The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype

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Abstract | The emergence of drug resistance in pathogenic microorganisms provides an excellent example of microbial evolution that has had profound consequences for human health. The widespread use of antimicrobial agents in medicine and agriculture exerts strong selection for the evolution of drug resistance. Selection acts on the phenotypic consequences of resistance mutations, which are influenced by the genetic variation in particular genomes. Recent studies have revealed a mechanism by which the molecular chaperone heat shock protein 90 (Hsp90) can alter the relationship between genotype and phenotype in an environmentally contingent manner, thereby 'sculpting' the course of evolution. Harnessing Hsp90 holds great promise for treating life-threatening infectious diseases.

The emergence of drug resistance is an evolutionary process that is based on selection for organisms that have an enhanced ability to survive and reproduce in the presence of a drug[1–5]. In competitive and communicative microbial communities in nature, microorganisms invest considerable energy in the production and elaboration of antimicrobial agents[6–8]. Consequently, the evolution of drug resistance is ubiquitous in nature, and microorganisms explore diverse strategies to outcompete their neighbours. Antimicrobial agents are widely used for treating infectious disease — for example, in animal husbandry and the management of agricultural pests — which has accelerated the evolution of drug resistance in many microorganisms[9].

Drug resistance not only poses a major threat to human health, but also has striking economic consequences. For example, a conservative estimate of the annual 'evolution bill' in the United States is US$33 billion[9]; this figure includes the annual cost of treating patients who have drug-resistant infections, the additional pesticides that are required to manage resistant pests and the loss of crops owing to resistant pests. Increases in the frequency of drug resistance can be attributed to multiple factors, including an increase in the frequency of intrinsically resistant species[10,11]; the indiscriminate exchange of mobile resistance determinants, particularly among bacteria[7,12,13]; and the *de novo* accumulation of mutations that confer resistance[1,14,15]. The evolutionary dynamics depend on the biology and population size of the microorganism in question, the properties of the drug that is deployed and other factors that influence the opportunity for the genetic exchange of resistance determinants. The fact that the emergence of drug resistance outpaces the development of new antimicrobial agents underscores the crucial importance of understanding the evolutionary mechanisms that lead to the development of resistance.

Drug resistance has traditionally been approached from a mechanistic perspective, in terms of identifying the cellular determinants that prevent a drug from entering a cell, remove a drug from the cell, inactivate a drug or prevent a drug from inhibiting its target. From an evolutionary perspective, it is clear that none of these mechanisms acts alone. The phenotypic effects of mutations that confer resistance depend on the genetic variation that has accrued in particular genomes. This is illustrated by the fact that the development of resistance is often accompanied by a fitness cost or deleterious effect on growth in the absence of the drug. In viruses, bacteria and fungi, this cost is often mitigated by the accumulation of compensatory mutations that enhance the fitness of the resistant genotype in the absence of the drug[3,16]. In a broader context, as with fitness in most environments, fitness in the presence of a drug is a complex trait, and many loci affect the fitness landscape[17,18].

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doi:10.1038/nrmicro1835
Published online
4 February 2008

Fungal pathogens pose a particularly interesting challenge, because they are eukaryotes and share close evolutionary relationships with their human hosts[19]. The number of drug classes that have distinct targets in fungi is limited and the usefulness of most antifungal drugs is compromised owing to either severe host toxicity or diminished efficacy in killing fungal pathogens[20]. Fungi are renowned for causing life-threatening disease in immunocompromised individuals, but can also pose a threat to healthy humans[21]. The frequency of fungal infections has increased in recent years — for example, in the United States, bloodstream infections caused by fungi increased by 207% between 1979 and 2000 (REFS 22,23) — along with the number of individuals who have become immunocompromised owing to the treatment of malignancies, organ transplantation and autoimmune disorders. Most of the deaths that are attributed to fungal pathogens are caused by *Candida albicans* and *Aspergillus fumigatus*[23,24]. Fungal infections are notoriously difficult to treat owing to high mortality rates, and cost the health-care system billions of dollars each year in the United States alone[25]. New strategies are therefore required to predict and prevent the evolution of fungal drug resistance. As eukaryotes, fungi also provide tractable model systems to study evolution[1,26,27], cellular signalling[28] and the genetic architecture of complex traits[29–32].

This Review focuses on the mechanisms that potentiate the evolution of fungal drug resistance, with an emphasis on the central role of the molecular chaperone heat shock protein 90 (Hsp90) in remodelling the relationship between genotype and phenotype. A brief survey of the key classes of antifungal drugs and canonical resistance mechanisms will be provided, before a discussion on the salient principles of Hsp90-mediated phenotypic diversity and how these principles apply to the evolution of fungal drug resistance. Additional mechanisms by which alterations in cellular states can influence the emergence of drug resistance, including the effects of prions, biofilms and persister cells, will then be highlighted. Finally, the possibility of harnessing evolution for therapeutic benefits through combinatorial cellular perturbations will be explored.

Mode of action of antifungal drugs

Investigating how fungi evolve drug resistance requires an appreciation of how drugs exert their toxic effects. The current arsenal of antifungal drugs targets a limited number of cellular processes (FIG. 1). Most of the antifungal drugs that are in clinical use target the biosynthesis of ergosterol, which is the major sterol in fungal cell membranes and is analogous to cholesterol in the mammalian cell. Ergosterol is the target of the polyenes, which include amphotericin B, a drug that has been clinically exploited for over 50 years. The usefulness of amphotericin B has been compromised, however, by its toxicity to the host, probably owing to effects on cholesterol-containing host-cell membranes. Other steps in the ergosterol biosynthesis pathway are targeted by the allylamines, thiocarbamates, morpholines and azoles[11,20,33]. The azoles are well tolerated, have activity against diverse fungi and have been one of the most widely used classes of antifungal drugs for decades; they target lanosterol 14α-demethylase (encoded by *ERG11*), and therefore block the production of ergosterol and cause the accumulation of a toxic sterol intermediate[33].

The only new class of antifungal drug to have reached the clinic in recent decades is the echinocandins. The echinocandins block cell-wall synthesis by inhibiting β-(1,3)-D-glucan synthase and have favourable safety profiles and a broad spectrum of activity[34].

Canonical resistance mechanisms

A basic understanding of the key mechanisms of antifungal drug resistance is a prerequisite to further discussion of more complex modulations of genotype to phenotype. Although fungal responses to antifungal drugs are complex, and not easily categorized as resistant versus sensitive[1,4,35], several molecular alterations enable fungi to survive exposure to antifungal drugs. Rather than a comprehensive review of the mechanisms of fungal drug resistance, which can be found elsewhere[11,33,36,37], here, the focus is on salient themes and recent developments as they apply to two of the most widely used classes of antifungal drugs, the azoles and echinocandins (FIG. 2).

Increased efflux of a drug from a cell is a ubiquitous resistance mechanism in a wide range of cells, from bacteria to cancer cells, and is also important in fungi (FIG. 2). Constitutive upregulation of a multidrug transporter of the major facilitator class has been shown to confer resistance to the azole fluconazole in species of *Candida* and *Aspergillus*[38–42]. Similarly, constitutive upregulation of multidrug transporters of the ATP-binding cassette (ABC) family confers resistance to multiple azoles, as well as other drugs[37,43]. Recent progress has been made in deciphering the regulatory circuitry that governs the expression of both classes of transporters[44–50]. Upregulation of these efflux pumps can result from either the accumulation of hyperactivating mutations or amplification of relevant transcription factors. In *C. albicans*, an intriguing mechanism of gene amplification that confers azole resistance was recently identified[51]. This mechanism involves the formation of an isochromosome, in which a chromosome arm that harbours both a transcription factor that regulates ABC transporters and the target of the azoles, Erg11, is duplicated. In contrast to the central role of multidrug

Figure 1 | **Antifungal drugs and their targets.** The main classes of antifungal drugs that are in clinical use and how they exert their effects on the fungal cell. **a** | Azoles, such as fluconazole, voriconazole and posaconazole, inhibit Erg11, block the production of ergosterol and cause the accumulation of a toxic sterol intermediate, which results in cell membrane stress. Sterol synthesis occurs in the endoplasmic reticulum. **b** | Polyenes, such as amphotericin B, bind to ergosterol, thereby forming pores in cell membranes. **c** | 5-flucytosine inhibits DNA and RNA synthesis. **d** | Echinocandins, such as caspofungin and micafungin, inhibit β-(1,3)-D-glucan synthase (the catalytic subunit is encoded by *FKS1* and *FKS2* in *Saccharomyces cerevisiae*), and thus disrupt cell-wall integrity. ►

Persister cell
A metabolically quiescent cell that neither grows nor dies when exposed to cidal concentrations of antimicrobial compounds.

Polyene
A class of antifungal drug that intercalates into ergosterol-containing fungal membranes, thereby forming membrane-spanning channels that lead to the leakage of cellular components and cell death.

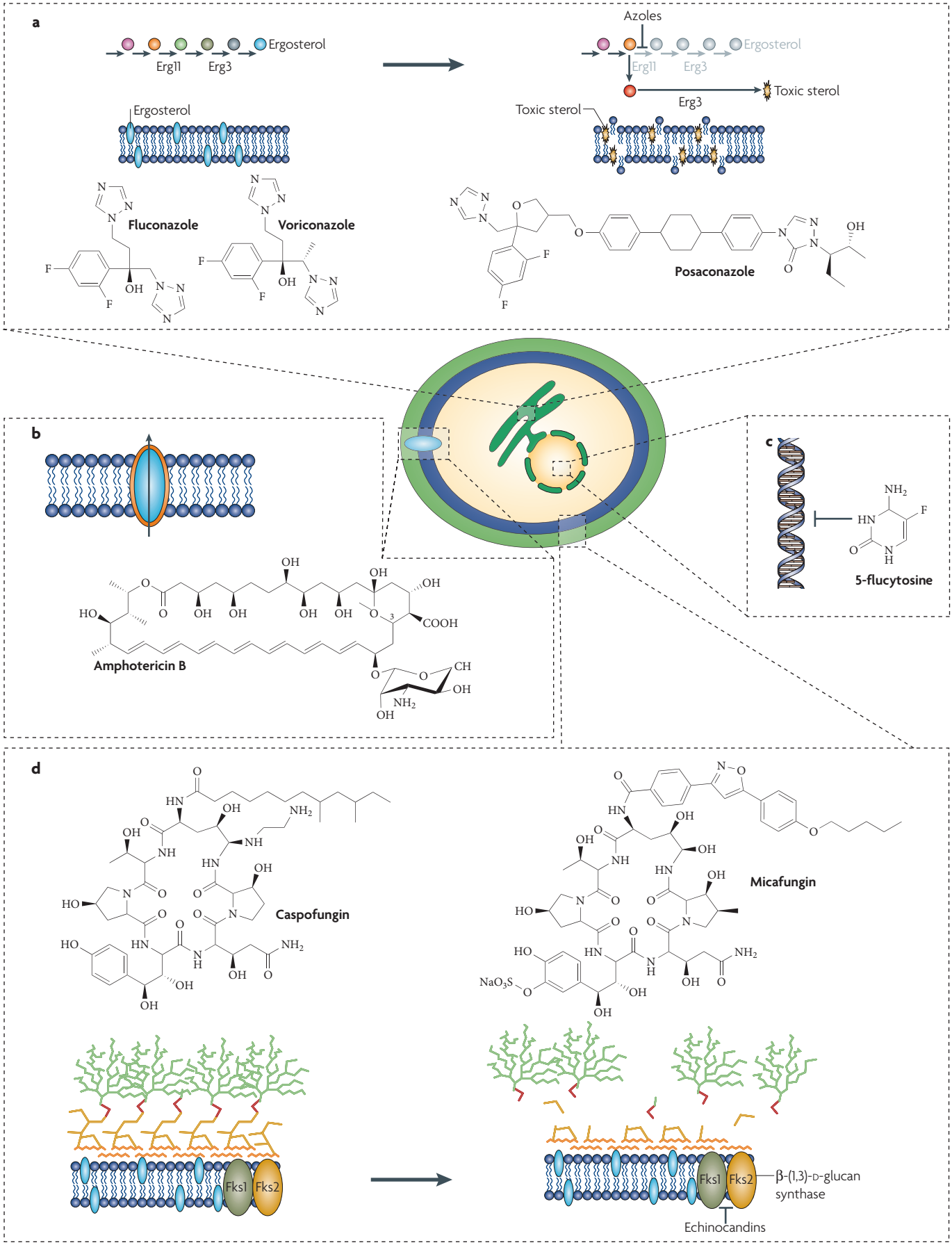
Azole
A class of antifungal drug that inhibits fungal cytochrome $P450_{14DM}$ (also known as lanosterol 14α-demethylase), which is encoded by *ERG11* and catalyses a late step in the biosynthesis of ergosterol; includes the triazoles (for example, fluconazole, voriconazole and posaconazole) and the imidazoles.

Echinocandin
A class of antifungal drug that interferes with fungal cell-wall biosynthesis by inhibiting β-(1,3)-D-glucan synthase; includes caspofungin and micafungin.

Major facilitator class
A large family of proteins that uses the energy that is provided by the proton motive force of the membrane to transport substrates across the membrane.

ATP-binding cassette family
A member of a large family of proteins that uses the energy that is provided by the hydrolysis of ATP to transport substrates across membranes.

Isochromosome
An abnormal chromosome that possesses a median centromere and two identical arms.



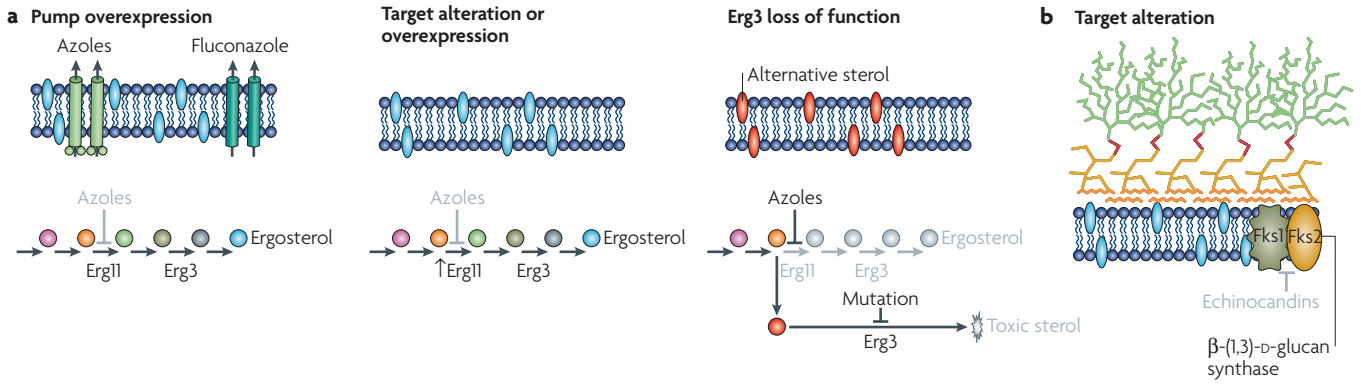


Figure 2 | **Azole and echinocandin resistance mechanisms. a** | Resistance to azoles can result from the upregulation of two classes of efflux pump that remove the drug from the cell, through the mutation or overexpression of Erg11, which minimizes the impact of the drug on the target, or alterations in ergosterol biosynthesis, such as the loss-of-function mutation of Erg3, which blocks the accumulation of a toxic sterol intermediate that is produced when Erg11 is inhibited by azoles. **b** | Resistance to echinocandins can result from mutations in Fks1 that minimize the impact of the drug on the target.

transporters in resistance to azoles, echinocandin resistance does not seem to be tightly coupled to the expression of efflux pumps[52].

A second common mechanism by which cells acquire resistance is the alteration or amplification of the drug target, which minimizes the impact of the drug on the cell (FIG. 2). Mutation or overexpression of Erg11 has been associated with azole resistance in *Candida* and *Aspergillus* species[37,43]. Mutation of the target of the echinocandins, Fks1, is a common mechanism of resistance to echinocandins in fungi[53].

A third category of resistance mechanism involves cellular alterations that minimize the toxicity of the drug. Most examples involve alterations in the ergosterol biosynthetic pathway that are associated with azole resistance (FIG. 2). For example, upregulation of ergosterol biosynthetic genes confers azole resistance[54,55], which is expected, given that a reduction in the target or pathways that are affected by a drug often confers hypersensitivity to the drug, a principle that has been widely exploited in drug-target identification[56–58]. One specific alteration in ergosterol biosynthesis that confers azole resistance is loss of function of Erg3, which blocks the accumulation of the toxic sterol that would otherwise occur if Erg11 was inhibited[33].

Fitness effects of resistance mutations

The dissemination of resistant organisms is crucially dependent on the fitness of resistant mutants. In a clinical context, drug resistance often emerges through the accumulation of multiple mechanisms of resistance[42,59]. There are three possible phenotypic effects of combining resistance mutations: they might not interact (the fitness effect of the combined mutations is the product of their individual fitness effects); they might interact synergistically (the fitness effects are greater than expected), which is termed positive epistasis; or they might interact antagonistically (the fitness effects are less than expected), which is termed negative epistasis[1]. In one study, combining azole resistance mechanisms that evolved under different selection regimens revealed strong antagonism[60]. The phenotypic effects of resistance mutations depend not only on other resistance mutations but also on additional genetic variation. For example, the initial acquisition of a mutation that confers drug resistance often also confers a fitness disadvantage in the absence of that drug, which then drives selection for compensatory mutations that ameliorate the deleterious effects of the resistance mutations[1,3,16]. Several experimental populations of *C. albicans* that evolved fluconazole resistance and overexpressed a multidrug transporter, as well as numerous other genes, initially showed a fitness cost of resistance[61,62]. This fitness cost was ameliorated through further evolution as many genes returned to basal levels of expression[63].

Sculpting fitness landscapes

Because natural selection acts on the phenotypic consequences of genetic variation, it stands to reason that mechanisms that modulate the relationship between genotype and phenotype would have a profound impact on the evolution of drug resistance. Recent studies have revealed that an evolutionarily conserved mechanism of protein folding can modulate the phenotypic consequences of mutations that confer antifungal drug resistance[64,65]. The central player is Hsp90, an essential molecular chaperone that regulates the form and function of diverse signal transducers[66–69]. Alterations in the relationship between genotype and phenotype can be mediated by inhibiting the function of Hsp90 in distinct ways (BOX 1), including genetic alterations[70,71], pharmacological inhibitors[72,73] and environmental stress[74].

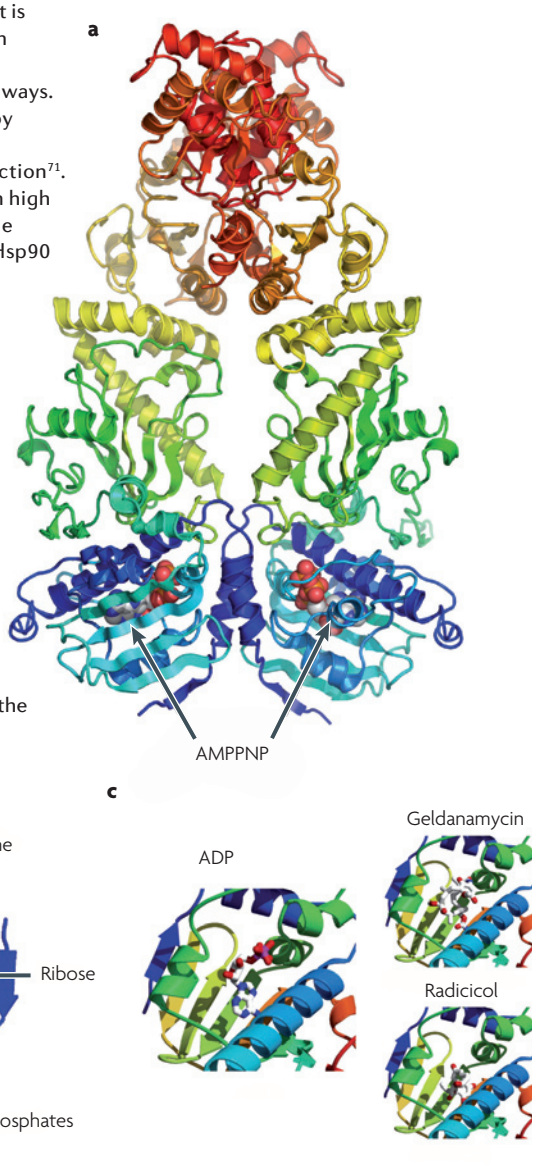
Hsp90-mediated genetic capacitance. Hsp90 was first implicated in modulation of the relationship between genotype and phenotype in evolutionarily distant species, including flies and plants. An emergent property of the role of Hsp90 in stabilizing key regulators of cellular signalling is its function as a capacitor for the storage and release of genetic variation[75,76]. Hsp90 can allow variation to accumulate in a silent state that is revealed only when the function of Hsp90 is compromised, for

Box 1 | **Inhibiting Hsp90 function**

Heat shock protein 90 (Hsp90) cooperates with a plethora of co-chaperones and cycles dynamically through complexes with client proteins to enable the maturation of these key signal transducers. It resides primarily in the cytoplasm as a homodimer (see the figure, part **a**) and, in many organisms, is produced in vast excess of the amount that is required for normal growth. Under basal conditions, it is one of the most abundant proteins and is strongly induced in response to environmental stress[143].

Hsp90 function can be inhibited in three complementary ways. First, its function can be compromised genetically, either by drastically reducing expression levels[70] or by destabilizing mutations that cause a temperature-dependent loss of function[71]. Second, structurally diverse small molecules that bind with high affinity to the unusual ATP-binding pocket in Hsp90 (see the figure, parts **b** and **c**) can inhibit Hsp90 function. Because Hsp90 inhibitors, such as geldanamycin and radicicol, bind with higher affinity than natural nucleotides, they block the ATPase-dependent conformational changes that are required for chaperone activity[72,73]. Drug-bound Hsp90 recruits E3 ubiquitin ligases to client proteins, which results in their proteasome-mediated degradation[144]. Third, the most relevant mechanism to compromise Hsp90 function in nature is environmental stress, such as increased temperature. Although Hsp90 is induced in response to stress, the global problems in protein folding that ensue can increase the number of cellular targets for Hsp90 and titrate Hsp90 away from key client proteins, thereby compromising Hsp90 function[74].

Part **a** of the figure shows the crystal structure of full-length *Saccharomyces cerevisiae* Hsp90 in complex with a non-hydrolysable ATP analogue (AMPPNP) and the co-chaperone p23/Sba (not shown)[145]. Part **b** of the figure shows the ribbon structure of the amino-terminal domain of *S. cerevisiae* Hsp90 and the binding site for ATP and ADP. Part **c** of the figure compares the co-crystal structures of the *S. cerevisiae* Hsp90 amino domain and bound ADP with complexes of geldanamycin and radicicol. Structure **a** is courtesy of L. Pearl and C. Prodromou, Institute of Cancer Research, Chester Beatty Laboratories, London, UK . Panels **b** and **c** reproduced, with permission, from REF. 66 © 2006 Annual Reviews.



example, during stress (FIG. 3). Hsp90 also buffers epigenetic variation, which results in heritable chromatin alterations[77]; this is consistent with recent data that established a connection between Hsp90 and the DNA helicases that are involved in chromatin remodelling[78]. Conceivably, Hsp90 could modulate the phenotypic consequences of variation in Hsp90-dependent client proteins, in proteins that acquire destabilizing mutations that render them dependent on Hsp90 or in other components of Hsp90-dependent pathways[74,79,80]. Traits that are initially dependent on Hsp90 can evolve Hsp90 independence (FIG. 3), thereby providing an environmentally contingent mechanism that couples the emergence and fixation of new traits[76]. In the context of cancer, Hsp90 also has a role in enabling somatic evolution, probably through multiple mechanisms, for example, buffering the effects of the genetic alterations that are associated with malignant transformation (BOX 2). Functionally compromising many genes in complex genetic networks can expose phenotypic variation[81,82], which thus provides a broad framework in which alterations in cellular circuitry can promote the emergence of new traits[83].

Epigenetic variation
Variation that is caused by heritable changes that are not a result of a change in the DNA sequence.

Hsp90 and fungal drug resistance

The model yeast *Saccharomyces cerevisiae* provides the ideal 'experimental palate' to explore the effects of Hsp90 on the relationship between genotype and phenotype, and investigate the specific molecular mechanisms that are involved[65]. In *S. cerevisiae* strains in which

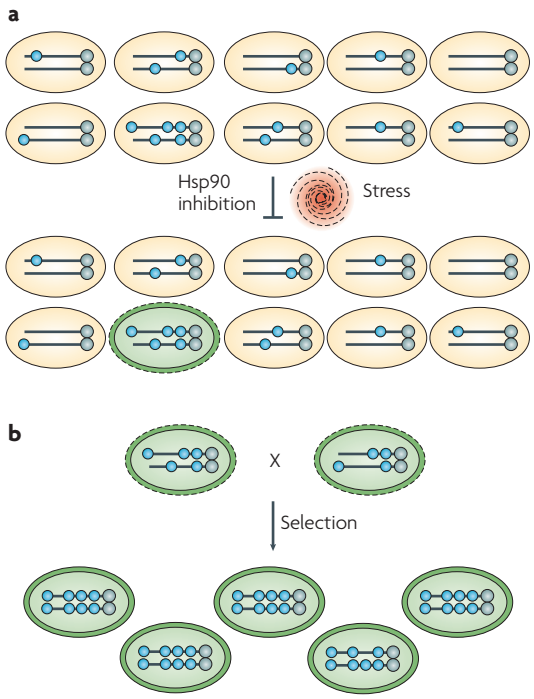


Figure 3 | **Hsp90-mediated genetic capacitance.** **a** | Heat shock protein 90 (Hsp90) can buffer the expression of genetic variation by keeping mutations in a silent state until they are revealed during Hsp90 inhibition or stress. A hypothetical population is shown that contains one pair of homologous chromosomes harbouring genetic polymorphisms (represented by blue circles) that do not affect phenotype if Hsp90 is fully functional. When Hsp90 is compromised, for example, during stress, rare individuals that are enriched in predisposing polymorphisms (represented by a green oval with a dashed perimeter) express a novel phenotype. **b** | Traits that are initially dependent on Hsp90 can evolve Hsp90 independence. Several generations of crossing individuals that express new traits when Hsp90 function is compromised can enrich underlying polymorphisms, such that a threshold is passed and the phenotype is expressed even when Hsp90 is fully functional (represented by a green oval with a solid perimeter). Figure adapted, with permission, from REF. 74 © (2004) John Wiley & Sons.

Hsp90 levels could be regulated either before or after drug selection, it was shown that Hsp90 potentiated the rapid emergence of azole resistance. Hsp90 is not only required to survive the stress that is associated with acute exposure to a high drug concentration, but is also intimately involved in the mechanism of resistance that is selected under these conditions — reducing Hsp90 levels in resistant mutants abrogates resistance. The Hsp90-dependent mechanism of resistance that is favoured by this selection regimen is loss of function of Erg3. Notably, Hsp90 is required both for Erg3-mediated resistance and the resistance that is acquired through diverse mutations in the genome[65].

The trivial explanation that a reduction in Hsp90 levels simply compromises growth, and therefore impairs the ability to adapt to new conditions, has been ruled out by the finding that strains that have low levels of Hsp90 can evolve azole resistance under a selection regimen that favours an Hsp90-independent mechanism of resistance[65]. The resistance mechanism that is favoured under this selection regimen is hyperactivating mutations in a transcription factor that causes the upregulation of multidrug transporters. Resistance that is acquired by this mechanism is stable even when Hsp90 function is compromised. Notably, in yeast and many other organisms, Hsp90 is expressed in vast excess of the level that is required for growth, which renders it ideally positioned to buffer genetic variation and regulate the function of key signal transducers.

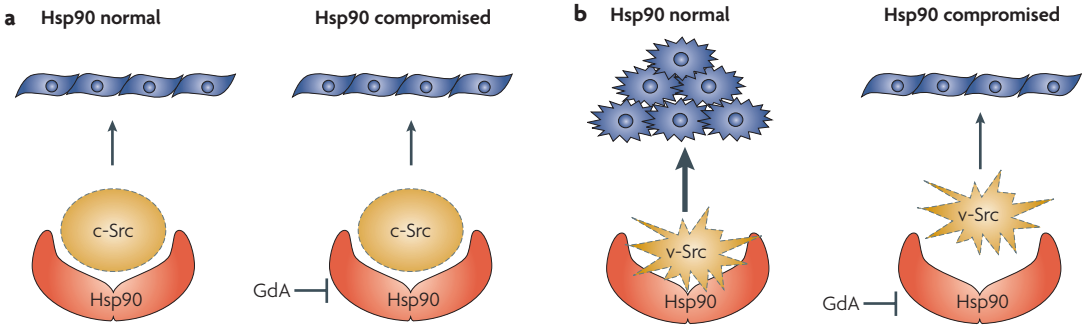
Hsp90 has a key role in the evolution of azole resistance; it enables the specific cellular signalling circuits that are required for cells to survive the membrane stress that is exerted by the antifungal drug (FIG. 4). The phenotypic effect of specific resistance mutations is contingent on these Hsp90-dependent cellular responses[65]. For example, the survival of Erg3 loss-of-function mutants is increased in the presence of azoles owing to a block in the ergosterol biosynthesis pathway that prevents the accumulation of the toxic sterol which is produced by the inhibition of Erg11. These mutants have an altered membrane sterol composition and can grow in the presence of azoles, although this is dependent on Hsp90-mediated cellular responses. However, if a resistance mechanism averts a toxic effect of the drug on the cell, such as overexpression of the multidrug transporters that remove the drug or mutations in the target enzyme that block drug activity, then it could be predicted that the phenotypic effects of the resistance mutations would be independent of Hsp90. The role of Hsp90 in the evolution of azole resistance is conserved in the pathogenic yeast *C. albicans*[65]. Inhibiting Hsp90 function blocks the emergence of azole resistance under a rapid selection regimen.

The calcineurin connection

How does Hsp90 modulate the phenotypic effects of diverse resistance mutations? In the simplest model, Hsp90 enables a common cellular response that is required for phenotypic effects. As the Hsp90-dependent resistance mutations were loss-of-function mutations, this is inconsistent with Hsp90 enabling the activity of these proteins. Rather, the effects of compromising Hsp90 function were similar to the phenotypic consequences of impairing calcineurin, a protein phosphatase and key regulator of cellular signalling[84–86]. Compromising calcineurin function genetically or pharmacologically enhances the susceptibility of *C. albicans* to azoles and *A. fumigatus* to echinocandins[87–89]. Calcineurin inhibitors are synergistic with these antifungals against wild-type cells. Although the effects of calcineurin inhibitors have also been observed with some azole-resistant strains, the specific mechanisms by which calcineurin enables resistance remain largely unknown.

Calcineurin has been shown to be an Hsp90 client protein in diverse species[90–92]. In *S. cerevisiae*, Hsp90 chaperones the unstable catalytic domain, which enables calcineurin-dependent stress responses. As expected if calcineurin is the key mediator of Hsp90-dependent azole

Box 2 | **Hsp90 and somatic evolution**



Cancer provides a particularly revealing example of how somatic evolution can be enabled by the chaperone machinery. By stabilizing key regulators of cellular signalling that are prone to misfolding, heat shock protein 90 (Hsp90) plays a central part in enabling malignant transformation. Together with other chaperones, Hsp90 is overexpressed in many tumours, which maintains protein homeostasis and enhances cell survival during external stress, such as exposure to hypoxia and acidosis[146,147]. In addition to mediating crucial stress responses, Hsp90 also enables malignant transformation by allowing cells to tolerate the genetic alterations that are characteristic of cancer cells, such as aberrant signalling and mutations that would otherwise be lethal[148,149]. Many key oncogenic regulators acquire mutations that make them prone to misfolding but activate their oncogenic potential. For example, most oncogenic mutations of the normal cellular Src tyrosine kinase (c-Src; see the figure, part **a**) involve truncations that result in loss of the regulatory domain, thus rendering the mutant protein (v-Src; see the figure, part **b**) constitutively active but exquisitely dependent on Hsp90 for function[150–152]. Compromising Hsp90 can reverse the oncogenic phenotype that arises from this and many other underlying genetic alterations. On an even more global scale, the master regulator of the heat shock response in eukaryotes, heat shock factor 1, enables malignant transformation by orchestrating adaptation to the complex network of cellular functions that support proliferation, survival, protein synthesis and metabolism[153].

resistance, pharmacological inhibition of calcineurin phenocopies the inhibition of Hsp90 in *S. cerevisiae*, *C. albicans* and species of *Aspergillus*[65] (FIG. 5). In *S. cerevisiae*, deleting the immunophilin that the calcineurin inhibitor cyclosporin A (CsA) must bind to in order to inhibit calcineurin blocks the effects of CsA on resistance; this confirms that the CsA-mediated reduction of azole resistance is indeed a result of the inhibition of calcineurin[65]. The connection between Hsp90 and calcineurin is conserved in diverse fungi, and these proteins seem to mediate responses to divergent stresses — cell-membrane stress in *S. cerevisiae* and *C. albicans* and cell-wall stress in *Aspergillus* species.

The genetic architecture of Hsp90-dependent azole resistance is complex, and has been explored using the power of *S. cerevisiae* genetics and *erg3* mutants as a model[64]. There is evidence that multiple effectors downstream of calcineurin mediate azole resistance, including Crz1, Hph1, Hph2, and others that remain to be determined. Crz1 mediates calcineurin-dependent transcription of a suite of genes that are involved in signalling pathways, ion and small-molecule transport, cell-wall integrity and vesicular trafficking[93–95]. Crz1 has a minor role in the azole tolerance of wild-type cells and resistant mutants[64,93,96]. Hph1 is not widely distributed in the fungal kingdom, but in *S. cerevisiae* it plays a major part in azole resistance, at least in certain strain backgrounds[64]. Hph1 is a resident protein of the endoplasmic reticulum and is dephosphorylated by calcineurin. Hph1 also has a redundant role, together with Hph2, in the promotion of survival during specific stresses, such as alkaline pH, high salt levels and cell-wall stress[97]. The mechanism by which Hph1 mediates azole resistance remains elusive. Although Hph1 is not found in *C. albicans*, Crz1 still plays only a partial part in azole resistance, which suggests that additional calcineurin-dependent effectors that mediate resistance remain to be identified.

Immunophilin
A family of *cis–trans* peptidylprolyl isomerases that was originally studied as a cellular receptor for immunosuppressive drugs, such as cyclosporin A and FK506; includes cyclophilins and FK506-binding proteins.

Role of Hsp90 in signalling beyond calcineurin

Although calcineurin might be the central regulator of cellular signalling through which Hsp90 potentiates the evolution of azole resistance, it is only one of the many regulators that are Hsp90 client proteins. Hsp90 is one of the most highly connected hubs in cellular networks. A recent high-throughput physical, genetic and chemical–genetic study of *S. cerevisiae* suggested that approximately 10% of the yeast proteome interacts with Hsp90 (REF. 78). A chemical–genetic screen of heterozygous *S. cerevisiae* deletion mutants revealed distinct connectivities for Hsp90 at 30 and 37°C[98]. The relationship between connectivity in genetic networks and effects on fitness and phenotypic variation remains complex[99]. However, the impact of Hsp90 on many different signalling pathways suggests that it might enable adaptation to diverse stresses through distinct cellular regulators. The global effects of Hsp90 on cellular signalling position this chaperone uniquely at the interface of the environment, genotype and phenotype.

Importance of Hsp90-dependent drug resistance

The evolution of drug resistance is a more complex process in a human host than in the laboratory, owing to pharmacokinetic variables, interactions with other microorganisms, the effects of immune cells and quiescent pathogen reservoirs. In a clinical context, increases in fungal drug resistance are often caused by multiple

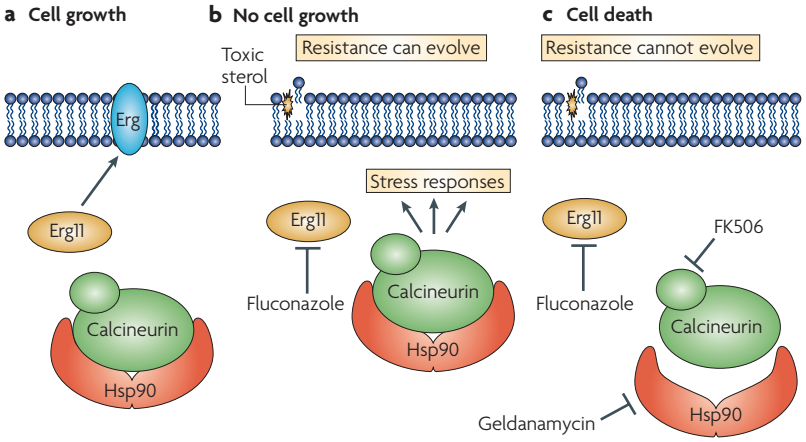


Figure 4 | **The role of Hsp90 in fungal drug resistance. a** | Under normal physiological conditions, fungal cells contain ergosterol in their cell membranes and calcineurin-dependent stress responses are not required for most species, including *Saccharomyces cerevisiae* and *Candida albicans*. **b** | Azole antifungals (for example, fluconazole) inhibit Erg11 and block the synthesis of ergosterol, which results in the accumulation of a toxic sterol intermediate that disrupts membrane integrity. Heat shock protein 90 (Hsp90) chaperones calcineurin, thereby enabling the signal transduction networks that are required for the emergence and maintenance of drug resistance. **c** | Hsp90 inhibitors (for example, geldanamycin) or calcineurin inhibitors (for example, FK506) block these signalling networks and, therefore, prevent the evolution of resistance and abrogate resistance once it has evolved.

mechanisms that operate in the same cell[42,59]. Strikingly, inhibiting Hsp90 has profound effects on the resistance that evolves in this context (FIG. 5). One study examined a series of clinical isolates that were recovered over a 2-year period from an HIV-1-infected patient who was treated with fluconazole. It was found that early in the course of treatment the basal fluconazole resistance phenotypes were exquisitely dependent on Hsp90, but this dependence gradually evolved towards Hsp90 independence[65]. These findings recall the early seminal studies of Waddington[100,101], who suggested that selection on traits that are initially expressed only in response to stress can lead to genetic assimilation, with loss of environmental sensitivity and a trait becoming fixed. Intriguingly, these steps towards Hsp90 independence correspond with the upregulation of expression of multidrug transporters. In *C. albicans*, Hsp90 inhibitors can enhance the efficacy of azoles even against drug-sensitive clinical isolates, which often show tolerance to the azoles, as Hsp90 inhibitors can render the fungistatic azoles into a fungicidal combination. In pathogenic *Aspergillus* spp., Hsp90 seems to have a key role in mediating cellular responses to the cell-wall stress that is exerted by the echinocandins.

The natural environmental stress of febrile temperatures that are reached in an infected human phenocopies the effects of Hsp90 inhibition, and therefore reduces fungal drug resistance[65]. This suggests a specific mechanism for a therapeutic benefit of fever, which sensitizes the pathogen to drug-induced changes in cellular signalling. Notably, fever could provide optimal selective conditions for the evolution of fungal drug resistance from Hsp90-dependence to Hsp90-independence, as was observed in the series of clinical isolates discussed above. Although fevers have been shown to be beneficial in clearing infectious disease for many decades, they are rarely allowed to persist owing to the difficulty of establishing the benefits versus the potential costs[102].

Altered cellular states

Although Hsp90 provides one of the most explicit examples of a mechanism that can alter the relationship between genotype and phenotype, and potentiate evolution, there are several other ways in which alterations in the cellular state can affect resistance phenotypes. One extremely different mechanism by which changes in protein folding can reveal cryptic variation in a genome is provided by fungal prions, proteins that can adopt an altered conformation that is self-perpetuating and that are transmitted as a protein-based element of inheritance. A fungal prion that uses a particularly fascinating mechanism to modulate phenotypic diversity is [PSI$^+$][103,104], a prion that is an epigenetic modifier of the fidelity of translation termination. In some genetic backgrounds, conversion to the [PSI$^+$] prion state confers increased resistance to antibiotics such as bleomycin, anisomycin and benomyl, whereas for others it confers increased sensitivity[104]. These traits are mediated largely by the read-through of nonsense codons, which reveals cryptic variation in a genome[103]. Importantly, [PSI$^+$]-dependent traits are complex, and genetic re-assortment can cause them to become stable in the absence of [PSI$^+$], thereby providing a mechanism for the acquisition and assimilation of new traits.

Biofilms provide another intriguing example of how a single genotype can give rise to a spectrum of resistance phenotypes that are based on altered cellular states. Biofilms represent complex architectures of different cell types that form when free-moving (planktonic) cells interact with particular surfaces, such as plastics and catheters. The mechanisms by which these interactions initiate remodelling of the cellular state, alteration of the transcriptional programme, induction of morphological changes, production of an extracellular matrix and alteration of cell-to-cell communication have generated considerable interest, largely owing to the extraordinary resistance of biofilms to many antifungal agents[105–108]. *Candida* spp. biofilms are resistant to most antifungal drugs, including the azoles and amphotericin B, but are susceptible to the echinocandins. To date, the evidence suggests that many different factors contribute to biofilm drug resistance, including the upregulation of multidrug transporters, reduced drug diffusion and growth rate, and alterations of the plasma membrane and cell wall[109,110]. Cells that are recovered from biofilms often maintain elevated drug resistance when placed in planktonic conditions; consistent with transient epigenetic reprogramming, this resistance is not maintained during subsequent propagation under planktonic conditions[111,112]. Notably, both genetic and epigenetic regulation can impact on the heterogeneity of expression of cell-surface proteins that contribute to phenotypic variation in cell–cell and cell–surface interactions[113].

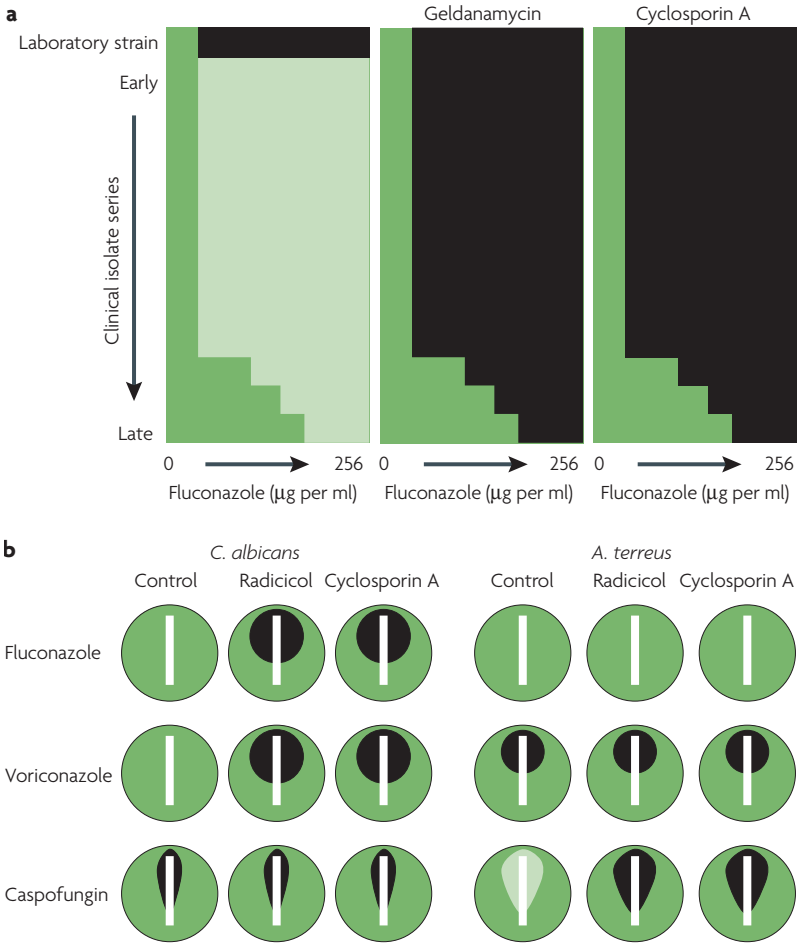


Figure 5 | **Inhibition of Hsp90 or calcineurin function abrogates drug resistance of pathogenic fungi. a** | The schematic on the left shows the fluconazole sensitivity of a *Candida albicans* laboratory strain and the resistance of serial clinical isolates that were recovered from an HIV-1-infected patient who received treatment with fluconazole over a 2-year period (isolates that were recovered at an early stage of treatment are at the top; those that were recovered at a late stage in treatment are at the bottom). Growth differences in liquid medium with different concentrations of fluconazole are colour coded as follows: dark green, maximal growth; light green, intermediate growth; and black, no growth. Although the laboratory strain was unable to grow in any concentration of fluconazole tested, the clinical isolates that were recovered at an early stage of treatment showed intermediate growth at all concentrations and the clinical isolates that were recovered at a later stage showed increased resistance and growth at increasing concentrations. The schematics on the middle and right show how the inhibition of Hsp90 by geldanamycin (5 μM) and calcineurin by cyclosporin A (20 μM) reduce the growth of all clinical isolates in the presence of fluconazole and affect isolates at the early stages of infection to a greater extent than isolates from the later stages. **b** | Schematic of the resistance of a *C. albicans* clinical isolate and *Aspergillus terreus* soil isolate to two azoles (fluconazole and voriconazole) and an echinocandin (caspofungin) on solid medium; growth differences are colour coded as for **a**. Antifungal test strips (white rectangle) produce a gradient of drug concentration, with the highest appearing at the top. Plates contained a control, radicicol (5 μM) or cyclosporin A (20 μM), as indicated. Radicicol or cyclosporin A reduced the growth of *C. albicans* in the presence of azoles and reduced the growth of *A. terreus* in the presence of echinocandins. Figure adapted, with permission, from REF. 65 © (2005) American Association for the Advancement of Science.

A biofilm that is composed of a single genotype consists of cells that have distinct phenotypes, including variants that have enhanced resistance and are known as persister cells. For example, fractionation of a *C. albicans* biofilm revealed biphasic killing by amphotericin B, in which most of the cells were killed, but a small fraction survived[114]. Persister cells are not mutants, but are phenotypic variants that give rise to a new biofilm that has a comparable biphasic killing pattern. In bacterial biofilms, persister cells are often dormant cells that are produced in a culture during specific phases of growth[115,116]. In *C. albicans*, persister cells are only produced in biofilms and it is unknown whether dormancy is involved[114]. Although the role of these altered cellular states in the evolution of heritable resistance phenotypes has yet to be explored, it is clear that phenotypic heterogeneity can enhance survival during stress[117].

Combinatorial cellular perturbations

Because microorganisms use many mechanisms to evade killing by antimicrobial drugs, it might be possible to use strategic drug combinations to enhance killing and impede the evolution of drug resistance. Interacting combinations of molecules are numerous in nature, probably because of the benefits to the producing organism in competitive and communicative microbial communities[8,118]. These molecules can have many effects on species interactions. For example, a rhizosphere fungus can enhance *Arabidopsis thaliana* thermotolerance by the production of an Hsp90 inhibitor[119]. Like their genetic counterparts, two drugs can have no interaction or they can interact synergistically or antagonistically[29,120,121].

Combination therapy has been an important strategy for treating diverse pathogens and diseases[122–125]. Combinatorial strategies for cancer therapy have been mainly empirical to date, and the key principle has been the non-overlapping toxicities of the individual agents; such strategies are often based on non-crossreactive mechanisms of action[126]. From the pathogen's perspective, using two drugs can have additive or synergistic effects on pathogen killing in the short term. This strategy also has the potential to impede the evolution of drug resistance, both owing to a reduction in pathogen-population size, and thus the probability of acquiring resistance mutations, and the reduced probability of acquiring multiple resistance mutations simultaneously. Drugs can also be used together with conventional antimicrobials to block the emergence of resistance by inhibiting mutation or the genetic exchange of resistance determinants[127]. An intriguing benefit can even arise from combining two drugs that normally have strongly antagonistic effects such that the combination has less effect than that of either drug alone (also called suppression). In this case, resistance to one of the drugs can remove the suppression, thereby rendering the combination more effective against the resistant mutant than the wild type[128].

Although there is considerable interest in the development of effective combination strategies, few studies have shown a clear therapeutic benefit of combination therapy for the treatment of fungal disease. One notable example is the use of 5-flucytosine, which targets nucleic-acid synthesis and is deployed only in combination with other drugs because of the high incidence of resistance to this drug that is observed when it is used alone[124,129]. An

intriguing strategy for combination therapy involves the inhibition of calcineurin. Pharmacological inhibitors of calcineurin function are synergistic with azoles or echinocandins against different fungi, and can impair the growth and virulence of pathogenic fungi[86,130]. The profound immunosuppressive effects of calcineurin inhibitors complicate their usefulness in antifungal therapy. Promising strategies may involve non-immunosuppresive analogues or the targeting of fungal-specific components of the calcineurin pathway[86].

The role of Hsp90 in the emergence and maintenance of fungal drug resistance suggests an exciting new avenue for combination therapies. Notably, therapeutic benefit of a recombinant antibody against fungal Hsp90 has been observed in combination with amphotericin B, although the mechanism by which these effects are mediated remains unknown[131]. Perhaps as a consequence of its function in chaperoning a panoply of key regulators of cell signalling, Hsp90 has emerged as a tantalizing target for the treatment of diverse diseases. Hsp90 inhibitors were initially advanced into clinical trials for anticancer applications[132–136]. Recent studies suggest that many other therapeutic applications remain to be explored, including their potential as combination agents with antifungals for fungal disease[64,65], their potential as antimalarials[91] and their possible use against several neurodegenerative diseases[137,138]. The complexity of the role of Hsp90 in maintaining cellular protein-folding homeostasis might indeed minimize the probability of the evolution of resistance to Hsp90 inhibitors, thereby prolonging their therapeutic usefulness[139]. The future challenge will lie in discovering how to selectively modulate Hsp90 in the desired context without detrimental effects to the host. In cancer cells, this might be achieved by an altered conformation of Hsp90 in tumours that has higher drug-binding affinity[140–142]. For fungal applications, the ultimate goal would be to identify fungal-selective Hsp90 inhibitors.

Conclusions

Deciphering how genotype and the environment interact to yield phenotype continues to pose a central challenge in biology. Hsp90 has multifaceted roles in modulating the translation of genotype to phenotype that range from buffering genetic and epigenetic variation to stabilizing mutant oncogenic regulators and enabling the phenotypic effects of mutations through unmutated cellular regulators. The standard laboratory practice of producing monocultures under controlled conditions has led to a profound under-appreciation of the central role of stress responses in organism proliferation and adaptation to the harsh and fluctuating environments that are found in nature. It is likely that many different mechanisms for modulating fitness landscapes will continue to be revealed. Ultimately, basic insights into signalling, stress responses and evolution could shed new light on the treatment of many life-threatening diseases.

1. Anderson, J. B. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nature Rev. Microbiol.* **3**, 547–556 (2005).
An excellent introduction to fungal drug resistance from an evolutionary perspective.
2. Antonovics, J. *et al.* Evolution by any other name: antibiotic resistance and avoidance of the e-word. *PLoS Biol.* **5**, e30 (2007).
3. Cowen, L. E. Predicting the emergence of resistance to antifungal drugs. *FEMS Microbiol. Lett.* **204**, 1–7 (2001).
4. Cowen, L. E., Anderson, J. B. & Kohn, L. M. Evolution of drug resistance in *Candida albicans*. *Annu. Rev. Microbiol.* **56**, 139–165 (2002).
5. Levin, B. R., Lipsitch, M. & Bonhoeffer, S. Population biology, evolution, and infectious disease: convergence and synthesis. *Science* **283**, 806–809 (1999).
6. Riley, M. A. & Wertz, J. E. Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol.* **56**, 117–137 (2002).
7. Wright, G. D. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Rev. Microbiol.* **5**, 175–186 (2007).
8. Yim, G., Wang, H. H. & Davies, J. Antibiotics as signalling molecules. *Phil. Trans. R. Soc. Lond. B* **362**, 1195–1200 (2007).
9. Palumbi, S. R. Humans as the world's greatest evolutionary force. *Science* **293**, 1786–1790 (2001).
10. Pfaller, M. A. & Diekema, D. J. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J. Clin. Microbiol.* **42**, 4419–4431 (2004).
11. White, T. C., Marr, K. A. & Bowden, R. A. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11**, 382–402 (1998).
12. Alekshun, M. N. & Levy, S. B. Molecular mechanisms of antibacterial multidrug resistance. *Cell* **128**, 1037–1050 (2007).
13. Hastings, P. J., Rosenberg, S. M. & Slack, A. Antibiotic-induced lateral transfer of antibiotic resistance. *Trends Microbiol.* **12**, 401–404 (2004).
14. Rambaut, A., Posada, D., Crandall, K. A. & Holmes, E. C. The causes and consequences of HIV evolution. *Nature Rev. Genet.* **5**, 52–61 (2004).
15. Woodford, N. & Ellington, M. J. The emergence of antibiotic resistance by mutation. *Clin. Microbiol. Infect.* **13**, 5–18 (2007).
16. Maisnier-Patin, S. & Andersson, D. I. Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res. Microbiol.* **155**, 360–369 (2004).
17. Perlstein, E. O. *et al.* Revealing complex traits with small molecules and naturally recombinant yeast strains. *Chem. Biol.* **13**, 319–327 (2006).
18. Perlstein, E. O., Ruderfer, D. M., Roberts, D. C., Schreiber, S. L. & Kruglyak, L. Genetic basis of individual differences in the response to small-molecule drugs in yeast. *Nature Genet.* **39**, 496–502 (2007).
19. Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. & Doolittle, W. F. A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* **290**, 972–977 (2000).
20. Odds, F. C., Brown, A. J. & Gow, N. A. Antifungal agents: mechanisms of action. *Trends Microbiol.* **11**, 272–279 (2003).
21. Fraser, J. A. *et al.* Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature* **437**, 1360–1364 (2005).
22. Martin, G. S., Mannino, D. M., Eaton, S. & Moss, M. The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J. Med.* **348**, 1546–1554 (2003).
23. Pfaller, M. A. & Diekema, D. J. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20**, 133–163 (2007).
24. McNeil, M. M. *et al.* Trends in mortality due to invasive mycotic diseases in the United States, 1980–1997. *Clin. Infect. Dis.* **33**, 641–647 (2001).
25. Wilson, L. S. *et al.* The direct cost and incidence of systemic fungal infections. *Value Health* **5**, 26–34 (2002).
26. Elena, S. F. & Lenski, R. E. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Rev. Genet.* **4**, 457–469 (2003).
27. Zeyl, C. Experimental evolution with yeast. *FEMS Yeast Res.* **6**, 685–691 (2006).
28. Bahn, Y. S. *et al.* Sensing the environment: lessons from fungi. *Nature Rev. Microbiol.* **5**, 57–69 (2007).
29. Boone, C., Bussey, H. & Andrews, B. J. Exploring genetic interactions and networks with yeast. *Nature Rev. Genet.* **8**, 437–449 (2007).
30. Hartman, J. L., Garvik, B. & Hartwell, L. Principles for the buffering of genetic variation. *Science* **291**, 1001–1004 (2001).
Provides an important perspective on the complex relationship between genotype and phenotype and mechanisms of buffering variation in eukaryotic genomes.
31. Rockman, M. V. & Kruglyak, L. Genetics of global gene expression. *Nature Rev. Genet.* **7**, 862–872 (2006).
32. Steinmetz, L. M. & Davis, R. W. Maximizing the potential of functional genomics. *Nature Rev. Genet.* **5**, 190–201 (2004).
33. Lupetti, A., Danesi, R., Campa, M., Del Tacca, M. & Kelly, S. Molecular basis of resistance to azole antifungals. *Trends Mol. Med.* **8**, 76–81 (2002).
34. Turner, M. S., Drew, R. H. & Perfect, J. R. Emerging echinocandins for treatment of invasive fungal infections. *Expert Opin. Emerg. Drugs* **11**, 231–250 (2006).
35. Rex, J. H. & Pfaller, M. A. Has antifungal susceptibility testing come of age? *Clin. Infect. Dis.* **35**, 982–989 (2002).
36. Sanglard, D. Resistance of human fungal pathogens to antifungal drugs. *Curr. Opin. Microbiol.* **5**, 379–385 (2002).
37. Sanglard, D. & Odds, F. C. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect. Dis.* **2**, 73–85 (2002).
38. Coste, A. *et al.* Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryot. Cell* **6**, 1889–1904 (2007).
39. da Silva Ferreira, M. E. *et al. In vitro* evolution of itraconazole resistance in *Aspergillus fumigatus*

involves multiple mechanisms of resistance. *Antimicrob. Agents Chemother.* **48**, 4405–4413 (2004).
40. Nascimento, A. M. *et al.* Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob. Agents Chemother.* **47**, 1719–1726 (2003).
41. Pasrija, R., Banerjee, D. & Prasad, R. Structure and function analysis of CaMdr1p, a major facilitator superfamily antifungal efflux transporter protein of *Candida albicans*: identification of amino acid residues critical for drug/H^+ transport. *Eukaryot. Cell* **6**, 443–453 (2007).
42. Perea, S. *et al.* Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **45**, 2676–2684 (2001).
43. Chamilos, G. & Kontoyiannis, D. P. Update on antifungal drug resistance mechanisms of *Aspergillus fumigatus*. *Drug Resist. Updat.* **8**, 344–358 (2005).
44. Coste, A. *et al.* A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics* **172**, 2139–2156 (2006).
45. Coste, A. T., Karababa, M., Ischer, F., Bille, J. & Sanglard, D. *TAC1*, transcriptional activator of *CDR* genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. *Eukaryot. Cell* **3**, 1639–1652 (2004).
46. Hiller, D., Stahl, S. & Morschhauser, J. Multiple *cis*-acting sequences mediate upregulation of the *MDR1* efflux pump in a fluconazole-resistant clinical *Candida albicans* isolate. *Antimicrob. Agents Chemother.* **50**, 2300–2308 (2006).
47. Liu, T. T. *et al.* Genome-wide expression and location analyses of the *Candida albicans* Tac1p regulon. *Eukaryot. Cell* **6**, 2122–2138 (2007).
48. Morschhauser, J. *et al.* The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans*. *PLoS Pathog.* **3**, e164 (2007).
49. Riggle, P. J. & Kumamoto, C. A. Transcriptional regulation of *MDR1*, encoding a drug efflux determinant, in fluconazole-resistant *Candida albicans* strains through an Mcm1p binding site. *Eukaryot. Cell* **5**, 1957–1968 (2006).
50. Rognon, B., Kozovska, Z., Coste, A. T., Pardini, G. & Sanglard, D. Identification of promoter elements responsible for the regulation of *MDR1* from *Candida albicans*, a major facilitator transporter involved in azole resistance. *Microbiology* **152**, 3701–3722 (2006).
51. Selmecki, A., Forche, A. & Berman, J. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* **313**, 367–370 (2006).
Established a novel mechanism of fungal drug resistance that involves aneuploidy and isochromosome formation; also showcased the genomic plasticity that underpins phenotypic variability.
52. Niimi, K. *et al.* Overexpression of *Candida albicans CDR1*, *CDR2*, or *MDR1* does not produce significant changes in echinocandin susceptibility. *Antimicrob. Agents Chemother.* **50**, 1148–1155 (2006).
53. Perlin, D. S. Resistance to echinocandin-class antifungal drugs. *Drug Resist. Updat.* **10**, 121–130 (2007).
54. MacPherson, S. *et al. Candida albicans* zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. *Antimicrob. Agents Chemother.* **49**, 1745–1752 (2005).
55. Silver, P. M., Oliver, B. G. & White, T. C. Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. *Eukaryot. Cell* **3**, 1391–1397 (2004).
56. Giaever, G. *et al.* Chemogenomic profiling: identifying the functional interactions of small molecules in yeast. *Proc. Natl Acad. Sci. USA* **101**, 793–798 (2004).
57. Hu, W. *et al.* Essential gene identification and drug target prioritization in *Aspergillus fumigatus*. *PLoS Pathog.* **3**, e24 (2007).
58. Xu, D. *et al.* Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. *PLoS Pathog.* **3**, e92 (2007).
59. White, T. C. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. *Antimicrob. Agents Chemother.* **41**, 1482–1487 (1997).
60. Anderson, J. B., Ricker, N. & Sirjusingh, C. Antagonism between two mechanisms of antifungal drug resistance. *Eukaryot. Cell* **5**, 1243–1251 (2006).
61. Cowen, L. E., Kohn, L. M. & Anderson, J. B. Divergence in fitness and evolution of drug resistance in experimental populations of *Candida albicans*. *J. Bacteriol.* **183**, 2971–2978 (2001).
62. Cowen, L. E. *et al.* Evolution of drug resistance in experimental populations of *Candida albicans*. *J. Bacteriol.* **182**, 1515–1522 (2000).
63. Cowen, L. E. *et al.* Population genomics of drug resistance in *Candida albicans*. *Proc. Natl Acad. Sci. USA* **99**, 9284–9289 (2002).
64. Cowen, L. E., Carpenter, A. E., Matangkasombut, O., Fink, G. R. & Lindquist, S. Genetic architecture of Hsp90-dependent drug resistance. *Eukaryot. Cell* **5**, 2184–2188 (2006).
65. Cowen, L. E. & Lindquist, S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science* **309**, 2185–2189 (2005).
Describes a new mechanism by which Hsp90 can enable the evolution of fungal drug resistance and modulate the relationship between genotype and phenotype.
66. Pearl, L. H. & Prodromou, C. Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annu. Rev. Biochem.* **75**, 271–294 (2006).
67. Picard, D. Heat-shock protein 90, a chaperone for folding and regulation. *Cell. Mol. Life Sci.* **59**, 1640–1648 (2002).
68. Pratt, W. B. & Toft, D. O. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med.* **228**, 111–133 (2003).
69. Young, J. C., Moarefi, I. & Hartl, F. U. Hsp90: a specialized but essential protein-folding tool. *J. Cell. Biol.* **154**, 267–273 (2001).
70. Chang, H. C. & Lindquist, S. Conservation of Hsp90 macromolecular complexes in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**, 24983–24988 (1994).
71. Nathan, D. F., Vos, M. H. & Lindquist, S. *In vivo* functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. *Proc. Natl Acad. Sci. USA* **94**, 12949–12956 (1997).
72. Roe, S. M. *et al.* Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J. Med. Chem.* **42**, 260–266 (1999).
73. Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E. & Neckers, L. M. Inhibition of heat shock protein HSP90–pp60v–src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl Acad. Sci. USA* **91**, 8324–8328 (1994).
74. Sangster, T. A., Lindquist, S. & Queitsch, C. Under cover: causes, effects and implications of Hsp90-mediated genetic capacitance. *Bioessays* **26**, 348–362 (2004).
75. Queitsch, C., Sangster, T. A. & Lindquist, S. Hsp90 as a capacitor of phenotypic variation. *Nature* **417**, 618–624 (2002).
Demonstrated that the molecular chaperone Hsp90 buffers development from the destabilizing effects of stochastic processes and also buffers the expression of genetic variation in *A. thaliana*.
76. Rutherford, S. L. & Lindquist, S. Hsp90 as a capacitor for morphological evolution. *Nature* **396**, 336–342 (1998).
This groundbreaking study established that Hsp90 could buffer the expression of genetic variation in *Drosophila melanogaster*, and therefore allow cryptic variation to accumulate until revealed by environmental stress.
77. Sollars, V. *et al.* Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. *Nature Genet.* **33**, 70–74 (2003).
78. Zhao, R. *et al.* Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the Hsp90 chaperone. *Cell* **120**, 715–727 (2005).
Provides a high-resolution map of the physical, genetic and chemical–genetic interactions of Hsp90 in *S. cerevisiae*.
79. Ruden, D. M., Garfinkel, M. D., Sollars, V. E. & Lu, X. Waddington's widget: Hsp90 and the inheritance of acquired characters. *Semin. Cell Dev. Biol.* **14**, 301–310 (2003).
80. Rutherford, S. L. Between genotype and phenotype: protein chaperones and evolvability. *Nature Rev. Genet.* **4**, 263–274 (2003).
81. Bergman, A. & Siegal, M. L. Evolutionary capacitance as a general feature of complex gene networks. *Nature* **424**, 549–552 (2003).
82. Hughes, T. R. *et al.* Functional discovery via a compendium of expression profiles. *Cell* **102**, 109–126 (2000).
83. Kirschner, M. & Gerhart, J. Evolvability. *Proc. Natl Acad. Sci. USA* **95**, 8420–8427 (1998).
An insightful perspective on evolvability — the capacity to generate heritable phenotypic variation.
84. Fox, D. S. & Heitman, J. Good fungi gone bad: the corruption of calcineurin. *Bioessays* **24**, 894–903 (2002).
85. Heitman, J. Cell biology. A fungal Achilles' heel. *Science* **309**, 2175–2176 (2005).
86. Steinbach, W. J., Reedy, J. L., Cramer, R. A. Jr, Perfect, J. R. & Heitman, J. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nature Rev. Microbiol.* **5**, 418–430 (2007).
An excellent review of calcineurin biology in fungal pathogens and our potential to harness this cellular regulator in the treatment of fungal infections.
87. Cruz, M. C. *et al.* Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO J.* **21**, 546–559 (2002).
88. Sanglard, D., Ischer, F., Marchetti, O., Entenza, J. & Bille, J. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol. Microbiol.* **48**, 959–976 (2003).
89. Steinbach, W. J. *et al. In vitro* interactions between antifungals and immunosuppressants against *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **48**, 1664–1669 (2004).
90. Imai, J. & Yahara, I. Role of HSP90 in salt stress tolerance via stabilization and regulation of calcineurin. *Mol. Cell. Biol.* **20**, 9262–9270 (2000).
91. Kumar, R., Musiyenko, A. & Barik, S. *Plasmodium falciparum* calcineurin and its association with heat shock protein 90: mechanisms for the antimalarial activity of cyclosporin A and synergism with geldanamycin. *Mol. Biochem. Parasitol.* **141**, 29–37 (2005).
92. Someren, J. S., Faber, L. E., Klein, J. D. & Tumlin, J. A. Heat shock proteins 70 and 90 increase calcineurin activity *in vitro* through calmodulin-dependent and independent mechanisms. *Biochem. Biophys. Res. Commun.* **260**, 619–625 (1999).
93. Karababa, M. *et al. CRZ1*, a target of the calcineurin pathway in *Candida albicans*. *Mol. Microbiol.* **59**, 1429–1451 (2006).
94. Santos, M. & de Larrinoa, I. F. Functional characterization of the *Candida albicans CRZ1* gene encoding a calcineurin-regulated transcription factor. *Curr. Genet.* **48**, 88–100 (2005).
95. Yoshimoto, H. *et al.* Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277**, 31079–31088 (2002).
96. Onyewu, C., Wormley, F. L. Jr, Perfect, J. R. & Heitman, J. The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of *Candida albicans*. *Infect. Immun.* **72**, 7330–7333 (2004).
97. Heath, V. L., Shaw, S. L., Roy, S. & Cyert, M. S. Hph1p and Hph2p, novel components of calcineurin-mediated stress responses in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **3**, 695–704 (2004).
98. McClellan, A. J. *et al.* Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. *Cell* **131**, 121–135 (2007).
99. Siegal, M. L., Promislow, D. E. & Bergman, A. Functional and evolutionary inference in gene networks: does topology matter? *Genetica* **129**, 83–103 (2007).
100. Waddington, C. H. Canalization of development and the inheritance of acquired characters. *Nature* **150**, 563–565 (1942).
101. Waddington, C. H. Genetic assimilation of an acquired character. *Evolution* **7**, 118–126 (1953).
102. Hasday, J. D., Fairchild, K. D. & Shanholtz, C. The role of fever in the infected host. *Microbes Infect.* **2**, 1891–1904 (2000).
103. True, H. L., Berlin, I. & Lindquist, S. L. Epigenetic regulation of translation reveals hidden genetic variation to produce complex traits. *Nature* **431**, 184–187 (2004).
104. True, H. L. & Lindquist, S. L. A yeast prion provides a mechanism for genetic variation and phenotypic diversity. *Nature* **407**, 477–483 (2000).

Describes how the yeast prion [PSI$^+$] can uncover cryptic genetic variation and generate new heritable phenotypes.
105. Blankenship, J. R. & Mitchell, A. P. How to build a biofilm: a fungal perspective. *Curr. Opin. Microbiol.* **9**, 588–594 (2006).
106. Nett, J. & Andes, D. *Candida albicans* biofilm development, modeling a host–pathogen interaction. *Curr. Opin. Microbiol.* **9**, 340–345 (2006).
107. Nobile, C. J. & Mitchell, A. P. Genetics and genomics of *Candida albicans* biofilm formation. *Cell. Microbiol.* **8**, 1382–1391 (2006).
108. Wargo, M. J. & Hogan, D. A. Fungal–bacterial interactions: a mixed bag of mingling microbes. *Curr. Opin. Microbiol.* **9**, 359–364 (2006).
109. d'Enfert, C. Biofilms and their role in the resistance of pathogenic *Candida* to antifungal agents. *Curr. Drug Targets* **7**, 465–470 (2006).
110. Nett, J. *et al.* Putative role of β-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob. Agents Chemother.* **51**, 510–520 (2007).
111. Andes, D. *et al.* Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. *Infect. Immun.* **72**, 6023–6031 (2004).
112. Baillie, G. S. & Douglas, L. J. Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrob. Agents Chemother.* **42**, 1900–1905 (1998).
113. Halme, A., Bumgarner, S., Styles, C. & Fink, G. R. Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. *Cell* **116**, 405–415 (2004).
114. LaFleur, M. D., Kumamoto, C. A. & Lewis, K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob. Agents Chemother.* **50**, 3839–3846 (2006).
115. Levin, B. R. & Rozen, D. E. Non-inherited antibiotic resistance. *Nature Rev. Microbiol.* **4**, 556–562 (2006).
116. Lewis, K. Persister cells, dormancy and infectious disease. *Nature Rev. Microbiol.* **5**, 48–56 (2007).
117. Avery, S. V. Microbial cell individuality and the underlying sources of heterogeneity. *Nature Rev. Microbiol.* **4**, 577–587 (2006).
118. Keith, C. T., Borisy, A. A. & Stockwell, B. R. Multicomponent therapeutics for networked systems. *Nature Rev. Drug Discov.* **4**, 71–78 (2005).
119. McLellan, C. A. *et al.* A rhizosphere fungus enhances *Arabidopsis* thermotolerance through production of an HSP90 inhibitor. *Plant Physiol.* **145**, 174–182 (2007).
120. Lehar, J. *et al.* Chemical combination effects predict connectivity in biological systems. *Mol. Syst. Biol.* **3**, 80 (2007).
121. Yeh, P., Tschumi, A. I. & Kishony, R. Functional classification of drugs by properties of their pairwise interactions. *Nature Genet.* **38**, 489–494 (2006).
122. Blankson, J. N., Persaud, D. & Siliciano, R. F. The challenge of viral reservoirs in HIV-1 infection. *Annu. Rev. Med.* **53**, 557–593 (2002).
123. Griffith, K. S., Lewis, L. S., Mali, S. & Parise, M. E. Treatment of malaria in the United States: a systematic review. *JAMA* **297**, 2264–2277 (2007).
124. Mukherjee, P. K., Sheehan, D. J., Hitchcock, C. A. & Ghannoum, M. A. Combination treatment of invasive fungal infections. *Clin. Microbiol. Rev.* **18**, 163–194 (2005).
125. Wright, G. D. & Sutherland, A. D. New strategies for combating multidrug-resistant bacteria. *Trends Mol. Med.* **13**, 260–267 (2007).
126. Kwak, E. L., Clark, J. W. & Chabner, B. Targeted agents: the rules of combination. *Clin. Cancer Res.* **13**, 5232–5237 (2007).
127. Smith, P. A. & Romesberg, F. E. Combating bacteria and drug resistance by inhibiting mechanisms of persistence and adaptation. *Nature Chem. Biol.* **3**, 549–556 (2007).
128. Chait, R., Craney, A. & Kishony, R. Antibiotic interactions that select against resistance. *Nature* **446**, 668–671 (2007).
Demonstrates an intriguing feature of the fitness landscape — specific drug interactions can select against resistant populations.
129. Johnson, M. D. & Perfect, J. R. Combination antifungal therapy: what can and should we expect? *Bone Marrow Transplant.* **40**, 297–306 (2007).
130. Blankenship, J. R., Steinbach, W. J., Perfect, J. R. & Heitman, J. Teaching old drugs new tricks: reincarnating immunosuppressants as antifungal drugs. *Curr. Opin. Investig. Drugs* **4**, 192–199 (2003).
131. Pachl, J. *et al.* A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. *Clin. Infect. Dis.* **42**, 1404–1413 (2006).
132. Chiosis, G. Targeting chaperones in transformed systems — a focus on Hsp90 and cancer. *Expert Opin. Ther. Targets* **10**, 37–50 (2006).
133. Kamal, A., Boehm, M. F. & Burrows, F. J. Therapeutic and diagnostic implications of Hsp90 activation. *Trends Mol. Med.* **10**, 283–290 (2004).
134. Neckers, L. & Neckers, K. Heat-shock protein 90 inhibitors as novel cancer chemotherapeutics — an update. *Expert Opin. Emerg. Drugs* **10**, 137–149 (2005).
135. Workman, P. Combinatorial attack on multistep oncogenesis by inhibiting the Hsp90 molecular chaperone. *Cancer Lett.* **206**, 149–157 (2004).
136. Zhang, H. & Burrows, F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J. Mol. Med.* **82**, 488–499 (2004).
137. Luo, W. *et al.* Roles of heat-shock protein 90 in maintaining and facilitating the neurodegenerative phenotype in tauopathies. *Proc. Natl Acad. Sci. USA* **104**, 9511–9516 (2007).
138. Muchowski, P. J. & Wacker, J. L. Modulation of neurodegeneration by molecular chaperones. *Nature Rev. Neurosci.* **6**, 11–22 (2005).
139. Geller, R., Vignuzzi, M., Andino, R. & Frydman, J. Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. *Genes Dev.* **21**, 195–205 (2007).
140. Chiosis, G. & Neckers, L. Tumor selectivity of Hsp90 inhibitors: the explanation remains elusive. *ACS Chem. Biol.* **1**, 279–284 (2006).
141. Duvvuri, M., Konkar, S., Hong, K. H., Blagg, B. S. & Krise, J. P. A new approach for enhancing differential selectivity of drugs to cancer cells. *ACS Chem. Biol.* **1**, 309–315 (2006).
142. Kamal, A. *et al.* A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* **425**, 407–410 (2003).
143. Borkovich, K. A., Farrelly, F. W., Finkelstein, D. B., Taulien, J. & Lindquist, S. Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol. Cell. Biol.* **9**, 3919–3930 (1989).
144. Xu, W. *et al.* Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc. Natl Acad. Sci. USA* **99**, 12847–12852 (2002).
145. Ali, M. M. *et al.* Crystal structure of an Hsp90–nucleotide–p23/Sba1 closed chaperone complex. *Nature* **440**, 1013–1017 (2006).
146. Bagatell, R. & Whitesell, L. Altered Hsp90 function in cancer: a unique therapeutic opportunity. *Mol. Cancer Ther.* **3**, 1021–1030 (2004).
147. Whitesell, L. & Lindquist, S. L. HSP90 and the chaperoning of cancer. *Nature Rev. Cancer* **5**, 761–772 (2005).
148. Mosser, D. D. & Morimoto, R. I. Molecular chaperones and the stress of oncogenesis. *Oncogene* **23**, 2907–2918 (2004).
149. Takayama, S., Reed, J. C. & Homma, S. Heat-shock proteins as regulators of apoptosis. *Oncogene* **22**, 9041–9047 (2003).
150. Falsone, S. F., Leptihn, S., Osterauer, A., Haslbeck, M. & Buchner, J. Oncogenic mutations reduce the stability of SRC kinase. *J. Mol. Biol.* **344**, 281–291 (2004).
151. Xu, Y. & Lindquist, S. Heat-shock protein hsp90 governs the activity of pp60$^{v\text{-}src}$ kinase. *Proc. Natl Acad. Sci. USA* **90**, 7074–7078 (1993).
152. Xu, Y., Singer, M. A. & Lindquist, S. Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90. *Proc. Natl Acad. Sci. USA* **96**, 109–114 (1999).
153. Dai, C., Whitesell, L., Rogers, A. B. & Lindquist, S. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell* **130**, 1005–1018 (2007).

Acknowledgements
The author thanks L. Whitesell for invaluable comments on the manuscript. Work in the laboratory of L.E.C. is supported by a Career Award in the Biomedical Sciences from the Burroughs Wellcome Fund and by a Canada Research Chair in Microbial Genomics and Infectious Disease.

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