochondrial UPR is crucial for the longevity of worms with defective oxidative phosphorylation, as the long lifespan of *cco-1* worms, which are deficient in cytochrome c oxidase activity, was suppressed in the absence of a functional mitochondrial UPR, as induced by *ubl-5* RNA interference (RNAi)[201]. This response involves noncell-autonomous signalling engaging as yet unidentified mitokines, as selective RNAi of *cco-1* in sensory neurons was sufficient to activate the mitochondrial UPR in the worm intestine[201].

Interestingly, fibroblast growth factor 21 (FGF21) was recently identified as a biomarker that is secreted by muscle tissue in individuals with mitochondrial respiratory chain deficiencies, which suggests that such non-cell-autonomous signalling might also be conserved in humans[202–204]. Likewise, the mitochondrial-derived peptide (MDP) humanin, which was initially identified as having a protective effect against neurodegeneration, was shown to be elevated in the skeletal muscle of patients with mtDNA mutations, which indicates that it could be acting as a mitokine (reviewed in REF. 205). Although it is not yet clear what triggers the activation of the mitochondrial UPR, the evidence from *cco-1* worms[201] and worms treated with the mtDNA depleting agent ethidium bromide[206] suggests that it is crucial to maintain a balance in the levels of expression of different proteins in complexes and supercomplexes of the respiratory chain. This was further confirmed by a study in which stoichiometric imbalance between ETC subunits, induced either by inhibiting the translation of mtDNA-encoded proteins (for example, using doxycycline) or by stimulating the translation of nDNA-encoded proteins (for example, using resveratrol or rapamycin), resulted in an induction of the mitochondrial UPR in both worms and mammals[207]. Such an imbalance was shown to contribute to the beneficial effects of resveratrol and rapamycin, which induce mitochondrial biogenesis and extend fitness and lifespan[207].

Potential evidence for a beneficial role of the mitochondrial UPR comes from the inherited disease spastic paraplegia. This rare neurodegenerative disorder is caused by mutations in the *HSP60* gene[208] and in the paraplegin gene, which encodes a subunit of the m-AAA protease that degrades misfolded proteins and regulates mitochondrial ribosome assembly[209,210] (TABLE 2). Two groups have further reported that the mitochondrial UPR is induced in a *C. elegans* models of Friedriech's ataxia, a neurodegenerative disease in which the assembly and function of Fe–S cluster-containing subunits of ETC complexes I, II and III is impaired as a result of a mutation in the frataxin gene[211–213]. Whether it is possible to further increase the mitochondrial UPR in such disease contexts and whether this would be sufficient to restore proteostasis remains to be addressed. Such a strategy may potentially be beneficial for all inherited mitochondrial diseases in which mutations lead to proteotoxicity.

Identifying drugs to treat mitochondrial diseases

Several compounds that modulate mitochondrial function have been identified via target-based screens (FIG. 4), including compounds that bind to and modulate specific G protein-coupled receptors, nuclear receptors and other transcription factors as well as kinases. Hallmark compounds identified via such a targeted approach include natural, semisynthetic and synthetic agonists for the PPARs[128–130] and ERRs[137–141,144], SIRT1 (REFS 89,93,95,96, 98–100,214), TGR5 (REFS 215–221) and AMPK[222,223]. An important advantage of target-based strategies is that they enable structure–activity relationship (SAR) optimization of hits from primary screens. However, in the context of mitochondrial diseases, a target-based drug discovery approach has considerable limitations because a given target is only a small part of the large pleiotropic regulatory networks that govern mitochondrial homeostasis. By contrast, a phenotypic screen allows the identification of novel compounds that do not just modulate a single target or pathway but instead modulate a more global phenotype, without knowing the specific target (FIG. 4). In addition, phenotypic screens can be powerful in identifying novel pathways or proteins involved in the regulatory network of mitochondrial function (FIG. 4). Although phenotypic screens for mitochondrial function are only beginning to emerge, in the section below we assess the various models that may be used for such screens and consider the limitations associated with their design and application.

Models for mitochondrial phenotypic screens

Although immortalized cell lines are convenient because they can be grown in large quantities, these cell lines often lose the physiological features of an in vivo eukaryotic cell, including senescence and cell cycle regulation, and thereby instead resemble transformed cancer cells. This is a major drawback for the identification of drugs targeting mitochondrial function, as most transformed cells are metabolically reprogrammed to address their particular energetic needs: for example, overactivation of the phosphoinositide 3-kinase (PI3K)–mTOR pathway or inhibition of AMPK signalling[224]. Despite such caveats, immortalized cells have been used for the identification of mitochondrial modulators, as exemplified by the discovery of the fusion promoter M1 hydrazone using an image-based screen in mouse embryonic fibroblasts (MEFs)[171] (TABLE 3). Two other studies have identified the hitherto unknown mitochondrial effects of several US Food and Drug Administration (FDA)-approved drugs using phenotypic screens in human fibroblasts[206] and in C2C12 myotubes (a mouse muscle cell line)[205], as discussed below (TABLE 3).

Primary cell cultures represent an interesting alternative to immortalized cell lines, as they more closely resemble the in vivo metabolic milieu and can be isolated from patients or disease models and thereby even mimic complex genetic diseases (TABLE 3). An image-based screen in human umbilical vein endothelial cells (HUVECs) led to the identification of BRD6897, a compound that seems to govern mitochondrial biogenesis and turnover through a mechanism that is independent of known transcriptional programmes[225] (TABLE 3). Primary cells have also been used to identify mitochondrial toxins[226–228].

Other approaches using more primitive but intact organisms have proven to be valuable for identifying and functionally characterizing compounds that affect mitochondria. The budding yeast Saccharomyces cerevisiae is particularly interesting owing to the high level of conservation of mitochondrial genes and function (TABLE 3). For example, the mitochondrial division inhibitor MDIVI-1 was identified in a yeast screen by its capacity to suppress the growth defect of mitochondrial fusion mutants[186]. Likewise, chlorhexidine was identified as being able to rescue yeast ATP synthase mutants, which phenotypically resemble human inherited mitochondrial diseases such as the NARP (neuropathy, ataxia and retinitis pigmentosa) syndrome[229] (TABLE 3). Nevertheless, owing to the simplicity of the

unicellular yeast, it cannot be used to model a disease at the scale of an organ or an intact organism. However, this can be partly achieved in invertebrate animal models such as the nematode *C. elegans*, which has the advantage of modelling physiology at the multi-organ level while now being manageable at the scale of high-throughput screening[230] (TABLE 3). Like in yeast, most biological processes and genes involved in mitochondrial function are conserved in *C. elegans*, and the convenience of performing genome-wide RNAi screens aids the identification of the pathways that underlie the effects of the compounds[231] (TABLE 3).

Design of mitochondrial phenotypic screens

The use of phenotypic screens for drug discovery requires careful design, especially in the context of mitochondria. In fact, owing to the high metabolic flexibility of higher organisms, multiple readouts and complementary tests are required to elucidate the mechanism of action of a given compound. For example, glycolysis activators and oxidative phosphorylation inhibitors both reduce cellular respiration. If respiration is the only measured parameter, the only way to distinguish between these two compounds is to test them at different concentrations; a glycolysis activator should be harmless compared to an oxidative phosphorylation inhibitor at a high dose. Alternatively, these two types of compounds could be compared in a pro-glycolytic (for example, glucose) and pro-oxidative (for example, galactose) culture medium; a respiration inhibitor will be more toxic under pro-oxidative conditions but it may have no effect under pro-glycolytic conditions. This latter strategy (comparing compounds in pro-glycolytic or pro-oxidative culture medium) was used to assess the impact of compounds on the viability of human fibroblasts and led to the characterization of the metabolic effect of the antiemetic drug meclizine[232].

Another way to distinguish between two different classes of drugs that have the same effect on a given metabolic aspect is to analyse several parameters in parallel to obtain a so-called footprint of the effect of each compound on mitochondrial activity. For example, simultaneous measurement of mitochondrial and related viability parameters, combined with a gene expression survey, of mouse C2C12 myotubes treated with FDA-approved drugs led to the identification of negative modulators of mitochondrial function, such as the HMG-CoA reductase inhibitors, as well as stimulants of the transcription of genes involved in oxidative phosphorylation, such as microtubule destabilizers (for example, podophyllotoxin)[233].

A further improvement would be to obtain more comprehensive and complex mitochondrial footprints of compounds and drugs by measuring as many parameters as possible at a given time. For example, one of the gaps in our understanding of mitochondrial function is why both inhibition and stimulation of respiration can improve health and extend lifespan in primitive organisms such as yeast and worms. Such a question could be addressed by aligning respiration, biogenesis, mitochondrial dynamics and viability profiles in cells with metabolic characterization and lifespan assays in yeast and worms. In our own studies, we selected a set of mitochondrial function assays to construct a detailed atlas of mitochondrial footprints or signatures of known drugs with mitochondrial activity (BOX 3). This strategy could enable the accurate mapping of mitochondrial pathways affected by new compounds and provide a biochemical approach to further define mitochondrial regulatory networks.

Conclusion/Perspectives

Understanding how proteins that are encoded by mitochondrial and nuclear genomes work together with metabolites to maintain mitochondrial homeostasis continues to be a major research challenge. Mitochondrial function is regulated by a complex network of sensors and effectors, and so a multigenic and holistic approach is best suited for understanding the

physiology and the pathophysiology associated with this organelle. At present, by far the best-characterized aspect of the mitochondrial regulatory network is how sensors of the nutrient and energy status (such as AMPK, NAD^+ and SIRT1) and their downstream transcriptional effectors govern mitochondrial biogenesis. However, other key features that regulate mitochondrial function, such as fusion and fission, mitophagy and the mitochondrial UPR, have recently also been elucidated.

Several lines of evidence have suggested that mitochondrial biogenesis and other processes should not be seen as independent but instead as concomitant and interdependent phenomena. A few examples illustrate this interdependency. First, the mitochondrial UPR is induced during the L3–L4 stage of larval development of *C. elegans*, a phase during which mitochondrial biogenesis also takes place[4,234]. This is further supported by the fact that resveratrol and rapamycin, two compounds that induce mitochondrial biogenesis and extend fitness and lifespan, also induce the mitochondrial UPR207. Second, the balance between fusion and fission events affects the structure of the inner boundary membrane, which is a site that is enriched in proteins involved in translocation[235]. Therefore, processes that require massive protein translocation within mitochondria, such as mitochondrial biogenesis or the mitochondrial UPR, are also likely to be linked to fusion and fission events. The intertwined nature of these mitochondrial homeostatic processes also underscores that our understanding of the mitochondrial regulatory network is only in its infancy, and suggests that phenotypic screens, coupled with subsequent deconvolution of the targets of hit compounds, might be the best way to not only recognize new compounds that restore mitochondrial function but also identify existing compounds that are toxic to mitochondria. Using this approach, we can expect the emergence of new targets and compounds that could lead to the development of drugs for both rare and common diseases in which dysfunctional mitochondria are implicated.

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Glossary

Mitochondrial DNA (mtDNA)	A 16.5kb circular DNA sequence carried within mitochondria, composed of a light and heavy strand. Both strands contain 37 genes, including 13 that encode protein subunits of the OXPHOS complexes, while the remaining code for rRNA and tRNA molecules that are essential for transcription and synthesis of mitochondrially encoded proteins.
Oxydative phosphorylation	Enzymatic phosphorylation of ADP to ATP, which is coupled to electron transfer from a substrate to molecular oxygen in the electron transport chain.
Heteroplasmy	A mixture of different forms of mitochondrial DNA within a single cell
Quantitative trait loci (QTLs)	Genetic loci contributing quantitatively to a trait or phenotype. A QTL partly explains the genetic contribution to a given phenotype.

Proteostasis	Homeostasis of the protein folding landscape.
Caloric restriction diet	A diet that involves the consumption of 20-50% less calories than normal without causing malnutrition: that is, with maintainance of proper vitamin and mineral intake.
NAD^+	The oxidized version of NAD; serves as a co-factor in oxidation-reduction reactions. NAD^+ also acts as an obligatory co-substrate for sirtuin-mediated deacylation reactions.
Inverse agonists	Compounds that bind to the same receptor binding-site as a prototypical agonist for that given receptor. Through this binding, inverse agonists reverse the basal or constitutive activity of the receptor. Oestrogen-related receptor-α is an example of constitutively active nuclear receptor for which inverse agonists exist.
mtDNA haplogroup	A group sharing the same single nucleotide polymorphism (SNP) on mitochondrial DNA (mtDNA). Some haplogroups have been suggested to have a greater likelihood of predisposing individuals to Alzheimer's disease.
Mitokine	A signalling molecule that is produced by mitochondria in a given tissue and affects mitochondrial function in a distinct tissue.
Myokine	A signalling molecule that is produced by the muscle and has cytokine-like properties.

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Box 1

Towards a network approach for mitochondrial diseases

The symptoms and age of onset of mitochondrial diseases caused by mutations in mitochondrial DNA (mtDNA) can be variable even within the same family. This can be partially explained by the variation in the number of copies of normal and mutated forms of mtDNA within a cell (termed heteroplasmy). This is not observed for diseases caused by nuclear DNA mutations because they are inherited in a Mendelian fashion. Furthermore, the variability among patients from the same family carrying the same mutation is affected by environmental contributions, epigenetic factors and the presence of other genetic polymorphisms that ultimately modify the nature and expression of the disease phenotype. These modifier genes often work within large interaction networks, similar to those observed in complex multigenic diseases.

Complex diseases are associated with changes at several genetic loci, termed quantitative trait loci (QTLs), and each locus contributes quantitatively to the phenotype. For example, genetic studies suggest that common diseases in which mitochondrial dysfunction is involved, such as type 2 diabetes, are caused by small changes in many genes rather than large effects produced by mutations in a few genes[236–238]. Likewise, in the case of Parkinson's disease, mutations in PTEN-induced putative kinase 1 (PINK1) and parkin are only associated with the early-onset form of the disease, which represents a small fraction of all Parkinson's disease cases (TABLE 2), whereas other forms of Parkinson's disease follow a complex disease inheritance pattern that is modified by gene–gene and gene–environment interactions. One could therefore hypothesize that although rare inherited mitochondrial diseases differ from common diseases in which mitochondrial dysfunction is implicated, with respect to the severity of impact of the mutations (affecting either one or several genes), both types of diseases could benefit from a network approach to identify novel target genes and pathways that modulate mitochondrial function.

Box 2

Metabolic flexibility and the upstream sensors of mitochondrial biogenesis

Evolution favoured the survival of organisms that were capable of adapting to environmental challenges, such as a non-continuous supply of energy. This explains why proteins that are involved in pathways governing metabolic flexibility and the shift between anabolism and catabolism, such as AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR), are incredibly well conserved from yeast to mammals[239]. When energy is abundant, mTOR stimulates anabolic pathways[110], whereas sufficient ATP levels keep AMPK activity low[107]. On the contrary, when energy is limited, mTOR activity is low[110] and AMPK is activated by the high AMP/ATP ratio, which promotes catabolic energy-generating pathways[107]. AMPK activates mitochondrial energy production by activating transcription factors, such as the forkhead box O (FOXO) proteins, through both direct (that is, phosphorylation) and indirect mechanisms, which involve metabolic changes leading to increased NAD^+ levels and subsequent sirtuin 1 (SIRT1)-mediated FOXO deacetylation[78].

Aside from these well-conserved kinases, which act as cell-autonomous fuel sensors and effectors, other pathways involving transcription factors and their co-factors regulate metabolic flexibility at the organismal scale. In vertebrates, mitochondrial biogenesis and activity are controlled in part by a ‘yin-yang’ between co-repressors, such as nuclear receptor co-repressor 1 $(NCOR1)_{70}$, and co-activators, such as steroid receptor co-activator protein 1 (SRC1; also known as NCOA1)[151] and PPARγ co-activator 1α (PGC1α)[240,241], which fine-tune transcriptional networks controlling oxidative metabolism. For example, increased cellular NAD^+ levels during energy stress lead to deacetylation and activation of PGC1α[74,77,78,105], which, together with reduced activity of NCOR1 (REF. 70), favours oxidation and enhances the use of stored energy during caloric restriction. These processes are reversed by excessive caloric intake at both the enzymatic and gene expression level. In fact, a high-fat diet leads to high ATP and low NAD^+ levels, which attenuates AMPK and SIRT1 activity[242] and induces the expression of SRC3 (also known as NCOA3) and the histone acetyltransferase GCN5 (REF. 152). This results in the acetylation and inhibition of transcription factors, such as the tumour suppressor p53 (REF. 69), FOXO[71–73] and PGC1α[74], which in turn attenuates mitochondrial activity[242]. Furthermore, NCOR1 is activated[70], which accentuates the decreased transcription of genes governing mitochondrial activity and contributes to the storage of excess calories[152,243]. These co-factor networks target and fine-tune a limited number of transcription factors, including nuclear respiratory factor 1 (NRF1), NRF2 and several nuclear receptors, such as the peroxisome proliferator-activated receptors (PPARs) and oestrogen-related receptors (ERRs)[244–246]. Each of these transcription factors binds to a specific motif, promoting the transcription of different gene sets in the tissues where they are expressed.

One interesting point to underscore is that in invertebrates, including yeast and worms, mitochondrial biogenesis and metabolic flexibility are regulated through similar transcriptional regulatory networks that govern these processes in vertebrates, even though invertebrates lack homologues of some of the transcription factors (for example, nuclear receptors in yeast) and co-factors (for example, PGC1α in yeast and worms) that are present in vertebrates. By contrast, the kinases (mTOR and AMPK), sirtuins and NCOR1 are conserved throughout evolution.

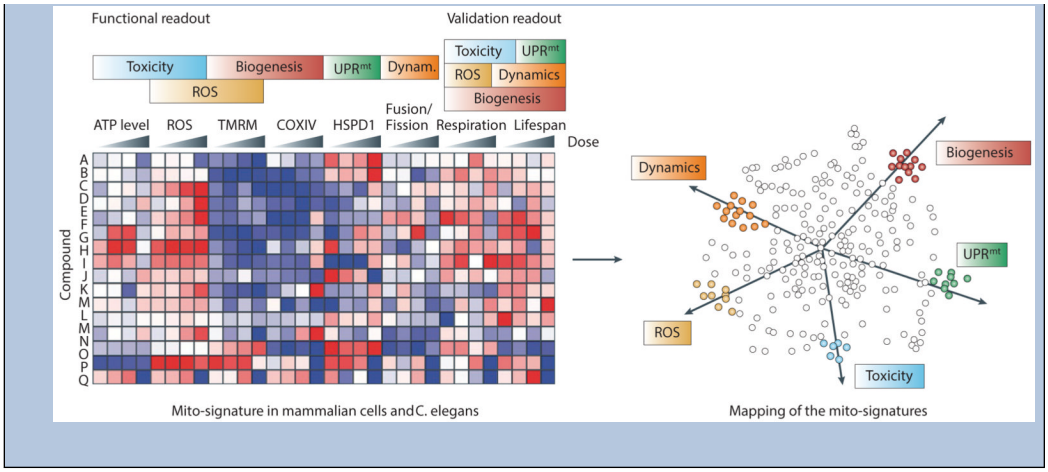
Box 3

Functional readouts to obtain mitochondrial fingerprints

Phenotypic screens offer the possibility to simultaneously record several parameters and hence obtain a more global footprint of the activity of a compound on mitochondria (see the figure). Ideally, these different parameters should be representative of the array of functions of interest and should first be performed with mitochondrial benchmark compounds. Below, we highlight a compendium of mitochondrial tests that could be used to define a multiscalar mitochondrial signature for a given compound. A representative example of results obtained using this array of tests performed in our laboratory is shown in the left panel of the figure. The heat map represents changes (blue for decrease and red for increase compared to vehicle control) in the different signals after treatment with increasing doses of mitochondrial benchmark compounds.

Determination of ATP levels is an effective and robust way to assess compound toxicity in cells[233]. Reactive oxygen species (ROS) are important signalling molecules but high levels are deleterious. ROS can therefore be used to assess both toxicity and ROS homeostasis. Several methods to directly measure ROS using fluorescent probes have been described in cells[247]. The most promising tools for specific and quantitative ROS measurements are fluorescent protein-based redox probes[248], but their use requires a transfection step, which can be limiting. The mitochondrial membrane potential ($\Delta\psi M$) can be assessed by fluorescent probes, such as tetramethylrhodamine (TMRM), that accumulate inside mitochondria proportionally to $\Delta\psi M$ without having any impact on mitochondrial respiration. TMRM can also provide information about mitochondrial abundance and thereby both mitochondrial biogenesis and efficiency. Several parameters of mitochondrial function, including mitochondrial biogenesis and the mitochondrial unfolded protein response (UPR^{mt}) can also be monitored either directly by measuring gene expression or by using luciferase reporter assays. In the case of mitochondrial biogenesis, appropriate readouts include levels of expression of one of the respiratory complex subunits, such as the cytochrome c oxidase subunit IV (COXIV), measured either directly (for example, at the mRNA level) or indirectly (for example, using a promoter reporter activity assay). A similar approach can be applied to measure the activation of the UPR^{mt} by measuring changes in mRNA levels of mitochondrial chaperonin heat shock protein 60 (HSP60) or *HSP60* promoter reporter activity. The only way to assess mitochondrial dynamics is by cellular imaging. Systematic image analysis software now makes it possible to quantify mitochondrial networks in cells transfected with mitochondrially targeted green fluorescent protein (GFP) in a high-throughput manner[185]. Furthermore, through the development of multiwell format oxygen-dependent fluorescence quenching systems, it has become possible to evaluate respiration in a high-throughput format[225,232]. Importantly, this technology can also be applied to measure respiration in *Caenorhabditis elegans*[207]. Finally, lifespan, activity and fitness assays in worms are good surrogates to assess the general impact of compounds on mitochondria and physiological homeostasis[249]. Importantly, such tests already indicate the potential effect of a compound at the scale of an entire organism.

It is important to underscore that the combination of several of these assays is desirable and most valuable to characterize new compounds. By combining the results of several tests for all compounds, it is possible to define a comprehensive set of mitochondrial signatures that enable the clustering of novel chemical entities according to their footprint on mitochondrial function (see right panel of figure). Such a signature will also provide insight into the particular mitochondrial signalling pathways that are affected, facilitating downstream target deconvolution.



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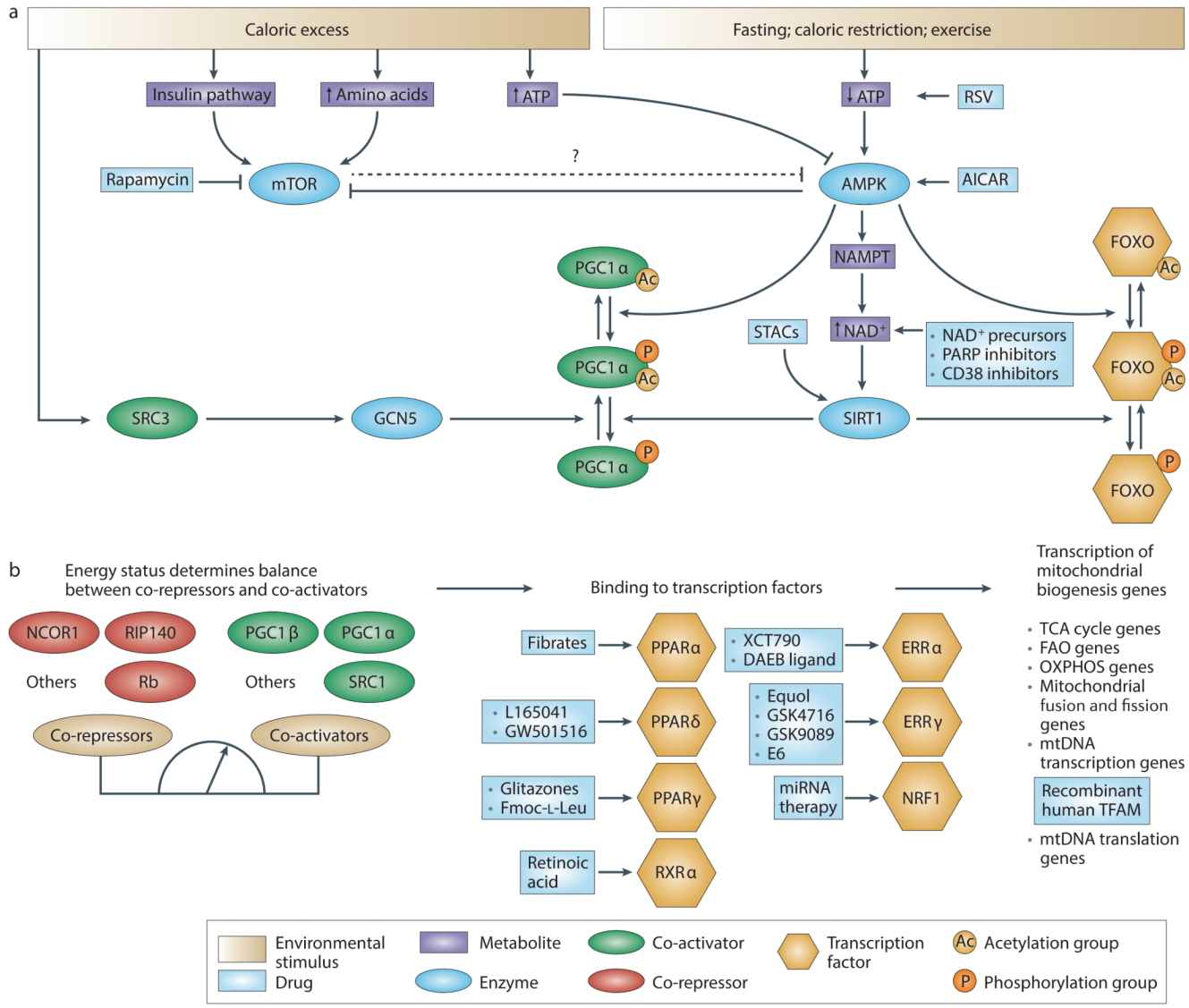

Figure 1. Pharmacological approaches targeting mitochondrial biogenesis

Pharmacological approaches for targeting mitochondrial biogenesis. a | Upstream sensors of energy status. Energy stress promotes the activation of AMP-activated protein kinase (AMPK) via an increase in the AMP/ATP ratio, whereas energy excess activates the mammalian target of rapamycin (mTOR) pathway either via an increase in levels of amino acids or via the activation of insulin signalling. Rapamycin, 5-aminoimidazole-4-carboxamide riboside (AICAR) and resveratrol (RSV) simulate energy crisis by inhibiting mTOR or activating AMPK. AMPK-mediated phosphorylation of co-factors (such as PPARγ co-activator 1α (PGC1α)) and transcription factors (such as forkhead box O (FOXO) proteins) is a preliminary step required for their activation. AMPK also increases levels of NAD^+, which is a substrate for sirtuins. NAD^+ levels can be increased either by supplying precursors or by inhibiting NAD^+-consuming enzymes such as CD38 and poly(ADP-ribose) polymerases (PARPs). The increase in NAD^+ levels activates sirtuin 1 (SIRT1), which subsequently deacetylates PGC1α and FOXO. The acetylation status of PGC1α is counterbalanced by the activity of the histone acetyltransferase GCN5, which is activated by feeding and via the recruitment of steroid receptor co-activator protein 3

(SRC3). Sirtuin-activating compounds (STACs) can also activate SIRT1 directly. b | Downstream transcriptional factors and co-factors. The balance between the activity of co-repressors and co-activators determines the activation of transcription factors involved in mitochondrial biogenesis. Nuclear receptors such as peroxisome proliferator-activated receptors (PPARs) are ideal drug targets as they are activated upon ligand binding. Likewise, agonist ligands for retinoid X receptor-α (RXRα), the heterodimerization partner of the PPARs, enhance mitochondrial function. Also, several compounds can induce the transcriptional activity of the oestrogen-related receptors (ERRs). The only approach described to modulate the activity of the transcription factor nuclear respiratory factor 1 (NRF1) was a microRNA (miRNA)-based approach. Finally, a recombinant form of human mitochondrial transcription factor A (TFAM) was designed to stimulate the processing of mitochondrial DNA (mtDNA). FAO, fatty acid oxidation; NAMPT, nicotinamide phosphoribosyltransferase; NCOR1, nuclear receptor corepressor 1; OXPHOS, oxidative phosphorylation; RIP140, receptor-interacting protein 140; Rb, retinoblastoma protein; TCA, tricarboxylic acid.

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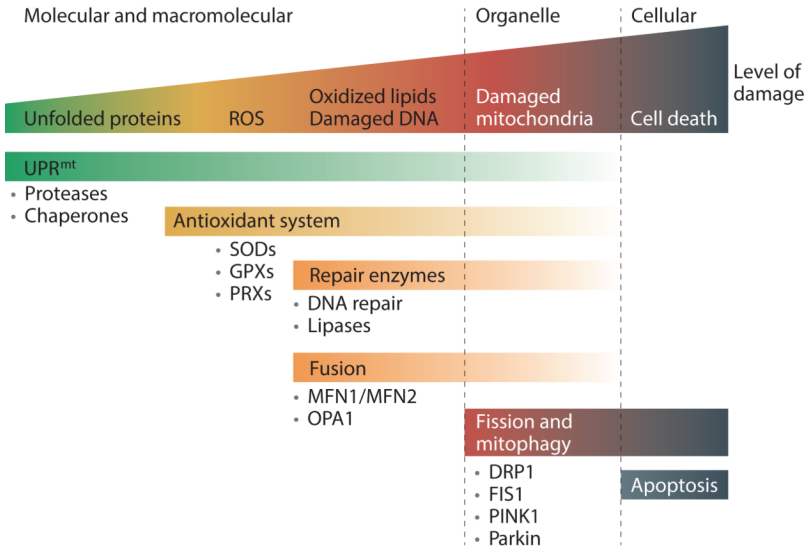

Figure 2. Mitochondrial quality control processes

A graphical model of the different steps involved in mitochondrial quality control. At a basal level, cells maintain proteostasis within their mitochondria with the help of the mitochondrial unfolded protein response (UPRmt) machinery. If proteostasis is not properly maintained, levels of reactive oxygen species (ROS) might increase following electron transport chain dysfunction and be downregulated by antioxidant enzymes such as superoxide dismutases (SODs), which convert superoxide (O2 −•) into hydrogen peroxide (H2O2), or peroxiredoxins (PRXs) and glutathione peroxidases (GPXs), which remove H2O2. When antioxidant levels are not sufficient, and/or ROS levels are excessive, ROS damage the mitochondrial microenvironment (for example, proteins, mitochondrial DNA (mtDNA) and lipids), resulting in the loss of membrane potential and ATP synthesis efficiency, thereby increasing ROS production in a feedforward reaction. A second line of defence consists of enzymes that repair or eliminate the damaged components: that is, DNA repair enzymes for mtDNA, and lipases for the digestion of oxidized lipids. In parallel, mitochondria can restore their efficiency through fusion– fission cycles[235]. One hypothesis is that fusion enables mitochondria to exchange their content, and thereby dilute the damaged materials to facilitate their repair or removal. When these mechanisms are insufficient and damage has affected a great portion of the mitochondrion, fission helps to eliminate damaged mitochondria that are beyond repair through mitophagy. Finally, when damage reaches too many mitochondria, these undergo fragmentation followed by apoptosis. DRP1, dynamin-related protein 1; FIS1, mitochondrial fission 1 protein; MFN1, mitofusin 1; OPA1, optic atrophy protein 1; PINK1, PTEN-induced putative kinase 1.

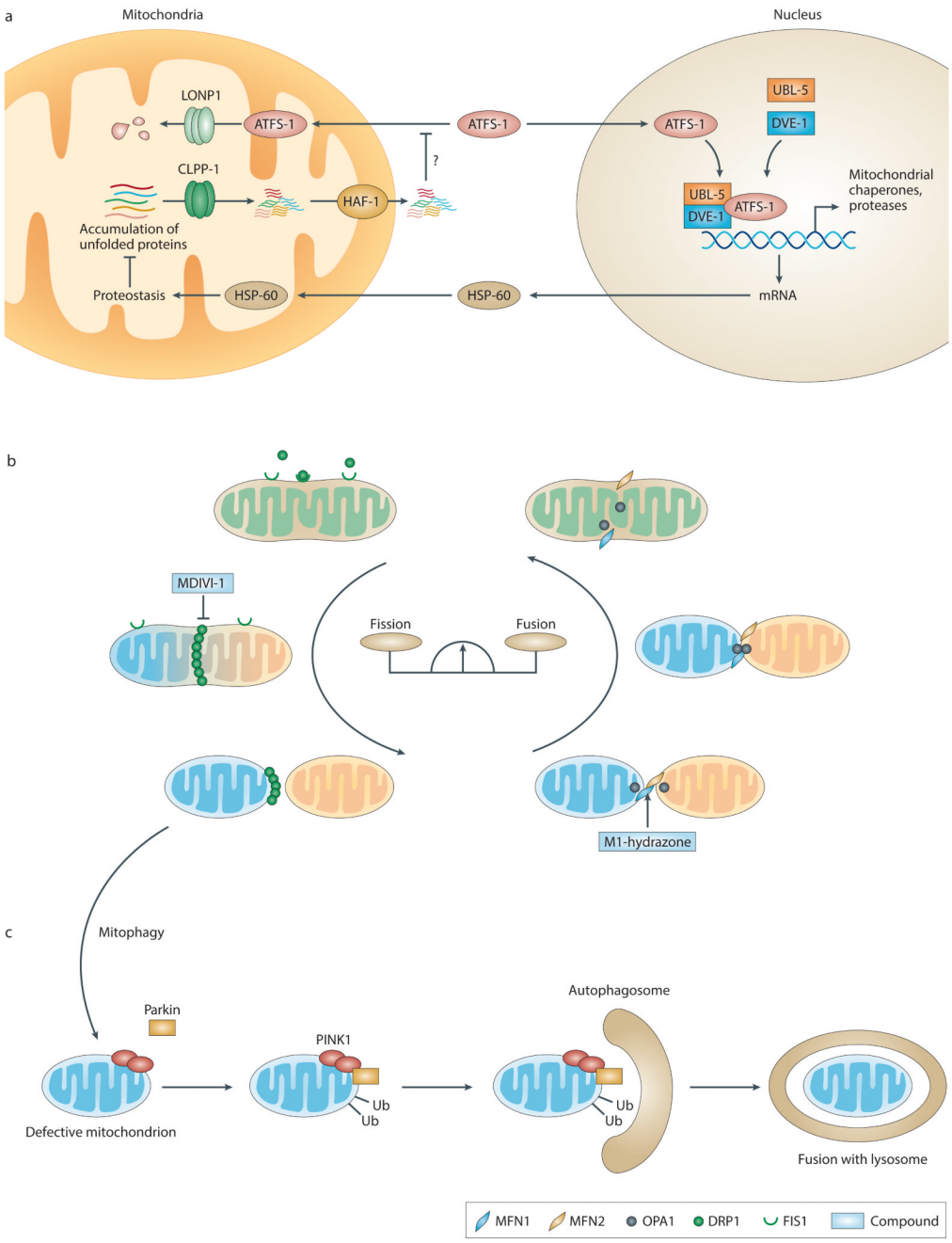

Figure 3. Current models of mitochondrial quality control processes

a | A model of the mitochondrial unfolded protein response in *Caenorhabditis elegans*. In basal conditions, the transcription factor ATFS-1 (activating transcription factor associated with stress 1) is imported into the mitochondrial matrix via the translocase of the outer membrane (TOM) complex and the translocase of the inner membrane 23 (TIM23) complex, where it is degraded by the mitochondrial Lon peptidase 1 (LONP1). When unfolded proteins accumulate, the CLPP-1 protease generates peptide fragments, which are exported to the cytosol via the HAF-1 transporter. The presence of these peptides in the cytosol inhibits the import of ATFS-1 into mitochondria, which subsequently accumulates in the nucleus through an as yet uncharacterized translocation step. In complex with ubiquitin-like

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protein 5 (UBL-5) and DVE-1, ATFS-1 then induces the transcription of proteases and mitochondrial chaperones that translocate back into the mitochondria to restore local proteostasis. b | A model of fusion and fission in mammalian cells. The balance between fusion and fission events determines the shape of the mitochondrial network. Fusion of the outer mitochondrial membrane is achieved by the tethering of mitofusin 1 (MFN1) and MFN2, whereas optic atrophy protein 1 (OPA1) is required for the fusion of the inner mitochondrial membrane. The compound M1-hydrazone promotes fusion in an MFN1- and/or MFN2-dependent manner. Fission occurs through the GTPase dynamin-related protein 1 (DRP1), which is recruited by mitochondrial fission 1 protein (FIS1) and is specifically inhibited by the compound MDIVI-1. c | Model of mitophagy in mammalian cells. Defective mitochondria are isolated from the mitochondrial pool by fission (as marked by the arrow). They are characterized by a loss of mitochondrial membrane potential, which is accompanied by the accumulation of PTEN-induced putative kinase 1 (PINK1) at the surface of the mitochondrion. This leads to the recruitment of parkin, which ubiquitylates mitochondrial outer membrane proteins, thereby triggering the recruitment of autophagosomes. The defective mitochondrion is then engulfed in an autophagosome before its fusion with a lysosome and digestion. HSP-60, mitochondrial chaperonin heat shock protein 60 (*C. elegans*).

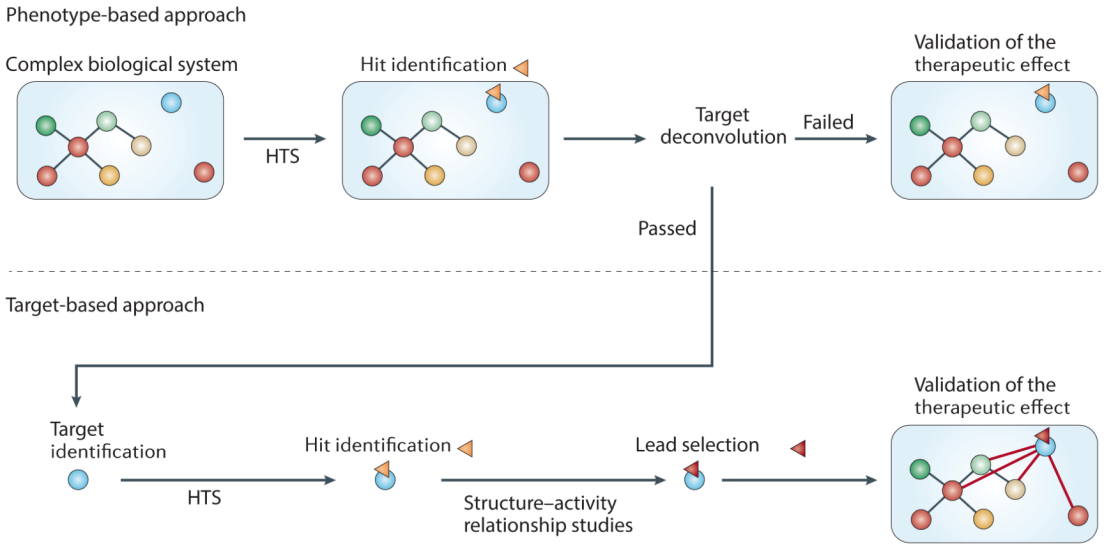

Figure 4. Models and approaches for mitochondrial screens

Phenotype-based versus target-based screening approaches are shown. A phenotypic screen aims to identify hit compounds that are able to modulate the activity of a complex biological system that is only partially characterized. When a hit (yellow triangle) is identified, its target may not always be known. Validation of its therapeutic effect can be performed without knowledge of the pathway. If the target can be identified, this can aid the optimization of hits into lead compounds (red triangle) with improved properties and eventually expand knowledge of the pathway, as depicted by the new connections in the network. See TABLE 3 for drawbacks and advantages of model systems for mitochondrial screens. HTS, high-throughput screening.

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Table 1
Selected typical inherited mitochondrial diseases

Genome	Gene	Gene function	Clinical description
mtDNA	*MTND1*, *MTND2*, *MTND4* and *MTND6*	Complex I subunits	Leber hereditary optic neuropathy (LHON)
	MTATP6	Complex V subunit	Neuropathy, ataxia, retinis pigmentosa (NARP) or maternally inherited Leigh's syndrome (MILS)
	MTTL1	Mitochondrial protein translation	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS)
	MTTK	Mitochondrial protein translation	Myoclonic epilepsy, muscle weakness and wasting with red-ragged fibres (MERFF), cerebellar ataxia, deafness and dementia
	MTCO1, *MTCO2* and *MTCO3*	Complex IV subunits	Complex IV deficiency: clinically very variable, from single-organ defects (such as motor neuron deficiency or myopathy) to multisystem defects (for example, encephalomyopathy)
nDNA	*COX10*, *COX6B1*, *SCO1* and *SCO2*	Complex IV subunit or assembly	
	SURF1 *COX15*	Complex IV biogenesis or assembly	Leigh syndrome characterized by loss of motor milestones, hypotonia, movement disorders, nystagmus, breathing disorders, ophthalmoplegia, peripheral neuropathy and cardiomyopathy
	MFN2	Mitochondrial fusion	Charcot–Marie–Tooth disease type 2A; peripheral sensorimotor neuropathy characterized by motor and sensory deficits and postural tremors
	OPA1	Mitochondrial fusion	Optic atrophy

COX10, cytochrome c oxidase assembly homolog 10; *COX15*, cytochrome c oxidase assembly homolog 15; *COX6B1*, cytochrome c oxidase subunit 6B1; *MFN2*, mitofusin 2; *MTATP6*, mitochondrially encoded ATP synthase 6; *MTCO1*, mitochondrially encoded cytochrome c oxidase 1; *MTND1*, mitochondrially encoded NADH dehydrogenase subunit 1; *MTTK*, mitochondrially encoded tRNA lysine; *MTTL1*, mitochondrially encoded tRNA leucine 1 (UUA/G); *OPA1*, optic atrophy protein 1; *SCO1*, cytochrome c oxidase assembly protein SCO1; *SURF1*, surfeit 1.

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Table 2

Selected rare diseases associated with mitochondrial dysfunction*

Gene	Gene function	Clinical description
Parkin *PARK7* (also known as DJ1) *PINK1*	Mitophagy and/or mitochondrial dynamics	Early onset form of Parkinson's disease, characterized by tremor, bradykinesia, rigidity and postural instability
FXN	Mitochondrial iron metabolism	Friedreich's ataxia, characterized by difficulties in coordinating movements, associated with neurological signs, cardiomyopathy and (sometimes) type 1 diabetes
Paraplegin	Mitochondrial protease	Spastic paraplegia; characterized by progressive spasticity and weakness of the lower legs owing to axonal degeneration
HSP60	Mitochondrial quality control, mitochondrial UPR	

FXN, frataxin; *HSP60*, mitochondrial chaperonin heat shock protein 60; *PARK7*, Parkinson's disease protein 7; *PINK1*, PTEN-induced putative kinase 1; *UPR*, unfolded protein response.

* Nuclear DNA only.

Table 3

Drawbacks and advantages of model systems for mitochondrial screens*

	Suitability for high-throughput screening format	Conservation with human proteins	Multi-organ physiology readout	Feasibility of genome-wide screen	Examples of drug discovery
Target-based approach					
Isolated target	++++	++++	NA	NA	• ERR ligands[137–141,144] • PPAR ligands[128–130] • SIRT1 activators[89,93,95–100,214] • GPBAR1 agonists[215–221] • AMPK agonists[222–223]
Phenotype-based approach					
Yeast	+++	++	+	++++	• MDIVI-1 (REF. 186) • Chlorhexidine[229]
Immortalized cell lines	+++	+++	+	++	• M1 hydrazone[185] • Meclizine[232] • Podophyllotoxins[233]
Primary cells	−/+	++++	+	−/+	• BRD6897 (REF. 225)
Caenorhabditis elegans	++	++	++++	+++	

AMPK, AMP-activated protein kinase; ERR, oestrogen-related receptor; GPBAR1, G protein-coupled bile acid receptor 1; NA, not applicable; PPAR, peroxisome proliferator-activated receptor; SIRT1, sirtuin 1.

* Although targeted approaches use isolated targets and directly assess the change in target activity, phenotypic screening approaches can use different models with variable degrees of complexity. Models are ranked by degree of complexity from top to bottom in this table. Several criteria can help to determine the choice of the model. For example, the worm *C. elegans* is less suitable for high-throughput screening than an assay using isolated proteins or immortalized cell lines, but it models multi-organ physiology and is amenable to high-content screens. The last column provides examples of compounds and drugs that have been discovered using the different models and that are discussed in this article. The '−' and '+' signs are relative comparisons of the different models for each criteria, ranging from not suitable (−/+) to highly suitable (++++).