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NOTE

Genetic Architecture of Hsp90-Dependent Drug Resistance▽

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Hsp90 potentiates the evolution of azole resistance in the model yeast *Saccharomyces cerevisiae* and the opportunistic pathogen *Candida albicans* via calcineurin. Here, we explored effectors downstream of calcineurin regulating this Hsp90-dependent trait. Using *S. cerevisiae erg3* mutants as a model, we determined that both Crz1 and Hph1 modulate azole resistance.

The molecular chaperone Hsp90 is essential, abundant at normal temperatures, and induced by stress in eukaryotes. Under physiological conditions, Hsp90 dynamically interacts with a diverse set of inherently unstable, or metastable, client proteins (20, 21, 28). It is involved in the maturation and intracellular transport of many regulators of growth and development, including transcription factors and kinases. Under stressful conditions, Hsp90 is induced, but the increased cellular demand for its chaperone functions can exceed its induction (24). This likely occurs because associations between Hsp90 and client proteins are less stable during stress and because Hsp90 is diverted to assist stress-damaged proteins.

By chaperoning regulators of cell signaling in an environmentally contingent manner, Hsp90 is poised to influence the evolution of new traits. In fungi separated by ~800 million years of evolution, we found that Hsp90 potentiates the rapid evolution of drug resistance (7). In both the model yeast *Saccharomyces cerevisiae* and the opportunistic pathogen *Candida albicans*, Hsp90 enables the rapid evolution of resistance to the azole antifungal drugs. The azoles inhibit Erg11 and thereby block the biosynthesis of ergosterol, the predominant sterol of fungal membranes (17). Inhibition results in the accumulation of toxic intermediates in ergosterol biosynthesis, culminating in severe membrane stress. Resistance to azoles is a complex trait that can arise by multiple mutations whose phenotypic consequences are contingent on cellular stress responses (7) and interactions with genetic variants in particular genomes (1, 2).

We determined that the key mediator of Hsp90-dependent azole resistance is calcineurin (7), an Hsp90 client protein and a conserved calcium-activated protein phosphatase. In fungi, calcineurin regulates cell cycle progression, morphogenesis, and virulence (10). Hsp90 binds the catalytic subunit of calcineurin, keeping it stable and poised for activation (12, 14). Calcineurin activation is required for tolerance of a myriad of environmental stresses, including the membrane stress exerted by azoles (8, 22). By chaperoning calcineurin, Hsp90 regulates membrane stress responses that are crucial for cells to survive in the presence of azoles, thereby enabling the phenotypic consequences of new resistance mutations.

Here, we explored the genetic architecture of Hsp90-dependent drug resistance by dissecting the contribution of downstream effectors of calcineurin, Crz1 and Hph1. *S. cerevisiae* strains with resistance to the widely deployed azole fluconazole acquired by loss of function of the ergosterol biosynthetic enzyme Erg3 provide the ideal model due to the exquisite dependence of their resistance on both Hsp90 and calcineurin (7). This resistance mechanism blocks the accumulation of toxic sterol intermediates, resulting in altered membrane sterol composition (1). To validate this model, we used a selection regimen favoring Erg3 mutations. Plating ~10^4 cells of a strain with wild-type levels of Hsp90 (Hi90) on medium with a high concentration of fluconazole (128 μg/ml) yields three classes of colonies (Fig. 1A) (7): (i) large colonies (≥1.6 mm^2) with increased fluconazole resistance (Hi90-R; Fig. 1B), defined by an increase in the MIC at which growth is inhibited by 50% (MIC_{50}) relative to the drug-free growth control (all tested had mutations in Erg3 [7]); (ii) intermediate colonies with no change in resistance but with increased tolerance (Hi90-T; Fig. 1B), defined by growth at drug concentrations above the MIC_{50}; and (iii) small abortive colonies (≤0.7 mm^2). A concentration of an Hsp90 inhibitor that did not impair growth on its own (7) completely blocked the emergence of both fluconazole resistance and tolerance (Fig. 1A). As expected if Hsp90 enables the evolution of azole resistance by chaperoning calcineurin, an inhibitor of calcineurin function also blocked the evolution of both resistance and tolerance to fluconazole (Fig. 1A). Furthermore, inhibitors of Hsp90 or calcineurin abolished both resistance and tolerance phenotypes that had been acquired in their absence (Fig. 1C).

MIC testing and selection experiments were performed as previously described (7). Selection plates were photographed under standard conditions, and images were processed using the free software program CellProfiler (www.cellprofiler.org [15]) in order to measure colonies and classify them as resis-

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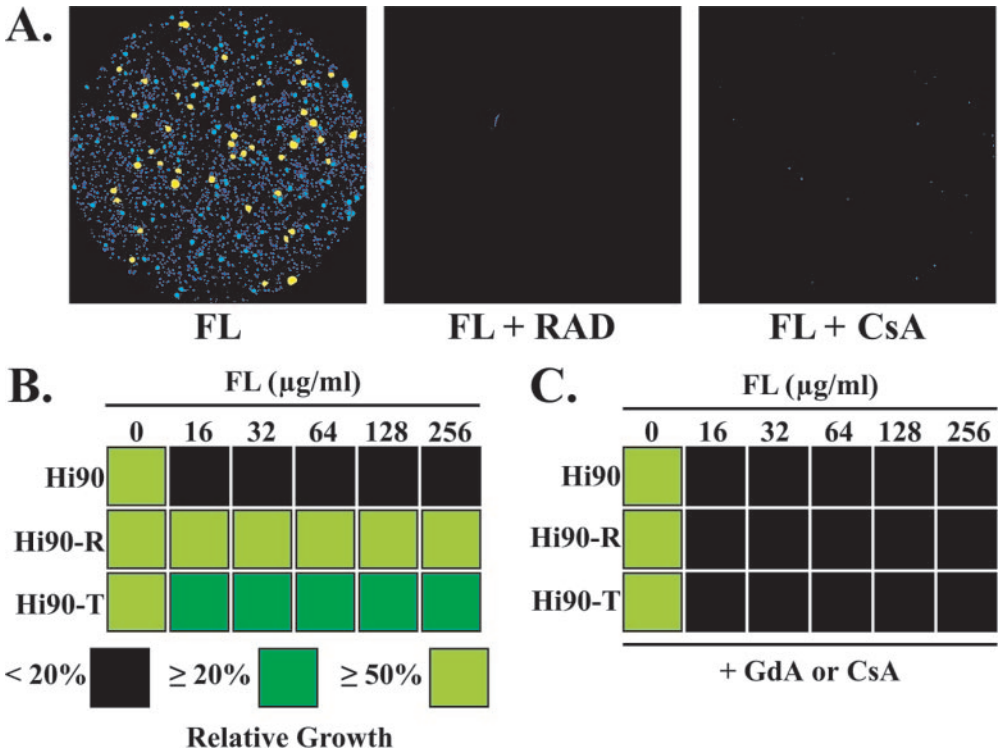

FIG. 1. Hsp90 and calcineurin mediate both fluconazole (FL) resistance and tolerance acquired by acute selection. (A) Pharmacological inhibitors of Hsp90 (radicicol [RAD]) or calcineurin (cyclosporine [CsA]) block the emergence of both FL resistance and tolerance. Approximately 10^4 cells of a strain with wild-type levels of Hsp90 (Hi90) were plated onto synthetic defined (SD) medium with 128 μg/ml FL supplemented with 1 μM RAD or 20 μM CsA, as indicated, and incubated for 7 days at 23°C. Photographs of selection plates were analyzed with CellProfiler software. The large colonies (≥1.6 mm^2, shown in yellow) had resistant phenotypes (Hi90-R, Fig. 1B), the intermediate colonies (turquoise) had tolerant phenotypes (Hi90-T, Fig. 1B), and the smallest colonies (≤0.7 mm^2, purple) were abortive. (C) Inhibitors of Hsp90 (geldanamycin [GdA]) or calcineurin (CsA) abrogate both FL resistance and tolerance. Resistance was measured at 23°C by broth microdilution in SD medium supplemented with 5 μM GdA or 20 μM CsA, as indicated. Optical densities at 595 nm of MIC test plates were averaged for duplicate measurements and normalized relative to the FL-free controls (see color legend).

tant, tolerant, or abortive. The following steps were carried out automatically (without user intervention) on all images: (i) the red channel was extracted from each color image; (ii) the plate was located by alignment with a template; (iii) aligned images were cropped to standard dimensions; (iv) gradients in illumination across each plate were corrected, and background was subtracted; (v) the plate rim was removed by cropping; (vi) colonies were identified using an algorithm detecting local maxima in the distance transform of the image; (vii) dividing lines between clumped colonies were identified using a watershed on the distance-transformed image; (viii) the area of the identified colonies was measured; and (ix) colonies were classified and color coded by size. CellProfiler software provides a powerful analytical tool for high-throughput selection experiments.

The cellular circuitry underlying *erg3*-mediated resistance is of particular interest as azole-resistant clinical isolates of *C. albicans* harboring this resistance mechanism have been recovered from patients treated with azoles (23). Since *erg3* mutants were not recovered in *C. albicans* in response to the acute selection regimen described above (7), we constructed a *C. albicans* homozygous *erg3* deletion mutant by disrupting the remaining *ERG3* allele in a heterozygous deletion mutant, as previously described (7). Strikingly, the exquisite Hsp90- and calcineurin-dependent azole resistance phenotype of *erg3* mutants identified in *S. cerevisiae* was conserved in *C. albicans* (Fig. 2).

The best-characterized downstream effector of calcineurin is the zinc finger transcription factor Crz1. When calcineurin is activated by stress, it dephosphorylates Crz1, causing translocation of the transcription factor from the cytosol to the nucleus (26). Crz1 is the major effector of calcineurin-regulated gene expression in both *S. cerevisiae* and *C. albicans*, activating a suite of genes involved in signaling pathways, ion/small molecule transport, cell wall integrity, and vesicular trafficking (13, 25, 27). In *S. cerevisiae*, azoles stimulate Ca^{2+} influx, activating calcium signaling pathways and upregulating genes controlled by Crz1 (4, 9). While Crz1 mediates most Ca^{2+}- and Na^{+}-induced calcineurin-dependent gene expression (13, 27), cells lacking Crz1 function are less sensitive to alkaline pH or to high salt concentrations than cells lacking calcineurin function (11, 13, 25). If Crz1 were the sole downstream effector of calcineurin modulating azole resistance, then deletion of *CRZ1* in *erg3*Δ mutants would abolish resistance. Double mutants ($n = 15$), constructed by mating single mutants in the BY4741/2 background followed by sporulation, all showed a partial loss of resistance but were not as sensitive as *erg3*Δ mutants lacking the regulatory subunit of calcineurin required for its activation,

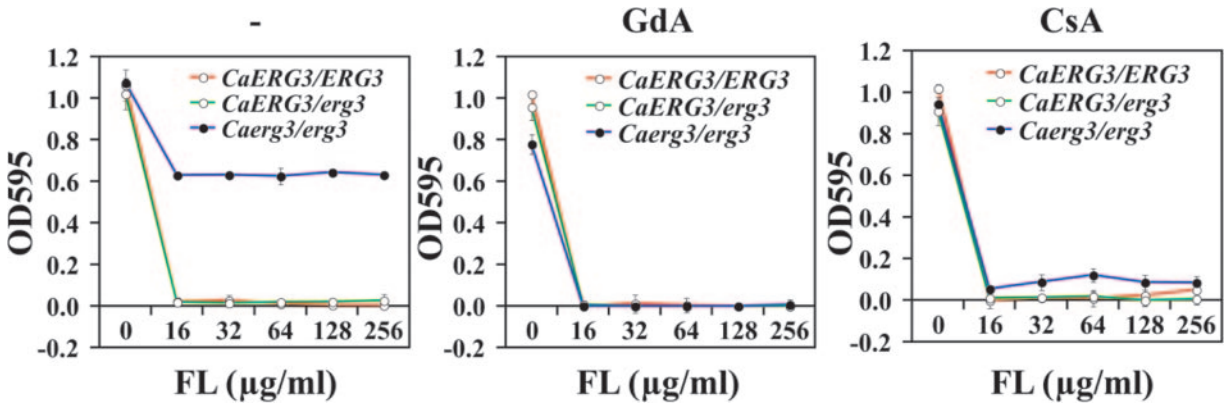

FIG. 2. Hsp90- and calcineurin-dependent fluconazole (FL) resistance phenotype of *S. cerevisiae erg3* mutants is conserved in *C. albicans*. FL resistance of a *C. albicans* parental strain (Ca*ERG3*/*ERG3*) and both heterozygous (Ca*ERG3*/*erg3*) and homozygous (Ca*erg3*/*erg3*) *erg3* deletion mutants was measured at 30°C in synthetic defined medium supplemented with inhibitors of Hsp90 (geldanamycin [GdA], 5 μM) or calcineurin (cyclosporine [CsA], 20 μM), as indicated. Optical densities at 595 nm (OD595) of MIC test plates were averaged for three replicates. Error bars are standard deviations.

Cnb1 (Fig. 3A). Pharmacological impairment of Hsp90 or calcineurin function abolished the residual tolerance of *erg3*Δ *crz1*Δ double mutants (Fig. 3A). Related findings for *C. albicans* cells with wild-type Erg3 function indicate that Crz1 has only a modest effect on azole tolerance (13, 18). These results suggest that additional downstream effectors of calcineurin modulate cellular responses to azoles.

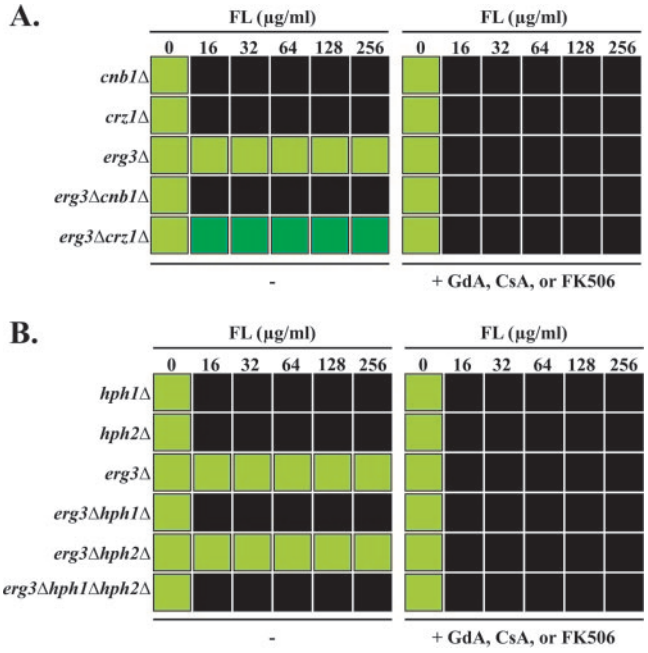

FIG. 3. Downstream effectors of calcineurin Crz1 and Hph1 modulate fluconazole (FL) tolerance and resistance. (A) FL resistance of mutants in the BY4741/2 background lacking the activating regulatory subunit of calcineurin Cnb1, the calcineurin downstream effector Crz1, or the ergosterol biosynthetic enzyme Erg3 and double mutants. Resistance was measured at 30°C in synthetic defined (SD) medium (left panel) and SD medium supplemented with an inhibitor of Hsp90 (geldanamycin [GdA], 5 μM) or calcineurin (cyclosporine [CsA], 20 μM, or FK506, 10 μM) (right panel), and was analyzed as in Fig. 1B. (B) FL resistance of mutants in the BY4741/2 background lacking the calcineurin downstream effector Hph1, its homolog Hph2, or Erg3 and double and triple mutants, as above.

An ideal candidate for a second downstream effector of calcineurin mediating Hsp90-dependent azole resistance is Hph1. The tail-anchored integral membrane proteins Hph1 and Hph2 serve redundant roles in promoting survival during stress, including alkaline pH, high salt, and cell wall stress; they function independently of Crz1 and have a greater impact on growth under alkaline conditions than does Crz1 (11). Calcineurin directly dephosphorylates Hph1, altering its distribution within the endoplasmic reticulum, but does not interact with or dephosphorylate Hph2 (11). Strikingly, *erg3*Δ *hph1*Δ double mutants ($n = 4$), constructed by mating single mutants in the BY4741/2 background followed by sporulation, all showed a complete loss of resistance comparable to that observed with loss of calcineurin function in the *erg3*Δ background (Fig. 3B). Deletion of *HPH2* did not alter the sensitivity of the *erg3*Δ *hph1*Δ double mutant ($n = 2$) or the resistance of the *erg3*Δ mutant ($n = 4$); this resistance was abrogated by inhibition of Hsp90 or calcineurin (Fig. 3B). These findings provide the first evidence for a key role for Hph1 in azole resistance in *S. cerevisiae*. With no apparent Hph1/2 homologs in *C. albicans*, the key effectors of Hsp90- and calcineurin-dependent azole resistance remain to be identified in this organism.

Drug resistance is often a complex trait affected by natural genetic variation in yeast (19). We examined the potential for genetic variation affecting these signaling pathways among *S. cerevisiae* strains by creating the double and triple mutants in hybrid backgrounds between BY4741 and W303. The striking result was that meiotic segregants of the same genotype showed two different growth responses to fluconazole. For example, three of the seven *erg3*Δ meiotic progeny exhibited the expected resistance phenotype, while the remainder exhibited sensitivity (Fig. 4). The *erg3*Δ *crz1*Δ progeny segregated between tolerance ($n = 5$) and sensitivity ($n = 6$). The *erg3*Δ *hph1*Δ progeny consisted of both resistant ($n = 2$) and sensitive strains ($n = 4$), as did the *erg3*Δ *hph2*Δ progeny. Finally, the *erg3*Δ *hph1*Δ *hph2*Δ progeny consisted of both tolerant ($n = 6$)

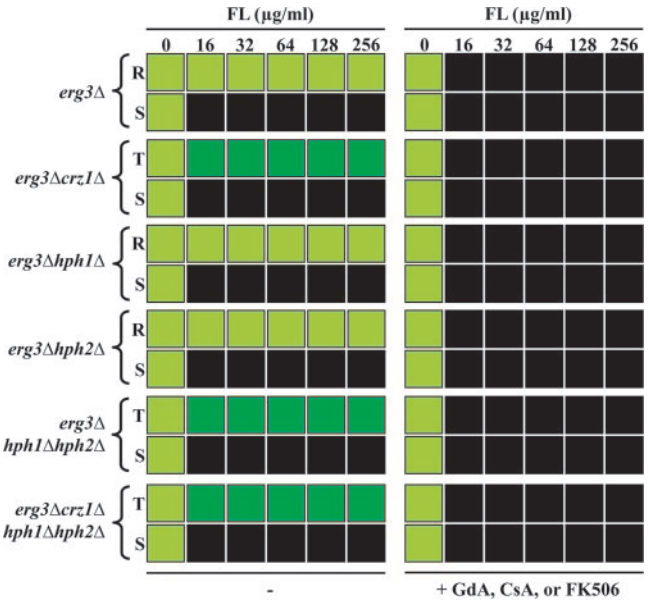

FIG. 4. Genetic variation between two *S. cerevisiae* strains affects fluconazole (FL) resistance and tolerance. Shown is the response to FL in synthetic defined (SD) medium (left panel) and SD medium supplemented with an inhibitor of Hsp90 (geldanamycin [GdA], 5 μM) or calcineurin (cyclosporine [CsA], 20 μM, or FK506, 10 μM) (right panel) of haploid single, double, triple, and quadruple mutants created by crossing BY4741 and W303 backgrounds, analyzed as in Fig. 1B. R indicates the resistant class of progeny, T indicates the tolerant class, and S indicates the sensitive class.

and sensitive ($n = 6$) strains. To determine if the genetic variation affecting the response to azoles that was segregating between the genetic backgrounds was in a single gene or was multigenic, we crossed an *erg3*Δ *hph1*Δ resistant haploid with a sensitive one. Only 2 out of the 13 meiotic tetrads tested showed the 2:2 ratio of resistance to sensitivity expected for a single segregating locus, suggesting that the functional variation between backgrounds was multigenic (data not shown).

The tolerant *erg3*Δ *hph1*Δ *hph2*Δ triple mutants enabled us to determine if Crz1 and Hph1/Hph2 were the only two pathways directly downstream of calcineurin modulating azole resistance. If this were the case, then deletion of *CRZ1*, *HPH1*, and *HPH2* would abolish the resistance of the *erg3*Δ mutant. Three out of seven of the haploid *erg3*Δ *crz1*Δ *hph1*Δ *hph2*Δ quadruple mutants retained tolerant phenotypes; Hsp90 or calcineurin inhibitors abrogated this tolerance (Fig. 4). Taken together, these results demonstrate that both Crz1 and Hph1/Hph2 modulate azole resistance and further suggest that additional resistance determinants work through this Hsp90- and calcineurin-dependent cellular circuitry. One candidate would be protein kinase CK2, which has recently been reported to modulate azole resistance in *C. albicans* through a calcineurin-dependent pathway distinct from Crz1 (5). The functional genetic variation between strains examined here could also be identified through analysis of novel calcineurin substrates (6) or through genome-wide mapping studies (19).

Understanding the genetic architecture of Hsp90- and calcineurin-dependent drug resistance is of broad therapeutic importance. Hsp90 and calcineurin have emerged as promising new targets for therapeutics directed against diverse fungal pathogens (3, 7, 16). Inhibitors of Hsp90 or calcineurin function may enhance the efficacy of existing antifungals, rendering recalcitrant pathogens more responsive to treatments. Hsp90 inhibitors are in currently in phase II clinical trials as anticancer agents, and calcineurin inhibitors are widely deployed as immunosuppressants. Given that Hsp90 and calcineurin are highly conserved regulators of cell signaling in eukaryotes, successful deployment of inhibitors of their function in antifungal therapy may be compromised by toxicity to the host. Identifying fungus-specific components of this cellular circuitry with a global impact on drug resistance and virulence would provide novel therapeutic targets.

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