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ORIGINAL ARTICLE

Aif1p inhibits development of fluconazole resistance in yeast

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Abstract Apoptosis-inducing factor 1 (AIF1) was originally considered to induce apoptosis in yeast through a partially caspase-dependent pathway. In our research work, we demonstrated that aif1p could also inhibit fluconazole resistance in yeast. Several ABC transporters such as PDR5, PDR10 and HXT11 were upregulated in an *aif1* mutant. A rhodamine 6 G (R6G) efflux study demonstrated increased extrusion of R6G through efflux pumps in the *aif1* mutant. Therefore, we concluded that aif1p may inhibit the development of FLC resistance in yeast via some ABC transporters induction.

Keywords AIF1 · Fluconazole sensitivity · Yeast

Introduction

In mammals, apoptosis-inducing factor (AIF) is a flavoprotein with oxido-reductase activity which is localized in the mitochondrial intermembrane space (Susin et al. 1999; Miramar et al. 2001). Through apoptotic induction, AIF translocates to the nucleus, where it leads to chromatin condensation and DNA degradation (Susin et al. 1999). AIF has been proved to control a caspase-independent pathway of apoptosis, which is important for neurodegeneration and normal development (Susin et al. 1999; Cregan et al. 2002). It represents a phylogenetically old sequence. Aside from mammals, AIF-like proteins can be identified in frogs, flies, worms, yeasts, and higher plants (Lorenzo et al. 1999). *Saccharomyces cerevisiae* has an AIF/AMID/AIFL homologue (AIF-1; gene AIF1) with high identity to mammalian AIF mainly in the oxidoreductase domain (Wissing et al. 2004). This study showed that yeast AIF1 gene disruption renders cells resistant to oxygen stress and delays age-induced apoptosis (Wissing et al. 2004). The enhanced resistance to oxidative stress of AIF1-disrupted cells appears opposite to the situation in mammals, where the loss of AIF renders many cell types less able to deal with oxidative stress (Lipton and Bossy-Wetzel 2002; Klein et al. 2002; Cande et al. 2004; van Empel et al. 2005). Furthermore, orthologues of core regulators of mammalian apoptosis such as caspases, HtrA2/Omi, and the proteasomal death pathways have been shown to be conserved in yeast (Blanchard et al. 2002; Madeo et al. 2002; Fahrenkrog et al. 2004).

Azole antifungals are commonly used for fungal infections, but azole resistance can be a problem for some patient groups. High-level, clinically significant azole resistance usually involves overexpression of plasma membrane efflux pumps belonging to the ATP-binding cassette (ABC) or the major facilitator superfamily class of transporters (Cannon et al. 2009). *Saccharomyces cerevisiae* represents a practical and conventional system for studying the properties of antifungal compounds, not only against fungal human pathogens to which they are closely related (e.g., *Candida albicans*) (Barns et al. 1991) but also with those that are evolutionarily more distant (e.g., filamentous fungi). In this study, we found that the aif1p mutant and wild-type strain showed different fluconazole sensitivity. Several ABC transpoters such as PDR5, PDR10 and HXT11 were upregulated in the *aif1* mutant. Rhodamine 6 G (R6G) efflux study demonstrated increased extrusion of R6G through efflux pumps in the *aif1* mutant. So we next investigated the role of aif1p in the development of fluconazole resistance in yeast.

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Materials and methods

Antifungals

Fluconazole (FLC) was obtained from Pfizer (New York, NY, USA). Miconazole (MCZ) and Rhodamine 6 G were purchased from Sigma Chemical (St. Louis, MO, USA). Itraconazole was provided as a sponsor by Prof. Liu (The Second Military Medical University, Shanghai, China). Terbinafine (TRB), Amphotericin B (AmB) and Rhodamine 6 G were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml, 1 mg/ml and 10 mM, respectively.

Strains and growth media

Saccharomyces cerevisiae BY4741 and aif1 mutant 6233 were purchased from invitrogen (CA, USA). The strains were cultivated at 30°C under constant shaking (200 rpm) in a complete liquid medium YPD consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose. For agar plates, 2% (w/v) Bacto agar (Difco; BD Biosciences) was added to the medium. Uridine (20 mg/ml) was added to ensure the growth of the strains.

Growth curve study

The effect of FLC exposure related to the time and concentration on the strains was determined in the YPD liquid medium. FLC was added to the cultures for different concentration ladders, the final concentration of which was 16 μg/ml. The growth was monitored by measuring the optical density (600 nm) of the cultures during the subsequent 72 h.

Time–kill curves

Saccharomyces cerevisiae was grown in YPD medium with vigorous shaking at 30°C for 16 h. Then, RPMI 1640 medium was added at the starting inoculum of 10^6 CFU/ml, diluting it to 10^3 CFU/ml. Cell suspensions were incubated with fluconazole concentrations of 1, 8, 16, and 64 μg/ml, respectively. DMSO comprised <1% of the total test volume. After incubation with agitation at 30°C for 24 h, a 100-μl aliquot was removed from each solution and serially diluted 10-fold in sterile water. A 100-μl aliquot from each dilution was streaked on the Sabouraud dextrose agar (SDA) plate. Colony counts were determined after the incubation at 30°C for 48 h. The experiment was performed in triplicate. Antifungal activity were defined as a respective increase or decrease of ΔlogCFU. ΔlogCFU= logarithm of CFU after 24 h of exposure − logarithm of CFU at time zero.

Susceptibility testing

The sensitivities of the mutant strains were also tested on agar plates containing different of azoles (fluconazole, miconazole) or other agents (terbinafine, amphotericin B). Five microliters of tenfold serial dilutions of each yeast culture (OD_{600}=1.0) were spotted on the appropriate medium plates and then incubated at 30°C for the indicated times.

Rhodamine 6 G efflux by S. cerevisiae cells

Passive efflux of Rhodamine 6 G (Sigma) was determined by adapting the method described by Kolaczkowski et al. (1996). Approximately 1.0×10^7 cells in the absence or presence of fluconazole (16 μg/ml) from an overnight culture were incubated in 200 ml of YPD medium and grown for 5 h at 30°C. The cells were pelleted and washed three times with phosphate buffered saline (PBS) buffer without glucose. The cells were resuspended in PBS buffer to a concentration of 1.0×10^8 cells/ml and shaken at 30°C for 1 h to exhaust the energy, after which Rhodamine 6 G was added at a final concentration of 10 mM and the suspension was incubated for 2 h at 30°C. Then, the cells were washed three times with PBS before adding glucose (final concentration, 2 mM) to initiate Rhodamine 6 G efflux. At the specific intervals after adding glucose, the cells were centrifuged and 100 μl of supernatant was transferred to the wells of 96-well flat-bottom microtiter plates (BMG Labtechnologies, Offenburg, Germany). Rhodamine 6 G fluorescence densities of the samples were measured with a Universal Microplate Spectrophotometer (TECAN M200; Switzerland). The excitation wavelength was set at 515 nm, and an emission wavelength was set at 555 nm.

Quantitative Real-Time RT-PCR

RNA isolation and real-time RT-PCR were performed as described previously (Wang et al. 2006). The isolated RNA was re-suspended in diethyl pyrocarbonate-treated water. The OD_{260} and OD_{280} were measured and the integrity of the RNA was visualised by subjecting 2–5 μl of the samples to electrophoresis through a 1% agarose-MOPS gel. First-strand cDNA was synthesised from 3 μg of total RNA in a 60-μl reaction volume using the cDNA synthesis kit for RT-PCR (TaKaRa Biotechnology, Dalian, P.R. China) in accordance with the manufacturer's instructions. Triplicate independent quantitative real-time PCRs were performed using the LightCycler® System (Roche Diagnostics, Mannheim, Germany). SYBR Green I (TaKaRa Biotechnology) was used to visualize and monitor the amplified product in real time according to the manufacturer's protocol. Gene-specific primers were designed for the genes of interest and the 18S rRNA (Table 1) according to the manufacturer's protocol.

Table 1 The sequences of the primers used in this study [a]

Primer name	Sequence
PDR1-F	CTGAAGTGGGTATTTGC
PDR1-R	TAGGTCATTATCCGTGTC
PDR5-F	AAAGCGGTAGCGGAAT
PDR5-R	CGTATCAAGGGTGTCG
SNQ2-F	CTTTGACGGATGCTGTG
SNQ2-R	TTTTGAGGAAGGGTGGA
YOR1-F	CATTTCGGGCTTTCTAT
YOR1-R	GAGCCTTTCCTATTTCC
PDR10-F	TCTCCTCTTCCCATTCT
PDR10-R	CATCTTCTGCGTCTCAA
PDR15-F	CTCCCAAGAACAACCAACAG
PDR15-R	GCTTACGAAAGGGCTCAG
HXT11-F	AACCCATTCTTACTTTCG
HXT11-R	GAGCCACCTATTGATTAC
YAP1-F	CTCGGAACATAAACCAT
YAP1-R	AAGCGAAATCTCACAA
ACT1-F	GTCGGTAGACCAAGACAC
ACT1-R	AGAAGGTATGATGCCAGA
18S-F	GTGCCAGCAGCCGCGGTA
18S-R	TGGACCGGCCAGCCAAGC

F Forward primer, *R* reverse primer

The PCR protocol consisted of denaturation (95°C for 1 min), 40 cycles of amplification and quantification (95°C for 10 s, 60°C for 20 s and 72°C for 30 s with a single fluorescence measurement), melting (60–95°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement) and finally a cooling step to 40°C. The change in fluorescence of SYBR Green I dye in every cycle was monitored by the LightCycler system software (Roche Diagnostics) and the threshold cycle (CT) above background for each reaction was calculated. ACT1 RT-PCR products were used as internal standard. The CT value of ACT1 was subtracted from that of the gene of interest to obtain a △CT value. The △CT value of an arbitrary calibrator was subtracted from the △CT value of each sample to obtain a △△CT value. The gene expression level relative to the calibrator was expressed as $2^{-\triangle\triangle CT}$.

Results

Disruption of AIF1 resulted in resistance to FLC in time-growth

We observed the effect of FLC on the strains. The growth tendency of the *aif1* mutant 6233 and the wild-type BY4741 showed little difference in the absence of FLC. In the presence of 16 μg/ml FLC, both two strains were inhibited within 20 h. After 36 h incubation, the *aif1* mutant showed more growth than the wild-type BY4741. It was proved that the *aif1* mutant 6233 was more resistant to fluconazole comparing with the wild-type BY4741 (Fig. 1).

Further time–kill studies were conducted with FLC against Wild Type and △aif1 Strain

As shown in Fig. 2, two strains showed no differences in the absence of FLC. When 1 μg/ml FLC was added, the concentration of the wild-type strain BY4741 was dramatically decreased under the starting inoculum of 10^3 CFU/ml while the △*aif1* strain 6233 showed little inhibition. After FLC was added at the concentrations of 8 μg/ml and 16 μg/ml, respectively, the CFU produced by the △*aif1* strain 6233 gradually declined, but was still higher than CFU produced by the wild -type strain BY4741. When the fluconazole concentration changed gradually from 8 to 16 μg/ml, the inhibition for both two strains kept stable. The study further demonstrated that AIF1 inhibited fluconazole resistance more than the wild-type BY4741 did.

Disruption of AIF1 resulted in resistance to fluconazole in spot assay

Spot assay showed that the △*aif1* strain 6233 was more resistant than the wild-type strain when 8 and 16 μg/ml FLC were added. Moreover, after incubation with 8 μg/ml FLC for 22 days, the strain 6233 still showed more resistance to fluconazole than BY4741. Both strains showed more resistance than the untreated strains. This indicated that Aif1p could inhibit the development of fluconazole resistance. The strain 6233 also showed cross-resistance to miconazole. Meanwhile, it showed no obvious effect on the

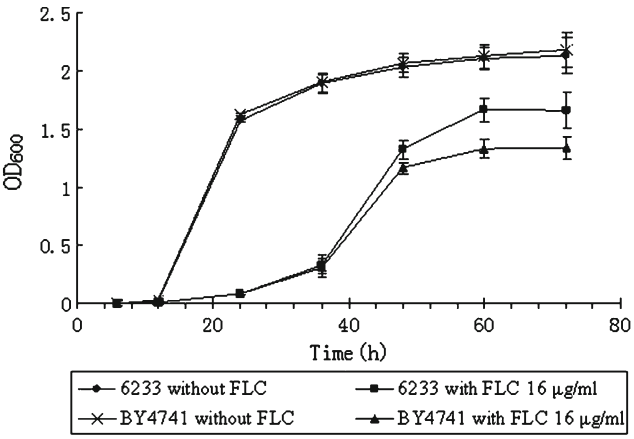

Fig. 1 Effects of FLC with 16 μg/ml on the growth of wild-type BY4741 and *aif1* mutant 6233 cultured in YPD. The growth was monitored by measuring the optical density (600 nm) of the cultures during the subsequent 72 h

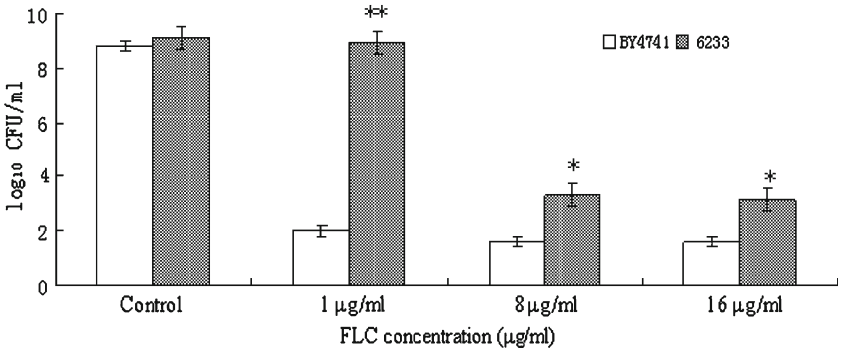

Fig. 2 Antifungal activity of fluconazole after 24 h exposure. The isolate was exposed to 1, 8, and 16 μg/ml of fluconazole, and the number of viable organisms was determined at 48 h by plate counts. ΔlogCFU=logarithm of CFU after 24 h exposure − logarithm of CFU at time zero

susceptibility to other agents, including terbinafine and amphotericin B (Fig. 3).

Increased efflux of Rhodamine 6 G in △aif1 strain

The fluorescene dye Rhodamine 6 G is known to be the substrate of multidrug resistance pumps, and its increased efflux has been demonstrated in a number of organisms that maintain multidrug resistance. Therefore, we tested the Rhodamine 6 G efflux n these two strains. As shown in Fig. 4, without glucose supply, the function of efflux transporters was weak, and neither the strains 6233 nor BY4741 had a marked effect on the efflux ability. When glucose was added, the function of efflux transporters was enhanced significantly in both groups: the mutant 6233 increased the efflux of Rhodamine 6 G compared with the strain BY4741. Fluconazole inhibited rhodamine 6 G efflux from both 6233 and BY4741 following the addition of glucose.

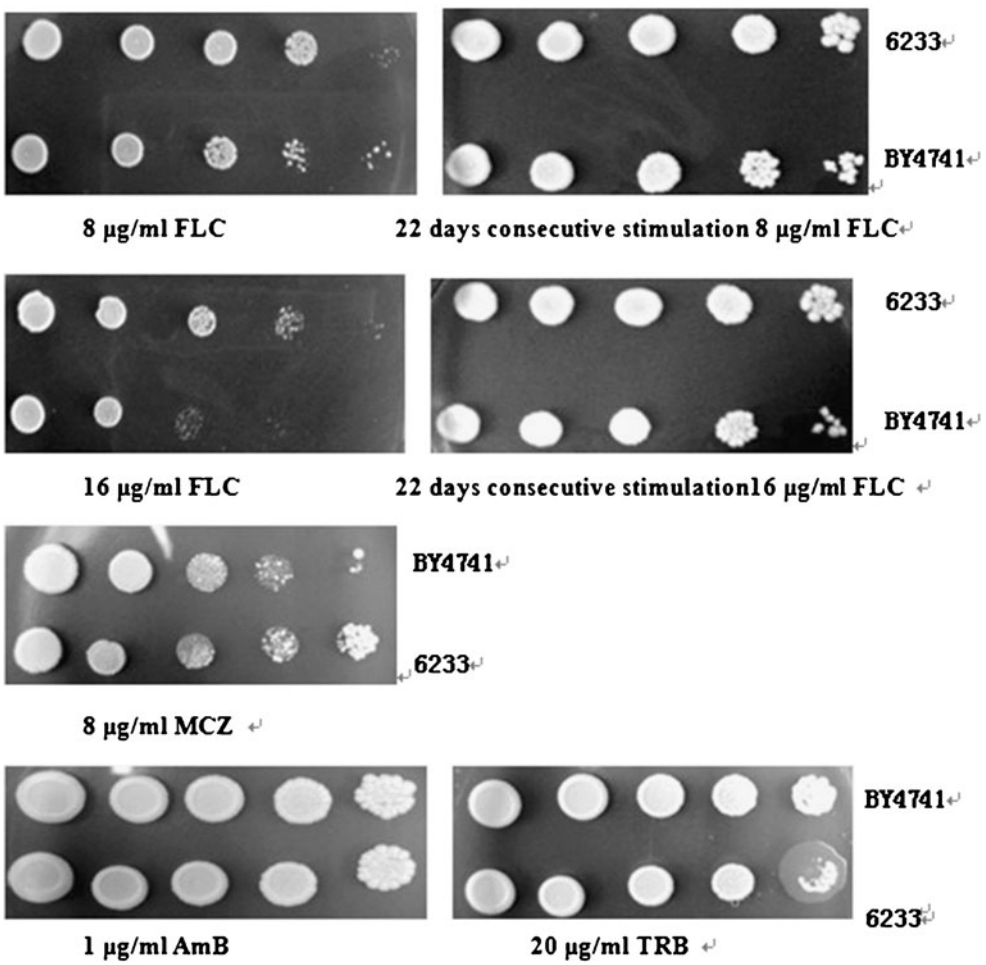

Fig. 3 Drug susceptibility profiles of *S cerevisiae* as determine by spot assays. Wild-type strain BY4741 and Δ*aif1* strain 6233 were spotted on YPD agar plates supplemented with 0.0025% uridine with or without different antifungal agents at the indicated concentrations. Plates were incubated for 48 h at 30°C. The names of strains are indicated on the *right*

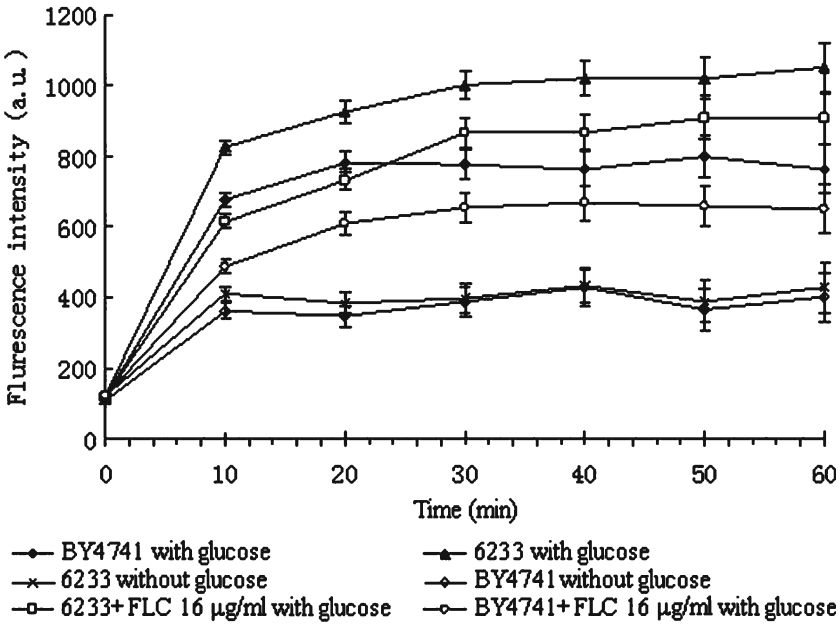

Fig. 4 Function of the efflux pumps in wild-type strain BY4741 and Δ*aif1* strain 6233 in the absence or presence of FCZ, as indicated through determining flourescence intensities (FI) from the level of Rhodamine 6 G transported out in the presence or absence of glucose. Each datum point represents the mean± SD of three measurements of the extracellular concentration of Rhodamine 6 G at the specified time interval. Glucose (2 mM) was added at the starting time point for groups with glucose supply

Up-regulation of PDR5, PDR10 and HXT11 in △aif1 strain

To discover the relationship between AIF-deleted strain and other genes known as drug resistance-related ones, quantitative RT-PCR were performed for seven genes of interest to compare their transcription level. The results are shown in Fig. 5, which showed drug resistance-related genes PDR5, PDR10 and HXT11 were above 4-fold changes in the *aif1* mutant 6233 compared with the wild-type BY4741. The expression of genes PDR1, SNQ2, YOR1, and PDR15 had slightly changeds between the mutant and wild-type strains.

Discussion

In the previous study, Aif1p was reported to induce apoptosis in yeast through a partially caspase-dependent pathway. In this work, we demonstrated that aif1p could also inhibit the fluconazole resistance in yeast. Here, our findings on AIF1 suggested a multi-function of the apoptosis-inducing factor related to the drug sensitivity.

We investigated the wild-type strain BY4741 and the *aif1* mutant strain 6233 in the development of antifungal drugs resistance in yeast (Shen et al. 2007). The results of our study indicated that Aif1p inhibited the development of FLC resistance in yeast, and that it was accelerated as a result of deletion of AIF1: after 22 days' FLC exposure for the development of drug resistance, spot assays indicated that the *aif1* mutant strain without AIF1 expression was markedly more resistant to FLC than the parent strain (Fig. 1, 2 and 3), but it produced no obvious differences between the two strains when using terbinafine (TRB) or amphotericin B (AmB).

In this research, we also found that PDR5, PDR10 and HXT11 were markedly upregulated in yeast *aif1* mutant. PDR5, PDR10 (pleiotropic drug-resistant 10) and HXT11 (hexose transporter 11) are from the ABC efflux pump family and could cause azole resistance. *S. cerevisiae* Pdr1p and Pdr3p, two homologous transcription factors belonging to the Zn_2C_6 binuclear zinc cluster family, have been shown to be encoding transporters of the ABC efflux pump family and then upregulate these ABC transporters, which can transport the drugs out of the cell and decrease the incellular fluconazole accumulation accordingly causing azole resistance (Dexter et al. 1994; Katzmann et al. 1996; Fardeau et al. 2007; Wendler et al. 1997). It is well known that enhanced extrusion of drugs through efflux pumps, which is observed in all cells from microorganisms to mammalians, constitutes a major cause of multidrug resistance

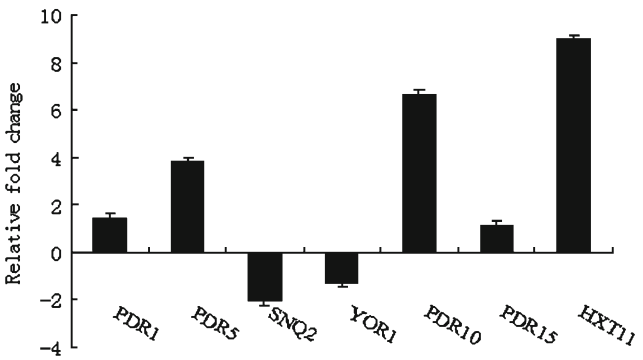

Fig. 5 Real-time RT-PCR analysis of genes which are related with drug-sensitivity in the *aif1* mutant 6233 compared with the wild-type BY474. Gene expression is indicated as a fold change relative to that of the control group. Data are shown as means±SD from four experiments

(MDR) (Sanglard et al. 1995). We measured Rhodamine 6 G in the supernatant of yeast, which represents the function of efflux pumps. The increased efflux of Rhodamine 6 G of *aif1* mutant cells grown in the presence of glucose indicates that aif1p might reduce the extrusion of drug out of the yeast cells by inhibiting efflux pumps, thus increasing the susceptibility of the cells to fluconazole .

Taken together, we conclude that Aif1p may inhibit the development of FLC resistance in yeast via some ABC transporters induction.

Further studies are planned to uncover the exact mechanism of AIF1 involved in fluconazole resistance by regulating a drug resistance pathway. Hence, our findings may open a new doorway for the development and design of new effective agents for the treatment of microbial infections.

Competing interests None declared.

Ethical approval Not required.

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