



# *Candida albicans* Zn Cluster Transcription Factors Tac1 and Znc1 Are Activated by Farnesol To Upregulate a Transcriptional Program Including the Multidrug Efflux Pump *CDR1*

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**ABSTRACT** Farnesol, a quorum-sensing molecule, inhibits *Candida albicans* hyphal formation, affects its biofilm formation and dispersal, and impacts its stress response. Several aspects of farnesol's mechanism of action remain incompletely uncharacterized. Among these are a thorough accounting of the cellular receptors and transporters for farnesol. This work suggests these processes are linked through the Zn cluster transcription factors Tac1 and Znc1 and their induction of the multidrug efflux pump *Cdr1*. Specifically, we have demonstrated that Tac1 and Znc1 are functionally activated by farnesol through a mechanism that mimics other means of hyperactivation of Zn cluster transcription factors. This is consistent with our observation that many genes acutely induced by farnesol are dependent on *TAC1*, *ZNC1*, or both. A related molecule, 1-dodecanol, invokes a similar *TAC1-ZNC1* response, while several other proposed *C. albicans* quorum-sensing molecules do not. Tac1 and Znc1 both bind to and upregulate the *CDR1* promoter in response to farnesol. Differences in inducer and DNA binding specificity lead to Tac1 and Znc1 having overlapping, but nonidentical, regulons. Induction of genes by farnesol via Tac1 and Znc1 was inversely related to the level of *CDR1* present in the cell, suggesting a model in which induction of *CDR1* by Tac1 and Znc1 leads to an increase in farnesol efflux. Consistent with this premise, our results show that *CDR1* expression, and its regulation by *TAC1* and *ZNC1*, facilitates growth in the presence of high farnesol concentrations in *C. albicans* and in certain strains of its close relative, *C. dubliniensis*.

**KEYWORDS** *Candida albicans*, farnesol, zinc cluster transcription factor

*Candida albicans* is a major opportunistic human fungal pathogen that can cause life-threatening systemic infections in immunocompromised individuals (1–3). Multiple important *C. albicans* virulence-related traits, including the morphological switch between yeast and hyphal growth (4, 5), biofilm formation and dispersal (6), interspecies communication with bacteria (7), and response to oxidative stress (8), can be modulated by its quorum-sensing molecule (QSM), farnesol, the first identified QSM for eukaryotes (9–14).

Among multiple *Candida* species, *C. albicans* has been found to produce the most significant amounts of farnesol, followed by its close relative, *C. dubliniensis* (15, 16). Dense cultures of *C. albicans*, in certain media, can accumulate farnesol to concentrations as high as 50  $\mu\text{M}$  (15, 16). The known mechanisms underlying the biological activity of farnesol in *C. albicans* include modulation of signaling pathways, such as the Ras1-Cyr1/cAMP-PKA cascade, in part via direct inhibition of Cyr1 (17–19). Farnesol

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exposure also results in a transcriptional response in *C. albicans* in both sessile and planktonic cells (12, 20–23).

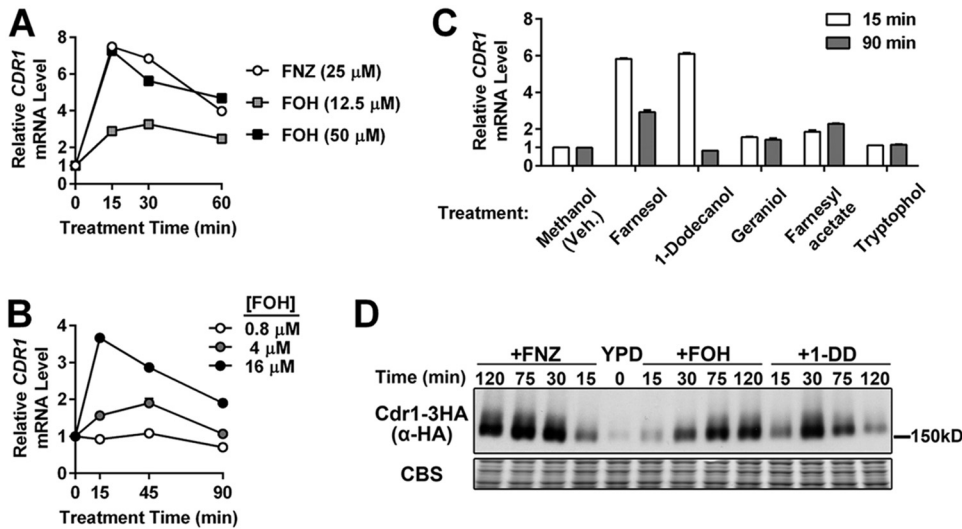
One of the outstanding questions regarding farnesol activity in *C. albicans* is the existence of specific farnesol receptors and transporters (13). Adenylyl cyclase Cyr1 is a cytoplasmic target of farnesol, as it binds and is inhibited by farnesol (18). Transcription factors that directly respond to farnesol as a nuclear receptor/effector to regulate gene expression, however, have not been identified. Growth of *C. albicans*, in the white cell form, is remarkably resistant to growth inhibition by high concentrations of farnesol compared to other fungal species (14, 24). This property might result from an efficient farnesol efflux by a certain transporter(s). The ABC (ATP-binding cassette) transporter Cdr1 was found upregulated upon 2 to 24 h of farnesol treatment (21, 22) and has been proposed to play a role in farnesol efflux (22). Expression of *CDR1* and another ABC transporter, *CDR2*, in *C. albicans* is regulated by the Zn(II)Cys6 transcription factor Tac1 (25). Gain-of-function (GOF) mutations in *TAC1* are often found in clinical isolates of *C. albicans* that are resistant to treatment with azole drugs due to high levels of *CDR1* expression (25–27). Tac1 binds to a 13-bp drug-responsive element (DRE) at the *CDR1* and *CDR2* promoters and activates transcription upon acquisition of gain-of-function mutations or treatment with certain xenobiotics, such as fluphenazine (25, 26, 28). The *C. albicans* *TAC1* gene is located in a zinc cluster region on chromosome 5 (25), where it neighbors two other transcription factors from its family, Hal9 and Znc1. Interestingly, Znc1, when activated artificially, also increases *CDR1* expression (29).

In this work, we investigated whether Tac1 functions as a farnesol nuclear receptor/effector to activate *CDR1* expression and searched for other transcription factors with a similar function. Our work showed that Tac1 and Znc1 contributed individually and in tandem to the transcriptional activation response to farnesol. We also found that *CDR1* expression, and its regulation by *TAC1* and *ZNC1*, facilitates growth in the presence of farnesol in both *C. albicans* and *C. dubliniensis*.

## RESULTS

**Farnesol and 1-dodecanol rapidly induce *CDR1* expression.** Since specific xenobiotic inducers evoke an acute activation of the *C. albicans* *CDR1* promoter (28, 30), we tested whether the known physiologic inducer of *CDR1*, farnesol (FOH), also led to a rapid transcriptional induction of the efflux pump gene. FOH addition to exponentially growing cells led to an increase in *CDR1* mRNA expression with an amplitude and temporal pattern comparable to that of fluphenazine (FNZ), a well-studied inducer of *CDR1* (Fig. 1A). FOH induces *CDR1* expression in a dose-dependent manner, starting at concentrations as low as 4  $\mu$ M (Fig. 1B). The 12-carbon backbone and hydroxyl group of FOH are required for its full inhibition of *C. albicans* hyphal growth (9, 31). Several different terpene alcohols and FOH derivatives were tested for their ability to rapidly induce *CDR1*. Geraniol and farnesyl acetate are unable to induce levels of *CDR1* expression comparable to those of FOH (Fig. 1C). 1-Dodecanol (1-DD), however, another 12-carbon molecule that inhibits hyphal growth (32), induces *CDR1* expression at concentrations similar to those of FOH (Fig. 1C). Tryptophol (Fig. 1C), an aromatic amino acid-derived alcohol with fungal quorum-sensing activity (33, 34), and tyrosol (see Fig. S1 in the supplemental material), another *C. albicans* quorum-sensing molecule (33), do not induce *CDR1*. Tracking the expression of a 3 $\times$  hemagglutinin (HA)-tagged *CDR1* allele by immunoblot analysis confirmed that FOH and 1-DD also induced Cdr1 at the protein level (Fig. 1D). Cdr1 protein levels, especially in response to FNZ and FOH, appear to stay at the peak induced level longer than the mRNA, indicating that Cdr1 is fairly stable.

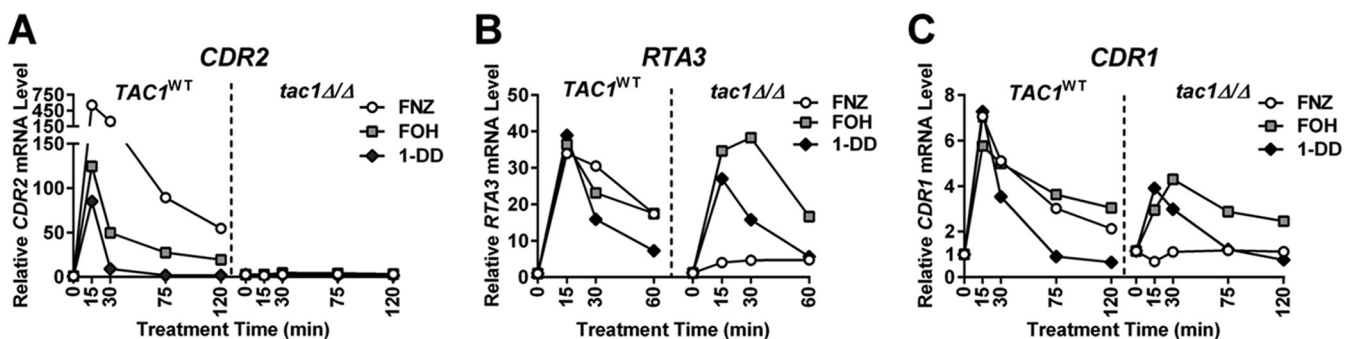
**Tac1 is required for the induction of some, but not all, FOH and 1-DD target genes.** Induction of *CDR1*, *CDR2*, and *RTA3* by xenobiotics, such as FNZ and estradiol, is dependent on the zinc cluster transcription factor Tac1 (25, 28, 29, 35). The observation that *CDR1*, *CDR2*, and *RTA3* expression was induced by FOH and 1-DD treatment (Fig. 1A and 2A and B) suggested Tac1 hyperactivation as a mechanism for FOH- and 1-DD-induced transcription. Unlike FNZ induction of *CDR1*, *CDR2*, and *RTA3*, which was



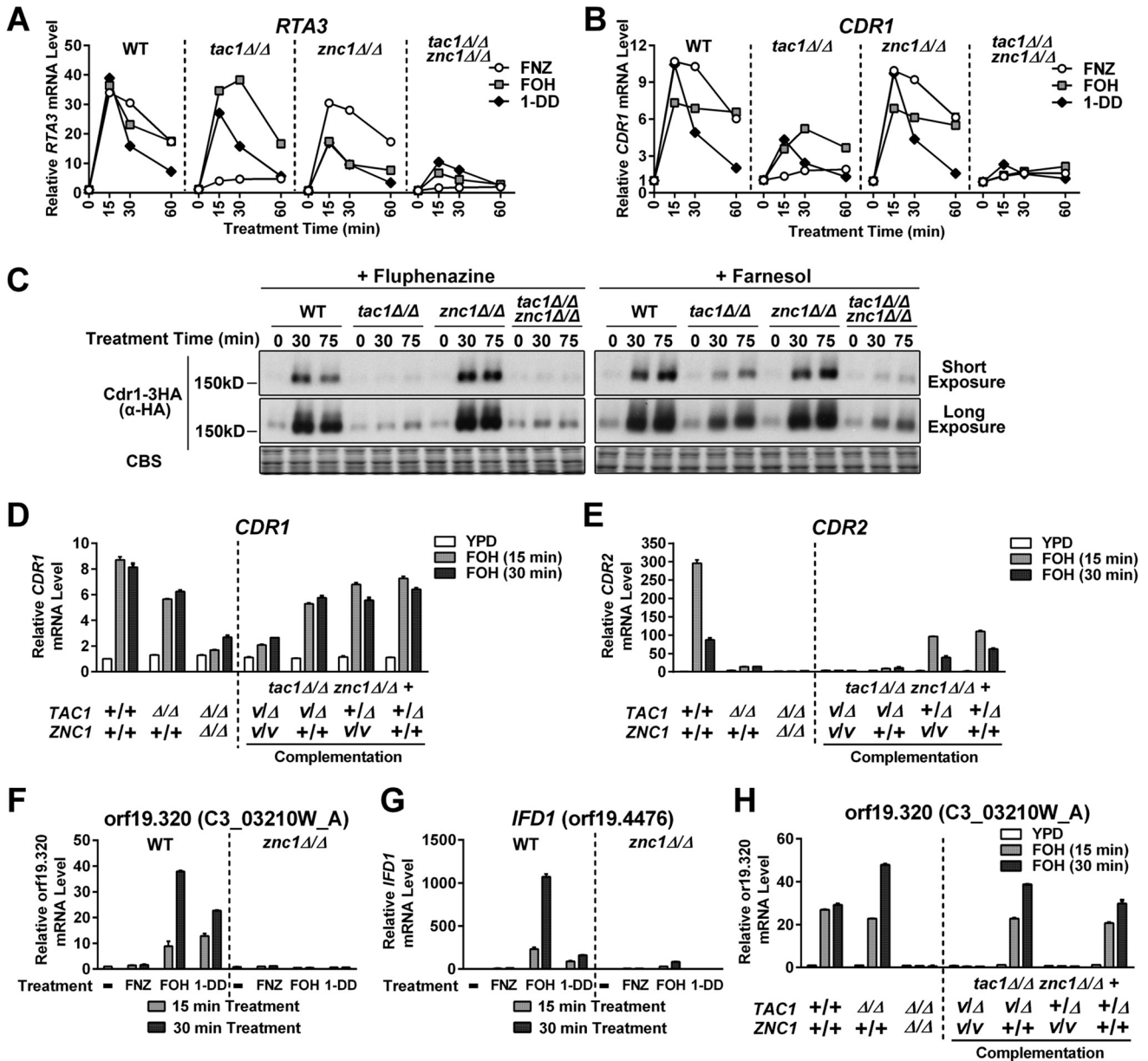
**FIG 1** *CDR1* induction by farnesol and 1-dodecanol treatment. (A) RT-qPCR analysis of *CDR1* mRNA expression in a *C. albicans* wild-type strain (yLM167) grown in YPD and treated with farnesol (FOH) or fluphenazine (FNZ). *CDR1* basal expression (mRNA level prior to treatment; 0 min) was set to 1 to calculate the relative *CDR1* level across conditions. *ACT1* level was used as an internal reference. Results from one representative experiment were presented by the means and standard deviations (values may not be large enough to give a visible error bar) of two qPCR measurements on the same set of cDNA samples. (B) RT-qPCR analysis of *CDR1* mRNA expression induced by increasing concentrations of FOH. *CDR1* expression, in the absence of treatment, in the tested strain (yLM167) was set to 1. (C) RT-qPCR analysis of changes in *CDR1* expression upon exposure to molecules structurally or functionally related to FOH. Each compound tested (or an equal volume of methanol [vehicle]) was added into log-phase cultures of a wild-type strain (yLM167) at a final concentration of 50 μM. The *CDR1* mRNA level in the methanol-treated samples (15 min) was set to 1. (D) Immunoblot analysis of whole-cell extracts made from a strain expressing C-terminally 3×HA-tagged Cdr1 (yLM505) treated with FNZ (25 μM), FOH (50 μM), or 1-DD (50 μM) for the indicated amount of time. Extracts were resolved on a 6% SDS-PAGE gel and probed by an α-HA antibody or stained by Coomassie blue (CBS) as a loading control.

entirely Tac1 dependent, only FOH and 1-DD induction of *CDR2* was entirely dependent on Tac1 (Fig. 2A to C). The residual *CDR1* induction, and virtually unaffected *RTA3* induction, suggest that additional transcription factors respond to FOH and 1-DD at these promoters.

**Znc1 contributes to the induction of multiple FOH and 1-DD target genes.** The first candidate transcription factor that we tested for Tac1-independent induction of *CDR1* and *RTA3* by FOH (and 1-DD) was Znc1. Znc1 is a zinc cluster transcription factor that is encoded adjacent to *TAC1* and whose sequence bares the greatest similarity to Tac1 of all other members of the *C. albicans* zinc cluster transcription factor family (Candida Genome Database [25]). Znc1 was previously identified as a potential regulator of *CDR1* and *RTA3* in an experiment in which a potent activation domain was fused

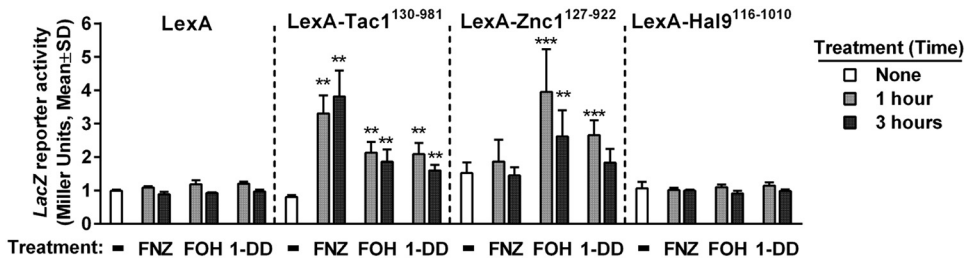


**FIG 2** *TAC1* dependence of *CDR2*, *RTA3*, and *CDR1* induction by FOH and 1-DD. RT-qPCR analysis of *CDR2* (A), *RTA3* (B), and *CDR1* (C) mRNA expression after treatment with FNZ (25 μM), FOH (50 μM), and 1-DD (50 μM) in a wild-type (*TAC1*<sup>WT</sup>; yLM167 or yLM660) and *tac1* deletion strain (*tac1*Δ/Δ; yLM166 or yLM663). The expression level of each gene, in the absence of treatment (0 min) in the wild-type background, was set to 1.



**FIG 3** Znc1 and Tac1 contribute individually and in tandem to the regulation of specific FOH- and 1-DD-induced promoters. (A and B) RT-qPCR analysis of *RTA3* (A) and *CDR1* (B) mRNA expression in *tac1Δ/Δ* and *znc1Δ/Δ* strains treated with FNZ, FOH, or 1-DD. A wild-type strain (yLM660) and mutants carrying a *tac1* deletion (yLM663), *znc1* deletion (yLM661), or double deletion (yLM664) were treated with FNZ (25 μM), FOH (50 μM), or 1-DD (50 μM). *CDR1* and *RTA3* basal expression in the untreated wild-type strain (yLM660) was individually set to 1. (C) Immunoblot analysis of Cdr1 protein levels in *tac1Δ/Δ*, *znc1Δ/Δ*, and double deletion strains treated with FNZ or FOH. Wild-type (yLM665), *tac1Δ/Δ* (yLM666), *znc1Δ/Δ* (yLM667), and *tac1Δ/Δ znc1Δ/Δ* (yLM668) strains expressing C-terminally 3×HA-tagged Cdr1 were treated with FNZ (25 μM) or FOH (50 μM). Cell lysates were resolved on 6% SDS-PAGE gels and probed with an anti-HA antibody. Blot images, acquired at two exposure times, are presented, and Coomassie blue staining (CBS) images are presented as a loading control. (D to E) RT-qPCR analysis of *CDR1* (D) and *CDR2* (E) mRNA expression in *tac1Δ/Δ* and *znc1Δ/Δ* strains, and complementation controls, treated with FOH. A *tac1Δ/Δ znc1Δ/Δ* strain was complemented by *ZNC1* (yLM676), *TAC1* (yLM677), or both (yLM675) and treated with FOH (50 μM). Parallel experiments were also conducted in wild-type (yLM660), *tac1* deletion (yLM663), parental *tac1 znc1* double deletion (yLM664), and mock complementation (yLM678) strains for comparison. A plus sign labels a native or restored gene locus, while Δ and V (vector) mark an unrestored gene disruption and a mock complementation by introducing an empty vector. (F and G) RT-qPCR analysis of *orf19.320* (F) and *IFD1* (G) mRNA expression in the wild-type *ZNC1* or *znc1* deletion background (yLM660 and yLM661, respectively) treated with FNZ (25 μM), FOH (50 μM), and 1-DD (50 μM) for the indicated period of time. *orf19.320* and *IFD1* basal expression in the wild-type strain was individually set to 1. (H) qPCR analysis of *orf19.320* expression in the complementation strains using cDNA samples tested in panels D and E. Noninduced expression levels in the wild-type strain (yLM660) were set to 1.

to the full-length wild-type Znc1 (29). *RTA3* induction by FOH and 1-DD was decreased in a *znc1Δ/Δ* strain, while *RTA3* induction by FNZ was largely unaffected (Fig. 3A). FNZ, FOH, and 1-DD induction of *CDR1* was largely unaffected in the *znc1Δ/Δ* strain; however, FOH and 1-DD induction of *CDR1* and *RTA3* was decreased in a *tac1Δ/Δ*



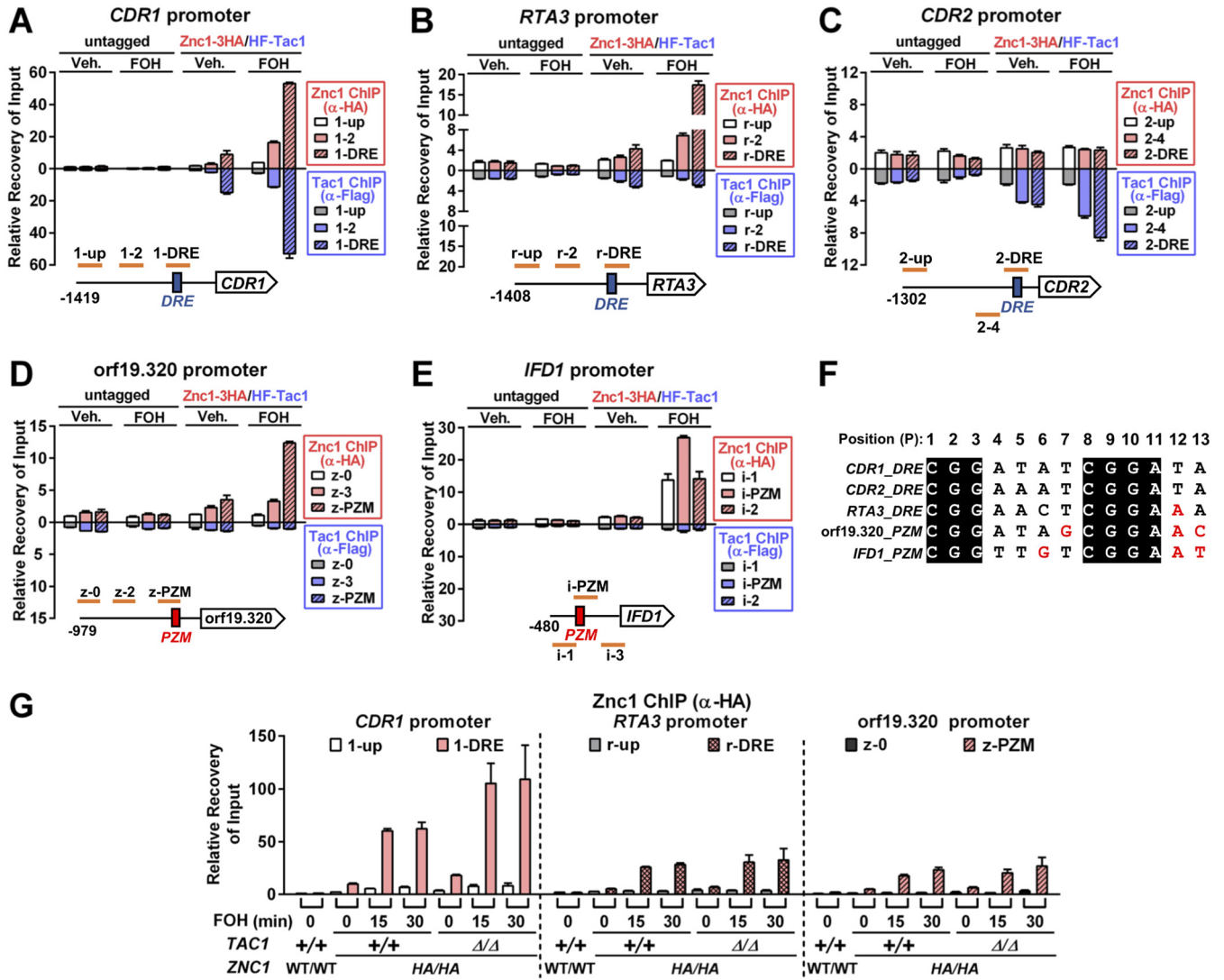
**FIG 4** Direct hyperactivation of Tac1 and Znc1 by FOH and 1-DD. *C. albicans* one-hybrid assay assessing Tac1- and Znc1-dependent reporter activation under multiple inducing conditions. A *C. albicans* one-hybrid strain expressing the LexA DNA binding domain (yLM567) or the fusion of the LexA DBD with Tac1, Znc1, or Hal9 fragments (yLM568, yLM680 and yLM681, respectively) were treated with FNZ (25  $\mu$ M), FOH (50  $\mu$ M), and 1-DD (50  $\mu$ M) for 1 h and 3 h before measurement of LacZ activity. Untreated (none) and methanol-treated (not shown) cultures showed the same level of basal activity. Statistical significance for reporter activation was determined by comparing the basal and induced LacZ activity in *t* tests (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

*znc1* $\Delta/\Delta$  strain compared to that of either single mutant (Fig. 3A and B). The pattern of Cdr1 protein expression was consistent with the epistasis analysis of *CDR1* mRNA expression in the *tac1* and *znc1* strains (Fig. 3C). FOH induction of *CDR1* in the *tac1* $\Delta/\Delta$  *znc1* $\Delta/\Delta$  strain was restored by complementation with either *TAC1* or *ZNC1* (Fig. 3D), while *CDR2* induction was only restored upon *TAC1* complementation (Fig. 3E).

In addition to *CDR1* and *RTA3*, the previous Znc1 activation domain fusion analysis (29) suggested that several other genes, including *orf19.320* and *IFD1* (*orf19.4476*), were direct Znc1 targets. Both *orf19.320* and *IFD1* are induced by FOH and 1-DD but not by FNZ (Fig. 3F and G). Induction of *orf19.320* and *IFD1* by FOH and 1-DD was *ZNC1* dependent but not affected by *TAC1* (Fig. 3H). Consistent with these results, FOH induction of *orf19.320* in a *tac1* $\Delta/\Delta$  *znc1* $\Delta/\Delta$  strain was restored by reintroduction of *ZNC1* but not *TAC1* (Fig. 3H).

**Hyperactivation of Tac1 and Znc1 by farnesol and 1-dodecanol.** The mechanism by which small molecules regulate the ability of Tac1, as well as other members of the *PDR1* family, to activate transcription has not been clearly defined. Direct binding to these small molecules/xenobiotics has been shown, in the case of *C. glabrata* *PDR1* (36), to play an important role in this process. Among the strongest pieces of evidence for direct xenobiotic hyperactivation in *C. albicans* is the finding that a heterologous DNA binding domain (DBD) fused to Tac1 (minus the DBD) activated reporter genes in response to xenobiotic (30, 37). We used a *C. albicans* one-hybrid assay to test whether Tac1 or Znc1, with its native DBD replaced by the LexA DBD, could activate a LacZ reporter in response to FOH and 1-DD. The LexA-Tac1 fusion protein activated its reporter gene in response to both FOH and 1-DD at levels comparable to, although somewhat lower than, the levels previously observed (30) for FNZ (Fig. 4A). The LexA-Znc1 construct induces the LacZ reporter in response to FOH and 1-DD but does not respond to treatment with FNZ. The fold activation by FOH and 1-DD are similar for LexA-Znc1 and LexA-Tac1, while LexA-Znc1 has a basal activation potential that is slightly higher than that of the Tac1 construct. Hal9, the *C. albicans* transcription factor with next highest similarity to Tac1 and Znc1, fused to LexA does not activate the reporter in response to FNZ, FOH, or 1-DD (Fig. 4).

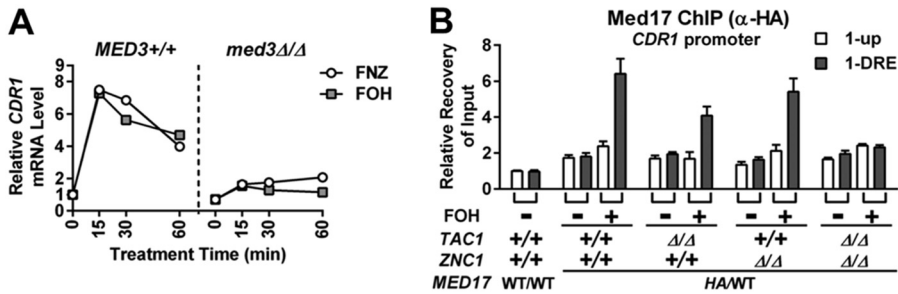
Hyperactivation of Tac1 tightly correlates with its phosphorylation by the Mediator complex and can be detected by a decrease in gel mobility (30). FOH and 1-DD treatment both result in an N-terminal His Flag-tagged Tac1 mobility shift that is slightly lower than the shift caused by FNZ (Fig. S2A). The Tac1 band shift by FOH is unaffected in the *znc1* deletion mutant (Fig. S2B), ruling out a potential competitive effect by hyperactivated Znc1. The variability of Tac1 phosphorylation pattern suggests inducer-specific conformations of hyperactive Tac1 that lead to differential phosphorylation by Mediator. To test if hyperactivated Znc1 is also subject to phosphorylation, we generated strains expressing C-terminally 3 $\times$ HA-tagged Znc1. The tagging does not compromise Znc1 activation competence at the *CDR1* and *orf19.320*



**FIG 5** Tac1 and Znc1 occupancy at FOH target promoters. (A to E) ChIP analysis of Tac1 and Znc1 occupancy at *CDR1* (A), *RTA3* (B), *CDR2* (C), *orf19.320* (D), and *IFD1* (E) upon treatment with FOH. A strain carrying two copies of N-terminally 6His3Flag-tagged *TAC1* and two copies of C-terminally 3×HA-tagged *ZNC1* (Znc1-3HA/HF-Tac1; yLM686) and a strain with native *TAC1* and *ZNC1* (untagged; yLM660) were treated with FOH (50 μM) or vehicle (Veh.; methanol) for 15 min before fixation. Each sample was immunoprecipitated by an anti-Flag antibody and an anti-HA antibody in separate reactions. Promoter regions tested for Tac1 and Znc1 binding and their relative positions to the known Tac1 *cis* elements at the *CDR1*, *RTA3*, and *CDR2* promoters (DRE; blue boxes) or the CGG triplet motifs found at the *orf19.320* and *IFD1* promoters (PZMs; red boxes) are schematically shown in each panel. Percent recovery of input (Input%) at the *CDR1* promoter 1-up region in the anti-Flag/anti-HA ChIP products obtained from the methanol-treated untagged strain was set to 1 to normalize Tac1/Znc1 binding across conditions and promoter regions. Hence, the strength of ChIP signals (y axis value) can be compared across panels. (F) Alignment of the *CDR1*, *CDR2*, and *RTA3* DREs and the CGG triplet motifs (PZMs) at the *orf19.320* and *IFD1* promoters. The 13 nucleotide positions are numbered in order. Individual nucleotide substitutions that have been found to be deleterious in the *CDR2* DRE (26) are highlighted in red. (G) ChIP analysis of Znc1 occupancy at target promoters in a *tac1* deletion background. Wild-type (yLM684) and *tac1* deletion (yLM685) strains expressing two copies of C-terminally 3×HA-tagged *ZNC1* (*HA/HA*) were treated with 50 μM FOH for the indicated period of time before fixation for an anti-HA ChIP assay. Percent recovery of input (Input%) at the 1-up region in a wild-type strain with native Tac1 (+/+) and Znc1 (WT/WT) was set to 1 to normalize recoveries across conditions and promoters.

promoters (Fig. S2C and S2D). As opposed to Tac1, FOH or 1-DD treatment does not induce detectable changes in Znc1-3HA mobility in either the wild-type or *tac1* deletion background (Fig. S2A and E).

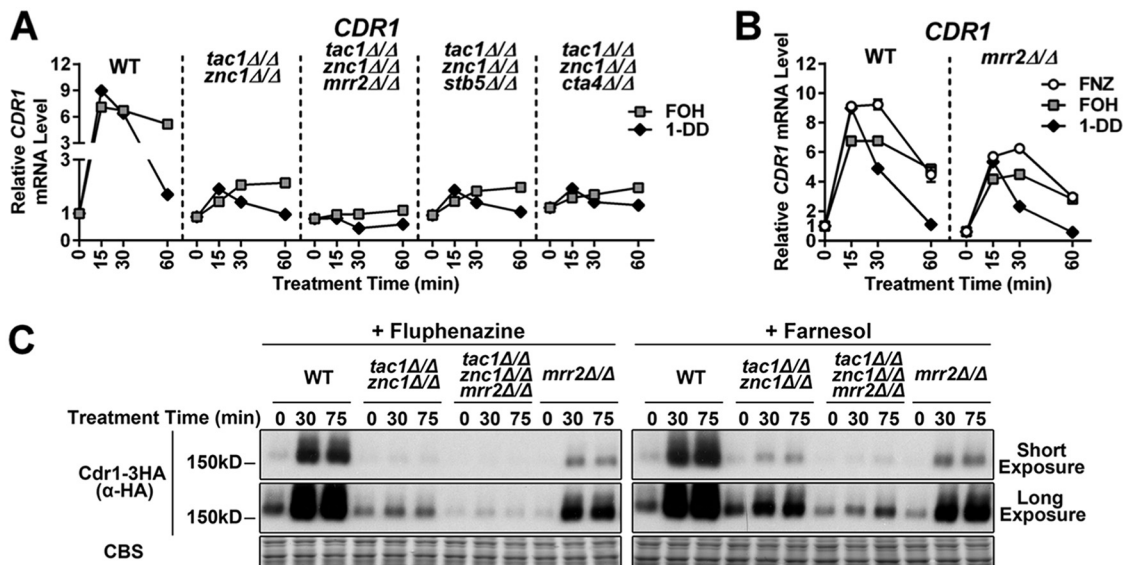
**Tac1 and Znc1 promoter occupancy correlates with their impact on target gene induction by FOH.** Chromatin immunoprecipitation (ChIP) analysis was performed to test whether Tac1 and Znc1 promoter occupancy determined their FOH-induced target gene specificity. Tac1 and Znc1 occupancy are enriched at the *CDR1* DRE in the absence of inducer, and this occupancy is enhanced by treatment with FOH (Fig. 5A). There is also a weak but reproducible enrichment of Tac1 and Znc1 occupancy at the *RTA3* DRE



**FIG 6** Mediator requirement for, and recruitment by, Tac1- and Znc1-dependent induction of *CDR1* by farnesol. (A) RT-qPCR analysis of *CDR1* mRNA expression in a wild-type strain (yLM167) and a *med3* null strain (yLM232) after treatment with 50  $\mu$ M FOH. FNZ (25  $\mu$ M) induction (30) was performed as a reference. (B) Anti-HA ChIP analysis of Mediator occupancy at the *CDR1* promoter in wild-type (yLM695), *tac1* $\Delta/\Delta$  (yLM696), *znc1* $\Delta/\Delta$  (yLM697), and *tac1* $\Delta/\Delta$  *znc1* $\Delta/\Delta$  (yLM698) strains expressing C-terminally 3 $\times$ -HA-tagged Med17 treated with 50  $\mu$ M FOH. Percent recovery of input (Input%) at the 1-up region in a ChIP product obtained from a strain with native *MED17* (WT/WT; yLM660) was set to 1.

under noninducing conditions. Similar to our observation that Znc1 was the primary regulator of *RTA3* expression in response to FOH, only Znc1 occupancy at the *RTA3* DRE was increased by treatment with FOH (Fig. 3B). Tac1 and Znc1 occupancy was specific to the *CDR2* and *orf19.320* promoters, respectively, under noninducing conditions and was enhanced by induction with FOH (Fig. 5C and D). Another Znc1-dependent promoter, *IFD1*, is exclusively bound by Znc1, but only after FOH treatment (Fig. 5E). The ChIP assay results allowed identification of potential *cis* elements for Znc1 at the tested promoters. High Znc1 occupancy at the *CDR1* and *RTA3* DRE suggests DNA binding preference of Znc1 similar to that of Tac1. Thirteen-bp DRE-like CGG triplet sequences were found in the *orf19.320* and *IFD1* promoter regions, whose locations correlated to the region of highest local enrichment for Znc1 ChIP signal. Thus, we refer to these 13-bp elements as potential Znc1 binding motifs (PZMs). DREs and PZMs in the tested genes share a core consensus of CGGNNNNCGGAN (Fig. 5F). Multiple bases found in PZMs (labeled red in Fig. 5F) have been reported to impair *CDR2* DRE function (26) and may specifically reduce Tac1 binding. The ChIP (Fig. 5B) and expression analysis (Fig. 3A) suggest that one such nucleotide in the *RTA3* DRE, P12 A, is better tolerated by the Znc1 DBD than the Tac1 DBD. One model for the partially redundant function of Tac1 and Znc1 at the *CDR1* promoter is that both transcription factors competently bind to the *CDR1* promoter in the absence of the other. To test this hypothesis, we performed Znc1 ChIP in a *tac1* $\Delta/\Delta$  strain. Znc1 occupancy is observed at the *CDR1*, *RTA3*, or *orf19.320* promoters in a *tac1* $\Delta/\Delta$  strain and even increases at the *CDR1* DRE (Fig. 5G) compared to that of a wild-type *TAC1* strain. This last finding indicates Tac1 and Znc1 compete for DRE binding at promoters where we detected cooccupancy. Additionally, we have found in ChIP assays that FNZ treatment has only a minor effect on Znc1 occupancy compared to its effect on Tac1 at the *CDR1* promoter (Fig. S3A) or the effect of FOH/1-DD on Znc1 occupancy at the *RTA3* promoter (Fig. S3B). Previous studies have shown that Tac1 GOF mutants can confer fluconazole resistance through a mechanism that relies on the ability of Tac1 to bind and activate the *CDR1* promoter (25, 26, 28, 30). We have found that the fluconazole MIC in *TAC1*<sup>GOF</sup> mutant strains does not decrease in the *znc1* $\Delta/\Delta$  strain (Table S1). Collectively, this evidence supports the hypothesis that Tac1 and Znc1 bind promoters independently of each other.

**FOH-induced Znc1 works through a Mediator-dependent coactivator mechanism.** Our previous work showed recruitment of Mediator complex is critical to FNZ-induced Tac1-dependent *CDR1* activation (30). Here, we found the Mediator tail module is also important for FOH-induced *CDR1* expression (Fig. 6A) and that either Tac1 or Znc1 is competent for Mediator recruitment at the *CDR1* DRE under these conditions (Fig. 6B). Therefore, Tac1 and Znc1 both show DRE binding and Mediator

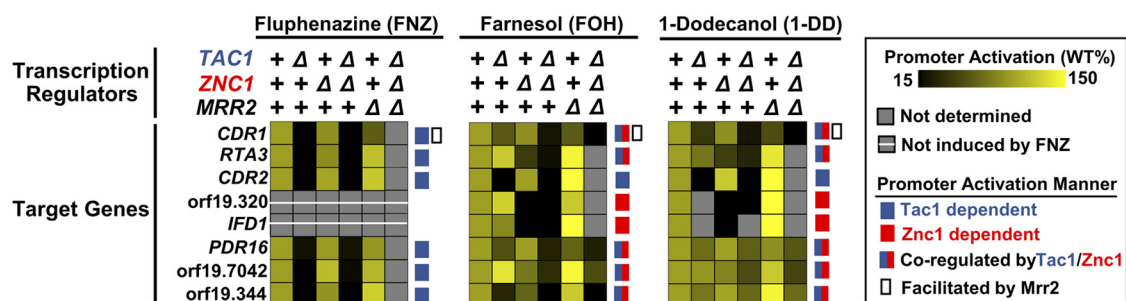


**FIG 7** Influence of Mrr2 on FOH and 1-DD target gene expression. (A) RT-qPCR analysis of *CDR1* mRNA expression in a wild-type strain (yLM660) and *tac1Δ/Δ znc1Δ/Δ* strains (yLM701 to yLM704) generated from an otherwise wild-type, *mrr2Δ/Δ*, *stb5Δ/Δ*, or *cta4Δ/Δ* background and treated with FOH (50  $\mu$ M) and 1-DD (50  $\mu$ M). *CDR1* expression, in the absence of treatment, in yLM660 was set to 1. (B) RT-qPCR analysis of *CDR1* mRNA expression in wild-type (yLM660) and *mrr2* null (yLM662) strains treated with FNZ (25  $\mu$ M), FOH (50  $\mu$ M), or 1-DD (50  $\mu$ M). *CDR1* expression level, in the absence of treatment, in the wild-type strain was set to 1. (C) Anti-HA immunoblot analysis of C-terminally 3 $\times$ HA-tagged *CDR1* protein expression in *MRR2* wild-type strains (yLM665 and yLM705) and deletion mutants (yLM707 and yLM706) in the presence and absence of *TAC1* and *ZNC1* and treatment with FNZ (25  $\mu$ M) or FOH (50  $\mu$ M). Blot images acquired at different exposures are presented with a Coomassie blue stain (CBS) as a loading control.

recruitment at the *CDR1* promoter in the presence of FOH that is independent of the other.

**Additional transcription factors regulate FOH- and 1-DD-induced transcription in a promoter-specific manner.** Despite the FOH induction of *CDR1* being severely compromised in the *tac1Δ/Δ znc1Δ/Δ* strain, a small residual induction was observed in this background (Fig. 7A). This finding suggested that another transcription factor was involved in *CDR1* activation by certain inducers. A genetic screen of zinc cluster transcription factors identified Mrr2, Stb5, and Cta4 as potential regulators of *CDR1* (29). The *tac1 znc1 cta4* and *tac1 znc1 stb5* triple deletion mutants showed unaffected FOH and 1-DD induction of all genes tested compared to that of a *tac1 znc1* double null strain (Fig. 7A). Deletion of *mrr2*, however, largely eliminates the residual induction of *CDR1* mRNA by FOH (and 1-DD) in the *tac1Δ/Δ znc1Δ/Δ* strain (Fig. 7A), while deletion of *mrr2* also compromises *CDR1* activation by FNZ, FOH, and 1-DD when Tac1 and Znc1 are both present (Fig. 7B). Deletion of *mrr2* in the *tac1Δ/Δ znc1Δ/Δ* background also decreases induced Cdr1 protein levels (Fig. 7C). Interestingly, *mrr2* deletion appears to cause a greater decrease in the induced Cdr1 protein levels than in mRNA levels (Fig. 7B and C), suggesting Mrr2 also regulates *CDR1* posttranscriptionally. In addition to *CDR1*, the genes *CDR2* (Fig. 2A), *PDR16* (Fig. S4A), orf19.7042 (Fig. S4B), and orf19.344 (Fig. S4C) are dependent on *TAC1*, *ZNC1*, or a combination of the two under different induction conditions (29). Deletion of *mrr2*, *stb5*, or *cta4* did not impact the expression of these additional Tac1/Znc1 target genes when we assayed their induction in a *tac1Δ/Δ znc1Δ/Δ* background (Fig. S4D to F). Likewise, deletion of *mrr2* in an otherwise wild-type background did not compromise induction of the tested Tac1/Znc1 target genes, other than *CDR1*, by FNZ, FOH, or 1-DD (Fig. S4G to K). Among the genes tested, Mrr2 functions as a specific modulator of *CDR1* expression rather than a broad regulator of FOH and 1-DD induction. The transcription factor(s) responsible for the residual FOH or 1-DD induction of *PDR16*, orf19.7042, and orf19.344 in the *tac1Δ/Δ znc1Δ/Δ* strains remain unidentified. Figure 8 provides a heat map summary of the impact of Tac1, Znc1, and Mrr2 on expression of the fluphenazine-, farnesol-, and 1-dodecanol-induced genes analyzed in this work.

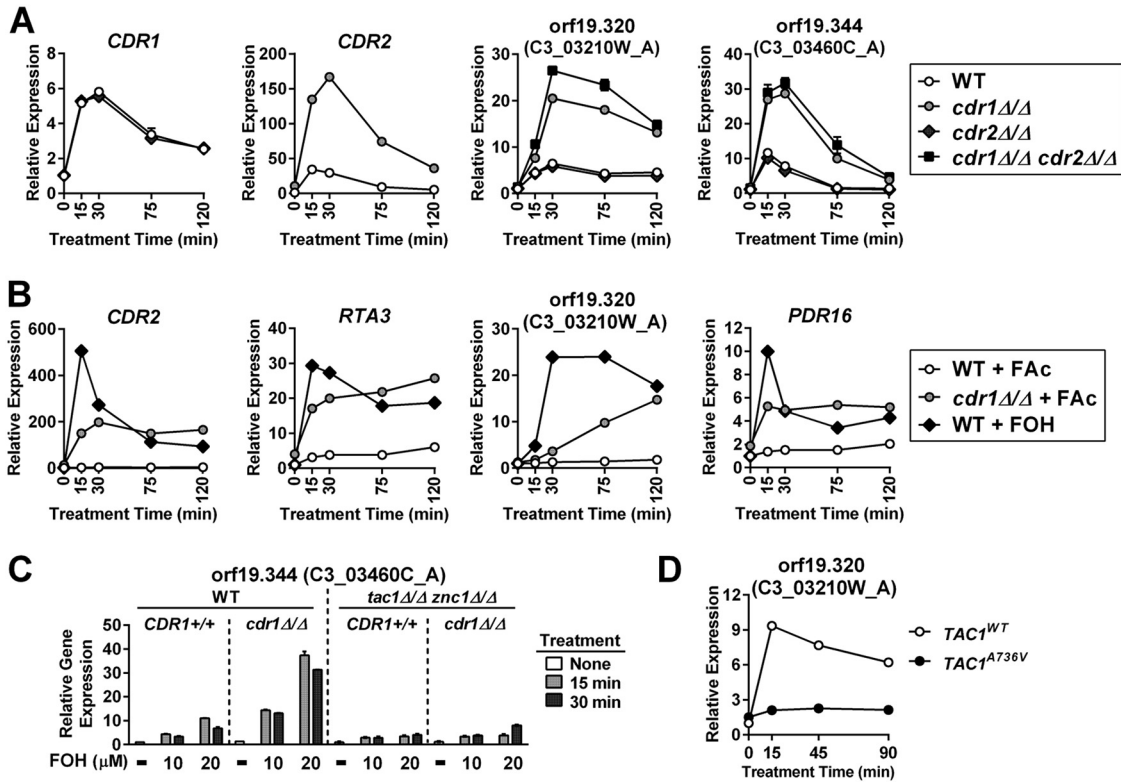




**FIG 8** Combinatorial control of gene expression by Tac1, Znc1, and Mrr2. A summary of the effect of *TAC1*, *ZNC1*, and *MRR2* on gene expression induced by FNZ (25  $\mu$ M), FOH (50  $\mu$ M), and 1-DD (50  $\mu$ M) is shown. The means of the relative gene expression after 15 and 30 min of exposure to the inducer were used to compare promoter activation in different strain backgrounds after being normalized to the induced expression level in the wild-type strain as percentages. Effects of transcription factor deletion on gene activation are visualized by a black-yellow chromatic scale. Transcription factor(s) dependence for each promoter was illustrated by the colors and symbols as coded in the legend box.

**Cdr1-mediated feedback regulation of the transcriptional FOH response.** It has been proposed that *C. albicans* Cdr1 can decrease intracellular FOH concentration via its efflux pump activity (22). This led us to hypothesize that FOH-induced *CDR1* expression is part of a negative feedback mechanism to downregulate the cellular response to FOH. To test whether increased *CDR1* expression downregulated the transcriptional response to FOH, we compared FOH-induced gene expression in a wild-type and *cdr1* deletion background. In a *cdr1* $\Delta/\Delta$  strain, *CDR2*, orf19.320, and orf19.344 are all expressed at higher levels by the same concentration of exogenous FOH (Fig. 9A). *cdr2* deletion does not affect FOH induction and had a minimal effect when combined with *cdr1* deletion, suggesting *CDR1* plays a specific role in modulating this response. Based on this finding, we sought to determine whether the efficiency of Cdr1-mediated xenobiotic transport governed the transcriptional response to other small molecules. Farnesyl acetate, an FOH-like molecule, causes little to no induction of FOH-inducible promoters in a wild-type strain (Fig. 9B). In the absence of *CDR1*, however, farnesyl acetate and FOH result in comparable induction of several FOH target genes (Fig. 9B). This finding suggests that the Cdr1-dependent transport of farnesyl acetate, rather than an inability to activate the relevant transcription factors, is the limiting factor in response to farnesyl acetate. Compared to an FOH induction curve that peaks and then decreases, the induction curve for farnesyl acetate in the *cdr1* null shows a plateau or gradual increase (Fig. 9A and B). Similar to FOH, 1-DD treatment induces higher levels of target gene expression in the absence of *cdr1* (Fig. S5A and B), while geraniol and tryptophol do not (Fig. S5C). Deletion of *cdr1* only enhances the FOH/1-DD response to specific inducers rather than a pool of broadly related molecules. The *CDR1*-facilitated negative feedback model predicts that the increase in FOH target gene expression in the *cdr1* null strain will also be dependent on Tac1 and Znc1. Indeed, FOH induction in wild-type and *cdr1* $\Delta/\Delta$  strains exhibits a similar dependence on *TAC1* and *ZNC1* (Fig. 9C and Fig. S4D to F). The negative feedback model also predicts that overexpression of Cdr1 would increase farnesol efflux and desensitize *C. albicans* to FOH induction. A strain carrying a *TAC1* GOF allele, which overexpresses *CDR1*, showed a dramatically lower degree of Znc1 target gene induction than a wild-type strain at identical concentrations of FOH (Fig. 9D). These results further support a model where FOH-induced Cdr1 expression facilitates FOH clearance.

**Cdr1 facilitates *C. albicans* and *C. dubliniensis* resistance to FOH exposure.** *C. albicans* is able to tolerate significantly higher levels of exogenous FOH than other ascomycetes through a mechanism that is not entirely understood (13, 14, 24). To determine whether the *CDR1* transcriptional response pathway described above could detoxify FOH, we investigated the role of Cdr1 in *C. albicans* FOH survival. Under the conditions tested, our *C. albicans* wild-type strain did not exhibit a major decrease in colony size when grown on YPD agar containing 200  $\mu$ M FOH (Table 1).



**FIG 9** Feedback modulation of FOH induction by Cdr1. (A) RT-qPCR analysis of *CDR1*, *CDR2*, *orf19.320*, and *orf19.344* mRNA expression in a wild-type strain (yLM660) and mutant strains carrying a deletion in *cdr1* (yLM708), *cdr2* (yLM709), or both (yLM710) after treatment with FOH (20  $\mu$ M). Expression of each gene in the wild-type strain, in the absence of treatment, was set to 1. Symbol legends are in the box on the right. (B) RT-qPCR analysis of *CDR2*, *RTA3*, *orf19.320*, and *PDR16* mRNA expression in wild-type (yLM660) and *cdr1* null (yLM708) strains treated with 50  $\mu$ M farnesyl acetate (FAC) or 50  $\mu$ M FOH. Expression of each gene in the wild-type strain, in the absence of treatment, was set to 1. Symbol legends are in the box on the right. (C) RT-qPCR analysis of *orf19.344* mRNA expression in *TAC1/ZNC1* wild-type (WT; yLM660 and yLM708) and *tac1Δ/Δ znc1Δ/Δ* (yLM664 and yLM711) strains, derived from a wild-type or *cdr1Δ/Δ* background, after treatment with increasing concentrations of FOH. Expression of *orf19.344* in the wild-type strain (yLM660), in the absence of treatment, was set to 1. (D) RT-qPCR analysis of *orf19.320* mRNA expression in strains carrying a wild-type (*TAC1*<sup>WT</sup>; yLM167) or a GOF mutant *TAC1* allele (*TAC1*<sup>A736V</sup>; yLM169) treated with 16  $\mu$ M FOH. Expression of *orf19.320* in the *TAC1*<sup>WT</sup> strain, in the absence of treatment, was set to 1.

The growth of a *cdr1* deletion strain, however, was compromised or completely inhibited at 50 or 100  $\mu$ M FOH (Table 1). Colony growth, within the FOH concentration range tested, was not affected in a *cdr2* null strain. FOH tolerance, however, was further decreased in a *cdr1Δ/Δ cdr2Δ/Δ* strain versus a *cdr1Δ/Δ* strain. Consistent with this finding, deletion of *tac1* further sensitizes a *cdr1* null mutant to FOH, while *TAC1* GOF mutants, which overexpress Cdr2, increase FOH tolerance in a *cdr1Δ/Δ* strain in a manner that is dependent on *CDR2* (Table 1). A killing assay was performed to determine if FOH inhibits growth of *cdr1* deletion mutants through a fungistatic or fungicidal effect. The *cdr1Δ/Δ cdr2Δ/Δ* strain showed decreased viability upon treatment with 200  $\mu$ M FOH, as only ~25% of mutant cells survived after 6 h of treatment (Fig. 10A). Identical treatment with vehicle had no effect on viability (Fig. S6A). The viability of the *cdr1Δ/Δ cdr2Δ/Δ* and *cdr1Δ/Δ* strains decreases at a similar rate during the first 6 h of FOH exposure. Deletion of the transcription factors that drive FOH induction of *CDR1* and *CDR2* had a lesser impact on viability after FOH exposure than complete deletion of *CDR1* and *CDR2* (Fig. 10A). Deletion of *tac1*, *znc1*, and *mrr2* individually, or *tac1 znc1* simultaneously, does not affect FOH tolerance (Table 1). The *tac1Δ/Δ znc1Δ/Δ mrr2Δ/Δ* strain, however, showed mildly compromised colony formation and decreased cell growth upon FOH exposure (Table 1 and Fig. 10B). There was no difference in growth sensitivity of the wild-type and the *cdr1Δ/Δ cdr2Δ/Δ* strains to SDS, indicating that the mutants did not impact the membrane integrity of the cells (Fig. S6B).

**TABLE 1** Relative colony radius of *C. albicans* strains grown on farnesol-containing YPD agar<sup>a</sup>

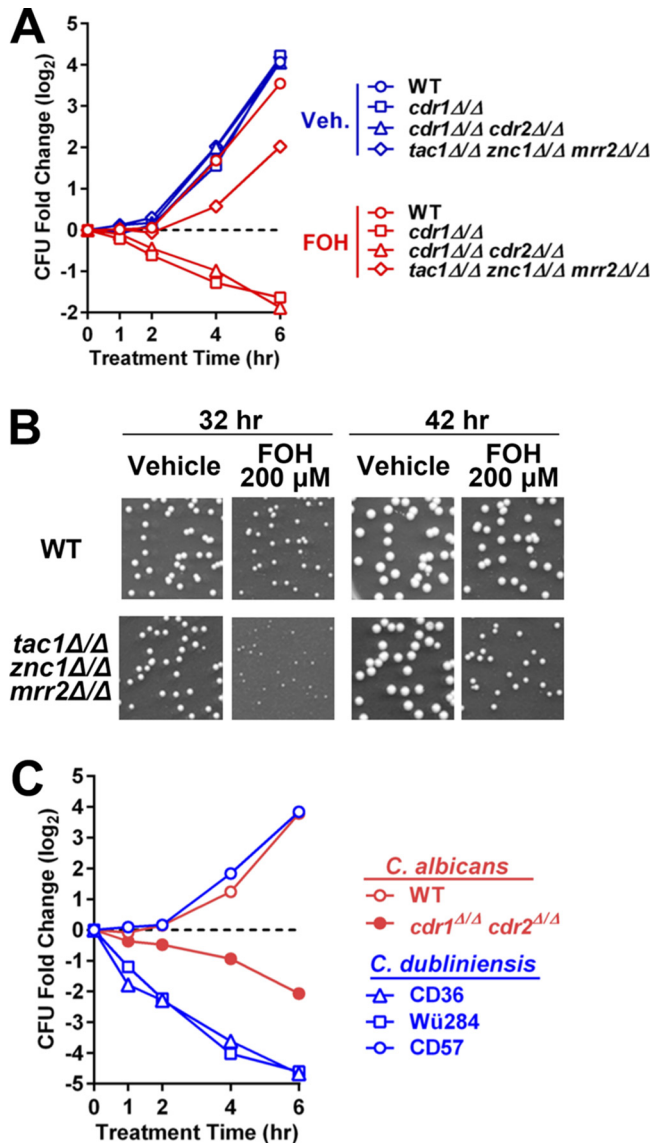
Strain <sup>b</sup>	Vehicle	[FOH] ( $\mu$ M)			
		25	50	100	200
Wild type	++++	+++	+++	+++	+++
<i>cdr1</i> $\Delta/\Delta$	++++	+++	+	NG	NG
<i>cdr2</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
<i>cdr1</i> $\Delta/\Delta$ <i>cdr2</i> $\Delta/\Delta$	++++	++	NG	NG	NG
<i>tac1</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
<i>tac1</i> $\Delta/\Delta$ <i>cdr1</i> $\Delta/\Delta$	++++	+++	NG	NG	NG
<i>znc1</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
<i>znc1</i> $\Delta/\Delta$ <i>cdr1</i> $\Delta/\Delta$	++++	+++	+	NG	NG
<i>tac1</i> $\Delta/\Delta$ <i>znc1</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
<i>tac1</i> $\Delta/\Delta$ <i>znc1</i> $\Delta/\Delta$ <i>cdr1</i> $\Delta/\Delta$	++++	+++	NG	NG	NG
<i>mrr2</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
<i>tac1</i> $\Delta/\Delta$ <i>znc1</i> $\Delta/\Delta$ <i>mrr2</i> $\Delta/\Delta$	++++	+++	+++	++	++
<i>TAC1</i> <sup>WT</sup>	++++	+++	+++	+++	+++
<i>TAC1</i> <sup>WT</sup> <i>cdr1</i> $\Delta/\Delta$	++++	+++	++	NG	NG
<i>TAC1</i> <sup>WT</sup> <i>cdr1</i> $\Delta/\Delta$ <i>cdr2</i> $\Delta/\Delta$	++++	+++	NG	NG	NG
<i>TAC1</i> <sup>A736V</sup>	++++	+++	+++	+++	+++
<i>TAC1</i> <sup>A736V</sup> <i>cdr1</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
<i>TAC1</i> <sup>A736V</sup> <i>cdr1</i> $\Delta/\Delta$ <i>cdr2</i> $\Delta/\Delta$	++++	+++	NG	NG	NG
<i>TAC1</i> <sup>N977D</sup>	++++	+++	+++	+++	+++
<i>TAC1</i> <sup>N977D</sup> <i>cdr1</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
<i>TAC1</i> <sup>N977D</sup> <i>cdr1</i> $\Delta/\Delta$ <i>cdr2</i> $\Delta/\Delta$	++++	+++	NG	NG	NG

<sup>a</sup>Each entry in the table represents the colony size observed for a particular combination of strain and treatment. The average radius of colonies formed by a wild-type reference strain (wild type or *TAC1*<sup>WT</sup>) on non-FOH-containing plates (vehicle) was set to 100% to normalize colony size of its derivative mutants under each growth condition. Data were symbolized by the following transformation: 90 to 110%, +++++; 70 to 90%, ++++; 50 to 70%, ++; 30 to 50%, +. NG, no visible colony formation was seen after 40 h of incubation at 30°C.

<sup>b</sup>Strains tested in this table are marked by their genotypic feature in Table S2. Strains (and their growth data) are divided (horizontal line) with respect to their parental wild-type background (designated wild type or *TAC1*<sup>WT</sup>).

The *C. dubliniensis* genome-sequencing strain CD36, a close relative of *C. albicans*, has been reported to have much higher sensitivity to FOH-induced cell death (24). It is also known that multiple *C. dubliniensis* strains within genotype group I, including CD36, do not express functional full-length Cdr1 protein due to a homozygous variant that creates a stop codon at *CDR1* amino acid 756 (38). All *cdr1*<sup>756stop/756stop</sup> *C. dubliniensis* strains (CD36, CD38, and Wü284) we tested showed complete or partial growth inhibition by 50  $\mu$ M FOH (Table 2 and Fig. 10C). There are multiple *CDR1*<sup>+/+</sup> *C. dubliniensis* strains (such as CD57), and we have observed that they exhibit resistance comparable to that of *C. albicans* at FOH levels as high as 200  $\mu$ M (Table 2 and Fig. 10C). Additionally, we have found that deletion of *cdr1* sensitizes CD57 to farnesol, while deletion of *cdr1* in the *cdr1*<sup>756stop/756stop</sup> strain Wü284 does not further sensitize it to farnesol (Table 2).

**Tac1 and Znc1 mediate the *CDR1* transcriptional FOH response in *C. dubliniensis*.** Our standard protocol showed that FOH induces *CDR1* expression in CD57 with kinetics and amplitude similar to those of *C. albicans* (Fig. 11A). Although CD57 showed an extremely low expression level of *C. dubliniensis* *CDR2* compared to that of its *C. albicans* ortholog, FOH treatment results in a comparable fold induction of *CDR2* in the two species (Fig. 11A). The ortholog of orf19.320, a *C. albicans* Znc1-dependent promoter, in *C. dubliniensis* (Cd36.83180) had an expression pattern in which induction was only observed after 1 h of FOH treatment (Fig. 11A). A LexA-CdTac1 fusion protein, constructed in a fashion similar to that for the earlier LexA-CaTac1 fusion (Fig. 4), activated LacZ expression upon FOH and FNZ treatment when tested in a one-hybrid reporter assay in *C. albicans* (Fig. 11B). Representatives of the three different classes of FNZ/FOH-induced promoters defined in *C. albicans*, Tac1/Znc1 dependent (*CDR1*), Tac1 dependent (*CDR2*), and Znc1 dependent (Ca orf19.320/Cd36\_83180), have a very similar dependence on these same transcription factors in *C. dubliniensis* (Fig. 11C). An



**FIG 10** Dependence of *C. albicans* and *C. dublinienseis* farnesol sensitivity on *CDR1* expression. (A) Colony formation analysis comparing cell viability between wild-type and *cdr1* null strains after 1 to 6 h of FOH or vehicle (methanol) exposure. Wild-type (yLM660), *cdr1* null (yLM708), *cdr1 cdr2* double deletion (yLM710), and *tac1 znc1 mrr2* triple deletion (yLM702) strains were each diluted from overnight YPD cultures and treated with 200  $\mu\text{M}$  FOH in YPD medium. Aliquots, after an appropriate dilution (if needed), were spread on YPD plates at the indicated time points. Fold change in CFU was calculated by setting the CFU before treatment (not shown) to 1 and is presented in a logarithmic form (base 2). (B) Representative plate scans showing a high FOH concentration in agar medium reduces colony growth of a *tac1* $\Delta/\Delta$  *znc1* $\Delta/\Delta$  *mrr2* $\Delta/\Delta$  strain. Dilutions of a wild-type strain (yLM660) and a *tac1 znc1 mrr2* triple deletion mutant (yLM702) from overnight cultures were plated on YPD agar supplemented with 200  $\mu\text{M}$  FOH or the same volume of vehicle (methanol). Plates were imaged after incubation for the indicated amounts of time at 30°C. (C) Colony formation analysis comparing cell viability between three *C. dublinienseis* strains (CD36, Wü284, and CD57) after 1 to 6 h of FOH or vehicle (methanol) exposure. Wild-type (yLM660) and *cdr1 cdr2* double deletion (yLM710) *C. albicans* strains were tested in parallel as a control. The strains were each diluted from overnight YPD cultures and treated with 200  $\mu\text{M}$  FOH in YPD medium. Aliquots, after an appropriate dilution (if needed), were spread on YPD plates at the indicated time points. Fold change in CFU was calculated by setting the CFU before treatment (not shown) to 1 and is presented in a logarithmic form (base 2).

exception was the observation that deletion of *znc1* in the *tac1* $\Delta/\Delta$  CD57 strain did not decrease FOH *CDR1* induction as strongly as it does in *C. albicans*, suggesting the existence of another regulator(s) governing FOH induction of the *C. dublinienseis CDR1* (*CdCDR1*) promoter.

**TABLE 2** Relative colony radius of *C. dubliniensis* strains grown on farnesol-containing YPD agar<sup>a</sup>

Strain <sup>b</sup>	Vehicle	[FOH] ( $\mu$ M)			
		25	50	100	200
CD36 (I; <i>cdr1</i> <sup>756stop</sup> / <i>cdr1</i> <sup>756stop</sup> )	++++	+++	++	NG	NG
CD38 (I; <i>cdr1</i> <sup>756stop</sup> / <i>cdr1</i> <sup>756stop</sup> )	++++	++	+	NG	NG
Wü284 (I; <i>cdr1</i> <sup>756stop</sup> / <i>cdr1</i> <sup>756stop</sup> )	++++	++	NG	NG	NG
Wü284 <i>cdr1</i> $\Delta/\Delta$	++++	++	NG	NG	NG
CD57 (I; <i>CDR1/CDR1</i> )	++++	+++	+++	+++	+++
CD57 <i>cdr1</i> $\Delta/\Delta$	++++	++	++	++	++
CD57 <i>tac1</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
CD57 <i>tac1</i> $\Delta/\Delta$ <i>znc1</i> $\Delta/\Delta$	++++	+++	+++	+++	+++
CM1 (I; <i>CDR1/CDR1</i> )	++++	+++	+++	+++	+++
CBS8500 (I; <i>CDR1/CDR1</i> )	++++	+++	+++	+++	+++
CD506 (II; <i>CDR1/CDR1</i> )	++++	++	++	++	+++
CAN6 (II; <i>CDR1/CDR1</i> )	++++	+++	+++	+++	+++
p7718 (IV; <i>CDR1/CDR1</i> )	++++	+++	+++	+++	+++

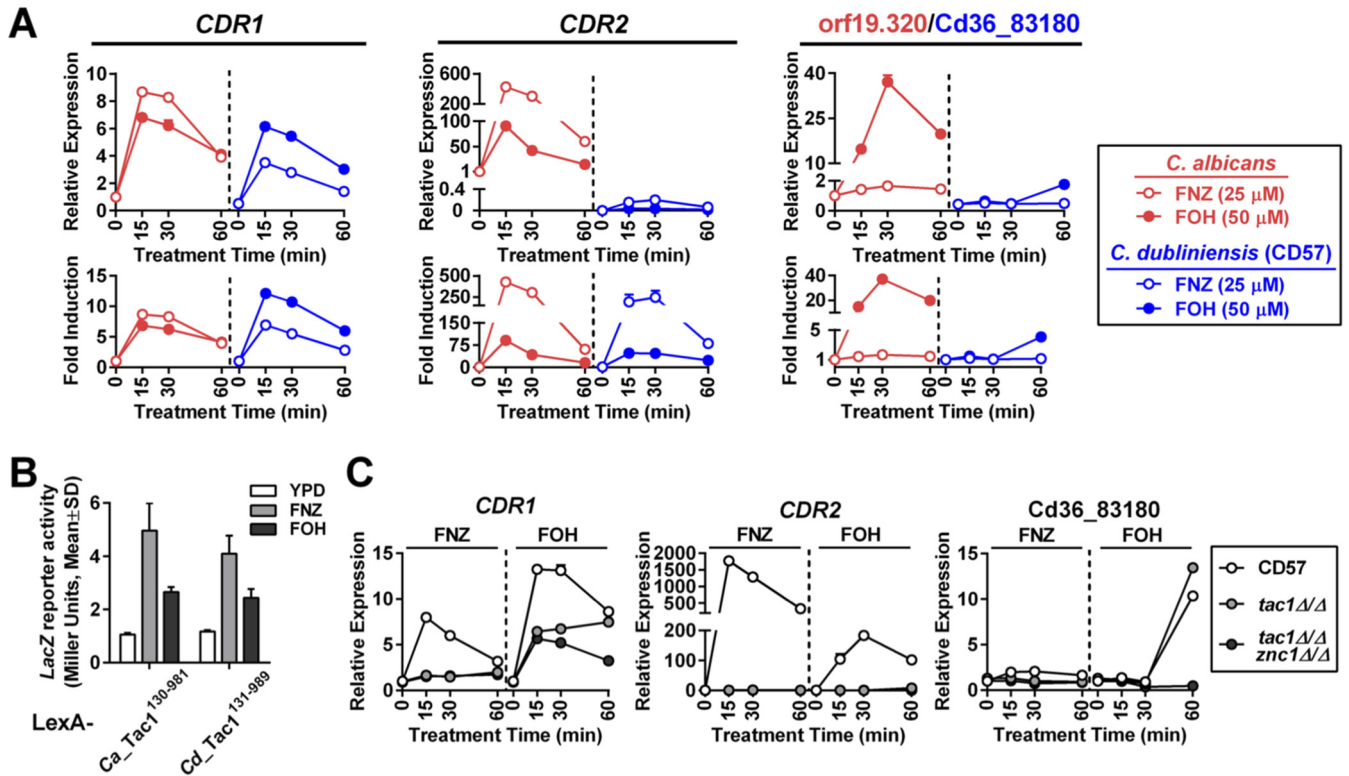
<sup>a</sup>The average radius of colonies formed by each wild-type *C. dubliniensis* isolate on non-FOH-containing plates (vehicle) was set to 100% to normalize its colony size or its derivative mutants colony size under each growth condition. Data were symbolized by the following transformation: 90 to 110%, +++++; 70 to 90%, +++; 50 to 70%, ++; 30 to 50%, +. NG, no visible colony formation was seen after 40 h of incubation at 30°C.

<sup>b</sup>The genotype group (I, II, or IV) of each *C. dubliniensis* isolate and the presence or absence of the *cdr1*<sup>756stop</sup> allele are denoted in parentheses. The genotype of each mutant tested in this table is described in detail in Table S2.

## DISCUSSION

Our characterization of Tac1, Znc1, and Mrr2 as essential signal targets (direct or indirect) for farnesol provides a new framework for thinking about how the *C. albicans* cell coordinates its transcriptional response to the quorum-sensing molecule. The demonstration that multiple Zn cluster transcription factors can be activated by overlapping yet nonredundant small molecules reveals a previously underappreciated combinatorial complexity that allows these factors to regulate complex patterns of gene regulation. Additionally, the dependence of *CDR1* expression on this transcriptional response, combined with the regulation of this response by the action of this important efflux pump, supports the mounting evidence for *CDR1* serving as a farnesol transporter and suggests the presence of a negative feedback loop modulating its action.

**Tac1 and Znc1 act as targets in *Candida* for farnesol.** Earlier work had indicated that *CDR1* expression was upregulated upon treatment with farnesol (21, 22) and that Tac1 was an important regulator of *CDR1* (25, 27). This work demonstrates that farnesol can activate an acute transcriptional response via the hyperactivation of Tac1 and Znc1. The kinetics and amplitude of this response are very similar to the well-characterized response of Tac1 to xenobiotics, such as fluphenazine and estradiol (28), and expands the range of *C. albicans* Tac1 hyperactivators into the realm of physiological small molecules. Our discovery that farnesol can lead to the upregulation of *CDR1* through the hyperactivation of Znc1 reveals that the control of efflux pump expression is more complex than previously appreciated. The finding that Tac1 and Znc1 share overlapping but nonidentical targeting to sequences in the promoter elements in farnesol-induced genes extends our knowledge of how a diverse set of genes can be upregulated via farnesol. It is uncertain, at this point, whether farnesol activates Tac1 and Znc1 through a direct binding mechanism, similar to the activation of Pdr1 in *C. glabrata* by azoles (36), or through an indirect mechanism, such as posttranslational modification. The observed subcellular localization of exogenously added farnesol includes nuclear enrichment (39), which would allow farnesol to hyperactivate Tac1 and Znc1 through direct binding. Parallels have previously been drawn between the mechanism of action of the *C. glabrata* Pdr1 zinc cluster transcription factor and mammalian nuclear receptors



**FIG 11** Tac1 and Znc1 regulate gene induction by FOH in *C. dubliniensis*. (A) RT-qPCR analysis comparing FOH induction of *CDR1*, *CDR2*, and *orf19.320* orthologs in *C. albicans* and *C. dubliniensis*. A wild-type *C. albicans* strain (yLM660) and a *C. dubliniensis* isolate (CD57) were treated with 25  $\mu$ M FNZ and 50  $\mu$ M FOH in YPD culture for the indicated amount of time before collection for RNA extraction. Expression of each pair of orthologs was measured by pan-primers and compared across species by setting the basal expression in the *C. albicans* strain to 1 (relative expression; upper). Gene fold induction in each species is shown in the lower panels by setting gene basal expression in yLM660 and CD57 individually to 1. (B) LacZ reporter activation by CdTac1 under FNZ and FOH treatment conditions. *C. albicans* one-hybrid strains expressing LexA-CdTac1<sup>131-989</sup> (yLM766) or LexA-CaTac1<sup>130-980</sup> (yLM568) fusion proteins were treated with 25  $\mu$ M FNZ or 50  $\mu$ M FOH for 2 h and measured for  $\beta$ -galactosidase activity. (C) RT-qPCR analysis showing the effect of *tac1* deletion and *tac1 znc1* double deletion on FNZ and FOH induction of *CDR1*, *CDR2*, and Cd36.83180 in *C. dubliniensis*. CD57 and its *tac1* $\Delta/\Delta$  (yLM764) and *tac1* $\Delta/\Delta$  *znc1* $\Delta/\Delta$  (yLM765) derivatives were treated with 25  $\mu$ M FNZ and 50  $\mu$ M FOH. Basal expression of each gene in CD57 was individually set to 1.

(36, 40), and it is interesting that the mammalian bile acid receptor FXR (farnesoid X-activated receptor) is also activated by farnesol (41). However, strong physiological FXR agonists, such as chenodeoxycholic acid (CDCA) and deoxycholic acid (DA) (42, 43), do not activate *CDR1* expression in *C. albicans* (see Fig. S1 in the supplemental material), suggesting the lack of a broader overlap between Tac1/Znc1 and mammalian FXR inducers. If direct binding of inducers to the Zn cluster transcription factors is the mechanism of hyperactivation, the structural differences between Tac1 inducers (farnesol, dodecanol, flufenazine, and estradiol) suggest a low-affinity/specificity interaction similar to that observed for the FXR receptor. The observation that Znc1 can respond to farnesol (and dodecanol) but not flufenazine indicates that it is possible for the Zn cluster transcription factors to make structural distinctions between these molecules. Although we cannot rule out posttranslational modification as a mechanism, the absence of a mobility shift in Znc1 upon farnesol treatment compared to the hyperactive Tac1 phosphorylation mobility shift (30) suggests that phosphorylation is not a requirement for farnesol activation of Tac1 and Znc1.

**Mode of Tac1- and Znc1-DNA interaction.** In fungi, paralogous zinc cluster transcription factors have been reported to form both homo- and heterodimers, for example, Pdr1/Pdr3 (44) and Oaf1/Pip2 (45, 46) in *Saccharomyces cerevisiae*. Our observation of distinct binding of Tac1 and Znc1 at promoters where they show nonredundant activation, as well as the binding of Znc1 to promoters in a *tac1* null strain, does not support the idea that Tac1 and Znc1 bind promoters as a stable heterodimer. The previous observation that Tac1 requires the presence of both CGG triplet elements

in a DRE for gene activation (26) largely rules out the possibility that monomeric Tac1 and Znc1 each bind to one CGG triplet in a single DRE, a mode of DNA binding infrequently observed for zinc cluster transcription factors (47). The evidence presented here supports a model where Tac1 homodimers and Znc1 homodimers competitively bind to coregulated promoters at a single paired CGG triplet element. Given the variation in DRE and PZM sequences at Tac1-dependent promoters (i.e., CDR2) and Znc1-dependent promoters (i.e., orf19.320), it is likely that Tac1 and Znc1 have overlapping but nonidentical sequence specificity. The DNA binding specificity of zinc cluster transcription factors, namely, the sequence between and surrounding the CGG triplet(s), is thought to be determined by the amino acid sequence in the linker region between the zinc clusters and the dimerization domain (48–50). Divergence in Tac1 and Znc1 in their linker regions may contribute to their different occupancies at the DREs and PZMs. Since it has previously been noted that Tac1 binds to DREs regardless of promoter status (35), it was reasonable to think that hyperactivation of Tac1 did not work by a mechanism that increased DNA binding of the transcription factor. The ChIP analysis shows, however, that DRE/PZM occupancy is clearly increased in an inducer-specific manner. It is unclear whether this reflects a direct increase in binding affinity of the transcription factor or the formation of a more stable transcription factor complex involving interactions with coactivators and enhanced chromatin remodeling.

**The complex regulation of the *CDR1* promoter.** The new regulatory mechanisms revealed by our study further demonstrate the complexity of the *CDR1* promoter. Previous studies of activation of the *CDR1* promoter focused on gain-of-function mutants in Tac1 or xenobiotic hyperactivation of Tac1 (25, 26, 28, 51). Our study now adds farnesol and 1-dodecanol treatment to the limited number of conditions where *CDR1* expression can be induced chemically in the absence of Tac1 (37). Tac1-independent *CDR1* induction by farnesol can be largely attributed to Znc1 (and Mrr2) function. Interestingly, unlike Tac1 (27) and Mrr2 (52), no gain-of-function mutants of Znc1 have been reported to drive azole resistance in *C. albicans*. Of the well-characterized *CDR1* inducers, farnesol is the only one considered to be a *C. albicans* metabolite.

Identification of Tac1 and Znc1 as farnesol-induced transcription activators of the *CDR1* promoter allowed us to test whether the proposed *CDR1*-mediated farnesol efflux (22) provided feedback to the transcriptional response. The amplification of the Tac1- and Znc1-driven transcriptional response to farnesol in cells lacking Cdr1 function, as well as the dampening of the Znc1-driven transcriptional response to farnesol in *TAC1* gain-of-function cells that overexpress *CDR1* (Fig. 9), support the idea that Cdr1 serves to regulate intracellular levels of farnesol via an efflux mechanism. However, the observation that the transcriptional response to farnesol still exhibits attenuation after rapid induction in a *cdr1* null strain (Fig. 9) indicates that there are additional farnesol transporters in *C. albicans*. In contrast to this response, the plateau observed for the transcriptional response to farnesyl acetate in *cdr1* null strain indicates that Cdr1 is the sole efflux pump for farnesyl acetate (Fig. 9B).

**The role of *CDR1* in modulating farnesol sensitivity.** Although the phenotypic regulation of *C. albicans* by farnesol is not the central focus of the work presented here, the suggested circuit involving farnesol, Zn cluster transcription factors, and Cdr1 prompted us to begin to investigate how these factors might interact to influence *Candida* biology. Under our experimental conditions (cells grown in YPD), as well as synthetic medium conditions tested in several other studies (53–55), farnesol concentrations as high as 300  $\mu$ M showed minor effects on the growth or viability of wild-type *C. albicans*. These concentrations of farnesol are typically toxic to most other fungi (14, 24). Our analysis of farnesol toxicity to *C. albicans* and *C. dubliniensis* strains with and without functional Cdr1 consistently suggests Cdr1-mediated farnesol efflux plays a protective role under these growth conditions (Fig. 10 and Tables 1 and 2). It is somewhat surprising that transcription factor mutants in *C. albicans* and *C. dubliniensis* do not show a more dramatic change in farnesol sensitivity given the decrease in *CDR1*

induction. It is possible that expression of other genes that influence farnesol sensitivity was affected by *tac1*, *znc1*, and/or *mrr2* deletion in a way that compensated for the compromised *CDR1* induction in these mutants. Elsewhere it has been reported that farnesol treatment under a non-medium condition (phosphate-buffered saline) induced apoptosis in *C. albicans* through a Cdr1-dependent mechanism (22), suggesting Cdr1 regulates *C. albicans* farnesol sensitivity in either direction depending on the treatment condition. Since activity of Cdr1, an ATP-dependent transporter, is strongly affected by cellular energy status (56, 57), the availability of nutrients may affect the role of Cdr1 during farnesol exposure.

The results of this study lead to the ability to ask new questions about both the general role and mechanism of zinc cluster transcription factors in the response to physiological fungal metabolites, as well as the action of farnesol as a quorum-sensing molecule. For instance, how do Tac1 and Znc1 achieve specificity in their response to farnesol? Are there other metabolite ligands? Do the other genes in the Tac1/Znc1 farnesol regulon play an important role in quorum sensing? It has been observed that an increase in *CDR1* and *CDR2* expression in sessile *C. albicans* cells compared to that in planktonic cells (58, 59) contributes to drug resistance in early *C. albicans* biofilm, in cooperation with the upregulation of the major facilitator transporter, *MDR1* (60). The mechanism underlying the induction of these pumps in biofilm has not been clarified. Farnesol induction of *CDR1* and *CDR2* suggests accumulation of a quorum-sensing molecule favored by static growth as a possible answer.

## MATERIALS AND METHODS

**Strains and plasmids.** Strains and plasmids used in this study are listed in Tables S2 and S3, respectively, in the supplemental material. Construction of the strains and plasmids are described in detail in the supplemental material. Primers used for generating the strains and plasmids are listed in Table S4. *C. albicans* transformation was performed by electroporation and selected by Clonate resistance (1% yeast extract, 2% peptone, 2% glucose, 2% agar, 0.1 mM uridine, 100  $\mu$ g/ml Clonate) or complementation of auxotrophy (6.7 g/liter Difco YNB without amino acids [BD], appropriately supplemented with 1.5 g/liter drop-out mix synthetic medium lacking uracil, histidine, arginine, and leucine [US Biological] and with 2% glucose and 2% agar). Expression of flippase was induced by growth in YPMal (1% yeast extract, 2% peptone, 2% maltose, 0.1 mM uridine) liquid medium for 24 h. Successful eviction of the *SAT1* marker was selected by sensitivity to 100  $\mu$ g/ml Clonate.

**Cell growth and drug treatment.** Cells were grown in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose, 0.1 mM uridine) at 30°C if not specified otherwise. Drug treatment was performed by adding fluphenazine (6 mg/ml aqueous stock; Alfa Aesar), farnesol (mixed isoforms; biweekly 50 mM or fresh 200 mM methanolic dilution; MP Bio), 1-dodecanol (50 mM methanolic dilution; Sigma), farnesyl acetate (50 mM methanolic dilution; Fluka [mixed isoforms]), geraniol (50 mM methanolic dilution; Sigma), tryptophol (50 mM methanolic stock; Sigma), tyrosol (50 mM aqueous stock; Alfa Aesar), chenodeoxycholic acid (50 mM dimethyl sulfoxide [DMSO] stock; Cayman), or deoxycholic acid (50 mM DMSO stock; Fisher) into mid-log-phase *C. albicans* or *C. dubliniensis* culture to the final concentration specified for each experiment in the figure legends. To test cell growth in the presence of farnesol, an overnight culture, after appropriate dilution, was spread on YPD plates supplemented with each concentration of FOH or the same volume of methanol. Colony number and size were analyzed by OpenCFU (61) after 40 h of incubation at 30°C. In farnesol killing assays, an overnight culture of each strain to be tested was diluted to an optical density (OD) of 0.05 in fresh YPD medium for treatment with 200  $\mu$ M farnesol or the same volume of methanol. Aliquots taken at each of the indicated time points, after an appropriate dilution (if needed), were plated on YPD agar (no farnesol). Colony number was counted after 40 h of incubation at 30°C.

**Reverse transcription-quantitative PCR (RT-qPCR).** RNA samples were prepared from collected frozen cell pellets and reverse transcribed as described previously (62). qPCR was performed using the relative standard curve method (StepOne; Life Technologies). *ACT1* abundance, measured by ZL712/ZL713, was used as an internal reference to compare *CDR1* (ZL540/ZL541), *CDR2* (ZL542/ZL543), *RTA3* (ZL544/ZL545), orf19.320 (ZL951/ZL958), *IFD1* (ZL823/ZL824), *PDR16* (M2PT-1/M2PT-2), orf19.7042 (M2PT-23/M2PT-24), orf19.344 (M2PT-15/M2PT-16), or expression across strains and conditions. ZL540/ZL541, ZL1008/ZL1009, and ZL951/ZL958 were used as respective pan-primers to compare expression and induction of *CDR1*, *CDR2*, and orf19.320 homologs in *C. albicans* and *C. dubliniensis*.

**Immunoblotting.** Immunoblot analysis was used to compare Cdr1-3HA expression or 6His3Flag-Tac1 or Znc1-3HA gel mobility. Cell lysates were prepared, resolved by SDS-PAGE, and probed by an  $\alpha$ -HA (3F10; Roche) or  $\alpha$ -Flag (F7425; Sigma) antibody as described previously (30). A lower-molecular-weight region of a gel, which typically did not contain the immunoblotting signals of interest in this study, was stained by Coomassie blue as the loading reference.

**ChIP.** ChIP experiments were performed as described previously (30) to analyze 6His3Flag-Tac1, Znc1-3HA, and Mediator (Med17-3HA) occupancy at target promoters. Results of ChIP experiments are presented in the form of relative recovery of input. The absolute recovery at the *CDR1* promoter 1-up



region in a non-farnesol-treated untagged strain (as specified in the figure legends) was set to 1 to normalize recoveries at other promoter regions across conditions. Primers used in the ChIP assay are listed in Table S4, with their probing region denoted.

**Liquid  $\beta$ -galactosidase activity assays.** *C. albicans* one-hybrid strains were diluted from overnight culture, grown for 3 to 4 h in fresh YPD medium, and treated with fluphenazine (~25  $\mu$ M), farnesol (50  $\mu$ M), 1-dodecanol (50  $\mu$ M), or methanol for 1 h or 3 h before collection for  $\beta$ -galactosidase activity measurement by an SDS-chloroform method (30, 63).  $\beta$ -Galactosidase activity, in Miller units, was calculated by the following simplified formula:  $1,000 \times A_{420}/(T \times C)$ , where  $A_{420}$  is the absorbance of the reaction product at 420 nm,  $T$  is the reaction time in minutes, and  $C$  is the total amounts of cells in total OD at 600 nm ( $OD_{600}$ ) used in the reaction.  $A_{420}$  and  $OD_{600}$  values were measured with a Beckman Coulter DU-7300 spectrophotometer. The activity of each LexA fusion protein was tested in at least three confirmed transformants.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00968-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 6.1 MB.

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