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Review

Pathological Mutations of the Mitochondrial Human Genome: the Instrumental Role of the Yeast *S. cerevisiae*

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Abstract: Mitochondrial diseases, which altogether represent not so rare diseases, can be due to mutations either in the nuclear or mitochondrial genomes. Several model organisms or cell lines are usually employed to understand the mechanisms underlying diseases, yeast being one of them. However, in the case of mutations within the mitochondrial genome, yeast is a major model because it is a facultative aerobe and its mitochondrial genome can be genetically engineered and reintroduced *in vivo*. In this short review, I will describe how these properties can be exploited to mimic mitochondrial pathogenic mutations, as well as their limits. In particular; pathological mutations of tRNA, cytb, and ATPase genes have been successfully modeled. It is essential to stress that what has been discovered with yeast (molecular mechanisms underlying the diseases, nuclear correcting genes, import of tRNA into mitochondria or compounds from drug screening) has been successfully transferred to human patient lines, paving the way for future therapies.

Keywords: mitochondrial diseases; mitochondrial transformation; tRNA import; tRNA mutants; ATPase mutants; correcting genes; drug screening; mitochondrial therapies

1. Introduction

1.1. Mitochondrial Functions and Dysfunctions

Mitochondria are the vital center of cell function. In addition to their primary function, *i.e.*, as the power plant of the cell and controler of the cell redox balance, mitochondria are involved in many essentiel cellular processes. They mediate apoptosis via the permeability transition pore, are involved in many catabolic and anabolic pathways such as beta-oxidation, heme biosynthesis, steroidogenesis,

amino acid metabolism, assembly of Fe/S clusters and gluconeogenesis. They also control stress responses, in particular the oxidative stress response via reactive oxygen species (ROS) and calcium Ca^{2+} storage (rewiewed in [1] and references therein). They play a central role in aging [2] and are subject to mitophagy, the pathway that mediates the selective elimination of dysfunctional or unwanted mitochondria and is critical to proper cellular functions [3].

Without a proper mitochondrial structure, the cell is not viable, and mitochondrial dysfunctions underly many types of human diseases, even though the causal relation between mitochondrial functions and these multifactorial diseases is still open to debate [4].

Mitochondria are associated with diseases such as diabetes [5], cardiac [6] and vascular [7] diseases, cancer [8,9], and also neurodegenerative diseases. A disorder of endoplasmatic reticulum (ER)-mitochondrial communication is now suspected to be the basis of Alzheimer's disease [10]. Junctions between the ER and mitochondria are indeed essential to the exchange of metabolites such as lipids and Ca^{2+} and play a role in mitochondrial dynamics [11]. Mitochondria are also highly suspected to be involved in Parkinson's [12] and Huntington's diseases [13].

Because of these numerous pathways and interconnected cellular functions, many genes are involved, and mutations in these genes may lead to the various diseases mentioned. Almost all these genes are encoded by the nuclear genome, and only a few, coding for some components of the respiratory chain, tRNAs and rRNAs, are encoded by the mitochondrial genome. In humans, this amounts to a total of 37 genes [14]. Despite the very small number of genes involved, pathologies due to mitochondrially-encoded mutations cannot be considered as rare diseases since they are found in approximately one in 200 individuals, with clinical expression in 1:5000 to 1:10,000 individuals [15,16].

In this short review, I will focus only on mitochondrially-encoded mutations since they exhibit very peculiar properties compared to nuclearly-encoded mutations. Reviews on mitochondrial diseases in general can be found in [17] for historical aspects, [18] for bioenergetics, [19] for nucleo-mitochondrial intergenomic cross-talk as well as in [20] and [21] for the role played by yeast.

1.2. The Particular Properties of Mitochondrially-Encoded Mutations

The mitochondrial system presents some specificities which are briefly summarized below: (i) The mutation rate of mitochondrial DNA is significantly higher than that of nuclear DNA; (ii) the cell contains many mtDNA molecules, and mtDNA does not follow Mendelian rules of inheritance but is maternally inherited. In the case of deletions and many mtDNA point mutations, a mixture of wild-type and mutated molecules co-exist in the cell, a situation which is called heteroplasmy. Their respective proportions may vary in the progeny and in different tissues, the symptoms appearing only when a high proportion of mutated molecules is reached, called the "threshold"; (iii) since some tissues (heart, brain and muscle, for example) are more energy-dependent than others, one expects more deleterious effects in these tissues; (iv) the relation between genotype and phenotype is not straightforward, with one mutation leading to different syndromes in different individuals (even within the same family) and the same syndrome being due to different mutations in different genes; (v) since the nuclear and mitochondrial genomes interact genetically for mitochondrial function, the variation of nuclear background among individuals must also be considered. More details on these caracteristics can be found in [16] and references therein.

This complexity is far from being understood. Mitochondrial involvement in cell programs which control oogenesis and differentiation, as well as the basic mechanisms which distribute mtDNA molecules to cell progeny such as the bootleneck that takes place during oogenesis, remain to be uncovered (reviewed in [22]). These caracteristics do not facilitate genetic counseling or the quite frequent establishment of a mutation as being mitochondrial nor do they cause a disease.

2. Choice of Suitable Models to Study Mitochondrial Diseases

Because of the complexity of the human organism and the difficulties associated with direct experimentation, it is necessary to make studies and analyses in model organisms. As will be seen, there is no perfect model system.

2.1. Mammalian Systems

Mammals come to mind as the first choice. Mouse models seem well adapted since they are relatively simple mammals, which can be easily manipulated by many genetic and molecular biology techniques, although they are labor-intensive. They offer several advantages because mice and humans display quite similar gene contents, comparable types of internal organs and physiological processes.

However, for a long time, establishment of mice with pathogenic mutant mtDNA was impeded by the impossibility to reintroduce an *in vitro* engineered form of a mitochondrial gene into mitochondria. Progress has been made with the mutator mouse which carries a form of gamma DNA polymerase devoid of proofreading [23], a strategy based on the detailed analysis of the yeast mitochondrial polymerase [24,25]. These mice generate mitochondrial genome mutations at high frequency, among which the desired mutations can be searched for, if they exist. Alternatively, the impressive mito-mice system (reintroduction of mitochondria with a somatic mtDNA mutation into mouse zygotes [26]) permits precise mutations to be created, but is still a long and costly procedure. In addition, one should add that there is no guarantee that the resulting mice will reflect the human phenotype [23,27–29]. The precise reasons for such situations are unclear but the prime suspect might well be the nuclear background, a parameter, which reappears in different aspects of these diseases (see [30,31] and Section 3). Several reviews cover these important points in more detail [32–35].

An alternative mammalian system such as patient cell lines, is commonly used, but cell lines which are immortalized are not physiological and do not reproduce the characteristics of differentiated cells in the body. Even primary cell lines from patients, usually derived from fibroblasts or myoblasts, which are not necessarily the affected tissues, do not retain all characteristics [36]. Analyzing the same mutation, discrepancies appear among results obtained in different cell lines (see for example the case of the A3243G mutation of tRNALeu(UUC) [37,38]).

Cybrids, which fuse an enucleated cell carrying a suspected pathological mitochondrial mutation with cells lacking mitochondrial DNA, suffer from the same limitations as cultured cells in general. However, they have been essential for the process of uncovering whether the mitochondrial genome is responsible for a given mitochondrial disease and for the study of its effects [39].

2.2. Non Mammalian Organisms

Several non-mammalian organisms have also been exploited. The interest in studying mitochondrial functions in the worm *C. elegans*, which is easy to manipulate and has a very simple RNAi gene knock-down protocol by feeding, has considerably increased in the past years. Mitochondrial dysfunction confers phenotypes related to longevity, sterility, developmental larval arrest in L3 or ethidium bromide sensitivity ([40–43] reviewed in [44]). A mitochondrial deletion exists which has been used to study heteroplasmy [45], and it has been shown that, while the proportion of heteroplasmy is variable from one animal to another, the proportion of heteroplasmy in the whole population is constant (60%) during a hundred generations. The molecular mechanism is not known, and this study only reflects what has been found with a specific deletion, isolated by chance. The situation is quite similar in the fly *D. melanogaster*. As a genetic model system it offers many advantages, and has been widely used, in particular to study neurodegenerative disorders. Mutants in nuclear-encoded mitochondrial genes (ribosomal proteins, some respiratory complex components) have been identified as "bang"-sensitive or deficient in eye-development (reviewed in [44]). One interesting mitochondrial-encoded mutation in the ATP6 subunit has, however, been identified as a suppressor of the SesB mutant (localized in the mitochondrial adenine nucleotide translocator or ANT) and mimics pathological features seen in Leigh syndrome [46]. No specific mitochondrial DNA mutations have been described otherwise, except for a large-scale deletion in the mitochondrial DNA of the fly *D. subobscura*, which was obtained by chance. As was the case for the mitochondrial deletion of the nematode, this discovery helped to analyze heteroplasmy and gene expression, but was not direcly relevant to known human mitochondrial pathologies [47].

2.3. Yeast, Saccharomyces Cerevisiae

2.3.1. General Properties

Yeast (unless mentioned otherwise, this means the budding yeast *Saccharomyces cerevisiae*) has been extensively used for understanding human diseases. As a simple and unicellular microrganism, it may seem quite different from humans and their diseases, but several important properties have made yeast a pivotal organism for many human studies and, as will be seen in Section 2.3.2, in particular for mitochondrial diseases:

(i) Simplicity, rapidity and low cost.

(ii) Highly developed genetics with a diverse and well-adapted toolbox. Thousands of mutations exist in practically every nuclear gene of the genome, and any new mutation can be created *in vitro* and reintroduced into the genome thanks to the rich collection of tools, which has been developed.

(iii) A wealth of functional studies organized into an efficient database. When the complete genome was published [48], we had some functional knowledge of about 30% of the roughly 5800 genes; the figure now reaches practically 85%. All information obtained is centralized in the Saccharomyces Genome Database or SGD, which is easy to use and kept up to date [49].

(iv) Protein sequence conservation and heterologous complementation. All the properties I have described can be usefully exploited because bioinformatic analyses have revealed a high

conservation thoughout eucaryotic evolution for many genes involved in basic cellular functions. As a consequence, we have gone a long way since the pioneering work of [50] on heterologous complementation of a yeast (*S. pombe*) mutation by a human gene. Recent orthology studies now show that nearly 1000 yeast genes are associated with human diseases [51], and in many cases the mammalian ortholog is functional and complements the yeast deletion mutant.

(v) The "omics" approaches. Not only has the thorough annotation of the *S. cerevisiae* genome, performed by the yeast biologists themselves, served as basis for annotation of many other organisms, but the global approaches which have been developped to study regulatory networks, protein and gene interaction networks have served as pilots for applications to other organisms. This was also the case for computational analyses and the development of systems biology.

More information can be obtained from two reviews, both by Botstein and Fink [52,53], written 23 years apart. They put the possible use of yeast as a reference experimental organism in perspective. This time lapse is large enough to follow the impressive evolution of knowledge, concepts and techniques developped in yeast, "an experimental organism for the 21st century biology" [53].

2.3.2. Why Is Yeast Particularly Well Suited for Mitochondrial Studies?

There are several specific properties which make this organism invaluable for mitochondrial research: (i) *S. cerevisiae* is a facultative anaerobe and can grow by fermentation when devoid of any mitochondrial genetic information; this means that any mutation which affects mitochondrial function and would be essential in a strict aerobe can be maintained and analyzed on fermentable medium (usually glucose). (ii) There is a very strong conservation, from yeast to humans, of the genes encoded within the mitochondrial genome, *i.e.*, components of the respiratory chain, tRNA and rRNA genes. There is only one exception, which is the absence of complex I. (iii) It is the only organism, together with the algae *C. reinhardii*, in which mitochondria can be transformed. This means that all techniques relative to reverse genetics can also be applied to the mitochondrial genome; mutations can be created *in vitro* and reintroduced at will *in vivo*, at their proper site, in the mitochondrial DNA. Originally described by two groups [54,55], the biolistic transformation (shooting microprojectiles layered with recombinant mitochondrial DNA at high velocity into yeast cells) has been efficiently developed to the point of now being a routine technique [56]. This approach is greatly facilitated by the very high homologous recombination frequency of the mitochondrial genome, which allows efficient integration. With this technique, many kinds of modification can be introduced and, in particular, mutations which precisely mimic the human pathological mutations (Section 3). (iv) Finally, we should mention that in contrast to what is observed in other organisms, *S. cerevisiae* is homoplasmic, which means that in a mutated cell, all mtDNA molecules possess the same mutation; the phenotype is therefore independent of any threshold effect, and the presence or absence of respiratory growth defects in cells carrying a presumed pathological mutation provides strong evidence for the classification as pathological or not in humans.

3. Mimicking Mitochondrial Pathogenic Mutations in Yeast Mitochondrial Genome

Human mutations found in patients have been reported in all genes (producing proteins or RNA) encoded by the mitochondrial genome, and some of them have been confirmed to be causative of the disease; more information can be found in MITOMAP [57].

The biolistic transformation has a low yield since it kills most of the cells, which means that positive selection has to be applied. This is done in two steps: The first one selects the surviving cells and the second the mitochondrial transformants among them (usually around one in 1,000 survivors). The first pathological mutations were created in the COXI and COX3 mitochondrial genes (coding for Subunit I and Subunit III of cytochrome oxydase, respectively). The I280T and M273T COX1 mutations have been observed in hematopoeitic cells from patients suffering from acquired idopathic sideroblastic anemia [58], but their biochemical effect is unclear. In yeast, these mutations caused similar effects to those reported in humans (mild effects on respiration) and the biochemical study deepened the analysis and allowed to propose a biochemical hypothesis. A short deletion in COX3 was also designed in yeast and had as strong an effect as in humans [59].

Several CYT b mutations have also been created with biolistic transformation. Many of them were focused on modeling regions of the mammalian Qo site in yeast cyt b or on further understanding the differential efficacy of Qo site inhibitors on mammalian and pathogen bc1 complex [60–64]. However, pathological human mutation equivalents were also constructed and analyzed ([65–68] and references therein). In humans, their biochemical properties have not been seriously investigated and their effects have mostly been deduced from what is know of the enzyme structural properties. Interestingly, a detailed comparative analysis of the molecular effects of the G15699C mutation (Lys319Pro in yeast) has been reported [65]. In humans, it showed a dramatic loss of steady state levels of Complex III and a strong decrease in enzyme activity. In yeast, it showed that the mutation hindered the assembly of the complex but that the assembled complex was stable. The catalytic activity of the enzyme was decreased (as in humans) and a lower Km for quinol substrates, and thus a slower rate of electron transfer from the Q0 site, was observed.

Complex I does not exist in *S. cerevisiae* and therefore cannot be manipulated the same way despite the fact that there are several well established pathological mutations in the mitochondrial subunits. Since the green alga *Chlamydomonas reinhardii*, which possesses a Complex I rather similar to the human one, is the other organism for which mitochondrial transformation is possible, it should be possible to develop it into a model system for Complex I. A recent attempt has been made in this direction with one pathological ND4 mutation (L158P) introduced into the mtDNA of the algae [69]. This mutation has an impact on Complex I activity and leads to a clear *in vivo* phenotype, as respiration in the dark is impaired.

The work done on ATPase subunit 6 and tRNAs (which represent in fact quite a high proportion of the pathological mutations [70]) is more specifically focused on mitochondrial diseases.

Five pathological mitochondrial mutations in the ATPase 6 subunit have been mimicked in yeast. T8993G [71], T8993C [72], T9176G [73] and T9176C [74] have been found in patients presenting a NARP (neuropathy ataxia retinitis pigmentosa) or MILS (maternally-inherited Leigh's Syndrome) syndromes. These mutations, in conserved regions of subunit *a*, all impair the ATP synthase. Examination of the same mutations in yeast has shown that these mutations have a similar impact on

the ATPase as they have on the human ones, *i.e.*, a decrease in mitochondrial ATP synthesis rate, confirming yeast to be a useful model for molecular and biochemical studies. In addition, because the biochemical studies have been thoroughly performed in yeast, some new information, not yet obtained in humans, has been uncovered (review in [75]). For exemple, while in humans the T8993G mutation presents a decrease in ATP synthesis, there are still debates about the reason for this. Contradictory experimental results have proposed this decrease to be due either to defects in ATPase assembly/stability or the functioning of the enzyme proton channel. In yeast, it has clearly been shown that the enzyme is correctly assembled but that the reverse functioning of ATP synthase (proton translocation out of mitochondria) is significantly compromised.

This new information gathered from yeast experiments was quite evident for the fifth mutation, T8851C. This mutation is associated with a bilateral striatal lesion, a rare neurological disorder with largely unknown biochemical consequences [76]. The analysis of the same mutation in yeast revealed a functional impairment of the ATP synthase, seemingly at the level of proton translocation. As the assembly/stability of the ATP synthase is not affected, a functional impairment of the enzyme must be responsible for the respiratory growth deficiency. The authors indeed showed that there was a very low F1-mediated ATP hydrolytic activity. Considering the high level of sequence conservation between the proteins, the block of proton translocation is probably the cause of this neurological disease [77].

Finally tRNA mutations mimicking human ones have been constructed, mostly in tRNALeu(UUR), which is an observed hot spot for mutations. In this case, the screening is more complex: (i) No positive screeening is possible, and this entails searching for rare phenotypically defective colonies among normal ones; (ii) mutations of the mitochondrial translation apparatus lead to a very rapid loss of mtDNA (rho°) so that the interesting point mutation—with a respiratory deficient phenotype—is hidden among a high frequency of rho°—which presents the same respiratory deficient phenotype. Although ATPase 6 mutations also cause a high frequency of rho°, the ATPase 6 studies have taken advantage of a clever genetic tool (expression of an allotropic form of the ARG8m expressed into mitochondria, which requires functional mitochondrial translation [78]). This is unfortunately not amenable to studies of mt-tRNA mutations.

To demonstrate that is was feasible to generate specific tRNA mutations, our lab successfully recreated a known tRNA mutation by biolistic transformation [79]. With this possibility at hand, three MELAS (Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode) mutations in mt tRNALeu(UUR)—3243G, C3256T and T3291C—were created in yeast through systematic tRNALeu gene sequencing of cells with a respiratory deficient phenotype and were shown to have very serious phenotypic consequences [80]. The yeast cells carrying those mutations were all unable to grow on respiratory substrates, and a large proportion of them lost their mitochondrial genome. A variety of pathological mutations covering different phenotypes in humans were then generated, their presence being screened by ACRS (artificially created restriction site) independently of the presumed phenotype. They exhibited different types of phenotypes: tRNALeu(UUR) T3250C is present in a family with mt myopathy (MM/CPEO, mitochondrial myopathy/chronic progressive external ophtalmoplegia; [81]) and A3260AG is associated with adult onset maternally inherited myopathy and cardiomyoapthy (MMC patients [82,83]. As opposed to the MELAS yeast mutations previously described which could not grow on respiratory substrates, these two mutants grew slowly and reached a lower plateau than the wild-type strain. Accordingly, they showed a significant decrease

of O_2 consumption, with a more limited effect on the T3250C equivalent mutation. In contrast, yeast cells carrying the G3249C mutation (with the severe Kearns-Sayre syndrome in humans) are as drastically affected as the MELAS mutation type (unpublished). Two other mutations, the tRNALys G8328A [84] found in a case of sporadic encephalomyopathy and the tRNAVal C1624T (Leigh syndrome and encephalopathy; [85]) both presented a conditional growth defective phenotype (heat-sensitivity) in yeast. From this study, it is apparent that very severe syndromes such as MELAS or Kearns-Sayre, produce very severe defects in yeast resulting in complete absence of growth on respiratory medium, while other mutations leading to less severe phenotypes in humans (CPEO, MM, encephalopathy) have milder effects when mimicked in yeast, with different degrees paralleling the degrees observed in humans [86]. In the same publication, the nuclear context was checked very carefully, taking advantage of a yeast mutation (*kar1-1*, [87]), which allows the placement of the same mitochondrial genome in different nuclear backgrounds. This study indeed revealed that the same mitochondrial mutation had a more or less severe phenotype according to the nuclear background in which it was placed, but that the rank in phenotypic effect of the different mutations was always the same in a given background, confirming previous results [88].

The molecular basis of the various respiratory defects was investigated. The structure, relative amount and aminoacylation of the tRNAs were checked, and it was found that the defects reflected what was observed in human cells [89], thus validating yeast once again as a tool to study mitochondrial disease. This system, although valuable, is limited by the fact that while there is a rather good sequence conservation between humans and yeast, not all human mutations can be mimicked in yeast. In order to circumvent this problem, my group attempted to replace the yeast tRNA by its human counterpart. To determine if it was feasible, we first introduced the human tRNALeu(UUR) into an otherwise wild-type mitochondrial genome. In such a context, we could examine the biochemical functions of the human tRNA (tRNA maturation and aminoacylation) in cells which grow normally since they have the wild-type yeast tRNA. All properties were found to be normal. In a second step, we placed the wild-type human tRNALeu(UUR) in the presence of one of the pathological mutations (with a temperature-conditional phenotype) and observed that the human gene could support yeast cell growth at the defective temperature. The same experiment was performed with the human tRNA Asp which could also functionally replace a mutated version of the yeast tRNA Asp. These results show that the human tRNAs can functionally replace their yeast counterparts and open the way to the possibility of creating and investigating any human mit tRNA mutation in the future [90].

In conclusion, modeling mutations in yeast has been informative; it allowed to either deepen or elucidate some biochemical mechanisms (as for several ATPase mutations), bring arguments in favor of pathogenicity in humans (such as in the apparent contradiction of the tRNAVal C1624T mutation phenotypic effects observed in the mother and her offspring [85]) or understand how to compensate the defective phenotype (see Section 4).

4. Impact on Possible Therapies

Among possible therapies against these deadly diseases, one immediately thinks of new chemical drugs or peptides, which could be delivered to the human body. As far as cell or gene therapies are concerned, the first difficulties lie with the fact that the “good gene” cannot be imported as such into the mitochondria since no nucleic acids are imported. We either have to find nuclear genes, which can

correct the mitochondrial mutation since they are imported naturally (the geneticist calls them "suppressor") or find a way to develop an import system for nucleic acids into mitochondria. For all these situations, yeast has been extremely useful in investigating these different approaches, taking advantage of the mutations, which have been modeled.

4.1. Identifying Correcting Genes and Derived Peptides

Creating pathological human mutations in yeast indeed provides a wealth of information on the molecular basis of these mutations, but the real strength of the yeast model is the power of its genetics. Starting with mutations, which have a defective phenotype on respiratory substrates, it is quite easy to select for secondary mutations, which can alleviate this phenotype. Since mitochondrial diseases involve two players (the nuclear genome cross-talking with the mitochondrial one) these secondary mutations (or suppressors) could *a priori* be localized in either of the two genetic compartments; in yeast, it is very easy to discriminate the two categories, in particular because one can lose the mitochondrial genome at will. Alternatively, one can manipulate the gene dosage and search for wild type genes, which could correct the initial mutation when their gene dosage is increased (overexpression). Both approaches have been successfully used.

In the case of *cyt b* mutations, many suppressors have been obtained and most were secondary mutations in the CYT b gene itself. However, some extragenic nuclear suppressors were also obtained and turned out to be mutations of the Iron-Sulfur protein. They provide important information on the relationship between structure and function of this protein and its interaction with Complex III [66,91].

Direct suppressors of the NARP mutations have never been searched for. However, a gene has been identified which, when overexpressed, induces a metabolic bypass of the defective ATPase; *ODC1* (coding for the oxodicarboxylate carrier) is able to correct the growth deficiency of some ATPase mutants as long as a few functional ATPases are still present [92]. It seems that under such conditions, the *ODC1* gene can improve the respiratory capacity of the yeast NARP T8993G mutation [75].

In the case of the tRNA mutations, the strategy was more direct. Correction by gene overexpression was deliberately searched for, with the rationale that overexpressing a wild-type gene should be transposable to other systems, in particular to humans. The *TUF1* gene, encoding the mitochondrial translation elongation factor EF-Tu, was initially isolated for its ability to correct, when overexpressed, the defective phenotype of some randomly obtained tRNA mutations during a large library screening [93]. It was therefore the first gene tested on pathological mutations, and it was found to restore all the phenotypic defects of the yeast mutant equivalent to C3256T [80]. It was later shown that *TUF1* corrected many different pathological tRNA mutations. The same type of experiments was performed with the cognate tRNA synthetase, which was also shown to be efficient when overexpressed [88]. More recently, new correcting gene candidates have been identified, and their effect remains to be caracterized [94]. It is interesting to note that serious arguments exist that support the fact that these suppression effects are not linked to the catalytic activity of these two proteins in protein synthesis but more probably to a chaperone effect [86,94]. Their correcting effects are also dependent upon the nuclear genetic background, perhaps in relation to the intrisic level of the correcting gene naturally present within the cell [88,95].

At this point, it is important to evaluate whether modifying the expression in nuclear genes can correct mitochondrial defects in human cells, which was the initial reason behind this screen. In 2008,

based on the yeast data, Sasarman and coworkers [38] tested the effect of EF-Tu (and EFG2) in patient human tissues and observed that there was some correcting effect. As in yeast, the same effect also existed for the cognate synthetases [96–98].

Since a point mutation in mtLeuRS that strongly reduces the catalytic activity did not impair the correcting ability [86] and since the effect is not restricted to the cognate synthetase [99], the region of the aaRS involved in this potential chaperone-like interaction with tRNA was searched for. It turned out that a *C*-terminal region of 66 aminoacids in yeast (67 in humans) had full suppressing activity, and moreover that this region was necessary and sufficient to rescue the defective phenotype of human equivalent mutations in yeast mt-tRNA Leu, mt-tRNA Val and mt-tRNA Ile [100]. It remained to be seen if such peptides had the same corrective effect on patient cell lines. This has proven to be the case based on the recent finding [101] that three human mt-aaRS (mt-ValRS, mt-LeuRS and mt-IleRS) are all able to suppress, in human transmitochondrial cybrid cells, the mitochondrial functional defects associated with pathogenic homoplasmic mutations in the mt-tRNA Ile gene. This effect was observed independent of the strength of the original phenotype. This suppression effect, as in yeast, can be restricted to the non-catalytic carboxy-terminal domain. Reciprocally, one specific synthetase (leucyl tRNA synthetase) was able to partially rescue defects due to mutations in non-cognate tRNAs, and this effect was restricted to the C-terminal peptide which can enter mitochondria and interact with the same spectrum of mt-tRNA [102].

4.2. Import of RNA

We now know that, besides well-described protein-import pathways into mitochondria, RNA import also exists as a quasi-ubiquitous process. Situations and mechanisms vary widely between organisms (reviews in [103–107]) but the import concerns only small non-coding RNA and mostly tRNAs. Once again, studies in yeast were the starting point in improving the understanding of the phenomenon and developing new strategies.

The presence of a cytoplasmic tRNALys(CUU)—also called tRK1—within the mitochondria was found more than thirty years ago [107,108] but not understood at that point in time since the mitochondrion encodes its own tRNALys(UUU)—called tRK3—and was proposed to be able to decode both AAA and AAG codons. It is only very recently that the role of this supposedly redundant tRNA was understood. tRK1 is imported into mitochondria in several steps. First, it is charged by the cytosolic LysRS, it is then recognized by one of the two isoforms of enolase, a glycolytic enzyme, and the complex then moves to the mitochondrial surface where the charged tRK1 is taken up by the pre-mitochondrial LysRS [109,110], and more specifically by its N-terminal domain [111]. This basic knowledge was exploited in an elegant way to show that tRK1 is essential for mitochondrial protein synthesis at high temperatures. Under such conditions, tRK3 is not modified properly and cannot recognize mRNAs containing an AAG codon, a function then ensured by tRK1 [111]. From this work and studies run on the tRNA determinants for aminoacylation and import [112] the following questions immediately arose: Is it possible to manipulate these determinants to import cytosolic tRNAs with altered aminoacylation identities? Could these engineered tRNAs be functionally active to correct diseases as explained below?

It was first shown in yeast that tRK1(CUU) changed into tRK1(CAU) could charge methionine, be imported into isolated mitochondria and incorporated into mtDNA encoded proteins. This *in vitro*

approach was followed by an *in vivo* test in which a COX2 amber mutation was suppressed by modified (both for aminoacylation and import properties) tRK2(CUA). It was also shown that, *in organello*, an imported modified yeast tRNA was functional on the human mitochondrial translation apparatus [113]. These important results were then transposed to patient human cell lines and the imported tRNA was shown to restore mitochondrial function in transmitochondrial cybrid cells bearing the MERFF mutation [114]. More recently, the defective phenotype of a patient cell line carrying a MELAS mutation was partially rescued by an importable tRNAsLys with Leucine aminoacylation identity [115].

This very basic research demonstrated that specifically designed tRNAs can address human mitochondria *in vivo*, be active in mitochondrial translation, and (partially) compensate the defective phenotype of human patient cell lines with potential applications for therapy.

While no "natural" tRNA import has been found in mammalian mitochondria, several small RNAs are probably imported (5S RNA [116]; the RNA moiety of RNAseP [117] and the mitochondrial RNA processing endonuclease [118]). More recent works now aims to develop possible applications. The appropriate selection of aptamers [119] and the understanding of the role of human tRNA synthetase in human tRNA import [120] have allowed progress to be made. Synthetically engineered modified oligonucleotides carrying a nucleotide sequence complementary to the mutated region can be targeted at human mitochondria *in vivo* and change the heteroplasmy ratio [121]. While other systems are now being studied (based for example on the Leishmania RNA imported complex—or RIC complex—which enters human cells by a caveolin-1 dependent pathway [122]), the work undertaken with yeast has pioneered and stimulated the topic of RNA import into mitochondria.

4.3. Drug Screening

Alternatively to the genetic approach, one can also attempt to identify drugs, which could alleviate the defects induced by the mutation. Because mitochondrial defects impair the proper functioning of the mitochondrial respiratory chain, one might expect that some chemicals, which act upon respiration functions, might relieve the symptoms. The positive effect of idebenone (a derivative of Coenzyme Q10) on patients with Coenzyme Q10 deficiency was first described [123]. This product was also successfully used later to cure the cardiomyopathy, which often develops in Friedriech Ataxia patients [124]. Other metabolic products have also been tested. The possibilities of treating the metabolic disorders of mitochondrial diseases were based on the following rationales: "(i) removal of noxious metabolites such as dichloroacetate; (ii) administration of electron acceptors; (iii) administration of vitamins and cofactors; (iv) administration of oxygen radicals scavengers; (v) increase of mitochondrial biogenesis with reverastrol; (vi) increase of autophagy activity with lithium". They have been reviewed in [125].

Based on this, a systematic screen of metabolic products able to improve the viability of MELAS models was done [126] using yeasts as the primary test substrate. These studies convincingly showed that supplementation with riboflavin (vitamin B2) or coenzyme Q10 effectively counteracted the respiratory defect in MELAS yeast and improved the pathologic alterations in MELAS fibroblast and cybrid cell models. Despite these encouraging results, no attempt has been made to apply this knowledge to large-scale drug screening, in part because of the lack of appropriate models. The possibility to mimic, in yeast, mitochondrial pathological mutations (see above) and the versatility, cheap price and fast growth associated with the important knowledge accumulated on *S. cerevisiae*

over the years, should make this organism particularly suitable for a first step in large screening. It is only recently that such an approach has been described [127]. The authors screened about 12,000 chemical compounds in a yeast strain where the *FMC1* gene had been deleted. In this mutant, there are much fewer assembled ATP synthase complexes than in a wild-type strain, whereas the ones that are assembled are fully functional. The Δfmc1 strain does not grow on glycerol [128]. Presumed active compounds were then tested on the yeast models of atp6-NARP mutants described in Section 3. Three compounds, chlorhexidine, oleic acid, and dihydrolipoic acid, were identified, which turned out to also be effective in a human cybrid-based model of NARP. This work, although it did not reveal completely unexpected compounds, is important, as it constitutes a proof of principle that yeast can be efficiently used for large-scale screening of new compounds active on ATPase deficiencies. In addition, once the drug is identified, it can itself become an efficient tool to understand the mechanism of action, opening ways to new information about cell pathways, as exemplified by prion studies [129,130].

Recently, large-scale screening has been undertaken to correct defective mutations of the mitochondrial gamma-polymerase (encoded by the *MIP* gene in yeast). While this gene is nuclear-encoded, its effects impact the mitochondrial DNA and any drug, which would act upon its defective forms, is of great importance. Screening different *mip* mutants, six unforeseen compounds answered the criteria; three of these are now being further analyzed [131].

5. Conclusions

Despite the strong reductive aspects of using a small unicellular eucaryote to undestand some dysfunctions of the complex human organism, yeast has proven invaluable for a better understanding of mitochondrial diseases. Basic research progress has been made to understand the molecular mechanisms which underly the proper function of mitochondria in the cell. This in turn has allowed further steps, which have been validated later in human patient cell lines and could help with possible therapies. One can bet that we have not yet heard the last of yeast!

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Conflicts of Interest

The author declares no conflict of interest.

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