Review

The Power of Yeast in Modelling Human Nuclear Mutations Associated with Mitochondrial Diseases

Camilla Ceccatelli Berti [†] [ID], Giulia di Punzio [†] [ID], Cristina Dallabona, Enrico Baruffini [ID], Paola Goffrini [ID], Tiziana Lodi and Claudia Donnini *[ID]

Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy; camilla.ceccatelliberti@unipr.it (C.C.B.); giulia.dipunzio@unipr.it (G.d.P.); cristina.dallabona@unipr.it (C.D.); enrico.baruffini@unipr.it (E.B.); paola.goffrini@unipr.it (P.G.); tiziana.lodi@unipr.it (T.L.)
* Correspondence: claudia.donnini@unipr.it; Tel.: +39-0521-905602
† These authors contributed equally to this work.

Abstract: The increasing application of next generation sequencing approaches to the analysis of human exome and whole genome data has enabled the identification of novel variants and new genes involved in mitochondrial diseases. The ability of surviving in the absence of oxidative phosphorylation (OXPHOS) and mitochondrial genome makes the yeast *Saccharomyces cerevisiae* an excellent model system for investigating the role of these new variants in mitochondrial-related conditions and dissecting the molecular mechanisms associated with these diseases. The aim of this review was to highlight the main advantages offered by this model for the study of mitochondrial diseases, from the validation and characterisation of novel mutations to the dissection of the role played by genes in mitochondrial functionality and the discovery of potential therapeutic molecules. The review also provides a summary of the main contributions to the understanding of mitochondrial diseases emerged from the study of this simple eukaryotic organism.

Keywords: yeast model; mitochondria; diseases

1. Introduction

Mitochondrial diseases (MDs) are inherited disorders that, through various mechanisms, lead to mitochondrial dysfunction. The genetic cause of MDs includes mutations in either mitochondrial DNA (mtDNA) or nuclear DNA genes. Due to the dual genetic control of mitochondrial function (nuclear and mitochondrial), MDs display different inheritance pattern: sporadic, maternal, autosomal dominant, autosomal recessive or X-linked [1].

Mitochondria provide energy to cells by oxidative phosphorylation (OXPHOS) carried out by a series of multi-heteromeric complexes embedded in the mitochondrial inner membrane. These complexes constitute the mitochondrial respiratory chain (MRC) that, through sequential reactions of reduction and oxidation, performs "cellular respiration" [2]. The OXPHOS process is responsible for the supply of energy to cells; any defect or alteration in the process leads to pathological consequences, mainly in tissues and organs that have a high energetic request such as the brain, skeletal muscles, and heart. The consequences of an impairment of the OXPHOS system are a decrease in ATP production and an increase in reactive oxygen species (ROS). Mitochondria are mainly involved with the production of ATP by OXPHOS and play a role in other bioenergetic pathways such as the tricarboxylic acid cycle (TCA)—[3] and fatty acids β-oxidation [4]. In addition to these primary functions, mitochondria are also involved in biosynthetic pathways, including but not limited to amino acids and nucleotides [5], iron sulphur cluster [6], and in cell signalling with a determinant role in apoptosis [7], and calcium homeostasis [8].

Given the complexity of mitochondrial genetics and biochemistry, mitochondrial inherited diseases may present extremely heterogeneous clinical manifestations, ranging

from lesions in single tissues, such as the optic nerve in Leber's hereditary optic neuropathy, to more diffuse lesions including myopathies, encephalomyopathies, cardiopathies and hepatopathies, all the way to complex multisystem syndromes characterised by a vast range of symptoms, severity, age of onset and outcome [9–12]. Genetic defects of OXPHOS, the group of mitochondrial disorders predominantly identified to date, have a prevalence at approximately 1:5000 [13–15] making these the most common pathologies with a genetic basis. However, more recently, the term "mitochondrial disease" has also been extended to another series of pathologies, known as secondary mitochondrial dysfunction (SMD), caused by mutations in genes that are not involved in the production or functionality of respiratory complexes [16]. For example, defects in the mitochondrial fission/fusion processes are implicated in the onset of age-related human disease, such as Alzheimer's and Parkinson [17] or cardiovascular disease [18], thus underlining the pivotal role of the mitochondrion in many cellular functions, beyond energy production.

To date, thanks also to the increasingly widespread application of the next generation sequencing (NGS) to whole exome sequencing (WES) and whole genome sequencing (WGS), pathogenic variants in approximately 300 disease genes have been described [1,19] most associated with dysfunction of mitochondrial energetics [20]. However, in a large fraction of patients with MDs, the genetic basis is still unknown. This is not surprising since there are approximately 1200 human genes encoding mitochondrial localised proteins [21,22].

On the other hand, the identification of numerous nuclear variants of unknown significance requires functional validation to confirm pathogenicity and an in-depth analysis to assess the mechanisms through which they are associated with mitochondrial disease [1].

Model systems (yeast, *Caenorhabditis elegans*, *Drosophila,* zebrafish, mouse) have proved their usefulness to validate the pathogenicity of variants, to assess the disease progression and the mechanisms associated with mitochondrial dysfunction. They therefore represent a powerful tool to study new disease genes, in particular when the gene function is unknown, when there is only a single patient, patient samples cannot be obtained or when cell lines, derived from patient fibroblasts, are aphenotypic (see for recent review [1,20,23]). The yeast *Saccharomyces cerevisiae* is the organism that more than any other has contributed to our understanding of mitochondria functionality. In fact, it was in this organism that the cytoplasmic factor *rho* (ρ), later identified with the mtDNA, was initially detected, and its role in the generation of respiratory enzymes was proven [24,25]. *Saccharomyces cerevisiae* is a facultative anaerobe yeast, able to grow on fermentable and non-fermentable (i.e., oxidative) carbon sources. This eukaryote has the peculiarity to survive on fermentable carbon sources in the absence of mtDNA, which makes it a major player in our understanding of the mitochondrial biogenesis. Mutations affecting mitochondrial functions are, in fact, easily identifiable in media containing an oxidative carbon source such as glycerol, ethanol, or lactate. More generally, the success of this experimental single cell organism is linked to its efficient homologous recombination properties, which allowed the creation of genetic knockouts collections [26], to the easiness of its manipulation [27,28] and to the high degree of similarity in cellular activities, including those related to mitochondria, with higher, and more complex, eukaryotes [29,30]. Remarkably, more than 50% among the 1000 protein species estimated in yeast mitochondria [31,32] have a human homolog [21], and 70% of the nuclear genes involved in human mitochondrial diseases are conserved in yeast [20]. Thanks to these features, *S. cerevisiae* has significantly contributed to the identification of the molecular basis of numerous mitochondrial diseases, as reviewed elsewhere [20,33–36]. The aim of this review was to summarise the advantages that the yeast model offers in the validation of mutations, in the determination of their heritability, in deepening the role of genes and of specific mutations in mitochondrial functionality, in the discovery of potential therapeutic molecules and to give a few examples of their application. In particular, this review intends to highlight the main advantages offered for the diagnostics of mitochondrial disorders caused by mutations in nuclear genes, whose list increases day by day due to the advent and application of NGS technologies.

2. Modelling Putative Disease Variants

Considering the huge number of novel genetic variants identified by NGS, a major challenge is to determine if these are the cause of the pathology of interest. Bioinformatic tools can certainly help but they are not sufficient to confirm the causality that must be biologically proven through functional analyses. The experimental workflow changes depending on whether the variants identified are novel and appear in a gene that has previously been associated with disease or in a gene not previously linked to disease and in this case, if the function of the gene is known or not. The workflow for the creation of the yeast models of mitochondrial diseases is represented in Figure 1.
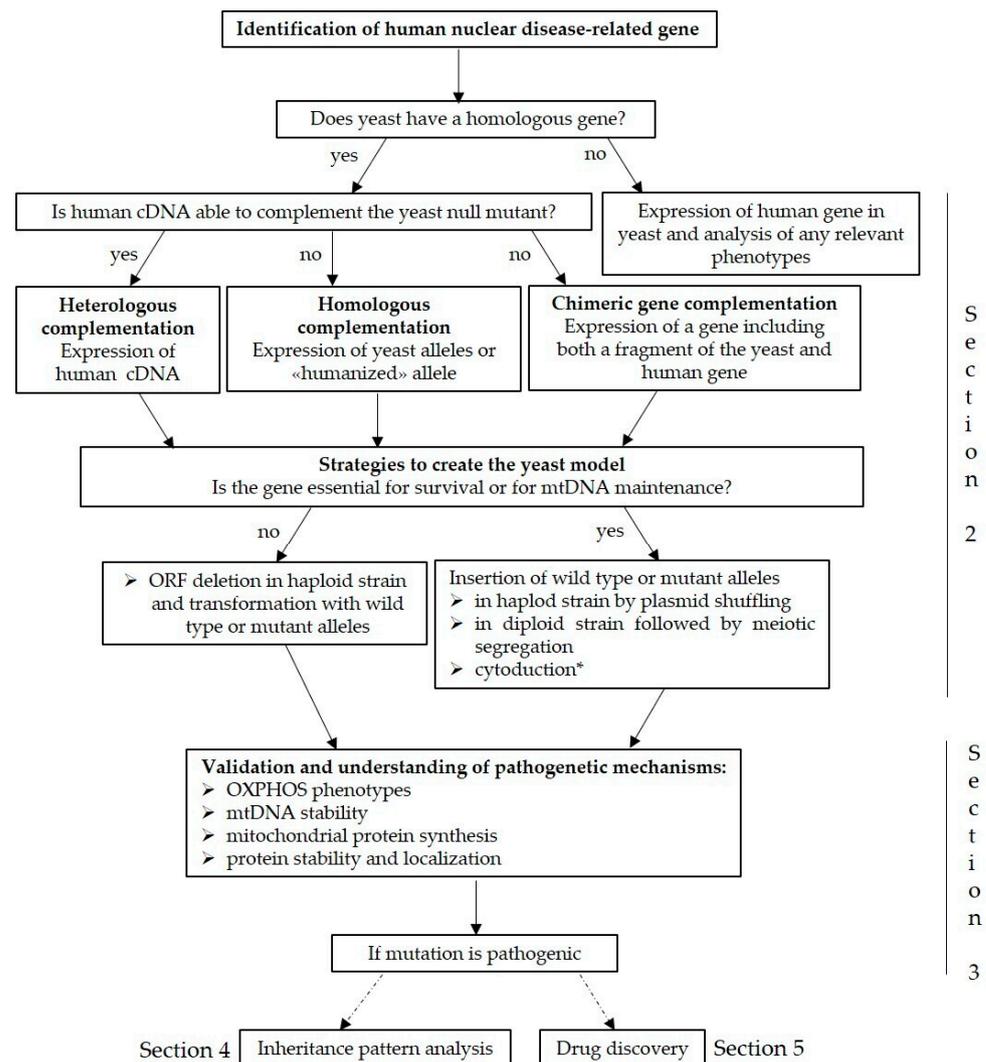
Figure 1. Workflow for the creation of the yeast models of mitochondrial diseases. * cytoduction is a strategy only used in case the gene is essential for mitochondrial DNA (mtDNA) maintenance. cDNA, complementary DNA; ORF, open reading frame; OXPHOS, oxidative phosphorylation.

For the creation of the model, the first question to ask is whether yeast has a gene orthologous to the human one. When a human disease-related gene is not present in the yeast genome, the human gene can be expressed in yeast under the control of a regulatable promoter to modulate the levels of the heterologous protein. The analysis of any relevant phenotypes associated to the expressed gene is then performed. Examples of "humanised" yeast models comprise neurodegenerative disorders in which the pathogenesis is associated to protein misfolding with the consequent formation of aggregates or oligomers. Disorders s such as Parkinson's, Huntington's and polyglutamine (poly(Q)) diseases have

been modelled in yeast and have been extensively documented in the review by Khurana and Lindquist [37].

When a homolog of the gene involved in the disease is present in the yeast genome, heterologous, homologous or chimeric gene complementation approaches can be used depending on the ability/inability of the human cDNA to complement the yeast null mutant strain. As reported in Table 1, to validate the pathogenicity of novel genetic variants, several yeast models of diseases have been constructed using the different approaches.

Table 1. List of genes linked to mitochondrial diseases categorised according to their primary role. This table includes nuclear genes associated to mitochondrial disorders in humans with an orthologous gene in *S. cerevisiae* * for whom the yeast model was reported in the literature to have been used to validate the pathogenic role of the variants associated with disease.

Function		Human/Yeast Gene	References *
OXPHOS subunits	CII	*SDHA/SDH1*	[38]
		SDHB/SDH2	[39,40]
		SDHD/SDH4	[41,42]
	CIV	*COX6B1/COX12*	[43]
	CV	*ATP5E/ATP15*	[44]
OXPHOS assembly factors	CII	*SDHAF1/SDH6*	[45]
	CIII	*BCS1L/BCS1*	[46–50]
		LYRM7/MZM1	[51,52]
	CIV	*COX10/COX10*	[53,54]
		SURF1/SHY1	[55,56]
	CV	*ATPAF2/ATP12*	[57,58]
Protein import and processing		*AFG3L2/AFG3*	[59–64]
		GFER/ERV1	[65,66]
		MIPEP/OCT1	[67]
		PITRM1/CYM1	[68,69]
		PMPCB/MAS1	[70]
		SPG7/YTA12	[60,62,63]
		TIMM50/TIM50	[71]
		TIMM8A/TIM8	[72,73]
mtDNA replication, transcription and maintenance		*MPV17/SYM1*	[74,75]
		POLG/MIP1	[76–90]
		POLR2A/RPB1	[91]
RNA maturation/modification		*ELAC2/TRZ1*	[92]
		GTPBP3/MSS1	[93]
		MRM2/MRM2	[94]
		MTO1/MTO1	[95,96]
		TRIT1/MOD5	[97]
		TRMT5/TRM5	[98]
		TRMU/MTO2	[99,100]
		TRNT1/CCA1	[101,102]

Table 1. *Cont.*

Function	Human/Yeast Gene	References *
Mitochondrial aminoacyl tRNA synthetases	*AARS2 / ALA1*	[103,104]
	GARS/GRS1	[105–107]
	GATB/PET112	[108]
	HARS2/HTS1	[109]
	KARS/MSK1	[110]
	LARS2/NAM2	[111]
	QRSL1/HERS2	[108]
	RARS2/MSR1	[112]
	TARS2/MST1-THS1	[113]
	VARS2/VAS1	[114,115]
	WARS2/MSW1	[116]
	YARS2/MSY1	[117–119]
Translation	*GFM1/MEF1*	[120]
	TUFM/TUF1	[120–122]
Membrane dynamics and composition	*DNM1L/DNM1*	[123–125]
	MFN2/FZO1	[126]
	OPA1/MGM1	[127,128]
	TAZ/TAZ1	[129–131]
	VPS13C/VPS13	[132–134]
	APOO/MIC26 paralog MIC27	[135]
	GDAP1/	[136]
	ACO2/ACO1	[137–140]
	IDH3A/IDH2	[141]
	MDH2/MDH1	[142]
	MECR/ETR1	[143]
	MPC1/MPC1	[144]
	PDHA1/PDA1	[145]
	PDHX/PDX1	[146]
	PPA2/PPA2	[147]
	SLC25A13/AGC1	[148]
	SLC25A3/PIC2-MIR1	[149,150]
Fe–S cluster biogenesis	*ABCB7/ATM1*	[151–153]
	FDXR/ARH1	[154]
	FXN/YFH1	[155–160]
	ISCU/ISU1 paralog ISU2	[161,162]
	LYRM4/ISD11	[163]
	NFU1/NFU1	[164]

Table 1. *Cont.*

Function		Human/Yeast Gene	References *
Enzyme co-factors		*COASY/CAB5*	[165,166]
		LIPT1/LIP3	[167]
		LIPT2/LIP2	[168]
		PANK2/CAB1	[169]
Metabolite transport		*SLC25A1/CTP1*	[170]
		SLC25A4/AAC2	[171–179]
		SLC25A19/TPC1	[180]
		SLC25A32/FLX1	[181]
Electron carriers	CoQ	*COA6/COA6*	[182,183]
		COQ2/COQ2	[184–187]
		COQ4/COQ4	[188,189]
		COQ5/COQ5	[190]
		COQ6/COQ6	[191,192]
		COQ8A/COQ8	[193]
		COQ8B/COQ8	[194]
		COQ9/COQ9	[195,196]
		PDSS1/COQ1	[187]
	Cytc	*CYCS/CYC1 paralog CYC7*	[197,198]
		HCCS/CYC3	[199–201]

In order to use the heterologous complementation approach, the human cDNA is inserted in a specific yeast expression vector, under the control of an appropriate promoter, and containing a yeast selectable marker.

An example of a heterologous complementation approach refers to the GRACILE syndrome-related gene *BCS1L*, which encodes a mitochondrial chaperone required for the correct assembly of complex III being necessary for the incorporation of the Rieske FeS protein Rip1 [46–48,50,202]. Another example of validation where human cDNA was directly used is that of *COASY* [165,166], a gene encoding for the mitochondrial bifunctional enzyme, coenzyme A synthase [203], whose mutations are associated with the development of a form of neurodegeneration with brain iron accumulation (NBIA), namely CoPAN (COASY protein-associated neurodegeneration) characterised by iron accumulation in the brain and the impairment of mitochondrial energy generation [165,204].

It must be underlined that using the human cDNA to evaluate the consequence of a mutation has the advantage that a direct demonstration of the role of the amino acid substitution is obtained. However, this approach could not be suitable if the complementation is fair, preventing further phenotypic analysis, or if the expression levels are not optimised.

When human cDNA is unable or unsatisfactorily to complement the yeast null mutant, the homologous complementation approach is performed. This approach is based on the fact that if an amino acid is conserved or semi-conserved, it should perform the same role in yeast and human protein, so that a detrimental effect in the yeast protein should replicate what happens in the human protein. At first, the conservation of the amino acid during the evolution from yeast to human is evaluated by the alignment of proteins. If the residue is conserved, it could be directly mutagenized, thus producing the "pathological" allele. When the mutation affects a non-conserved residue but the surrounding stretch is conserved, a general role of this region is suggested; in this case, it is possible to replace the yeast amino acid with the corresponding wild-type residue present

in human protein to serve as the "humanised" control. The yeast mutant allele and the humanised control are then expressed in a yeast strain deleted of the gene under analysis to evaluate the ability to complement the mutated phenotype. In this approach, the gene copy present in the yeast genome can be directly mutagenized, for example, through the "delitto perfetto" technique [205]. Alternatively, the wild-type and mutant genes can be cloned in a plasmid and inserted in the null mutant. An example of homologous complementation approach regards the disease-related gene *ISCU* which encodes a scaffold protein needed for the assembly of iron–sulfur (Fe–S) clusters and whose recessive mutations lead to myopathy or skeletal and cardiac myopathy [206–208] in human. The modelling of a heterozygous missense mutation in the corresponding yeast gene *ISU1* allowed to confirm both pathogenicity and dominance of the new variant [162] (see also paragraph 4). The same approach also allowed to validate the pathogenic role of a mutation in *COX6B* gene, encoding a subunit of complex IV, associated to severe infantile encephalomyopathy [43].

If the human cDNA does not complement the yeast deletion, and the amino acid under investigation is present in a region which is not conserved, a third strategy can be attempted, based on the construction of a chimeric gene which includes a fragment of the yeast gene and a fragment of the human cDNA. One of the causes of the lack of complementation of genes encoding for mitochondrial proteins is that the human mitochondrial targeting sequence (MTS) necessary for the import into the mitochondria is not recognised by the yeast import machinery, since the MTS sequences are partially different between mammals and yeast [209]. In this case, the chimera can be constructed by changing the region encoding the MTS of the human gene with its yeast counterpart, or with a generic yeast MTS. Such an approach has been used, for example, for constructing a model for studying mutations in *POLG*, encoding the mitochondrial DNA polymerase [89], or in *SPG7* and *AFG3L2*, which encode for two subunits of the human mitochondrial inner membrane m-AAA protease [63]. However, in some cases, it is necessary to replace other parts of the human protein with that of yeast to allow complementation, creating a true chimeric polypeptide. This approach has been used for studying mutations in *OPA1*, which encodes for a mitochondrial dynamin like GTPase involved in mitochondrial fusion [128,210] and in *ANT1*, which encodes for a mitochondrial ADP/ATP carrier [174,211].

When the deletion of the yeast gene leads not only to an OXPHOS phenotype but also to the lethality or to the irreversible loss of mtDNA, other strategies can be used to create a relevant model. The most used is the plasmid shuffling strategy [212]. To this end, the gene is disrupted in a strain containing a plasmid with the selectable marker *URA3* and a wild-type copy of the gene. This strain is then transformed with a plasmid harbouring a different selectable marker and expressing the mutant allele. The treatment with 5-fluoroorotic acid (5-FOA) allows the growth of only those strains expressing hypomorphic mutant alleles. This is the case of some mutations in *GFER*, which encodes for a disulphide relay system protein [65]. When the deletion is associated to defects or loss of mtDNA, the selection on 5-FOA will allow to obtain cells containing only the mutant allele and assess the pathogenic role of the mutant variant. This approach has been used to study pathogenic mutations in *MIP1*, the yeast ortholog of *POLG* [213]. The advantage of this technique is that it is rapid, and the wild-type strain and the mutant ones are isogenic. A second strategy that can be used is based on the insertion of the mutant allele in a heterozygous diploid strain and performing tetrad analysis after sporulation. This approach has been used to study the effects of mutations in *MEF1* and *TUF1*, which encode for mitochondrial translation elongation factors [120]. A third strategy that can be used when gene deletion is associated to mtDNA loss relies on cytoduction, i.e., the fusion of the cytoplasm of the mutant strain devoid of mtDNA with the cytoplasm of a second strain [214]. This technique has been used for finding *MIP1* mutant alleles which behave as antimutators of the mtDNA [215]. However, these two last techniques have some limitations, mainly the non-isogenicity and the request of haploid strains with complementary auxotrophies, respectively.

3. Validation of Mutations and Understanding of Pathogenetic Mechanisms

3.1. Analysis of OXPHOS Phenotypes

Once the model of the disease is obtained, the first, very quick way to validate (or not) an alleged pathological mutation is to test the ability of the mutant strain to grow in the presence of oxidative carbon sources such as glycerol, ethanol, acetate or lactate by a spot assay analysis. The growth of the mutant strain is then evaluated by comparison with the corresponding wild-type strain both at 28 °C, the optimal temperature for yeast growth, and at 37 °C, a temperature at which *S. cerevisiae* is still able to grow but often makes more easily evident the detrimental role of an amino acid substitution.

Alternatively to the spot assay, a growth curve can be obtained to precisely and quantitatively evaluate the mutant growth performance, allowing the identification of minor defects not detectable with a semi-quantitative test such as a spot assay. Irrespective of the used test, if the strain carrying the mutant allele shows a complete absence or a reduction in oxidative growth, it is possible to conclude that the mutation is detrimental, thus validating it as pathological. In the first case, the mutation is very severe; in the second case, the mutation is leaky and it is possible to conclude that in this case the protein function is partially maintained. In contrast, if the mutant strain does not show a reduction in oxidative growth, it is not possible to exclude a causative role for the mutation tested and a deeper analysis must be carried out. Alternatively or in addition to an oxidative growth test, oxygen consumption rate (OCR) can be measured on whole cells grown in non-repressing conditions in which mitochondrial respiration is active. This analysis also allows to determine the severity of the damage, which in most cases correlates with the phenotypic severity in humans.

Since one of the predominant roles of mitochondria is the production of energy through oxidative phosphorylation (OXPHOS), the enzymatic activity of the respiratory complexes and the rate of ATP synthesis could also be measured to compare mutants with the wild-type strains [216,217]. Additionally, mitochondrial membrane potential ($\Delta\Psi$m) and the production of reactive oxygen species (ROS) could be evaluated because they are intimately connected to OXPHOS [218,219]. Moreover, to understanding exactly what is affected in the mutant strain, it is possible to explore a variety of phenotypic and molecular defects. The specific analyses appropriate to perform depend on the specific function of the protein encoded by the gene under investigation, as, for example, iron content measurement, mitochondrial dynamics or mitophagy [162,220].

A particular case of validation refers to a mutation found in a gene of unknown function and previously not associated with mitochondrial pathologies. This is, for example, the case of gene *LOC644096*, now termed *SDHAF1*, whose mutations lead to infantile leukoencephalopathy. The biochemical analysis of mitochondrial respiratory chain complexes performed in muscle and fibroblasts have shown a specific reduction in SDH and SCoQR. Because the transfection of fibroblasts with the gene *LOC644096* was not suitable to examine whether the disease-segregating missense mutations of *SDHAF1* were indeed causing cII deficiency, *S. cerevisiae* was used as a model. The putative *SDHAF1* yeast ortholog, *YDR379c-a*, an uncharacterised ORF of 239 bp was disrupted, and the null mutant resulted OXPHOS incompetent because of a profound and specific reduction in SDH activity. This suggested that *YDR379c-a*, named *SDH6*, encoded a protein which was specific for complex II. When yeast mutant alleles carrying the equivalent human mutations were created and introduced into the *sdh6Δ* mutant, the transformant strains behaved like the null mutant indicating the pathological effect of the mutations [45].

3.2. Determination of mtDNA Stability

Like its human counterpart, yeast contains several copies of mtDNA molecules, from 10–50 to 50–200 copies per cell, depending on the carbon source, growth temperature and haploid/diploid status [221,222]. As in human mitochondria, several copies of yeast mtDNA are packaged into 10–40 protein–DNA complexes, called nucleoids. These are anchored to the mitochondrial inner membrane [223–225] and contain proteins involved

in packaging, replication, transcription, repair and recombination but also heat shock or Krebs cycle proteins [223,225–229].

One of the most peculiar characteristics of *S. cerevisiae* is *petite* positivity, i.e., it can survive without mtDNA. In this case, ATP is produced through alcoholic fermentation, provided that a fermentable carbon source is added in the medium, a condition resulting in colonies of small size called "*petite*". The "*petite* phenotype" can be caused by mutations in nuclear genes (pet mutants) [230], or directly by mtDNA mutations (cytoplasmic *petite* mutants) [24]. Cytoplasmic *petite* mutants, called "*petites*", arise spontaneously at high frequency even in a nuclear wild-type background (around 1–10% depending on the strain), and can be devoid of mtDNA (rho^0 cells) or carry long deletions of mtDNA (rho^- cells); in the latter case, the mtDNA often contains several tandem repeats of the same sequences [231]. Cells containing whole mtDNA are called rho^+. Rho^- mtDNA genomes are not very stable and may result in the loss of mtDNA, making the cell rho^0. Mutations in several nuclear genes involved in the replication, recombination and repair of the mtDNA, but also in its maintenance and integrity, can affect the *rho* status of the cells (reviewed in [232]).

When a mutation in a mtDNA molecule occurs, the cell is heteroplasmic. Contrary to what happens in mammals, heteroplasmy is just a transient condition in yeast, giving rise, in a few generations, to two homoplasmic populations of cells, each with only a kind of mtDNA genome [233–235]. The effects of nuclear mutation on mtDNA stability can be measured through the determination of the *petite* frequency, i.e., the ratio between the number of *petites* colonies and the number of total colonies. Although yeast cells are homoplasmic, and human cells are mainly heteroplasmic, a population of yeast cells recapitulates the heteroplasmic status of a single human cell. The higher the frequency of *petites* is, the higher the detrimental effect of the mutation is on the maintenance of the integrity and on stability of the mtDNA. It must be underlined that the *petite* frequency depends on two factors: an intrinsic factor, which depends on both the strain background, in particular the nuclear mutation under investigation, which influences the onset of *petites* per generation, and on the growth rate of rho^+ vs. *petite* cells, which is generally different depending on the strain; an extrinsic factor which depends on the growth conditions, such as the medium, carbon source, and temperature, which can influence both the onset of *petite* cells and the growth rates [232]. Then, it is critical that the comparison between mutant and wild type is performed in the same genetic background and in the same growth conditions. All the methods used to measure the *petite* frequency, described in [212], are based on a pre-growth in an oxidative carbon source to minimise the presence of *petites*. This is followed by growth in a medium supplemented with a fermentable carbon source, such as glucose, for several generations (at least 10–15), to allow the onset and the growth of *petite* cells. This analysis can be conducted on a cell population or on cells deriving from single colonies. The first method offers the advantage that the onset of *petites* occurs independently several times in several cells, resulting in a frequency that is rather constant, whereas in the second case, the *petite* frequency of each colony is highly variable since the number of *petites* is strongly influenced by the time of the onset of the first *petite* cell and thus the results must be analysed as in a fluctuation test based on the median.

Moreover, to discriminate between rho^- and rho^0 cells, and then to distinguish if a nuclear mutation results primarily in deletions or in depletion of mtDNA, three main methods can be applied [212]: (i) crossing a number of *petite* cells with different mit^- tester strains, harbouring a single point mutation in a mitochondrial gene encoding for a respiratory complex subunit; if at least one of the diploids obtained is respiratory proficient, it means that the tested cell retained a mtDNA fragment encompassing the mit^- mutation and then it was rho^-; (ii) analysis by the Southern Blot of the mtDNA extracted from *petite* colonies using an *ori* fragment as a probe; (iii) staining of mtDNA with DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride), which, in yeast, binds both the nuclear DNA and the mtDNA. By these techniques, it was shown that some mutations in *POLG/MIP1*

increase primarily the frequency of *rho*⁻ colonies, whereas others mostly increase the frequency of rho^0 colonies [77,78].

Some nuclear mutations also result in a decrease in the number of intact mtDNA molecules, i.e., the cells are respiratory proficient, but contain less mtDNA. The relative mtDNA levels can be measured through qPCR, amplifying a region of one of the mitochondrial protein genes as the target and a region of the nuclear DNA, such as *ACT1*, as the control [236]. By comparing the mtDNA/nuclear DNA ratio of a nuclear mutant strain and of the corresponding wild-type strain, it is possible to evaluate whether the mutation is associated to the depletion of mtDNA. An example of this approach is the study of the effects of polymorphisms/mutations in *POLG/MIP1* on the mtDNA [237].

Mitochondrial fusion and fission have a critical role in several aspects of the mitochondrial metabolism, among which are the replication and fidelity of the mtDNA [238,239]. Indeed, the absence of fusion results in the complete loss of mtDNA in yeast [240,241] and the partial loss of mtDNA in mammalian cells [242]. Mitochondrial fission prevents the clustering of nucleoids resulting in an unbalanced distribution of mtDNA copies within the mitochondria. Interesting enough, concomitantly inhibiting fission and fusion can suppress cellular dysfunction, including in yeast and in human cells [243]. Due to the evolutive conservation of genes also associated to fission and fusion, the consequences of human mutations have been evaluated in yeast. As an example, expression of a the *MGM1-OPA1* chimeric construct was used to model both dominant and recessive human pathological mutations in *OPA1*, associated to optic atrophy (DOA), to DOA⁺ and to pathologies associated to mtDNA depletion [127,128]. However, in mammalian cells, mitochondrial fusion, allowing mtDNA genomes with distinct mutations to complement each other, seems to ameliorate the detrimental effects of heteroplasmic mtDNA mutations and then the clinical severity of inherited mtDNA encephalomyopathies [242].

3.3. Analysis of Mitochondrial Protein Synthesis (MPS)

A correct OXPHOS metabolism may also depend on the correct gene expression and translation of the mtDNA. In particular, mutations in all the nuclear-encoded proteins involved in mitochondrial transcription, the processing and maturation of the RNAs, the aminoacylation of the tRNA and translation can result in defects of the MPS and, in turn, in defects of the OXPHOS system. In addition to specific analyses linked to specific genes under investigation, such as the determination of the maturation of the RNA, the presence of specific modifications in the tRNAs and the levels of aminoacylated tRNA, [100,103,244,245], the main analysis used to evaluate such defects is the measurement of the MPS.

The two main methods for measuring the MPS are based on an SDS-PAGE gel electrophoresis of proteins labelled in vivo or in organello [246]. In both cases, cells are grown in medium supplemented with a non-fermentable carbon source, if the strain is respiratory proficient, or in non-repressing conditions, such as with galactose, or with glucose at low concentrations until exhaustion. The presence/absence and the quantity of the eight mitochondrial-encoded proteins (in order, the mitochondrial subunit Var1, and the OXPHOS complexes subunits Cox1, Cox2, Cob, Cox3, Atp6, Atp8/Atp9) can thus be assessed and the comparison between the mutant strain and the corresponding wild-type allows the identification of those mutations which affect the protein synthesis. Mutations in some genes, such as those encoding for aminoacyl-tRNA synthetase, generally reduced the levels of all the mitochondrial proteins, since the whole protein synthesis is compromised. Mutations in other genes, on the contrary, mostly or specifically affect the synthesis of specific proteins. For example, mutations equivalent to the human ones in *MTO1*, which encodes for a subunit of the complex which catalyses the 5-carboxymethylaminomethyl modification of the wobble uridine base in mitochondrial tRNAs, affected specifically the synthesis of Cox1, Cox2 and Cob; on the contrary, mutations in *TRZ1*, which encodes for the tRNA 3′-end processing endonuclease tRNase Z, affected the synthesis of all the mitochondrial proteins, though at a different extent [92,95].

3.4. Evaluation of Protein Stability

Another aspect that can be evaluated in the yeast models of diseases is the effect of missense mutations on the stability/quantity of the protein. A reduction in the protein steady-state level could be the molecular cause of the disease or at least a major contributor. Unfortunately, only a few commercial antibodies are available to date that specifically recognise yeast mitochondrial proteins. To overcome this limitation, the addition of a polypeptide tag enables revealing the protein under analysis with an antibody against the tag sequence. However, it is necessary to exclude that the tag addition interferes with protein import and protein function [70].

To assess the functionality of the fusion protein, a complementation test to evaluate oxidative growth is performed comparing the phenotype of the strain expressing the tagged vs. untagged wild-type protein. The steady-state level of mutated proteins is then analysed by Western blot and immunodetection, directly on whole cell protein extract and using cytosolic or mitochondrial markers as the loading control. Interestingly, the overexposure of the signals could allow to evidence a degradation product pointing out that the mutant protein is unstable. Such a situation was, for example, observed by studying the human mutation R183Q in the pitrilysin metallopeptidase 1 encoded by *PITRM1* in yeast, taking advantage of the presence of the orthologous gene, *CYM1*. This autosomal recessive missense mutation, associated with protein instability, was found in two patients presenting a slowly progressive neurodegenerative disease characterised by mental retardation, spinocerebellar ataxia, cognitive decline and psychosis [68].

3.5. Analysis of Mutant Proteins Localisation

The pathogenic variant can also interfere with the correct protein localisation into mitochondria. To assess this possibility and take advantage of the available antibody or a tag antibody, Western blot analysis could be performed. This approach was used to analyse mutations in *MPV17*, associated to the hepatocerebral form of mitochondrial DNA depletion syndromes (MDDS) and Navajo neuro-hepatopathy, using the yeast orthologous gene *SYM1*. Both genes encode for a small protein localised to the inner mitochondrial membrane, whose function is not yet fully understood. The impact of seven pathological missense mutations, localised in different protein domains, on correct mitochondrial localisation was assessed demonstrating that the mutated residues do not compromise protein import [75].

By exploiting yeast strains expressing pathogenic variants, it is also possible to evaluate if they interfere with the ability of the mutated protein to be part of a complex. A relevant fraction of mitochondrial proteins is in fact localised in the inner mitochondrial membrane [70] and some of these are organised into complexes. This is, for example, the case for the proteins of the electron transport chain complexes, except for complex I that is not present in *S. cerevisiae*, where it is replaced by a non-proton-translocating NADH dehydrogenase activity performed by a single protein: Ndi1p [247]. Blue native polyacrylamide gel electrophoresis (BN-PAGE) technique, initially set up to study principally mitochondrial respiratory chain enzymes [248], can be used to analyse any protein complex [249] as respirasomes (supercomplexes derived by different stoichiometric aggregates of the respiratory complexes) [250]. For example, this technique was used to assess the effects of pathogenic mutations in the cIV assembly factor SURF1, Shy1 in yeast, demonstrating that pathological variants compromise the cIV assembly and the formation of the supercomplexes with the cytochrome bc1 complex [55,56]. With the same technique, it was demonstrated that Sym1, the equivalent of MPV17, takes part in a high molecular–weight complex of which the composition is still unknown [251]. Furthermore, the impact of seven *MPV17/SYM1* missense mutations was assessed showing that six of them compromised the formation of the fully assembled complex [75].

4. Inheritance Pattern Analysis: Dominance/Recessivity and Gene Interactions Analysis

A great advantage offered by yeast is the possibility to have information on the dominance/recessivity of mutations, which is not always easy to collect in patients, especially in sporadic cases with no familial history. The dominance/recessivity of a mutation can be established by comparing the phenotype of a diploid heterozygous strain harbouring the mutation under analysis with an isogenic diploid homozygous strain. If the phenotype of the heterozygous strain is similar to that of the homozygous strain, this means that the mutation is recessive, although it is not possible to discriminate whether the allele is null or hypomorphic and, in the latter case, the degree of hypomorphism. On the contrary, if the heterozygous strain shows a detrimental phenotype compared with the homozygous strain, it means that the human mutation is dominant, resulting in an antimorphic allele or in a neomorphic allele and causing a negative dominance or a gain-of-function dominance, respectively. However, the dominance can also be due to haploinsufficiency.

Several yeast genes, when disrupted in a single copy in a diploid strain, cause haploinsufficiency (www.yeastgenome.org, [252,253]). For genes encoding mitochondrial proteins, haploinsufficiency, if present, generally results in a decrease in respiratory growth, of respiratory activity and/or of mtDNA stability, i.e., the hemizygous shows a detrimental phenotype compared to the wild-type homozygous strain.

If haploinsufficiency occurs for the gene under investigation, more detailed information can be inferred by comparing the diploid heterozygous with both a wild-type diploid homozygous and the hemizygous strain. Depending on the heterozygous strain genetic background, the hemizygous can be a diploid deleted in one gene copy or a null strain transformed with two plasmids, one empty and the other harbouring the wild-type allele.

When the heterozygous strain shows a phenotype similar to that of the hemizygous one or intermediate between the homozygous strain and hemizygous one, it means that the allele is null or hypomorphic, respectively, and the mutation causes a dominant pathology due to haploinsufficiency. However, if in humans no haploinsufficiency is associated to the gene under analysis, the mutation will likely behave as recessive and the pathology occurs only when in homozygosis or in compound heterozygosis with a second mutation. When the phenotype of the heterozygous strain is more detrimental than that of the hemizygous strain, it suggests that the human mutation alone can be the cause of the pathology due to a negative dominance or to a gain-of-function dominance.

On the assumption that the hemizygous strain shows haploinsufficiency, when heterozygous and homozygous strains show similar phenotypes, the mutation is recessive; this means that the allele is hypomorphic and the human mutation is pathological in compound with a second mutation or is a phenotypic modifier.

An example of this genetic analysis is the study on mutations in *MIP1*, the ortholog of the human *POLG* (reviewed in [213]). Some mutations in the polymerase domain abolish the maintenance of the mtDNA in the haploid strain, whereas the heterozygous strain shows an increase in the *petite* frequency compared to the hemizygous strain: these mutations cause dominant pathologies in patients. Instead, other mutations strongly increase the *petite* frequency or make the haploid strain *rho*^{0}, whereas the behaviour of the heterozygous is intermediate or similar to that of the hemizygous strain. Considering that *POLG* does not show haploinsufficiency in humans, these mutations are typically recessive and are found in homozygosis or in compound heterozygosis in patients. Regarding *MIP1*, it should also be noted that some mutations have a slight effect in the haploid background and any effect when compared with heterozygous and wild-type homozygous strains. These mutations are typically polymorphisms which behave as phenotypic modifiers worsening the effects of the pathological mutations in compounds. Another example of dominance/recessive analysis concerns the gene *ISCU*, whose model has been shown in paragraph 2, and whose recessive mutations have been associated to diseases in humans. The analysis performed in yeast allowed the identification of the first dominant mutation, as highlighted by the fact that the heterozygous strain was associated with a respiratory deficient phenotype [162].

Yeast can also be useful when more mutations are present in the patients, in order to understand which mutations are responsible for the pathology and if an additive/synergistic effect occurs, suggesting a functional interaction of the mutated amino acids. Indeed, some patients show two mutations in cis and/or in trans. This is rather common for genes in which several SNPs/mutations are present in the population, especially those which encodes for long mRNA such as *OPA1* (https://databases.lovd.nl/shared/genes/OPA1 (accessed on 18 February 2021)) [254] or POLG (https://tools.niehs.nih.gov/polg/ (accessed on 18 February 2021)) [255], in which more than 250 pathological variants have been identified. If two mutations are present in cis, the role of each mutation can be dissected in yeast by introducing the single mutation in the gene under analysis. In this case, three mutant alleles are constructed: an allele containing one of the two mutations, an allele with the other mutation and the double mutant allele. By comparing the phenotype of each haploid strain harbouring these mutant alleles with the wild-type haploid strain, four main cases are possible. First, both single mutant alleles are neutral, but the double mutant allele is associated to a detrimental phenotype: in this case the mutations are not pathological alone, but just in compound, suggesting a functional interaction. Second, a single mutant allele is associated to a detrimental phenotype and the double mutant allele has the same behaviour: one mutation is pathological whereas the other is a neutral SNP. Third, a single mutant allele is associated to a detrimental phenotype, and the double mutant allele leads to a worse phenotype: one mutation is pathological whereas the other is a phenotypic modifier which can influence the phenotype of the pathological mutation. Fourth, both single mutant alleles are associated to a detrimental phenotype, and in the double mutant, the phenotypic effects are additive or synergistic: both mutations are pathological alone, and when in compound, negatively affect the effect of the other one. Examples of such analyses, with different outcomes, have been performed for *MIP1* and *DNM1*, which encodes for a dynamin-related GTPase involved in mitochondrial organisation [78,124].

If two mutations are present in trans, suggestive of a recessive pathology, the analysis to distinguish the role of each mutation can be performed by comparing the heterozygous diploid strain harbouring both mutant alleles with the wild-type homozygous strain, and with two heterozygous strains each harbouring a single mutant allele and a wild-type allele, and with two homozygous mutants each harbouring one of the two mutations in both alleles. If the mutations were both recessive, it is expected that the compound heterozygous strain has a detrimental phenotype compared to the homozygous wild type as well as to the single heterozygous ones. However, other information can be inferred by the comparison with both the single heterozygous and the homozygous mutant strains. For example, thank to this comparison, we demonstrated that a mutation in *MIP1* found in compound heterozygosis with a second mutation was dominant, and acted synergistically with a second, recessive mutation in trans [78].

5. Yeast as A Model for Mitochondrial Diseases Drug Discovery

To date, no effective treatments exist for mitochondrial diseases [256]. In recent years, phenotype-based screenings have been proposed in yeast models to find drugs able to suppress OXPHOS phenotypes associated with mitochondrial diseases mutations.

High throughput drug-screening (HTS) was performed in the case of Friedreich's ataxia (FRDA), caused by mutations in the nuclear gene *FXN* that encodes the highly conserved frataxin, a chaperone for iron-sulphur cluster (ISC) assembly in the mitochondrial matrix [257]. More than 100,000 compounds were screened for their ability to improve mitochondrial functions in yeast lacking the expression of *YFH1* gene, the functional orthologous of *FXN*. The rescue was recorded by a colorimetric assay, quantitatively monitoring cell metabolic activity on respiratory substrates [258].

Alternatively, a phenotype-based screening, named "drug drop test" [20,259] has been developed, in which yeast mutants, defective in oxidative growth due to the alteration of mitochondrial functionality, are initially spread on a solid medium. The mutants are then exposed to compounds from chemical libraries, spotted on small sterile filters placed on

the agar surface. The appearance of a halo of enhanced growth around a filter indicates the effect of the corresponding drug. The strength of this method is that the diffusion of the molecule around the filter in the agar medium creates a concentration gradient, making it unnecessary to find the optimal drug concentration by testing different dilution of the compound. The screen allows to rapidly analyse chemical libraries, like those containing FDA-approved drugs (i.e., Prestwick or Selleck), in a repurposing approach. The drug drop test was used for the first time to identify drugs active in the yeast model for ATP synthase disorders, associated with neurodegenerative syndromes including neuropathy, ataxia and retinitis pigmentosa (NARP) [259]. Active compounds were then confirmed as effective in cybrid-based model of NARP.

Phenotype-based screenings enable the identification of potential therapeutic compounds in the absence of validated drug targets and independently on the knowledge of their mechanism of action. Yeast offers additional experimental tools, like the collection of homozygous and heterozygous deletant mutants, by which chemical–genomic experiments can be performed obtain an indication on the molecular mechanism of active drugs. In the haploinsufficiency profile (HIP) approach, the reduced fitness of a heterozygous deletion mutant to the inhibitory concentration of a drug indicates that the deleted gene is the molecular target of the drug [260]. By a chemical genomic analysis of haploinsufficiency, heterozygous Tim17 or Tim23, the components of the translocase inner mitochondrial membrane involved in mitochondrial protein import, displayed sensitivity to sodium pyrithione (NaPT) [261]. In vitro experiments indicated that NaPT specifically influences the import of pre-sequence proteins via Tim23 complex and indicates the machinery of the mitochondrial import as a potential target for a therapeutic approach.

By the drug drop test, two molecules, the antibiotic pentamidine and clarithromycin, have been found to actively restore oxidative growth and OXPHOS phenotypes due to mutations in the yeast *BCS1* gene, the orthologue of the human *BCS1L* [262]. It has been demonstrated that pentamidine and clarithromycin target mitochondrial rRNA, thus altering the synthesis of mitochondrial encoded OXPHOS subunits, with the consequent alteration of the OXPHOS complex assembly. However, the two antibiotics were able to rescue the respiratory phenotypes of *bcs1* mutants carrying missense mutations, that not completely compromise the Bcs1 activity, but did not restore phenotypes due to point mutations or deletion that fully abolish Bcs1 function. In vivo experiments performed in yeast also allowed to propose a model of action of the two drugs.

Very recently, taking advantage of the impaired oxidative growth of a strain carrying a mutation in the *CAB1* gene, the ortholog of the human *PANK2* that encodes the panthotenate kinase (PANK), a screening of the Selleck chemical library has been performed. Two molecules in particular, nalidixic acid and 5,7 dichloro-8-hydroxyquinoline, were found to be able to restore the multiple defects associated with PANK deficiency, with the rescue not being allele-specific [169].

Yeast-based screenings have also been used to determine therapeutic strategies against mitochondrial diseases affecting mtDNA stability. This is the case of DOA, and of the more severe form named DOA-plus, the most common mitochondrial optic neuropathies, characterised by the gradual loss of vision as a result of the degeneration of the optic nerve cells. These pathologies are mainly caused by mutations in the nuclear gene *OPA1*, encoding a mitochondrial GTPase implicated in mtDNA maintenance, [263–265] whose functional orthologue in yeast is *MGM1* gene [266]. Drugs were first selected as able to restore the thermal sensitive (ts) growth on respiratory substrates of a yeast mutant harboring $mgm1^{I322M}$ mutation. This mutation is equivalent to I382M mutation in *OPA1* [127], one of the few pathological mutations that can be modelled in yeast, due to the low similarity between *MGM1* and *OPA1* sequences. Positive hits were then subject to a subsequent screening, using a strain harbouring the ts $chim3^{S646L}$ mutation in the *MGM1/OPA1* chimeric allele, encoding the N-terminal region of Mgm1 and the whole GTPase, middle and GED domains of OPA1 [128] (see paragraph 2) identifying six effective drugs. Five of them also ameliorated, to a different extent, the pathological OXPHOS phenotypes of *Opa1* null

mouse embryo fibroblasts (MEFs), that express the human OPA1 isoform 1, bearing R445H and D603H mutations, associated with DOA-plus and DOA, respectively. The analysis in patient's fibroblasts bearing the same mutations allowed to identify the tolfenamic acid, a non-steroidal anti-inflammatory drug, as the most promising therapeutic compound and to propose the repurposing of this drug in a clinical trial for neurodegenerative diseases associated with *OPA1* mutations [267].

Therapeutic molecules were also found via yeast-based screening for a broad spectrum of mitochondrial pathologies, including Alper syndrome, ataxia neuropathy, dominant and recessive progressive external opthalmoplegia (arPEO and adPEO), characterised by mtDNA deletions or depletion consequent to the mutation in the mtDNA polymerase POLG [255].

Two chemical libraries of FDA-approved molecules were screened, taking advantage of one of these *mip1* mutant (G651S equivalent to POLG G848S) whose ts mutation conferred an evident but not irreversible phenotype. The clofilim tosylate (CLO), belonging to a class of anti-arrhythmic agents, displayed the best rescuing activity, suppressing the respiratory growth defect and preventing mtDNA loss in all *mip1* mutants tested [268]. The rescuing effect of CLO was later validated in two animal disease models, *C. elegans* and zebrafish [269], as well as in the fibroblasts of a patient carrying compound heterozygous *POLG* mutations. The molecular mechanism by which CLO exerts the rescuing activity is not yet known, however, the successful application in this four-model approach indicates that CLO is acting by a rescuing mechanism conserved through the evolution.

6. Conclusions

Early studies and more recently, NGS techniques, have made possible the identification of a huge number of novel human genetic variants whose causality in determining mitochondrial syndromes was demonstrated through functional analyses, often in model systems. Because of the evolutionary conservation of genes and systems, the study of human genetic defects associated with mitochondrial dysfunction has often been directly addressed in the model organism *Saccharomyces cerevisiae,* the molecular and genetic workhorse for much of our understanding of mitochondrial biogenesis in eukaryotes. As shown in Table 1, *S. cerevisiae* helped to resolve the cause of OXPHOS diseases for about a third of the disease genes known today. It can also be noted that the contribution to the identification of causative genes-concerned variants associated to genes with a wide range of mitochondrial functions, from those that have a specific role in OXPHOS biogenesis, as defects in respiratory complexes or in DNA maintenance and expression, to those that have a secondary impact on OXPHOS caused by deficiency in protein import and processing, metabolite and electron transport, membrane dynamics and composition, TCA cycle and metabolism, Fe–S cluster biogenesis and protein quality control.

Today, one of the major challenges in the field of mitochondrial diseases is the identification of the genetic basis of the conditions of patients lacking a diagnosis, generally because of the clinical and genetic heterogeneity of these pathologies. Certainly, international collaboration within the mitochondrial disease field will improve the identification of additional cases with similar clinical phenotypes and above all, of new disease genes. Model systems will therefore continue to prove being fundamental in proving the pathogenicity of the variants and in improving our understanding of the role played by different genes. In this context, yeast remains a powerful model for discovering the function of mitochondrial proteins, especially for those yet to be characterised. In fact, it should be remembered that many proteins present in the mitochondrial proteome still lack any detailed characterisation. Furthermore, another aspect still minimally considered is the indirect effect of cytoplasmic proteins on mitochondrial biogenesis. A screening recently done in yeast, on the negative effect of heat stress on respiratory capacity, allowed to expand the repertoire of genes affecting mitochondrial function, allowing the identification of 105 new genes and novel pathways, whose corresponding proteins are predominantly present in the cytoplasmic proteome [270].

Another major challenge relative to the mitochondrial disease is their treatment. Their heterogeneity, the intrinsic variability of mitochondrial genetics, the fact that they directly or indirectly affect several organs via ATP production, and the impossibility for many drugs to reach the brain, which is often affected in mitochondrial disorders, represent the main problems in the development of effective therapies [256]. A radical cure for these diseases will probably only come from gene therapy. However while "tailored", personalised therapeutic approaches, such as gene therapy, cell therapy and organ replacement can be useful for individual conditions [256], their costs cannot be easily supported by health authorities, given the large number of patients affected by mitochondrial diseases. For this reason, the identification of therapeutic molecules effective on a broader spectrum of mitochondrial pathologies remains an important objective to alleviate, if not eliminate, the defects responsible for the diseases. In this context, given the long times required for drug development and the numerous and different targets of drugs, repurposing available therapeutic molecules remains an interesting way forward. Even from this point of view, yeast disease models have demonstrated to be useful, thanks to the techniques here reported, allowing to quickly analyse a large number of molecules. Some of these compounds were found to be effective also in the corresponding animal models and in patients fibroblasts, despite the more complex genetic interactions present in animals, including humans, than in yeast. The numerous disease models available in yeast can also be explored to identify beneficial broad-spectrum molecules.

In addition to the great advantages of the yeast model, it should be noted that some limitations exist: (i) yeast cannot be used to model a disease at the scale of an organ or an intact complex multicellular organism; (ii) yeast does not allow to assess tissue specificity and disease progression; (iii) some functions fulfilled by human mitochondria do not exist in *S. cerevisiae* such as the respiratory complex I which, however, is present in the yeast *Yarrowia lipolytica*, used to model mutations related to this complex [271]. Moreover, cell division and mitochondrial replication in human development could lead to much greater variation in the relative levels of the mtDNA mutation in a largely stochastic system [23]. This could induce compensatory biogenesis mechanisms to maintain the cell's mitochondrial function, an effect not observable in yeast where the heteroplasmic condition is rapidly lost. Despite these limitations, mitochondrial function conservation between yeast and humans renders yeast a key model for mitochondrial medicine.

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