A yeast-based assay identifies drugs active against human mitochondrial disorders

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Due to the lack of relevant animal models, development of effective treatments for human mitochondrial diseases has been limited. Here we establish a rapid, yeast-based assay to screen for drugs active against human inherited mitochondrial diseases affecting ATP synthase, in particular NARP (neuropathy, ataxia, and retinitis pigmentosa) syndrome. This method is based on the conservation of mitochondrial function from yeast to human, on the unique ability of yeast to survive without production of ATP by oxidative phosphorylation, and on the amenability of the yeast mitochondrial genome to site-directed mutagenesis. Our method identifies chlorhexidine by screening a chemical library and oleate through a candidate approach. We show that these molecules rescue a number of phenotypes resulting from mutations affecting ATP synthase in yeast. These compounds are also active on human cybrid cells derived from NARP patients. These results validate our method as an effective high-throughput screening approach to identify drugs active in the treatment of human ATP synthase disorders and suggest that this type of method could be applied to other mitochondrial diseases.

budding yeast | drug screening | transcription profiling | NARP cybrid

Although our understanding of the molecular mechanisms underlying mitochondrial diseases has considerably improved, the development of effective treatments has still been extremely limited (1). The insufficiency of relevant disease models in conjunction with the absence of high-throughput drug screening assays may, at least in part, explain this failure.

Among these disorders, some are associated with primary deficiencies in the mitochondrial ATP synthase (2), an enzyme that catalyzes the final steps of mitochondrial ATP production. To date, seven point mutations in the mitochondrial *ATP6* gene encoding subunit *a* of ATP synthase have been associated with a group of maternally inherited neurodegenerative syndromes with onset in early infancy (1, 3), including NARP (neuropathy, ataxia, and retinitis pigmentosa). In diseases resulting from mutations in mitochondrial DNA (mtDNA), wild-type and mutated mtDNA coexist in patient mitochondria, a characteristic called heteroplasmy. The severity of the disease depends on the proportion of mutant alleles, with a minimal critical proportion of ~70% for penetrance of a clinical phenotype; this property is known as the threshold effect (4, 5).

The budding yeast *Saccharomyces cerevisiae* serves as a good model for mitochondrial disease because (*i*) mitochondrial genes and function are particularly well conserved from yeast to human (1); (*ii*) yeast are genetically tractable; and (*iii*) yeast have the ability to survive either by fermentation or by respiration, where only the latter requires oxidative phosphorylation (OXPHOS). This last point is particularly beneficial because mutant strains in which ATP synthase activity is impaired can be easily maintained on fermentable media (e.g., glucose); therapeutic strategies can then be tested on media where respiration is required (e.g., glycerol, ethanol, or lactate). In addition, along with *Chlamydomonas reinhardtii* (6), yeast is the only eukaryote in which site-directed mutagenesis of the mitochondrial genome has been established (7). Because of the natural instability of heteroplasmy in yeast, homoplasmic populations in which 100% of mitochondria contain mutated mtDNA can be easily generated. Thus, yeast models of the five most common *ATP6* mutations found in NARP patients (T8993G, T8993C, T9176G, T9176C, and T8851C) have been generated and characterized (8–11). Other patients exhibiting ATP synthase deficiency have been found to carry mutations in two nuclear genes, *ATP12* (12) and *TMEM70* (13), which encode proteins that are required for ATP synthase assembly. An appropriate yeast model of such disorders is the deletion mutant for the nuclear gene *FMC1* that encodes a protein required at high temperatures (35–37 °C) for assembly of the F_1 sector of ATP synthase (14). When the *fmc1Δ* mutant is grown at high temperatures, its mitochondria contain far fewer assembled ATP synthase complexes than a wild-type (*WT*) strain, whereas the ones that assemble are fully functional. This heterogeneity is also found in patients with decreased levels of ATP synthase due to *ATP12*, *TMEM70*, or heteroplasmic *ATP6* mutations. Therefore, the *fmc1Δ* mutant constitutes an appropriate model of these disorders.

In this study, we establish a two-step screening assay designed to identify drugs active against inherited ATP synthase disorders modeled in yeast. In the primary screen, ~12,000 compounds from various chemical libraries were tested for their ability to suppress the respiratory growth defect of the *fmc1Δ* mutant. In the secondary screen, active compounds were tested on the five yeast *atp6*-NARP mutants. Our screen identified chlorhexidine (CH) and oleate (OA); further experiments confirmed that they improve various respiratory phenotypes of both the *fmc1Δ* and NARP mutants. Dihydrolipoic acid (DHLA), which has previously been reported as active against mitochondrial encephalopathies and is currently being tested in patients (15, 16), was also active in our yeast-based method. Moreover, we show that CH, OA, and DHLA are effective in a human cybrid-based model of NARP. These results validate our yeast-based approach as a method for identifying compounds with potential to treat inherited mitochondrial diseases affecting ATP synthase.

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Results

Development of a Yeast-Based Screen for Drugs That Suppress NARP Phenotypes. The *fmc1Δ* mutant and the five yeast *atp6*-NARP mutants exhibit growth defects on glycerol-based medium at 35 and 37 °C (Fig. 1*A*). At 35 °C, the temperature used for the screening assay, the different mutants present growth defects of varying severity, with the *atp6*-NARP T9176G, T8851C, and T8993G mutants (the latter being the most frequent mutation in human) exhibiting the most severe phenotypes. Our measurements of mitochondrial ATP synthesis in these mutants (Fig. 1*A Right*) correlate well with their fitness on glycerol. They also confirm previous observations that defects in ATP synthesis must be severe (estimated at ~80% or more; refs. 8 and 17) before a clear growth defect on respiratory medium can be observed in yeast. Even more significant is the fact that these observations also correlate with what is known about the relative severity of the corresponding *atp6*-NARP mutations in human (2, 18, 19), which likely reflects a high level of evolutionary conservation within the region of subunit *a* affected by these mutations. These observations constitute a preliminary validation of the use of yeast to model human inherited mitochondrial diseases affecting ATP synthase. Because the *fmc1Δ* strain displays an intermediate respiratory growth phenotype and is a good model of heteroplasmy, we selected it for our primary screen.

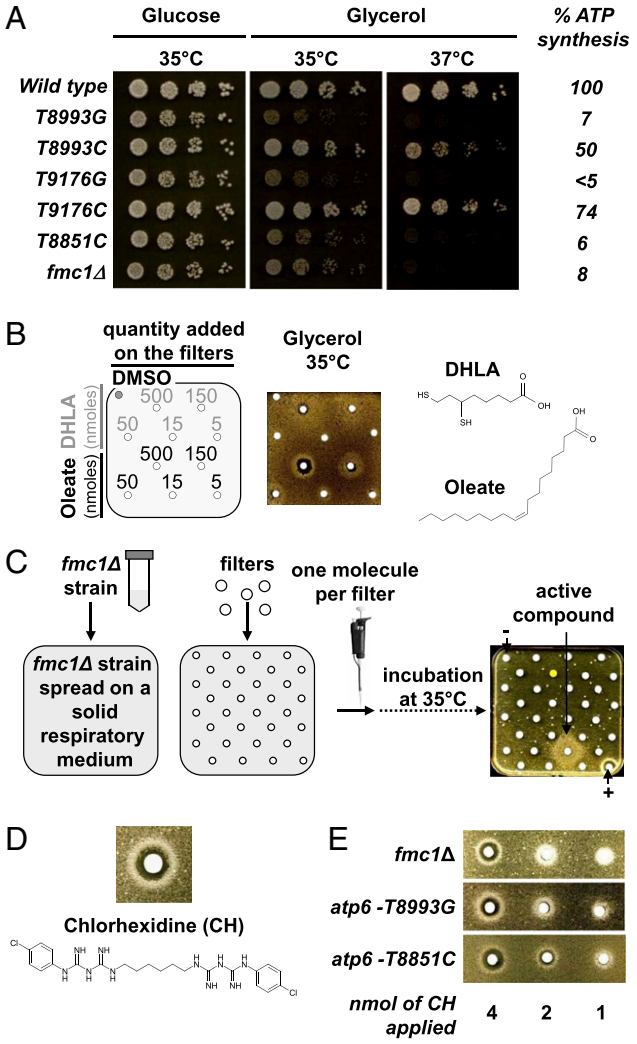

Fig. 1. Development of a yeast-based screening assay for NARP-related diseases and identification of CH. (*A*) Serial dilutions of the *WT* strain, five *NARP* mutants, and the *fmc1Δ* strain were spotted onto glucose and glycerol plates. The plates were incubated at the indicated temperature for 3 d (glucose) or 7 d (glycerol). Rates of ATP synthesis relative to *WT* are indicated in the right-hand column. (*B*) Candidate screen. The *fmc1Δ* strain was spread onto rich glycerol plates. Small sterile filters were then placed on the agar surface and DHLA or OA (chemical structures depicted at *Right*) were added to the filters at the indicated quantities. The plates were then incubated at 35 °C for 7 d. (*C*) Screening assay carried out as described in *B* except that DMSO was added to the upper left filter (negative control, –) and DHLA to the bottom right filter (positive control, +). At the remaining positions, compounds from the chemical libraries were added, and plates were incubated for 7 d at 35 °C. (*D*) The improvement of fmc1Δ growth obtained with CH is shown, and its molecular structure is depicted. (*E*) The dose-dependent effect of CH on three yeast mutants is shown.

Our next tested candidate compounds that could hold therapeutic potential for mitochondrial disease in our yeast-based assay. We selected DHLA because it has already been used as a treatment for patients presenting mitochondrial encephalopathies (15, 16). We also selected OA, a fatty acid known to induce expression of the mitochondrial Odc1p protein (20), a carrier for various Krebs cycle intermediates encoded by the *ODC1* gene in yeast (21). We have previously isolated *ODC1* as a multicopy suppressor of the respiratory growth defect of the *fmc1Δ* strain (22). By using our simple assay described below, we found that both DHLA and OA partially suppress, in a dose-dependent manner, the growth defect of the *fmc1Δ* strain (Fig. 1*B*). The fact that DHLA, a compound displaying therapeutic benefits for patients affected by mitochondrial encephalopathies, also suppresses the respiratory growth phenotype of the *fmc1Δ* strain further validates our yeast-based method.

Primary Screen of Chemical Libraries Using Yeast-Based Assay. We then performed our primary screen by testing 12,000 compounds from various chemical libraries for their ability to suppress the respiratory growth defect of the *fmc1Δ* mutant strain. Similarly to an assay we previously developed (23, 24), *fmc1Δ* cells are spread on solid glycerol medium and exposed to filters spotted with the compounds. Active compounds are then identified by a halo of enhanced growth around a filter (example in Fig. 1*C Right*). The advantage of this method is that, in one simple experiment, it allows numerous compounds to be tested across a large range of concentrations due to diffusion of the drugs in the growth medium. This design improves the sensitivity of the screen drastically because many compounds (including OA and CH, see below) are toxic at high concentrations (Fig. 1*B* and *C*). The positive hits obtained were then tested in a secondary screen by using the yeast *atp6*-NARP mutants in the same experimental procedure.

Our screen used, among others, the Prestwick Chemical Library, a collection of drugs for which bioavailability and toxicity studies have already been carried out in humans; therefore, active compounds from this library can directly enter drug optimization programs. The percentage of active compounds in our primary screen was quite low (only ~10 of 12,000 molecules tested, corresponding to <0.1%), indicating that the screening assay is specific and stringent.

Identification of CH. Among the hits from our primary screen was CH, an antiseptic compound from the Prestwick Chemical Library (Fig. 1*D*). The secondary screen (Fig. 1*E*) showed that, in addition to its activity in the *fmc1Δ* strain, CH elicits a dose-dependent partial suppression of the respiratory growth defects of the *atp6*-NARP T8993G and T8851C strains. In contrast, CH had no visible effect on the *atp6*-NARP T9176G strain, which is the most severe NARP mutation in both human and yeast (Fig. 2*A*). The severity of this phenotype is due to an almost complete lack of incorporation of subunit *a* into ATP synthase (10), whereas substantial residual mitochondrial ATP synthesis is observed in the mutants that are rescued by CH (11).

CH Rescues Multiple Mitochondrial Defects in *fmc1Δ* Cells. To characterize the effects of CH, we used the *fmc1Δ* strain because it responds best to the drug. The *fmc1Δ* strain exhibits a low rate of ATP synthase assembly and low levels of respiratory complexes

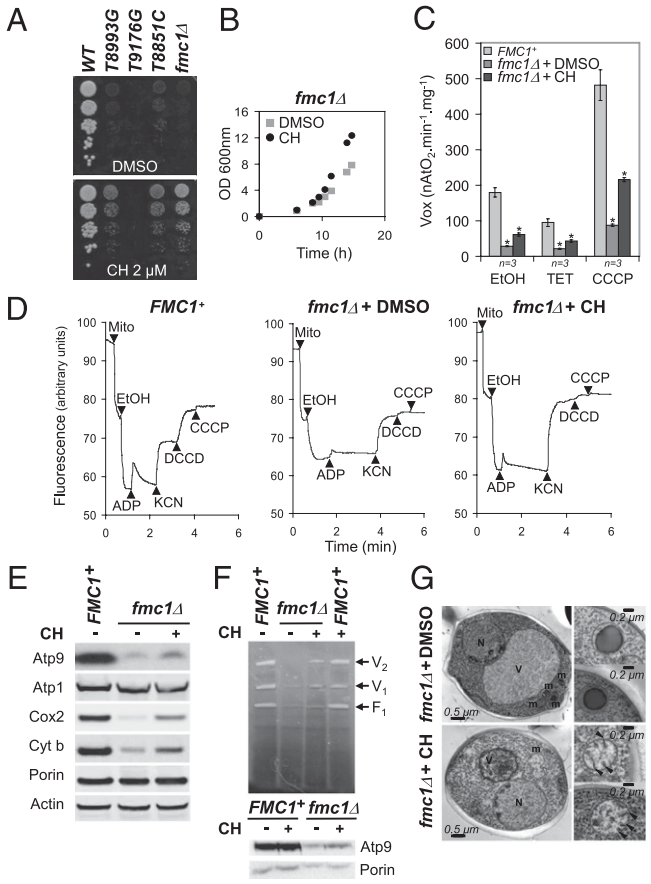

Fig. 2. CH suppresses multiple phenotypes of yeast models of ATP synthase disorders. (*A*) Serial dilutions of the *WT* strain, three *NARP* mutants, and the *fmc1Δ* strain were spotted onto glycerol plates supplemented with CH (2 μM final concentration) or the equivalent quantity of DMSO (– control). The plates were incubated at 35 °C for 7 d. (*B*) Growth curves of the *fmc1Δ* strain grown at 35 °C in liquid galactose medium supplemented with 1.25 μM CH (the optimal concentration) or DMSO. (*C*) Mean respiration rates of the *WT* and *fmc1Δ* strains treated in vivo with 1.25 μM CH or DMSO were determined using three different substrates as indicated. Error bars represent SD. * represents significant difference compared with untreated cells (P = 0.05, one-sided Wilcoxon rank test). (*D*) Energization of the mitochondrial membrane was determined by rhodamine 123 fluorescence quenching with intact mitochondria from the *fmc1Δ* strain treated in vivo with 1.25 μM CH (*Right*) or DMSO (*Center*), and the *WT* isogenic strain (*FMC1*$^+$; *Left*). The contents were added as follows: mitochondrial proteins (Mito), ethanol (EtOH), ADP, potassium cyanide (KCN), DCCD, and CCCP. (*E*) SDS-PAGE and Western blot analysis of mitochondrial proteins from the mitochondria used in *D*. Porin was used as a loading control. (*F*) BN-PAGE, ATPase activity, and Western blot analysis of extracts from isolated mitochondria. V_2 and V_1 indicate the dimeric and monomeric forms of the F_1F_0 ATP synthase complex, respectively; F_1 indicates the free F_1 particles. (*G*) Ultrastructure electron micrographs of *fmc1Δ* cells grown at 35 °C in presence of 1 μM CH (*Lower*) or DMSO as control (*Upper*). Arrowheads indicate mitochondrial cristae.

III and IV when grown at high temperatures (35-37 °C; ref. 25). In addition, its mitochondria are devoid of cristae and contain large inclusion bodies composed mostly of aggregated, unassembled α and β subunits of the F_1 moiety of ATP synthase. Finally, its inner mitochondrial membrane energization and respiration rate are impaired. We thus set out to determine whether CH treatment could rescue these phenotypes. For growth in liquid, we selected galactose as a carbon source to allow faster growth of the mutant strains while retaining their mitochondrial activity; although galactose is a fermentable substrate, unlike glucose it does not elicit repression of mitochondrial biogenesis. In addition, the growth defect of *fmc1Δ* yeast as well as its rescue by CH could be clearly observed in liquid galactose medium (Fig. 2*B*).

Oxygen consumption rates. Using ethanol as an electron donor, we measured the oxygen consumption levels of *WT* (*FMC1*$^+$) and *fmc1Δ* whole cells grown at 35 °C in rich galactose medium with or without CH. In the absence of CH, *fmc1Δ* cells exhibited respiration activity six times lower than *WT*; treatment with CH resulted in a significant (2.2-fold; P = 0.05, *SI Methods*) increase in respiration (Fig. 2*C*). We also measured respiration in the presence of triethyltin (TET), an inhibitor of ATP synthase (basal or state 4 respiration), and the mitochondrial membrane uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), i.e., in conditions where respiration is maximal (uncoupled state). In these conditions, treatment of *fmc1Δ* cells with CH also resulted in significantly improved respiration rates (2.1- and 2.5-fold, respectively; P = 0.05; Fig. 2*C*).

Respiratory enzyme abundance. The respiration data suggest that CH treatment increases the amount of respiratory enzymes in *fmc1Δ* cells, which we confirmed by Western blot analysis (Fig. 2*E*). We observed a partial restoration of steady-state levels of complex III–V subunits except for Atp1p, whose abundance is unaffected by the *FMC1* deletion as previously observed (14). In contrast, CH treatment did not affect the amount of respiratory enzymes in *WT* cells (Fig. S1).

Energization of mitochondrial membrane. We assessed the proton pumping activity of isolated mitochondria prepared from *WT* (*FMC1*$^+$) and *fmc1Δ* cells grown with or without CH using the fluorescent membrane potential (ΔΨ)-sensitive dye rhodamine 123 (26). Consistent with their low oxygen consumption and our previous results (14), mitochondria from untreated *fmc1Δ* cells were poorly energized with ethanol relative to *WT*, whereas those from *fmc1Δ* cells grown in the presence of CH were energized almost as efficiently as *WT* mitochondria (Fig. 2*D*). In *WT* mitochondria, a further addition of ADP, as expected, led to a transient decrease of fluorescence quenching, reflecting the use of ΔΨ by ATP synthase to phosphorylate the added ADP. Because of the low levels of ATP synthase in *fmc1Δ* mitochondria, the addition of ADP had little effect on membrane potential. In contrast, a significant ΔΨ decrease was induced by ADP addition in mitochondria isolated from CH-treated *fmc1Δ* cells. This observation reflects a higher level of ADP phosphorylation in mitochondria from *fmc1Δ* cells upon CH treatment.

ATP synthesis rates. We measured ATP synthesis rates of mitochondria isolated from both *WT* and *fmc1Δ* cells grown with or without CH, using NADH as a respiratory substrate and an excess of external ADP. In good agreement with the partial suppression of the respiratory growth phenotype, we observed a modest but reproducible effect of CH on ATP synthesis rates in *fmc1Δ* cells, whereas CH had almost no effect on *WT* mitochondria (Table S1).

Blue native (BN)-PAGE and ATPase activity. We evaluated the effect of CH on both assembly and activity of ATPase in isolated mitochondria from both *fmc1Δ* and *WT* cells grown with or without CH. The BN-PAGE and ATPase activity stain demonstrate that CH treatment led to a significant increase in fully assembled ATP synthase in the *fmc1Δ* strain (Fig. 2*F*).

Mitochondrial morphology. We evaluated the effect of CH on the mitochondrial morphology of *fmc1Δ* cells using electron microscopy. Consistent with previous observations (25), 77% of cell sections of *fmc1Δ* cells grown at 35 °C display matrix-localized, electron-dense inclusion bodies consisting of ATP synthase subunits α and β. Strikingly, this proportion was reduced to 6% when *fmc1Δ* cells were grown in the presence of CH (Fig. 2*G* and Table S2). Moreover, mitochondrial cristae were clearly discernible in some of these cells, whereas they were completely absent in untreated *fmc1Δ* cells. The restoration of cristae is consistent with the CH-induced increase in oxygen consumption and respiratory chain subunits, because cristae allow higher amounts of respiratory enzymes to be assembled within mitochondria.

Transcription Profile of *fmc1Δ* Strain and Its Response to CH Treatment. To investigate the global effects of CH on cellular function in the *fmc1Δ* mutant, we carried out genome-wide

comparative analyses of the transcriptional responses to the *FMC1* gene deletion and the addition of the drug using high-resolution tiling microarrays (ref. 27; Fig. 3*A*). Overall, 336 of 5,446 expressed genes [defined in *SI Methods*; results in Dataset S1 and available from ArrayExpress (details in *SI Methods*)] showed at least 1.5-fold differential expression in response to the deletion of *FMC1* (Fig. 3*A*, triangles). We analyzed these genes according to Gene Ontology (28) and transcription factor targets (ref. 29; *SI Methods*; results in Dataset S2). As expected, these genes are mostly related to mitochondrial respiration; in particular, the down-regulated genes are enriched for subunits of the respiratory chain complexes (30/47; $P = 6 \times 10^{-17}$; P values corrected for multiple testing; *SI Methods*) and targets of the respiratory gene expression activator Hap4p (25/38; $P = 5 \times 10^{-24}$). Additionally, enrichment for targets of the transcription factors Rtg3p (5/31; $P = 8 \times 10^{-2}$) and Gcn4p (15/121; $P = 5 \times 10^{-3}$) indicate that the retrograde pathway, a transcriptional program that responds to mitochondrial dysfunction by modulating metabolism, was activated in the *fmc1Δ* mutant (30–32). Finally, iron homeostasis was also perturbed in the *fmc1Δ* strain (11/38; $P = 6 \times 10^{-7}$).

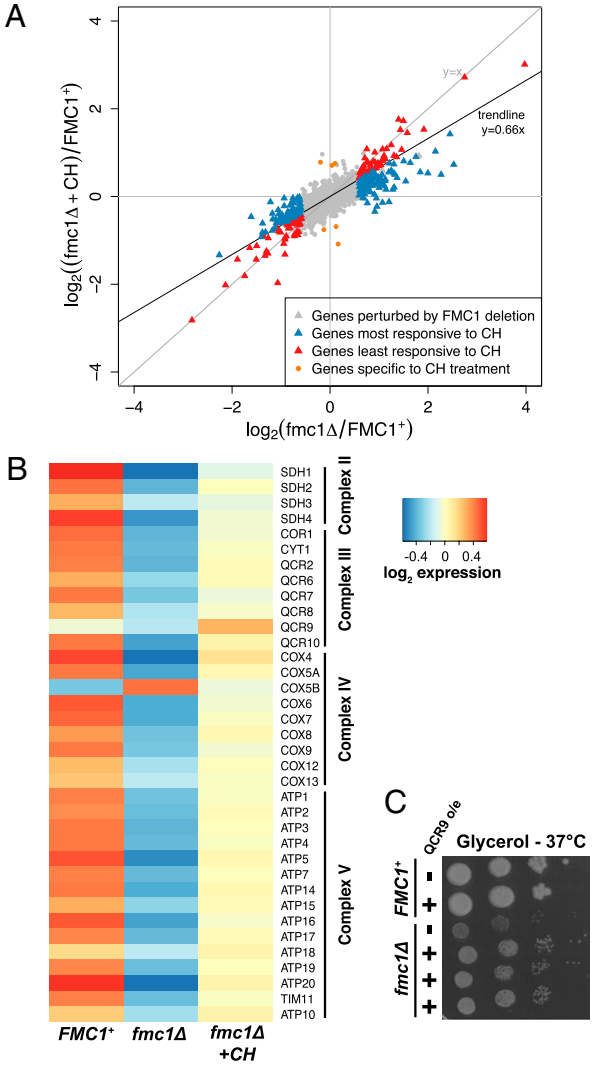

Fig. 3. Transcription profiles of *fmc1Δ*, *fmc1Δ* + CH, and *WT* (FMC1$^+$). (*A*) Scatterplot of $\log_2$-fold changes in gene expression of CH-treated (*y* axis) and untreated (*x* axis) *fmc1Δ* yeast relative to *WT* in rich galactose medium at 35 °C. CH treatment induces a genome-wide shift toward *WT* expression levels of ~1/3 as evidenced by the trendline $y = 0.66x$. (*B*) Expression of genes encoding respiratory chain complex subunits. $\log_2$-scale normalized expression values were centered for each gene by subtracting the mean expression level from all values per gene. Subunits show a general pattern of down-regulation in *fmc1Δ* yeast relative to *WT* and partial rescue by CH treatment. (*C*) Overexpression of the *QCR9* gene in the *fmc1Δ* strain (bottom three rows, compared with the *fmc1Δ* strain in third row and *WT* strain in first two rows) results in partial recovery of growth on rich glycerol medium at a nonpermissive temperature.

The drug CH induced a partial rescue of the *fmc1Δ* mutant at the transcriptional level, with gene expression fold changes compared with *WT* reduced genome-wide by ~1/3 (Fig. 3*A*; trendline $y = 0.66x$, where complete rescue would be ~0 and no drug effect would be ~1). Almost all genes perturbed in the *fmc1Δ* mutant responded positively to CH treatment, although to varying extents. The most responsive genes (perturbed genes whose fold change relative to *WT* was reduced by >1/3; Fig. 3*A*, blue), included all 30 respiratory chain complex subunits and 23/25 ($P = 3 \times 10^{-21}$) of the Hap4p targets down-regulated in the mutant; they were also enriched for genes involved in cristae formation (3/4; $P = 10^{-3}$). Accordingly, signals of the retrograde response largely disappeared after CH treatment. Among the genes least responsive to CH treatment (perturbed genes whose response to the drug was lower than the genome-wide trend; Fig. 3*A*, red) were those involved in iron homeostasis. The small number of genes (six) specific to CH treatment (those whose treatment-induced expression was significantly different from both other conditions) and their lack of functional enrichments suggests that the drug has no major side effects unrelated to the mutant phenotype.

The transcriptional behavior of the respiratory chain subunits provides important insight into the regulatory impact of CH on the *fmc1Δ* mutant (Fig. 3*B*). Among the subunits, there was a clear pattern of down-regulation in the *fmc1Δ* mutant relative to *WT*, with CH treatment recovering expression to intermediate levels (corroborating the measurements of protein levels; Fig. 2*E*). The only genes that did not follow this pattern were *COX5B* (encoding an "anaerobic" isoform of cytochrome *c* oxidase subunit 5) and, more interestingly, *QCR9*, encoding a component of cytochrome bc_1 complex, whose expression was up-regulated beyond *WT* levels by CH treatment. The unique response of *QCR9* prompted us to investigate whether overexpression of this gene alone was sufficient to suppress the respiratory growth defect of the *fmc1Δ* strain. Indeed, a partial rescue was observed in *fmc1Δ* cells overexpressing the *QCR9* gene (Fig. 3*C*). These findings suggest that *QCR9* could be a key regulatory target for determining the cellular levels of bc_1 complex (*Discussion*). Together, our transcription data correspond well to our biochemical data and strongly indicate that the main effect CH has on the *fmc1Δ* strain is improvement of its respiratory function, in which *QCR9* may play a role. Notably, *WT* cells displayed a very limited response to CH at the transcriptional level that included neither activation of respiratory pathways nor up-regulation of *QCR9* expression (Dataset S1). These results suggest that CH does not act via a general transcriptional induction of respiratory pathways; rather, it requires specific conditions to exert its beneficial effects (*Discussion*).

Drugs Active in the Yeast-Based Assay are also Active in Human NARP Cells. We next tested the compounds that were active in our yeast-based assay in a human cell-based model of NARP syndrome; in particular, we used a cybrid-based model nearly homoplasmic for the NARP T8993G mutation (33). To encourage the cells to rely on OXPHOS rather than glycolysis, we used glucose-deprived medium (34), in which the NARP cybrids exhibit a significant growth defect (33). Strikingly, DHLA, CH, and OA all significantly increased the growth rate of NARP cybrids (Fig. 4*A*). In agreement with its effects in mitochondrial encephalopathy patients, treatment with 200 μM DHLA increased the survival of NARP cybrid cells by 2.2-fold, recovering their survival to nearly the rate measured in control cybrids (Fig. 4*A*). Remarkably, CH (Fig. 4*A Right*) and OA (Fig. 4*A Left*) treatments resulted in a clear dose-dependent improvement of NARP cybrid survival (the maximal increases were 1.5- and 1.6- fold, respectively). As in yeast, however, CH did not affect growth of WT cybrids (Fig. S2). The improved survival of

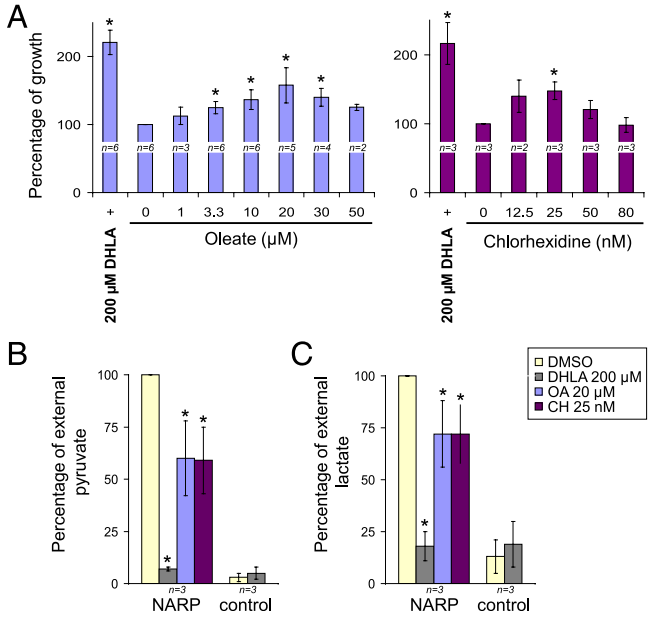

Fig. 4. DHLA, CH, and OA are active in a human cybrid-based model of NARP syndrome. NARP and control cybrids were grown in glucose-deprived medium with DHLA, CH, and OA at the indicated final concentrations or with the equivalent quantity of DMSO as a control (0). (*A*) Percentage of growth after 3 d in glucose-deprived medium containing OA or CH at the indicated concentrations, compared with treatment with DHLA at 200 µM final concentration (+), a compound being tested in clinical trials for the treatment of mitochondrial encephalopathies. * represents conditions with growth significantly different from in DMSO ($P < 0.05$; *Methods*). (*B*) Mean percentage of external pyruvate in NARP cybrids treated with DHLA, CH, OA (left-hand side of graph) compared with control cybrids (right-hand side of graph). Error bars represent SD. * represents significant difference compared with the DMSO condition ($P = 0.01$). (*C*) Mean percentage of external lactate as in *B*.

the NARP cybrids suggests that DHLA, OA, and CH improve OXPHOS production of ATP in these cells.

As one of the main symptoms of NARP-related mitochondrial disorders is lactic acidosis, which is most likely due to a shift toward glycolytic metabolism, it would be particularly valuable if these compounds could reduce glycolysis. Indeed, CH, OA, and DHLA effected a reduction in extracellular levels of the glycolytic byproducts pyruvate and lactate in NARP cybrids (Fig. 4*B* and *C*), comparable to their amelioration of growth: DHLA reduced external pyruvate and lactate to the levels observed in control cybrids, and OA and CH each reduced pyruvate by ~40% and lactate by ~30% ($P = 0.01$; *Methods*). These observations demonstrate that DHLA, OA, and CH reduce glycolysis in NARP cybrids, indicating that they induce a metabolic shift toward OXPHOS.

Discussion

Our results validate the use of a yeast-based assay to identify compounds potentially active in the treatment of inherited mitochondrial diseases caused by ATP synthase deficiency. Firstly, there is a strong correlation between the severity of mutations in patients and the respiratory defects caused by the homologous mutations in yeast. Secondly, DHLA, a compound previously found to be active against mitochondrial encephalopathies in humans, is also active in yeast. Thirdly, CH and OA, two compounds identified by our yeast-based assay, are also active in human cybrids derived from NARP patients.

>CH has remarkable suppressor activity in our yeast model (*fmc1Δ*) of ATP synthase assembly disorders, with a substantial (more than twofold) increase in respiration due to higher amounts of respiratory complexes III and IV. Consistent with this result, the *fmc1Δ* mutant recovered an effective capacity to energize the inner mitochondrial membrane upon CH treatment. Another striking effect of CH is the restoration of mitochondrial cristae and elimination of matrix-localized inclusion bodies formed by aggregation of α and β ATP synthase subunits. In agreement with these suppressive effects, CH partially restored the expression of most components of the OXPHOS pathway perturbed in the *fmc1Δ* mutant. The transcription data, which provide insight into the cellular response to CH treatment, tell us that the strongest beneficial effect of CH is at the level of the respiratory chain. The fact that CH did not exert this effect on *WT* cells indicates that (*i*) the transcriptional response of *fmc1Δ* cells to CH is more likely a downstream rather than a direct effect of treatment, and (*ii*) CH requires specific conditions to improve respiratory function, including but not limited to ATP synthase deficiency (otherwise it would have rescued all of the *atp6*-NARP mutants).

The expression pattern of *QCR9*, which encodes a subunit (Qcr9p) of complex III, suggests that this gene plays a role in the rescue mediated by CH. The possibility that increased Qcr9p synthesis alone could result in a higher abundance of complex III is in line with previous studies showing that such a regulatory mechanism is commonly used in the expression of mitochondrial and chloroplastic energy-transducing enzymes (35–37). Because complexes III and IV are mainly present together in the form of supercomplexes (38), increasing complex III abundance may allow more complex IV to be incorporated into mitochondria. *QCR9* overexpression does indeed improve respiratory growth of the *fmc1Δ* mutant, but to a lesser extent than CH treatment; CH treatment also effected a partial increase in ATP synthase assembly (Fig. 2 *E* and *F*). It is therefore possible that CH works by increasing both the number of ATP synthase complexes and the efficiency with which they are used (via the stronger protonmotive force produced by complexes III/IV, whose increase in quantity may be mediated by up-regulation of *QCR9*). The combination resulted in a modest but significant improvement of ATP production in *fmc1Δ* cells, leading to restoration of respiratory growth. The modest improvement of ATP production by CH has significant therapeutic potential when considering the threshold phenomenon: Small increases in ATP production can be sufficient to restore a healthy state (4, 5). Further investigations into how exactly CH improves the abundance of respiratory chain complexes and ATP synthase are needed to more thoroughly characterize the therapeutic potential of this drug. In addition, evidence of CH displaying detrimental effects at high concentrations (39) will be important to consider as CH continues to be developed as a therapeutic.

In addition to providing a simple and powerful screening assay for identifying drugs active against ATP synthase disorders, the system presented here constitutes a proof of principle that yeast can be used as a pharmacological model for the study of mitochondrial diseases. The drugs identified can be used in various reverse screening strategies (40) to identify their intracellular targets, potentially revealing novel cellular mechanisms involved in disease pathologies (41, 42). In addition, the use of multiple *atp6*-NARP mutants in our secondary screen, which resulted in candidate compounds with allele-specific efficacies, holds promise for the development of personalized therapeutics for mitochondrial diseases.

As yeast models of inherited mitochondrial disorders continue to be developed, we believe the screening approach presented here will continue to yield promising chemical therapeutics and insights into disease mechanisms (1, 43).

Methods

Yeast Strains and Culture Medium. The *S. cerevisiae* strains used and their genotypes are listed in Table S3. For details on growth procedures, see *SI Methods*.

Yeast-Based Drug Screening Assay. This assay was adapted from an existing test (23, 24). Two hundred forty microliters of exponentially growing cell cultures, adjusted to an OD_{600} of 0.2, was spread homogeneously with sterile glass beads (~3 mm diameter) on a square Petri dish (12 cm × 12cm) containing YPAGly solid medium. Sterile filters (similar to those used for anti-

GENETICS

biograms) were placed on the agar surface, and 2.5 μl of individual compounds from the various chemical libraries were applied to each filter in addition to DMSO, the vehicle, as a negative control, and a DHLA solution in DMSO as a positive control. Plates were then incubated at 35 °C for 7 d and scanned using a Snap Scan1212 (Agfa). For information on compounds screened, see *SI Methods*.

Isolation of Yeast Mitochondria and Subsequent Experiments. Mitochondria were prepared by the enzymatic method as described (44) from cells grown for 7–8 generations in YPAGal medium at 35 °C in the presence of CH or DMSO. For details on the experiments with isolated mitochondria, see *SI Methods*.

Ultrastructural Studies. Please see *SI Methods* for details.

Transcription Profiling. Two biological replicates of strains MC1 and MC6 were cultured in YPAGal + DMSO/CH at 35 °C and harvested; total RNA was isolated and reverse-transcribed into cDNA, which was hybridized to whole-genome tiling arrays. For more details, see *SI Methods*.

Statistical Analysis of Transcription Profiles. Raw tiling array data were processed to provide normalized intensity values for each probe in each hybridization. The expression level of each transcript was estimated by the median value of the probe intensities of the transcript across both arrays per strain and condition (27). For more details, see *SI Methods*.

Human Cell Lines and Culture Conditions. The cybrid cell lines JCP213 (control) and JCP239 (NARP T8993G) (33) were cultivated in high-glucose DMEM; growth measurements were performed in glucose-deprived DMEM supplemented with CH, DHLA, OA, or DMSO. For each treatment condition, four wells were used. After 3 d of incubation with the drugs, cell proliferation was estimated by using Neutral Red staining (45) and also by cell counting with an Adam cell counter. The cells were then assayed for lactate and pyruvate by using kits from DiaSys-Poles. For more details, see *SI Methods*.

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