The Ca-loop in thymidylate kinase is critical for growth and contributes to pyrimidine drug sensitivity of Candida albicans

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The yeast Candida albicans is the most prevalent opportunistic fungal pathogen in humans. Drug resistance among C. albicans isolates poses a common challenge, and overcoming this resistance represents an unmet need in managing this common pathogen. Here, we investigated CDC8, encoding thymidylate kinase (TMPK), as a potential drug target for the management of C. albicans infections. We found that the region spanning amino acids 106–123, namely the Ca-loop of C. albicans TMPK (CaTMPK), contributes to the hyperactivity of this enzyme compared with the human enzyme (hTMPK) and to the utilization of deoxyuridine monophosphate (dUMP)/deoxy-5-fluorouridine monophosphate (5-FdUMP) as a substrate. Notably, expression of CaTMPK, but not of hTMPK, produced dUTP/5-FdUTP–mediated DNA toxicity in budding yeast (Saccharomyces cerevisiae). CRISPR-mediated deletion of this Ca-loop in C. albicans revealed that the Ca-loop is critical for fungal growth and susceptibility to 5-fluorouridine (5-Furd). Of note, pathogenic and drug-resistant C. albicans clones were similarly sensitive to 5-FUrd, and we also found that CaTMPK is essential for the growth of C. albicans. In conclusion, these findings not only identified a target site for the development of CaTMPK-selective drugs, but also revealed that 5-FUrd may have potential utility as drug for managing C. albicans infections.

Candida albicans is a yeast species and is the most prevalent fungal pathogen in humans (1). In general, C. albicans growing as budding yeast are tolerated by the host immune system. This fungus becomes pathogenic upon switching to hyphal growth in response to changes in environmental temperature, pH, and nutrient limitation (2). Four classes of antifungal drugs have been developed to treat candidiasis: polyenes, azoles, 5-flucytosine (5-FC), and echinocandins. However, major challenges in candidiasis treatment are the acquisition of drug resistance. It has been suggested that drugs such as azoles generate stress in fungus to drive genome evolution, thus selecting gene mutation for developing drug resistance (3–6). New therapeutic strategies are needed to overcome these obstacles for clinical treatment during infection outbreaks.

Biochemical differences between hosts and pathogens can be exploited to develop selective drugs that have cytotoxic effects on the pathogen but not the host. We targeted an essential enzyme in C. albicans, thereby selectively suppressing fungal growth and overcoming drug resistance. Thymidylate kinase (TMPK) is the key enzyme in the biosynthesis of dTTP, catalyzing the conversion of thymidine monophosphate (dTMP) to thymidine diphosphate (dTDP), which is subsequently phosphorylated by nucleoside diphosphate kinase (NDPK) to form thymidine triphosphate (dTTP) (7, 8). CDC8, which encodes TMPK, is an essential gene in yeast; budding yeasts carrying temperature-sensitive mutations in cdc8 are not viable at nonpermissive temperatures (9–12). In C. albicans, TMPK (CaTMPK) is also encoded by CDC8, but the essentiality of this gene has not been previously characterized. In this study, we used genetically engineered clones to demonstrate CDC8 as an essential gene in viability of C. albicans. The main catalytic modules of TMPK include the LID region, P-loop, DR motif, and elements involved in dTMP binding. The sequences of the P-loop, DR motif, and TMP-binding elements are highly conserved among TMPK orthologues, whereas the sequences of the LID region that contribute to the closed conformation for catalysis are divergent. Given the key function of TMPK in dTTP synthesis and the sequence divergence between humans and pathogens, this study investigated the potential application of CaTMPK from C. albicans in antifungal drug development.

The abbreviations used are: 5-FC, 5-flucytosine; TMPK, thymidylate kinase; hTMPK, human TMPK; CaTMPK, C. albicans TMPK; 5-FdUMP, deoxy-5-fluorouridine monophosphate; 5-FUMP, 5-fluorouridine monophosphate; 5-FdUTP, 5-fluorodeoxyuridine triphosphate; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; 5-FU, 5-fluorouracil; PDB, Protein Data Bank; tTA, tetracycline-regulatable transactivator; Nat, nourseothricin; T5, thymidylate synthase; NDPK, nucleoside diphosphate kinase; DO, dropout; PEI, polyethyleneimine; MIC, minimum inhibitory concentration.

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It has been shown that CaTMPK in complex with ADP and dTMP (PDB code 5UIV) at a resolution of 2.45 Å has a unique surface-exposed loop (termed Ca-loop) (13). However, the functional significance of the Ca-loop in CaTMPK and the molecular mechanism by which the Ca-loop affects catalysis remain unexplored. Here, our data suggest that the Ca-loop mediates hyperactivity of CaTMPK. CRISPR-mediated deletion of this Ca-loop markedly slowed the growth of *C. albicans*, highlighting this region as a new target site for the design of fungi-specific inhibitors. In addition, we provide in vitro and in vivo evidence that CaTMPK is highly efficient at using deoxyuridine monophosphate (dUMP) as substrates, provoking a possible role of dUTP in stress-induced genetic instability of fungi. Moreover, CaTMPK is also capable of utilizing deoxy-5-fluorouridine monophosphate (5-FdUMP) as the substrate, thus mediating 5-FUrd toxicity. We further showed that 5-FUrd is useful for the treatment of infections caused by *C. albicans* strains that are resistant to 5-FC and azoles.

### Results

#### Biochemical differences between hTMPK and CaTMPK

Members of the TMPK enzyme family have been categorized into type I and type II enzymes. Both hTMPK and CaTMPK are type I enzymes (7). The sequences in P-loop, dTMP binding, and the DR motif in catalysis are highly conserved between CaTMPK and hTMPK (Fig. 1A). The structure-based alignment (PDB codes 5UIV and 1E2D) (13, 14) shows that the *Candida*-specific Ca-loop starts from Phe-107 to Lys-118. We have previously developed a series of hTMPK inhibitors (15, 16). Here, we showed that two compounds, YMU1, and 3b, at 1 μM effectively suppressed hTMPK activity, but at 10 μM had no inhibitory activity to CaTMPK (Fig. 1B). We compared the enzyme activities of CaTMPK and hTMPK. The kinetic parameters of CaTMPK and hTMPK are shown in Table 1. The *k*_cat of CaTMPK for dTMP was 15-fold higher than that of hTMPK, with similar *K*_m values observed for ATP and dTMP. Thus, the catalytic efficiency of CaTMPK is much higher than that of

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**Figure 1. Differences between CaTMPK and hTMPK.** A, sequence alignment of CaTMPK and hTMPK. Identical and similar residues are shown in red and yellow, respectively. Key catalytic elements, including the P-loop, DR motif, and LID region, are labeled. The sequence alignment was generated by MUSCLE (37) and presented by using the ESPript 3.0 program (38). B, hTMPK (0.4 μg) and CaTMPK (0.015 μg) were preincubated with hTMPK inhibitors, YMU1, and 3b compounds at the indicated concentration for 10 min, followed by NADH-coupled assay. Data are expressed in % of activity relative to the control reaction without inhibitor treatment; *n* = 3. Bar denotes mean ± SD.
Specific features of TMPK in C. albicans

Table 1

Kinetic parameters of hTMPK, CaTMPK, and CaTMPKΔ107–118 in dTMP-based reaction

|       | $K_{ATP}$ | $K_{dTMP}$ | $k_{cat}$ | $k_{cat}/K_{dTMP}$ |
|-------|-----------|------------|-----------|-------------------|
| hTMPK | 2.53 ± 3.2| 16.3 ± 2.3 | 21.1 ± 0.6| 1.3 ± 0.2         |
| CaTMPK| 4.43 ± 4.2| 24.1 ± 3.2 | 320.8 ± 7.1| 13.5 ± 1.0        |
| CaTMPKΔ107–118 | 100.4 ± 15.5| 37.3 ± 9.0 | 86.8 ± 11.6| 2.5 ± 0.7         |

hTMPK. Taken together, the results suggest that these two TMPKs exhibit distinct properties.

Ca-loop confers hyperactivity on CaTMPK and determines the growth rate of C. albicans

To verify the contribution of the Ca-loop to the catalytic rate of CaTMPK, we generated a Ca-loop-deleted mutant, Δ107–118. Recombinant WT and Δ107–118 CaTMPK proteins were purified for activity assays. The 107–118 deletion led to a 70% reduction in activity (Fig. 2A). The kinetic analysis of this deleted form of CaTMPK showed changes in ATP- and dTMP-binding affinity. $k_{cat}/K_m$ indicates that the catalytic efficiency was decreased about 6-fold by the deletion of the 107–118 loop (Table 1). Because CaTMPK lacking the Ca-loop exhibited decreased activity, we asked whether deletion of this Ca-loop would affect the growth of C. albicans. CaTMPK is the gene product of CDC8. Therefore, we performed CRISPR-mediated deletion of the 319–354 region of the CDC8 gene locus (cdc8Δ107–118) in the nonpathogenic C. albicans HLC54 strain (17). The cells were transformed by a cassette containing Cas9, sgRNA targeting the Ca-loop sequence for Cas9 cleavage, and the donor template from plasmid pGEX-2T-CaTMPKΔ107–118, followed by selection with nourseothricin (Nat) (Fig. 2 and C). The selected clone was analyzed by PCR amplification of the region spanning the Ca-loop sequence of the CDC8 gene locus (Fig. 2D). Sanger sequencing further confirmed that the cdc8Δ107–118 clone had a 36-bp in-frame deletion in the Ca-loop (Fig. 2E). We then compared the growth rates of HLC54 and HLC54[cdc8Δ107–118]. The results showed that HLC54[cdc8Δ107–118] grew much slower than the parental strain HLC54 (Fig. 2F). Taken together, these results show that the Ca-loop is a critical structural element of CaTMPK in the rate of dTDP formation, which is indeed controlling the growth of C. albicans.

Ca-loop allows CaTMPK to use dUMP as a substrate

Given the unique structural feature of the dTMP/LID/Ca-loop of CaTMPK, we further asked whether there is a difference in substrate selectivity between hTMPK and CaTMPK. Here, we performed an isotope labeling–based γ-phosphate transfer assay to analyze the capacity of purified CaTMPK and hTMPK to phosphorylate different dNMPs. As expected, both enzymes were capable of phosphorylating dTMP to dTDP, and dAMP, dGMP, and dCMP were not used as substrates by these enzymes (Fig. 3A). However, there was a striking difference in the capability of CaTMPK to transfer [γ-32P]phosphate from ATP to dUMP, so as 5-FdUMP (Fig. 3B). Analysis of the steady-state kinetics revealed a large difference in the rate of dUMP phosphorylation between hTMPK and CaTMPK (Fig. 3C). The $k_{cat}$ of CaTMPK for dUMP was 6-fold higher than that for dTMP, but the catalytic efficiency ($k_{cat}/K_m$) of CaTMPK for dTMP remained higher than that for dUMP (Table 1 and Fig. 3C). This finding indicates that CaTMPK normally prefers dTMP as a substrate. However, the elevation of the dUMP/dTMP ratio can promote the production of dUDP by CaTMPK.

Next, we asked whether the Ca-loop is involved in the utilization of dUMP as a substrate. Similar to the results of the dTMP-based assay, the Δ107–118 was defective in dUMP utilization (Fig. 3D), indicating that the Ca-loop determines the utilization of dUMP as a substrate in the enzymatic reaction. Comparison of the substrate specificity between WT and Ca-loop-deleted CaTMPK by [γ-32P]ATP transfer assay using different dNMPs further confirmed the contribution of the Ca-loop in dUMP phosphorylation (Fig. 3E).

Distinct dUTP/5-FdUTP–mediated DNA toxicity in CaTMPK- and hTMPK-expressing Saccharomyces cerevisiae

Thymidylate synthase (TS) is the enzyme responsible for converting dUMP to dTMP. Upon uptake, 5-fluorouracil (5-FU) and 5-fluorouridine (5-FUrd) are metabolically converted to 5-FUMP and 5-FdUMP (18), the latter of which is a suicide inhibitor of TS. Therefore, treatment with 5-FU or 5-FUrd leads to dUMP accumulation. A previous study has shown that 5-FU–induced death in S. cerevisiae is reversed by deletion of uracil–DNA-glycosylase (UNG1) (19). This finding suggests that 5-FU treatment causes misincorporation of dUTP, the removal of which by Ung1 leads to DNA toxicity and cell death. Because CaTMPK is hyperactive in the conversion of dUMP to dUDP, we then asked whether CaTMPK and hTMPK can mediate the differential toxicity of 5-FU and 5-FUrd in S. cerevisiae. RWY-42-22B, harboring a temperature-sensitive mutation of cdc8α, is an S. cerevisiae strain that can grow at 23°C normally but is not viable at 30°C (12). The growth of this strain at nonpermissive temperatures was rescued by expression of hTMPK or CaTMPK without a significant difference in growth rate (Fig. S1). To avoid complications associated with endogenous cdc8α, two strains expressing hTMPK or CaTMPK were further engineered by replacement of cdc8α with HIS3. We tested the sensitivity of these two strains to 5-FU. Interestingly, the growth of yeast expressing CaTMPK was obviously suppressed along a gradient increase in 5-FU concentration (0–2.5 μM) in agar plates; in contrast, only a slight response was observed in hTMPK-expressing yeast (Fig. 4A). To determine whether 5-FU–induced death in the CaTMPK-expressing strain was associated with misincorporation of dUTP/5-FdUTP, ung1 was deleted from this strain for the 5-FU sensitivity assay. The results showed that deletion of ung1 allowed the CaTMPK strain to survive in 5-FU–containing medium (Fig. 4A). Similar results were observed in 5-FUrd gradient (0–50 μM) agar plates. A high concentration of 5-FUrd was required for suppression of CaTMPK-expressing yeast growth, most likely due to the lower activity of uridine permease than uracil permease in S. cerevisiae. Nevertheless, ung1 deletion abrogated the 5-FUrd response in CaTMPK-expressing yeast.
conclusion, expression of CaTMPK increases the susceptibility of yeast to 5-FU and 5-FUrd via dUTP/5-FdUTP-mediated DNA toxicity (Fig. 4B).

Sensitivity of C. albicans to 5-FUrd with other drugs

5-FC is an antifungal pyrimidine drug used for the treatment of Candida infection. Upon uptake, 5-FC is converted to 5-FU by cytosine deaminase in fungi. We compared the cytotoxic effects of 5-FU, 5-FC, and 5-FUrd on C. albicans growth. The concentration of 5-FUrd that suppressed the growth of the HLC54 strain was in the nanomolar range, whereas that of 5-FC was in the micromolar range. Thus, the antifungal activity of 5-FUrd is more potent than that of 5-FC. Notably, C. albicans growth was unaffected by 50 nM 5-FU (Fig. 5A). The low 5-FU sensitivity of C. albicans is probably due to the poor uracil permease activity (20, 21).

Given the contribution of the Ca-loop to the utilization of dUMP and 5-FdUMP as substrates of CaTMPK, we next investigated whether deletion of the Ca-loop could change the susceptibility of C. albicans to 5-FUrd. The 5-FUrd susceptibility was then compared in the HLC54 and HLC54[cdc8Δ107–118] strains. Consistent with the growth rate analysis performed in liquid culture (Fig. 2B), the spot assay with serial dilutions on a control plate demonstrated that the growth rate of HLC54[cdc8Δ107–118] was slower than that of HLC54. In contrast to the control plate, the presence of 100 nM 5-FUrd caused more pronounced growth suppression of the parental HLC54 strain than of HLC54[cdc8Δ107–118]. Like S. cerevisiae, C. albicans also lack thymidine kinase. Therefore, thymidylate synthase, which converts dUMP to dTMP, is the only enzyme responsible for dTMP formation. Treatment of 5-FUrd inhibits thymidylate synthase in C. albicans, thereby resulting in dTMP depletion and dUMP accumulation. Although the affinity of CaTMPK to dTMP is 31-fold higher than dUMP, kcat to dTMP is 6-fold lower than dUMP. The decrease of dTMP and the rise in dUMP in 5-FUrd–treated cells can increase the CaTMPK reaction to dUDP formation. The NDPK reaction in dUTP formation would further drive CaTMPK converting more dUMP toward dUDP. Ultimately, the depletion of dTTP with the increase in dUTP leads to DNA toxicity due to dUTP misincorporation. Because 5-FC is intracellularly metabolized to 5-FU, a similar difference was observed between these two strains on the plate containing 50 μM 5-FC (Fig. 5B). Flucona-
zole and amphotericin B are antifungal drugs that target ergosterol (21). Thus, the Ca-loop of CaTMPK makes a unique contribution to fluoropyrimidine susceptibility in C. albicans.

The growth responses of HLC54 and HLC54[cdc8Δ107–118] to fluconazole were similar, whereas in the amphotericin B plate, growth suppression of HLC54[cdc8Δ107–118] was more pronounced than that of the parental strain. A recent study has reported that amphotericin B causes oxidative stress and DNA damage to kill fungus (22). Insufficient supply of dTTP for DNA repair might cause the HLC54[cdc8Δ107–118] strain to be more susceptible to amphotericin B.

Different pathogenic C. albicans isolates from patients are sensitive to 5-FUrd

5-FUrd has not been considered as a drug for the treatment of Candida infection, probably because a high dosage of 5-FU is required. The acquisition of drug resistance in C. albicans has been a challenge in medical care (4, 23–26). Given the high potency of 5-FUrd in suppressing HLC54 by the CLSI method (27), we then assessed 5-FUrd sensitivity in pathogenic C. albicans strains. The MIC50 values of 5-FUrd for ATCC90028 and SC5314 by the CLSI method (27) were 0.03 and 0.04 µg/ml (Table 2), respectively.

We further tested the 5-FUrd sensitivity of a number of pathogenic C. albicans isolates from patients. The MIC50 of 5-FUrd was 0.03 µg/ml for the drug-susceptible isolate and was 0.01–0.08 µg/ml for five 5-FC–resistant isolates, designated non-wildtype strains. For azole-resistant isolates, 5-FUrd continued to efficiently inhibit growth in the range 0.02–0.07 µg/ml.

Because 5-FC is intracellularly metabolized to 5-FU, we asked why the 5-FC–resistant isolates remained sensitive to 5-FUrd. Several mutations in 5-FC–resistant strains were identified in the enzymes involved in the formation of 5-FUMP from 5-FC,
including purine-cytosine permease (Fcy2), cytosine deaminase (Fca1), and uracil phosphoribosyltransferase (Fur1) (25). These 5-FC–resistant but 5-FUrd–sensitive strains might have mutations in these genes. 5-FUrd is transported into \textit{C. albicans} through uridine permease (Fui1) and is then phosphorylated to 5-FUMP by uridine kinase (Urk1) (28). Presumably, activation of 5-FUrd metabolism by the Fui1 and Urk1 pathways can overcome the defects in genes mediating 5-FC activation, namely Fcy2, Fca1, and Fur1, leading to toxicity, as depicted in Fig. 6. One important effect of antifungal drugs is host liver toxicity. We then evaluated the potential toxicity of 5-FUrd in human HepG2 cells and found that the IC$_{50}$ of 5-FUrd was 4 \mu g/ml (Fig. S2). Taken together, these results suggest that 5-FUrd might be a suitable option for antifungal treatment.

\textbf{CaTMPK is essential for the growth of \textit{C. albicans}}

Finally, we verified whether CaTMPK is essential for the growth of pathogenic \textit{C. albicans}. To this end, we generated Tet-Off–regulated CaTMPK clones in a pathogenic strain, \textit{THE1}, which has a tetracycline-regulatable transactivator (tTA) inserted at the \textit{ENO1} locus (29). After disruption of one allele of \textit{CDC8} using the Nat$^R$-flipper cassette (30), the promoter region of another \textit{CDC8} allele was replaced with the Tet-Off promoter (Fig. 7A). Growth curve analysis of clones in doxycycline-containing medium showed that these Tet-Off clones, \textit{H9004 cdc8/p97TetoffCDC8} and \textit{H9004 cdc8/p99TetoffCDC8}, stopped proliferation at 16 h and lost viability after 18 h, whereas one allele-deleted clone (\textit{H9004 cdc8/CDC8}) grew normally (Fig. 7B). The lag time required for completely abolishing viability is probably due to the residual dTTP pool present after repression of CaTMPK that still supported a small fraction of growth. Spot assay confirmed that the viability of these two Tet-Off clones requires \textit{CDC8} in the plate containing doxycycline (Fig. 7C). Thus, CaTMPK is an essential factor for the growth of \textit{C. albicans}, strengthening its potential as a drug target.

\textbf{Discussion}

Members of the TMPK enzyme family have been categorized into type I and type II enzymes. Both hTMPK and CaTMPK are type I enzymes (7). The \textit{in vitro} analyses of this study highlight two major differences between the purified hTMPK and CaTMPK. First, the catalytic efficiency of CaTMPK is 15-fold higher than hTMPK. Second, CaTMPK is highly active in the conversion of dUMP to dUDP and 5-FdUMP to 5-FdUDP. Consistent with the data from biochemical analyses, we provide evidence that this Ca-loop–mediated hyperactivity determines the growth rate of \textit{C. albicans}, suggesting the potential of targeting this site to control infection of this pathogen. Furthermore, the differences in the capacity to use dUMP and

\textit{Figure 4. 5-FU and 5-FUrd sensitivity of yeast expressing hTMPK and CaTMPK. A, S. cerevisiae strains lacking endogenous cdc8$^{\text{ts}}$ alone or in combination with ung1, as indicated, and expressing hTMPK and CaTMPK were used for spot assays on agar plates in the absence or presence of an increasing gradient of 5-FU and 5-FUrd concentrations. Each spot contained 200 cells, and the plates were incubated at 30 °C for 3 days. B, biochemical pathway responsible for 5-FU/5-FUrd–induced DNA toxicity in yeast via CaTMPK. CaTMPK mediates dUTP/5-FdUTP formation in yeast cells treated with 5-FU or 5-FUrd, leading to uracil misincorporation in DNA. Treatment with uracil-DNA glycosylase 1 (Ung1) led to the generation of excess DNA breaks, resulting in toxicity.}

\textit{Figure 5. Ca-loop affects the sensitivity of \textit{C. albicans} to 5-FUrd and 5-FC. A, spot tests of the sensitivity of the HLC54 strain to fluoropyrimidine drugs. Two hundred cells were spotted on SD-uracil plates containing increasing concentrations of 5-FU, 5-FC, and 5-FUrd and incubated at 30 °C for 3 days. B, 5-fold serial dilutions of the parental HLC54 and the HLC54[cdc8$^{\text{ts107-118}}$] mutant were spotted on SD-uracil plates containing the vehicle, 5-FUrd (100 \mu M), 5-FC (50 \mu M), fluconazole (1 \mu g/ml), or amphotericin B (0.05 \mu g/ml).}
Specific features of TMPK in C. albicans

Table 2
Minimum inhibitory concentrations of 5-FUrd and antifungal drugs of clinical use for pathogenic and drug-resistant isolates of C. albicans

Minimum inhibitory concentrations (MICs) of 5-FUrd were measured by the broth microdilution method as described under “Experimental procedures” and determined in accordance with the guidelines in Clinical and Laboratory Standards Institute (CLSI) document M27(27). The abbreviations used are as follows: 5-FC, 5-flucytosine; (R), resistance; (NW), non-wildtype strain.

| Strain          | 5-FUrd   | 5-FC   | Posaconazole | Voriconazole | Itraconazole | Fluconazole |
|-----------------|----------|--------|--------------|--------------|--------------|-------------|
| ATCC90028       | 0.030 ± 0.011 | 0.5 | 0.06 | 0.015 | 0.06 | 1 |
| SC5314          | 0.044 ± 0.003 | 0.06 | 0.015 | 0.015 | 0.06 | 0.5 |
| F2016a067       | 0.049 ± 0.001 | 0.03 | 0.03 | 0.03 | >16(NW) | 64(R) |
| F2016d024       | 0.031 ± 0.003 | 0.03 | 0.25 | 0.5 | >8(R) | 8(R) |
| F2016g048       | 0.063 ± 0.012 | 0.03 | 1(R) | >8(R) | 1 | 256(R) |
| F2015f019       | 0.036 ± 0.008 | 0.03 | 0.12 | 0.06 | 0.12 | 4 |
| F2014a093       | 0.021 ± 0.001 | 0.03 | >8(R) | >8(R) | >16(NW) | >256(R) |
| F2014f090       | 0.028 ± 0.002 | 0.12 | >8(R) | 0.25 | >16(NW) | 2 |
| F2015b076       | 0.026 ± 0.001 | 0.03 | 1 | >8(R) | 0.5 | 256(R) |
| F2017g023       | 0.043 ± 0.001 | >64(NW) | 0.03 | 0.004 | 0.06 | 0.5 |
| F2016e087       | 0.021 ± 0.008 | >64(NW) | 0.06 | 0.015 | 0.12 | 0.5 |
| F2016e085       | 0.076 ± 0.020 | 8(NW) | 0.015 | 0.004 | 0.03 | 0.06 |
| F2017h022       | 0.018 ± 0.002 | >64(NW) | 0.015 | 0.004 | 0.03 | 0.25 |
| F2017h071       | 0.027 ± 0.003 | 4(NW) | 0.015 | 0.004 | 0.03 | 0.25 |

5-FdUMP as substrates support the finding that CaTMPK, but not hTMPK, can mediate dUTP/5-FdUTP toxicity under 5-FU and 5-FUrd treatment in S. cerevisiae. Although 5-FU is not useful for the treatment of C. albicans infection probably because of the lack of transporter, we observed that nonpathogenic and pathogenic strains of C. albicans are very sensitive to 5-FUrd. We propose that the utilization of dUMP and 5-FdUMP as substrates by CaTMPK increases the 5-FUrd susceptibility of C. albicans. In addition, our finding also opens a new question whether the high activity of CaTMPK in dUMP utilization might participate in the mechanism of stress-induced genome evolution (31, 32) of C. albicans by uracil misincorporation in DNA, thereby facilitating the development of drug resistance.

By establishing clones genetically engineered for Tet-Off control of CaTMPK in a pathogenic strain, we provide the first evidence that CaTMPK is essential for supporting viability of C. albicans. CRISPR-mediated deletion was performed to generate a C. albicans strain lacking the Ca-loop of CaTMPK, which led to a markedly reduced growth. This result indicated that the hyperactivity of CaTMPK in dUMP synthesis is an important factor affecting the growth rate of C. albicans. Notably, in this study, we also used the S. cerevisiae expression system to evaluate differences between the effects of hTMPK and CaTMPK on growth and drug sensitivity. Despite the considerable differences in catalytic rates, the growth rates of S. cerevisiae expressing CaTMPK and hTMPK were very similar. This observation is consistent with a previous report that demonstrated that decreased thymidylate kinase activity did not affect the growth of S. cerevisiae (33). Apparently, C. albicans and S. cerevisiae have different requirements for TMPK activity for growth maintenance. Notably, we observed a striking difference in 5-FU and 5-FUrd toxicity between S. cerevisiae strains expressing hTMPK or CaTMPK. Intracellular 5-FU is converted to 5-FdUMP, which is an inhibitor of TS, thereby blocking the synthesis of dTMP from dUMP, which in turn results in the accumulation of dUMP. Because dUMP is not a good substrate for hTMPK, cells expressing hTMPK would have less dUTP accumulation. In contrast, dUMP and 5-FdUMP are good substrates for CaTMPK, and dUDP formation by this
enzyme might facilitate dUTP accumulation, leading to misincorporation in DNA. Uracil is removed from DNA by the action of Ung1, thereby generating DNA breaks. Excess uracil incorporation therefore leads to DNA toxicity. Given that deletion of ung1 abolished 5-FU and 5-FUrd toxicity in S. cerevisiae expressing CaTMPK, it is very clear that high activity of CaTMPK in the utilization of dUMP and 5-FdUMP is responsible for DNA toxicity of 5-FU and 5-FUrd.

In this study, our data revealed 5-FUrd to be a potent drug for the treatment of C. albicans infection. This proof-of-concept
study showed that a number of *C. albicans* isolates that were resistant to azoles or 5-FC were susceptible to 5-FUrd at a dose that had little toxicity in human hepatoma cells. We proposed that 5-FUrd could overcome 5-FC resistance in these strains because the metabolic activation pathways of 5-FUrd and 5-FC are different. Therefore, 5-FC resistance resulting from mutations in genes mediating the metabolic activation of 5-FC might not affect 5-FUrd toxicity.

In summary, this report reveals several novel points. First, there are biochemical differences between the essential enzyme TMPK from *C. albicans* and host (human) TMPK due to the Ca-loop. CaTMPK is essential for the growth of the pathogenic strain of *C. albicans*, highlighting this enzyme as a new drug target. Second, 5-FUrd is a potent inhibitor of *C. albicans* growth due to the involvement of the Ca-loop in 5-FUrd toxicity. Third, 5-FUrd is able to overcome 5-FC and multidrug resistance in pathogenic *C. albicans* isolates.

**Experimental procedures**

**Media and chemicals**

All *S. cerevisiae* strains in this study were grown in synthetic defined (SD) medium containing 2% glucose, 6.7 g/liter yeast nitrogen base without amino acids, and 0.77 g of dropout (DO) supplements — Ura (Clontech). *C. albicans* were grown in YPD (1% yeast extract, 2% bactopeptone, and 2% glucose), synthetic complete medium (SC medium: 6.7 g/liter yeast nitrogen base without amino acids, 0.77 g of DO supplements — Ura, and 80 mg/liter uridine), or SD medium at 30 °C. For drug susceptibility analysis, *C. albicans* were grown in RPMI 1640 medium (Sigma) and buffering with MOPS (Sigma). For solid media, 2% yeast nitrogen base without amino acids, 0.77 g of dropout (DO) supplements — Ura, and 80 mg/liter uridine, or SD medium at 30 °C. For drug susceptibility analysis, *C. albicans* were grown in RPMI 1640 medium (Sigma) and buffering with MOPS (Sigma). For solid media, 2% yeast nitrogen base without amino acids, 0.77 g of dropout (DO) supplements — Ura, and 80 mg/liter uridine, or SD medium at 30 °C. For drug susceptibility analysis, *C. albicans* were grown in RPMI 1640 medium (Sigma) and buffering with MOPS (Sigma).

**Plasmids construction**

*C. albicans* CDC8 in pUC57 was purchased from GenScript (catalog no. 5001191-1). Because of the difference in CTG codon usage for serine in *C. albicans* and leucine in *Escherichia coli* and *S. cerevisiae*, the expression of the CDC8 gene in *E. coli* and *S. cerevisiae* would cause S68L mutation. This codon of the CDC8 gene was mutated to TCG by site-directed mutagenesis to maintain the correct protein sequence termed CaTMPK when expressed in *E. coli* and *S. cerevisiae*. CaTMPK and hTMPK cloned to pGEX-2T were used to produce recombinant protein CaTMPK and hTMPK in *E. coli*. CaTMPK deleted of 107–118 amino acids were generated by site-directed PCR mutagenesis in pGEX-2T-CaTMPK. For expression in *S. cerevisiae*, PCR-amplified hTMPK and CaTMPK fragments were each cloned to *pRS416* plasmid (gift from M. Y Chen, National Yang-Ming University, Taiwan) containing the *TEF1* promoter and *CYC1* terminator. The plasmids pV1025 and pV1090 (kindly provided by Valmik K. Vyas, Cambridge, MA) were used for CRISPR genome editing in *C. albicans*. The plasmid pV1090-17 carrying the sgRNA sequence targeting *CDC8* in *C. albicans* was cloned at BsmBI restriction sites of the plasmid pV1090, and the plasmid pV1025 contains Cas9 for expression in *C. albicans*. Plasmid pSF2AS was provided by Jia-Ching Shieh (Chung Shan Medical University, Taiwan) (30). p97CAU1 and p99CAU1 were from Chung-Yu Lan (National Tsing Hua University, Taiwan) (29). These plasmids were used for construction of the Tet-Off CDC8 system in *C. albicans*. The plasmid for deleting one allele of the CDC8 cassette was constructed. The 5′-flanking region (nucleotides −1 to −470) and the 3′-flanking region (nucleotides +676 to +1195) of the coding sequence of *CDC8* were PCR-amplified from the genomic DNA of strain *SC5314* and cloned into plasmid pSF2AS at KpnI/Xhol and NotI/Sacl sites, respectively, to generate pSF2AS-CDC8. Plasmids p97CAU1-CDC8 and p99CAU1-CDC8, used for replacing the promoter of *CDC8* with the Tet-Off promoter, were generated by insertion of the 5′-flanking region (nucleotides −106 to −579) and the 3′-flanking region (nucleotides +1 to +479) of *CDC8* coding sequence into plasmids p97CAU1 and p99CAU1 at the KpnI/Xhol and SpeI/XbaI sites, respectively. Plasmids and primers in this study are listed in Tables S2 and S3.

**Establishment of *S. cerevisiae* and *C. albicans* strains**

*S. cerevisiae* strains RWY42-22A (Mata CDC8) and RWY42-22B (Mata cdc8-1) were provided by Raymund J. Welling (Université de Sherbrooke). RWY42-22B (Mata cdc8-1) contains temperature-sensitive (ts) mutation allele of cdc8 (cdc8-1), which is able to grow at 23 °C but not at 30 °C. The isogenic *CDC8* strain (WT) (12), RWY42-22A, is able to grow at both temperatures. RWY42-22B was further transformed with plasmids pRS416-TEFI-hTMPK-CYC1 and pRS416-TEFI-CaTMPK-CYC1, respectively, and replaced the endogenous *cdc8* with the HIS3 gene, thus generating RWY42-22B-1A and RWY42-22B-1B. The endogenous *UNG1* gene of RWY42-22B-1A and RWY42-22B-1B was replaced by the TRP1 marker, which was amplified from the plasmid *pRS314* (provided by M.Y. Chen, National Yang-Ming University, Taiwan), thus generating RWY42-22B-2A and RWY42-22B-2B strains.

Nonpathogenic *C. albicans* strain, *HCLS4* (34) (provided by Hsiu-Jung Lo, National Health Research Institutes, Taiwan), was used to generate endogenous Δ107–118 mutation in endogenous *CDC8* locus by a CRISPR system (17). Briefly, pV1090-17 and pV1025 (20 μg) were linearized before transformation by a modified LiAC method (35). For Ca-loop deletion in the endogenous *CDC8* locus, donor templates (378 bp) were amplified from the plasmid pGEX-2T-CaTMPKA107–118 and purified for transformation along with linearized pV1090-17 and pV1025. The transformants were spread on the YPD plate containing Nat at 250 μg/ml and incubated at 30 °C for 3 days. The colonies grown on the Nat plate were isolated for genomic DNA extraction followed by PCR examination.

Clones in which one allele of CDC8 deleted and another allele of CDC8 coding gene under Tet-off promoter were generated in a pathogenic *C. albicans* THE1 strain carrying tTA (29). One allele of *CDC8* was first deleted by transforming *THE1* cells with KpnI/Sacl linearized plasmid pSF2AS-CDC8 to generate *THE1-CDC8*, which is Δ*cdc8/CDC8* (Table S1). The promoter
replacement cassettes were PCR-amplified from plasmids p97CAU1-CDC8 and p99CAU1-CDC8 by primer pairs: 5'-GGTACGGTTTCTGTTAAGTACTGTTGCG (nucleotides -579 to -554 of the CDC8 coding sequence, forward primer in Table S3) and 5'-GTTATACACCCATCTTTTCTTTGT (nucleotides +479 to +451 of CDC8 coding sequence, reverse primer in Table S3). PCR products were purified and transformed into the THE1-CDC8 strain to generate Δcdc8/p97Tet-offCDC8 and Δcdc8/p99Tet-offCDC8 (THE1-p97CDC8 and THE1-p99CDC8 in Table S1). All strains in this study are listed in Table S1.

**Purification of recombinant proteins**

pGEX-5X-HSV-TK1, pGEX-2T-hTMPK, and pGEX-2T-CaTMPK (WT/Ca-loop mutants) were each transformed into an E. coli BL21 strain. A single clone was inoculated in 15 ml of LB broth and cultured overnight at 37 °C. 10 ml of the overnight culture was diluted to 1000 ml, and cell growth was continued at 37 °C until the A600 reached 0.5. To induce hTMPK and CaTMPK expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the culture was incubated for another 3.5 h at 37 and 32 °C, respectively. For HSV-TK1 induction, 0.5 mM IPTG was added, and the culture was incubated at 27 °C for 16 h. Cells were harvested by centrifugation and resuspended in 20 ml of lysis buffer containing 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1 mM PMSF, and 1 mM protease mixture before being disrupted by sonication. Following centrifugation, 2 ml of GST-Sepharose beads was added to the clarified cell lysate and incubated with gentle shaking at 4 °C for 1.5 h. Beads were washed five times with a buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 mM protease mixture. The GST-tag was then cleaved from the target proteins by thrombin.

**In vitro activity assay (NADH-coupled assay)**

Activity assay was measured using a spectrophotometric method by coupling ADP formation to the oxidation of NADH catalyzed by pyruvate kinase and lactate dehydrogenase. Reaction mixture for each set in 100 μl contained 500 μM ATP, 500 μM dTMP (for measurement of dUMP activity, 2 mM ATP, and 10 mM dUMP), 100 mM Tris-HCl, 100 mM KCl and 10 mM MgCl2, 500 μM phosphoenolpyruvate, 250 μM NADH, 4 units of pyruvate kinase, and 5 units of lactate dehydrogenase. Reaction was initiated by adding purified proteins (0.4 μg of hTMPK or 0.015 μg of CaTMPK) and measured the reduction of NADH by 340 nm. For the hTMPK inhibitor assay, both hTMPK (0.4 μg) and CaTMPK (0.015 μg) were preincubated with compounds at the indicated concentrations for 10 min, followed by adding reaction mixtures as described above and measuring A340. The Km values for dTMP and dUMP were measured by various concentrations of substrates using 500 μM ATP (2 mM for dUMP). This concentration followed previous work (16). The Km values were obtained by varying concentrations using 250 μM dTMP. The Km values were obtained by fitting the Michaelis-Menten equation.

**[32P]phosphate transfer assay**

The substrate selectivity of hTMPK and CaTMPK was measured by the [32P]phosphate transfer assay. TMPK (0.2 μg) and CaTMPK (0.02 μg) were added to the TMPK reaction mixture containing 0.05 μM [γ-32P]ATP (10 μCi/μl), 50 μM ATP, 100 mM Tris-HCl, 100 mM KCl, and 10 mM MgCl2, for 30 min.

The coupled reaction was performed by preincubating 1 μg of HSV-TK1, 0.2 μg of hTMPK (or 0.02 μg of CaTMPK) and 500 μM nucleosides for 5 min, followed by adding TMPK reaction mixtures containing 0.1 μM [γ-32P]ATP (10 μCi/μl), 100 μM ATP, 100 mM Tris-HCl, 100 mM KCl, and 10 mM MgCl2. Reaction mixtures were incubated for 30 min. To analyze the substrate selectivity of WT and Ca-loop deletion of CaTMPK, reaction mixtures containing 0.1 μM [γ-32P]ATP (10 μCi/μl), 100 μM ATP, 100 mM Tris-HCl, 100 mM KCl, and 10 mM MgCl2 were added to CaTMPK (0.02 μg) or CaTMPKΔ107–118 (0.06 μg) with 1 mM dNMP and incubated for 15 min. The assay was terminated by heating at 95 °C. 2 μl of reaction mixture was spotted onto PEI-cellulose thin-layer chromatography (TLC). After air drying, 2 μl of the corresponding dNMP (20 mM) was also spotted on TLC for separation by 2 M acetic acid, 0.5 mM LiCl. The position of nucleotides on TLC sheet was visualized by UV, followed by autoradiography for assessing [32P]phosphate transfer.

**Yeast growth curve and drug susceptibility tests**

Cells at 10^4 cells/ml in SD – Ura medium or SC medium were plated onto a clear flat-bottom 96-well plate and incubated at 30 °C in a TECAN SPARK plate reader to obtain growth curve by A600 reading every 30 min.

MICs of 5-FUrd were determined by the broth microdilution method in accordance with the guidelines in Clinical and Laboratory Standards Institute (CLSI) document M27 (27), using RPMI 1640 medium containing 0.165 M MOPS, pH 7.0, and 0.2% glucose, an inoculum of 10^3 cells/well, and incubating for 24 h at 35 °C, followed by A600 reading by TECAN SPARK plate reader. The MIC of 5-FUrd was determined as the lowest dosage of 5-FUrd causing 50% inhibition of the growth by using the concentrations in the range of 0.004 – 2 μg/ml. MICs of nine antifungal agents of human use (5-FC; four azoles: posaconazole, voriconazole, itraconazole, and fluconazole; and three echinocandins: anidulafungin, caspofungin, and micafungin) were determined by the microdilution colorimetric Sensititre YeastOne SYO-09 panel (TREK Diagnostic Systems, Cleveland, OH), in accordance with the manufacturer’s instructions. MIC values were determined visually, after 24 h of incubation, as the lowest concentration of drug that caused complete inhibition (amphotericin B) or a significant diminution (≥50% inhibition; flucytosine, azoles, and echinocandins) of growth relative to that of the growth control.

For drug susceptibility assay on a solid medium plate, S. cerevisiae or C. albicans colonies were inoculated in 5 ml of SD selection media and grown overnight to logarithmic phase at 30 °C. For gradient plate, cells were counted, and 200 cells were spotted onto solid medium containing an increasing concentration of drugs in the agar. For plates with fixed concentra-
tions of drug, the cultures were adjusted to 0.1 by A₆₀₀ and then serially diluted 5-fold before spotting on plates. The plates were incubated at 30 °C for 2–3 days.

**Statistical analysis**

Data are presented as the mean ± S.E. of the mean. Statistical comparison of means was performed using a two-tailed unpaired Student's t test.

**Author contributions**—C.-Y. H., Y.-C. C., and Z.-F. C. data curation; C.-Y. H. formal analysis; C.-Y. H. validation; C.-Y. H. investigation; C.-Y. H. and Z.-F. C. methodology; C.-Y. H. and Z.-F. C. writing-original draft; B. A. W.-H. and J.-M. F. resources; Z.-F. C. conceptualization; Z.-F. C. writing-review and editing.

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