Resistance to miltefosine results from amplification of the *RTA3* floppase or inactivation of flippases in *Candida parapsilosis*

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Running title: Miltefosine resistance in *Candida parapsilosis*

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Abstract

Flippases and floppases are two classes of proteins that have opposing functions in the maintenance of lipid asymmetry of the plasma membrane. Flippases translocate lipids from the exoplasmic leaflet to the cytosolic leaflet, and floppases act in the opposite direction. Phosphatidylcholine (PC) is a major component of the eukaryotic plasma membrane and is asymmetrically distributed, being more abundant in the exoplasmic leaflet. Here we show that gene amplification of a putative PC floppase or double disruption of two PC flippases in the pathogenic yeast *Candida parapsilosis* results in resistance to miltefosine, an alkylphosphocholine drug that affects PC metabolism that has recently been granted orphan drug designation approval by the US FDA for treatment of invasive candidiasis. We analysed the genomes of 170 *C. parapsilosis* isolates and found that 107 of them have copy number variations (CNVs) at the *RTA3* gene. *RTA3* encodes a putative PC floppase whose deletion is known to increase the inward translocation of PC in *Candida albicans*. *RTA3* copy number ranges from 2 to >40 across the *C. parapsilosis* isolates. Interestingly, 16 distinct CNVs with unique endpoints were identified, and phylogenetic analysis shows that almost all of them have originated only once. We found that increased copy number of *RTA3* correlates with miltefosine resistance. Additionally, we conducted an adaptive laboratory evolution experiment in which two *C. parapsilosis* isolates were cultured in increasing concentrations of miltefosine over 26 days. Two genes, *CPAR2_303950* and *CPAR2_102700*, gained homozygous protein-disrupting mutations in the evolved strains and code for putative PC flippases homologous to *S. cerevisiae DNF1.* Our results indicate that alteration of lipid asymmetry across the plasma membrane is a key mechanism of miltefosine resistance. We also find that *C. parapsilosis* is likely to gain resistance to miltefosine rapidly, because many isolates carry loss-of-function alleles in one of the flippase genes.

Author summary

Miltefosine was developed as an anticancer drug but is commonly used to treat infections with the protozoan parasites *Leishmania* and *Trypanosoma cruzi*. More recently, it has been used to treat fungal infections, and in 2021 it was designated as an orphan drug by the US Food and Drug Administration for treatment of invasive candidiasis. Miltefosine is a derivative of phosphatidylcholine (PC), a major constituent of the cell membrane. PC and other phospholipids are asymmetrically distributed across the cell membrane. The mechanism of action of miltefosine is unknown. Here, we show that either increasing the activity of a putative floppase, which controls outward “flop” movement of phospholipids, or decreasing the activity of flippases, which control inward “flip” movement, results in

increased resistance of the fungal pathogen *Candida parapsilosis* to miltefosine. This result suggests that miltefosine acts by controlling the localisation of PC or other phospholipids in the membrane. Importantly, we find that many *C. parapsilosis* isolates carry mutations in one flippase gene, which renders them partially resistant to miltefosine, and prone to easily acquiring increased resistance.

43 **Introduction**

44

45 Lipids in yeast plasma membranes belong to three main families: sterols, sphingolipids, and
46 phospholipids (or glycerophospholipids) [1]. Phospholipids (including phosphatidylcholine
47 (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine
48 (PS)) and sphingolipids (including the long chain bases (LCBs) dihydrosphingosine (DHS)
49 and phytosphingosine (PHS)) are distributed asymmetrically in the plasma membrane
50 bilayer [2, 3]. Plasma asymmetry is determined by the action of lipid translocators via rapid
51 inward (flip) and outward (flop) movements [4]. Most flippases are members of the P4-
52 ATPase family [5], and most floppases are ABC-type transporters [6]. The Rta1 family of 7
53 transmembrane domain proteins are also predicted lipid transporters [7].

54

55 Several antimicrobial drugs are targeted against lipid synthesis, such as azole drugs, which
56 target ergosterol synthesis in fungi [8-10]. Miltefosine (hexadecylphosphocholine) is an
57 alkylphosphocholine derivative that inhibits PC biosynthesis or localization [11]. Miltefosine
58 was initially investigated as an antitumor agent, but it was found to have a very limited
59 anticancer effect [12]. However, it has been used successfully to treat infections with
60 *Leishmania* and *Trypanosoma cruz*i [13-15]. Recently, miltefosine has also been shown to
61 have antifungal activity, including against *Aspergillus* [16], *Cryptococcus* [17]
62 and *Paracoccidioides* [18]. Anti-*Candida* activity has also been reported, including against
63 *Candida auris* [19, 20], *Candida krusei* [21] and *Candida albicans*, where it inhibits growth of
64 both planktonic and biofilm cells [22]. In 2021, miltefosine was granted orphan drug
65 designation approval by the US Food and Drug Administration (FDA) for treatment of
66 invasive candidiasis
67 (https://www.accessdata.fda.gov/scripts/opdlisting/oopd/detailedIndex.cfm?cfgridkey=843921).

68

69 The mechanism of action of miltefosine is not completely understood. In liver cells,
70 miltefosine is proposed to reduce the biosynthesis of PC by inhibiting the activity of
71 CTP:phosphocholine cytidylyltransferase (CT) and PE N-methyltransferase activity [23].
72 Treatment of *Leishmania* with miltefosine decreases the amount of PC and increases PE in
73 the membrane, because of a reduction of intracellular choline [24]. In addition, cytochrome c
74 activity in the mitochondria is inhibited, and the plasma membrane Ca^{2+} channel is disrupted
75 [25]. Studies in *Saccharomyces cerevisiae* suggest that related drugs, such as edelfosine
76 (lysophosphatidylcholine) also disrupt lipid rafts in the cell membrane, perhaps by directly
77 interacting with sterols [26]. In *Aspergillus fumigatus*, miltefosine affects metabolism of lipids
78 and fatty acids, as well as by increasing mitochondrial fragmentation [16].

Most flippases belonging to the P4-ATPase family, which consist of two subunits, a catalytic alpha-subunit with 10 transmembrane alpha-helices, and a beta-subunit with two transmembrane alpha-helices belonging to the Cdc50 family [5]. Flipping of PE, PS and PC is specifically associated with Class 3 P4-ATPases, represented by Dnf1 and Dnf2 in *S. cerevisiae* [27]. Disruption of *DNF1* or *DNF2*, or of *LEM3* (*CDC50*), results in miltefosine resistance in *S. cerevisiae* [28]. Similarly, mutations in P4-ATPases in *Leishmania* species confer resistance to miltefosine [29-32].

In *Candida* species, flippases have not yet been associated with miltefosine resistance. However, deletion of an unrelated gene, *RTA3*, results in increased miltefosine sensitivity in *C. albicans* [33]. Rta3 is a member of the Rta1/Rsb1-like family in *S. cerevisiae*, which encode putative transporters with seven transmembrane domains (TMs). ScRsb1 controls the localization of sphingoid bases in *S. cerevisiae*, including phytosphingosine and dihydrosphingosine [34, 35]. Rsb1 is localized to the membrane and is likely to act as a membrane transporter (a floppase) [7], or possibly as a regulator of transporters [36]. Overexpression of another family member, *ScRTA1*, correlates with increased resistance to 7-amino cholesterol, an inhibitor of 8-7 sterol isomerase and 14-sterol reductase [37]. The Rsb1 family members have not been fully characterised in *Candida* species. As well as showing that deleting *C. albicans RTA3* (*CaRTA3*) increases sensitivity to miltefosine, Srivastava et al [33] also showed that CaRta3 localizes to the plasma membrane, and that a fluorophore-labeled PC accumulates in the inner leaflet of the membrane in an *CaRTA3* deletion. They proposed that CaRta3 is either a floppase, or that it inhibits the activity of a flippase.

Some previous reports suggest that *CaRTA3* is also associated with resistance to azoles. Rogers and Barker [38, 39] found that transcription of *CaRTA3* is up-regulated in fluconazole-resistant isolates from AIDS patents, and Coste et al. [40] showed that Tac1p, a transcription factor regulating expression of the MDR transporters *CDR1* and *CDR2*, also regulates expression of *CaRTA3* via binding to the drug-response element. Whaley et al [41] reported that deleting *CaRTA3* reduced fluconazole resistance of a clinical isolate of *C. albicans*, and that overexpressing *CaRTA3* increased resistance. However, Srivastava et al. [33] showed that deleting *CaRTA3* in a reference isolate had no effect on fluconazole resistance. The role of Rta3 in azole resistance is therefore unclear.

In 2020, we found that the *RTA3* gene has undergone amplification in 23 clinical isolates of *Candida parapsilosis*, resulting in increased copy number of 24-33x [42]. Variable copy numbers of *RTA3* were also found by West et al [43] in four isolates. Here, by genome sequencing, we report that *RTA3* amplification is very common in *C. parapsilosis*. We find 16 distinct amplifications of *RTA3* with unique endpoints, indicative of independent and parallel amplification events. We also find a fusion to a related neighboring gene, *RTA2*. Copy number of *RTA3* is correlated with resistance to miltefosine, but not to azoles. In addition, we find that resistance to miltefosine induced by exposure to the drug is associated with loss-of-function mutations in two P4-ATPase flippase genes.

Results

C. parapsilosis phylogeny shows independent origins of *RTA3* CNVs

We explored the relationships of 170 *Candida parapsilosis* isolates using a genome-wide SNP alignment approach, generating the largest phylogeny of *C. parapsilosis* isolates to date (Fig 1). Isolates were obtained from several locations, including some previously published, and 127 that are sequenced and described here for the first time (S1 Table). Most isolates originated from the Memorial Sloan Kettering Cancer in New York (designated by MSK) and the Centre Hospitalier Universitaire de Nantes, France (designated by FM) (Fig 1).

Figure 1. SNP-based unrooted phylogeny of 170 *C. parapsilosis* isolates.
Isolates harboring a CNV at *RTA3* are marked with a thick gray bar and a letter (A-P) corresponding to one of the 16 CNVs found. Isolates from Memorial Sloan Kettering Cancer Center (MSK) are named in blue (the prefix MSK is omitted from these strain names), and isolates from CHU de Nantes (FM) are named in red. Green dashed lines label each of five apparent clades. Fifty similar isolates in Clade 1, and 19 similar isolates in Clade 4, were grouped and are shown as blue triangles. The phylogeny was constructed by calling SNPs for each sample using the GATK HaplotypeCaller tool [44]. Filtered heterozygous sites were resolved using 100 iterations of Random Repeated Haplotype Sampling to provide haploid inputs for tree construction [45]. The tree was then constructed using RAxML with the GTRGAMMA model of nucleotide substitution.

148 Isolates fall into five major clades, with almost half (84/170), belonging to clade I. Most clade
149 I isolates were isolated at MSK. Half (8/16) of the FM isolates are found in Clade 4. There
150 are 49 highly similar isolates in Clade 1, and 19 highly similar isolates in Clade 4, all from
151 patients at MSK. These may have recently originated from two single isolates, and they are
152 indicated with blue triangles in Fig 1. Some other isolates (e.g. MSK2233 and MSK2234 in
153 Clade 2) may also share a recent origin. However, overall there is little evidence for
154 geographical clustering. Each clade includes at least one isolate from both MSK and FM (Fig
155 1). Three isolates from a clinical setting in Kuwait [46] are each located in a different clade
156 (designated by Kw in Fig 1). Interestingly, an environmental sample isolated from Irish soil,
157 UCD321, groups with clinical isolates from both MSK and FM (in Clade 5). The diversity of
158 isolates obtained from the same clinical setting, and the close relationship between isolates
159 from different geographical settings, highlight the global nature of *C. parapsilosis* as a
160 human pathogen.
161
162 We previously showed that the *RTA3* gene, encoding a putative floppase, had undergone
163 extensive copy number amplification in 23 *C. parapsilosis* isolates [42]. This was also
164 observed in a small number of isolates by West et al [43]. We therefore characterized copy
165 number variants (CNVs) at the *RTA3* locus in all sequenced isolates. We identified
166 increased *RTA3* copy numbers in 104 of 170 isolates. Sixteen CNVs with unique endpoints
167 were observed (assigned letters A-P, Figs. 1, 2). Nine different CNVs were observed in
168 isolates from Clade 1, whereas only CNV-L is found in isolates in Clade 3, and there are no
169 CNVs in Clade 2 isolates (Fig 1). Most (15/16) of the CNVs have a single evolutionary origin,
170 and some are present in only a single isolate (Fig 1). However, CNV-A has three separate
171 origins on the tree (one in Clade 1, and two in Clade 5).
172
173 **16 unique CNVs amplify *RTA3***
174
175 We found that *RTA3* has been amplified in 16 different types of CNV, each with unique
176 endpoints. Thirteen CNV patterns result from tandem duplications that amplify the entire
177 *RTA3* coding sequence (Fig 2A), and these repeat units range in size from 2.3 to 4.5 kb.
178 Some of these CNVs extend into the upstream neighbouring gene *MAK16,* and one (CNV-N)
179 includes the first 17 bp in the coding sequence of the downstream gene *RTA2*. The copy
180 number of *RTA3* varies both in isolates that share the same CNV, and in isolates with
181 different CNVs (Fig 2B). Isolate MSK807 (CNV-G) has the lowest estimated copy number of
182 *RTA3* among isolates with CNVs, at four copies, whereas isolate FM16 (CNV-J) has the
183 highest copy number, at 50 copies.

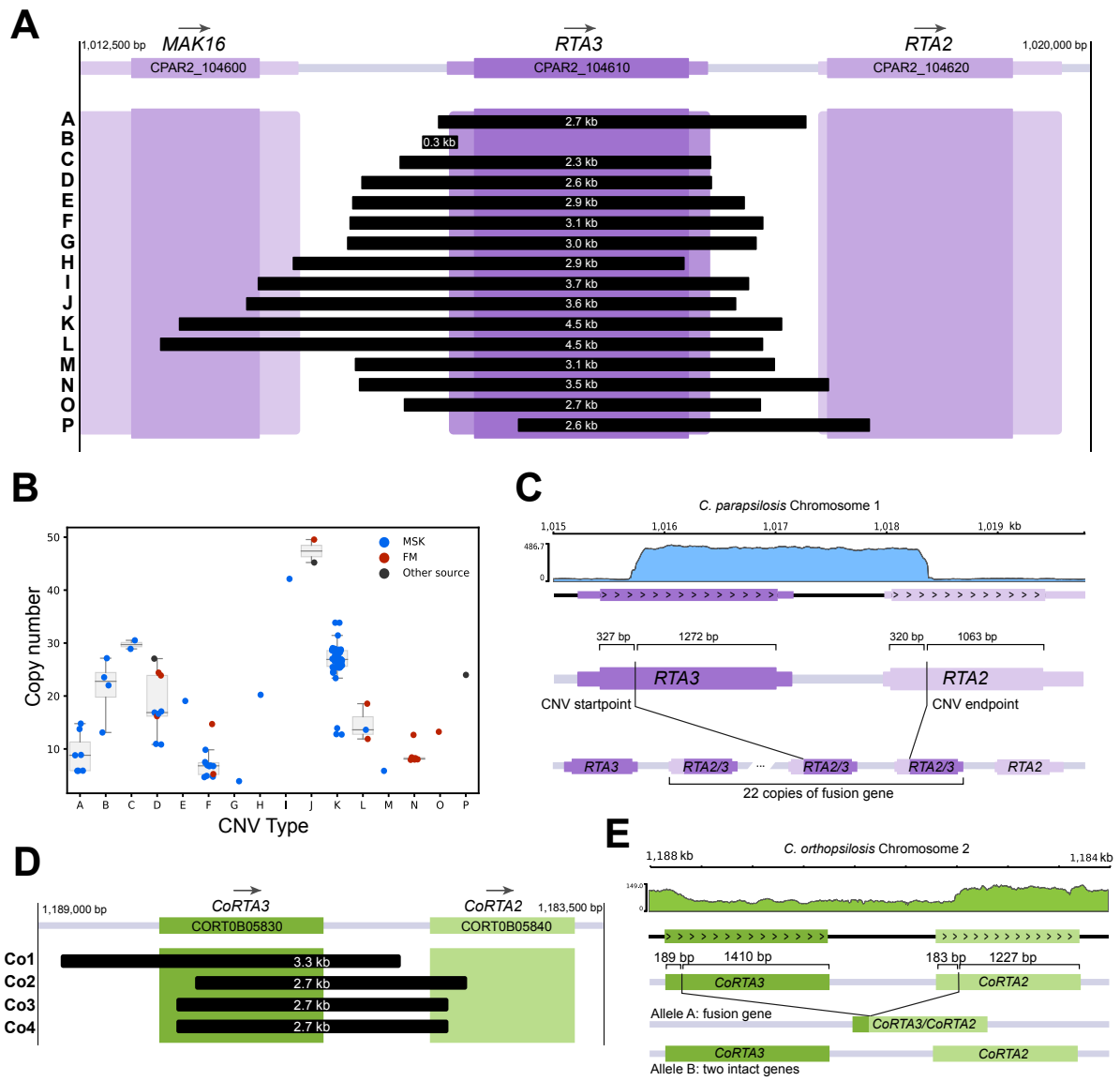


Figure 2. CNVs amplify different sequences at the *RTA3* locus

A. Span of 16 different CNVs at the *RTA3* locus in *C. parapsilosis*. The coding sequence of *RTA3* is shown by a dark purple box, with the flanking UTRs in a lighter color. The neighbouring genes *MAK16* and *RTA2* are also shown. The extents of the tandemly repeating units in each of 16 CNVs are shown by black boxes, labelled (A-P) on the left, and their lengths are indicated in white. Exact breakpoints were identified by interrogating split reads from the Illumina data, and the array structure of CNVs D and K was verified by MinION sequencing of isolates UCD321, MSK478, and MSK812.

B. Copy number of *RTA3* in each *C. parapsilosis* isolate. Each dot represents a single isolate: blue isolates from MSK, red from CHU de Nantes, and dark grey from other sources. Median and interquartile ranges are shown for CNVs present in more than one strain. Dots are jittered for clarity.

C. *RTA2/3* fusion gene in strain 434 containing CNV-P. A coverage plot created by PyGenomeTracks [47] is shown at the top. The schematic shows the position of CNV breakpoints in relation to the CDS of both genes, and the structure of the inferred array of fusion genes.

D. Amplifications of *RTA3* in *C. orthopsilosis*. Orientation of the chromosome has been flipped to highlight the similarities to *RTA3* CNVs in *C. parapsilosis*.

E. A deletion in *C. orthopsilosis* generates a *CoRTA3/CoRTA2* fusion. A coverage plot of the locus is shown at the top. The schematic shows CNV breakpoints in both *CoRTA3* and *CoRTA2* and how the resulting fusion gene is formed at one allele, leaving the other allele intact.

For CNV-K, three isolates have roughly half the *RTA3* copy number of other isolates with the same CNV (~13x instead of ~26x; Fig 2B; S1 Table). We considered the possibility that only one allele of *RTA3* was amplified in these isolates (*C. parapsilosis* is diploid), and that both alleles were amplified in other isolates. We explored this issue by using long read sequencing (Oxford Nanopore) of *C. parapsilosis* MSK812 which is one of the CNV-K isolates with fewer copies. We found that it has 8 copies of *RTA3* at one allele and 6 copies at the other (S1A Fig). Similarly, sequencing of isolate MSK478 (which also has CNV-K, with higher copy number of *RTA3*) showed that it has at least 11 copies at both alleles (S1B Fig). Therefore the variation in copy number among isolates with CNV-K is due to expansion or contraction of the array in both alleles, and not due to hemizygosity for the amplification.

Two of the *RTA3* CNVs alter the structure of the protein it encodes. The amplification in CNV-P starts within the *RTA3* open reading frame, and ends within the related adjacent gene *RTA2* (Fig 2A,C). This repeat structure generates an array of in-frame *RTA2/RTA3* gene fusions, with the N-terminus derived from *RTA2* and the C-terminus from *RTA3* (Fig 2C). *RTA3* and *RTA2* are very similar genes, which likely facilitated the fusion event. In CNV-H, the Rta3 protein is slightly truncated because this CNV consists of a tandem duplication with an endpoint 31 bp upstream of the stop codon of *RTA3*, resulting in a protein that is 10 amino acids shorter than the wild type.

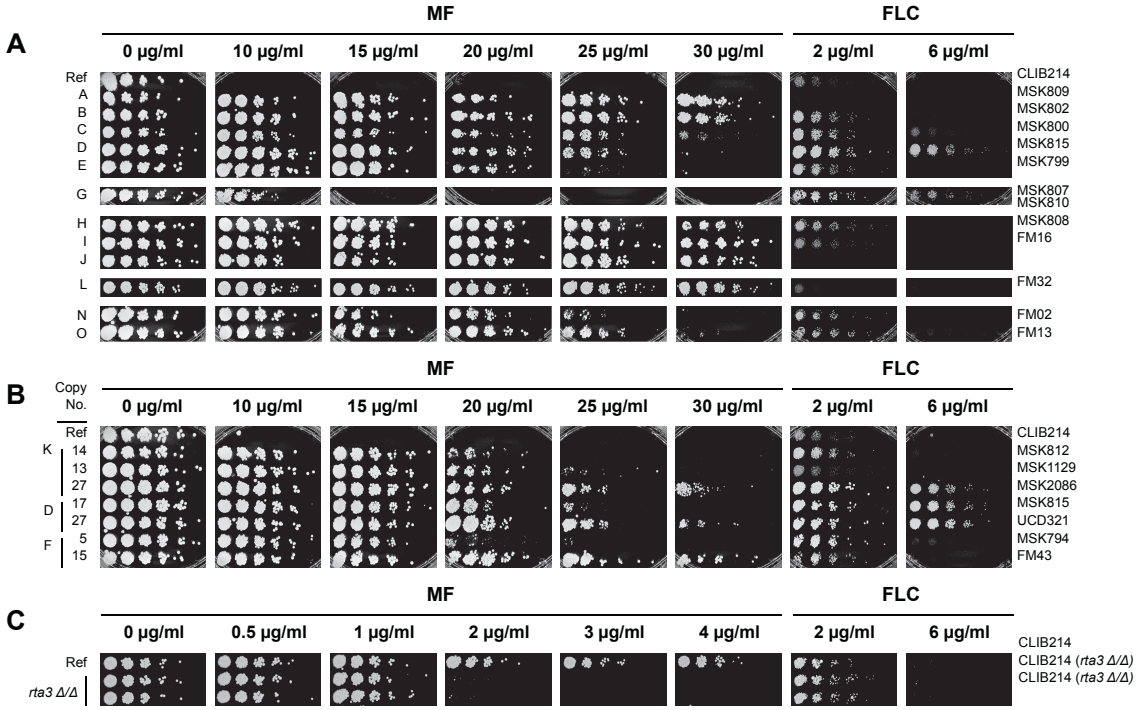
CNV-B is substantially different from the others because it is an amplification of only a small region (269 bp) that is upstream and completely outside the *RTA3* coding sequence (Fig 2A). Sequencing read coverage analysis suggests that the repeat has a complex organization in which direct repeats are interspersed with inverted copies of a central segment flanked by inverted-repeats, resulting in an N:(2N-1):N copy number pattern (S2A Fig). This repeat structure was confirmed by MinION sequencing of isolates MSK802 and MSK803. The structure of the CNV-B repeat is similar to the DUP-TRP/INV-DUP structure seen in some human CNVs [48] and is reminiscent of amplifications formed via Origin-Dependent Inverted Repeat Amplification (ODIRA) [49]. The ODIRA model proposes that replication errors at origins that are surrounded by short inverted repeats generate extrachromosomal intermediates, which are then integrated at the original site [49]. This results in complex CNVs with repeat units in head-to-head and tail-to-tail arrangements that are similar to the CNV-B structure. To date, ODIRA has only been observed in *S. cerevisiae*,

237 and it is unclear whether it occurs in other yeasts [49]. If CNV-B were caused by ODIRA, we
238 would anticipate that the central region of the repeat would contain an origin of replication
239 (S2B Fig). However, determined the temporal order of replication in *C. parapsilosis* using
240 Sort-seq (S2C Fig), and we found no evidence that there is an origin near *RTA3* [50].
241
242 We also looked for evidence of *RTA3* amplification in other *Candida* species. *RTA3* is not
243 amplified in 200 sequenced *C. albicans* genomes [51, 52]. However, we identified four
244 different *RTA3* CNVs among 36 strains of *Candida orthopsilosis*, a close relative of *C.*
245 *parapsilosis* (Fig 2D). Only one of these (CNV-Co1) consists of a simple tandem
246 amplification of the entire *CoRTA3* ORF. Two others, CNV-Co2 and CNV-Co3, amplify a
247 fusion of the *CoRTA2* and *CoRTA3* genes, similar to CNV-P in *C. parapsilosis* (Fig 2D).
248 Interestingly, the final *C. orthopsilosis* CNV (CNV-Co4) involves a deletion. In the two strains
249 containing CNV-Co4, the 3' end of *CoRTA3*, the 5' end of *CoRTA2,* and the intergenic space
250 between them are deleted at one allele (Fig 2E). This results in a new fusion gene, with the
251 N-terminus derived from *CoRTA3* and the C-terminus derived from *CoRTA2*. Single copies
252 of *CoRTA2* and *CoRTA3* are intact on the other allele (Fig 2E). This is the only example we
253 have seen of an *RTA3* deletion in *C. parapsilosis, C. orthopsilosis* or *C. albicans*.
254
255 **Copy number of *RTA3* is correlated with miltefosine, but not fluconazole, resistance**

256 Deletion of *RTA3* in *C. albicans* has been shown to increase susceptibility to miltefosine [33]
257 and possibly to fluconazole [41]. We therefore investigated the effect of copy number on
258 resistance of *C. parapsilosis*. Fig 3A shows that amplification of *RTA3* is associated with
259 miltefosine resistance. For example, *C. parapsilosis* strains with only two copies of *RTA3*
260 (one at each allele, e.g. the reference strain CLIB214) fail to grow at miltefosine
261 concentrations of 10 µg/ml, whereas all the strains with *RTA3* amplifications can grow at this
262 concentration. Strains with CNVs A, H, I, J and L can tolerate miltefosine concentrations up
263 to at least 30 µg/ml, as can isolates with CNV-B (which amplifies only the region upstream of
264 *RTA3*). The CNV with the weakest effect on MF resistance is CNV-G, which can tolerate 10-
265 15 µg/ml, which is still higher than the reference strain CLIB214.

266 When different isolates carrying the same CNV are compared, miltefosine resistance is seen
267 to be correlated with the copy number of *RTA3* (Fig 3B). For example, *C. parapsilosis*
268 isolates MSK812 and MSK1129, which have 13-14 copies of CNV-K, can tolerate miltefosine
269 concentration up to ~20 µg/ml, whereas *C. parapsilosis* MSK2086 which has 27 copies
270 survives up to 30 µg/ml. Similarly, *C. parapsilosis* MSK815 with 17 copies of CNV-D and
271 MSK794 with 5 copies of CNV-F tolerate miltefosine concentrations up to ~20-25 µg/ml

272 whereas UCD321 and FM43 with 27 and 15 copies of CNV-D and CNV- F respectively
273 survive up to ~30 µg/ml (Fig 3B).



274

275 **Figure 3. CNVs of *RTA3* correlate with resistance to miltefosine**

276 A. A representative isolate of most CNV patterns (Fig 2) was grown on YPD with increasing
277 concentrations of miltefosine (MF) or fluconazole (FLC) as shown. Cells were serially diluted (1/5).
278 The CNV pattern is indicated on the left, with the strain name shown on the right. MF and FLC plates
279 were incubated for 48 hr. "Ref" indicates the reference strain *C. parapsilosis* CLIB214.

280 B. Copy number is directly correlated with miltefosine resistance. Isolates with different copy
281 numbers of CNV-K, CNV-D and CNV-F were grown as in (A). The *RTA3* copy number of each isolate
282 is shown on the left.

283 C. Deleting *RTA3* reduces resistance to miltefosine. *RTA3* was deleted in *C. parapsilosis*
284 CLIB214 using CRISPR-Cas9. The growth of two biological replicates is shown, as in (A), except that
285 incubations on FLC were for 72 h.

286

287 The isolates have variable levels of sensitivity to fluconazole (Fig 3), but the copy number of
288 *RTA3* does not correlate with susceptibility to this drug. For example, isolates MSK815 and
289 UCD321 which have 17 and 27 copies of CNV-D differ in their susceptibility to miltefosine,
290 but they both tolerate fluconazole levels of 6 µg/ml.

291 We also found that deleting *RTA3* in the reference isolate of *C. parapsilosis* (CLIB214) by
292 CRISPR-Cas9 editing [53] results in increased sensitivity to miltefosine (Fig 3C). Very low

293 levels of miltefosine were used (up to 4 µg/ml), because the parental strain, without any
294 *RTA3* amplifications, is very sensitive. However, susceptibility to fluconazole was not
295 affected by deleting *RTA3* (Fig 3C).

296 Increasing the *RTA3* copy number correlates with increased expression, as shown by West
297 et al [43]. However we wondered whether amplifying the upstream region (CNV-B) has the
298 same effect as amplifying the entire open reading frame. To explore this further, we
299 measured *RTA3* expression by RT-PCR in one isolate of *C. parapsilosis*, MSK808, with
300 approximately 42 copies of *RTA3* (CNV-I), and two isolates (MSK802 and MSK803) in which
301 only the promoter region is amplified (CNV-B). We found that *RTA3* expression is
302 approximately 22-fold higher in *C. parapsilosis* MSK808, and 2.8- to 6.6-fold higher in *C.*
303 *parapsilosis* MSK802 and MSK803 than in the reference strain CLIB214 (Table 1). There are
304 28 copies of the direct repeat in CNV-B in *C. parapsilosis* MSK802 and 24 in *C. parapsilosis*
305 MSK803. Thus, *RTA3* promoter region amplification (CNV-B) can lead to increased
306 expression.
307

308 **Generation of miltefosine-resistant strains by experimental evolution**

309 The prevalence of *RTA3* amplification in *C. parapsilosis* isolates suggests that there may be
310 some strong selective pressure inducing amplification. To determine if miltefosine is the
311 driving force, we characterized the effect of exposing isolates to increasing concentrations of
312 miltefosine, in an adaptive laboratory evolution approach. We started with two isolates with
313 only one copy of *RTA3* at each allele that were in the same clade as other isolates that had
314 undergone amplification - *C. parapsilosis* MSK795 from Clade 1, which is related to isolates
315 that have undergone 4 different CNVs (B, C, E, and F), and *C. parapsilosis* MSK247 from
316 Clade 5, which is related to isolates with CNVs A, D, J and M (Fig 1; Fig 4). Five lineages
317 were evolved from MSK247 (247A to 247E) and one from MSK795 (795B). Isolates were
318 cultured in YPD with increasing concentrations of miltefosine, up to a maximum of 32 µg/ml,
319 over a 26-day period (Fig 4A). Sixteen randomly picked evolved colonies from each lineage
320 tolerated miltefosine levels of 40 µg/ml (Fig 4B). The genomes of ten isolates derived from
321 MSK247 and two from MSK795 were sequenced, together with the parental strains. The
322 sequenced strains are listed in Methods.
323

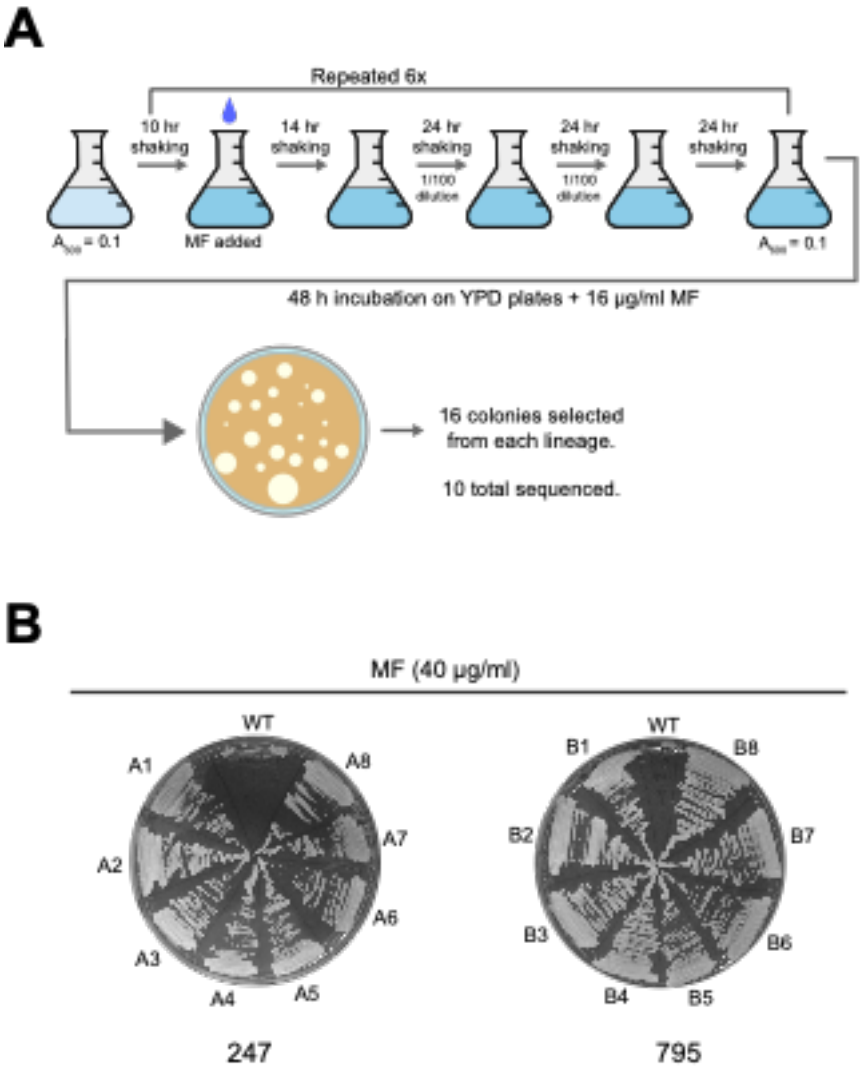


Figure 4. Generation of miltefosine-resistant *C. parapsilosis* isolates by adaptive laboratory evolution

A. Six colonies (5 derived from *C. parapsilosis* MSK247 and one from *C. parapsilosis* MSK795) were inoculated in YPD at A_{600}=0.1,and incubated at 30℃ for 10 h. Miltefosine (indicated with a blue drop) was added to a final concentration of 1 µg/ml, the cultures were incubated for a further 14 h, and then re-inoculated into the same media using a 1/100 dilution and incubated for 24 h. The dilution was repeated every 24 h for 3 days. Cells were then inoculated in fresh media, grown for 10 h, and the miltefosine concentration was doubled. The process was repeated until the concentration of miltefosine reached 32 µg/ml (24 days). On day 25, the overnight cultures were plated on YPD with 16 µg/ml miltefosine. Sixteen colonies picked from each lineage for further analysis, and the genomes of 10 were sequenced.

B. Growth of representative isolates evolved from *C. parapsilosis* MSK247 and *C. parapsilosis* MSK795 on 40 µg/ml miltefosine. WT = parental strain.

341 **Miltefosine-resistant isolates acquired homozygous disruptions in two flippase genes**

342 We did not find any evidence of amplification of the *RTA3* locus in any of the evolved
343 miltefosine-resistant strains. However, by comparing the sequences of the evolved strains to
344 those of the parental strains, we identified two genes with homozygous loss-of-function
345 variants in all 10 resistant isolates: *CPAR2_102700* and *CPAR2_303950*. These variants
346 include frameshifts, nonsense mutations, and missense mutations that are predicted to be
347 deleterious (Fig 5A). *CPAR2_102700* and *CPAR2_303950* encode putative Class 3 P4-
348 ATPases and are homologs of the PC flippase genes *DNF1* and *DNF2* in *S. cerevisiae* (Fig
349 5B).

350 The two sequenced isolates derived from *C. parapsilosis* MSK795 (795B1 and 795B16)
351 acquired mutations in both *CPAR2_102700* and *CPAR2_303950*, whereas lineages derived
352 from *C. parapsilosis* MSK247 acquired mutations only in *CPAR2_303950* (Fig 5A). However,
353 subsequent analysis revealed that *C. parapsilosis* MSK247 contains a homozygous natural
354 variant in *CPAR2_102700*, resulting in a Trp-to-Stop nonsense mutation (W1280X; Fig 5A).
355 *C. parapsilosis* MSK247 tolerates miltefosine concentrations of 5 µg/ml, whereas *C.*
356 *parapsilosis* MSK795 fails to grow (Fig 5C). Derivatives of both parents that carry
357 homozygous inactivating mutations in both *CPAR2_30395* and *CPAR2_102700* can grow up
358 to 30 µg/ml.

359

360

361

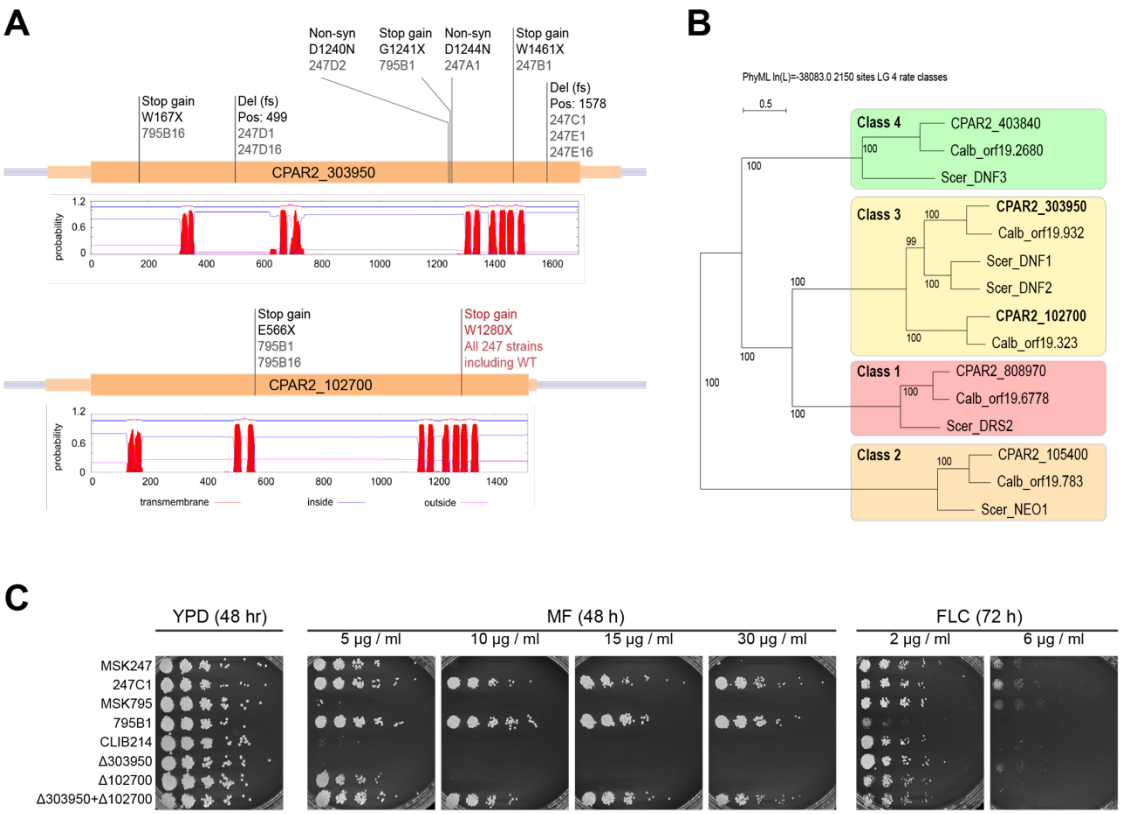


Figure 5. Protein-disrupting mutations in two flippase genes confer resistance to miltefosine in laboratory evolved strains

A. Schematic showing homozygous mutations in P4-ATPases *CPAR2_102700* and *CPAR2_303950*. Mutations named in black were acquired during the laboratory evolution experiment, in the evolved strains named below the mutation name. The mutation named in red is a natural homozygous variant found in the *C. parapsilosis* MSK247 isolate and all strains derived from it. The predicted transmembrane topology of each protein (from TMHMM [54]) is shown, where red peaks show predicted transmembrane domains.

B. Phylogeny of P4-ATPases in *C. parapsilosis*, *C. albicans*, and *S. cerevisiae*. The P4-ATPase genes in three yeast species were identified by BLAST, aligned and a tree was constructed using SeaView [55]. Monophyletic groups of genes were assigned to established classes (1-4) [56]. Both *CPAR2_102700* and *CPAR2_303950* (in bold) belong to Class 3 P4-ATPases.

C. Deleting *CPAR2_102700* and *CPAR2_303950* increases resistance to miltefosine, but not to fluconazole. *CPAR2_102700* and *CPAR2_303950* were deleted singly or together in the *C. parapsilosis* CLIB214 background, and growth was compared to isolates evolved from *C. parapsilosis* MSK247 (247C1) and *C. parapsilosis* MSK795 (795B1), as in Figure 3.

This result suggests that maximum levels of miltefosine resistance requires inactivation of both genes, *CPAR2_30395* and *CPAR2_102700*. To test this hypothesis, we deleted them, both separately and together, in the *C. parapsilosis* CLIB214 genetic background using

383 CRISPR-Cas9 editing (Fig 5C). Deleting *CPAR2_102700* alone in *C. parapsilosis* CLIB214
384 allows growth up to 5 µg/ml miltefosine, whereas deleting *CPAR2_303950* alone has no
385 effect (Fig 5C). However, strains in which both *CPAR2_303950* and *CPAR2_102700* are
386 deleted tolerate at least 30 µg/ml miltefosine, a similar concentration to strains derived from
387 the experimentally evolved isolates (MSK795B1, MSK247C1) (Fig 5C). Deleting
388 *CPAR2_102700* alone, or in combination with *CPAR2_303950*, slightly increases sensitivity
389 to fluconazole (Fig 5C).
390

391 **Discussion**

392 There is some debate over whether Rta3-like proteins are transporters or signaling
393 receptors. Rta3 is a member of the Rta1-family, which encodes proteins with 7
394 transmembrane domains, similar to the structure of G-protein-coupled receptors (GPCRs)
395 [7]. Within this family, Rta3 is more closely related to *S. cerevisiae* Rsb1 than to other
396 members. Most of the early evidence suggested that Rsb1 directly flips LCBs in the plasma
397 membrane [35]. However, Johnson et al [36] suggested that rather than acting as a
398 transporter, Rsb1 determines resistance to the LCB phytosphingosine by regulating
399 endocytosis of the tryptophan transporter Tat2, either by signalling through a G-protein (as a
400 GPCR), or by an arrestin-mediated effect on targeting of ubiquitin ligases. In addition,
401 Srivistava et al (2017) found that deleting *RTA3* in *C. albicans* resulted in increased flipping
402 of a fluorescently labelled PC, possibly by controlling activity of an unknown flippase [33].

403 However, several pieces of evidence suggest that Rsb1 is a direct lipid transporter,
404 specifically a floppase. Firstly, Rsb1 shares no sequence similarity with other yeast GPCRs
405 [7]. Secondly, Makuta et al [57] showed that Rsb1 (and not other Rta1-family members)
406 regulates LCB transport, and that this activity is dependent on a loop region following TMS5,
407 and not the C-terminus as expected for a GPCR. Rsb1 in *S. cerevisiae* and Rta3 in *C.*
408 *albicans* are located in the plasma membrane [33, 34, 41]. The increased flipping observed
409 by Srivistava et al (2017) [33] may be due to crosstalk between sphingolipids and
410 glycerophospholipids (e.g. PC), as shown in *S. cerevisiae* by Kihara and Igarashi (2004)
411 [35]. Changes in the distribution of one may be compensated by changing the distribution of
412 the other [7]. The association of *RTA3* copy number amplification with miltefosine resistance
413 in *C. parapsilosis* is also consistent with a role as a transporter. It is therefore likely that Rta3
414 is a transporter of LCBs, similar to Rsb1, and that its overexpression and deletion cause
415 changes in the localization of both PC and LCBs. Our results also suggest that *C.*
416 *parapsilosis CPAR2_102700* and *CPAR2_303950*, members of the class 3 family of P4-
417 ATPases, are the direct flippases of PC. Our data however cannot rule out the alternative

418 model of Johnson et al [36] whereby Rta3 regulates the function of *CPAR2_102700* and
419 *CPAR2_303950*, rather than directly acting as a transporter.

420 *RTA3* is adjacent to a paralogous gene (called *RTA2*) in many species in the *Candida* (CUG-
421 Ser1) clade [58, 59]. In fact, *C. parapsilosis* CNV-P results from amplification of an
422 *RTA3/RTA2* fusion, with the N-terminus derived from Rta2 and the C-terminus from Rta3
423 (Fig 2C). Deleting *RTA2* in *C. albicans* increases sensitivity to azole drugs, and
424 overexpression increases resistance [60]. Whaley et al [41] showed that deleting *RTA3*
425 increases sensitivity of an azole-resistant isolate of *C. albicans*, but does not have any effect
426 on a reference isolate. Our results show that *C. parapsilosis RTA3* is unlikely to play any role
427 in azole resistance: deleting the gene does not increase sensitivity, and levels of resistance
428 are not associated with *RTA3* copy number in several different genetic backgrounds.
429 However, the sequence similarity between *RTA2* and *RTA3* in *C. parapsilosis* (and in the
430 related species *C. orthopsilosis* and *C. metapsilosis*) is much higher than between *C.*
431 *albicans RTA2* and *RTA3* (S3A Fig). This similarity, and the phylogenetic relationship among
432 these sequences (S3A Fig), indicates that in the ancestor of *C. parapsilosis*/*C.*
433 *orthopsilosis*/*C. metapsilosis*, a gene conversion event occurred in which *RTA3* overwrote
434 *RTA2*. The biggest difference between Rta3 and Rta2 in *C. parapsilosis* is that the former
435 has a longer C-terminus (S3B Fig). Because we have never observed amplifications of
436 *RTA2* in miltefosine-resistant *C. parapsilosis* isolates, we suggest that the two genes may
437 have different functions, and that the difference may be related to the C terminus. In *C.*
438 *albicans*, *RTA2* (and possibly *RTA3*) also contribute to tunicamycin-induced ER stress,
439 which has not been tested in *C. parapsilosis* [61, 62].

440

441 Of the 14 CNVs resulting in tandem array amplification of *RTA3,* only CNV-A and CNV-E
442 have obvious repeat sequences at the CNV endpoints, direct repeats of 19 bp and 10 bp
443 respectively. The other 12 either have no repeats, or the repeat is <4 bp. The longer repeat
444 sequence at the breakpoints of CNV-A may explain why it has originated independently
445 three times (Fig 1). CNV-A and CNV-E may result from non-allelic homologous
446 recombination between the direct repeats [48]. It is likely that CNV-P also originated by non-
447 allelic homologous recombination due to the high sequence similarity between *RTA3* and
448 *RTA2*. CNVs with shorter direct repeats at their endpoints may result from microhomology-
449 mediated break induced replication [63]. However, there is no obvious mechanism for CNVs
450 with no repeats at the breakpoints.

451 CNV-B (upstream from the coding sequence) is particularly intriguing, because it results in
452 inversions and triplications of part of the repeated sequence (S2A Fig). This structure is

453 reminiscent of the origin-dependent ODIRA model for CNV generation [49]. We did not find
454 any evidence of a replication origin in the amplified sequence (S2A Fig), but it remains
455 possible that a rarely used origin is present. It is likely that the amplified region contains a
456 promoter because expression of *RTA3* is increased (Table 1).

457 Our results strongly suggest that there is selective pressure driving amplification of *RTA3* in
458 *C. parapsilosis*. This conclusion is based on our observation that amplification occurred in
459 isolates from diverse genetic and environmental backgrounds, distributed across the *C.*
460 *parapsilosis* phylogeny (Fig 1). We also found that there have been multiple independent
461 events of amplification, with at least 16 unique endpoints. West et al (2021) previously
462 described *RTA3* amplifications in 4 *C. parapsilosis* isolates, including one from the New York
463 subway [43]. Those amplifications also have different endpoints, but because the data
464 comes from metagenomics analyses, we cannot determine if their endpoints differ from the
465 16 CNVs we describe.

466 The CNVs sometimes encompass parts of the adjacent genes, but *RTA3* is the only
467 complete gene amplified in all of them (except for CNV-B, where its likely promoter is
468 amplified, and CNV-H where its 3' end is slightly truncated in the amplified copies). The
469 majority of the isolates in this study originated from clinical sources, although one with an
470 *RTA3* amplification (*C. parapsilosis* UCD321) has an environmental origin (it was isolated
471 from soil). There are several known examples of environmental conditions that induce or
472 selecting for CNVs, such as copper resistance resulting from amplification of *CUP1* in *S.*
473 *cerevisiae* [64, 65], and CNVs associated with growth of *C. albicans* in the presence of
474 fluconazole [66]. It is highly unlikely that the *RTA3* amplifications we discovered were driven
475 by exposure to miltefosine because it is not a commonly used antifungal drug and, as we
476 have shown, exposure to miltefosine is more likely to result in loss-of-function mutations in
477 flippase genes. At present, we do not know what kind of selective pressure led to the
478 widespread amplifications of *RTA3* in *C. parapsilosis*.
479
480 Miltefosine has potential as an antifungal drug [20-22], and it has recently been designated
481 as an orphan drug for the treatment of invasive candidiasis
482 (https://www.accessdata.fda.gov/scripts/opdlisting/oopd/detailedIndex.cfm?cfgridkey=843921
483). However, our analyses suggest that it would be a poor choice, at least for invasive
484 *Candida parapsilosis* infections. Many isolates are naturally resistant because of
485 amplifications of *RTA3*, and in others, resistance rapidly arises due to loss-of-function
486 mutations in the flippase genes. Although mutations in two flippase genes are required for
487 resistance, we note that many of the *C. parapsilosis* samples we examined (56 of 170) have

488 predicted loss-of-function variants in *CPAR2_102700*, meaning that acquiring a mutation in
489 *CPAR2_303950* would be sufficient to render them highly resistant to miltefosine.
490

491 **Materials and methods**

492

493 **Strains and growth**. Isolates used are listed in S1 Table. Isolates were maintained on YPD
494 agar (1% Bacto Yeast Extract (212750, Sigma), 2% Bacto Peptone (211677, Sigma), 2%
495 Bacto Agar (214010, Sigma), 2% D-(+)-Glucose (G8270, Sigma)), and liquid cultures were
496 grown in 5 ml YPD broth without agar at 30ºC and 200 rpm shaking overnight. For serial
497 dilutions, 0.5 ml of the overnight cultures were harvested at 13,000 rpm at room temperature
498 for 1 min, washed twice in 0.5 ml of phosphate-buffered saline (PBS) buffer (BR0014G,
499 Thermofisher), resuspended in 0.5 ml PBS, and diluted to A_{600}= 0.0625 (approximately 6.25 x
500 10^5 cells/ml) in PBS. Five-fold serial dilutions were made in PBS and transferred with a
501 pinner to YPD agar plates containing miltefosine (M5571, Sigma Aldrich) or fluconazole
502 (F8929, Sigma Aldrich) at the indicated concentrations. Pinned plates were incubated at
503 30ºC for the indicated times and photographed using a Singer PhenoBooth.
504

505 **Gene deletions/disruptions.** The entire open reading frames of *CPAR2_104610* (*RTA3*)
506 and the flippases *CPAR2_303950* and *CPAR2_102700* were deleted using CRISPR-Cas9
507 with the pCP-tRNA system, as described in Lombardi et al [53]. All primers used for gRNA
508 and repair template synthesis are listed in S2 Table.
509

510 **Microevolution of miltefosine resistant isolates**. The microevolution method was
511 modified from Papp et al [67] and Ene et al. [68]. Three colonies from *C. parapsilosis*
512 MSK247 and *C. parapsilosis* MSK795 were originally chosen. Subsequent analysis showed
513 that 5 evolved lineages were derived from *C. parapsilosis* MSK247 (247A to E), and one
514 from *C. parapsilosis* MSK795 (795B). Colonies were incubated for 10 h in 5 ml YPD at 200
515 rpm and 30ºC. Miltefosine was added to a final concentration of 1.0 µg/ml, and the cultures
516 were incubated for a further 14 h. The cultures were diluted (1:100) into fresh media
517 containing the same concentration of miltefosine every 24 h for 3 days. Overnight cultures
518 were then diluted to an A_{600} = 0.1, incubated for 10 h, and the miltefosine concentration was
519 doubled. The 5-day cycle was repeated until a concentration of 32 µg/ml of miltefosine was
520 reached (see Fig 4A). Cultures were diluted and plated on YPD agar plates containing 16
521 µg/ml miltefosine, yielding 100-200 colonies each plate, and were incubated at 30ºC for 48
522 h. The genomes of ten resistant isolates (247A1, 247B1, 247C1, 247D1, 247D2,

523 247D16,247E1, 247E16, 797B1 and 795B16), together with the parental strains were
524 sequenced by Beijing Genomic Institute via DNBseq.
525
526 **Illumina sequencing.** Illumina sequencing of MSK isolates was carried out as described in
527 Zhai et al [42]. 45 *C. parapsilosis* isolates from Centre Hospitalier Universitaire de Nantes,
528 France were screened for resistance to miltefosine, and 16 isolates were chosen for
529 sequencing. Genomic DNA was isolated from 5 ml overnight cultures in YPD at 30℃ using
530 phenol-chloroform-isoamyl alcohol extraction. Cell pellets were resuspended in 200 µl of
531 extraction buffer (Triton X 100 2% m/v, NaCl 100mM, Tris 10mM pH 7.4, EDTA 1mM, SDS
532 1% m/v), transferred to screw cap tubes, and approximately 0.3 g of acid-washed beads and
533 200 µl of Phenol-chloroform-isoamyl alcohol (25:24:1) was added. Cells were lysed using a
534 1600 MiniG bead beater from Spex SamplePrep for 6 x 30 seconds with 30 s pauses in
535 between, and then centrifuged at 14000 rpm for 10 min at room temperature. The
536 supernatant was extracted twice more with 200 µl of TE and 200 µl of phenol-chloroform-
537 isoamyl alcohol and one 30 s agitation in the bead beater. DNA was precipitated using 80 µl
538 of ammonium acetate (7.5M) and 1 ml of 100% isopropanol, washed with 1 ml of 70%
539 ethanol and air-dried. Pellets were resuspended in 400 µl of TE with 1 µl of RNAse A (100
540 mg/ml) incubated overnight at 37℃, DNA was precipitated again, and resuspended in 150 µl
541 water. Illumina sequencing was performed by the UCD Conway Genomics Core using a
542 NextSeq 500. 1 ng of gDNA was tagmented (fragmented and tagged with adapter
543 sequences) using the Nextera kit transposome, Dual-indexed paired-end libraries were
544 prepared using Nextera XT DNA Library Prep kit. An Illumina NextSeq500 mid output 300
545 cycle sequencing kit was used to prepare and run the flowcell (HVGWJAFX2).

546 **Oxford Nanopore sequencing.** Strains were grown overnight in 50 ml YPD broth and
547 genomic DNA was extracted from approximately $4x10^9$ cells using a QIAGEN Genomic-tip
548 100/G kit (10223, QIAGEN) with minor modifications. The lyticase incubation was extended
549 to 2 h, and the proteinase K incubation was extended to overnight (~15 h). DNA libraries
550 were prepared using three different kits as per manufacturer's instructions. Libraries from *C.*
551 *parapsilosis* MSK812 and UCD321 were prepared using a Ligation Sequencing Kit (SQK-
552 LSK109, Oxford Nanopore) using 1 µg of DNA per strain. DNA was repaired using a
553 NEBNext FFPE DNA Repair Mix (M6630, New England Biolabs) and NEBNext Ultra II End
554 repair/dA-tailing Module (E75460, New England Biolabs). Adapters were ligated using
555 NEBNext Quick Ligation Module (E60560, New England Biolabs). Libraries were sequenced
556 on a MinION Mk1C device. Priming, loading and washing was carried using the EXP-
557 FLP002, and SQK-LSK109 and EXP-WSH002 Oxford Nanopore kits respectively as per
558 manufacturer's instructions. The genomes were sequenced in parallel on one flow cell; the

559 first isolate (*C. parapsilosis* MSK812) was sequenced for 24 h, and the flow cell was
560 washed, re-primed and loaded with the *C. parapsilosis* UCD321 isolate for an additional ~48
561 h. Libraries from *C. parapsilosis* MSK802 and MSK803 were prepared using a Rapid
562 Barcode Sequencing Kit (SQK-RBK004, Oxford Nanopore) using 400 ng of DNA per
563 sample. Samples were multiplexed using barcodes R04 and R05 respectively. Libraries
564 were sequenced on an original MinION device for 72 h. Priming and loading was performed
565 using the EXP-FLP002 and SQK-RBK004 kits respectively. The *C. parapsilosis* MSK478
566 library was prepared using a Rapid Sequencing Kit (SQK-RAD004, Oxford Nanopore) using
567 400 ng of DNA, and sequenced for 72 h using an original MinION device. Basecalling was
568 performed for all samples using guppy_basecaller with the following parameters: "--
569 input_path fast5 --save_path fastq -c dna_r9.4.1_450bps_fast.cfg --verbose_logs --
570 cpu_threads_per_caller 5 --num_callers 10". Guppy v.4.2.2+effbaf8 was used for samples
571 MSK812 and UCD321 and Guppy v.3.6 was used for samples MSK802 and MSK803. For
572 multiplexed samples MSK802 and MSK803, demultiplexing was performed using
573 guppy_barcoder with the following parameters: "--barcode_kits SQK-RBK004 -t 30 --
574 verbose_logs --trim_barcodes". Reads were filtered using NanoFilt with the following
575 parameters: "-l 1000 -q 7" [69].

576 **Sequence analysis**. Illumina reads were trimmed with Skewer version 0.2.2 using tags "-m
577 pe -t 4 -l 35 -q 30 -Q 30" [70]. The trimmed reads were aligned to the *C. parapsilosis*
578 reference genome using bwa mem version 0.7.12 . The resulting BAM files were sorted and
579 duplicate reads were marked using GenomeAnalysisToolkit (GATK version 4.0.1.2) SortSam
580 and MarkDuplicates tools respectively [44]. Variants were called with GATK HaplotypeCaller
581 using the tag "--genotyping_mode DISCOVERY", combined using GATK CombineGVCFs
582 and joint-genotyped using GATK GenotypeGVCFs. Variant files were filtered for read depth
583 (<15) using and genotype quality (<40) using GATK VariantFiltration. Additionally, clusters of
584 SNPs (5 SNPs in a 100bp window) were filtered using GATK VariantFiltration. A custom
585 script was used to remove variants that were flanked on either side by a long string of mono-
586 or dinucleotide repeats, and variants that were called as heterozygous but had an allele
587 depth ratio <0.25 or >0.75 (https://github.com/CMOTsean/milt_variant_filtration).
588 Additionally, for tree construction, indels were excluded using GATK SelectVariants with the
589 tag "--select-type-to-include SNP". For analysis of the evolved strains, a custom script was
590 used to filter out variants in the evolved strains that were also present in the respective
591 parent strain (https://github.com/CMOTsean/milt_variant_filtration).
592

593 **SIFT4G analysis**. A SIFT prediction database was created for *C. parapsilosis* using the
594 SIFT4G algorithm [71], and the recommended Uniref90 database as a reference for protein

595 sequences [72]. The *C. parapsilosis* prediction database was used to annotate variants from
596 the evolved strains with whether they are likely to be deleterious to protein function.
597 For each annotated gene in *C. parapsilosis*, the number of evolved strains which carried a
598 variant predicted to be protein function-affecting by SIFT in that gene was tallied. Variants
599 were also visualised using Integrative Genomics Viewer (IGV) to manually check results
600 [73].
601
602 **Phylogeny construction.** Called SNPS were concatenated and heterozygous sites were
603 resolved randomly to either allele by 1000 iterations of Random Repeated Haplotype
604 Sampling [45]. SNP trees were then constructed from each of the 1000 haploid inputs, using
605 RAxML (v8.2.12) with the GTRGAMMA model of nucleotide substitution and the random
606 number seed "-p 12345" [74]. The tree with the highest maximum likelihood score was
607 chosen, and the remaining 999 trees were used to generate branch support values.
608
609 **Estimating copy number.** For each strain with a CNV at *RTA3*, the average coverage
610 across the *RTA3* ORF was found using BEDTools coverage (v2.29.2) [75]. This value was
611 divided by the average genome coverage (found with BEDTools genomecov) and multiplied
612 by two to adjust for ploidy, to calculate an estimate for the copy number of *RTA3*. For CNVs
613 that did not cover the entire *RTA3* ORF, the average coverage of a representative section of
614 the CNV was used instead.
615 MinION read sequences were used to identify the exact CNV copy number for a set of
616 strains. The respective CNV sequences plus 1 kb flanking sequence on either side, were
617 searched against the set of MinION reads for strains MSK478, MSK802, MSK803, MSK812,
618 and UCD321 using BLASTN (v2.10.0) [76]. The search outputs were parsed with RECON-
619 EBB (https://github.com/CMOTsean/recon-ebb) to estimate and visualise copy number from
620 reads which include hits for multiple copies of the CNV sequence and both regions of
621 flanking sequence, i.e the reads which covered the entirety of the repeat region.
622
623 **Replication timing profiles.** Relative replication time was determined by sort-seq as
624 described previously [77]. Briefly, replicating (S phase) and non-replicating (G2 phase) cells
625 were enriched from an asynchronously growing culture by FACS based on DNA content. In
626 each sample, genomic DNA was extracted and subjected to Illumina sequencing to measure
627 relative DNA copy number. Replication timing profiles were generated by normalizing the
628 replicating (S phase) sample read count to the nonreplicating (G2) sample read count in 1-kb
629 windows.
630

631 **Quantitative RT-PCR**. Cell harvesting and RNA extraction methods were adapted from
632 Cravener and Mitchell, 2020 [78]. Cells were inoculated from overnight cultures to an A_{600} of
633 0.2 in 25 ml of pre-warmed YPD broth and incubated at 30°C for 6 h at 200 RPM using an
634 orbital shaker. Cells were then harvested via vacuum filtration using MicroPlus-21 Sterile
635 0.45µm filters (10407713, Whatman) and stored at -80°C for at least 24 h prior to RNA
636 extraction.Cells were lysed by mechanical disruption as recommended in the QIAGEN
637 RNeasy Mini Kit (74104, QIAGEN) with some modification [78]. Cells were lysed in RLT lysis
638 buffer (QIAGEN) and Phenol:Chloroform:Isoamyl Alcohol (25:24:1)(P3803, Sigma) in a 1:1
639 ratio. Lysis was performed using a 1600 MiniG from Spex SamplePrep using 30 s lysis
640 followed by 30 s chilling on ice for a total of 6 min. RNA was extracted using a QIAGEN
641 RNeasy Mini Kit followed by two rounds of DNase digestion: one on-column using the
642 QIAGEN RNase-Free DNase Set (79254, QIAGEN); and one off-column using Invitrogen's
643 TURBO DNA-free Kit (AM1907, ThermoFisher). cDNA was synthesized from 1 µg RNA
644 using M-MLV reverse transcriptase (9PIM170, Promega) and Oligo(dT)15 primers (C110A,
645 Promega), following manufacturer's instructions. qPCR was performed in 20 µl reactions
646 using 50 ng cDNA using FastStart Universal SYBR Green Master (Rox) (4913850001,
647 Sigma) as per manufacturer's instructions on an Agilent Technologies Stratagene Mx3005p
648 machine using default "two-step" settings. All primers are listed in S2 Table. Relative
649 quantification was performed using the 2(-Delta Delta C(T)) method by comparing
650 expression to *ACT1*, and using *C. parapsilosis* CLIB214 as the calibrator strain. Calculations
651 and statistics were performed in R using the pcr package [79].

652 **Data availability**. All sequencing data is deposited at NCBI under BioProject number XXXX
653 (awaiting accession numbers).
654

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672 Table 1. Expression of *RTA3* in strains with different amplifications

Isolate	Relative Expression	Range	P Value
MSK802 (CNV-B)	6.57	2.12 - 20.35	7.37E-03
MSK803 (CNV-B)	2.79	1.24 - 6.25	3.39E-02
MSK808 (CNV-I)	22.50	6.52 - 77.62	6.92E-05
CLIB214 (Reference)	1.00	0.19 - 5.27	N/A

673

674 **References**

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902

903 **Supplementary Table 1** List of strains used.

904 **Supplementary Table 2**. List of primers used

905 **Supplementary Figure 1. Copy number determination of CNV-K repeat**
906 Visualisations of BLASTN results using the CNV-K repeat unit plus 1 kb flanking sequence
907 as query against MinION reads for isolates MSK478 and MSK812 where each plot
908 represents hits against a single read. Each line represents a hit, and adjacent hits are
909 separated vertically for clarity. Read identifiers are shown on the y-axis.
910 A. The exact copy number of CNV-K at both alleles was identified in isolate MSK812. (i)
911 Seven reads in the MSK812 MinION dataset have 8 copies of the CNV-K repeat unit, one is

912 shown as an example. (ii) Eighteen reads in the MSK812 dataset have 6 copies of the CNV-
913 K repeat unit, one is shown as an example.
914 B. MSK478 has at least 11 copies on both alleles. No reads in the MSK478 dataset
915 covered the entirety of the repeat array, i.e. no reads had sequence matching both sides of
916 the query flanking DNA. The read with the highest number of copies of the CNV-K repeat
917 contains 11 copies, establishing a likely lower bound for copy number at both alleles.
918
919 **Supplementary Figure 2. Structure of CNV-B**
920 A. (i) CNV-B consists of a central region (B) flanked by two regions (A and C) bounded
921 by inverted repeat pairs (inward-facing triangles). The CNV occurs upstream of the *RTA3*
922 coding sequence. (ii) CNV-B resolves as a repeat array of regions ABC interspersed with
923 inverted copies of region B.
924 B. The ODIRA model of complex CNV generation, adapted from Brewer et al (2015)
925 [49] under the terms of the Creative Commons Attribution Licence. The topmost diagram has
926 been labelled to demonstrate relationship to the observed CNV in (A).
927 C. Replication profile of strain MSK802 mapped to the *C. parapsilosis* reference
928 genome. Relative DNA copy number, as a proxy for replication time, is on the Y-axis, where
929 high values denote earlier replication. The region containing *RTA3* on chromosome 1 is
930 denoted by a red bar.
931
932 **Supplementary Figure 3. Comparison of Rta2 and Rta3 proteins.**
933 A. A phylogenetic tree was generated from Muscle alignments of Rta2 and Rta3
934 sequences from CUG-Ser species (CGOB) using PhyML, implemented in SeaView
935 [55]. Bootstrap values are shown. The pink box highlights the Rta2 and Rta3 sequences
936 from the *Candida parapsilosis* complex. The gene names are taken from the Candida Gene
937 Order Browser.
938 B. Alignment of *C. parapsilosis* Rta2 and Rta3 generated using Muscle implemented in
939 SeaView.
940