

Mitochondrial Targets for Pharmacological Intervention in Human Disease

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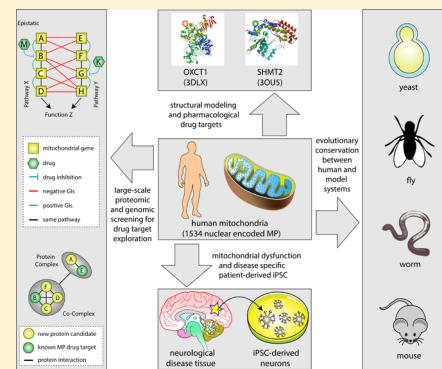
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S Supporting Information

ABSTRACT: Over the past several years, mitochondrial dysfunction has been linked to an increasing number of human illnesses, making mitochondrial proteins (MPs) an ever more appealing target for therapeutic intervention. With 20% of the mitochondrial proteome (312 of an estimated 1500 MPs) having known interactions with small molecules, MPs appear to be highly targetable. Yet, despite these targeted proteins functioning in a range of biological processes (including induction of apoptosis, calcium homeostasis, and metabolism), very few of the compounds targeting MPs find clinical use. Recent work has greatly expanded the number of proteins known to localize to the mitochondria and has generated a considerable increase in MP 3D structures available in public databases, allowing experimental screening and in silico prediction of mitochondrial drug targets on an unprecedented scale. Here, we summarize the current literature on clinically active drugs that target MPs, with a focus on how existing drug targets are distributed across biochemical pathways and organelle substructures. Also, we examine current strategies for mitochondrial drug discovery, focusing on genetic, proteomic, and chemogenomic assays, and relevant model systems. As cell models and screening techniques improve, MPs appear poised to emerge as relevant targets for a wide range of complex human diseases, an eventuality that can be expedited through systematic analysis of MP function.

KEYWORDS: Drug–protein interactions, human disease, mitochondria, model system, network, pharmacological target, protein complex, pathways, small molecules, systems biology



1. INTRODUCTION

Mitochondria are essential organelles responsible for diverse functions, including ATP production, ion homeostasis, and the initiation of apoptosis.^{1–3} These functions are exercised not only within the mitochondria itself but also through its interaction with other organelles such as the endoplasmic reticulum (ER),^{4,5} for example, in coordinating interorganellar tethering and lipid and Ca²⁺ exchange. While ancestral mitochondria had distinct genomes, over evolutionary time the majority of proteins required for mitochondrial function have been transferred to the nuclear genome and are imported via mitochondrial localization signals.⁶

Estimates suggest that as many as 1 in 5000 individuals suffer from an illness with mitochondrial etiology,⁷ making the mitochondria an appealing pharmacological target. These illnesses include Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS).^{8,9} Mitochondrial protein (MP) dysfunction has also been linked to schizophrenia and autism,^{10,11} cancer,¹² and metabolic disorders.¹³ For example, mutations in the mitochondrial-encoded superoxide dismutase *SOD1*, which functions to

protect mitochondria from oxidative damage, have been linked to the progression of ALS;¹⁴ NADH dehydrogenase 4, to Leber hereditary optic neuropathy;⁵ *PARKIN*, to the familial form of PD;⁶ and Krebs tricarboxylic-acid cycle enzymes, to oncogenesis.⁷ These are just few examples of disease-associated mutations that affect mitochondrial function; for a more detailed summary of the role of various MPs in disease, readers are urged to consult any of several recently published reviews.^{2,3,15}

Currently, there are 327 mitochondrial-targeted small molecules (as annotated in Drug Bank¹⁶ and various literature sources, including Wagner et al.;¹⁷ see Table S1), suggesting that targeting the mitochondria is an effective avenue for therapeutic modulation. These include the triaminopyridine flupirtine, a nonopioid analgesic drug with mitochondria-dependent antioxidant and free radical scavenging activity that

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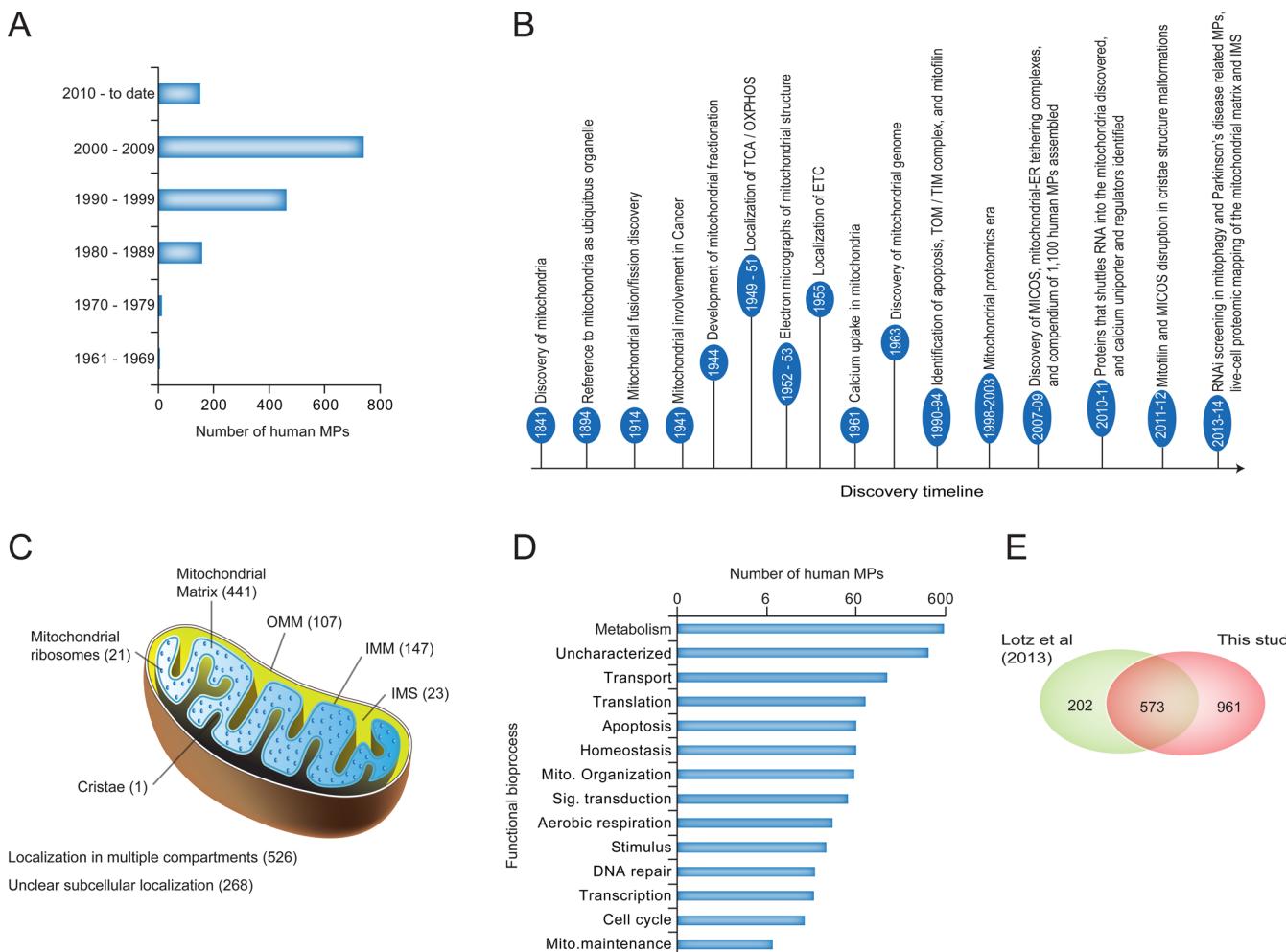


Figure 1. Composition of mitochondria and timeline of important discoveries. (A) Human MPs show a steady increase in the number of annotated genes over time. (B) Key milestones and discoveries in mitochondrial research. (C) Subcellular localization of MPs, including the inner and outer mitochondrial membrane (IMM and OMM, respectively), intermembrane space (IMS), and matrix, with highly organized cristae structures forming from the inner membrane into the matrix. These subcompartments contain many unique MPs. (D, E) Functional grouping (D) and overlap of our compiled MP catalogue with that from a recently published study (E) of MPs in human heart function.⁴¹

has been shown to be effective against ischemic neuronal damage, apoptosis, and age-associated brain disorders.^{18–20} Similarly, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors such as the commercially available statins (e.g., atorvastatin and simvastatin) have been shown to exhibit neuroprotective effects against the pathologies of PD, AD, traumatic brain injury, secondary progressive multiple sclerosis, and several other neuropathological conditions.^{21–24} Other bioactive molecules targeting mitochondria (for more detail, see Table S1) and the mechanisms underlying their efficacy have been reviewed elsewhere.^{15,25–28}

While our compendium of currently reported small molecules (Table S1) serves as a useful resource for gross indication of targeted mitochondrial pathways and processes, determining whether the physiological effect of individual drugs is elicited exclusively within the mitochondria is, in many cases, unknown and requires further in-depth experimentation that is beyond the scope of this review. Given the involvement of MPs in a myriad of essential functions, it is possible that the listed small molecules either diffuse out of the mitochondria to improve the efficacy of their action on extramitochondrial targets,^{29,30} or interact with related extramitochondrial pathways. For this reason, certain mitochondria-targeting small

molecules, such as adenosine triphosphate or dimethyl sulfoxide (Table S1), are unsuitable for therapeutic intervention, as they are prone to broader effects on cellular metabolism. Because mitochondrial protein interactions are incompletely characterized at present,³¹ further examination of mitochondrial pathways, specifically as they integrate with extramitochondrial processes, may help to anticipate these extramitochondrial effects.

In this review, we examine MPs targeted by current therapeutics, presenting a literature-compiled census of known drug interactions for 1534 human MPs. We also discuss the high-throughput discovery of drugs that target nuclear-encoded MPs from a network pharmacology standpoint. We discuss how such an approach can accelerate the pace of drug discovery by identifying candidates that may be further studied to derive more specific mechanistic details using standard pharmacological methods. Finally, we outline how shifting experimental platforms from model organisms to human cell lines can accelerate compound discovery and drug target identification and how the application of developing technologies and improvements in three-dimensional (3D) structural analysis could spur future pharmacological discovery.

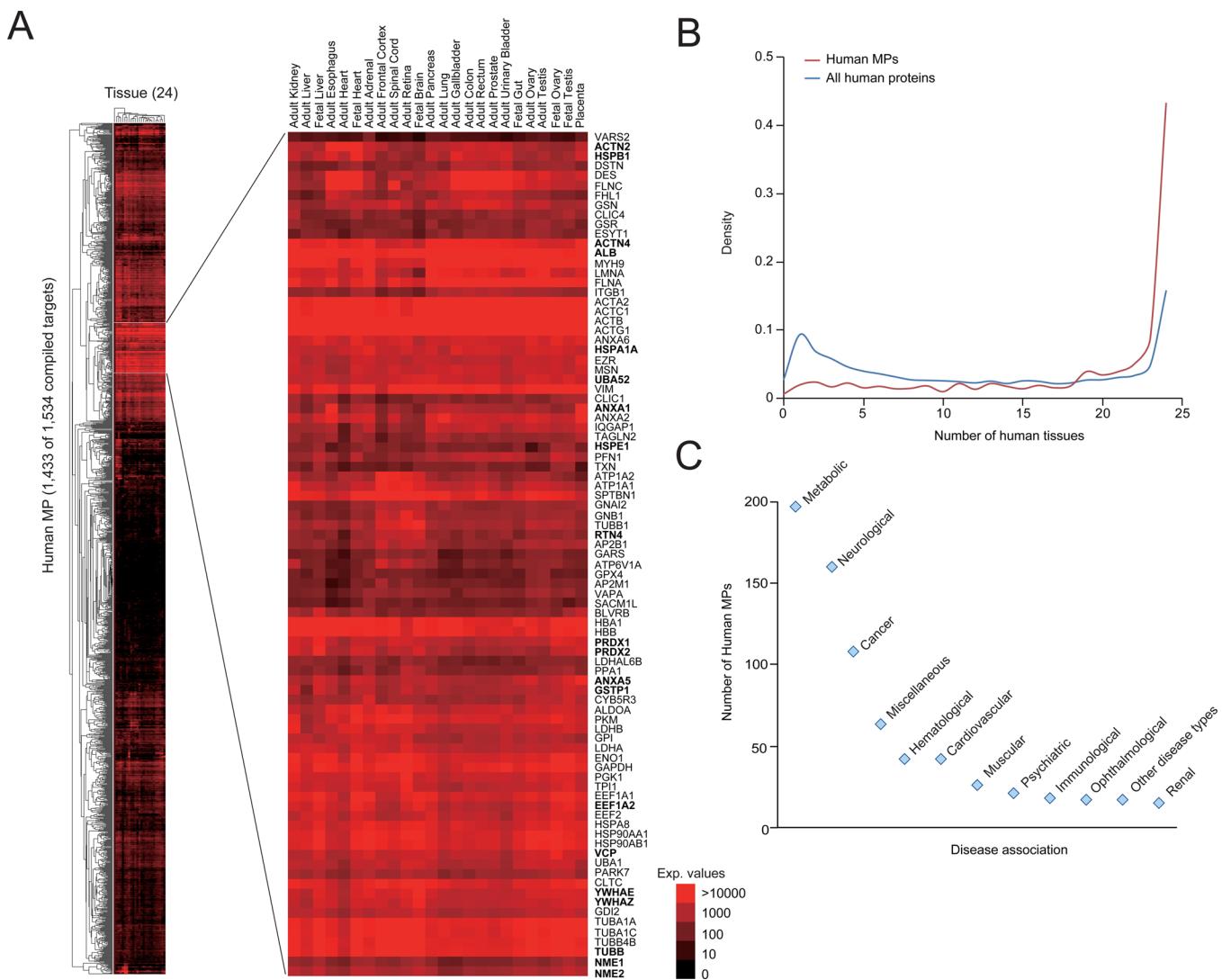


Figure 2. Disease association for human MPs. (A, B) Heat map showing expression of 93% of the human MPs (1433 of 1534) from our target index (A) and their distribution in tissue expression as compared against non-MPs (B) that are expressed in at least one of the 24 histologically healthy tissue samples, extracted from a recently published large-scale human proteome draft map.⁴² The bold text in the zoomed-in view of the inset indicates proteins constitutively expressed in the majority of human tissues examined and enriched specifically for processes encoding for programmed cell death (p -value $\leq 1.3 \times 10^{-6}$; significance computed using Fisher's exact test.). (C) Distribution of disease associations of human MPs. In the case of MPs associated with multiple diseases, assignment was made only to one disease type (see Table S1 for details).

2. SYSTEMATIC CHARACTERIZATION OF THE MITOCHONDRIAL PROTEOME

While the biochemical and structural characteristics of the mitochondria have been well-known for decades, the emergence of high-throughput proteomic screening efforts around the turn of the millennium has greatly accelerated the pace of characterizing MPs, pathways, and complexes. For example, in the model yeast *Saccharomyces cerevisiae*, more than 400 additional MPs have been identified (1200 total; up from ~750)^{32,33} during the past decade, and in human, more than 450 have been identified (~1100 total; up from 615)^{34,35} (Figure 1A). Representative discoveries, shown in Figure 1B, have informed our understanding of basic mitochondrial biology, and modern proteomics and integrative systems biology have helped to address how MP dysfunction can lead to human diseases.

Based on published proteomics studies, the total number of MPs in humans is estimated to approach 1500.^{34,36} To generate

a census of currently described MPs, we systematically annotated human proteins as being mitochondrial on the basis of experimentally supported database annotations, manual curation of the peer-reviewed literature, and a hand-picked set of well-established and widely used subcellular localization databases (Table S1). While our analysis allowed us to define a comprehensive catalogue of 1534 putative nuclear-encoded human MPs, establishing localization of these proteins within the mitochondria has been more challenging, as only 48% (750 of 1534) of these MPs have been experimentally identified to specifically localize to a mitochondrial subcompartment in mammalian cells (147 inner membrane, 107 outer membrane, 23 intermembrane space, 441 mitochondrial matrix, 1 cristae, and 21 mitochondrial ribosome proteins). 34% of MPs (526 of 1534) have been found to localize to both mitochondria and other compartments,^{37,38} leaving 268 proteins with unclear submitochondrial localization (Figure 1C and Table S1). This suggests that more experimentation, such as that being conducted for the ongoing Human Protein Atlas³⁹ project, is

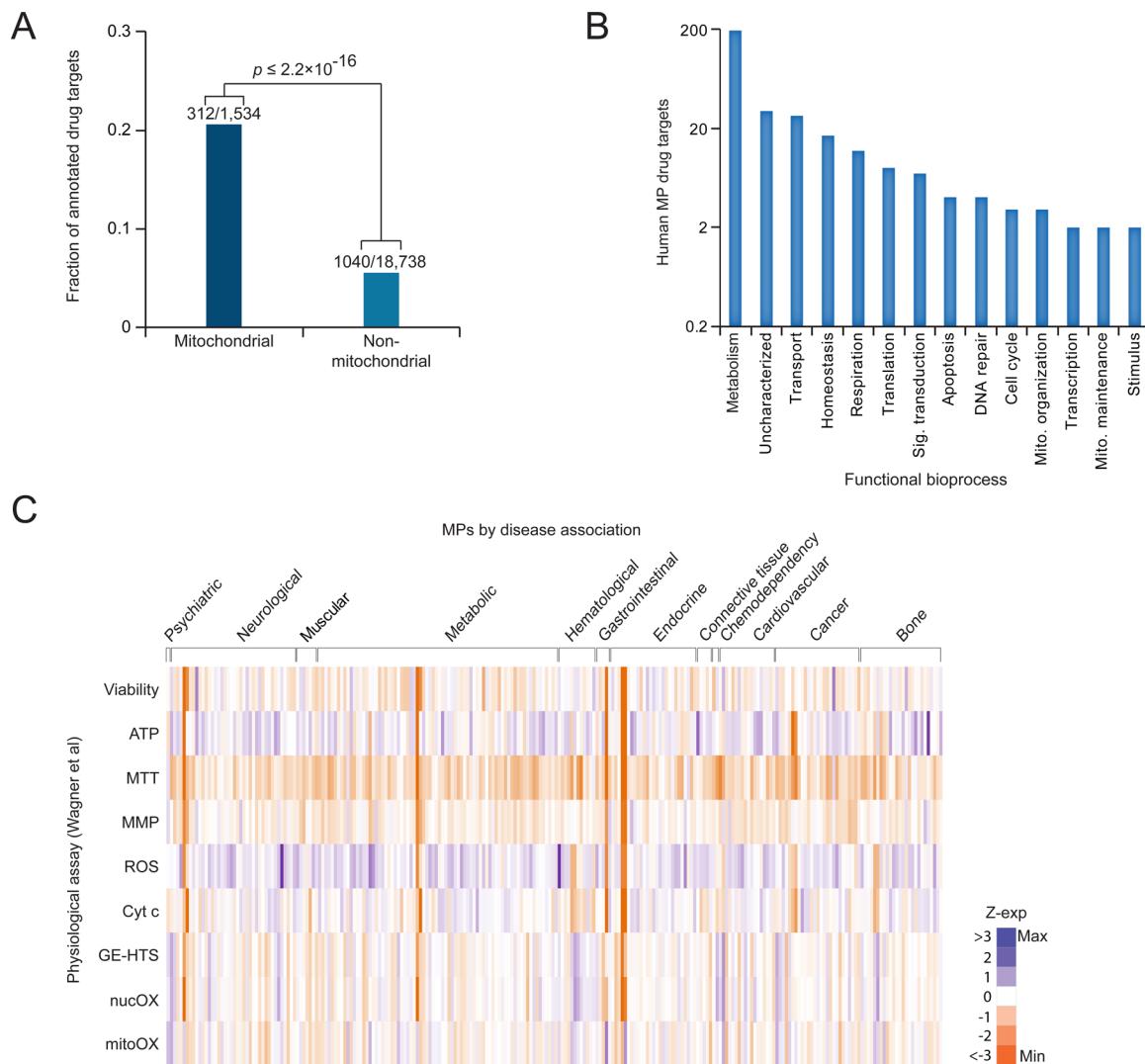


Figure 3. Small molecules targeting MPs and their associations to protein complexes and pathways. (A) Fraction of mitochondrial and non-MPs that are potential drug targets; *p*-value was computed using Fisher's exact test. (B) Functional grouping of MP drug targets as annotated by Gene Ontology bioprocess terms. (C) Gene expression profiles of various mitochondrial physiological activities measured for each of the 56 MPs with disease evidence against 125 distinct chemical perturbations compiled from the study of Wagner et al.¹⁷ The physiological measurements were performed for mitochondrial oxidative damage (MitOX), nuclear oxidative damage (NucOX), gene expression-based high-throughput screening (GE-HTS), cytochrome C activity (CytC), reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], a measure of mitochondrial dehydrogenase activity. The expression values are represented as *z*-scores; score details are shown in Table S3.

required to understand organellar functionality comprehensively.⁴⁰ Additionally, 373 human MPs are of unknown function and thus provide an opportunity for novel functional discovery (Figure 1D and Table S1).

As an independent assessment to measure the quality of our annotation efforts, we compared the MP target index to a recently published study focusing on annotating MPs expressed in human heart tissue.⁴¹ While our analysis captured about 74% of the current mitochondrial annotations (573 of the 775 MPs reviewed in UniProt; Figure 1E), the remainder of the annotations we report are new and well-supported by other sources of information (Table S1), providing a valuable resource for future studies on mitochondrial biology.

Finally, we examined protein abundance for the 1534 human MPs using a recently published large-scale human proteome draft map, in which protein abundance was measured for 16 570 annotated human proteins in 24 histologically healthy

tissue samples.⁴² Our analysis indicated that nearly 43% (619 of 1433 detected) of the human MPs are constitutively expressed in all tissues, versus ~13% (1991 of 15 134) of non-MPs (Figure 2A,B and Table S2). Thus, while there appears to be considerable tissue-specific variation in MP expression (consistent with previous reports^{43,44}), we feel that this set of proteins represents a constitutive current estimate of MP composition.

3. MITOCHONDRIAL DYSFUNCTION IN DISEASE PATHOLOGIES

Establishing an accurate and complete list of mammalian MPs, as well as characterizing submitochondrial localization, is complicated by the fact that MP localization can be tissue- or condition-specific.^{43,45,46} Thus, both the experimental model and survey conditions play substantial roles in the elucidation of MP function.⁴⁷ Fortunately, tissue lineages are being

established to model specific effects of mitochondrial dysfunction. For example, familial PD, AD, and other neurodegenerative disorders caused by the impairment of mitochondrial processes, including oxidative stress and quality control factors (e.g., PINK1 and PARKIN), can be modeled in vitro using disease-specific patient-derived induced pluripotent stem cells (iPSC) to elucidate unknown disease mechanisms.⁴⁸ Reprogramming of differentiated somatic cells provides an avenue for exploring various neurological diseases and allows for patient stratification, which will inform specific molecular disease etiology.^{48,49} Small molecules can then be screened for efficacy in specific molecular disease states.⁴⁸ This has been well-demonstrated in two recent publications^{50,51} wherein the drug targets identified in a yeast model of α -synuclein toxicity (a small lipid binding protein involved in several neurodegenerative disorders, including PD) led to the identification of early pathogenic phenotypes in iPS neurons derived from patients harboring an α -synuclein mutation.

Next, to clarify our 1534 compiled human MPs as being either causal or associated with mitochondrial diseases, we collected large sets of disease association data from databases (e.g., CORUM, GAD, OMIM, and CGP), and literature sources (from both full-text articles and abstracts), including our previous study.³ This effort resulted in 514 disease-linked and 9 disease-causing MPs, of which 210 were deemed to be both disease-linked and disease-causing (Table S1 in the Supporting Information). Strikingly, ~63% (465 of 733) of the human MPs with disease evidence are associated with cancer, metabolic, and neurological disorders (Figure 2C and Table S1). These disorders can arise either from mutations in a mitochondrial gene (whether encoded by the nuclear or mitochondrial genome⁵²) or from the indirect disruption of a mitochondria-dependent metabolic or homeostatic processes.^{53–55}

Disruptions in mitochondrial function that have been subsequently linked to disease can be broadly classified as those that result in the increased generation of reactive oxygen species (ROS), the dyshomeostasis of calcium buffering and storage, or the disruption of ATP production,³⁰ although alterations in ATP production and calcium homeostasis are inherently linked. For example, damage to the mitochondrial membranes affects the ability of mitochondria to generate ATP, which, in turn, leads to a lack of efficient pumping of calcium outside the cell or into other intracellular calcium stores (such as the ER).⁵⁶ Excessive intracellular calcium leads to the formation of so-called mitochondrial permeability transition pores that bypass both mitochondrial membranes.⁵⁷ This further disrupts mitochondrial function, ultimately causing swelling, rupture, and activation of apoptosis through the release of cytochrome C from the mitochondrial intermembrane space into the cytosol, resulting in apoptosis observed in ischemia/reperfusion injury.⁵⁶

Modifications in mitochondrial function have also been linked to cancer through several lines of evidence. For example, a shift in mitochondrial metabolism from primarily oxidative phosphorylation (OXPHOS) to a near sole dependence on glycolysis, a phenomenon termed the Warburg effect, is a long-known hallmark of cancer cells.^{58,59} As a result of being less dependent on OXPHOS, cancerous cells are able to survive in microenvironments with poor blood supply, such as those frequently encountered in rapidly growing tumors. Additionally, increased production of reactive oxygen and nitrogen species alters cell signaling in a manner that promotes cancer cell

survival.⁶⁰ This can occur via inhibition of mitogen-activated protein kinase phosphatases and through numerous transcription factors, including ETS1 (V-Ets Avian Erythroblastosis Virus E26 oncogene homologue 1), which controls the differentiation, survival, and proliferation of lymphoid cells.⁶¹ Excessive production of reactive oxygen and nitrogen species, as well as oxidative and nitrosative stress, are also major contributing factors to AD and Type 2 Diabetes Mellitus.^{62–67}

Finally, remodelling of mitochondrial structures through fusion (joining of mitochondria involving the coordinated activity of both outer and inner mitochondrial membranes) has contributed greatly to the pathogenesis of human disorders, especially in neurodegeneration.⁶⁸ Alternately, mitochondrial fission (or division) rarely results in human disease, principally due to the essentiality of this process for cell survival.²⁶ This is evidenced by the known embryonic lethality of fission mediator DRP1 knockout in mice.⁶⁹

4. PHARMACOLOGICAL TARGETING OF MITOCHONDRIA

Of the 1534 compiled human MPs, 312 are known targets of one or more existing small molecules (Figure 3A and Table S1). This represents almost 20% of the human mitochondrial proteome, significantly more than the ~5% of targeted non-MPs ($p \leq 2.2 \times 10^{-16}$). As mitochondria are key sites for the production of ATP, it is not surprising that the bulk of mitochondrial drug targets, almost 200, are involved in energy metabolism (Figure 3B). The remaining targets are widely distributed across a variety of biological processes (e.g., mitochondrial transport, respiration, transcription, and genome maintenance; Figure 3B), reflecting the importance of mitochondria in diverse cellular functions.

When grouped according to broad disease classes, the gene expression profiles of MPs involved in various physiological activities targeted by small molecules (from the study of Wagner et al.¹⁷) are largely distributed to metabolic, neurological, or cancer-related illnesses, consistent with mitochondrial function in energy production and apoptosis (Figure 3C and Table S3). The most prominent category of mitochondria-targeted small molecules are antioxidants or free radical scavengers. While antioxidant therapy has been typically limited due to difficulties in accessing the mitochondria, recent advances have allowed mitochondrial targeting of such molecules as the lipophilic cation-associated drug MitoQ, the antioxidant Szeto–Schiller peptides, or drugs encapsulated in dequalinium-containing liposomes.^{71–73}

Another category of pharmacological agents exploit the fact that mitochondria regulate the apoptotic cascade. One such group of drugs (including ABT-737, A-385358, and gossypol) reduce the ability of cancer cells to resist chemotherapeutic agents by inhibiting the sequestration of pro-apoptotic proteins (such as BCL2) by antiapoptotic proteins (such as BAK).²⁷ Additionally, as mentioned above, the Warburg effect in cancer cells makes glycolysis an attractive pharmacological target. For instance, phloretin or 2-deoxy-D-glucose interferes with glucose uptake and has shown promising anticancer activity.⁷⁴ Currently, there are several inhibitors of glycolysis that enhance cancer cell sensitivity to chemotherapeutic agents and are undergoing preclinical trials.²⁷ Likewise, mitochondrial metabolic modulators are undergoing clinical trials for improvement of ischemic heart disease and cardiomyopathy.⁷⁵

Increasing the NAD⁺/NADH ratio directly, as is done by nicotinamide, enhances mitophagy (mitochondrial degrada-

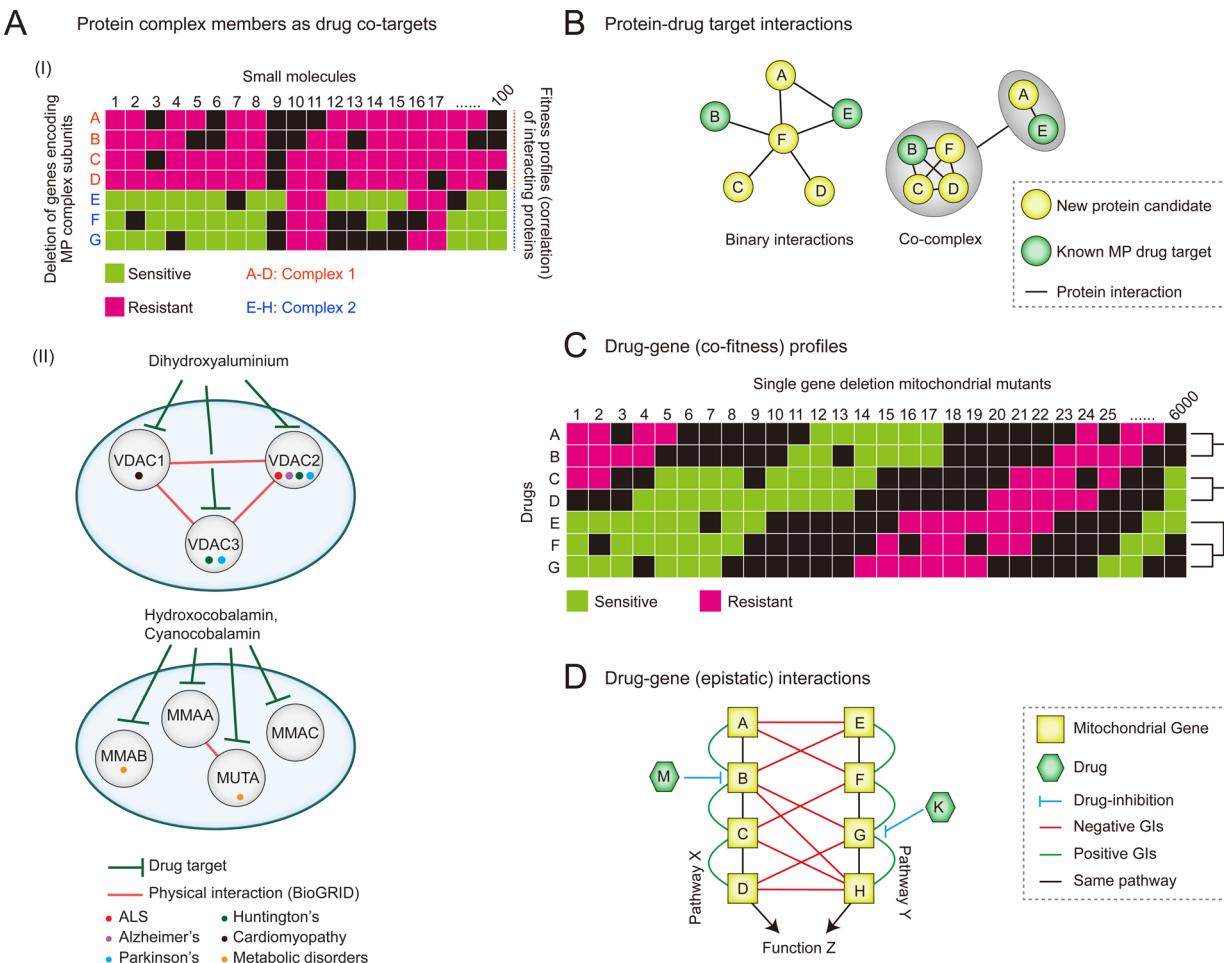


Figure 4. Fitness profiling and interaction networks in mitochondrial drug target discovery. (A) Fitness profiles of interacting proteins complex members sharing phenotypic responses (I) and subnetworks of physically connected disease-linked MP complex subunits (II), targeted by small molecules. (B) Subnetworks depicting the physical connectivity, either directly (left) or within a complex (right), between a new and known MP drug target. (C) Illustration of several possible co-fitness profiles of single gene deletion mutant strains grown in the presence small molecules targeting related mitochondrial processes or pathways. (D) Epistatic interactions for two redundant pathways (X and Y). In pathway X, gene B that exists with A, C, and D in a linear pathway is inhibited by the drug M, whereas gene G in pathway Y (which is in a pathway with E, F, and H) is inhibited by drug K. Here, the additional pathway information provided by genetic interaction (GI) mapping enabled the discovery that drugs M and K inhibit parallel pathways, which may suggest, for example, combination drug therapies involving both M and K.

tion) and the subsequent removal of dysfunctional mitochondria.⁷⁶ This and other effects on mitochondrial function make nicotinamide useful in treating a range of disorders, including AD.⁷⁷ Indirect increase of the NAD⁺/NADH ratio activates AMP-activated protein kinase (AMPK), as is seen with resveratrol.⁷⁸ While resveratrol has limited health benefits, a synthetic activator of AMPK, 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICAR), corrected cytochrome c-oxidase deficiency in a knockout mouse model, improving motor performance.⁷⁹ Similarly, direct activation of the downstream effectors of NAD⁺/NADH, namely, the sirtuin family of histone deacetylases, results in enhanced mitophagy.⁸⁰ It is unclear whether mitochondrial dysfunction is the cause⁸¹ or the result of metabolic syndromes;^{82,83} however, improvement of mitochondrial function apparently results in an overall improvement of patient condition. Compounds that enhance a specific step in the electron transport chain can also help to improve mitochondrial function.⁸⁴ For example, methylene blue, which enhances cytochrome C oxidase and mitochondrial respiration, has been used to counteract respiratory defects associated with AD.⁸⁵

Several nuclear proteins that increase the expression of mitochondrial metabolic enzymes and enhance mitochondrial function are reported to ameliorate metabolic disorders upon drug-based stimulation. These include the retinoic acid receptor,⁸⁶ peroxisome proliferator-activated receptors,⁸⁷ estrogen-related receptors,⁸⁸ and mitochondrial transcription factor A.⁸⁹ Some of these transcription factors are already targets of clinically used drugs.⁹⁰

Recently, impaired mitophagy was implicated in PD,⁹¹ and chemical inhibitors like nicotinamide that can enhance removal of defective mitochondria may slow the progression of neurodegenerative disorders.⁹² A genome-wide high-throughput RNA interference (RNAi) screen identified a diverse set of genes that affect mitophagy rates (e.g., TOMM7, HSPA1L, BAG4, and SIAH3),⁹³ suggesting the existence of multiple pathways that influence this process. Compounds that inhibit mitochondrial fission⁹⁴ and fusion⁹⁵ were also shown to attenuate apoptosis in models of neurodegenerative disorders. These include MDIVI-1, which blocks outer membrane permeabilization via inhibition of DNM1 and which is currently undergoing clinical evaluation.^{94,96}

The ability of FDA approved drugs to be repurposed for mitochondrial diseases has also been an area of intense interest. For example, cyclosporine A (CsA), a clinical immunosuppressant used to prevent rejection of transplanted organs, has been used for years to inhibit mitochondrial permeability of the transition pore through its molecular target cyclophilin D.⁹⁷ CsA was shown to reduce the size of myocardial infarctions in humans⁹⁸ and is also being tested for its ability to ameliorate neuronal damage after stroke,⁹⁹ with both diseases involving mitochondrial dysfunction through extra-mitochondrial pathology. CsA analogues that specifically act on mitochondrial cyclophilin D, but not on cytosolic isoforms, would have the added advantage of lacking immunosuppressant side effects.¹⁰⁰

However, currently there are few clinically approved drugs that directly influence disease pathologies that are linked to MPs compiled in this study, whereas others may be palliative and treat only symptoms and not the cause of the actual disease. Furthermore, many of the clinical drugs that affect MP function lack the specificity to allow them to be employed for clinical intervention in mitochondrial diseases. This is largely due to the inaccessibility of the inner mitochondrial membrane to small molecules.¹⁰¹ However, as our understanding of mitochondrial pathways is still incomplete,¹⁰² emerging methods aimed at systematically understanding mitochondrial gene function may yet uncover new avenues for therapeutic modulation.

5. IDENTIFICATION OF POTENTIAL PHARMACOLOGICAL TARGETS THROUGH SYSTEMATIC SCREENING

The recent prevalence of large-scale genomic and proteomic data sets has facilitated a paradigm shift in therapeutic design towards an approach that integrates pathway and systems data and that is increasingly tailored to individual patients.¹⁰³ In examining large-scale protein–protein interaction (PPI) data, interacting proteins have been found to be more likely to share similar fitness profiles across tested drugs (i.e., deletion of genes encoding physically connected subunits of a protein complex result in similar sensitivities to structurally diverse compounds, as evidenced by correlated fitness similarity profiles),^{104,105} making protein complex members likely drug co-targets (Figure 4A).

Conversely, members of functionally related disease- or non-disease-linked MPs can be used to predict relevant complexes or pathways targeted by a drug (Figure 4A). For example, loss-of-function of any one of the three functionally and physically connected voltage-dependent anion channel (VDAC) MP members VDAC1–3, associated with a variety of diseases (e.g., ALS, PD, AD, Huntington's disease (HD), and cardiomyopathies^{106,107}) has been shown to cause sensitivity to the small molecule dihydroxyaluminum.^{104,108–110} Similarly, methylmalonic acidemia (MMA) is a hallmark of genetic metabolic disorders and is caused by mutations in genes (MMAA, MMAB, MMAC, and MUT) involved in the translocation of cobalamin (vitamin B12) into the mitochondria. These proteins are all targeted by hydroxocobalamin and cyanocobalamin. Also, physical interactions have been observed between the human GTPase MMAA (methylmalonic aciduria type A) and MUT (methylmalonyl-CoA mutase),¹¹¹ and while these MPs are not expected to have comparable binding affinities for the same compounds, it seems likely that members of this pathway may be targeted to achieve the same physiological effect (Figure 4A).

Physical (i.e., protein–protein) interaction data can provide information on protein complex composition, thus identifying potential additional drug targets through guilt-by-association (Figure 4B).¹¹² Moreover, drugs often function by perturbing physical interactions between proteins. For example, melatonin has been shown to interfere with the apoptotic pathway by impeding the dimerization of the pro-apoptotic protein BAX (the interacting partner of BCL2).¹¹³ Also, the small molecule 10058-F4, known to bind to the C-terminal domain of MYC, inhibits a family of transcriptional factors including c-MYC, MYCN, and MAX. The subsequent disruption of the c-MYC/MAX and MYCN/MAX heterodimeric interactions leads to protein degradation, apoptosis, and lipid formation in neuroblastoma cells.^{114,115} Some drug treatments use the opposing strategy of enhancing physical associations. For example, the free radical scavenger edaravone, which has neuroprotective effects, causes an increase in binding stability between the pro-apoptotic protein BAD and the conserved regulatory protein 14-3-3 in response to oxidative stress, thereby reducing apoptosis.¹¹⁶

Large-scale protein interaction data has been generated mostly using scalable platforms such as yeast two-hybrid,^{117,118} protein–fragment complementation,¹¹⁹ and affinity purification–mass spectrometry.^{31,120–123} Currently, due to difficulties in accessing MPs using standard laboratory conditions, their interactions are considered to be under-surveyed. To improve coverage in MP interaction screens, the currently employed practices such as growth in standard fermentative culturing conditions (YPD media) and growth of culture to saturation (which induces mitophagy) will likely need to be revisited, as they tend to inhibit MP gene expression.

Genetic interaction screening presents a useful alternative for definition of complexes. Of specific relevance is the identification of drug targets through systematic competitive growth of gene deletion mutant strains in the presence of a given small molecule and subsequent identification of sensitive strains (i.e., haploinsufficiency profiling and homozygous deletion profiling, or HIP–HOP; Figure 4C).^{124,125} HIP screening in yeast has revealed that gene deletions in mitochondrial translation confer sensitivity to tigecycline, which led to the discovery of tigecycline's anti-leukemic action.¹²⁶

Similarly, the enzyme kynureneine 3-monooxygenase (KMO), which is targeted by the small molecules lanthellamide A and UPF648,^{127,128} was identified as a potential therapeutic target for HD based on loss-of-function yeast screens.¹²⁹ KMO inhibition has also recently been observed to improve neurodegenerative conditions in mouse models.¹³⁰ Also, yeast genetic screens assaying growth of loss-of-function alleles under high temperature and using glycerol as a carbon source identified the antibacterial chlorhexidine as a potential candidate for ameliorating mitochondrial dysfunction.¹³¹ This was later confirmed in mammalian tissues.¹³¹

As HOP profiling depends on an epistatic relationship between the drug target and a second gene with a loss of function allele, characterization of the yeast genetic interaction network has been highly useful in deciphering drug target pathways. Synthetic genetic array (SGA) screening^{132,133} has allowed near-comprehensive surveys of genetic interactions regardless of protein localization and thus is particularly useful in charting mitochondrial gene function (Figure 4D). Briefly, SGA quantifies the growth of thousands of yeast deletion strains, systematically identifying instances wherein double

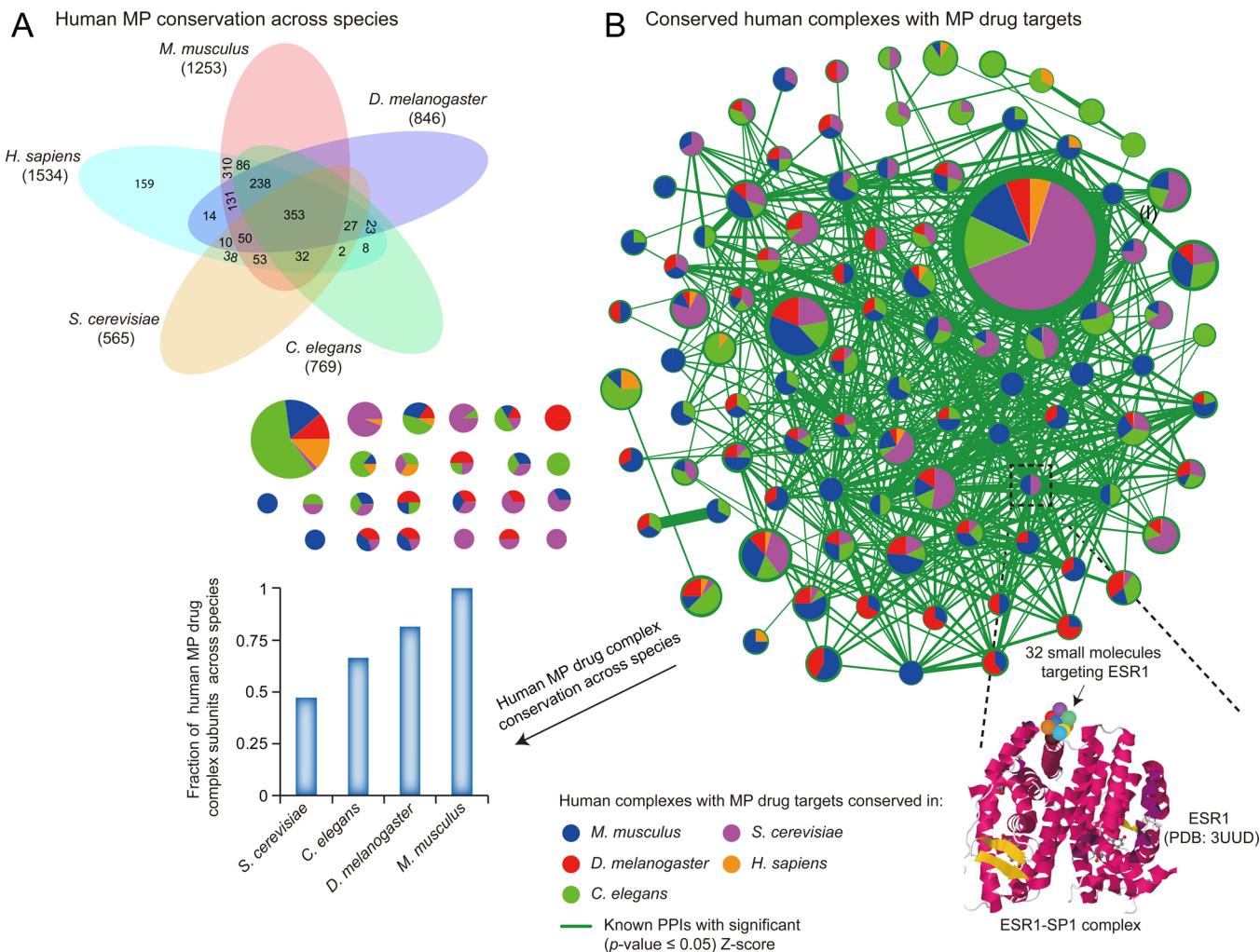


Figure 5. Human MP and complex conservation across species. (A) Venn diagram showing the overlap of 1534 human MPs with four other eukaryotes. The numbers in parentheses show the extent of human MP conservation in other species. (B) Evolutionary conservation map showing 119 (of the 1788) curated human protein complexes containing at least one drug-targeted MP in additional model species. As an example, the conserved ESR1-SP1 complex in the bottom inset highlights ESR1, as 32 drugs are known to target this MP. Node size is proportional to the number of subunits comprising the complex, and the colored wedges are sized according to the proportion of the human complex containing an MP drug target conserved in yeast, fly, worm, and mouse. The fraction of conserved MP drug complex subunits across species is shown as a bar graph. Edges in the network graph indicate significant PPIs ($|z\text{-score}| \geq 1.961$ versus random permutation; $p\text{-value} \leq 0.05$) compiled from BioGRID (ver. 3.2.111), whereas the edge width reflects the degree of PPI connectivity between conserved complex subunits.

mutant strains grow either more or less than expected based on the growth of constitutive single deletion strains. Deviation from expected growth rate in the double mutant strain is indicative of a synergistic genetic relationship between the two genes. Resulting genetic interaction profiles have been used in combination with HOP data to identify novel drug targets,¹³³ although this approach has not yet been specifically applied to mitochondrial genes.

Alternately, recent large-scale screening efforts in human cell lines have monitored fluorescent indicators of mitochondrial function to examine process-specific perturbation effects. For example, large-scale chemical screening at various stages of apoptotic processes in Jurkat cells identified compound (norgestrel and diclofenac)-specific profiles that activated mitochondrial annexin binding.¹³⁴ The use of recently developed large-scale RNAi-mediated epistatic or chemogenomic screens through short hairpin RNA (shRNA), small interfering RNA (siRNA), and endoribonuclease-prepared siRNA (esiRNA) plasmid libraries in mammalian cells^{135–139}

have raised the possibility of high-throughput assays of functional interactions, as has been done in yeast.^{133,140,141}

Recently, an assessment of ATP production in various RNAi-mediated knockdowns revealed adenylate kinase to be a potential target for therapeutic intervention to counter electron transport chain or bioenergetic disruptions characteristic of cancers and neurodegenerative diseases.¹⁴² Similarly, analysis of PARKIN (a component of the E3 ubiquitin ligase complex associated with PD) in RNAi-treated HeLa cells using high-content microscopy has revealed regulators associated with mitochondrial damage.⁹³ A similar screen in *Drosophila melanogaster* tissues identified a novel regulator of calcium transport, LETM1,¹⁴³ whereas a *Caenorhabditis elegans* RNAi screen combined with the mitotoxic drug antimycin has identified additional genes important for mitochondrial protection.¹⁴⁴

While RNAi may present an attractive approach for the systematic survey of mitochondrial gene function and chemogenomic analysis, off-target effects, uneven or limited gene

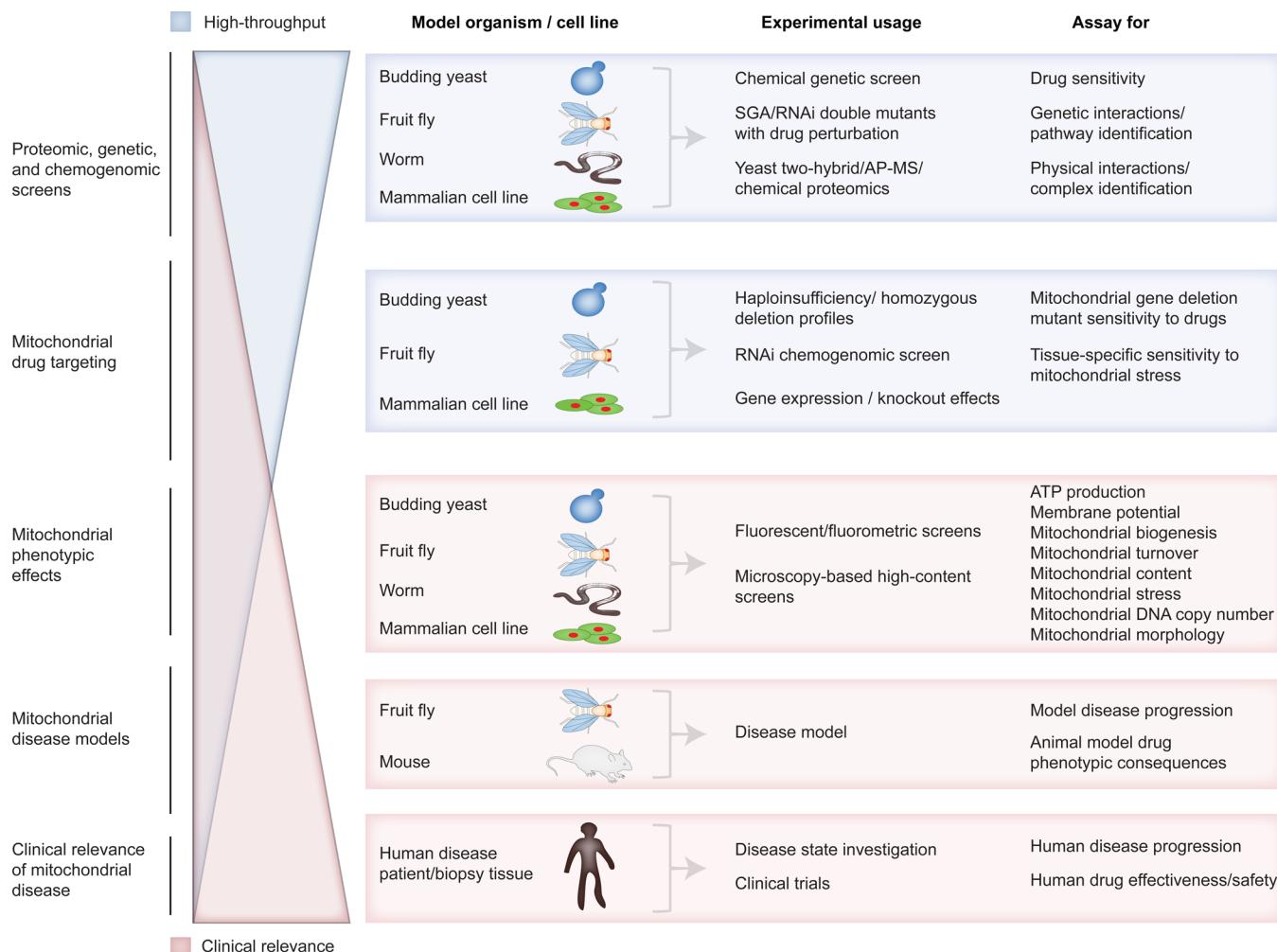


Figure 6. Relationship between the scale of research in tractable model systems and its relevance to clinical application. At each research level, the model organisms in use, methodologies available, and assays typically conducted are listed.

coverage, and imperfect suppression of the target gene may obscure interpretation.^{145–147} The recent advent of RNA-guided CRISPRs (clustered regularly interspaced short palindromic repeats) for targeted gene disruption^{148,149} offers a promising strategy for gene deletion assays in mammalian cells. However, as with RNAi, potential off-target effects of CRISPRs would present a limitation to large-scale screening. More recent adaptations, such as the use of truncated sgRNAs (short or single-guide RNAs),¹⁵⁰ seek to limit these off-target effects.

6. INTERPRETING TARGET ASSOCIATION DATA

Although much of the large-scale protein and genetic interaction data generated over the past decade has come from model organisms such as yeast, fly, and worm,¹⁴¹ the high conservation of MPs and complexes (Figure 5A,B and Table S4) allows these results to be particularly transferable to humans through cross-species orthologue mapping. This strategy has been reported widely by us^{31,151} and others^{152–157} to inform human protein function.

These lower eukaryotic models are not only generally more rapid and cost-effective for small molecule screening than their mammalian counterparts, but also can be vital when the phenotypic effect of certain mutations cannot be experimentally tested directly in disease tissues.¹⁵⁷ For example, ample

literature evidence supports the complex formation of an E3 ubiquitin ligase (PARKIN), a mitochondrial kinase (PINK1), and a protein of unknown function (DJ-1) based on their functional similarity in preserving mitochondrial integrity and promoting ubiquitination of Parkin substrates in human brain lysates.¹⁵⁸ Knockout of both PARKIN and PINK1 in fly has drastic phenotypic effects due to mitochondrial damage, causing muscle degeneration, male infertility, and the loss of dopaminergic neurons.^{159,160}

However, despite the utility of such highly tractable model organisms for identifying fundamental pathways and processes (Figure 6), they are inevitably limited in terms of modeling specific human disease states. For example, while neurotransmitter systems in fly mediate many behaviors (i.e., learning and memory) that are conserved in humans,¹⁵⁷ the fly brain has no substantia nigra, which is pertinent to understanding how clinical features mediated by dopaminergic neuron loss in Parkinson's disease correlate with behavioral phenotypes.¹⁵⁷ Likewise, while essential molecular mechanisms underlying tumorigenesis and metastasis can be probed in fly, it is not feasible to model many types of malignancies that are common in humans, such as those related to specific tissues (e.g., prostate, ovarian, or breast cancer).¹⁵⁷ Since cellular and molecular processes can vary between model species and humans, careful consideration of the model system is required

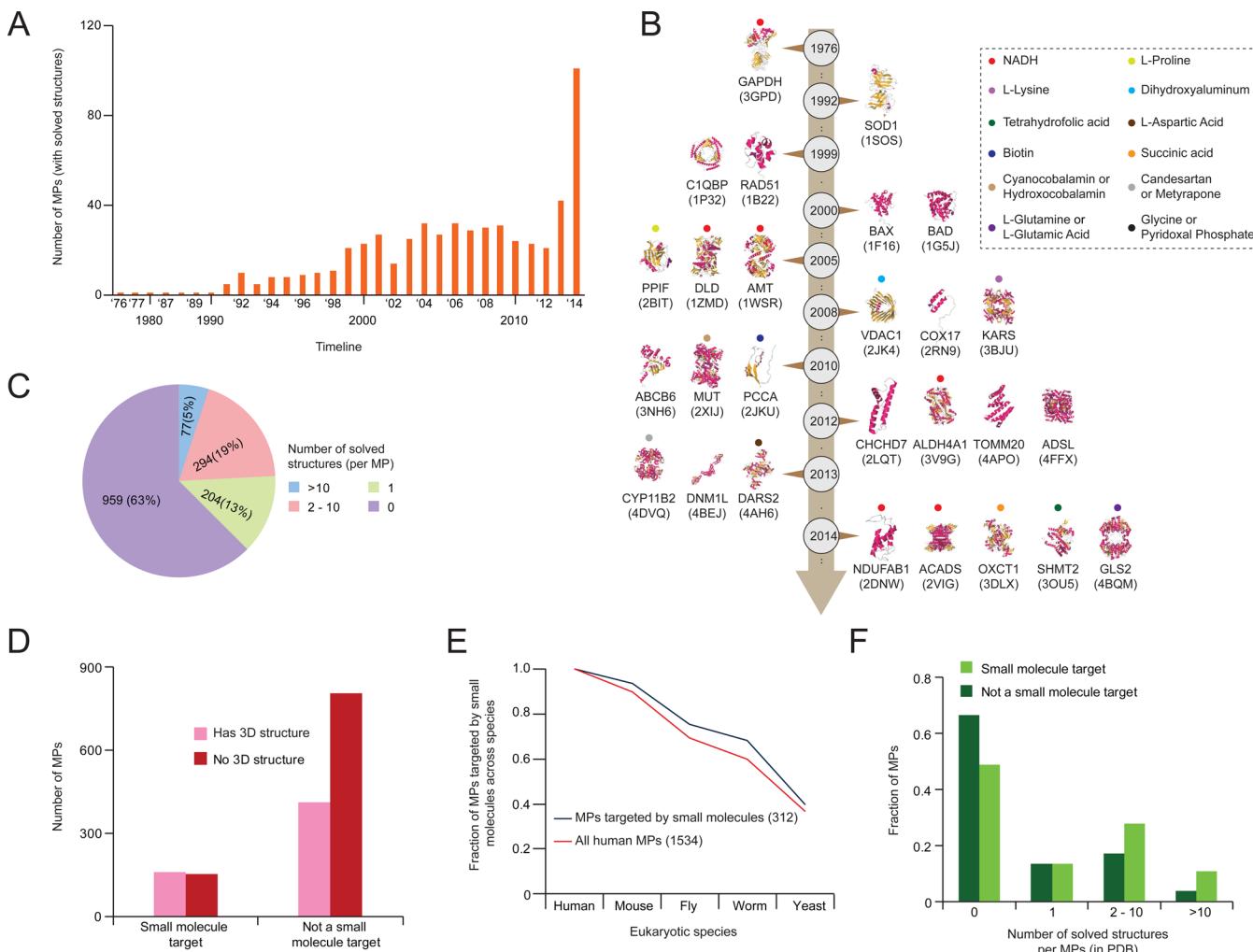


Figure 7. Human MP structures and their relationships to small molecule inhibitors. (A) Bar graph showing the number of MPs with solved 3D structures (compiled from public databases) over time. (B) Timeline showing representative 3D MP structures (with year of publication) along with known small molecule inhibitors (denoted by dots). (C) Number of solved structures per human MP. Multiple solved 3D structures for the same human MP can arise in separate research publications. (D) Comparison of MPs with known targeted drugs and with solved 3D structures. (E, F) Fraction of human MPs targeted by small molecules conserved across eukaryotic species (E) and with known small molecule targets (F) by the number of solved 3D structures.

when designing screens and interpreting data. Orthology mapping is therefore best suited as a means to generate new testable hypothesis or to lend ancillary support to an existing one.

Mammalian *in vivo* models offer a more faithful modeling of disease but at the cost of scalability (Figure 6). For example, mouse models that closely replicate specific forms of mitochondria-associated neurodegenerative diseases are constantly being generated, such as *SOD1*-deficient mice for ALS¹⁶¹ or mice that model PD.¹⁶² However, these disease models lack the throughput needed for lead compound discovery.

7. STRUCTURAL MODELING OF DRUG TARGETS FOR THERAPEUTIC DISCOVERY

In silico prediction of small molecule–protein interactions potentially provides an efficient alternative to experimental screening.^{163–167} This is particularly relevant for MPs, as they can be difficult to assay experimentally. For example, docking analysis of mitochondrial monoamine oxidase B facilitated the design of novel inhibitors of the protein.¹⁶⁸ In addition to

predicting novel drug binding interactions, in silico screening has been useful in the discovery of potential therapeutic effects for existing small molecules¹⁶⁹ and in the optimization and development of small molecule agonists or antagonists from previously identified interactions.¹⁷⁰ In silico target prediction typically depends largely on characterized 3D protein structures, which have increased dramatically for MPs over the past decade (Figure 7A,B and Table S1).

Approximately 37% of MPs have at least one solved 3D structure, as compiled by the Protein Data Bank (Figure 7C). However, as protein docking analysis has centered around identifying small molecule binding mechanisms, drug targets make up a significantly higher proportion (162 of 575; 28%) of solved 3D structures, with half (162/312) of all known mitochondrial drug targets having solved 3D structures (Figure 7D). Furthermore, a higher fraction of MP drug targets are conserved within other model species (Figure 7E) and have multiple solved structures when compared to non-drug-targeted MPs (Figure 7F), suggesting an intense research focus on MP–small molecule docking.

For those proteins that fail to crystallize properly for structural analysis, potentially due to low yield or protein instability, the high conservation of MPs (Figure 5A) provides relevant alternatives from other model organisms. For example, when the mammalian neurodegeneration-associated protein kynureine 3-monooxygenase failed to crystallize, the homologous yeast protein provided the crystal structure of both the protein and the protein–small molecule (UPF 648) binding complex.¹²⁸ Notably, this example may be somewhat unique, as use of alternative model organisms may not improve acquisition of high-quality crystals. This is especially true for membrane-bound MPs, where obtaining concentrated pure solution is often demanding and requires appropriate crystallization and solubilization conditions.⁵⁰ Expressing a mutated form or altering the surface properties of the protein⁵¹ and improving the solubility of the native proteins in a different host⁵² can ease some of the associated problems, but this remains a slow process. Consequently, there is a pressing need to predict the structure of proteins in an informed way, which may involve the detection of homologues of known 3D structures using template-based structural homology modeling and fold recognition.⁵³

In the absence of a solved structure, prospective drug targets can also be proposed based on amino acid conservation. For example, the structure of DRP1, modeled using similarity to dynamin, was queried for potential antagonists using automated docking analysis, finding multiple candidates with potential antiapoptotic effects.¹⁷¹ Compound activity can also be estimated through protein secondary structure¹⁷² and examination of protein interaction¹⁷³ or expression¹⁷⁴ data. For example, small molecules can be designed to specifically target interface sites between interacting protein pairs, to inhibit or stabilize important protein–protein interactions, or to disrupt or strengthen a protein complex^{175,176} (for a more in-depth review on protein–protein interaction interface targeting, see Duran-Frigola et al.¹⁷⁶). As well, gene network expression changes characteristic of disease states can be identified using systematic transcriptional analysis,^{177–179} an approach useful for mitochondrial targets.¹⁷⁴ Thus, *in silico* screening, in combination with dedicated experimental efforts aimed at elucidating mitochondrial gene function on a large scale,¹³² presents an attractive combination for future drug targeting studies.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

The majority of drugs discovered to date that target MPs have been revealed through either target-based screens,¹⁸⁰ systematic competitive growth assays,¹⁴⁰ or phenotype-based screens.¹⁸¹ While our curation efforts have generated a list of 327 compounds targeting 312 unique MPs, future discovery of novel therapeutics can benefit from a greater systematic understanding of mitochondrial disease etiology and of mitochondrial gene function. Yeast, worm, and fly are scalable, easily manipulated systems that have been invaluable in assaying the molecular functions of thousands of genes, many of which are directly conserved across eukaryotes.^{124,151} However, these organisms are limited in terms of modeling tissue-dependent diseases in humans. The specific cellular environment of a disease state may play a significant role in disease progression, and understanding these effects will be critical in determining how a drug restores health to a dysfunctional mitochondrial network. This is particularly true for diseases that exhibit tissue specificity, such as PD,⁴⁸ which

requires specialized differentiated tissue culture types to examine.

Recent advances in mammalian cell lines, specifically using high-content screening of mitochondrial reporters, have made it possible to perform targeted lead compound discovery at high throughput. However, delivery of drugs *in vivo* still presents a substantial hurdle, as more rigorous optimization procedures are required with respect to testing drug–target specificity and selectivity in preclinical models to avoid clinical trial failure. Future combinations of existing drug information with structural modeling, gene network analysis, expression profiling, and gene deletion/knockdown assay in multiple model organisms, holds the promise to provide more effective and more easily deliverable therapeutics.

■ ASSOCIATED CONTENT

S Supporting Information

Human MP index (including gene annotation, biological process, subcellular localization, and disease association) and known small molecule inhibitors are listed in Table S1. Protein composition of MPs from this study mapped against human proteins measured in 24 histologically healthy tissue samples (compiled from a recently published large-scale human proteome draft map⁴²) are shown in Table S2. Bioactive compounds targeting MPs linked with disease (sheet 1) and the gene expression profiles (sheet 2) of various physiological effects of MPs against chemical perturbations (compiled from the study of Wagner et al.¹⁷) are shown in Table S3. Compilation of human protein complexes containing MPs that are known drug targets (sheet 1) and conservation of these MPs to other eukaryotic species (sheets 2 and 3) are indicated in Table S4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CRISPR, clustered regularly interspaced short palindromic repeats; CsA, cyclosporine A; ER, endoplasmic reticulum; HD, Huntington's disease; HIP, haploinsufficiency profile; HOP, homozygous deletion profile; iPSC, induced pluripotent stem cells; MP, mitochondrial protein; OXPHOS, oxidative phosphorylation; PPI, protein–protein interaction; PD, Parkinson's disease; RNAi, RNA interference; RNS, reactive

nitrogen species; ROS, reactive oxygen species; SGA, synthetic genetic array; sgRNA, short or single-guide RNA; shRNA, small hairpin RNA; siRNA, silencing RNA; VDAC, voltage-dependent anion channel

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